Methods in Molecular Biology™

VOLUME 124

Protein Kinase Protocols

Edited by

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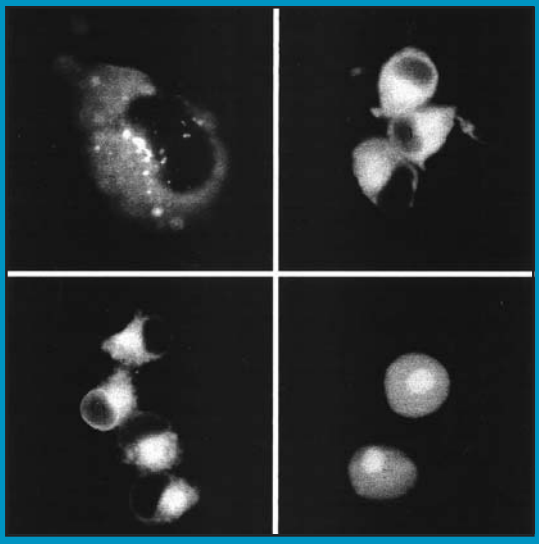

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Protein Kinase-Mediated Signaling Networks

Regulation and Functional Characterization

Alastair D. Reith

1. Introduction—Regulation of Membrane Receptor Kinase Complexes

Many aspects of cellular metabolism are regulated by reversible phosphorylation of proteins. Several amino acid residues within proteins are subject to such posttranslational regulatory events, the best characterized of which are tyrosine, serine, and threonine. Transfer of phosphate to such residues is mediated by protein kinases that catalyze the transfer of phosphate from adenosine triphosphate (ATP) to specific relevant amino acid residues. This fundamental property of protein kinases is utilized and elaborated upon in many different contexts to generate protein kinase mediated signaling cascades by which extracellular stimuli are accurately perceived and elicit appropriate cellular responses.

Protein kinases constitute the largest single enzyme family in the human genome, with an estimated total number estimated around 2000, and are highly conserved across species. This latter aspect has enabled great strides to be made in our understanding of the functions of these proteins through genetic analysis in tractable model organisms such as yeast, *C. elegans*, and *Drosophila*. Together with a diverse range of complementary techniques, including biophysical and crystallographic studies, an integrated view is emerging that facilitates our understanding of this fundamentally important aspect of cellular function.

In this chapter, I shall briefly review current concepts of the molecular mechanisms by which both receptor and intracellular protein kinases are regulated and coordinated within signaling cascades. The value of pharmacological

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

tools for molecular dissection of protein kinase signaling pathway function is also considered.

1.1. Protein Kinase-Mediated Recruitment of Receptor Complexes

Several distinct classes of cell-surface receptor are known to utilize protein kinase activity, either directly or indirectly, to transduce extracellular stimuli across the plasmamembrane to the cytoplasm. Receptor tyrosine kinases (e.g., EGFR, PDGFR) and receptor serine/threonine kinases (e.g., TGFβ receptors) bear intracellular protein kinase domains that are covalently linked with extracellular ligand-binding domains. In contrast, membrane-spanning cytokine receptors (e.g., erythropoietin receptor, G-CSFR) lack intrinsic protein kinase activity, but utilize the closely associated JAK family of intracellular kinases. For all three classes of receptors, specific and high-affinity interaction with extracellular ligands is thought to stimulate the stabilization of receptor dimers or oligomers that, in turn, mediate activation of the associated protein kinase catalytic domain ***(1)***. Functional receptor ser/thr kinases constitute a heteromeric complex between type II receptors (bearing ser/thr kinase domain) and type I receptors. Ligand binding stimulates the catalytic activity of the type II receptor, resulting in phosphorylation of specific residues on the type I receptor. This promotes transient interaction and phosphorylation of a subset of SMAD proteins. A membrane-associated adaptor protein, SARA, likely serves to recruit SMADs to the membrane and stabilize receptor complexes. Once phosphorylated, SMADs dissociate from the receptor complex, heterodimerize with other SMAD proteins, and translocate directly to the nucleus to evoke extracellular ligand induced transcriptional change ***(2)***.

For both receptor tyrosine kinases and cytokine receptor–JAK complexes, ligand-mediated kinase activation results in transphosphorylation of specific tyrosine residues on the intracellular domain of the receptor protein. In turn, these phosphorylated residues contribute to phosphotyrosine-containing docking motifs for recruitment and activation of a variety of intracellular signaling proteins that constitute a functional receptor signaling complex.

1.2. Modular Binding Domains Mediate Receptor Complex Assembly

The repertoire of intracellular signaling proteins known to associate with specific phosphotyrosine recognition motifs are characterized by the presence of one or more conserved modular domains. In addition, a number of additional protein–protein interaction domains have been identified within receptor signaling complex proteins. Together, such modular motifs facilitate assembly of specific intracellular signaling complexes. Proteins bearing such motifs fall into two broad classes: those found covalently linked with catalytic activities

(e.g., kinases, phosphatases), and so-called adaptor or scaffold proteins that lack defined catalytic function ***(3)***. Modular motifs used in this regard include the following:

1.2.1. SH2 Domains

First identified within src family kinases ***(4)***, src homology 2 domains specifically interact with phosphotyrosine containing peptide motifs defined by the phosphotyrosine and 3–5 C-terminal residues. Importantly, distinct classes of SH2 domains associate selectively with different phosphopeptide motifs. Screening degenerate phosphopeptide libraries has provided an indication of preferential recognition motifs for different SH2 domains ***(5)***. However, "optimal" phosphopeptide motifs defined in this way do not include all high-affinity sites. For example, fynSH2, but not those of GAP or GRB2, interacts with a YEDP phosphotyrosine-containing motif of EphA family receptor tyrosine kinase ***(6,7)***. This differs markedly from the optimal src family SH2 phosphopeptide-binding motif YEEI defined from degenerate phosphopeptide library screens.

1.2.2. PTB Domains

Identified initially in SHC and IRS1 adaptor proteins, PTB domains recognize phosphotyrosine motifs that are preceeded by a β-turn — typically as a NP×Y motif. Hydprophobic residues located 5–8 residues N-terminal to the phosphotyrosine help to confer selectivity of such interactions. Unlike SH2 domains, phosphotyrosine is not essential for PTB domain binding to all target recognition motifs ***(8,9)***.

1.2.3. SH3 Domains

SH3 domains optimally recognize a left-handed polyproline type II helix. The primary function of SH3 domains is thought to be in generating oligomeric complexes. As exemplified by analysis of the Grb2-sos complex, there is some evidence that ser/thr phosphorylation within such motifs can promote dissociation of such interactions ***(10,11)***.

1.2.4. PDZ Domains

PDZ domains recognize short carboxy terminal sequences, typically E(S/T)DV. As with SH3 domains, there is some evidence that phosphorylation of serine/threonine residues can promote dissociation of interaction ***(12)***.

Clearly, the combination of such domains within a given protein can have a major impact on signaling properties. For example, PDZ domains are often found in multiple copies, so enabling adaptors to promote aggregation of target proteins. Similarly, the presence of nine SH2 binding sites for PI-3K in the

adaptor protein IRS1, is likely to facilitate signal amplification within the insulin receptor signaling complex.

In addition to roles in assembly of receptor complexes, phosphorylation-modulated binding domains and recognition motifs are also utilized for intramolecular interactions by which the activity of protein kinases is regulated. An illustration is provided by studies of the src and hck protein tyrosine kinases. These kinases bear a C-terminal catalytic domain along with a single SH2 and a single SH3 domain. Two key tyrosine residues are known to be involved in regulation of src family kinase catalytic activity. The autophosphorylation site Y^{416} is located in the activation loop and is necessary for full activity of src, whereas the C-terminal residue Y^{527} is phosphorylated by the src negative regulator CSK. An understanding of the mechanism underlying this regulation came from crystallographic analyses of inactive conformations of src and hck ***(13,14)***. In the inactive state, the SH2 and SH3 domains bind to the surface of the catalytic domain lying distal to the activation loop. The SH2 domain specifically interacts with the CSK-mediated $pTyr^{527}$ motif, whereas the SH3 domain associates specifically with a left-handed polyproline type II helix that is located between the SH2 and catalytic domains. This has the consequence that the active site conformation is disrupted. More recent higher resolution analysis indicates that, in contrast to the active enzyme where the activation loop is in an open conformation, the intramolecular SH2-Y^{527} and SH3-pro rich domain interactions within inactive Src result in Tyr^{416} within the activation loop adopting a conformation that blocks binding of peptide substrate ***(15)***. Dephosphorylation of Y^{527} or juxtaposition with competing SH2 or SH3 ligands ***(16)*** provides the necessary conformational change to faciliate phoshphorylation of Y^{416}, and hence stabilize a catalytically active conformation.

1.3. Kinase-Regulated Endocytosis of Receptor Complexes

Internalization of activated receptor complexes plays a key role in regulation and specification of signaling cascade events. Amongst G protein-coupled receptors (GPCRs), attentuation of signaling is faciliated by the activity of a family of GPCR ser/thr kinases (GRKs). GRK-mediated phosphorylation of agonist-occupied receptors stimulates receptor association with βarrestins which, in turn, promotes disassociation of receptor-G protein complexes and receptor internalization ***(17,18)***. This endocytosis has been found to be necessary for GPCR-mediated mitogenic signaling via the mitogen-activating protein kinases (MAPK)/ERK cascade (*see* **Subheading 2.1.**). Interestingly, blocking internalization has no effect on shc-*ras* or Raf, but specifically inhibits the ability of Raf to activate MEK ***(19)***. Normal endocytosis is also required for maximal tyrosine phosphorylation of activated EGFR and ligand-mediated

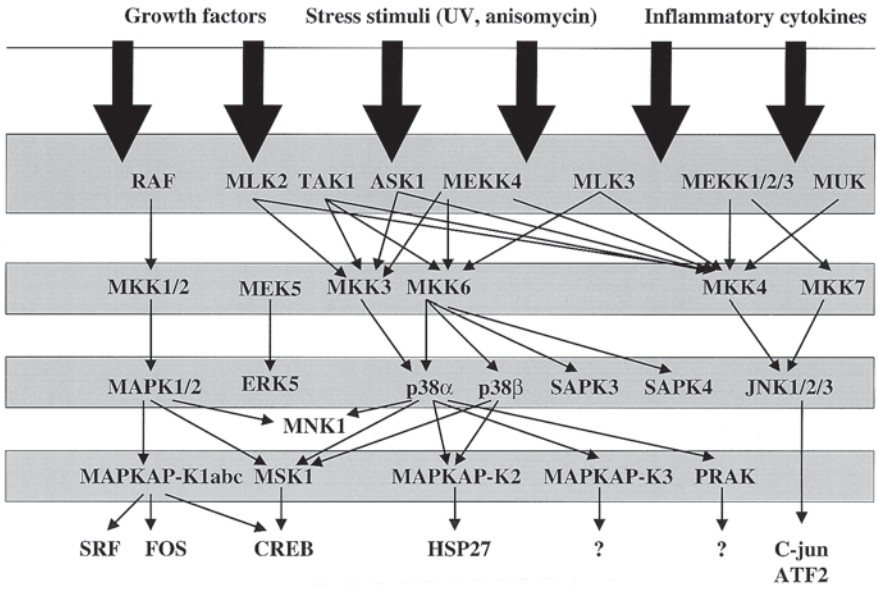

Fig. 1. Mitogen and stress activated signaling cascades.

activation of MAPK/ERK ***(20)***. In contrast, other effectors of activated EGFR exhibit hyperphosphorylation in the absence of normal endocytic trafficking, suggesting that regulation of receptor trafficking could play a key role in intracellular pathway regulation. In support of this, it has also been found that NGF signaling from axon terminal to activate the transcription factor CREB within the cell body of sympathetic neurons requires both internalization and retrograde transport of an NGF-TrkA ligand-receptor complex ***(21)***.

2. Organization of Intracellular Kinase Signaling Complexes

2.1. Intracellular MAP Kinase Cascades

Although activation of receptor ser/thr kinases results in a fairly direct route to activation and translocation of transcription factors (**Subheading 1.1.**), receptor tyrosine kinases and GPCRs utilize more elaborate intracellular kinase transduction cascades to modulate transcriptional activity. By far the best characterized of these are those involving MAPKs, components, and organization of which are conserved from yeast to mammals ***(22)***. There are three well-defined MAPK pathways in mammals — MAPK/ERK, p38/SAPK2, and JNK/SAPK1. The core MAPK cascade module is composed of three distinct kinases that function in a hierarcheal manner. MAPKs are proline-directed ser/thr kinases that recognize and phosphorylate S/T-P motifs in target proteins. MAPKs are phosphorylated, and hence activated, by MKKs — a relatively

small group of dual specificity kinases that phosphorylate T×Y motifs within the activation loop of target MAPKs. In turn, MKKs are phosphorylated and activated by MKKKs – a larger group of ser/thr kinases characterized by the presence of a variety of additional regulatory domains. MKKKs themselves can be activated by additional upstream kinases (so-called MKKKKs), or interaction with *ras* or *rho* family small GTP-binding proteins. Distinct MAPK cascades are preferentially activated by a variety of extracellular stimuli, including cellular stresses such as irradiation, osmotic shock, heat shock, as well as growth factors and cytokines, and the diversity of regulatory motifs within MKKKs is likely to play a role in this respect. MAPK cascades activate a wide variety of substrates that include additional protein kinases, transcription factors, and cytoskeletal proteins.

2.2. Scaffold Proteins Define Functional Kinase Cascades

The complexity of MAPK signaling cascades offers a capacity for signal amplification, as well as providing scope for modulation of activity and integration of cellular response to diverse stimuli. Clearly, such a system demands tight regulation of the mutiplicity of potential kinase associations and activations. This is achieved by scaffolding mechanisms of which there are two types; kinases themselves can function as scaffolds through docking motifs that interact directly with other kinases of the cascade, and disticnt scaffold proteins that lack catalytic activity but mediate selective association between two or more kinases.

Initially identified in yeast ***(23–25)***, evidence has accumulated for roles of both direct kinase–kinase interaction and scaffold proteins in the formation of functional and selective intracellular signaling complexes in other systems, including mammals. The following are selected examples:

2.2.1. MAPK/ERK Pathway

Kinase suppressor of *ras* (KSR) was identified initially through genetic screens in *Drosophila* and *C. elegans* for *Ras* suppressors, and is conserved in mammals ***(26–28)***. Genetic analysis suggested that KSR normally acts upstream of or parallel to Raf. Consistent with this, KSR was also identified as ceramide-activated protein (CAP) kinase that is involved in phosphorylation-mediated activation of Raf-1 in response to a subset of stimuli that activate the MAPK/ERK pathway ***(29)***. Additional studies indicated that distinct regions of KSR associate with Raf, MEK1, and ERK, suggesting that KSR may also act as a scaffold protein to link *ras* with MAPK pathway ***(30,31)***. Whereas KSR-MEK complexes appear stable in the absence of pathway activation, those with ERK are more transient, perhaps reflecting a requirement for ERK translocation to the nucleus.

The ability of 14-3-3 proteins to interact with a variety of signaling proteins, including PKC, PI-3 kinase, Raf-1, and KSR, make members of this family of dimeric molecules likely key modulators of intracellular signaling complexes. Evidence suggests that the interaction of 14-3-3 proteins with both Raf and KSR protein kinases may require a phosphoserine-containing motif (RSxpSxP) ***(30,32)***, but the precise roles of 14-3-3 proteins in MAPK/ERK pathway remain unclear.

A noncatalytic scaffold protein of the MAPK/ERK pathway, Mek partner-1 (MP1), was identified in yeast two-hybrid screen of MEK interactors ERK ***(33)***. Consistent with a scaffolding role, MP1 overexpression enhances ERK1 activation and reporter gene expression and enhances association of MEK and ERK. Direct interaction between Raf and MEK has also been observed ***(34)***. Interestingly, a phosphorylation site within the proline-rich region of MEK1 that is necessary for association with B-Raf was found to be required for sustained MEK1 activation. As discussed (**Subheading 3.2.**), this can have profound consequences on biological consequences of MAPK/ERK pathway activation. A MAPK-binding motif has also been defined for the MAPK substrate MAPKAP-K1 ***(35)***. Conservation of this motif in some other MAPK substrates, such as MNK and MSK kinases, suggests that this may represent a docking site that contributes toward regulation of a number of MAPK signaling complexes.

2.2.2. JNK/SAPK Pathway

Both direct kinase–kinase interactions and noncatalytic scaffold proteins have been identified as playing roles in specifying and regulating JNK/SAPK pathway activity. JNK interacting protein (JIP)-1 was first identified by yeast two-hybrid screening for proteins that interact with JNK ***(36)***. Of the many upstream kinases with potential to activate JNK, JIP-1 would appear to offer selectivity of signaling because it forms stable complexes with MLK3, DLK, and MKK7, but not MEKK1, MEKK4, Raf, MKK4, MKK3/6, or MEK1 ***(37)***. As such, JIP serves to scaffold MLK3/DLK-MKK7-JNK as a distinct signaling complex, so promoting signaling selectively through this cascade. Consistent with this model, DLK and MKK7 have been reported to be expressed preferentially in neurons where they are observed to colocalize, unlike MKK4 that exhibits a distinctive distribution ***(38)***. Overexpression of recombinant JIP1 results in retention of both MKK7 and JNK within the cytoplasm, with consequent inhibition of JNK pathway activity. However, whereas this reveals a potentially powerful regulatory function for this scaffold protein, the physiologic relevance of such an observation is currently unclear.

In contrast to MKK7, present evidence indicates that MKK4 can utilize direct kinase–kinase docking motifs to constitute a functional signaling com-

plex with the upstream regulator MEKK1 and downstream substrate JNK ***(39)***. MEKK1 stably interacts with MKK4, but this association is disrupted as a consequence of MKK4 activation. Both JNK and *p38* (but not ERK1) interact competitively with the MKK4 N-terminal region to which MEKK1 also interacts. JNK has also been reported to interact directly with the N-terminal region of MEKK1 ***(40)***. Together, these data suggest that MEKK1 signaling to JNK via MKK4 utilizes a series of sequential high-affinity interactions. Such direct interactions may, of course, operate in conjunction with noncatalytic scaffold proteins.

2.3. Regulation of Nuclear-Cytoplasmic Distribution

Key substrates of intracellular MAP kinase cascades are found both within the cytoplasm and nucleus. As such, it is perhaps not too surprising that regulation of kinase distribution across the nuclear membrane serves as an effective strategy in controlling MAP kinase signaling cascades.

Consistent with its activity toward transcription factors, MAPK/ERK acquires a nuclear location following activation by the upstream kinase MEK, despite the absence of an obvious nuclear localization signal (NLS), and can remain in the nucleus for several hours. MEK itself lacks an NLS but does bear functional nuclear export signal (NES) ***(41)***, mutation of which confers distinct biological properties to MEK ***(42)***. Together with the recent finding that MEK phosphorylation promotes nuclear localization ***(43)***, it is evident that a dynamic equilibrium between nuclear-cytoplasmic location is key to biological regulation in this pathway, where the primary role of nuclear MKK may be to maintain MAPK activity.

The same principle underlies the emerging regulatory mechanisms that operate on MAPKAP-K2, a p38/SAPK2 stress pathway substrate. MAPKAP-K2 bears a functional NLS that confers predominantly nuclear localization in resting cells. However, an activation-dependent NES has also been identified that results in MAPKAP-K2 assuming a cytoplasmic location following *p38* activation by stress stimuli ***(44)***. The significance of such signaling-dependent nuclear-cytoplasmic shuttling may lie in the recent finding that cytosolic MAPKAP-K2 promotes stabilization of IL-8 mRNA ***(45)***, providing a likely mechanism for the well-established function of the *p38* stress pathway in cytokine induction.

A more direct example of regulation of protein kinase signaling cacades by control of nuclear-cytoplamsic distribution is provided by the NF-κB signaling pathway. In nonstimulated cells, the NF-κB family of transcription factors are located in the cytoplasm in an inactive form in complex with IκBs. These inhibitory proteins maintain NF-κBs in an inactive state by masking an NLS of NFκBs. Stimulation of cells with TNFα or IL-1 activates a signaling cascade leading to activation of ser/thr kinases IKKs that phosphorylate IκB-NF-κB

complex on specific serine residues in IκB. Such phosphorylated Iκbs are targeted for ubiquitination and subsequent degradation serves to unmask the NLS of NF-κB, so facilitating TNFα or IL-1 nuclear translocation and stimulating characteristic transcriptional responses ***(46)***.

3. Integration of Pathway Activation and Cellular Responses

It is apparent that components of kinase mediated signaling cascades are utilized in combinatorial and permutable ways to evoke the wide diversity of cellular responses by which cells respond appropriately to environmental change. As the examples below indicate, ligand-activated receptors are used in multiple combinations to ensure accurate perception of specific extracellular stimuli. Moreover, intracellular kinase pathways can operate as common links between diverse receptor types. Evidence is also emerging as to how the cascade nature of intracellular pathways facilitates integration of this multiplicity of inputs. Clearly, the outcome of such integrative functions is dependent upon the wider cellular context — for example, activation of the MAPK/ERK pathway can be mitogenic in proliferative cell types, but clearly has distinct functions in postmitotic cells such as neurons.

3.1. Receptor Crosstalk and Pathway Activation

Activation by dimerization provides considerable scope for potential crosstalk between receptor tyrosine kinases through formation of distinctive heterodimers. Heterodimeric complexes within the EGFR/ErbB subfamily of RTKs that facilitate assembly of distinctive receptor signaling complexes have been well documented ***(1)***. More recently, EGFR- βPDGR heterodimers have been reported that may account for the ability of EGF to stimulate βPDGFR activation in some cell types ***(47)***.

A number of ligand-activated GPCRs have been found to activate the *ras*-Raf-MEK-MAPK intracellular cascade through the use of protein kinase intermediaries. One route to this end is through transactivation of receptor tyrosine kinases. Three distinct RTKs have been reported to be activated following GPCR stimulation ***(48)*** and it would appear that a given GPCR can utilize distinct RTKs according to cell type. Linkage of GPCR-activated RTKs to MAPK via Ras is implicated to occur by one or more of PI3-K, src family kinases, or PKC.

Available evidence indicates Gβγ subunits may play a role, but the precise mechanism of GPCR-mediated RTK activation is currently unclear. However, Ras-mediated recruitment of c-Raf to the plasmamembrane has been reported to sequester Gβγ subunits to Raf ***(49)***. Whereas this has no apparent consequence on Raf activity, such sequestration does downmodulate GPCR signaling to PLCβ. As such, this mechanism could provide a feedback loop for GPCR

signaling or facilitate crosstalk between RTK and GPCR mediated extracellular ligands. At least two additional mechanisms can link GPCRs with the MAPK/ERK intracellular cascade. GPCRs themselves can provide scaffolds for assembly of signaling complexes in a manner analogous to that defined for receptor tyrosine kinases. For example, JAK2 associates specifically with angiotensin II type I receptors via a YIPP receptor motif, the integrity of which is essential for angiotensin-mediated phosphorylation of JAK2 ***(50)***. The FAK family kinase, PYK2 has also been implicated as a mediator of GPCR induced activation in neuronal cells ***(51)***. In this case, GPCR activation of Pyk2 is thought to stimulate PYK2 mediated recruitment of *ras* via Shc-grb2-sos complex.

3.2. Temporal Regulation and Integration Within Intracellular Cascades

The duration of activation of a signaling cascade is a key variable by which distinct cellular reponses are evoked. For example, temporal regulation of Raf-MAPK pathway is thought to underlie the markedly different responses of PC12 cells to EGF, PDGF, insulin, or NGF. All these growth factors stimulate MAPK/ERK pathway activation in PC12 cells. However, only NGF induces PC12 differentiation, and this is correlated with sustained MAPK/ERK pathway activation and nuclear localization of MAPK/ERK, whereas the other growth factors induce only a transient activation of MAPK. Overexpression of EGFR ***(52)*** or insulin receptors ***(53)*** confers sustained activation and nuclear localization of MAPK in response to respective growth factor, concommitant with the ability of the relevant factors to induce differentiation of receptor overexpressing PC12 cells. Thus, it would seem that differentiation in this model requires a threshold of MAPK activation to promote nuclear localization and consequent modulation of transcriptional regulation, either directly, or indirectly through other kinases.

The molecular mechanism by which sustained MAPK/ERK activity is achieved is not yet fully defined. Transient activation in this system is thought to operate through a feedback loop involving phosphorylation-dependent dissociation of grb2-sos complex ***(10,11,54)***. Sustained activation of MAPK/ERK has been associated with a B-raf mediated pathway that utilizes the small GTPase Rap1 ***(55,56)*** although another report ***(57)*** indicates that Rap1 activation is not essential for NGF-induced differentiation of PC12 cells.

Although MKKs are dual specificity kinases (**Subheading 2.1.**), a recent report suggests that differential activity towards specific residues within the T×Y motif may offer a novel mechanism of regulation. JNK activation requires phosphorylation of both Thr^{183} and Tyr^{185} by the upstream kinases MKK4 or MKK7. However, MKK4 preferentially phosphorylates JNK in vitro at Tyr^{185},

whereas MKK7 preferentially phosphorylates the Thr^{183} residue ***(58)***. Together with the distinctive complexes within which MKK4 and MKK7 are known to phosphorylate JNK in vivo (**Subheading 2.2.**), preferential phosphorylation potentially provides a means for close-controlled regulation of JNK activity. For example, there may be a requirement for additional stabilizing proteins to facilitate JNK activation by a single MKK. Alternately, JNK activation could operate as a function of two distinct pathway inputs, via MKK4 and MKK7. A number of "dual responsive" kinases have also been reported recently that can be activated by either MAPK/ERK or *p38*/SAPK2 intracellular cascades ***(59–61)***. The mechanisms by which these kinases are regulated at the interface between such cascades remains to be elucidated. Evidence of crosstalk between TGFβ-SMAD and MAPK/ERK and JNK pathways is also emerging ***(2)***.

3.3. Transcriptional Targets of Protein Kinase-Mediated Signaling Pathways

Analysis of regulation of transcriptional targets of specific signaling pathways has historically focused on specific target genes such as c-*fos* and c-*myc* ***(62,63)***. Further insights to the roles of pathway multiplicity in response to extracellular stimuli has come from recent global analysis of transcriptional targets by use of oligonucleotide array technology ***(64–66)***. Application of such approaches are not yet commonplace, although the recent commercial availability of defined arrays now makes this a readily accessible technology. First reports indicate that the ability to screen steady-state RNA changes of a large number of genes simultaneously represents a very powerful tool for evaluation of crosstalk between pathways in modulating changes in gene expression. Of particular interest is analysis of immediate early gene (IEG) expression induced by βPDGFR signaling pathways in NIH3T3 cells ***(67)***. In this study, a screen of approximately 6000 genes identified 66 IEGs induced by βPDGFR activation. Interestingly, mutation of up to five tyrosine residues representing known SH2 binding motifs within bPDGFR had only quantitative, not qualitative, effects on expression of 64/66 of these IEGs. FGF induced similar induction profiles, whereas EGF induced only a subset of these IEGs. Thus, early evidence would suggest that although induction of some genes is dependent on activation of specific pathways, many signaling cascades focus on a small set of overlapping genes. The point of convergence in such responses is currently unknown, but could operate through; (1) parallel pathways acting on common transcription factor complexes; (2) crosstalk between intracellular pathways; or (3) membrane proximal signaling components activating common intracellular pathways. Regardless, such array technologies hold great promise as a new tool for elucidating global changes

in RNA induction by specific pathways and identifying changes in response to distinct stimuli or as a consequence of modulating specific pathway components by genetic means and/or treatment with selective pharmacological agents. Such approaches are likely to prove particularly informative in relation to cell-type differences in the roles of particular signaling pathways.

4. Pharmacological Approaches to Analysis of Protein Kinase Function

Given the fundamental functions of protein kinase-mediated signaling cascades in evoking cellular responses to environmental stimuli, it is perhaps not surprising that subversion of protein kinase function is observed in a variety of disease states. Historically, this is reflected most clearly in oncology where several kinase components of signaling pathways were identified initially on the basis of their oncogenic or protooncogenic properties in cell culture or animal models and human cancers. However, as key mediators of noxious or inappropriate stimuli, such as those that evoke inflammatory responses or induce cell death, modulation of protein kinase function is of considerable therapeutic potential across a wide variety of clinical indications. This incentive to develop therapeutics within the commercial sector is also having a major positive impact in providing both knowledge and novel reagents.

4.1. Protein Kinase Inhibitors as Experimental Tools

A number of natural products, such as staurosporine, have been known for many years to act as inhibitors of protein kinase activity by competing with ATP for binding to the nucleotide binding pocket. However, such compounds show broad activity across a variety of protein kinases, making them of little value as tools. Such problems with selectivity reflect the highly conserved nature of the ATP binding pocket. More recently, a variety of ATP-competitive small molecule kinase inhibitors have been identified that have demonstrable selectivity for particular kinase classes. Although the majority of reported kinase inhibitors are ATP competitive, this may reflect a bias towards screening compound libraries by direct enzymatic assays. In this respect, it is interesting to note that screening strategies based on whole-cell assays using reporter-gene constructs have been successful in identifying kinase inhibitors that act in a noncompetitive manner for either ATP or protein substrate ***(68)***.

Whereas the criteria for developing such compounds as drugs are many and varied, some that exhibit appropriate pharmacokinetic properties have been shown to be efficiacious in a variety of relevant animal models, and a growing number are currently under evaluation in a clinical context ***(69)***. More importantly, in relation to the current volume, it is clear that nontoxic, potent and selective small-molecule inhibitors of a given protein kinase represent powerful tools for

Table1
Published Selective Small Molecule Inhibitors of Protein Kinases

Kinase	Compund	IC^{50}	Reported selectivity	Ref.
EGFR	PD15305	29 p*M*	>10^5-fold vs 6 kinases	***86***
FGFR	PD166866	52 n*M*	>1000-fold vs 7 kinases	***72***
VEGFR	SU5416	20 n*M*	>20-fold vs 4 kinases	***81***
FGFR/	PD173074	25 n*M*	>800-fold vs 6 kinases	***82***
VEGFR		100–200 n*M*		
TrkA	CEP-701	4 n*M*	17-fold vs VEGFR; 60-fold vs PKC >200-fold vs 3 other kinases	***87***
PDGFR	AG1296	1 μ*M*	>10-fold vs 3 kinases	***84***
SCF-R		1.8 μ*M*		
MEK1/2	PD98059	2-7/50 μ*M*	No activity vs 18 kinases	***88***
	U0126	60/70 n*M*	>100-fold vs 9 kinases	***68***
	PD184352	17 n*M*	>500-fold vs 7 kinases	***70***
p38	SB-203580	600 n*M*	No activity vs 12 kinases	***83***
	SB-220025	60 n*M*	>50-fold vs 5 kinases	***71***
JAK2	AG490	n.r.	No cellular activity reported vs 5 kinases	***85***

Examples from the literature of potent and selective tool compounds are given. Details of assays for IC_{50} determination and selectivity profiling can be found in the original reference.

the molecular dissection of signaling pathways in physiologically relevant cell culture and animals models ***(70–72)***. However, given the potential for crossreactivity with other kinases, interpretation of data generated with a given tool inhibitor needs to be supported with additional biochemical correlates in relation to other kinases/pathways that may impact on the biology of the system under investigation. For example, the compound Ro-31-8220 was used for many years as a potent PKC inhibitor before demonstration of similar potency against MAPKAP-K1, p70S6 kinase, and MSK1 ***(61,73)***. However, despite such crossreactivity, it can be usefully employed along with tool inhibitors selective for other kinase(s) to provide insights of kinase pathway integration and crosstalk ***(61)***. With broadening repertoires of selectivity screens, and availability of selective inhibitors acting on distinct targets in the same kinase cascade, such problems are likely to be more easily circumvented in the future. Examples of some currently useful tool inhibitor compounds are given in **Table 1**.

4.2. Generation of Inhibitor-Sensitive Protein Kinases

An alternative experimental approach to the difficulties in developing inhibitors selective for a given protein kinase is to mutate key residues within

that kinase to generate mutant protein with sensitivity to existing tool compounds. A converse strategy, in which resistant forms of a previously sensitive kinase are generated, can be of value in investigation of the molecular basis of action of a given compound. To date, different experimental approaches have demonstrated that both src family and MAPK family kinases are amenable to such mutational strategies.

For src family kinases, modelling of the ATP-binding pocket of v-src identified Ile^{338} as presenting a bulky side chain, present in all eukaryotic protein kinases, that was predicted to block a pocket not normally utilized by ATP ***(74)***. Because mutation of this residue to glycine had little detrimental effect on enzyme activity, an $Ile^{338}Gly$ mutant protein provided an ideal tool with which to seek to identify a mutant selective src inhibitor from a panel of structural analogs of the previously defined src family inhibitor PP1 ***(75)***. By this route, an analog selective for mutant src or mutant fyn in relation to the relevant normal proteins, that retained selectivity against five other kinases, was identified as an effective tool compound for cell culture studies ***(76)***. The size of the amino acid side chain at the Ile^{338} equivalent across the family of protein kinases correlates strongly with potency of inhibition by PP1. As a further elaboration of manipulating kinase selectivity by mutational approaches, the replacement of phenylalanine with glycine at this site in CaMKII and cdk2 creates mutant kinase proteins with >100-fold increased sensitivity to inhibition by PP1 ***(77)***.

Interestingly, cocrystals of p38 with the p38 inhibitor SB-203580 identified the same ATP-binding pocket residue (Thr^{106} in p38) as a key determinant in the activity of this compound. Consistent with this, other MAPK kinases insensitive to SB-203580 (e.g., JNK1, SAPK3, SAPK4) bear amino acids with bulkier side chains at this site. As predicted from such models, a $Thr^{106}Met$ mutant p38 became insensitive to SB-203580 ***(78)***, whereas mutation of Met residue to Thr or Ala in SAPK3, SAPK4, or JNK renders these MAPK family members sensitive to SB-203580 ***(79)***. However, although Thr^{106} is crucial to conferring sensitivity, generation of a potency equivalent to that of SB-203580 toward p38 requires additional mutation of adjacent residues ***(79,80)***. Together, these examples illustrate how knowledge of the molecular basis of inhibitor activity can facilitate development of more potent and selective inhibitor compounds. Such information provides a basis for rational design and is of value not only to the molecular dissection of the complexity of synergy and crosstalk within intracellular kinase signaling cascades, but also to the development of therapeutics.

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2

Cloning Protein Tyrosine Kinases by Screening cDNA Libraries with Antiphosphotyrosine Antibodies

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1. Introduction

Protein tyrosine kinases (PTKs) play prominent roles in the regulation of fundamental biological processes including normal cell growth and survival, cell differentiation, and development. Across vertebrate and invertebrate species, both nonreceptor (cytoplasmic) and receptor (transmembrane) type PTKs have been identified, making them one of the most extensively examined family of proteins. Currently, genes encoding at least 50 receptor and 33 nonreceptor vertebrate PTKs have been cloned ***(1,2)***, several by techniques that exploit the structural and functional conservation of the kinase catalytic domain.

The catalytic domain of PTKs is comprised of approx 250 amino acids that can be divided into 11 highly conserved sequence motifs ***(3)***. This homology has been successfully utilized in the molecular-based cloning of novel and known PTKs. These stratagies have included the low stringency screening of cDNA libraries with probes homologous to the catalytic domain of preexisting PTK clones, the use of degenerate oligonucleotides as hybridization probes, and the use of degenerate oligonucleotides as primers for polymerase chain reaction (PCR)-based screening.

In addition to sequence similarity, the kinase domains of PTKs possess phosphotransferase activity, making them functionally related. The transphosphorylation and autophosphorylation activities of PTKs have been well documented ***(1,2)*** and interestingly, the expression of just the catalytic domain in *Escherichia Coli* results in an active tyrosine kinase ***(4,5)***. The technique of

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

detecting protein phosphorylation on tyrosine residues by immunoblotting with phosphotyrosine-specific antibodies has proven highy sensitive in Western blot analysis ***(6)***. Because endogenous PTK activity in bacteria is negligible, the premise on which expression cloning functional PTKs is based is the use of antiphosphotyrosine antibodies to detect active tyrosine kinases that are expressed from cDNA clones introduced into bacteria.

The main advantage of this functional screening approach is confirmation of the catalytic activity of the cloned PTK gene. This method also allows for the potential cloning of novel kinases that phosphorylate tyrosine residues, because there is no sequence bias in this procedure. PTK cDNAs that diverge from the normal sequence would not be found by nucleic acid hybridization techniques. Moreover, this functional screen has facilitated the identification of an emerging family of dual specificity protein kinases that phosphorylate serine, threonine, and tyrosine residues ***(7–9)***.

The use of antibodies in general to screen expression libraries has been described previously ***(10,11)***, and modifications suitable for the use of antiphosphotyrosine antibodies will be described here. The most important aspects of this procedure are the possession of an expression library that is ready to screen and an antiphosphotyrosine antibody, either of which can be commercially obtained or generated according to already published procedures ***(12–14)***. For simplicity, we will describe the use of a lambda gt11 cDNA expression library, which is commonly used for immunological screening. However, other types of equally suitable expression libraries will be described below.

In principle, a lambda *gt11* cDNA expression library allows for the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible production of cDNA-encoded proteins fused to β-galactosidase in bacteria. A simple procedure is used for plating libraries on an expression host, which results in a single plaque arising from a single phage that has infected one bacterium (*see* **Note 1**). An active tyrosine kinase produced could phosphorylate itself and bacterial proteins on tyrosine, and this is detectable on nitrocellulose filters probed with antiphosphotyrosine antibodies. Positive clones are visualized by either radioactive or nonradioactive methods. A cDNA encoding a potential PTK is further characterized molecularly and biochemically to confirm its identity. The efficacy of this approach has been demonstrated in the cloning of both nonreceptor and full-length receptor tyrosine kinases ***(15–20)***.

2. Materials

2.1. The Bacteriophage Lambda cDNA Expression Library

Optimally, the library should contain inserts no smaller than 1.0 kb, which is the minimal size required to comprise the catalytic domain of a PTK ***(3,15)***. Several innovative modifications of the conventional lambda *gt11* cDNA ex-

pression library are now available, as well as other bacteriophage lambda libraries such as Lambda Zap (Stratagene) and Lambda EXlox (Novagen). These libraries contain features that not only increase the efficiency of detecting positive clones, but also eliminate the need to eventually subclone cDNA from lambda into prokaryotic or eukaryotic vectors. These features include an increased cloning capacity and unidirectional cloning of cDNA into lambda, and in vivo excision systems of cDNA from the recombinant lambda DNA. In addition, for biochemical analysis of a cloned PTK, the cDNA may be subcloned into vectors which allow its expression as a fusion protein with an epitope tag (*myc*, *T7*, *HA*, *His*) to which antibodies are commericially available ***(21)***. Alternatively, the vector insert in lambda may itself contain sequences encoding either an epitope tag or glutathione S-transferase ***(22)***, and the cloned PTK may be expressed as a fusion protein from the plasmid on its in vivo excision and isolation from lambda DNA. We recommend consideration of other library constructions that contain these additional features.

2.2. Antiphosphotyrosine Antibodies

The production of effective polyclonal antibodies (PAb) and monoclonal antibodies (MAb) that recognize phosphotyrosine (PY) has a rich history, and although commercially available, procedures for their generation have been well documented ***(12–14)***. Anti-PY antibodies have been raised against a variety of antigens including phosphotyrosine, structural analogues such as phosphotyramine or p-aminobenzylphosphonic acid, polymerized mixtures of phosphotyrosine, alanine and glycine/threonine, and the bacterially expressed catalytic domain of the PTK v-abl. These antibodies are broadly reactive and recognize phosphorylated tyrosine in the context of many peptide sequences. Alternatively, it is possible to produce polyclonal antiphosphopeptide antibodies that recognize a specific PTK in its phosphorylated state. An oligopeptide containing a phosphorylated tyrosine residue can be synthesized based on the tyrosine phosphorylation site in the PTK of interest ***(23)***. This strategy was successfully employed to generate antibodies to the tyrosine-phosphorylated form of the PTK neu ***(24)***. Ultimately, you need a stock of anti-PY antibody that will detect tyrosine-phosphorylated proteins by Western blot analysis (*see* **Note 2**).

Some preliminary tests of your stock anti-PY antibody are recommended and are relatively simple. For a commercial antibody, Western blot analysis of a cell lysate that contains tyrosine-phosphorylated proteins of known molecular weights (this is also commercially available), under the conditions you will use to screen the library, will verify its specificty and effective concentration. It is assumed that anti-PY antibodies generated yourself have been extensively characterized already. In general, a concentration of 1–3 μg/mL is suitable for

Western blots and library screening. Because serum often contains anti-*E. Coli* reactive antibodies, these can be removed before you begin library screening by presorption onto *E. Coli* protein lysates. *E. Coli* protein-coated filters (*see* **Subheading 3.1.1.**), obtained from plated lambda *gt11* phage that do not contain inserts, can be reacted with anti-PY serum under library screening conditions. It may be necessary to do this several times, but the same lambda *gt11* plate can be used to produce several filters. You will have a cleaner antibody to screen with as a result. In general, MAbs have less background reactivity with *E. Coli* proteins.

Because not all tyrosine-phosphorylated proteins that bind to monoclonal anti-PY antibodies bind to polyclonal anti-PY antibodies ***(14)***, MAbs may have a lower binding constant, and sensitivity may be compromised. However, specific signals detected by a monoclonal anti-PY antibody may be amplified by altering the secondary detection reagent (*see* **Subheading 3**). Finally, although PAbs may be reused several times, we do not suggest this as their properties may change with reuse (*see* **Note 3**). In contrast, MAbs can be reused several times. For storage and reuse of antibodies, sodium azide should be added to 0.02% (*see* **Note 4**). Antibodies can be kept at 4°C for up to 1 mo and used 5–10 times.

2.3. Reagents

2.3.1. Library Plating and Plaque Isolation

1. *E. coli* strain Y1090 (Stratagene, genotype: Ä(lac)U169 araD139 strA supF mcrA trpC22::Tn10 (Tet^r) [pMC9 Amp^r Tet^r]).
2. LB media: 10 g/L bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, final pH 7.5. Autoclave, then add filter sterilized 1 *M* $MgSO_4$ to 10 m*M* final concentration.
3. Ampicillin: 100 mg/mL in distilled water, sterile filtered with Millipore 0.22 μm filter, and stored at –20°C.
4. 10% (w/v) maltose in distilled water. Sterile filtered and stored at 4°C.
5. 10 cm and 15 cm Petri dishes.
6. Bottom agar: 15 g agar in 1 L LB, autoclaved. Used to make LB plates.
7. Top agarose: 0.75 g agarose in 100 mL LB, autoclaved.
8. Phage buffer: 0.1 *M* NaCl, 0.05 *M* Tris base, 0.1% gelatin, final pH 7.5, autoclaved. Add sterile-filtered $MgSO_4$ to a final concentration of 10 m*M*.
9. T *M* buffer: 20 m*M* Tris-HCl, pH 8.0. Autoclave and add sterile-filtered $MgSO_4$ to a final concentration of 10 m*M*.
10. Chloroform.
11. 1 *M* $CaCl_2$, autoclaved.
12. 10 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG): Made in double-distilled water and stored at –20°C.
13. 10% sodium azide: Made in double-distilled water and stored at room temperature.

14. Tris-buffered saline (TBS): 0.17 *M* NaCl, 0.01 *M* Tris base, final pH 7.5.
15. TBST: TBS with 0.05% Tween-20.
16. 1 *M* $MgSO_4$: sterile filtered with Millipore 0.22 μm filter and stored at room temperature.
17. Nitrocellulose filters for 10 and 15 cm Petri dishes (Stratagene #420106 and #420107).
18. Sterile Pasteur pipets.
19. Sterile 10–15 mL glass or polypropylene tubes.
20. Markers: Syringe needle (Becton-Dickinson 20G needle, #305175), water insoluble ink pen (VWR Scientific Products #52877-150), or fluorescent markers (VWR Scientific Products #52878-180).

2.3.2. Screening Filters

1. Blocking Solutions:
   a. Block Type A: 5% bovine serum albumin (BSA) (Sigma #A-2153) in TBST.
   b. Block Type B: 5% BSA and 1% ovalbumin (Sigma #A-5503) in TBST.
   c. Block Type C: 20% fetal calf serum (heat inactivated) (Gibco-BRL #16000-036) in TBST.
   d. Block Type D: 2% goat serum (heat inactivated) (Gibco-BRL #16210–064), 1% fish gelatin (Norland Products), and 1% BSA in TBST.
2. Secondary screening reagents (*see* **Note 5**) ***(25)***:
   a. Radioactive: ^{125}I coupled to protein A or protein G (30 mCi/mg specific activity) (NEN; Amersham; ICN) or coupled to an appropriate secondary antibody, X-ray film, intensifying screen, Saran wrap.
   b. Nonradioactive:

      Type A: Horseradish peroxidase (HRP) coupled to protein A (Boehringer-Mannheim #605-295 at 1:5000) or protein G (Bio-Rad #170-6467) or coupled to an appropriate secondary antibody (Boerhinger-Mannheim #1814-141 at 1:20,000 dilution and #1812-168 at 1:10,000 dilution). Visualization solution is comprised of 5 mL of 100 m*M* Tris-HCl, pH 7.5 containing 100 μL of DAB (40 mg/mL of 3,3'-diaminobenzidine in H_2O), 25 μL $NiCl_2$ (80 mg/mL in H_2O), and 15 μL of 3% H_2O_2. Solutions are also commercially available (Pierce; Biorad; Boerhinger-Mannheim). Alternatively, other chromogenic substrates may be used, and are described elsewhere ***(26)***. For enhanced chemiluminescence (ECL)-based detection, commercial kits are available (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim) in which equal volumes of luminol reagent and oxidizing agent are mixed for use. Otherwise, ECL visualization solution can be made by mixing 0.5 mL of 10× luminol solution (4 mg luminol/mL dimethyl sulfoxide [DMSO]), 0.5 mL 10× p-iodophenol stock (10 mg/mL in DMSO), 2.5 mL of 100 m*M* Tris-HCl, pH 7.5, and 25 μL of 3% H_2O_2, in a 5-mL final volume (with H_2O).

      Type B: Alkaline phosphatase (AP) coupled to protein A (Boerhinger-Mannheim #100-052 at 1:1000), or coupled to an appropriate secondary antibody (Boerhinger-Mannheim #1814-206 at 1:5000 and #1814-214 at 1:5000).

For chromogenic detection, AP buffer consists of 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, and 5 m*M* $MgCl_2$. BCIP/NBT visualization solution is comprised of 5 mL of AP buffer containing 33 μL of NBT (50 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate in 70% dimethyl formimide) and 17 μL of BCIP (50 mg/mL of nitroblue tetrazolium in 100% dimethyl formimide).

Type C: Biotinylated secondary antibody (Boerhinger-Mannheim #605-100 at 1:1000 and #605-195 at 1:15,000; Bio-Rad #170-6401) and avidin conjugated to alkaline phosphatase (Boerhinger-Mannheim #100-200 at 1:2500; Bio-Rad #170-6533) or HRP (Bio-Rad #170-6528).

2.3.3. Clone Identification

1. dNTPs (Boerhinger-Mannheim #104035, #104094, #104272).
2. α-^{32}P-dATP (NEN #Blu-Neg512H).
3. DNAse I (Stratagene #600031).
4. DNA polymerase I (Boerhinger-Mannheim #642711).
5. 0.5 m*M* ethylenediaminetetracetic acid (EDTA) (autoclaved).
6. tRNA (Boerhinger-Mannheim #109495).
7. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0, autoclaved.
8. Phenol (Ambion #9730).
9. Sephadex G-50.
10. Klenow fragment of *E. coli* DNA polymerase I (Stratagene #600071).
11. Nitrocellulose membrane (Stratagene #420115).
12. Base denaturing solution: 1.5 *M* NaCl and 0.5 *M* NaOH.
13. Neutralization solution: 1 *M* NaCl and 0.5 *M* Tris-HCl, pH 7.0.
14. Hybridization solution: 5× SSC, 5× Denhardt, 1% SDS, and 100 μg/mL denatured salmon sperm DNA.
15. 20× SSC solution: 3 *M* NaCl and 0.3 *M* Na_3 Citrate, pH 7.0.
16. 100× Denhardt solution: 2 g/L ficoll, 20 g/L polyvinylpyrrolidone, 20 g/L BSA.
17. 0.4 *M* NaOH.
18. Wash solution: 200 m*M* Tris-HCl, pH 7, 0.1× SSC,and 0.1% SDS.
19. Denatured salmon sperm DNA (Stratagene #201190).

2.3.4. Kinase Activity Analysis

1. 4× Laemmli reducing sample buffer: 0.25 *M* Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, and 0.25% bromophenol blue. Store at 4°C.
2. Lysis buffer: 1% Triton X-100, 150 m*M* NaCl, 10% glycerol, 1 m*M* sodium orthovanadate, 2 m*M* EDTA, and 20 m*M* Tris-HCl, pH 7.5.
3. Ripa buffer: lysis buffer including 0.1% sodium dodecyl sulfate (SDS) and 0.5% sodium deoxycholate.
4. γ-^{32}P-ATP (NEN #BLU-NEG502A).
5. Kinase buffer: 20 m*M* HEPES, pH 7.5, 10 m*M* $MgCl_2$, and 10 m*M* $MnCl_2$.
6. Protein A-Sepharose (Pharmacia Biotech #17-0780-010).
7. GammaBind G-Sepharose (Pharmacia Biotech #17-0885-01).

8. 10× Phosphate-buffered saline (PBS), calcium- and magnesium-free (Gibco-BRL #70011-044).
9. ^{32}P-orthophosphate (NEN #NEX053S).
10. M9 media: 0.5% casamino acids, 0.1 m*M* $CaCl_2$, 0.02% glucose, 10 μg/mL thiamin, 6 g/L Na_2HPO_4-$7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl; add sterile filtered $MgSO_4$ to 1 m*M* final concentration.

3. Methods

3.1. Screening the Expression Library with Antiphosphotyrosine Antibody

3.1.1. Plating the Library for Screening

1. A culture of *E. coli* Y1090 should be grown overnight at 37°C, with moderate shaking (2500 rpm), in LB media containing 50 μg/mL of ampicillin and 0.2% maltose. Subsequently, this culture may be stored at 4°C and used later to grow cultures (*see* **Note 6**).
2. For each 15 cm LB plate, 1 mL of the *E. coli* Y1090 overnight culture is centrifuged (4000*g*, 15 min, 4°C) and the bacterial pellet is resuspended in 0.5 mL of T *M* buffer, or in 10 m*M* $MgSO_4$.
3. The bacterial suspension is then infected with 1×10^4 to 5×10^4 lambda *gt11* recombinant phage for 15 min at 37°C. We found 2×10^4 pfu/plate to be convenient. Use phage buffer to make appropriate dilutions of the stock lambda *gt11* library (*see* **Note 7**).
4. Meanwhile, prewarm the 15-cm LB plates at 42°C. Each plate should have an identification mark on its base (*see* **Note 8**).
5. Top agarose should be well dissolved and kept at 45–50°C.
6. For each 15-cm LB plate, 5 mL of top agarose is removed into a sterile tube and the infected Y1090 bacteria is added. Mix gently and quickly pour onto the LB plate, without forming air bubbles. This is best achieved by pouring the agarose-cell suspension along the inside wall of the agar plate and gently shaking plate in a cirular motion on the bench top to get an even overlayer. Let the plate set for 5 min with the lid slightly off.
7. Incubate the plates, inverted, for 3 to 5 h at 42°C, or until clear plaques, of approx 1 mm in diameter, are detectable.
8. Meanwhile, soak nitrocellulose filters in 10 m*M* IPTG (*see* **Note 9**). Let them air-dry on Saran Wrap™. Wear gloves and use forceps to handle the filters. When dry, mark each filter with a water insoluble ink marker to correspond to its LB plate.
9. Carefully overlay each LB plate with an IPTG-impregnated filter. Do not form air bubbles. This is best accomplished by bending the filter in the center and placing the midline of the filter in the middle of the plate. Then slowly allow the filter to make contact with the agarose surface. Do not lift and move the filter once it has contacted the surface. Incubate plates, inverted, at 37°C for 8–10 h. Reasonable protein expression occurs by 4 h.

10. Before removing the filter, mark its position on the LB plate by poking small holes in an assymetric pattern through the filter and into the agar. You can turn the plate over and mark where the holes are on the base of the plate with a pen.
11. Carefully, lift the nitrocellulose off without removing the top agarose. If top agarose sticks to the filter, cool plates at 4°C for 15 min before lifting the filter. Place the filter, agarose-contact side up, into a Petri dish containing TBS. For duplicate screening, a second IPTG-impregnated filter may be placed on the plate for 4 h to overnight at 37°C (*see* **Note 10**). Remember to mark the second filter in the same places as the first.
12. Rinse the filters four times in TBS, 10 min each time at room temperature, with gentle rocking.
13. If filters are not to be screened immediately, the last wash should be done with TBST containing 0.02% sodium azide. Filters can be stored in individual Petri dishes with TBST/azide at 4°C, or they can be air dried on Saran Wrap™, wrapped, and stored at toom temperature. The LB plates with plaques may be wrapped in parafilm and stored at 4°C.

3.1.2. Screening Filters with Antiphosphotyrosine Antibodies

1. After a final wash in TBST, filters are incubated with 15 mL of blocking solution (Types A–D; *see* **Subheading 2.3.2.**) with gentle rocking, for at least 2 h at room temperature or overnight at 4°C (*see* **Note 11**). Block type D is the most effective for reducing background signals.
2. Remove blocking solution and wash once in TBST for 10 min.
3. Incubate filters with 10–12 mL of blocking solution containing antiphosphotyrosine (PY) antibody (1–3 μg/mL) for at least 2 h at room temperature or overnight at 4°C, rocking gently (*see* **Note 12**). If screening a large number of filters ***(30–40)***, a container with a diameter slightly larger than the filter should be used to conserve volume. A 2-L beaker with 15-cm filters that are individually separated by nylon mesh works well.
4. Filters are then washed four times in TBST, 10 min each time at room temperature, with gentle rocking. Bound anti-PY antibodies can be detected on the filter by either a radioactive method (*see* **step 5A**) or by a nonradioactive method (*see* **step 5B**).

5A. Radioactive detection: Treat filters with 10–12 mL of blocking solution containing ^{125}I-labeled protein A or an ^{125}I-conjugated secondary antibody (at approx 0.1–0.5 μCi/mL) for at least one hour at room temperature (or 4°C overnight), with gentle rocking. Filters should then be transferred to a new Petri dish and washed four times in TBST as in **step 4**, above. Filters are then air-dried on Saran Wrap™, wrapped in Saran Wrap™, and marked with a radioactive pen or fluorescent marker for later alignment. The filters should be exposed to X-ray film under an intensifying screen at –70°C for a few days before developing.

5B. Nonradioactive detection: Treat filters with 10–12 mL of blocking solution containing HRP or AP conjugated to either protein A or protein G, or to an appropriate secondary antibody, using a dilution either recommended by the

manufacturer or previously determined by Western blot analysis (*see* **Subheadings 2.2.** and **2.3.2.**) for 1 h with gentle rocking. Filters should then be transferred to a new Petri dish and washed four times in TBST as described in **step 4**. An appropriate substrate is then added for chromogenic or chemiluminescence detection (*see* **Note 13**).

a. AP-based assay: Rinse the blot in AP buffer (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$). Add BCIP/NBT visualization solution and rock gently. When staining is apparent (indigo/dark blue color in 10 to 30 min), stop the reaction by washing the filter several times with water and air-dry.
b. HRP-based assay: Add DAB/$NiCl_2$ visualization solution to filter and rock gently. When staining is apparent (dark brown), wash the filter several times with water and air-dry. Alternatively, other chromogenic substrates may be used (*see* **Subheading 2.3.2.**) ***(26)***.
c. ECL-based assay: If using a commercial kit (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim), mix equal volumes of the luminol reagent and oxidizing agent. Otherwise, ECL visualization solution can be made as described (*see* **Subheading 2.3.2.**). Add to filter and agitate gently for 1 min. Drain excess liquid from filter before wrapping in saran or placing between clear plastic sheets. Mark plastic or saran with a fluorescent marker for later alignment. Expose to X-ray film in the dark for 5 s to 30 min. Develop film.
d. Biotinylated secondary antibody: Add biotinylated secondary antibody to the filter for 1 h with gentle rocking. Wash the filter three times with TBST as in **step 4**. Transfer into TBST containing an avidin-HRP complex or an avidin-AP complex for 30 min with gentle rocking. Wash the filter three times with TBST as in **step 4**. Visualize by the addition of an appropriate chromogenic substrate as described in **steps a–c**. This procedure amplifies the signal derived from a single plaque, but may also increase background staining as well.

3.1.3. Isolation and Rescreening of Positive Plaques

1. Once positive plaques have been identified, the filters or X-ray film should be matched to their corresponding LB plates.
2. The large end of a sterile Pasteur pipet can be used for removing agar plugs that contain the positive phage by stabbing it through the top agarose into the hard agar beneath.
3. The agar plug is released by shaking the pipet end into a sterile tube containing 1 mL of phage buffer and one drop of chloroform. Let phage particles diffuse out for 1–2 h at room temperature. This phage stock solution may be stored at 4°C. Typically, a plaque has 10^6–10^7 infectious particles.
4. Each phage stock solution is diluted 10^2–10^4 in phage buffer and each dilution is plated onto an LB plate as described above (*see* **Subheading 3.1.1.**, **steps 1–6**).
5. Filters are screened again with anti-PY antibody as described above (*see* **Subheading 3.1.1.**, **steps 7–13**). A third rescreen is done on 10-cm LB plates for the final isolation of single positive phage.

3.2. Molecular Analysis of Positive Clones

3.2.1. Isolation of Recombinant Bacteriophage Lambda DNA from Positive Plaques

1. A Y1090 culture is grown overnight at 37°C in LB containing maltose and ampicillin as described in **Subheading 3.1.1.**, **step 1**.
2. Bacteria from 1 mL of culture is pelleted (4000*g*, 15 min, 4°C) and resuspended in the same volume of T *M* buffer. This is infected with 10^6- 10^7 phage particles from a purified single phage stock, for 30 min at room temperature.
3. Infected bacteria is then transferred to 40 mL of LB containing 5 m*M* $CaCl_2$ and 50 μg/mL ampicillin, and the culture is shaken vigorously for 1 h at 37°C.
4. Bacteria from 20 mL is pelleted (4000*g*, 15 min, 4°C).
5. Recombinant bacteriophage can be purified from the bacterial pellet using a commercially available preparatory kit (Stratagene, Promega) according to the manufacturer's instructions (*see* **Note 14**).

3.2.2 Identification of Independent Clones

1. If you have many positive clones, we recommend that they first be classified according to their cDNA inserts. Inserts may be excised from recombinant lambda *gt11* phage DNA by restriction enzyme digestion, and subsequently subcloned into prokaryotic or eukaryotic expression plasmids for large scale propagation and further molecular analysis. As mentioned above (Section 2.1), some libraries offer a convenient in vivo excision system of plasmids from the recombinant lambda phage, which eliminates the need to subclone.
2. Cross-hybridization analysis: This method may be useful if a large number of phage are isolated, to quickly determine that recombinant phage harbor different genes. cDNA inserts can be used to make radioactive or nonradioactive probes for use in a hybridization procedure which screens all of the positive recombinant lambda phage DNA with each cDNA insert ***(27)***. cDNA inserts may be labeled with ^{32}P by either nick translation or random oligonucleotide primed synthesis (*see* **Note 15**) ***(28)***. Screening of lambda phage DNA can be accomplished using a dot blot technique ***(29)*** in which the recombinant phage DNA are immobilized on a nitrocellulose or nylon membrane for hybrization with each probe ***(30)***. Clones that hybridize with a single probe are scored as different isolates or different portions of the same gene. Filters can be washed and sequentially reprobed with each cDNA insert probe.
   a. Labeling cDNA by nick translation: Mix 0.25 μg of a gel-purified fragment of the cDNA (100–1000 bases long) with 2.5 μL of 0.5 m*M* 3dNTP mix (no dATP), 100 μCi of $\alpha^{32}P$-dATP, 1 μg of DNAse I, and 1 μL of DNA polymerase I (25 μL final volume), and incubate at 14°C for 30–45 min. Stop the reaction by adding 1 μL of 0.5 m*M* EDTA, 3 μL of tRNA (10 mg/mL stock), and 100 μL of TE buffer. Phenol extract the mix and apply the aqueous (top) phase to a Sephadex G- 50 column to remove unincorporated nucleotides. The specific activity of the probe should be approx 10^8 cpm/μg.

b. Labeling cDNA by random oligonucleotide primed synthesis: Mix 100 ng of gel-purified cDNA that has been heat-denatured (100°C for 10 min, then chilled on ice) with 1 ng of random sequence hexanucleotides as described in **step2a** above, but substitute Klenow for DNAse I and DNA pol I. Phenol extract, and purify as described in **step2a**.
c. Dot blotting the cDNA: This can be done manually, but a vacuum/manifold device gives the most consistent results. Heat-denature DNA (100°C for 10 min, then chilled on ice) or base denature DNA (2 μL DNA in 100 μL of 1.5 *M* NaCl/0.5 *M* NaOH at 37°C for 20 min) and apply to the nitrocellulose (or nylon) membrane (in "dots"). Place the membrane in a glass dish and treat for 10 min in denaturing solution and then for 10 min in neutralization solution. If using nitrocelluose, bake the membrane for 2 h at 80°C to immobilize the DNA. If using a nylon membrane, immobilize the DNA by crosslinking with ultraviolet (UV) light. We recommend using a modified nitrocelluose membrane (Stratagene) which combines the strength of nylon with the lower background of nitrocelluose.
d. Hybridization analysis: Treat the dot blot with 6¥ SSC and then hybridization solution for 3 h at 55–68°C (use a heat-sealable polyethylene bag, or use a hybridization bottle for a rotary style oven). Add hybridization solution containing the ^{32}P-labeled probe (2.5×10^5–1×10^6 cpm/mL) and incubate overnight at 55–68°C. There are also commercially available quick hybridization solutions that allow hybridization in 1–2 h (Stratagene). Wash in 2× SSC/0.1% SDS and 0.2× SSC/0.1% SDS in succession at 55–68°C. Air dry the blot and expose to film. The dot blot may be stripped in either boiling water for 5 min or in 0.4 *M* NaOH for 30 min at 45°C. Wash the blot with wash solution at room temperature with gentle agitation before reprobing.

3. Restriction Enzyme Digestion Analysis: Alternatively, once inserts are subcloned into a plasmid, restriction enzyme mapping can be used to identify identical and independent clones.

3.2.3. Sequence Identification of Cloned PTKs

1. Sequence analysis is the most direct method to identify a cloned PTK. If few positive clones are identified in the library screen, then the insert cDNA sequences can be subcloned and sequenced immediately. Once multiple cDNAs have been categorized into groups that represent single clones, the cDNA from one member of each group can be sequenced for identification. Using primers specific to the lambda *gt11* vector, one can sequence without subcloning. Such primers are commercially available (Clontech). Finally, the nucleic acid and the translated amino acid sequence can be compared to those deposited in sequence databases (GenBank, EMBL) and be identified as known or novel.
2. While full-length receptor-type PTKs have been successfully isolated from a single positive clone, it is possible that you will isolate only a partial cDNA clone of a PTK. It will then be necessary to use the partial cDNA to screen a nucleic acid library ***(18,31)***. If the 5-prime end of the clone is missing, you may try using

5-prime rapid amplification of cDNA ends (RACE) ***(32)*** to isolate the missing part of the gene.

3.3. Functional Analysis of Positive Clones

3.3.1. Analysis of Bacterial Lysates

Expression of a cloned PTK cDNA in bacteria is a convenient way to verify its kinase activity since there is no bacterial background PTK activity ***(7)***. After protein synthesis is induced, bacterial lysates can be examined biochemically by Western blot analysis using an anti-PY antibody and an antibody to the protein that is fused to the PTK (such as anti-β-galactosidase antibody). The PTK-fusion proteins are usually the proteins most heavily tyrosine-phosphorylated in lysates because of autophosphorylation.

1. Induce protein expression either in bacteria that harbor the recombinant bacteriophage lambda, or in bacteria that have been transformed with an expression plasmid containing the cDNA insert from the bacteriophage, under the appropriate conditions. For example, grow transformed bacteria by shaking (2500 rpm) overnight at 37°C in 3 mL of LB media with the appropriate antibiotic (such as 50 μg/mL ampicillin). Dilute the overnight culture 1:100 in LB media, grow for 2–3 h at 37°C with shaking, and then induce protein expression for 2–3 h at 37°C by adding the appropriate agent (addition of IPTG to 10 m*M* final concentration for example). If inducing expression from bacteria infected with a recombinant phage, grow and infect bacteria as in **Subheading 3.2.1.**, **steps 1–3**. Protein expression is induced by adding IPTG (10 m*M* final concentration) and shaking the culture for another 2–3 h.
2. Bacteria are harvested by centrifugation at 4000*g* for 10 min at 4°C.
3. The bacterial pellet is lysed by resuspending in 1× PBS (0.5 mL of 1× PBS for 1 mL of bacterial culture) and sonicating with a microprobe-equipped sonicator, or by freezing on dry ice and then thawing. Lemmli sample buffer is added to a 1¥ final concentration and the sample is boiled for 2 min. Samples may be examined by Western blot analysis (10–20 μL out of a 500-μL sample is sufficient) immediately, or frozen at –20°C.
4. Alternatively, the cloned PTK-fusion protein may be immunoprecipitated from the bacterial pellet for Western blot analysis. After protein expression is induced, bacteria can be pelleted and resuspended in ice-cold lysis buffer that does not contain 1% Triton X-100. Sonicate or freeze/thaw the suspension to lyse the bacteria as described in **Subheading 3.3.1.**, **step 3**. Add Triton X-100 to a final 1% concentration and mix thoroughly. Clear the lysate by centrifugation at 10,000*g* for 5 min at 4°C. Adjust the lysate supernatant to 0.1% SDS and 1% sodium deoxycholate for RIPA conditions, if desired. Add the precipitating antibody (approx 1–5 μg) and incubate for 4 h to overnight, at 4°C, with gentle rotation. Add protein A or protein G conjugated to

Sepharose for 1 h at 4°C with rotation. Pellet the Sepharose by brief centrifugation and wash three times with either lysis buffer or RIPA buffer. Resuspend the Sepharose in 1× Lemmli sample buffer and boil for 2 min. The samples may be analyzed immediately or frozen at –20°C.

3.3.2 Kinase Activity Analysis

As described in **Subheading 3.3.1.**, PTK-fusion proteins can be immunoprecipitated with either an anti-PY antibody or with an antibody to the protein that is fused to the PTK. This immunocomplex can be subjected to an in vitro kinase reaction using γ-^{32}P-ATP to validate its identity as a PTK.

1. Following immunoprecipitation, the complex is washed three times with ice-cold lysis buffer or RIPA buffer, and then two times with kinase buffer.
2. Resuspend the complex in 50 µL to 100 µL of kinase buffer containing 5–50 µCi of γ-^{32}P-ATP (greater than 5000 Ci/mmole specific activity) and incubate at 30°C for 20 min.
3. Add 4× Lemmli sample buffer to 1× final concentration and incubate at 100°C for 2 min. Samples can be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography to functionally validate its identity as a PTK.

Alternatively, the PTK-fusion protein that is expressed in bacteria can be metabolically labeled in vivo with ^{32}P-orthophosphate and subjected to phosphoamino acid analysis to verify autophosphorylation on tyrosine ***(33)***. This method is labor intensive and is probably more beneficial when dual kinase activity is suspected ***(7)*** (*see* **Note 16**).

1. After protein expression is induced, pellet the bacteria by centrifugation at 4000*g* for 10 min at 4°C. Resuspend the pellet in M9 media (0.1 mL media for each 10 mL of the original bacteria culture) containing 500 µCi of ^{32}P-orthophosphate, and incubate for 30 min at 37°C. You can also induce expression and label proteins with ^{32}P-orthophosphate simultaneously in M9 media, for several hours to overnight.
2. Collect bacteria by centrifugation at 4000*g* for 10 min at 4°C, wash three times with M9 media by resuspending and pelleting, and lyse as described in **Subheading 3.3.1.**, **step 3**.
3. Alternatively, the ^{32}P-labeled PTK-fusion protein can be immunoprecipitated as described in **Subheading 3.3.1.**, **step 4**.
4. For phosphoamino acid analysis, lysates or immunoprecipitates containing the ^{32}P-labeled PTK fusion protein can be resolved by SDS-polyacrylamide gel electrophoresis and isolated either directly from the gel, or transferred and immobilized onto nitrocelluose for isolation. The isolated protein is then subjected to acid hydrolysis and products resolved by two-dimensional electrophoresis on cellulose thin-layer plates (*see* **Note 16**).

4. Notes

1. The genetics and lytic cycle of bacteriophage lambda will not be described here and we refer you to a detailed description elsewhere ***(34)***.
2. Commercially available anti-PY antibodies: rabbit polyclonal (UBI) and mouse monoclonal (PY20: ICN, Zymed, Transduction Laboratories; 4G10: UBI).
3. For Western blots, staining of protein bands may become more or less prominent with each reuse of anti-PY antibodies. This may be because of the loss of high-affinity antibodies during early uses ***(25)***.
4. Sodium azide is toxic.
5. Reagents for chromogenic detection based on HRP or AP are available as commercial kits (Pierce; Bio-Rad; Boerhinger-Mannheim), as are the ECL reagents (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim).
6. When using specialized bacteriophage cDNA expression libraries, it may to necessary to use specific bacterial host strains and conditions for plating.
7. Because it is desirable to have space between individual plaques, you may need to try plating several different dilutions of the stock lambda *gt11* library to determine a reasonable plaque density.
8. It is best to pour LB plates 2–4 d in advance and store them inverted at room temperature. Try to avoid condensation formation in the Petri dish and on the lid, as moisture may accumulate on the top agar and cause plaques to streak together. Moisture can be absorbed carefully with filter paper.
9. Nitrocellulose should be initially moistened according to the manufacturer's recommendations.
10. Plates are often screened in duplicate for the primary screen in order to avoid false positives.
11. A variety of blocking solutions have been successfully employed. Do not use nonfat dry milk in the blocking solution, because it contains constituents which bind to anti-PY antibodies.
12. Most antibodies produce a good signal at room temperature. Incubation times can be varied and in general, 2–4 h is good. An 8–10 h incubation may give you a signal that is up to ten times stronger. If you are using a low-affinity anti-PY antibody, then incubate filters overnight with antibody at 4°C.
13. If an ECL-based detection system is used, do not use sodium azide in any solutions, as it interferes with chemiluminescence chemistry. HRP and AP catalyze the formation of insoluble colored precipitates directly on the surface of the filter and positive plaques may be located more accurately than by X-ray film. The signal produced by AP remains active slightly longer than that produced by HRP.
14. Lambda DNA purification kits offer rapid methods which produce high quality DNA for restriction enzyme digestion, cDNA insert mapping, and sequencing. However, a detailed description of bacteriophage lambda DNA isolation using polyethylene glycol (PEG) precipitation and phenol/chloroform extraction is available ***(34)***.

15. For nonradioactive alternatives using biotin and digoxigenin, and detection by AP, HRP, ECL, and immunoflourescence, *see* **ref. *35***.
16. Phosphoamino acid analysis is described in Chapter 4.

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3

Immunoprecipitation and Western Blotting of Phosphotyrosine-Containing Proteins

Kathleen M. Woods Ignatoski

1. Introduction

Changes in the tyrosine phosphorylation state of a protein in response to external stimuli can have profound effects on cellular signal transduction. The addition of a phosphate group to a tyrosine residue can change a protein's activation state or create a high affinity binding site for other proteins. Conversely, removal of a phosphate group can also change the catalytic activity of an enzyme. Tyrosine phosphorylation of cellular proteins is a rare event that can be increased growth factor addition or cellular attachment to extracellular matrix. Therefore, it is important to be able to observe changes in tyrosine phosphorylation of particular proteins under the influence of different stimuli. Tyrosine phosphorylation of proteins is difficult to detect unless external stimuli are present; even then, many proteins are phosphorylated only in response to one stimulus. Therefore, it is necessary to concentrate the protein of interest in order to observe the phosphorylation state changes between stimulated and unstimulated cells. ^{32}P-labeling of cellular proteins can be used; however, phosphoserine and phosphothreonine are also detected along with phosphotyrosine. Phosphoamino acid analysis can be helpful, but it is not quantitative because acid hydrolysis, which breaks down the proteins into individual amino acids, can remove the phosphate group from the tyrosine. Therefore, other methods of detecting changes in tyrosine phosphorylation states have been developed.

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

1.1. Antiphosphotyrosine Antibodies

In 1981, the first antiphosphotyrosine antibodies were developed by immunizing animals with r-aminobenzylphosphonic acid *(1,2)*. Since then, many different antigens have been used to generate antiphosphotyrosine antibodies, including phosphorylated v-*abl* protein *(3)*, phosphotyrosine or phosphotyramine conjugated to keyhole limpet hemocyanin *(4)*, and phosphotyrosine conjugated to bovine serum albumin (BSA) *(5)*. These antibodies have been used in a variety of methods; among them are Western blotting, immunoprecipitation, localization by immunofluorescence or electron microscopy, and phosphoprotein purification.

Because antiphosphotyrosine antibodies are generated to different antigens *(6)*, their specificities are different *(7)*. One example of this can be seen in **Fig. 1** where the three antiphosphotyrosine antibodies, generated to different antigens, recognize different subsets of proteins. This illustrates the need to determine the specific antiphosphotyrosine antibody with the greatest affinity for the protein of interest.

1.2. Optimizing Lysis Conditions

In addition to determining the correct antiphosphotyrosine antibody, the optimal buffer in which to lyse the cells needs to be determined. An example of this can also be seen in **Fig. 1**. Three different lysis buffers having various pHs and containing different combinations of detergents and salts were used to generate whole cell lysates. Each of these components can have different effects on protein solubility *(8–11)*. Phosphotyrosine-containing proteins in the lysates were detected by Western blotting with antiphosphotyrosine antibodies. Data in **Fig. 1** indicate that identification of the optimal lysis conditions and of the correct antiphosphotyrosine antibody can enhance the detection of changes in phosphotyrosine content of the protein of interest.

1.3. Detection of Phosphotyrosine-Containing Proteins

A specific tyrosine phosphorylated protein can be detected in one of two basic ways: (1) the protein can be immunoprecipitated with antiphosphotyrosine antibodies and used on a Western blot probed with an antibody specific for that protein, or (2) the protein can be immunoprecipitated with a specific antibody then probed with antiphosphotyrosine antibodies on a Western blot. Proteins immunoprecipitated with antiphosphotyrosine antibodies and viewed by Western blot are predominantly tyrosine phosphorylated proteins. Therefore, this approach detects different amounts of phosphorylated protein and not changes in the relative percent of a specific protein, which is tyrosine phosphorylated. Immunoprecipitation with an excess of specific antibody, allowing all of a particular protein to be precipitated, followed by probing a

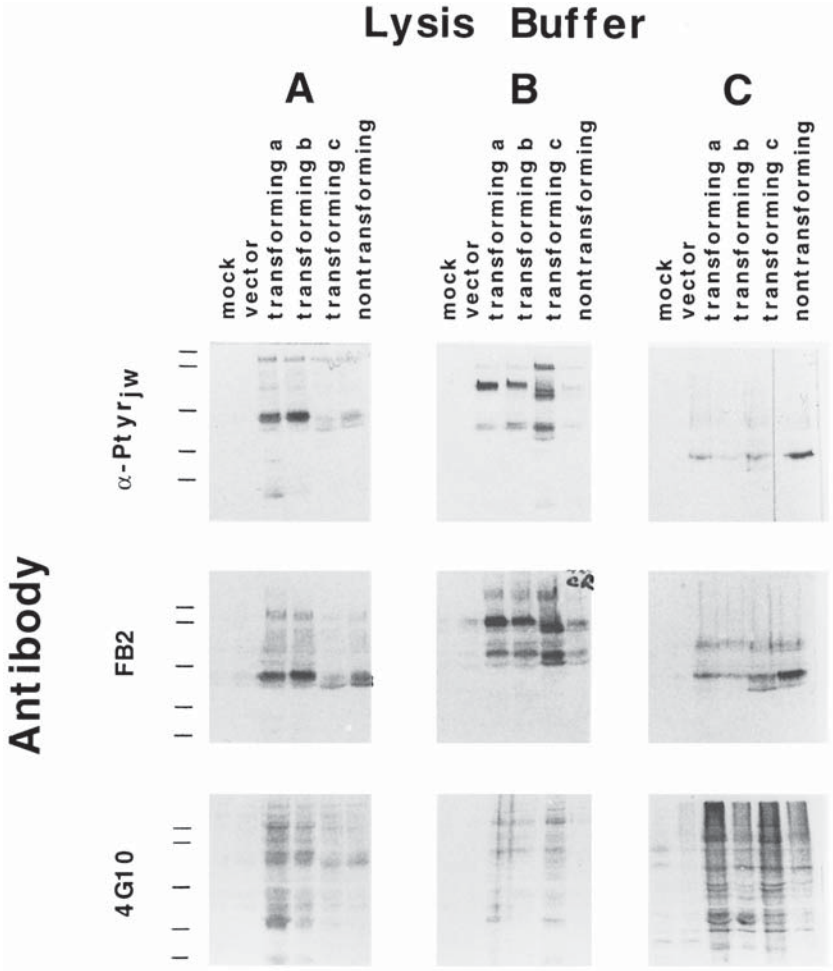

Fig. 1. Protein blots to test lysis conditions and antiphosphotyrosine antibody specificity. Cells expressing various transforming or nontransforming v-*src* alleles and cells that were mock or vector alone transfected were lysed in the three different lysis buffers. Proteins from the whole cell lysates were separated on 10%-polyacrylamide gels containing SDS, transferred to nitrocellulose, and probed with three different anti-phosphotyrosine antibodies (α-Ptyr$_{jw}$, FB2, and 4G10). Blots shown are from a single experiment designed to observe all blots under the same conditions. Individual blots were repeated at least three times with similar results. Transforming a = wild-type v-*src*, transforming b = v-*src*-Y416F, transforming c = v-*src*-F172Δ, and nontransforming = v-*src*-F172Δ/Y416F ***(19)***. Molecular weight markers are depicted on the left side of the A column; they correspond to the following molecular weights: 97.4 kDa, 66 kDa, 45 kDa, 31 kDa, and 21.5 kDa. Figure reprinted with permission from *BioTechniques (7)*.

blot with the appropriate antiphosphotyrosine antibody is the preferred method of determining the changes in a protein's tyrosine phosphorylation state. The total amount of the protein of interest can be determined by probing the same or an identical blot with the protein-specific antibody. These two steps together allow for determination of the percentage of protein that becomes tyrosine phosphorylated or dephosphorylated in response to stimuli. Antiphosphotyrosine antibodies can detect between 0.04 and 40 ng of bound phosphate on a Western blot ***(3)***. This should be sufficient to detect most tyrosine phosphorylation events.

The methods presented here are basic immunoprecipitation and Western blotting protocols that have been modified to better detect phosphotyrosine-containing proteins. The following methods will be discussed in this chapter: cell lysis, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, detection of phosphotyrosine containing proteins.

2. Materials

2.1. Cell Lysis

1. Phosphate-buffered saline (PBS): 116 m*M* NaCl, 12 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4
2. Cell lysis buffer. Some examples of appropriate lysis buffers include the following:
   a. 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% Nonidet P-40 (NP40), 0.25% Na^+deoxycholate, and 10 μg/mL BSA.
   b. 10 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 50 m*M* NaF, 30 m*M* $Na_4P_2O_7$, 150 m*M* Na_3 VO_4, 5 m*M* ethylenediaminetetracetic acid (EDTA), and 1% Triton X-100.
   c. 30 m*M* Tris-HCl, pH 6.8, 150 m*M* NaCl, 1% NP40, 0.5% Na^+ deoxycholate, and 0.1% SDS.

   All of these lysis buffers should be supplemented with the following inhibitors immediately prior to use:
   300 μg/mL phenylmethysulfonyl fluoride.
   20 μg/mL aprotinin.
   10 μg/mL leupeptin.
   100 μ*M* Na_3 VO_4.
3. Løwry protein assay reagents:
   a. Biuret reagent (Sigma, cat. no. 690-1).
   b. Folin and Ciocalteu's reagent (Sigma, St. Louis, MO, cat. no. F-9252).

2.2. Immunoprecipitation

1. Protein A/G Sepharose beads (Calbiochem Oncogene Research Products, Cambridge, MA, cat. no. IP10X).

2. Primary antibody appropriate for signaling protein of interest.
3. PBS + 0.5% Triton X-100.
4. PBS + 0.1% Triton X-100.
5. 3× sample buffer: 10% glycerol, 15%($^{v}/_{v}$) β-mercaptoethanol, 3% SDS, 37.5% $^{v}/_{v}$ upper Tris (1.5 *M* Tris-HCl, pH 8.8, 0.4% SDS), and 2.34 mg/mL bromophenol blue.

2.3. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

1. Lower Tris: 0.5 *M* Tris-HCl, pH 8.3, 0.4% SDS.
2. 29:1 acrylamide:*bis*-acrylamide: 29 g acrylamide and 1 g *bis*-acrylamide in 100 mL dH_2O.
3. TEMED (Sigma).
4. 10% Ammonium persulfate.
5. Upper Tris: 1.5 *M* Tris-HCl, pH 8.8, 0.4% SDS.
6. Electrophoresis buffer: 25 m*M* Tris-HCl, pH 8.3, 192 m*M* glycine, 0.1% SDS.
7. Protein stain, such as:

a. Coomassie brillant blue R-250 (Bio-Rad, Hercules, CA, cat. no. 161-0400).
b. Ponceau S (Sigma, cat. no. P-7170).
c. Silver stain (Bio-Rad, cat. no. 161-0449).

2.4. Western Blotting

1. Nitrocellulose sheets (0.45 μ*M*) (VWR Scientific, Detroit, MI, cat. no. 27376-991).
2. Whatman 3M paper (Schleicher and Schuell, Keene, NH, cat. no. GB003).
3. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol, 0.1% SDS (*see* **Note 1**).
4. Electroblotting transfer apparatus: Trans-blot cell system (Bio-Rad, cat. no. 170-3939).

2.5. Detection of Phosphotyrosine-Containing Proteins

1. Bovine serum albumin (BSA), Fraction V (Sigma, cat. no. A-2153).
2. TBST: 50 m*M* Tris-HCl, pH 7.7, 150 m*M* NaCl, 0.05% Tween-20.
3. Appropriate anti-phosphotyrosine antibody (primary Ab). For example:
   a. 4G10 (UBI, Lake Placid, NY, cat. no. 05-321).
   b. PY20 (Calbiochem Oncogene Research, Cambridge, MA, cat. no. PT04).
   c. FB2 (conditioned medium from ATCC CRL 1891 cells, ATCC, Rockville).
4. Appropriate secondary antibody conjugated to alkaline phosphatase (Promega, Madison, WI, cat. no. S3731, S3721, S3831, or S3821).
5. BCIP/NBT substrate for alkaline phosphatase reaction (Sigma, cat. no. B-5655). Dilute 1 tablet in 10 mL distilled water.
6. Microscope immersion oil, if quantification is needed.

3. Methods

3.1. Cell Lysis

1. Wash cells three times with PBS at room temperature.

2. Lyse cells on ice in 300 µL of lysis buffer per 100-mm dish. Incubate lysate on ice for 5 min, scrape the cells and transfer to a 1.5-mL Eppendorf tube. Following a further incubation on ice for 10 min, clear the debris from the whole cell lysate by centrifugation in a microcentrifuge at full power for 10 min at 4°C.
3. Equalize amounts of protein between samples by using the Løwry protein assay (*see* **Note 2**) ensuring that the starting amount of total protein is 100 µg to 2 mg, depending the cellular amount of the protein of interest.
4. If visualization of phosphotyrosine-containing proteins in the whole cell lysate is required, add sample buffer to final 1× concentration and skip to **Subheading 3.3.**

3.2. Immunoprecipitation

1. Wash protein A/G Sepharose beads three times with lysis buffer. Resuspend beads in 1× starting volume of lysis buffer.
2. Preclear nonspecific binding proteins by incubating the lysate in a 1.5-mL Eppendorf tube with 50 µL washed protein A/G Sepharose beads (*see* **Note 3**) on a rotating wheel for 20 min at 4°C. Pellet the Sepharose beads by centrifugation for 10 s at full speed in a microcentrifuge at room temperature and transfer the supernatant to a clean tube (*see* **Note 4**).
3. Add about 1 µg of antibody specific for the protein of interest to the lysate and incubate at 4°C for at least 1 h (*see* **Note 5**).
4. Add 50 µL of washed protein A/G Sepharose beads (*see* **Note 6**) and incubate for 40 min at 4°C with constant mixing.
5. Centrifuge briefly (10 s) at maximum speed in a microcentrifuge at room temperature, carefully remove supernatant and wash the pellet as follows. First wash twice with PBS + 0.5% Triton X-100, then wash twice with PBS + 0.1% Triton X-100, and finally twice with PBS (*see* **Note 7**).
6. Elute protein from beads by resuspending in 1× sample buffer and boiling for 3 min. Samples can be stored at –20°C until needed.

3.3. SDS-PAGE

1. Immunoprecipitated proteins or proteins from whole cell lysates can be separated on SDS-PAGE. Prepare gel as follows.

   Separating gel:

   a. 7.5 mL lower Tris.
   b. 7.5 mL 29:1 acrylamide:*bis*-acrylamide solution.
   c. 15 mL distilled water.
   d. 15 µL TEMED.
   e. 150 µL 10% ammonium persulfate.

   Pour and allow to set.

   Stacking gel:

   a. 3.75 mL upper Tris.
   b. 1.65 mL 29:1 acrylamide:*bis*-acrylamide.
   c. 9.6 mL distilled water.

d. 15 μL TEMED.
e. 150 μL 10% ammonium persulfate.

Pour, add comb, and allow to set.

2. Remove comb, set the gel on the electrophoresis rig, and add electrophoresis buffer to reservoirs.
3. Heat samples to 100°C for 3 min, centrifuge briefly, and load sample to preformed wells within the stacking gel. Run at 120 kW hours per gel (e.g., 30 mA for 4 h). Above 30 mA, gels require cooling.
4. The proteins can be visualized by different staining techniques, including Coomassie brillant blue, Ponceau S, or silver staining or by Western blot. New stains, such as SYPRO orange (Bio-Rad, Richmond, CA), allow for protein staining in the gel then protein transfer to a membrane for Western blotting.

3.4. Western Blotting

1. Cut a piece of nitrocellulose that is slightly larger than the gel and four pieces of Whatman 3M paper and soak in transfer buffer for at least 10 min at room temperature (*see* **Note 8**).
2. Remove the stacking gel from the separating gel by blotting it onto a paper towel. Soak separating gel in transfer buffer for at least 10 min at room temperature.
3. Place gel carefully on two pieces of soaked Whatman 3M paper. Overlay the gel with the presoaked nitrocellulose sheet, followed by two further pieces of presoaked 3MM paper. Be sure to remove any air bubbles trapped within this "sandwich."
4. Place sandwich in the electroblotting apparatus for transfer of proteins to nitrocellulose, being sure that the nitrocellulose lies close to the anode (positive electrode). The length of time and amount of current needed for transfer depends on the thickness of the gel, size of the gel, and the type of transfer apparatus (*see* **Note 9**; **refs.** ***12*** and ***13***). For a 0.75-mm-thick gel of 15 cm × 15 cm, transfer in the Bio-Rad trans-blot cell system can be done at 1.2 A in 1 h at room temperature.

3.5. Detection of Phosphotyrosine-Containing Proteins

1. Trim the nitrocellulose to the size of gel, and place membrane in a 500-mL centrifuge bottle. Alternatively, blot can be placed in a plastic dish slightly larger than itself (*see* **Note 10**).
2. Block membrane with 20 mL 5% BSA in TBST (*see* **Note 11**) for at least 1 h at room temperature by rolling the bottle inside a large Pyrex dish on top of a rocking platform.
3. Incubate the blot with 1 μg/mL antiphosphotyrosine antibody in 10 mL 5% BSA/TBST at room temperature for 1 h-with agitations (*see* **Note 12**).
4. Wash blot twice in 50 mL TBST with agitation (rolling) for 5 min each time.
5. Incubate blot with secondary antibody for 1 h at room temperature with agitation. Secondary antibody should be used at 1:7500 dilution (manufacturer recommended concentration) in 10 mL TBST. For antiphosphotyrosine Westerns, use alkaline-phosphatase-conjugated secondary antibody (*see* **Note 13**).

6. Wash the blot three times in 50 mL TBST with agitation (rolling) for 5 min each time.
7. Blot excess TBST from membrane with Whatman 3M paper and rinse the vessel with distilled water (*see* **Note 14**).
8. Add 10 mL BCIP/NBT substrate solution and incubate for 5–30 min (or until membrane starts to turn purple) at room temperature. Stop the reaction by rinsing the membrane with distilled water and blotting dry with Whatman 3M paper.
9. A second probe of the original blot may be performed at this time to detect the specific protein of interest and to determine the relative amounts of the protein present. There is no need to reblock the membrane (*see* **Note 15**).
10. Quantitate the blot with xylene-based oil and subsequent densitometry. Drop oil onto, and around, the bands to be quantified. Place the blot between transparencies (to protect the scanner) and scan immediately. Drying of the oil on the nitrocellulose leaves the blot in a less than desirable condition. Be sure that all pictures and subsequent probings have been done before quantitation (*see* **Note 16**).

4. Notes

1. Transfer buffers are based on the original recipe from Towbin ***(14)***. SDS should be included in the buffer because it facilitates easier transfer of high molecular-weight protein species ***(15)***.
2. The Løwry assay for protein quantitation can be used with almost any buffer; whereas, the Bradford assay, which is a shorter assay, does not work if NP40 or Triton X-100 is present in the buffer. Recently, Bio-Rad has added a modifier to the Bradford assay kit that allows the assay to work in the presence of NP40 or Triton X-100.
3. Protein A/G Sepharose beads are prepared by centrifugation of 1-mL bead slurry at maximum speed in a microcentrifuge for 10 s. Discard supernatant and add resuspend pellet in 1 mL of lysis buffer. Repeat centrifugation and washing of beads two more times, resuspend in 1 mL lysis buffer and store on ice.
4. Preclearing will decrease nonspecific protein binding to the beads in **step 8**.
5. The primary antibody incubation may be done at room temperature with constant mixing. However, leaving the antibody, or other proteins, at room temperature could lead to denaturation and degradation. If the immunoprecipitation is to be done on ice, be sure to mix every so often.
6. Some antibodies cannot bind protein A, but can bind protein G, so a mixture of both can be used. Protein A/G Sepharose beads are more expensive than protein A Sepharose beads, so check if protein A will bind the antibody. Instead of using protein A/G Sepharose beads, a secondary antibody of the correct isotype can be used to bridge the primary antibody to protein A Sepharose. If necessary, add the secondary antibody to the immunoprecipitate for 1 h at 4°C before adding protein A Sepharose beads. Protein A can also be added as fixed *Staphlococcus aureus*; this is less expensive, but requires extensive washing in lysis buffer before use. It is more difficult to lose some of the precipitate during washing if *S. aureus* is used instead of protein A Sepharose beads.
7. Other washing methods can be used. Examples include washing five or six times in lysis buffer or in plain PBS. Triton X-100 adds stringency to the washes.

8. There are several choices of membranes for Western blotting: nylon, nitrocellulose, and polyvinylidene fluoride (PVDF). PVDF is stronger than nitrocellulose, but more expensive; however, reinforced nitrocellulose is now available. Even though PVDF has a higher protein-binding affinity, nitrocellulose is recommended because proteins visualized by Western blot can be quantitated if it is used (*see* **Note 11**).
9. There are two common methods of electroblotting proteins to membranes, wet and semidry. Semidry blotting is quicker than wet blotting and uses much less buffer; however, wet blotting is more effective if the proteins of interest are of high molecular weight (>100 kDa) ***(13)***.
10. Use of the roller bottle facilitates even coverage of the membrane.
11. BSA is more expensive, but nonfat dry milk used as a blocking agent can cause a high background because of phosphotyrosine-containing proteins in the milk. Milk can also strip some of the proteins from PVDF membrane ***(16)***.
12. The primary antibody usually can be reused about five times. It should be frozen in the presence of 5% BSA/TBST to prevent denaturation on freeze-thawing.
13. The colorimetric reaction for alkaline phosphatase-conjugated secondary antibody involving the reactants NBT/BCIP should be used to detect phosphotyrosine. The horseradish peroxidase-facilitated colorimetric reaction is not as sensitive as the alkaline phosphatase system, and the colored product fades if the blot is not kept in the dark ***(17)***. Chemiluminescent detection of phosphotyrosine antibodies on a Western blot often produces a high background. The alkaline phosphatase colormetric reaction is easier to quantitate than chemiluminescence because the film used to detect chemiluminescence needs to be a particular optical density in order to be in the linear range for quantification.
14. TBST at pH 7.7 is not at the correct pH for the alkaline phosphatase NBT/BCIP reaction and will quench the signal ***(18)***. Therefore, the excess TBST should be blotted from the membrane before substrate addition.
15. A second probing of the original blot using chemiluminescent detection can determine the relative amount of the specific protein that was loaded. It is helpful if the antibody used to detect the protein of interest is from a different species than the antiphosphotyrosine antibody so there is no cross-reaction between the secondary and primary antibodies giving elevated readings. If this cannot be achieved, two identical blots can be performed, one for phosphotyrosine and one for the protein of interest.
16. Xylene or a xylene-based oil, such as microscope immersion oil, will clear the opaque background of nitrocellulose, but not the colored bands. Clearing the background allows for densitometry similar to analysis of X-ray film ***(19)***.

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4

Two-Dimensional Phosphoamino Acid Analysis

Peter Blume-Jensen and Tony Hunter

1. Introduction

Signals transmitted between cells activate intracellular signaling pathways that are precisely regulated, controlled, and organized. Ultimately, the intracellular biochemical events culminate in a specific cellular response(s). A major mechanism for intracellular signal transduction in both eukaryotic and prokaryotic cells is protein phosphorylation ***(1)***. Beside being fast and reversible, protein phosphorylation is tightly regulated and highly specific, and allows signals to be sustained or attenuated via amplification, feedback, and crosstalk ***(2)***. The specificity in signaling is, in part, achieved by two means. Catalytic specificity of protein kinases and phosphatases provides the basis for site-specific phosphorylation and dephosphorylation, respectively ***(3)***. Specific tyrosine and serine/threonine phosphorylation sites and their surrounding sequences, in turn, provide selective binding sites for conserved protein modules found in most cytoplasmic signaling molecules ***(2,4,5)***. Identifying the sites of protein phosphorylation and the nature of the phosphorylated residue is therefore paramount in the study of signal transduction.

Protein phosphorylation can occur: (1) on the hydroxyl group of serine, threonine, or tyrosine, forming a phosphate ester; (2) on the nitrogen of histidine, arginine and lysine, forming a phosphoramidate linkage; (3) on the sulphur of cysteine forming a phosphate thioester; or (4) on the carboxyl group of aspartate, forming an acid anhydride. In eukaryotic cells, hydroxyl-linked phosphorylation is by far the most studied. N-linked phosphorylation is acid-labile and therefore most often goes undetected in conventional protein chemical studies that often employ acidic conditions during sample treatment and analysis. It has

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

been estimated that tyrosine phosphorylation constitutes less than 0.05% of total cellular protein phosphorylation with serine/threonine phosphorylation constituting most of the remainder ***(1)***. Phosphoramidate bonds and phosphotyrosine, however, are stable to alkaline hydrolysis conditions that break peptide bonds, such as 3N KOH, 105°C for 4 h. Based on this, it has been estimated that histidine phosphorylation constitutes 5–10% of total phosphorylation, or 10- to 100-fold more than tyrosine phosphorylation in eukaryotic cells, whereas arginine and lysine phosphorylation constitutes less than 0.1% ***(6,7)***. For a description of phosphoamino acid analysis of such N-linked phosphorylation by alkali treatment, *see* Chapter 5 (*see* also **refs. *6–9***).

Phosphopeptide mapping (*see* Chapter 7) and phosphoamino acid analysis are often used in conjunction to determine the identity of sites of phosphorylation and estimate the stoichiometry of phosphorylation at particular sites. In this chapter, we will describe phosphoamino acid analysis of serine, threonine, and tyrosine phosphorylation by two-dimensional electrophoresis on cellulose thin-layer chromatography (TLC) plates. Briefly, this involves labeling of the protein of interest with [^{32}P]orthophosphate either in vivo or in vitro, followed by partial acid hydrolysis of the whole protein, or of individual phosphopeptides derived by proteolytic digestion. The released phosphoamino acids are then separated by electrophoresis in two dimensions on TLC plates and visualized by exposure to X-ray film or a PhosphorImager screen. This is an extremely sensitive technique that requires a few disintegrations per minute (dpm) of ^{32}P-labeled amino acids.

2. Materials

2.1. General Equipment and Materials

1. HTLE 7000 thin-layer electrophoresis apparatus (CBS Scientific).
2. LCT-100 large chromatography tanks (CBS Scientific).
3. Trans-Blot apparatus (Bio-Rad).
4. Semi-Dry Blot apparatus (Bio-Rad).
5. Polyvinylidene difluoride (PVDF) membrane (Immobilon-P; cat. no. IPVH 000 10) was obtained from Millipore.
6. SpeedVac sample concentrator (Savant; cat. no. SC-100).
7. Microcentrifuge tubes (Kontes; cat. no. 749510-1590).
8. Disposable pestles for tissue grinding (Kontes; cat. no. 749521-1590).
9. Domestic variable speed electrical drill (Black and Decker).
10. 1.5-mL screw-cap tubes (Sarstedt; cat. no. 72.692).
11. 1.5-mL microcentrifuge tubes (Sorenson; cat. no. 16070).
12. Plastic microtransfer pipets (Research Products Int.; cat. no. 147500).
13. Oxford ultramicropipets and capillary tips (Fisher Scientific; cat. no. 21-199-9).
14. 20 cm × 20 cm cellulose thin-layer (100-μm) chromatography glass plates (E × Science; cat. no. 5716).

15. Whatman 3MM paper (Whatman; cat. no. 303 0917).
16. 6.5-mm polyethylene disks (Kantes; cat. no. 420162-0020).
17. Phosphoserine (Sigma; cat. no. P-0753).
18. Phosphothreonine (Sigma; cat. no. P-1003).
19. Phosphotyrosine (Sigma; cat. no. P-5024).
20. ε-dinitrophenyl (DNP)-lysine (Sigma; cat. no. D-0380).
21. Xylene cyanole FF (Sigma; cat. no. X-4126).
22. India ink with 10μci/mL ^{35}s or, fluorescent ink (numerous sources).

2.2. Solutions

Buffers, solvents, and reagents should all be reagent grade and can be obtained from various distributors.

2.2.1. Elution and Precipitation of [^{32}P]-Labeled Proteins from SDS-Polyacrylamide Gels

1. 50 m*M* NH_4HCO_3: This solution is made up fresh by dissolving 0.4 g NH_4HCO_3 in 100 mL deionized water. The final pH should be approx 7.3. On prolonged storage, the pH of the solution will rise to approx 8.3.
2. Sodium dodecyl sulfate (SDS): Prepare 20% SDS solution by dissolving 20 g SDS in 100 mL deionized water. This solution can be stored at room temperature for extended periods of time.
3. Carrier protein stock solution: Dissolve pancreatic RNase A or immunoglobulins (pure fraction) in deionized water to a final concentration of 1 mg/mL. Boil the RNaseA solution for 5 min to inactivate any contaminating proteases. The stock solutions are stored as 500-μL aliquots at –20°C and can be reused several times.
4. Trichloroacetic acid (TCA) solution: A 100% TCA solution is prepared by dissolving 100 g of TCA in deionized water to give a final volume of 100 mL of solution. Store at 4°C.

2.2.2. Electrotransfer of Proteins from SDS Polyacrylamide Gels to PVDF Membranes

1. Transfer buffer without SDS: Dissolve 12 g Tris base and 57.6 g glycine in deionized water and 0.8 liter methanol to give a final volume of 4 L.
2. Transfer buffer with SDS: Dissolve 12 g Tris base and 57.6 g glycine in deionized water and 0.8 L methanol to give a final volume of 4 L. Degas the solution for 10 min and add 20 mL 20% SDS.

2.2.3. Partial Acid Hydrolysis of Phosphoproteins

6 N HCl: Add one part concentrated (37%) HCl to one part deionized water. Keep in a 15-mL screw-cap tube at room temperature. Wearing gloves, make the solution up in a fumehood. Concentrated HCl can result in severe burn

injuries on direct contact with the skin and the vapors are highly irritating and damaging to the mucosal lining of the respiratory tract.

