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Integrin Protocols

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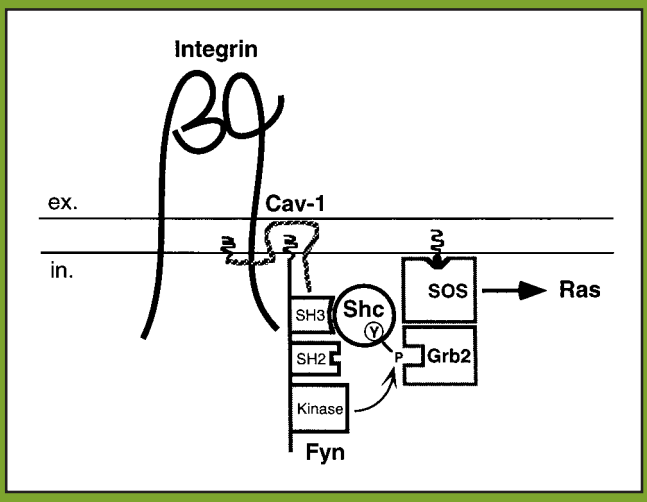

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Integrin-Binding Peptides Derived from Phage Display Libraries

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1. Introduction

1.1. Use of Phage Display Libraries in Integrin Research

Integrins are a family of cell-surface receptors that recognize extracellular matrix proteins and mediate cell adhesion. Certain integrins bind to proteins containing the sequence Arg-Gly-Asp (RGD; **ref.** ***1***). Such RGD-binding integrins include $\alpha_5\beta_1$, $\alpha v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ ***(2,3)***. Peptide libraries displayed on filamentous phage have provided a straightforward method to characterize the peptide-binding specificity of these integrins. In phage display-based strategies, libraries of random peptides are expressed on the surface of bacteriophage. Each individual phage carries a single peptide. The peptide is fused to the phage capsid protein pIII by insertion of its encoding DNA sequence in the *pIII* gene of the phage genome. Libraries displaying peptides fused to the major capsid protein pVIII have also been used. Libraries using the pIII protein display 3–5 copies of each individual peptide whereas libraries using the pVIII protein display approx 3000 copies ***(4,5)***. Analysis of the phage-displayed peptides found during screenings on purified integrins revealed that the majority of the integrin-binding peptides contain an RGD motif ***(6–11)***. Other interesting ligand motifs were also found in the remaining population of integrin-binding peptides. Conversely, panning on immobilized fibronectin fragments containing the RGD sequence revealed peptide motifs present within integrins ***(12)***. We describe here strategies for the use of phage display libraries

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in cell-adhesion research, with emphasis on the peptide-binding specificity of different members of the integrin family.

1.2. Display of Peptide Libraries on Bacteriophage

A major advantage of phage display-based strategies is that peptides capable of binding to integrins can be isolated quickly by using a simple general procedure (**Fig. 1**). Samples of libraries displaying different degenerate peptides can be analyzed in the same experiment. It is useful to employ a series of libraries each displaying peptide inserts of different lengths, compositions, and structures. This approach enhances the probability of finding a specific ligand to an integrin. Phage clones that show the highest avidity to integrins will gradually enrich following each round of "biopanning," resulting in the isolation of novel peptide ligands ***(10)***. The libraries can be used individually or they can also be pooled and used in a single experiment. However, because clones having a lower growth rate in bacteria may be lost by amplifying a mixture of heterogeneous phage libraries, we currently tend not to pool the different libraries in a single screen.

The first peptides to be displayed on phage libraries were degenerate linear hexapeptides and decapeptides ***(13–15)***. Subsequent work indicated that the binding affinity of phage-displayed peptides to integrins, as well as to other targets, were markedly improved if the peptides were conformationally constrained by a disulfide bond formed by cysteines flanking the peptide insert ***(6–11)***. Integrin-binding peptides containing up to four cysteines, thus potentially capable of forming two disulfide bonds, have also been isolated during panning on integrins ***(10)***. Libraries engineered to display such double-cyclic peptides have then been used successfully to find ligands to integrins and other receptors ***(6–11)***. One such peptide, RGD-4C ***(10)***, has been shown to bind to αv integrins in vitro ***(10)*** and in the angiogenic endothelium of tumor blood vessels ***(16)***, in which αv integrins are upregulated. RGD-4C has also been used to deliver a cytotoxic drug to the tumor vasculature in an animal model ***(17)***.

The number and diversity of individual clones present in a given library will influence the success of the screen. With current library-making techniques, the maximum number of individual clones obtained is approx 10^9. This means that the diversity of the peptide sequences present in a library is limited. If the random insert is seven residues or longer, only a portion of all possible permutations of such peptides is actually displayed. The preparation of the libraries—a critical factor—has to be optimized so that the number of recombinants is as high as possible. It is important to ensure that practically every phage in the library carries an insert. Insertless phage cannot be totally avoided, but they usually cause no problems and can be eliminated in the display systems and biopanning by using stringent phage selection procedures.

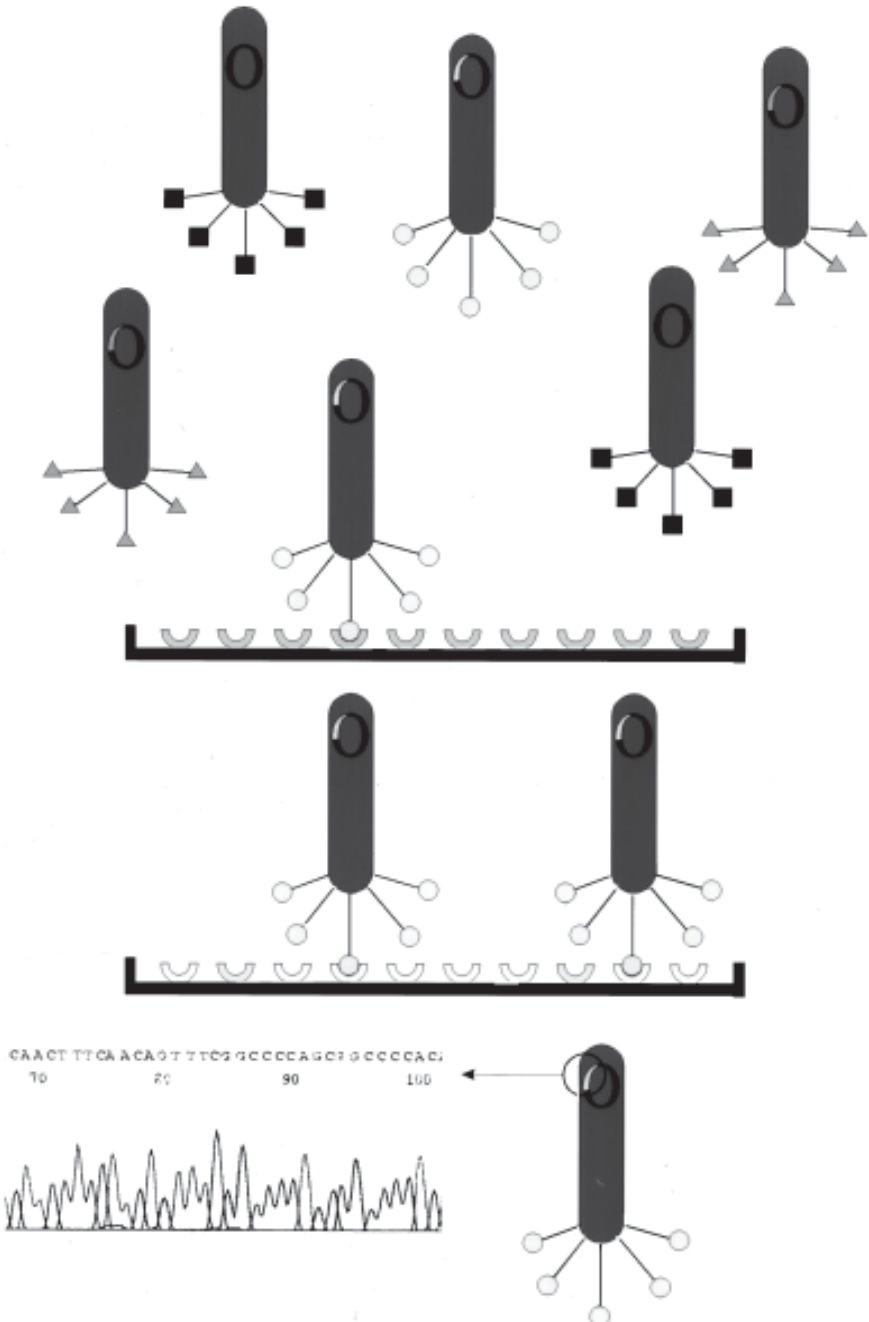

Fig. 1. Phage display peptide library screening. The procedure consists of the following sequential steps: screening the library on a specific target such as an integrin (upper panel); washing to remove nonspecific interactions, elution of the bound phage from the target, and amplification of the eluted phage (middle panel); and identification of the binding peptide by sequencing of the phage DNA (lower panel).

1.3. Purification of Integrins

Integrins isolated in their active state and free of contaminating proteins are suitable targets for phage library screenings. Placenta has been the tissue most commonly used to purify human integrins *(18)*. For preparation of RGD-directed integrins, two affinity chromatography-based methods have been described. In one method, the integrin-binding peptides KGRGDSP or CRRETAWAC are coupled to Sepharose, and the integrins that bound to the affinity matrix are eluted with EDTA or a competing integrin-binding peptide *(9,18)*. In an alternative method, integrins are captured by using specific antibodies coupled to Sepharose *(8)*. Integrins can be eluted from the antibody matrix by low- or high-pH buffers, followed by immediate neutralization of the eluted fractions. If the antibodies are directed against the cytoplasmic portion of an integrin, the corresponding cytoplasmic peptide provides an effective elution strategy *(8)*. As integrins are glycoproteins, further purification—if needed—can be accomplished with wheat germ agglutinin lectin affinity chromatography *(18)*.

Integrins retain activity when coated on microtiter wells. The activity of the immobilized integrins can be tested by using a labeled ligand. For example, iodinated fibronectin has been used with the $\alpha_5\beta_1$ integrin *(7)*.