2.2.4. Two-Dimensional Electrophoresis

1. Marker dye mixture: Dissolve 5 mg ε-dinitrophenyl (DNP)-lysine (yellow) and 1 mg xylene cyanol FF (blue) in 1 mL of a 1:1 (v/v) mixture of pH 4.72 electrophoresis buffer and deionized water. Store at 4°C.
2. Phosphoamino acid marker mixture: Dissolve 1 mg of each of phosphoserine, phosphothreonine, and phosphotyrosine in 1 mL deionized water. Store at 4°C.
3. pH 1.9 buffer: 50 mL formic acid (88% w/v), 156 mL glacial acetic acid, 1794 mL deionized water.
4. pH 3.5 buffer: 100 mL glacial acetic acid, 10 mL pyridine, 1890 mL deionized water.
5. pH 4.72 buffer (20 mL)*:* 1 mL *n*-butanol, 0.5 mL pyridine, 0.5 mL glacial acetic acid, 18 mL deionized water.
6. Ninhydrin staining solution: Dissolve 0.5 g ninhydrin in 200 mL acetone. Keep in a spray bottle at room temperature.

Electrophoresis buffers can be stored for some time in glass bottles with air-tight lids. Check the pH after preparation and also before electrophoresis, if the buffers are stored before being used. The pH should not vary by more than 0.2 pH unit when using reagent grade solvents. If it does, do not adjust the pH, but remake the buffer. Note, that pyridine should be kept under an atmosphere of nitrogen. When oxidized it turns yellow and can no longer be used.

3. Methods

Phosphoamino acid analysis of hydroxyl-linked phosphorylation (i.e., phosphorylation on serine, threonine, and tyrosine residues) is accomplished by partial acid hydrolysis of the purified ^{32}P-labeled protein. Typically, the protein of interest is labeled by incubation of cells with [^{32}P]orthophosphate or by phosphorylation in vitro in the presence of [γ-^{32}P]ATP. Conditions for radioactive labeling are often empirically determined, and have to be optimized in each individual case. The ^{32}P-labeled protein is then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and recovered from the gel in either of two ways before partial acid hydrolysis (*see* **Note 1**). The protein can be eluted from the SDS gel, precipitated with TCA or acetone, washed in ethanol, and air-dried and then subjected to hydrolysis in solution (*see* **Note 2**). Alternatively, it can be electrotransferred to a PVDF membrane (e.g., Immobilon-P) and hydrolyzed *in situ* (*see* **Note 3**). Finally, phosphopeptides derived by proteolytic digestion and separated by two-dimensional electrophoresis on TLC plates or by high-performance liquid

chromatography (HPLC) can be isolated and analyzed by phosphoamino acid analysis (*see* also Chapter 7, this volume).

3.1. Elution and Precipitation of [^{32}P]-Labeled Proteins from SDS-Polyacrylamide Gels

Several methods have been described for both recovery and concentration of proteins from SDS gels, and as long as the protein is eluted in a denatured and reduced form, and the concentrated sample is freed of any salts and chemicals, it can, in most instances, be used for subsequent phosphoamino acid analysis. The following method, based on elution in boiling SDS and precipitation with TCA, works reproducibly for us.

1. After separating the ^{32}P-labeled protein samples by SDS-PAGE, the gel is dried onto paper or dialysis membrane, and the edges of the dried gel are marked with ^{35}S-labeled India ink or fluorescent ink for later alignment. Expose the gel to X-ray film and/or a PhosphorImager screen.
2. The gel is aligned with the image on the film or the printed copy (1:1) of the phosphorimage and stapled together with the gel on top.
3. Place the gel and image on a light box on top of a glass plate. Using a razor blade, mark and cut out the protein bands of interest from the individual lanes. Check by Cerenkov counting the amount of radioactivity incorporated into the protein in each gel piece.
4. Peel the paper or membrane backing from the gel and remove any remaining paper by gentle scraping with a razor blade. Alternatively, peel the paper after rehydration (*see* **step 5** below).
5. Transfer each of the gel pieces to a 1.5-mL tissue grinder microcentrifuge tube and hydrate in 500 µL of freshly prepared 50 m*M* NH_4HCO_3, pH 7.3–7.5, for 5 min at room temperature.
6. Using an ordinary domestic-type electric drill, grind the swollen gel pieces with disposable tissue grinder pestles. When gel bits are fine enough, pass them a few times through a disposable yellow tip connected to a 200-µL pipetman, and transfer the samples to a 1.5-mL screw-cap microcentrifuge tubes (*see* **Note 4**). Rinse the tissue grinder and tube with another 500 µL of 50 m*M* NH_4HCO_3, and pool with the sample in the micro screw-cap tube. Add 20 µL β-mercaptoethanol and 5 µL 20% (w/v) SDS, boil for 3 min, and then shake for ≥ 2 h at 37°C, or for ≥ 4 h at room temperature.
7. Precipitate the gel by centrifugation for 2 min at 14,000*g* in a microcentrifuge at room temperature and immediately transfer the supernatant to a new 1.5 mL microcentrifuge tube before the gel precipitate reswells. Extract the gel pieces once more by adding 300 µL of 50 m*M* NH_4HCO_3 containing 6 µL β-mercaptoethanol and 2 µL 20% SDS, vortex, and incubate at 37°C for another 1 h or at room temperature for 90 min.
8. Centrifuge for 2 min at 14,000*g* in a microcentrifuge and combine the supernatant with the first supernatant. Then centrifuge for ≥ 5 min at 14,000*g* to remove any

residual gel fragments, and transfer the combined supernatant to a new microcentrifuge tube. Check by Cerenkov counting. At least 60–80% of the starting radioactivity in the gel piece should be recovered in the supernatant (*see* **Note 4**).

9. Place the tube containing the cleared supernatant on ice. Add 20 μg of the carrier protein stock solution (20 μL of a 1 mg/mL solution) (*see* **Note 5**), mix, and then add 250 μL ice-cold 100% TCA, mix well, and leave for 1 h on ice. Centrifuge for ≥ 20 min at 4°C at 14,000*g*, and remove the supernatant to a new tube with a disposable plastic pipet. A small white pellet should be visible in the bottom of the tube. Check by Cerenkov counting that the majority of the ^{32}P label is in the precipitated pellet before discarding the supernatant. If this is not the case, one can try to recover the labeled protein by incubating the supernatant on ice for another hour, and centrifuging for ≥ 30 min at 4°C at ≥ 25,000*g*, e.g., in a Beckman centrifuge fixed-angle rotor.
10. Centrifuge the tube containing the protein pellet for 60 s in a microcentrifuge and remove the last traces of liquid with a yellow tip. Add 500 μL ice-cold 99% absolute ethanol, vortex, and centrifuge at 14,000*g* for 5 min at 4°C. Remove the ethanol and air-dry the pellet by leaving the tube open on the bench. Alternatively, lyophilize for a short time in a SpeedVac, but make sure that the pellet does not dry out completely. For partial acid hydrolysis, go to **Subheading 3.3**.

3.2. Electrotransfer of Proteins from SDS Polyacrylamide Gels to PVDF Membrane

Instead of recovering the ^{32}P-labeled protein from an SDS gel by elution and TCA precipitation, the protein can be transferred to a membrane and phosphoamino acid analysis carried out directly on a membrane piece with the protein bound to it. This method is recommended for phosphoamino acid analysis whenever possible, because it is simpler and usually gives better yields of ^{32}P-labeled phosphoamino acids, provided the protein is transferred efficiently to the membrane ***(10)***. Bear in mind that only PVDF (e.g., Immobilon-P) membrane can withstand the harsh conditions used for phosphoamino acid analysis by partial acid hydrolysis (*see* **Note 3**). We usually use the Bio-Rad Trans-Blot apparatus for quantitative electrotransfer of proteins to membrane (*see* **Note 6**).

1. Remove the SDS polyacrylamide gel from the glass plates and soak it for a few minutes in transfer buffer (*see* **Note 7**). Meanwhile, wet the membrane briefly in methanol, and then leave it submerged in deionized water or transfer buffer.
2. Assemble the transfer sandwich in a small buffer reservoir with a little transfer buffer in it. Place a Scotch Brite pad, a piece of Whatman 3MM paper cut slightly bigger than the gel piece, the gel, the prewetted PVDF membrane, another Whatman 3MM paper piece, and finally, a Scotch Bride pad. Make sure no air is trapped in between the layers before closing the plastic holder. Place the transfer sandwich in the buffer-filled Bio-Rad Trans-Blot apparatus with the membrane towards the anode, and the gel toward the cathode. Transfer conditions depend on

the protein, but typically a constant current of 400 mA for 3.5 h will ensure quantitative transfer of a protein of a relative molecular mass of 150 kDa (*see* **Note 8**).

3. After transfer, rinse the membrane in two changes of deionized water to remove salts and detergents, place it on a Whatman 3MMM paper and cover with plastic wrap, mark the membrane for later alignment, and expose to film or on a PhosphorImager.
4. Align the membrane with the film or with a copy of the phosphorimage and cut out the piece of membrane that contains the protein of interest from each lane. If desired, one can stain with India ink to detect proteins on the membrane without affecting the subsequent acid hydrolysis.

3.3. Partial Acid Hydrolysis of Phosphoproteins

Phosphoamino acid analysis of hydroxyl-linked phosphorylation is typically done by partial acid hydrolysis. Phosphodiester bonds are relatively stable under these conditions, so that phosphorylation on serine, threonine, and tyrosine can be detected by release of free phosphoamino acids. The incubation of phosphoproteins or phosphopeptides in concentrated acid leads to hydrolysis of the peptide bonds and, eventually, free amino acids are released. However, some hydrolysis of phosphodiester bonds will also occur after extended time periods, leading to release of free [^{32}P]phosphate from the phosphoamino acids. Consequently, hydrolysis times are critical. As mentioned earlier, both phosphoproteins eluted from SDS gels and membrane-bound phosphoproteins can be used for the partial hydrolysis, but hydrolysis of membrane-bound protein is recommended, except when transfer efficiency to the PVDF membrane is low.

3.3.1. Partial Acid Hydrolysis of Membrane-Bound Phosphoproteins

1. Each of the membrane strips containing the ^{32}P-labeled protein are cut into pieces of approx 2 × 2 mm with a razor blade, and transferred to a Sarstedt screw-cap microcentrifuge tube.
2. A minimal volume (10 µL) of methanol is added to each tube to wet the membrane pieces. Spin the tubes briefly in a microcentrifuge to ensure that the membrane pieces are completely wetted in the bottom of the tubes.
3. Optional: The membrane pieces can be rinsed once in deionized water to remove most of the methanol. Do this by adding 500 µL of water to each tube, vortex, spin shortly, and then aspirate the liquid with a yellow tip attached to a vacuum line.
4. Add 200 µL of 6 *N* (or 5.7 *M* constant boiling) HCl to each tube, screw the cap on tightly, vortex, and place the tubes in an oven at 110°C for 60 min to hydrolyze.
5. Centrifuge the tubes for 1 min in a microcentrifuge and transfer the supernatant to a new screw-cap tube. Add 50 µL deionized water to the filters, vortex, spin down briefly, and transfer the supernatant to the hydrolyzate. Check by Cerenkov counting that at least 90% of the radioactivity has been released from the membrane.

6. Place the samples in a SpeedVac to evaporate the HCl. The samples can now be saved at –20°C or they can be processed immediately as described in **Subheading 3.4**. Before analyzing samples that have been kept at –20°C, thaw the samples at room temperature, add 70 µL deionized water, vortex, and centrifuge at 14,000*g* for 5 min to sediment any particulate matter that might have formed during the freezing and thawing. Carefully transfer the supernatant to a new screw-cap tube. Lyophilize the samples in a SpeedVac and check the amount of radioactivity by Cerenkov counting. Then separate the phosphoamino acids as described in **Subheading 3.4**.

3.3.2. Partial Acid Hydrolysis of Eluted, Precipitated Phosphoproteins

One can use either TCA-precipitated proteins that are lyophilized after the ethanol wash stage or proteins oxidized in performic acid and lyophilized (*see* **Note 2**).

1. Resuspend the dried protein sample in the screw-cap microcentrifuge tube in 50–100 µL of 6 *N* (or constant boiling 5.7 *M*) HCl, and incubate at 110°C for 60 min to hydrolyze the sample.
2. Place the sample in a SpeedVac to evaporate the HCl. Check the amount of radioactivity by Cerenkov counting. Proceed then as described in **Subheading 3.4**.

3.4. Separation of Phosphoamino Acids from Hydrolyzates of Phosphoproteins or Phosphopeptides by Two-Dimensional Electrophoresis

The phosphoamino acids contained in the phosphoprotein or phosphopeptide hydrolyzate, phosphoserine (P.Ser), phosphothreonine (P.Thr), and phosphotyrosine (P.Tyr), are now separated by electrophoresis in two dimensions on cellulose TLC plates. Electrophoresis in the first dimension is performed in pH 1.9 buffer, which leads to good separation of P.Ser from P.Thr and P.Tyr. Electrophoresis in the second dimension in pH 3.5 buffer, then, separates P.Thr efficiently from P.Tyr, so that all three phosphohydroxy amino acids are separated after electrophoresis in two dimensions. The electrophoresis will also resolve the three ^{32}P-labeled phosphoamino acids from free [^{32}P]phosphate and from ^{32}P-labeled phosphopeptides released during the partial hydrolysis. Finally, electrophoresis in two dimensions, rather than one, also serves to separate the ^{32}P-labeled phosphoamino acids from derivatives of ^{32}P-labeled RNA, like [^{32}P]ribose 3'-phosphate and [^{32}P]3'-UMP, which are major contaminants derived by acid hydrolysis from RNA when ^{32}P-labeled protein is obtained from in vivo labeling (*see* **Note 9** and **refs. *11*** and ***12***).

3.4.1. Plate Preparation and Sample Loading

1. Prepare the cellulose TLC plate for electrophoresis. Orient the plate so that the direction in which the cellulose was poured (seen as streaks in the cellulose layer when the plate is placed on a light box) is parallel to the direction of

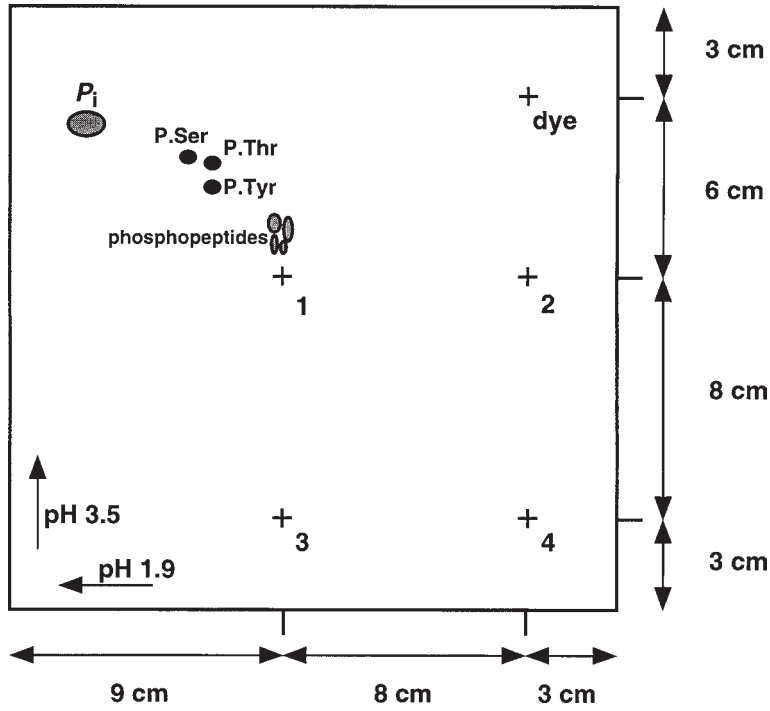

Fig. 1. Cellulose TLC plate indicating sample and dye origins and ^{32}P-labeled products from a sample applied to origin 1. Four samples are typically applied on a TLC plate at the indicated sample origins, shown as 1, 2, 3, and 4. Before electrophoresis in the first dimension, a marker dye mixture of ε-DNP-lysine and xylene cyanol FF is applied to the dye origin depicted in the upper right corner. The plate is rotated 90° counterclockwise for second dimension electrophoresis at pH 3.5. The positions of phosphoserine (P.Ser), phosphothreonine (P.Thr), phosphotyrosine (P.Tyr), released orthophosphate (P_i) and partially hydrolyzed phosphopeptides are shown for a sample applied to origin 1.

electrophoresis in the first dimension (*see* **Note 10**). Using a blunt-ended soft pencil, gently mark four spots on the cellulose layer where the protein samples will be applied and one where the marker dye will be applied (*see* **Fig. 1**).

2. Dissolve the samples in 6 µL of a 5:1 mix of pH 1.9 buffer and phosphoamino acid marker mixture. Vortex and make sure that the sides near the bottom of the tube are covered with the liquid as well.
3. Immediately prior to loading the samples on the cellulose plate, centrifuge for 3 min at 14,000*g* to sediment any particulate matter. Check the amount of radioactivity by Cerenkov counting and decide how much of each sample is to be applied. Note that only very few dpm are required to obtain a signal.

4. Apply the desired amount of counts from each sample to the marked origins on the plate, carefully avoiding transfer of any particulate matter from the bottom of the tubes (*see* **Note 11**). One can use a yellow tip attached to a 2-μL Gilson pipet and apply the smallest possible volume at a time. Alternatively, an Oxford micropipet fitted with a capillary tip can be used, applying between 0.2 to 0.5 μL of the sample at a time. It is important to air-dry between each application. This can be done by focusing the airflow from an airline fitted with a filter at the end. Typically, one applies four samples on each plate as indicated (**Fig. 1**). The marker dye (1 μL) is applied at the top, right-hand corner, where indicated (**Fig. 1**).

3.4.2. Two-Dimensional Electrophoresis Using the HTLE 7000 Thin-Layer Apparatus

The HTLE 7000 electrophoresis system has been optimized for the separation of ^{32}P-labeled phosphopeptides and phosphoamino acids using cellulose thin-layer chromatography plates. The HTLE 7000 apparatus must be connected to a power supply that can deliver at least 1500 V at 100 mA, to running tap water at approx 16°C, and to an air pressure line with an adjustable valve that can deliver a constant pressure of at least 10 lb/in^2. The apparatus is closed during electrophoresis using a clamping system. An inflatable airbag squeezes away excess buffer from the plate during runs, as well as prevents buffer from running up onto the cellulose plate via the electrophoresis wicks. A bottom cooling plate connected to running tap water prevents overheating of the system during the run. The assembled apparatus with the TLC plate is shown in **Fig. 2**.

3.4.2.1. Assembly of Apparatus Without TLC Plate

The apparatus is first assembled as shown in **Fig. 2**, but without the TLC plate. The purpose of this is to squeeze out excess buffer from the electrophoresis wicks while preparing the TLC plate.

1. Fill both buffer tanks with 500 mL of the appropriate electrophoresis buffer.
2. Cut two 25 × 35-cm pieces of thin polyethylene sheeting. They serve to protect the TLC plate, to protect against radioactive contamination of the apparatus, and finally to prevent (buffer) contact between the TLC plate and any parts of the HTLE 7000 apparatus.
3. Place one of the polyethylene protector sheets on the bottom Teflon insulating sheet and tuck the ends in between the bottom cooling plate and the buffer tanks (**Fig. 2**).
4. Electrophoresis wicks are made from two 20 × 28-cm pieces of Whatman 3MMM paper. The paper sheets are then folded lengthwise to give two 14 × 20-cm electrophoresis wicks of double thickness, and wetted in the electrophoresis buffer. The wicks are then inserted into the buffer tanks, and the folded ends bended over the bottom cooling plate on top of the first polyethylene protector sheet. The upper polyethylene protector sheet is then placed over the wicks,

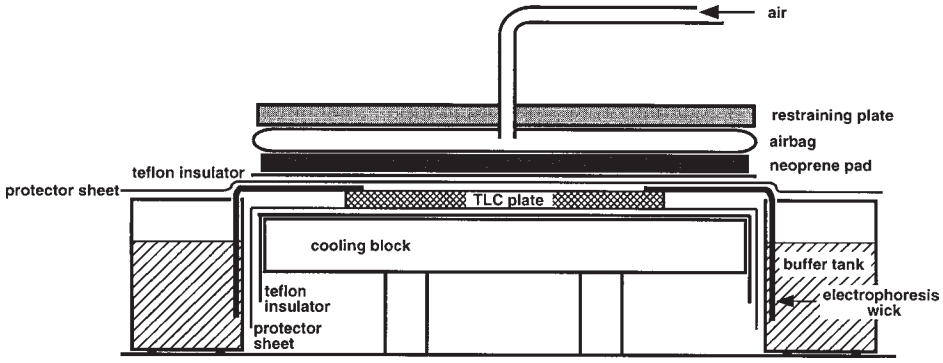

Fig. 2. The HTLE 7000 thin-layer high-voltage electrophoresis apparatus. A cross section through the assembled apparatus is shown. The Teflon insulator sheet on the bottom cooling plate is covered with a protecting polyethylene sheet, and the TLC plate is placed on top. The double-layer electrophoresis wicks are placed in the buffer tanks so that the folded ends overlap approx 1 cm at the edges of the plate. A second polyethylene protector sheet covers the plate and the wicks, followed by a Teflon insulator sheet and the neoprene pad. The apparatus is closed with securing pins (not shown), the airbag inflated at 10 lb/in^2 and tap water flows through the bottom cooling plate. A typical run is from 20–25 min at 1.3 to 1.5 kV (*see* text for details).

followed by the Teflon insulating sheet and finally the neoprene pad on top. Close the restraining plate, secure it with the pins, and turn the air pressure up to 10 lb/in^2. This will inflate the airbag and press out excess buffer from the electrophoresis wicks, while the thin-layer plate with the samples spotted on it is being wetted. The apparatus is now assembled exactly as shown in **Fig. 2**, but without any TLC plate.

3.4.2.2. First Dimension Electrophoresis

1. Before electrophoresis in the first dimension, the TLC plate is wetted in pH 1.9 buffer using a prewetted Whatman 3MM paper with five holes corresponding to the position of the sample origins. To make the blotter, cut a Whatman paper into a 25 × 25-cm square, and then make five circular 1.5-cm holes with a cork borer at positions corresponding to the sample and marker dye origins (*see* **Fig. 3A**).
2. To prepare for the first dimension electrophoresis, wet the blotter with the five holes in pH 1.9 buffer and drag it over the edge of the buffer-containing reservoir to remove excess buffer. The blotter is then carefully placed on the plate so that the sample and marker origins are in the center of the holes (**Fig. 3A**). Press gently with your fingertips around the holes, so that buffer will run out from the blotter and concentrate the samples in the center of the holes at the position of the origins. Then press with your palms on the remainder of the blotter to wet the plate evenly all over, avoiding excess buffer. If buffer puddles are present, remove them carefully by blotting with tissue paper.

3. The TLC plate is now ready to be electrophoresed. The air pressure is shut off, the apparatus opened and the neoprene pad, Teflon insulator sheet and upper polyethylene sheet removed, and excess liquid removed from both the polyethylene sheets with a tissue.
4. The prewetted plate is placed, cellulose side up, on top of the lower polyethylene sheet, and the wicks are folded over so that there is a 1 cm in overlap at the edge of the plate. The upper polyethylene sheet is placed on top of the plate and the apparatus reassembled in the same order and manner as described above, carefully avoiding movement of the upper polyethylene sheeting. The apparatus is closed and secured with the pins, the air pressure turned up to 10 lb/in^2, and the cooling water flow turned on. Now the assembled apparatus looks exactly like shown in **Fig. 2**.
5. Switch on the high-voltage supply and carry out the first dimension electrophoresis with the HTLE 7000 thin-layer electrophoresis apparatus for 25 min at 1.5 kV.
6. After the first dimension electrophoresis the plate is dried in front of a fan for at least 30 min, or until the smell of acetic acid from the plate is minimal.

3.4.2.3. Second Dimension Electrophoresis

1. To prepare for the second dimension electrophoresis, the TLC plate is wetted with pH 3.5 buffer, containing 0.1 m*M* EDTA to prevent streaking. Soak three strips of Whatman 3MM paper, 3 cm, 6.5 cm, and 10 cm wide × 25 cm long, in the pH 3.5 buffer, and place them on the plate on each side of the samples and parallel to the first dimension electrophoresis direction (**Fig. 3B**). Press gently with your fingertips on the edges of the strips, so that the buffer moves toward the center between adjacent strips and thereby concentrates the samples on a line going through the sample origins. A sharp, brown line will appear on this line. The plate should be evenly wetted using as little buffer as possible, because excess buffer may result in fuzzy maps.
2. The plate is now rotated 90° counterclockwise and placed on the HTLE apparatus, and the apparatus assembled exactly as described above for first dimension electrophoresis. Electrophoresis in the second dimension is carried out for 20 min at 1.3 kV.
3. After electrophoresis, dry the plate in a fumehood for 10 min with warm air from a fan or in an oven at 65°C.

3.4.2.4. Detection of Phosphoamino Acids

1. Spray the plate with 0.25% ninhydrin in acetone and develop under warm air using a fan or in an oven at 65°C for at least 15 min. Upon heating, ninhydrin reacts with the primary amino groups so that the phosphoamino acid standards will appear as purple spots on the cellulose plate.
2. Mark the plate with ^{35}S-labeled radioactive India ink and expose to X-ray film at –70°C with an intensifying screen or on a PhosphorImager screen.
3. After developing, the identity of the radioactive spots is revealed by aligning the film or a copy of the phosphorimage with the stained phosphoamino acid

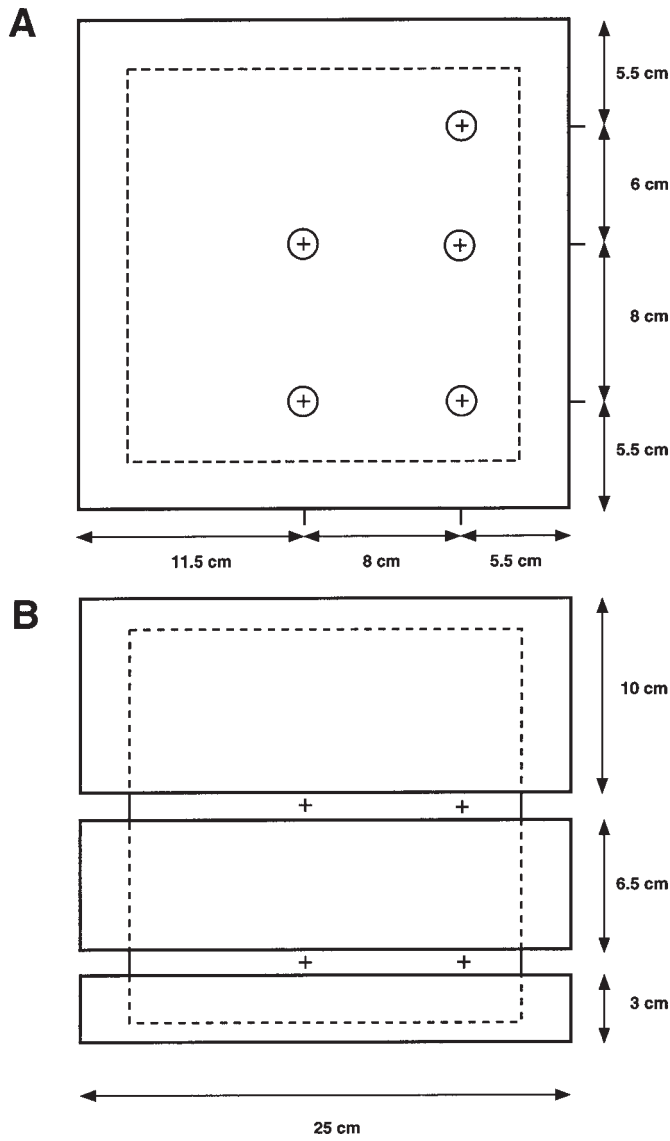

Fig. 3. Whatman 3MM paper blotters for wetting the plates before separation in the first **(A)** and second **(B)** dimensions. The holes in the blotter for the first dimension are made with a sharp cork borer. The stippled line indicates the outline of a TLC plate.

standards on the plate. An example of an experiment where phosphoamino acid analysis was performed on a phosphoprotein that was labeled with [^{32}P]orthophosphate in vivo is shown in **Fig. 4**.

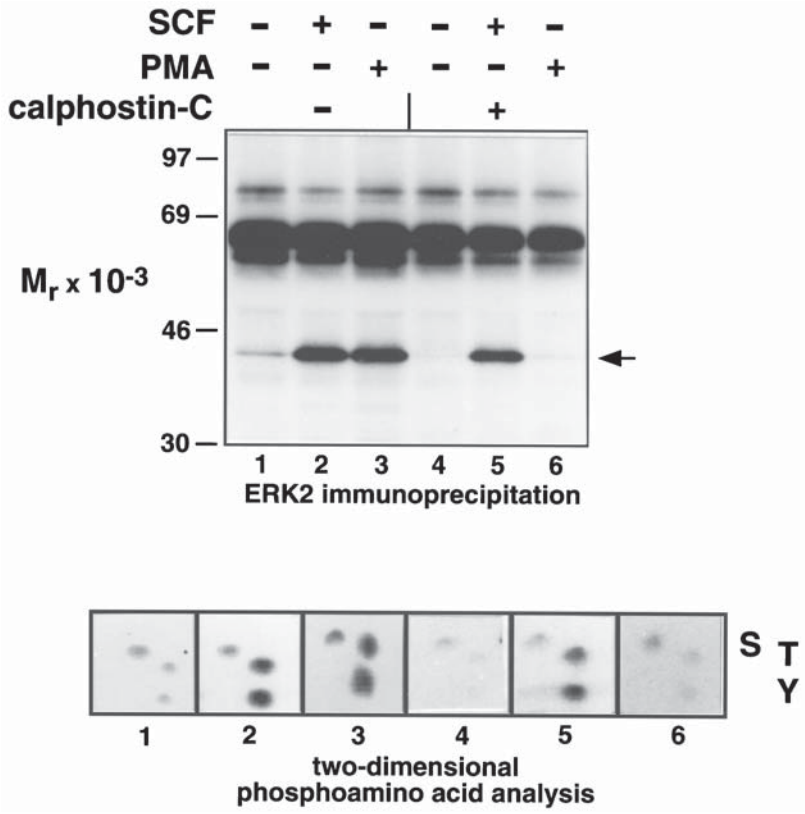

Fig. 4. SCF-stimulated ERK2 phosphorylation. **(A)** ERK2 was immunoprecipitated from Kit-transfected porcine aortic endothelial cells labeled in vivo with [^{32}P]orthophosphate. Proteins were resolved by SDS-PAGE and electrotransferred to a PVDF membrane using a Bio-Rad Trans-Blot apparatus. The filter was exposed on a PhosphorImager and then to film. The arrow indicates p42^{ERK2}. **(B)** ERK2 from each lane was subjected to partial acid hydrolysis and phosphoamino acid analysis, exactly as described in the text. The letters to the right illustrate the relative position of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) on the map. It is evident that SCF stimulates phosphorylation on threonine and tyrosine. (Reproduced from **ref.** ***15*** with permission from the publisher).

4. Notes

1. The same two basic strategies are used to recover radiolabeled proteins before proteolytic digestion for two-dimensional peptide mapping. Both peptide mapping and phosphoamino acid analysis can therefore be performed on parts of the same sample, which is useful whenever quantitation of phosphorylation is important (**Notes 2** and **3**; *see*, e.g., **refs.** ***13*** and ***14***).

2. If a TCA-precipitated protein sample is to be used for both peptide mapping and phosphoamino acid analysis, simply take a part of the ethanol-washed sample containing the desired number of dpm and resuspend it directly in 6 *N* (or constant boiling 5.7 *M*) HCl. Alternatively, it can easily be resuspended in HCl after the performic acid oxidation step. Usually only a smaller fraction is taken for the phosphoamino acid analysis because only very few dpm (<100 dpm) are required for phosphoamino acid analysis.
3. Membrane-bound protein can be used directly for both tryptic digestion and hydrolysis in 6 *N* (or constant boiling 5.7 *M*) HCl, *in situ* ***(10)***. However, the choice of membrane is important. Although nitrocellulose, nylon, and PVDF membrane can all be used for tryptic digestion *in situ*, only PVDF membrane can be used for partial acid hydrolysis because both nylon and nitrocellulose will dissolve in strong acid. Only nitrocellulose can be used for cyanogen bromide cleavage of proteins *in situ*.
4. We recommend to use screw-cap microcentrifuge tubes from Sarstedt and flip-cap microcentrifuge tubes from Sorenson, because protein losses caused by nonspecific binding are smaller with these tubes than with tubes from other manufacturers tested. If phosphoprotein or phosphopeptide losses are unacceptably high despite using these brands of tubes, one can try to reduce losses by coating tubes with the siliconizing agent Sigmacote (Sigma). Simply fill the tubes with Sigmacote, aspirate, and leave them open on the bench to air-dry. Wash two times in deionized water prior to usage (Lincoln R. Potter, personal communication).
5. We prefer to use RNase A as carrier protein because it will degrade contaminating ^{32}P-labeled RNA, which can be present when the protein was isolated from ^{32}P-labeled cells.
6. Usually, proteins, especially high-molecular-weight proteins, are transferred most efficiently with a wet-blotter apparatus, like the Bio-Rad Trans-Blot apparatus, but a semidry blotting apparatus can be used in cases where one has tested beforehand that the protein is transferred efficiently that way. Transfer times are shorter and 10 times less buffer is required than for a wet blotter.
7. Most proteins will transfer efficiently to membrane in transfer buffer without SDS, but sometimes high-molecular-weight proteins are transferred poorly. Addition of SDS will usually lead to better transfer in these cases.
8. Optimal transfer conditions have to be determined empirically for each protein. Make sure to cool the apparatus during fast transfers by either blowing on it with a fan or by performing the transfer in the cold room.
9. When one is separating phosphoamino acids obtained by partial acid hydrolysis of a ^{32}P-labeled protein obtained by in vitro labeling, and it is in a relatively pure form, one gets adequate resolution by electrophoresis in one dimension at pH 3.5. Only released free phosphate and phosphopeptides are the major contaminating labeled species in the sample, and they separate well from the phosphoamino acids. This has the advantage that one can analyze up to 20 samples at the same time by spotting them in a row near one edge of the cellulose plate.

10. Empirically, the phosphoamino acids are found to be better concentrated, and less streaking is seen when electrophoresis in the first dimension is done in a direction parallel to that in which the cellulose was poured onto the plate.
11. Particulate matter or debris is the main source of streaking and poor resolution of phosphoamino acids separated by two-dimensional electrophoresis.
12. The times and voltages given for electrophoresis are for the HTLE 7000 system. We have found that the quality of each batch of cellulose plates varies slightly; therefore, the indicated electrophoresis times and voltages for first- and second-dimension electrophoresis may have to be adjusted for every new batch that is used. Choose electrophoresis conditions so that the migration of the free [^{32}P]phosphate will be confined to the quadrant reserved for each sample (**Fig. 1**).

Acknowledgments

The methods outlined above are the cumulative effort of numerous people who are, or have been, working in the Molecular Biology and Virology Laboratory at The Salk Institute. Special thanks go to Jill Meisenhelder and Lincoln R. Potter for helpful comments, and Lars Rönnstrand, Ludwig Institute for Cancer Research, Uppsala, Sweden, for numerous discussions about technical improvements and tips on phosphopeptide mapping and phosphoamino acid analysis. P.B.-J. is a Special Fellow of The Leukemia and Lymphoma Society of America. T.H. is an American Cancer Society Professor.

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5

Two-Dimensional Phosphopeptide Mapping of Receptor Tyrosine Kinases

Jeremy M. Tavaré and Tarik Issad

1. Introduction

A bewildering array of receptor and nonreceptor protein tyrosine kinases have been uncovered during the past two decades. During this time it has become clear that the autophosphorylation of these protein kinases on tyrosine residues has two major effects on their function. First, the phosphorylation event often enhances the activity of the protein kinase such that it may then phosphorylate other intracellular substrates on tyrosines; this is the case for the insulin receptor and pp60$^{c\text{-}src}$, for example. However, a more widespread role among the tyrosine kinases is the generation of a phosphotyrosine binding site for effector proteins that contain either src homology 2(SH_2) or "Phospho Tyrosine Binding" (PTB) domains. It is these latter effector proteins that then further transmit the signal into the cell interior ***(1,2)***.

As a result of complex modes of regulation, tyrosine kinases are, by necessity, phosphorylated on multiple tyrosine residues. Indeed, many (if not all) receptor and nonreceptor tyrosine kinases are also phosphorylated on serine and threonine residues. The role of these latter phosphorylations is considerably less well known than for the tyrosine phosphorylation events but, at least in the case of the epidermal growth factor (EGF) receptor, they may be involved in downregulation of the signal ***(3)***.

Various methodologies have been developed that allow the experimenter to examine the state of phosphorylation of individual sites on protein kinases and protein substrates. These include digestion of the protein with trypsin (or other protease or chemical methods) and separation of peptides by Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (useful for

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

peptides of 1–100 kDa; *see* **ref. *4***), reverse-phase high-performance liquid chromatography (HPLC), or thin-layer chromatography. Here, we will restrict ourselves to the use of the latter - two-dimensional phosphopeptide mapping. We will discuss its application to the study of the phosphorylation of receptor tyrosine kinases in intact cells.

Two-dimensional phosphopeptide mapping is arguably the most sensitive of the various methodologies, allowing the detection of only very small amounts (a few cpm) of ^{32}P-labeled material, an important consideration when studying receptor tyrosine kinases that are not abundant proteins. It requires relatively inexpensive equipment, but does need particularly good sample preparation. With knowledge of the primary sequence of a particular protein, it can be used to identify precisely individual phosphorylation sites (based on the charge and relative hydrophobicity of the peptides), but more often than not this requires additional experimental manipulation of the isolated peptides.

In this chapter, we discuss, first, how to metabolically label your protein of interest both in vivo and in vitro. We will then outline the most common approaches for the isolation and generation of phosphopeptides by various digestion techniques, with emphasis on the use of trypsin. Finally, we discuss the separation of phosphopeptides, as well as their characterization and identification. Our discussion is biased by our interest of the insulin receptor tyrosine kinase, but can be modified for use with other systems.

2. Materials

Unless otherwise stated, all chemicals are of reagent grade.

2.1. Metabolic Labeling

1. Radiolabeled inorganic phosphorus (*Pi*) can be obtained from Amersham International plc or NEN at 370 MBq/mL (10 mCi/mL).
2. Phosphate-free Dulbecco's modified Eagle's medium (DMEM) can be purchased as a custom-made medium from Life Techologies (usually requires 4–6 wk notice).
3. Cell lysis buffer: For cultured cell lines — 50 m*M* HEPES, pH 7.4, 1 m*M* Na_3VO_4, 10 m*M* NaF, 30 m*M* tetra-sodium pyrophosphate, 2.5 m*M* benzamidine, 0.5 m*M* phenylmethyl sulphonyl fluoride (PMSF), 1 μg/mL each of peptstatin, antipain, and leupeptin and 10 m*M* ethylenediaminetetracetic acid (EDTA) supplemented with 1% (v/v) or 0.1% (v/v) Triton X-100 (*see* **Note 1**). Often, it is best to make up a solution without the vanadate, adjust the pH to approx 7.2, and then add the vanadate as a solid. The solution pH will readjust to pH 7.4 as the vanadate dissolves. This can prevent problems associated with changes in the oxidation state of the vanadate on exposure to concentrated acids/alkalis. This solution is stable for at least 1 mo at 4°C. The PMSF has a short half-life in aqueous solution and should be added just prior to use.

4. Protein A-Sepharose: 2.5 mg protein A-Sepharose (Sigma) is preswollen by incubation in 100 μL of cell lysis buffer. After letting stand for 5 min at 4°C, the mixture is centrifuged for 30 s at 10,000*g* and the supernatant removed. The procedure is repeated. Resuspend the final pellet in 50 μL cell lysis buffer and add the appropriate amount of antibody. Store at 4°C until required.
5. Collagenase for liver or adipose cell preparation can be obtained from Boehringer (for hepatocytes) or Sigma (for adipocytes). Bovine serum albumin (BSA) is from Sigma.
6. 2× concentrated cell lysis buffer: For freshly isolated cell suspensions — 100 m*M* HEPES, pH 7.6, 2% Triton X-100, 20 m*M* NaF, 60 m*M* tetrasodium pyrophosphate, 4 m*M* benzamidine, 2 m*M* $Na_3 VO_4$, 2 m*M* PMSF, and 2 μg/mL each of pepstatin, antipain, and leupeptin.
7. Kinase reaction buffer: 40 m*M* (3-[N-Morpholino] (MOPS) propane sulfuric acid), pH 7.4, 2 m*M* $Na_3 VO_4$, 24 m*M* $MgCl_2$, 4 m*M* $MnCl_2$. Can be stored at 4°C for at least 1 mo.

2.2. SDS-PAGE and Tryptic Digestion

1. SDS-PAGE sample buffer: 125 m*M* Tris-Cl, pH 7.5, 100 m*M* dithiothreitol (DTT), 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, 20% (w/v) sucrose.
2. Transfer buffer: 25 m*M* Tris-Cl, 190 m*M* glycine, 20% methanol.
3. Transfer membranes: Nitrocellulose is obtained from Schleicher and Schuell. Immobilon membrane is obtained fromMillipore.
4. Electroelution buffer: 20 m*M* Tris-Cl, pH 8.0, 0.1% SDS, 0.1% 2-mercaptoethanol, 2 m*M* EDTA (make up fresh).
5. Trypsin digestion buffers: Use either (1) 50 m*M* ammonium bicarbonate, pH 8.2 (make up fresh on the day), or (2) 50 m*M* N-ethyl morpholine, pH 8.2 (can be stored for up to 1 mo). The latter buffer generally results in less residue after lyophilization of peptides.
6. Trypsin: Some sources of trypsin are contaminated with protein phosphatase activity. For this reason we routinely use sequencing grade To Sylphenylalanyl-Chloromethane (TPCK)-treated trypsin from Boehringer (cat. no. 1 418 475). The trypsin is supplied TPCK-treated to remove contaminating chymotryptic activity.

2.3. Two-Dimensional Separation

1. Thin layer apparatus: Pharmacia LKB Multiphor flat-bed apparatus.
2. Thin layer chromatography plates: Eastman-Kodak (cat. no. 13255) 20 cm × 20 cm.
3. Markers: Freshly made up 10 mg/mL DNP-lysine (Sigma), 10 mg/mL xylen cyanol FF solution (Sigma) in 50% ethanol.
4. Whatman 3M paper.
5. First-dimension separation buffers (electrophoresis): For a pH 1.9 separation use pyridine/glacial acetic acid/H_2O (10:100:890 v/v). For a pH 3.5 separation use pyridine/glacial acetic acid/H_2O (5:50:945 v/v). Use deionized or MilliQ water,

and only a recently opened bottle of analytical grade pyridine (pyridine absorbs CO_2 and can change pH on storage). Make all buffers up fresh.
6. Second-dimension separation buffer (ascending chromatography): pyridine/acetic acid/butanol/H_2O (60:18:90:72 v/v). This can be stored in a tightly sealed glass chromatography tank for at least 1 wk, depending on how many times it is used and how good the seal on the tank is (*see* **Subheading 3.**).
7. Chromatography tank: A standard glass chromatography tank sufficient to take two 20 cm × 10 cm thin-layer plates is suitable. Ensure that the glass top has a good seal. It is advisable to use a small amount of silicone oil or vaseline to ensure an air-tight seal is maintained.

3. Methods

3.1. In Vivo Labeling of Receptor Tyrosine Kinases in Cultured Adherent Cells

In general, because of the low specific radioactivities of adenosine triphosphate (ATP) that can be achieved in intact cells, a cell type should be chosen that expresses at least 20,000 receptors per cell. However, this estimate will vary considerably between cell lines and is also governed by other factors, such as the efficiency of the method of receptor isolation (*see* below) and the number of phosphorylation sites present.

3.1.1. Metabolic Labeling

1. Plate cells in 60-mm culture dishes and grow to confluence.
2. Wash cells twice with 5 mL serum- and phosphate-free DMEM.
3. Incubate cells in 2 mL of serum- and phosphate-free DMEM supplemented with 1 mCi (37 MBq) of radioactive [^{32}P]P_i (*see* **Note 2**).
4. Incubate for 2–4 h at 37°C and 5% CO_2 (*see* **Note 3**), and then stimulate the cells with ligand as required (usually 1–5 min for maximal tyrosine phosphorylation of the insulin receptor) (*see* **Note 4**).
5. Remove the radioactive medium by aspiration with a Pasteur pipet linked to a vacuum line, and scrape the cells into 0.5 mL of ice-cold cell lysis buffer supplemented with 1% (v/v) Triton X-100 using a rubber policeman (a piece of rubber bung, 12 mm × 10 mm × 3 mm, with a straight edge, and attached to a syringe and needle is usually sufficient).
6. To extract the cells efficiently, pipet the lysate repeatedly through a 1-mL pipet tip about 3–five times.
7. Incubate the lysates on ice for 5 min and then centrifuge at 10,000*g* for 5 min to pellet insoluble (generally cytoskeletal) material.

3.1.2. Immunoprecipitation of Receptors from Cell Lysate

At all stages during receptor isolation, it is crucial to keep the samples at 4°C and to carry out all procedures as rapidly as is safely possible.

1. Add 500 μL of cell lysate to a predetermined volume of antisera specific to the protein of interest in a 1.5-mL Eppendorf tube also containing 2.5 mg protein A-Sepharose (or protein G-Sepharose depending on the antibody species and subtype).
2. Wrap the tube in Parafilm and tumble end-over-end for 2 h at 4°C. Tumbling can be achieved either by encasing the tubes in lead pots and taping this to a "paddle-stirrer" or by putting the tubes into a commercially available blood-tube rotator (e.g., from Stuart Scientific).
3. Centrifuge at 10,000*g* and 4°C for 5 s.
4. Remove the supernatant with a 5-mL syringe and 23-gage needle (take care not to remove any Sepharose pellet).
5. Discard the supernatant and wash the pellet twice with 1 mL ice-cold cell lysis buffer supplemented with 1% (v/v) Triton X-100, and then twice with 1 mL ice-cold cell lysis buffer supplemented with 0.1% (v/v) Triton X-100 (this helps the final elution of protein from the pellet).
6. Elute the ^{32}P-labeled proteins from the protein A-Sepharose by boiling with 10 μL sample buffer for 2 min. Then add 90 μL water and boil for a further 3 min.
7. Save the supernatant and reboil the pellet with an additional 100 μL of water.
8. The supernatant from **steps 5** and **6** are combined and lyophilized (using a Speedvac concentrator) down to a volume small enough to load onto an SDS-PAGE gel (usually approx 20 μL).

3.2. In Vivo Labeling of Freshly Isolated Cells in Suspension

The use of cultured cells presents many advantages including analysis of receptor phosphorylation after site-directed mutagenesis (*see* **Subheading 3.7.**), or the possibility of cotransfecting the cells with other components of the signaling pathway studied. However, it is also important to be able to analyze receptor tyrosine kinases in a more physiological context where all the natural regulatory proteins, for instance, kinases or phosphatases that control the phosphorylation state of the receptor, are present in a more natural proportion. Moreover, in such target cells it is also possible to correlate the phosphorylation status of a receptor with a measure of the relevant biological effects of the ligand in the cell type studied.

In general, cells isolated from tissues express considerably lower levels of receptor than is possible by transfection of cultured cells or in transformed cell lines. This makes phosphopeptide mapping considerably more challenging as the levels of ^{32}P incorporation are much lower; hence, large amounts of [^{32}P]Pi phosphate must be used (up to 20 mCi per experiment).

Because of the large amounts of radioisotope required, good screening of the worker is imperative. We have developed a procedure in which most of the radioactivity throughout the cell incubation, extraction, and the immunopre-

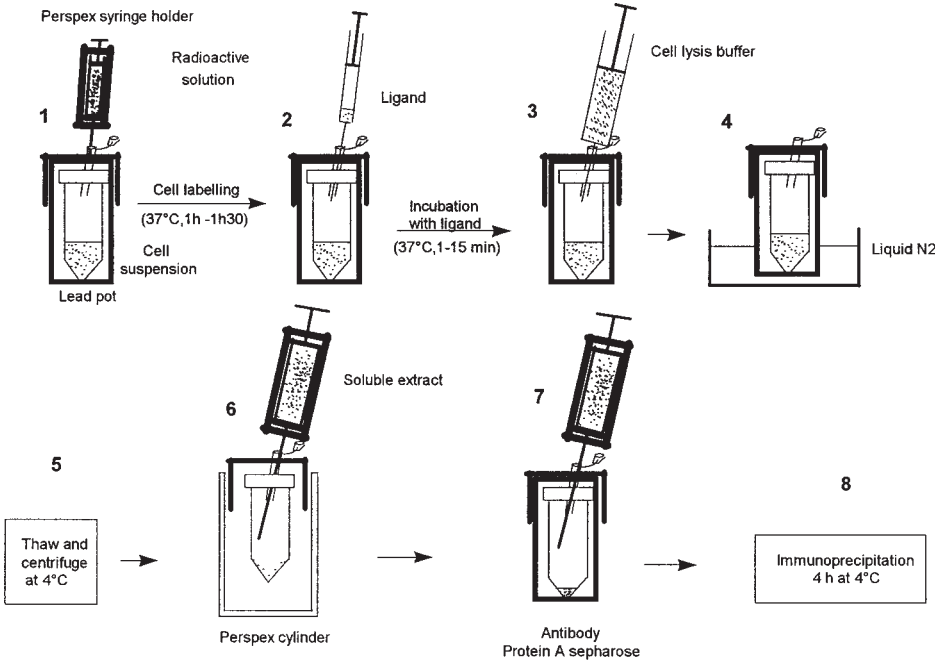

Fig. 1. Apparatus for the incubation of suspended cells in radiolabeled ^{32}P-phosphorus. *See* text for details.

cipitation steps is kept inside special chambers made of lead or Perspex. The details of the procedure are given in **Fig. 1** (*see* **Note 5**).

We have performed these experiments in hepatocytes ***(5)*** and adipocytes ***(6)***, but the procedure described here can be easily adapted for other cell types.

3.2.1. Metabolic Labeling

1. Prepare freshly isolated hepatocytes and adipocytes by collagenase digestion in Krebs–Ringer buffer using standard methods ***(7,8)***.
2. Resuspend hepatocytes (30×10^6 cells in 2 mL) or adipocytes (lipocrit 10% in 6 mL) in low phosphate (0.2 m*M*) Krebs-Ringer buffer containing 5 m*M* HEPES, 2 m*M* $CaCl_2$ and 2%–4% defatted BSA.
3. Cells are placed into 50-mL Falcon plastic tubes. These tubes are placed into lead pots and will remain in these pots throughout the incubation procedures (*see* **Fig. 1**).
4. Access to the cells is made possible through a 3–4-mm diameter hole in the top of the lead pot and in the top of the plastic tube. In this hole, a Beckman tube is inserted. The bottom of the Beckman tube is cut allowing syringe needles to be introduced for gassing the cells, adding the radioactive solution, the ligands, and

the cell lysis buffer. The Beckman tube can be closed after each of these operations with its original top.

5. Add [^{32}P]Pi to the incubation medium to give 1–2 mCi/mL (**step 1** in **Fig. 1**) using a syringe screened by a Perspex holder. After gassing each vial for 1 min with 5% O_2/95% CO_2, incubate the cells for 1–2 h (*see* **Note 6**) at 37°C in a shaking water bath and stimulate the cells with ligands as required (**step 2** in **Fig. 1** and *see* **Note 7**).
6. Cells are extracted by adding an equivalent volume of 2 × concentrated cell lysis buffer (**step 3** in **Fig. 1**). The incubation vials are then rapidly frozen in liquid nitrogen (**step 4** in **Fig. 1**) and can either be stored at –20°C for 24–48 h or thawed immediately for subsequent immunoprecipitation of the receptors.

3.2.2. Immunoprecipitation of Receptors from Cell Lysate

1. Centrifuge the lysate for 10 min (10,000*g* at 4°C) and rapidly transfer the vials to a Perspex cylinder (*see* **Note 8**, **step 6** in **Fig. 1**). Remove the soluble extract with a syringe needle, leaving the insoluble material in the incubation vial.
2. The soluble extract obtained (4–10 mL) is incubated for 4 h with an appropriate antiserum and 10 mg protein A-Sepharose. After centrifugation for 1 min (10,000*g*, 4°C), the vials are once again transferred into a Perspex cylinder and the supernatant is discarded. The amount of radioactivity remaining is now fairly low and the antibody-Sepharose pellet can be resuspended in 1 mL of ice-cold cell lysis buffer and transferred into 1.5-mL Eppendorf tubes for subsequent washes and elution of the ^{32}P labeled proteins as described in the previous section.

3.3. In Vitro Labeling of Tyrosine Kinases

Receptor tyrosine kinases, and any other tyrosine kinase for that matter, can be partially purified (e.g., by using wheat germ lectin chromatography or immunoprecipitation from a cell lysate with specific antisera). These receptor preparations can be incubated in vitro in the presence of radiolabeled ATP and subjected to phosphopeptide mapping. This technique sometimes allows much greater incorporation of ^{32}P into the protein and thus the production of improved peptide maps. However, it should be noted that some sites phosphorylated in vitro may well not be physiologically relevant in vivo tyrosine phosphorylation sites (e.g., **ref. *9***).

The method below provides assay conditions, which are suitable for many receptor and nonreceptor tyrosine kinases. However, the amount of protein incubated and appropriate concentrations of Mn^{2+} and Mg^{2+} will require some initial characterization.

1. Preincubate 20 µL of partially purified receptor, or receptor attached to antibody-coated protein A-Sepharose beads, with 20 µL kinase reaction buffer for 5 min at 30°C.
2. Add 5 µL of [γ-^{32}P]ATP (try 100 µ*M* final concentration and 200 cpm/pmol as a starting point) and incubate for between 5 and 30 min at 30°C.

3. Add 10 μL SDS-PAGE sample buffer and boil for 5 min to stop the reaction.
4. Process the samples by SDS-PAGE as described below.

3.4. Isolation of ^{32}P-Labelled Proteins from SDS PAGE Gels and Tryptic Digestion

Proteins are separated by SDS-PAGE using standard techniques. For the best results, we would recommend using a Bio-Rad Miniprotean II gel with 1.5-mm thick spacers and 10-well combs. After separation, keep the gels hydrated, but do not stain or destain the gels as this prevents subsequent elution of proteins and peptides.

The aim is to identify the band corresponding to the ^{32}P-labeled receptor for subsequent elution. This can be achieved by one of three methods. The first method is perhaps the most suitable, as it avoids copurification of contaminants and generation of nonvolatile ammonium salts. These salts can adversely affect the migration of peptides by thin-layer electrophoresis.

3.4.1. Transfer to Nitrocellulose and Digestion

1. Electrophoretically transfer the proteins to nitrocellulose membrane using either a semidry transfer apparatus, or using a Transblot Cell™, (both are manufactured by Bio-Rad Laboratories). The membranes are placed up against the gels which are, together sandwiched between three layers of Whatman 3M filter paper, soaked in transfer buffer.
2. If using a semidry transfer apparatus, perform the transfer for 30 min at 15 V and at 20°C. If using a Transblot Cell, the same sandwich setup is followed, but the tank is submersed in 2 L of transfer buffer and the transfer is performed for 2.5 h at 90 V and at 4°C.
3. Wrap the membrane in a plastic bag to keep moist (a simple, domestic bag sealer is adequate for this purpose). Do not allow membrane to dry out at any time.
4. Identify the ^{32}P-labeled band of interest by autoradiography and cut out with a razor blade. Keep the size of membrane as small as possible.
5. Place the membrane in a 1.5-mL Eppendorf tube with 100 μL of 50 m*M* ammonium bicarbonate, pH 8.2, or 50 m*M* N-ethyl morpholine, pH 8.2. In both cases, it is advisable to add acetonitrile (to 10% v/v) to prevent the peptides sticking to the membrane. Add 10 μL of a freshly prepared 1 mg/mL stock of TPCK-trypsin.
6. Incubate for 16 h at 30°C.
7. Add an additional 10 μL of a freshly prepared stock of 1 mg/mL TPCK-trypsin and incubate for a further 5 h at 37°C.
8. Save the eluate, wash the membrane with 200 μL of 5% acetonitrile, 0.1% trifluroacetic acid (TFA), and pool the eluates.
9. Count the pooled eluates and residual membrane by Cerenkov counting (*see* **Note 9**).

10. Lyophilize the eluate in a Speedvac concentrator. Resuspend in 100 μL water and relyophilize. Repeat at least twice, or until the white residue has fully reduced in size (*see* **Note 10**).

3.4.2. Elution by Digestion

1. Identify the area corresponding to the ^{32}P-labeled protein by sealing the gel in a plastic bag (a simple, domestic bag sealer is adequate for this purpose) followed by autoradiography or exposure to a PhosphorImaging screen.
2. Cut out the band of interest and finely mince the gel chip with a razor blade.
3. Bake the gel chip on a glass microscope slide for 1 h at 70°C. Reconstitute the gel pieces in 1 mL of 50 m*M* NH_4HCO_3, pH 8.2, and place in a 1.5-mL Eppendorf tube. Add 100 μg TPCK-treated trypsin (100 μL of a 1 mg/mL freshly prepared stock). Incubate at 30°C for 16 h.
4. Add an additional 100 μg TPCK-trypsin and incubate at 30°C for a further 6 h.
5. Recover the eluate. Count the eluate and residual gel pieces by Cerenkov counting (*see* **Note 11**).
6. Lyophilize the elute in a Speedvac concentrator. Resuspend in 100 μL water and relyophilize. Repeat this process at least three times or until the white residue (nonvolatile ammonium salts) does not reduce in volume any further (*see* **Note 10**).

3.4.3. Elution by Electrophoresis (Electroelution)

1. Take the isolated wet gel slice from **step 2** above, mince, and place it in a dialysis bag with 500 μL of electroelution buffer.
2. Place the dialysis bag in a Bio-Rad Transblot Cell, immersed in 2 L of electroelution buffer.
3. Electrophorese at 50 mA, 10 V for 16 h at room temperature.
4. Reverse the current for the final 5 min of the elution to recover protein that adheres to the dialysis sac.
5. The eluate is saved and the remaining gel chips washed with 100–200 μL of water.
6. The eluates from **step 5** are pooled and centrifuged for 5 min at room temperature to remove residual gel chips. Count the eluate and remaining gel pieces by Cerenkov counting to check elution efficiency (*see* **Note 12**).
7. Lyophilize the supernatant to a volume of 200 μL.
8. Incubate with 800 mL acetone at –80°C for 60 min. Use an acetone stock that is stored at –20°C.
9. Thaw the sample at room temperature for approx 10 min, or until all precipitated SDS has redissolved.
10. Precipitate the protein by centrifugation at 10,000*g* for 10 min at room temperature.
11. Lyophilize the pellet in a Speedvac and resuspend in a total volume of 100 mL 50 m*M* ammonium bicarbonate, pH 8.2, or 50 m*M* N-ethyl morpholine, pH 8.2, as appropriate.

12. Add 10 μg TPCK-trypsin (i.e., 10 μL of a 1 mg/mL freshly prepared solution in ammonium bicarbonate or N-ethyl morpholine) and incubate for 16 h at 30°C.
13. Add a further 10 μg TPCK-trypsin and continue incubation at 30°C for another 5 h.
14. Boil the digest for 5 min and lyophilize to dryness in a Speedvac concentrator.
15. Resuspend the peptides in 100 μL of water and lyophilize. Repeat the latter procedure at least twice, or until the white residue does not reduce in volume (nonvolatile ammonium salt) (*see* **Note 10**).

The TPCK-trypsin can be replaced by any other protease, e.g., V8 protease, Lys-C, chymotrypsin, and so on. However, the aforementioned methods have been characterized for the use of trypsin and may require some adaptations for other digestion protocols (*see* **Note 13**).

3.5. Two Dimensional Separation of ^{32}P-Phosphopeptides

1. Resuspend the lyophilized tryptic phosphopeptides in 8–10 μL of MilliQ H_2O. Subject to a 5-s spin in a microfuge to ensure the sample is at the base of the tube (*see* **Note 14**).
2. Make a template, on a 20 cm × 20 cm acetate sheet (or other clear plastic sheet) using the example shown in **Fig. 2**. Mark the positions of the sample origin of application with a marker pen. (Note these positions differ depending on the pH used for separation.)
3. Place a thin-layer cellulose chromatography plate (Eastman-Kodak; cat. no. 13255, 20 cm × 20 cm) over the template, and place this on a light box. Carefully tape the corners to prevent movement of the plate relative to the template during sample application.
4. Using a 10-μL glass Hamilton syringe or a 2 μL Gilson pipet, spot approx 1-μL aliquots of sample onto the cellulose chromatography plate such that the applied spot does not exceed 5–7-mm diameter. A gentle stream of warm (but not hot) air from a hair drier can be blown across the sample to aid drying.
5. Apply 1 μL of a solution of 10 mg/mL DNP-lysine (Sigma) plus 10 mg/mL xylene cyanol FF (Sigma) in 50% ethanol, over the top of the sample as internal markers.
6. Cover the plate with two pieces of Whatman 3M chromatography paper such that they address the sample approx 1 cm away on both sides (*see* **Fig. 3**). Using a 10-mL syringe, apply electrophoresis buffer to the filter paper such that it soaks through and wets the plate underneath. Do not apply excess buffer. When the filter paper is completely wet, the buffer will begin to advance from the edge of the filter paper and meet the sample.
7. To ensure that the sample does not diffuse when the buffer reaches it, make sure that buffer approaches from both sides simultaneously. Remove the filter papers and gently remove excess buffer from the plate surface with a tissue.

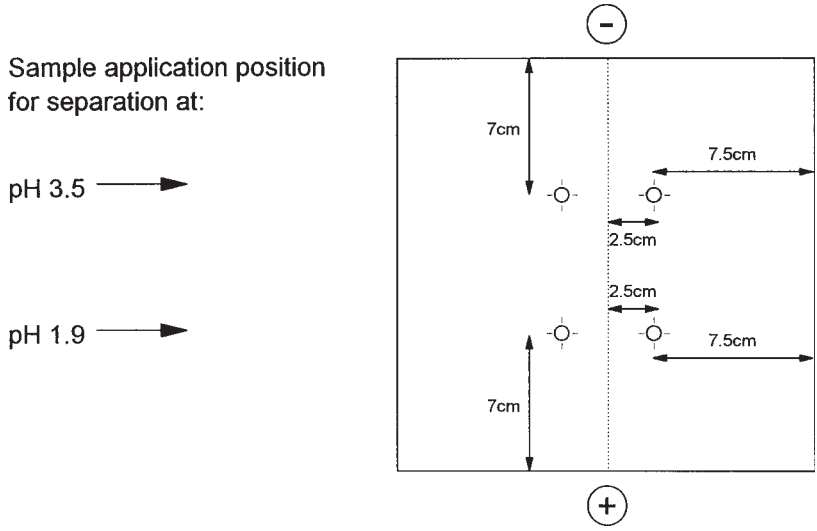

Fig. 2. Template for the application of samples to thin-layer chromatography plates. A transparent plastic sheet (e.g., acetate or overhead projection sheet) is cut to 20 cm × 20 cm. A series of circles is drawn, as indicated, using a permanent marker pen. This is placed over a light box and under the thin-layer chromatography plate, to allow application of the sample.

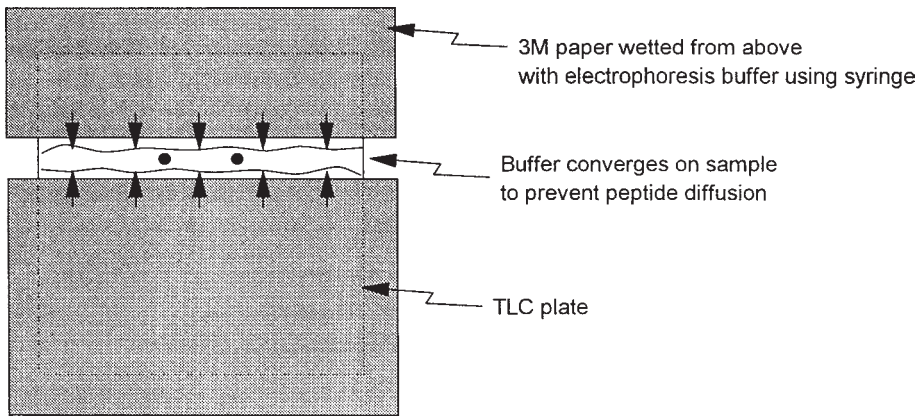

Fig. 3. Use of Whatman 3M wicks to apply chromatography buffer to the thin-layer plate. Two pieces of Whatman 3M paper are cut to the approximate size indicated, and placed gently onto the surface of the plate. The chromatography buffer is slowly applied using a syringe and gentle pressure, at the edges of the wicks closest to the region of sample application (two small black circles). The solvent front will then approach the samples simultaneously from both directions thus preventing diffusion of the sample into surrounding cellulose.

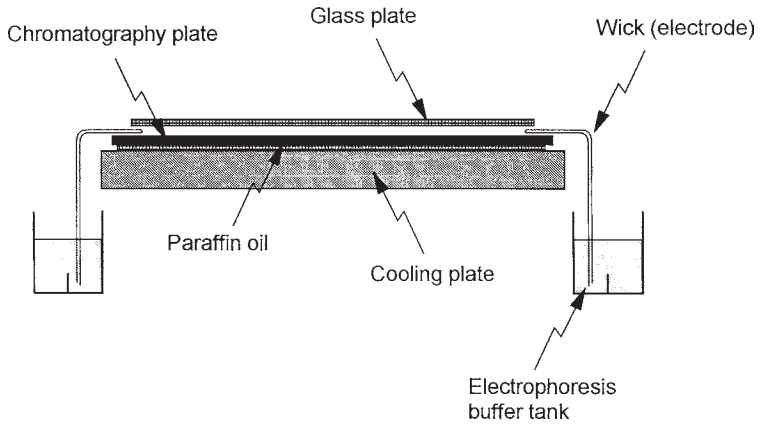

Fig. 4. Setting up the first dimension. A thin layer of paraffin oil is placed between the cooling plate and the thin-layer plate. This allows efficient cooling and thus prevention of condensation build up. The wicks are placed as shown (doubled up and soaked in electrophoresis buffer), and over the top of this is positioned a glass plate with firm downward pressure. The latter provides a humid atmosphere for the plate during separation, and also a firm contact between the electrode and thin-layer plate.

8. Place the thin-layer plate on the cooling surface of the thin-layer electrophoresis tank. Ensure a good contact between the thin-layer plate and the cooling plate using 2–3 mL paraffin oil (apply an even coverage over the cooling plate using a tissue). Ensure that the paraffin does not soak into the cellulose surface of the plate as this is seriously detrimental to the second-dimension separation.
9. Place approx 200 mL electrophoresis buffer in each reservoir and place wicks of Whatman 3M paper (doubled up) presoaked in electrophoresis buffer, to provide electrodes (*see* **Fig. 4**). Place a glass plate over the wicks, gently exerting a downward force, to ensure that a good contact between the plate is made, and that a humid atmosphere is maintained above the thin-layer plate.
10. Electrophoresis is carried out at 400 V until the DNP-lysine has moved approx 2 cm from the origin (*see* **Note 15**).
11. Remove the plate and dry in a fume cupboard overnight (*see* **Note 16**).
12. The plate is cut down the middle, bisecting the two areas of sample application (*see* **Fig. 2**).
13. Place each plate in a chromatography tank preequilibrated with 120 mL of second-dimension buffer (i.e., should be approx 1 cm depth of buffer (*see* **Note 17**).
14. The chromatographic dimension is run until the solvent front is 1 cm from the edge of plate. This usually takes approx 1 h.
15. Dry the plate for at least 2–3 h in a fume hood. This should be sufficient time to remove residual pyridine.

16. Autoradiograph the plate or subject to analysis using a PhosphorImager.

3.6. Recovery of Peptides and Further Analysis

Peptides can be recovered from the plate for further analyzes including phosphoamino acid analysis, HPLC, or digestion with alternative proteases.

1. Mark the position of the thin-layer plate relative to the autoradiograph. Score a line around the peptide of interest with a fine needle. Scrape the cellulose off this region of the plate using a small metal spatula and onto a piece of paper.
2. Place the cellulose into a 1.5-mL Eppendorf tube.
3. To elute the peptide, first estimate the approximate Rf of the peptide in the second dimension. Assuming the Rf of DNP-lysine to be 1.0, if the Rf of the peptide is <0.75 it is likely that incubation of the cellulose with 0.5 mL water will be sufficient to elute the peptide. With a peptide of Rf>0.75, try using a solution of 30% (v/v) acetonitrile in water. Incubate under the chosen conditions for 5 min at room temperature. 0.1% TFA can be included in the buffers to aid elution.
4. After incubation, pellet the cellulose by centrifugation for 1 min at 10,000*g* (*see* **Note 18**).
5. Lyophilize the eluate and wash the residue three times with 100 µL water to remove all volatile material.

The peptide sample can now be resuspended in buffer for further protease digestion (e.g., *see* below) or in 6 *M* HCl for phosphoamino acid analysis. Alternatively, the peptides can be further purified by reverse phase HPLC. The latter step is important if you wish to sequence the peptides, as contaminants appear to be present in the cellulose that interfere with sequencing. It should also be noted that the recovery of peptides at this stage can be quite low when compared to what was originally applied to thin-layer plate.

3.7. Identification of Peptides

Sequencing of peptides eluted from the plates may be performed to identify which sites in the protein correspond to which tryptic peptides present in the maps. However, as the amounts of peptide present on the plates are usually limiting (femtomoles), and the cellulose plates often carry contaminants that interfere with the sequencing reactions, sequencing is often not feasible.

There are two alternatives to sequencing peptides. One is to perform manual radio-sequencing. A detailed discussion of the methodology for this is beyond the scope of this chapter, however, the methods have been described elsewhere ***(10)***. A second approach is to compare the experimentally determined migration of a tryptic peptide with that predicted for tryptic peptides derived by inspection of the known primary amino acid sequence. The success of this approach depends on the size of the protein and the number of phosphorylated residues. To further aid identification, the migration can be compared before

and after digestion with another protease, such as *S. aureus* V8 protease or Glu-C (*see* **Note 19**), which cleaves on the C-terminal side of glutamate, or cyanogen bromide, which cleaves at methionine residues.

3.7.1. Identification of Peptides Based on Charge and Hydrophobicity Predictions

To predict the charge on a peptide:

1. Begin by listing the predicted tryptic peptides generated by cleavage of the protein. Include only those peptides that possess potential phosphorylation sites. Assume trypsin to completely cleave at all lysine and arginines, but remember that some Basic-Basic sequences may not be efficiently cleaved, nor will basics close to a phosphorylation site (as the negative charge disrupts trypsin binding and cleavage). Basic residues followed by a proline are also poorly cleaved.
2. For the pH used for the separation, summate the charges on the side chains, and the N-terminal amine and C-terminal carboxylate according to the rules set out in an excellent review on this topic by Boyle et al. ***(11)***. In summary:
   a. At pH 1.9, the charges on the N-terminal amino group, plus side chains of Arg, His, and Lys are +1. At this, pH the charges on P-Ser, P-Thr, and P-Tyr are –1. All other charges are neutral (except sulfated Cys which has a charge of –1, if performic acid oxidation was performed).
   b. At pH 3.5, the charges on the N-terminal amino group, plus side chains of Arg, His, and Lys are again +1. However, the charge on the C-terminal carboxylate is now approx –0.5. Whether Glu and Asp have a charge at this pH is debatable, but probably depends on the context of the peptide; we generally consider them to be essentially neutral at this pH. The charges on P-Ser, P-Thr, and P- Tyr are all –1. Again, all other charges are neutral (except sulfated Cys, which will have a charge of –1 if performic acid oxidation was performed).
   c. These rules may not be strictly followed. For example, electroendosmosis will cause a gradual migration of neutral molecules toward the cathode. For this reason, the migration of your peptide of interest should be compared to that of one of the internal markers DNP-lysine (which is neutral at pH 1.9 and 3.5) and xylene cyanol FF (which has a charge of –1 at both these pH s).
4. Prediction of the migration of the peptide in the chromatographic dimension is even more fraught with problems. The extent of migration obviously depends on its hydrophobicity. A value for the Rf of a peptide can be generated by comparing the extent of migration of the peptide of interest with that of DNP-lysine. An approximate comparison of experimentally determined and predicted hydrophobicities can be performed using a table of individual Rf values for the constituent amino acids given in ***(11)***. However, again it should be stressed that gives only a very approximate prediction, as many factors affect the hydrophobicity of a peptide.
5. Remember, too, that some peptides may be multiply phosphorylated. This will yield a series of related peptides that are phosphorylated on one, two, or more residues.

Often such peptides migrate in a diagonal with the monophosphorylated peptide being the least hydrophilic, and thus migrates furthest in the vertical chromatographic dimension and furthest towards the cathode in the electrophoretic dimension. The most highly phosphorylated peptide is the most hydrophilic and thus runs least far in the chromatographic dimension and furthest towards the anode. A good example of this behavior can be found in the insulin receptor ***(12)***.

6. Some peptides can be singly phosphorylated, at the same residue, but cleaved at different basics by trypsin, especially if these lie close to the site of phosphorylation. For example, in the case of the insulin receptor the sequence YYRK, where both tyrosines can be phosphorylated, can lead to cleavage at either the arginine or at the lysine. This yields two peptides of identical phosphate content (i.e., *bis*-phosphorylated) that differ by the absence (i.e., Y(P)Y(P)R) or presence (i.e., Y(P)Y(P)RK) of the extra lysine. Thus, two *bis*-phosphorylated products appear from the same peptide that differ by a positive charge. Also, the peptide with the extra lysine will be more hydrophilic. Thus, these peptides appear to lie on a diagonal (*see* **ref. *12***).

3.7.2. Site-Directed Mutagenesis

A powerful method for identifying phosphorylation sites is to mutate predicted sites. For example, tyrosines are routinely mutated to phenylalanine, serines and threonines to alanines. Such changes are relatively conservative, and will minimally disrupt protein conformation.

These mutants can be cloned into a mammalian expression vector and transiently overexpressed in a suitable cell line (e.g., COS cells or HEK 293 cells). Phosphopeptide maps of the mutant receptor can then be compared to those of the wild-type for the absence of specific phosphopeptides.

3.7.3. Identification of Peptides Using Antiphosphopeptide Specific Antisera

Another route to confirming the identity of a phosphopeptide is to attempt to raise antisera specific to a phosphopeptide sequence. We have successfully carried out experiments to identify a serine phosphorylation site within the insulin receptor using this method ***(13)***.

1. After tryptic digestion of the protein as in **Subheading 3.4.**, the trypsin must be inactivated by boiling for 5 min, and then addition of 0.5 m*M* PMSF to inactivate residual trypsin.
2. Incubate, by tumbling end-over-end, for 2 h at 4°C, with a predetermined dilution of antiphosphopeptide specific antiserum coupled to protein A-Sepharose in a final volume of 500 µL of 50 m*M* NH_4HCO_3, pH 8.2, or 50 m*M* N-ethyl morpholine, pH 8.2.
3. Centrifuge for 30 s at 10,000*g* to pellet the Sepharose. Retain the supernatant, and wash the pellet twice with 1 mL 50 m*M* NH_4HCO_3, pH 8.2, or N-ethyl morpholine, pH 8.2.

4. The phosphopeptide is eluted by resuspending the Sepharose pellet in 500 μL 1 *M* acetic acid containing 10 μg of an appropriate carrier peptide (the sequence of which does not matter) to prevent sticking of the phosphopeptide to plastic and glass. Tumble end-over-end at room temperature for 30 min.
5. Lyophilize the phosphopeptide eluate and wash three times with water.
6. Subject the phosphopeptide eluate, unbound material (supernatant in **step 3**) and original tryptic digest to two-dimensional thin-layer chromatography as described in **Subheading 3.5**.
7. If the antibody is effective at binding a short tryptic fragment of protein, the phosphopeptide should be removed from the supernatant and appear in the pellet.

4. Notes

1. The Na_3VO_4 is a potent and nonselective tyrosine phosphatase inhibitor. The inclusion of NaF, tetra-sodium pyrophosphate, and EDTA should effectively inhibit most serine/threonine phosphatases. To inhibit proteases a cocktail of benzamidine, PMSF, pepstatin, antipain, and leupeptin (as well as EDTA) is found to be most effective in our laboratories. Sometimes we include okadeic acid and/or microcystin, although this will greatly increase the cost of the buffer with relatively little gain in quality of the data.
2. When radiolabeling cells considerable attention should be paid to protection of the worker from exposure. Most of the hazard involves handling the stock isotope. This should be carried out using forceps, and the radiolabel should be removed with a syringe encased in Perspex (minimum thickness of 1.5 cm). Subsequently, all work must be performed behind a 1.5-cm thick Perspex screen. To avoid direct handling of the 60-mm Petri dishes of cells, we place them within a 70 mm × 70 mm × 15 mm Perspex holder, which has a central 4-mm deep well within which the dish sits. Once the cells have been labeled, and the medium removed, approximately only 10% of the radioactivity remains.
3. During the labeling period, it is essential that the cells are incubated with [^{32}P]P_i for a period sufficient to allow the intracellular ATP-specific activity to label to equilibrium. For a detailed discussion of this aspect, as well as methodology for measuring ATP-specific radioactivity, the reader is referred to Hopkirk and Denton ***(14)*** (*see* **Note 6**). In a minority of cell types (e.g., muscle) insulin can promote a rise in the specific radioactivity of ATP, probably via increasing P_i transport across the plasma membrane; this considerably complicates interpretation of the results.
4. To avoid exposure to radioactive medium, as well as to ensure homogenous mixing of the ligand in the medium, the ligand is usually placed at the very bottom of a 1.5-mL Eppendorf tube. 0.5 mL of incubation medium is removed from the cells, mixed with the ligand in the tube, and returned to the cells for simple mixing by gentle swirling of the dish.
5. It is good practice to perform a trial run of the experiment using nonradioactive medium to identify areas of special difficulty with the technique. Recovery of

the receptors at the end of the nonradioactive experiment can be evaluated by Western blotting with an antiphosphotyrosine antibody or an antireceptor antibody ***(16)***.

6. It is important that steady-state labeling of the cells is achieved. This can be assessed in preliminary experiments, using identical incubation conditions, but using only 60–100 mCi/mL [^{32}P]P_i). After different times of incubation (from 0.5–3 h) the cells are separated from the medium by centrifugation and the rate of uptake of radioactivity by the cells estimated by counting an aliquot of the incubation medium. For liver cells, steady state [^{32}P]P_i uptake is achieved after 45 min of incubation, and remains stable for at least 2 h. The labeled cell pellet can also be analyzed by SDS-PAGE and autoradiography to evaluate the rate at which steady-state protein labeling with [^{32}P]P_i is achieved.
7. The ligands are introduced by the use of a 1-mL syringe. Ligands are generally in a small volume (50–100 μL) to avoid decreases in specific activity of the medium.
8. A transparent Perspex cylinder is necessary in order to be able to see the tube contents while collecting the soluble material.
9. Using this method, we routinely achieve an elution of >90% of the counts from the membrane. For very hydrophobic peptides, a solution of 60% acetonitrile in 0.1% TFA may be preferable for elution at **step 7**.
10. It is essential for two-dimensional separation of peptides that there are no traces of SDS or ammonium bicarbonate left in the sample. If you find that you get large amounts of the white residue left after freeze-drying the tryptic peptides, you should reduce the size of the gel chip to as small as possible. This will prevent salt carry-through. Alternatively, use 50 m*M* N-ethyl morpholine pH 8.2 for the tryptic digestion (less residue carry-over).
11. This is often not the most efficient method for recovery of phosphoprotein from the gel. The efficiency varies from 50–90% depending on the protein and the percentage acrylamide in the gel.
12. We generally get recoveries from 7% SDS polyacrylamide gels of about 90% (Mr < 100,000) and 70–80% (Mr > 150,000).
13. For Glu-C, use 25 m*M* $(NH_4)_2CO_3$, pH 7.8, 5% acetonitrile at 30°C. For Asp-N, use 50 m*M* NaH_2PO_4, pH 7.8, at 37°C. For chymotrypsin, use 100 m*M* Tris-HCl, pH 7.8, 10 m*M* $CaCl_2$ at 37°C. For Lys-C use same buffers as for trypsin, but adjust pH to 7.8.
14. Sometimes the peptides can be oxidised with performic acid (prepared by adding 0.9 mL formic acid and 0.1 mL hydrogen peroxide [30% stock]). Incubate for 60 min at room temperature. We usually do not find that this is a crucial requirement for consistent maps, but may be a help if using DTT during electroelution of peptides from gel chips, or if prolonged periods of vacuum drying are going to be encountered.
15. If the DNP-lysine does not appear to move or smears, do not worry because you have probably applied too much salt with the sample. Often the migration of peptides is unaffected.
16. It is critical to ensure that the plate dries completely before separation by ascending chromatography. If the plates are moist, smearing in the second dimension will be observed.

17. Ensure that the chromatography tank is well equilibrated with buffer before the run. This is best achieved by placing two pieces of Whatman 3M paper on each side of the tank, such that they dip into the buffer at the bottom. Once this paper is saturated with buffer, by capillary action, the tank will be ready to use. Make sure you have a securely fitting top to the tank.
18. The cellulose pellet packs better in the presence of acetonitrile. Take care when removing supernatant not to disturb the pellet. Follow the recovery of peptide by Cerenkov counting the eluate and remaining cellulose. Repeat the procedure until 80–90% of counts are eluted.
19. To cleave with *S. aureus* protease V8, add to the peptide 50 µL of 10 µg/mL preparation of V8 and incubate for 16 h at 30°C in 50 m*M* NH_4HCO_3 pH 7.8. Add a further 50 µL of freshly prepared 10 µg/mL V8 for 5 h at 30°C. Lyophilize and separate by one-dimensional thin-layer electrophoresis (at either pH 1.9 or 3.5) in parallel with a sample of the noncleaved peptide.

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6

Identification of the Sites of Phosphorylation in Proteins Using High Performance Liquid Chromatography and Mass Spectrometry

A. Grey Craig

1. Introduction

After incubation of cells with ^{32}P-labeled inorganic phosphate, it is possible to identify in vivo radiolabeled phosphoproteins. Generally, after the cells are lysed the phosphoprotein can be separated by sodium dodecyl sulfate (SDS) gel electrophoresis and a rough estimate of the size of the phosphoprotein can be gained. In order to determine the phosphorylated residues in the protein, the radiolabeled band can be transferred to a membrane, hydrolyzed with trypsin (or another suitable enzyme), and the two-dimensional (2D) map established. The phosphopeptides observed on the 2D map can be tentatively correlated with expected tryptic fragments, based on their hydrophobicity and charge. A number of protocols have been developed to refine the correlation of the expected fragments to phosphopeptides present on the 2D map. For example, phospho-amino acid analysis of individual species present on the 2D map can be used to identify the type of phosphorylated residues present. In addition, manual Edman degradation can be performed on phosphopeptides after they are removed from the 2D map in order to identify the position of the ^{32}P-containing residue. This information can be used to help determine the position of the phosphorylated residue when more than one such residue is present in the tentatively assigned fragment sequence. Armed with this information, mutational analysis can confirm the site of phosphorylation, again using 2D map analysis. Although the above protocols have been successfully utilized, difficulties can arise. For example, the 2D map may not have sufficient resolution to separate two different phosphopeptides. As a result, the manual Edman

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

analysis may not take into account the heterogeneous nature of the sample extracted. Alternatively, ambiguous results can be obtained from manual Edman when more than five cycles are required to discriminate between two possible "tentatively assigned" fragments. Partial oxidation of cysteine, methionine and tryptophan residue-containing peptides may also complicate the interpretation of the 2D map. Finally, the correlation between the pI and the hydrophobicity of the fragments may not be sufficient information to direct the mutational analysis. Many of these problems can be resolved by high-sensitivity mass spectrometry (MS) analysis. Analysis of peptide mixtures with MS will generally clarify whether a sample is heterogeneous and delineate modifications such as phosphorylation and oxidation.

Several approaches for mapping phosphopeptides with MS have been proposed based on ionization techniques available in the 1980s ***(1,2)***. The introduction of matrix-assisted laser desorption ***(3,4)*** and electrospray ***(5,6)*** ionization techniques a decade later has led to substantial reductions in the amount of sample needed for MS analysis. However, the sensitivity with MS remains significantly lower than that available with radioactive labeling. As a result, it is generally not possible to use MS to analyze directly the minute amounts of phosphopeptides that can be visualized by autoradiography. MS mapping strategies for identifying the phosphopeptides generated by proteolysis of phosphoproteins generally make use of reverse phase high-performance liquid chromatography (RP-HPLC) to remove buffer salts, because their presence inhibits MS ion formation. RP-HPLC also separates the peptides based on hydrophobicity, thereby reducing the bias in the MS ionization process that may lead to inhibition of a peptide that is not readily ionized. Peptides are eluted from RP-HPLC columns using an aqueous mobile phase containing an ion pairing agent such as trifluoroacetic acid (buffer A) and an organic modifier, typically acetonitrile, containing the same ion pairing agent (buffer B). In conventional RP-HPLC, the peptides are detected by their ultraviolet (UV) absorption. Recently, Verma et al. used an alternative means of detecting phosphopeptides to identify the site of phosphorylation in S-phase Cdk inhibitor (Sic1p) ***(7)***. In this method, developed by Carr and coworkers ***(8–10)*** an electrospray triple quadrupole instrument operated in the negative ionization mode is used to analyze tryptic hydrolysis fragments, separated by RP-HPLC ***(11,12)***. MS identification of the phosphopeptide-containing fractions uses 10% of the eluent from the RP-HPLC (first pass), whereas the remainder of the stream is split for collection to be analyzed subsequently. In the first pass, fragmentation of all species exiting the RP-HPLC is induced and the instrument is set to monitor solely for the presence of a single ion, the m/z 79 fragment ion. Because this fragment ion is a specific marker for phosphopeptides, it unequivocally identifies the phosphopeptide-containing fraction. The high sensi-

tivity available with this technique is due, in large part, to using this nonUV method of phosphopeptide detection. Because all peptides absorb in the UV, only the phosphopeptides produce this intense fragment ion. This advantage is important, as the remaining material is required for second and third dimension passes to fully characterize the phosphopeptide ***(7)***. Another advantage of Carr's protocol is that it obviates the need for ^{32}P labeling. The electrospray triple quadrupole instrument applied in the above method has also been used by other investigators identifying the sites of phosphorylation in phosphoproteins (e.g., Mann and coworkers characterized IKK-2) (NF-KB inhibitory subunit kinase-2) ***(13)***. Because these accomplishments are impressive, the instruments used are expensive, and not always readily available, compared to other MS instruments.

The method described herein utilizes more generally available MS instrumentation, RP-HPLC and the classical ^{32}P label to identify the phosphopeptide-containing fraction(s). Typically, biological observations are made on experiments that utilize ^{32}P. As such, the ^{32}P radioactive trace offers an excellent marker to substitute for monitoring the m/z 79 fragment ion. In addition, successive steps of enzymatic hydrolysis are incorporated as a means of localizing the site of phosphorylation. In order to carry out additional stages of enzymatic hydrolysis, without requiring elaborate scale-up procedures, use is made of the significant improvements in sensitivity that have recently been made with reduced diameter columns for RP-HPLC ***(14,15)***. The products of enzymatic hydrolysis are analyzed by MALD and/or nanospray MS ***(16,17)***. In Matrix Assisted Laser Description (MALD), a UV laser is used to irradiate the sample dispersed in a matrix. The matrix is typically a small organic molecule which absorbs at the wavelength of the laser so that rapid volatilization occurs. The sample molecules trapped within the matrix are ionized and mass analyzed based on the time it takes to travel a known distance (time-of-flight). Alternatively, using nanospray ionization, a solution flows (0.02 μL/min–0.2 mL/min) through a fine capillary needle which sprays clusters made up of sample molecule ions and solute molecules. After the solute molecules are stripped away, the ions are transported into a mass spectrometer and collected in an ion trap where different mass to charge ratio (m/z) ions are ejected from the trap and detected. MALD time-of-flight and nanospray ion trap instruments are techniques which are inexpensive to commercialize and therefore have the potential to become more widespread in biochemistry/biology laboratories ***(18)***.

In the strategy presented, the labeled phosphoprotein is immunoprecipitated and separated by SDS gel electrophoresis prior to being transferred onto a membrane where enzymatic hydrolysis is performed. The primary isolation step is accomplished using RP-HPLC with a millibore diameter column. Based on the presence of the radioactive label, phosphopeptide-containing fractions

are analyzed with MS to identify the general site of phosphorylation. In order to localize the site of phosphorylation further, an additional enzymatic hydrolysis step using a different specificity enzyme is often required. The fragments generated from the secondary digest are again purified with RP-HPLC, utilizing a smaller microbore diameter column, and analyzed with MS. By using MS to determine the intact mass of phosphopeptides present in the map, it is possible to obtain accurate masses that will augment the identification of phosphopeptide fragments present in the 2D map. In the strategy outlined below, this information is used in combination with phospho-amino acid analysis, manual Edman protocols and the peptide pI to interpret 2D maps.

2. Materials

2.1. Extraction and Millibore RP-HPLC Equipment (see Note 1).

1. HP1090 (Hewlett Packard, Palo Alto, CA) 200 µL injection loop, 200 µL/min flow rate, 2.1 × 150 mm C_{18} Vydac (5 µ 300 Å particle size) column.
2. Ultrasonic bath, FS3 (Fischer Scientific).
3. Filter pipet tips (Molecular Bio-Products, San Diego, CA).
4. Microcentrifuge tubes (1.5 and 0.5 mL) (Sarstedt, Germany).
4. Trifluroacetic acid (TFA) (redistilled in house).
5. A-buffer : 0.055% TFA in DI H_2O (Millipore).
6. B-buffer: 0.05% TFA in acetonitrile:DI H_2O (9:1).
7. Extraction buffer: formic acid, acetonitrile, isopropanol, and DI H_2O mixture (1:1:1:1).

2.2. Phosphoprotein (Primary) Proteolysis

1. Membrane pretreatment buffer: 0.5% PVP360 (Sigma Chemical Co., St Louis, MO) in 100 m*M* acetic acid.
2. Trypsin stock: 25 µg sequencing grade trypsin (Boehringer Mannheim, Germany, cat. no. 1418475) in 50 µL 0.055% TFA.
3. Lys-C stock: 5 µg Asp-N (Boehringer Mannheim, Germany, cat. no. 84212321) in 50 µL DI H_2O.
4. Trypsin and Lys-C hydrolysis buffer: pH 8.5, 50 m*M* NH_4HCO_3.
5. 10% aqTFA.
6. 100 m*M* sodium acetate.
7. 100 m*M* TCEP (Diagnostic Chemicals Ltd., Oxford, CT) in 50 m*M* sodium acetate.

2.3. Mass Spectrometry Equipment (see Note 2)

1. Reflex MALD time-of-flight instrument (Bruker Daltonics, Billerica, MA) operated at +31 kV accelerating voltage and +30 kV reflector voltage, 100 MHz digitizer, mass accuracy better than 500 ppm.
2. Esquire nanospray quadrupole ion trap instrument (Bruker Daltonics, Billerica, MA) operated at 800 V, mass accuracy better than 200 ppm.

3. Nanospray capillaries (Bruker Daltonics, Billerica, MA).
4. Electrospray tuning/calibration mix (G2421, Hewlett Packard, Palo Alto, CA).
5. Adjustable volume (0.5–10 μL) pipet (Eppendorf varipette 4710, Germany) and pipet tips (Eppendorf geloader 0030 001.222, Germany).
6. UV absorbing matrix: Nitrocellulose (1 mg/mL) (Schleicher and Schuell, Germany) is added to a saturated solution of α-cyano-4-hydroxy cinnamic acid (Aldrich Chemical Co., Milwaukee, WI) in isopropanol and acetone (1:1).
7. 1% formic acid in methanol:DI H_2O (1:1).

2.5. Phosphopeptide (Secondary) Proteolysis and Microbore RP-HPLC

1. Michrom Bioresources UMB100 (Michrom Bioresources, Auburn, CA) 100 μL injection loop, 30 μL/min flow rate, 0.5 × 150 mm C_{18} Vydac (5 μ 300 Å particle size) column.
2. Vacuum centrifuge (Savant Speed-Vac, Farmingdale, NY).
3. Asp-N stock: 2 μg Asp-N (Boehringer Mannheim, Germany, cat. no. 1054589) in 50 μL DI H_2O.
4. Chymotrypsin stock solution: 25 μg chymotrypsin (Boehringer Mannheim, Germany, cat. no. 1418467) in 50 μL DI H_2O.
5. Asp-N hydrolysis buffer: 100 m*M* Na_2HPO_4, pH 7.5.
6. Chymotrypsin hydrolysis buffer: 50 m*M* $Et_3NH_4HCO_3$ (Aldrich Chemical Co., Milwaukee Wisconsin), pH 8.2.
7. 10% aqTFA.

3. Methods

3.1. Determining an Appropriate HPLC Gradient and Identifying the Retention Time of the Phosphopeptide of Interest

In this protocol we rely on detection of the radioactively labeled phosphopeptide eluting from RP-HPLC. The first step, while the background counts on the HPLC are low, is to determine an appropriate gradient for analysis of the phosphopeptide of interest. This can be accomplished by carrying out 2D map analysis of a tryptic (or other compatible) enzyme digest (*see* **Note 3**) as described in detail in this volume, and elsewhere ***(19)***. From the 2D map between 500 and 1000 cpm of the cellulose powder containing a phosphopeptide is collected, the peptide is extracted from the cellulose and the sample applied to RP-HPLC. The retention time of the phosphopeptide eluting fraction is thereby determined.

1. Phosphopeptides eluted on a 2D map are localized by autoradiography and removed from the plate and collected (the cellulose is loosened from the plate by scraping with a clean spatula and collected in a filter-containing pipet tip attached to a vacuum system). The pipet tip (including filter and cellulose) is placed in a microcentrifuge tube and centrifuged to transfer the cellulose powder to the tube.

2. The extraction buffer (30–40 μL) is added to the cellulose powder containing the phosphopeptide and the microfuge tube is floated in the sonication bath and sonicated for (30–60 min).
3. The decant is removed and counted. Between 500 and 1000 counts are sufficient to carry out **steps 4** and **5**. The decant is diluted (1:5) with A-buffer prior to **step 4**.
4. The sample is injected onto RP-HPLC with a standard gradient (isochratic at 100% A for 5 min followed by a 40-min gradient from 0% B to 80% B). The eluent from the column, while under isochratic conditions, is collected in one 1.5-mL microfuge tube; followed by gradient fractions collected at 30-s intervals in 0.5-mL microfuge tubes (for the first 20–25 min of the gradient) and then at 5-min intervals in 1.5 mL microfuge tubes (over the remainder of the gradient and the column wash) (*see* **Note 4**).
5. The retention time of the phosphopeptide is determined by counting the fractions (*see* **Note 5**).
6. The dilution of the extracted peptide (**step 3**), or the collection times, may need to be adjusted and the procedure repeated with a phosphopeptide from a fresh 2D map.

3.2. Isolation and Hydrolysis of Phosphoprotein

SDS gel electrophoresis (*see* **Note 6**) is used to isolate the ^{32}P labeled phosphoprotein of interest (a sample containing approx 0.2–2.0 μg of a 50 kDa phosphoprotein and 100,000 cpm ^{32}P labeled protein is required, *see* **Note 7**), the phosphoprotein is transferred to a PVDF membrane as described elsewhere ***(20)*** and hydrolyzed with trypsin or another suitable enzyme.

1. The PVDF membrane is exposed to X-ray film and the region containing the labeled protein excised using a clean disposable razor blade. Other regions are excised (*see* **Note 8**) as control samples. While keeping the membrane pieces moist with DI H_2O, use the razor blade to cut the excised membrane into 1 mm^2 or smaller squares. Using clean pinceps place these membrane pieces into a 1.5-mL microfuge tube.
2. Add 1 mL of the pretreatment buffer to the membrane pieces, shake the microfuge tube for 15 min at 32°C, remove the buffer, and rinse three times with 1 mL DI H_2O.
3. After removing the final DI H_2O rinse, add the enzyme buffer (100 μL) and measure the total cpm in the membrane pieces. An aliquot of the trypsin stock solution (3 μL equivalent to 1.5 μg) (or Lys-C, 0.5 μg) is then added every 8 h to the microfuge tube incubated at 37°C (*see* **Note 9**). The same amount of buffer and enzyme are added to the control samples at appropriate intervals.
4. The release of phosphopeptides is checked by removing the membrane pieces and counting the hydrolysis buffer after 16 and 24 h incubation (*see* **Note 10**).
5. When the digestion is complete (*see* **Note 11**), the membrane pieces are removed.
6. A small aliquot of the protein hydrolysate (corresponding to approx 100–500 cpm) is removed and a 2D map analyzed to verify that the map is comparable with that obtained previously.

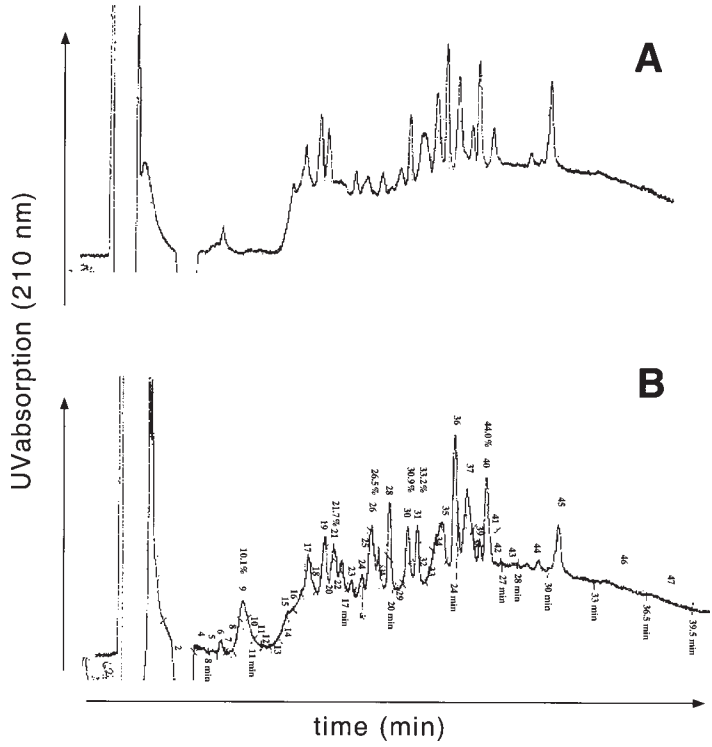

Fig. 1. The UV trace observed from milli-bore RP-HPLC using the standard gradient (see 2.1) of (A) enzyme blank and (B) approximately 0.5 μg of a phosphoprotein. Some UV absorption is observed (Figure 2 shows the ^{32}P label observed for these fractions).

7. The remainder of the hydrolysis solution is acidified by adding sufficient volume (typically 10–20 μL) of 10% TFA to adjust the pH of the hydrolysis buffer solution to approx pH 4 and then sufficient (typically 50–80 μL) 100 m*M* NaOAc to adjust to pH 5.0 (*see* **Note 12**).
8. Reduction of the protein is carried out by adding 10 μL of 100 m*M* TCEP in 50 m*M* NaOAc and incubating at 25°C for 60 min.

3.3. Millibore RP-HPLC Isolation of Phosphopeptide(s)

The resulting hydrolysis mixture containing the phosphopeptide(s) of interest are separated by RP-HPLC using the predetermined conditions. Fractions collected are prepared for MS and 2D map analysis.

1. The hydrolyzed phosphoprotein sample is analyzed with RP-HPLC using gradient conditions and manually collecting fractions with the same time intervals as determined in **Subheading 3.1.** (*see* **Note 13** and **Fig. 1**). The fractions collected are stored directly on ice.

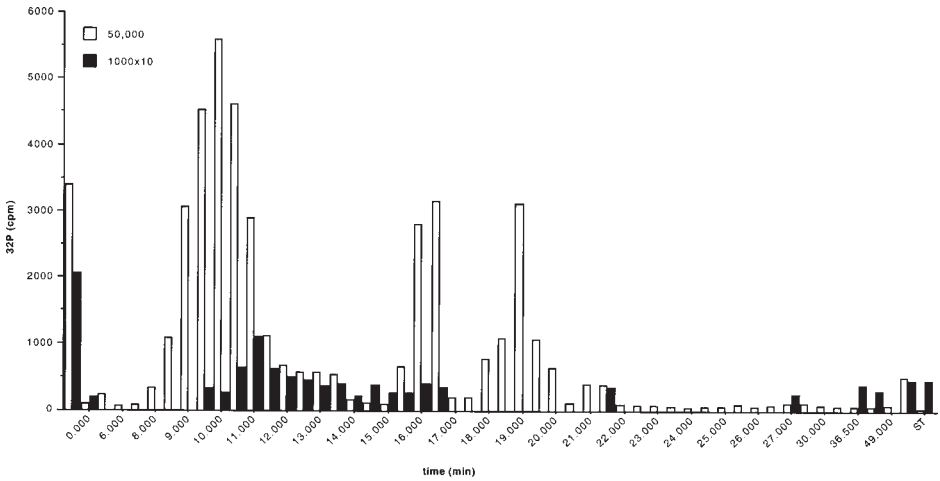

Fig. 2. Comparison of the ^{32}P label observed from (■) a phosphopeptide removed from a 2D tryptic map containing 1000 counts (cpm × 10) and (□) and a phosphoprotein hydrolyzed with trypsin. The retention time of the extracted phosphopeptide (11.0 minutes) was used to direct the investigation to the equivalent retention time fraction from the phosphoprotein digest. ST is the empty sample tube and the reading before that is the injector rinse.

2. Based on the RP-HPLC retention time of the phosphopeptide (as determined in **Subheading 3.1.**), the fraction in which the phosphopeptide of interest elutes is identified (*see* **Fig. 2**).
3. Using a hand held counter it is quickly verified that this fraction contains the phosphate label (*see* **Note 14**).
4. After the phosphopeptides of interest have eluted (but not necessarily before the gradient and collection are finished) a 1-μL aliquot of the samples of interest (representing 1% of the total sample) is transferred onto a preprepared MALD target (*see* Notes 15 and 16).
5. In order to confirm that the phosphopeptide isolated corresponds with the species identified in the 2D map, the RP-HPLC fraction from the primary digest and the phosphopeptide mixture are analyzed alone on separate 2D maps, and mixed together on a third 2D map. This mixture represents a coelution experiment and is used to verify that the intensity of the spot of interest is increased and that no new spots appear (*see* **Note 17**).

3.4. MS Analysis and Interpretation

The phosphopeptide(s) of interest, and both earlier and later eluting fractions, are analyzed with MALD and subsequently with nanospray MS.

1. All samples prepared in **step 6** are analyzed with MALD (*see* **Note 18** and **Fig. 3**). Those fractions eluting prior to, or after, a fraction are also analyzed in order to identify other closely eluting peptide fragments (*see* **Note 19**).

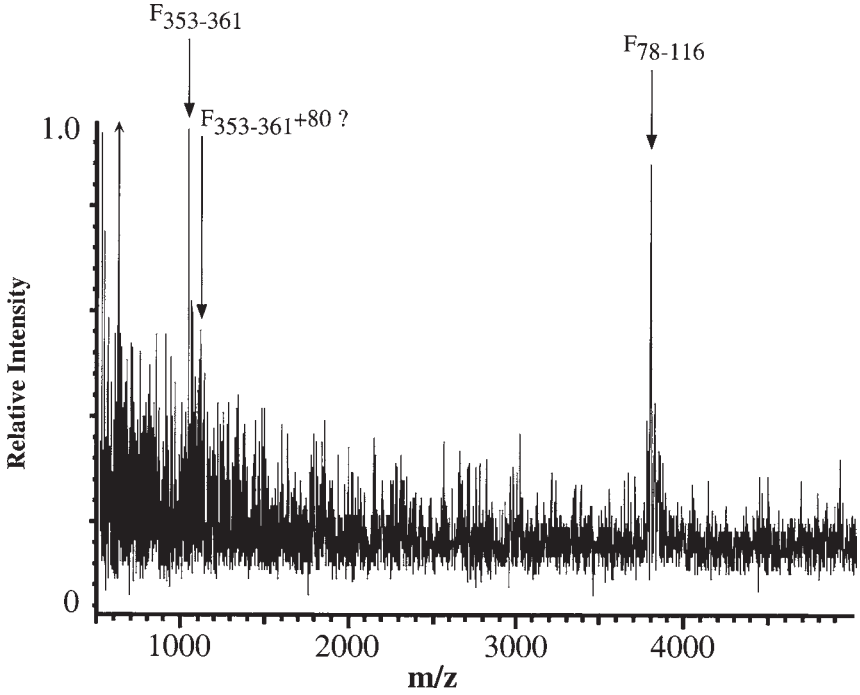

Fig. 3. The MALD mass spectrum of the ^{32}P containing fraction with retention time of 19 min shown in **Fig. 2**. In the RP-HPLC fractions recovered from the primary digest it is not unusual to observe closely eluting nonphosphorylated peptides as indicated.

2. Tentative assignments are then made between species observed in the MALD mass spectra and expected hydrolysis fragments of the protein generated by a suitable computer program (*see* **Note 20**). Allow a suitably wide tolerance range of masses (approximately ± twice the expected accuracy of mass measurement) to ensure that all possible candidates are considered.
3. It is important to determine whether there are any other possible ambiguous assignments conflicting with the tentative assignments made in **step 2**. The observed species should be compared with singly phosphorylated and singly oxidized fragments (this is easily done by subtracting either 80 or 16 Da from the observed species and checking against the computer generated nonphosphorylated fragments). Multiply phosphorylated or oxidized fragments (subtracting multiples of either 80 or 16 Da from the observed masses) must also be considered. Searches should also be carried out to identify fragments corresponding with combinations of these modifications. In addition, for tryptic digests, compare the expected trypsin autolysis fragments with the species observed in the MALD mass spectra (*see* **Note 21**). Based on these calculations some of the tentative assignments made in **step 2** may be considered ambiguous

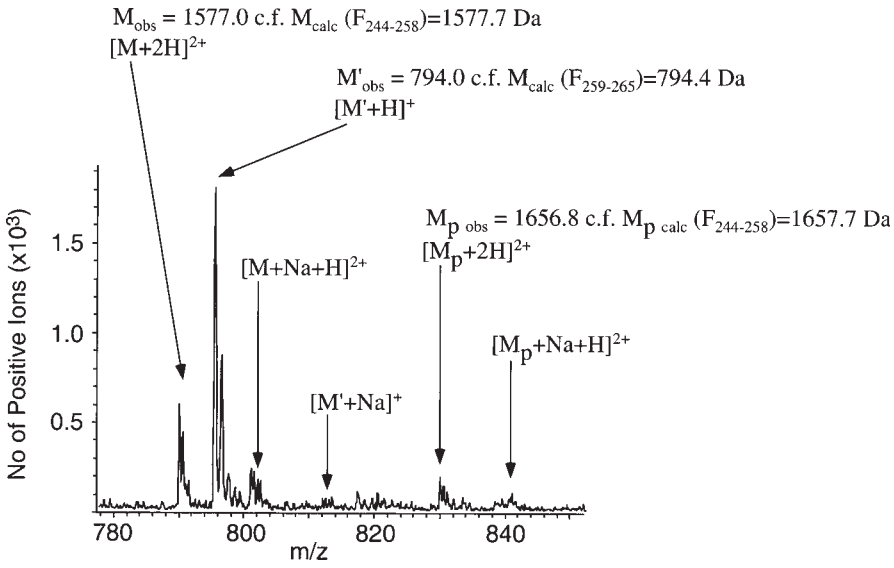

Fig. 4. Nanospray mass spectrum of a ^{32}P containing fraction recovered from a secondary digest (approximately 1.0mg of phosphoprotein was hydrolyzed with trypsin (primary proteolysis), purified with milli-bore RP-HPLC and then hydrolyzed with Asp-N (secondary proteolysis) and purified with milli bore RP-HPLC), in which M, Mp and M' species were clearly observed. It is unusual to observe a peptide (indicated as M') which is not the non-phosphorylated form of a phosphopeptide closely eluting with the ^{32}P label in the purification after the second proteolysis, but it does occur! M and M' were generated from the same precursor phosphopeptide isolated after the primary proteolysis step but not separated on micro bore RP-HPLC because both M and M' have the same theoretical HPLC retention time.

(e.g., an observed species may be consistent with either a singly phosphorylated fragment and a trypsin autolysis fragment).

4. Based on the MALD results, the expected mass and approximate charge based on the tentative assignments (charge = 1 for free amino terminus + 1 for every arginine or lysine residue in the proposed sequence) of the fragments can be calculated and the electrospray MS instrument calibrated (using electrospray tuning/calibration mix) over an appropriate mass range. Next, the instrument focusing can be optimized for detection of a control peptide having similar mass and charge compared to that of the proposed species.
5. A 1-μL aliquot of the sample (representing 1% of the total sample) is transferred to a nanospray capillary, using an adjustable volume pipet with geloader tips, for Nanospray MS analysis (*see* **Note 22**).
6. Nanospray MS analysis of fractions (*see* **Note 23**), particularly after tentative assignments have been made, allow the presence of a particular mass and charge species to be verified/identified (*see* **Fig. 4**).
7. The information gained in **steps 1–6** and **Subheading 3.3.**, **step 5** is used to supplement the predicted positions of phosphopeptides on the 2D map. For

example, based on size, charge, pI and phospho-amino acid analysis certain fragments may be predicted in regions of the 2D map. The successful MS analysis of one or more of these phosphopeptides will help allow reinterpretation of the expected positions of other fragments in the map. Even the analysis of nonphosphorylated peptides, in phosphopeptide containing fractions, gives additional information. The observed species may be the nonphosphorylated form of the phosphopeptide or it may have a very similar retention time to the phosphopeptide. If the latter is the case, then a computer search of expected phosphoprotein hydrolysis fragments with similar retention time to the observed species will include the phosphopeptide and this information will help direct an assignment. All the information gained can be used in an iterative fashion to help refine other assignments in different regions of the 2D map.

8. Resolution of remaining ambiguities may require further information. Approximately 2% of the phosphopeptide sample is consumed for MS analysis (in **steps 1** and **6**) whereas a further 5–10% of the sample is reserved for 2D map analysis (*see* **Subheading 3.**5). Rather than attempting to preconcentrate the remaining phosphopeptide solution (reducing the volume typically results in large sample losses) the remaining sample is used to perform a secondary digest (*see* **Note 24**).

3.5. Microbore RP-HPLC Isolation of Secondary Enzyme Hydrolysis Phosphopeptide(s)

For reasons identified in **Subheading 3.4.**, a secondary enzyme hydrolysis (with differing enzyme specificity compared to the first hydrolysis) is performed to conclusively identify the phosphopeptide. It is also advantageous to carry out a secondary enzyme hydrolysis when an intact nonphosphorylated fragment is observed from the ^{32}P containing fraction, but not the corresponding phosphopeptide fragment (*see* **Note 25**). By reducing the size of the phosphopeptide, while retaining the approximate concentration of the phosphopeptide in the solution, the intact phosphopeptide ion is often observed. In order to keep the phosphopeptide concentration roughly comparable, the secondary digest is purified with microbore RP-HPLC at a lower flow rate while retaining the same time interval for collection. The choice of enzyme depends on the alternative assignments that need to be distinguished. For example, if one or more assignments involve sequences containing aspartic acid or glutamic acid residues (*see* **Note 26**), then an Asp-N digest may be an appropriate means of confirming an assignment. Other factors to consider are the size of the fragments that would be produced (*see* **Note 27**) and whether an alternative enzyme would better localize the site of phosphorylation (*see* **Note 28**). Chymotrypsin is one such alternative enzyme which has a complementary, if somewhat broader, specificity compared to either trypsin or Asp-N. The resulting hydrolysis of the phosphopeptide(s) of interest, after separation by microbore RP-HPLC, is prepared for MS analysis.

1. The HPLC purified fraction(s) isolated in **Subheading 3.3.** is reduced in volume to 5–10 μL by drying in a vacuum centrifuge. An earlier eluting fraction with low counts is also reduced in volume for use as an enzyme blank, and an appropriate control peptide is prepared in a separate microfuge tube as a positive control.
2. An aliquot (100 μL) of the enzyme buffer solution is added to the fraction of interest and the control samples.
3. Asp-N (0.2 μg) or alternatively, chymotrypsin (2.0 μg) are added and the solutions incubated at 37°C.
4. The enzyme hydrolysis of the positive control peptide is stopped after 16 h by addition of 10 μL of 10% TFA. RP-HPLC analysis and MALD MS analysis are used to verify enzyme hydrolysis of the control peptide.
5. Provided satisfactory hydrolysis is observed in **step 4**, the secondary enzyme hydrolysis of the phosphopeptide and the enzyme blank are stopped by addition of 10 μL of 10% TFA.
6. The hydrolyzed phosphoprotein sample is analyzed with microbore RP-HPLC using a standard gradient (isochratic at 100% A for 5 min followed by a 20-min gradient from 0% B to 60% B; *see* **Note 29**). The eluent from the column while under isochratic conditions is collected in one 0.5-mL microfuge tube followed by separate gradient fractions collected at 30-s intervals. The phosphopeptide-containing fractions are identified as in **Subheading 3.2**. A 1 μL aliquot of this sample (representing 6% of the total sample) is immediately transferred onto a preprepared MALD target(s) prepared as in **Subheading 3.3**.
7. Repeat steps in **Subheading 3.4.**, as applicable.

3.6. Further Interpretation

Usually, the second enzyme hydrolysis will have resolved ambiguities and a single site of phosphorylation is present. For example, a ^{32}P-containing fraction may again be isolated in which the MS species observed is assigned as a phosphorylated peptide fragment. This fragment is consistent with hydrolysis of a fragment observed after the first hydrolysis, and the specificity of the enzyme used in the second hydrolysis step. It is worthwhile to compare the results obtained with other information, such as the fragment pI, the phosphoamino acid analysis or the manual Edman analysis. Provided these are all consistent, the site of phosphorylation can be considered identified.

However, a second hydrolysis step may not resolve all ambiguities. Consider that a nonphosphorylated peptide fragment that meets the above criteria (i.e., that it is consistent with further hydrolysis and the specificity of the second enzyme) is observed from the ^{32}P-containing fraction. The method presented is designed, in part, to address this situation. If the level of incorporation of phosphate is sufficiently low (*see* **Note 30**), in conjunction with the ionization bias (*see* **Note 25**), then we may isolate and observe a nonphosphorylated peptide after both the first and second hydrolysis steps (**Subheadings 3.3.** and **3.5.**). Because the millibore RP-HPLC step (**Subheading 3.3.**) is localizing the

^{32}P label, we deduce that the nonphosphorylated species observed is closely eluting with the phosphopeptide (which is not observed). After the second proteolysis step (**Subheading 3.5.**), if the fragment observed from the ^{32}P-containing fraction corresponds with proteolysis of this first hydrolysis fragment, then we have a critical piece of information. Because the second proteolysis step is again localizing the ^{32}P label, and the second proteolysis fragment is generated from the first hydrolysis fragment, it is proposed that the observed fragments are the nonphosphorylated forms of the phosphopeptide fragments. The site of phosphorylation is thereby localized to the sequence of the second hydrolysis fragment.

1. The mass observed from analysis of the ^{32}P label-containing second hydrolysis fraction should be compared with expected hydrolysis fragments from the species observed in the primary hydrolysis fraction (*see* **Note 20**). The question asked is "does the observed second hydrolysis species correspond ONLY to an observed and expected fragment formed from a primary hydrolysis fragment ?"
2. If the answer is "yes," then go to **step 5**.
3. If the answer is no, then all the possibilities cannot be detailed herein, and even if they were, the interpretation may still not be clear cut. Therefore, check for ambiguities, i.e., compare the observed species with the intact phosphoprotein sequence and trypsin autolysis fragments (as in **Subheading 3.4.**, **step 3**) since it is possible that coeluting impurities were present in the first hydrolysis fraction. The question asked is "is it plausible that the observed species is a hydrolysis fragment of a species coeluting with the phosphopeptide in the millibore RP-HPLC, which coincidentally coelutes with the phosphopeptide fragment on the microbore RP HPLC ?"
4. 2D map analysis of the phosphopeptide fractions obtained from RP-HPLC purification after the first and second hydrolysis reactions (each alone and mixed together) can be used to confirm that the second proteolysis reaction cleaved the phosphopeptide and help answer the question posed in **step 3**.
5. Next, ask if the secondary hydrolysis fragment contains an amino acid consistent with the phosphoamino acid analysis and whether the results are consistent with the manual Edman analysis (carried out on either the primary or secondary hydrolysis fragment).
6. Test your assignment wherever possible. For example, by changing the primary enzyme from trypsin to Lys-C you should change the 2D map position (and RP-HPLC retention time) of a fragment which incorporates an arginine residue at either the N- or C- terminal cleavage site. If the fragment you observe is the nonphosphorylated form of the phosphopeptide then the change of primary enzyme should be reflected in the mass observed. Alternatively, if a tentative assignment contains a residue which may be oxidized, then confirming the presence of an oxidized residue in the isolated phosphoprotein (by analyzing the 2D map of the fraction with and without performic acid treatment) may allow this assignment to be confirmed.

4. Notes

1. It is necessary to have access to a millibore HPLC, such as that described in **Subheading 2.**, for **Subheadings 3.1.** and **3.2.** (to obtain reproducible retention times for comparisons). In order to carry out a secondary enzyme digest and localize the site of phosphorylation (*see* **Subheading 3.3.**), without requiring scale up of the starting material, it is necessary to have access to a microbore HPLC such as that described in the Methods section.
2. It is not necessary to have access to both MS instruments described in **Subheading 3.** to perform phosphopeptide mapping successfully. In **Subheading 3.4.**, omitting the nanospray MS analysis (**steps 5** and **6**) reduces the chances of identifying the phosphopeptide and thereby increases the reliance on the deductive arguments discussed in **Subheading 3.6**.
3. Lys-C can be a useful alternative to trypsin when trying to distinguish between different possible assignments (*see* **Subheading 3.6.**, **step 6**). The buffer concentration and volatility are important constraints in this protocol.
4. Always condition the column and allow it to equilibrate. Avoid elution conditions where a peptide from one run may be eluted in the following run (e.g., if a higher percentage of organic modifier is used). HPLC initialization protocol: an A buffer injection is measured first to verify that the chromatogram is blank, followed by an enzyme blank, another A buffer blank and the phosphoprotein/phosphopeptide hydrolysate. Record and mark the UV trace to indicate fraction number, when the fraction cut started (even though there is no UV absorbance) and time after injection start at suitable intervals.
5. If most of the radioactivity eluted in the column crash, then the peptide is hydrophilic. It can typically be dried down to 0 μL and taken up in 100% A buffer and reinjected onto the column with minimal losses (check this by counting the empty tube afterwards). If it again crashes off the column, then a different ion pairing agent (e.g., hexaflurobutyric acid) can be used, or an alternative column designed for hydrophilic peptides (e.g., Alltech Lichrosorb Select B 5U) may be used. The collection time can be adjusted but it should remain as narrow (30 s) as possible between 0–40% B or 0–50% B before it is widened out (above 40% B or as determined in **Subheading 3.1.**) to collect the column wash.
6. It may be advantageous to determine the minimal SDS gel electrophoresis time that will separate the phosphoprotein of interest from other immunoprecipitated proteins. Longer duration SDS gel electrophoresis will increase the area of the phosphoprotein in the gel, and therefore, the area of the phosphoprotein on the membrane. The physical size of the membrane determines the volume of the enzymatic digestion buffer that should be kept 100 μL.
7. The success in measuring the intact phosphopeptide is also dependent on the degree of phosphorylation. However, with this method it may be possible to identify the site of phosphorylation even when the phosphopeptide is not directly detected (as explained in **Note 28** and **Subheading 3.6.**).
8. Based on autoradiogram exposure of the membrane, excise the phosphoprotein-containing portion of the membrane. From the same membrane, excise a similar

size area of membrane either above or below phosphoprotein being careful to avoid the phosphoprotein region (and the antibody light or heavy chain regions). A second control region can be excised from the membrane from an area which is not in contact with the gel.

9. To minimize the contribution of enzyme autolysis fragments in the HPLC chromatograms and in the MS, the enzyme stock is kept at 4°C for a maximum of 12 h (or a fresh stock is used). In addition, enzyme to substrate conditions that maximize hydrolysis of the phosphoprotein (as indicated by elution of ^{32}P from the membrane), and minimize interference of the enzyme in the subsequent chromatographic isolation and MS analysis, are employed.
10. Intermittent shaking and gentle centrifugation of the membrane pieces is carried out to ensure that the membrane pieces are dispersed and completely immersed in the enzyme buffer solution.
11. If significant counts remain in the membrane (>50% of the total number of counts prior to hydrolysis) return the membranes to the buffer and add the third aliquot of enzyme.
12. The actual volumes required to adjust the pH should be determined first using the enzyme blank. The same volumes are then employed for the phosphoprotein.
13. Ideally, the volume to be injected should be less than that which can be loaded in a single injection (and the same as that used in **Subheading 3.1.**). Multiple sample injections may increase the loss of hydrophilic peptides. Fractions are collected while marking the UV trace to indicate fraction number, when the fraction cut started (irrespective of the UV absorbance) and time after injection start at suitable intervals. These records will help when comparing these results to those obtained in **Subheading 3.1**.
14. In addition to rapid scanning with a handheld counter, using a scintillation counter accurately determines the number of cpm in all fractions and relevant material. The total cpm for the eluent of the HPLC column (i.e., including the column crash and the collected fractions) plus the total cpm lost (i.e., counts left in the sample microfuge tube, syringe, and injector wash) should approximate to the cpm of the hydrolyzed phosphoprotein sample. Counting all of these microfuge tubes, or the closest equivalent (e.g., rinse the syringe and count the rinse), allows minimization of losses and optimization of the recovery of the phosphopeptide. Less than 10% of the hydrolyzed phosphoprotein counts should be lost. You can also evaluate the benefit of rinsing the membrane vs counts lost (in the column crash) as the total injection volume increases.
15. MALD targets are first cleaned by sonicating in 10% HNO_3 for 15 min and then in DI H_2O (15 min). The targets are removed from the H_2O, rinsed with methanol and dried. The target is pretreated with a (0.5 μL) aliquot of the UV absorbing matrix.
16. Take care that the matrix is not disturbed (with the pipet tip) when applying the sample aliquot. If the phosphopeptide is sufficiently hydrophobic that the solution contains more than approx 25% acetonitrile, drying of the sample can be facilitated by aiming a stream of nitrogen gas at the solution on the matrix.

17. When a number of consecutive HPLC fractions have significant levels of ^{32}P, then presumably the phosphopeptides have similar retention times under the RP-HPLC conditions employed. It is essential to carry out the 2D map analysis to verify that the fraction of interest contains the appropriate 2D map phosphopeptide and also determine the purity of this fraction. Even when a single or isolated ^{32}P label fraction is recovered, it is important to confirm the homogeneity of this fraction.
18. The MALD target is inserted into the MS instrument vacuum chamber and analyzed, then withdrawn from the instrument and rinsed by adding 10 μL of DI H_2O to the matrix surface and immediately blowing the solution off the matrix surface with a stream of N_2 gas. Extensive (50–200 independent laser shots) analysis of the sample from different positions on the matrix surface are carried out both prior to, and after, rinsing of the sample target.
19. By analyzing pre- and posteluting fractions, closely eluting peptides can be distinguished from coeluting peptides. If a peptide is observed in a preeluting fraction, then it is presumed that its presence in the later fraction is because of "tailing" of the species as it elutes off the column. If it is observed in a posteluting fraction with equal or greater intensity, it is presumed that the fraction was cut while the peptide was eluting.
20. A number of computer programs can be used to generate the calculated masses of hydrolysis fragments from a known protein, e.g., MacBioSpec ***(21)*** (available through PESciex Internet address: http://www2.perkin-elmer.com/sc/index.htm) or MS-Digest (available from UCSF http://prospector.ucsf.edu/htmlucsf/msdigest.htm) or GPMAW (http://130.225.147.138/gpmaw/default.htm).
21. Checking whether any species observed could be owing to autolysis fragments can be done with programs listed in **Note 20**, and the appropriate enzyme sequence. For example, loading the trypsin sequence and setting trypsin as the enzyme will generate trypsin autolysis fragments. In order to check for alternative specificity cleavage sites, it is often preferable to determine whether the observed mass species would correspond to any peptide fragment generated from the precursor and then subsequently determine if any of these fragments make "sense" on a chemical proteolysis basis. This is accomplished with a search based on "mass."
22. Prior to transfer of the sample for analysis, the nanospray capillary is rinsed with isopropanol, and then dried with a stream of N_2 gas. To check that a valuable sample is not loaded into a damaged or contaminated capillary, a 1-μL aliquot of 1% formic acid in methanol is loaded into the capillary using the geloader tips. The sample is inserted into the MS instrument and positioned. Once inserted into the source housing, the capillary is examined with a 25× microscope and, if necessary, the aperture enlarged by carefully touching the capillary against the end plate. The mass spectrum is measured in both positive and negative ionization modes (which will serve as background spectra), the position of the capillary is optimized and the flow rate checked.

23. An aliquot (1 μL) of the HPLC purified sample is inserted into the capillary using the geloader tips. The capillary is inserted into the source housing, taking care not to damage or contaminate the capillary. Final repositioning of the capillary may be necessary. Both positive and negative ionization mode spectra are measured.
24. No discussion of PSD or MS^n capabilities available with certain MALD time-of-flight or nanospray MS instruments is included. The ability to discriminate between more than one possible assignment, or further localize the site of phosphorylation with PSD or MS^n techniques has been well documented but is considered "icing on the cake." The method presented here is applicable for researchers who have access to MALD time-of-flight, but not necessarily to PSD or alternatively, access to a nanospray single quadrupole instrument (i.e., without MS^n).
25. An ionization bias is often observed in MS where the relative intensity of a mass in the mass spectrum does not reflect the overall relative abundance of this species. This ionization bias is not only observed for different peptides (with differing sequences), but also peptides of the same sequence where one form of the peptide is modified (e.g., peptide and phosphopeptide mixtures). For example, a mixture of a peptide and the corresponding phosphopeptide (9:1) may not result in a detectable signal for the phosphopeptide ***(22,23)***.
26. Using V8 S. aureas or Asp-N at high enzyme to substrate conditions will hydrolyze peptide bonds at the carboxy or amino-terminal side of glutamic acid residues, respectively.
27. Low-mass fragments are often difficult to detect in MALD time-of-flight mass spectra because of the presence of matrix ions.
28. Selection of an appropriate enzyme may be made on the basis of the peptide observed in the fraction, irrespective of whether a phosphopeptide is observed. A low level of phosphorylation, or the ionization bias (*see* **Note 25**), may prohibit observation of the phosphopeptide.
29. As illustrated in **Subheading 3.1.**, for the millibore RP-HPLC system the retention time of the phosphopeptide can be determined on the microbore RP-HPLC prior to injection of the actual sample. Although this strategy works very well with the millibore RP-HPLC, it is less reliable due to the reduced reproducibility of the retention times with microbore systems and is mentioned here only as an option to be considered.
30. An underlying axiom when using MS to identify phosphopeptides is that MS requires a high level of incorporation of phosphate at a particular site to identify that particular phosphopeptide. The level of incorporation of phosphate at individual sites is not readily revealed by autoradiography after SDS gel electrophoresis of a phosphoprotein. If the phosphoprotein contains multiple sites of phosphorylation many of the protein molecules may contain phosphate, but an isolated phosphopeptide may be present together with a significant excess of the corresponding nonphosphorylated peptide. For example, a protein which has ten or more sites of phosphorylation and a stoichiometry of incorporation of

phosphate at each site of 30% will, on average, generate at least one phosphate (and therefore a label) on every molecule. However, MS analysis of one of the phosphopeptides will be attempting to detect a phosphopeptide in the presence of an excess amount of the corresponding nonphosphorylated peptide.

Acknowledgments

This work was supported by National Institute Health grants NIH-GM 48677, NIH-NCRR Shared Instrument Grant 1S10RR-8425, NSF Major Research Instrumentation program DDBI-972450 and conducted in part by the Foundation for Medical Research. The author wishes to thank R. Craig for careful reading and helpful comments and D. Johns for preparation of the manuscript.

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7

Phosphorylation of Smad Signaling Proteins by Receptor Serine/Threonine Kinases

Serhiy Souchelnytskyi, Lars Rönnstrand, Carl-Henrik Heldin, and Peter ten Dijke

1. Introduction

Transforming growth factor-β (TGF-β) family members, which include TGF-βs, activins, and bone morphogenetic proteins (BMPs), elicit their multifunctional effects by binding to and complex formation of type I and type II serine/threonine kinase receptors (*see* **Fig. 1**). Each family member signals via distinct combinations of type I and type II receptors, both of which are required for signaling. Upon formation of the heteromeric receptor complex, the type I receptor is phosphorylated by the type II receptor kinase. Phosphorylation occurs predominantly in a region rich in glycine and serine residues (GS domain) in the juxtamembrane domain of the type I receptor, which possibly leads to a conformational change and thereby activates the type I receptor kinase (*see* **Fig. 1**) ***(1–3)***. The activated type I receptor propagates the signal downstream through transient interaction with, and phosphorylation of, particular Smoe- and mad related protein (Smad) molecules ***(1–3)***. Certain Smads are phosphorylated directly by activated type I receptors in a differential manner; they are therefore termed pathway-restricted Smads. Whereas Smad2 and Smad3 act in TGF-β and activin pathways, Smad1, Smad5, and Smad[8] are thought to act in BMP pathways. Phosphorylation occurs at the two most C-terminal serine residues in a conserved C-terminal Ser-Ser-X-Ser motif (*see* **Fig. 2**). Pathway-restricted Smads oligomerize with Smad4, which acts as a common mediator in TGF-β, activin, and BMP signaling. After translocation to the nucleus, the oligomers interact with DNA directly, or in complex with other DNA-binding proteins, and control transcription of target genes (*see* **Figs. 1** and **2**). Recently,

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

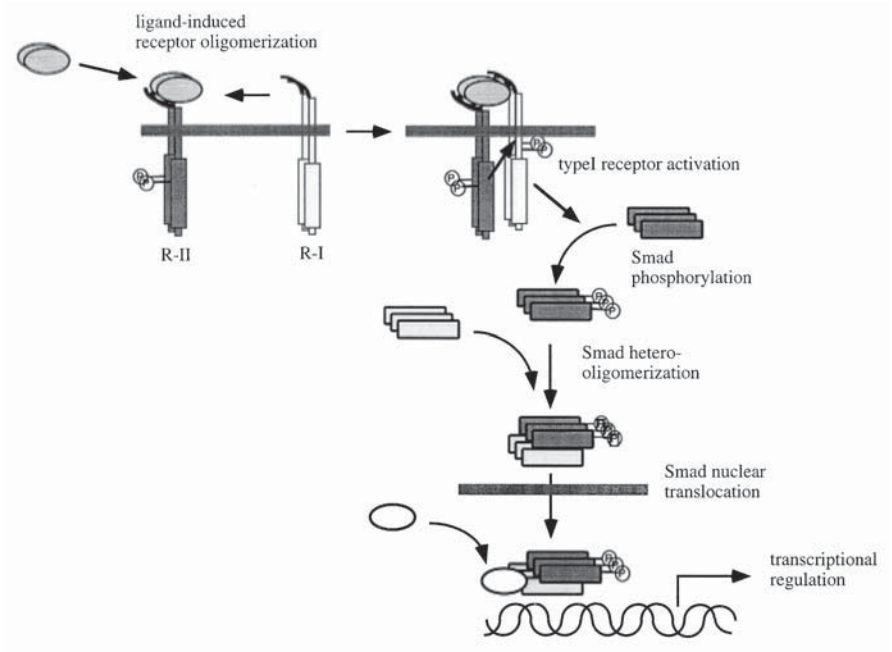

Fig. 1. TGF-β signaling through Smad proteins. A hypothetical signal transduction pathway for TGF-β family members through their serine/threonine kinase receptors and downstream effector molecules of the Smad family, is depicted.

inhibitory Smads, Smad6, and Smad7, have been identified that antagonize TGF-β family signaling ***(3)***.

Here we describe a method to study Smad phosphorylation using in vivo [^{32}P]orthophosphate-labeled cells followed by immunoprecipitation of Smad proteins from cell lysates. Smad phosphorylation in transfected COS cells, as well as in nontransfected cells, is described. The intensity of the signals are much higher in transfected COS cells vs the nontransfected cells. In particular, use of transfected cells is benificial when the effect of a particular substance, or mutation in receptor or Smad, on receptor-mediated Smad phosphorylation needs to be analyzed. However, overexpression of receptors and Smads in COS cells may lead to interactions and phosphorylations that may not occur at physiological levels. Therefore, to determine whether a particular Smad is a physiological substrate in ligand-mediated activation of a particular receptor complex, we recommend to analyze phosphorylation of endogenous Smads in nontransfected cells. Moreover, Smad proteins are components of an intracellular regulatory network, that appear not only to be phosphorylated

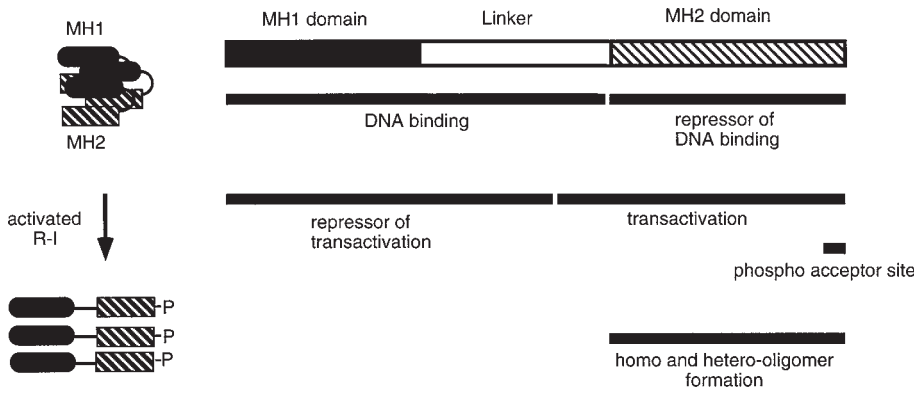

Fig. 2. Functional domains in Smad proteins. Type I receptor-induced phosphorylation of pathway-restricted Smads may induce an unfolding of the N-and C-terminal domains (shown on left side), allowing heteromeric complex formation and transcriptional activation mediated by C-terminal domain and direct DNA-binding via N-terminal domain (shown on right side). The phospho-acceptor site of type I receptor kinase is indicated. The conserved regions in the N-terminal domain (MH1 domain) and C-terminal domain (MH2 domain), are indicated by black and hatched boxes, respectively.

by up-stream serine/threonine kinase receptors, but also by other kinases *(4)*. It may thus be interesting to examine the Smad phosphorylation under a variety of physiological conditions. As phosphorylation by receptors and other kinases may occur on different sites, it is of interest to examine which amino acid residues are phosphorylated in Smad proteins. This may be achieved, in part, by two-dimensional separation of Smad phosphopeptides.

2. Materials

2.1. Preparation of Cells

1. COS cells and mink lung epithelial (Mv1Lu) cells (as well as many other cell types) can be obtained from cell and tissue collections, e.g., American Type Culture Collection (Bethesda, MD) (*see* **Note 1**). COS and Mv1Lu cells are cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 50 µg/mL streptomycin.
2. Cell culture media, e.g., DMEM, FBS, antibiotics, and cell culture equipment can be obtained from various suppliers of cell culture reagents and equipment, e.g., Life Technologies (Paisley, UK) or Sigma (St. Louis, MO).

3. Common cell culture and biochemistry facilities are needed for preparation of cells and DNA transfection.

2.2. DNA Transfection of Cells

1. Plasmid DNA for transfection needs to be of high purity; DNA preparations using Qiagen resin (Qiagen, Valencia, CA) gives satisfactory results.
2. For DNA transfection the following stock solutions are used:
   a. 50 mg/mL diethylaminoethyl (DEAE)-dextran (Pharmacia LKB, Uppsala, Sweden) in water.
   b. 10 m*M* chloroquine (Sigma) in water.
   c. DMEM with 10% FBS, serum-free DMEM and DMEM with 10% dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany).

2.3. In vivo [^{32}P]orthophosphate Labeling of Cells

1. [^{32}P]orthophosphate (40 mCi/mL) can be obtained from Amersham (Aylesbury, UK).
2. Ham's F-12 phosphate-free medium or phosphate-free DMEM can be obtained from SVA, (Uppsala, Sweden.)
3. TGF-β can be obtained from various suppliers (e.g., R/D Systems, Minneapolis, MN).
4. Activin can be obtained from National Hormone and Pituitary Program, (Rockville, MD).
5. Bone morphogenetic proteins can be obtained from Creative Biomolecules (Hopkinton, MA) or Genetics Institute (Boston, MA).
6. Means of protection against radioactive radiation are strongly recommended; plexiglas protection shields and boxes for samples, special lead-protection coats, separate set of automated pipets and plugged aerosol-protection tips, separate centrifuge for radioactive samples, and separate shaker. Radioactivity protection rules should be followed strictly.

2.4. Cell Lysate Preparation and Smad Immunoprecipitation

1. Cell lysis buffer: 20 m*M* Tris-HCl, pH 7.4, 150 m*M* sodium chloride, 0.5% Triton X-100, 50 m*M* sodium fluoride, 10 m*M* sodium pyrophosphate, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin (Bayer, Leverkusen, Germany).
2. Phosphate-buffered saline (PBS): potassium phosphate monobasic 0.2 g/L, potassium chloride 0.2 g/L, sodium chloride 8 g/L, sodium phosphate dibasic 1.15 g/L.
3. Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden).
4. Antibodies to epitope tags and Smads can be obtained from different suppliers, e.g., Santa Cruz Biotechnology (Santa Cruz, CA).
5. RIPA buffer: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% NP40, 0.5% Deoxycholic acid sodium salt (DOC), 0.1% SDS, 5 m*M* ethylenediaminetetracetic acid (EDTA).

6. Equipment and reagents for performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): For separation of Smads polyacrylamide (National Diagnostics, Atlanta, GA) gels with 8% concentration (or 7% to 12% concentration gradient) can be used.
7. Lemmli sample buffer: 35 m*M* Tris-HCl, pH 6.8, 2.5% SDS, 10% sucrose, 0.005% bromophenol blue.
8. Reagents and equipment for radioactivity detection: X-ray films are available from Amersham (Aylesbury, UK). Image analysis can be performed with FujiX Bas 2000 Bio Image Analyser (Fuji Photo Film Co, Japan) or Phosphorimager (Molecular Dynamics Limited, Chesham, UK).
9. Gel fixation buffer: 7.5% acetic acid, 20% methanol, in water.
10. Gel drying buffer: 4.5% glycerol, 20% methanol, in water.

2.5. Two-Dimensional Separation of Smad Phosphopeptides

1. Western blotting equipment is available from Bio-Rad Laboratories (Hercules, CA) or Hoefer Pharmacia Biotech (San Francisco, CA).
2. Transfer buffer: 25 m*M* Tris, 125 m*M* glycine, pH 8.2, 20% methanol, 0.1% SDS.
3. Nitrocellulose membrane (Hybond-C extra) can be obtained from Amersham (Aylesbury, UK).
4. Membrane blocking solution: 200 μL 0.5% polyvinylpyrrolidon K30 (Aldrich, Germany) in 0.6% acetic acid. Can be stored at room temperature.
5. 50 m*M* ammonium hydrogen carbonate. Must be prepared fresh.
6. Trypsin (Promega, Madison, WI) for digestion should be of sequence grade. Trypsin stock is prepared by dilution of 20 μg of modified sequence grade trypsin with 200 μL of 1 m*M* HCl, and can be stored at –20°C.
7. Performic acid is prepared by incubating 450 μL of formic acid with 50 μL of 30% hydrogen peroxide for 1 h at room temperature before use for a peptide oxidation.
8. For two-dimensional phosphopeptide mapping, Speed-Vac (Savant Instruments), high-voltage electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA), sonicator, and a tank for ascending chromatography are needed.
9. Cellulose thin-layer chromatography plates (20 × 20 cm) can be obtained fromMerck KGaA.
10. Electrophoresis buffer pH 1.9: formic acid:acetic acid:water/50:156:1794/v:v:v/.
11. Thin-layer chromatography buffer: isobutyric acid:n-butanol:pyridine:acetic acid:water/1250:38:96:58:558/v:v:v:v:v.

3. Methods

An important step in the activation of pathway-restricted Smads is their type-I receptor-mediated phosphorylation, which precedes and allows for the subsequent steps in Smad signaling e.g., heteromeric complex formation of Smads and nuclear accumulation of Smad complexes (*see* **Note 2**). Here we describe a protocol for in vivo [^{32}P]orthophosphate labeling of cells to determine Smad

phosphorylation levels; the preparation of cells, DNA transfection of cells, and immunoprecipitation of phosphorylated Smads from cell lysates and analysis thereof. In addition, we describe a method for two-dimensional separation of Smad phosphopeptides.

3.1. Preparation of Cells

The way that cells are treated and cultured prior to assay may affect Smad activation. Thus, to obtain reproducible results in Smad phosphorylation assays, like any other method involving cell culture, it is important to maintain the cells which are used for assay under optimal growth conditions. A routine with a regular splitting schedule (every 3–4 d for COS and Mv1Lu cells) should be followed. Splitting cells to very low densities, or keeping cells too long at high density, should be avoided. In addition, culture conditions during the phosphorylation assay are important. Exponentially growing cells should be seeded at a density that will provide a 70–80% confluent cell monolayer at the day of assay.

3.2. DNA Transfection of Cells

Smad phosphorylation assays are often performed on transfected cells, in which particular receptors and Smads are ectopically expressed. Use of epitope-tagged Smads (*see* **Note 3**) facilitates their detection and allows for distinction from endogenous Smads, which is particulary useful when mutated Smads are analyzed. To obtain strong signals on Smad phosphorylation, COS cells are often used as expression levels achieved in COS cells are very high. However, on their overexpression, TGFβ-superfamily receptors have a tendency to form ligand-independent complexes that are active and induce Smad phosphorylation, thus decreasing the ligand effect (*see* **Note 4**). DNA is introduced into COS-1 cells by the DEAE-dextran transfection method (*see* **Note 5**).

1. Seed COS cells (2–5 × 10^5 cells/10-cm dish) 1 d before transfection in DMEM with 10% of FBS.
2. Add DNA in serum-free DMEM medium, start by using 0.1–0.3 μg DNA for each receptor/mL of medium. The total amount of DNA added should be within the range 2–4 μg DNA/mL of medium (*see* **Note 6**); 7 mL for a 10 cm dish, or 2 mL for a 40 mm dish. Subsequently, add DEAE-dextran to a final concentration of 250 μg/mL (add 50 μL of a 50 mg/mL stock solution in water to 10 mL of medium), followed by chloroquine to a final concentration of 100 μ*M* (add 10 μL of a 100 m*M* stock solution in water to 10 mL of medium).
3. Aspirate medium from cells, wash them once with serum-free DMEM and add DNA (prepared as described in **step 2**, above) to the cells, and incubate for 3.5 h in a CO_2 incubator at 37°C.
4. Aspirate the medium and incubate cells for 2 min in 10% dimethyl sulfoxide (DMSO) in serum-free DMEM (37°C), then wash twice with serum-free DMEM

and subsequently add 2 mL (for a 40-mm dish) or 10 mL (for a 10-cm dish) DMEM supplemented with 10% FBS.

5. After 20–24 h of recovery, the cells can be trypsinized and reseeded. In parallel to analysis of Smad phosphorylation level, the expression of receptors and Smads needs to be examined. We therefore often perform COS transfections in 10-cm plates, and split these afterward into three 40-mm dishes; one dish to examine Smad phosphorylation and two other dishes for receptor and Smad expression, respectively (*see* **Note 7**).
6. 40–48 h after transfection the cells can be used for [^{32}P]orthophosphate labeling.

3.3. In Vivo [^{32}P]orthophosphate Labeling of Cells

For a [^{32}P]orthophosphate labeling a 40-mm dish of transfected COS cells (*see* **Subheading 3.2.**) is sufficient, whereas for nontransfected cells or for phosphopeptide mapping a 10-cm dish is required to obtain sufficient quantity of phosphorylated Smad for analysis. Note that in this method high amount of radioactivity is used. Thus, safety rules should be followed strictly.

1. Warm phosphate-free medium at 37°C (*see* **Note 8**).
2. Calculate how much medium you need for labeling. We use 4 mL/10-cm dish, or 1 mL/40-mm dish with the cells placed on a shaker; for labeling without a shaker, increase the volume of medium to 7 mL/10-cm dish. Add [^{32}P]orthophosphate to the medium (0.5 to 1.0 mCi/mL of medium (*see* **Note 9**)).
3. Change the culture medium to phosphate-free medium containing [^{32}P]orthophosphate and incubate at 37°C for 2.5–3.0 h (*see* **Note 10**).
4. For incubation with a ligand, add TGFβ (10 ng/mL), activin (50–100 ng/mL), or BMPs (100–500 ng/mL) to cells for 1 h at 37°C.

3.4. Cell Lysate Preparation and Smad Immunoprecipitation

1. Wash cells three times with cold (4°C) PBS, prior to addition of lysis buffer (1 mL/dish). Lyse cells for 20–30 min with shaking. Collect the lysate in an Eppendorf tube (*see* **Note 11**).
2. Centrifuge at 15,000*g* for 10 min at 4°C. Preclean the supernatant by incubating with nonimmune serum and Protein A Sepharose (100 µL of 50% slurry) for 1 h at 4°C with end-to-end shaking, followed by centrifugation for 1 min at 15,000*g*. Transfer supernatant to a new tube.
3. Add antibodies for immunoprecipitation of Smads. If the transfected Smad protein is epitope-tagged, antibodies directed against tags can be used according to manufacturer's recommendations. Antibodies that are able to immunoprecipitate endogenous Smads have also been reported ***(5,6)*** .
4. Incubate for 2–4 h, add Protein A Sepharose (100 µL of a 50% slurry) and continue incubation with end-to-end shaking for 30–60 min at 4°C (*see* **Note 12**).
5. Wash the Protein A Sepharose beads four times with the lysis buffer, once with distilled water, and add 60 µL of 1.5 times concentrated Laemmli sample buffer. The radioactivity of the sample, measured by a portable β-detector should be

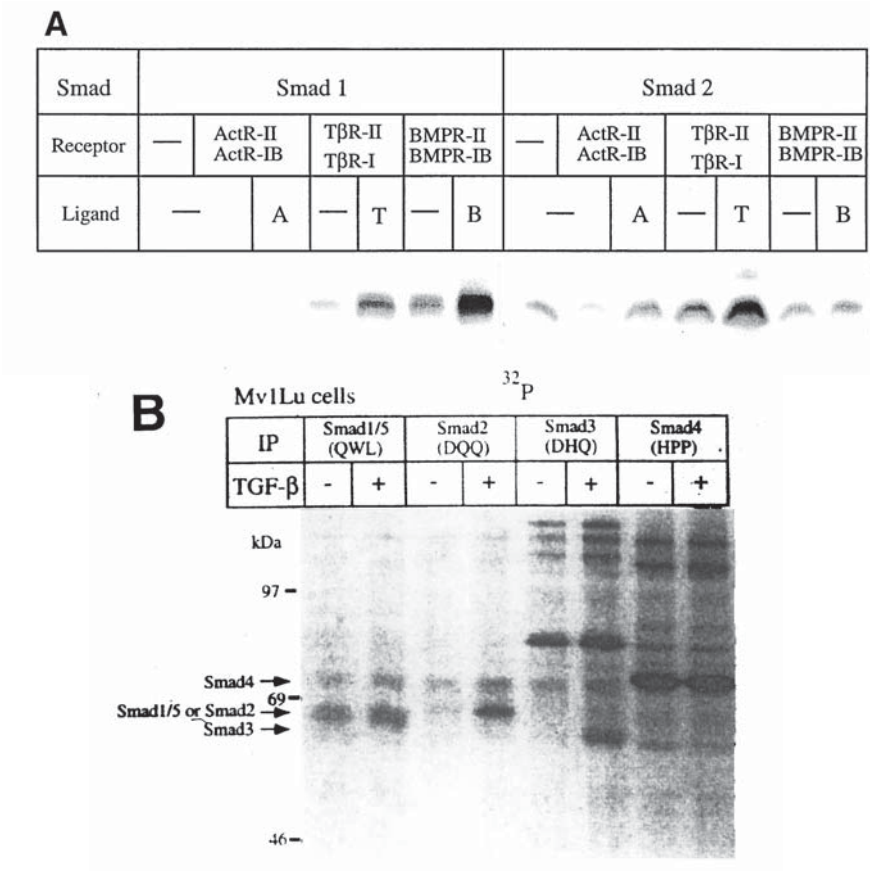

Fig. 3. Ligand-induced phosphorylation of Smads. **(A)** Smad phosphorylation by TGF-β family members in COS cells. COS cells transfected with Flag-Smad1 or Smad2 alone, or the indicated type I and type II receptors were labeled with [^{32}P]orthophosphate, immunopreciptated with anti-Flag antibodies or SED antiserum, and analyzed by SDS-PAGE. COS cells were treated with 10 ng/mL TGF-β1 (T) or 100 ng/mL activin (A) or 100 ng/mL BMP-7 (B), where indicated. **(B)** TGF-β1-induced phosphorylation of Smad2 and Smad3 and constitutive phosphorylation of Smad4 in nontransfected mink lung epithelial (Mv1Lu) cells. Mv1Lu cells were labeled with [^{32}P]orthophosphate in the absence or presence of TGF-β. Cell lysates were subjected to immunoprecipitation (IP) with DQQ, DHQ, HPP, or QWL antisera that recognize Smad2, Smad3, Smad4, or Smad1/5, respectively, and analyzed by SDS-PAGE and autoradiography.

from 20 to 500 cpm. Higher activity (more than 1500 cpm) suggests insufficient washing of beads, or strong interaction of nonspecific labeled material with beads. In the first case, more stringent washing conditions should be used, e.g., RIPA

lysis buffer, or 0.5 *M* NaCl. In the second case, pretreatment of Protein A Sepharose beads with 0.5% bovine serum albumin (BSA) for 10 h before use is recommended.

6. Boil the samples for 5 min and proceed with SDS-PAGE using 7–12% gradient, or 8% homogeneous, gels.
7. Following SDS-PAGE, the gel is incubated in gel fixation buffer for 30 min and gel drying buffer for 15 min, both at room temperature. The gel is then dried on a slab gel dryer and exposed to X-ray film, or to a Fuji Image Analyzer plate. An illustration of Smad phosphorylation in transfected COS cells and in nontransfected mink cells is shown in **Fig. 3**. If two-dimensional separation of Smad phosphopeptides is to be performed, separated proteins must be transfered from a nonfixed gel to a nitrocellulose membrane.

3.5. Two-Dimensional Separation of Smad Phosphopeptides

To obtain further insight to the pattern of phosphorylation at distinct sites in Smads, two-dimensional phosphopeptide mapping can be performed *(7,8)*. In the first dimension, proteins are subjected to high-voltage electrophoresis, and in the second dimension phosphopeptides are separated by ascending chromatography (*see* also Chapter 7 for two-dimensional mapping of receptor tyrosine kinases).

1. Transfer ^{32}P-labeled proteins, separated by SDS-PAGE (*see* **Subheading 3.4.**) to a nitrocellulose membrane. We transfer in a transfer buffer for 3.5 h in Bio-Rad transfer unit at 400 mA with cooling (*see* **Note 13**).
2. After transfer, put the membrane in a plastic bag. The membrane should be kept wet all the time, because drying will interfere with trypsin digestion. Expose the membrane to an X-ray film, or to a Image Analyzer plate for 3–12 h depending on signal intensity. Mark the membrane, or the plastic bag, with radioactive ink. This facilitates alignment of the exposed and developed X-ray film, or print-out from Image Analyzer, with the membrane to localize protein bands of interest. Mark bands of interest on the membrane with a pencil, and cut them out. Reexpose the membrane to confirm that the bands of interest have been cut out properly.
3. A piece of membrane with band of interest should be incubated in 200 µL of 0.5% polyvinylpyrrolidon K30 (Aldrich) in 0.6% acetic acid at 37°C for 30 min to block the membrane. This is to prevent binding of trypsin to the membrane during the next steps.
4. Wash the membrane three times with water and add 200 µL of freshly prepared 50 m*M* ammonium hydrogen carbonate. Add 1 µg of trypsin (Promega), and incubate for 12–16 h at 37°C. Trypsin is prepared by dilution of 20 µg of modified sequence grade trypsin with 200 µL of 1 m*M* HCl. 10 µL of this stock solution contain 1 µg of trypsin (*see* **Note 14**).
5. Transfer solution to a new tube. Add 200 µL of 50 m*M* ammonium hydrogen carbonate to the membrane (a solution prepared 1 d before may be used; pH of

the solution should be 7.3 to 7.6), and combine it with the previous aliquot in a new tube. Freeze the sample at –70°C and vacuum dry it, preferentially in a Speed-Vac (*see* **Note 15**). After drying, a small quantity of white powder may be present in the tubes, but the sample should not appear yellowish or "caramel-like" (*see* **Note 16**).

6. Prepare performic acid by incubating 450 μL of formic acid with 50 μL of 30% hydrogen peroxide for 1 h at room temperature. Oxidize dried samples with performic acid for 1 h on ice.
7. Add 500 μL of water, mix, freeze, and dry in a Speed-Vac under deep vacuum (*see* **step 5**, above) for 4–5 h.
8. Add 50 μL of 50 m*M* ammonium hydrogen carbonate, vortex thoroughly, sonicate for 3 min, and add 1 μg of trypsin (10 μL of trypsin stock solution). Continue the digestion for 6–12 h at 37°C.
9. Add 150 μL of electrophoresis buffer, pH 1.9, and centrifuge the samples at 15,000*g* for 1 min at room temperature. Transfer 190 μL of the supernatant to a new tube, freeze it, and vacuum dry in a Speed-Vac. If a white powder appears, redissolve it in 10 μL of electrophoresis buffer, and vacuum-dry again. The presence of a white powder, or especially caramel-like substances, would make efficient separation of phosphopeptides impossible (*see* **Note 16**).
10. Add 10 μL of electrophoresis buffer, vortex, and centifuge at 15,000*g* for 1 min at room temperature.
11. Take out the supernatant with an automatic pipet, carefully avoiding insoluble material. Apply in small portions (0.5–1.0 μL) to a cellulose thin-layer chromatography plate (20 cm × 20 cm; Merck). In order to obtain the best resolution of Smad phosphopeptides, the application points for different Smads should be as shown in **Fig. 4**. Use of a fan with no heating will accelerate the sample application.
12. Carefully wet the loaded plate by applying a filter paper with a hole at the place of application point that has been moistened with pH 1.9 electrophoresis buffer. Migration of the buffer from the hole border to the center will also facilitate concentration of the sample. The plate has to be wet, but no liquid should be on the surface. Perform high voltage electrophoresis. For HTLE system, apply 2000 V for 25 min (Smad2) to 30 min (Smad4).
13. Dry plate extensively in a fume hood. A fan (without heating) is useful for this purpose. Drying should be at least 45 min, with a fan, or 3 h without. Noncomplete drying may lead to different migration pattern of phosphopeptides on chromatography.
14. With a scalpel blade, make a line 2.5 cm from the top of the plate. Chromatography, which takes about 12 h, will stop at this line. Note the time of chromatography for every plate. That may help in analysis and comparison of migration pattern of phosphopeptides. Usually, the chromatography time should be similar for all plates in the same experiment. For ascending chromatography we use isobutyric acid:n-butanol:pyridine: acetic acid:water buffer (*see* **Note 17**).
15. After chromatography, dry plates in a fume hood for at least 3 h. Use of a fan with heating should be avoided if phosphoamino acid analysis, or sequencing of separated phosphopeptides, will follow. For identification of the phosphopeptides

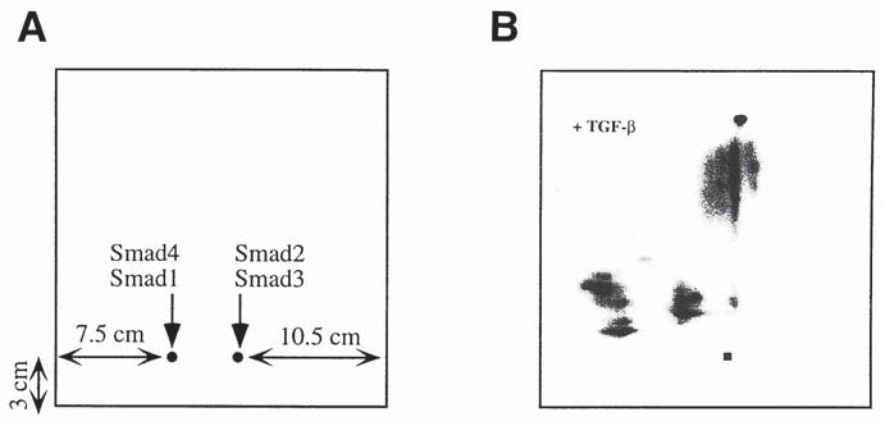

Fig. 4. Two-dimensional phosphopeptide map of Smad protein. **(A)** Scheme of sample application points for Smad1, Smad4, and for Smad2, Smad3. **(B)** Two-dimensional phosphopeptide map of Smad2, purified from cells treated with TGF-β.

of interest, mark the plates with radioactive ink to allow alignment with X-ray film or an Imager Analyzer print-out. We recommend the use of a Fuji Image Analyzer equipment for detection purposes because the intensities of radioactive signals of many phosphopeptides are very low.

16. Analyze the print-out from the Image Analyzer or developed X-ray films. For identification of phosphorylation site(s) in a particular phosphopeptide, align the image with the two-dimensional plate and mark the position of the phosphopeptide of interest with a pencil.
17. Scrape the phosphopeptide spot from the plate to a tube and extract twice with 200 mL of pH 1.9 electrophoresis buffer. Pool supernatants in a new tube. Dry it in a Speed-Vac. At this stage a phosphopeptide of interest can be used for phosphoamino acid analysis, radiochemical sequencing, flow mass-spectrometry, or for other analysis. Based on the sequence of peptides their migration in the 2-D system described above can be predicted ***(9)***.

4. Notes

1. The provided information on suppliers for reagents and equipment is not comprehensive and is not meant to reflect an endorsement by us for a particular supplier.
2. After receptor-mediated phosphorylation of pathway-restricted Smads, they form a complex with Smad4 that accumulates in the nucleus. Heteromeric complex formation of two Smads has been demonstated in transfected COS cells using epitope tagged Smads and by immunoprecipitation with one antisera followed by immunoblotting with another ***(10)***. Ligand-induced nuclear accumulation has

been demonstrated in transfected COS cells, as well as nontransfected cells, by immunofluorescence with antisera to endogenous and transfected, epitope tagged Smads ***(5,6,11)***.

3. Epitope tagging at the C-terminus of pathway-restricted Smads should be avoided because it interferes with phosphorylation by receptor kinases. When expressed to sufficiently high levels, such molecules act in a dominant negative fashion. Epitope tagging at the N-terminus of pathway-restricted Smads does not intefere with receptor-mediated phosphorylation, and is less likely to interfere with Smad function. Use of different expression vectors for Smads and TGF-β superfamily receptors may result in different expression levels for these proteins.
4. Upon overexpression of type I and type II receptors in COS cells, ligand-independent complex formation may occur because of the intrinsic affinity between receptors. This leads to a ligand-independent activation of type I receptors and, thereby, phosphorylation and activation of Smads. Usually ligand-addition after receptor expression leads to a further increase of type I receptor activation. As a control, it is recommended to also investigate COS cells transfected with Smads in absence of receptors.
5. Different transfection protocols, e.g., using lipofection or calcium phosphate precipitation may be better for other cell lines.
6. Exceeding a total DNA concentration of 4 μg/mL of medium may result in a dramatic decrease of transfection efficiency.
7. Receptor expression can be analyzed by crosslinking with appropriate iodinated ligand ***(12)***, by immunoprecipitation on metabolically labeled cells, or by Western blotting ***(13)*** of cell extracts with antireceptor antibodies. Smad expression can be analyzed by metabolic labeling with [^{35}S]methionine, followed by immunoprecipitation with anti-Smad ***(7,8)***, or antitag antibodies, or by Western blotting with these antibodies.
8. We use phosphate-free Ham's F-12, but other types of medium, e.g., phosphate-free DMEM, can also be used.
9. Lower concentrations of [^{32}P]orthophosphate result in weakened Smad phosphorylation signals.
10. Special attention should be given to prevent evaporation of liquid during incubation. This can be achieved by careful wrapping of dishes with parafilm, or incubation in a closed Plexiglas protection box in the presence of wet paper. Use of Plexiglas box is preferable, because it provides an additional protection against radioactive radiation.
11. Original (soft-plastic) Eppendorf tubes should be used, as they provide much better protection against contamination by spilling through tube cap.
12. In the aforementioned time intervals, no significant differences in Smad phosphorylation have been observed with different cell types, overexpressed or endogenous Smads, or different antisera.
13. Nylon membrane is not recommended.
14. Trypsin solution can be stored at –20°C.

15. Vacuum should be enough deep to keep the liquid in the tubes frozen. If the samples are dried as nonfrozen, it may affect the quality of the maps.
16. If a caramel-like substance appears, 100 μL of water should be added and sample should be dried under deep vacuum again. If another electrophoresis buffer is used, the solubilization should be performed in this buffer.
17. The chromatography buffer has a very strong smell, and chromatography should be performed in a fume hood. Other chromatography buffers may be used as well, depending on the resolution desired (*9*).

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8

Assays for Mitogen-Activated Protein Kinase (MAPK) Subtypes and MAPK Activating Protein Kinase-2 (MAPKAP K-2) Using a Common Cell Lysate

Rozen Le Panse, Navita Rampersaud, and Louis C. Mahadevan

1. Introduction

The mitogen-activated protein kinases (MAPKs) correspond to a family of serine/threonine kinases that can be divided into three subtypes, ERK, JNK/SAPK, and p38 MAPK. They are activated by a complex set of upstream kinases organized into parallel kinase cascades. This introduction will not discuss these upstream kinases, but **Fig. 1** illustrates the complexity of these cascades ***(1–4)***. Note that there appears to be cell-line specific variation within this circuitry, especially in relation to upstream activators of these cascades, although this remains a contentious issue.

The first group of MAPKs, called extracellular-regulated kinases (ERKs), was discovered at the end of the 1980s by their ability to phosphorylate microtubule-associated protein, MAP2, and were molecularly cloned from peptide sequence ***(5)***. ERK1 and ERK2 isoforms (44 and 42 kDa, respectively) have been well studied and are activated in response to various mitogenic signals (growth factors, hormones, etc.). These ERK isoforms are directly activated by two protein kinases MEK1 and MEK2 (MAPK/ERK kinase 1 and 2), which phosphorylate ERKs on both tyrosine and threonine residues, the two residues being separated by glutamic acid, thus delimiting a phosphorylated sequence TEY ***(6)***. Once activated, ERK isoforms can phosphorylate further downstream kinases (e.g., MAPKAP K-1, etc), as well as many enzymatic and transcription factor substrates, thereby modulating cellular behavior ***(1,2)***.

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

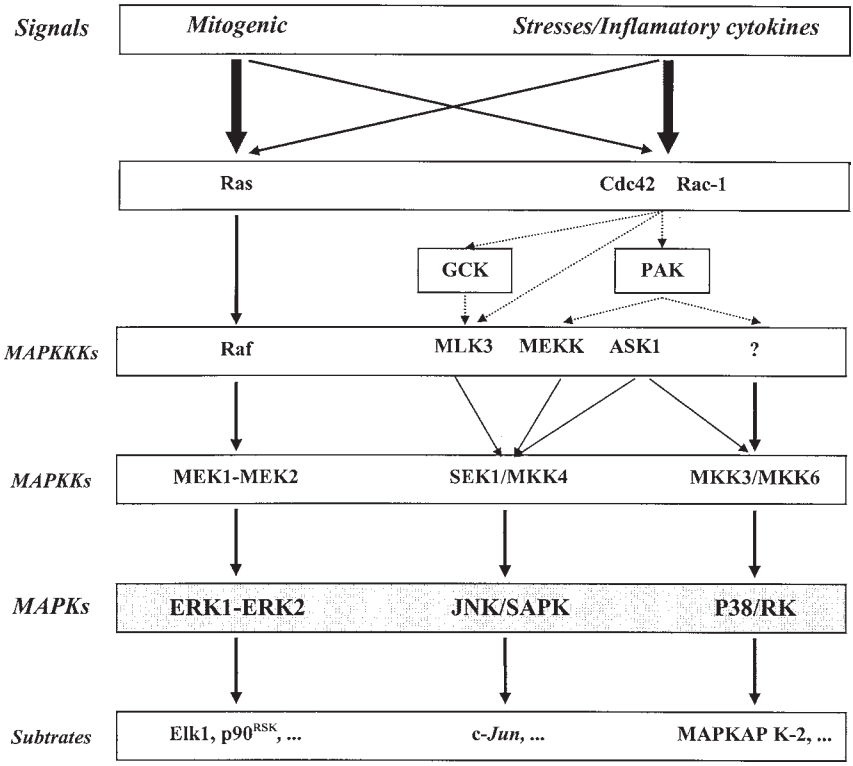

Fig. 1. Schematic representation of the different MAPK signaling pathways. MAPK: mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPKK kinase; ERK: extracellular-regulated kinase; JNK/SAPK: c-Jun NH2-terminal kinase; p38 MAPK stress-activated protein kinase: *p38*/reactivating kinase; MEK: MAPK/ERK kinase; MKK: MAP kinase kinase; SEK1: Stress-activated protein kinase-ERK kinase 1; MEKK: MEK kinase; MLK3: mixed lineage kinase 3; ASK1: apoptosis signal-regulating kinase 1; PAK: *p21*-activated kinase; GCK: germinal center kinase; MAPKAP K-2: MAPK-activated-protein kinase-2.

More recently, another group of MAPKs, JNK (c-Jun NH_2-terminal kinase), was identified by its ability to phosphorylate the NH_2-terminal domain of c-Jun. This domain possesses a region called the delta region to which JNK binds specifically and phosphorylates two serine residues stimulating transactivation of c-Jun *(7)*. This category of MAPK, also called SAPK (stress-activated protein kinase) *(8)*, is activated weakly in response to growth factors but strongly in response to extracellular stresses (UV, osmotic shock, and heat shock). Like the ERKs, JNK/SAPKs are activated by dual phosphorylation within a

sequence TPY by the MEK homologs, SEK1/MKK4 (SAPK-ERK kinase 1/MAP kinase Kinase 4) ***(9)*** and MKK7 ***(18)***.

The last group of MAPKs corresponds to a protein kinase which, similar to JNK/SAPKs, is also strongly stimulated by extracellular stresses. This protein kinase, called p38MAPK, is the mammalian homolog of the HOG1 protein that is involved in the osmolarity response in *Saccharomyces cerevisiae* ***(10)***. p38MAPK has been identified by its ability to phosphorylate and activate *in vivo* another serine/threonine kinase, the MAPK-activated protein kinase-2 (MAPKAP K-2) ***(11)***. p38MAPK is activated by dual phosphorylation on a sequence TGY by two MEK homologs, MKK3 and MKK6 ***(12)***.

In general, the activities of the different MAPKs have been studied using different lysis procedures and methods for ERK, JNK/SAPK, or p38MAPK cascades. Here, we describe a method that facilitates a direct comparison of the state of activation of all three cascades within a single cell lysate. In the following sections, the use of a defined lysis buffer to harvest monolayer cell cultures is first described. We then describe three different assays to analyze the state of activation of ERK, JNK/SAPK, and p38MAPK cascades in this lysate.

2. Materials

2.1. General Reagents

1. [γ-^{32P}]adenosine triphosphate (ATP): NEN Research Products or Amersham.
2. All other general reagents and chemicals can be obtained from Sigma.
3. Protease inhibitor mixtures ***(14)***:
   a. Mixture A (100×): 200 µg/mL aprotinin, 500 µg/mL leupeptin, 5 mg/mL soybean trypsin inhibitor, and 5 mg/mL tosyl lysyl chloromethyl ketone made up in distilled water (*see* **Note 1**).
   b. Mixture B (100×): 10 mg/mL tosyl phenylalanyl chloromethyl ketone and 10 mg/mL phenylmethanesulphonyl fluoride made up in ethanol (*see* **Note 1**).
4. Prestained markers (New England BioLabs).
5. Cell lysis buffer: 25 m*M* HEPES, pH 8.0, 0.2 m*M* ethylenediaminetetraacetic acid (EDTA), 1.5 m*M* $MgCl_2$, 0.3 *M* NaCl, 0.1% Triton X-100, 20 m*M* β-glycerophosphate, 0.1 m*M* orthovanadate, 1 µ*M* microcystin-LR, and 1× protease inhibitor mixture A and B.
6. 4× Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 40% v/v glycerol, 8% SDS, 250 m*M* Tris-HCl, pH 6.8, 20% w/v β-mercaptoethanol, 0.004% v/v bromophenol blue. This is diluted with water to give 2× SDS- PAGE sample buffer.
7. Gel running buffer: 0.25 *M* Tris, 1.92 *M* glycine, and 1% w/v SDS.

2.2. Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM).
2. Fetal calf serum (FCS).

3. Glutamine.
4. Phosphate-buffered saline (PBS).

All cell culture reagents obtained from Life Technologies.

2.3. JNK Assay

1. GSH-agarose (Sigma).
2. Glutathione-*S*-transferase (GST)-c-Jun$_{1-79}$: now provided by companies such as New England BioLabs and Alexis Biochemicals. GST–c-Jun$_{1-79}$ can also be prepared as described by Smith and Corcoran ***(13)*** (*see* **Note 2**).
3. Dilution buffer: 20 m*M* HEPES, pH 8.0, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, 0.05% Triton X-100, 20 m*M* β-glycerophosphate, 0.1 m*M* orthovanadate, and 1× protease inhibitor mixture A and B.
4. HEPES binding buffer (HBIB): 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 0.1 m*M* EDTA, 25 m*M* $MgCl_2$, 0.05% Triton X-100.
5. JNK/SAPK kinase buffer: 20 m*M* HEPES, pH 8.0, 2 m*M* dithiotreitol (DTT), 20 m*M* β-glycerophosphate, 20 m*M* $MgCl_2$, and 0.1 m*M* orthovanadate.

2.4. ERK Assay

1. Mouse antibody against ERK1 and ERK2: Zymed Laboratories Inc. (cat. no. 13-6200).
2. Goat peroxidase-conjugated antimouse IgG antibody (Amersham).
3. ECL kit (Amersham).
4. Polyvinylidene difluoride (PVDF) membrane (Immobilon-P) (Millipore).
5. 15% polyacrylamide minigel (0.75-mm thickness).

Stock solutions (*see* **Note 3**)	**Resolving gel** (for 10 mL)	**Stacking gel** (for 5 mL).
30% w/v acrylamide 5 ml	0.833 mL	
1% w/v *bis*-acrylamide	0.860 mL	0.666 mL
1.5 *M* Tris-HCl (pH 8.8)	2.5 mL	—
1 *M* Tris-HCl (pH 6.8)	—	0.618 mL
Distilled water	1.585 mL	2.821 mL
10% ammonium persulfate	50 µL	50 µL
TEMED	5 µL	5 µL

6. Transfer buffer 0.05% w/v SDS, 12.5 m*M* Tris, 96 m*M* glycine, and 10% v/v methanol.
7. TBST: 20 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, and 0.05% v/v Tween-20.

2.5. MAPKAP K-2 Assay

1. Random copolymer of L-glutamic acid and tyrosine (4:1, poly-Glu-Tyr, Sigma).
2. SDS removal buffer: 50 m*M* Tris-HCl, pH 8.0 plus 20% v/v isopropanol (250 mL/gel).
3. Equilibration buffer: 50 m*M* Tris-HCl, pH 8.0 plus 1 m*M* DTT (250 mL/gel).
4. Denaturation buffer: 50 m*M* Tris-HCl, pH 8.0, 6 *M* Guanidine-HCl, 20 m*M* DTT and 2 m*M* EDTA (50 mL/gel).

5. Renaturation buffer: 50 m*M* Tris-HCl, pH 8.0 containing 1 m*M* DTT, 2 m*M* EDTA, and 0.04% v/v Tween-20.
6. MAPKAP K-2 kinase buffer: 40 m*M* HEPES, pH 8.0, 1 m*M* DTT, 0.1 m*M* ethylene glycol-*bis* N,N,N',N'-tetraacetic acid (EGTA), 20 m*M* MgC_{l2}, 100 μ*M* orthovanadate.

3. Methods

3.1. Cell Preparation

C3H 10T 1/2 mouse fibroblasts are used in the experiments described here. The lysis buffer used here allows the analysis of many kinases within the three MAPK cascades (ERK, JNK/SAPK, and p38 MAPK) in the same lysate. As an illustration, we describe analysis of JNK/SAPK activation by "pull down" assay (*see* **Subheading 3.2.**), ERK activation by Western blotting (*see* **Subheading 3.3.**) and MAPKAP K-2 by "in gel" kinase assay (*see* **Subheading 3.4.**).

1. Confluent fibroblast monolayer cultures (60-mm diameter dishes) grown in DMEM containing 2 m*M* glutamine and 10% (v/v) FCS are rendered quiescent by a 24-h incubation in DMEM containing 2 m*M* glutamine and 0.5% (v/v) FCS.
2. The fibroblasts are then stimulated with appropriate stimuli and incubated for a given time at 37°C. Cells prepared in 60-mm diameter dishes are then scraped in 100 μL ice-cold cell lysis buffer (*see* **Subheading 2.1.**) and placed immediately on ice (*see* **Note 4**).
3. The cell suspension is rotated at 4°C for 30 min and the extract cleared by centrifugation at 13,000*g* for 10 min at 4°C. Depending on the size of the plates used, there may be a substantial pellet at this stage.
4. The supernatant is collected taking care not to disturb the pellet and divided in two fractions:
   a. 30 μL are mixed with 10 μL of 4× SDS-PAGE sample buffer (*see* **Subheading 2.1.**). Samples can then be stored at –20°C for the analysis of ERK and MAPKAP K-2 activity at a later point (*see* **Subheadings 3.3.** and **3.4.**).
   b. 70 μL are used directly to determine the activation state of JNK/SAPK; this is used fresh and not frozen (*see* **Subheading 3.2.**).

3.2. JNK/SAPK Assay: GST Fusion Protein-Associated In Vitro Kinase Assay

The activation of JNK/SAPK isoforms is analyzed by their ability to bind to and phosphorylate the NH_2-terminal domain of c-Jun *(7)*. We use an NH_2-terminal fragment of c-Jun ($c\text{-}Jun_{1\text{-}79}$) containing the delta region (necessary for JNK/SAPK binding) and the two serine residues. This fragment is linked to GST protein and the recombinant protein can thus be recovered on glutathione-agarose beads *(13)*. The phosphorylation of this GST-c-Jun by bound kinases is then detected using [γ-^{32}P] ATP. In this assay all samples and buffers should be kept on ice as much as possible.

1. The supernatant (70 µL) collected after cell lysis (*see* **Subheading 3.1.**) is diluted with three volumes of dilution buffer (*see* **Subheading 2.3.**) and rotated at 4°C for 30 min (*see* **Note 5**).
2. The cell extract is centrifuged at 13,000*g* for 10 min at 4°C to remove any precipitation caused by the altered salt concentration. The supernatant is mixed with 10–20 µg of glutathione-agarose beads linked to GST–c-Jun_{1-79} (usually suspended in 20 µL of dilution buffer) and rotated overnight at 4°C to allow protein interactions.
3. The glutathione-agarose beads with GST–c-Jun_{1-79} and bound JNK/SAPKs are recovered from the lysates by brief centrifugation at a low speed (10 s at 380*g*.) and washed four times with similar centrifugations with 1 mL of HEPES binding buffer (HBIB).
4. The agarose beads are finally resuspended in 30–50 µL of JNK/SAPK kinase buffer and incubated with 20 µ*M* unlabeled ATP and 3–5 µCi [γ-3^{2P}]ATP for 40 min at 30°C (*see* **Note 6**). The reaction is terminated by two washes with 1 mL of HBIB buffer. After the final wash, as much supernatant as possible is removed. An additional brief centrifugation can help at this stage.
5. The phosphorylated proteins are then eluted in 30 µL of 2× SDS-PAGE sample buffer and can be stored at –20°C at this stage. When required, samples are boiled for 5 min. This separates the beads from the GST-fusion proteins. Samples should be briefly spun to separate the beads from the GST-fusion proteins which remain in the supernatant. This supernatant is then resolved on a 10% SDS-polyacrylamide minigel and detected by autoradiography of the dried gels (*see* **Fig. 2**).

3.3. ERK Assay: Western Blotting with an Antibody Against ERK1 and ERK2

MAPKs are activated by phosphorylation of two residues, threonine, and tyrosine. In the case of ERK1 and ERK2, the phosphorylated isoforms possess a slower electrophoretic mobility in polyacrylamide gels and a shift between the inactive (nonphosphorylated) and active (phosphorylated) ERK isoforms can be observed (*see* **Fig. 3**) ***(15)***.

1. The cell extract (30 µL) mixed with 4× SDS-PAGE sample buffer (from **Subheading 3.1.**) is boiled for 5 min, centrifuged briefly to remove insoluble material and 10 µL are electrophoresed on a 15% polyacrylamide minigel (*see* **Subheading 2.4.**) (*see* **Note 7**).
2. ERK1 and ERK2 possess molecular weights of 44 kDa and 42 kDa, respectively. Using prestained markers, electrophoresis can be performed on a minigel until the 32.5-kDa marker reaches the bottom of the resolving gel.
3. Proteins in the gel are then transferred onto PVDF membrane by electroblotting in transfer buffer for 3 h at 300 mA or overnight at 70 mA, at 4°C.
4. The membrane is "blocked" for 1 h at room temperature or overnight at 4°C in TBST (*see* **Subheading 2.4.**) plus 5% nonfat dried milk, and then incubated for 1

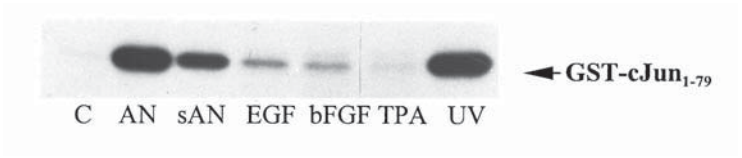

Fig. 2. Phosphorylation of GST–c-Jun$_{1-79}$ by JNK/SAPK. GST–c-Jun$_{1-79}$ protein-associated kinase assay from confluent and quiescent C3H 10T 1/2 cells treated with anisomycin 60 min (AN = 10 μg/mL and sAN = 50 ng/mL), epidermal growth factor (EGF) 15 min (50 ng/mL), basic fibroblast growth factor (bFGF) 15 min (20 ng/mL), 12-O-tetradecanoylphorbol 13-acetate (TPA) 15 min (100 μ*M*), UV 30 min (200 J/m^2) and a control (C).

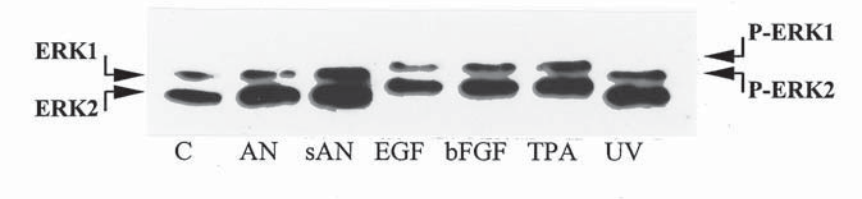

Fig. 3. Activation of ERK1 and ERK2. Western blot analysis with a monoclonal antibody against ERK1 and ERK2 of lysates from confluent and quiescent C3H 10T 1/2 cells treated with anisomycin 60 min (AN = 10 μg/mL and sAN = 50 ng/mL), EGF 5 min (50 ng/mL), bFGF 5 min (20 ng/mL), TPA 5 min (100 μ*M*), UV 30 min (200 J/m2) and a control (C).

h at room temperature with an antibody against ERK1 and ERK2 diluted 1:5000 in TBST (*see* **Note 8**). After four washes of 5–10 min each in TBST, the membrane is incubated for 1 h at room temperature with a goat peroxidase-conjugated antimouse IgG antibody diluted 1:5000 in TBST. The membrane is finally washed four times for 5–10 min in TBST before the detection of the immune signal using the enhanced chemiluminescence (ECL) method (*see* **Fig. 3**).

3.4. MAPKAP K-2 Assay: In-Gel Protein Kinase Assay

To analyze the state of activation of the p38 MAPK cascade, we use its downstream kinase MAPKAP K-2 as an indicator. The activity of MAPKAP K-2 is analyzed using an in-gel protein kinase assay. This assay consists of separating proteins in a SDS-polyacrylamide gel in which polypeptides are copolymerized. After electrophoresis, the proteins are renatured in the gel to recover kinase activity, and if appropriate, they can phosphorylate the polypeptide copolymerized in the gel; this is detected by incubating the gel with [γ-^{32}P]ATP (*see* **Note 9**). Here, the MAPKAP K-2 activated isoforms are detected using a

random copolymer of L-glutamic acid and tyrosine (poly-Glu-Tyr) in the SDS-polyacrylamide gel. MAPKAP K-2 isoforms do not phosphorylate poly-Glu-Tyr in this in-gel protein kinase assay, but the ability of the active kinases to autophosphorylate is enhanced in the presence of this polymer ***(16,17)***.

1. SDS-polyacrylamide minigels are made as normal with the exception that 200 μg/mL of poly-Glu-Tyr is included in the resolving gel mixture prior to casting. The stacking gel is prepared as normal.
2. The cell extract (30 μL) mixed with 4× SDS-PAGE sample buffer (*see* **Subheading 3.1.**) is boiled for 5 min, centrifuged briefly to remove insoluble material, and 20 μL are electrophoresed on the 14% polyacrylamide minigel copolymerized with poly-Glu-Tyr. Using prestained markers, samples are electrophoresed until the 32.5-kDa marker has migrated to the bottom of the resolving gel (*see* **Note 10**).
3. After electrophoresis, SDS is removed by incubating the gel in SDS removal buffer (250 mL/gel) followed by another incubation in equilibration buffer (250 mL/gel) (*see* **Subheading 2.5.**) Then, the proteins are denatured by incubating the gel in denaturation buffer (50 mL/gel)(*see* **Subheading 2.5.**). All these incubations are carried out with gentle shaking for 1 h at room temperature. Finally, the proteins are renatured by an overnight incubation without agitation at 4°C in renaturation buffer (*see* **Subheading 2.5.**).
4. For the kinase assay, the gel is equilibrated for 1 h at room temperature in MAPKAP K-2 kinase buffer (*see* **Subheading 2.5.**). The kinase assay is carried out in fresh MAPKAP K-2 kinase buffer containing 3 μ*M* unlabeled ATP and 10 μCi [γ-^{32}P]ATP for 1 h at room temperature. The gel is then washed extensively in 5% (w/v) trichloroacetic acid (TCA) plus 1% (w/v) sodium pyrophosphate until washes are free of radioactivity. Autoradiography of dried gels is then performed (*see* **Fig. 4**).

4. Notes

1. When required, the protease inhibitor solutions A and B are added to the buffers just prior to use.
2. The amount of purified fusion protein is determined by the BCA (Pierce) protein assay. The GST-fusion proteins are then stored in 0.02% sodium azide and 20% glycerol at –20°C and when required beads are washed four times with dilution buffer.
3. To obtain the best separation of the proteins, all these solutions are made fresh and filtered through 0.45-μm Millipore filters.
4. Assays can be scaled up to 100-mm-diameter dishes, using 250 μL of lysis buffer.
5. This step is performed to dilute the high salt concentration present in the lysis buffer that can affect protein interactions.
6. During this incubation, beads are resuspended by gentle agitation occasionally (twice in 40 min).

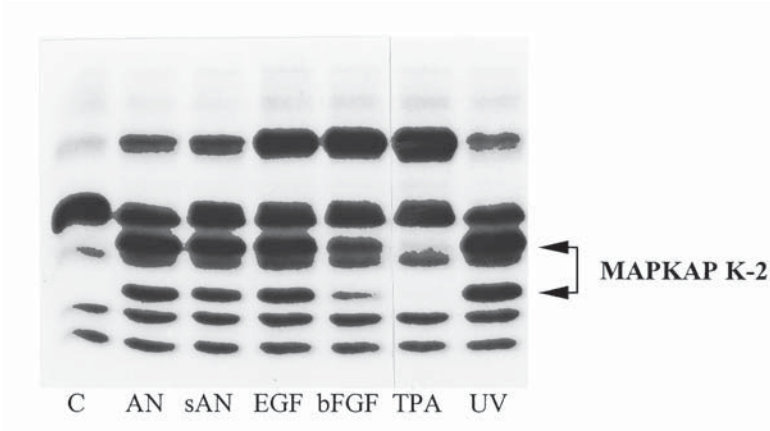

Fig. 4. Autophosphorylation of MAPKAP K-2. In-gel protein kinase assay using SDS-polyacrylamide gel copolymerized with a poly-Glu-Tyr copolymer. Analysis of MAPKAP K-2 phosphorylation from confluent and quiescent C3H 10T $^1/_2$ cells treated with anisomycin 60 min (AN = 10 μg/mL and sAN = 50 ng/mL), EGF 5 min (50 ng/mL), bFGF 5 min (20 ng/mL), TPA 5 min (100 μ*M*), UV 30 min (200 J/m^2) and a control (C).

7. In the ERK assay, a modified SDS-PAGE system is used. The gel itself does not contain SDS, though SDS is present in the gel running buffer. This provides better resolution of active and inactive forms of the ERKs.
8. High background can be reduced by incubating the blot with the antibodies diluted in TBST plus 5% nonfat dried milk
9. The in-gel protein kinase assay can also be used to analyze ERK1 and ERK2 or JNK activity when the gel is copolymerized with 500 μg/mL MBP (myelin basic protein) or 100 μg/mL GST–c-Jun$_{1\text{-}79}$, respectively ***(7,16)***. Note that in contrast to the effect of poly-Glu-Tyr in the MAPKAP K-2 assays, these proteins are utilized as substrates by the renatured kinases in the gel.
10. In mouse fibroblasts, there are two MAPKAP K-2 isoforms of 46 and 55 kDa, whereas human fibroblasts yield a single predominant 50 kDa form ***(16,17)***.

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9

JAK-Mediated Phosphorylation and Activation of STAT Signaling Proteins

Analysis by Phosphotyrosine Blotting and EMSA

Nicola Broughton and Mark S. Burfoot

1. Introduction

The JAK/STAT pathway is activated by a wide range of ligands including cytokines and growth factors (***1***,***2***; *see* **Table 1**). This pathway was discovered by two independent approaches that initially identified the role of JAKs and STATs in interferon signaling. The first, a biochemical approach, used promoter elements to isolate and purify STATs ***(3)*** and the second, a genetic approach, led to the isolation of mutants defective in their response to interferon ***(4)***. The latter resulted in eight mutant cell lines (summarized in **Table 2**) which were complemented by components of the interferon pathway ***(5–9)***. These mutants have been fundamental in demonstrating a role for JAKs and STATs in cytokine signaling and in understanding the mechanisms of activating the JAK/STAT pathway.

In humans, the JAK family consists of four members: JAK1, JAK2, JAK3, and TYK2. With the exception of JAK3, which is only expressed in hematopoietic cells, the JAKs are universally expressed ***(10)***. Initial studies on JAKs identified them as a novel family of tyrosine kinases ranging from 120 to 140 kDa in size. These proteins do not possess a transmembrane region and were, therefore, placed in the cytoplasmic family of tyrosine kinases, but unlike other kinases they contain no known classical SH2, SH3, PTB or PH domain. There are seven homology domains, JH1–JH7, conserved between the JAKs, numbered sequentially from the C-terminus. JH1 is a catalytically active kinase domain. JH1 and JH2 are highly conserved but JH2 lacks key residues involved in kinase activation and is, therefore, catalytically inactive and known as a pseudokinase domain. Little is known about the exact function of JH3–JH7,

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

Table 1
JAKs and STATs Activated by Various Cytokines

Cytokine	Shared subunit	JAK	STAT
IL-12	None	JAK2, Tyk2	STAT3/4
G-CSF		JAK1, JAK2	STAT1/3
EPO		JAK2	STAT5
PRL		JAK1, JAK2	STAT5
GH		JAK2	STAT5
TPO		JAK2	STAT1/3/5
IFNα/β		JAK1, Tyk2	STAT1/2/3
IFNγ		JAK1, JAK2	STAT1
IL-10		JAK1, Tyk2	STAT1
Serotonin		JAK2	STAT3
IL-3	βc	JAK1, JAK2	STAT5/6
GM-CSF		JAK1, JAK2	STAT5
IL-5		JAK1, JAK2	STAT5
IL-15	γc	IL-213	JAK1, JAK3
STAT3/5			
IL-2		JAK1, JAK3	STAT3/5
IL-7	γc	JAK1, JAK3	STAT1
IL-9		JAK1, JAK3, Tyk2	STAT1/3
IL-4	γc IL-4α	JAKI, JAK3	STAT6
IL-13	IL-4α	JAK1	STAT6
IL-6	gp130	JAKi, JAK2, Tyk2	STAT1/3
LIF/CNTF		JAK1, JAK2	STAT3
OSM		JAK1, JAK2, Tyk2	STAT1/3

but these domains are implicated in receptor binding and interaction with other proteins required for a biological response ***(10)***.

Upon ligand binding, JAKs, which are constitutively bound to receptor chains, are juxtaposed and activated by auto- and transtyrosine phosphorylation. Notably for JAK activation, two key residues in the KEYY motif of the kinase domain are thought to be tyrosyl-phosphorylated ***(11)***. Activation of STATs, downstream of JAK activation, is crucial to cytokine mediated gene induction. STATs are recruited to cytokine receptors and phosphorylated on tyrosine and serine residues ***(12)***. STATs 1, 3, 4, and 6 are recruited to specific motifs through their SH2 domains. STAT2, however, is only recruited to its target receptor after STAT1 has bound and STAT5 may be recruited to either motifs in target receptor chains and/or to JAKs directly ***(13)***. Hetero/homodimerization occurs through SH2 and tyrosine motifs and STATs translocate to the nucleus to activate gene transcription ***(14)***.

The JAK/STAT pathway can be analyzed in a variety of ways, utilizing a range of techniques that provide slightly different types of information. JAK

Table 2
Mutant Cell Lines Defective in their Response to Interferon

Response to Mutant Groups	Response to IFNα/β	Complementing IFNγ	Protein
U1	—	+	TYK2
U2	—	–/+	P48
U3	—	—	STAT1
U4	—	—	JAK1
U5	—	+	INFAR2c
U6	—	+	STAT2
γ1	+	—	AF1/JAK2
γ2	+	—	JAK2

kinases can be analyzed using an in vitro kinase assay following stimulation of a cell line with an appropriate ligand (such as interleukin 6 (IL-6) or oncostatin M) and subsequent immunoprecipitation of the specific JAK to determine kinase activity. Although this approach has its merits, the in vitro assay of JAK kinase activity does present technical difficulties not apparent for other kinases. As a consequence, one of the most frequently used techniques is Western blotting of either total cell lysate from an activated extract, or immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Phosphorylation can be identified either by metabolic labeling of cells with ^{32}P, or Western blotting of unlabeled lysates followed by detection of the JAK proteins with antiphosphotyrosine antibodies. The latter procedure is the favored method, and is described in this chapter.

Most cell types can be used for stimulation of JAKs and STATs, although tissue specificity may limit the choice of ligand used. An example provided here is of the activation of JAK1, STAT1, and STAT3 in response to IL-6 and JAK1, STAT1, and STAT5 in response to oncostatin M in fibrosarcoma cells (HT1080) (*see* **Fig. 1** that demonstrates an electrophoresis mobility shift analysis [EMSA] analysis of STATs and **Fig. 2** that shows an antiphosphotyrosine analysis of JAK1 and STAT5).

In essence, cells are harvested and lysed in an appropriate buffer and the desired JAK immunoprecipitated from the lysate. Precipitated protein is then fractionated by SDS-PAGE and Western blotted. The phosphorylated protein is detected using phosphotyrosine antibodies with subsequent verification of identity by use of JAK antibodies on the same blot. It is also possible to coimmunoprecipitate using this assay. Inducible associations can be identified such as the coimmunoprecipitation of JAK1 with the gp130 receptor chain after IL-6 or oncostatin *M* stimulation.

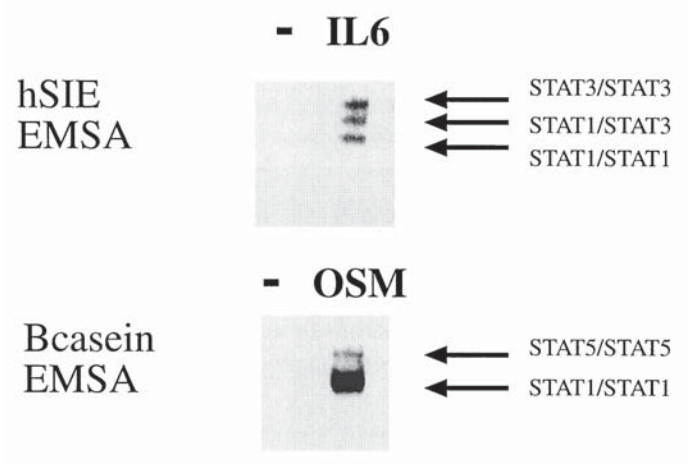

Fig. 1. Electrophoresis mobility shift analysis of stats. The LSIE EMSA shows the activation and DNA binding activity of stats 1 and 3 after treatment with H71080 cells IL6. The β-casein EMSA shows the activation of stats 1 + 5 after treatment with OSM.

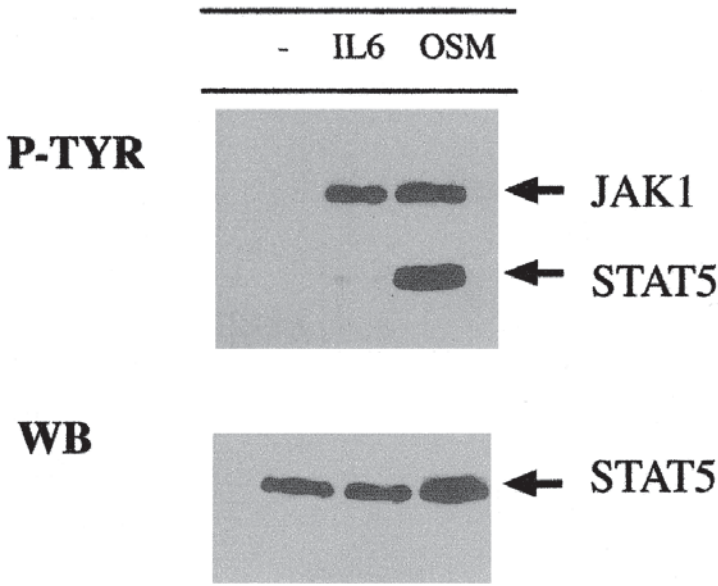

Fig. 2. Activation of JAK1 and STAT5 in response to OSM (and IL6). The P-TYR panel shows JAK1 and STAT5 phosphorylation using Anti P-TYR Antibodies. The bottom panel is a reprobe with STAT 5 antibodies to show even loading.

Table 3
Consensus STAT Binding Sites (GAS)

STAT1	TTCC[G>C]GGAA
STAT2	STAT2 does not bind to GAS elements
STAT3	TTCC[G=C]GGAA
STAT4	TTCC[G>C]GGAA
STAT5	TTCC[A>T]GGAA
STAT6	TTCC[A>T/N]GGAA

Being tyrosyl-phosphorylated, analysis of STAT proteins is also amenable to assay using antiphosphotyrosine Western blotting (*see* **Fig. 2** that shows phosphorylation of STAT5 in response to oncostatin M). As transcription factors, STATs can also be detected using other assays. These include ligand stimulation of cells transfected with reporter constructs bearing upstream binding sites for STAT proteins. In addition, immunohistochemistry using STAT specific antibodies can identify nuclear translocation of a STAT(s) following activation. A commonly used method is EMSA. Here, lysates from stimulated cells are prepared and analyzed for STAT/DNA binding using a radiolabeled oligonucleotide, which represents a STAT consensus binding sequence. A fraction of the lysate is incubated with a radiolabeled probe, electrophoresed on a nondenaturing polyacrylamide gel, and STAT complexes identified by subsequent autoradiography (*see* **Fig. 1**). STAT proteins bind to GAS elements within genes and, as such, these motifs are commonly used to provide oligonucleotide sequences for EMSA of STAT activation. A comprehensive list of GAS elements utilized by the STATs is illustrated in **Table 3** and **Subheading 2.6**.

This chapter describes two techniques for analysis of JAK-STAT signaling in cell culture models; Western blotting of SDS-PAGE with phosphotyrosine detection of activated JAKs and STATs, and electrophoresis mobility shift analysis (EMSA) of STAT activation.

2. Materials

Materials and antibodies described here were used routinely by the authors and their colleagues and are, therefore, only suggestions for the less experienced user. Unless otherwise stated, reagents can be obtained from Sigma.

2.1. Preparation of Whole Cell Extracts

Lysis buffers used for detection of JAK/STAT proteins: these can be stored at –20°C.

1. General purpose lysis buffer: 1% Triton X-100 (v/v), 10% glycerol (v/v), 50 m*M* HEPES, 150 m*M* NaCl, 1 m*M* ethylenediaminetetracetic acid (EDTA), pH 8.0,

200 μ*M* sodium orthovanadate (*see* **Note 1**), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 2**), 10 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride 1.5 m*M* magnesium chloride, 10 μg/mL aprotinin, 10 μg/mL leupeptin.

This buffer can be used routinely to look at JAK and STAT activation by Western blotting. It may also be used to detect some coimmunoprecipitations, such as the IL-6 induced association of the receptor chain gp130 and JAK1, but cannot be used for EMSA, as it results in high backgrounds.

2. General purpose lysis buffer for Western blotting and EMSA: 0.5%(v/v) NP40, 10%(v/v) glycerol, 25 m*M* Tris-HCl, pH 7.5, 75 m*M* NaCl, 50 m*M* EDTA, pH 8.0, 100 μ*M* sodium orthovanadate (*see* **Note 1**), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 2**), 50 m*M* sodium pyrophosphate, 50 m*M* NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin.

   This buffer can be used for Western blotting of JAKs and STATs and for EMSA of STATs, but cannot be used for coimmunoprecipitations.

3. Coimmunoprecipitation lysis buffer: Some coimmunoprecipitations, such as the association of the receptor chain gp130 and JAK1 in response to IL-6, may be detected using buffer 1 but for unstable or weak associations, such as gp130 and STAT1 or JAK1 and STAT5, it is often wiser to use a gentle detergent such as Brij. Two such buffers are listed below. The only difference between the lysis buffers is the pH of the solution. Either buffer would be useful for gp130 and STAT1 or JAK1 and STAT5 interactions but for protein interactions that have not been tested it may be wise to try both buffers (*see* **Note 3**).
   a. 0.25% (v/v) Brij, 50 m*M* Tris-HCl, pH 8.0, 10%(v/v) glycerol, 0.1 m*M* EDTA, pH 8.0, 200 μ*M* sodium orthovanadate (*see* **Note 1**), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 2**), 10 μg/mL aprotinin, 10 μg/mL leupeptin.
   b. 0.5% (v/v) Brij, 75 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA, pH 8.0, 200 μ*M* sodium orthovanadate (*see* **Note 1**), 0.5 m*M* PMSF (*see* **Note 2**), 100 m*M* sodium fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin.

4. PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4.7H_2O$.

2.2. Immunoprecipitation

1. Antibodies for immunoprecipitation and Western blotting:
   a. JAK1 (Santa Cruz; HR785).
   b. JAK3 (UBI; 05-406 [human] 06–342 [murine]).
   c. TYK2 (Santa Cruz; Sc169).
   d. STAT1 (Santa Cruz; Sc417).
   e. STAT2 (Santa Cruz; Sc476).
   f. STAT3 (Santa Cruz; Sc482).
   g. STAT4 (Santa Cruz; Sc486).
   h. STAT5A/B (Santa Cruz; Sc835).
   i. STAT6 (Santa Cruz; Sc621).

2. Protein A Sepharose/Protein G Sepharose (Pharmacia): To make a 50% slurry, take an equal volume of protein A and protein G Sepharose and pellet in a

microcentrifuge for 5 min at 4°C, maximum speed. Wash pellet two times in lysis buffer and then resuspend the bead pellet in an equal volume of lysis buffer.
3. Western wash buffer (*see* **Note 4**): 0.1% Triton X-100 (v/v), 10% glycerol (v/v), 50 m*M* HEPES, 150 m*M* NaCl, 1 m*M* EDTA, pH 8.0, 200 μ*M* sodium orthovanadate, 0.5 m*M* PMSF, 10 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride 1.5 m*M* magnesium chloride, 10 μg/mL aprotinin, 10 μg/mL leupeptin.
4. Western loading buffer: 100 m*M* Tris-HCl, pH 6.8, 2%(w/v) sodium dodecyl sulfate (SDS), 100 m*M* dithiothreitol (DTT), 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating gel buffer: 375 m*M* Tris-HCl, pH 8.8, 0.1% (w/v) SDS.
2. Stacking gel buffer: 12 m*M* Tris-HCl, pH 6.8, 0.1% (w/v) SDS.
3. Protein markers (Amersham RPN 756): 1 μg in 25 μL Western loading buffer.
4. 30% (w/v) Acrylamide/*bis*-acrylamide stock solution (37:5:1) (Anachem).
5. Running buffer: 25 m*M* Tris-HCl, 186 m*M* glycine, 0.1% (w/v) SDS.
6. TEMED (Sigma).
7. Ammonium persulfate (APS) (Sigma): 10%(w/v) aqueous solution.
8. Electrophoresis gel rig (ATA) with glass plates and 1-mm spacers and appropriate comb. The size of the comb will depend on the dimensions of the gel apparatus used and the number of samples to be loaded. The comb should be washed once in water and then wiped with a 1% SDS solution (1 g of SDS in 10 mL water)

2.4. Western Transfer of Proteins

1. Western transfer apparatus: semidry apparatus available from Bio-Rad and wet transfer apparatus from Ideal Scientific.
2. Transfer buffer: 25 m*M* Tris-HCl, 150 m*M* glycine, 10–20% (v/v) methanol.
3. Polyvinylidene difluoride (PVDF) membrane (Millipore).
4. TBST buffer: 10 m*M* Tris-HCl, pH 7.4, 75 m*M* NaCl, 1 m*M* EDTA, pH 8.0.
5. Whatmann paper (3M) (VWR Scientific).