Phage library selections have been performed with integrins coated on plastic. Alternatively, integrins can be immunocaptured on microtiter wells precoated with antibodies directed against the integrin cytoplasmic domain. Such immunocapture procedure is not suited for the primary selections with phage libraries, as antibodies avidly bind phage. Thus, an antibody-binding rather than integrin-binding phage may be enriched. During the selection process, however, the immunocapture assay helps to demonstrate the specific binding of an individual phage to a particular integrin species *(8,9)*.

1.4. Selection for Integrin-Binding Phage

In the first round of selection, optimal results are achieved by incubating the phage libraries with the immobilized integrins overnight at 4°C. This longer incubation time will enhance the probability to select multiple integrin-binding clones. In subsequent rounds of pannings, the incubation time can be decreased to 1 h because the selected population is amplified so that multiples copies of each clone are present. The bound phage can be eluted specifically by an RGD-containing peptide or by the cation-chelator EDTA. Both methods will dissociate integrin-bound ligands. The phage can also be eluted nonspecifically with a buffer solution of pH 2.2. The eluted phage do retain their infectivity after neutralization of the pH.

The binding affinity of different phage clones to integrins can be compared in a phage-attachment assay *(7–9)*. High-affinity clones are favorably selected when the integrin coating concentration is decreased in each panning because

the phage clones compete for a limited number of binding sites. A way to recover the best binders is to search, in each round of selection, for the minimal integrin concentration yielding at least a 10-fold enrichment above background. Usually, the best binders become evident by appearing more frequently during the third and fourth rounds of pannings when a representative number of clones (100 or more) is sequenced.

1.5. Sequencing of the Phage Insert

The sequence of the integrin-binding peptide is determined by sequencing the region of the phage genomic DNA that encodes the displayed peptide ***(5)***. In most display systems, peptides are inserted near the amino terminus of the minor capsid protein pIII. When the phage single-stranded DNA (ssDNA) is directly used for sequencing, the primer used for the sequencing reactions should be located approx 100 bp from the peptide insert site. However, the direct sequencing of phage ssDNA prepared by standard methods often gives high background, and many sequences may be unreadable. An alternative approach is to sequence a double-stranded DNA (dsDNA) PCR product of the phage rather than the ssDNA itself. The sense and antisense primers used in the PCR are at 100 bp from the DNA sequence encoding the peptide. Sequencing is then carried out using the same sense PCR primer, or a nested primer.

1.6. Reproduction of Phage Displayed Motifs as Synthetic Peptides

Only those phage-displayed peptides that show at least 100-fold stronger binding to integrins relative to albumin may be worthy of reproduction as recombinant or synthetic peptides. The solubility properties of a peptide are not always predictable based on the amino acid sequence. Aromatic and hydrophobic residues tend to decrease solubility of peptides in aqueous buffers, whereas charged residues tend to have an enhancing effect. Tryptophan is an amino acid residue that is relatively rare in naturally occurring protein sequences but is encountered repeatedly in highly active phage library-derived peptides. The integrin-binding peptides CRRETAWAC and CRGDGWC are two examples of such peptides ***(8,10)***. Tryptophan may be favored in phage library-derived peptides because it provides a hydrophobic scaffold and it improves the binding activity of a displayed peptide.

Cyclic peptides can be restricted to a structure favorable for integrin binding because the presence of a disulfide bridge constrains its conformation. However, making the disulfide bridge complicates the peptide synthesis. Disulfide bonds usually form by air oxidation, but some peptides may need a treatment with an oxidant or dimethyl sulfoxide (DMSO). Fortunately, oxidation of phage library-derived synthetic peptides usually proceeds rapidly, because the peptides often adopt the energetically favorable conformation they have on the

surface of phage. In fact, in our experience, it has been far more problematic to prepare scrambled versions of the phage library-derived cyclic peptides; the scrambled sequences may not possess a similar solubility or structural features as the parent peptide. A unique problem occurs when a peptide contains four cysteines, such as CDCRGDCFC, which is capable of forming alternative disulfide bonds ***(10,16,17)***. We have found that multiple forms of the peptide can be obtained by random oxidation of the peptide without directing the formation of specific disulfide bonds. However, we have found that one arrangement of the disulfide bonds is more active than the other conformers when each form is synthesized individually (Assa-Munt et al., in preparation).

1.7. Testing the Anti-Adhesive Activities of Integrin-Binding Peptides

Integrin-binding peptides can be analyzed in two different types of cell-adhesion assays ***(3–5)***. In the first experimental design, the peptide is tested for its ability to inhibit cell adhesion because it competes for the ligand-binding site of the cell surface receptor. The peptide is added to the culture media under serum-free conditions. Depending on the integrin studied, the adhesion substratum can be fibronectin, vitronectin, or other matrix proteins. The peptide is then tested for inhibition of cell attachment. In the second type of assay, the peptide is immobilized on a microtiter plate and used as the adhesion substratum. Other cellular functions can be tested in vitro such as cell migration, proliferation, and apoptosis. Phage-display derived integrin-binding peptides can also be tested for their ability to block integrin function in vivo. For example, we have shown that certain integrin-binding peptides isolated by using phage display have an antimetastatic activity ***(19)***.

2. Materials

2.1. Construction of Phage Display Peptide Libraries

1. TE: 10 m*M* Tris-HCl at pH 7.5, 1 m*M* EDTA, autoclave.
2. SOB media: 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 10 mL of 250 m*M* KCl. Adjust pH to 7.0, the volume to 1 L, and autoclave.
3. SOC media: Add 20 mL of sterile-filtered 1 *M* glucose solution/L of the SOB media.
4. LB media: 10 g bacto-tryptone, 10 g NaCl, 5g yeast extract/L. Autoclave. Add tetracycline to 20 μg/mL. Check the genotype of the host bacteria for other drug resistance (e.g., streptomycin, kanamycin). If possible, a second antibiotic in addition to tetracycline should be added to the media and plates to help prevent contamination.
5. QIAquick PCR Purification and Nucleotide Removal Kits (Qiagen, Valencia, CA).
6. Wizard Plus Miniprep Kit (Promega, Madison, WI).
7. Electrocompetent bacteria of choice. The host bacteria must be pilus negative (F'-minus) and sensitive to the selectable antibiotic used (e.g., tetracycline in the case of fUSE5).

8. StrataClean Resin (Stratagene, La Jolla, CA).
9. LB agar plates with tetracycline (20 μg/mL).
10. Ethanol 100%.
11. 3 *M* NaOAc (pH 5.2), autoclave.
12. Glycogen solution, 20 mg/mL (Boehringer Mannheim, Germany).
13. *Sfi*I and *Bgl*I restriction enzymes (New England Biolabs, Beverly, MA; Promega; or equivalent).
14. Sequenase Kit (Amersham, Arlington Heights, IL).
15. PEG/NaCl: 100 g of polyethylene glycol (PEG 8000), 116.9 g of NaCl, 475 mL double-distilled H_2O (ddH_2O). Rock in a 1-L bottle until solutes dissolve (it may be necessary to heat to 65°C). This solution is stored at 4°C and it may be autoclaved. Final volume is 600 mL.

2.2. Coating Integrins on Microtiter Wells

1. Flat-bottom 96-well microtiter plates (Linbro/Titertek, ICN Biomedicals, Costa Mesa, CA).
2. Tris-buffered saline (TBS): 50 m*M* Tris-HCl at pH 7.5, 100 m*M* NaCl, autoclave.
3. 25 m*M* n-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA) in TBS. This detergent keeps integrins solubilized.
4. TBS/1 m*M* $MnCl_2$. Mn^{+2} cations activate integrins and enhance the binding of peptides to purified integrins.

2.3. In Vitro Panning

1. Phage display peptide library of choice.
2. Starved K91kan bacteria grown in TB/Kanamycin to OD_{600} = 1–2.
3. LB or NZY media containing tetracycline (20 μg/mL) and kanamycin (100 μg/mL).
4. Kanamycin/tetracycline LB agar plates. We adjust the concentration of kanamycin to 100 μg/mL and of tetracycline to 40 μg/mL.
5. PEG/NaCl solution (as described in **Subheading 2.1.**).
6. TBS/gelatin: 50 m*M* Tris-HCl at pH 7.5, 150 m*M* NaCl. Autoclave 0.1 g of gelatin in 100 mL of TBS. After autoclaving, swirl to dissolve the molten gelatin. Store at RT.
7. TBS, 3% BSA; TBS, 1% BSA.
8. TBS/0.5% Tween.
9. Elution buffer 1: 0.1 *M* glycine-HCl at pH 2.2/0.1% BSA/0.05% phenol red.
10. Elution buffer 2: 10 m*M* EDTA in TBS, 0.1% BSA.
11. Elution buffer 3: 500 μ*M* GRGDSP synthetic peptide in TBS, 0.1% BSA.

2.4. Sequencing of Phage Insert

1. LB or NZY medium containing tetracycline and kanamycin (*see* **Subheading 2.3.**).
2. PEG/NaCl (*see* **Subheading 2.1.**).
3. StrataClean Resin (Stratagene).
4. Glycogen (Boehringer Mannheim).
5. 3 *M* NaOAc (pH 5.2).

6. 70% Ethanol.
7. 100% Ethanol.
8. Primer for direct cycle-sequencing of phage ssDNA: 5' CCCTCATAGTTAGC GTAACG 3'.
9. Primers used when a double-stranded PCR product of the phage insert is prepared for sequencing: 5' TAATACGACTCACTATAGGGCAAGCTG ATAAACCGATACAATT 3' (sense) and 5' CCCTCATAGTTAGCGTAACGA TCT 3' (antisense).
10. Automated sequencing kit (ABI system, Perkin Elmer, Norwalk, CT) or conventional sequencing by the dideoxy method with a Sequenase kit (Amersham).

2.5. Cell-Adhesion Assay

1. Flat-bottom 96-well microtiter plates (Linbro/Titertek, ICN Biomedicals).
2. Microtiter plate reader (Technika Reader 520, Organon, Durham, NC or equivalent).
3. Extracellular matrix proteins (e.g., fibronectin, vitronectin, collagen).
4. TBS/3% BSA.
5. DMEM serum-free medium.
6. Integrin-expressing cell lines.
7. 2.5 m*M* EDTA.
8. Synthetic peptides to be tested: stock solutions up to 1 *M* can be made in ddH_2O or DMSO, depending on the solubility of the peptide. Adjusting the pH may increase the solubility considerably. Use 1 *M* Tris at the desirable pH (range will depend on the amino acid composition of the peptide).
9. 3.5% Paraformaldehyde/PBS.
10. 20% Methanol.
11. 5% Crystal violet.
12. 0.1% SDS.