2.5. Antiphosphotyrosine Detection

1. BSA-TBST blocking solution: TBST buffer supplemented with 5% BSA 0.1 m*M* sodium vanadate, 0.01% (w/v) sodium azide (*see* **Note 5**).
2. Antiphosphotyrosine antibodies: PY20 (ICN) or 4G10 (UBI).
3. Secondary antibodies: Donkey antirabbit Ig horseradish peroxidase (HRP)-linked F(ab')2 fragment (Amersham, NA9340). Sheep antimouse Ig HRP-linked F(ab')2 fragment (Amersham, NA9310).
4. Antibody Dilution Buffer: TBST buffer supplemented with 1% BSA.
5. ECL detection kit (Amersham).
6. Stripping buffer: 2 *M* glycine, pH 2.5, 0.25% SDS (w/v).

2.6. EMSA Analysis of STAT Activation and DNA Binding

1. Commonly used GAS probes:

a. hSIE (GTCGACATTTCCCGTAAATC): binds STAT1 and STAT3 ***(15)***.
b. β-Casein (AGATTTCTAGGAATTCAATCC): binds STAT1 and STAT5 ***(16)***.
c. IRF1 (CCTGATTTCCCCGAAATGACG): binds STATs 1, 3, 5, and 6 ***(17)***.
d. FCγR (CCTGATTTCCCCGAAATGACG): binds STATs 1, 3, 5, and 6 ***(18)***.
e. ISRE (AGGAAATAGAAACTT): binds STAT2 (*see* **Note 6**) ***(19)***
f. 9–27$^{(TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA)}$: binds^{ISGF3} ***(20)***.

These oligonucleotide sequences represent the first strand for radiolabeling. Complementary second strand oligonucleotides are also required.

2. T4 Polynucleotide Kinase buffer (New England Biolabs).
3. 10 U of T4 Polynucleotide kinase (New England Biolabs).
4. γ^{32}P. ATP (Amersham) 1 mCi/mL.
5. DNA loading buffer: 30% (v/v) glycerol, 5 m*M* EDTA, pH 7.4, 0.1% (w/v) bromophenol blue (BDH Ltd.), 0.1% (w/v) xylene Cyanol (Bio-Rad).
6. 1.5 *M* NaCl.
7. Electrophoresis gel rig (ATA) with glass plates and 1-mm spacers and appropriate comb. The size of the comb will depend on the dimensions of the gel apparatus used and the number of samples to be loaded.

2.7. Purification of the Oligonucleotide Probe

2.7.1. Gel Extraction

1. Acrylamide gel: 15% (v/v) Anachem gel mix/0.5× TBE
2. 1×TBE: 0.9 *M* Tris, 0.9 *M* boric acid, 20 m*M* EDTA.
3. TE: 10 m*M* Tris, pH 8.0, 0.1 m*M* EDTA, pH 8.0.
4. DNA loading buffer: 30% (v/v) glycerol, 5 m*M* EDTA, pH 7.4, 0.1% (w/v) bromophenol blue (BDH, Ltd.), 0.1% (w/v) xylene Cyanol (Bio-Rad).
5. Extraction buffer: 1% SDS (w/v), 0.5 *M* ammonium acetate, 1 m*M* EDTA.
6. Filters — Spin-x filters (Costar).
7. Probe resupension buffer TE, 75 m*M* NaCl.
8. Absolute ethanol.
9. Scintillation fluid.

2.7.2. Column Purification

1. Sephadex G-25 columns (NAP 5 columns, Pharmacia).
2. TE: 10 m*M* Tris, pH 8.0, 0.1 m*M* EDTA, pH 8.0.
3. Probe resupension buffer TE, 75 m*M* NaCl.

2.8. Preparation of Extracts and EMSA Reactions

1. Cell lysis buffer (**Subheading 2.12.** recommended for the detection of STATs).
2. 100 U Poly dI.dC (Pharmacia) dissolve in 1.6 mL of water to give a final concentration of 62.5 U/mL. Store at –20°C.
3. Wu binding buffer: Prepare a solution of 10 m*M* HEPES, pH 7.9, 1.5 m*M* magnesium chloride, 0.1 m*M* ethylene glycol-*bis* N,N,N',N'-tetraacetic acid (EGTA), 100 m*M* NaCl and 5% (v/v) glycerol.

4. 10% (w/v) Ficoll in Wu binding buffer.
5. PBS for washing cells: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4.7H_2O$.
6. Probe resupension buffer TE, 75 m*M* NaCl.
7. Electrophoresis gel rig (ATA) with glass plates and 1-mm spacers and appropriate comb. The size of the comb will depend on the dimensions of the gel apparatus used and the number of samples to be loaded.
8. BSA (Bohringer Mannheim) (10 mg/mL) in water (store at –20°C).
9. tRNA (Pharmacia) (10 mg/mL) in water (store at –20°C).

3. Methods

3.1. Preparation of Whole Cell Extracts

The choice of lysis buffer is largely dependent on the scope of detection that you wish to obtain. Varying the salt condition, type of detergent, and pH greatly affect the success of the technique and/or the ability to coimmunoprecipitate. **Subheading 2.1.** outlines varying buffers used for altering specificity. For the detection of JAK and STAT phosphorylation by antiphosphotyrosine Western blotting, for example JAK1 and STAT5 activation in response to oncostatin M (*see* **Fig. 2**) cell lysis buffers described in 2.1.1 and 2.1.2 work effectively. Coimmunoprecipitations are most successful in buffers described in **Subheadings 2.1.1.** and **2.1.3.** (*see* **Note 3**). For EMSA of STATs (*see* **Fig. 1**), the cell lysis buffer described in **Subheading 2.1.2.** works best.

1. Cells are grown in tissue culture to approx 80% confluence. For each desired immunoprecipitation point 2–3 × 10^6 cells (e.g., Hela, Cos 7 or HT1080 cells) are treated or untreated with an appropriate ligand such as oncostatin M: 80ng/mL for 15 min (R and D systems). **Figure 2** shows JAK1 and STAT5 activation in fibrosarcoma cells (HT1080) stimulated with oncostatin M at 80 ng/mL for 15 min.
2. Cells are washed twice in ice-cold PBS and then lysed in 500 µL Western lysis buffer, and kept on ice for 30 min (*see* **Note 7**) with occasional vortexing. Cellular debris is then removed by centrifugation in a microcentrifuge at 20,000*g* for 5 min at 4°C.

3.2. Immunoprecipitation

From each cell, extract multiple immunoprecipitations can be performed. However, cleaner Western blots and hence more information is obtained with a single JAK and STAT immunoprecipitation. An example of this is shown in **Fig. 2** where JAK1 and STAT5 have been immunoprecipitated from oncostatin *M* treated fibrosarcoma (HT1080) cells.

1. Incubate lysate with 1–10 µL of antibody (for the example in **Fig. 2**, 10 mL of JAK1 and STAT5 antibodies were used: **Subheading 2.2.**) with 40 µL 50% protein A:protein G (1:1) Sepharose slurry (Pharmacia) which has been equilibrated in the appropriate lysis buffer (for the example in **Fig. 2**, cell lysis

buffer described in **Subheading 2.1.1.** was used) (*see* **Note 8**). Place the immunoprecipitations on a rotating wheel at 4°C for 2–16 h.
2. Spin lysates in a microcentirfuge at 20,000*g* for 5 min at 4°C and transfer the supernatant to a fresh 1.5-mL Eppendorf tube.
3. Wash the Sepharose pellet twice in 1-mL Western wash buffer. Centrifuge briefly at 20,000*g*, 30 s, 4°C after each wash. Remove Western wash buffer and add a further 1-mL fresh Western wash buffer. After final wash remove excess buffer using a Hamilton syringe.
4. Resuspend the Sepharose pellet in 40-µL Western loading buffer. This can now be stored at –20°C, if desired.
5. The whole cell lysate may now be precleared of excess antibody by reincubation with 40 µL 50% protein A:protein G (1:1) Sepharose slurry for a further 2 h at 4°C. Following microcentrifugation at 20,000*g* for 5 min at 4°C, the lysate is transferred to a fresh Eppendorf tube for further immunoprecipitations, if necessary.

3.3. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Cast the 6.5% acrylamide (v/v) separating gel. A 30-mL gel solution is sufficient for two plates in the gel rig. For a 6.5% gel add: 6.5 mL protogel (30% solution), 15 mL separating gel buffer (**Subheading 2.3.**, **step 1**) and 8.5 mL water. 250 µL of APS and 25 µL of TEMED should be added to the gel mix, thoroughly mixed, and then poured into the assembled gel plate approx 3/4 of the way up. Overlay the gel with 500 µL of water to achieve a flat interface.
2. Once the gel has set (approx 30 min), pour off the water, and cast a 5% (v/v) stacking gel over the separating gel. 15 mL of gel solution will be adequate. For a 5% stacking gel, add: 2.5 mL protogel (30% solution), 7.5 mL stacking gel buffer (**Subheading 2.3.**, **step 2**) and 5 mL of water. Use 90 µL APS and 10 µL TEMED to aid polymerization of the acrylamide gel.
3. Insert a comb into the stacking gel before it has polymerized.
4. After the gel has completely polymerized (this usually takes 30 min) assemble the gel rig according to the manufacturer's instructions, add running buffer and remove the comb.
5. Boil the loading samples and protein markers for 5 min, and microcentrifuge for 20,000*g* at 4°C for 5 min.
6. Using a fine pipet or Hamilton syringe, load the protein samples into the wells of the stacking gel taking care not to load the Sepharose beads. Keep the end of the loading tip below the level of the sample when loading to create an even dye front.
7. Run the gel at 150–250 V for approx 2 h, until the leading dye front reaches the bottom of the gel.

3.4. Western Transfer of Proteins

The nature of the apparatus used for the transfer of proteins is important in the detection of some proteins. Semidry transfer is commonly used with satis-

factory results but often requires practice to achieve blots with even transfer and low background. In the author's experience, wet blotting is more user-friendly and better for achieving good transfer of large proteins of 130 kDa and greater. The steps below outline the procedures used for wet blotting.

1. Equilibrate the wet-blot apparatus in transfer buffer, soaking the sponges for at least 10 min.
2. Cut two pieces of 3M Whatmann to the size of each gel and an equivalent sized piece of PVDF (usually ~7.5 × 14 cm). The PVDF must be activated by placing in methanol and then equilibrated in transfer buffer with considerable shaking to thoroughly remove the methanol.
3. Cut the separating gel to an appropriate size to include the range of visible rainbow protein markers and to equal that of the size of PVDF already cut (usually ~7.5 × 14 cm) and then remove the gel from the glass plate and equilibrate in transfer buffer in a clean plastic tray.
4. Assemble the wet-blot apparatus: Take the transfer container and fill with transfer buffer. Add two plastic spacers and the first electrode followed by a further two spacers. Next, add three of the presoaked sponges, one piece of 3M Whatmann, the gel, PVDF, and then the second piece of 3M Whatmann (at this point, care should be taken to avoid trapped air that may cause uneven transfer. To avoid this use a glass pipet to roll the layers flat and remove air bubbles). Add another three sponges, a spacer, and the second electrode. Put the lid on this and place the apparatus at 4°C (usually in a cold room) and run at 25 V for 1 h.
5. Disassemble the apparatus and quickly transfer the PVDF membrane to a clean tray filled with TBST buffer and equilibrate for 10 min at room temperature.

3.5. Antiphosphotyrosine Detection

It is important that all apparatus used is thoroughly cleaned by washing several times in distilled water to remove any contaminating phosphates.

1. Incubate the PVDF membrane in BSA-TBST blocking solution, for 2–16 h at 4°C with continual agitation. If the membrane is sealed into a plastic bag then this allows agitation to be achieved on a rotary wheel. Alternatively, the membrane may be placed in a plastic tray and incubated on a shaker. This step removes the nonspecific binding of antibody to the membrane.
2. Add the membrane to a plastic tray and wash twice on a shaker in TBST for 5 min at room temperature. Alternatively, the membrane can be sealed each time in a plastic bag and agitated using a rotary wheel.
3. Incubated the membrane with antiphosphotyrosine monoclonal antibodies 4G10 (UBI) or PY20 (ICN) at 1:2000 in 1% BSA (w/v), 0.01% (w/v) sodium azide in TBST for 2 h at 4°C with constant agitation (*see* **Note 9**).
4. Wash the membrane three times in TBST for 5 min at room temperature as described in **step 2**.
5. Incubate the membrane with secondary antibody HRP-conjugated sheep antimouse $F(ab')_2$ diluted 1:2000 in 1% BSA (w/v) TBST for 40 min at 4°C with

constant agitation. It is important that no sodium azide is present at this step as it may inhibit the HRP (*see* **Note 9**).

6. Wash the membrane at least three times in TBST for 20 min at room temperature as described in **Subheading 3.5.2**.
7. Identify the positive signals by autoradiography following detection using the Amersham ECL kit as recommended by the manufacturer. In brief, remove TBST from the blot and wash once with distilled water. Place blot in a plastic tray. In the ECL kit there will be two solutions that need to be added sequentially. Add approx 1 mL solution 1 and wash gently by shaking the plastic tray. Add the same amount of the second solution and wash gently for 2 min. Add the membrane to a plastic bag or a sheet of Saran Wrap™; maintain some of the ECL solution over the membrane and cover with remaining Saran Wrap™ or seal plastic bag. Maintaining some ECL solution on the membrane during detection prevents the substrate from being used up completely, leading to a false negative result. Following antiphosphotyrosine detection, it is usually desirable to determine the protein levels on the blots of the JAK/STAT proteins especially in negative controls. Therefore, the membrane must be stripped of antibody before redetection.
8. Incubate the membrane in stripping buffer for 2 h at room temperature in a plastic tray with one change of buffer or overnight with no change of buffer.
9. Wash the membrane extensively in distilled water and reequilibrate in TBST buffer for 10 minutes at room temperature.
10. Incubate the membrane with specific JAK or STAT antibodies (in **Fig. 2** the membrane was reprobed with a 1:2000 dilution of STAT5 antibody in 1%BSA/TBST) to detect the protein of interest and repeat **steps 3–7** above (*see* **Notes 9** and **10**).

3.6. EMSA Analysis of STAT Activation and DNA Binding

EMSA analysis of STAT complexes provides a functional assay to detect STAT activation. It also has advantages over phosphotyrosine detection as this technique is less time consuming, more sensitive and utilizes only a fraction of the prepared cellular lysate.

3.7. Radiolabeling the Oligonucleotide Probe

1. In a 1.5-mL screw-cap vial on ice add the following: 2 μL 10× kinase T4 polynucleotide kinase buffer; 800ng of single stranded of oligonucleotide DNA GAS probe; 130 μCi γ^{32}P ATP; 10 U T4 polynucleotide kinase.
2. Incubate reaction mixture at 37°C for 30 min.
3. The first oligonucleotide strand is now labeled. To make a double-stranded probe add 800 ng complementary oligonucleotide, 1 mL of 1.5 *M* NaCl and incubate at 90°C for 5 min in a preheated water bath.
4. Turn off the water bath and allow strands to anneal in a slowly cooling water bath for 4 h or overnight.

3.7. Purification of the Labeled Probe

There are several methods for purifying labeled probes. Gel extraction and sephadex column purification are described later. Both produce very clean probes and choice of method is entirely dependent on personal preference and time commitment.

3.7.1. Gel Extraction

1. Take the labeled probe and add 3 μL DNA loading buffer. Run probe on 15% acrylamide gel at 150 V, 1–2 mA until bromophenol blue reaches the bottom. GAS probes run between bromophenol blue and xylene cyanol and ISRE probes run with xylene cyanol.
2. Cover gel in plastic wrap and expose to X-ray film for 30 s. Mark position of gel and use developed film to cut out labeled probe. Macerate gel slice in Eppendorf tube with 500 mL extraction buffer (**Subheading 2.7.1.**, **step 5**) and leave at 37°C for 2 h or overnight with continual agitation achieved by placing tubes on a shaker.
3. Microcentrifuge probe/gel mix through a Spin-X filter at 20,000*g* for 5 min at 4°C and precipitate probe with 2 vol absolute ethanol by incubating on dry-ice for 20 min. Centrifuge at 20,000*g* for 20 min at 4°C. Resuspend in 100 μL of probe resuspension buffer (**Subheading 2.7.1.**, **step 7**) Add 1 μL of labeled probe to 500 μL of scintillation fluid and count the activity using a scintillation counter. Specific activity of the probe should be at least 3×10^5 cpm/mL.

3.7.2. Column Purification

1. Equilibrate sephadex G-25 (NAP5-pharmacia) column with 5 mL TE. Add probe mix to the top of the column and add a further 400 mL TE to allow the probe mix to run into the column.
2. Add 800 mL TE to the column and collect fractions of 100 μL. The probe should elute in fraction 3, 4, or 5.

3.8. Preparation of Extracts for EMSA Reactions

1. Making an EMSA extract requires smaller amounts of cells than Western blotting. Typically 1×10^5 Hela or Cos or HT1080 cells are plated in each well of a 6-well dish (3 cm diameter per well) and left overnight. The following day the cells are treated with cytokine for 15 min or left untreated (In **Fig. 1**, HT1080 cells were treated with oncostatin M or IL-6, both at 80 ng/mL for 15 min).
2. Wash cells 2× in PBS.
3. Add 100 μL EMSA cell lysis to each well on the plate. Scrape cells from the plate and place in a 1.5-mL Eppendorf tube. Incubate at 4°C for 15 min.
4. Centrifuge lysates at 20,000*g* for 5 min at 4°C in a microcentrifuge to remove cellular debris. Transfer supernatant to a fresh Eppendorf tube. This cleared lysate is now ready to be used in an EMSA reaction.
5. To 10-μL cleared lysate add 1 μL Poly dI.dC. Incubate for 5 min at room temperature.

6. Make probe mix of sufficient quantity to add 10 μL to each of the reactions.
   1 vol Wu Binding Buffer.
   1 vol Ficoll 400.
   0.1 vol BSA (10 mg/mL).
   0.1 vol tRNA (10 mg/mL).
   1 mL probe/10 reactions.
7. Add 10 μL probe mix to 10 μL cleared lysate and incubate 20 min at room temperature.
8. Cast a 6% acrylamide gel in 0.5× TBE. Prerun gel at 150 V. Load EMSA reactions and DNA loading buffer as a marker. Run gel at 150–200 V until xylene cyanol reaches the bottom. Dry gel and autoradiograph 6h overnight (*see* **Note 11**).
9. To identify STAT complexes in an EMSA assay, antibodies can be used to retard the STAT protein in the gel. To carry out a supershift preincubate 10-μL cleared cell lysate with antibody (In **Fig. 7**, 5 μL of STAT5 antibody could be used to retard the upper complex) for 10 min at room temperature. Add Poly dI.dC and proceed with the EMSA assay as outlined in **steps 5–7** above.

5. Notes

1. A stock of 20 m*M* sodium orthovanadate is preferable. Preparation of the solution requires boiling until the yellow color disappears.
2. PMSF is very harmful and should be weighed in a hazardous hood and then dissolved in isopropanol to obtain a final concentration of 50-m*M* stock solution.
3. For most JAK/receptor, STAT/receptor and JAK/STAT interactions the pH of the cell lysis buffer is irrelevant. However, for the association of, e.g., JAKs and insulin receptor 1 substrate, the pH of the lysis buffer may be important.
4. Wash buffer for immunoprecipitations from general purpose lysis buffer (**Subheading 2.1.**, **step 1**). For all other immunoprecipitations lysis buffer can be used as wash buffer.
5. Sodium azide is very toxic even in solution and care should be taken to avoid contact with skin.
6. STAT 2 binds to DNA as part of a complex with STAT1 and *p48* known as ISGF3. This complex does not bind to GAS sites but binds to ISRE (Interferon Stimulable Response Element).
7. Lysates should always be kept on ice. If frozen, the lysates must be thawed on ice.
8. The amount of Sepharose used for immunoprecipitation can be important as proteins can bind nonspecifically. If background proteins become a problem add less Sepharose to each immunoprecipitation or add the Sepharose for only 30 min at the end of the incubation with antibody.
9. The primary antibody solution (i.e., phosphotyrosine or JAK antibody) may be used repeatedly for up to 6 mo if sodium azide is added. The secondary antibody (this antibody is coupled to HRP) should be used fresh each time. Neither antibody solution should contain vanadate as it may inhibit further antibody interactions.
10. Blots can be reprobed up to six times with good results. Alternate between rabbit and mouse secondary whenever possible to minimize background.

11. Typically, STAT complexes will be visible on an overnight exposure although this will vary depending on cell type or ligand used.

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10

Assays for Glycogen Synthase Kinase-3 (GSK-3)

Darren Cross

1. Introduction

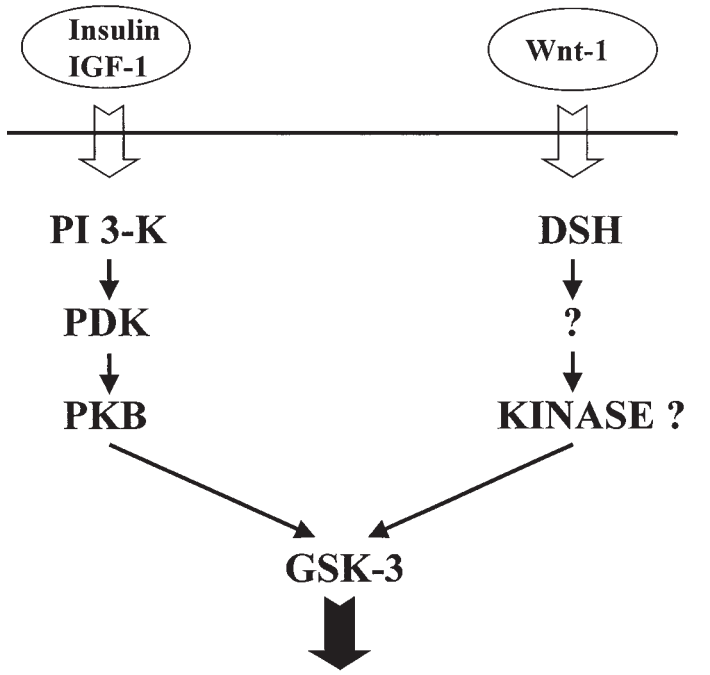
Two isoforms of the serine/threonine kinase glycogen synthase kinase-3 (GSK-3) occur in mammalian cells, GSK-3α (53 kDa) and GSK-3β (47 kDa), which are 85% identical over the entire protein and 95% identical within the catalytic domain ***(1)***. GSK-3 is ubiquitously expressed in mammalian tissues and GSK-3 homologs have been identified in every eukaryotic species tested to date ***(2)***, strongly suggesting that it plays a central role in cellular regulation.

In contrast to the regulation of most protein kinases, GSK-3 is highly active in its basal state and, in turn, its substrates are highly phosphorylated in unstimulated cells. Moreover, the activity of GSK-3 is inhibited in response to diverse extracellular stimuli including insulin, growth factors, and wnts, with consequent dephosphorylation of target proteins.

Many GSK-3 substrates have been identified in vitro (*see* **Fig. 1**), representing a diverse range of cellular functions. These include enzymes involved in metabolism (e.g., glycogen synthase ***[3]***, eIF-2B ***[4]***, and ATP-citrate lyase ***[5]***), transcription factors (e.g., c-Jun ***[6]***, NF-AT ***[7]***, and CREB ***[8]***), regulatory subunits (e.g., inhibitor-2 ***[9]*** and G-subunit of protein phosphatase-1 ***[10]***) and proteins involved in cell morphology (e.g., β-catenin ***[11]***, τ ***[12]***, and MAP-1B ***[13]***). However, it remains unclear whether all substrates of GSK-3 identified in vitro are physiologically relevant targets in vivo.

The activity of GSK-3 is dependent on the phosphorylation of a conserved tyrosine residue (Tyr-279 in GSK-3α and Tyr-216 in GSK-3β) ***(14)***. However, insulin, growth factor, or wnt-induced inhibition of GSK-3 is completely reversed by treatment with serine/threonine-specific protein phosphatases ***(15–17)***, indicating that this inhibition is because of an increase in serine/threonine

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith



Metabolism	Transcription	Morphology
Glycogen synthase	c-Jun	MAP-1B
eIF-2B	CREB	Tau
ATP-citrate lyase	NF-AT	β-Catenin
G-subunit of PP1		

Fig. 1. Signaling pathways leading to GSK-3 inhibition, and in vitro substrates of GSK-3.

phosphorylation, and not from the decreased phosphorylation of tyrosine residues.

Indeed, the insulin- and growth factor-induced inhibition of GSK-3 is because of the phosphorylation of a conserved N-terminal serine residue, Ser-21 in GSK-3α and Ser-9 in GSK-3β ***(16,18)***. Phosphorylation of this residue is catalyzed by numerous kinases in vitro, including MAPKAP kinase-1, p70 S6 kinase, and protein kinase B ***(18,19)***. However, it is likely that protein kinase B is responsible for the phosphorylation of these sites in response to insulin and IGF-1 (***18***; *see* **Fig. 1**).

There is increasing evidence to indicate that GSK-3 plays an essential role in many different cellular processes. For example, by targeting glycogen synthase and eIF-2B, GSK-3 is probably a key modulator of insulin action. In addition, GSK-3 has been implicated in various developmental processes in a diverse range of organisms including *Xenopus*, *Drosophila*, and *Dictyostelium* ***(2)***. Finally, emerging evidence suggests that GSK-3 has an important role in neuronal function, because it phosphorylates various neuronal proteins such as τ and it mediates neuronal outgrowth ***(13)***.

This chapter describes methods to isolate GSK-3 isoforms specifically from cells in culture. In addition, because GSK-3 plays a central role in insulin signaling, methods to isolate GSK-3 from the major insulin-sensitive tissues (liver, skeletal muscle, and adipose tissue) is described. Techniques are then given to assay changes in GSK-3 activity in these samples. The assays described can be easily adapted to enable GSK-3 to be isolated and assayed from other sources.

2. Materials

2.1. Producing Crude Extracts

1. Phosphate-buffered saline (PBS; tablets from Sigma; cat. no. P4417).
2. Microcystin-LR (Calbiochem, cat. no. 475815).
3. "Complete" Protease Inhibitor cocktail (Boehringer, cat. no. 1697498).
4. Lysis buffer: 25 m*M* Tris-HCl, 3 m*M* ethylenediaminetetracetic acid (EDTA), 3 m*M* ethylene glycol-*bis* (β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 50 m*M* NaF, 2 m*M* sodium orthovanadate, 0.27 *M* Sucrose, 2 m*M* microcystin-LR, 10 m*M* sodium β-glycerophosphate, 5 m*M* sodium pyrophosphate, 0.5% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, protease inhibitors, pH 7.4 (*see* **Note 1**).
5. Homogenization buffer: 50 m*M* NaF, 4 m*M* EDTA, 1 m*M* sodium orthovanadate, 0.1% (v/v) β-mercaptoethanol, protease inhibitors (*see* **Note 1**).
6. Adipocyte buffer: 30 m*M* HEPES, 120 m*M* NaCl, 10 m*M* $NaHCO_3$, 1.2 m*M* $MgSO_4$, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 2.5 m*M* $CaCl_2$, 40 mg/mL BSA, 1 mg/mL glucose, 0.2 μ*M* adenosine, pH 7.4.
7. Adipocyte lysis buffer: 10 μL of 0.2 *M* EDTA, 5 μL of 0.2 *M* EGTA, 10 μL of 0.1 *M* orthovanadate, 1 μL of β-mercaptoethanol and 0.5 μL of microcystin-LR (*see* **Note 1**).
8. Liquid nitrogen.
9. Pestle and mortar.

2.2. Immunoprecipitation

1. PBS; tablets from Sigma; cat. no. P4417).
2. Buffer A: 50 m*M* Tris-HCl, 1 m*M* EGTA, 0.15 *M* NaCl, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol, pH 7.4.
3. Protein G-Sepharose (Pharmacia; Cat No. 17-0618-01).

4. Anti-GSK-3α antibody (Upstate Biotechnology, cat. no. 06–391).
5. Anti-GSK-3β antibody (Transduction Labs, cat. no. G22320).
6. Shaking platform (Vibrax-VXR; BDH, cat. no. 330/0360/00 and 330/0360/12).

2.3. GSK-3 Activity Assay

1. Buffer A: 50 m*M* Tris-HCl, 1 m*M* EGTA, 0.15 *M* NaCl, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol, pH 7.4.
2. [γ-^{32}P]ATP (10 mCi/mL; Amersham). Dilute 10-fold in H_2O and 50 m*M* ATP to give a stock concentration of 1 m*M* [γ-^{32}P]ATP (1 mCi/mL).
3. ATP (Boehringer Mannheim, cat. no. 127531).
4. Phospho-GS Peptide-2 (YRRAAVPPSPSLSRHSSPHQS(P)EDEEE) and GS Peptide-2 (YRRAAVPPSPSLSRHSSPHQAEDEEE) GSK-3 substrate peptides (Upstate Biotechnology, cat. no. 12-241 and 12-242, respectively).
5. cAMP-dependent protein kinase inhibitor (PKI; Sigma, cat. no. P210).
6. Orthophosphoric acid.
7. P81 phosphocellulose chromatography paper (Whatman, cat. no. 3698 915).
8. $PP2A_1$ (Upstate Biotechnology, cat. no. 14-165).
9. Microcystin-LR (Calbiochem, cat. no. 475815).

2.4. Immunoblotting

1. Sodium dodecyl sulfate (SDS)-sample buffer: 25 m*M* Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, bromophenol blue, pH 6.8.
2. Transfer buffer: 25 m*M* Tris, 190 m*M* glycine, 20% (v/v) methanol.
3. Transblot transfer apparatus (Bio-Rad).
4. Tris-buffered saline and Tween (TBST): 20 m*M* Tris-HCl, 0.15 *M* NaCl, 0.1% (v/v) Tween-20, pH 7.4.
5. Dried skimmed milk powder.
6. Anti-GSK-3α/β antibody (Upstate Biotechnology, cat. no. 05-412).
7. Antiphospho (Ser-21) GSK-3α antibody (Upstate Biotechnology, cat. no. 06–733).
8. Antiphospho (Ser-9) GSK-3β antibody (Quality Controlled Biochemicals, cat. no. 44-600).
9. Antiphospho (Tyr-279/216) GSK-3α/β antibody (Upstate Biotechnology, cat. no. 05-413).
10. ECL kit (Amersham, cat. no. RPN 2106).
11. Autoradiography paper (Kodak; Amersham).

3. Methods

3.1. Isolation of GSK-3 from Crude Extracts

3.1.1. Producing Crude Extracts

3.1.1.1. From Cultured Cells:

1. Aspirate cell media and rinse cells once with a suitable volume of ice-cold PBS. 3 mL PBS is sufficient for a 6 cm dish.

2. Add a suitable volume of ice-cold lysis buffer (0.2, 0.5, and 1 mL for 6-, 10-, and 15-cm dishes, respectively) and place cells on ice.
3. Scrape cells and transfer lysate to a microcentrifuge tube.
4. Clear lysate of cell debris by centrifugation for 5 min at 13,000*g* at 4°C.
5. Use cell lysate for analysis (*see* **Subheadings 3.1.2.** and **3.2.**), or snap freeze lysate in liquid nitrogen and store at –80°C until required (*see* **Note 2**).

3.1.1.2. From Muscle and Liver Tissue:

1. Obtain fresh sample of tissue and snap freeze in liquid nitrogen (*see* **Note 2**).
2. Grind tissue sample into a fine powder under liquid nitrogen, using a precooled pestle and mortar, and store powder at –80°C until required.
3. Homogenize powder at 4°C in 3 vol (3 mL of buffer per 1 g of powder) of ice-cold homogenization buffer.
4. Centrifuge the homogenate for 10 min at 13,000*g* at 4°C.
5. Use extract for analysis (*see* **Subheadings 3.1.2.** and **3.2.**), or aliquot and snap freeze in liquid nitrogen and store at –80°C until required (*see* **Note 2**).

3.1.1.3. From Rat Primary Adipocytes

1. Dissect epididymal fat pads from a fed male 180 g Wistar rat, and place pads in a plastic beaker (*see* **Note 3**) containing adipocyte buffer prewarmed to 37°C.
2. Transfer fat pads to a fresh beaker containing 3 mL of prewarmed adipocyte buffer supplemented with 3 mg of collagenase, and chop pads using scissors into small pieces.
3. Digest pads for 1 h in a shaking water bath at 37°C.
4. Strain the digested pads twice through a 0.5-mm^2 plastic sieve and wash the isolated adipocytes with prewarmed adipocyte buffer.
5. Resuspend primary adipocytes in adipocyte buffer to give a final volume of about 5 mL.
6. Use 1-mL aliquots of adipocyte suspension for stimulations.
7. Following stimulation, add 26.5 μL of adipocyte lysis buffer to the adipocyte suspension, mix, and lyse the adipocytes immediately by snap freezing in liquid nitrogen. Store the lysates at –80°C until required (*see* **Note 2**).
8. After thawing, centrifuge the lysates for 10 min at 13,000*g* at 4°C, and transfer the supernatants (discarding the pellet and fat layer) to fresh microcentrifuge tubes.
9. Centrifuge lysates for a further 5 min at 13,000*g* at 4°C, and immediately use extract for analysis (*see* **Subheadings 3.1.2.** and **3.2.**).

3.1.2. Immunoprecipitation

3.1.2.1. Conjugation of Anti-GSK-3 Antibody to Protein G-Sepharose:

1. Use 5 μL of Protein G-Sepharose resin for each individual immunoprecipitation (*see* **Note 4**). Wash required amount of Protein G Sepharose into PBS (*see* **Note 5**) and resuspend pellet in an equal volume of PBS.

2. Add required amount of the GSK-3 antibody to the Protein G-Sepharose (*see* **Notes 4** and **6**).
3. Conjugate antibody to Protein G-Sepharose by incubation on a shaking platform for at least 1 h at 4°C (*see* **Note 7**).
4. Wash unbound or weakly bound antibody away from pellet with PBS (*see* **Note 8**).
5. Resuspend pellet in an equal volume of PBS, and store at 4°C until required (*see* **Note 7**).

3.1.2.2. Optional Covalent-Coupling of Antibody to Protein G-Sepharose

If GSK-3 immunoprecipitation is going to be followed by immunoblot analysis of GSK-3, it is necessary to carry out an extra coupling step as described in the following section (*see* **Note 9**).

1. Conjugate antibody to Protein G-Sepharose as described in **Subheading 3.1.2.**A, above.
2. Wash beads in 10 vol of 0.2 *M* sodium borate pH 9.0 (*see* **Note 8**).
3. Resuspend beads in an equal volume of sodium borate pH 9.0, and add fresh dimethylpimelimidate to a final concentration of 20 m*M*.
4. Incubate resin on a shaking platform for 1 h at room temperature.
5. Wash resin in 5 vol of 0.2 *M* ethanolamine pH 8.0, and incubate on a shaking platform for a further 2 h at room temperature.
6. Wash resin in 10 vol of PBS, resuspend pellet in an equal volume of PBS, and store at 4°C until required (*see* **Note 7**).
7. Monitor effectiveness of coupling by subjecting samples of resin taken before and after coupling to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. No antibody heavy chain (55 kDa) should be apparent on the gel after coupling. If coupling is inefficient, *see* **Note 10**.

3.1.2.3. Immunoprecipitation

1. For each immunoprecipitation, aliquot 10 µL of the 50% (v/v) Protein G-Sepharose stock suspension into 1.5-mL microcentrifuge tubes (*see* **Note 5**).
2. Add the required amount of crude extract to each 5.0-µL pellet (*see* **Note 4**).
3. Incubate the suspension on a shaking platform for 1–2 h at 4°C.
4. Centrifuge the samples for 1 min at 13,000*g* at 4°C, and carefully remove the extract without disrupting the pellet (*see* **Note 8**).
5. Add 1 mL of ice-cold Buffer A containing 0.5 *M* NaCl to each pellet and resuspend pellet.
6. Centrifuge the samples for 1 min at 13,000*g* and carefully remove the wash without disrupting the pellet (*see* **Note 8**).
7. Repeat **steps 5** and **6** once with Buffer A containing 0.5 *M* NaCl and twice with 1 mL of Buffer A.
8. Remove as much buffer as possible without disturbing the pellet (*see* **Note 8**).

9. Assay the immunoprecipitate for GSK-3 activity (*see* **Subheading 3.2.**) or denature pellet in SDS-sample buffer for immunoblotting analysis (*see* **Subheading 3.3.**).

3.2. Assay of GSK-3 Activity

The assay of GSK-3 is based on its ability to incorporate radioactive [^{32}P]-phosphate from [γ-^{32}P]ATP into specific peptide substrates. The source of GSK-3 dictates which peptide can be used for the assays (*see* **Note 11**). Another unusual characteriztic of GSK-3 is that it generally requires a "priming" phosphorylation event in its substrates. Thus, it phosphorylates a Ser/Thr residue (shown in bold type) which is N-terminal to a phosphorylated Ser/Thr residue (shown underlined) in the sequence –**Ser/Thr**-Xaa-Xaa-Xaa-Ser/Thr(P)- (*see* **Note 12**). GSK-3 is unable to phosphorylate peptides lacking such a "priming" phosphate (*see* **Note 13**).

3.2.1. Assaying GSK-3 Activity

1. The standard GSK-3 assay volume is 50 µL.
2. Take the GSK-3 sample (usually 5 µL; either from immunoprecipitation, fractionation, crude extract or purified enzyme; *see* **Note 11**) and place in a microcentrifuge tube on ice.
3. Add 25 µL of ice-cold Buffer A.
4. Add 5.0 µL of substrate peptide (300 µ*M* in water; *see* **Notes 11–13**).
5. Add 5.0 µL of PKI (10 µ*M* in water; *see* **Note 14**).
6. Initiate the kinase assay by the addition of 10 mL of 50 m*M* MgCl/0.5 m*M* [γ-^{32}P]ATP (1 mCi/mL).
7. Incubate assay for 15 min at 30°C (*see* **Note 15**).
8. Remove a 40-µL aliquot of the reaction and spot on to a 2-cm^2 square of P81 phosphocellulose paper, and immerse the square in a beaker of 75 m*M* orthophosphoric acid.
9. Wash the P81 squares at least four times (2 min per wash with shaking) in orthophosphoric acid.
10. Wash squares once in acetone, dry, place in 1.5-mL microcentrifuge tubes, and count the amount of radioactive phosphate incorporated (*see* **Note 16**).
11. Express the activity of GSK-3 as a percentage of activity compared to GSK-3 activity measured in unstimulated cells (for example see **Fig. 2**), or express GSK-3 activity as a total specific activity.

3.2.2. Phosphatase Treatment of GSK-3

GSK-3 activity is inhibited by phosphorylation on certain Ser/Thr residues (*see* **Subheading 1.**), and so this inhibition can be fully reversed by treatment with protein phosphatase-2A ***(15)***. For this reason, GSK-3 activity can also be expressed as a reactivation ratio, i.e., GSK-3 activity measured without phos-

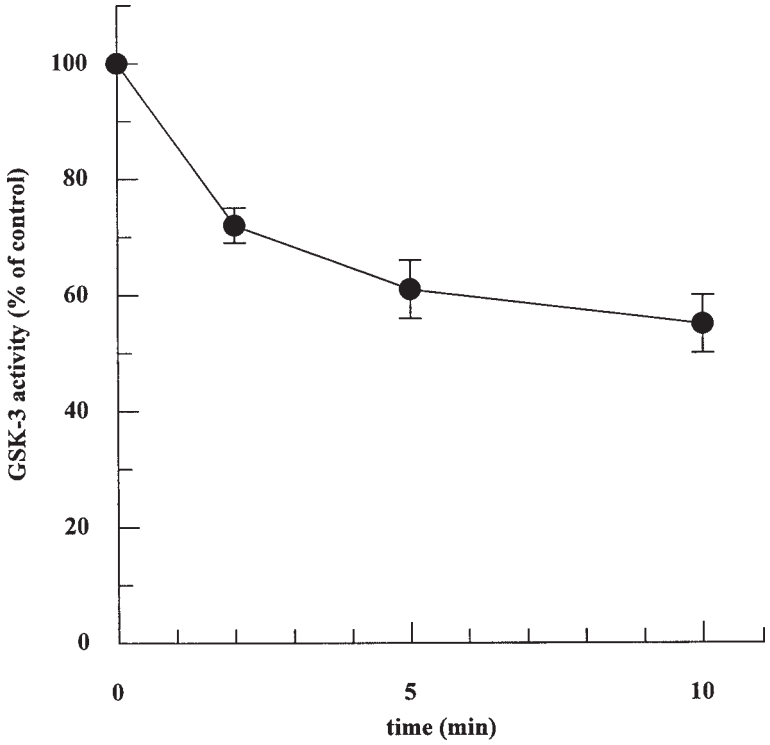


Fig. 2. Inhibition of GSK-3 in response to insulin in L6 myotubes. Rat L6 myotubes were treated for the indicated times with 100 n*M* insulin. The cells were lysed and both GSK-3 isoforms were coimmunoprecipitated from 25 mg of lysate as described in **Subheadings 3.1.1** and **3.1.2**. The immunoprecipitates were assayed for GSK-3 activity using phospho-GS Peptide-2 as the substrate, as described in **Subheading 3.2.1**. Results are expressed relative to the specific activity of GSK-3 in lysates from unstimulated myotubes (100%, 60 mU/mg). The data are given as +/– S.E.M. (n = 10) for five separate experiments at each time point, where each experiment was carried out on duplicate sets of cells. No activity towards GS Peptide-2 was detected in the immunoprecipitates.

phatase treatment divided by GSK-3 activity measured after phosphatase treatment.

1. Immunoprecipitate GSK-3 as described in **Subheading 3.1.2**. Twice the usual number of immunoprecipitates are required because the GSK-3 activity has to be assayed in parallel with and without phosphatase treatment.
2. Incubate immunoprecipitate with 2.0 µL of $PP2A_1$ (50 mU/mL; diluted in Buffer A) for 20 min at 30°C (*see* **Note 15**), and then terminate reaction by adding 2.0 µL of okadaic acid (25 µ*M* in water; *see* **Note 17**).

3. Assay for GSK-3 kinase activity as described in **Subheading 3.2.1**.
4. Express GSK-3 activity as a reactivation ratio: activity measured without phosphatase treatment divided by activity measured after phosphatase treatment

3.3. Immunoblot analysis of GSK-3 Phosphorylation State

Phosphorylation of a conserved N-terminal serine residue in GSK-3 is known to trigger its inhibition (*see* **Subheading 1.**). In addition, phosphorylation of a conserved tyrosine residue in GSK-3 is known to be essential for its activity (*see* **Subheading 1.**). It is, therefore, possible to determine the activity status of GSK-3 using antibodies that specifically recognize the phosphorylated forms of these regulatory sites. Furthermore, if the antibodies are used in conjunction with activity assays, it is possible to examine the mechanism of GSK-3 inhibition.

3.3.1. SDS-PAGE and Transfer of Protein to Nitrocellulose Membrane

1. Denature GSK-3 samples in a suitable volume of SDS-sample buffer by boiling for 5 min, and store denatured samples at –20°C until required.
2. Subject denatured samples to 10% SDS-PAGE.
3. Transfer proteins from gel on to nitrocellulose membrane using a Bio-Rad Trans-blot apparatus. Assemble the transfer sandwich using components presoaked in transfer buffer. Place a Scotch Brite pad, two sheets of 3M chromatography paper, the gel, the nitrocellulose membrane, two more sheets of 3M paper and another Scotch Brite pad in the transfer cassette. Place the cassette in the apparatus with the membrane side toward the anode (red).
4. Transfer protein onto membrane for 200–300 V hours (*see* **Note 18**).

3.3.2. Immunoblotting

1. Block nonspecific binding sites on the nitrocellulose membrane by incubating it on a rocking platform for 1 h at room temperature with 10% (w/v) dried skimmed milk in TBST buffer (*see* **Note 19**).
2. Rinse away excess blocking solution and incubate membrane on a rocking platform for 1 h at room temperature with 2 mg/mL of the relevant anti-GSK-3 antibody in TBST buffer (*see* **Note 19**).
3. Wash the membrane at least four times (5 min per wash) on a rocking platform with 100–200 mL of TBST buffer (*see* **Note 19**).
4. Incubate membrane on a rocking platform for 1 h at room temperature with the relevant secondary-HRP-coupled-antibody diluted (1:5000) in TBST.
5. Wash the membrane at least four times (5 min per wash) on a rocking platform with 100–200 mL of TBST buffer (*see* **Note 19**).
6. Develop blot with the Amersham ECL kit, using protocol recommended by the manufacturer.

4. Notes

1. The buffers contain EDTA to inhibit protein kinase activity, various serine/threonine phosphatase inhibitors (fluoride, pyrophosphate, β-glycerophosphate, microcystin-LR) and a tyrosine phosphatase inhibitor (orthovanadate), to ensure that the phosphorylation state of the proteins is not altered on lysis. Sodium orthovanadate stock solution must be prepared in a specific manner, to ensure its effectiveness as a phosphatase inhibitor. To prepare orthovanadate: adjust a 0.1 *M* stock solution to pH 10.0, boil, cool to room temperature, and readjust to pH 10.0. Continue this cycle until the solution is stable at pH 10.0, and store the stock solution at 4°C.
2. Extracts may be snap frozen in liquid nitrogen and stored at –80°C until required. However, extracts should only be freeze/thawed once to ensure that their integrity remains.
3. Only plasticware should be used when handling adipocytes, because glass causes them to shear.
4. It is necessary to optimize both the amount of antibody coupled to the Protein G-Sepharose pellet and the amount of crude extract used, so that the immunoprecipitation depletes 80–100% of target GSK-3 isoform(s). Usually a 5 μL pellet of Protein G-Sepharose is sufficient to bind enough antibody to deplete GSK-3 from 50 μg of cell culture lysate and 300 μg of tissue extract. There are two simple techniques for determining the extent of immunodepletion; immunoblotting the sample before and after immunoprecipitation, and/or analyzing the amount of GSK-3 isolated in sequential rounds of immunoprecipitation.
5. For accuracy and ease of handling of Protein G-Sepharose, it is essential to cut off the ends of pipet tips.
6. Because there are two mammalian isoforms of GSK-3, GSK-3α, and GSK-3β, it may be necessary to immunoprecipitate specifically either isoform or both isoforms simultaneously, depending on the study.
7. Because Protein G and antibodies are reasonably stable, it is possible to conjugate the antibody to the Protein G-Sepharose overnight. Moreover, after the Protein G/antibody complex has been washed, it is possible to store the pellet at 4°C for many weeks. This means that large stocks of resin with antibody bound can be made at once.
8. The most careful part of immunoprecipitation is during the washing of the pellets, because it is of critical importance that none of the pellet is lost. To wash pellets, repeat the following procedure at least four times. Resuspend the pellet in 1 mL of buffer (use greater volumes of buffer if the pellet is larger than 0.5 mL), pellet the resin by centrifuging for 1 min at 13,000*g*, aspirate off the buffer, and resuspend pellet in fresh buffer. A good way of ensuring that the pellet remains intact is to always leave a cushion of approx 100 μL of buffer above the pellet after aspirating. After the final wash step, the 100 μL of buffer is removed carefully using a suitable pipet.

9. If GSK-3 immunoprecipitates are going to be immunoblotted for GSK-3, the antibody must be covalently coupled to the Protein G-Sepharose resin. The reason for this being that the antibody heavy chain (55 kDa) migrates at a similar position to GSK-3 on a SDS-PAGE gel, and thus may interfere with the GSK-3 immunoblot.
10. There are a couple of obvious reasons why the covalent coupling is inefficient. First, it is important to ensure that all unbound or loosely bound antibody is washed away following the initial coupling step (**Subheading 3.1.2.1.**). In addition, the covalent coupling reaction only occurs if the pH is greater than 8.3, therefore the pH may have to be adjusted after adding the dimethylpimelimidate.
11. The source of the GSK-3 to be assayed dictates which substrate peptide can be used. The peptide usually used to assay GSK-3, termed phospho-GS Peptide-2, is also a substrate for other kinases, and thus GSK-3 must be immunoprecipitated prior to its assay. However, Proud and colleagues have developed a specific peptide substrate for GSK-3, termed 2B-(SP) (RRAAEELDSRAGS(P)PQL), which can be used to assay GSK-3 activity directly in crude cell extracts ***(20)***.
12. The "priming" phosphate is incorporated into GSK-3 substrate peptides by either incubation in vitro with a suitable kinase (such as CK2 for phospho-GS Peptide-2) or synthetically. Peptides lacking this "priming" phosphate are not substrates for GSK-3 (*see* **Note 13**).
13. GSK-3 cannot phosphorylate a peptide in which the "priming" phosphorylated residue has been substituted with an alanine residue. Such peptides should therefore be used as negative control substrates, to determine the level of phosphate incorporated into the substrate peptides by kinases other than GSK-3. This is particularly important when using 2B-(SP) to assay GSK-3 in crude extracts (*see* **Note 11**).
14. PKI is a synthetic peptide inhibitor of cAMP-dependent protein kinase.
15. The kinase reaction can be carried out in any constant-temperature water bath or heating block. However, because Sepharose beads rapidly settle to the bottom of the tube and thus are partitioned from the reaction substrates, it is advisable to assay immunoprecipitates on a shaking platform at 30°C.
16. One unit of kinase-specific activity is defined as that amount of kinase that incorporates 1 nmol of phosphate into substrate in 1 min at 30°C.
17. For a reactivation ratio to be determined, each GSK-3 sample has to be treated in parallel with and without phosphatase, and then assayed in parallel. The control (phosphatase untreated) incubations are carried out by incubating one set of samples with okadaic acid prior to the addition of $PP2A_1$.
18. Transfer conditions depend on the protein. However, transferring for about 300 V hours is sufficient for the quantitative transfer of GSK-3. The transfer can be carried out overnight, e.g., 16 h at 20 V, or faster, e.g., 3 h at 100 V. However, if the transfer is fast, i.e., 50 V and above, it will have to be carried out at 4°C to prevent over-heating.
19. Generally, use 10 mL of buffer in a plastic (approx 10 cm × 10 cm) dish for blocking and probing the membrane sheet. If the antibody is in short supply, the probing can be carried using smaller volumes in a sealed bag. To ensure that the background signal on an immunoblot is as minimal as possible, it is important to

wash the membrane thoroughly after each round of probing. The membrane should therefore be washed vigorously in relatively large volumes of TBST on an orbital-shaking platform.

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11

Cyclin-Dependent Kinases and Cyclin-Dependent Kinase Inhibitors

Detection Methods and Activity Measurements

Gavin Brooks

1. Introduction

Normal cellular proliferation is under the tight control of both positive and negative regulators that determine whether a particular cell can progress through the cell cycle (*see* **refs. *1–3*** for reviews). This carefully ordered progression of the mammalian cell cycle is controlled by the sequential formation, activation, and deactivation of specific cell cycle-regulatory molecules (**Fig. 1**) that exist as a series of complexes consisting of a catalytic kinase subunit (known as the cyclin-dependent kinase or CDK) and a regulatory cyclin subunit. These cyclin:CDK complexes form part of the positive regulatory machinery that drives the cell through the cycle. Most cyclin mRNAs and proteins show a dramatic fluctuation in their expression during the cell cycle. For example,G1/s cyclin, cyclin, cyclin E is unstable, peaks during *late*G_1 and disappears rapidly thereafter, whereas cyclins A and B accumulate transiently at the onset of *S* phase and in late G_2 respectively, followed by their rapid degradation ***(1,2)***. In contrast, expression of the various CDK molecules remains relatively constant throughout the cell cycle.

The CDKs can interact with a variety of cyclin partners, with each specific complex displaying its kinase activity at a particular point in the cell cycle (**Fig. 1**). These cyclin:CDK complexes are able to bind to other regulatory proteins to form large, heterologous multicomplexes that can act as regulatory elements in the control of cellular events. For example, cyclin D:CDK4 can bind proliferating cell nuclear antigen (PCNA) and the cyclin-dependent kinase inhibitor (CDKI) protein, $p21^{CIP1}$ (***4,5***; *see* below). The binding of other proteins to these cyclin:CDK complexes, such as the CDKIs, can regulate the ki-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

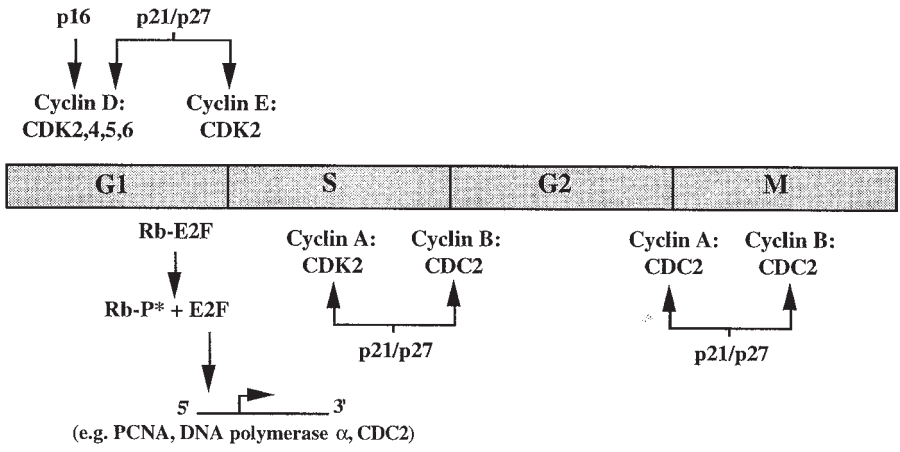

Fig. 1. The mammalian cell cycle showing where various cell cycle-regulatory molecules act.

nase activity of the complexes throughout the cell cycle such that different cell cycle-regulatory molecules are activated at specific times to "drive" the cell through successive checkpoints, i.e., G_1, *S*, G_2, and *M* phases of the cycle (**Fig. 1**).

The cyclin-dependent kinase inhibitors (CDKIs) are a class of cell cycle regulatory molecules that exert a negative regulatory effect on the cell cycle machinery by binding to, and inhibiting the activities of, specific cyclin:CDK complexes ***(6)***. Primarily, these proteins cause arrest in the G_1 phase of the cell cycle and it is likely that they may play a role in the development of the terminally differentiated phenotype. Currently, two structurally distinct groups of CDKIs exist in mammals: (1) the INK4 family, which includes p14, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ and (2) $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (*see* **ref. *6*** for review and references therein).

The expression and activities of certain cyclins, CDKs and CDKIs have been shown to be aberrant in certain proliferative diseases, e.g., cancer and restenosis. For example, an amplification of the cyclin D_1 gene has been reported in breast carcinoma cells ***(7,8)*** whereas CDK2 and CDC2 complex activities have been shown to be upregulated, and certain CDKI molecules downregulated, during the intimal hyperplastic response induced following balloon injury to arteries in animal models of restenosis ***(9,10)***. Thus, cell cycle-regulatory molecules are pivotal in modulating both normal and aberrant cellular proliferation, suggesting that their expression and activity status may serve as suitable prognostic markers for certain proliferative diseases.

This chapter outlines the purification of various CDK and CDKI molecules from tissue homogenates and cellular lysates and describes the subsequent measurement of their enzyme activities in vitro.

2. Materials

1. Chemicals to be used in these procedures should be of the best grade available commercially. Solutions should be prepared with double-distilled sterile water, unless indicated otherwise.
2. Cyclin, CDK and CDKI antipeptide antibodies, and the GST-retinoblastoma fusion protein (GST-pRb) can be obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. The various immunizing peptides for each antibody also are available for use in competition studies.
3. Adenosine trisphosphate (ATP) (Sigma-Aldrich): Prepare as a 10 m*M* stock solution in water and store at –20°C in aliquots.
4. [γ-^{32}P] ATP redivue (3000 Ci/mmol) (Amersham Life Sciences). Alternatively, [γ^{32}P] ATP of the same specific activity may be obtained from other suppliers (e.g., ICN Biomedicals Ltd, Sigma-Aldrich Company, Ltd).
5. Histone H1 substrate protein (Boehringer-Mannheim Diagnostics and Biochemicals, Ltd).
6. Protein A Sepharose beads (Pharmacia Biotech).
7. 0.1% w/v Coomassie brilliant blue solution: 50% methanol, 10% acetic acid, 0.1% w/v Coomassie brilliant blue R-250 (Sigma-Aldrich Company, Ltd.), 40% water. Store at room temperature. Can be used many times (20–40) before replacing.
8. Destain Buffer: 5% methanol; 7% acetic acid; 88% water. Store at room temperature.
9. CDK lysis buffer (*see* **Note 1**): 50 m*M* Tris-HCl (pH 7.4), 0.25 *M* sodium chloride, 0.1% v/v Nonidet P-40 (NP-40), 5 m*M* ethylenediaminetetraacetic acid (EDTA), 50 m*M* sodium fluoride, 1 m*M* sodium orthovanadate, 1 m*M* sodium pyrophosphate, 10 m*M* benzamidine, 50 μg/mL phenyl methyl sulphonyl fluoride (PMSF), 10 μg/mL N-toysl-phenylalanine chloromethyl ketone (TPCK), 10 μg/mL soybean trypsin inhibitor (STI), 1 μg/mL aprotinin, 1 μg/mL leupeptin. Prepare fresh every time and store on ice.
10. Wash buffer**:** 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol. Stable for up to 1 mo. Store at 4°C.
11. ATP buffer (*see* **Note 1**): Wash buffer plus 10 μ*M* ATP, 1 μCi/μL [γ-^{32}P] ATP. Prepare fresh every time and store on ice.
12. 2× sample buffer: 120 m*M* Tris-HCl, pH 6.8, 4% v/v sodium dodecylsulfate (SDS), 20% v/v glycerol, 10% v/v β-mercaptoethanol (or 200 m*M* dithiothreitol), 0.002% bromophenol blue (Sigma-Aldrich Company, Ltd.). Stable for many months at 4°C.
13. Rb buffer (*see* **Note 1**): 20 m*M* sodium-β-glycerophosphate, pH 7.3, 15 m*M* $MgCl_2$, 5 μg/mL leupeptin, 1 m*M* benzamidine, 0.5 m*M* PMSF, 0.1 m*M* sodium orthovanadate, 1 m*M* dithiothreitol. Prepare fresh every time and store on ice.

14. Rb assay buffer (*see* **Note 1**): Rb buffer plus 50 μ*M* ATP, 10 μCi [γ^{32}P]ATP, 0.5 μg GST-pRb fusion protein. Prepare fresh every time and store on ice.
15. 12% SDS-polyacrylamide gel compositions — based on 14 cm × 14 cm gel using 0.75-mm spacers (*see* **Note 2**):
    a. Stacking gel: 0.65 mL 30% acrylamide: 0.8% *bis*-acrylamide, 1.25 mL 1.5 *M* Tris-Cl (pH 6.8), 50 μL 10% SDS, 3.05 mL water, 25 μL 10% ammonium persulfate solution (*see* **Note 3**), 5 μL TEMED (Sigma).
    b. Resolving gel: 6 mL 30% acrylamide:0.8% *bis*-acrylamide, 3.75 mL 1.5 *M* Tris-Cl (pH 8.8), 150 μL 10% SDS, 5 mL water, 150 μL 10% ammonium persulfate solution (*see* **Note 3**), 6 μL TEMED.

    Both gel solutions should be prepared fresh every time. Do not add the ammonium persulfate and TEMED until the gel is ready to pour.
16. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer: prepare a 5× stock solution of SDS-PAGE running buffer as follows: 15.1 g Tris base, 72 g glycine, 5 g SDS, water to 1000 mL. This solution is stable at room temperature for many weeks. Do not adjust the pH of this stock solution because the pH will be 8.3 when it is diluted to a 1× working solution. The 1× working solution should be prepared, by dilution with water, immediately prior to electrophoresis.

3. Methods

3.1. CDK Kinase Activity Assays

The following procedure is a modification of the method of Draetta et al. ***(11)*** and has been used routinely in my laboratory for determining CDK activities in CDC2-, CDK2-, CDK4-, CDK5-, and CDK6- containing complexes in cardiac myocytes ***(12)***. It also can be used for determining CDK activities in tissue homogenates. Each kinase has a different specificity for a particular substrate protein, although the individual kinases can be placed into one of two groups depending on their abilities to phosphorylate either histone H1 or a GST-pRb fusion protein. Thus, the G_1 acting CDKs, CDK4, and CDK6, phosphorylate a GST-pRb fusion protein efficiently whereas the G_1/S and G_2/M kinases, CDK2 and CDC2, and the G_1-acting kinase, CDK5, efficiently phosphorylate histone H1. It should be noted that these substrates are not specific for any one group of kinases since it is possible for CDC2, CDK2, and CDK5 to phosphorylate a GST-pRb fusion protein and similarly for CDK4 and CDK6 to phosphorylate histone H1. However, the extent of phosphorylation in these latter cases is much less than that observed with their more specific substrates.

3.1.1. Preparation of Tissue Homogenates and Cellular Lysates

Tissue homogenates and cellular lysates are prepared as follows:

1. To prepare a tissue homogenate, cut the freshly dissected tissue into small pieces with sharp scissors and, as quickly as possible, place into a polypropylene tube

cooled to 4°C. Immediately add sufficient ice-cold CDK lysis buffer to just cover the tissue mass (e.g., 1.5–3-mL buffer should be sufficient for ~500 mg tissue) and homogenize the tissue using a polytron homogenizer, e.g., T8 Ultra-Turrax (IKA). Transfer homogenate to an appropriate number of Eppendorf tubes and proceed to **step 3**.
2. To prepare a cellular lysate, transfer freshly isolated or trypsinised cells to an Eppendorf tube and lyse on ice in ice-cold CDK lysis buffer. Use the minimum volume of buffer necessary to obtain lysis of cells. This is approx 100–200 μL buffer per 1×10^6 cells for large cells, such as adult rat cardiac myocytes, or 100–200 μL buffer per $0.5–1 \times 10^7$ cells for smaller cells, such as ovarian carcinoma cells. Proceed to **step 3**.
3. Sonicate samples on ice (3 pulses of 5 s each on maximum setting) and incubate on ice for 30 min. Centrifuge sonicated samples at 12,000*g* in a benchtop refrigerated centrifuge at 4°C. Transfer supernatant to a fresh tube and remove a 5-μL aliquot for protein concentration determination (*see* **Note 4**). Snap freeze supernatants on dry-ice and store overnight in liquid nitrogen prior to assaying for CDK complex activity (*see* **Note 5**).

3.1.2. Immunoprecipitation and CDK Kinase Assays

1. Preswell protein A Sepharose beads (0.1 g/mL) in ice-cold phosphate-buffered saline (PBS), pH 7.4, and then equilibrate in CDK lysis buffer for 1 h at 4°C with gentle mixing on a rotating blood wheel.
2. Add 40 μL preswollen protein A Sepharose beads to 250 μg of cell lysate protein in an Eppendorf tube. Make up to a final volume of 750 μL using CDK lysis buffer and mix gently on a rotating wheel for 1 h at 4°C. Pellet the beads using a 15-s pulse at 16,500*g* in a benchtop microcentrifuge and remove the supernatant to a clean Eppendorf tube ("precleared cell lysate").
3. Immunoprecipitate the precleared cell lysate with the appropriate CDK antibody (e.g., 1 μg/sample as determined by titration) for 1 h at 4°C (*see* **Note 6**). Add 50 μL of preswollen protein A Sepharose beads and mix gently for 1 h at 4°C on a rotating wheel.

The protocol then differs slightly depending on which CDK is being assayed:

CDC2, CDK2 and CDK5 Kinase Assays:

4. Wash the immunocomplex bound beads four times with 1 mL CDK lysis buffer each time, then once in 1 mL wash buffer. Pellet beads using a 15-s pulse at 16,500*g* in a benchtop microcentrifuge and resuspend the pellet in 20 μL wash buffer containing 125 μg/mL histone H1 substrate protein. Incubate for 5 min at 30°C.
5. Add 5 μL ATP buffer to each reaction tube and incubate for 10 min at 30°C. Terminate the reaction by adding 25 μL 2× sample buffer to each sample and go to **step 8**, below.

CDK4 AND CDK6 KINASE ASSAYS:

6. Wash the immunocomplex bound beads four times with 1 mL CDK lysis buffer each and then once in 1 mL of Rb buffer. Pellet beads using a 15-s pulse in a microcentrifuge and resuspend the pellet in 30-μL of Rb assay buffer per sample.
7. Incubate the reaction at 30°C for 1 h and terminate by the addition of 30 μL 2× sample buffer.
8. For all kinase assay products, samples are boiled for 3 min and proteins separated by 12% SDS-PAGE (*see* **Note 2**). Separated proteins can be visualized by staining the gel in 0.1% w/v Coomassie brilliant blue solution (*see* **Note 7**). The gel is then dried and exposed to X-ray film overnight at –70°C using intensifying screens. The resultant autoradiograph can be either scanned using a laser densitometer or the phosphorylated bands excised directly from the gel and counted in a scintillation counter. **Figure 2A** and **B** shows representative autoradiographs of CDK2 (**Fig. 2A**) and CDK6 (**Fig. 2B**) kinase activities in developing rat cardiac myocytes (*see* **Note 8**). It also is possible to use the above method to determine specific cyclin-associated kinase activities (*see* **Note 9**).

3.2. CDKI Inhibitory Activity Assays

The following procedure is a modification of the methods of Draetta et al. ***(11)*** and Guo et al. ***(13)*** and has been used successfully in my laboratory to determine the inhibitory activity of $p21^{CIP1}$ present in adult rat cardiac myocytes against neonatal rat cardiac myocyte CDK2 activity ***(14,15)***.

1. Cell lysates are prepared as detailed in **Subheading 3.1.1.** in ice-cold CDK lysis buffer. Boil lysates to obtain a heat stable protein preparation (*see* **Note 10**) and snap-freeze samples on dry ice and store in liquid nitrogen prior to use (*see* **Note 5**). Prior to freezing, remove a 5-μL volume of supernatant containing the sample of interest for protein analysis (*see* **Note 4**).
2. Thaw samples and incubate 50 μg of neonatal myocyte lysate protein (containing activated CDK2) with 50 μg of boiled adult myocyte lysate (*see* **Note 11**) overnight at 4°C with mixing on a rotating wheel.
3. Immunoprecipitate CDK2 from the mix using an anti-CDK2 antibody as described in **Subheading 3.1.2**.
4. Wash immunocomplex bound beads four times with 1 mL CDK lysis buffer each time, and once with 1 mL wash buffer. Pellet beads using a 15-s pulse at 16,500*g* in a benchtop microcentrifuge and resuspend the pellet in 20 μL wash buffer containing 125 μg/mL histone H1 substrate protein and incubate for 5 min at 30°C.
5. Add 10 μL of ATP buffer to each sample tube and incubate for 10 min at 30°C.
6. Terminate the reaction by the addition of 30 μL 2× sample buffer.
7. Boil samples for 3 min and separate proteins by 12% SDS-PAGE (*see* **Note 2**). Separated proteins can be visualized by staining the gel in 0.1% w/v Commassie brilliant blue solution (*see* **Note 7**). The gel is then dried and exposed to X-ray film overnight at –70°C using intensifying screens. The

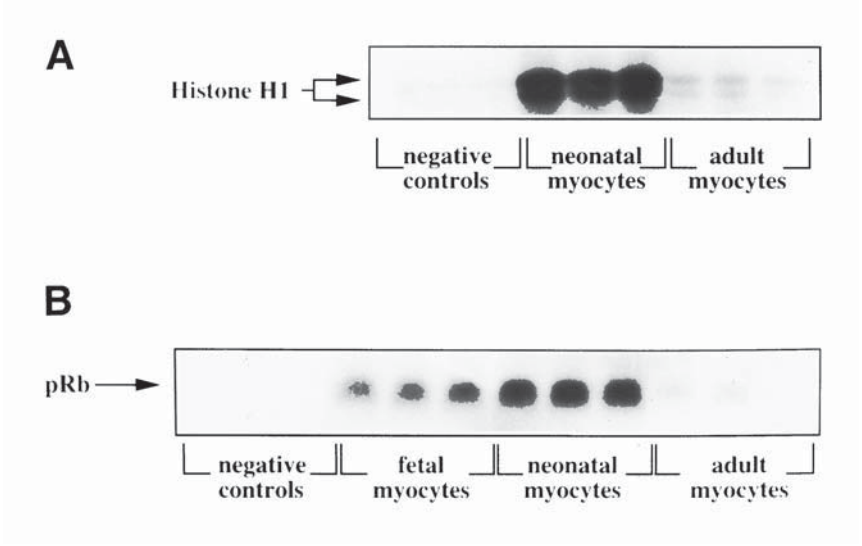

Fig. 2. Representative autoradiographs of: **(A)** CDK2-mediated phosphorylation of histone H1 and **(B)** CDK6-mediated phosphorylation of GST-pRb fusion protein in rat cardiac myocytes prepared from hearts of animals at different developmental ages. The negative control lanes represent: no antibody, cellular lysate (left hand lane); CDK antibody, no cellular lysate (middle lane); and, CDK antibody plus immunizing peptide, cellular lysate (right hand lane).

phosphorylated histone H1 bands then are excised from the gel and counted in a liquid scintillation counter.

4. Notes

1. All assay buffers should be prepared fresh on the day of assay. We have found that storage of such solutions overnight at 4°C leads to significant loss of measurable kinase activity.
2. Protein samples may be separated by SDS-PAGE as follows: pour a 12% SDS-PAGE resolving gel into a glass plate sandwich separated by 0.75-mm spacers (use, e.g., Protean-II or Hoefer gel electrophoresis equipment) and allow gel to polymerize for 30 min at room temperature. Once the resolving gel has set, pour the stacking gel, and immediately insert a Teflon comb into the layer of stacking gel, ensuring that no air bubbles are present. Add additional stacking gel to completely fill the spaces in the comb if necessary. Allow the gel to polymerize for 30–45 min at room temperature then gently remove the comb. Fill the wells with 1× SDS-PAGE running buffer and, using a Gilson pipet, load the protein sample(s) into one or more wells. Add 1× SDS-PAGE running buffer to the top and bottom reservoirs and connect the apparatus to a power supply. Run the gel at 10 mA constant current until the bromophenol blue tracking dye

in the sample buffer enters the resolving gel then increase the current to 15 mA. When the tracking dye reaches the bottom of the resolving gel, disconnect from the power supply, remove the gel from the glass plates and dry under vacuum prior to autoradiography. The time required for running a typical 14 cm × 14-cm gel is ~4 h.

3. 10% Aqueous ammonium persulfate solution can be stored at 4°C for a maximum of 5 d.
4. Protein concentrations may be determined by the method of Bradford. Prepare a series of dilutions (e.g., 1, 2, 5, 10, 20 μL) of a protein standard, e.g., bovine serum albumin (BSA), type V (Sigma-Aldrich Company, Ltd.) in 800 μL double-distilled water. Add 200 μL of Bradford Reagent (Bio-Rad Protein Assay), measure the absorbance value at 595 nm and construct a standard curve from the A_{595} values. Prepare a similar dilution of the 5-μL aliquot taken from the protein sample of interest in 800 μL double-distilled water, add 200 μL of Bradford Reagent (Bio-Rad Protein Assay) and measure the absorbance value at 595 nm. Determine the protein concentration of the sample from the standard curve. This assay is a simple colorimetric assay for measuring total protein concentration based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of proteins. It is important to note, however, that many detergents and basic protein buffers are known to interfere with this assay and researchers should refer to the manufacturer's instructions for advice on acceptable reagents and concentrations.
5. CDK activities in frozen lysates remain measurable for 2–3 d following preparation when stored in liquid nitrogen. However, the degree of measurable activity deteriorates rapidly, such that only 50–75% of original activity (i.e., that present in freshly prepared cell lysates) remains after one day of storage and 25–50% after 2 d.
6. It is advisable to include a series of control immunoprecipitations in all kinase assays to ensure that the results obtained are specific for the CDK of interest. Suggested controls include: no CDK antipeptide antibody, cellular lysate; CDK antipeptide antibody plus immunizing peptide (mix together for 5–10 min at room temperature prior to addition to lysate), cellular lysate; CDK antibody, no cellular lysate; and, no antibody, no lysate. All immunoprecipitations and kinase assays should be carried out in triplicate and each individual experiment should be repeated at least three times to obtain good statistical results.
7. It is advisable to stain all SDS-PAGE gels with Coomassie brilliant blue solution for 30–60 min at room temperature, followed by destaining until blue bands and a clear background are obtained (typically 1–2 h). Gels should then be dried down prior to autoradiography. This approach facilitates clear observation of the band(s) of interest directly on the gel and also reduces significantly the amount of background radioactivity that could affect the final results.
8. Phosphorylated histone H1 protein migrates as two distinct bands with M_r 32 and 34 kDa by 12% SDS-PAGE. Phosphorylated GST-pRb fusion protein (Santa Cruz Biotechnology, Inc.) migrates with a M_r of 40 kDa under the same conditions.

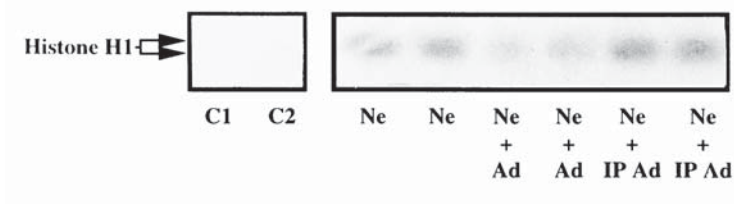

Fig. 3. Representative autoradiograph of $p21^{CIP1}$-mediated inhibition of CDK2 activity in rat cardiac myocytes. The autoradiograph shows histone H1 phosphorylation by immunoprecipitated CDK2 complexes from neonatal myocytes in the presence or absence of adult myocyte lysates that contain $p21^{CIP1}$. C1 = no CDK2 antibody, cellular lysate; C2 = CDK2 antibody, no cellular lysate; Ne = neonatal myocyte lysate; Ne + Ad = 50 μg neonatal and 50 μg heat stable adult myocyte lysate; Ne + IP Ad = neonatal and $p21^{CIP1}$ immunodepleted heat stable adult myocyte lysate.

9. To measure the kinase activity associated with a particular cyclin, e.g., cyclin E in a cellular extract, immunoprecipitate cyclin E-containing complexes from a cellular lysate using a specific anticyclin E antibody at the appropriate concentration, e.g., 1 μg/sample as determined by titration. The resultant immunoprecipitated protein then is analyzed for CDK2 activity (cyclin E associates specifically with CDK2), using a similar protocol to that described above, with histone H1 as a substrate. This method can be used to determine the kinase-associated activity of any cyclin molecule providing the appropriate substrate protein for the CDK molecule that complexes with the immunoprecipitated cyclin is used in the in vitro kinase assay.
10. Most CDKI molecules described to date are heat-stable proteins. The use of a heat-stable cellular extract in these studies enriches for those CDKI molecules that may be expressed at relatively low levels, compared to other heat labile proteins. Thus, if a heat stable extract was not used, it might prove difficult to demonstrate inhibitory activity of CDKI molecules in a particular cellular lysate.
11. To demonstrate that a particular CDKI molecule is present in a cellular lysate and responsible for CDK inhibitory activity, it is necessary to compare CDK activity in mixes of CDK-containing lysate with CDKI-containing lysate that has been: (1) depleted of a particular CDKI molecule by prior immunoprecipitation with a CDKI-specific antibody; and (2) not depleted of any CDKI molecule. *See* **Fig. 3** for an example.
12. A typical time frame, from start to finish, for the above assays is 1–2 d.

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12

Protein Histidine Kinase

Harry R. Matthews and Karina Chan

1. Introduction

Protein phosphorylation on histidine occurs in a large number of processes in prokaryotes and in an unknown number of processes in eukaryotes. The functions of protein phosphohistidine in prokaryotes are much more clearly understood ***(1,2)***. The methods presented here have been developed for the study of protein histidine phosphorylation in eukaryotes where phosphohistidine is found as an intermediate in some enzyme reactions and may also be involved in signaling mechanisms ***(3)***.

In prokaryotes, two types of protein phosphorylation on histidine have been characterized in detail. The first, historically, is the phosphoenolpyruvate-sugar phosphotransferase system ***(4,5)***. In this system, phosphohistidine acts as a phosphate donor and the initial phosphate donor is phosphoenolpyruvate, so this is not a protein kinase system. The second prokaryotic system is the two-component regulatory system ***(1,2)***. The core of this system comprises three protein domains, which may or may not be on different polypeptides. The first domain is the protein kinase domain, the second is the histidine substrate for this kinase and the third contains an aspartate residue that removes the phosphate from phosphohistidine. Genes homologous to those of the two-component system have been identified in lower eukaryotes ***(1,6,7)***.

Phosphohistidine in eukaryotic proteins was reported in the 1960s ***(8)*** and the first protein histidine kinases were described in the 1970s ***(9)***. After a short hiatus, work resumed in the late 1980s ***(10)*** and the first purification of a eukaryotic protein histidine kinase was reported in 1991 ***(11)***. Methods for the assay of protein histidine kinase ***(12)*** and the identification of phosphohistidine in proteins were also developed ***(13)***. Cloning of the purified kinase showed that it represents a novel class of kinases ***(14)***. Protein histidine phosphoryla-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

tion of the protein, p-selectin, was reported, in human platelets, at a site that is not homologous to the two-component phosphorylation site ***(15)***. Human platelets have substantial protein histidine kinase activity in cell lysates (F. Tablin and H. R. Matthews, unpublished) but the mechanism of phosphorylation of p-selectin has not been reported. The timing of the phosphorylation, the first one to two minutes after stimulation of the platelets to aggregate, suggests that the phosphorylation may be involved in signaling or translocation of p-selectin.

Special methods are needed for working with phosphohistidine because it is unstable in acidic conditions. Of the two isomers, 1-phosphohistidine is much less stable than 3-phosphohistidine, at least when they are studied as the free amino acid ***(16,17)***. However, stability of phosphohistidine in proteins appears to be quite variable, depending on the site of phosphorylation.

Two critical methods for the study of the eukaryotic protein histidine kinases are presented below. The first is an assay, which is fairly specific for alkali-stable protein phosphorylation. Protein histidine phosphorylation is by far the most common alkali-stable protein phosphorylation in yeast, *S. cerevisiae*, (H. R. Matthews, unpublished) and the true slime mould, *Physarum polycephalum* ***(18)***. Thus, the assay normally detects and measures histidine kinase activity. In outline, the kinase is incubated with radioactive Mg-adenosine triphosphate (ATP), substrate and buffer, to allow the kinase to transfer the gamma phosphate of ATP to histidine in the substrate. The mixture is then subjected to a mild alkaline hydrolysis, spotted onto Nytran paper, washed, and the remaining radioactivity determined in a phosphorimager or liquid scintillation counter. Alternatively, the reaction mixture can be analyzed by SDS gel electrophoresis.

The second method provides unequivocal identification of a radioactive alkali-stable phosphoamino acid and can be used directly, or in conjunction with the first method, to show protein phosphorylation on histidine. The protein is digested to its constituent amino acids by alkaline hydrolysis (or enzyme proteolysis) and the digest is analyzed by ion-exchange chromatography. A 20-min run separates the alkali-stable amino acids, allowing identification of the particular one(s) present in the sample. Typically, internal standards of the phosphoamino acids, phosphoarginine, phospholysine, phosphohistidine, phosphotyrosine, and phosphothreonine, are added to the radioactive sample before chromatography. The elution position of the radioactivity is compared with that of the standards and the nature of the radioactive amino acid can be deduced. The main advantage of this method is that it is largely independent of sample volume because the sample is bound to the top of the column before elution is begun. In our laboratory, a sample volume of 2 mL is routinely employed. The method requires that the unknown phosphoamino acid be radioactively labeled.

Both methods take advantage of the alkali-lability of phosphoserine to remove any background from this, the major phosphoamino acid found in proteins.

2. Materials

2.1. Histidine Kinase Assay

1. [^{32}P]ATP stock solution: 10 mCi/mL [γ-^{32}P]ATP in phosphate or tricine buffer at neutral pH diluted with 1 vol of 2 m*M* ATP to give a stock solution of 5 mCi/mL, 1 m*M* ATP (the initial specific activity of the [γ-^{32}P]ATP makes little difference). Note that the label must be in the gamma position.
2. 10× kinase buffer: 500 m*M* Tris-HCl, 150 m*M* $MgCl_2$, pH 7.5.
3. Stop solution: 100 m*M* ATP, 3 *N* NaOH.
4. 10× wash solution: 0.5 *M* Na_2HPO_4, 10 mm ATP.
5. Nytran membrane, presoaked overnight in 1× wash solution and air-dried (can be kept indefinitely after presoaking).
6. Substrate solution: 5 mg/mL histone H4 in water (*see* **Note 1**).

2.2. Identification of Phosphohistidine

1. Mono Q HR 5/5 FPLC column obtained from Pharmacia Biotech (ion exchanger type: quaternary ammonium exchange strong anion exchanger, ionic capacity: 320 ± 50 mmol (Cl^-)/mL beads, binding capacity: 65 mg/mL HAS, bed volume: 1 mL).
2. Liquid chromatograph, liquid chromatograph terminal, interface, multimeter, absorbance detector, fluorescence detector, fraction collector, O-phthaldialdehyde pump, scintillation counter, and nitrogen tank.
3. Prederivatising agent: Prepare a solution of 3 mL Brij, 50 g H_3BO_3, 44 g KOH, 1 L distilled water. Store at room temperature.
4. Derivatising agent: Must be freshly made for each run. Prepare solution of 400 µL 2-mercaptoethanol (toxic), 200 mL of prederivatizing agent, 80 mg O-phthaldialdehyde (dissolve completely in 1.0 mL of 100% methanol). Filter (0.2 µm) and degas.
5. Elution buffer: Must be made freshly. Prepare a solution 0.75 *M* $KHCO_3$ (pH 8.5 with 5 *N* KOH). Filter (0.2 µm) and degas. The pH is very critical.
6. 100% Ethanol. Filter (0.2 µm) and degas.
7. 1 *M* KOH. Filter (0.2 µm) and degas.
8. 3 *N* KOH.
9. 3 *N* Perchloric acid.
10. Ecolite, liquid scintillation "cocktail" for aqueous samples.
11. Nytran membrane.
12. Distilled water. Filter (0.2 µm) and degas.
13. Phosphoamino acid standards: 0.5 mg/mL phospholysine; 0.5 mg/mL phosphoarginine; 0.5 mg/mL phosphoserine; 0.5 mg/mL phosphothreonine; 0.5 mg/mL phosphotyrosine; 0.5 mg/mL phosphohistidine.

Except for phospholysine and phosphohistidine, these phosphoamino acids are available from suppliers such as Sigma Chemical Co. Phosphohistidine and phospholysine can be synthesized with phosphorus oxychloride and polyhistidine or polylysine, respectively ***(19)***. This method produces mainly 3-phosphohistidine. The 1-isomer can be produced by the reaction of phosphoramidate with histidine, at short reaction times ***(19)***. Preparative separation of isomers of phosphohistidine can be accomplished on disposable silica columns ***(20)***.

3. Methods

Steps 3.1. through **3.2.** or **3.3.** are needed for the protein histidine kinase assay. The subsequent steps, which may be carried out independently, are for unequivocal identification of phosphohistidine.

3.1. Kinase Reaction

1. Calculate the volumes of the reagents (*see* **Note 2**). The typical reaction volume is 50 μL of which 1/10 volume is 10× kinase buffer and 1/10 volume is 5 mg/mL histone H4 (substrate solution). Sufficient [γ^{32}P]ATP stock solution is diluted with 1 m*M* ATP to give 375,000 to 1,500,000 cpm/μL and 1/10 volume is used per 50-μL reaction. The volume of enzyme solution can vary from 1 to 20 μL and water is added to make the final volume 50 μL. Keep a small amount of diluted [γ^{32}P]ATP to use as a standard after the Nytran membrane has been washed.
2. Mix the solutions on ice. After vortexing, incubate them for 15 min at 30°C.
3. Stop the reactions by adding 10 μL of the stop solution and vortexing them. Incubate the reaction mixtures at 60°C for 30 min to hydrolyze any phosphoserine. Take care that evaporation or condensation on the lid of the tube does not significantly affect the volume of the solution. Cool the tubes on ice.

3.2. Nytran Assay

1. If the reaction mixtures are to be assayed for the total histidine kinase activity, mark a section of Nytran membrane into 2-cm squares with a pencil, and mark the membrane to indicate the first square. You need two more squares than you have reaction mixtures.
2. Carefully blot each reaction mixture onto one square per 50-μL reaction, spreading it over the whole area of the square. This should be done behind a screen to provide for protection against radiation. If a large number of samples is involved, it may be advisable to screen the squares already blotted by placing a screen over them, supported just above the membrane. Some unbound [^{32}P]ATP may soak through the membrane, so place a paper towel or other disposable material beneath the membrane.
3. Place the blotted membrane in a dish of 1× wash solution and occasionally agitate it gently. After about 5 min, drain the membrane and transfer to a fresh dish of 1× wash solution. Repeat this for four washes, total. Then wash the membrane in water and dry it under a lamp.

4. Put 3 μL of the diluted [^{32}P]ATP solution, saved from preparing the reaction mixture (**Subheading 3.1.**, **step 1**), into a separate tube, on ice, and add 47 μL water and mix. Dilute 2 μL of this solution into 48 μL water and mix. Blot 5 μL of this solution onto one of the unused squares of the Nytran membrane and dry it. If the original diluted [^{32}P]ATP solution contained 375,000 cpm/μL, then this square contains 15,000 cpm. Whatever the original radioactivity in the original diluted [^{32}P]ATP solution, this square contains 20 pmols of ATP and is used to convert the phosphorimager's integrated "counts" into pmols of ATP.
5. Expose a phosphorimager screen to the membrane. Exposure time can vary from about 1 h to several days, depending on the level of kinase activity. Read the screen in a phosphorimager and integrate the radioactivity in each square. The phosphorimager is by far the most convenient way to analyze the membrane. However, cutting out each square and determining its radioactivity in a scintillation counter or Geiger counter can produce similar results. Autoradiography can be used for qualitative analysis, but is not recommended for quantitative studies because of the nonlinearity of the film response.
6. The kinase activity of each sample, in pmols/s, is calculated as: (integrated counts from sample square)*20/{(integrated counts from ATP square)*15*60}.

3.3. SDS Gel Electrophoresis

1. As an alternative to the use of the Nytran membrane (**Subheading 3.2.**), the reaction mixtures from **Subheading 3.1.** may be analyzed by SDS gel electrophoresis. The hydrolyzed reaction mixtures can be added to SDS sample buffer and loaded directly onto the gel. However, this tends to give poor resolution, probably caused by the NaOH in the stop solution. If phosphoserine is not a problem, or the gel is washed with alkali after fixing, the stop solution and subsequent hydrolysis may be omitted. However, we have found that precipitation of proteins from the reaction mixture, after hydrolysis, is an effective preliminary to SDS gel electrophoresis (*see* **Note 3**).
2. Add 2 vol (120 μL) of cold acetone to each cooled reaction mixture, mix, and allow to sit on ice for 10 min.
3. Centrifuge the samples at 10,000*g* for 15 min. Remove the supernatant. Dissolve each pellet in 7 μL of SDS sample buffer and load 5 μL onto one lane of an SDS gel. Run the electrophoresis. Do not fix or stain the gel in acetic acid or trichloracetic acid.
4. Wrap the damp gel in Saran Wrap™ and expose it to a phosphorimager screen. This works well for short exposure times, up to a few hours or even overnight, but for longer exposure times, we fix the gel in formalin ***(21)*** before exposing it to the screen.
5. This method gives the lowest backgrounds and valuable qualitative information about the protein(s) that are acting as substrate(s) in the reaction. It is more time consuming for large numbers of samples and doesn't have the convenient internal standard for the ATP specific activity that the Nytran membrane method does.

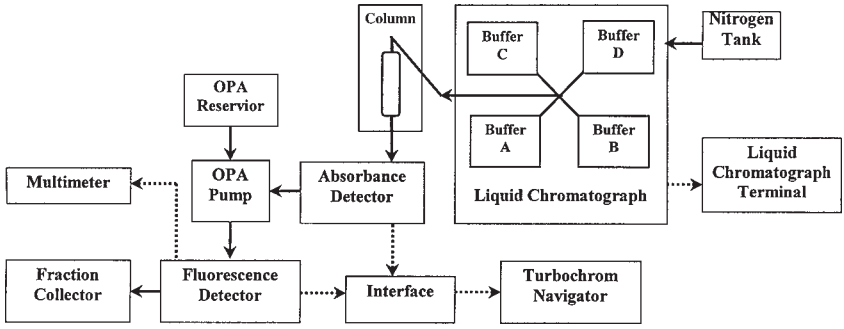

Fig. 1. Schematic of phosphoamino acid analyzer. The dashed lines represent electrical connections. The parameters of the run are entered in and displayed by the liquid chromatograph. The multimeter displays the fluorescence readings. The interface collects the data from the fluorescence detector and the absorbance detector. The data is then transferred to the turbochrom navigator, which translates and displays the data onto a graph. The eluent from the column passes first through the ultraviolet absorbance detector, which is not used directly in this method although it is useful for testing the column and we often include an internal standard of AMP to provide an independent test of proper column operation. From the absorbance detector, the eluent is mixed with a derivatizing solution and passes through a short (0.2 mL) loop to allow the derivatizing solution to react with primary amino groups in the eluent, producing a fluorescent compound. The fluorescence is measured with the fluorescence detector.

3.4. Preparation of Sample for Chromatography

1. The washed Nytran membrane, preferably not dried, can be used directly for chromatographic analysis of the phosphoamino acid present. If the sample is available as a band on an SDS gel, it may be blotted, electrophoretically, to Nytran (*see* **Note 4**). Alternatively, digestion may be carried out directly in the gel slice. It is not recommended that the gel be dried.
2. The sample must be hydrolyzed. In a small test tube, place 100 µL 3 *N* KOH and the Nytran paper or gel fragments. Larger volumes of KOH may be used if necessary to cover the sample. Heat at 105°C for 5 h (*see* **Note 5**).
3. Vortex each tube thoroughly and cool it on ice (*see* **Note 6**). Remove the liquid and neutralize it with 3 *N* perchloric acid. Approximately 100 µL is required to bring the pH of the sample to 8.5. Avoid overshooting by checking the pH with pH indicator strips.
4. Mix the solution and allow it to stand on ice for 10 min. Centrifuge each tube at 10,000*g* for 5 min and remove the supernatant. Discard the potassium perchlorate pellet (*see* **Note 6**).
5. The sample is diluted with water before chromatography and filtered through a 0.2-µm filter. The amount of dilution is usually determined by the size of the

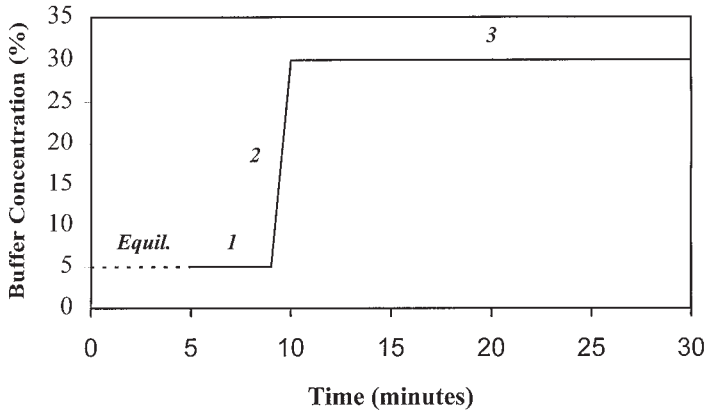

Fig. 2. Elution parameters. The run begins with an equilibration sector during which 5% buffer passes through the column for at least 5 min at a flow rate of 0.7 mL/min. With the column equilibrated, the sample and standards can be injected into the column. The first sector of the run is 4 min long. The buffer concentration remains at 5% but the flow rate is decreased to 0.6 mL/min. During this period, the phosphoamino acids adhere to the column. The second sector is 1 min long and continues at a flow rate of 0.6 mL/min; however, during this sector, the concentration of the buffer is increased to 30%. The third sector of the run is the gradient, which is an isocratic elution of the phosphoamino acids over 20 min. The 30% buffer concentration and the 0.6 mL/min flow rate remain constant. During the fourth and fifth sectors of the run, the column is washed. The fourth sector is one minute long and runs a 50% concentration of KOH through the column. The fifth sector removes the KOH and the flow rate is increased to 0.7 mL/min. To prepare the column for another run, the sixth sector is 10 min long and runs a buffer concentration of 5% through the column at 0.7 mL/min.

sample loop — the final volume after dilution equals the sample loop volume — but if the perchloric acid step is omitted, dilution of at least 50-fold is recommended.

3.5. Preparation of the HPLC and Column

1. The liquid chromatograph used in our laboratory has four elution buffer reservoirs (A, B, C, and D) (*see* **Note 7**). Add the filtered and degassed water, elution buffer, ethanol, and 1 *M* KOH to reservoir A, B, C, and D, respectively. Open the pressure valve of the nitrogen tank and pressurize the reservoir chamber to 5 psi (*see* **Note 8**) to maintain the solutions in their degassed state.
2. Elute the column with water and check the backpressure (*see* **Note 9**). If the backpressure is excessive, wash the column as recommended by the supplier. We have found the methanol wash particularly effective in this application. Add the derivatizing solution to the *o*-phthaldialdehyde (OPA)

Table 1
The Gradient Program

Sector	Flow rate Time	H_2O (mL/min)	$KHCO_3$ A%	KOH B%	C%
Equil	5.0	0.7	95.0	5.0	0.0
1	4.0	0.6	95.0	5.0	0.0
2	1.0	0.6	70.0	30.0	0.0
3	20.0	0.6	70.0	30.0	0.0
4	1.0	0.6	50.0	0.0	50.0
5	0.5	0.7	95.0	5.0	0.0
6	10.0	0.7	95.0	5.0	0.0

Table 2
A Sample Set of Elution Times[a]

Phosphoamino acid	Average elution time (minutes)	Standard deviation of elution time
P-Arginine	9.37	—
P-Lysine	14.43	0.10
P-Threonine	16.29	0.08
P-Serine	17.10	—
P-Histidine	19.52	0.18
P-Tyrosine	21.96	0.61

[a]The standard deviations for phosphoarginine and phosphoserine were not measured in this series. The larger variability in the position of phosphotyrosine is typical and depends on the other components in the mixture.

reservoir and make sure the absorbance detector, fluorescence detector, OPA pump, and the Turbochrom Navigator have been turned on (*see* **Fig. 1**).

3. Set the fraction collector to a rate of 30 s between each fraction. The fraction collector rack must have at least 60 vials because the sample and standards will run through the column in 30 min.

3.6. Loading and Running the Sample and Standards

The following procedures and gradient (**Fig. 2**, **Table 1**) were used to determine elution times of phosphoamino acids from the HPLC column. The elution of standards is shown in **Fig. 3** and a sample set of elution times is given in **Table 2**.

1. Once the gradient parameters have been set and the column is equilibrating, the sample can be loaded into the loop. Set the absorbance detector reading to zero,

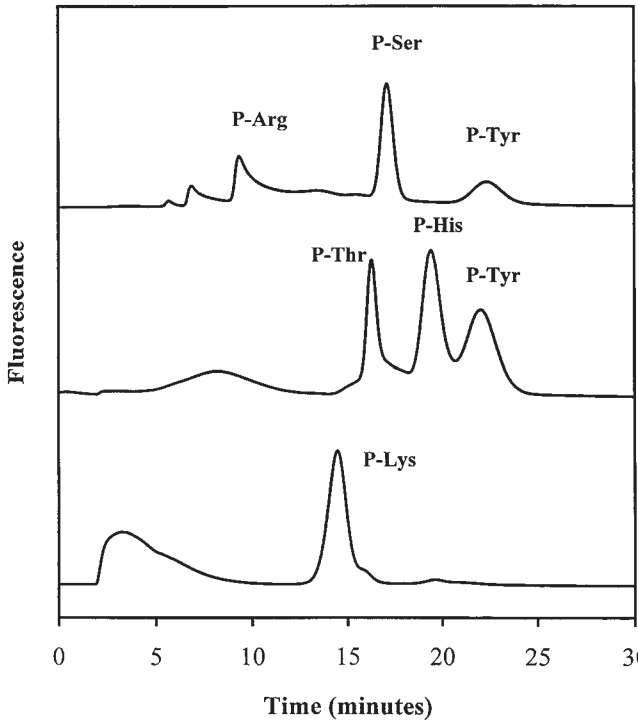

Fig. 3. Elution times of standards. The turbochrom navigator program generated the graph. It shows the fluorescence of standard amino acids eluted from the column.

and check the Turbochrom Navigator is ready. Inject the sample, turn on the fraction collector, and set the HPLC to Run, which switches the gradient program from the equilibration step to the first step of the gradient and initiates data collection by the TurboChrom Navigator program.

2. Allow the column to run for 30 min. After all the fractions have been collected, 4 mL of Ecolite must be added to each fraction and shaken. Use a scintillation counter to measure the radioactivity of the fractions.

3.7. Interpretation of Data

After running the column, two important measurements must be compared: the counts per minute taken from the scintillation counter and the fluorescence profile taken from the Turbochrom Navigator. Through the comparison of these measurements, the phosphoamino acid phosphorylated in the sample can be determined.

1. Using the data taken from the scintillation counter, plot the CPM vs elution time in minutes. On a secondary axis, plot the fluorescence vs time in minutes using the data taken from the Turbochrom Navigator (*see* **Note 11**).

2. It can be determined which phosphoamino acid is radioactive by comparing the peaks on the graph. Typically, the observed fluorescence comes from the standards and each fluorescence peak is identified by its elution time. If a fluorescence peak coelutes with a radioactivity peak the identity of the radioactive phosphoamino acid can be deduced. On rare occasions, fluorescence from the sample obscures one or more of the standards. In this case, it is necessary to rely solely on the elution time of the radioactive peak. Although this is probably satisfactory, given the reproducibility of elution times (**Table 2**), the presence of the internal standards greatly increases the reliability of the identification.

4. Notes

1. Histone H4 can be obtained from biochemical suppliers or purified from total histone by chromatography on BioGel P-10 in 10 m*M* HCl. A column at least 1 m long is necessary and histone H4 elutes after the other histones, partly because of its small size and partly because of a small ion-exchange effect.
2. In our laboratory we use a spreadsheet in the computer program, Microsoft Excel, Office97 version. This spreadsheet may be downloaded from `http://moby.ucdavis.edu/HRM/PKP/`.
3. This method was introduced to our laboratory by Paul Besant, University of Western Australia.
4. Do not attempt the alkaline hydrolysis on proteins blotted to nitrocellulose. The nitrocellulose chars during the hydrolysis and produces a gray/black solution that destroys chromatography columns.
5. Take care that evaporation or condensation on the lid of the tube does not significantly affect the volume of the solution, as the resulting higher concentration of KOH can lead to reduced yields of phosphohistidine ***(22)*** (P. G. Besant and P. V. Attwood, unpublished).
6. The perchloric acid step may be omitted if the sample is diluted sufficiently (about 50 times) before chromatography.
7. Four reservoirs are not essential. Three would suffice because the fourth reservoir, containing ethanol, is not used in the gradient.
8. When pressurizing the chamber, ensure that O-rings on each container are properly placed. An improperly placed or broken O-ring will keep the chamber from pressurizing.
9. If the pressure decreases drastically, the reservoir containers should be purged again or the frits should be checked to ensure they are properly attached.
10. If the pressure increases beyond the maximum pressure, the column could be inverted and run backwards with methanol to reduce the pressure.
11. It is critical that the correct time corresponding to the CPM is plotted because a shift in the peaks can affect the interpretation of the results.

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Cloning and Expression of Recombinant HEXA-HIS Tagged ZAP-70 Using the Baculovirus System

Tracey Purton, Sandra Wilkinson, and Edward J. Murray

1. Introduction

The activation of T cells results in both tyrosine and serine/threonine phosphorylations of a number of substrates within minutes of T-cell receptor (TCR) ligation ***(1,2)***. The phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the (ζ-chain within the TCR complex act as binding sites for the N-terminal SH2 domains of ZAP-70 ***(3)***. This relocation brings ZAP-70 proximal to the CD4-associated $p56^{lck}$ kinase, which activates the kinase activity of ZAP-70 by phosphorylation at Y493 ***(4)***. The activated ZAP-70 is then able to transduce a signal via an as yet uncharacterized cascade, which may involve SLP-76 ***(5)***, vav ***(6)*** PLC-γ(***(7)*** p120/130 ***(8)*** and LAT ***(9)***. The major role of ZAP-70 in T-cell signal transduction has been highlighted in ZAP-70 deficient patients who suffer severe combined immunodeficiency disease (SCID) like symptoms ***(10)***.

We have cloned and expressed ZAP-70 in several different hosts as a first step to studying the in vitro properties of this pivotal kinase in T-cell activation. The baculoviral system was found to be most appropriate for generating sufficient yields of pure active kinase for both assay development and crystallographic study. A number of peptide substrates of this enzyme have now been described in the literature ***(5)***. In this chapter, we describe procedures used in cloning and purifying hexa-his-tagged ZAP-70. In addition, we detail two methods for assessing the activity of the enzyme, first, via autophosphorylation and also by assaying the phosphorylation of a peptide substrate. In this respect, a peptide, modeled around the Y113ESP motif from the human SLP-76 sequence encompassing amino acids 107 to 120), has proved both an efficient

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

and selective substrate for ZAP-70 ($K_{m\ app.}$ = 20 m*M*) when compared with another T-cell nonreceptor tyrosine kinase, $p56^{lck}$ ($K_{m\ app.}$ = 550 m*M*).