3. Methods

3.1. Construction of Phage Display Peptide Libraries

3.1.1. Isolation and Purification of fUSE5 Vector

The fUSE5 plasmid ***(5)*** is propagated in F'-minus host bacteria *Escherichia coli* MC1061. These bacteria should be grown in LB media (100 μg/mL streptomycin and 20 μg/mL tetracycline) (*see* **Notes 1** and **2**). We recommend growing at least a 1 L culture to obtain a reasonable working amount of this vector. fUSE5 is replication deficient and gives a low yield ***(5)***. A standard plasmid preparation protocol or a commercial plasmid purification kit (Qiagen, Promega, or Stratagene) may be used to isolate the plasmid.

3.1.2. Digestion of fUSE5 Vector

The fUSE5 vector was engineered to contain a 14-bp "stuffer" that renders the phage noninfective by disrupting the gene III reading frame ***(5)***. Restora-

tion of infectivity occurs only when the stuffer is replaced by an in-frame insertion. The stuffer sequence of fUSE5 within gene III can be excised with the restriction enzyme *Sfi*I. The overhanging ends left by the two *Sfi*I sites are designed to be incompatible, leaving the vector unable to religate. This feature also allows for unidirectional cloning of a *Bgl*I DNA insert ***(4,5)***. It is recommended to use at least 30 μg of fUSE5 vector for the *Sfi*I digestion because of losses during the purification steps. The *Sfi*I-digested fUSE5 vector must be separated from the stuffer. A commercial kit (Qiagen, Promega, or Stratagene) may be used for this step. To confirm that the ~9.5-kb vector has been dilinearized, an aliquot may be run on an 0.8% agarose gel. Once fUSE5 digestion has been adequately obtained, the vector is ready for ligation.

3.1.3. Preparation of Insert

The synthetic inserts are purchased or synthesized as single-stranded degenerate oligonucleotides. The sequence of the template is:

5' CAC TCG GCC GAC GGG GCT $(NNK)_X$ GGG GCC GCT GGG GCC GAA 3'

where N indicates an equimolar mixture of all four nucleotides; K indicates an equimolar mixture of G and T, preventing the introduction of a stop codon into the sequence. X represents the number of repeats. The oligonucleotide is converted into dsDNA using the Sequenase 4.0 Kit (Amersham). A complementary primer at the 3' end of the template (5' TTCGGCCCCAGCGGCCCC 3') is used for the reaction. Next, the resulting reaction mixture is purified from any unbound primers and single-stranded oligonucleotides by using the QIAquick Nucleotide Removal Kit (Qiagen). Finally, the double-stranded oligonucleotide is digested with *Bgl*I. This produces overhanging ends that are compatible to those on the fUSE5 vector digested with *Sfi*I.

3.1.4. Test Ligations

Before the final ligation between the *Sfi*I-digested fUSE5 vector and the *Bgl*I-digested insert is carried out, test ligations should be done to optimize the vector to insert molar ratio. Approximately 500 ng of vector is needed. A mock test ligation without adding the *Bgl*I insert fragment must be included as a negative control. To expedite the test, ligations may be carried out at room temperature for 3–4 h, then ethanol precipitated for at least 1 h at –20°C. The DNA pellet is then resuspended in 50 μL of ddH_2O. For the electroporation of the host bacteria (F'-minus strain), use 1 μL of DNA solution and 25 μL of electrocompetent bacteria. Add samples to 1 mL of SOC media and shake at 225 rpm for 1 h at 37°C. Plate serial dilutions (e.g., 2 μL and 200 μL) on LB/tetracycline plates. Count the number of colonies and determine the optimal vector-to-insert molar ratio, transformation efficiency, and background from the negative control ligation.

3.1.5. Library Ligation

After determining the optimal molar ratio for the ligation and ensuring that ligation of the vector alone yields a low or no background, the final library ligation may be performed. At least 10 μg of *Sfi*I-digested fUSE5 vector should be used for production of the library. For best results, the ligation should be carried out overnight at 16°C in a final volume of 500 μL. In order to exchange the ligation buffer to ddH_2O, the ligation mixture must be purified by using a commercial buffer exchange kit (Promega, Qiagen, or Stratagene) or by ethanol precipitation. The final volume should then be approx 200 μL. We use 1 μL of DNA per 20–25 μL of electrocompetent bacteria for the electroporation. Pool every 25 electroporations into 25 mL of SOC media, shake for 1 h at 37°C at 225 rpm and add to 500 mL of LB media with tetracycline (plus streptomycin if MC1061 is the host or a second antibiotic according to the chosen bacterial host genotype). From each 500-mL flask, plate serial dilutions (for example, 0.5 and 50 μL) on LB/tetracycline plates. The following day, count the colonies from the plates to determine the diversity of the library (in transducing units). Ideally, it should be approx 10^9. To amplify the phage library, incubate the bacteria in a 37°C shaker overnight (final volume of 2 L). The titer should be 10^{11}–10^{12} (*see* **Note 3**). Proceed with the protocol for precipitation of the phage with PEG/NaCl as discussed in **Subheading 3.3.2.**

3.2. Coating Integrins on Microtiter Wells

1. To a 96-well microtiter plate, add 25 μL of purified integrin diluted at various concentrations (from 10–500 ng per well) in TBS containing 25 m*M* n-octyl-β-D-glucopyranoside.
2. Add 200 μL of TBS containing 1 m*M* $MnCl_2$.
3. Incubate at 4°C for 16–24 h.
4. Remove the integrin solution from the well. If desired, the solution can be mixed with fresh integrin and reused for coating.
5. Saturate the well by incubating with 350 μL of TBS, 3% BSA, 1 m*M* $MnCl_2$ for 2 h at RT.
6. Wash the well three times with TBS/1 m*M* $MnCl_2$ by inversion and removing most of the liquid by pressing against a paper towel.

3.3. In Vitro Panning

3.3.1. First Round of Panning

1. Add 80 μL of 1% BSA, TBS, 1m*M* $MnCl_2$ to a well coated with integrin.
2. Add 20 μL of phage library containing 10^{10}–10^{11} transducing units (tu)/mL.
3. Incubate at 4°C for 16–24 h with gentle shaking.
4. Wash 10 times with 400 μL of TBS containing 0.5% Tween 20. Allow washing solution to stand for 1 min, invert the plate, and then press against a paper towel to remove most of the washing buffer.

5. To elute the phage, incubate in 100 μL of 0.1 *M* glycine–HCl at pH 2.2/0.1% BSA/0.05% phenol red, for 10 min with gentle shaking. Alternatively, integrin-bound phage can be eluted with 10 m*M* EDTA or 500 μ*M* GRGDSP peptide in TBS (pH 7.4).
6. Transfer the low-pH elution buffer to a sterile 50-mL tube containing 8 μL of 1 *M* Tris at pH 9.0. Note neutralization of the solution by color change to red. Do not yet discard the pipet tip. In the first round of panning, it is important to rescue the phage from both the pipet tip and the well.
7. Add 100 μL of starved K91kan bacteria to the 50-mL tube. Also add 100 μL of bacteria directly to the well to harvest the phage from the well and the pipet tip (*see* **Note 4**).
8. Mix gently the bacteria with the phage and let the tubes stand for at least 15 min (but no more than 1 h) at RT. In the meantime, warm LB media containing kanamycin (100 μg/mL) to 37°C.
9. Add 20 mL of the prewarmed LB medium kanamycin (100 μg/mL) and tetracycline (0.2 μg/mL; it has been suggested that this lower concentration of tetracycline induces the promoter of the tetracycline resistance (tet^R) gene of fUSE5). Shake the tubes at 37°C for 40–60 min.
10. Adjust tetracycline to a final concentration of 20 μg/mL. Spread 200 μL and 2-μL aliquots of the bacterial culture onto LB plates containing 40 μg/mL tetracycline to determine the amount of phage clones recovered from the panning procedure. Incubate the plates overnight at 37°C.
11. Amplify the phage by growing the remaining of the bacterial culture in a 37°C shaker for 16–24 h.

3.3.2. Successive Rounds of Panning

1. Pellet the bacteria by centrifuging at 6000g in 40-mL Sorvall tubes in a SS-34 rotor for 15 min. Transfer the supernatant containing the phage to a clean centrifuge tube, and repeat the centrifugation. Finally, transfer the supernatant to another clean centrifuge tube containing 3 mL of PEG/NaCl solution to precipitate the phage. Vortex briefly, and then rock gently at 4°C for at least 4 h. If the pannings are not continued on the same day, the tube may be kept overnight at 4°C.
2. Pellet the phage by centrifuging at 20,000g for 15 min. Carefully pour out or aspirate and discard the supernatant; keep the phage pellet.
3. Dissolve the phage pellet in 1 mL of TBS by shaking at 37°C for 10 min. Vortex for 1 min, and centrifuge at 2000g for 1 min. Transfer the supernatant to an Eppendorf tube containing 150 μL PEG/NaCl for a second phage precipitation. Vortex briefly, and rock gently at 4°C for 1 h. Centrifuge at 18,000g for 10 min. Aspirate the supernatant. Centrifuge 5 s and remove the remaining liquid.
4. Dissolve the phage pellet in 100 μL TBS/0.02% NaN_3. Allow the solution to stand for 5 min at 37°C and then vortex until the pellet is dissolved. A pipet may also be used for solubilization of the phage. Centrifuge briefly and collect the supernatant.