2. Materials

2.1. Construction of Recombinant ZAP-70 Transfer Vector

1. ZAP-70 cDNA (*see* **Note 1**).
2. pAcHLT transfer vector (Pharmingen).
3. Competent DH5-α (Life Technologies).
4. T4 DNA ligase (NEB).
5. 10× Ligase buffer; 500 m*M* Tris-HCl, pH 7.2, 7.5 m*M* $MgCl_2$, 10 m*M* adenosine triphosphate (ATP), 100 m*M* dithiothreitol (DTT), and 200 µg/mL gelatin.
6. LB medium: 10 g Bactotryptone (DIFCO), 5 g yeast extract (DIFCO), and 5 g NaCl in 1 L distilled water. Autoclave.
7. Ampicillin (sodium salt) Sigma; 1000× solution is 50 mg/mL in distilled water.
8. Phenol/HS; Phenol:chloroform:isoamyl alcohol at 25:24:1 (Gibco-BRL) saturated with 0.3 *M* NaCl made in 50 m*M* Tris-HCl, pH 7.5.
9. Chloroform: (BDH) GPR grade.
10. 2 *M* sodium acetate: 164 g anhydrous sodium acetate (Sigma Molecular bioloy Grade) in 1 L distilled water made to pH 5.2 with glacial acetic acid.
11. Ethanol: (BDH) absolute ethanol Analar grade.

2.2. Generation of Recombinant hexa-his ZAP-70 Baculovirus

1. Complete TC 100 medium (Gibco-BRL): 10% fetal calf serum (FCS), 50 U/mL penicillin, 50 U/mL streptomycin, 100 µg/mL kanamycin, and 2 m*M* glutamine.
2. Spodoptera frugiperda (Sf9) cells.
3. BaculoGold genomic DNA (Pharmingen).
4. Transfection buffer A (Pharmingen) : Graces medium with 10% FCS.
5. Transfection buffer B (Pharmingen): 25 m*M* HEPES, pH 7.1, 125 m*M* $CaCl_2$, 140 m*M* NaCl.
6. 28°C Incubator and tissue-culture hood.

2.3. Viral Plaque Isolation, Amplication and Calculation of Viral Titer by Plaque Assay

1. TC 100 medium and Sf9 cells: as in **Subheading 2.2.** above.
2. Low gelling temperature agarose (Sigma).
3. 40°C Water bath.
4. Six-well tissue-culture dishes (Nunc).
5. Perspex boxes with lids.
6. Sterile wide nozzle plastic disposable pipets.
7. Neutral red (Sigma): Diluted 1/20 with PBS.

2.4. Purification of Hexa-His ZAP-70 from Infected Sf9 Cell Lysates

1. TC 100 medium and Sf9 cells: as in **Subheading 2.2** above.
2. High titer recombinant ZAP-70 viral stock.

3. Phosphate-buffered saline (PBS; Dulbecco): 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 dissolved in 800 mL distilled water.
4. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 200 m*M* NaCl, 150 m*M* KCl 5 m*M* β-mercaptoethanol, 1 m*M* sodium vanadate, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 5 m*M* benzamidine, 10 μg/mL aprotinin.
5. Wash buffer/20 m*M* imidazole: as in **Subheading 2.4.4.** above with 20 m*M* imidazole.
6. Elution buffer: Wash buffer (as in **Subheading 2.4.4.** above) with 500 m*M* imidazole.
7. Ni^{++}-chelate affinity matrix (Qiagen).
8. 10-mL syringe barrel.
9. Glass wool.
10. Dialysis buffer: 50 m*M* Tris-HCl, pH 8.0, 200 m*M* NaCl, 10 m*M* β-mercaptoethanol, and 30% glycerol in distilled water.

2.5. Autophosphorylation Assay of Recombinant hexa-his ZAP-70 Activity

1. 500 m*M* HEPES, pH 7.5, 200 m*M* $MnCl_2$, 1% Triton X-100.
2. 5 μ*M* adenosine-5'-triphosphate (ATP) (CalBioChem): 10 m*M* stock made in MilliQ water and stored in aliquots at –20°C**.**
3. [γ-^{32}P ATP] (Amersham Pharmacia Biotech): Specific activity at reference date > 92.5 Tbq/mmol (>2500 Ci/mmol).
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
5. Protein disruption buffer: 2 mL 20% SDS, 1 mL 1 *M* Tris-HCl, pH 7.0, 1.2 mL glycerol, 1 mL β-mercaptoethanol, and 4.8 mL distilled water.
6. X-Omat Scientific Imaging Film (Kodak).
7. ClingFilm™.

2.6. Assay of the Ability of Recombinant Hexa-His ZAP-70 to Phosphorylate a Peptide Substrate

1. 500 m*M* HEPES, pH 7.5, 200 m*M* $MnCl_2$, 1% Triton X-100.
2. 10 m*M* ATP.
3. [γ-^{32}P]-ATP (Amersham Pharmacia Biotech): 250 μCi/25 μL.
4. 1 *M* dithiothreitol (DTT) (Boehringer).
5. 0.01% Triton X-100 (protein grade, CalBioChem) in water.
6. 100 m*M* sodium orthovanadate, stored at –20°C.
7. ZAP-70 enzyme preparation.
8. [γ-^{33}P]-ATP (Amersham Pharmacia Biotech): 250 μCi/25 μL.
9. 10 m*M* SLP76 peptide substrate - SFEEDDYESPNDDQRRR (*see* **Note 2**).
10. Stop mix containing 100 m*M* ethylenediaminetetracetic acid (EDTA), 6 m*M* adenosine, pH 7.0.
11. P81 chromatography paper (Whatman).
12. 0.5% orthophosphoric acid (Merck).

3. Methods

3.1. Construction of Recombinant ZAP-70 Transfer Vector

The first stage of generating a recombinant baculovirus is the construction of a transfer vector that provides flanking baculovirus DNA for the ZAP-70 insert to act as target sites for a double homologous recombination event.

1. Digest 5 μg pAcHLT transfer vector DNA with *Nde*1 and *Bgl*II restriction enzymes (*see* **Note 3**).
2. Load the pAcHLT digest on a 0.6% low-melting-point gel with a 3-cm preparative gel comb. Run the gel at 100 V for 1 h to separate the low-molecular-weight linker from the linearized vector.
3. Visualize the vector DNA band under short-wave UV light and excise with a scapel blade (*see* **Note 4**).
4. Place the gel slice in a 1.5-mL Eppendorf tube and estimate the volume. Add 5 *M* NaCl to approx 0.3 *M* final concentration. Melt the gel at 65°C for 15 min.
5. Quickly add an equal volume phenol/HS and vortex for 15 s.
6. Centrifuge for 15 min at 10,000*g* in an Eppendorf microcentrifuge. Aspirate the aqueous phase to a clean 1.5 mL tube and repeat the phenol/HS extraction.
7. Repeat centrifugation and remove the aqueous phase. Add equal volume of phenol/chloroform, vortex, and spin again.
8. Remove aqueous phase to a clean 1.5-mL tube and extract with equal volume chloroform. Repeat centrifugation as in **step 6**.
9. Remove aqueous phase to a clean tube. Add 1/10 volume 2 *M* NaAc, pH 5.2, and 2 vol ethanol.
10. Place tube on dry ice for 30 min until frozen.
11. Pellet the linearized pAcHLT DNA by centrifugation at 10,000*g* for 20 min at 4°C in an Eppendorf microcentrifuge.
12. Aspirate the supernatant and add 100 μL 70% ethanol to rinse away excess salt.
13. Remove all traces of 70% ethanol. Air-dry the pellet for 10 min and add 20 μL sterile distilled water. This should give a DNA solultion of approx 0.2 μg/μL.
14. Set up following series of ligation reactions:

*Nde*1-*Bgl*II linearized pAcHLT	1 μL
ZAP70 insert (0.1 μg/μL)	[0 μL, 0.2 μL, 1 μL]
Sterile distilled water	[7 μL, 6.8 μL, 6 μL]
10× ligation buffer	1 μL
T4 DNA ligase (200 U/mL)	1 μL

15. Incubate 15°C overnight.
16. Transform "Subcloning Efficiency competent DH5-α" bacteria (*see* **Note 5**).

3.2. Generation of Recombinant hexa-his ZAP-70 Baculovirus

The recombinant ZAP-70 transfer vector DNA and genomic baculoviral DNA are cotransfected into Sf9 cells to permit the homologous recombination event that transfers the ZAP-70 insert into the baculoviral genome.

The pAcHLT vector contains a partial copy of a vital baculovirus gene within the 3' flanking viral DNA. Therefore, viable viral particles are only reconstituted if the recombination event has occured and this provides a powerful selection process for recombinant virus. We use the BaculoGold expression system in which >95% of generated plaque forming units represent recombinant virus.

1. Seed a 750-mL tissue-culture flask with 5×10^6 exponentially growing low passage Sf9 cell in a 40-mL volume complete TC-100 medium (*see* **Note 6**).
2. Incubate for 42 h at 29°C.
3. Aspirate the medium and replenish with 10 mL fresh complete TC-100 medium. Harvest the adherent cells with cell scraper using light movements that cover the whole surface of the flask. Pipet the suspended Sf9 cells across the flask surface and dispense into clean sterile 50-mL centrifuge tube.
4. Count the cells using a hemocytometer and make the suspension to 10^6 cells/mL with TC-100 complete medium.
5. Seed a 60-mm tissue-culture dish with 2.5 mL Sf9 cells and incubate 1 h at 29°C.
6. Mix 0.5 μg BaculoGold DNA and 2.0 μg recombinant transfer vector. Add 1 mL transfection buffer A and mix gently.
7. Aspirate medium from 60-mm tissue-culture dish containing incubated Sf9 cells (from **step 5** above) and add 1 mL transfection buffer B.
8. Slowly add the DNA mix onto the cells, 5 drops at a time with gentle shaking of the dish.
9. Incubate for 4 h at 29°C.
10. Aspirate transfection mix and rinse the cell monolayer gently three times with 3 mL complete TC-100 medium each time (*see* **Note 7**).
11. Incubate for 5 d at 29°C with 5 mL complete TC-100 medium in a closed perspex box lined with tissue paper moistened with sterile distilled water.
12. Harvest the supernatant and clarify by centrifugation at 3K 2000*g* for 15 min in a bench-top centrifuge (*see* **Note 8**).

3.3. Viral Plaque Isolation, Amplication, and Calculation of Viral Titer by Plaque Assay

It is advisable to purify a single plaque prior to maintaining a high-titer viral stock. Safety issues relating to this procedure are given in **Note 9**.

1. Seed a 6-well tissue-culture dish with 1.4×10^6 Sf9 cells in 2 mL complete TC-100 medium. Incubate for 1 h at 29°C.
2. Generate a dilution series of transfection supernatant from undiluted to 10^{-5} dilution in 1-mL aliquots complete TC-100 in sterile 1.5-mL Eppendorf tubes.
3. Aspirate the medium from the 6-well dish and add 0.2 mL diluted virus to each well. Incubate for 1 h at 29°C.
4. During the incubation period, prepare a 1.5% gel overlay as follows. Weigh 1.5 g low-melting agarose in a 250-mL conical flask and add 50 mL sterile distilled

water. Microwave to dissolve the gel and cool to hand-hot (i.e., 50–60°C). Add 50 mL complete TC-100 medium and place in water bath at 40°C for 40 min.
5. Aspirate viral supernatants and add a 2-mL aliquot of gel overlay carefully to each well (*see* **Note 10**).
6. Let gel overlay set on vibration-free surface and add 2 mL complete TC-100 to each well.
7. Incubate for 6 d at 29°C.
8. Aspirate medium and add 2 mL 10% neutral red/PBS-A.
9. Incubate for 3 h at 29°C and aspirate all traces of neutral red/PBS. Take care not to disturb the overlay.
10. Incubate stained dishes inverted for at least 2 h at 29°C. The plaques appear as white on a red background.
11. Pick a single well isolated plaque by sucking up a plug of agar around the plaque using a wide-bore plastic disposable sterile pipet.
12. Expel the agar plug into a 1.5-mL Eppendorf tube containing 1 mL TC-100 complete medium and agitate gently for at least 6 h to allow the baculovirus to diffuse away from the agarose.
13. Seed a 100-mm tissue-culture dish with 6×10^6 Sf9 cells in 10 mL TC-100 complete medium and incubate for 1 h at 29°C to ensure adherence. Add the 1 mL of baculovirus-containing supernantant from **step 12** above and incubate cells for 3 d to permit infection and amplification of the baculovirus (*see* **Note 11**).
14. Harvest the infected cells by scraping and pellet in a 15-mL tube at 2000 rpm 1500*g* in a bench-top centrifuge at room temperature (*see* **Note 12**).
15. Filter the supernatant containing the baculovirus through a 0.22-μ*M* filter and set up 1-mL aliquots of serial dilutions to 10^{-6}.
16. Repeat steps 1–10 to perform a plaque assay on each of the serial diluted baculoviral supernatant. Set up duplicates for each dilution. A high titer should reach $1–5 \times 10^7$ plaque forming U/mL (pfu/mL).

3.4. Purification of hexa-his ZAP-70 from Infected Sf9 Cell Lysates

Many factors influence the yield and purity of hexa-his proteins isolated from infected Sf9 cells, including multiplicity of infection (MOI), duration of infection, and toxicity of cloned product. The late viral promoter used in pAcHLT necessitates lengthy time-courses for optimal yields, at which time the host Sf9 cells are exhibiting severe cytopathic effects caused by the attritional nature of viral replication and life cycle. Clearly, the host cell innate biological processes for posttranslational modification, active secretion or processing are severely impaired (although this maybe be redressed by the use of early viral vectors).

1. Seed four 750-mL tissue-culture flasks with 2.5×10^7 Sf9 cells per flask (*see* **Note 13**) in 25 mL complete TC100 medium. Incubate for 1 h at 29°C to allow the cells to adhere.

2. Add 2.5×10^7 pfu of recombinant virus to each flask and incubate for 66 h at 29°C.
3. Scrape any adherent cells from the flask and pellet the infected cells in a bench-top centrifuge at 2000*g* 10 min at 4°C (*see* **Note 14**).
4. Wash the cell pellet in 10 mL ice-cold PBS and pellet cells as above.
5. Resuspend in 2 mL lysis buffer and dounce 50 strokes on ice with type A pestle.
6. Transfer the lysate to a 15-mL centrifuge Corex tube and pellet the debris at 109,000*g* for10 min at 4°C in SS34 rotor.
7. Prepare a Ni^{++} column as follows. Add 3 mL of 50% slurry of Ni^{++}-chelate affinity matrix to a 10-mL syringe barrel blocked with a glass wool plug. Pass 10 mL resuspension buffer over the resin to equilibrate.
8. Harvest the supernatant from **step 6** above, and apply to the column. Collect flowthrough and reapply to the column.
9. Wash column with 10 mL resuspension buffer, followed by 10 mL wash buffer 1 and 10 mL wash buffer 2.
10. Elute with 3 × 1 mL elution buffer ensuring each aliquot has 5 min equilibration time on the column.
11. Pool the 3 mL of eluate and dialyze against dialysis buffer overnight at 4°C **(Fig. 1).**
12. Store at –80°C in 30% glycerol, activity of enzyme stored at –20°C is labile.
13. Check aliquots by PAGE and western analysis (*See* **figs. 1A** and **1B**)

3.5. Autophosphorylation Assay of Recombinant hexa-his ZAP-70 Activity

1. Purified enzyme is diluted with 0.01% Triton X-100 to 10^{-5} as appropriate.
2. Prepare a premix containing 10 µL assay buffer, 30 µL unlabeled ATP, 25 µL [γ-^{33}P] ATP (*see* **Note 15**).
3. Add 10 µL hot premix to 15 µL enzyme.
4. Allow assay to proceed for 60 min at 30°C. Stop reaction by addition of 25 µL protein disruption buffer followed by boiling for 2 min.
5. Separate proteins by SDS-PAGE and visualize phosphorylated proteins by autoradiography of the dried gel under a layer of Clingfilm™(*see* **Note 16**).

3.6. Assay of the Ability of Recombinant hexa-his ZAP-70 to Phosphorylate a Peptide Substrate

1. Purified enzyme is diluted with 0.01% Triton X-100 as appropriate (*see* **Note 17**).
2. Prepare a premix of 1700 µL distilled water, 250 µL assay buffer, 25 µL 1 *M* DTT (final concentration in assay 10 m*M*), 2.5 µL unlabeled 10 µ*M* ATP (final assay concentration 10 µ*M*), 5 µL 100 m*M* vanadate (final assay concentration 20 µ*M*), 12.5 mL 10 m*M* SLP76 peptide substrate (final assay concentration 50 µ*M*), and 5 µL [γ-^{33}P] ATP giving a total volume of 2 mL suitable for assaying 100 samples. Aliquot 20 µL premix into each assay tube.
3. Preincubate tubes for 5 min at 30°C. Start reaction by the addition of 5 µL of enzyme and incubate for a further 15 min at 30°C.

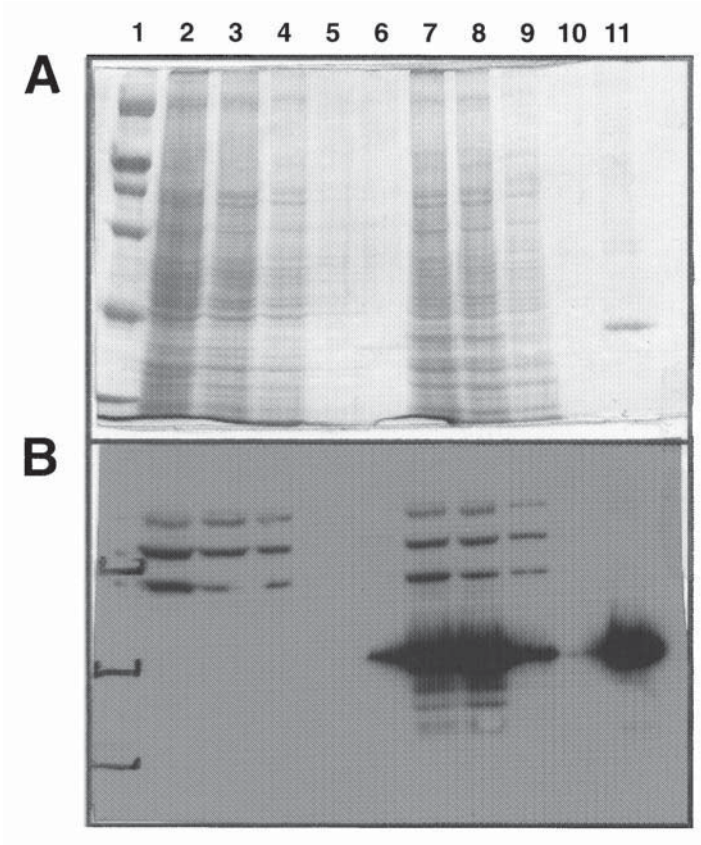

Fig. 1. **(A)** PAGE/SDS analysis of uninfected Sf9 lysate (lanes 1–6) and recombinant ZAP-70 baculoviral infected Sf9 lysates (lanes 7–11) followed by staining with Coomassie blue. **(B)** Same lysates transfered to nitrocellulose and probed with anti-ZAP-70 polyclonal.

4. Stop the reaction by the addition of 10 μL stop mix. Separate peptide from unreacted ATP by spotting a 30-μL aliquot of reaction mix onto squares of P81 paper and wash in three changes of 0.5% orthophosphoric acid (*see* **Notes 18** and **19**).
5. Wash P81 squares in ethanol, dry, and determine incorporated radioactivity by liquid scintillation spectroscopy. Plot data as shown in **Fig 2.**
6. Plot concentration of substrate against incorporation of radioactivity (*see* **fig. 2**)

4. Notes

1. ZAP-70 inserts can be generated quickly using PCR primers and template T-cell cDNA obtained from either commercial sources (Clontech Quick-Clone for

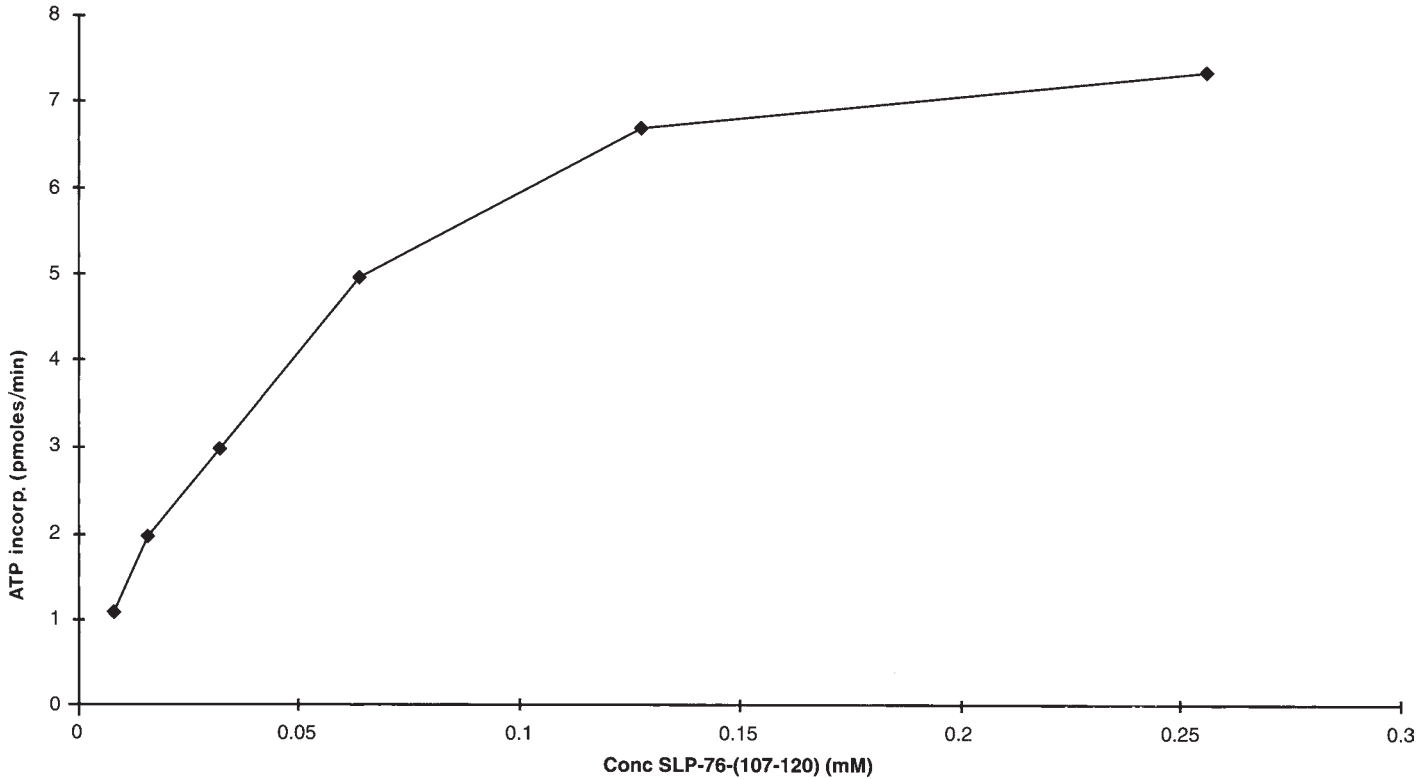

Fig. 2. Graph showing a representative experiment detailing the phosphorylation of the exogenous substrate SLP-76-(107–120) by ZAP-70 catalytic domain (untagged enzyme).

example) or prepared in house. We find that cDNA derived from MOLT-4 and/or Jurkat T cells is a good source for ZAP-70. We find that either full length or truncated catalytic domain of ZAP-70 are equally well expressed in Sf9 cells.
2. The peptide has been tagged at the C-terminus with three arginine residues to facilitate binding to P81 paper.
3. Other sites in the polylinker may used. However, it is clearly important to get the reading frame correct with respect to the N-terminal hexa-his tag on the pAcHLT and the ZAP-70 coding sequence.
4. Take time to ensure that the thinnest possible slice of gel is taken to give the highest yield of DNA.
5. The colonies are assayed for presence of recombinant DNA by using the miniprep. procedure as detailed in other laboratory manuals (*see* vol. 1, this series).
6. The cells must be maintained in a healthy state. We passage the cells immediately after they reach confluence (approx 4×10^7 cells per 750-mL flask) and split them 1 in 5 every third day. We discard cells with passage numbers higher than 150. Poorly maintained cell lines do not adhere well to the plastic.
7. Excessive force during this rinse step can result in substantial loss of cell monolayer.
8. The clarified supernatant may be stored for at least 1 yr at 4°C without any loss of viral titer. Aliquots may be stored under liquid nitrogen in 10% DMSO in FCS.
9. Recombinant baculovirus represents no hazard to human workers because of the restricted insect host range. However, there have been some reports of viral uptake by human liver cell lines ***(11)***. The main hazard is to the environment and thus class 2 containment must be adhered to throughout. This may be increased to class 3 if the nature of the insert instigates either extra hazard or host range alterations. Because of the mobile nature of the GMO, the local GMSC must be notified of such experiments. We find that 10,000 ppm available chlorine renders all discarded virus nonviable. This is equivalent to 5 Haz-tabs in 250 mL water.
10. We prewarm the pipettes before use to prevent gel solidifying in the nozzle. It is imperative that the gel overlay is not too hot (not over 40°C), otherwise the cell monolayer will die.
11. We observe cytopathic effects on the host cells on day 2 of infection. A successful amplification results in an initial elongated (sausage-shaped) phenotype and later to enlarged rounded nonadherent cells, which float around in clumps.
12. It is usual to harvest the host cells from an amplification and use the soluble lysates for Western analysis with appropriate antibodies to the cloned protein. This provides an early indication that the protein has been successfully expressed.
13. Sf9 cells are easily adapted to suspension growth in roller bottles or spinner flasks, if necessary.
14. If the MOI is low (<1), then the supernatant may be saved as a source of more recombinant virus.
15. ^{32}P labeled ATP may be used in preference to ^{33}P, if so reduce the volume of radiolabel in the assay and increase safety precautions accordingly.

16. If available, a Phosphorimager may be used in preference to standard autoradiography to visualize phosphorylated protein. This has the advantage of being both quicker and quantifiable.
17. While the specific activity of a given preparation is unknown at this stage, we usually expect around 10 nmol P-33 incorpration/min/mg enzyme.
18. This methodology can easily be adapted to high throughput format by separating peptide from unreacted ATP on Millipore anionic phosphocellulose filter paper MultiScreen 96-well plates. In this case, the reaction should be stopped with 2% orthophosphoric acid and filters then loaded and washed as before. 10 μL Scintillation fluid is then added to each well and plates counted directly in a Scintiallion counter suitable for 96-well plate format.
19. If inhibitor is to be added to the reaction, appropriate solvent controls must be included and the volume of distilled water equal to the total volume of solvent removed from the premix.

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Analysis of Protein Kinase Subcellular Localization by Visualization of GFP Fusion Proteins

Ralph A. Zirngibl and Peter Greer

1. Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is fast becoming the marker of choice for protein localization and gene expression studies. The rapid rise in popularity stems from several advantages over traditional markers such as β-galactosidase and alkaline phosphatase, which typically require the addition of exogenous substrates for detection. The unique intrinsic spectral properties of GFP allow it to be directly visualized in fixed or live cells by fluorescence microscopy without the addition of cofactors or exogenous substrates ***(1–8)***.

GFP is a 27-kDa β-sheet barrel-shaped monomeric protein with a centrally located helix containing a peptide chromophore that forms by cyclization and oxidation of a Ser-Tyr-Gly sequence ***(9,10)***. The absorption and emission spectra of GFP proteins have been modified extensively by mutagenic studies ***(2,11–14)***. There are now a number GFP variants with greatly improved spectral characteristics, including the pEGFP vectors available from Clontech (CLONTECH Laboratories, Inc. Palo Alto, CA). The GFP coding sequence in pEGFP has been "humanized" for better expression in mammalian cells by silent mutations, which incorporate the preferred human codons ***(15)***. In addition, amino acid substitutions of Phe64Leu and Ser65Thr in the peptide chromophore shift the excitation maxima to 488 nm, increase the fluorescence, and shift the emission maxima to 507 nm ***(16)***. Other GFP variants with distinct spectral characteriztics are also available from Clontech, including red-shifted variants with maximal absorption near 490 nm and emission maxima at different wavelengths ranging from green, blue, red, yellow, or cyan. These excita-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

tion and emission wavelengths are well within the working parameters of most confocal fluorescence microscopes and cell sorters ***(11,17,18)***. In addition to a number of generic vectors for expression of GFP fusion proteins, there are also plasmids available which employ GFP has a reporter for promoter studies (Clontech, Invitrogen, Carlsbad, CA). Finally, when GFP alone is expressed in eukaryotic cells, the fluorescence is diffusely localized throughout the cell. In contrast, when GFP is expressed as a fusion protein, the fluorescence distribution typically reflects the normal subcellular localization of the fusion partner, and fusions to either the amino or carboxyl termini of a number of proteins do not interfere with their normal subcellular localizations ***(1,3–5,8,19–21)***. The choice of where the fusion is made will become important if the protein is posttranslationaly modified. These modifications could include, but are not limited to, removal of a signal peptide, fatty acid acetylation, and proteolytic processing of the protein. The fusion protein should be tested for retention of normal function, as loss function can lead to mislocalization ***(22)***. The localization of the GFP fusion protein should also be compared to the native protein whenever possible before making conclusions about the correct localization. The availability of GFP proteins that emit fluorescence of different colors has also enabled the simultaneous detection of multiple GFP fusion proteins within the same cell ***(11,13)***, and even more sophisticated fluorescence resonance energy transfer (FRET) studies to examine direct molecular interactions ***(23,24)***. Traditional FRET studies have been used to show dimerization of receptor tyrosine kinases ***(25,26)*** and assembly of multiprotein complexes ***(27,28)***. These studies were complicated by the need for chromophore-linked monoclonal antibodies, or other means of directly labeling the proteins of interest. These potential problems, such as the availability of nonactivating monoclonal antibodies, can be overcome by expressing the proteins of interest as GFP fusions. One recent report describes the use of GFP fusions and FRET to study the assembly and topology of ATP synthase subunits ***(29)***.

We were interested in the subcellular localization of two closely related nonreceptor tyrosine kinases called Fps/Fes and Fer. These two kinases make up a distinct subclass characterized by a unique amino terminal domain followed by a central Src homology 2 (SH2) and C-terminal kinase domain ***(30)***. The localization of these two kinases was previously examined by subcellular fractionation techniques or conventional indirect immunocytochemistry and immunofluorescence. The results of these studies suggested that Fps/Fes and Fer could be localized to both the cytoplasm and the nucleus ***(31–35)***. However, more recent analysis using confocal fluorescence microscopy in combination with direct visualization of GFP fusion proteins suggested a different localization of these two kinases, which is similar to that of known markers for the Golgi apparatus ***(33)***.

In this chapter, we describe the generation and use of expression plasmids encoding fusions of GFP with the nonreceptor tyrosine kinases Fps/Fes and

Fer for subcellular localization studies. In principle, this procedure can be applied to the study of any protein. The precise subcellular localization of these GFP fusion proteins in transfected Cos-1 cells can be determined with very high confidence using confocal fluorescence microscopy.

2. Materials

2.1. Vector Construction

1. Generic eukaryotic expression vectors and the GFP cDNAs can be obtained from commercial suppliers (Clontech) or research groups working in the field. The GFP vectors described here are freely available from the authors.
2. Restriction enzymes, alkaline phosphatase, T4 DNA ligase, and their respective buffers were obtained from New England Biolabs (New England Biolabs). *Pfu* polymerase and buffer was from Stratagene (Stratagene).
3. Electrocompetent bacteria from a recA$^-$ strain of *Escherichia coli*: We routinely use the XL-1 blue bacterial strain (Stratagene) already competent for transformation or made competent as described in ***(36)***.
4. FISHERbrand Petri plates (Fisher Scientific).
5. LB media: 10 g bacto-tryptone (BDH), 5 g bacto-yeast extract (BDH), and 10 g of NaCl (FISHER Scientific) to 900 mL of deionized water and stir until solutes are dissolved. Adjust the pH to 7.0, make up to 1 L with deionized water and autoclave for 20 min in the liquid cycle. To make agar plates, add 15 g of Agar (ICN) per liter to the LB media and autoclave for 20 min in the liquid cycle. To make LB with ampicillin, let the media cool to 50°C before adding 250 μg/mL of ampicillin (Sigma-Aldrich).
6. For DNA minipreps and CsCl banding of DNA, we follow the protocol as described in ***(36)***. Alternatively, several companies (i.e., Qiagen, GIBCO Life Technologies, etc.) sell kits for plasmid purification.

2.2. Transfection of Mammalian Cells

1. Cos-1 cells (American Type Culture Collection, Rockville, MD).
2. Dulbeccos Modified Eagle Media (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (HyClone Laboratories).
3. OPTI-MEM (Life Technologies).
4. LIPOFECTAMINE (Life Technologies).
5. Gelatin (0.1%w/v): prepared by adding 0.5 g Type A gelatin from porcine skin (Sigma) to 500 mL distilled water and autoclaving. Store at 4°C.
6. Phosphate-buffered saline (PBS): To make 1× PBS, add 10 g NaCl, 0.25 g KCl, 1.44 g $Na_2HPO_4{\cdot}12H_2O$, and 0.25 g KH_2PO_4 to 900 mL of water and dissolve. Once the salts are dissolved, adjust the pH to 7.2 and bring the volume up to 1 L. Autoclave the solution in the liquid cycle.
7. Fixative: 4%w/v paraformaldehyde in PBS. To prepare fixative, add 4 g paraformaldehyde (BDH) to 100 mL of PBS (*see* **Note 1**). Add 80 μL 5 *M* NaOH and heat to 65°C. Once the paraformaldehyde is completely dissolved, let the

solution cool to room temperature, add 40 µL of concentrated HCl and adjust to pH 7.4. The solution should be stored in the dark at 4°C and used within a week.
8. Polystyrene tubes, 4 mL (Canlab).
9. Polypropylene tubes, 15 mL, screw-top (Sarstedt).
10. Mounting media: 50% glycerol (BDH), 50% 1× PBS.
11. Trypsin: To 1.9 L deionized water add 5 g Trypsin (Difco), 0.8 g ethylenediaminetetraacetic acid (EDTA), 14.0 g NaCl, 0.6 g $Na_2HPO_4 \cdot 12\ H_2O$, 0.48 g KH_2PO_4, 0.74 g KCl, 2.0 g D-glucose and 6.0 g Tris. Adjust the pH to 7.6 and make up to 2 liters. Filter sterilize through a 0.22-µm filter (Corning). Aliquot the filtered trypsin into 40-mL aliquots in a laminar flow hood and store at –20°C for long-term storage. Keep a working solution at 4°C.
12. 100×15 mm tissue culture dishes (Sarstedt).
13. 6-well plates (Falcon 3046, flat bottom) (Canlab).
14. Microscope slides (3 × 1 in. × 1 mm) cover slips (22×22 mm No.1). (Fisher Scientific.)
15. 70% Ethanol.

2.3. Visualization of GFP

We used an Olympus InSIGHT Plus inverted confocal microscope with an Omnichrome Argon laser. For the GFP constructs, we used the FITC filter sets provided with the microscope. A CCD solid state camera and DSP-200 Digital processor (DAGE-MTI, Inc.) along with MCID imaging software (Imaging Research, Inc.) were used to record the images as TIF files. The TIF files were imported into CorelDRAW! 3.0 (Corel Corporation, Ottawa, Ont., Canada) for the generation of figures.

3. Methods

The GFP-encoding sequences used were derived from the original GFP cDNA provided by Douglas Prasher ***(37)***, into which we introduced a Ser65Thr by polymerase chain reaction (PCR) mutagenesis. Using the eukaryotic expression plasmid, pECE ***(38)***, and the Ser65Thr mutant version of the original GFP, three generic vectors were generated that allow for both amino and carboxyl terminal GFP fusions (**Fig. 1**). The GFP-encoding sequences were amplified by PCR using primers that incorporated restriction sites to facilitate cloning into pECE and the generation of in-frame fusions. The pECE plasmid contains a bacterial origin of replication and an ampicillin resistance gene for propagation in bacteria. It also has a SV40 promoter, origin of replication, multiple cloning site and a polyA signal sequence. The SV40 origin promotes episomal amplification of the plasmid in Cos-1 or Cos-7 cells. The resulting higher expression levels greatly facilitate detection of the GFP fusion proteins.

To provide a marker of the trans-Golgi network the complete cDNA of rat TGN38 ***(39)*** was cloned by reverse transcription and PCR and expressed as a

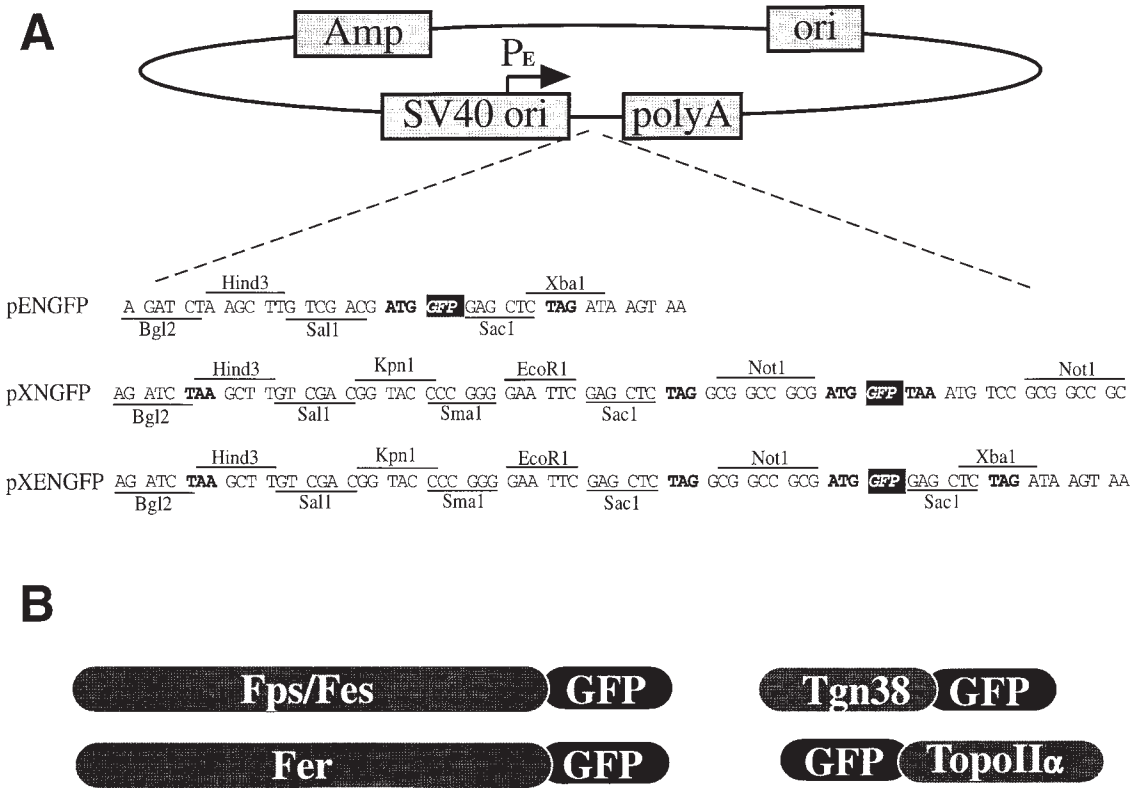

Fig. 1. Plasmids for expression of GFP fusion proteins. **(A)** Three generic constructs for the generation of either C- or N-terminal fusions with GFP. In pENGFP, the GFP open reading frame was amplified with primers containing *Sal*1 and *Sac*1 sites and cloned between the *Sal*1 and *Sac*1 sites of pECE. In pXNGFP, the GFP open-reading frame was amplified with primers containing *Not*1 sites and cloned into the *Not*1 site of a modified version of pECE, called pXN, in which the Xba1 of pECE was converted to a Not1 site. pXENGFP is a hybrid of pENGFP and pXNGFP. Note the presence of an inframe termination codon between the Sac1 and Not1 sites of the latter two plasmids when planning N-terminal fusions. **(B)** Structures of the four fusion proteins expressed in **Fig. 2**.

fusion to the amino terminus of GFP. Similarly, the carboxyl terminal nuclear localization signal of topoisomerase IIα ***(40)*** was generated by PCR and fused to the carboxyl terminus of GFP to provide a nuclear marker.

These methods give a detailed description of how the murine *fer* cDNA is cloned to the pXENGFP vector. All manipulations can be modified to suit your gene of interest.

3.1. Cloning of Murine fer into pXENGFP

Because the murine *fer* cDNA did not have suitable restriction sites for cloning into our vector, a PCR strategy was adopted to introduce the necessary cloning sites.

1. Two PCR primers were designed for amplification of the complete murine Fer coding sequence. The 5' primer also introduced a *Sal*1 site upstream of the translational initiation codon. The 3' primer replaces the *fer* termination codon with a *Not*1 site to allow inframe fusion with the 5' end of the GFP coding sequences in the pXENGFP vector shown in **Fig. 1** (*see* **Note 2**).
2. Amplify the open reading frame of *mfer* with the two primers using a thermostable DNA polymerase with proofreading ability (*see* **Note 3**).
3. Remove unincorporated primers, deoxyribonucleotides, and polymerase from the PCR reaction using an anion exchange column (*see* **Note 4**).
4. Digest PCR product with *Sal*1 and *Not*1 (*see* **Note 5)**.
5. Digest the pXENGFP vector with *Sal*1 and *Not*1, and dephosphorylate with alkaline phosphatase (*see* **Note 6**).
6. Run the digested vector and insert on an agarose gel. Isolate the appropriate fragments from the gel (*see* **Note 6**).
7. Check one-tenth of the isolated DNA on an agarose gel with a known amount of marker DNA. This will allow you to see if the fragments have been recovered and also to estimate the amount of DNA.
8. Ligate the digested PCR product into the vector using T4 DNA ligase following the supplier's protocol. For the ligation, a molar ratio of 1:2 (vector to insert) is recommended (*see* **Note 7**).
9. Electroporate 1 μL of the ligation into electrocompetent XL-1 Blue bacteria (*see* **Note 8**).
10. Recombinants are selected on LB plates with 250 μg/mL of ampicillin, and miniprep plasmid DNA ***(36)*** is analyzed for the presence of the correct insert.
11. Plasmid DNA is prepared for transfections (*see* **Note 9**).

3.2. Transfection of the Expression Vector into Cos-1 Cells

Cos-1 cell transfections provide a very rapid means of testing for expression of GFP expression plasmids. Using the transient transfection method described here, results can be obtained within four days. Alternatively, stable expressing lines can be selected. However, we have observed that visualization of GFP fusions is greatly facilitated by the high levels of expression achieved by tran-

sient expression in Cos-1 cells, and stable lines rarely achieve the same levels of expression. Although other methods of DNA transfection are suitable, we find the LIPOFECTAMINE reagents from Gibco-BRL Life Technologies give very reproducible results. Cos-1 cells are first seeded onto gelatin-coated coverslips and grown to approx 40% confluence prior to transfection. After a couple of days to allow the fusion protein to accumulate, the cells are fixed and viewed on the confocal fluorescence microscope. Similar levels of fluorescence can be seen in live, unfixed cells.

We routinely subculture Cos-1 cells in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere incubator. When cells reach 80–90% confluence, they are passaged as 1:10 and 1:20 dilutions. Cos-1 cells grow rapidly and should not be allowed to reach confluence as in our hands their growth and transfection characteristics appear to change. All media and reagents used for tissue culture should be warmed to room temperature before use.

Day 1

1. Add 22×22 mm glass microscope coverslips to 35 mm tissue-culture dishes (*see* **Note 10**).
2. Sterilize the coverslips by washing with 70% ethanol. Aspirate and dry.
3. Coat the coverslips with 0.1% gelatin solution (*see* **Note 11**).
4. Aspirate off the gelatin and let the coverslips dry in the laminar flow hood.
5. Aspirate media off the cells.
6. Rinse cells with 10 mL prewarmed 1×PBS.
7. Add 1 mL of trypsin to cells and tap on side of plate to completely cover all cells and let sit for one or 2 min until cells lift off.
8. Add 10 mL warm DMEM + 10% FBS to cells, resuspend the cells by pipeting the media up and down 2–3 times, and transfer to a 15-mL screw-top tube.
9. Spin cells in a bench-top centrifuge at 350*g* for 3 min at room temperature.
10. Aspirate off media; resuspend cells in 10 mL of fresh DMEM +10% FBS and count cells using a hemocytometer.
11. Seed 1×10^5 Cos-1 cells per 35 mm tissue-culture dish containing gelatin-coated coverslips. This is enough cells to achieve approx 40% confluence after overnight culture.

Day 2

1. Dilute 1–2 μg of plasmid DNA into 100 μL of OPTI-MEM in a 4-mL polystyrene tube.
2. In a separate polystyrene tube, dilute 3 μL of lipofectamine reagent into 100 μL of OPTI-MEM (*see* **Note 12**).
3. Decant the diluted DNA into the tube containing the diluted lipofectamine reagent and mix gently by inverting the tube several times.

4. Let the mixture sit at room temperature for 45–60 min.
5. Rinse the cells twice with 2 mL 1×PBS and once with 2 mL of OPTI-MEM.
6. Dilute the DNA/lipofectamine mixture with an additional 800 µL of OPTI-MEM and add it to the washed cells.
7. Culture the cells for 4–5 h.
8. Add 1 mL of DMEM+10%FBS and continue culturing the cells overnight.

Day 3

1. Aspirate the media and add 2 mL of fresh DMEM+10%FBS. Continue culturing the cells overnight (*see* **Note 13**).

Day 4

1. Aspirate media and add 2 mL of fixative. Incubate at 4°C for 5 min (*see* **Note 14**).
2. Aspirate fixative and rinse cells three times with 2 mL 1×PBS.
3. Use forceps to remove coverslips and place (cells down) onto one drop of mounting media on a microscope slide (*see* **Note 15**). Avoid trapping air bubbles.

3.3. Visualization of GFP Fusion Proteins

Because different confocal fluorescence microscopes have different requirements and computer software for capture and analysis of the images, the appropriate reference manuals for your system should be consulted. An example of Fps/Fes-GFP, Fer-GFP, TGN38-GFP and GFP-Topoisomerase IIα localization in transfected Cos-1 cells is seen by confocal fluorescence microscopy in **Fig. 2** (panels A, B, C, and D, respectively).

4. Notes

1. Paraformaldehyde is toxic and hazardous. Follow the handling instructions provided by the manufacturer.
2. Two DNA primers are designed to amplify the desired cDNA sequence by PCR using a thermostable DNA polymerase. We have good results using *Pfu* from Stratagene. The primers should consist of at least 18 nucleotides of precise homology to the target cDNA at their 3' ends, preceded by a restriction endonuclease recognition sequence (6–8 nucleotides), and an extension (3–7 nucleotides) at the immediate 5' end. Primers forming the junction with GFP must be designed to produce an inframe fusion. For fusing GFP to the C-terminus of your protein of interest, it is convenient to end the primer homology immediately before the termination codon. The extension at the 5' end of the primer can be designed to basepair with the original template cDNA after the engineered restriction endonuclease site. This will allow for slightly higher annealing temperatures during the PCR amplification. The 5' extension also promotes complete digestion of the DNA by the restriction endonuclease ***(41)***. The primer design for m*fer* is given below:
   The 5' primer is in the 5' untranslated region (UTR).
   5'-GCT GTC GAC CAC AGT GTG GAG GAT AAG-3' m*fer* 5'UTR *Sal*1 primer

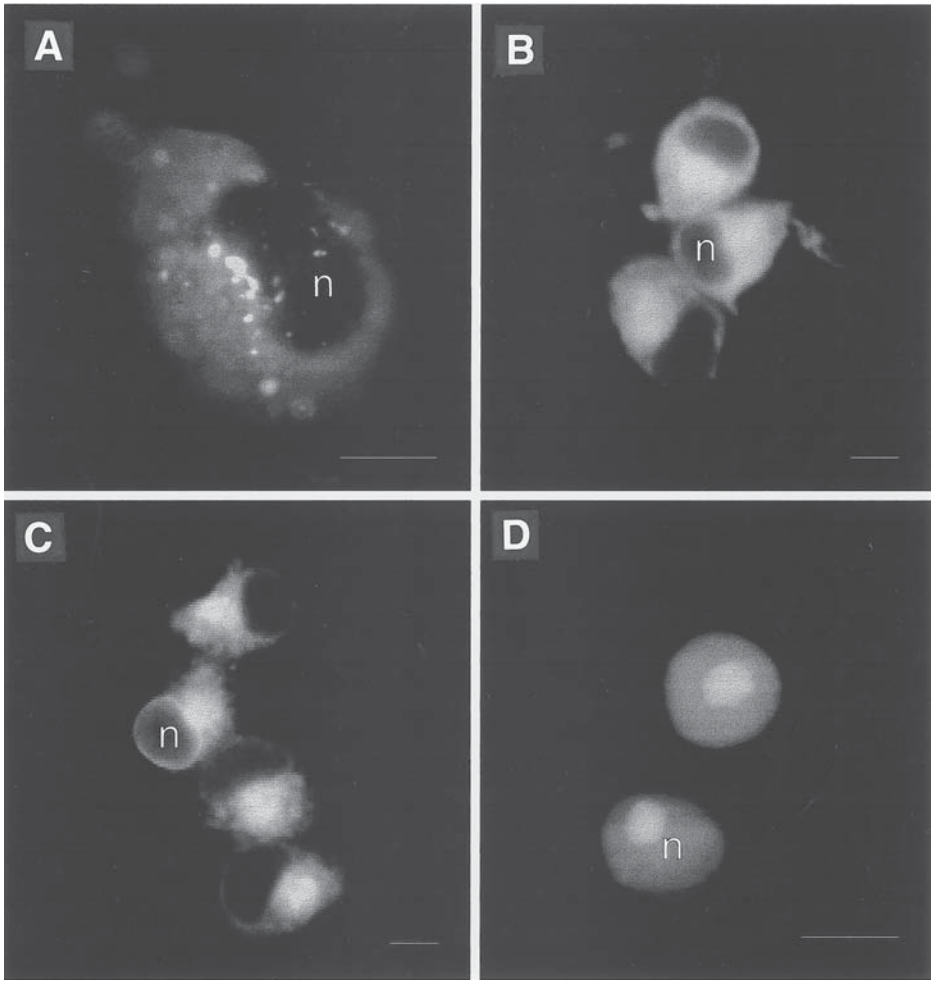

Fig. 2. Confocal images of Cos-1 cells expressing GFP fusion proteins. Cos-1 cells were transfected with expression plasmids encoding: A, Fps/Fes-GFP; B, Fer-GFP; C, TGN38-GFP; or D, GFP-Topoisomerase IIα. The positions of some nuclei are indicated, (n). The scale bars indicate 20 μm. Note the images in panels A and D were captured at twice the magnification as those in panels B and C.

The 3' primer is at the termination codon.

```
   Ile Lys Lys Met Ile Thr ***
5'-ATC AAG AAG ATG ATC ACA TAG TGC AGC CAG GGC-3' mfer cDNA
3'-TAG TTC TTC TAC TAG TGT ATC ACG TCG GTC CCG-5'
3'-TAG TTC TTC TAC TAG TGT CGC CGG CGG GTC CCG-5' mferStopNot
primer
```

Table 1
Typical PCR Reactions

Component	Final concentration	Per 100 µL reaction
10× PCR buffer[a]	1×	10 µL
Primer 1 (50 pmol/µL)	0.5 µ*M*	1 µL
Primer 2 (50 pmol/µL)	0.5 µ*M*	1 µL
2 m*M* dNTP mix	200 µ*M*	10 µL
Template DNA (10 pg/µL)	1pg/µL	10 µL
Pfu polymerase	2.5 U	1 µL
Water		67 µL

[a]The buffer is supplied by the company.

The *Sal*1 and *Not*1 restriction endonuclease sites are underlined in the 5' and 3' primers respectively.

3. The use of a thermostable DNA polymerase with proofreading capabilities, such as *Pfu*, is highly recommended when amplifying protein-coding sequences for expression purposes. The PCR product should also be sequenced to make certain no mutations have been introduced. A typical PCR reaction is outlined in **Table 1**. Some optimization of conditions may be necessary for your gene of interest ***(36)***.

   Thermal cycling conditions for the *mfer* PCR were: Thirty cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 5 min.
4. Removal of primers and unincorporated nucleotides may be important, because they can interfere with subsequent restriction endonuclease digestion. This may be achieved by ethanol precipitation, alternatively, several different kits are available commercially (e.g., Qiagen) that remove unincorporated primers and nucleotides. We have found that several enzymes (e.g., *Eco*R1, *Bam*H1, and *Xba*1) work even when the unincorporated primers and nucleotides are still present. This allows one to digest the crude PCR product directly; however, other enzymes may not cut the PCR product under these conditions.
5. For digesting DNA with two different enzymes the suppliers instructions should be consulted to see if the two enzymes have a compatible buffer. In the example given here, *Sal*1 and *Not*1, work in the *Sal*1 buffer supplied by the company (New England Biolabs). If there is no buffer compatible with both enzymes, digest the DNA first with one enzyme, then dilute the reaction to 200 µL with water, add 200 µL of 5 *M* ammonium acetate, mix, and centrifuge at 14,000*g* for 20 min at room temperature. Most proteins will precipitate under these conditions. Transfer the supernate (containing the nucleic acid) to a fresh Eppendorf tube, add 5 µL of 1 mg/mL yeast tRNA as a carrier, and mix with 1 mL of 100% ethanol. Pellet the nucleic acid by centrifugation at 14,000*g* for 20 min at room temperature. Discard the supernate, add 400 µL of 70% ethanol to the pellet and centrifuge at 14,000*g* for 5 min. Carefully remove and discard the 70% ethanol wash and dry the nucleic acid pellet. Dissolve in 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5. Make up in the appropriate reaction for the second restriction digestion.

6. For dephosphorylating the vector and inactivating the alkaline phosphatase follow the manufacturers instructions. We have found that the following modification of this protocol gives satisfactory results. Add 1 μL of calf intestinal phosphatase directly to the restriction digestion, and continue incubating the DNA at 37°C for an additional 20 min. Add sodium dodecyl sulfate (SDS) and EDTA to 0.5% and 10 m*M*, respectively, and heat inactivate at 65°C for 5 min. Resolve the cut PCR product, as well as the cut and dephosphorylated vector by agarose gel electrophoresis and purify. We use the QIAquick Gel Extraction Kit from Qiagen. Other DNA isolation protocols ***(36)*** and kits can be used.
7. A typical 10 μL ligation consists 100–200 ng of vector and the appropriate amount of insert to provide a 2 molar equivalent. Electroporation can be performed immediately with 1 μL of the ligation reaction; however, we have found that some ligations give a low yield of colonies on electroporation. If this occurs, the remaining 9 μL of the ligation can be purifed using the ammonium acetate/ethanol precipitation method described above in **Note 5**. Dissolve the isolated ligation in 5 μL of water and use 1 μL in a second electroporation.
8. We routinely prepare electrocompetent bacteria ***(36)*** from rec A^- strain of *E. coli* (e.g., XL-1 Blue, DH10B). Alternatively, these can be purchased from commercial suppliers (e.g., Stratagene).
9. Miniprep DNA can be used for the transfection of Cos-1 cells, but better results are obtained with more highly purified DNA preparations. We typically purify plasmid DNA by equilibrium density centrifugation on a CsCl gradient ***(36)***, but commercial ion exchange resin kits also yield high-quality DNA suitable for transfections.
10. Depending on your microscope, it may be possible to view cells grown directly on tissue-culture dishes. However, we have obtained better results using coverslips.
11. The gelatin provides a substrate to which the cells can attach and spread. Other substrates such as laminin, collagen, and fibronectin can be substituted, but these are generally more expensive.
12. Polystyrene tubes are recommended for use with lipofectamine because it may bind to other surfaces such as polypropylene.
13. It may be possible to observe fluorescence in the cells on day three, but an additional day of culture is recommended.
14. GFP fluorescence can also be viewed without fixing the cells, which does allow one to view movement of the tagged proteins by time lapse photography ***(1)***. Do not use glutaraldehyde as a fixative, because this leads to autofluorescence of the cells.
15. The cover slip should not be sealed with Permount or other organic solvents as they can lead to autofluorescence and interference with the GFP signal.

Acknowledgments

The authors are grateful to Douglas Prasher for the GFP cDNA sequence, Shelagh Mirski, and Susan Cole for the GFP-topoisomerase IIα construct, and

Derek Schulze for performing the confocal analysis. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

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Detection of Phosphorylation-Dependent Interactions by Far-Western Gel Overlay

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1. Introduction

The far Western gel overlay assay is a highly sensitive tool for the detection of direct protein–protein interactions. The ability to distinguish direct associations between two proteins makes this technique ideal not only for identifying potential binding partners of a protein, but also for characterizing interaction domains and binding sites. The assay relies on the ability of the protein of interest to interact with target proteins that have been immobilized on nitrocellulose filters. Potential interactions can be monitored under various types of conditions by manipulation of the protein that is used as the probe. Such manipulations may include mutagenesis, or protein modifications such as phosphorylation. Detection of phosphorylation-dependent interactions is particularly relevant in light of the fact that many signaling proteins are modified by phosphorylation, which in turn affects activity, localization, and most importantly, the ability to form protein complexes (*see* **refs. *1–4***). We describe here the standard protocol for the far-Western gel overlay assay, with modifications that enable the detection of phosphorylation-dependent protein interactions. This technique has been used successfully by our laboratory, as well as others to identify potential phosphorylation-dependent interactions between a number of signaling proteins ***(5–7)***.

The assay can be broadly divided into two major steps: (1) preparation of the nitrocellulose filters containing potential binding proteins and (2) generation of differentially phosphorylated fusion proteins that will be used to probe the filters (*see* **Fig. 1**). The probe is produced as a glutathione-*S*-transferase (GST) fusion protein, and then modified in a two-step reaction that generates differentially phosphorylated protein probes that are radiolabeled to a high sto-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

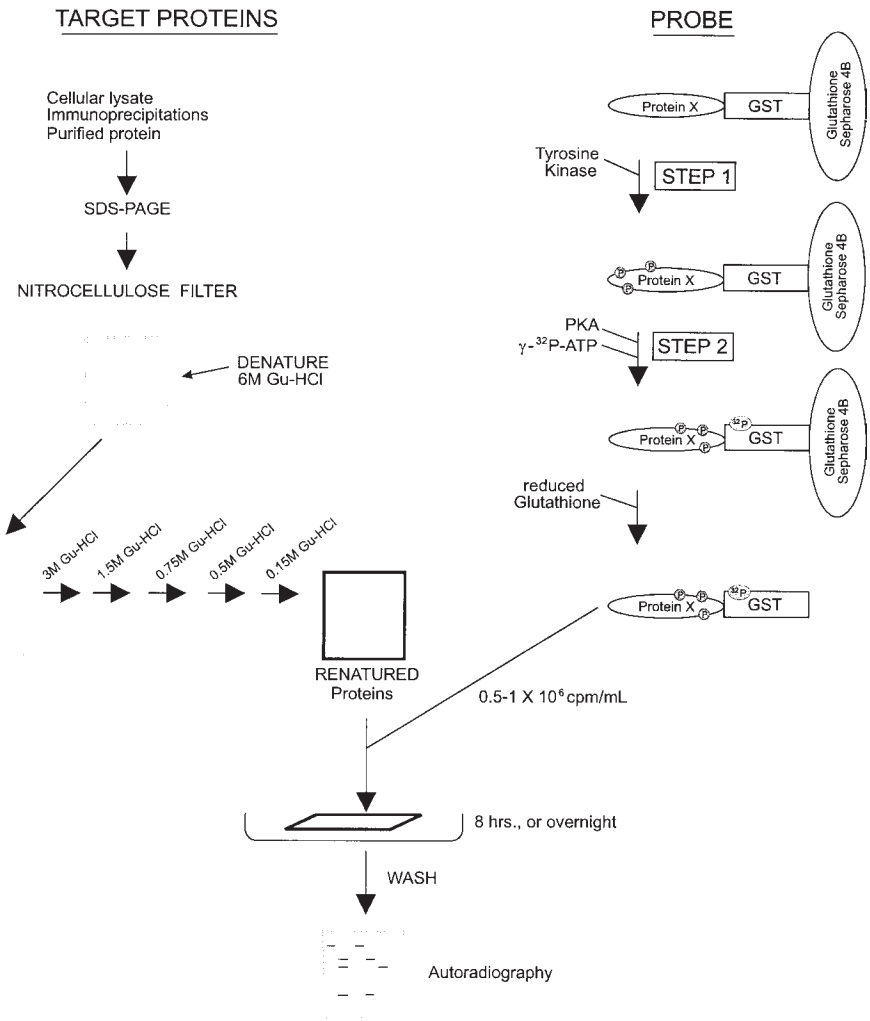

Fig. 1. Generation of filters and preparation of the probe for detecting phosphorylation-dependent protein interactions. *Left:* Denaturation and renaturation of nitrocellulose filters containing target proteins. *Right:* Two-step process for generating phosphorylated probes. Step 1: Phosphorylation reaction using kinase of interest. Step 2: Radiolabeling phosphorylated probes with PKA.

ichiometry. The first step is a "cold" reaction, in which the fusion proteins may be phosphorylated by a single kinase, differentially phosphorylated using distinct kinases, or left unphosphorylated. This is followed by a second step, the "hot" reaction, in which all fusion proteins, regardless of their phosphorylation

state, are efficiently radiolabeled at a site engineered into the expression vector that is outside the protein sequences of interest. This is achieved in a simple, one-step kinase reaction using commercially available protein kinase A (PKA). In this way, potentially relevant sites in the protein probe can be phosphorylated independently of radiolabeling the probe for detection purposes. The advantage of this technique is that the probe is radiolabeled to a high stoicheometry at the PKA phosphorylation site, thus conferring a greater degree of sensitivity and specificity to the assay. This is in contrast to other methods such as biotinylation of the probe followed by Western blot detection ***(8)***, or detection of the probe by anti-GST antibodies ***(9)***, which tend to be less sensitive.

The technique for generating differentially phosphorylated probes was developed initially to detect protein–protein interactions that were dependent on phosphorylation by tyrosine kinases, but the protocol can be adapted to accommodate the use of any kinase. The choice of kinase should be made bearing in mind a few general considerations. First, the protein used as a probe should be a good in vitro substrate of the kinase. Second, it is important to determine that the residues that are phosphorylated by the kinase in vitro correspond to sites that are phosphorylated in vivo. Differences between the array of sites that are phosphorylated in vivo and in vitro may affect the ability to detect certain protein–protein interactions, or allow the detection of interactions that do not occur physiologically. The protocol described here to generate tyrosine phosphorylated protein probes was developed using the cellular counterpart of the Rous sarcoma transforming gene product $pp60^{v\text{-}src}$. Any steps in the assay that may be different if using a different kinase are noted.

2. Materials

2.1. Reagents

1. Expression vector pGEX-2TK (available from Pharmacia Biotech).
2. Restriction enzymes *Bam*H1, *Sma* I, *Eco*R1 (commercially available).
3. Bacterial strain for the expression of recombinant protein, such as *Escherichia coli* AG1 cells (Stratagene).
4. Isopropyl-β-D-thiogalactoside (IPTG, Boehringer Mannheim): Add distilled water to yeild a 100-m*M* stock solution. Store at –20°C.
5. Glutathione Sepharose 4B (Pharmacia Biotech).
6. Glutathione (reduced form, Sigma).
7. Catalytic subunit of protein kinase A (PKA, Sigma).
8. γ^{32}P-ATP, 7000 Ci/mmol (ICN).
9. Dithiothreitol (DTT, Sigma): Add distilled water to yeild a 1-*M* stock solution. Store at –20°C.
10. Protease inhibitors:

a. Aprotinin: supplied as a solution in 0.9% NaCl and 0.9% benzyl alcohol (Sigma). Store at 4°C.
b. Phenylmethylsulfonyl fluoride (PMSF, Sigma): add 95% ethanol to yield 200 m*M* stock solution (200×). Store at 4°C.
c. Leupeptin (Boehringer Mannheim): add distilled water to yield 10 mg/mL stock solution (200×). Store at –20°C.

11. Ampicillin (sodium salt, Sigma).
12. 2-Mercaptoethanol (electrophoresis grade, Sigma).
13. Nitrocellulose (Protran, Schleicher, and Schuell; pore size 0.2 μm).

2.2. Buffers

1. Phosphate-buffered saline (PBS): 10 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 0.14 *M* NaCl, 3 m*M* KCl, pH 7.2.
2. LB Medium: per liter of distilled water, add: 10 g bacto-tryptone (Difco Laboratories), 5 g bacto-yeast extract (Difco Laboratories), and 5 g NaCl. Adjust pH to 7.0, sterilize by autoclaving and store at room temperature.
3. L-Amp medium: LB medium plus ampicillin (100 μg/mL). Add ampicillin just prior to use. L-Amp medium can be stored at 4°C for 1–2 wk.
4. NETN: (NP40, EDTA, Tris, Nacl buffer) 200 m*M* Tris, pH 7.5, 100 m*M* NaCl, 1 m*M* ethylenediaminetetracetic acid (EDTA), 0.5% NP40. Store at 4°C.
5. Kinase buffer: 20 m*M* PIPES, pH 7.2, 100 μ*M* ATP, 0.4 m*M* vanadate. Mix well, then add $MnCl_2$ to a final concentration of 10 m*M* (*see* **Note 1**). Prepare 10 mL of kinase buffer on the day of use, and store on ice.
6. HMK buffer: 20 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, and 12 m*M* $MgCl_2$. Can be prepared in advance and stored at room temperature.
7. STOP buffer: 10 m*M* sodium pyrophosphate, 10 m*M* sodium phosphate, pH 8.0, 10 m*M* EDTA. Can be prepared in advance and stored at room temperature.
8. Hyb-75 buffer: 20 m*M* HEPES, pH 7.7, 75 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), and 0.05% NP40. Store at room temperature or 4°C. We have found it convenient to prepare a 10× stock of Hyb75 and store it until use at 4°C. Omit DTT from the 10× stock, and add to 1 m*M* on dilution of the stock to 1× (*see* **Note 2**).
9. Denaturing buffer: 6 *M* Guanidine-HCl (GuHCL) in Hyb75 (*see* **Note 3**).
10. 5% Blocking buffer: 5% milk powder in Hyb75.
11. 1% Blocking buffer: 1% milk powder in Hyb75.
12. Reduced Glutahione solution: 50 m*M* reduced glutathione, 100 m*M* Tris, pH 8.0, 120 m*M* NaCl. Store for up to 1 wk at 4°C.
13. 1× Sodium dodecyl sulfate (SDS) Sample buffer: 50 m*M* Tris-HCl, pH 6.8, 1% SDS (electrophoresis grade), 5% sucrose, 0.5% 2-mercaptoethanol. Add approx 0.01 mg/mL bromophenol blue, store at room temperature.
14. SDS electrophoresis buffer: 25 m*M* Tris, 0.2 *M* Glycine, 0.1% SDS; store at room temperature.
15. Transfer buffer: 25 m*M* Tris, 0.2 *M* glycine, 20% methanol; prepare up to one day in advance and store at 4°C.

2.3. Equipment

1. Sonicator equipped with a microprobe (Model W–375, Heat Systems Ultrasonics, Inc.).
2. Vertical slab electrophoresis unit (Hoefer SE 400 Series, Pharmacia Biotech).
3. Immunoblot tank transfer apparatus (Trans-Blot Cell, Bio-Rad Laboratories).

3. Methods

Cloning a gene, or portion of a gene of interest, into pGEX-2TK using standard techniques is briefly described, followed by a short description of the methods used to express and purify the fusion protein. For more detailed information on bacterial expression and purification, *see* Chapter 10 of this volume. The far-Western assay itself, consisting of preparation of the nitrocellulose filters containing putative target proteins, generation of differentially phosphorylated probes, and finally probing the filters, is then described. A representative far Western assay using phosphorylated and unphosphorylated probes is presented in **Fig. 2**.

3.1. Cloning Foreign Genes into pGEX-2TK

Use of the expression vector pGEX-2TK allows one to synthesize a fusion protein containing a phosphorylation site for radiolabeling that is independent of any phosphorylation sites in the protein itself. When a gene of interest is cloned into pGEX-2TK, the resulting construct encodes a protein containing a PKA phosphorylation site located between the GST moiety and the coding sequence of the gene of interest (*see* **Fig. 3**, **ref. *10***).

1. Prepare the cDNA fragment of interest such that it contains restriction enzyme sites that are compatible with those in the multiple cloning site of pGEX2TK. This can be achieved either by using sites within the gene that are compatible, or by treating incompatible ends with nucleases or polymerases to generate blunt ends that can be cloned into the *Sma*I site of the vector. Alternately, the cDNA fragment of interest can be synthesized by polymerase chain reaction (PCR) using primers that contain the appropriate restriction sites.
2. Digest vector with appropriate enzymes, followed by phosphatase treatment with calf intestinal alkaline phosphatase (CIAP) to dephosphorylate the 5' ends.
3. Ligate the DNA fragment to the vector using T4 DNA ligase 4 h at room temperature or overnight at 15°C. Transform into an appropriate strain of competent *E. coli* and select recombinants on ampicillin plates. Pick single colonies and isolate plasmid DNA to analyze inserts.
4. Clones containing the gene of interest in the correct orientation should be cultured from a single ampicillin-resistant colony in L-Amp medium, and frozen as a 50% glycerol stock at –70°C for long term storage.

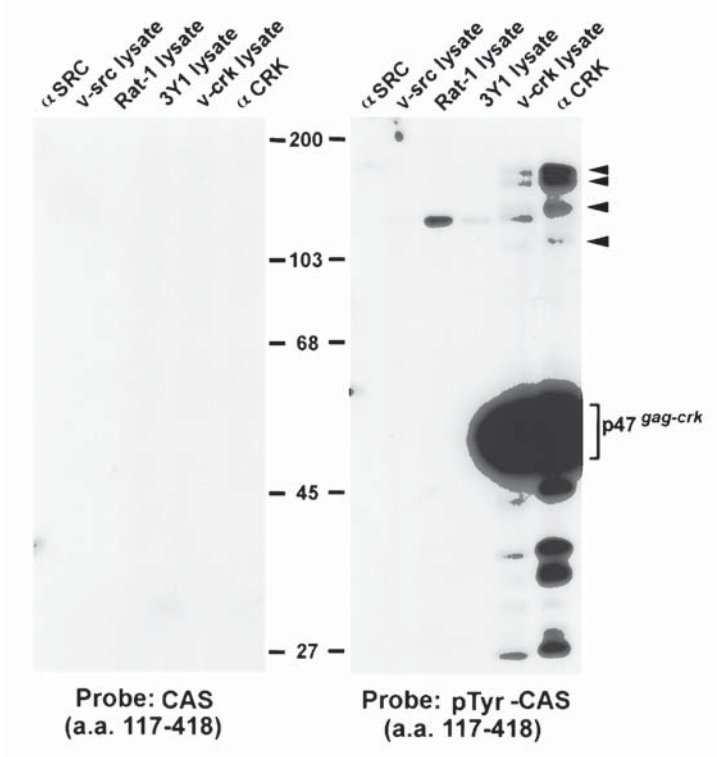

Fig. 2. Far-Western assay detecting the phosphorylation-dependent interaction of *CAS* with cellular proteins. *Right:* The substrate binding domain (amino acids 117–418) of *CAS (7,11)* was phosphorylated in vitro on tyrosine residues using *SRC* kinase, then radiolabeled with PKA, and used to probe filters containing proteins from whole cell lysates and immunoprecipitations. *Left:* The substrate binding domain of *CAS* was left unphosphorylated, radiolabeled with PKA, and used to probe filters containing cellular and immunoprecipitated proteins.

3.2. Preparation of GST-Fusion Protein

Standard procedures are used for the expression and purification of GST-fusion proteins. Because this topic is covered elsewhere in this volume, this section consists of a brief outline of the procedure used to isolate GST fusion proteins. Any steps that deviate from standard procedures will be noted.

1. Inoculate 2-mL of L-Amp medium with a single ampicillin-resistant colony of the clone of interest. Incubate overnight at 37°C with shaking. Inoculate a fresh 5-mL culture of L-Amp medium with 200 μL of this overnight culture, and incubate at 37°C with shaking for 2 h. Add 5 μL 100 m*M* IPTG and incubate for an additional 2 h.

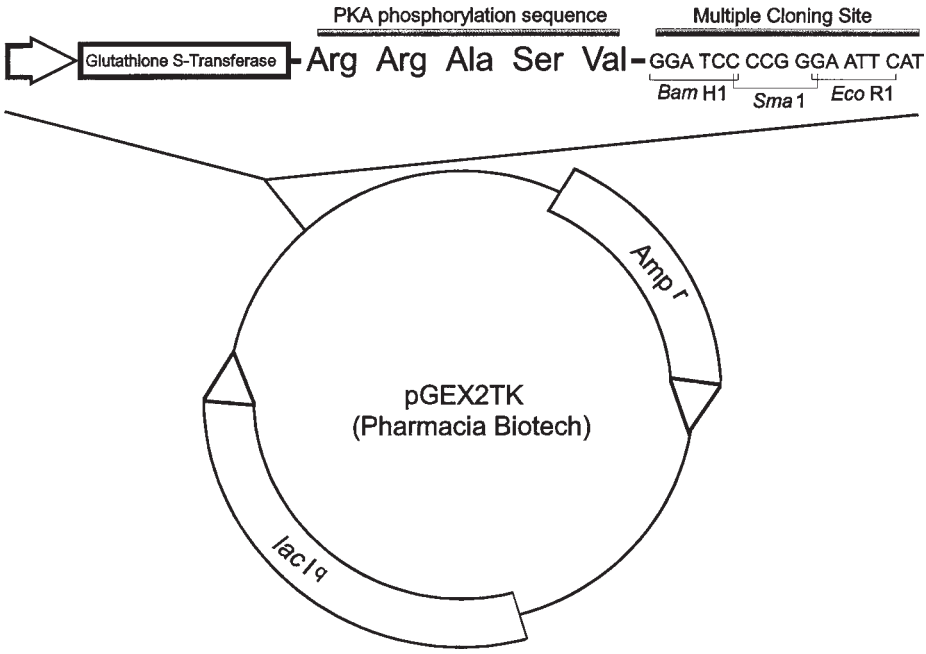

Fig. 3. Bacterial expression vector (pGEX2TK) for the expression of GST fusion proteins in which a peptide recognition sequence for PKA is inserted between GST and the protein encoded by the insert DNA. Adapted from Pharmacia Biotech product information.

2. Pellet the bacteria by centrifugation at 7000*g* in a table top centrifuge for 5–10 min at 4°C.
3. Resuspend the pellet in 1 mL of ice-cold NETN containing protease inhibitors: 50 μg/mL leupeptin, 0.5% aprotinin, 1 m*M* PMSF (final concentrations) (*see* **Note 4**).
4. Sonicate for 20–30 s on ice using a continuous pulsemat 30% output, avoiding the production of foam.
5. Centrifuge at 14,000*g* in a microcentrifuge for 10 min at 4°C to pellet bacterial cell debris. Transfer the supernatant to a fresh microcentrifuge tube. Store the supernatant on ice while preparing the glutathione Sepharose.
6. Glutathione Sepharose 4B is supplied by the manufacturer as a slurry and stored at 4°C. Transfer 300–400 μL of the glutathione bead slurry to a microcentrifuge tube. Wash the beads in ice-cold PBS three times and resuspend to the original slurry volume in PBS.
7. Add 300–400 μL of the washed glutathione bead slurry to the bacterial supernatant from **step 5**. Incubate end-over-end at 4°C for 15 min.
8. Pellet the beads by centrifugation at 14,000*g* for 2 min at 4°C. Aspirate the supernatant and wash the beads three time with ice-cold PBS.

9. Resuspend the beads to the original slurry volume in PBS plus protease inhibitors (*see* **step 3** above). GST fusion proteins can generally be stored at 4°C for up to 2 wk without significant degradation, but storage time will vary depending on the stability of the protein.
10. Quantitate protein by analyzing an aliquot of the Sepharose slurry on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with known protein standards, followed by staining with Coomassie blue.

3.3 Generation of Filters

Prior to incubation with a phosphorylated protein probe, nitrocellulose filters are generated that contain putative target binding proteins (*see* **Fig. 1**, and **Note 5**). The filters are then taken through a series of incubations to allow target-binding proteins to renature following SDS-PAGE and Western transfer ***(7,10)***. This promotes faithful protein–protein interactions and increases the efficiency of detection, as target proteins are allowed to refold to their native conformations.

1. The day before the assay, prepare samples to be analyzed in SDS Sample buffer and separate proteins by one-dimensional (1D) denaturing gel electrophoresis (*see* **Note 6**).
2. Blot proteins onto nitrocellulose overnight (*see* **Note 7**).
3. Immediately following transfer, place the filter directly in 100 mL of denaturing buffer for 5 min at 4°C with constant rocking. All of the subsequent washes are done at 4°C with constant rocking.
4. Remove and save the 100-mL denaturing buffer. Add another 100 mL of denaturing buffer to the filter for 5 min. Again, save the final 100 mL and add it to the first 100 mL.
5. Dilute the 200 mL of denaturing buffer from **steps 3** and **4** with 200 mL Hyb-75. This generates 400 mL of Hyb-75 + 3 *M* GuHCl. Wash filters twice in 200 mL each of 3 *M* GuHCl for 5 min. Save the final 200 mL of wash buffer.
6. Dilute 3 *M* GuHCl wash buffer to 1.5 *M* GuHCl with 200 mL Hyb75. Wash filters twice in 200 mL of 1.5 *M* GuHCl for 10 min per wash. Save the final 200 mL of wash buffer.
7. Dilute 1.5 *M* GuHCl wash buffer to 0.75 *M* with 200 mL Hyb75. Wash filters twice with 200 mL of 0.75 *M* GuHCl for 15 min per wash. Save the final 200 mL of wash buffer.
8. Dilute 0.75 *M* GuHCl to 0.5 *M* GuHCl with Hyb75. Wash filters twice in 200 mL of 0.5 *M* GuHCl for 15 min per wash. Save the final 200 mL of wash buffer.
9. Dilute 0.5 *M* GuHCl to 0.1 *M* GuHCl with Hyb75. Wash filter twice in 200 mL of 0.1 *M* GuHCl for 20 min per wash.
10. Place the filter in a clean dish, and incubate in 200 mL of Hyb-75 for 30 min at 4°C.
11. Block filter for 30–60 min at 4°C in 5% blocking buffer.
12. Block filter for 60 min at 4°C in 1% blocking buffer.

13. Add probe (*see* **Subheading 3.4.**) to 10 mL 1% blocking buffer to a final concentration of 0.5–1 x 10^6 cpm/mL. Incubate filter with probe for 8 h, or overnight, at 4°C.
14. Wash the filter three times in 100 mL 1% blocking buffer for 15 min per wash at room temperature.
15. Expose the filter to X-ray film at –70°C.

3.4. Preparation of the Probe

Preparation of the probe should be performed on the same day as denaturation and subsequent renaturation of the filter. It is best to begin preparation of the probe during the step in which the filter is in 5% blocking buffer (*see* **Subheading 3.3.**, **step 11**).

1. Transfer 1–3 µg of fusion protein (*see* **Subheading 3.2.**) to a microcentrifuge tube (*see* **Note 8**).
2. Wash once by adding 500 µL of kinase buffer, pelleting the beads at 14,000*g*, 4°C, and aspirating the supernatant.
3. Add fresh kinase buffer containing active kinase directly to the beads in a total volume of 50 µL. At this step, the amount of kinase that is added will vary depending on the activity of each kinase being used (*see* **Notes 9** and **10**).
4. Incubate at room temperature for 15 min, occasionally tapping the tube to resuspend the beads.
5. Pellet the beads by centrifugation for 1 min in a microcentrifuge at 14,000*g*, 4°C. Remove the supernatant and add 1 mL of STOP buffer to stop the reaction. Vortex.
6. Pellet the beads by centrifugation in a microcentrifuge for 1 min at 14,000*g*, 4°C. Remove the supernatant, and wash three times in 500 µL ice-cold PBS, followed by one wash in 500 µL ice-cold HMK buffer.
7. Aspirate the HMK buffer and store the fusion protein on ice while preparing the PKA (*see* **Note 11**).
8. PKA is supplied as a lyophilized powder and stored at –20°C (*see* **Note 12**). Remove one vial (250 U) from the freezer approx 10 min prior to use, and let it stand at room temperature.
9. During this 10 min, prepare HMK/DTT by adding 1 µL 1 *M* DTT to 1 mL of HMK buffer. Prepare γ^{32}P-ATP by diluting manufacturers stock to a final concentration of 10 µCi/µL in water.
10. Resuspend PKA in 200 µL HMK/DTT for a final concentration of 1.25 U/µL.
11. To the fusion protein from **step 7**, add 10 µL (100 µCi) γ^{32}P-ATP plus 40 µL (50 U) PKA (*see* **Note 13**).
12. Incubate the reaction on ice for 45 min with occasional tapping.
13. Pellet the beads by centrifugation in a table top microcentrifuge for 1 min at 14,000*g*, 4°C. Remove the supernatant, and wash once in 1.0 mL of STOP buffer, followed by five washes in 500 µL ice-cold PBS.

14. Elute the fusion protein from the agarose beads by adding 200 μL reduced glutathione solution and incubating at room temperature for 1–5 min with occasional tapping (*see* **Note 14**).
15. Pellet the beads by centrifugation in a microcentrifuge for 1 min at 14,000*g*, 4°C. Remove the supernatant to a fresh microfuge tube and SAVE; the supernatant contains the radiolabeled fusion protein. Repeat steps 14 and 15, combining the second 200 μL eluate with the first for a total of 400 μL.
16. Calculate cpm/μL of the probe by Cerenkov counting (*see* **Note 15**).
17. Add probe to nitrocellulose filter in 10 mL 1% blocking buffer, 0.5–1 x 10^6 cpm/mL (*see* **Note 16**).
18. Follow **steps 13–15** in **Subheading 3.3.** to incubate probe with filter, wash filters, and expose to X-ray film.

4. Notes

1. The order in which the reagents are added is critical; always add $MnCl_2$ last. Important: The composition of this buffer may be slightly different depending on the enzymatic requirements of the particular kinase being used. This may be determined by performing a titration experiment as outlined in **Note 9**, keeping the amount of kinase constant, and altering and/or changing the components of the buffer to determine the optimum reaction conditions.
2. The pH and the percentage of NP40 in Hyb-75 are critical, as both significantly affect the binding efficiency of the probe.
3. 500 mL of denaturing buffer may be made as follows: 50 mL 10× Hyb-75 plus 200 mL of water in a 1-L beaker. Add Guanidine-HCl to 6 *M* (286.5 g) with constant stirring. Bring volume up to 500 mL with water and add DTT to 1 m*M*. Store at 4°C until use.
4. Protease inhibitors should be added just prior to use. Buffer plus protease inhibitors must be stored on ice.
5. Sources of potential target proteins include whole cell lysates, subcellular fractions, immune complexes, or purified protein. Alternatively, proteins can be isolated from cells in different states of activation, such as cells that have become transformed, or cells that have been stimulated with growth factors. Essentially, any sample that can be analyzed by SDS-PAGE is amenable to this technique.
6. In general, a final acrylamide concentration of 8% in the separation gel is appropriate for separating proteins in the molecular weight range of 40–200 kD. Electrophoresis is performed in a vertical slab electrophoresis unit in SDS electrophoresis buffer, at constant current (25–30 mA for a slab gel 1.0 mm thick).
7. We recommend carrying out electrophoresis the day before the assay, and blotting overnight, because preparation of filters and generation of the phosphorylated probe is in itself a full day experiment. For blotting, we utilize a tank transfer system and assemble the immunoblot sandwich as specified by the manufacturer. Briefly, assemble the sandwich on a plastic support in the

following order: one fiber pad or sponge, Whatman filter paper, gel, nitrocellulose, Whatman filter paper, and a second fiber pad or sponge. Immnoblot overnight at 4°C, at a constant current of 0.15 A. Blotting can also be performed at 0.55 A for 2–3 h at 4°C.

8. Depending on the concentration of fusion protein in the Sepharose slurry (*see* **Subheading 3.2.**, **step 10**), the volume of Sepharose beads needed for 1–3 μg of protein may be too small to visualize and manipulate in the microcentrifuge tube. If this is the case, add 25–50 μL of unconjugated glutathione beads that have been washed three times in PBS to the microcentrifuge tube before proceeding. This will facilitate subsequent manipulations of the fusion protein.
9. The kinase used in this step must be titrated to determine the amount needed to achieve the highest stoichiometry of phosphorylation. This can be done by carrying out a kinase assay as outlined in **Subheading 3.4.**, **steps 1–6**, with certain modifications. To parallel samples containing a constant amount of fusion protein (1–3 μg), add increasing amounts of kinase and 100 μCi γ^{32}P-ATP. Adjust the amount of kinase buffer in the reaction to maintain a final reaction volume of 50 μL. Incubate the reaction for 15 min, stop the reaction by adding 1 mL STOP buffer, and wash the Sepharose beads three times with PBS. Analyze the products on SDS-PAGE, followed by autoradiography to determine the amount of kinase that catalyzed maximal incorporation of γ^{32}P-ATP.
10. The kinase should not be taken out of the freezer until just before use in order to maintain maximum activity.
11. At this point, the protein should not be stored on ice more than 30–60 min.
12. PKA is supplied in vials of 250 or 2500 U/vial. It is most convenient for this assay to order individual vials of 250 U/vial, as opposed to a large single quantity from which one measures an appropriate amount.
13. The order in which the components are added is important.
14. The reduced glutathione solution should be made up fresh, but can be reused for up to 1 wk if stored at 4°C.
15. The efficiency of Cerenkov counting is 50–60%, depending on the type of vial used. We typically obtain cpm of 2 μL radiolabeled protein and divide by 2 to calculate cpm/μL.
16. Any unused probe can be stored at –20°C for up to 1 wk.

Acknowledgments

This work has been supported by the National Science Foundation (MCB-9210188 and 9723820), the Jeffress Memorial Trust (J-261 and J-421), the Charlotte Geyer Foundation, and the American Cancer Society (IRG-149L).

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16

Purification of Tyrosine-Phosphorylated Proteins by Immunoaffinity Chromatography and Direct Cloning of Their cDNAs from Bacterial Expression Libraries

Pier Paolo Di Fiore and Francesca Fazioli

1. Introduction

Growth factor receptors endowed with intrinsic tyrosine-kinase activity (receptor tyrosine kinases, RTKs) are capable of intracellular signal transduction through their ability to autophosphorylate and to phosphorylate cellular proteins, globally referred to as substrates (**refs. *1–3*** and references therein).

In general, two types of signals, that are not mutually exclusive, appear to emanate from RTKs, as the consequence of activation of their intrinsic enzymatic activity. First, as the receptors undergo autophosphorylation they become able to stably bind src homology (SH)2-containing (or PTB-containing) molecules, through a specific SH2:phosphotyrosine (pTyr) interaction (**refs. *4*** and ***5***, and references therein). In turn, this leads to propagation of signals by directing the subcellular localization of the domain-containing proteins or of their targets, or by recruiting these proteins as substrates for the receptor itself ***(4,5)***. Second, RTKs can phosphorylate intracellular substrates that do not contain SH2 domains, thus affecting their activity or interaction with other proteins (**refs. *1–3*,*6***, and references therein).

Molecular genetic studies have demonstrated that the tyrosine-kinase activity of RTKs is indispensable for biological action ***(1–3)***. High-resolution two-dimensional analysis of pTyr-containing proteins, in cells treated with RTK-activating growth factors, has revealed hundreds of proteins, many of which still await characterization (e.g., *see* **ref. *7***). Thus, the acquisition of the complete repertoire of intracellular transducers recruited by RTKs remains one of the paramount issues in the elucidation of early events in RTK-mediated signaling.

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

In recent years, the availability of high-quality anti-pTyr antibodies has enormously aided analytical studies of pTyr-containing proteins. However, the classical approach to the characterization of unknown pTyr-containing proteins, entailing protein purification followed by microsequencing and cloning with degenerate oligonucleotides, remained a laborious and time-consuming task. Therefore, several laboratories have directed their efforts at the development of alternative methods, that would allow direct cloning of molecules that can propagate RTK-mediated signals (e.g., *see* **refs. *8–15***).

Whereas a comprehensive review of these methodologies would exceed the scope of the present chapter, they can be grouped into two broad categories that target different types of intracellular transducers. On one hand, methods like CORT (cloning of receptor targets) were developed, which allowed for cloning of intracellular transducers based on their ability to interact with tyrosine-phosphorylated intracellular domains of RTKs (***12,14***; *see* Chapter 17). These interactors are not necessarily substrates for the kinase activity of RTKs, as shown in the case of GRB-2 and that of the p85 subunit of the phosphatidylinositol 3-kinase (PtdIns-3K). Alternate approaches have been developed that are based directly on the pTyr content of RTK substrates, entailing affinity chromatography onto immobilized anti-pTyr antibodies ***(8–11,13,15)***.

Our laboratory developed such a method. In brief, the approach relies on stimulation of cells with an appropriate growth factor, followed by batch purification of an entire set of substrates by immunoaffinity chromatography using immobilized antiphosphotyrosine antibodies ***(13)***. No attempt is subsequently made to isolate individual proteins. Instead, polyclonal sera are generated against the entire pool of purified proteins, and used for screening of cDNA expression libraries ***(16)***. A general scheme of this approach is depicted in **Fig. 1** (*see* **Note 1**). We have applied this methodology to the analysis of events triggered in vivo by activation of the epidermal growth factor receptor (EGFR) and isolated several cDNAs encoding proteins that are phosphorylated on tyrosine following activation of the EGFR and other receptor-tyrosine kinases (***16–18***; *see* **Note 2**).

The remainder of this chapter provides a detailed description of the experimental protocol implemented in cloning substrates of the EGFR, including the following (*see* **Fig. 1**).

1. Optimization of substrate phosphorylation by the receptor in vivo.
2. Affinity purification onto immobilized anti-pTyr antibodies.
3. Analysis of the purified pTyr-containing protein preparation.
4. Generation of polyclonal sera directed against the purified pTyr-containing protein preparation.
5. Testing the polyclonal sera.
6. Screening of bacterial expression libraries with the polyclonal sera.

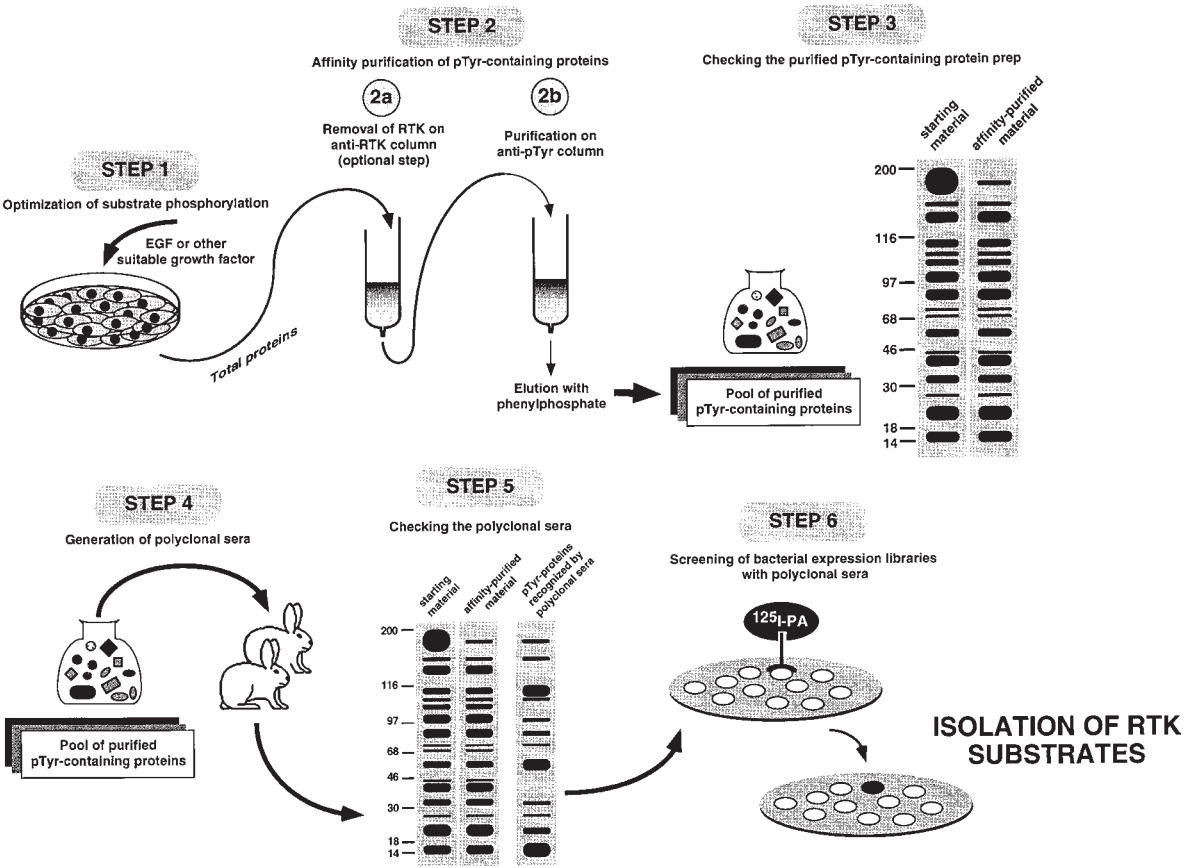

Fig. 1. Purification of RTK substrates and direct cloning of their cDNAs. A schematic diagram of the method employed for the purification of putative EGFR substrates and for direct cloning of their cDNAs is depicted. More details can be found in **refs. *11*** and ***16–18***. The subdivision into **steps 1–6** parallels the stepwise description of the method in this chapter.

A general knowledge of basic procedures in protein analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and immunoblotting) and cell-culture manipulations is assumed.

2. Materials

1. High-quality anti-pTyr monoclonal antibodies can be purchased from several commercial sources. We have extensive experience with anti-pTyr monoclonals (MAb) from Upstate Biotechnology (UBI, anti-pTyr hybridoma 4G10), Oncogene Science (P-Tyr Ab-1-Clone 1G2), and ICN (PY 20 and PY 69 monoclonals). All of these MAbs worked reproducibly well in our hands (*see* **Note 3**). Immobilized (agarose conjugated) anti-pTyr MAbs are also available from the same commercial sources (*see* **Note 4**).
2. Tissue-culture materials and reagents: Disposable sterile plasticware (plates and pipets) were purchased from Falcon. In our experiments, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with 10% calf serum (Colorado), antibiotics and L-Glutamine. Antibiotics (penicillin and streptomycin) can be purchased as a 100 × stock solution (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) from HyClone. L-glutamine can be purchased also from HyClone as a 100× stock solution (200 m*M*). Indication of commercial sources reflects our own experience. The described culturing conditions are optimal for NIH-3T3 or other murine fibroblasts. If different cell lines are used, culture conditions should be changed accordingly.
3. Human serum fibronectin (Upstate Biotechnology): Fibronectin can be diluted in sterile PBS at a concentration of 40 μg/mL (stock solution), and used at ~1 $\mu g/cm^2$ of surface.
4. EGF (receptor grade, Upstate Biotechnology): EGF can be made as a stock solution in distilled water (or phosphate-buffered saline [PBS]) at a concentration of 100 μg/mL. The stock should be kept at –20°C in aliquots and repeated freeze/thawing should be avoided.
5. Lysis buffer: 1% Triton X-100 (*see* **Note 5**), 10 m*M* Tris-HCl, pH 7.6, 5 m*M* ethylene glycol-*bis* N,N,N',N'-tetraacetic acid (EGTA), 50 m*M* NaCl plus phosphatase inhibitors (30 m*M* sodium pyrophosphate, 50 m*M* sodium fluoride and 100 μM sodium orthovanadate, *see* **Note 6**), and protease inhibitors (2 m*M* phenylmethylsulfonylfluoride [PMSF], 50 μg/mL aprotinin, *see* **Note 7**). Lysis buffer is made fresh every time, starting from stock solutions and stored on ice, for the duration of the required manipulations (*see* **Note 8**). Inhibitors should be added to ice-cold lysis buffer immediately prior to usage.
6. Columns: Disposable glass or plastic low-pressure chromatography columns can be purchased from different commercial sources. Bio-Rad Low Pressure EconoColumns were used in our experiments.
7. Column elution buffer: Lysis buffer supplemented with 10 m*M* phenylphosphate.
8. Column regeneration buffer: 1.5 *M* NaCl, 1% Triton X-100, 10 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA.

9. Column storage buffer: 50 m*M* sodium phosphate buffer pH 7.6, 0.15 *M* NaCl supplemented with 0.2% sodium azide.
10. PBS: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.2. Adjust volume to 1 L. Dispense in convenient volumes and sterilize by autoclaving. Store at room temperature.
11. TBS buffer (for immunostaining): 20 m*M* Tris-HCl, pH 7.4, 0.9% w/v NaCl.
12. Towbin buffer (for transfer of gels onto nitrocellulose or immobilon): 100 m*M* glycine, 25 m*M* Tris base.
13. High-performance liquid chromatography (HPLC)-grade water (Fischer).
14. Ultrapure Triton X-100: Surfact-Amps X-100, 10% v/v solution (Pierce).
15. Freund's adjuvant complete and incomplete (Sigma or ICN).
16. Molecular-size markers for protein gels can be purchased from several commercial sources. The methodologies described in this chapter frequently require comparison of identical blots in which proteins are identified either with color-based stainings or by autoradiography. For this purpose it is useful to employ a mixture of prestained markers and [^{14}C]-labeled standards (14,300–200,000 range), which can be purchased from Gibco-BRL-Life Technologies.
17. Protein-determination kits can be purchased from Bio-Rad (Bio-Rad Protein Assay) or Pierce (BCA Protein Assay) and used according to the manufacturer's instructions.
18. Auro Dye Gold staining kit can be purchased from ISS-Enprotech and used according to the manufacturer's instructions.
19. Blotting membranes can be purchased from Schleicher and Schuell (Nitrocellulose, BA85, 0.45-μm pore) or from Millipore (Immobilon-P).

3. Methods

3.1. Optimization of Substrate Phosphorylation by RTKs in vivo

The selection of the best model system (cell line) and experimental conditions to utilize is critical. The final goal of the entire procedure is to purify enough pTyr-containing proteins to directly immunize rabbits. Thus, conditions have to be experimentally set to yield high stoichiometry of tyrosine phosphorylation and to allow for quantitative recovery of pTyr-containing proteins. The approach described here was developed to isolate substrates of ligand activated EGFR. The same approach should, in principle, be applicable to any other RTK. In our experience, the important variables to consider are: (1) the number of receptors expressed in the cell line; and (2) the time of exposure to the growth factor, dose of the growth factor, and temperature at which the stimulation is performed (*see* **Note 9**).

To test several conditions of treatment:

1. Coat tissue-culture dishes (*see* **Note 10**) with fibronectin (~1 μg/cm^2 of surface, or less). Fibronectin can be diluted in sterile PBS at a concentration of 40 μg/mL (*see* **Note 11**). Dispense 2–3 mL of the solution into a 100-mm plate or 5–6 mL

into a 150-mm plate. Make sure that the fibronectin solution covers the entire surface of the plate. Incubate at 37°C (in a tissue-culture incubator) for at least 30 min. Aspirate the fibronectin. Plates are ready to be used.

2. Plate cells (*see* **Note 12**) and let them grow until just confluent. Wash twice with PBS and refeed with prewarmed serum-free medium (*see* **Note 13**). Incubate in a tissue-culture incubator, for at least 6 h (*see* **Note 14**).
3. Treat with various amount of EGF, for different lengths of time, at different temperatures (*see* **Note 9**). EGF can be added directly to the tissue-culture dish at the final desired concentration.
4. After treatment, rapidly wash cells three times with ice-cold PBS. After the third wash, make sure to aspirate all PBS (*see* **Note 15**).
5. Lyse cells with ice-cold, freshly prepared lysis buffer. Minimize the volume used to ensure a high protein concentration. 4–10 μL/cm^2 is sufficient. Allow lysis buffer to cover the entire plate with gentle rocking and place onto an ice bed for 2–5 min.
6. Collect lysate with the aid of a cell scraper and transfer to a 1.4-mL microcentrifuge tube (Eppendorf). Vortex for a few seconds. Clarify lysate by high-speed centrifugation (14,000*g* for 10 min at 4°C) in a microcentrifuge.
7. Measure protein concentration by any standard colorimetric assays. We routinely use kits from Bio-Rad (Bio-Rad Protein Assay) or Pierce (BCA Protein Assay).
8. Total lysates can be stored at –80°C for long periods of time. Aliquoting is recommended because freeze-thawing accelerates degradation.
9. Analyze samples by Western blotting with anti-pTyr antibodies (*see* **Note 16**).
10. The same protocol can be implemented, with adequate scaling up for larger volumes, to prepare total proteins for the preparative affinity purification onto immobilized anti-pTyr antibodies (*see* **Note 17**).