5. Transfer 20 μL of the solubilized phage per integrin-coated well containing 80 μL of 1% BSA/TBS/1 m*M* $MnCl_2$. Use three wells, each coated with a different amount of integrin, (e.g., 500, 50, and 5 ng). A fourth well, coated with BSA only, must be included as a negative control. Incubate the phage for 1 h at RT.
6. Follow the procedures outlined in the previous section to wash, elute, and amplify the phage. At least two or three rounds of panning should be performed. Sequence the DNA inserts starting from round II as described in **Subheading 3.4.**

3.4. Sequencing of the Phage Insert

1. Pick well-separated colonies from agar plates and transfer them to 5 mL of LB media containing 20 μg/mL tetracycline and 100 μg/mL kanamycin. Grow 14 mL of the bacterial culture in a 50-mL Falcon tube. Incubate in a 37°C shaker for 16 h.
2. Centrifuge the Falcon tube at 6000g for 15 min to pellet the bacteria.
3. Transfer the supernatant to an Eppendorf tube containing 750 μL PEG/NaCl, vortex for 1 min, and incubate the tubes on ice at least for 1 h.
4. Centrifuge at 6000g for 15 min. Aspirate the supernatant, centrifuge briefly, and remove the rest of the liquid.
5. Resuspend phage in 1 mL of TBS by vortexing. Store the phage at –20°C.
6. For preparation of ssDNA, transfer 200 μL of the phage to an Eppendorf tube containing 10 μL of StrataClean resin. Vortex for 1 min. Centrifuge at 2000g for 4 min.
7. Transfer 180 μL of the supernatant to a new Eppendorf tube containing 10 μL of StrataClean resin. Repeat vortexing and centrifugation.
8. Transfer 150 μL to an eppendorf tube containing 1 μL of glycogen, 150 μL of TE, and 40 μL of 3 *M* NaOAc. Add 1 mL of 100 % ethanol. Incubate the tubes on ice for 1 h.
9. Centrifuge at 8000g for 30 min at 4°C. Aspirate the supernatant, centrifuge briefly, and remove the rest of liquid.
10. Wash with 1 mL of 70% ethanol. Centrifuge at 18,000g for 15 min and aspirate the supernatant. Centrifuge again briefly, and remove the rest of the liquid.
11. Resuspend the DNA in 5 μL of ddH_2O. The dissolved DNA is ready for cycle sequencing.
12. Sequence the DNA directly using the 5' CCCTCATAGTTAGCGTAACG 3' primer. An alternative method is to first prepare a double-stranded PCR product containing the insert site, and then sequence the dsDNA product.
13. If you use an automatic Perkin Elmer (Norwalk, CT) ABI PRISM sequencing system, the following protocols apply.

3.4.1. PCR Mix

1. 100 ng Template ssDNA.
2. 8 μL Terminator Ready Reaction Mix.
3. 3.2 pmol Primer (5' CCCTCATAGTTAGCGTAACG 3') at 20 ng/reaction.
4. Add ddH_2O to 20 μL.

3.4.2. PCR Setting

1. 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Repeat for 25 cycles.
2. Precipitate the DNA, wash with 70% ethanol, and air dry.

3.5. Testing Integrin-Binding Activity of Peptides

Once the insert sequences are determined, motifs can be found and synthetic or recombinant peptides obtained.

1. Coat microtiter wells with integrins as described in **Subheading 3.2.**
2. The ability of the synthetic peptide to prevent binding of iodinated extracellular matrix proteins to the integrin can be determined ***(7)***.
3. You can also determine the inhibition of phage binding to the integrin by addition of the peptide. Incubate the phage displaying the peptide of interest with increasing concentrations of the cognate synthetic or recombinant peptide for 1 h at RT.
4. After washing, determine the ability of each peptide to inhibit phage binding or ligand binding to the integrins. Calculate the IC_{50} values ***(7,8)***.

3.6. Cell-Adhesion Assays

1. Coat microtiter wells with an appropriate extracellular matrix protein (such as fibronectin, vitronectin, or collagen) and block with TBS/3% BSA.
2. Detach cultured cells with 2.5 m*M* EDTA, wash once with serum-free DMEM. Count the cells and resuspend at the appropriate concentration (approx 10^6 cells/mL) in DMEM containing 1% BSA. Divalent cations can be added (1 m*M* Mn^{2+}, Mg^{2+}, and/or Ca^{+2}).
3. Add 10^5 cells/well with different concentrations of the peptide in serum-free media. Keep in the tissue culture incubator for 1 h at 37°C.
4. Remove unbound cells by washing a few times with serum-free DMEM. The bound cells may be stained by 5% crystal violet and the IC_{50} value of the peptide can be estimated as described ***(12)***.
5. In a reverse application, coat microtiter wells with the peptide alone or the peptide coupled to a carrier protein. The capacity of cells to attach to the bound peptide can be estimated ***(12)***.

4. Notes

1. Biological specimens: Destroy bacteria and phage before disposal to sink.
2. Maintain the fUSE5 vector in a pilus-negative bacteria such as K802 or MC1061. Restreak the bacteria in minimal media plates every 2 wk and keep a glycerol stock of the bacterial clone. When restreaking, always streak the bacteria in a LB/tetracycline plate as a negative control. The presence of tetracycline-resistance colonies indicates possible cross-contamination with fUSE5 phage.
3. Contamination risk: K91kan is infected easily by phage present in the lab. Be careful when handling bacteria and phage. Use only sterile single-use or autoclaved labware. Avoid spills.

4. It is important to try to collect most bound phage in the first panning. This is the reason for adding bacteria to both microtiter well and pipet tip.

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2

Production of Rat Monoclonal Antibodies Specific for Mouse Integrins

Marc Delcommenne and Charles H. Streuli

1. Introduction

Monoclonal antibodies (MAbs) have proved to be an important tool for identifying novel cell-adhesion molecules and for determining their ligand binding specificity, function, and structure. Consequently, they have been instrumental in defining three families of adhesion receptors: the cadherin, immunoglobulin, and integrin families. Integrins include over 20 adhesion receptors that react with the extracellular matrix or cell-surface molecules. Integrins are composed of two distinct transmembrane glycoprotein subunits, α and β, which are noncovalently linked to each other. At least 15 α chains and 8 β chains have been observed in several possible combinations that determine the ligand specificity and function of the complex ***(1)***.

To this date, MAbs have been produced against virtually all human integrin chains and most of them are now commercially available from several companies. However, MAbs specific for integrins of other species are still scarce and unquestionably needed. In addition, production of novel MAbs against human integrins remains important for epitope mapping ***(2–4)*** and dissecting integrin-mediated signal transduction pathways ***(5)***. Finally, some applications require large amounts of MAbs and preparing home-made anti-integrin MAbs can be very cost effective. Therefore, we shall detail below the procedure we followed to produce and screen rat MAbs against mouse integrins that was successful in our hands and that can be adapted for specific purposes.

1.1. Strategy

Four factors are important for the successful generation of MAbs: the species of the animal used for immunization, the type of the antigen, the immunization scheme, and the screening procedure.

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Mice are generally used for hybridoma generation but are seldom used for raising antibodies against mouse receptors because of their tolerance to most syngeneic antigens. Hamsters or more often, rats, are currently used instead. Rats are in easy supply and provide a high number of spleen lymphocytes available for fusion with myeloma cells. Moreover, hamster hybridomas grow slowly initially and were reported to sometimes be overgrown by fibroblast-like cells *(6)*.

Affinity-purified receptors, purified membranes, or cells have been used as immunizing material for anti-integrin MAb production. Immunization with purified receptors increases the probability of obtaining specific hybridomas. However this strategy necessitates obtaining the receptor in sufficient quantities for immunization, either by direct purification or by immunoaffinity purification on a first generation mono- or polyclonal antibody column. In addition, MAbs raised against purified integrins are frequently not function-blocking and are therefore of limited use. Immunization with purified membranes has been used successfully for hybridoma production *(7)* but this procedure requires additional material and we shall not describe this protocol here. We have chosen the injection of whole cells, which are highly immunogenic and are simple to obtain and use. Although this procedure presents a disadvantage in that hybridomas are produced against virtually all cellular antigens and require several screening steps to select for anti-integrin hybridomas, the immunization protocol is easy and function-blocking antibodies can be obtained. Choice of the cell type used for immunization is also particularly important in this respect since cell lines expressing high levels of the integrins of interest will increase the chance of generating hybridomas against these receptors. Cell types expressing immunodominant antigens such as mucins have to be avoided as they have been shown to hamper the immune response against other cell surface antigens (P. J. Kilshaw, pers. comm.).

Although immunization schedules can vary widely, most of them have been devised to promote both amplification of high-affinity antibodies producing B-cell clones and the availability of proliferating B cells on the fusion day, as only these cells can fuse with the myeloma. Thus, multiple injections of the antigen emulsified with adjuvant are performed; this constantly releases antigen into the circulation, promoting continuous proliferation of specific B cells. In addition, the fusion is performed 3 d after the final boost, when the activated B cells are still proliferating but not yet differentiated into secreting plasma cells.

The screening protocol has to be established prior to the fusion so that hybridomas reacting with the antigen of interest can be selected rapidly and amplified (*see* **Note 1**). In our procedure, MAbs were first selected by indirect immunofluorescence of cell-surface staining followed by flow cytometry. Next, we focused our attention to MAbs that immunoprecipitated heterodimeric

complexes showing two migrating bands of 90–110 and 140–180 kDa on nonreducing SDS-PAGE gels.

1.2. Outcome

To produce MAbs against mouse β_1 and α_v integrins, we selected the mouse melanoma K1735-M2 cell line as an immunogen because it expresses most β_1 integrins ***(8)*** and also $\alpha_v\beta_3$ at a high level. Spleen cells from rats immunized with this melanoma cell line generated a hybridoma according to the screening protocol described below, which was specific for mouse α_v integrin. This MAb, C8F12, is now being used to monitor the physiology and differentiation of various cell lineages ***(9)***. This antibody is being used further to generate antisera against $\alpha_v\beta_3$ and second generation MAbs against mouse β_1, β_3, β_5, β_6 and β_8 chains as they all combine with the α_v subunit.

2. Materials

2.1. Animals and Cell Lines

1. Animals for immunization: 6-wk-old female Lou rats, purchased from OLAC (Shaw's Farm, Blackthorn, Bicester, UK) (*see* **Note 2**).
2. IR983F rat myeloma cells, deficient in hypoxanthine phosphoribosyltransferase (HPRT) and adenosine phosphoribosyltransferase (APRT) (*see* **Note 3**) ***(10)***.
3. K-1735 M2 mouse melanoma cells. These cells are passaged at 1:10 or 1:20.
4. Human embryonic lung cells, MRC-5 (ATCC #CCL 171). These cells are passaged at 1:3, but should be used before passage 40 ***(11)***.

2.2. Material for Routine Cell Culture

1. Cell culture medium for routine culture of MRC5, IR983F, and K-1735 M2 cells: Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley, Scotland #12100-046) prepared according to the manufacturer's directions supplemented with 1 x non essential amino acids solution (Life Technologies cat. no. 11140-050), 10% fetal calf serum (FCS) (Life Technologies cat. no. 16000-044), solution of 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies cat. no. 15140-122), 50 μg/mL gentamycin (Life Technologies cat. no. 15750-029), 3.7 g/L sodium bicarbonate (Sigma, St. Louis, MO, cat. no. S 5761). Media supplements are stored as concentrated stocks at –20°C, and media are stored at 4°C.
2. Phosphate-buffered saline (PBS): to make 1 L of 10× stock solution, dissolve 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4, and 11.25 g of Na_2HPO_4, adjust to pH 7.4, and bring to a total volume of 1 L. Autoclave the solution of 1× PBS at 121°C for 20 min and store it at room temperature.
3. PBS–EDTA 0.2%: to make 1 L, dissolve 2 g of EDTA in 800 mL distilled water and adjust the pH to 8.0 with 5 *M* NaOH to help dissolve EDTA. Then add 100 mL of 10× PBS, adjust the pH to 7.4, and bring to a total volume to 1 L. Sterilize by autoclaving at 121°C (15 psi) for 20 min, and keep at room temperature.

4. 0.25% Trypsin, 1 m*M* EDTA solution (Life Technologies cat. no. 25200-056). Store in aliquots at –20°C.
5. Cell storage solution: Mix 5 mL of DMSO (Sigma cat. no. D 8779) with 10 mL of FCS and 35mL of DMEM and keep at 4°C.
6. 100-, 250-, and 500-mL glass bottles for medium.
7. Sterile 5-, 10-, and 25-mL glass pipets.
8. Multichannel pipet.
9. 50-, 100-, and 150-mm tissue-culture dishes (Nunc, Roskilde, DK).
10. 25- and 75-cm^2 tissue-culture flasks (Falcon, Los Angeles, CA).
11. 1.5-mL sterile Eppendorf tubes.
12. Sterile 15- and 50-mL centrifuge tubes (Falcon).
13. Refrigerated bench-top centrifuge (with holders for 15, 50-mL tubes and 96-well microplates).

2.3. Material for Immunization and Boosts

1. Complete Freund adjuvant for the first injection (Sigma cat. no. F 5881) (*see* **Note 4**).
2. Incomplete Freund adjuvant for the boosts (Sigma #F 5506).
3. 1-mL syringes.
4. 21-gage needles.

2.4. Material for Feeder Layer Preparation and Cell Fusion

1. Mitomycin C solution: Dissolve mitomycin C (Sigma cat. no. M 0503) at 1 mg/mL in H_2O, filter sterilize, and store at 4°C. This solution is stable for at least 4 mo.
2. Hybridoma culture medium (H medium): To 500 mL of cell culture medium described above (DMEM, 10% FCS), add 50 mL of horse serum (Life Technologies cat. no. 16050-122) and 5.5 mL of hypoxanthine 100× stock solution. This stock solution is prepared by dissolving 136 mg of hypoxanthine (Sigma cat. no. H 9636) in 100-mL H_2O heated to 70°C. The 100× stock solution is filter sterilized, aliquoted, and stored at –20°C.
3. Hybridoma selection medium (HA medium): To 250-mL hybridoma culture medium (H medium), add 250 µL of 1000× stock solution of azaserine. This solution is prepared by dissolving 10 mg of azaserine (Sigma cat. no. A 1164) in 10 mL of H_2O. The azaserine 1000× stock solution is filter sterilized, aliquoted, and stored at –20°C (*see* **Note 5**).
4. Spleen cell resuspension medium: DMEM containing 2.5% FCS and antibiotics (*see* **Subheading 2.2.1.**) kept at 4°C.
5. Polyethylene glycol (PEG): 50% solution in PBS (Sigma cat. no. P7181).
6. 1-L and 200-mL beakers.
7. 500 mL of 70% ethanol.
8. Dissection board.
9. Medium-sized scissors, fine scissors, and fine forceps autoclaved for 20 min at 121°C.
10. Sterile 21-, and 25-gage needles.
11. Sterile 20-mL syringes.
12. Water bath at 40°C.
13. Timer.

14. Sterile 1-mL glass disposable pipets (Volac, John Poulten Ltd, Barking, UK).
15. Sterile 96-well flat-bottomed plates (Nunc cat. no. 1-67008A).
16. Sterile 24-well flat-bottomed plates (Nunc cat. no. 1-43982).

2.5. Additional Material for Screening

1. 20% NaN_3 stock solution: dissolve 20 g of NaN_3 (Sigma cat. no. S 2002) in 100 mL of H_2O.
2. 96 conical-well microplates (Nunc cat. no. 442587).
3. FITC-conjugated Rabbit anti-rat IgG antiserum (Sigma cat no. F 1763).
4. PBS–2% formaldehyde: Dilute 5 mL of formaldehyde 40% (Merck, Rahway, NJ, cat. no. B10113-76) in 95 mL of PBS and keep at 4°C.
5. Flow cytometer (FACSscan, Becton Dickinson, Rutherford, NJ).
6. [^{35}S]-Methionine-labeling reagent: Tran^{35}S-label (ICN Biomedicals, Thame, UK cat. no. 51006) at 10 mCi/mL.
7. Low-methionine DMEM: Prepare this medium by adding 3.5 g of glucose and 3.7 g of sodium bicarbonate to 1 L of cystine–methionine-deficient, low-glucose DMEM (Sigma cat. no. D3916) and filter sterilize it. Mix 900 mL of this medium with 100 mL of the regular DMEM to obtain the low-methionine medium.
8. Dialyzed FCS: Sterilize a dialysis membrane by autoclaving at 121°C for 20 min. Pour 20 mL of FCS into this membrane in a laminar flow hood and dialyze it at 4°C against 3× 1 L of autoclaved 150 m*M* NaCl saline solution. Filter sterilize, aliquot the dialyzed FCS, and store at –20°C.
9. Lysis buffer: This buffer contains 150 m*M* NaCl, 50 m*M* Tris-HCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1% NP_40, pH 7.4, and is supplemented just prior to use with the protease inhibitors 1 m*M* PMSF (Sigma #P 7626), 0.2 trypsin inhibitor units (TIU)/mL aprotinin (Sigma #A 6279), 10 μg/mL leupeptin (Sigma #L 2023), 10 μg/mL pepstatin (Sigma #P 4265). Keep this buffer at 4°C.
10. Activated charcoal (Sigma cat. no. C 5260).
11. Protein G Sepharose beads (Zymed Labs Inc., South San Francisco, CA, cat. no. 10-1242).
12. [^{14}C]-labeled molecular weight markers (Amersham cat. no. CFA 756).
13. 30% solution of trichloroacetic acid, stored at 4°C.
14. GF/C glass microfiber filters (Whatman International, Maidstone, UK).
15. Scintillation fluid.
16. Scintillation counter.
17. Fluorographic reagent: Amplify, (Amersham, Arlington Heights, IL cat. no. NAMP 100).

3. Methods

3.1. Protocol of Rat Immunization with K1735-M2 Cells

1. Plate K1735-M2 cells on 150-mm dishes at 10^6 cells/dish and grow until subconfluent.
2. To detach the cells, wash the monolayers with PBS–EDTA, and harvest the cells by incubating them with 5 mL of PBS–EDTA per 150-mm dish for 5–10 min at 37°C.

3. Transfer the pooled detached cells to a 50-mL tube and wash them three times with serum-free DMEM.
4. Resuspend 20×10^6 cells in 250 µL of PBS and aspirate them in a 1-mL syringe.
5. Transfer 250 µL of complete Freund adjuvant for the first injection to an Eppendorf tube.
6. Mix cell suspension with adjuvant and then emulsify by passing the mixture rapidly back and forth between the syringe, fitted with a 12-gage needle, and the Eppendorf tube.
7. Remove some blood from each rat before the first injection to have a preimmune control antiserum. Then prime the rats by multiple subcutaneous injections.
8. Boost the rats 2–3 times at monthly intervals by injecting intraperitoneally 20×10^6 cells emulsified in Freund incomplete adjuvant.
9. Boosting should continue until the antiserum titer, assessed by immunofluorescence, is at least $1/10^4$ (*see* **Subheading 3.3.1.**). Remove approx 200 µL of blood from rat by tail bleed. After letting the clot form by incubation for 1 h at 37°C, recover the antiserum from the clot and spin it at 1500g in a minifuge to discard residual cells. Store the aliquots of antiserum at –20°C.
10. For the final boost, inject 20×10^6 cells resuspended in 500 µL of PBS intraperitoneally 3–4 days prior to fusion.

3.2. Fusion Protocol

3.2.1. Preparation of MRC5 Cells Feeder Layer

The feeder layer is prepared 3 d before fusion. This period allows the medium to be conditioned for optimal survival and growth of the hybridomas.

1. Three days before cell fusion, discard the medium from exponentially growing MRC5 cells in 150-mm dish and incubate the cells for 1 h with 15 mL of fresh culture medium plus 30 µL of mitomycin C stock solution (*see* **Note 6**).
2. Wash the cells 3× with DMEM serum-free medium, 1× with PBS–EDTA, and then detach them with 2 mL of trypsin-EDTA.
3. Resuspend MRC5 cells in hybridoma selection medium (HA medium) at the density of 8×10^4 cells/mL.
4. Distribute 100-µL aliquots in a 96-microwell plate using a multichannel pipet (*see* **Note 7**).

3.2.2. Preparation of Myeloma Cells

1. Maintain the IR983F myeloma cells in strict exponential growth for at least 1 wk before fusion to maximize their fusion capacity. To maintain exponential growth, adjust the cell density to 0.5×10^5 cells/mL (i.e., 1.5×10^6 cells/30 mL in 150-mm dish) 2, 4, and 6 d before fusion.
2. Because IR983F cells are rather adherent, detach them with PBS–EDTA 0.2%, transfer the cell suspension to a 50-mL tube, and fill the rest of the tube with DMEM.