3.2. Affinity Purification onto Immobilized Anti-PTyr Antibodies

A number of preliminary considerations apply:

1. It is important to establish how many mg of total proteins will be needed. The final goal is to immunize at least two rabbits with a nearly pure preparation of pTyr-containing proteins. A minimum of 100 μg of pTyr-proteins per rabbit is needed, although larger amounts may be desirable. pTyr-containing proteins will represent at best 0.1% of total proteins and a final recovery of 50% might be expected. Thus, in the best case, a yield of 0.05% of the starting material can be obtained. It follows that approx 400 mg of total cellular proteins should be processed to yield enough antigen (*see* **Note 18**).
2. A strategic decision has to be made about whether to deplete the protein preparation of the tyrosine phosphorylated RTK. In our experimental scheme for EGFR substrates, depletion of the EGFR was performed, because we estimated that it constituted approx 50% of the pTyr-containing proteins in the preparation (*see* **Note 19**).

A step-by-step protocol for the affinity purification onto immobilized anti-pTyr antibodies follows. The entire procedure should be performed at 4°C (*see* **Note 20**).

1. Pack a Bio-Rad Low Pressure EconoColumn with 3 mL (packed volume) of agarose-conjugated anti-pTyr MAb (*see* **Note 21**). We used columns with a diameter of 1.5 cm and a length of 10 cm with a fitted reservoir that could accommodate up to 60 mL of buffer. Such an arrangement overcomes the need for a flow adaptor (Bio-Rad, if needed) that would be indispensable if smaller columns or no reservoir are used.
2. Wash the column with 20 bed volumes (60 mL) of ice-cold lysis buffer to equilibrate.
3. Thaw the total protein preparation in a 37°C water bath, clarify it by maximum speed centrifugation for 10 min at 4°C in a microcentrifuge. Unclarified preparations will block the column.
4. Apply 100 mg of the protein sample at a concentration of approx 2 mg/mL, in a volume of 50 mL (*see* **Note 22**). Collect the flowthrough and recycle it through the column, at least three times to maximize binding (*see* **Note 23**).
5. Wash the column with 50 bed volumes (100 mL) of ice-cold lysis buffer (*see* **Note 24**). Let the column drain completely, but do not allow it to dry out.
6. Overlay 12 mL of elution buffer (*see* **Note 25**). Fit a 25 ¥ 5/8" needle at the bottom of the column to reduce flow rate and to facilitate dropwise collection. Harvest 0.5-mL fractions. pTyr-containing proteins should start eluting with the second bed volume (fraction 7). Store fractions on ice, if Western blot analysis is to be performed immediately, or at –80°C for long-term storage.
7. The anti-pTyr column can be regenerated by washing with 10 bed volumes (30 mL) of regeneration buffer, followed by washing with 10–20 bed volumes of PBS. Columns can be stored in storage buffer in an upright position at 4°C (*see* **Note 26**).
8. Measure protein concentration of the fractions, by a standard colorimetric assay. We routinely use kits from Bio-Rad (Bio-Rad Protein Assay) or Pierce (BCA Protein Assay) (*see* also **Note 27**).
9. Analyze fractions by Western blot with anti-pTyr antibodies. Load 500 ng of proteins from each fraction, if using standard 16 × 10 cm gels, or proportionally less for minigels. Load 100 μg of the starting material as a positive control and for a rough estimation of enrichment and yield (*see* **Note 28**).
10. Save and pool only those fractions showing the highest pTyr-protein content. Normally >80% of the pTyr-containing proteins should elute in fractions 7–12 (*see* **Note 29**).

3.3. Analysis of the Purified pTyr-Containing Protein Preparation

The aim of this part of the protocol is to check for the purity of the affinity purified pTyr-containing preparation. Because the preparation will to be used as immunogen, a rather high degree of purity is desirable. Checking purity is achieved by comparing phosphotyrosine content (determined by anti-pTyr Western blotting) with total protein detected by staining with Auro Gold Dye.

1. Load twice, onto the same gel, 500 ng of the purified proteins. Leave a couple of blank lanes in between to allow cutting of the membrane after transfer (*see* **Note**

30). Fill empty lanes with loading buffer (or molecular-size markers) to avoid lane distortion.
2. Transfer the gel onto Immobilon or Nitrocellulose and cut the blotted membrane, such as to recover two replica blots.
3. Stain one blot with anti-pTyr and one with Auro Gold Dye. Compare the signals in the two staining to estimate the degree of purification (*see* **Notes 31** and **32**). Densitometric scanning of the blots may aid in reaching a quantitative assessment.

3.4. Generation of Polyclonal Sera Directed Against the Purified pTyr-Containing Protein Preparation

A minimum of 75–100 μg of purified pTyr-containing proteins are needed to immunize one rabbit. It is advisable to immunize at least two rabbits, because variability in immune response is to be expected. The immunization protocol reported here has been widely used in our laboratory with excellent results. Several commercial sources can also effectively perform the immunization procedure, if desired.

Some attention should be paid to the protein concentration in the protein preparation used for injection, because concentrations of at least 30–50 μg/mL are required. If necessary, concentration of the protein samples can be obtained by any microconcentrating device (e.g., Model 1750 sample concentrator, ISCO; *see* also **Note 33**). Make sure to save at least 10 μg of the preparation (preferably more) for subsequent monitoring steps (*see* below).

1. Prebleed New Zealand rabbits. Acquisition of a good prebleed (at least 10 mL of serum) is critical for the subsequent phases of the project. It is advisable not to allow much time to elapse between the prebleeding and the first immunization (a few days are acceptable).
2. Mix 25 μg of the purified protein preparation (in a volume of 500–750 μL) with an equal amount of complete Freund's adjuvant. Inject close to the inguinal lymph nodes of the rabbit (Initial Injection, "II").
3. Obtain the first test bleed, of approx 5 mL of serum, 1 wk later.
4. Two weeks after the initial injection (**step 2**, above), inject 10 μg of purified proteins, mixed with an equal (v/v) amount of incomplete Freund's adjuvant, into two or three shaved areas along the back of the rabbit (First boost).
5. Subsequent boosts will follow (as in **step 4**) every other week. Normally 5–6 boosts are required.
6. Test bleeds are taken (as in **step 3**), 1 wk after each boost.
7. Test bleeds (at 1:500 or 1:1000 dilutions) are analyzed by immunoblotting of the original purified pTyr-protein prep (500 ng/Lane). Alternatively a dot-blot procedure can be used (*see* **Notes 28** and **34**).
8. Satisfactory titers should be observed from bleed 4/5 onward. Sacrifice the animal following the first bleed that does not show increase in titer and/or intensity of staining (*see* **Note 35**).

3.5. Testing the Polyclonal Sera

Prior to screening bacterial expression libraries with the polyclonal sera, it is useful to characterize them for their ability to recognize pTyr-containing proteins independently of pTyr content (*see* **Note 34**). Two approaches can be employed: *[^{32}P]-labeled proteins.* In this case, sera are used to immunoprecipitate [^{32}P]-labeled proteins from cell lysates, followed by phosphoamino acid analysis. This approach can be very useful for analytical purposes, but it is less suitable for the purpose of assessing the performance of the polyclonal sera in library screening. Detailed experimental methodologies can be found in Chapter 5 of this volume. *Unlabeled lysates.* Here, total cellular extracts are used to test (1) recognition of pTyr-containing proteins by the polyclonal sera; (2) dilutions of the sera which are optimal for screening of expression libraries. This approach is described below.

1. Prepare a total cell lysate of serum-starved cells (unphosphorylated state, henceforth referred to as – lysate) and from growth factor-stimulated cells (phosphorylated state, henceforth denoted as + lysate). Use treatment conditions and protocol outlined in **Subheading 3.1.** (*see* **Note 36**).
2. In a pilot experiment, establish by Western blotting the best serum dilution to be used. Generate replica blots of the – and + lysates (100 μg of protein/lane) and blot with polyclonal sera at 1:100, 1:250, 1:500, and 1:1000 dilutions. Perform parallel immunostainings with the prebleed, to control for specificity.
3. Set up an immunoprecipitation experiment, from both - and + lysates, with 3 mg of total proteins and 10–20 μL of the polyclonal serum directed against the pTyr-proteins. Set up identical immunoprecipitations (3 mg of lysates) with 10–20 μL of prebleed. Set up identical immunoprecipitations with an anti-pTyr MAb. Divide the immunoprecipitates into three identical aliquots (representative of 1 mg of total cellular proteins) and analyze by SDS-PAGE followed by immunoblotting. Immunostain the three sets with (1) anti-pTyr MAb; (2) polyclonal anti-pTyr-proteins (at a dilution established as in point 2); and (3) prebleed (at identical dilution as the immune serum).
4. Comparison of patterns among the various immunostainings will provide an indication about the specificity of the polyclonal sera for pTyr-containing proteins.

3.6. Screening of Bacterial Expression Libraries with the Polyclonal Sera

A comprehensive discussion of expression screening with antibodies exceeds the scope of this chapter. Efficient protocols can be found elsewhere in this volume (*see* Chapter 3) and in step-by-step instructions of many commercially available kits for detection. Here, we provide hints that may facilitate troubleshooting of frequently encountered problems.

1. It is useful to check the "library background" of the polyclonal sera. This is accomplished by preparing 1 plate of library phages (under the same conditions that will be used for the actual screening). Following lifting, the filter can be cut into several pieces to test various conditions of blocking and/or antibody dilutions.
2. High "library background" can be reduced substantially by preincubation of the antisera with filters saturated with bacteria/phage lysates (Clontech). Background can vary substantially with the detection system employed. In our experience color-based detection employing alkaline phosphatase-conjugated reagents (CLICK-II, Clontech) yielded superior signal:noise ratios, when compared to detection with [^{125}I]-Protein A ***(16–18)***.
3. Finally, background can also be reduced substantially by purifying the antibody fraction of the total polyclonal sera by affinity chromatography onto immobilized Protein A.
4. False "screening" positives are frequent, especially when signals are weak. Duplicate filters of the library should always be prepared and screened with the polyclonal sera. Pick only those phage that are positive on both filters.
5. If more than one rabbit has been immunized, it is useful to combine sera for the purpose of library screening.
6. After phage isolation, false positives can be sorted out by immunostaining with prebleed. It is also recommended to use unrelated immune sera (any antiserum available and directed against unrelated proteins) as an additional specificity control of detection (*see* **Note 37**). Discard phage that react with prebleed or with unrelated immune sera.
7. Expect multiple positives for the same gene. A cross-hybridization scheme can be easily set up, using the phage gridding method described in **Note 37**, in order to group identical and/or overlapping clones. We found it useful to proceed in a stepwise fashion according to the following scheme:
   a. Isolate cDNA inserts from a few phage (5 or 6). A PCR-based approach can be easily devised for this step.
   b. Hybridize the inserts to the gridded phages (*see* **Note 37**).
   c. Isolate a few more phage inserts from those phages that did not hybridize in the previous step.
   d. Hybridize to the gridded phages.
   e. Repeat the above steps if necessary.

4. Notes

1. An effective alternative to the scheme that we employed is one employed by Parsons's group. In their protocol, following the affinity purification onto anti-pTyr column, the purified preparation was used to generate monoclonal antibodies (MAb) ***(10,15)***. Pros and cons of the polyclonal sera approach (PSA) compared with the MAb approach (MAA) are as follows:
   a. PSA is faster than MAA and much less labor-intense. It does not require expertise with generation and maintenance of hybridomas.

   b. MAA facilitates characterization of individual substrates (phosphorylation state, subcellular localization, coimmunoprecipitation with other proteins) before a cDNA clone is available. With the PSA approach, one must first obtain cDNAs in order to generate specific antibodies directed against individual proteins.
   c. Sucessful cloning from bacterial expression libraries is more likely with PSA, than MAA, given the superiority of polyclonal sera to monoclonals in screening of expression libraries.
2. We have named the novel genes isolated by our approach *eps* genes (EGFR Pathway Substrate). In one round of direct cloning, we isolated three novel cDNAs, *eps*8, *eps*15, and *eps*10 (later renamed radixin), three cDNAs encoding known pTyr-containing proteins *fyn*, PLC-γ, and *shc*. We also isolated two cDNAs for proteins in which we were never able to show pTyr content or association with pTyr-containing proteins in vivo, likely representing "cloning noise" of our approach. Thus, in 6 of 8 (75%) cases, the direct cloning approach identified cDNAs encoding *bona fide* pTyr-containing proteins.
3. There are important differences among anti-pTyr antibodies. MAbs from UBI and Oncogene Science do not work well in the presence of SDS (which is a common component of many lysis buffers), whereas PY 20 and PY 69 do not show appreciable differences in binding to pTyr-containing proteins in the presence or absence of 0.1% SDS. We also have evidence that patterns of detected pTyr-containing proteins differ, to some extent, with the different MAbs, indicating different specificity ranges.
4. The amount of MAb conjugated per mL of beads varies from company to company. In general, the conjugated anti-pTyr MAbs come at a concentration of 4, 10, and 15 mg of MAb per mL of packed beads, for UBI, ICN, and Oncogene Science Mabs, respectively. Despite these differences, the amount of MAb in 2–3 mL of beads should be vastly in excess of antigen (pTyr-containing proteins), for all practical purposes connected with large-scale purifications.
5. High-purity Triton X-100 is recommended. It can be purchased from Pierce (Surfact-Amps X-100) as a 10% solution. This offers the additional advantage of ease in pipeting (undiluted Triton X-100 is viscous and difficult to aliquot).
6. Phosphatase inhibitors: Sodium pyrophosphate can be made as a 1-*M* stock in water and stored at 4°C. Sodium fluoride can be made as a 0.5-*M* stock in water and stored at 4°C. Sodium orthovanadate must be dissolved in a strong buffer and made fresh every time. We make 1 mL (or less) of a 0.5-*M* stock in 1 *M* HEPES (pH 7.4) (it may require vigorous vortexing and heating at 37°C) and use the appropriate amount to make the lysis buffer. Concentration of sodium orthovanadate might have to be increased, even substantially (10–20-fold) depending on the cell line. We have noticed, however, that concentrations above 5 m*M* will yield viscous lysates, possibly caused by nuclear membrane lysis, for unknown reasons.
7. Protease inhibitors: PMSF can be prepared as a 100-m*M* stock in ethanol and stored at 4°C for not longer than 1 mo. Be careful, PMSF is extremely destructive

to mucous membranes of the respiratory tract, eyes and skin. Aprotinin is usually solubilized in water at 5 mg/mL. Because aprotinin aggregates on repeated freeze/thawing, the stock solution should be stored in small aliquots at - 20°C.

8. SDS to a final concentration of 0.1% (from a 10% stock, filter-sterilized, and stored at RT) can be added, provided compatibility with the anti-pTyr MAb (*see* **Note 3**).
9. A detailed description of the optimization procedure is beyond the scope of this chapter and can be found in Fazioli et al. ***(13)***. In brief, we found that for our purposes, a stimulation with EGF at a concentration of 100 ng/mL for 30 min at 4°C was optimal. The cell line utilized in our experiments was a genetically engineered NIH/3T3 cell overexpressing the EGFR at approx 1.0×10^6 receptors/cell ***(19)***. Conditions for other RTKs may vary.
10. Coating of the plates with fibronectin is necessary because most monolayer cells tend to detach from the plate, under conditions of serum starvation. Conditions are optimized for mouse fibroblasts, other cell lines might not require fibronectin coating or might do fine on cheaper substrates, such as gelatin or poly-L-lysine. For analytical purposes, as in the case of testing different conditions of treatment, 35-mm plates should yield enough protein for analysis. For large-scale preps, we routinely use 150-mm plates.
11. Fibronectin is difficult to dissolve. Pipet directly into the vial (normally containing 10 mg of dried powder) 5–10 mL of sterile PBS and allow for spontaneous solubilization for 1–2 h at room temperature. Do not attempt to dissolve by pipeting at this stage, because fibronectin will stick to the pipet. After 1 h, the solution can be pipeted, brought to final volume and filter sterilized.
12. In selecting the cell line to use, some variables should be considered. First, a cell line (even if genetically engineered) with high receptor number per cell is preferable. Second, it is best to select a cell line that grows at high-saturation density, to maximize the protein yield per plate (this is especially important in the preparative phase). Third, it is important to select a cell line with low-tyrosine phosphorylation background in the absence of serum stimulation (i.e., after starvation). Many cell lines (particularly tumor cell lines) harbor activated tyrosine kinases, resulting in high background, which may interfere with experimental purposes.
13. For our fibroblast cell line, optimal condition for serum starvation were DMEM medium supplemented with transferrin (Collaborative Research, final concentration 5 μg/mL), and sodium selenite (Sigma, 10^{-8} *M* final). Transferrin can be dissolved in sterile PBS at the concentration of 5 mg/mL and stored in aliquots at –20°C. Sodium selenite can be made as a 10^{-5} *M* stock solution in water, filter-sterilized, and stored at room temperature for long periods of time.
14. Duration of starvation might be longer for different cell lines and should be experimentally determined by checking the residual level of pTyr-containing proteins, at different time-points of serum deprivation.
15. Cells can be washed rapidly by pouring PBS directly out of the bottle and emptying the plate by inversion. After the third wash, let the plate stand in a tilted

position (on an ice bed) for 1 min, to allow for all residual PBS to accumulate on one side, to facilitate aspiration.

16. An extensive description of Western blotting procedure is outside the scope of this chapter and can be found elsewhere in this volume. The following are a few tips, from our experience, for anti-pTyr blotting. Fractionate total cellular lysates (100 μg/lane) by SDS-PAGE, transfer to Nitrocellulose or Immobilon and probe with anti-pTyr (UBI anti-pTyr diluted 1:100 in TBS + 0.5% w/v BSA (TBS: 20 m*M* Tris-HCl, pH 7.4, 0.9% w/v NaCl). To better resolve proteins of a wide range of molecular weights, we suggest to perform electrophoresis using a standard (16 × 10 cm) 3–27% acrylamide gradient gel. Blocking of nonspecific binding sites on the membrane can be performed in 3% w/v nonfat dry milk in TBS for 2 h at room temperature, under agitation. A more expensive (and qualitatively superior) alternative is blocking in 5% w/v BSA (Ultrapure, ICN) in TBS, for 2 h at room temperature, under agitation. In our experience, optimal signal:noise ratios of detection are obtained using [^{125}I]-protein A (Amersham, ≥ 30 mCi/mg) at 0.2 μCi/mL in TBS + 0.5% w/v BSA.
17. It is advisable to check the quality of the large-scale preparation before processing it in the affinity chromatography step. Signals in an anti-pTyr Western blot should be comparable to those obtained in the small scale prep, which gave optimal results (always save an aliquot of the small scale preps, for further use as positive control).
18. For fibroblasts, this can be achieved by a large preparation of 50 plates (150 mm diameter), thus making the entire procedure reasonably manageable. Different cell lines might yield less protein per plate (or have lower pTyr-protein content) making the procedure more time consuming and expensive (*see* also **Note 12**).
19. Depletion of the EGFR was needed because it would have constituted the major antigen in our protein prep, with undesired effects on the subsequent generation of polyclonal sera. In other cases, depending on the number of receptors/cell in the model system utilized, this step may not be necessary. The methodology for removing the EGFR, onto immobilized anti-EGFR antibody, parallels that described for the anti-pTyr column and is explained in detail in Fazioli et al. ***(13)***. A major potential problem is that RTK-bound substrates (or other bound molecules, whose purification might be desired) might be lost during the RTK depletion. However, under our conditions ***(13)*** this loss was minimal, as evaluated by analyzing the material bound to the anti-RTK column by immunoblot with anti-pTyr MAb.
20. Some general guidelines about the handling of a column: (1) never let a column run dry; (2) apply samples and washing buffer gently, without stirring or disturbing the surface of the bed; and (3) if capping/uncapping of the column is needed, always cap bottom first and uncap top first, to prevent air bubble entry into the column.
21. It might be advisable to use a mixture of available conjugated MAbs, for example 1 mL of UBI anti-pTyr and 1 mL of Oncogene Science anti-pTyr, to cover for possible differences in specificity towards various pTyr-proteins. Make sure that

the lysis buffer used is compatible with all used antibodies, as far as SDS content is concerned.

22. A compromise is made between the amount of material to apply, and the concentration to be used. Concentration of proteins above 2–3 mg/mL can result in a slower flow rate and possible clogging of the column. On the other hand, volumes in excess of approx 50 mL will be difficult to handle on a small column (even with a fitted reservoir). As an alternative, larger columns can be used (also increasing the amount of agarose-conjugated anti-pTyr). In our experience, larger columns have lower total yield, and also often result in a lower final concentration of the purified material, imposing a consequent concentration step with likely loss of material, and are more time consuming. For these reasons, smaller columns are preferable, even if multiple runs are required to purify an entire large-scale preparation.
23. This procedure can be rather time consuming. An effective alternative for the pTyr-protein-binding phase is to perform it in batch, by simply adding the anti-pTyr beads to the cell lysate and rotating the mix for at least 3 h at 4°C. In this case, larger amounts of total cellular lysate can be processed onto 2–3 mL (packed volume) of beads. If the batch procedure is used, simply apply the mix, at the end of the incubation, to the column, in order to pack the beads. Washing and elution are always best performed on the column, rather than in batch.
24. The washing procedure may also be monitored by collecting the last mL of the wash and reading it at A280, against a blank made of lysis buffer. It should be < 0.01 OD.
25. Phenylphosphate specifically competes the pTyr binding to the antibody, thus allowing for specific recovery of pTyr-containing proteins. Elution with phenylphosphate is preferred to harsher procedures, such as 0.1 *M* glycine, pH 2.8, which would also remove nonspecifically bound proteins.
26. Reduction in binding capacity at each regeneration of the column should be expected.
27. Standard kits (Bio-Rad or Pierce) can be used. In some cases, depending on protein concentration in the eluate, a sensitive micromethod variation (5–200-ng range) is preferable (for example Protein-Gold, ISS-Enprotech). Step-by-step instructions for micromethods are provided by the manufacturer.
28. When several affinity purifications are conducted at the same time, gel analysis of many fractions may be cumbersome. Dot-blot analysis can be a practical alternative ***(13)***.
29. Particular attention must be paid to avoiding external contamination of the purified pTyr-protein preparations with other proteins. The most common and abundant source of contamination are keratins from the operator's hands. Keratin-contamination will affect subsequent interpretation of the Auro-gold stainings, and generation of polyclonal sera. In addition to the usage of disposable, sterile glassware and plasticware (preferably individually wrapped items), we recommend wearing and frequently changing gloves at all times. We also recommend use of HPLC-grade water for the preparation of all solutions.

30. A blank sample prepared and treated for loading exactly as the protein sample, but containing no protein, should be included as a negative control. This facilitates monitoring of protein contamination of the sample during the manipulation.
31. One cycle of affinity-purification should yield approx 80% pure pTyr-containing proteins (with a 500–1000-fold enrichment with respect to the original preparation). We recommend against using less-pure material for immunization. If necessary, a second cycle of affinity purification can be performed to increase enrichment.
32. An alternative to the Auro Gold Dye staining is to perform a small-scale affinity purification of pTyr-containing [^{35}S]-methionine-labeled proteins, in parallel to the large-scale purification, and checking purity of this "pilot" prep. This can be achieved on a single blot, by first exposing it to X-ray film and collecting the total protein signals, followed by Western blotting with anti-pTyr antibodies. In this latter step, if [^{125}I]-protein A is used, the [^{35}S] radiation can be shielded with several layer of aluminum foil.

    Another alternative is to mix the unpurified starting material with trace amounts of total [^{35}S]-labeled proteins (1:50, for example) and subject the entire mix to affinity purification, followed by purity checking as above. In our experience [^{35}]S-methionine-based protocols yield better resolution. However, they are time consuming and cumbersome and require radioactive handling throughout the procedure.
33. Some degree of planning in the initial phases of the affinity purification step should make this step unnecessary. In general, concentrations of approx 30–50 μg/mL should be obtained, following our protocols. If possible, we recommend against concentration of the purified protein preparation because, in our experience, this is a step in which major losses can be encountered. Dialysis of the purified protein preparation should not be necessary because adverse effects of the chemical components of the elution buffer on the immunization procedure have not been observed.
34. It is critical, in all experiments performed with the polyclonal sera raised against the pTyr-proteins, to preabsorb the sera with 10 m*M* phenyl phosphate. A good proportion of the generated antibodies (especially in the initial bleeds) will have anti-pTyr specificity. These antibodies will allow detection of pTyr-containing bands, but will be of no value in the screening of bacterial expression libraries.
35. It is not advisable to keep boosting the animal after the peak of reactivity is reached because titers will tend to decrease with time. In our experience, an animal that does not show good titers by bleed 5, will never do so.
36. A certain amount of total lysate will be needed for standardization of the polyclonal sera. It is recommended to prepare at least 10–15 mg of total lysate and to store it in aliquots of 1 mg/each, in a way to perform the entire standardization on the same test material.
37. An entire collection of phage can be quickly checked by arraying them onto a bacterial lawn with the aid of a grid. Simply plate a bacterial lawn as for phage plating, but without adding phages to it. Incubate at 37°C for a couple of hours.

Place the plate onto a numbered grid (mark the plate and the grid on corresponding spots on the edges, to allow orientation) and spot 1 μL of pure phage in each square of the grid. Reincubate to allow phage to grow and then lift the plate, making sure to reproduce the alignment marks on the filter. Several replica plates can be easily prepared to allow for multiple screening with polyclonal sera, prebleeds and unrelated immune sera.

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17

Identification of Receptor Tyrosine Kinase (RTK) Substrates by the Cloning of Receptor Targets (CORT) Strategy

Roger J. Daly

1. Introduction

Activation of receptor tyrosine kinases (RTKs) leads to autophosphorylation of the intracellular region of the receptor on specific tyrosine residues, thus creating binding sites for a variety of signaling proteins ***(1)***. These interactions are mediated by protein modules such as src homology (SH)2 and phosphotyrosine binding (PTB) domains, which target specific tyrosine phosphorylated peptide sequences ***(2)***. This chapter describes how the physical association of an RTK with particular substrates can be exploited in an expression cloning procedure and novel receptor targets identified.

The cloning of receptor targets (CORT) strategy was based on the observation that the C-terminal tail of the epidermal growth factor receptor (EGFR), which contains the five known autophosphorylation sites, could be cleaved from the intact receptor using cyanogen bromide. When phosphorylated, this fragment bound to the SH2 domains of Ras-GTPase activating protein (Ras-GAP) and phospholipase C-γ (PLC-γ) ***(3)***. Consequently, this fragment represented an ideal probe for screening cDNA expression libraries. The first CORT screens were performed on a λ*gt11* library and identified proteins were assigned the prefix Grb (for growth factor receptor bound) and then numbered consecutively. This led to the cloning of Grb1, the p85 subunit of PI3-kinase, and Grb2, an adaptor protein that couples tyrosine kinases to Ras via recruitment of the Sos GDP-GTP exchange factor ***(4–6)***. A schematic representation of the CORT strategy is presented in **Fig. 1**. Presumably, this strategy could be applied to receptors other than the EGFR. However, it should be noted that

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

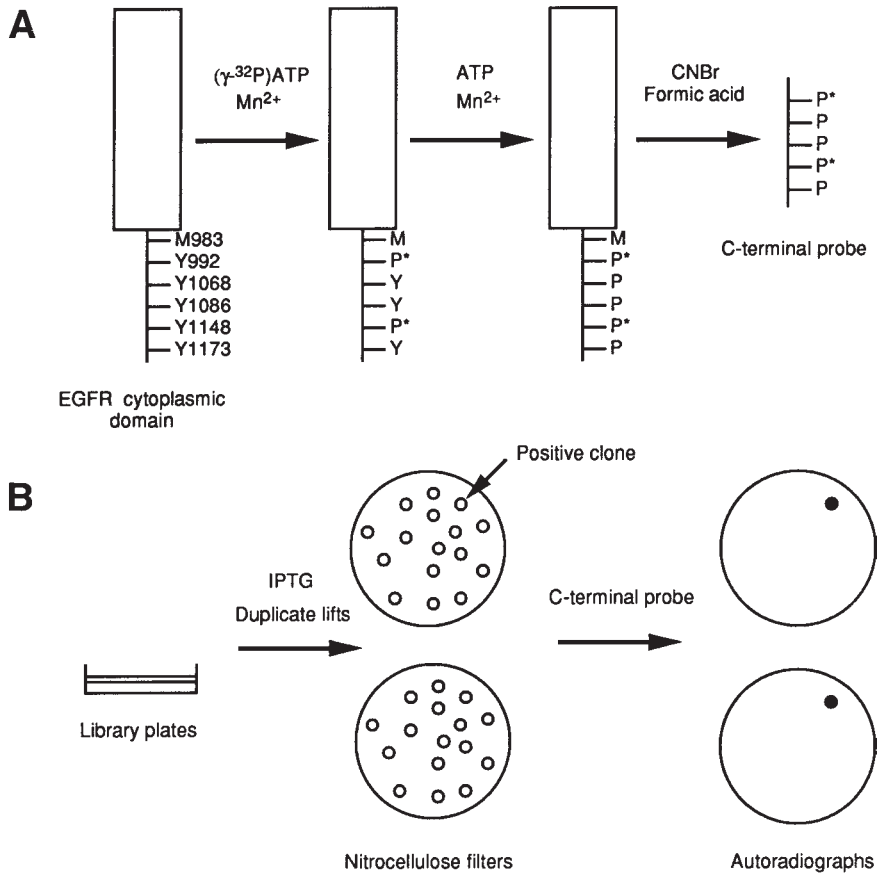

Fig. 1. Identification of RTK substrates by the CORT strategy. **(A)** Preparation of a radiolabeled probe corresponding to the C-terminus of the EGFR. The intracellular region of the EGFR is represented schematically with the site of cyanogen bromide cleavage (methionine 983) and the five major tyrosine autophosphorylation sites indicated. Autophosphorylation in the presence of (γ-^{32}P) ATP results in the incorporation of radiolabeled phosphate (P*). Incubation with cold ATP ensures complete phosphorylation of the probe. Cleavage with CNBr generates a radiolabeled 203 amino acid fragment containing the five phosphorylated tyrosine residues. **(B)** Plating and screening of the λEXlox cDNA library. Following plating, duplicate lifts are performed using IPTG-impregnated nitrocellulose filters which are then incubated with the C-terminal probe. Duplicate positive signals on the autoradiographs are represented by shaded circles (*see* also **Fig. 2**).

screening with the C-terminal probe generated by cyanogen bromide cleavage gave a lower background than that performed using the intact intracellular domain. If suitable chemical or enzymatic cleavage sites are not available and cannot be engineered in a particular receptor, then phosphorylation of a recom-

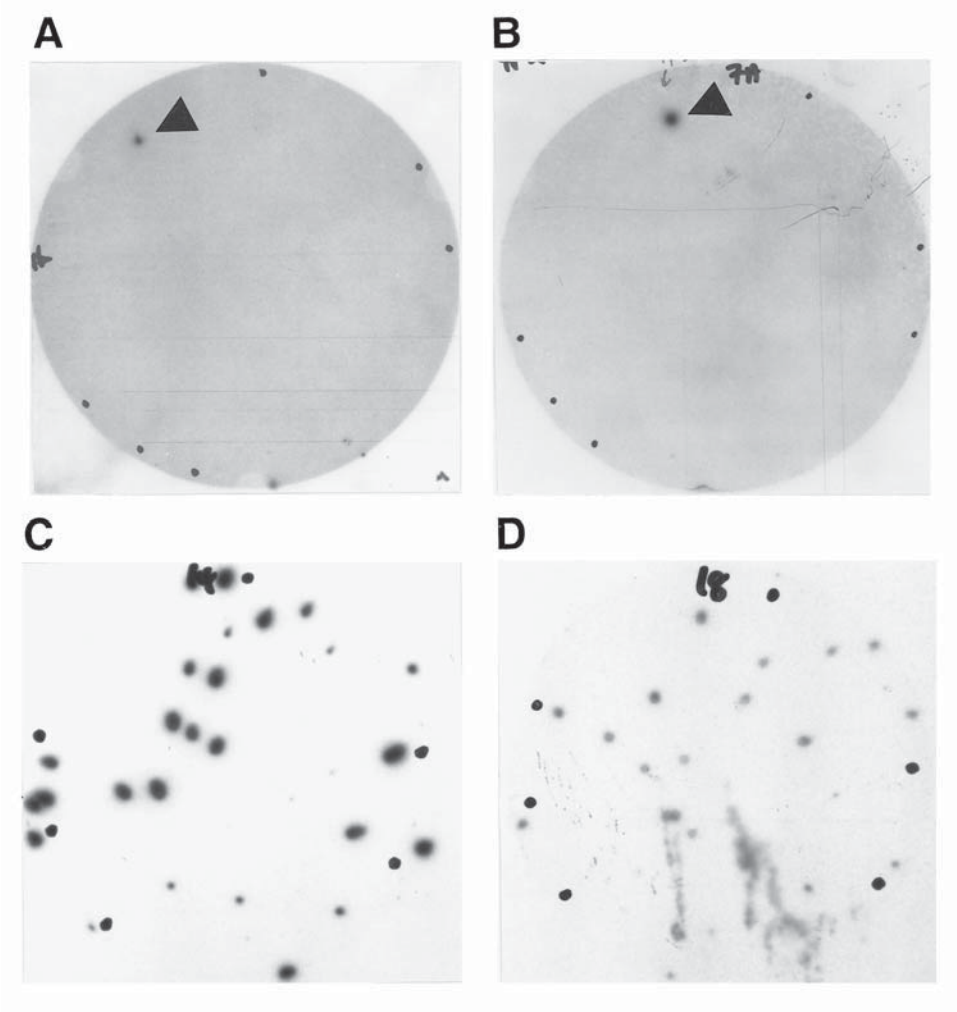

Fig. 2. Identification and isolation of positive clones by the CORT strategy. A λEXlox cDNA expression library prepared from the 184 normal breast epithelial cell line was screened with the radiolabeled EGFR C-terminal probe ***(14)***. The upper panels show autoradiographs of duplicate filters from the primary library screen. **(A)** and **(B)** are plaque lifts derived from plates induced with IPTG for 4 h and overnight, respectively. Duplicate positive signals derived from a Grb2 cDNA clone are indicated by arrowheads. These signals superimpose on alignment of the filters using the orientation marks. The exposure time was 48 h at –70°C with one intensifying screen. The lower panels show tertiary screening results for cDNA clones corresponding to Grb2 **(C)** and Ras-GAP/Grb13 **(D)**. The exposure time was 4.5 h at –70°C with one intensifying screen. Note that the intensity of the signal can vary because of differences in binding affinity.

binant fusion protein corresponding to the receptor region under investigation represents an alternative strategy for probe generation.

A subsequent modification utilizing λEXlox cDNA libraries *(7)* significantly improved the efficiency of the CORT technique *(8)*. In this vector system, expression of cloned cDNAs is driven by T7 RNA polymerase, which in the *Escherichia coli* strain BL21(DE3)pLysE, is under lacUV5 control and is, therefore, inducible by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture *(9)*. The clones are expressed as fusions with 260 amino acids of the T7 capsid protein T10. Use of λEXlox leads to higher levels of recombinant protein in the bacteriophage plaque and hence increased screening sensitivity compared to that obtained with λ*gt11*. Presumably, this is because of the higher activity of T7 RNA polymerase compared to *E. coli* RNA polymerase and the smaller size of the expressed fusion proteins (with λ*gt11* the fusion partner is β-galactosidase, which is 110 kDa). Therefore the protocol described in this chapter uses λEXlox or related vectors.

CORT screening of λEXlox libraries from a variety of sources led to the isolation of a further 12 Grb clones *(8,10–14)* (**Table 1**), most of which represent cDNAs for previously identified signaling proteins. However, it should be noted that all three members of the Grb7 family of SH2 domain-containing proteins were initially identified using this technique. Also, not all Grbs were isolated by virtue of an SH2 domain-mediated interaction, because Grb12 corresponds to the N-terminal region of Shc that contains a PTB domain, and the binding of Grb11 appears not to involve either an SH2 or PTB module.

The potential advantages of the CORT technique vs conventional approaches for identifying RTK targets are that it avoids laborious purification and microsequencing procedures, and it may detect interactions which occur at a low stoichiometry in vivo or which are unstable during detergent-based extraction. Furthermore, the DNA sequence homology between different SH2 or PTB domains is often too low for the use of reduced stringency hybridization procedures. However, one drawback to the CORT technique is that the identified proteins may not associate in vivo with the activated receptor despite a detectable association in vitro. Examples of such proteins are Grb10 and Grb14 *(13,14)*. Presumably, this occurs because the technique is sufficiently sensitive to detect relatively weak interactions. Other factors, such as conformation of the full-length protein, posttranslational processing, and/or subcellular localization, may also affect the ability of the two proteins to associate in the living cell.

In this chapter, the CORT protocol is divided into three stages: (1) the preparation of a radiolabeled probe corresponding to the C-terminus of the EGFR, (2) the plating and screening of λEXlox cDNA expression libraries, and (3) the isolation and further characterization of positive clones.

Table 1
Proteins Isolated by the CORT Technique

Clone	Description/Original name	Reference
Grb1	p85 subunit of PI3-kinase	4
Grb2	Grb2	5
Grb3	Crk	8
Grb4	Nck	8
Grb5	Fyn	8
Grb6	PLC-γ	8
Grb7	Grb7	8
Grb8	Drk (Drosophila Grb2)	10
Grb9	SH-PTP2	11
Grb10	Grb10 (Grb7-related)	13
Grb11	Under characterization	13
Grb12	Shc	12
Grb13	Ras-GAP	14
Grb14	Grb14 (Grb7-related)	14

2. Materials

2.1. Preparation of a Radiolabeled Probe Corresponding to the C-Terminus of the EGFR

1. Recombinant EGFR cytoplasmic domain. The relevant region of the EGFR (amino acids 644-1186) has been expressed and purified sucessfully in baculoviral systems ***(15)***. However, an almost identical recombinant protein (amino acids 647-1186), produced by the same methodology, is now available from Stratagene (La Jolla, CA). This can be diluted in 20 m*M* N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) pH 7.5 to 50 μg/mL and stored in reaction size aliquots (10 μL), which are stable for several months at –70°C.
2. (γ-^{32}P) ATP, 6000 Ci/mmol, 10 mCi/mL (Dupont-NEN).
3. 150 m*M* $MnCl_2$.
4. HNTG buffer: 20 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 0.1% v/v Triton X-100, 10% v/v glycerol. Store at 4°C, but warm to room temperature prior to use.
5. ATP (Boehringer Mannheim). Freeze aliquots of a 1 m*M* stock at –20°C.
6. Bovine serum albumin (BSA), Fraction V, RIA Grade (Sigma): Dissolve at 20 mg/mL in water and store in aliquots at –20°C.
7. 20 m*M* HEPES, pH 7.5: Dissolve in water and store at room temperature.
8. Centricon 30 concentrators (Amicon, Beverly, MA).
9. Cyanogen bromide crystals (Sigma). Store at 0–5°C in a dessicator and handle under a fume hood because the vapor is toxic.
10. Formic acid (approx 90% v/v).

11. Probe buffer : 50 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 1% v/v Triton X-100, 10% v/v glycerol. Store at 4°C. Add sodium orthovanadate to a final concentration of 200 μ*M* just prior to use from 1000× stock aliquots (dissolved in water and stored at –20°C).

2.2. Plating and Screening of cDNA Libraries

1. A cDNA library in λEXlox or the related λSCREEN-1. A variety of cDNA libraries in these vectors are available from Novagen (Madison, WI), as well as the corresponding cDNA synthesis kits (*see* **Note 1**).
2. Bacterial strains ER1647, BM25.8, and BL21(DE3)pLysE (Novagen).
3. Bacteriological Petri dishes (150 and 90 mm).
4. Nitrocellulose filters: Hybond C-extra, 137 and 82 mm (Amersham).
5. IPTG (Boehringer Mannheim): Prepare a 0.1 *M* stock in water, filter sterilize through a 0.22 μm filter, and store at –20°C. Dilute to 1 m*M* in sterile water immediately prior to use.
6. L-broth: Bacto-Tryptone 10 g/L, Bacto-Yeast extract 5 g/L, NaCl 5 g/L, pH 7.0. Add 15 g agar/L for L-broth-agar.
7. 2×YT broth: Bacto-Tryptone 16 g/L, Bacto-Yeast extract 10 g/L, NaCl 5 g/L, pH 7.0. Add 7 g agarose/L for 2×YT-top agarose.
8. SM: 0.1 *M* NaCl, 8 m*M* $MgSO_4$, 0.01% w/v gelatin, 0.05 *M* Tris-HCl, pH 7.5. Sterilize the above media by autoclaving and store at room temperature.
9. Maltose (20% w/v). Filter sterilize and store at 4°C.
10. $MgSO_4$: 1 *M* aqueous solution. Filter-sterilize and store at room temperature.
11. Tetracycline: 5 mg/mL in ethanol. Store in aliquots at –20°C.
12. Chloramphenicol: 34 mg/mL in ethanol. Store in aliquots at –20°C.
13. Streptomycin: 10 mg/mL in water. Store in aliquots at –20°C.
14. Kanamycin: 10 mg/mL in water. Store in aliquots at –20°C.
15. Carbenicillin: 50 mg/mL in water. Store in aliquots at –20°C.
16. Ampicillin: 50 mg/mL in water. Store in aliquots at –20°C.
17. Block buffer: 20 m*M* HEPES, pH 7.5, 5 m*M* $MgCl_2$, 1 m*M* KCl, 5 m*M* DTT, 5% w/v nonfat dried milk, 0.02% w/v sodium azide. Store at 4°C for up to 2 wk.
18. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl containing 0.05 or 0.1% (v/v) Triton X-100, as appropriate.
19. Pyrex or Tupperware containers for filter probing and washing. Containers for the former procedure should be circular and approx 150 mm in diameter.
20. Fluorescent markers for autoradiography (Stratagene).

2.3. Characterization of Positive Clones

1. STET buffer: 8% w/v sucrose, 5% v/v Triton X-100, 50 m*M* ethylenediaminetetracetic acid (EDTA), 50 m*M* Tris-HCl, pH 8.0. Filter-sterilize and store at 4°C.
2. Lysozyme/RNase: 10 mg/mL lysozyme, 1 mg/mL DNase-free RNase A, 50 m*M* Tris-HCl, pH 8.0. Store at –20°C in small aliquots. Do not refreeze.
3. 5 *M* ammonium acetate.

4. Isopropanol.
5. Chloroform.
6. 70% (v/v) ethanol.
7. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

3. Methods

3.1. Preparation of a Radiolabeled Probe Corresponding to the C-Terminus of the EGFR

1. Add to a 1.5 mL screw-capped tube the following: approx 500 ng (10 µL) of EGFR cytoplasmic domain, 22.5 µL of HNTG buffer, 16 µL (160 µCi) of (γ-^{32}P) ATP, and 1.5 µL of 150 m*M* $MnCl_2$. Incubate at room temperature for 40 min with constant agitation (*see* **Note 2**).
2. Add 2.5 µL of 1 m*M* ATP and incubate for a further 5 min (*see* **Note 3**).
3. Dilute 5 µL of 20 mg/mL BSA in 1 mL of 20 m*M* HEPES, pH 7.5. Add the probe reaction to this solution, mix gently, and pipet into the upper chamber of a Centricon 30. Spin at 5000*g* in an appropriate fixed angle rotor (e.g., Sorvall SM24) for 30 min or until the volume of liquid in the sample reservoir (upper chamber) is approx 50 µL. Add 1 mL of 20 m*M* HEPES, pH 7.5 (without BSA), and repeat the centrifugation. Remove the filtrate cup (lower chamber), seal, and dispose of as radioactive waste. Attach the retentate cup to the sample reservoir, invert the Centricon and seal the membrane support base with Parafilm to avoid radioactive contamination. Centrifuge at 5000*g* for approx 2 min to collect the concentrate. Transfer the probe to a 1.5-mL screw-capped tube.
4. Measure the volume of the concentrate and add formic acid to a final concentration of 70% (v/v).
5. Using forceps, add a small (1–2 mm diameter) crystal of cyanogen bromide. Mix to dissolve the crystal and then gently flood the tube with nitrogen gas, taking care not to displace the solution, which is highly radioactive. Close the tube and allow the cleavage reaction to proceed overnight in the dark at room temperature.
6. Spin the sample in a Speed-Vac concentrator under vacuum until dry. Resuspend the sample in 300 µL of deionized water and dry down again. Repeat this washing step twice more (*see* **Note 4**).
7. Resuspend the sample in 50–100 µL of probe buffer by vigorous agitation for approx 10 min. Transfer to a fresh tube. Rinse the tube with a similar volume of probe buffer and combine with the first aliquot. Transfer to approx 40 mL of blocking buffer. Determine the specific activity of the probe by performing a Cerenkov count on a 2-µL aliquot of the probe solution. This should indicate approx 6×10^2 cpm/µL, i.e., approx 5×10^7 cpm/µg of starting material. The probe solution is stable for approx 1 wk and can be used twice (e.g., for primary and secondary library screens).

3.2. Plating and Screening of cDNA Libraries

1. Streak out BL21(DE3)pLysE cells on L-broth-agar plate containing 34 µg/mL chloramphenicol. Grow overnight at 37°C.

2. Prepare plating bacteria by inoculating a single colony into L-broth containing 0.2% (w/v) maltose, 10 m*M* $MgSO_4$, and 34 μg/mL chloramphenicol. Place in a shaking incubator at 37°C and grow to an OD_{600} of approx 0.5. The culture can be stored for several days at 4°C prior to use.
3. Prepare 2×YT-agar plates (150 mm) and 2×YT-top agarose for plating the library. Ensure the plates are dry and number them for identification. Prewarm the plates to 37°C. Equilibriate the molten top agarose to 48°C and then add chloramphenicol to 170 μg/mL.
4. For each 150 mm plate, mix 5×10^4 phage (*see* **Note 5**) with 600 μL plating bacteria in a 14-mL polypropylene tube and incubate for 30 min at 37°C. Add 7 mL of molten 2XYT-top agarose and pour immediately on to a plate.
5. Once the top agarose has set, incubate the plates for 6–8 h at 37°C until the plaques reach a diameter of 0.5–1 mm.
6. Whilst the plates are incubating, soak individual nitrocellulose filters for approx 1 min in 1 m*M* IPTG using a large bacteriological Petri dish, and then allow them to air-dry on aluminium foil. Prepare two filters per plate for duplicate plaque lifts. Once dry, number the filters for identification using a permanent marker pen (*see* **Note 6**).
7. Carefully place a filter on each plate and make an asymmetric series of orientation marks by piercing vertically through the nitrocellulose and bottom agar using a 21-gage needle (*see* **Fig. 2**). Indicate the positions of the holes on the bottom of the plate using a marker pen.
8. Incubate at 37°C for 4 h.
9. Remove filters and place in TBS/0.05% Triton.
10. Place the duplicate filters on the plates. Make orientation marks using a 21-gage needle at the same positions as previous, using the pen marks on the bottom of the plate as a guide. A light box may assist this process, which simplifies the identification of duplicate positive clones (**Fig. 2**). Incubate the plates overnight at 37°C.
11. Wash the first set of filters 3–4 times in TBS/0.05% Triton on an orbital mixer until the washes are clear of debris (*see* **Note 7**). Wash in TBS for 5 min and then transfer the filters individually into blocking buffer using forceps. Incubate on an orbital mixer at 4°C until the duplicate set are ready for probing.
12. Remove and wash the duplicate filters and then incubate in blocking buffer for approx 6 h.
13. Transfer the filters individually into the probe solution (*see* **Subheading 3.1.**) using forceps. Work behind a perspex screen to avoid exposure to the radioactive probe.
14. Incubate the filters overnight at 4°C on an orbital mixer.
15. Transfer the filters individually into TBS/0.05% Triton X-100. Be careful not to splash radioactive probe during this process. Perform three 15 min washes in TBS/0.05% Triton X-100 followed by one 15 min wash in TBS/0.1% Triton X-100.
16. Dry the filters on 3MM paper and enclose in plastic wrap. Apply fluorescent markers to orientate the X-ray film on the filters. Expose for autoradiography at –70°C, usually for 48 h (*see* **Note 8**).

3.3. Isolation and Further Characterization of Positive Clones

1. Align the film on the filters and record the position of the orientation holes. Use these marks to identify the position of the positive plaques on the plates (*see* **Note 9**).
2. Trim the narrowing end from an appropriate number of 1 mL pipet tips using clean scissors or a scalpel blade to leave an aperture of approx 0.5 cm diameter. Use these in combination with an automatic micropipet to pick positive plaques. Add each agar plug to 1 mL of SM containing a drop of chloroform. Elute the phage for 1–2 h at room temperature or overnight at 4°C. Store the phage stocks at 4°C.
3. Prepare serial 10-fold dilutions of the phage stocks in SM. Secondary screens are performed on plates containing approx 1000 plaques, which are usually obtained by plating 100 µL of 10^{-2} or 10^{-3} dilutions of the phage stock. Add 100 µL of phage dilution to 100 µL of BL21(DE3)pLysE plating bacteria and incubate as previously described. Plate on 2×YT-agar in 90-mm Petri dishes using 3 mL of 2×YT-top agarose.
4. Incubate for 6–8 h until plaques appear and perform lifts overnight using IPTG-impregnated filters. Duplicate lifts are not necessary.
5. Process, probe, and wash the filters as described in **Subheading 3.2.**, **steps 9–16**.
6. Pick secondary positives using a sterile Pasteur pipet fitted with a bulb.
7. Plate the secondary stocks to achieve 10–100 plaques per dish and perform a tertiary screen to isolate single positive plaques.
8. Excision of pEXlox plasmids from the phage is performed using bacterial strain BM25.8. Streak out BM25.8 cells on L-Broth-agar containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Inoculate a single colony into L-broth containing 50 µg/mL kanamycin, 34 µg/mL chloramphenicol, 0.2% (w/v) maltose, and 10 m*M* $MgSO_4$ and grow to an OD_{600} of approx 0.5.
9. Mix 100 µL of bacteria with 100 µL of phage and incubate for 30 min at 37°C. Discrete colonies are usually obtained by incubating the bacteria with 100 µL of either a 10^{-1} or 10^{-2} dilution of the phage stock.
10. Plate on L-broth agar containing 50 µg/mL carbenicillin (*see* **Note 10**).
11. Inoculate an individual colony derived from each phage clone into 5 mL L-broth containing ampicillin (50 µg/mL). Grow overnight at 37°C.
12. Pellet the bacteria in a microcentrifuge and resuspend in 200 µL of STET buffer (*see* **Note 11**).
13. Add 10 µL of lysozyme/RNase mix and incubate at 100°C for 3 min.
14. Centrifuge at 12,000*g* in a microcentrifuge for 10 min.
15. Transfer the supernatant to a fresh tube and add 200 µL of 5 *M* ammonium acetate and 400 µL of isopropanol. Mix and then centrifuge at 12,000*g* for 2 min in a microcentrifuge.
16. Wash the pellet in 70% (v/v) ethanol, briefly dry, and resuspend in 100 µL TE buffer.
17. Transform into a standard bacterial host strain (e.g., DH5α) for restriction enzyme analysis and sequencing (*see* **Note 11**).

4. Notes

1. As noted by the Margolis laboratory ***(16)***, random-primed cDNA libraries may aid the cloning of proteins with SH2 or PTB domains localized towards their N-termini.
2. The C-terminal probe derived from one such reaction is sufficient for 40 mL of blocking buffer for incubation of approximately ten 137 mm filters. A standard library screen involves duplicate lifts from 10 plates and therefore requires two probe reactions.
3. The addition of 50 μ*M* ATP ensures complete phosphorylation of the probe.
4. This step can be time-consuming. Allow approx 90 min to dry down each wash.
5. The titer of the library should be determined using BL21(DE3)pLysE bacteria because the plating efficiency varies depending on the host bacterial strain.
6. A GST fusion protein containing an EGFR-binding module (e.g., the SH2 domain of Grb2) provides a useful positive control for probing ***(16)***. Apply 100 and 10 ng aliquots of the fusion protein to a piece of nitrocellulose filter and allow to dry. Use GST alone as a control. Block and then probe as for the plaque lifts. The procedure should readily detect 10 ng of the fusion protein. However, a λEXlox phage clone encoding an interacting protein or module is subjected to the same plating, induction, and transfer steps as the library and therefore controls for the entire procedure.
7. Do not rotate the filters so fast that they adhere to each other and consequently do not wash properly. This also applies when washing the filters following probing.
8. It can help to mark the position of the orientation holes with a pen prior to exposure, and to place duplicate filters alongside each other in the same orientation to assist the identification of positive plaque signals (*see* **Fig. 2**).
9. Although it is tempting to pick plaques that give positive signals only on one filter, these are usually artefacts if the duplicate lifts have been performed correctly.
10. The use of carbenicillin, rather than ampicillin, reduces the number of satellite colonies.
11. Plasmid excision produces a variety of multimers and the plasmid copy number in BM25.8 cells is low. Therefore, the pEXlox plasmids should be transformed into a standard host strain for further analysis. We find that the described "boiling lysis" technique for DNA minipreps gives the most reproducible transformation results. Standard plasmid preparation techniques can be used once the plasmids are transformed into *E. coli* DH5α. If further cDNA clones are required to assemble a full-length cDNA sequence, the λEXlox library should be plated on the bacterial strain ER1647 and subjected to standard plaque lift/DNA hybridization procedures ***(17)***.

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18

Identification of Receptor Tyrosine Kinase Associating Proteins Using the Yeast Two-Hybrid System

Sally A. Prigent

1. Introduction

Protein-protein interactions form the basis of most signal transduction pathways. In the case of pathways initiated by receptor tyrosine kinases (RTKs), phosphotyrosine residues present on the cytoplasmic domain of the activated receptor form docking sites for adaptor molecules and enzymes containing src homology (SH)2 and phosphotyrosine binding (PTB) domains ***(1)***. Association of these signaling components initiates cascades of interactions and reactions that determine the cell's response to the stimulus. Because these initial interactions are responsible for the biological properties of different receptors, there has been considerable interest in identifying potential binding proteins with a view to understanding the molecular basis of signaling diversity.

The yeast two-hybrid assay is a powerful technique for detecting protein–protein interactions. The assay is based on the fact that many transcription factors possess two distinct functional domains, a DNA binding domain and a transcriptional activation domain. These domains must be in close proximity to promote DNA transcription, but need not necessarily be present on the same protein. Thus, if the two domains are expressed separately in yeast, they are unable to promote transcription. However, if they are expressed as fusions with two interacting proteins, the activity of the transcription factor is restored. A number of modifications of the yeast two-hybrid system have been reported, and it is beyond the scope of this chapter to discuss each one ***(2–6)***. For a more detailed discussion of different DNA-binding domain and activation-domain vectors that have been used by various groups, *see* **ref.** *7*. The method described here is a modification developed by Stan Hollenberg ***(4)***, which has been used by our-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

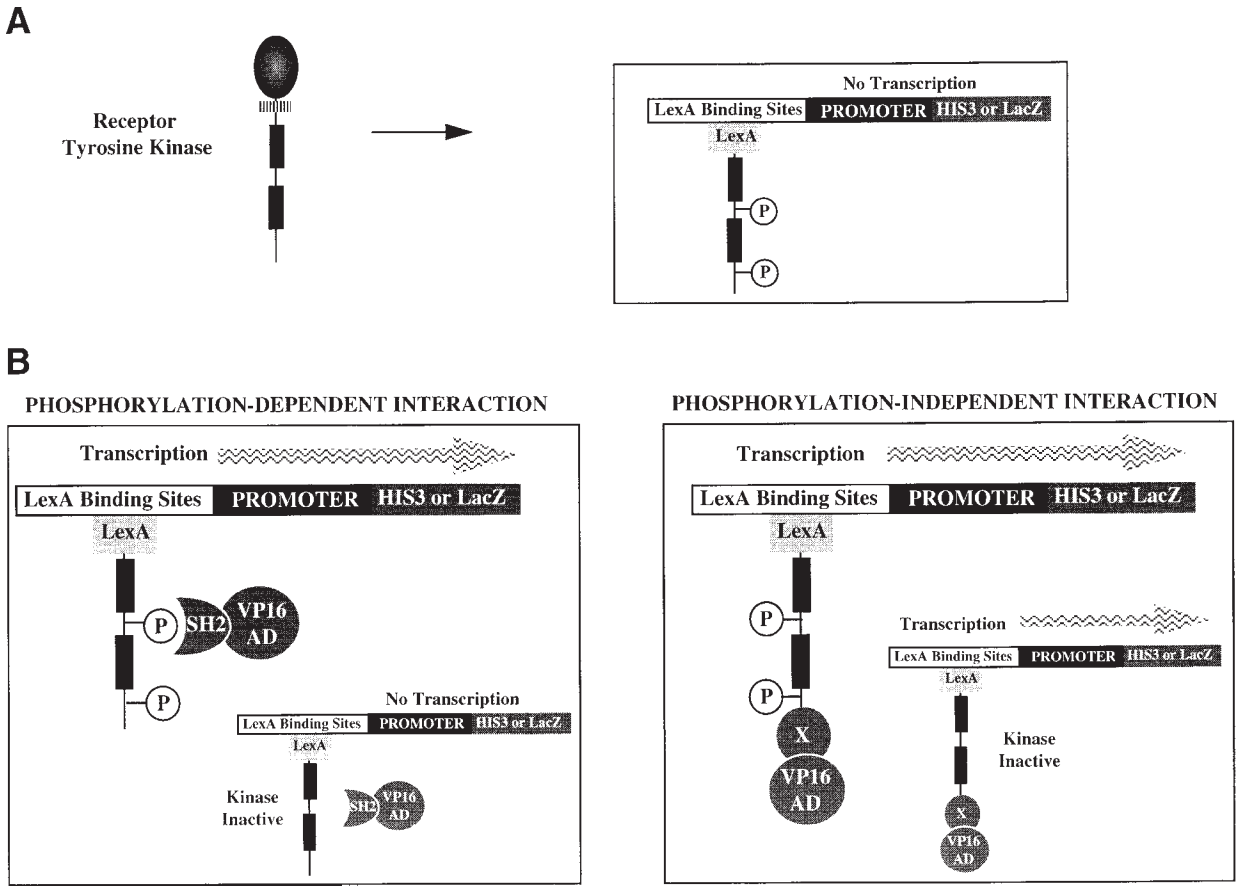

Fig. 1. Principle of Two-Hybrid Screen for Detection of Receptor Tyrosine Kinase Interacting Proteins

selves and others to detect phosphorylation-dependent and independent interactions with RTKs ***(8–10)***. A different system developed by Brent et al. ***(3)*** has also been used by others to look at interactions with tyrosine kinases, in particular with the insulin receptor and epidermal growth factor receptor (EGFR) ***(11–13)***, but will not be discussed here. The principle of the two-hybrid system, as applied to the detection of tyrosine–kinase interacting proteins is illustrated in **Fig. 1**.

Briefly, the method involves inserting the cDNA encoding the intracellular domain of the receptor of interest into a vector encoding the DNA binding domain of the *Escherichia coli* repressor LexA (pBTM116) (*see* **Fig. 2**). Expression of the LexA-receptor fusion is under the control of the *ADH1* promoter. This is commonly called the bait plasmid. The vector also encodes the *TRP1* gene to permit selection of yeast transformants on medium lacking tryptophan. The ability of the truncated receptor to autophosphorylate can be tested by immunoblotting of extracts from yeast expressing the LexA-kinase fusion with antiphosphotyrosine antibodies. The LexA-receptor plasmid is then transformed into yeast together with a plasmid library of cDNAs fused to the Herpes simplex virus (HSV) VP16 activation domain. The library used by ourselves, and others, was constructed by Hollenberg from day 9.5–10.5 mouse embryos and contains short inserts of 350–700 bp ***(4)***. This favors the folding of independent protein domains. Alternatively, commercially available libraries constructed in vectors encoding the GAL4 activation domain are compatible with the pBTM116 vector. The yeast strain, L40 {MATa *his*3Δ200 *trp*1-901 *leu*2-3112 *ade*2 LYS2::(4*lexAop*-HIS3) URA3::(8*lexAop-lacZ*)} contains two reporters stably integrated into the genome ***(14)***. These reporter genes are the *HIS3* gene and *lacZ* gene that are under the control of minimal GAL1 promoters fused to multiple LexA operators ***(4)***. Therefore, yeast expressing interacting proteins are able to grow in the absence of histidine in the medium permitting convenient selection. β-galactosidase activity can be detected by growth on plates containing substrate (X-gal), or by a more sensitive filter assay. The presence of two reporters increases the fidelity of the system.

Having identified colonies showing reporter activation, library plasmids from these colonies are tested for their ability to promote transcriptional activation with an unrelated control protein. Once it has been established that reporter activation is dependent on both the expression of the tyrosine kinase and "positive" library plasmid, the phosphorylation dependence of the interaction can be tested by using a kinase inactive mutant in the two-hybrid assay. The system lends itself readily to the further definition of interaction sites on the two proteins by construction of point, or deletion mutants. Ultimately, the significance of all novel interactions identified should be confirmed in vivo, e.g., by coimmunoprecipitation studies from cultured mammalian cells ***(15)***. The steps involved are summarized in **Fig. 3**.

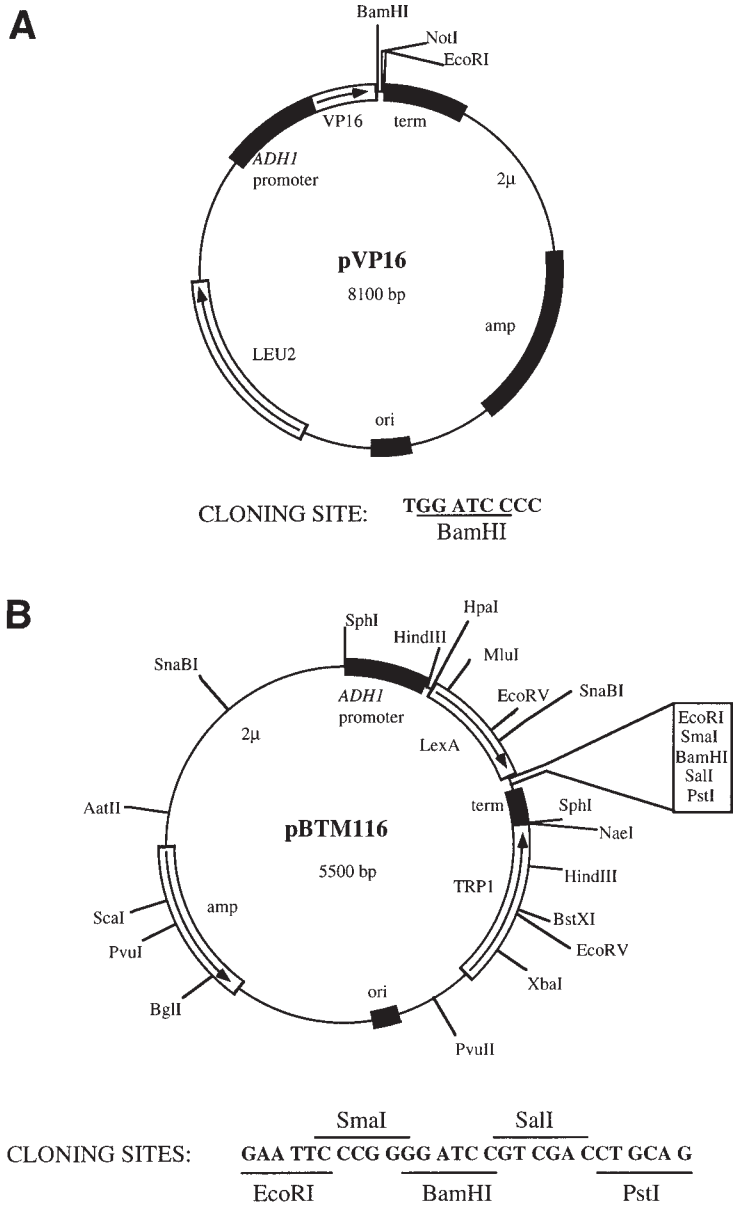

Fig. 2. Maps of DNA activation domain (**A**), and DNA binding domain (**B**) Vectors.

2. Materials

2.1. Plasmids and Libraries

The vector pBTM116 was constructed by Bartel and Fields (Dept. of Microbiology, State University of New York, Stony Brook, NY), and the VP16 mouse embryo library was constructed by Stan Hollenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). A commercially available alternative LexA binding domain vector, pH ybLex/Zeo, can be purchased from Invitrogen, which has a Zeocin selectable marker. LexA bait plasmids can be used in the L40 yeast strain with a variety of commercially available libraries from Clontech or Stratagene.

2.2. Yeast Culture

1. The yeast strain L40 *S. cerevisiae* can be purchased from Invitrogen (cat. no. C830-00).
2. YPD: 10 g yeast nitrogen base without amino acids (Difco, cat. no. 0919-15-3), 20 g bactopeptone (Difco, cat. no. 0118-17-0). Dissolve in water, pH to 5.8–6.0 and make up to 1 L. Autoclave, then add 50 mL filter-sterilized 40% glucose. For solid medium, add 18 g bactoagar (Difco, cat. no. 0140-01-0) per liter of medium prior to autoclaving.
3. Drop-out mixes: Drop-out mix is a combination of amino acids and essential nutrients listed below, excluding the appropriate selection supplement. It should be mixed thoroughly by end-over-end rotation for several minutes including a couple of marbles or magnetic flea to aid mixing. Mixes can be stored at room temperature. For the procedures described in the following sections two drop-out mixes should be prepared.
   a. Drop-out mix 1 ("triple" drop-out mix): leave out leucine, tryptophan and histidine.
   b. Drop-out mix 2: leave out leucine and tryptophan.

   All the following L-amino acids and supplements can be purchased from Sigma.

Adenine	0.5 g	Leucine	4.0 g
Alanine	2.0 g	Lysine	2.0 g
Arginine	2.0 g	Methionine	2.0 g
Asparagine	2.0 g	p-Aminobenzoic acid	0.2 g
Aspartic acid	2.0 g	Phenylalanine	2.0 g
Cysteine	2.0 g	Proline	2.0 g
Glutamine	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Glycine	2.0 g	Tryptophan	2.0 g
Histidine	2.0 g	Tyrosine	2.0 g
Inositol	2.0 g	Uracil	2.0 g
Isoleucine	2.0 g	Valine	2.0 g

4. Drop-out medium: Mix 6.7 g yeast nitrogen base without amino acids (Difco, cat. no. 0919-15-3), 2 g amino acid drop-out mix. Dissolve in water, adjust pH to approx 5.8–6.0 with NaOH and make up to 1 L (*see* **Note 1**). Autoclave and add

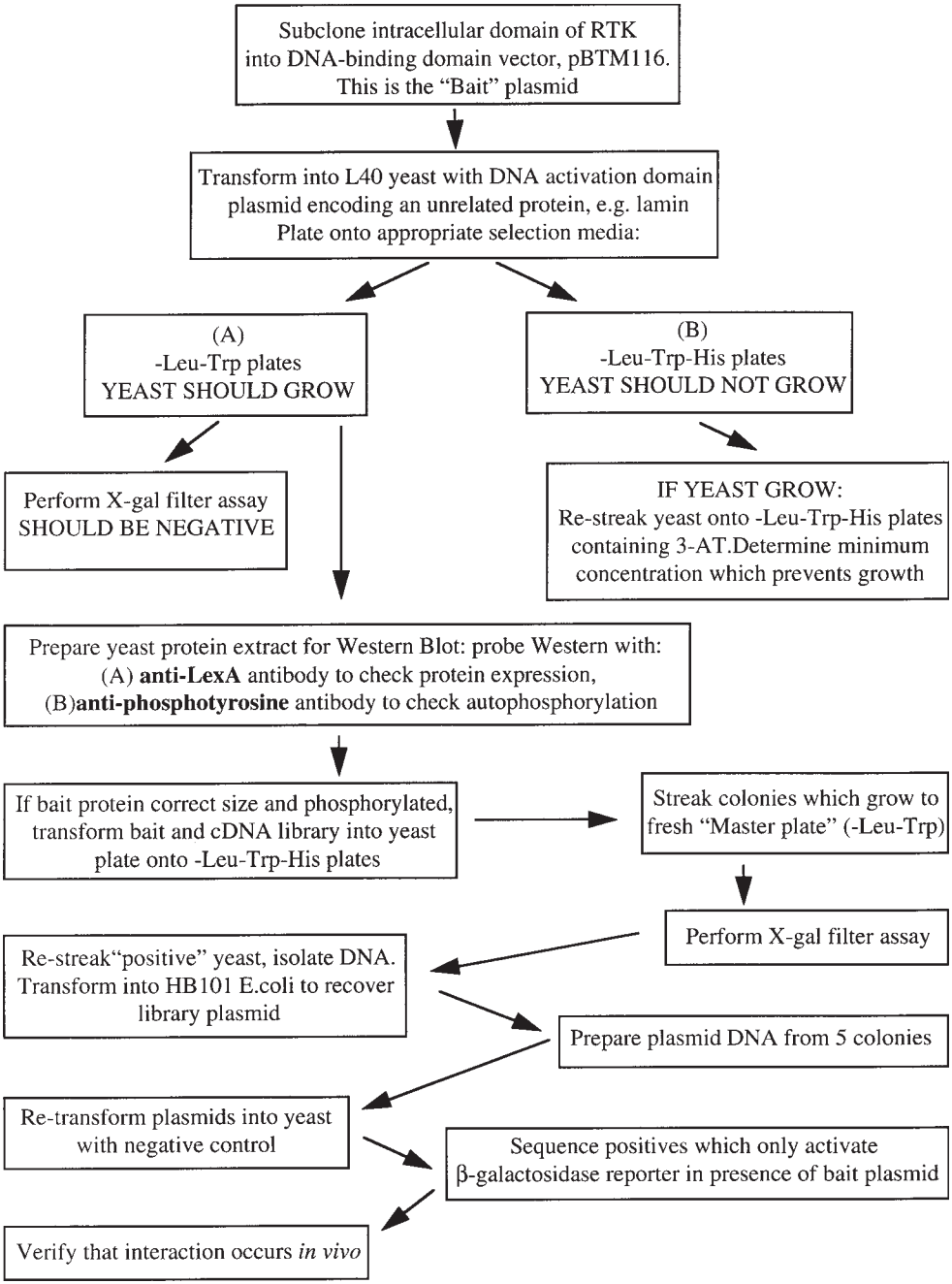

Fig. 3. Outline of Two-Hybrid Screen

50 mL 40% glucose (sterile filtered). For solid medium, add 18 g/L Bactoagar prior to autoclaving. For library screening prepare at least 50 × 15-cm plates per screen using drop-out mix 1. For subsequent analysis prepare as required 10-cm plates using drop-out mix 2.

2.3. Yeast Transformation

1. 0.1 *M* lithium acetate in TE (10 m*M* Tris pH 7.5, 1 m*M* ethylenediaminetetracetic acid [EDTA]).
2. 50% PEG 3350 (Sigma P 3640) in TE.
3. Phosphate-buffered saline (PBS): This can be purchased in tablet form from Gibco-BRL (cat. no. 18912-014). One tablet dissolved in 500 mL water.
4. 3-Amino-1, 2, 4-triazole (Sigma, cat. no. A8056): 1 *M* in water, store at –20°C.

2.4. Preparation of Yeast Lysates for Westerns

1. 0.25 *M* NaOH/1% β-mercaptoethanol.
2. 50% trichloroacetic acid (TCA) (Sigma T 9159).
3. Sodium dodecyll sulfate (SDS) sample buffer: 50 m*M* Tris-HCl, pH 6.8, 100 m*M* dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. The buffer should be made without dithiothreitol, which should be added just before use.

2.5. Expression and Phosphorylation of Bait Protein

1. 5% milk powder in tris-buffered saline and Tween (TBST): 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.1% Tween-20.
2. 5% Bovine serum albumin in TBST.
3. Anti-LexA goat polyclonal antibody (Santa Cruz Biotechnology, sc-1725).
4. Antiphosphotyrosine antibody: Rc20 horseradish peroxidase conjugate, or monoclonal antibody PY20 (Transduction Laboratories cat. nos. E120H; P11120).
5. Horseradish peroxidase conjugated antigoat IgG (for detection of and anti-LexA antibody) and antimouse IgG For detection of PY20 (Santa Cruz Biotechnology).
6. ECL reagents (Amersham RPN 2108).

2.6. Beta-Galactosidase Assays

1. X-gal stock: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 mg/mL in N,N-dimethylformamide. Store at –20°C covered in foil.
2. Z buffer/X-gal substrate solution: Prepare 1 L of Z buffer containing 16.1 g $Na_2HPO_4 \cdot 7H_2O$; 5.5 g $NaH_2PO_4 \cdot H_2O$; 0.75 g KCl; 0.25 g $MgSO_4 \cdot 7H_2O$. Check for pH is around 7.0, autoclave. Prepare the substrate solution immediately prior to use by mixing 100 mL Z-buffer, 0.27 mL β-mercaptoethanol, and 1.67 mL X-gal stock solution. β-mercaptoethanol may be omitted if maximum sensitivity is not required.
3. Filters: Whatman grade-50 and grade-3 filters, 8.5-cm diameter.
4. Yeast X-gal plates: Prepare a base medium and salt solution (10× BU) as follows:
   a. Base: 20 g yeast nitrogen base without amino acids, 2.1 g drop-out mix 2 (-Leu-Trp), 60 g Bactoagar, 1.8 L water.

b. 10× BU: 70 g $Na_2HPO_4 \cdot 7H_2O$, 30 g KH_2PO_4, water to 1 L, pH 7.0. Filter sterilize.
c. Autoclave base for 16 min at 15 lb/sq in and allow to cool to around 60°C. To 600 mL of base add 100 mL 10 × BU, 100 mL 20% glucose, 5 mL X-gal (20 mg/mL stock in dimethyl formide [DMF]), and water to 1 L.

5. o-Nitrophenyl b-D-Galactopyranoside (ONPG), 4 mg/mL in Z buffer.

2.7. Recovery and Preparation of Library Plasmid from Positive Colony

1. Yeast lysis buffer: 2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
2. Glass beads: 425–600 microns, acid-washed (Sigma, cat. no. G8772).
3. Phenol/chloroform/isoamyl alcohol (25:24:1) (Gibco-BRL).
4. Sterile water.
5. LB/ampicillin plates: Dissolve in 800 mL water, 10 g Bactotryptone, 5 g Bacto-yeast extract, 10 g NaCl. Adjust pH to approx 7.0, make up to 1 L, add 15 g/L Bactoagar, autoclave 15 min at 15 lb/sq in on liquid cycle. Cool to approx 55°C, add ampicillin from liquid stock (100 mg/mL) to final concentration 100 μg/mL.
6. M9 plates: Minimal medium: Prepare 10× M9 as follows: Dissolve in 950 mL water 58 g Na_2HPO_4 , 30 g KH_2PO_4 , 5 g NaCl, 10 g NH_4Cl. Use sodium hydroxide to adjust pH to 7.2–7.6, make up to 1 L with water and autoclave. Add 2 mL thiamine (2 mg/mL) per L. For M9 plates, dilute 50 mL 10× M9 with 450 mL water, add 10 g Bactoagar and autoclave. Cool to around 60°C, then add 5 mL 20% glucose, 5 mL 10 m*M* $CaCl_2$, 0.5 mL 1 *M* $MgSO_4$, 0.5 mL thiamine (2 mg/mL stock).
7. Solutions for plasmid minipreps:
   a. Solution 1: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0. Autoclave and store at 4°C.
   b. Solution 2: 0.2 *M* NaOH, 1% SDS. Store at room temperature.
   c. Solution 3: Mix 60 mL 5 *M* potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL water. Autoclave.
8. 5 *M* lithium chloride.
9. 70% ethanol.
10. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5.

2.8. Primers for DNA Sequencing

For the pVP16 vector the following primers can be used for automated sequencing of "positive" clones:

1. Sense: 5'-GAGTTTGAGCAGATGTTTA-3'
2. Antisense: 5'-TGTAAAACGACGGCCAGT-3'.

For sequencing using Sequenase version 2.0 (Amersham), the following sense primer should be used: 5'-GGTACCGAGCTCAATTGCGG-3'.

3. Methods

3.1. Construction of Tyrosine Kinase Bait Plasmid

Proteins investigated using a two-hybrid approach should not contain transmembrane sequences or sequences that could potentially anchor the protein in the yeast cell membrane, e.g., farnesylation sequences ***(16)***. Therefore, to study receptor tyrosine–kinase interactions using this system, one should express the isolated intracellular domain as a LexA fusion. Where interactions with phosphotyrosine residues are sought, it is important that the kinase should be active and able to autophosphorylate sites on the cytoplasmic domain of the protein. Although there is evidence that many receptor tyrosine kinases autophosphorylate when expressed without an extracellular domain, it is not clear whether this is true for all tyrosine kinases, or whether the isolated cytoplasmic domains possesses the same spectrum of phosphorylation sites as the full-length ligand-activated receptor. In our hands, we have been able to detect phosphorylation of the intracellular domain of the VEGF receptor KDR and to detect phosphorylation-dependent interactions (S.A.P., manuscript in preparation). Others have succesfully detected phosphorylation-dependent interactions with the insulin receptor ***(11)***, M-CSF receptor ***(10)*** and c-Met ***(8)***. In the case of the Ret tyrosine kinase, phosphorylation-dependent interactions were detected when the kinase was expressed as a fusion with the type Ia dimerization domain of cAMP-dependent protein kinase ***(9)***. This fusion protein is expressed in some papillary thyroid carcinomas and results in constitutive activation of the kinase ***(17)***. It may be possible to fuse this dimerization domain to other kinases to produce constitutively active enzymes in yeast where the intracellular domain alone does not autophosphorylate efficiently.

cDNA corresponding to the cytoplasmic tyrosine kinase domain can be generated conveniently by PCR from a full-length cDNA clone, if available, or by RT-PCR from a suitable mRNA source. Care must be taken to ensure that the insert is in the correct reading frame (*see* **Fig. 2**). The most convenient cloning sites for pBTM116 are *Bam*H1 and *Eco*R1, but their use will depend on the absence of internal *Bam*H1 or *Eco*R1 sites in the amplified cDNA of interest (*see* **Note 2**).

3.2. Choice of Library

A library should be chosen from a tissue that normally expresses the kinase of interest. A large number of libraries are commercially available from Clontech (http://www.clontech.com/clontech/Catalog/MATCHMAKER/MMlibraries.html) and Stratagene in vectors encoding the GAL4 activation domain, and *LEU*2 gene, which are compatible with pBTM116. Invitrogen sells a range of libraries in a B42 activation domain vector pYESTrp, which can be

use together with pH ybLex/Zeo, but not pBTM116 (*see* Web site for more information: http://www.invitrogen. com/cat_hybrid.html#hybridlib). Alternatively, libraries may be constructed from a suitable tissue using kits from Clontech or Stratagene, or can be custom-made by these companies. For the purpose of this chapter, it is assumed that a library is already available (*see* **Note 3**).

3.3. Yeast Culture

L40 are stored as a frozen glycerol stock containing 20% glycerol at –80°C. We routinely culture L40 on YPD plates, on which they should appear pink in color. There is a risk of contaminating the stock by growth on YPD, especially if other yeast strains are in use in the laboratory, so prior to performing any transformations we streak yeast onto drop-out plates lacking uracil and lysine, on which they should grow. Plates can be kept at 4°C for several weeks, however, for preparation of competent yeast we always use a colony from a fresh plate.

3.4. Test Transformation

Before beginning an extensive library screen it is important to establish a number of factors:

1. that the bait does not activate transcription on its own;
2. that it is expressed;
3. that it is phosphorylated on tyrosine residues.

The kinase/pBTM116 contstuct is transformed into yeast together with an unrelated protein in the corresponding activation domain vector using the following protocol, which can be scaled up or down as necessary. Lamin is commonly used as a control protein as it is a generally sticky nuclear protein.

3.4.1. Small-Scale Test Transformation

All solutions, tips, pipets, centrifugation tubes, and so on should be sterile, and aseptic technique should be applied at all times.

1. Inoculate 5 mL YPD with a single yeast colony. Grow overnight at 30°C.
2. Dilute 100 µL in 50 mL YPD and grow for approx 12 h at 30°C until the OD_{600} is about 1–2 (approx 2×10^7 cells/mL).
3. Pellet yeast by centrifugation at 3000*g* for 5 min at room temperature in a sterile 50-mL Falcon tube using a bench-top centrifuge.
4. Wash pellet with 50 mL 0.1 *M* lithium acetate in TE. Pellet yeast as in **step 3** above.
5. Resuspend yeast in 1 mL 0.1 *M* lithium acetate in TE, incubate with shaking at 30°C for 1 h.

6. Dispense two 100-μL aliquots in Eppendorf tubes.
7. Premix 1 μg of pBTM116/kinase construct with 1 μg of an unrelated cDNA (e.g., lamin) in the pVP16 vector. Add DNA to one aliquot of competent yeast and mix with pipet tip.
8. To a second aliquot of yeast, add 1 μg each of positive control plasmids. We use Ras and Byr2 in DNA binding domain and activation domain vectors, respectively ***(16)***.
8. Add 400 μL 50% PEG 3350 (Sigma P3640) in TE, mix by inversion.
9. Place at 30°C for 30 min and then at 42°C for 20 min (shaking not necessary).
10. Centrifuge in Eppendorf centrifuge for a few seconds. Remove supernatant.
11. After a few seconds, reaspirate any remaining drops of PEG.
12. Resuspend yeast in 100 μL sterile PBS.
13. Spread half of each transformation mixture onto a drop-out plate lacking leucine and tryptophan to calculate transformation efficiency. Spread the other half onto a drop-out plate lacking leucine, tryptophan, and histidine. Only yeast expressing the positive control plasmids should grow in the absence of histidine.

3.4.2. Suppression of Background Transcriptional Activation Using 3-AT

When the control transformations are plated onto triple drop-out plates, colonies should not be visible after 1 wk. If colonies do grow, they should be restreaked onto plates containing 1–30 m*M* 3-AT, an inhibitor of the enzyme encoded by the *HIS*3 gene. For subsequent library screens, the minimum concentration should be used which just suppresses growth.

3.4.3. Whole Yeast Lysates for Westerns

This protocol originated from David Bowtell, University of Melbourne (*see* http://grimwade.biochem.unimelb.edu.au/bowtell/molbiol/molbiol.ht mL for more useful protocols).

1. Inoculate 2.5 mL selection medium with a yeast colony containing the plasmid of interest. Grow with shaking overnight at 30°C.
2. Centrifuge cells at 3000*g* in bench-top centrifuge for 5 min at room temperature. Resuspend in 1 mL 0.25 *M* NaOH/1% β-mercaptoethanol. Transfer to 1.5-mL Eppendorf tube.
3. Incubate on ice for 10 minutes.
4. Add 0.16 mL 50% trichloroacetic acid.
5. Incubate on ice for 10 min.
6. Pellet cells by centrifugation at 12,000*g* for 10 min at room temperature.
7. Resuspend pellet in 1 mL ice-cold acetone by vortexing vigorously.
8. Pellet cells by centrifugation at 12,000*g* for 10 min at room temperature.
9. Air dry pellet and resuspend in 200–500 μL SDS sample buffer.

3.4.4. Expression and Phosphorylation of Bait Protein

Analyze 40 μL of extract, prepared as above, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using an anti-

LexA binding-domain antibody. The presence of phosphotyrosine can be determined using antiphosphotyrosine antibody, of which many are commercially available, but we use Rc20, or PY20 antibody from Transduction Labs. We routinely use Tricine gels for analysis of proteins ***(18)***, and use ECL detection reagents for visualizing bands on Westerns. Best results are obtained using 5% Marvel in TBST for blocking and antibody incubation steps when probing blots with the LexA antibody. For antiphosphotyrosine immunoblots we use 5% BSA in TBST for blocking, and perform subsequent antibody incubations in TBST. An example of a typical Western is shown in **Fig. 4**.

3.5. Library Screening: Identification of Positive Clones

3.5.1. Large-Scale Library Transformation

We routinely transform sufficient yeast to plate onto 50 × 15-cm plates using a scaled-up version of the test transformation described above (*see* **Note 4**).

1. Dilute 1 mL of overnight yeast culture in 500 mL YPD and grow for approx 12 h at 30°C until the OD_{600} is about 1–2 (2×10^7 cells/mL).
2. Pellet yeast by centrifugation for 5 min at 3000*g* in autoclaved 250-mL or 500-mL centrifugation buckets for Sorvall centrifuges, or sterile 50-mL Falcon tubes for bench-top centrifuges.
4. Wash with 250 mL 0.1 *M* lithium acetate in TE. Pellet yeast as above.
5. Resuspend yeast in 10 mL 0.1 *M* lithium acetate in TE. Incubate with shaking at 30°C for 1 h.
6. Dispense yeast into 200-µL aliquots in Eppendorf tubes.
7. Premix equal quantities of pBTM116/kinase DNA and library plasmid and add 4 µg total DNA (i.e., 2 µg DNA binding domain and activation domain) per 200 µL aliquot of yeast.
8. Add 800 µL 50% PEG 3350 in TE. Mix by inversion.
9. Incubate at 30°C for 30 min and then at 42°C for 20 min.
10. Centrifuge in Eppendorf centrifuge for a few seconds. Remove supernatant.
11. After a few seconds, reaspirate any remaining drops of PEG.
12. Resuspend yeast in 200 µL sterile PBS.
13. Plate 20 µL from one aliquot onto a drop-out plate lacking leucine and tryptophan to calculate transformation efficiency. Each of the remaining aliquots are plated onto separate 15-cm "triple" drop-out plates lacking leucine, tryptophan, and histidine and incubated at 30°C (*see* **Note 5**). Where initial test-transformation has indicated some constituutive activation of the *HIS*3 reporter gene, an appropriate amount of 3-AT should be included in the medium.

Transformation efficiency is calculated as the number of colonies growing on double-drop-out plates per µg DNA transformed (*see* **Note 6**). Depending on the library, 10^6–10^8 transformants should be screened. Colonies may be obvious on the triple drop-out plates by 1 wk, but we frequently leave the plates

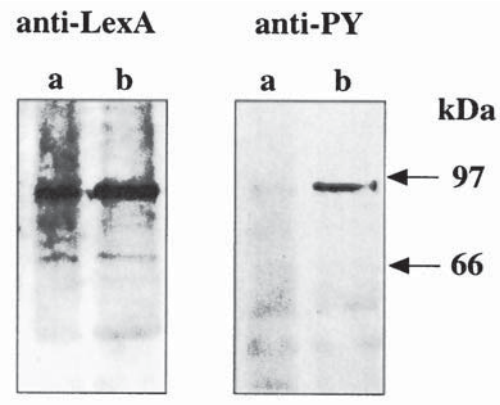

Fig. 4. Expression of LexA/kinase fusion in yeast. Extracts were prepared from yeast expressing the KDR intracellular domain **(A)** and a point mutation of the kinase in which the ATP-binding site was mutated **(B)**, as fusions with LexA. Proteins were analyzed by SDS-PAGE and immunoblotting with anti-LexA (Santa Cruz) and antiphosphotyrosine (PY) (Rc20, Transduction labs) antibodies. Immunoblots demonstrate that both proteins were expressed as LexA fusions, but only the receptor with native kinase activity was able to autophosphorylate.

for up to 3 wk to allow slow growing yeast to become apparent (*see* **Note 7**). Putative positives are restreaked onto double drop-out "master plates," grown overnight at 30°C and stored at 4°C. β-Galactosidease filter assays are then performed on these colonies, as described below (*see* **Note 8**).

3.5.2. Beta-Galactosidase Filter Assay

1. Lay Whatman grade-50 filters onto appropriate selection plates avoiding air bubbles, and allow to moisten.
2. Streak colonies onto filters and grow overnight at 30°C. Include a positive and negative control.
3. The following day, place Whatman grade-3 filters inside Petri dishes and saturate with Z-buffer/X-Gal substrate, carefully smoothing out air bubbles.
4. Remove filters containing yeast from selection plates and plunge into liquid nitrogen for 10 s to permeabilize cells.
5. Place filters containing permeabilzed yeast, colony-side up on top of filters soaked in substrate.
6. Place at 30°C for 15 min–24 h, until blue color appears.

3.5.3. Recovery of Library Plasmid from Positive Colony

1. Scrape off yeast from a colony patch (approx 1 cm × 0.5 cm) into 100 µL yeast lysis buffer + 200 µL phenol/chloroform/isoamyl alcohol (25:24:1 mixture). Add a small spatula full of glass beads.

2. Vortex for 2 min at maximum speed.
3. Centrifuge at room temperature for 5 min at 12,000*g*.
4. Transfer aqueous phase to an Eppendorf tube.
5. Add 0.1 vol 5 *M* potassium acetate + 2 vol of ethanol.
6. Incubate at room temperature for 2 min.
7. Centrifuge at room temperature for 5 min at 12,000*g*.
8. Rinse pellet with 70% ethanol, centrifuge, and remove supernatant.
9. Air-dry pellet and resuspend in 30 µL water.
10. Use 1 µL to electroporate *Leu-* bacteria (HB101) (*see* **Note 9**).
11. Spread bacteria onto LB/Ampicillin plates. Allow to grow overnight.
12. Spot 10–20 colonies onto M9 plates. Usually, approx 50% of colonies grow (*see* **Note 10**).
13. Pick five colonies from each positive, inoculate 5 mL LB containing 100 µg/mL ampicillin with each colony, grow overnight at 37°C.

3.5.4. Plasmid Miniprep

Any standard method for small-scale plasmid preparation can be used. Because the quality of the DNA is not too important for subsequent yeast transformation, we use the following modification of the alkaline lysis protocol using solutions 1, 2, and 3 as follows:

1. Pellet 1.5 mL each culture in Eppendorf tubes. Add 100 µL solution I. Resuspend by vortexing, incubate 5 min at room temperature.
2. Add 200 µL solution 2, invert 10 times, incubate 5 min at room temperature.
3. Add 300 µL solution 3, vortex, incubate 5 min at room temperature.
4. Add 600 µL 5 *M* LiCl, spin 5 min at room temperature. Remove 1 mL of supernatant into a fresh tube containing 600 µL isopropanol. Mix well and spin 5 min at room temperature.
5. Wash pellet with 1 mL 70% ethanol, air-dry for 2–5 min.
6. Resuspend in 40 µL TE.

3.5.5. Retransform Isolated Library Plasmid with Original Bait Plasmid and Negative Control to Verify Interaction

Purified library plasmids are retransformed into yeast together with the original bait, and with an unrelated protein. We use lamin in the pBTM116 vector as a negative control. Several plasmids recovered from each positive should be analyzed to ensure that the library plasmid giving the positive interaction has been isolated, as yeast are able to take up more than one library plasmid. Use 5 µL of miniprep DNA per transformation, together with 1 µg bait or control plasmid. A positive control transformation should also be performed at this stage. We use Ras and Byr2, which have previously been shown to interact in the two-hybrid system ***(16)***. Four colonies from each transformation are streaked onto filters for X-gal assay. Frequently, all five plasmids isolated give a positive in-

teraction, and only one of these need be sequenced. For sequencing, plasmids prepared as above should be purified on Qiagen "Quick-spin" columns.

3.6. Further Characterization of Positive Clones

Isolated plasmids that interact with the bait protein, but not the control protein, should be analyzed further by double-stranded DNA sequencing. A description of the procedure is beyond the scope of this review, however we routinely use Sequenase version 2.0 (Amersham). The primer sequence used will depend on the vector used for library construction. A comparison of the DNA sequence with the Genbank database frequently reveals the identity of the protein, and the identity of the protein may influence subsequent analysis. Whatever the identity of the protein, it is important to establish that the interaction is one which forms in vivo, for which additional complementary experiments are necessary.

3.6.1. Generation of GST-Fusion Proteins to Precipitate Receptor Tyrosine Kinase From Tissue-Culture Cells

We routinely express a GST-fusion protein of the protein fragment isolated in the yeast two-hybrid screen, and use this to precipitate ligand-stimulated, or unstimulated receptor from a suitable tissue-culture cell system. cDNA inserts from the pVP16 library can be conveniently isolated by *Bam*H1 and *Eco*R1 digestion and subcloned in-frame into the same sites in pGEX3x (assuming inserts do not contain these sites). For other libraries it may be necessary to isolate inserts by PCR to generate appropriate restriction sites.

3.6.2. Construction of Receptor Mutants to Determine Phosphorylation Dependence of Interaction and Binding Site

If the receptor appears to associate with the GST-fusion protein specifically in its ligand-activated form, or if the isolated protein contains SH2 or PTB domains, it is likely that the interaction is phosphorylation-dependent. This can be confirmed in the two-hybrid assay by generating a kinase-inactive mutant of the receptor kinase. The critical lysine residue at the ATP-binding site can be mutated (e.g., to alanine) and retested in the two-hybrid assay (*see* **Fig. 5**). The position of the critical lysine is usually documented in the Swiss Protein Database entry for the tyrosine kinase. We find the "Quick-Change" mutagenesis kit from Stratagene to be convenient for site-directed mutation. If the receptor autophosphorylation sites are known, these can be similarly mutated and the point-mutants assessed for their ability to interact with the protein of interest in the two-hybrid assay. Some caution should be used when interpreting the results, as some tyrosine kinases require phosphorylation at certain sites for kinase activity ***(19)***. When comparing the association of a putative effector

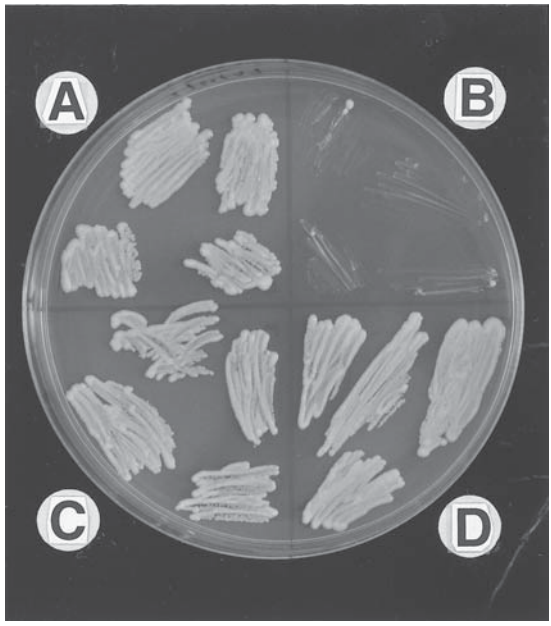

Fig. 5. Growth of L40 yeast expressing LexA-KDR and a VP16-SH2 protein on medium lacking histidine. KDR was shown to bind to an SH2-domain containing protein in a two-hybrid library screen (S. A. P., unpublished). When wild-type KDR is expressed as a LexA fusion together with the SH2-VP16 fusion, L40 yeast are able to grow in the absence of histidine **(A)**. A kinase-inactive KDR mutant is unable to bind the SH2-protein, and L40 yeast are unable to grow **(B)**. **(C)** and **(D)** represent yeast expressing phosphorylation-site point mutations of KDR that do not affect binding to the SH2-protein.

with various receptor mutants, we plate colonies onto X-gal plates and perform solution assays to assess the relative activation of the LacZ reporter.

3.6.2.1. Solution Assay for β-Galactosidase with ONPG Substrate

1. Seed 5 mL of drop-out medium lacking leucine and tryptophan with a single colony and shake overnight at 30°C.
2. Measure OD_{600}. Dilute culture to an OD_{600} of approx 0.2 and grow for 6 h at 30°C with shaking. Measure OD_{600}.
3. Wash, resuspend in 1 mL Z-buffer.
4. Remove 50 μL to fresh tube, freeze in liquid nitrogen, and thaw rapidly in water bath.
5. Set up blank containing 50 μL of Z-buffer.
6. Add 0.7 mL of Z-buffer containing β-mercaptoethanol, start timer, then add 0.16 mL ONPG solution (4 mg/mL in Z-buffer, prepared fresh, and dissolved by shaking for 1–2 h). Place at 30°C.

7. The solution should turn yellow in 20 min to 1 h. Weaker interactions could take as long as 24 h. When a distinct yellow color is attained, 0.4 mL of 1 *M* Na_2CO_3 is added to the tubes to stop the reaction, and the time elapsed recorded. The OD_{420} is then measured.

β-Galactosidase units are calculated by the following equation:

$$\text{Miller units} = 1000 \times OD_{420}/(t \times V \times OD_{600})$$

where t = time elapsed in minutes, V = 0.5 mL × concentration factor, and OD_{600} = absorbance at 600 nm of culture.

3.6.3. Coimmunoprecipitation of Receptor and Effector from Tissue-Culture Cells

Having prepared a GST-fusion protein of the receptor-associating protein (*see* **Subheading 3.6.1.**), a polyclonal antibody can be raised in rabbits. The antibody can be affinity-purified on a gluathione-agarose column containing bound fusion protein, and with a little luck will recognize the full-length protein in mammalian cells. The receptor can then be precipitated from appropriate ligand-stimulated and unstimulated mammalian cells using a receptor-specific antibody. Protein complexes are then analyzed by SDS-PAGE and immunoblotting, using the antibody raised against the fusion protein to detect the presence of the novel protein in receptor complexes.

4. Notes

1. Drop-out plates do not set if pH is not adjusted prior to autoclaving.
2. The pBTM116 vector has relatively few restriction sites for cloning. Where inserts have internal *Bam*HI and *Sal*I sites it is useful to remember that *Bgl*II and *Xho*I-digested fragments can be cloned into *Bam*HI and *Sal*I sites, respectively.
3. The protocols described can be used to determine whether known signaling proteins containing SH2 and PTB domains can interact with a tyrosine kinase of interest. cDNA for the putative signaling components can frequently be generated by RT-PCR from an mRNA source, or are sometimes available as EST clones. cDNAs are inserted into an appropriate activation-domain vector, transformed into yeast with the receptor bait construct, and reporter activation assessed as described.
4. Because plates do not contain antibiotics and colonies can take 2 wk to form on the library plates, contamination is frequently a problem. Contamination by bacteria is minimized by culturing yeast at pH of 5.8–6.0 as most bacteria will not grow well at this pH . Plates should, if possible, be poured in a sterile hood, and be properly dried before use. When plating yeast avoid working beneath air vents, and if possible use a dedicated yeast incubator for growth.
5. Other workers have recommended a recovery period following introduction of the plasmid constructs into the L40 yeast strain to allow expression of the

*HIS*3 gene. In this case yeast are grown in liquid culture overnight in selection medium lacking leucine, tryptophan and uracil prior to plating on plates lacking histidine ***(4)***. This has the possible disadvantage that the population of transformants could become skewed. We have omitted this step and have been able to detect interactions with various different baits, however it is possible that we have missed interactions by omitting this recovery period.

6. Most investigators use carrier DNA, or yeast RNA to increase the efficiency of transformation. From 500 mL culture using the protocol described here, 0.5 to 1 million transformants are usually obtained. To improve this efficiency, 100 µg single-stranded carrier DNA can be added per µg of plasmid DNA used in a transformation. Herring testes DNA "ready for use" can be purchased as a 10 mg/mL solution from Clontech, and is denatured prior to each use by incubating in a boiling water bath for 15 min. Transformations can also be performed in one batch to avoid extensive pipeting, however, we have found that by using large numbers of small tubes, contamination is reduced.
7. When peforming library transformations, we sometimes observe many small colonies growing on the selection plates (*-Leu*, *-Trp*, *-His*). These usually remain tiny and do not interfere with the detection of "positive" colonies, which continue growing to several millimeters in diameter.
8. The filter assay is the most sensitive method to detect β-galactosidase activity, however differences in the ability of two proteins to interact are more readily reflected in the solution assay, and by growth on X-gal plates. For example when comparing the ability of two mutant proteins to interact with a binding partner, a similar intensity may be observed in the filter assay, but differences may be detected in a solution assay or by growth on X-gal plates.
9. Electroporation should be used to introduce DNA isolated from positive yeast back into *Escherichia coli* for selection of library plasmid, as chemical transformation is not efficient enough.
10. Occasionally, it is not possible to isolate a positive library plasmid from the original positive clone. In some cases this may be caused by the plasmid integrating into the yeast genome, and it may be possible to recover the insert by PCR. In other cases there is no obvious explanation why a "positive" library plasmid cannot be recovered.

Acknowledgments

The author would like to thank Linda van Aelst, Cold Spring Harbour, from whom many of the protocols desribed here originated. She is also grateful to T. S. Pillay for reading the manuscript, and for his useful suggestions.

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19

cDNA Expression Cloning and Characterization of Phosphorylation Dependent Protein Interactors Using the Yeast Tribrid System

Christoph Volpers, Manuel Lubinus, Mark A. Osborne, and Jarema P. Kochan

1. Introduction

The yeast two-hybrid system has been utilized by many laboratories in the characterization of protein–protein interactions that occur in eukaryotic and prokaryotic cell systems. In addition, the two-hybrid system has been used to isolate and characterize novel interacting proteins to aid in the understanding of biochemical signaling pathways for various receptors and enzymes. The interacting components are fused to inert components of the transcriptional apparatus. One of the components is fused to a DNA-binding domain and is generally referred to as the "bait." The second protein is fused to a transcriptional activation domain and is referred to as the "fish" or the "prey." When there is an association between the "bait" and the "prey," the DNA-binding domain and the transcriptional-activation domain are brought into close proximity to activate the transcription of the reporter gene.