3.2.3. Preparation of Spleen Cells

1. Euthanize the rat by cervical dislocation and then dip it in a 1-L beaker filled with 500 mL of 70% ethanol.
2. Place the rat in a sterile laminar flow hood on its right hand side on a dissection board which has been cleaned with 70% ethanol.
3. Pinch up the superficial skin over the left side of the abdomen and make a 5-cm incision over the spleen. Then dissect the skin from the muscular wall and deflect the edges laterally to expose the abdominal wall through which the spleen is visible.
4. Using sterile fine forceps, incise the muscle layers and peritoneum over the spleen with fine scissors. Then, exteriorize the spleen by gently lifting its lower pole.
5. Release the spleen by cutting its mesentery and transfer it to a 50-mm Petri dish containing approx 5 mL of ice-cold DMEM–2.5% FCS.
6. After rinsing, transfer the spleen to a fresh 100-mm Petri dish and dislodge the spleen cells using two 20-mL syringes fitted with 26-gage needles, each containing 20 mL of DMEM–2.5% FCS. To achieve this, one syringe is used to anchor the spleen, while the other is used to flush fluid into successive small areas of spleen.
7. Transfer the cell suspension to a 50-mL tube leaving behind the spleen carcass and small lumps of tissue that may have settled on the base of the dish. Allow the remaining clumps and pieces of connective tissue to sediment for approx 5 min, then transfer the cell suspension to another 50-mL plastic tube.
8. Spin the tube at room temperature for 10 min at 400*g*.
9. Resuspend pellet in 10 mL of serum-free DMEM medium. To count the cells, take a 100-µL aliquot of the cell suspension and mix it with 810 µL of distilled water by gentle shaking for 30 s to lyse the erythrocytes, then add 90 µL of 10× PBS to restore the physiological osmolarity. The cell viability must be at least 95% as measured by Trypan blue exclusion.

*3.2.4. Fusion (***ref. *12*)

1. Mix 10^8 spleen cells with 2×10^7 IR983F myeloma cells in a 50-mL tube (5 spleen cells/1 myeloma cell). Add DMEM to a volume of 50 mL.
2. Spin the cells at room temperature for 8 min at 400*g*.
3. Aspirate the supernatant with a Pasteur pipet connected to vacuum. Make sure to remove the supernatant completely so as not to dilute PEG in the next steps.
4. Loosen the cells in the pellet by gently tapping the bottom of the tube. Place the tube in a 200-mL beaker containing water at 40°C and keep it there during the fusion.
5. Add 0.8 mL of 50% PEG prewarmed at 40°C to the pellet slowly over a period of 1 min using a 1-mL pipet, continuously stirring the cells with the pipet tip.
6. Continue stirring the cells in 50% PEG for a further 1.5–2 min.
7. With the same pipet, add 1 mL of DMEM, taken from a 50-mL tube containing 30 mL of DMEM kept at 37°C in another beaker at 40°C, to the fusion mixture, continuously stirring as before, over a period of 1 min.
8. Repeat **step 7**.

9. Repeat **step 7** twice, but add the medium in 30 s.
10. Always with the same pipet and continuously stirring, add 6 mL of DMEM over a period of approx 2 min.
11. With a 10-mL pipet add 12–13 mL of DMEM dropwise.
12. Spin down the aggregates at 400*g* for 5 min.
13. Discard the supernatant, break the pellet by gently tapping the tube, and resuspend the cells in hybridoma selection medium (H medium).
14. Adjust the cell density to 10^6 cells/mL and distribute 100 µL in each microwell of 96-well microplates containing MRC5 cells with a multichannel pipet.

3.2.5. Feeding Hybridomas

The medium is changed first on the fourth day after fusion. At this stage, all the nonfused myeloma cells are dead. Therefore, azaserine is no longer necessary and the cells are switched to hypoxanthine-containing culture medium (H medium). Half of the medium is removed from each microwell by suction using a Pasteur pipet connected to a vacuum and fresh medium is dispensed by using a multichannel pipet. Subsequently, medium is changed every 2 d, gradually diluting out residual azaserine and antibodies secreted by the unfused lymphocytes. Hybridomas will become apparent over the next 7–10 d, and should be screened before they become confluent (*see* **Note 8**).

3.3. Screening

The screening of supernatants generally begins 7–10 d after fusion, from hybridomas that cover a significant area of the microwell (at least one-fourth of the well area) and that have been refed at least three times.

3.3.1. Primary Screening of MAbs by Fluorescence Staining of M2 Cells

1. Detach K1735-M2 cells by PBS–EDTA treatment as described earlier, resuspend them at 20 × 10^6/mL in PBS–NaN_3 0.1%, and distribute them as 50-µL aliquots to 96-v-well microplates.
2. Dispense 50 µL of hybridoma supernatant to each microwell and incubate them with the cells for 1 h on ice.
3. Wash the cells by dispensing 100 µL of cold PBS 0.1% and centrifuging the microplates at 400*g* for 5 min at 4°C.
4. Aspirate the supernatants with a Pasteur pipet connected to a vacuum line.
5. Resuspend the cells in 200 µL of PBS and pellet them again by centrifugation at 400*g*.
6. Resuspend the cells in 50 µL of FITC-conjugated rabbit anti-rat IgG antiserum diluted 1/100 in DMEM, 10% FCS, and incubate them for 1 h on ice.
7. Add 100 µL of PBS to the cells and spin the microplates at 400*g* for 5 min at 4°C.
8. Resuspend the cells in 100 µL of cold PBS and then dilute them in 100 µL of PBS–2% paraformaldehyde.
9. Analyze the cells by flow cytometry or fluorescence microscopy and keep the positive supernatants for the secondary screening.

3.3.2. Secondary Screening by Immunoprecipitation

1. Plate 10^5 K1735-M2 cells in a 50-mm dish.
2. When the cells have reached 70% confluence, wash them three times with sterile, low-methionine DMEM.
3. Feed the cells with 2 mL of low-methionine DMEM,10% dialyzed FCS, and 200 μCi/mL of Tran^{35}S-label.
4. Place the 50-mm dish in a box with two other 50-mm dishes, one containing activated charcoal to adsorb the volatile [^{35}S]sulfur, and a second one filled with distilled water, to keep the atmosphere water saturated.
5. Transfer the culture dish-containing box to a cell incubator at 37°C.
6. After an overnight [^{35}S]-methionine incorporation, wash the cells three times with warm serum-free medium, lyse the cells on ice with 0.5 mL of lysis buffer at 4°C.
7. Scrape the lyzed cells and transfer the lysate to an Eppendorf tube. Repeat once.
8. Vortex the pooled lysates and leave them on ice for 30 min. Then pellet nuclei and insoluble material by a 15-min centrifugation at 21,000g in a minifuge at 4°C. Aliquot the lysate supernatant and store it at –80°C until use.
9. Assess the level of [^{35}S]-methionine incorporated in proteins by trichloroacetic acid (TCA) precipitation on glass filters. Transfer the filters to scintillation vials, add scintillation fluid, and count the cpm in a scintillation counter.
10. For each supernatant to test, dilute in an Eppendorf a volume of lysate corresponding to 5×10^6 cpm with lysis buffer to reach a final volume of 200–500 μL. Then react 20 μL of hybridoma supernatant with the lysate for 1 h at 4°C under gentle shaking or rotation.
11. Add 25 μL of 50% (v/v) Protein G Sepharose in lysis buffer to each eppendorf and rotate the samples for 1 h at 4°C.
12. Pellet the beads by a 21,000g pulse in the minifuge and wash them three times with the lysis buffer and once with 50 m*M* Tris-HCl at pH 7.4.
13. Elute the immunoprecipitated antigens from the beads by boiling them for 3 min in 30 μL of nonreducing 2× sample buffer.
14. Run the samples along side [^{14}C]-labeled molecular weight markers on a nonreducing 6% SDS-PAGE gel.
15. Fix the gel in a solution of 40% H_2O, 50% methanol, 10% acetic acid, for 30 min, wash the gel in distilled water for 30 min and incubate the gel with Amplify.
16. Dry the gel.
17. Expose the gel to autoradiographic film or phosphorimager plates (**Fig. 1**).

3.3.3. Subsequent Screening Tests

After selecting a MAb immunoprecipitating a dimeric molecular complex similar to integrins, it is necessary to confirm specificity of the antibody. An initial step is to deplete the integrin from the lysate by repeated immunoprecipitations with a polyclonal antiserum reacting with the α or β subunit cytoplasmic domain of the suspected integrin and then to proceed with the MAb

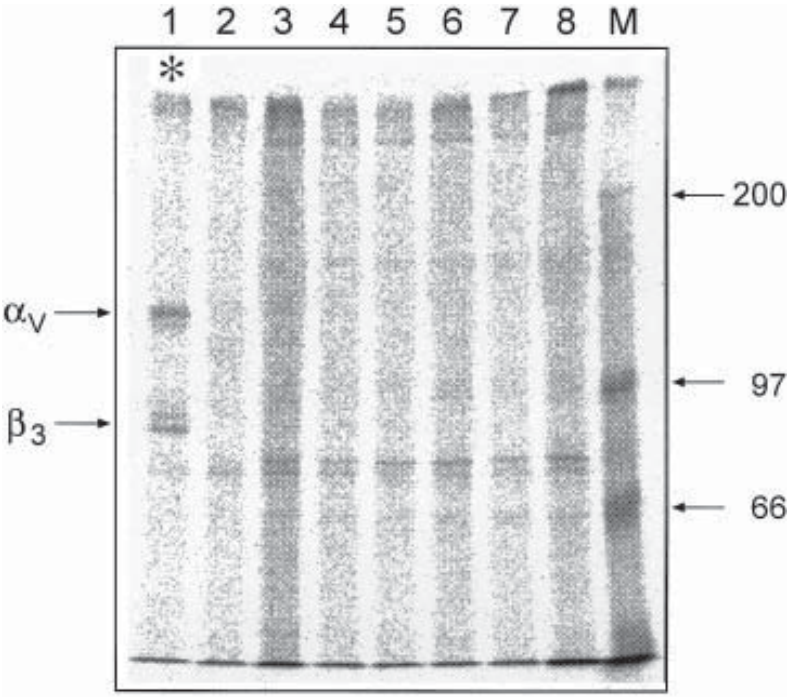

Fig. 1. Result of one initial screen to identify anti-integrin antibody. Hybridoma supernatants identified as positive in the primary fluorescence screen were tested for their ability to immunoprecipitate proteins with the approximate molecular mass of integrins. 5×10^6 cpm of a cell lysate of [^{35}S]-methionine-labelled K1735-M2 cells was immune precipitated with supernatants from eight hybridomas (lanes 1–8) and separated by SDS-PAGE. Sizes (in kDa) of the labeled molecular mass markers (Amersham cat. no. CFA 626) are shown in the margin. Only one supernatant-precipitated bands of the expected size (lane 1, indicated with an asterix). The antibody, MAb C8F12, subsequently turned out to recognize α_v integrin, and in this experiment β_3 integrin was coprecipitated.

(*see* **Note 9**). If this MAb no longer immunoprecipitates any material after immunodepletion, this will indicate that it reacts effectively with an integrin. The reverse experiment can also be performed: immunodepletion of the putative integrin with the MAb followed by immunoprecipitation with the polyclonal antiserum. The next step will be to ascertain the reactivity of the MAb with either one integrin subunit or an epitope formed by both α and β subunits. Determining the MAb specificity by Western blot should be attempted first. If the MAb does not work in Western blot, another method consists of performing immunoprecipitation after dissociation of the integrin heterodimer by increasing the pH ***(13)*** or SDS treatment ***(14)***. Concurrently,

the MAb should be tested for immunoprecipitation using different cell lines expressing various integrins. If the MAb appears to immunoprecipitate different integrin dimers of the same subfamily, this will indicate that this MAb recognizes a common subunit (**Fig. 2**). Tissue-section immunocytochemical staining of different organs is also a good complementary test, particularly for MAbs recognizing integrins expressed in restricted tissues such as $\alpha 4\beta_7$ ***(15)***.

Finally, an important part of the MAb characterization consists of assessing its capacity to block cell adhesion to the extracellular protein(s) interacting with the suspected integrin. This can be done by quantifying the percentage of adherent cells after antibody treatment using an ELISA reader ***(16,17)***.

3.4. Maintenance of Hybridomas

3.4.1. Expanding the Culture

During propagation of the positive hybridomas, it is advised to store a couple of samples of each in liquid nitrogen as soon as possible to have back-up cells should a contamination occur (*see* **Note 10**). Hybridomas are expanded as follows:

1. When the positive hybridomas cover at least 50% of the microwell area, 150 µL of medium are pipetted up and down to detach the cells. The cell suspension is then transferred to a 24-well dish containing 300 µL of H medium. Hybridoma cells left in the microwell are refed with 150 µL of H medium.
2. One day after the transfer, 1 mL of H medium is added if the cells are growing actively. Otherwise, this should be delayed.
3. When the cells cover the whole area of a 24-well dish, the medium is pipetted up and down to detach the cells and 1 mL of the cell suspension is transferred to a 25-mL flask already containing 3 mL of H medium.
4. One day later, 5 mL of fresh H medium are added to the cells.

3.4.2. Cell Storage

1. When the hybridomas are close to confluence, the 25-mL flask is shaken and the content is transferred to a 15-mL conical tube. An aliquot is taken and the cells are counted while the tube is spinning for 10 min at 400*g* (or 1500 rpm).
2. Cells are resuspended at $1–2 \times 10^7$ cells/mL in DMEM–10% FCS and mixed with an equal volume of DMSO storage solution, transferred to a –80°C freezer in a styrofoam box, and frozen to –80°C, then stored in liquid nitrogen.

3.4.3. Recovering Cells from Liquid Nitrogen Storage

1. Thaw the frozen cells by dipping the cryovial in a 37°C water bath.
2. Transfer the cell to a 50-mL tube containing 30 mL of warm DMEM, and spin the tube for 10 min at 400*g*.
3. Resuspend hybridoma cells into 1 mL of H medium and dispense them in a well of a 24-well dish. If many cells are viable, the medium will quickly become acid. Therefore, the cells will be transferred to a 25-mL flask. If cells are not numerous

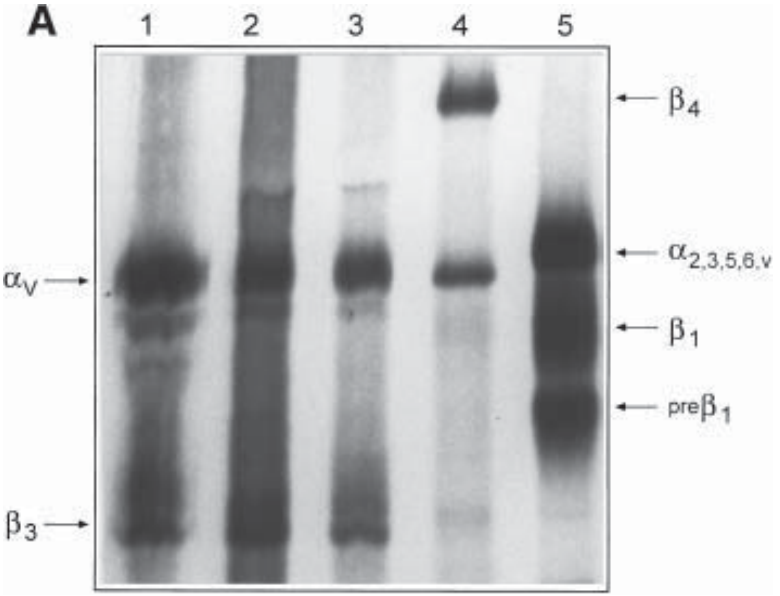

Fig. 2. MAb C8F12 recognizes α_v integrin. **(A)** Comparison of mouse integrin electrophoretic mobility with material immunoprecipitated by MAb C8F12. Primary mouse mammary epithelial cells were cultured overnight with [^{35}S]-methionine, after which they were lysed and subjected to immunoprecipitation with a rabbit anti-α_v cytoplasmic domain antiserum (lane 1), anti-mouse β_3 monoclonal antibody (lane 2), MAbC8F12 (lane 3), anti-mouse α_6 monoclonal antibody, GoH3 (lane 4), rabbit anti-β_1 cytoplasmic domain antiserum (lane 5). The immunoprecipitates were separated by SDS-PAGE on nonreducing 6% gels.

or healthy at thawing, it is safer to resuspend them to 1 mL of a mixture of MRC-5 cell conditioned medium diluted 1/4 in H medium.

3.4.4. Subcloning Hybridomas

After completion of hybridoma screening steps, it is necessary to ensure the monoclonality of the selected hybridoma. For this purpose, the hybridoma needs to be subcloned at least twice. Of the two methods currently in use, soft agar and limiting dilution cloning, we have adopted the latter for its simplicity.

1. Serially dilute the cells to a final density of 1 cell/mL.
2. Dispense cells as 100 µL aliquots in 96 well plates on a bed of mitomycin C–treated MRC5 cells (*see* **Subheading 3.2.4.**).
3. After the clones have grown sufficiently, test their supernatants by immunofluorescence and immunoprecipitation (*see* **Subheadings 3.3.1.** and **3.3.2.**).
4. Expand and clone positive clones a second time.

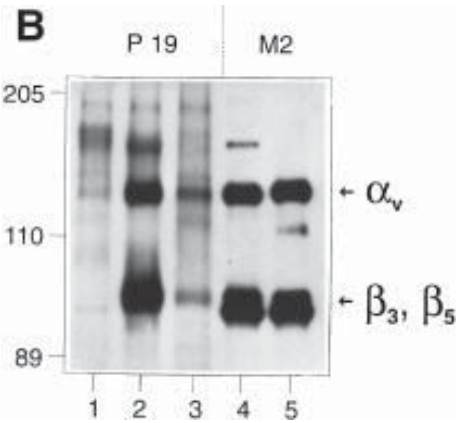

Fig. 2. *(continued)* MAb C8F12 recognizes α_v integrin. **(B)** MAb C8F12 was shown to recognize the mouse α_v integrin subunit by immunoprecipitation of α_v integrins from nondifferentiated mouse embryonal carcinoma P19 cells and K-1735 M2 cells. P19 cells express $\alpha_v\beta_5$ integrin but not $\alpha_v\beta_3$, whereas the reverse is true for K-1735 M2 cells. Lysates of cell surface biotinylated nondifferentiated P19 cells were reacted with anti-β_3 monoclonal antibody (lane 1), MAb C8F12 (lane 2) and rabbit anti-β_5 cytoplasmic domain antiserum (lane 3). Lysates of the cell-surface biotinylated M2 cells were reacted with MAb C8F12 (lane 4) or with anti-mouse β_3 monoclonal antibody (lane 5). Immunoprecipitates were separated on a 6% SDS-PAGE gel and transferred to PVDF membrane. This was incubated with streptavidin peroxidase and the cell-surface immunoprecipitated proteins were detected by ECL (Amersham) ***(15,18)***.

5. It is advisable to reclone the hybridomas from time to time to eliminate the nonsecretory variants that may appear and overgrow the antibody secretory cells.

4. Notes

1. Originally, the first anti-integrin MAbs were screened according to their capacity to inhibit cell adhesion to extracellular matrix molecules ***(19)***, cell–cell adhesion in immune response processes ***(20)***, phagocytosis ***(21)***, and clot formation ***(22)***. We chose not to follow this strategy considering that it would lead to the loss of valuable nonfunction-blocking anti-integrin MAbs.
2. Although any rat strain can be used for immunization and fusion with the IR983F myeloma, which is of LOU origin, it is more convenient to use compatible LOU rats for injection so that the resulting hybridomas can be used to generate ascites tumor, if necessary.
3. Mouse myelomas can be used conveniently instead of IR983F cells for fusions with rat lymphocytes and several rat anti-integrin MAbs have been produced this way ***(23,24)***. However, it has been claimed that rat hybridomas resulting from heterofusions happen to be unstable ***(25)***.
4. Alternative adjuvants are acceptable, for example Titre-Max Gold.