As the experience with the yeast two-hybrid system has grown, as have the needs to introduce various improvements and modifications to the original system described by Fields and Song in 1989 ***(1–3)***. In the study of cellular signaling, modification of proteins by various enzymes such as protein-tyrosine kinases (PTKs), serine/threonine kinases, glycosyltransferases, acylating enzymes, as well as other enzymes play critical roles. As a result, these modifications facilitate novel interactions and stimulate a series of biochemical cascades that follow extracellular and physiological stimuli. One of the best studied post-translational modifications is that of tyrosine phosphorylation of the intracel-

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

lular domains of cell-surface receptors and other signaling proteins following activation by ligands ***(4,5)***. There is very little, if any, protein tyrosine phosphorylation that occurs endogenously in *Saccharomyces cerevisiae*. Therefore, protein–protein interactions that are dependent on phosphorylated tyrosines will not be detected. Because the two-hybrid system utilizes *S. cerevisiae* as its host, only those activities present in the yeast cytosol or nucleus will be able to modify the two fusion partners. In addition, these modifying enzymes must be able to specifically modify the proteins of interest. Because of enzyme specificity, even modifications that occur endogenously in yeast may not be able to modify exogenous proteins. Therefore, the ability to specifically modify sites on proteins provides a tremendous advantage.

S. cerevisiae has been shown to express dual-specificity kinases ***(6,7)***, but to date, no monospecific tyrosine kinases have been described. Others have demonstrated that the two-hybrid system will work well with autophosphorylating tyrosine kinases as baits ***(8–12)***, but what of proteins that are tyrosine-kinase substrates but not kinases themselves? Mammalian two-hybrid systems have been described ***(13–15)***, but these are impractical for screening large numbers of transfectants, which is necessary to identify rare interacting clones. In addition, it is not clear whether the proteins of interest will be modified in order to facilitate the protein interactions desired in a two-hybrid system.

We and others have solved this problem by the introduction of a third component, a PTK, which then *trans*-phosphorylates substrates in the yeast cell (**Fig. 1**). The series of vectors that have been developed permits the investigation of phosphotyrosine-dependent signal transduction pathways, which are often critical in higher eukaryotic cell function ***(16,17)***. The design of the vectors will also accomodate virtually any other enzyme that is involved in posttranslational modification, or other proteins that are allosteric modulators resulting in the formation of multisubunit complexes.

In this chapter, we describe the three-component approach, the yeast-tribrid system, to investigate tyrosine phosphorylation. This modification is only a first example, as it should be possible to incorporate any posttranslational modification or allosteric regulation that is necessary to facilitate protein interactions. We hope that other approaches will be investigated in the future. The vectors described for the yeast-tribrid system are readily available to interested researchers.

2. Materials

2.1. Plasmid Constructs

Three different plasmid constucts are used in the tribrid system to direct the synthesis of the "bait" fusion protein, the cDNA-activation domain fusion protein, and the PTK in yeast cells (**Fig. 2**). All three plasmids contain a strong

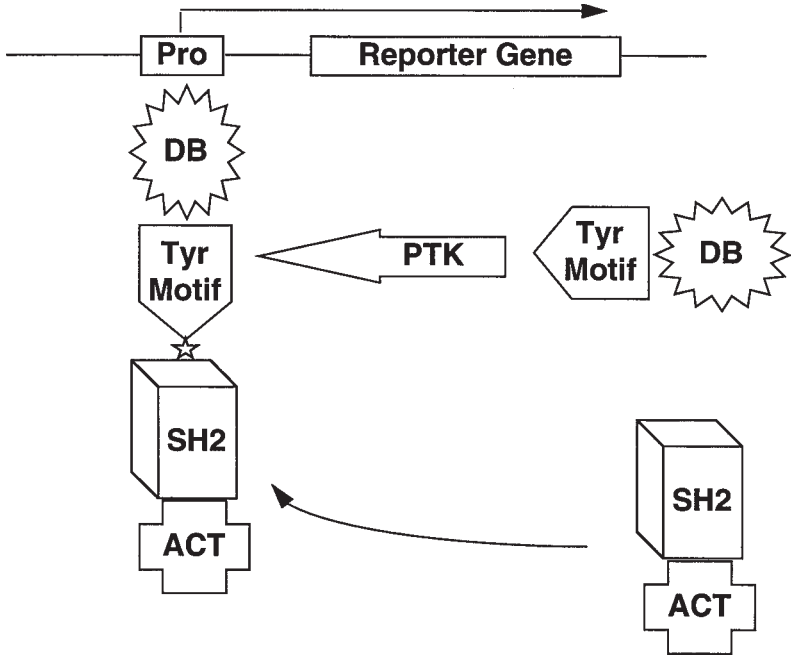

Fig. 1. Schematic description of the yeast tribrid system. Phosphorylation of a specific tyrosine motif within the fusion construct of the "bait" protein of interest and a DNA binding domain (DB, e.g., LexA) by a protein-tyrosine kinase (PTK) provides a binding site for a phosphorylation dependent interactor, typically an SH2 domain. (The phosphorylated tyrosine residue is represented by a star.) The bimolecular complex formed by interaction with the SH2-transcriptional activation domain (ACT, e.g., herpes simplex virus Vmw65) fusion protein binds to the promoter/operator (Pro) and activates transcription of a reporter gene as LacZ encoding β-galactosidase. The absence of tyrosine phosphorylation results in no interaction and therefore no β-galactosidase production in the assay.

inducible pGAL10/CYC1 hybrid promoter ***(18,19)***, a multiple cloning site, and the 3' transcriptional termination signal of the *CYC*1 gene ***(19,20)***. The galactose-inducible promoter allows recovery of transformants without the expression of their encoded proteins, a significant advantage for use with proteins that might be toxic or confer a growth disadvantage to the host cell. In the plasmid p4402, the DNA-binding domain of the bacterial *LexA* gene, and the nuclear localization signal of SV40 T antigen ***(21)***, is fused in-frame to the DNA encoding the protein of interest or "bait."

The cDNA library to be screened is cloned into the vector p4064 in frame with the transcriptional-activation domain. This activation moiety is amino ac-

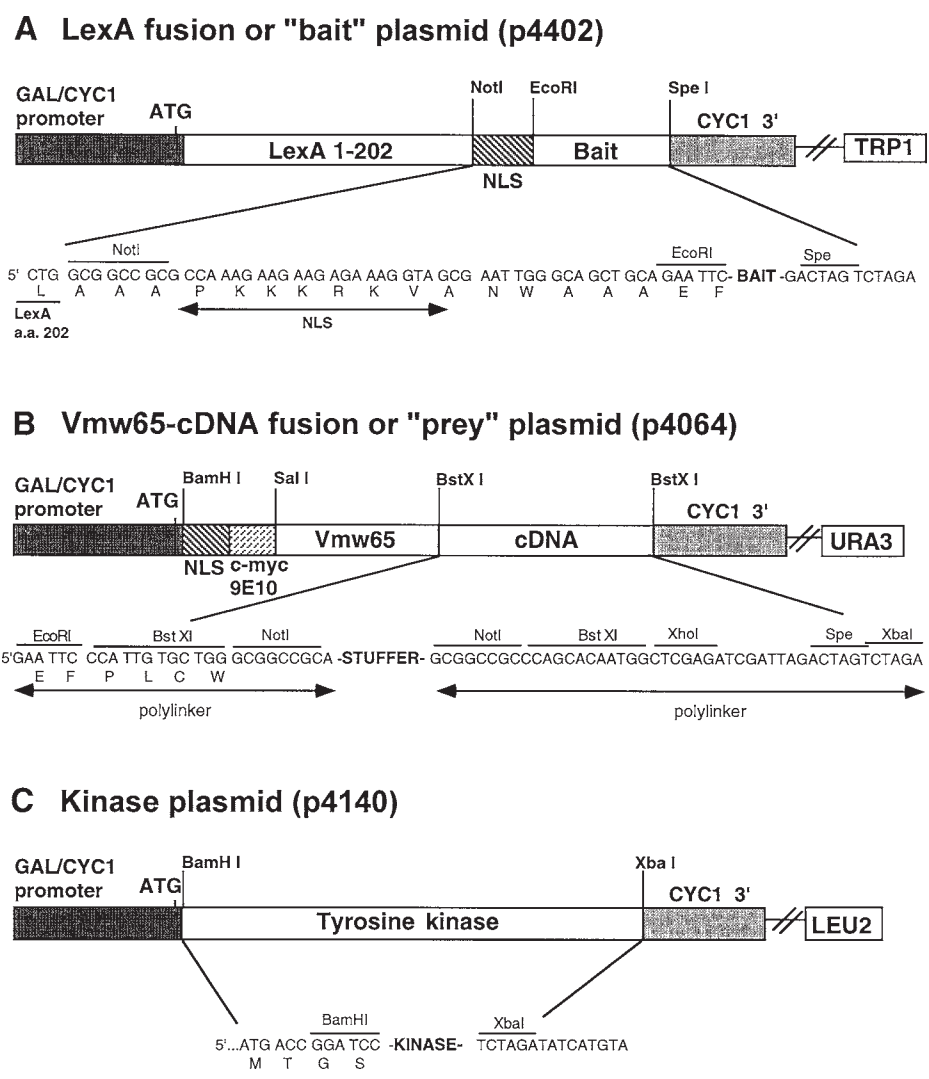

Fig. 2. Plasmids used for the galactose-inducible expression of the "bait" fusion protein (p4402), the cDNA-activation domain fusion protein (p4064) and the PTK (p4140) in yeast cells. For details, *see* **Subheading 2.1**. *CYC1*, transcription termination sequence of the yeast CYC1 gene; NLS, nuclear localization sequence from SV40 T antigen; c-*myc* 9E10, epitope tag.

ids 410–490 of the Vmw65 protein of herpes simplex virus type I (HSV-1) ***(22)***, which is simlar to the HSV2 protein VP16. The cDNA library can be inserted via 5'-phosphorylated *Bst*XI linkers; we use a 12-*mer* and a 8-*mer* (sequence: 5'-P-CTTTAGTGCACA-3' and 5'-P-CTCTAAAG-3', respectively)

which are blunt-end ligated to the cDNA. The fusion protein synthesized is targeted to the nucleus via the SV40 nuclear localization signal. p4064 is selected via the *URA3* marker.

The tyrosine kinase plasmid p4140 contains a *LEU2* selectable marker and unique *Bam*HI and *Xba*I restriction sites for insertion of the kinase encoding cDNA sequence. The details of the construction of these plasmids have been published ***(17,19,23)***.

For expression of Flag-tagged cDNA sequences in COS7 cells, the eukaryotic expression vector pDF-Flag, a pEF-BOS derivative, is used ***(24)***. The cDNA sequence is inserted into a *Sma*I site at the 3' end of a vector sequence encoding a 10 amino acid Flag epitope such that this epitope is at the N-terminus of the protein expressed.

2.2. Sequencing Primers

The following primers have been used to sequence the plasmid constructs described:

1. *LexA* primer: anneals approx 75 bp 5' to the polylinker - 5' TCGTTGACCTTCGTCAGCAGAGCTTCA 3'
2. Vmw65 primer: anneals approx 50 bp 5' to the polylinker - 5' TCGAGTTTGAGCAGATGTTTACCGATG 3'
3. GAL10/CYC1 5' primer: for kinase inserts. Anneals 30 bp 5' to *Bam*HI site - 5' TTACTATACTTCTATAGACACGCA 3'
4. CYC1 3' UTR primer: will sequence from the 3' end of the polylinker of all three plasmids. Anneals 25 bp 3' to the *Xba*I site - 5' GAGGGCGTGAATGTAAGCGTGAC 3'

2.3. Microbial Strains

1. The *S. cerevisiae* tribrid reporter strain is S-260 (MATα ura3::Col E1 operator (x6)-*LacZ leu*2-3,112 *trp*1-1 *ade*2-1 *can*1-100 ho).
2. For mating assays, W303-1a (*MATa leu*2-3, 112 *trp*1-1 *his*3-11, 15 *ura*3-52 *can*1-100 ho) is used.
3. The *Escherichia coli* strain DH10B (F^- mcrA Δ(mrr-hsdRMS-mcrBC) ø80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K *rps*L *nup*G) is used for generation of the cDNA library.
4. *E. coli* strain KC8 (pyrF::Tn5 *hsd*R *leu*B600 *trpc*9830 *Lac*D74 *str*A *gal*K *his*B436) is used for rescue of library plasmids from S-260 by electroporation.
5. *E. coli* strain XL-2 (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*[F' pro AB $lacI^q$ ZΔM15 Tn10 (Tet^r) Amy Cam^r]) is used for high-yield plasmid DNA preparation.

2.4. Media

1. YPDA (rich media): 10 g yeast extract, 20 g bacto peptone, 20 g glucose, 30 mg adenine sulfate per liter. 15 g agar added for plates.

2. Yeast selective media: modified from Sherman, 1991 ***(25)***:
   Per 900 mL:
   6.7 g yeast nitrogen base (without amino acids)
   1.2 g drop-out mix (*see* below)
   20 g agar (for plates)
   0.2 mL 10 *N* NaOH to raise the pH to approx 6.0 (which greatly assists the hardening of agar).
   After autoclaving, the carbon source is added to the media before use by the addition of 20% (w/v) glucose or 20% (w/v) galactose to a final concentration of 2%.
   Drop-out mix: Prepare a powder mix of:
   0.4 g adenine, 0.4 g uracil, 0.4 g tryptophan, 0.4 g histidine, 0.4 g arginine, 0.4 g methionine, 0.6 g tyrosine, 0.6 g lysine, 1 g phenylalanine4 g threonine, 0.6 g isoleucine, 2 g valine, 2 g aspartic acid, 1.2 g leucine, 2 g glutamic acid, 8 g serine.
   For Trp^- and/or Ura^- and/or Leu^- media, the respective components are omitted from the drop-out mix.
3. Uracil selection plates for KC8 cells: mix 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 g casamino acids, and 15 g agar in 1 L of water. Autoclave for 20 min, when cool add 1 mL 1 *M* $MgSO_4$, 10 mL 20% (w/v) glucose, 0.1 mL 1 *M* $CaCl_2$, and 5 mL 4 mg/mL tryptophan (all filter- sterilized). Ampicillin is added to 100 μg/mL.

2.5. Solutions

Solutions for yeast transformation (sterilized by filtration):

1. LiAcetate buffer: 1× TE, LiAcetate made fresh from:
   10× TE: 0.1 *M* Tris-HCl, 0.01 *M* ethylenediaminetetracetic acid (EDTA), pH 7.5; 10× LiAc: 1 *M* LiAcetate, pH 7.5
2. Polyethylene glycol (PEG) solution: 40% PEG, 1× TE, 1× LiAc made fresh from 50% PEG4000, 10× TE, 10× LiAc.
3. Phosphate-buffered saline (PBS): 150 m*M* NaCl, 16 m*M* NaH_2PO4, 4 m*M* Na_2HPO_4, pH 7.3.
4. TPBS: 0.1% Tween-20 in PBS.
5. Blocking buffer: 2.5% BSA in PBS.
6. Tris/dithiothreitol (DTT) solution: 0.1 *M* Tris-HCl, pH 9.4, 10 m*M* DTT.
7. Enzymatic lysis solution: 1.2 *M* sorbitol, 20 m*M* HEPES, pH 7.4, containing 0.25 mg yeast lytic enzyme (ICN # 360942; also known as Zymolyase), and 15 μL of glusulase (NEN # NEE154) per mL.
8. PBS/Triton: 1% Triton X-100 in PBS (protease inhibitors and 1 m*M* $NaVO_4$ as phosphatase inhibitor may be helpful).
9. Lysis buffer for plasmid preparation from yeast cells: 2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
10. X-Gal solution: 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethyl formamide (store in glass or polypropylene tube at –20°C).

11. Zeta buffer: mix 16 g Na_2HPO_4, 5.5 g NaH_2PO_4, 0.75 g KCl, 0.25 g $MgSO_4$, 2.7 mL β-mercaptoethanol in 1 L of water, adjust to pH 7.0. Store at 4°C. Make fresh 1/20 dilution of X-Gal in this buffer immediately before use.
12. Lemmli sample buffer (1×): 60 m*M* Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, 0.001% bromophenol blue.
13. Lysis buffer for COS cells: 10 m*M* CHAPS in PBS plus protease inhibitors (30 μg/mL aprotinin, 200 μg/mL PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin) and phosphatase inhibitors (1 m*M* Na-orthovanadate, 50 μ*M* pervanadate, 1 m*M* NaF).

2.6. Reagents and Materials Purchased from Commercial Sources

1. Large Bio-assay dishes (245 × 245 mm) used for the cDNA library construction and the primary screening are manufactured by Nunc (cat. no. 166508).
2. For the β-galactosidase filter assays, reinforced nitrocellulose must be used, because unsupported nitrocellulose shatter easily. Schleicher and Schuell (grade BA-S), Micron Separations (NitroPure) and Sartorius (reinforced cellulose nitrate) supported nitrocellulose have been used successfully.
3. Whatman 3M paper (Schleicher and Schuell).
4. Glass beads (425–600 microns, acid-washed) for yeast DNA isolation can be purchased from Sigma (cat. no. G-8772).
5. Antiphosphotyrosine antibodies: Upstate Biotechnology (cat. no. 05-321).
6. Anti-Flag antibodies: Eastman Kodak Co. (M2, cat. no. IB13010).
7. Peroxidase-conjugated antimouse IgG: Jackson Laboratories (cat. no. 115-035-150).
8. Peroxidase-conjugated antirabbit IgG: Jackson Laboratories (cat. no. 711-035-152).
9. Enhanced chemiluminescence (ECL) reagent: DuPont NEN.
10. RNAzol B: Biotecx Laboratories (Houston, TX).
11. Oligotex-dT Kit: Qiagen (Chatsworth, CA).
12. Superscript Choice System for cDNA Synthesis: Life Technologies (Gaithersburg, MD).
13. Sephacryl S-500 columns (cDNA size fractionation columns): Life Technologies.
14. Electroporation cuvets for *E. coli* (0.1-cm gap): Bio-Rad (Hercules, CA).

3. Methods

3.1. Expression of Bait Fusion Construct

The first step is usually the subcloning of the bait and the tyrosine kinase cDNAs into the lex-A vector p4402 and the kinase vector p4140, respectively. All subclonings can be performed by standard procedures ***(26)***. The correct in-frame fusion of the lex-A domain and the bait cDNA should be confirmed by sequencing and by Western blot analysis using an anti-LexA antibody or a bait-specific antibody if available (*see* **Note 1**).

After transformation into *S. cerevisiae* S-260 (*see* **Subheading 3.5.**), the expression of the lexA fusion in the yeast cells has to be confirmed.

1. Transform S-260 with the LexA-fusion protein expression vector. Select colonies on Trp^- glucose plates.
2. Pick a colony, inoculate 5 mL Trp^- glucose liquid medium, and grow overnight at 30°C.
3. Centrifuge cells at 3000*g* for 5 min. Wash cells in 10 mL sterile H_2O, and measure the OD_{600} of the cell suspension (*see* **Note 2**).
4. Centrifuge 2 OD units each in two tubes and resuspend the cells in 5 mL Trp^- glucose medium (as uninduced control) and 5 mL Trp^- galactose medium (for induction), respectively, to a final OD_{600} of 0.4.
5. Grow cells for 4–6 h at 30°C (shaker at 200 rpm or incubation wheel).
6. Pellet cells at 3000*g* for 5 min. Wash the cell pellet with 1 mL H_2O. Transfer cells to a microcentrifuge tube, spin 15 s at maximum speed and remove the supernatant (*see* **Note 3**).
7. Add 100–200 µL of 1× Lemmli sample buffer to each pellet, resuspend by pipeting up and down, and boil for 5 min.
8. Centrifuge the lysates 2–5 min in a microcentrifuge at maximum speed to remove unlysed cells and debris. Analyze the lysates for LexA-fusion protein expression by Western blotting *(27)*.

3.2. Activity of the Tyrosine Kinase

The expression and activity of the protein tyrosine kinase in yeast cells transformed with the kinase expression construct can be confirmed by probing Western blots of cell lysates, prepared as described above (**Subheading 3.**1), with antiphosphotyrosine antibodies. Most protein-tyrosine kinases will phosphorylate a wide variety of endogenous yeast proteins. In addition to Western blotting, we have also successfully used a rapid method for directly probing yeast cell colonies or patches for the expression of PTKs ("spot blot"). This technique allows us to determine whether yeast proteins are being modified by tyrosine phosphorylation.

1. Use the wide side of a toothpick to streak out a patch (about 1 cm in diameter) of S-260 cells transformed with the kinase plasmid on Leu^- glucose plate and grow overnight (you can also use the $Trp^-Ura^-Leu^-$ glucose plates from mating experiments if you want to reconfirm the kinase activity in mating experiments, *see* **Subheading 3.8.**).
2. Overlay with round reinforced nitrocellulose membrane, allow to completely wet from beneath, then transfer to Leu^- galactose plate for 18 h at 30°C.
3. Freeze nitrocellulose membrane for 5–10 s in liquid nitrogen, let it dry on paper towels, and wash in PBS to remove cell debris from the nitrocellulose.
4. Block for 30 min in 2.5% BSA in PBS, then wash 2 min in PBS (*see* **Note 4**).
5. Incubate for 1 h with antiphosphotyrosine antibody (1:7500 in TPBS), then wash 3× 5 min in TPBS.
6. Incubate for 45 min with peroxidase-conjugated antimouse IgG (1:20,000 in TPBS), then wash 3× 5 min in TPBS.
7. Develop with ECL detection reagent, and perform autoradiography.

As very little, if any, endogenous tyrosine phosphorylation is observed in yeast cells, a positive signal is caused by overexpression of an active exogenous kinase. **Figure 3A** shows the result of such a "spot blot" screening for enzymatic activity of the products of five different kinase constructs. After induction by galactose, tyrosine phosphorylation was detected in yeast cells transformed with an lck, a src kinase domain, and a jak3 kinase domain expression plasmid, respectively.

Our examples have focused on protein-tyrosine kinases, but different enzymes that modify proteins can be used. These can include serine/threonine kinases, glycosidases, acylases, ubiquinating enzymes, and so on. For each enzyme, different techniques can be used to confirm posttranslational modification. In some instances, endogenous enzymes may perform some of the modifications, whereas in others it may not be so.

3.3. Transphosphorylation and Suitability of the Bait

Protein-tyrosine kinases vary in their substrate specificity. We have observed that different kinases give different patterns of tyrosine-phosphorylated yeast proteins. That can be clearly seen in **Fig. 3B**. After it has been confirmed that the lexA fusion protein and an active tyrosine kinase are expressed, it is important to verify that the tyrosine kinase is phosphorylating the bait. **Figure 3C** demonstrates the result of a mating experiment in which three tyrosine kinase constructs (lck, src kinase domain, and jak3 kinase domain) are tested for their ability to phosphorylate immunoreceptor tyrosine-based activation (=ITAM) motifs in the FceRI beta subunit (LexA-beta) and the T-cell receptor zeta chain (LexA-zeta), respectively, as binding sites for the SH2 domain of fyn. Strains grown with the LexA nonfusion protein that contains no ITAMs were used as negative controls. All three kinases are active in yeast cells (*see* **Fig. 3A**), however, only lck provides phosphorylated binding sites for fyn in both baits, whereas the src kinase domain phosphorylates the respective site only in lexA-beta, and the jak3 kinase domain does not show specificity for either of these sites. These results highlight the importance of protein tyrosine kinase specificity, and demonstrate the need to test several PTKs for their ability to phosphorylate a specific bait (*see* **Note 5**). Similar control experiments will be necessary with other enzymes that posttranslationally modify other baits.

Immunoprecipitation with bait-specific or anti-LexA antibodies, followed by immunoblotting with antiphosphotyrosine antibodies, is the method of choice to show whether transphosphorylation of the LexA fusion protein has occurred. It may be necessary to test several different kinases in order to ascertain the best for phosphorylation of a particular bait (*see* **Note 6**).

1. Inoculate 5 mL Trp^-Leu^- glucose liquid medium with a yeast colony bearing the LexA fusion plasmid and kinase plasmid and grow overnight at 30°C.

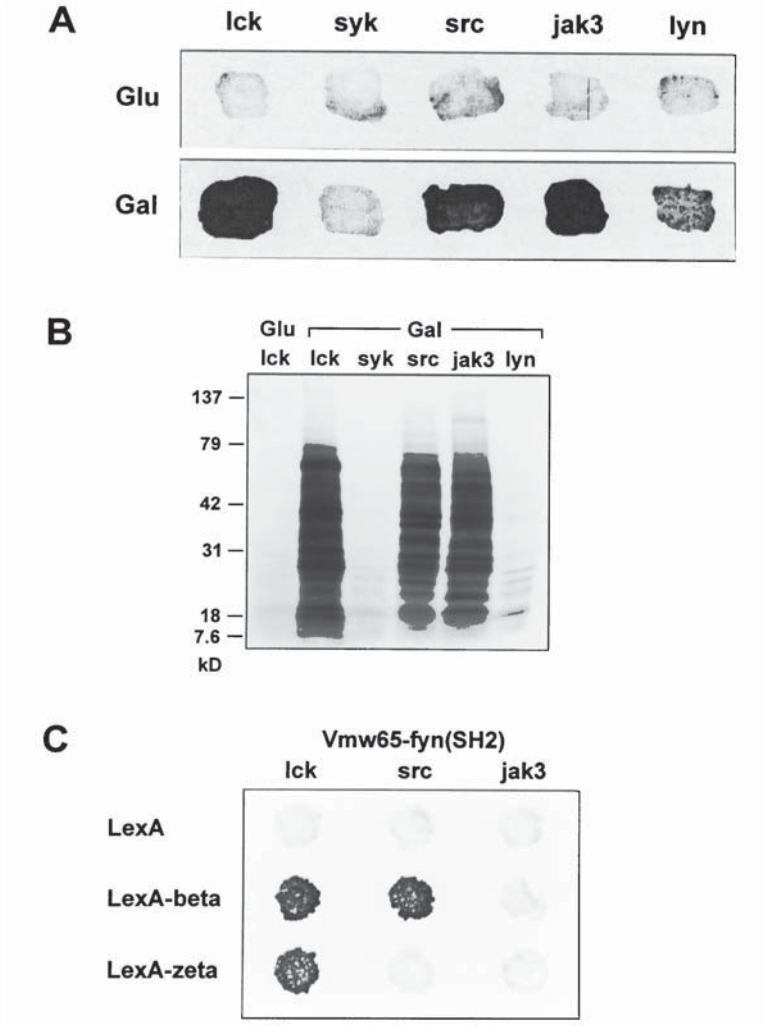

Fig. 3. Activity and specificity of protein tyrosine kinases. **(A)** "Spot blot" of yeast colonies with antiphosphotyrosine antibodies. S-260 yeast cell clones transformed with expression plasmids for lck, syk, src kinase domain, jak3 kinase domain, and lyn, respectively, were patched on a Leu$^-$ glucose plate, grown overnight, and then transfered via nitrocellulose filter to glucose (Glu) or galactose (Gal) plates. After incubation for 18 h at 30°C the nitrocellulose filters were processed as described in **Subheading 3.2**. **(B)** Western blot of cell lysates of S-260 clones transformed with expression plasmids for lck, syk, src kinase domain, jak3 kinase domain, and lyn, respectively, grown in liquid glucose media (Glu) or galactose media (Gal), as described in **Subheading 3.1**. The blot was developed with antiphosphotyrosine antibodies. *(cont.)*

2. Induce protein expression in a 10 mL Trp^-Leu^- galactose culture, as described in **Subheading 3.1.**, **steps 3–5**.
3. Pellet cells by centrifugation at 3000*g* for 5 min at room temperature and wash with 10 mL sterile H_2O.
4. Centrifuge again, wash with 1 mL sterile H_2O, and spin down in preweighed sterile microcentrifuge tubes. Remove the supernatant and weigh the tubes, recording the mass of the wet cell pellet.
5. Resuspend the pellet in 2 mL/g wet mass of Tris/DTT solution. Incubate at 30°C for 15 min.
6. Centrifuge for 2 min at 6000*g* and resuspend the pellet in 5 mL/g wet mass of enzymatic lysis solution. Incubate at 30°C with occasional rocking for 15–45 min.
7. Check for spheroplasting by microscopic inspection: mix 10 µL of cell suspension with 10 µL of H_2O and see if there are any cells remaining. There should be plenty of membrane ghosts and only few unlysed cells. If not, continue incubating for up to 1 h.
8. Pellet spheroplasts by centrifugation at 6000*g* for 5 min at room temperature in a microcentrifuge.
9. Resuspend pellet in PBS/Triton X-100 and pipet up and down to fully resuspend spheroplasts. Incubate on ice for 5 min. Suspension should be clear, not cloudly like a suspension of cells. Check for lysis by microscopic examination if desired.
10. Perform an immunoprecipitation using bait-specific or anti-LexA antibodies. Analyze immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with antiphosphotyrosine antibodies (*see* Chapter 2, this volume).

Finally, it is important to verify that the LexA fusion protein does not activate transcription of the reporter gene on its own. This must be done in the presence and absence of the kinase; some LexA fusion proteins do not activate transcription unless they are coexpressed with a tyrosine kinase. The self-activation test can be performed by a β-galactosidase filter assay (*see* **Subheading 3.6.**, **step 2**) using the original transformation plates of the lexA fusion and LexA fusion/kinase constructs, respectively.

Fig. 3. **(C)** Mating experiment demonstrating the substrate specificity of different tyrosine kinases. W303-1a strains carrying expression plasmids for LexA alone, a fusion protein of LexA and FceRI beta subunit (LexA-beta), and a fusion protein of LexA and the T-cell receptor zeta chain (LexA-zeta), respectively, were mated to S-260 transformants containing plasmids encoding a Vmw65-fyn kinase SH2 domain fusion protein and a kinase expression plasmid for lck, src kinase domain, or jak3 kinase domain. The diploids grown on full media were replica plated on glucose selection media plates, induced on galactose selection media, and a β-galactosidase assay was performed as described in **Subheading 3.6**.

3.4. cDNA Plasmid Library Construction

In many cases, it might be necessary to construct a new cDNA library in the Vmw65 fusion protein vector in order to screen successfully for interactors of a particular protein of interest. Libraries from tissues expressing the bait coding sequence can be assumed to contain its interacting partner(s) as well. For the construction of cDNA libraries from various cell lines and tissues, we used commercially available reagents and kits: After isolation of total RNA from the cells or tissues with RNAzol B, poly-A mRNA was prepared by use of Oligotex-dT affinity purification. First- and second-strand cDNA synthesis incorporating ^{32}P-dCTP were performed according to the instructions of the "Superscript Choice System for cDNA Synthesis." The cDNA fragments obtained were blunt-end ligated to *Bst*XI linkers, separated by size on Sephacryl S-500 columns, and aliquots of the fractions were run on an agarose gel to determine their size. After autoradiography, fractions with an average cDNA fragment size over 1 kb were pooled, precipitated, and aliquots ligated to *Bst*XI-cut Vmw65 fusion vector in various insert/vector ratios. The two *Bst*XI sites of p4064 are designed to contain different overhang sequences in order to avoid self-ligation. The ligation reactions are ethanol precipitated and electroporated into *E. coli* DH10B cells. The insert/vector ratio resulting in highest clone numbers per ng cDNA is scaled up for ligation and transformation of the remaining cDNA. After plating the cell suspension on large (245 × 245 mm) LB/Amp plates to allow for sufficient growth — and, therefore, representation within the library — of each individual clone, the colonies are rinsed from the plates with PBS and large-scale plasmid preparations are performed. For details and further considerations concerning cDNA plasmid library construction, *see* **refs. *28*** and ***29***.

3.5. Pilot and Large Scale Yeast Transformation

For screening of the cDNA library in the Vmw65 fusion vector for interactors of the protein of interest, the cDNA library has to be transformed into S-260 cells carrying the LexA fusion and kinase plasmids. A pilot transformation performed as a small-scale version of the protocol described below will give an estimate of how many colonies to expect per μg of library DNA. We have plated a 1:10, 1:100, and 1:1000 dilution of the cell suspension on small (100-mm diameter) plates and typically obtained 4000 to 5000 colonies per μg of cDNA library. This number can then be used to scale up the transformation for the screen. We have found that plating 20,000 colonies per large (245 × 245-mm) plate results in the best cell density to work with and usually have processed 10 to 20 plates at a time. If more than 400,000 colonies are to be screened, transformations performed on successive days may be advisable.

The following protocol has been used for the large-scale library transformation:

1. Inoculate 5 mL Trp^- Leu^- glucose liquid media with a S-260 transformant containing the LexA-bait fusion and the tyrosine kinase plasmid. Grow the culture at 30°C with shaking overnight.
2. Inoculate 300 mL Trp^- Leu^- glucose media in a 2-L flask with the overnight culture and grow for 4–5 h at 30°C and shaking at 220 rpm. The final cell density should be 3–5 × 10^6 cells/mL, corresponding to an OD_{600} of 0.9 to 1.1.
3. Centrifuge culture for 10 min at 500 g in six 50- mL polypropylene tubes in a bench-top centrifuge.
4. Wash each cell pellet in 20 mL sterile H_2O and centrifuge at 2000*g* for 5 min at room temperature.
5. Resuspend all cell pellets in a total volume of 1.5 mL LiAcetate buffer.
6. Mix the appropriate amount of library DNA to generate 200,000 colony forming units (20,000 cfu per plate × 10 plates), 60 µL salmon sperm DNA (10 µg/µL), and sterile H_2O to a total volume of 600 µL. Aliquot to three 15-mL polypropylene tubes.
7. Add 500 µL cell suspension to each of the three tubes. Add 3 mL PEG solution to each tube and mix gently.
8. Incubate at 30°C for 30 min on shaker at 200 rpm.
9. Heat shock by incubation at 42°C for 15 min.
10. Mix gently and plate 1 mL of the cell suspension per large (245 × 245 mm) Trp^- Ura^-Leu^- glucose plate, 10–12 large plates altogether (*see* **Note 7**). In addition, plate 100 µL of a 1:100 and a 1:1,000 dilution of the transformation mixture on small (10 cm) $Trp^-Ura^-Leu^-$ glucose plates, in order to estimate the total number of colonies screened.
11. Incubate the plates at 30°C for 24–36 h.

3.6. Primary Screening

In addition to the items discussed above, and the repertoire of the cDNA library, the success of the primary screening depends largely on the number and size of the yeast colonies on the plates. The colonies should be clearly visible but not touching, i.e., not exceeding about 1 mm in diameter.

1. Overlay plates with reinforced nitrocellulose (22 × 22-cm) sheets. Allow to wet completely from beneath. Then transfer nitrocellulose filters, colony-side up, to large $Trp^-Ura^-Leu^-$ galactose plates and incubate for 18 h at 30°C (*see* **Note 8**).
2. Perform β-galactosidase filter assay: Immerse nitrocellulose filter for 5–10 s in liquid nitrogen, then allow to dry at room temperature on paper towels for 10–15 min (*see* **Note 9**). Cut pieces of Whatman 3M paper to fit in the lid of the Nunc bioassay dishes and saturate with a 1:20 dilution of X-Gal in Zeta buffer (about 25 mL needed per plate). Place the nitrocellulose filter on the Whatman paper and cover with the bottom part of the plates so that the filters do not dry out. Add more X-Gal solution, if needed, in order to maintain an appropriate level of moisture during the color development step (*see* **Note 10**).

3. Pick blue colonies as they appear with a sterile toothpick and patch onto a small $Trp^-Ura^-Leu^-$ glucose plate (*see* **Note 11**). Blue colonies will typically appear 15 to 90 min from the start of the assay; afterward it becomes increasingly difficult to discern "true" positives from the emerging background of blue colonies (*see* **Note 12**).
4. Incubate the small $Trp^-Ura^-Leu^-$ glucose plates for 2–3 d at 30°C.

3.7. Colony Purification (Secondary Screening)

In general, it is not feasible to scrape the blue colony from the filter without touching neighboring colonies, especially as the colonies tend to "sweat" and slightly smear when they warm up after the liquid nitrogen permeabilization step. Each yeast patch grown on the plates generated at the end of the primary screening is therefore a mixed population of both the desired "positive" cells and contaminating noninteractors. The following protocol allows for the isolation of pure interactor clones.

1. Scrape cells from the yeast patches obtained in the primary screening with a toothpick into 1 mL of $Trp^-Ura^-Leu^-$ media lacking a carbon source. If there are a number of single, isolated colonies in the patch, make sure you take material from all of them.
2. Dilute 100 µL of the cell suspension in 900 µL of H_2O and determine the OD_{600}. Assume that 1 $OD_{600} = 1.5 \times 10^6$ colony forming units per mL, and calculate the volume needed to deliver 200 cells.
3. Dilute the cell suspension to 200 CFU/100 µL, and plate onto a 10-cm $Trp^-Ura^-Leu^-$ glucose plate. Incubate at 30°C for 1–2 d.
4. Overlay each plate with a circular nitrocellulose filter that has been labeled with the respective clone number and two asymmetric dot markings. Label the bottom of the Petri dish with two dots corresponding to the filter to make sure that plate and filter can be easily aligned in the correct orientation later. Transfer filter to a $Trp^-Ura^-Leu^-$ galactose plate and incubate for 18 h at 30°C.
5. Perform β-galactosidase filter assay (**Subheading 3.6.2.**). Identify a well-isolated blue colony on each filter, and pick the corresponding colony from the glucose plate. Patch the colony onto a fresh $Trp^-Ura^-Leu^-$ glucose plate. Incubate at 30°C for 1–2 d.
6. Scrape a portion of the patch into 1 mL of 50% (v/v) glycerol and freeze at –70°C as a long-term stock.

3.8. Kinase Dependence and Specificity of Interaction

Once pure clones for each original positive have been obtained, two important questions can now be adressed. The first question concerns the dependence of the interaction on PTK activity. The second question asks whether the Vmw65 fusion protein or "prey" interacts specifically with the LexA fusion protein used. To answer these questions, two different kinds of transformants are required.

To identify whether the interactions observed require the tyrosine kinase, the host strain should be cured of the tyrosine-kinase plasmid (*LEU2* marker) so that the resulting cells will be $Trp^+Ura^+Leu^-$. These double transformants can then be grown on Trp^-Ura^- glucose media, induced on galactose media, and screened by β-galactosidase filter assay, as described (*see* **Subheading 3.6.2.**). Transformants which retain β-galactosidase activity when induced do not require the kinase for the interaction to take place.

The second question, concerning the specificity of the interaction, can be addressed by selecting for transformants that have lost the LexA fusion plasmid (p4402; *TRP1* marker) and then mating the resulting strain (still carrying the Vmw65-cDNA fusion and tyrosine-kinase plamids) to W303-1a (MATa) transformed with a number of different LexA-fusion-protein constructs. The resulting diploids can then be tested individually by β-galactosidase assay following galactose induction (*see* **Note 13**).

These two different transformant profiles can be identified in the same plasmid segregation experiment. To do this, the triple-transformant yeast clones obtained in the secondary screening are grown under nonselective conditions (in rich media, such as YPDA) for several generations to allow for the loss of plasmids, which will occur randomly because they are no longer being subjected to nutritional selection (*see* **Fig. 4**).

1. For each positive interactor, inoculate 2 mL YPDA culture. Shake at 30°C for 24–48 h at 200–300 rpm.
2. Dilute the culture 1:6000 and plate 50 μL on a fresh YPDA agar plate. Incubate at 30°C overnight.
3. Replica plate each YPDA plate to two plates: one Trp^-Ura^- glucose plate and one Ura^-Leu^- glucose plate. Incubate at 30°C overnight.
4. Compare the two replicas. Identify two colonies on each plate that do not grow on the other replica:
   a. Two colonies that grow on the Trp^-Ura^- glucose plate, but not on the Ura^-Leu^- glucose plate ($Trp^+Ura^+Leu^-$, i.e., LexA/bait$^+$ Vmw65/cDNA$^+$, but kinase plasmid is lost, for kinase dependence analysis)
   b. Two colonies that grow on the Ura^-Leu^- glucose plate, but not on the Trp^-Ura^- glucose plate ($Trp^-Ura^+Leu^+$, i.e., Vmw65/cDNA$^+$ kinase$^+$, but LexA fusion plasmid is lost, for specificity screening).

   Patch these colonies to a fresh agar plate (with the appropriate amino acids).
5. For kinase dependence screening: Grow a sample of each clone from Trp^-Ura^- glucose plates along with positive and negative controls (if available) together on new plate, transfer to Trp^-Ura^- galactose plate via nitrocellulose filters, and perform β-galactosidase assay (**Subheading 3.6.2.**).
6. Mating assay for specificity of interaction:

   Transform the strain W303-1a with different LexA-fusion-protein bait constructs (including LexA alone) and select transformants on Trp^- glucose me-

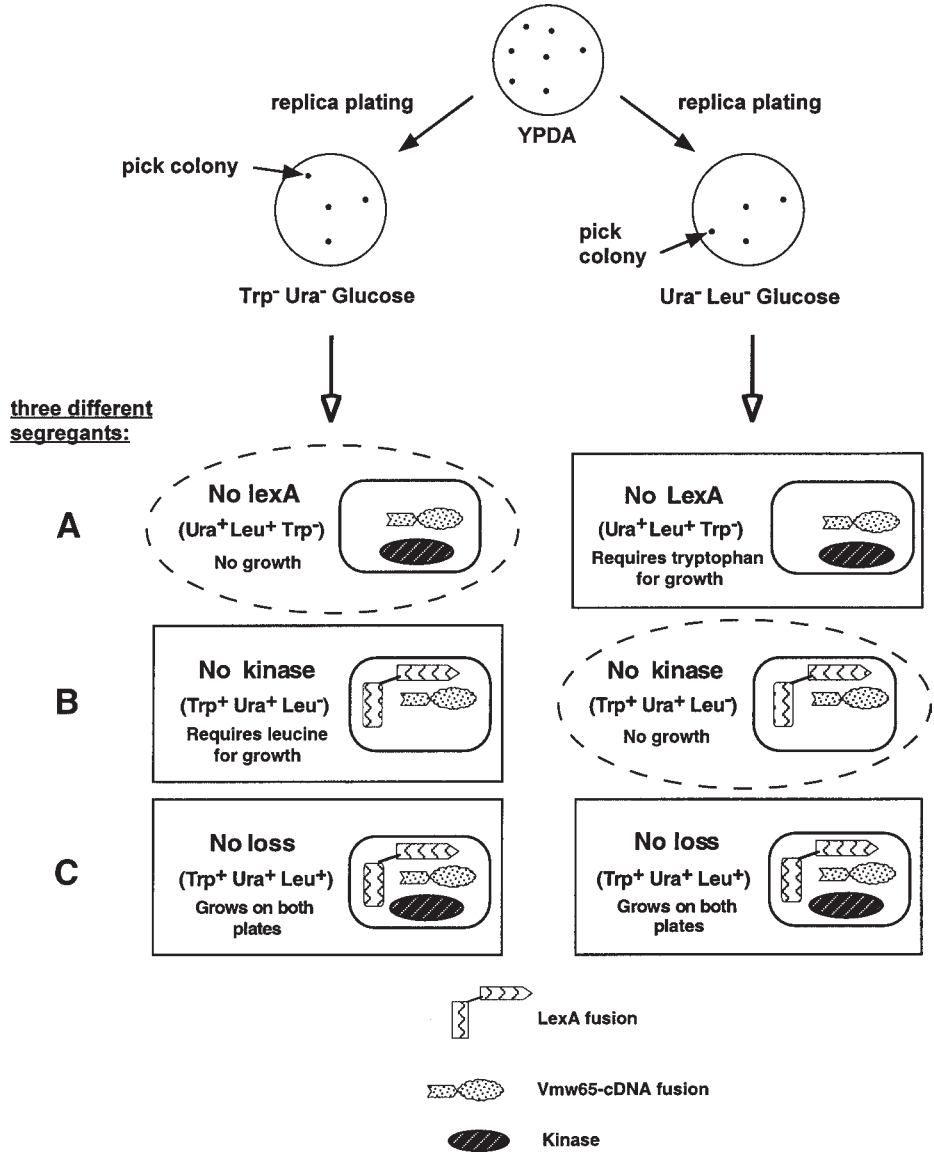

Fig. 4. Plasmid segregation to obtain yeast for kinase dependence and specificity assays. Positive isolates from the secondary screening are grown for 48 h in liquid full media, plated on YPDA agar, and then replica plated onto Trp$^-$Ura$^-$, as well as Ura$^-$Leu$^-$ selective media. Colonies growing exclusively on Trp$^-$Ura$^-$ media, i.e., lacking the kinase plasmid (segregant "b" on Trp$^-$Ura$^-$), can be used for the kinase-dependence assay; colonies growing exclusively on Ura$^-$Leu$^-$ media, i.e., lacking the LexA plasmid (segregant "a" on Ura$^-$Leu$^-$), are suitable for specificity tests.

dia. Mating is accomplished by mixing cell material of the LexA-fusion-protein transformant (W303–1a) with a sample from the library/kinase transformant (S-260, from Ura^-Leu^- glucose plate) on a YPDA plate with a sterile toothpick. After incubating for several hours to overnight at 30°C, replica plate to a $Trp^-Ura^-Leu^-$ glucose plate and incubate at 30°C overnight. Only the diploids will grow. This plate is then overlaid with nitrocellulose and the filter placed on a $Trp^-Ura^-Leu^-$ galactose plate for induction overnight, followed by a β-galactosidase filter assay.

3.9. Plasmid DNA Isolation and Retransformation

The Vmw65-cDNA fusion plasmid of the bait specific interactors can now be rescued from the yeast cells into *E. coli* for sequencing and further manipulation. For rescue, the uracil auxotrophic *E. coli* strain KC8 is used that is genetically complemented by the *Ura3* containing plasmid. As plasmid DNA yields and purity from the KC8 strain are usually low, we routinely transformed the DNA into the strain XL-2 and isolated plasmid preparations from these cells for seqence analysis.

Retransformation of the rescued plasmid back into the yeast strain S-260 with the appropriate bait and kinase to verify the interaction is particularly important, as we have observed blue colonies whose β-galactosidase activity is not linked to the plasmid and, therefore, has to be considered an artefact.

1. Inoculate 2 mL Ura^- glucose culture for each isolated patched interactor clone. Shake at 30°C for 24–36 h at 200 rpm.
2. Transfer 1.5 mL cells to a microcentrifuge tube and centrifuge at 12,000*g* for 30 s at room temperature. Wash cells with sterile H_2O.
3. Resuspend cell pellet in 200 µL lysis buffer and transfer to new microcentrifuge tube containing an equal amount of glass beads (ca. 200 µL volume).
4. Vortex for 2 min, then centrifuge at 12,000*g* for 5 min at room temperature in a microcentrifuge.
5. Transfer the supernatant to a fresh tube, add one volume (~150 µL) of saturated phenol and mix.
6. Centrifuge for 5 min at 12,000*g*, room temperature, take supernatant and perform ethanol precipitation. Often you will get a visible red pellet.
7. After washing with 70% ethanol, dry pellet and resuspend in 20 µL H_2O.
8. Centrifuge at 12,000*g* for 10 min at room temperature and transfer the supernatant to fresh tube (*see* **Note 14**).
9. Take 2 µL for electroporation into competent KC8 cells: mix 2 µL DNA and 40 µL competent KC8 cells, pipet into ice-cold electroporation cuvet, zap at 1.6 kV, 25 µF, 200 Ω (Bio-Rad Gene Pulser), immediately add 1 mL LB media to cuvet, transfer cell suspension to sterile microcentrifuge tube, and shake for 1 h at 37°C and 225 rpm.
10. Plate cells on Ura^-/Amp plates, incubate for 36 h at 37°C.

11. Pick one colony for each interactor and prepare plasmid DNA by standard methods.
12. Transform plasmid DNA into XL-2 cells and repeat DNA preparation for higher yields.

3.10. Sequence Analysis

Restriction digest analysis of the isolated Vmw65-cDNA plasmid (e.g., double digest with *Eco*RI and *Xba*I) provides information about the size of the cloned cDNA insert. After retransformation into S-260, the "prey" fusion can be expressed in liquid culture (*see* **Subheading 3.1.**) and Western blots of the cell lysates can be probed with vmw65-specific antibodies in order to confirm the size of the open reading frame in the interacting cDNA clone.

For quick and convenient analysis of the sequence data, we have used the GCG package of software tools including BLAST and FASTA searches for homologies with known sequences in available databases at the nucleotide and amino acid levels.

3.11. Confirmation of Interaction in Other Expression Systems: One Example

Even after exclusion of cDNA clones encoding nonspecifically binding polypeptides (*see* **Subheading 3.8.**), many two-hybrid, as well as tribrid, screens may yield a number of candidate interactors whose binding to the bait is confined to the specific conditions of the yeast expression system and constructs. One convenient and straightforward procedure that we have used successfully to confirm the authenticity of the specific interaction observed in two-hybrid experiments is the overexpression of the full-length protein of interest and an epitope-tagged interactor clone in the monkey kidney cell line COS7. We have amplified the interactor cDNA sequences by PCR and cloned them into the eukaryotic expression vector pDF-FLAG that directs the expression of the foreign sequence with a 10 amino acid FLAG epitope at the N-terminus under control of the elongation factor EF-1a promoter. After cotransfection with an expression construct for the full length protein of interest, the FLAG-tagged interactor is immunoprecipitated with a FLAG-specific antibody from cell lysates and the immunoblot probed with a bait specific antibody (*see* **Note 15**).

This approach should be also applicable to phosphorylation dependent interactions. If the required bait modification does not naturally occur in COS cells, coexpression of the kinase used for the tribrid screen might be necessary. However, limitations to this strategy might be implied by the potential deregulating and toxic effects of the tyrosine-kinase overexpression.

1. Cotransfect COS7 cells (1.5×10^5 cells per well in 6-well plate) with expression plasmids for protein of interest and FLAG-tagged interactor by standard methods ***(30)***.
2. After 48–72 h, wash cells twice with ice-cold PBS, remove supernatant.
3. Add 150 μL lysis buffer per well and incubate 10 min on ice.
4. Scrape cells, transfer to a microcentrifuge tube, and incubate on ice for 30 min.
5. Vortex for 1 min and centrifuge at 12,000*g* for 15 min at 4°C.
6. Take the supernatant and perform an immunoprecipitation with anti- FLAG antibody. Subject immunoprecipitates to SDS-PAGE and probe immunoblot with bait specific antibody.

4. Notes

1. Although it is ideal to have a bait-specific antibody at hand, commercially available LexA-specific immunoglobulins can also be used to confirm the expression of the bait construct. In this case, it is advisable to process cells expressing unfused LexA protein along with your samples for size comparison (LexA alone has a relative mobility of 25,000 Dalton). Lysates of uninduced cells grown with glucose as carbon source serve as negative control to eliminate confusion that may be caused by yeast proteins that immunoreact with the anti-LexA or bait-specific antibody.
2. Washing the cells with sterile water in **step 3** is necessary to remove all residual glucose, because glucose will interfere with induction of the pGAL promoter by galactose through the catabolite repression pathway ***(18)***.
3. S-260 cells will grow more rapidly in liquid cultures containing glucose as compared to galactose cultures. If you desire to subject the same amount of total protein to gel electrophoresis for uninduced and induced cultures, we recommend to check the OD again after induction and adjust for the volume of cell suspension to be used from the cultures.
4. "Spot blot" or Western blot membranes to be probed with antiphosphotyrosine antibodies must not be blocked with milk powder because of its content of phosphoproteins that will increase the background of the autoradiogram tremendously.
5. As we have found for one of our interactors (C. V. et al., manuscript in preparation), the interaction observed may be phosphorylation-dependent although the transphosphorylation of the "bait" cannot be demonstrated. In this case, tyrosine phosphorylation of the "prey" might be required for the interaction. In addition, there may be a variety of other reasons that protein-tyrosine kinases facilitate the interaction, including protein stability, intranuclear transport, expression levels, and others.
6. Preparing spheroplasts, although time consuming, is worth the effort because proteolysis is reduced relative to glass-bead lysis. It is important to include several controls: glucose induction to control for endogenous yeast proteins immunoreacting with the antibody used; a no-kinase control (transforming the LexA-fusion plasmid along with the kinase vector, pRS415, allows growth in

Trp$^-$ Leu$^-$ medium), and a control using the LexA fusion vector without an insert. LexA has no tyrosine residues, so it should not be modified by a tyrosine kinase.

7. Make sure that the Trp$^-$Ura$^-$Leu$^-$ glucose plates are not wet when plating the transformation mixture; air-dry in a sterile manner, if necessary. It is important to spread the mixture carefully to ensure even distribution of colonies, as a highly dense area of cells will give rise to very small colonies that may be difficult to detect when blue.
8. Do not autoclave the nitrocellulose before placing it on the transformation plates. Autoclaving causes wrinkling which interferes with the ability of the nitrocellulose to lie flat on the agar surface.
9. The liquid nitrogen permeabilization step (wear safety glasses!) is best accomplished in a large glass baking dish, after which the filters should be equilibrated to room temperature on paper towels. This allows the colonies, which tend to "sweat" as they warm up, to dry a little before becoming wet with Zeta buffer in the assay.
10. Be sure that the Whatman 3M paper is perfectly flattened in the lid, to ensure even contact with the nitrocellulose filter. Add more Zeta buffer as necessary; too much buffer results in runny colonies, too little results in poor color development.
11. As some cells that have been dipped in liquid nitrogen do actually survive, blue colonies can be picked by scraping directly from the nitrocellulose filter. Alternatively, they can be identified and picked from the glucose "master" plate. In this case, the cells picked have not been induced on galactose before. This may be preferable if one of the plasmids encodes a toxic protein such that the rescue of living cells from a colony exposed to galactose could be difficult. However, picking from the "master" plate can be tricky, as the colonies on the glucose plate are often small and easily smeared by the process of lifting to nitrocellulose. In addition, the positives identified on the filter after assay may be difficult to match later against the "master" glucose plate.

    Another method to consider is to plate the transformaton mixture directly onto nylon membranes (such as Hybond from Amersham) placed on the Trp$^-$ Ura$^-$ Leu$^-$ glucose plates. The colonies will then grow directly on the filters, which are transferred to Trp$^-$ Ura$^-$ Leu$^-$ galactose plates after 24–36 h. Although this method involves the fewest manipulations, we find that the overall transformation efficiency for plating directly on nylon filters is reduced fivefold, relative to plating onto agar plates.
12. It is helpful to note the time elapsed since the start of the assay when identifying blue colonies, so that they can be ranked according to intensity during subsequent analysis. We have not found any "true" positives that take longer than 90 min to turn blue during an initial screen.
13. It is convenient to maintain glycerol stocks of several different LexA fusion plasmid transformants in the strain W303-1a that can be used for the mating experiments.

    Screening the different interactor clones for kinase dependence on the same Trp$^-$Ura$^-$ plate, as well as mating with the bait and various other LexA constructs

on the same YPDA plate, permits evaluation of the relative intensities of the interactions (i.e., the binding affinities of the different interactors). To quantitate the interaction, β-galactosidase units may also be measured after galactose induction in liquid cultures ***(31)***.

14. The extra centrifugation step before electroporation of KC8 cells has been found to increase transformation efficiency considerably. This is probably caused by the removal of particulate material still contained in the preparation.
15. Colocalization of bait and FLAG-tagged interactor within COS cells by immunofluorescence microscopy is another method to provide evidence for the authenticity of their interaction ***(32)***.

Acknowledgments

The authors wish to thank Step Dalton for his contribution in designing the first generation of yeast expression plasmids, and Sharon Bowen for subcloning of the jak3 expression plasmid. Charles Burghardt and Mignon Nettleton are acknowledged for technical assistance. We thank Douglas Larigan, Ludmilla Foppiani, and John Duker for the preparation of oligonucleotides and DNA sequencing.

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20

Analysis of SH2 Domain — Phosphopeptide Interactions by Isothermal Titration Calorimetry and Surface Plasmon Resonance

George Panayotou and John Ladbury

1. Introduction

The interaction of activated growth factor receptors with signaling molecules has attracted enormous attention in the last few years because of the central role these processes play in growth differentiation and proliferation control in cells. The importance of src homology (SH)2 domains in mediating these interactions through binding to specific tyrosine phosphorylation sites on activated receptors has been well established. However, the complexity of intracellular signaling, and the apparent redundancy in some systems, has necessitated the development of assays for quantifying these interactions accurately. These in vitro studies have benefited from the linear nature of SH2 domain ligands, i.e., the ability of small synthetic phosphopeptides corresponding to receptor autophosphorylation sites to mimic, in most cases, the specificity and binding properties of the intact receptor. Several assays have been developed employing radioactive peptides or indirect detection of SH2 domains by immunological methods. These assays can determine, with varying degrees of accuracy, the equilibrium dissociation constant, K_D (or binding constant, $K_B = 1/K_D$) for an interaction, thus providing quantification of its strength. However, additional information about the kinetic rates and thermodynamics can provide a better characterization. Correlated with high-resolution structural detail, these data can provide insight into the balance of bonds that have been made or broken, whether the molecules have undergone conformational change on interacting, or whether water has been released from the binding surface on formation of the complex. Information of this nature can add a further level of

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

detail, helping to address issues such as the specificity of an interaction and the structural–thermodynamic relationship associated with forming the complex, all of which are factors that might be important when attempting pharmaceutical intervention. The use of isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) biosensors has provided a great deal of information on these issues.

1.1. Isothermal Titration Calorimetry (ITC)

In the process of going from the free to the bound (or complexed) state at a given temperature, every molecular interaction either gives out or takes in heat, i.e., has an exothermic or endothermic change in enthalpy (ΔH) associated with it. ITC uses the inherent change in thermal energy of a system to determine how much of the complex is formed in going from one equilibrium state to another under different concentrations. Thus, heat is used as the probe of the extent of interaction, much as a chromophoric property is used in spectroscopic determinations of thermodynamic parameters. In this case, however, the enthalpy change (ΔH) is directly measured. The change in concentration of the complex with respect to free ligand is monitored over a wide concentration regime by titrating one component of the interaction into the other, allowing determination of the binding constant, K_B. Therefore, in an experiment where the K_B and ΔH are determined, the change in Gibbs free energy, ΔG, and the change in entropy, ΔS, can also be determined at a given absolute temperature (T) : -RT ln $K_B = \Delta G = \Delta H - T\Delta S$. Thus, in one experiment a full thermodynamic characterization of an interaction can be obtained.

Details of the instrumentation and data processing have been described elsewhere ***(1,2)*** and are beyond the scope of this chapter. Briefly, typical instrumentation consists of two calorimeter cells that are housed in an isothermal jacket. The temperature outside is always kept cooler than the temperature at which the experiment is to be conducted. The cells are equipped with highly accurate electronic heating and sensitive temperature monitoring devices. One of these cells (the sample cell) is the vessel in which the titration is performed and initially contains one of the interacting components. The other cell is filled with buffer (or water) and acts solely as a reference with which the sample cell is continually maintaining thermal equilibrium.

The key to the method is that the two cells, through an electronic feedback system, are always kept at the same temperature (i.e., the temperature difference, $\Delta T = 0$). Because the external temperature is always lower than the experimental temperature there is always a requirement for heat into the two cells. Recording the energy input to the cells prior to any interaction taking place shows a constant value or a baseline. If the second component of the interaction is injected into the sample cell there will be a change in enthalpy of the system.

If the interaction is endothermic, then to maintain $\Delta T = 0$ the contents of sample cell have to be heated (equally the opposite applies if the interaction is exothermic, i.e., less heat is required by the sample cell). The amount of heat required per second (or power) by the cell in the time between the injection and the restoration of equilibrium is measured, and is equivalent to the heat of the interaction between the amount of material injected into the cell (titrant) and the cell contents (titrand). If the concentration of titrant and titrand are appropriate, the binding sites become saturated, over a series of injections, such that no further heat of interaction occurs. The output from the instrument can be displayed as a plot of the power against time. Thus, each injection is visualized by a peak on this plot. Integration of these peaks with respect to time gives the heat per injection. If the initial concentrations of the interacting components are known accurately, then the heat per injection obtained is a measure of the amount of complex formed. From this the amount of free ligand at any concentration can be determined. If, at a range of concentrations, the amount of complex formed and the amount of free ligand is known, the K_D can be determined. Typically, this is done by fitting the data over a complete titration in which the concentration regime is set up so as to lead to complete saturation of the binding sites on the interacting component in the calorimeter cell. The data are typically displayed as a plot of ΔH against mole ratio of interacting components.

1.2. Surface Plasmon Resonance (SPR) Biosensors

The BIAcore biosensor is the most commonly used instrument based on the phenomenon of surface plasmon resonance (SPR) ***(3–5)***. When light traveling within a medium of a given optical density reaches the interface with a medium of higher density, it will be total internally reflected if the incident angle is over a critical value. Despite total internal reflection, an evanescent wave penetrates for a short distance into the medium of higher density. If the optical interface is coated with a thin layer of gold, a resonance occurs between the evanescent wave and the outer shell electrons of the gold surface. Because of this resonance, a dip in the intensity of the reflected light is observed at a certain angle of the incident light, the value of which depends on the ratio of the refractive indices of the two media. A large range of incident angles is provided by focusing a wedge of monochromatic light beams onto the gold surface and an array detector is used to analyze the reflected light. The gold surface is usually coated with a hydrophilic dextran layer onto which a molecule can be immobilized. As a second analyte is passed over the surface and binds to the immobilized ligand, the refractive index of the medium close to the surface increases and therefore SPR occurs at a different angle of the incident light. These changes in the SPR angle values are recorded and converted into arbitrary resonance units (RU), which are plotted vs time. The binding curves obtained in this way can be ana-

lyzed for kinetic- and equilibrium-binding parameters. A more general description of BIAcore methodology can be found in a separate volume of this series ***(6)***.

Each one of the two techniques described in this chapter has distinct advantages and disadvantages. Both measure a wide range of dissociation constants, from m*M* to n*M*. Labeling with radioactive or other reporter compounds is not required and the procedures can be carried out automatically with both types of instrument. Calorimetry is the only technique that directly measures the enthalpy of an interaction. SPR biosensors, on the other hand, measure interactions in real time and can provide estimates of kinetic constants. Regarding the weaknesses of the two techniques, ITC often requires much larger amounts of material compared to SPR methods. The latter, however, require immobilization of one of the two interacting components, a procedure that can affect the properties of a macromolecule or introduce artefacts in the measurements caused by a high density of binding sites. ITC is performed with all components in solution, but it can be difficult to do in solvents with high heats of dilution (e.g., dimethyl sulfoxide [DMSO]). In general therefore, the two techniques can be seen as complementary, each providing distinct levels of information; ITC is the method of choice for a thermodynamic characterization, whereas SPR is better suited to kinetic analysis of an interaction. Ideally, the two methodologies should be combined for a complete characterization.

Both ITC ***(7–12)*** and the BIAcore biosensor ***(13–22)*** have been used in many studies for the analysis of the binding properties of SH2 domains. In most cases, small synthetic phosphotyrosine-containing peptides have been used as binding partners. This chapter describes basic procedures for analyzing these interactions, using the Fyn SH2 domain as an example. When both techniques are employed for the analysis of an interaction, the materials used (SH2 domain and phosphopeptides) should preferably be from the same source, in order to obtain comparative results. Given the diverse nature of the many different SH2-domain containing proteins and their respective ligands, these methods can only be seen as general guidelines and should be adapted and optimized for each specific application.

2. Materials

2.1. ITC Equipment

1. ITC instrument (MCS or VP. ITC fromMicroCal Inc.).
2. Filling syringe with 20-cm needle (Hamilton).
3. Dialysis tubing with suitable molecular weight cut-off.
4. Degassing apparatus (vacuum pump or helium gas cylinder).

2.2. ITC Reagents

1. Buffer solution: 10 m*M* potassium phosphate, pH 6.0, 30 m*M* NaCl, 5 m*M* dithiothreitol (DTT).

2. Sodium dodecyl sulfate (SDS) for cleaning cell after titration (10% aqueous solution).

2.3. BIAcore Equipment

1. BIAcore instrument, model 1000 or 3000 (Biacore AB).
2. Sensor chips (CM5 or SA; Biacore AB).
3. Disposable desalting columns (NAP5, Pharmacia).
4. Optional: high-performance liquid chromatography (HPLC) system with reversed-phase columns (e.g., Hewlett Packard 1090 system, Applied Biosystems C18 or C8 columns).

2.4. BIAcore Reagents

1. Amine coupling kit, comprising:
   a. N-Hydroxysuccinimide (NHS)
   b. N-Ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC)
   c. 1 *M* Ethanolamine, pH 8.5 (Biacore AB).
2. Acetate buffer, pH 4.5.
3. Avidin or streptavidin, disolved at 1 mg/mL in water (Boehringer).
4. NHS-Biotin (Gibco-BRL).
5. Standard running buffer: 20 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 3.4 m*M* ethylenediaminetetracetic acid (EDTA), 0.005% Tween-20, 4 m*M* DTT.
6. Standard regeneration buffer: 0.05% SDS.
7. Biotinylation buffer: 100 m*M* phosphate buffer, pH 5.8 - 7.8.
8. Direct immobilization solution: 50 m*M* HEPES, pH 7.4, 1 *M* NaCl.

2.5. Proteins and Peptides

1. Phosphotyrosine-containing peptides can be synthesized using standard methods with Fmoc-protected amino acids.
2. SH2 domains can be purified from bacteria after expression with suitable vectors. The Fyn SH2 domain used in this study was expressed as a Glutathione S-transferase (GST)-fusion protein, followed by affinity purification on glutathione-Sepharose and cleavage of GST using thrombin. The cleaved domain was dialyzed in 10 m*M* phosphate buffer, pH 6.0, 30 m*M* NaCl, 5 m*M* DTT, and stored at 4°C for several weeks without loss of binding activity.

3. Methods

3.1. Isothermal Titration Calorimetry

3.1.1. Preparation for a Titration

1. The experimental temperature should be selected, the water bath or peltier cooling system temperature should be set at approx 10°C below the experimental temperature, and the calorimeter should be allowed to adjust to the new temperature (*see* **Note 1**).

2. To obtain useful thermodynamic data the compounds under investigation should be as pure as possible. The concentrations of the solutions required will vary depending on the system and the expected binding constant (*see* **Notes 2–7**). The two components of the titration should be dialyzed in the same buffer solution. This ensures parity of the buffer solution in the calorimeter cell and the syringe, thus removing any heat of dilution of one buffer into the other (*see* **Note 8**). For the example shown in this Chapter, dialyze Fyn SH2 domain in 3-kDa cut-off dialysis tubing against 2 L of 10 m*M* potassium phosphate, pH 6.0, 30 m*M* NaCl, 5 m*M* DTT.
3. Set up experimental details (concentrations of interacting compounds, number and volume of injections, duration of each injection, time between injections, speed of rotation of titrating syringe [usually kept at 400 rpm] using the menu incorporated in the interactive computer software).

3.1.2. Performing the Titration

1. Fill the sample cell with one of the dialyzed interacting components (for example, Fyn SH2 domain) with the filling syringe (*see* **Note 9**).
2. Initiate equilibration of the calorimeter cells.
3. Fill the titrating syringe with the second interacting component (i.e., specific phosphopeptide). This should be done avoiding any air bubbles in the syringe. The syringe has an outlet near to the plunger outlet through which any trapped air can be discharged. The PTFE syringe holder can be removed to check that this is done.
4. When the calorimeter cells have reached thermal equilibrium, as indicated by a linear horizontal baseline (or constant cell feedback) insert the syringe (*see* **Note 10**). Insertion of the syringe disturbs the equilibrium so there will be a short delay to regain these conditions.
5. Start the titration using the software provided with the instrument. A typical titration will take between 1–2 h (*see* **Note 11**).
6. On completion of the titration, remove the contents of the cell and clean the sample cell using a dilute SDS solution and several liters of deionised water. In cases where samples have precipitated in the cell, the use of acidic or basic cleaning solutions can be used as recommended by the instrument manufacturers.
7. Determine the heats of dilution of the interacting components. First, titrate the sample from the syringe into a buffer solution in the calorimeter cell.
8. Repeat, titrating buffer into the sample solution.
9. The heats per injection from **steps 7** and **8** should be measured and subtracted from the raw data of the interacting components (using the computer software) prior to data analysis.

3.1.3. Data Analysis

The raw data are saved and automatically integrated to give the binding isotherm that plots ΔH against mole ratio of interacting compounds or against injection number. The shape of this isotherm will immediately suggest whether

the interaction is a simple one-step binding or a more complex series of interactions (*see* **Note 12**).

If the heats per injection are low, the titration could be improved by increasing the concentration or the injection volume. If this is insufficient to produce reliable data, the heats per injection can usually be changed by performing the experiment at a different temperature. Because there is a constant pressure change in heat capacity (ΔCp, the dependence of the ΔH on temperature) associated with all interactions, the heat per injection can often be improved by changing the temperature (*see* **Note 13**).

3.2. BIAcore Methods

In principle, SH2 domain–phosphopeptide interactions can be performed on the BIAcore in either of two orientations, i.e., with peptide or SH2 domain immobilized. However, there are two important considerations in SPR measurements that make immobilization of peptide the preferred method:

1. The response measured is directly proportional to the size of the bound macromolecule and, therefore, a protein will give a much better signal than a small peptide. Even though the sensitivity of the BIAcore 2000 and 3000 instruments are significantly improved over previous versions, reliable data for kinetic analysis are typically obtained using proteins of 10 kDa or more.
2. The stability of the immobilized analyte is also very important. When the specificity of different domains for binding to the same sequence is analyzed, it is essential that the same surface is used. The same is true when a range of protein concentrations is injected over a surface for equilibrium-binding assays. In these cases, the surface should be regenerated, i.e., the bound protein should be removed leaving the immobilized analyte ready for the next interaction. SH2 domain–phosphopeptide interactions are usually of high affinity and can be quite difficult to break. Therefore, a regeneration procedure would usually demand denaturation of the SH2 domain. In this respect, immobilizing phosphopeptides is preferable as they tend to be very stable, even to very harsh conditions. Two solutions that can be used reliably without affecting the binding ability of the immobilized peptide are 0.05% SDS or 2 *M*–6 *M* guanidine hydrochloride. Usually, a short pulse of 5 μL is enough, but the amount and time of exposure should be optimized for different interactions. It should be noted that while very acidic solutions (10–100 m*M* HCl) may break an interaction, they are also likely to hydrolyze the phosphate group of phosphotyrosine and should therefore be avoided. In case the above solutions are not suitable (for example, they result in reduction of the binding affinity of the phosphopeptide), the fast dissociation rates observed for SH2 domains can be exploited. Although allowing the SH2 domain to dissociate for a long time may not result in complete removal of all bound material, the injection of an excess of phosphopeptide during dissociation should take the response back to baseline levels in a very short time.

3.2.1. Phosphopeptide Immobilization

There are two ways in which a phosphopeptide can be immobilized; indirectly by biotinylation and binding to immobilized avidin or streptavidin; and directly through the amino-terminus.

For both cases there are some common considerations:

1. Although most specificity determinants for SH2 domain-phosphopeptide interactions are located C-terminally to the phosphotyrosine (+1 to +5), it is essential that a spacer of at least 5 amino acids is also present N- terminally to the phosphotyrosine to avoid steric hindrance problems when the peptide is immobilized.
2. The presence of internal lysine residues within the peptide sequence has to be taken into account as it can result in inappropriate immobilization or multiple biotinylation. In this respect, the pH of the solution in which the immobilization or biotinylation take place has to be adjusted to low values (preferably below pH 6.0) in order to ensure that ε-amino groups of lysine residues are protonated and therefore less able to react. In this case the reaction via the N-terminal amino group is favored (*see* **Note 14**).

3.2.1.1. Peptide Biotinylation

1. Purify the peptide, if necessary, using a reversed-phase column on an HPLC instrument.
2. Dry down the peptide peak under vacuum, resuspend in water, dry down, and repeat once.
3. Resuspend peptide in biotinylation buffer (final peptide concentration approx 0.1–1 mg/mL). Use pH 7.8 if no lysine residues are present in the peptide or pH 5.8 if there are.
4. Check that the pH of the final solution is correct by blotting a few microliters onto suitable pH paper. Adjust if necessary.
5. Add NHS-biotin, dissolved in dimethylformamide, to a final molar ratio of 100:1 (biotin:peptide) for biotinylation at pH 7.8 or a ratio of 1:1 for pH 5.8 (*see* **Note 15**). Incubate for 1 h at room temperature.
6. Add Tris-HCl, pH 7.8, to a final concentration of 0.1 *M*. Incubate for a further 30 min.
7. Purify the biotinylated peptide using reversed-phase HPLC (*see* **Note 16**).

3.2.1.2. Avidin or Streptavidin Immobilization

A standard NHS/EDC immobilization protocol can be used to immobilize avidin or streptavidin to the surface of a CM5 chip (*see* **Note 17**).

1. Mix equal volumes of 11.5 mg/mL NHS and 75 mg/mL EDC.
2. Immediately inject 40–60 μL over the surface at a flow rate of 10 μL/min.
3. Inject avidin or streptavidin at 0.1 mg/mL in acetate buffer, pH 4.5. Use the MANUAL INJECT command in order to adjust the immobilized level to approx

3000 to 5000 RU. If more than one surface will be used (as a control or for different peptides), then the immobilized amount should be similar for all flowcells.
4. Block excess unreacted sites with a 40-μL injection of 1 *M* ethanolamine.
5. Regenerate with a 5 μL pulse of 0.05% SDS or whichever regeneration solution will be used for subsequent experiments.
6. Inject a phosphopeptide solution in running buffer until a suitable level is immobilized (*see* **Note 18**).
7. Regenerate and check binding of SH2 domain. More peptide can then be injected until a satisfactory response is obtained (*see* **Notes 19** and **20**).

3.2.1.3. Direct Immobilization

1. Resuspend the purified peptide in direct immobilization solution.
2. Activate surface with NHS/EDS, as above.
3. Inject the peptide using the MANUAL INJECT command until a suitable response level is obtained.
4. Block excess sites and regenerate to remove noncovalently bound material, as above.

3.2.2. Binding of SH2 Domains

As the SPR technique measures changes in refractive index, small differences in composition between the running buffer and the protein solution can result in rather large, sudden "jumps" in the signal at the beginning and end of each injection. Moreover, if a protein is injected at a high concentration, a steady response will be given throughout the sensorgram by noninteracting protein ("bulk effect"). Additionally, in some cases, a certain level of nonspecific protein binding can also be observed. For these reasons, the subtraction of data obtained from injection of the same protein solution over a control surface (avidin only, or nonphosphorylated peptide or a phosphorylated, but nonspecific peptide) is essential in order to obtain data suitable for quantitative analysis. In the BIAcore 2000 for example, this is best achieved by allowing the solution to pass simultaneously over two or more flowcells, one of which is the control surface. Alternatively, the same solution can be injected separately over control and specific surface. The instrument's software can then be used to subtract the control set of data from the specific.

The responses attributable to buffer changes can be minimized by exchanging the buffer in which the protein is stored to running buffer, just prior to a set of experiments. This can easily be accomplished by using small, disposable desalting columns.

3.2.2.1. Equilibrium binding

For determination of the equilibrium dissociation constant (K_D) a broad range of SH2 domain concentrations is injected over the immobilized

phosphopeptide. It is essential that equilibrium is, indeed, reached during the course of the injection.

1. Prepare a series of SH2 solutions over a wide concentration range (*see* **Note 21**).
2. Inject over the immobilized phosphopeptide and over a control surface at a flow rate of 5 μL/min or higher.
3. Regenerate with a short pulse of regeneration solution.
4. Record the response at equilibrium for each concentration of SH2 domain and subtract that obtained with the control surface. Plot vs the concentration and fit the data to the equation

$$R = R_{max} * C / K_D + C$$

where R is the response and C is the concentration (*see* **Note 22**).

3.2.2.2. Competition Studies

In some cases, immobilization of peptides may not be suitable for measuring affinities, for example because of steric hindrance or because synthesis problems prevent the addition of a spacer N-terminally to the phosphotyrosine. Moreover, when many different peptides need to be compared, immobilization of each one to a different surface can be not only very expensive, but also not easy to adjust to the same effective immobilization level. In these cases, the relative potencies of phosphopeptides for binding to an SH2 domain are best determined by obtaining an IC_{50} value for competition in solution.

1. Immobilize a phosphopeptide at a level that will give a measurable, but not too high, response (200–300 RU) with a low concentration of SH2 domain (approx 10 times less than the K_D; *see* **Note 23**).
2. Mix the same amount of SH2 domain with a range of peptide concentrations (*see* **Note 24**) and allow to stand at room temperature for at least 15 min.
3. Inject the solution over the immobilized phosphopeptide and record the response at equilibrium.
4. Subtract the response obtained on a nonspecific surface and plot vs the log concentration of "competitor" peptide.
5. Fit the data to the equation

$$R = R_{max} / 1 + (C/IC_{50})^P$$

where R_{max} is the response obtained in the absence of competitor, C is the concentration of competitor, and P is the slope factor that determines the steepness of the curve (should be equal to one for a simple interaction).

3.2.2.3. Kinetic Analysis

The amount of peptide that is immobilized on the sensor-chip surface is extremely important for obtaining data that can be interpreted in a quantitative way. Obviously, there should be enough peptide to give a measurable response

with protein concentrations roughly an order of magnitude above and below the equilibrium disassociation constant K_D. As a general guideline, the response obtained with an average SH2 domain (15 kDa) should not exceed 200 RU for a saturating concentration. A higher density of phosphopeptide can result in experimental artefacts, including rebinding of dissociating material to the surface and mass-transport limited interactions, whereby the rate of binding depends on the rate of delivery of analyte to the surface. In this case, the kinetic analysis of the interaction becomes very complicated and it is difficult to fit simple mathematical models to the data.

The level of immobilized peptide can be adjusted by using the MANUAL INJECT command. Because very small volumes can be injected in this way and the process can be stopped and started at will, it is easy to control precisely the immobilized amount and adjust it to a level suitable for the desired SH2-binding response.

Although a GST-fusion SH2 domain will usually give a strong response for binding to a phosphopeptide and can be very useful for specificity studies, the use of nonfusion SH2 domains should be the choice for kinetic analysis. The main problem with GST-fusions arises from the well-established dimerization of the GST moiety ***(9)***. As a result, avidity effects can become prominent, especially at high levels of immobilized peptide, with inevitable difficulties in interpreting the data with simple kinetic models.

Rebinding of dissociating SH2 domain to the phosphopeptide can give artificially slower dissociation-rate constants. A simple way to check whether re-binding does indeed occur is to compare the dissociation in buffer flow with that obtained in the presence of an excess of phosphorylated peptide (nonbiotinylated). The peptide can be introduced immediately after the end of the protein injection using the COINJECT command. A faster dissociation rate would indicate that rebinding occurs and steps can be taken to reduce the problem, for example by immobilizing less phosphopeptide.

1. Immobilize peptide by any of the above methods (*see* **Subheading 3.2.1.**).
2. Inject SH2 domain, always using the KINJECT command, at a flow rate of at least 10 μL/min, and preferably higher (*see* **Note 25**). If possible, inject simultaneously over a control surface (BIAcore 2000).
3. In the KINJECT command, program at least several minutes of dissociation time, preferably until the response is close to the baseline.
4. Regenerate and repeat injections with different concentration of SH2 domain.
5. Analyse data, preferably with the software BIAevaluation v. 3.0, supplied with the instrument.

4. Notes

1. The water bath is set at a lower temperature so that there is always a requirement of heat to the calorimeter cells. The thermal adjustment time on going from one experimental temperature to another varies depending on several criteria; if low temperatures are

required (<10°C) equilibration will take longer (*see* also **Note 13**), the fluctuation of room temperature in the laboratory, the size of the change in temperature required from the previous experiment. For most instruments, the equilibration time should be no longer than 4 h.

2. The concentrations of the interacting solutions required are assessed based on the likely binding constant (K_B). A simple formula is used to determine the concentration of the component to be put into the calorimeter sample cell: $c = K_B \cdot [M]$ where $[M]$ is the concentration and c is a number that dictates the shape of the binding isotherm and for ideal titrations is between 10 and 100 (*see* **refs. *1*** and ***2***). So, for example, if a binding constant of approx 10^6 is expected the concentration of sample in the cell should be $10^{-5} - 10^{-4}$ *M*. The solution in the titrating syringe should be of sufficient concentration such that at the end of the titration saturation of the solution in the sample cell is ensured. At the end of the titration, the sample cell should contain two times as many moles of the solution from the syringe as the sample cell solution. Thus, in a typical experiment 250 μL of 10^{-5} *M* solution are injected into the solution in the sample cell (approximate volume 1.3 mL), which is at 10^{-6} *M*.
3. For a first experiment, when no idea of the likely binding constant exists, it is advisable to set up a concentration regime in which the most information can be obtained. Assume that the binding constant is in 10^5 to 10^6 M^{-1} range. This will require that the cell be filled with a solution in the μ*M* concentration range.
4. Sample concentrations should be known as accurately as possible. In a situation where one of the concentrations of the components of the interaction is unknown the thermodynamic data can still be obtained by floating this concentration as long as the stoichiometry of the interaction is known.
5. The interaction of compounds with high heats of dilution poses problems in ITC because, in some cases, these heats can be far greater than any heat of binding making accurate measurement impossible.
6. Insolubility of samples can cause problems in ITC experiments. It is recommended that the molecule with the lowest solubility is placed in the sample cell where the concentration is more dilute than the solution in the titrating syringe. In some cases, organic solvents such as DMSO are required. In these cases, the organic solvent should be kept at as low a concentration as possible (DMSO should be kept at below 1%, if possible). It is recommended that trial experiments to ascertain the heats of dilution of the prospective solvent are performed to judge their suitability.
7. There is no restriction on the size of the interacting components as long as the solutions can be handled effectively (i.e., injections can be made and rapid mixing effected). For example, ITC has been used to measure interactions with whole cells and sizing gels.
8. In some cases, it is not possible to dialyze the interacting components (e.g., if one of the components is a very small molecule). In such instances, both systems should be dissolved in the same buffer stock solution and the pH corrected to parity. If only one component can be dialyzed, the other should be dissolved in

the dialyzate. As a final resort, disparity of the buffer systems can be corrected for in the heat of dilution experiment.

9. Loading the calorimeter cell requires great care so as not to cause damage with the syringe needle (the cell walls are necessarily thin to improve thermal conductivity). The syringe should be lowered gently and vertically to the bottom of the cell and the solution injected until a meniscus is observed at the top of the filling tube.
10. Modern instruments with interactive computer software will have a built in routine program that checks the cell feedback and assesses when equilibrium between the calorimeter cell has been obtained. This is signified by the appearance of an icon on the screen.
11. The duration of a titration obviously depends on the number of injections and the time between injections. For an initial experiment, set up a titration with 15–20 injections with 4 min between each. In some cases, if a slow enthalpy change occurs (as shown by tailing of the titration peaks and a failure to return to the experimental baseline prior to the next injection the time between injections should be increased. Because the heat released (or taken up) in an interaction is a direct probe of the extent of an interaction the titration calorimeter can be used as a measure of the rate of reaction. Kinetic mode calorimetry using titration calorimeters, however, has not been widely reported to date.
12. A simple 1:1 binding interaction will always produce a sigmoidal titration plot when the axis are as shown (**Fig. 1**). The ITC software can be used to fit more complex interactions **(ref. 2)**.
13. Working at low temperatures can be problematic if the room temperature is significantly higher than the experimental temperature. This is usually manifested in a temperature drift over the course of an experiment. Modern calorimeters have additional cooling tubing to facilitate working at low temperatures. Newer calorimeters are adopting peltier cooling methods. In all cases, it is wise to make sure that all samples and cleaning solutions are kept at, or below, the experimental temperature to avoid long equilibration times.
14. In the case of direct immobilization, it has been shown that the presence of a single lysine residue at the N-terminus can help significantly the immobilization.
15. A relatively low amount of biotin is used at pH 5.8 to ensure that little biotinylation of lysine residues takes place. However, the combination of low pH and low biotin concentration also results in relatively inefficient biotinylation of the N-terminus. It may be necessary to experiment with the biotinylation conditions in order to get a satisfactory amount of correctly biotinylated peptide.
16. If no internal lysines are present, and therefore only one product is expected, it is possible to avoid this step and quickly purify the peptide using a disposable Sep-Pak reversed phase cartridge. It is essential, however, that all free biotin is washed away during this step. If possible, biotinylation should be confirmed by mass spectroscopy. An increase of 226 Dalton should be observed if one biotin molecule is attached.
17. Sensorchips are available with streptavidin already immobilized to a level of 3000 RU (Type SA, BIAcore). However, the substantially higher cost of these chips

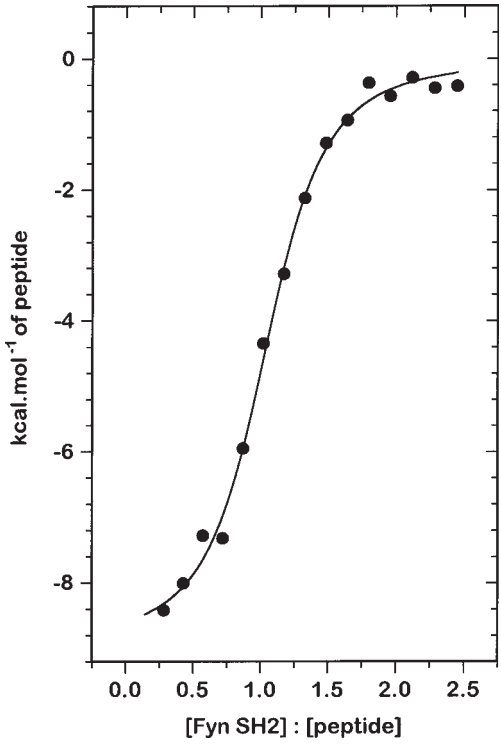

Fig. 1. ITC binding isotherm for the interaction of Fyn SH2 domain (0.021 m*M*) with pYEEI phosphopeptide (0.21 m*M*) at 25°C. The nonlinear least squares fit to the data points is shown. This fit gives a stoichiometry (*N*) of 1.02 ± 0.02, binding constant (K_B) of 9.82 ± (1.3) × 10^5 M^{-1} (or dissociation constant (K_D) of 1.02 m*M*) and ΔH of –37.41 ± –0.89 kJ × mol^{-1}. The heat of dilution for peptide is + 8 kJ × mol^{-1}.

and the ease of immobilizing avidin or streptavidin using standard methods makes the use of standard blank sensor chips preferable.

18. It is preferable to start with a very dilute phosphopeptide solution and increase the amount injected in small steps in order to avoid immobilizing too much peptide and thus render the surface useless for subsequent analysis. It is impossible to remove any significant amount of phosphopeptide once it has reacted with avidin/streptavidin.
19. The very high affinity of the avidin-biotin interaction ensures that no dissociation of immobilized phosphopeptide takes place even after many rounds of binding and regeneration.
20. If different surface will be used to immobilize distinct peptides, the relative amount of each one can be more accurately determined by injecting a monoclonal antibody against phosphotyrosine and comparing the response obtained at equilibrium.

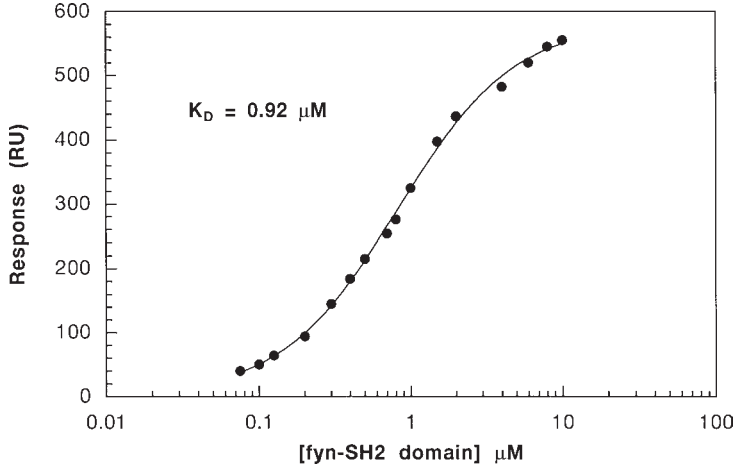

Fig. 2. Binding of the SH2 domain of Fyn to a pYEEI phosphopeptide immobilized on a BIAcore sensorchip. Note the sigmoidal shape of the fit, indicating that sufficient data points were included. The kinetic parameters for the same interaction were estimated in separate experiments using less immobilized peptide and were the following: $k_a = 2.5 \times 10^5\ M^{-1}\ s^{-1}$ and $k_d = 0.22\ s^{-1}$.

21. An accurate fit requires data over a wide range of concentrations. The best way to ensure this is to plot the data as response vs the logarithm of the protein concentration. In this case, a sigmoidal shape of the curve indicates that the data spread are sufficiently broad. In practice, concentrations should range at least one, and preferably two, orders of magnitude below and above the K_D (**Fig. 2**).
22. The amount of protein bound on the surface after a typical interaction is usually less than 1% of the total amount injected and therefore the total concentration is used here instead of the "free." More complicated equations can be used to fit interactions that do not follow a simple one-to-one model or when a heterogeneous analyte is used.
23. It is preferable to immobilize a peptide that has the highest affinity of those that will be compared. In this case low amounts of peptide and SH2 domain can be used, thereby avoiding possible artefacts (such as steep slopes representing high Hill coefficient numbers, *n*) which can distort the competition curve and make fitting difficult.
24. As aforementioned for equilibrium binding studies, it is best to use a wide range of concentrations, varying by one to two orders of magnitude above and below the IC_{50} value. Theoretically, for a simple one-to-one interaction, the response should drop from 90% to 10% of that obtained in the absence of competitor within a 81-fold concentration range.
25. A high flow rate (30 μL/min) ensures minimum perturbation of the signal at the beginning and end of the injection and fewer mass-transport problems. Moreover,

if a control surface is used simultaneously, there is very little delay between the two flowcells, facilitating the accurate subtraction of the control run from the specific interaction.

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Cloning and Characterization of RTK Ligands Using Receptor-Alkaline Phosphatase Fusion Proteins

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1. Introduction

Receptor tyrosine kinases (RTKs) bind to their ligands with high affinity and specificity. Soluble receptor approaches exploit these biological properties to make affinity probes that can be used to detect or to purify the cognate ligands ***(1,2)***. In many respects, these soluble receptor reagents resemble antibodies, and they can be used in almost all the same types of procedure. They can also have important advantages over antibodies. They can be used to identify and clone previously unknown ligands of orphan receptors ***(1–9)***. They can be produced much more quickly than antibodies. Also, because they exploit natural receptor–ligand interactions, they can give information not available with antibodies, for example permitting quantitative characterization of ligand–receptor binding interactions ***(1,2,10)***, or allowing the simultaneous detection of multiple cross-reacting ligands in an embryo ***(5,11,12)***.

Structurally, the RTKs consist of an extracellular ligand-binding region that is joined to the intracellular kinase domain via a single transmembrane domain. The principle of making a soluble receptor-affinity probe is to make a genetic construct encoding only the extracellular region, without the transmembrane or intracellular domains. This probe is expected to be soluble while retaining its ligand-binding properties.

These soluble receptor reagents are almost always produced with a tag at the carboxy-terminus. The tags that have generally been used are alkaline phosphatase (AP) ***(1)*** or the immunoglobulin Fc region ***(2)***. Both of these tags are dimeric, and both are expected to produce a fusion protein with a pair of receptor extracellular domains, both facing away from the tag in the same direction,

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

as if they were embedded in a cell membrane. This dimeric structure is likely to be an important feature in many experiments, because it may greatly increase the avidity of the fusion protein for ligands that are oligomeric, or are bound to cell surfaces or extracellular matrix. To produce a fusion protein, a cDNA sequence for the receptor extracellular domain is inserted in-frame into a vector containing the tag sequence (*see* **Subheading 3.1.**). The fusion protein is then expressed, generally in the supernatant of a mammalian cell line (**Subheadings 3.2.–3.4.**). The principles of using either AP or Fc fusion proteins are similar, and in this chapter we will focus on procedures for the AP tag.

The AP tag has the advantage of possessing an intrinsic enzymatic marker activity. It is therefore generally not necessary to purify the fusion protein, chemically label it, or use secondary reagents such as antibodies. This helps make detection procedures simple and extremely sensitive. Fusions can be made at either the N- or C-termini of AP. The human placental isozyme of AP ***(13)*** is used because it is highly stable, including a high heat stability that allows it to survive heat inactivation steps to destroy background phosphatase activities. The enzyme also has an exceptionally high turnover number (kcat), allowing sensitive detection. A wide variety of substrates for AP are available that allow either detection *in situ*, or quantitative assays in solution.

To identify and clone a new ligand, the first step is usually to identify a good source. Traditionally, it has been thought that control molecules are likely to be present at very low concentrations in the embryo, but we have noticed that many receptors and ligands are actually expressed in embryos at very high levels. Moreover, this expression is often highly localized. Embryos can therefore be an excellent source for the cloning of ligands, receptors, and other biological control molecules. An efficient procedure to identify a good source of ligand is to test embryos or tissues by affinity probe *in situ* staining using a receptor-AP fusion protein as a probe (*see* **Subheadings 3.5.** and **3.6.** ***[5,11]***). An alternative approach is to screen cell lines, either by quantitative binding of the receptor-AP fusion to the cell surface (*see* **Subheading 3.8.**) ***(1)***, or by coimmunoprecipitation of ligand with a receptor-AP fusion protein, which may be particularly useful in the case of soluble ligands (*see* **Subheading 3.9.**) ***(14,15)***.

Once a good source has been identified, the next step is typically to use an expression cloning approach. A cDNA library is prepared, expressed in a cell line, and screened for binding of the receptor-AP fusion protein (*see* **Subheading 3.11.**) ***(5)***. This type of procedure can be used to identify not only cell surface ligands, but also soluble ligands ***(9)***. To clone soluble ligands, an alternative approach is to coimmunoprecipitate enough ligand protein to obtain direct peptide sequence, then use this information to make corresponding oligonucleotides and isolate cDNA clones by polymerase chain reaction (PCR) or nucleic acid hybridization (*see* **Subheading 3.9.**) ***(15)***.

If a cDNA has been cloned that causes cells to bind a receptor-AP fusion protein, this cDNA probably encodes a ligand, though in principle it might instead encode a molecule that indirectly causes expression of a ligand. To establish direct binding between ligand and receptor, binding assays can be performed in solution with the receptor fused to an AP tag, and the ligand fused to an immunoglobulin (Ig) Fc tag (or vice versa) to demonstrate binding in a cell-free system (*see* **Subheading 3.10.**) ***(16)***.

In addition to cloning projects, receptor fusion proteins can be used for a wide variety of other experiments to characterize ligands and receptors. For example, the *in situ* detection of binding sites in tissues or embryos can give unique types of biological information not available from other approaches such as *in situ* hybridization or immunolocalization ***(5,11,12)***. Assays of the binding of receptor-AP fusions to ligands on cell surfaces, or in solution, can be used for the quantitative characterization of ligand–receptor interactions, including studies of the effect of mutations, cofactors, or antagonists ***(1,10,14)***. Also, while we focus here on fusion proteins made from receptors, essentially the same methods can be used with AP fusion proteins made from ligands ***(11,17–19)***, or presumably many other types of biological molecule that show specific binding interactions.

2. Materials

1. Vectors APtag-1, APtag-2, and APtag-4 (**Fig. 1**) can be obtained from GenHunterCorp. (tel. 800-311-8260; e-mail genhunt@telalink.net). APtag-2 and APtag-4 must be grown in the *Escherichia coli* strain MC1061/P3, which can be obtained from Invitrogen, Inc. or other commercial suppliers.
2. The NIH-3T3, COS, and 293T cell lines (*see* **Note 1**) can be obtained from the American Type Culture Collection.
3. Reagents for molecular cloning procedures and cell culture are standard and can be obtained from many commercial suppliers.
4. HBS buffer: 150 m*M* NaCl, 20 m*M* HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethane-sulfonic acid), pH 7.0.
5. TE: 1 m*M* Tris, 0.1 m*M* ethylenediaminetetracetic acid (EDTA), pH 8.0.
6. G418: Geneticin from Life Technologies. On each bottle, the activity is given in µg/mg. Calculate all G418 concentrations using activity not mass. Make a stock solution in water at 50 mg/mL, filter sterilize, and store at –20°C.
7. Complete culture medium: Dulbecco's modified Eagle's medium (DMEM) + 10% bovine calf serum + 1% penicillin/streptomycin.
8. Opti-MEM I (Life Technologies).
9. Lipofectamine (Life Technologies).
10. TBS buffer: 150 m*M* NaCl, 25 m*M* Tris (tris(hydroxymethyl) amino methane), pH 8.0.

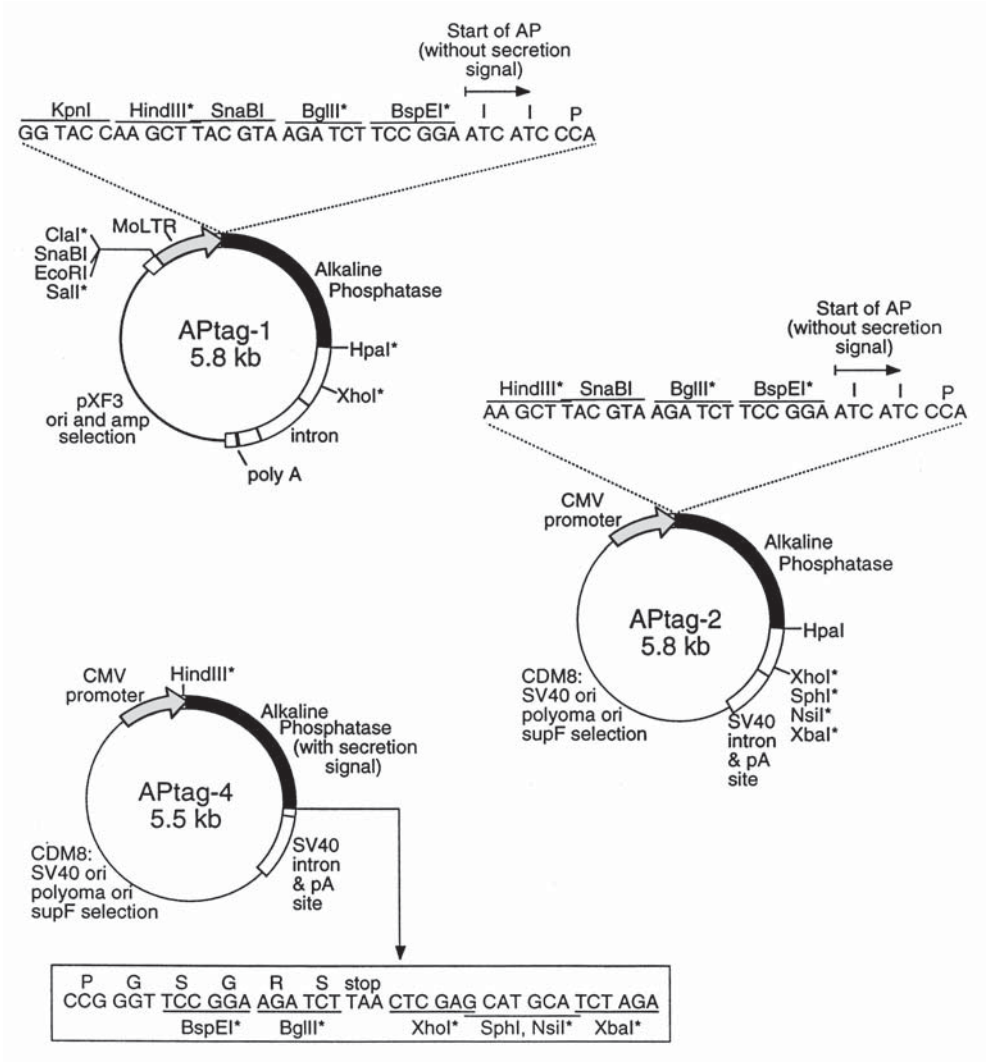

Fig. 1. Vectors to make AP fusion proteins. APtag-1 ***(1)*** is designed for stable transfection, whereas APtag-2 ***(11)*** and APtag-4 are for transient transfection. APtag-2 and APtag-4 have a supF selection marker and must be grown in the bacterial strain MC1061/P3. APtag-1 and APtag-2 are designed for fusions to the N-terminus of AP, whereas APtag-4 is for fusions to the C-terminus of AP. APtag-4 has its own secretion signal sequence so, in addition to making fusion proteins, it is useful as a source of unfused AP as an important negative control. MoLTR, long terminal repeat of the Moloney Murine Leukemia Virus. Asterisks indicate restriction sites that cut the vector only once.

11. Modified RIPA buffer: 0.5% Nonidet P-40 (NP40), 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1% NaN_3, 144 m*M* NaCl, 50 m*M* Tris-HCl, pH 8.0.
12. HBAH buffer: Hank's balanced salt solution, 0.5 mg/mL BSA (bovine serum albumin), 0.1% NaN_3, 20 m*M* HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethane-sulfonic acid), pH 7.0.
13. Triton-Tris buffer: 1% Triton X-100, 10 m*M* Tris-HCl, pH 8.0.
14. 2×AP substrate buffer: Add 100 mg Para-nitrophenyl phosphate (Sigma) and 15 µL 1 *M* MgCl2 to 15 mL 2 *M* diethanolamine, pH 9.8. This stock should be kept on ice, and can be stored frozen at –20°C in aliquots. To make up 2 *M* diethanolamine, take 20 mL liquid diethanolamine and make up final volume to 100 mL with water, adjusting pH with HCl.
15. Acetone-formalin fixative: 65% acetone, 8% formalin in 20 m*M* HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethane-sulfonic acid), pH 7.0. We routinely use this fixative to fix cells or embryos for AP *in situ* staining. However, 8% formalin can also be used alone if acetone should cause problems (*see* **Note 2**).
16. AP buffer for *in situ* staining (AP staining buffer): 100 m*M* NaCl, 5 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 9.5.
17. BCIP/NBT substrate: 0.17 mg/mL BCIP and 0.33 mg/mL NBT in AP staining buffer. Keep on ice. Can be stored frozen in aliquots at –20°C.
18. 4% paraformaldehyde: Add 2 g of paraformaldehyde powder to 50 mL phosphate-buffered saline (PBS). Add 5 µL 10 N NaOH. Heat in 55°C water bath for 30 min to dissolve the powder. Let it stand at room temperature to cool slowly. Filter through a 0.45-µm filter. Paraformaldehyde should be prepared fresh, or can be stored at –20°C and thawed before use.
19. Monoclonal antibodies to human placental AP can be bought from Genzyme Diagnostics (800-717-6314). Cat. no. MIA1801 has a relatively high affinity and is suitable for immunoprecipitations. Cat. no. MIA1802 has a lower affinity and can be used for affinity purification of AP fusions, although this is a procedure we rarely perform. Polyclonal antibodies can be purchased from several suppliers, including Zymed and DAKO.

3. Methods

3.1. Cloning of Foreign Gene Fragments into AP Vectors

APtag-1 or APtag-2 (**Fig. 1**) can be used to make a fusion protein with the receptor extracellular domain fused to the N-terminus of AP. This allows the AP tag to be fused to the position where the native receptor would enter the cell membrane, so it is unlikely the tag will interfere sterically with ligand binding. We generally position the fusion site immediately outside the hydrophobic transmembrane domain of the receptor.

For proteins that are not membrane anchored in their native state, such as soluble ligands, we recommend making both a fusion to the N-terminus of AP (with APtag-1 or APtag-2) and a fusion to the C-terminus of AP (with APtag-

4). In the case of fusions to the C-terminus of AP, secretion will be directed by the signal sequence of the AP, so any secretion signal in the inserted sequence should be eliminated. APtag-4 can also be used to produce unfused AP as a negative control (**Fig. 1**).

1. Digest the APtag vector of choice with the appropriate restriction enzymes (*see* **Note 3**).
2. Prepare a cDNA encoding the receptor extracellular domain, so it has sticky ends compatible with the vector (*see* **Note 4**).
3. Ligate the foreign gene into the restriction enzyme digested vector, transform them into competent *E. coli* and select recombinants. An *E. coli* strain containing the P3 plasmid, such as MC1061/P3, must be used for APtag-2 or APtag-4 (*see* **Note 5**). Check plasmid structure by restriction mapping and, if desired, by sequencing (*see* **Note 4**).

3.2. Preparation of AP Fusion Proteins from Transiently Transfected Cell Lines

AP fusion proteins are prepared by transfecting cultured cells (*see* **Note 6**). Depending on the situation, you may wish to use transient transfection (this section) or stable transfection (*see* **Subheading 3.3.**). Transient transfection is faster: it only takes about a week to obtain fusion protein ready for experiments, whereas it takes at least a month in a stable expression system. Also, in our experience transient transfection has been very reliable for expression of fusion proteins, whereas some proteins are expressed poorly following stable transfection. However, if you need a large amount of fusion protein over a long period of time, a stable cell line may save time and money in the long term. The APtag-2 and APtag-4 vectors are designed for transient expression, and have an SV40 origin so they will replicate in cell lines that express SV40 large T antigen, such as COS cells or 293T cells (*see* **Note 1**). We find that 293T cells give a several fold higher yield of fusion proteins than COS cells. Here we describe transfection with LipofectAMINE in a 100-mm tissue-culture plate as an example (*see* **Note 7**).

1. In a 100-mm tissue-culture plate, seed 7×10^5 cells per well in 10 mL complete medium. Incubate at 37°C in a CO_2 incubator until the cells reach 60–70% confluence. This will usually take about 13–16 h (*see* **Note 8**).
2. Prepare the following solutions in sterile tubes:
   Tube A: for each transfection, dilute 4 μg DNA (recombinant vector) in 400 μL Opti-MEM I reduced-serum medium.
   Tube B: for each transfection, dilute 24 μL LipofectAMINE reagent in 400 μL serum-free DMEM (*see* **Note 9**).
3. Combine the two solutions, mix gently, and incubate at room temperature for 30 min. The solution may appear cloudy, but this will not impair transfection.

4. Wash the cells once with 10 mL serum-free DMEM.
5. For each transfection, add 3.2 mL serum-free DMEM to each tube containing the DNA-LipofectAMINE complexes. Do not add antibacterial agents to media during transfection. Mix gently and overlay the diluted complex solution onto the washed cells.
6. Incubate the cells for 5 h at 37°C in a CO_2 incubator.
7. Add 6 mL DMEM with 20% bovine calf serum, without removing the transfection mixture. If toxicity is a problem, remove the transfection mixture and replace with complete medium.
8. Replace medium 24 h after the start of transfection.
9. Assay the AP activity of the supernatant 48 h after the start of transfection (*see* **Note 10**).

When a good AP fusion recombinant has been constructed and tested, you can scale up the procedure to collect larger amounts of AP fusion protein (*see* **Notes 11–13**). We suggest using 150 mm tissue culture plates for transfection. Adjust the amounts of all reagents in proportion to the surface area. Because the production of transfected protein increases rapidly around 48 h after the start of transfection, we generally change to fresh complete medium at 48 h after the start of transfection and condition it for a further 4 d. Spin the conditioned supernatant at maximum speed in a benchtop centrifuge, buffer the supernatant with 10 m*M* HEPES, pH 7.0, then 0.45 μm filter and store at 4°C. We generally also add 0.05% NaN_3 to prevent microbial growth.

3.3. Preparation of AP Fusion Proteins from Stable Cell Lines

As outlined in **Subheading 3.2.**, stable transfection may be useful for fusion proteins you will use in large amounts or over long periods. The APtag-1 vector (**Fig. 1**) is designed for stable transfection.

1. Linearize the expression vector (*Cla*I is usually a good site for plasmids based on APtag-1). Also linearize a selectable marker plasmid, such as pSV7neo, which confers resistance to G418. Phenol extract, ethanol precipitate and resuspend in sterile TE.
2. Plate NIH-3T3 cells so they are one-third confluent at the time of transfection. We usually do this by plating 4×10^5 cells on a 10-cm plate 2 d before transfection. Feed cells on the morning of transfection with 10 mL DMEM/10% calf serum.
3. Mix the following in two sterile 15-mL tubes. Amounts are for one 10-cm plate, increase all quantities in proportion to the surface area for more plates. When performing multiple transfections it may improve speed and accuracy to make larger premixes of A[carrier DNA + neo DNA + TE] and B[HBS + PO_4].
   Tube A:
   a. DNA: For APtag-1, 2 μg plasmid + 0.5 μg pSV7neo + 20 μg calf thymus carrier DNA are good amounts (*see* **Note 14**). Adding too much plasmid DNA

can severely reduce subsequent expression. When thawing carrier take care to disperse partitioned lumps.

b. Add TE to give total volume of 440 µL. Mix thoroughly.
c. Add 60 µL 2 *M* $CaCl_2$. Mix again.

Tube B:

a. 500 µL HBS
b. 7.5 µL $NaPO_4$ (35 m*M* NaH_2PO_4, 35 m*M* Na_2HPO_4)

Add solution A to tube B dropwise with Gilson P1000, while tube B is on vortexer in hood. Wait 5–15 min. The solution should be just detectably cloudy when carefully compared with clear buffer (*see* **Note 15**). If the precipitate looks good, add 1 mL to each 10-cm plate and swirl to mix. Leave plate (do not disturb) in incubator 6–12 h.

4. At the end of this time the cells should be covered with a carpet of particles, preferably so small that most of them do not have any discernible size when viewed with a 100× objective, but appear as tiny black dots. Wash cells gently two to three times with warm Hank's Balanced Salt Solution or DMEM. Add fresh complete medium.
5. After 12–36 h, split the cells and add medium containing G418 (400 µg/mL final activity for 3T3 cells). The following procedure works well to get clones of transfected NIH-3T3 cells. Harvest the cells from a 10 cm plate in 3 mL of trypsin. Transfer to a 50-mL tube, disperse well, and add 22 mL of complete medium containing serum and G418. Make the following dilutions in 15-mL tubes:

Cell suspension (mL)	Complete medium+G418 (mL)
5	7
2	10
1	11
0.5	11.5
0.2	12
0.1	12

Distribute each of these dilutions into a 96-well plate at 100 µL (or 2 drops) per well. Plate 10 mL of the remaining undiluted suspension in a 15-cm dish for a bulk uncloned population. Feed on about day 3 and day 9 after splitting, or sooner if the medium turns orange. Colonies should start to appear after about 5 to 10 d, depending on the cell line. Perform AP activity assays on the supernatant a few days later, well after the colonies have become confluent: transfer 100 µL of supernatant to a fresh 96-well plate, heat, and add 100 µL of 2×AP substrate solution (*see* **Subheading 3.4.1.**).

6. To collect AP supernatants in bulk, grow cells to confluence, then change the medium and condition it for a further 3 d. Harvest medium, spin out debris at maximum speed in a benchtop centrifuge, 0.45 µm filter, store at 4°C with 20 m*M* HEPES, pH 7.0, 0.05% NaN_3 (*see* **Notes 11–13**).

3.4. Verification of AP Fusion Protein

We verify the AP fusion protein in two different ways: (1) measuring the AP activity, and (2) immunoprecipitation to estimate the size of the fusion protein. Western blotting, using a polyclonal antibody to AP, can be used instead of immunoprecipitation.

3.4.1. Measuring the AP Activity

Because each fusion protein contains one AP tag, the concentration of fusion protein can be estimated according the AP activity (*see* **Note 16**). We measure the AP activity by adding the substrate para-nitrophenyl phosphate that is converted into a yellow product that can be quantitated in a spectrophotometer or plate reader at a wavelength of 405 nm.

1. Put 1 mL supernatant, or less, in an Eppendorf tube and heat-inactivate the endogenous AP activity in a 65°C water bath for 10 min.
2. Centrifuge the tube in a microcentrifuge at maximum speed for 5 min. Collect the supernatant.
3. Take some of the supernatant and add an equal amount of 2×AP buffer to check the AP activity (*see* **Note 16**). If the activity is reasonably high, it may be necessary to dilute the supernatant first, which can be done in HBAH, or in another buffer containing carrier protein.

3.4.2. Immunoprecipitation

1. Couple monoclonal antibody against AP to Sepharose beads (*see* **Note 17**).
2. For a six-well tissue-culture plate, label cells with 2 mL labeling solution (DMEM without methionine, containing 10% dialyzed serum, and 400 μCi ^{35}S-methionine) at 37°C for 3–6 h.
3. Collect the supernatant and concentrate to about 200 μL on Centricon-10 (Amicon).
4. Mix the supernatant with 20 μL beads coupled with anti-AP antibody for 30 min on a rotator at room temperature.
5. Wash beads twice in TBS/0.1% NP40, three times in modified RIPA buffer, and 1× in TBS/0.1% NP40. Use ice-cold buffers and do this quickly. After each wash, beads are pelleted by centrifugation at 5000 for 1 min in a microcentrifuge.
6. Add an equal volume of loading buffer and heat the sample for 2 min at 100°C. The size of AP fusion protein can be analyzed on an SDS-polyacrylamide gel. Unfused AP should migrate at an apparent molecular weight of approx 67 kDa.

3.5. Affinity Probe In Situ Using Receptor-AP Fusion Proteins on Whole-Mount Preparations of Embryos or Tissues

Whole mount AP *in situ* can be done either on whole embryos, or on parts of embryos or adult tissues that have been dissected out carefully. For penetration of the fusion proteins, whole embryos should not be too large: for mouse

embryos, they should not be older than approx day 10.5, and for chick embryos, day 4. For older embryos, you can dissect out the organ or tissue you are interested in, such as brain or other internal organs, and treat this as a whole-mount (*see* **Note 18**). You can also try sectioning the tissue, as described later. The protocol here describes a procedure without prefixation. This protocol works well for molecules that are located near the superficial layers of the embryo. To detect molecules that might be in deeper layers, you should consider sectioning, or you can try prefixing the embryo with either 4% paraformaldehyde or 8% formalin, and incubate embryos with AP fusion proteins in buffer containing a nonionic detergent such as NP40. However, depending on the protein, the signal detected may be reduced by these prefixation procedures.

1. Dissect embryos and transfer them to 2-mL microcentrifuge tubes. We use tubes with a round base, in which embryos are less likely to be trapped in the bottom.
2. Rinse embryos once with 1.5 mL HBAH buffer.
3. Incubate embryos with 1.5 mL AP fusion protein (*see* **Note 12**) for 75 min on a rotator at room temperature.
4. Remove AP fusion protein (*see* **Note 13**). Wash embryos six times with 1.5 mL ice-cold HBAH buffer. For each wash, leave the tube on a rotator for 5 min. If embryos later show a high background, it might be because this wash step was not sufficiently thorough. In our experience, washing 10 times or more, or even washing overnight at 4°C, can still give a good signal and the background may be reduced significantly.
5. Fix embryos with 1 mL acetone-formalin fixative for 2.5 min. A longer fixation time may reduce the signal. 8% formalin for 5 min can also be used alone, if acetone should cause any problem.
6. Wash out excess fixative with 1 mL HBS 3 × 5 min.
7. Incubate the tube containing embryos and 1 mL HBS in 65°C water bath for 15 min.
8. Put the tube on ice to bring it down to room temperature.
9. Rinse embryos once with 1 mL AP staining buffer.
10. Add 1 mL BCIP/NBT substrate. Incubate at room temperature on a rotator under a shade of aluminum foil (*see* **Note 19**). Staining can be monitored periodically. It only takes 5 to 10 min to see a strong signal. Weaker signals may take a few hours to develop. The sample can be incubated overnight, although background staining is then likely to become more significant.
11. Stop the reaction when it looks good by washing embryos with 1 mL PBS/10 m*M* ethylenediaminetetracetic acid (EDTA). Fix embryos in 8% formalin or 4% paraformaldehyde for 30 min. Wash and store embryos in PBS/10 m*M* EDTA in the dark.

3.6. Receptor-AP In Situ *on Tissue Sections*

The penetration of AP fusion proteins into unfixed whole mounts is likely to be limited. Sectioning allows access to deeper layers. For a good

survey of expression patterns, a combination of whole mounts and sections can be very helpful. The procedure below describes treatment of lightly fixed sections. An alternative is to cut thick unfixed sections with a vibratome and treat them essentially as wholemounts (*see* **Subheading 3.5.**).

1. Preparation of tissue sections:
   a. Dissect embryos or tissues and fix them in 4% paraformaldehyde. Depending on the size of the tissue, you can either do this at room temperature for 2 h, or at 4°C overnight. This protocol is good for tissues such as mouse embryos up to day 11.5 to 14.5. If the tissue is larger, you should fix it for longer or cut it open to let the fixative penetrate deeper.
   b. Rinse tissues with PBS once to remove fixative.
   c. Put tissues in 30% sucrose (in PBS) at 4°C on a rotator to mix them until they sink to the bottom of the tube when you take the tube out and let it sit upright.
   d. Pour out the sucrose solution until the surface is level with the upper part of tissue in the tube and add an equal amount of O.C.T. freezing solution (Tissue-Tek). Mix the tube on a rotator at room temperature for 2 h.
   e. Put the tissue in molds, add enough O.C.T. to cover the tissue, quick freeze the mold with tissue in liquid nitrogen, and transfer to –70°C freezer.
   f. Cryosection the tissue 1 d before the binding experiment and air-dry the sections at room temperature overnight. You can store the sections at –70°C after they have been dried.
2. Wash sections in a jar containing HBS for 10 min to remove O.C.T. on the slide.
3. Rinse twice in HBAH buffer.
4. Add AP fusion protein (*see* **Note 12**) to cover all sections on the slide and incubate at room temperature for 90 min.
5. Wash sections six times in cold HBAH.
6. Add acetone-formalin fixative on sections for 15 s exactly. Longer fixation might destroy some AP activity.
7. Wash sections twice in HBS.
8. Incubate sections in preheated HBS, kept in 65°C water bath, for 10 min. Increase the incubation time to 15 or 20 min if the background is high.
9. Wash sections 1× in AP staining buffer.
10. Add BCIP/NBT substrate to cover sections on the slide. Incubate at room temperature under a shade of aluminum foil. Staining can be monitored periodically against a white background under a dissecting microscope (*see* **Note 19**). Color should become visible in about 30 min to 2 h. Sometimes it takes a few hours, or even overnight, but background color is likely to appear after incubation of more than few hours.
11. Stop the reaction when it looks good by putting slides in PBS with 10 m*M* EDTA.
12. Fix sections in 8% formalin for 20 min.

13. Wash sections in PBS with 10 m*M* EDTA.
14. Mount the sections and keep them in the dark at room temperature.

3.7. Receptor-AP In Situ on Cultured Cells

In situ staining can also be done on cultured cells. This is a good method to identify individual positive cells when screening an expression library (*see* **Subheading 3.11.**). However, to screen cell lines for potential expression of a ligand, quantitative cell surface binding is much better, as described in **Subheading 3.8**.

The procedure described below can be used to detect either cell-surface ligands, or soluble ligands trapped within the cell in the secretory pathway, by a modification of the procedure at **step 2**.

1. Grow cells to be tested on a 10-cm tissure-culture plate until they are almost confluent, or have just reached confluence (*see* **Note 20**).
2. To detect a cell-surface ligand, wash cells 1× with 10 mL cold HBAH (*see* **Note 21**). Proceed to **step 3**. To detect a soluble ligand in the secretory pathway, wash cells once with 10 mL cold TBS (*see* **Note 21**), fix with TBS/4.5% formalin for 15 min, then incubate with HBAH containing 0.1% Triton X-100 for 15 min to permeablize the cells. Proceed to **step 3**.
3. Add 4 mL AP fusion protein (*see* **Note 12**) and incubate at room temperature for 90 min (*see* **Note 22**). Swirl briefly to mix at approximately the 30 min and 60 min time-points.
4. Remove the AP fusion protein solution with a pipet (*see* **Note 13**). Wash cells 6× with 10 mL cold HBAH (*see* **Note 21**). For each wash, incubate HBAH with cells for 5 min and gently swirl the medium by hand or on a platform shaker.
5. Aspirate the HBAH and add 10 mL acetone-formalin fixative slowly and swirl for 15 s exactly. Longer fixation might destroy some AP activity.
6. Aspirate the fixative and wash 2× with 10 mL HBS. Leave 10 mL HBS on the plate.
7. Incubate the plate containing 10 mL HBS on a flat shelf in a 65°C preheated oven for 100 min (*see* **Note 23**).
8. Wash with 10 mL AP staining buffer.
9. Add 4 mL BCIP/NBT substrate. Incubate at room temperature under a shade of aluminum foil. Staining can be monitored periodically against a white background under a dissecting microscope (*see* **Note 19**). Color should become visible in about 30 min. Sometimes it takes a few hours, or can even be incubated overnight, but background color will begin to appear.
10. Stop the reaction when it looks good by washing the plate with PBS and store the cells in 10 mL PBS with 10 m*M* EDTA at 4°C in the dark.

3.8. Quantitative Assay for Receptor-AP Fusion Binding to Cell Surfaces

This is the method of choice to screen cell lines for potential expression of a ligand that is cell surface associated (or extracellular matrix associated). It can

also be used to study quantitative aspects of ligand–receptor interactions, such as equilibrium constants or rate constants of binding, or the effects of antagonists, cofactors, or mutations.

1. Grow cells to be tested until they are almost confluent, or have just reached confluence. This can be done in a six-well tissue-culture plate.
2. Wash cells once with 3 mL cold HBAH (*see* **Note 21**).
3. Add 1 mL AP fusion protein solution (*see* **Note 12**) and incubate at room temperature for 90 min (*see* **Note 22**). Swirl briefly to mix at approx the 30 min and 60 min time-points.
4. Remove the AP fusion protein solution with a pipet (*see* **Note 13**). Wash cells 6× with 5 mL cold HBAH (*see* **Note 21**). For each wash, incubate HBAH with cells for 5 min and gently swirl the medium by hand or on a platform shaker.
5. Aspirate out all the remaining HBAH completely, and lyse cells with 300 μL Triton-Tris buffer at room temperature. It usually takes a few minutes at most for the cells to dissolve.
6. Collect all the lysate to an Eppendorf tube, rinse the plate with an additional 200 μL Triton-Tris, and pool this with the first lysate. Vortex for 30 s, allow to sit at room temperature for 5 min, and vortex again.
7. Spin down the lysate at maximum speed in a microcentrifuge, and transfer the supernatant to another Eppendorf tube.
8. Heat inactivate the supernatant in a 65°C water bath for 10 min.
9. Put the supernatant on ice to cool it.
10. Take 100 μL supernatant and add an equal amount of 2×AP substrate buffer to check the AP activity as described in **Subheading 3.4.** (*see* also **Note 24**).

3.9. Coimmunoprecipitation of Ligand with Receptor AP Fusion Protein

This can be used as an alternative method to screen cell lines, as a preliminary to cloning, and this can be particularly useful for soluble ligands. The protocol below includes steps to detect either cell associated or soluble ligand. If a soluble ligand is identified it can be cloned by expression methods (*see* **Subheading 3.11.**) or by scaling up the immunoprecipitation procedure to obtain peptide sequence which is then used to design oligonucleotides for PCR or library screening.

In addition to cloning projects, this coimmunoprecipitation method is also useful to characterize the molecular weight and other properties of a ligand, especially when an antibody is not available.

1. For a six-well tissue-culture plate, label cells with 2 mL labeling solution (DMEM without methionine, containing 10% dialyzed serum, and 400 mCi ^{35}S-methionine) at 37°C for 3–7 h.
2. Collect the supernatant and concentrate to about 200 μL on Centricon-10.
3. Wash the cells 5× with 5 mL HBAH buffer, and lyse in 200 μL Triton-Tris buffer containing 1 m*M* PMSF (phenylmethylsulfonylfluoride). Centrifuge for 10 min in a microcentrifuge at maximum speed to pellet nuclei.

4. Incubate the concentrated supernatants and the cell lysates (separately) with an equal volume of AP fusion protein solution (*see* **Note 12**) on a rotator at room temperature for 90 min.
5. Add 20 µL Sepharose beads coupled with excess anti-AP antibodies (*see* **Note 17**) to each tube and incubate for 30 min on a rotator at room temperature.
6. Wash beads 2× in TBS/0.1% NP40, 3× in modified RIPA buffer and 1× in TBS/0.1% NP40. Use ice-cold buffers and do this quickly. After each wash, centrifuge at 5000 for 1 min.
7. Add an equal volume of loading buffer and heat the sample for 2 min at 100°C. Any molecules that bind to the AP fusion protein are analyzed on an SDS-polyacrylamide gel, followed by autoradiography.

If a putative ligand is found by radioactive detection, the procedure can be scaled up for peptide microsequencing. Microsequencing usually requires amounts of protein in the microgram range, and (depending on the concentration of the ligand) it may be possible to isolate enough ligand from a liter, or so, of conditioned medium. The ligand concentration in conditioned medium can be estimated by staining gels for protein, for example with silver stain. To scale up the coimmunoprecipitation procedure, the anti-AP antibody coupled Sepharose beads are incubated with a saturating amount of AP fusion protein and are then crosslinked with dimethylpimelimidate to prevent the fusion protein from leaching off the beads. The conditioned medium is collected and concentrated with an Amicon pressure cell, and is then incubated on a rotator with the AP fusion protein crosslinked beads. The beads are loaded on a column, washed with modified RIPA buffer and then with 10 m*M* sodium phosphate, pH 6.8, and eluted with 100 m*M* glycine, pH 2.5. The eluted sample is TCA precipitated, run on an SDS-polyacrylamide gel, and transfered to a PVDF membrane for microsequencing. Peptide sequences obtained can be compared to the sequence databases. If the peptide does not correspond to any known sequence, it can be used to design oligonucleotides for PCR or library screening.

3.10. Cell-Free Binding of Fusion Proteins

To establish a direct interaction between ligand and receptor, binding assays in a cell-free system can be performed with receptors fused to an AP tag, in combination with ligands fused to an immunoglobulin (Ig) Fc tag, or vice versa (*see* **Note 25**). More generally, this provides a method to characterize ligand–receptor interactions in a cell-free system.

1. Prepare ligand-Ig fusion protein (*see* **Note 26**).
2. Incubate ligand-Ig fusion proteins with an equal volume of protein A-conjugated Sepharose beads (Pharmacia) on a rotator at room temperature for 1 h. Protein A binds the Ig Fc region.
3. Wash beads 2× with HBAH.

4. Add a 15-μL aliquot of beads to 500 μL receptor-AP fusion protein (*see* **Note 12**) and incubate on a rotator at room temperature for 2 h.
5. Wash beads 5× with HBAH and 1× with HBS.
6. Incubate beads with 100 μL HBS in 65°C water bath for 10 min, then transfer to ice.
7. Add equal amount of 2×AP substrate buffer to measure the AP activity as described in **Subheading 3.**4.

3.11. Expression Cloning

It is well worthwhile devoting some effort to find a good source material of RNA to make your expression library. Enriching the representation of the target molecule in this way at the outset can save a lot of work later at the screening stage, and can help to achieve a successful outcome. Sometimes a good guess about a ligand source can be made based on the biology of the receptor. A more direct approach is to detect ligand expression in cells or tissues, which can be done with one of the methods described above (**Subheadings 3.5.**, **3.6.**, **3.8.**, or **3.9.**). If a good source of ligand expression can be identified using a receptor-AP fusion protein, then one can have reasonable confidence that an expression cloning approach should work. A potential problem with all expression cloning approaches is that, in principle, they may fail if the target molecule consists of more than one polypeptide chain. However, in practice there are many examples of ligands or receptors that can heterodimerize, but can also bind reasonably well even without their normal heterodimerization partner, and have been successfully expression cloned.

Once a good source of RNA has been identified, a cDNA library can be prepared. The library is then expressed in a cell line, and screened for binding of the receptor-AP fusion protein. This approach can be used to clone either cell surface or soluble ligands.

1. Prepare total RNA and then poly(A)$^+$ RNA from a tissue or cell line with high expression of the ligand. Prepare double-stranded cDNA and insert into an appropriate expression vector to make a cDNA library for transient expression. Size-selecting your cDNA to remove molecules smaller than the predicted size of the molecule you are trying to clone is likely to greatly improve the quality of your library (*see* **Note 27**). Several manufacturers provide kits for RNA purification, cDNA synthesis, and library construction. A number of expression vectors are available; we have had good results with the CDM8 vector ***(20)*** (Invitrogen), which contains the SV40 origin and can replicate efficiently in cells containing the SV40 T antigen, such as COS cells or 293T cells.
2. Transfect the library into competent *E. coli* to make pools. Use a pool size of approx 1000–2000 clones per pool (*see* **Note 28**). For each pool, plate the transfected *E. coli* on a nitrocellulose filter on an agar plate, so that you can make a replica. Keep the original filter on agar at 4°C. Grow the bacteria on the replica and use this for the next step.

3. Collect the colonies from the replica and prepare plasmid DNA from it. For a 15-cm filter, we scrape off the bacteria in 15 mL LB medium, and make a conventional alkaline-SDS DNA minipreparation.
4. To screen the library, 4 μg DNA of each pool is transiently transfected into a 10-cm plate of COS cells with lipofectAMINE (*see* **Subheading 3.2.**).
5. 48 h after the start of transfection, perform affinity probe *in situ* on the cells with your receptor-AP fusion (**Subheading 3.7.**). At 48 h, the cells should be at or just before confluence and should also express large amounts of protein. Try to do AP *in situ* before cells are too confluent (*see* **Note 20**).
6. Staining should be monitored periodically against a white background under a dissecting microscope (*see* **Notes 19** and **29**).
7. When you get a positive pool, take the original bacterial filter, make a fresh replica, and cut the this into 10 segments that will be 10 subpools (make marks on the original so that you know the corresponding colonies for each subpool). Always keep the original filter and make DNA from the replica.
8. Collect DNA from each subpool and screen as described above (**steps 3–6**) to identify a positive subpool.
9. Pick individual colonies from the region of the original filter corresponding to the positive subpool (about 100–200 colonies). Transfer to a 96-well plate so there are one or two colonies in each well, with each well containing 200 mL growth medium. Grow bactrial colonies overnight. Take an aliquot of bacterial culture from each well, and pool aliquots of bacterial culture from each column of wells and each row of wells. Screen again (**steps 3–6**). By matching up the positive row and the positive column it should be possible to identify the positive well and subsequently isolate a positive clone in a final round of screening (*see* **Note 30**).

4. Notes

1. NIH3T3 cells are used with APtag-1 to make stable cell lines. COS cells or 293T cells are used for transient expression vectors APtag-2 and APtag-4. 293T cells seem to produce several-fold higher amounts of fusion protein than COS cells. However, COS cells might be preferred for expression cloning because they are larger and adhere to tissue culture plates better than 293T cells.
2. 100% formalin solution is approx 40% formaldehyde (commercially available stocks are usually 100% formalin or 10% formalin). Therefore, 8% formalin is approx 3% formaldehyde.
3. For APtag-1 and APtag-2, we generally use *Hin*dIII for the 5' end of the insert. At the 3' end, fusions at the *Bgl*II site will result in a 4 amino acid linker. Most often we fuse this to a *Bgl*II or *Bam*HI end on the insert. For example, joining a *Bam*HI site on the insert to the *Bgl*II site on the vector gives Gly-Ser-Ser-Gly, quite a long linker that should give plenty of conformational flexibility. However, fusion proteins linked at the *Bsp*EI site (which gives a 2 amino acid linker) have also worked well in our hands. If no appropriate

cloning site is available on your insert, you may want to consider blunt-end ligation. Note that *Bgl*II and *Bsp*EI both produce sticky ends that are compatible with several other enzymes.

For APtag-4, fusions can be made at the *Bgl*II or *Bsp*EI sites. In either case, the C-terminal peptide sequence of AP is likely to act as a good linker. The 3' end of the insert can be joined to the *Xho*I, *Nsi*I, or *Xba*I sites.

4. To make receptor-AP fusions, we generally make the fusion point precisely outside the hydrophobic stretch of residues that defines the transmembrane domain. If the gene happens to contain suitable restriction sites, they can be used. However, we generally use PCR to amplify the relevant region, while introducing artificial restriction sites at the ends of the insert. If PCR is employed, use conditions to minimize the introduction of mutations, for example, use a polymerase with a 3'–5' editing nuclease function, such as pfu polymerase (Stratagene), and keep the NTP concentrations low in accordance with the manufacturer's instructions. To ensure that mutations have not been introduced, you may wish to sequence the amplified gene. However, this may be too time consuming and a preferable insurance policy may be to prepare fusion proteins from two independent clones.
5. APtag-2 and APtag-4 have a supF marker, and must be grown in the MC1061/P3 bacterial strain (available from Invitrogen, Bio-Rad, and other suppliers) with selection in ampicillin (50 μg/mL) plus tetracycline (10 μg/mL). APtag-1 has an ampicillin marker and can be grown in commonly used competent *E. coli* strains with ampicillin selection.
6. We generally use mammalian cells to minimize the risk of inappropriate protein modification or folding, and because the transient expression protocols are fast and reliable. We have also used the Baculovirus expression system successfully. Expression in bacterial or yeast systems is likely to be more risky, and we know of several examples where this has not worked.
7. We generally prefer lipofectAMINE as a transfection reagent rather than DEAE-dextran, because it gives better transfection efficiencies. However, other commercially available transfection reagents may also be used. If you prefer a different size of plate, you can adjust the volumes of all solutions in proportion to the surface areas of the plates.
8. The transfection efficiency is sensitive to the confluence of cells. If the cells are either too sparse or too dense, poor expression may result.
9. The ratio of DNA to LipofectAMINE is somewhat empirical, and you may wish to try a titration. When using different-sized tissue-culture plates, adjust the amounts of all reagents in proportion to the surface area.
10. COS cells usually begin to express transfected protein 48 h after the start of transfection, but you can wait for one or a few more days if you want to have higher AP activity.
11. We usually find supernatants containing AP fusion proteins are stable for months, or even years, at 4°C. For many purposes, the supernatant should be ready to use as a reagent without further steps. If necessary, the protein can be concentrated

by ultrafiltration with an Amicon pressure cell and a PM30 or YM100 membrane (depending on the size of the fusion protein). We have also affinity purified using an anti-AP antibody, with elution by low pH ***(1)*** or 3 *M* $MgCl_2$ (M.-K. Chiang and J. G. Flanagan, unpublished), but this is laborious and should not generally be necessary. If serum in the complete medium is a problem for your subsequent experiments, the conditioned medium can be produced in serum-free conditions: for COS cells, Opti-MEM I can be used, and for NIH-3T3 cells, DMEM with insulin, transferrin, and selenium (Redu-SER; Upstate Biotechnology Inc.). The concentration of fusion protein produced is about one-tenth of that obtained from cells grown in complete medium, but can be concentrated as described above.

12. The concentration of receptor-AP fusion protein that will be optimal for final use depends on the experiment, and most importantly, it depends on the affinity of the ligand–receptor interaction. For the interaction of RTKs with their cognate ligands, the dissociation constant of binding (K_D) is generally in the range of approx 10^{-8} to 10^{-12} *M*. K_D is equivalent to the concentration of receptor-AP protein that will give half maximal occupation of ligand sites. In general, increasing the concentration of receptor-AP fusion is expected to increase the signal (saturably) and also increase the background (nonsaturably). For known ligands, we would typically use a receptor-AP concentration between one and ten times the K_D. When testing for an unknown ligand, we might typically try concentrations of receptor-AP fusion in the range of 2 n*M* to 40 n*M*. The optimal concentration for any particular experiment may need to be determined empirically. If necessary, supernatants can be concentrated by ultrafiltration (*see* **Note 11**) or can be diluted with HBAH buffer. For all types of binding experiment, we use unfused AP (at the same concentration as the fusion protein) as an important negative control.
13. For many types of experiment, such as staining of cells or tissues, the receptor-AP fusion protein can be saved after use and reused several times. The protein concentration remaining can be estimated from the AP activity.
14. Preparation of carrier DNA. (The purification may not be necessary if you can get good pure DNA.)
    a. Dissolve 100 mg calf thymus DNA at 1 mg/mL in TE. Suck hard five times through an 18 gage, 1.5-in needle to shear the DNA.
    b. Phenol-chloroform (1:1) extract until essentially no interface is left (about six times). Chloroform extract two times.
    c. Dialyze against 4 L of STE (10 m *M* NaCl, 10 m*M* Tris, 0.1 m*M* EDTA) for 2 d with one change of buffer.
    d. Ethanol precipitate, wash with 70% ethanol, evaporate the ethanol in a sterile hood, resuspend in sterile STE (resuspension takes a couple of hours at 50°C with occasional vortexing). Store frozen.
15. In our experience, the finer the precipitate the better. If there is no precipitate, try adding more phosphate: put 1 µL on the wall of the tube and vortex in immediately. If there are visible lumps of precipitate, throw away and start again with less phosphate solution.

16. When preparing samples for measurement of AP activity, avoid buffers containing phosphate, which is a competitive inhibitor of AP. The activity can be measured by the change of absorbance at 405 nm, either in a cuvet by spectrophotometer (OD/h) or in a 96-well plate with a microplate reader (*V*max in mOD/min). We perform all reactions at room temperature. For samples with low AP activity, compare the absorbance of your sample with a control containing AP substrate solution only, because the substrate gradually hydrolyzes spontaneously. Absorbance readings that are too low (<0.1) or too high (>0.8) may not be very accurate. To convert from *V*max in a microplate to OD/h in a cuvet, divide by an approximate conversion factor of 59 (this assumes a volume of 200 μL and a light path length of 0.713 cm for the microplate, vs a reaction diluted to 1 mL and a path length of 1 cm for the cuvet).

    To convert from OD/h in a cuvet to pmol of AP protein, divide by an approximate conversion factor of 36. Please note that this is an approximate and empirically determined value. To obtain accurate values for your particular protein, it would be necessary to measure the relevant activities and protein concentrations.
17. Coupling of monoclonal AP antibody to CNBr Sepharose beads.
    a. Weigh out about 3.5 g CNBr Sepharose powder (Pharmacia). Swell the gel for a few min by mixing with 1 m*M* HCl in a 50-mL tube. Wash in a sintered glass funnel over vacuum with about 500 mL of 1 m*M* HCl over a period of 15 min.
    b. Resuspend the washed gel in a small amount of 1 m*M* HCl and pipet some into a 15-mL tube. Centrifuge at 2 K for 5 min to estimate packed volume of beads. Adjust packed volume to 5 mL by suspending again and removing excess suspension. Centrifuge briefly to pack beads and remove supernatant.
    c. Set up the coupling reaction. The final concentrations should be as follows: gel at 40–50% v/v; 5 mg antibody; 0.25 *M* sodium phosphate, pH 8.3. Incubate at 4°C on a rotator overnight.
    d. For a 10-mL coupling reaction, add 5 mL of 1 *M* ethanolamine HCl, pH 8.0 to stop the reaction. Incubate on a rotator at 4°C for 4 h.
    e. Wash the beads once with 0.5 *M* sodium phosphate, pH 8.3, and then modified RIPA buffer 3×.
    f. Store beads in modified RIPA buffer in tightly closed tube at 4°C.
18. We find that embryonic tissues that are damaged during dissection or are cut through by knife may show AP staining nonspecifically, so data from dissected embryonic tissue should interpreted with caution. In addition, newly forming cartilage, bone, and nervous tissue might have higher endogenous AP activity, which can be difficult to heat-inactivate completely in a whole tissue preparation. We strongly suggest that in older embryos you should try sections for those tissues. If you should have problems with background staining, try a longer time of heat-inactivation and compare the staining carefully with negative controls.
19. Chromogenic AP substrates may darken significantly if exposed to light. During color development, samples should generally be kept in the shade. If you want to

view them under the microscope, do this for only a short time.

20. Different sizes of plates can be used, such as 6-well plates. For library screening, it is important to have a uniform carpet of cells, to minimize the risk of questionable or false positives. Smaller plates can give problems with a variety of edge effects, whereas a 10-cm dish gives a large uniform central area. It is also important to ensure a uniform density of cells over all parts of the plate, and to stain cells that are just under confluence, or just recently confluent. Overconfluent cells can pile up, trapping the fusion protein probe and sometimes causing unpredictable background staining.
21. Cells can dry and fall off very quickly if all the medium from the plate is aspirated. The problem is seen mainly around the edges, so becomes more severe as the plate size gets smaller. To minimize this effect, pipet the medium out, but leave enough to provide a thin covering at the center. With experience, this can be done quickly with a vacuum aspirator by withdrawing the tip of the pipet as soon as the liquid level reaches the bottom of the well.
22. The time is determined by the rate of reaction k_{on}. For the reaction of an RTK and its ligand, k_{on} can be quite slow, but 60 to 90 min at room temperature should be enough to give good binding. On ice, the reaction would be much slower.
23. For library screening, it is essential to have uniform heat inactivation across the plate, so it is important to ensure the shelf is exactly horizontal. An alternative to using an oven is to float the plate on a 70°C water bath for 30 min, but uneven heating is likely, so this may not be a good method for library screening.
24. If there are traces of background alkaline phosphatase activity even after heat inactivation, these can be removed by immunoprecipitating the fusion protein (*see* **Subheading 3.4.**, **steps 4** and **5**) and adding 1×AP substrate buffer to the Sepharose beads. However, we seldom perform these steps.
25. Instead of an Ig tag, other tags can be used such as *myc* or HA epitopes, or 6×*His*.
26. The ligand (or receptor) cDNA can be fused to the sequence of the human IgG1 Fc region in the pcDNAI vector ***(2,16)***. Prepare the Ig fusion protein in COS cells or 293T cells as described for the preparation of AP fusion protein.
27. Synthesis of cDNA usually results in a large proportion of short fragments. Moreover, smaller cDNAs are present at higher molar concentrations and also are likely to replicate preferentially after insertion into the vector. It is therefore recommended that cDNAs smaller than the molecule you are expecting to clone are eliminated. In our experience, gel purification methods work well and give an efficient separation of large and small molecules.
28. It is important not to make the pool size too large. If the pool size is in the range of 1000 to 2000 clones per pool, a positive pool should contain several positive cells and should be obvious at the screening stage. Larger pool sizes can cause difficulties in distinguishing between truly positive and false positive pools.
29. We prefer screening the plate under a dissecting microscope rather than other types of microscope, because it is easier and faster. In addition, it is usually easier to distinguish false positives such as clumps of cells, dead cells, and so on. Staining of positive cells may become visible in about 30 min, or may take a few

hours. False positives will tend to appear after 15 h. A positive pool should usually show 10 or more positive cells. The distribution of staining within the cell may also be a diagnostic feature: cell-surface staining should be distributed over the whole cell, whereas detection in the secretory pathway is likely to be concentrated in the perinuclear region. True positive cells should be distributed evenly over the plate: sometimes a cluster of two or a few cells may result from division of a genuine positive cell, but a large number of stained cells in only one region of the plate are more likely to result from locally inadequate heat inactivation, or too high a cell density.

30. In the case of a true positive clone, each successive screening should have an increased number of positive cells per plate. We have not experienced problems with false positives caused by heritable endogenous AP expression, or clones encoding a heat stable AP activity, but any such cells could be ruled out by a control where unfused AP, or no AP reagent, is added instead of the receptor-AP fusion probe.

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22

Isolation and Characterization of "Orphan-RTK" Ligands Using an Integrated Biosensor Approach

Martin Lackmann

1. Introduction

1.1. General Principles

The interest in biosensor-based ligand isolation strategies is to a large extent, a result of fundamental changes in the way novel proteins are isolated and characterized. Advances in genetic screening techniques have led to the identification of novel protein families with little knowledge of function or physiological context ***(1,2)*** and a rapidly increasing number of these so-called "orphan proteins" calls for complementary strategies to elucidate their structure and function. Over the past few years, different approaches have been developed to isolate ligands solely on the basis of their affinity for a particular orphan receptor. These include expression cloning strategies to detect cell-membrane bound ligands with tagged receptor extracellular domain (ECD) fusion proteins ***(3–6)*** and cell rescue assays to detect soluble ligands ***(7–9)***. BIAcore technology has been applied to search for suitable ligand sources of orphan receptors ***(10–12)***, but is also well suited to integrated use within a "classical" purification scheme ***(13,14)*** and has been exploited as an affinity detector to facilitate the isolation of orphan proteins ***(15,16)***. The advantages of this technology include a fast, robot-driven assay that is unaffected by sample toxicity, and the ability to assess specificity and kinetics of the observed interaction even in relatively crude samples, thus minimizing the risk of false positives.

1.2. Orphan Receptors

By exploiting the sensorchip-immobilized recombinant receptor ECD as sole monitor for the presence of a ligand, a critical emphasis is placed on the purity

From: *Methods in Molecular Biology*, Vol. 124: *Protein Kinase Protocols*
Edited by: A. D. Reith

and conformational integrity of the receptor preparation. While the purity can be assessed routinely with conventional methods, the most convincing monitor for conformational integrity (i.e., the physiological ligand) is not available. However, conformation-specific antireceptor monoclonal antibodies (MAbs), selected for their loss of reactivity to the immobilized denaturated receptor ECD on the BIAcore, can be used to monitor the conformation of the receptor protein during purification and use on affinity surfaces. Purification of the recombinant receptor ECD is performed under strictly nondenaturing conditions to minimize the possibility of protein modification or denaturation, and the use of marker peptides/polypeptides to which affinity reagents are available ***(17,18)*** greatly assist this task. The receptor used here, as an example, is the EPH family receptor tyrosine kinase (RTK) EphA3 (HEK), a pre-B-cell-derived transmembrane receptor, to which a conformation-specific MAb is available ***(19)***.

1.3. Receptor ECD Affinity Surfaces

The sample-exposed surface of the BIAcore sensorchip consists of a carboxymethylated dextran layer to which proteins and peptides can be coupled by a variety of strategies ***(20)***, the most common of which is coupling via primary amino groups. This coupling chemistry can be conveniently adopted for the preparation of Sepharose or agarose-based receptor ECD affinity columns. Optimization of coupling conditions and assessment of conformation/stability of the immobilized protein, and elution conditions for the putative ligand, can be performed on the BIAcore with minimal consumption of sample ***(13)***. Furthermore, the capacity of the affinity surface for the conformation-specific MAb as substitute for the ligand can be quantitated on a stoichiometric basis by using the BIAcore for concentration measurements ***(21)***. To ensure a uniform performance of sensorchips and affinity columns, this assessment protocol is used as an essential quality control throughout the ligand isolation strategy.

1.4. Principles of BIAcore Detection

BIAcore experiments are monitored via a photo-electric signal, the surface plasmon resonance wave ***(22)*** which detects molecular interactions on the protein-derivatized affinity surface of the sensor chip (**Fig. 1**). The resulting signal is obviously the sum of all interactions. These include not only ligand binding to a specific docking site on the immobilized receptor ECD, but also interactions between the ligand, or other components of the sample, and other available sites on the derivatized sensor chip. Depending on the sample composition, in particular its complexity, the ligand abundance and the type of "contaminants," ligand-derived specific or nonspecific signals will dominate this total

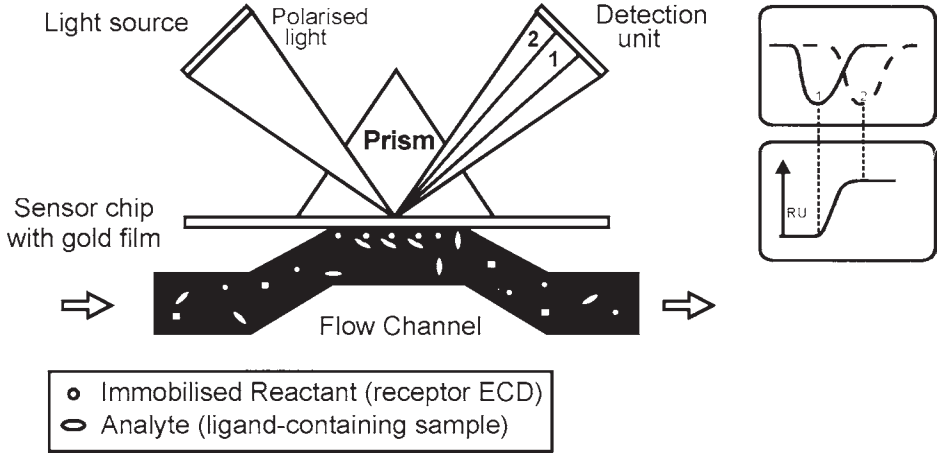


Fig. 1. Principle of BIAcore detection. The linear-polarized light focussed onto the gold film of the sensorchip will result in an evanescent wave (not shown) penetrating into the CM-dextran derivatized, sample exposed surface. In turn, the evanescent wave will cause in a discrete shadow in the reflected light ***(1,2)***, the angle of which changes on mass changes on the CM-surface. This angle, reflecting the sum of all interactions on the CM-dextran surface, is tranformed into the Reflectance Units (RUs) shown on the monitor.

BIAcore response. Dissection of this composite signal into receptor/ligand specific, and nonspecific, components is achieved by comparing binding of a parallel of the sample which is supplemented, prior to application, with a competing concentration of the soluble receptor ECD. BIAcore software-assisted substraction ***(21)*** of the nonspecific signal of the competed sample will yield the ligand/receptor specific response even at subnanomolar ligand concentrations in very complex samples ***(15)***.

1.5. Ligand Search and Isolation

The interaction of unrelated components with the affinity support is a major concern when ligands are present at low abundance in complex protein mixtures. Analysis of the candidate ligand sources at various concentrations, enrichment of receptor-binding activity by batch extraction procedures, and depletion of the activity with an affinity matrix are important control experiments to confirm the presence of a genuine ligand. Although "single-step" receptor affinity purification protocols have been reported ***(10,11)***, their success will depend on the ligand abundance and type of contaminants present in the source material. The response curves (sensorgrams) from the BIAcore experiments are often a good indicator of the complexity of the initial interactions. For complex samples, a conventional

protein purification strategy is necessary (preferably involving receptor affinity chromatography) in which the BIAcore assay is used to monitor the recovery and enrichment of the ligand throughout the procedure ***(15)***.

Once a putative ligand has been purified and identified by amino acid sequence determination, an estimation of its affinity for the receptor ECD will provide a first indication of the physiological relevance of the interaction. An estimate of an apparent affinity constant can be calculated, in the first instance, from the on- and off-rate constants of purified ligand by analyzing the BIAcore raw data of homogenous samples derived from the final purification steps ***(15)***. For more reliable analysis, sufficient quantities of the ligand or its recombinant homolog should be used to perform kinetic analysis under equilibrium conditions on the sensorchip, or in solution, to achieve an unambiguous assignment of the affinity and stochiometry of the interaction ***(23)***.

2. Materials

2.1. General

The manually operated surface plasmon resonance biosensors developed by BIAcore (BIAcore X, BIAcore AB, Uppsala, Sweden) or by Fisons Instruments (IAsys, Fisons Applied Sensor technology, Cambrige, UK) are not suited for applications with a large sample throughput. Two four-channel optical biosensors, BIAcore models 1000 and 2000 (Biacore Ab, Sweden) are equipped with an autosampler, whereby the BIAcore 2000 allows the simultaneous analysis of one sample on four parallel channels of the sensorchip, thus reducing sample consumption and assay time. Both grades (certified grade or research grade) of the carboxymethylated sensorchips (CM5, Biacore Ab, Sweden) are suitable for this application (*see* **Note 1**).

Unless indicated otherwise, all aqueous buffers used for liquid chromatography contain 0.02% Tween-20 (Pierce) to minimize losses because of nonspecific binding. All buffers are prepared from stock solutions that had been filtered through 0.2 μm filter units.

2.2. Assessment of Receptor Conformation

2.2.1. Homogeneity of Receptor ECD

1. Recombinant receptor ECD: purified from culture supernatants of an approptiately transfected mammalian cell line (*see* **Note 2**) by affinity chromatography under strictly nondenaturing conditions (*see* **Note 3**).
2. Liquid chromatography system: preferably a nonferrous “biocompatible” solvent delivery system. A Waters, Model 650 Protein purification system equipped with a Model 996 photodiode array detector has been used for the studies presented here.

3. SE-HPLC column: Superose 12 (10/30, Pharmacia). Running buffer: 20 m*M* phosphate, 150 m*M* NaCl, pH 7.4 phosphate-buffered saline (PBS).
4. Mono-Q Ion exchange column (5/5, Pharmacia): binding buffer: 20 m*M* Tris-HCl, pH 8.5; elution buffer: 1.0 *M* NaCl in binding buffer.
5. Centrifugal concentrators: Ultrafree-MC Filter Units (Millipore).

2.2.2. Immobilization to the Sensorchip

1. CM5 sensorchip: Certified or research grade (BIAcore AB, Sweden).
2. Antireceptor MAbs: a panel of antibodies, preferably tested for binding to the native receptor. Alternately, hybridoma supernatants (with a minimum MAb concentration of 1–10 μg/mL, buffer exchnaged in BIAcore running buffer — *see* **step 7**) from an immunization with the affinity-purified receptor ECD. To ensure conformational integrity the freshly prepared protein should be kept at 4°C in PBS containing 0.02% Tween-20.
3. FAST-Desalting column PC3.2/10 (Pharmacia).
4. N-hydroxysuccinimide (NHS): 0.05 *M* aqueous solution, stored in 100-μL aliquots at –20°C.
5. N-hydroxysuccinimide-N-ethyl-N'-(diethylaminopropyl)-carbodiimide (EDC): 0.2 *M* aqueous solution stored in 100 μL aliquots at –20°C.
6. 1,2 Di-aminoethane (1,2 DAE): 1 *M* aqueous solution, pH 9.0, stored in the dark at 4°C.
7. BIAcore running buffer: 10 m*M* HEPES, pH 7.4, 0.15 *M* NaCl, 3.4 m*M* EDTA, 0.005% Tween-20. Prepared as 10× stock without Tween-20. The 1× buffer is degassed prior to addition of the detergent.
8. Coupling buffer: 20 m*M* Na-acetate, pH 4.5, without Tween-20.
9. $MgCl_2$-desorption buffer: 3 *M* $MgCl_2$, 0.075 *M* HEPES/NaOH, 25% ethylene glycol, pH 7.2. Prepared from 4 *M* $MgCl_2$:solid $MgCl_2 \cdot 6H_2O$. dissolved in H_2O, 1 *M* HEPES/NaOH, pH 8.0 added (1/10 of final vol) and H_2O added to adjust the volume to 4 *M* $MgCl_2$. The pH should not be adjusted further ***(24)***.
10. Denaturation buffer: 6 *M* guanidine hydrochloride, 5 m*M* dithiothreitol, 5 m*M* ethylenediaminetetracetic acid (EDTA), 50 m*M* Tris, pH 8.0.
11. Wash buffer: 1 *M* NaCl, 20 m*M* Tris, pH 9.5.

2.2.3. Stability of Immobilized receptor ECD

1. CM5 Sensorchips: (*see* **Subheading 2.2.2.**).
2. EDC/NHS solutions: (*see* **Subheading 2.2.2.**).
3. Homogenous preparation of receptor ECD:
4. Antireceptor MAb, 0.1 mg/mL: (*see* **Subheading 2.2.2.**).
5. 1% Bovine serum albumin (BSA) in BIAcore buffer.
6. Desorption buffers:
   a. 4 *M* NaCl (not pH adjusted).
   b. 1 *M* glycine, pH 9.0.
   c. 0.1 *M* $NaHCO_3$, 1 *M* NaCl, pH 9.5.

d. 0.2 *M* Na_2CO_3, pH 11.5.
e. 1 *M* NH_4OH, pH 11.5 (not pH adjusted).
f. 50 m*M* 1,2 Di-ethanolamine (Fluka), pH 12.3.
g. 3 *M* $MgCl_2$-desorption buffer (*see* **Subheading 2.2.2.**). 0.1 *M* Na-acetate, 0.5 *M* NaCl, pH 4.0.
h. 0.5 *M* glycine-HCl, 0.5 *M* NaCl, pH 3.5.

2.3. Receptor ECD Affinity Columns

1. Cyanogen-activated Sepharose 4B (Pharmacia). Alternately, NHS-/ derivatized affinity gels: NHS-activated Sepharose (Pharmacia) or agarose (Affi-Prep 10, Bio-Rad) can be used.
2. Polypore DEAE columns (2.1 ¥ 30 mm, ABI/Brownlee).
3. CM5 Sensorchip: derivatized with native receptor ECD.
4. Dialysis tubing: M_r 10,000 cut off.
5. Homogenous receptor ECD preparation (0.5–1.0 mg).
6. Antireceptor MAb at 0.1 mg/mL in BIAcore buffer (*see* **Subheading 2.2.2.**)
7. Coupling buffer: 0.1 *M* $NaHCO_3$/0.5 *M* NaCl, pH 8.3
8. BIAcore running buffer: (*see* **Subheading 2.2.2.**).
9. Storage buffer: PBS (*see* **Subheading 2.2.2.**)/0.02% Tween-20/0.002% NaN_3

2.4. Identification of a Ligand Source

Although the expression pattern of the orphan receptor might suggest a certain preference for a ligand source, the absence of any information on its physiological context leaves the search for a ligand mostly to chance. Thus, a broad spectrum of different tissue culture and cell culture supernatants will be needed to provide a suitable starting point for screening purposes. It is obviously of critical importance to keep a complete record of the following:

1. The cell or tissue source;
2. Culture condition or extraction conditions (type of medium, additives, length of culture);
3. Treatment of sample (fold concentration, method of concentration).

Generally, a low ligand abundance should be expected. 10–50-fold concentrated samples (if possible from serum-free cultures) should be stored in multiple (minimally three) 0.3 mL aliquots in screw-cap tubes at –80°C.

2.4.1. Primary Screen

1. CM5 sensorchip: derivatized with native receptor ECD.
2. NAP-5 desalting columns: prepacked disposable Sephadex G25.
3. Aliquots of putative ligand sources: fresh-thawed, do not refreeze.
4. Soluble receptor ECD: prepared as in **Subheading 3.2.1**.
5. BIAcore running buffer: (*see* **Subheading 2.2.2.**).
6. Desorption buffer: as evaluated in **Subheading 1.3.2**.

2.4.2. Secondary Screen

1. Receptor ECD affinity column: prepared as described in **Subheading 3.2**.
2. Q-Sepharose* Fast Flow: binding buffer: 20 m*M* Tris, pH 8.0; elution buffer: 1 *M* NaCl in binding buffer.
3. S-Sepharose Fast Flow: binding buffer: 20 m*M* MES, pH 6.0; elution buffer: 1 *M* NaCl in binding buffer.
4. Phenyl-Separose Cl-4B: binding buffer: 20 m*M* Tris, pH 8.5, 4 *M* NaCl; elution buffer: 20 m*M* Tris, pH 8.5.
5. Wheat germ lectin Sepharose 6MB: elution buffer: 100 mg/mL N-acetyl glucosamine in PBS.
6. Lentil lectin Sepharose 4B: elution buffer: 100 mg/mL methyl-α-D-mannoside in PBS, 0.02% Tween-20.
7. Heparin Sepharose 4B: elution buffer: 1 *M* NaCl in BIAcore buffer.
8. $(NH_4)_2SO_4$: analytical grade (BDH).

2.5. Ligand Purification

1. Liquid chromatography system consisting of:
   a. Solvent delivery system (compatible with flow rates of 0.01–10.0 mL/min).
   b. Gradient controller.
   c. Absorbance detector (dual wavelength, i.e., 215 nm, 280 nm, or diode array).
   d. Fraction collector.
2. Spiral-wound cartridge ultrafiltration system: Amicon, molecular weight cut off 10 kDa.
3. Ultrafiltration cell and YM-10 membrane (200 mL capacity, Amicon).
4. Empty glass columns: XK 16/20 columns (Pharmacia or equivalent).
5. Empty EconoPac disposable Chromatography columns (Bio-Rad).
6. BIAcore sensorchips: derivatized with hEphA3 ECD, as described in **Subheading 1.2**.
7. NAP-5 Desalting columns: buffer — BIAcore running buffer.
8. Superose-12 SE-HPLC column (1.0 × 30 cm, Pharmacia): buffer - 50 m*M* $NaHPO_4$, 0.5 *M* NaCl.
9. μ-MonoQ column (PC1.5/50, Pharmacia): loading buffer; 20 m*M* Tris-HCl, pH 8.5; elution buffer; 1 *M* NaCl in loading buffer.
10. Q-Sepharose FF (Pharmacia): buffer as for μ-MonoQ column.
11. Phenyl Sepharose Cl–4 (Pharmacia): elution buffer; 20 m*M* Tris-HCl, pH 8.5; loading buffer; 4 *M* NaCl in elution buffer.
12. Protein Sepharose FF (Pharmacia): loading buffer; 20 m*M* Tris, 0.15 *M* NaCl, pH 7.4; elution buffer; 0.1 *M* glycine-HCl, pH 3.0.
13. hEphA3 (receptor) ECD-affinity gel: prepared as described in **Subheading 3.2.1**.
14. $(NH_4)_2SO_4$: analytical grade.
15. 1,2 Di-ethanolamine: 50 m*M* aqueous solution, pH 12.3.
16. HEPES: 1 *M* aqueous solution, no pH adjustment.

*All Sepharose-type column material can be obtained from Pharmacia.

17. Electrophoresis system (Phast system, Pharmacia).
18. Sodium dodecyl sulfate (SDS)-Phast gels, 8–25% acrylamide (Pharmacia).
19. 4× Sample buffer: 0.8 *M* Tris-HCl, pH 8.3, 4% SDS, 4 m*M* EDTA, 0.02% Bromophenol Blue.
20. Protein Silver Staining Kit (Pharmacia).

3. Methods

A detailed descripton of all technical and theoretical aspects details of BIAcore biosensor operation, the "BIAcore jargon" and common applications are beyond the scope of this chapter. Details can be found in the Instrument manuals and related volumes ***(21)***. Likewise, the production of the orphan receptor exodomains in suitable expression systems will not be discussed here. The methods described here have been optimized for the Eph family orphan receptor hEphA3 and may require modification for receptors with different structural and biochemical characteriztics.

3.1. Assessment of Receptor Conformation

Homogeneity of the receptor ECD is of critical importance and should be evaluated prior to coupling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/silverstaining (*see* **Note 4**). Contaminating proteins that may be present after affinity purification (*see* **Note 3**) should be removed by fractionation on SE-HPLC or ion-exchange columns, and a purity of >95% should be achieved. Coupling of the homogenous receptor ECD to the sensorchip is performed at 1–2 pH units below its isoelectric point in a low-salt buffer, to achieve concentration of the positively charged protein to the CM-coated sensorchip (*see* **Note 5**).

3.1.1. Homogeneity of Receptor ECD

1. The affinity-purified receptor ECD is analyzed by SDS-PAGE and silver staining. Sufficient protein should be loaded (100–200 ng/Lane on a Phast gel) and the gel should be slightly overstained to detect contaminating proteins.
2. Contaminating proteins, differing in apparent molecular size by 40–60 kDa can be separated by SE-HPLC at 0.25 mL/min. Alternatively, ion exchange high-performance liquid chromatography (HPLC) at 1 mL/min using a 1%/min gradient of NaCl in a suitable buffer over 60 min should yield an improved purity of the preparation. In some cases, a combination of ion-exchange HPLC and SE-HPLC may be necessary.
3. The purity is confirmed by SDS-PAGE/silverstaining and the preparation stored at 0.5–1.0 mg/mL, 4°C. If necessary, the concentration should be adjusted by centrifugal concentration using low-protein binding filter units.

3.1.2. Immobilization to the Sensorchip

1. The receptor ECD is buffer exchanged into Na-acetate buffer, pH 4.5 at 10–50 μg/mL (*see* **Note 5**) using a desalting column (300 μL is sufficient for four injections onto the sensorchip).

2. Using the INJECT command in the manual mode of the BIAcore (*see* **Note 6**), one channel of the chip surface is activated at 2 μL/min with 45 μL of NHS/EDC solution that had been mixed immediately prior to injection.
3. Without delay, 45 μL of the receptor preparation in coupling buffer is injected at 5 μL/min. The baseline is recorded at the start of the injection (note a baseline-drop because of refractive index change) and the injection stopped when an increase of 5000 RU is observed (*see* **Note 7**).
4. Inject 45 μL 1 *M* DAE, pH 9.0, at 2 μL/min immediately to block remaining activated sites (*see* **Note 8**).
5. The derivatized surface is washed at 10 μL/min with 35 μL of 1 *M* NaCl in 20 m*M* Tris, pH 9.5 (*see* **Note 9**) followed by the command EXTRA WASH.
6. The response level at this point should have reached 3500–4000 RU above the baseline. If the reading is significantly outside this range, the volume of the injected receptor ECD, or its concentration, in the coupling buffer should be altered and the immobilization repeated on a parallel channel.
7. After transferring the optimized conditions into a BIAcore method file, an identical surface should be prepared on a parallel channel in the automatic mode.
8. In manual mode, 35 μL of denaturation buffer is injected at 2 μL/min onto one of the parallel receptor-derivatized channels, followed by NEEDLE WASH and EXTRA WASH commands.
9. In programmed mode, the following samples are injected onto each receptor ECD-derivatized channel (*see* **Note 10**).
   a. BIAcore buffer.
   b. 1 μg/mL native-conformation specific antireceptor MAb (*see* **Note 11**).
   c. 1 μg/mL native-conformation specific antireceptor MAb containing 10 μg/mL of the soluble receptor ECD.
10. The surface is regenerated after each sample by injection of 25 μL $MgCl_2$-desorption buffer, followed by two NEEDLE WASH and one EXTRACLEAN commands (*see* **Note 12**).

A comparison of the sensorgrams should reveal the specific response of the MAb to the immobilized native receptor ECD. Whereas an obvious response is expected for binding of the MAb (sample ii) onto the "native channel (**Fig. 2A**, **B**), a baseline response should be observed for the MAb in the presence of competing soluble receptor ECD (sample iii, **Fig. 2B**) and for the MAb injected onto the "denatured channel" (*see* **Fig. 2A**). The difference between the total and competed responses yields a difference sensorgram which mirrors the total response (**Fig. 2B**).

3.1.3. Stability of Immobilized Receptor ECD

The coupling density of the receptor ECD often affects the "lifetime" of the native receptor ECD on the sensorchip. An optimal surface concentration should be determined empirically (*see* **Subheading 3.1.3.1.**). Furthermore, it

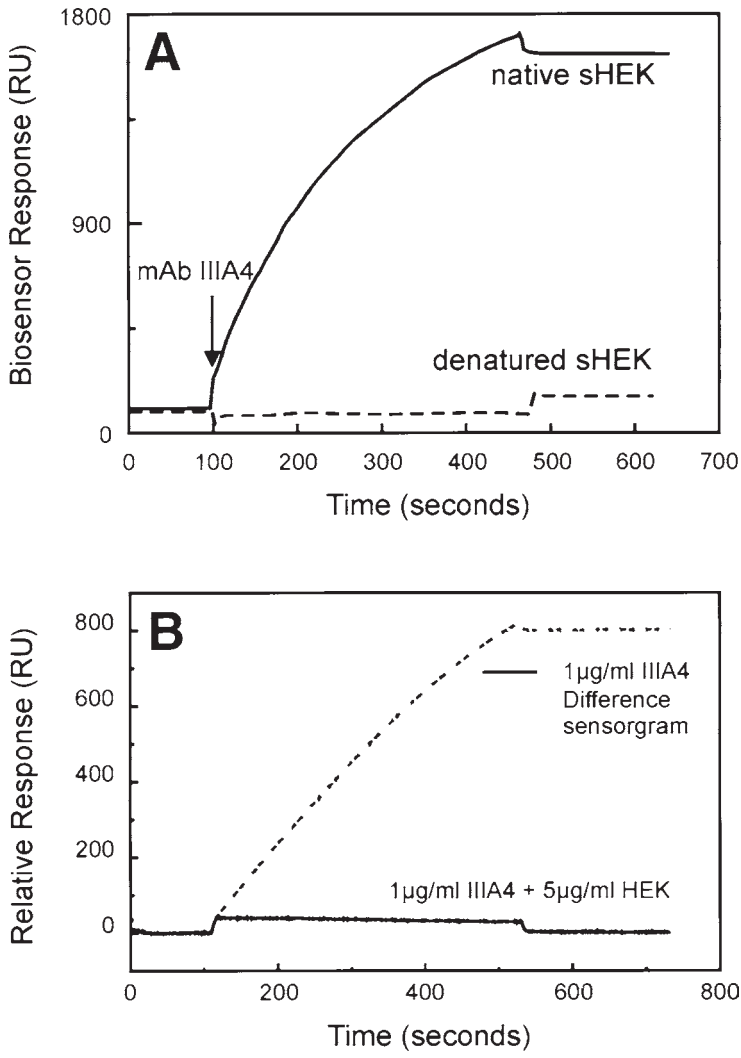


Fig. 2. BIAcore responses to native and denatured sensorchip-immobilized HEK-ECD. A sample (35 µL) of the conformation-specific anti-HEK MAb IIIA4 (2 µg/mL) in BIAcore running buffer was injected onto two parallel channel of the sensorchip surface that had been derivatized with the hEphA3-ECD. The receptor ECD on one of the channels had been denatured *in situ* by injection of 6 *M* guanidine hydrochloride, 5 m*M* dithiothreitol, 5 m*M* EDTA, 50 m*M* Tris, pH 8.0 **(A)**. Parallel samples of IIIA4 (1 µg/mL) with or without competing soluble hEphA3-ECD (5 µg/mL) are applied to the native-receptor sensorchip surface. The sensorgram of the hEphA3-ECD competed sample (1 µg/mL IIIA4 + 5 µg/mL hEphA3) is substracted from the un-competed sample (1 µg/mL IIIA4) to yield the difference sensorgram **(B)**.

is important to assess the stability of the receptor under various buffer conditions that may be used subsequently to regenerate the chip surface after sample application. The same buffers are suitable for elution of the receptor affinity column during ligand purification.

3.1.3.1. Optimization of Coupling Density:

1. A series of sensorchips comprising native receptor ECD surfaces of increasing surface densities is prepared by increasing the volume or the concentration of receptor solution used for immobilization. The maximum surface concentration, assessed by the relative response level, should be two- to threefold above the lowest concentration (2000–4000 RU optimal).
2. The stability of the various surfaces is now monitored daily by injecting a regime of samples including:
   a. BIAcore running buffer;
   b. Unrelated protein (1% BSA) in BIAcore running buffer;
   c. The conformation-specific MAb (1 μg/mL, *see* **Note 13**);
   d. The conformation-specific MAb containing 10 μg/mL of the soluble receptor ECD.
3. Each sample injection is followed by injection of $MgCl_2$-desorption buffer to regenerate the chip surface. The receptor constructs used in our laboratory exhibited a lifetime of 7–9 d before a 25–30% drop in reactivity towards the conformation-specific MAb was observed (*see* **Note 14**).

3.1.3.2. Evaluation of Desorption Conditions:

Although the $MgCl_2$-desorption buffer appears highly effective for most applications ***(24)***, its high viscosity causes serious carry-over problems and makes extensive washing regimes neccessary. This is a serious disadvantage for routine use in large-scale assays. Therefore, a thorough evaluation of alternative desorption conditions is recommended at an early stage.

1. A sensorchip carrying native receptor ECD surfaces at optimized densities is prepared as described above.
2. Each of the regeneration buffers is tested within the following regime of samples:
   a. 25 μL BIAcore buffer;
   b. 30 μL regeneration buffer;
   c. 25 μL BIAcore buffer;
   d. 35 μL antireceptor MAb;
   e. 25 μL $MgCl_2$ regeneration buffer.

The "mild" regeneration condition (physiological pH) should be tested first, followed by evaluating high-pH buffers and applying the low-pH buffer last (low pH will have the highest probability of denaturing the receptor ECD).

3.2. Receptor ECD Affinity Columns

The availability of preactivated NHS-derivatized affinity gels (Bio-Rad, Phamacia, Pierce) allows to use similar coupling chemistry for the preparation of the receptor ECD sensorchips and the receptor ECD affinity column. On the other hand, an array of different preactivated affinity matrices are available ***(25)*** and should be evaluated for optimal coupling efficiency and maximal retention of the native configuration of the receptor ECD (*see* **Subheading 3.2.1.**). CnBr-activated Sepharose (*see* **Note 15**) was used for the isolation of the hEphA3 ligand and its derivatization is described here.

3.2.1. Preparation

1. 0.5–1.0 mg of the homogenous receptor ECD (*see* **Note 16**) is dialyzed into coupling buffer with two buffer changes over 18 h.
2. Aliquots (20 µL) of the receptor preparation are taken before and after dialysis for estimation of protein concentration.
3. Weigh out sufficient dry beads to allow coupling of 2 mg receptor/mL of wet matrix.
4. Prior to use wash the beads with 10 vol of ice-cold 0.1 *M* HCl, followed by 2 × 10 vol of coupling buffer.
5. Aspirate the supernatant completely using a 23-gage needle. Add the dialyzed receptor solution and incubate on an end-over-end rotator for 4 h at room temperature or overnight at 4°C.
6. Aspirate the supernatant as in **step 5** and retain as the "nonbound" fraction. Incubate the beads with 1 *M* ethanolamine or 1,2 di-aminoethane, pH 9.5 for a minimum of 2 h at room temperature (or overnight at 4°C) to block remaining activated sites.
7. Wash the column with 20 vol of PBS, 0.02% Tween-20 and store at 4°C in PBS, 0.02% Tween-20, 0.02% NaN_3.
8. The amount of immobilized receptor ECD is estimated as the difference between the concentration and total volume of the supernatant before, and after, coupling. We routinely use the 215-nm absorbance of a 10-µL sample of the receptor ECD, fractionated on a micropreparative ion exchange column (2.1 × 30 mm) to estimate its concentration in the coupling buffer. A routine coupling efficiency of approx 95% should be achievable.

3.2.2. Evaluation of Column Capacity

The capacity of the receptor ECD affinity column for the native-conformation-specific MAb is used as the principal criterion for quality and performance, and has to be evaluated routinely after its preparation and during its use in the ligand isolation scheme. Denatured proteins have an increased tendency for nonspecific absorption, and when immobilized on the affinity matrix will result in increased absorption of contaminants. Thus, a thorough assessment of the

conformation of the immobilized receptor is important for an efficient use of the affinity column. This assessment should also be used to evaluate a suitable matrix among the available preactivated affinity gels (*see* above).

1. Transfer a 20-µL sample of the receptor ECD affinity beads (50% homogenous slurry) to a screw-cap reaction tube.
2. Dilute the MAb to 0.1 mg/mL in PBS, 0.02% Tween.
3. Collect the affinity beads by centrifugation at 6000*g* in a bench-top centrifuge (~ 1 min) and aspirate the supernatant completely using a 23-gage needle.
4. Add the MAb at a molar ratio, i.e.

$$\frac{\text{Volume}}{\text{MAb}} = \frac{\text{Volume}}{\text{beads}} \cdot \frac{\text{Concentration receptor ECD (packed beads)}}{\text{Concentration MAb}} \cdot \frac{Mr\ (\text{MAb})}{M_r(\text{receptor ECD})}$$

5. After 1 h incubation at room temperature, the 10,000*g* supernatant is recovered and the affinity beads washed with 3 ¥ 0.5 mL PBS/0.02% Tween-20. An aliquot of the supernatant at 1/10 and 1/100 dilution (BIAcore buffer) and aliquots of the washes (neat and 1/10 dilution) are quantitated for their MAb concentration on a native receptor ECD sensorchip, and the derived total response in the combined nonbound fractions is compared with the response of a sample taken prior to incubation on the affinity column. Estimate the bound MAb from the difference in MAb concentration of applied and recovered sample. In each case, the response, 20 s after sample injection relative to that of 10 s before injection, is used as relative response. With an equimolar concentration of MAb applied and two receptor binding sites per antibody available, the concentration in the affinity column supernatant should be ≥ 50% of the applied sample, if all immobilized receptor is in its native conformation.

3.3. Identification of a Ligand Source

The initial, or primary, screen of an array of biological samples may yield a number of putative ligand sources. A secondary, more extensive analysis of these initial "hits" will enable a reliable assessment of a reasonable starting material.

3.3.1. Primary Screen

Some 20–30 samples from the "ligand library" can be tested in one assay. Samples should be thawed quickly immediatly prior to analysis, and the sample rack of the BIAcore maintained at 8–10°C to minimize ambiguities because of sample degradation. Prior to the assay, samples should be desalted into BIAcore buffer (*see* **Note 17**).

1. 0.3 mL samples are loaded onto individual NAP-5 desalting columns that have been equilibrated with 3 vol of BIAcore running buffer. Rinse columns with 0.5 mL and elute with 0.4 mL BIAcore buffer.

2. Two parallel 0.1 mL samples are prepared, by adding to each 90 μL of buffer-exchanged sample 10 μL of buffer or 10 μL of a 0.05 mg/mL solution of receptor ECD in BIAcore buffer and incubation for 60 min at room temperature. The remainder of the sample is kept at 4°C and should be used to confirm the assay, should positive or ambiguous responses be observed (*see* **Subheading 3.3.2.**).
3. The BIAcore assay is performed by injecting samples onto a receptor ECD-derivatized sensorchip. The first and last samples are BIAcore running buffer followed by the antireceptor MAb in the absence, and presence, of 10 μg/mL soluble receptor ECD. This assesses the conformational integrity of the immobilized receptor during the assay.
4. The difference of the relative responses (*see* **Subheading 3.2.2.**, **step 5**) of each sample in the absence, or presence, of competing soluble receptor ECD yields the "receptor-specific" response (*see* **Fig. 3A**).

3.3.2. Secondary Screen

Once a putative ligand source has been identified, sufficient material should be prepared (*see* **Note 18**) for a series of pilot experiments to facilitate an initial chromatographic characterization of the putative ligand. At this stage, it is important to test a broad spectrum of fractionation modes to increase the chance of developing optimal purification procedures. An evaluation of this secondary screen will not only assess the reliability of the results from the primary screen, but also suggest an initial protocol for the purification of the ligand.

3.3.2.1. Batch Chromatography

1. Parallel 1-mL aliquots of the 2× concentrated source material are buffer exchanged into the various binding buffers suggested for ion-exchange and affinity chromatography (*see* **Subheading 2.4.2.**) using PD-10 desalting columns. Due to their increased capacity these columns should be used for samples > 0.5 mL instead of the NAP 5 columns. To achieve a similar sample recovery as on the NAP-5 columns (*see* **Subheading 3.3.1.**, **step 1**) following sample application the PD-10 columns are rinsed with 2 mL and eluted with 1.5 mL of the appropriate binding buffer.
2. The various ion exchange and affinity gels are prepared as 0.1-mL aliquots (only 10 μL of the receptor affinity column) in 1.5 mL reaction tubes by rinsing them with 1 mL elution buffer, followed by 3 × 1 mL binding buffer.
3. Apply 0.9 mL aliquots of the buffer-exchanged samples to each gel. Incubate on an end-over-end rotator for 30 min at room temperature. Centrifuge at 10,000*g* at room temperature for 1 min and collect supernatant.
4. Wash each gel with 3 × 0.5 mL BIAcore running buffer (*see* **Subheading 3.3.1.**) and elute with 0.3 mL elution buffer.
5. Desalt 0.3 mL aliquots of all samples, including the untreated starting material (but not the washes) into BIAcore buffer (*see* **Subheading 3.3.1.**) and analyze for the receptor-specific, relative response as described in **Subheading 3.2.2.**, **step 5**.

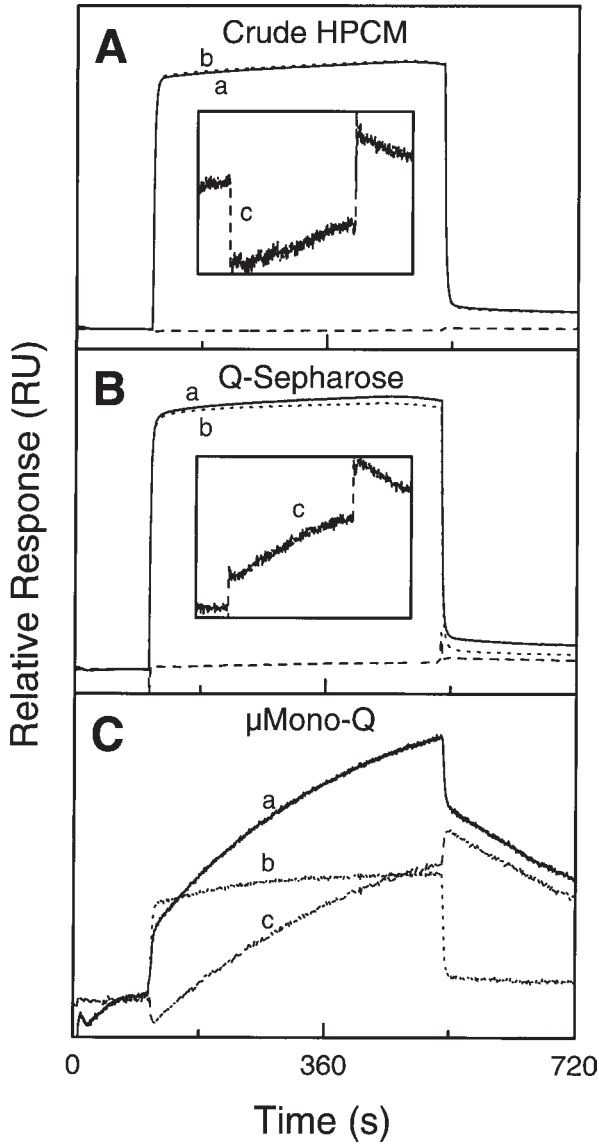


Fig. 3. Disection of BIAcore responses into total and receptor-ligand specific signals. The BIAcore response to crude HPCM **(A)**, the Q-Sepharose peak fraction **(B)**, and the μ-Mono Q peak fraction **(C)** in the presence of competing hEphA3 ECD (- - - b - - -) was substracted from the total response (—a—) to yield the difference sensorgram representing the specific response (····c····). For clarity, the difference sensorgram of the crude sample is shown at increased sensitivity (····c····) in the inset of panels A and B. Panel A and C have been redrawn, with permission, from **ref. *15***.

3.3.2.2. SE-HPLC:

1. Centrifuge a 0.8-mL aliquot of the 2× source material at 10,000*g*, 10 min at 4°C. Fractionate a 0.5-mL sample of the supernatant on a SE-HPLC column in BIAcore running buffer at 0.25 mL/min. Desalt the unfractionated sample into BIAcore buffer (*see* **Subheading 3.3.1.**) and assay for receptor-specific responses together with aliquots of 1 min fractions as described in **Subheading 3.2.2.**, **step 5**.

3.3.2.3. $(NH_4)_2SO_4$-Precipitation:

1. A 3-mL aliqout of 2× concentrated source material is adjusted at 4°C to 20% $(NH_4)_2SO_4$ by slowly adding 0.321 g of the solid salt to the rapidly vortexing sample and incubation on a rotating wheel for 1hour.
2. Collect precipiated proteins by centrifugation at 10,000*g* for 30 min at 4°C. Dissolve pellet in H_2O to a total volume of 0.3 mL. A 300-µL aliquot of the supernatant is taken for analysis, the remainder adjusted to 40% $(NH_4)_2SO_4$ (0.115 g/mL) and precipitated proteins recovered as described above.
3. Repeat the above protocol to obtain a 60% $(NH_4)_2SO_4$-pellet (0.112 g/mL) and supernatant.
4. 0.3 mL aliquots of all samples are buffer exchanged into BIAcore running buffer and analyzed on a receptor ECD-derivatized sensorchip as decsribed in **Subheading 3.3.1**.

3.4. Ligand Purification

The purification strategy for a ligand is guided by its particular biochemical properties and will vary from protein to protein. In all cases, receptor affinity chromatography should be a principle purification step. Sample preparation prior to affinity chromatography will depend on ligand abundance and complexity of the source material. The isolation of the hEphA3 ligand from human placenta conditioned medium (HPCM) ***(15)*** is outlined in the following protocol. Unless otherwise indicated, 0.5 mL samples are taken from each purification step and kept at 4°C prior to BIAcore and protein assays.

3.4.1. Concentration and Salt Precipitation

1. HPCM is stored at –20°C. Batches of 9–10 L are thawed rapidly at 37°C and the volume reduced 10-fold by ultrafiltration on a YM-10 membrane at 4°C.
2. Concentrated HPCM is centrifuged at 10,000*g*, for 30 min at 4°C to pellet insoluble material.
3. Solid $(NH_4)_2SO_4$ is added slowly to the cleared supernatant, under constant stirring on ice, to 30% saturation (166 g/L HPCM) (*see* **Note 19**). Incubate for 1 h.
4. Pellet precipitated proteins by centrifugation at 10,000*g* at 4°C and dissolve in a minimal volume of H_2O, 0.02% Tween-20. Take 0.5-mL aliquots of supernatant and solubilized pellet for assays.

5. Adjust the 30% $(NH_4)_2SO_4$-supernatant to 55% $(NH_4)_2SO_4$ (150 g/L 30% supernatant) and separate precipitated proteins from the 55% supernatant by 10,000*g* centrifugation. Take 0.5-mL aliquots of supernatant and solubilised pellet for assays.
6. To the 55%$(NH_4)_2SO_4$-supernatant add solid NaCl to 4 *M* final concentration and pellet precipitated proteins by centrifugation at 4°C, 10,000*g* for 30 min.
7. Buffer exchange 0.3 mL samples from each of these fractionation steps into BIAcore running buffer as described in **Subheading 3.3.1**. Analyze for receptor-specific binding on a hEphA3 sensorchip as described in **Subheading 3.**3.1. This assay should reveal enrichment of the hEphA3-ligand, first in the 30% $(NH_4)_2SO_4$-pellet and thereafter in the 55%$(NH_4)_2SO_4$ and the 4 *M* NaCl supernatants.

3.4.2. Phenyl-Sepharose Chromatography

1. Equilibrate a phenyl-Sepharose column (1.6 ¥ 5 cm) with 4 *M* NaCl, 20 m*M* Tris, pH 8.5, 0.02% Tween-20 as binding buffer.
2. Using a peristaltic pump, apply the 10,000*g* supernatant from **Subheading 3.4.1.**, **step 6**, onto the column at 2 mL/min. When all sample is loaded, rinse the sample container and tubing with binding buffer and continue to apply this wash to the column.
3. Disconnect the column from the peristaltic pump and rapidly connect to the HPLC system.
4. Apply loading buffer at 7 mL/min to wash the column and collect non bound material by monitoring the absorbance reading at 280 nm (*see* **Note 19**). Keep this nonbound fraction at 4°C until analysis by BIAcore assay.
5. When a baseline reading of the 280 nm absorbance is reached, elute the column at 7 mL/min with 20 m*M* Tris, pH 8.5, 0.02% Tween-20 and collect 2-min fractions for 60 min.
7. Desalt 0.3 mL aliquots of individual fractions into BIAcore running buffer and analyze together with aliquots of the nonbound and starting material for receptor-specific BIAcore responses as described in **Subheading 3.3.1**.
8. Estimate the NaCl concentration in column fractions by comparing their conductivity to NaCl standard solutions. An evaluation of these data should indicate the elution of the ligand at 0.6 *M* NaCl in a broad peak eluting at 17–42 min.

3.4.3. Q-Sepharose Chromatography

1. Concentrate and diafiltrate the combined active fractions (189 mL) to a volume of approx 50 mL in 50 m*M* NaCl using a YM-10 membrane and N_2 pressure.
2. Apply this preparation onto a Q-Sepharose column (1.6 × 5 cm) which had been equilibrated in 20 m*M* Tris, pH 8.5, 0.02% Tween-20, and wash the column with this binding buffer as described above (**Subheading 3.4.2.**) for Phenyl-Sepharose chromatography.
3. Elute bound proteins at 5 mL/min using a linear gradient from 0–60% elution buffer over 40 min and collect fractions every minute.

4. Exchange 0.3 mL samples of each fraction, the nonbound and loaded fraction, into BIAcore buffer and determine EphA3-specific responses on the BIAcore as described in **Subheading 3.3.1**. A comparison of the total and receptor ECD/ligand specific responses (*see* **Fig. 4B**) should indicate separation of the ligand, eluting after 20–30 min, from earlier-eluting proteins in the sample. These proteins contributed largely to the nonspecific BIAcore signal of the nonfractionated sample (*see* **Fig. 3A**).
5. Adjust the pool of these ligand-containing fractions (approx 350 m*M* NaCl) to 150 m*M* NaCl, pH 7.4 by adding diluted HCl and H_2O.
6. Pass this sample through a 10-mL Protein-G column at 2 mL/min to absorb contaminating immunoglobulins, which would bind nonspecifically to CnBr-activated Sepharose (*see* **Note 15**).
7. By monitoring the 280 nm absorbance, collect the nonbound fraction and regenerate the column by elution with 0.1 *M* glycine-HCl, pH 3.0. Discard the eluate.

3.4.4. hEphA3 ECD-Affinity Chromatography

1. Transfer the recovered ligand preparation to a 50-mL srew-cap tube and incubate for 1 h at room temperature with 0.5 mL packed hEphA3-ECD affinity gel on a end-over-end rotator.
2. Collected the affinity gel by centrifugation for 5 min at 5000*g* and transferring the beads into a 10-mL disposable Econo colunm. Retain the nonbound fraction at 4°C.
3. Wash the affinity column rapidly (*see* **Note 20**) with 9 vol of PBS, 0.02% Tween-20.
4. Elute bound proteins at gravity flow with six column volumes of elution buffer, by applying one column volume at a time to reduce the flow rate.
5. Neutralize eluting 0.5 mL fractions immediately by addition of 1 *M* HEPES (approx 50 μL per fraction).
6. For the BIAcore assay (*see* **Subheading 3.3.1.**) exchange aliqouts of the column load, the nonbound fractions and the column wash into BIAcore running buffer and dilute 4 μL aliquots of the eluate 25-fold into BIAcore buffer.

3.4.5. SE-HPLC

1. Apply the affinity-purified ligand preparation directly onto a Superose-12 SE-HPLC column, equilibrated at 0.25 mL/min in 50 m*M* phosphate buffer, pH 7.4, containing 0.5 *M* NaCl, 0.02% Tween-20 .
2. Collect eluting fractions every minute for 70 min and keep at 4°C.
3. Dilute aliquots of each fraction 40-fold into BIAcore buffer and assay for hEphA3-specific binding on the BIAcore (*see* **Subheading 3.3.1.**).
4. Determine the protein concentration in relevant fractions by comparison of the 215-nm absorbance of the eluate to the aborbance of known amounts of a standard proteins of similar apparent molecular size.
5. Prepare 3 μL-aliquots of fractions containing receptor ECD binding activity for SDS-PAGE by addition of 1 μL of 4¥ sample buffer. Fractionate samples on a 8–25% SDS PHAST gel and assess homogeneity of the ligand by silver staining.

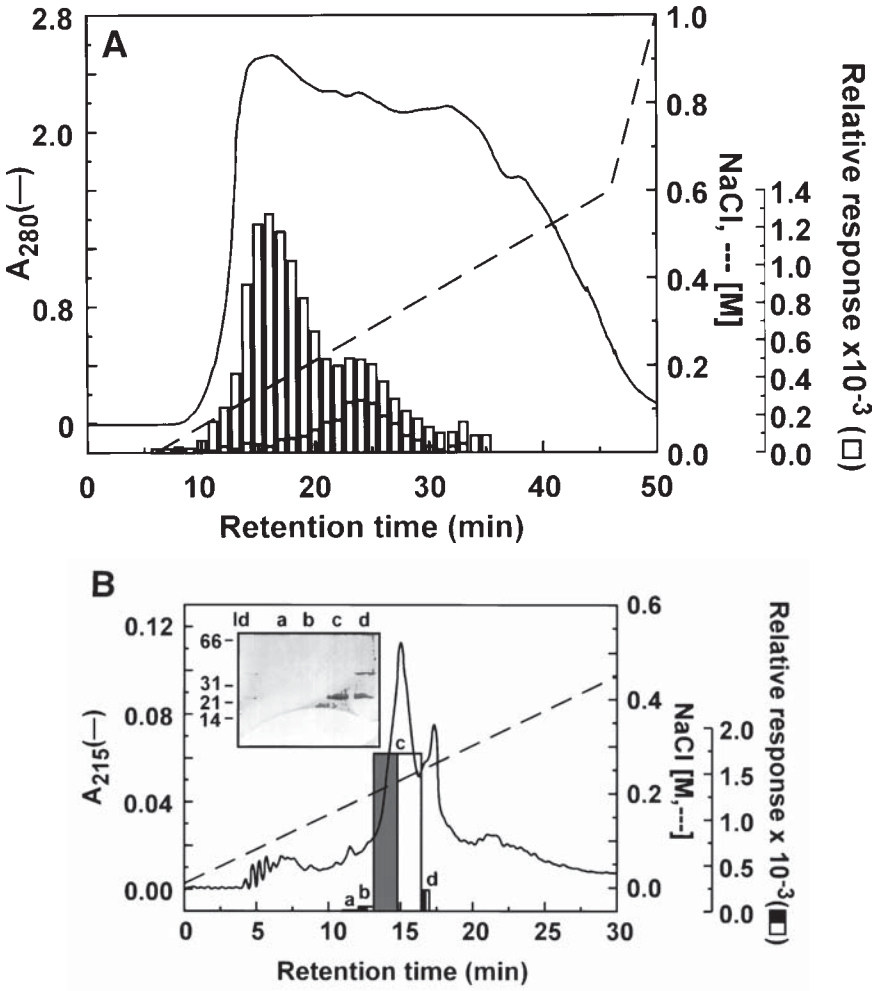


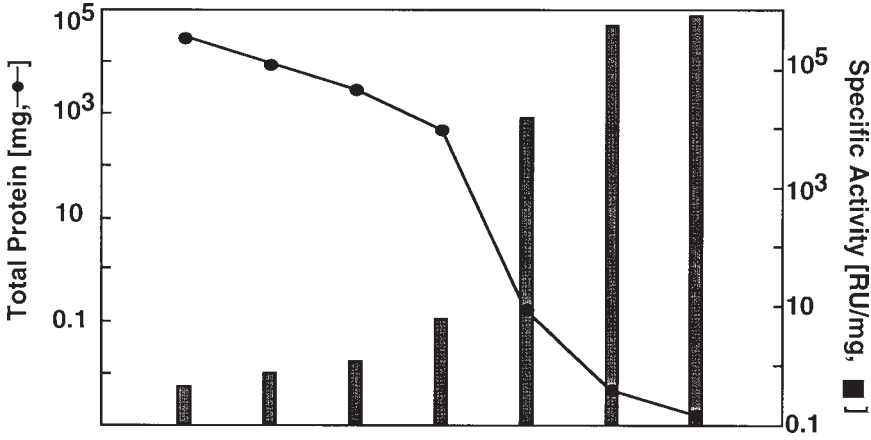
Fig. 4. Preparative and micropreparative ion exchange LC of the ligand preparation. **(A)** The dialyzed pool of ligand-containing fractions from Phenyl-Sepharose chromatography was fractionated on a preparative Q-Sepharose column eluted with a linear NaCl gradient (- - -). Eluting proteins were monitored at 280 nm (—) and HEK binding, in samples preincubated with or without 10 μg hEphA3 ECD, was determined on the BIAcore (q total response; n sHEK competable response). **(B)** The combined active peak fractions recovered from the SE-HPLC column were adjusted to 50 m*M* NaCl and fractionated on a μ-Mono-Q column with a 40-min gradient of 0–600 m*M* NaCl at a flow rate of 100 μL/min (- - -). Elution of proteins was monitored at 215nm (—) and HEK binding with or without competing hEphA3 measured on the BIAcore at 1/50 dilution. (□ total response, ◧ sHEK competable response) Proteins in the column load (ld) and in selected fractions were analyzed by SDS-PAGE with silver staining. The figure has been redrawn, with permission from **ref. *15***.

3.4.6. μ-Mono Q HPLC

1. A comparison of specific BIAcore responses and intensity of protein bands will indicate the ligand containing fractions, which are suitable for a final purification step.
2. Using 20 m*M* Tris, pH 8.5, 0.02% Tween dilute the pool of these fractions threefold to 50 m*M* NaCl .
3. Load the diluted sample in multiple 1 mL injections onto a μ-Mono Q column that had been equilibrated at 0.1 mL/min in binding buffer. To elute the bound proteins apply a 40-min gradient of 0–600 m*M* NaCl in binding buffer to the column.
4. Collecte the eluting proteins manually according to their absorbance at 215nm.
5. For the BIAcore assay on a hEphA3-ECD sensorchip dilute 2 μL-aliquots of these fractions 50-fold with BIAcore buffer.
6. Plot the hEphA3 ECD-specific and total BIAcore responses in each fraction. An overlay of the two graphs should indicate a homogenous ligand preparation with identical total and specific responses eluting in the major protein peak, which is separated from a contaminating protein with no response eluting in an adjacent peak (*see* **Fig. 3B**, **Fig. 4C**).
7. Assess the purity of the ligand by SDS-PAGE and silverstaining (*see* inset, **Fig. 3B**) using 1 μL of each fraction and 1 μL of 2¥ sample buffer for electrophoresis on a 8–25% SDS PHAST gel.
8. Estimate the protein concentration in the fractions from the 215-nm absorbance by comparison to the absorbance area of a known amount of a standard protein.
9. The sensorgram and the difference sensorgram of this ligand preparation should differ only by a (buffer-related) refractive index change (*see* **Fig. 4C**). Thus, the on-rates and off-rates illustrate the interaction of pure ligand with the immobilized receptor ECD. Use the BIAevaluation software to estimate the apparent affinity of the interaction; approximate values for the apparent size of the ligand can be derived from the SE-HPLC and SDS-PAGE analysis
10. Calculate the specific activity (in RU/mg total protein) by using the sum of the specific responses in each purification step together with a figure of the total amount of protein contained in the corresponding preparation. The increase of the specific activity from one purification step to the next will give an estimate of the achieved purification fold (*see* **Fig. 5**). Perform this calculation for every step during method development to ensure an effective purification strategy.

4. Notes

1. For the directional coupling of biotinylated or hexa-histidine tagged proteins Streptavidin-derivatized (SA) and NTA-derivatized sensorchips are available as affinity matrices. In our hands, the relatively weak NTA/hexa-histidine interaction results in constant loss of immobilized protein, whereas strong nonspecific binding problems are associated with the use of SA sensorchips.
2. Volumes 59 and 63 of this series suggest a number of mammalian expression systems. We have used chinese hamster ovary (CHO) cells transfected with the



Purification step	HPCM. pool	$NH_4(SO_4)_2$- precip.	Phenyl-Seph.	Q-Seph.	HEK-affi	SE-HPLC	μMono-Q HPLC
Purification fold	0	1.7	2.7	13.1	35205	$1.2x10^6$	$1.8x10^6$
Yield	100	54	26	21.3	18.4	18.0	9.0

Fig. 5. Estimation of purification fold for HEK ligand isolation. The sum of the hEphA3-compatable responses and the total protein estimate for each listed purification step were used to calculate the specific activity, fold purification, and yield of the ligand preparation during the purification scheme.

appropriate cDNA construct ligated to the IL3 sig-FLAG-pEFBOS vector ***(24)*** and selected for transfectant clones in medium containing 600 μg/mL of G418.

3. If native conformation-specific MAbs are avalilable, they should be used for affinity purification of the receptor ECD using the $MgCl_2$/ethylene glycol buffer system recommended by Tsang and Wilkins ***(24)*** as eluent. If a specific MAb is not available, but the receptor ECD has been produced as fusion protein containing a tag to which an MAb is available (*see* **Note 2**), the antitag MAb can be used as eluant.
4. We use routinely the PHAST electrophoresis sytem (Pharmacia) enabling a rapid analysis (3 h) with minimal sample consumption.
5. The ionic adsorption/concentration on the chip surface can be tested by injection of the receptor in the coupling buffer onto the nonactivated surface. A positive slope signifies the ionic interaction. To avoid coupling via the e- amino group of histidine, the pH of the coupling buffer should be at or below 6.0. For anionic proteins ($pI > 5.0$) the coupling buffer should contain NaCl to stop electrostatic repulsion. We have used successfully 0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3, and a protein concentration of 1–2 mg/mL.

6. To enable modifications of injection volume and flow rate, it is more convenient to use the manual mode during the initial experiments.
7. This value is an estimate for a 60–70-kDa protein and should be adjusted according to the size (i.e., approx 10,000 RU for a 120–140-kDa protein.
8. We observed less nonspecific binding of surfaces blocked with 1,2 DAE than with the ethanolamine that is recommended by the manufacturer. This is most likely because of charge-neutralization of the CM surface by the amino group remaining after coupling of 1,2 DAE.
9. Until the stability of the receptor ECD in acid or basic conditions is known, a "mild" desorption buffer (such as high-salt containing, neutral pH buffer) should be used.
10. On the BIAcore 2000 this can be done with a single injection in the multichannel mode.
11. If a native conformation-specific MAb has not been characterized, these two sensor surfaces can be used now to screen for a MAb (hybridoma supernatant) that binds to the "native" receptor ECD surface, but not to the *in situ*-denatured surface.
12. The $MgCl_2$-desorption buffer is extremely viscous and several washes are neccessary to clean the needle and flowpath of the BIAcore.
13. Prepare 200 µL of a 0.1-mg/mL stock solution of the MAb and the soluble receptor ECD. Keep at 4°C and prepare the samples from this solution daily.
14. Obviously the stability will depend on the type of samples and regeneration buffers that are injected across the affinity surface.
15. The various preactivated affinity gels will have different capacities for the target protein, and the characteriztics of the protein and the coupling chemistry determine the yield of coupled protein in its native configuration. In our experience, the CNBr-activated Sepharose has a slightly better coupling efficiency than the NHS-activated Sepharose (both are available from Pharmacia). In both cases, 90% of native configuration receptor were immobilized. On the other hand, CNBr- activated Sepharose is known to retain certain immunoglobulins strongly and nonspecifically ***(27)*** and so may may be unsuitable if a serum-containing source has been selected for the ligand isolation.
16. As with the receptor-derivatized sensorchips (*see* **Subheading 3.2.3.**), the Sepharose-immobilized receptor protein has a limited stability and the affinity gel should be prepared fresh to last for 1–2 mo.
17. The carboxymethylated dextran matrix of the BIAcore sensorhip surface will act as a chelator for metal ions, which in turn can bind certain proteins by metal affinity interaction ***(28,29)***. This effect has been noted with a number of iron-enriched bovine calf-sera used in tissue culture.
18. The amount of starting material depends on the concentration of the sample needed to detect a positive response. Sample preparation in these pilot experiments will result in dilution of the samples, but 10–20 mL of sample at 2× intitial concentration should be sufficient.
19. The nonbound fraction is discarded only after sufficient retention of ligand in the bound fraction has been confirmed. If the volume of this fraction is greatly

increased compared to the loaded sample, the assay should be performed on a concentration-adjusted (by YM-10 ultrafiltration) sample.

20. The hEphA3 ECD-specific BIAcore response profile of the ligand preparation indicates a significant off-rate of the ligand from the immobilized receptor (**Fig. 4B**), suggesting that extensive washing protocols on the affinity column would result in a considerable loss of ligand. To reduce the interaction time with the wash buffer, we used a 20-mL disposable syringe mounted to the stopper of the Econo column to increase the flow rate by air pressure.

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