5. Hybrids are commonly selected in a medium supplemented with HAT. Since IR983F cells are also deficient for adenine phosphoribosyltransferase, we preferred to use HA medium, principally for three reasons. First, selection with azaserine generates more hybridomas per fusion because loss of hybridomas with X chromosome segregation caused by culture in HAT medium does not happen in HA medium. Second, the proportion of IgG secreting hybridomas is higher when selected with azaserine as it eliminates hybridomas that are incapable of IgG heavy chain synthesis *(26)*. Thirdly, in case of hybridoma contamination by mycoplasmas, these microorganisms deplete thymidine from HAT-supplemented medium, causing the death of the hybridomas. However, hybridomas grown in HA medium will survive despite this contamination and can be treated with a suitable antimycoplasma drug.
6. Conventionally mouse peritoneal cells are used as feeder layer for hybridoma culture but are sometimes contaminated with pathogens. To minimize contamination risks, various cell lines have been used instead with mixed success *(27,28)*. Among the cell lines tested, MRC5 cells have been shown to provide an excellent feeder layer for hybridoma development partially because they secrete interleukin 6 *(29)*. Contrarily to what is stated in some methods books, it is crucial that MRC5 cells are treated with mitomycin C.
7. It is a good idea to set up 10× 96-well plates at this stage.
8. In a good fusion you might expect up to 60% of the wells to be showing signs of cell growth.
9. Antisera reacting with α and β chain cytoplasmic domains, available from Chemicon or Pharmingen can be used in these experiments. Because of the high conservation of sequence within the cytoplasmic domain, anticytoplasmic domain antibodies cross–react with integrins of most species. Incubation times, volumes of antibody to add to the lysate, and the number of sequential immunoprecipitations will depend on the antibody concentration and avidity and the level of integrin expression. Therefore, the immunodepletion protocol will have to be adapted for each case.
10. To avoid unnecessarily filling up a liquid nitrogen container, it is strongly recommended to discard frozen aliquots of hybridomas that are subsequently shown NOT to secrete useful antibodies.

Acknowledgments

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3

Biochemical Analysis of Integrin-Mediated Shc Signaling

Kishore K. Wary, Michael Dans, Agnese Mariotti, and Filippo G. Giancotti

1. Introduction

1.1. Membrane Proximal Events in Integrin Signaling

Upon binding to extracellular matrix (ECM) ligands, integrins aggregate on the plane of the plasma membrane and interact on the cytoplasmic side with elements of the cytoskeleton as well as signaling molecules. These events result in the organization of adhesive junctions, such as focal adhesions and hemidesmosomes, and the activation of signaling pathways that regulate gene expression ***(1,2)***.

Ligation of all β_1 and α_v-subunit containing integrins causes activation of the cytoplasmic tyrosine kinase focal adhesion kinase (FAK). This process requires the same segment of the integrin β subunit cytoplasmic domain which is known to interact with talin. Because talin binds to FAK, it is likely that the aggregation of integrins at the cell surface caused by ligand binding results in a correspondent oligomerization and thereby activation of FAK. Once activated, FAK undergoes autophosphorylation and combines with c-src or c-fyn. The FAK-src family kinase complex plays a role in regulating the assembly/disassembly of focal adhesions during cell migration and protecting cells from apoptotic death ***(3)***.

The results of recent studies have indicated that ligation of $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_6\beta_4$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$, causes recruitment and tyrosine phosphorylation of the adaptor protein Shc ***(4,5)***. Shc is a Src homology 2 (SH2)-phospho tyrosine binding (PTB) domain adapter that links various tyrosine phosphorylated signal transducers to Ras by recruiting the Grb2/mSOS complex to the

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plasma membrane. The recruitment of Shc to activated $\alpha_6\beta_4$ is likely to be mediated by the direct binding of Shc to tyrosine-phosphorylated sequences in the β_4 tail, because the SH2 and PTB domains of Shc can both bind directly to tyrosine phosphorylated β_4 ***(4)***. In contrast, the recruitment of Shc to β_1 and α_v integrins is indirect and mediated by the interaction of the integrin α subunit with caveolin-1 ***(5)***. Recent studies have indicated that caveolin-1, a protein implicated previously in the biogenesis of caveolae, links these integrins to the tyrosine kinase Fyn. Upon integrin-mediated activation, Fyn undergoes a conformational transition and recruits, via its SH3 domain, Shc ***(6)***. Shc then becomes phosphorylated on tyrosine and recruits the Grb2/mSOS complex (**Fig. 1A**). These events promote activation of the Ras-extracellular signal regulated kinase (ERK) pathway and progression through the G_1 phase of the cell cycle in response to soluble mitogens. In contrast, ligation of integrins that are not capable of recruiting Shc results in exit from the cell cycle and apoptotic death, even in presence of otherwise mitogenic concentrations of soluble growth factors ***(5–7)***. These observations suggest that FAK and Shc activate distinct intracellular signaling pathways in response to integrin ligation.

1.2. Outline of Experimental Procedures

In the following sections we describe methods to examine the recruitment and tyrosine phosphorylation of Shc and the association of Shc with Grb2 in response to integrin ligation. The recruitment of Shc to activated integrins can be detected by immunoprecipitating the integrins and probing the resulting samples by immunoblotting with anti-Shc antibodies. The tyrosine phosphory-

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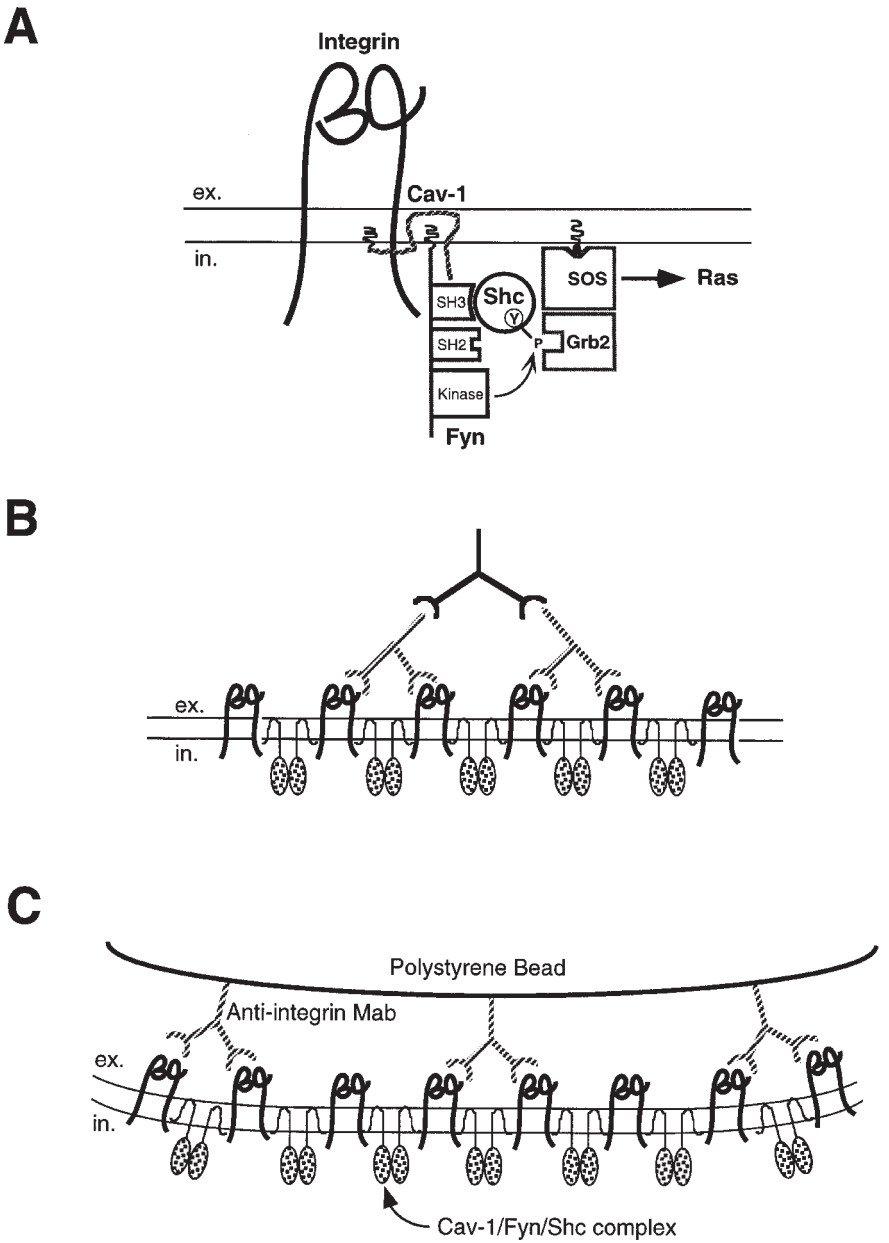
Fig. 1. *(continued on next page)* **(A)** Recruitment of Shc to activated β_1 and α_v integrins. The integrin α subunit transmembrane segment interacts with caveolin-1 which, in turn, interacts with Fyn. Integrin ligation results in activation of Fyn and exposure of its SH3 domain. The SH3 domain of Fyn recruits Shc and thereby allows the kinase domain to phoshorylate it at tyrosine 317. Upon phosphorylation, Shc tyrosine 317 binds to the Grb2/SOS complex causing activation of Ras ***(6)***. The biochemical mechanisms underlying the association of integrins with caveolin-1 and caveolin-1 with Fyn remain to be examined. Although all β_1 and α_v integrins combine with caveolin-1 and thereby, presumably, Fyn, only a subset of them is able to activate Fyn and recruit Shc. It is possible that only these integrins are associated with a required activator of Fyn or that the other ones combine with a suppressor of the kinase. **(B)** Formation of integrin clusters following incubation with anti-integrin monoclonal antibodies and rabbit anti-mouse IgGs. The clusters are small, but all activated integrins in them can be recovered by immunoprecipitation. **(C)** Formation of integrin clusters following incubation with polystyrene beads coated with anti-integrin monoclonal antibodies. The integrin clusters are larger, but a significant fraction of the



integrins ligated by the antibodies remain bound to the beads after solubilization and clarification of the extract. Given the ability of caveolin-1 to dimerize and oligomerize, it is likely that the integrin clusters formed beneath the polystyrene bead contain both antibody-ligated and unligated integrins. The latter subset of integrins can be efficiently recovered by immunoprecipitation.

lation of Shc and association of Shc with Grb2 are examined simultaneously by immunoprecipitating Shc and, after transfer to nitrocellulose, probing the top portion of the blot with anti-phosphotyrosine (anti-P-Tyr) and the bottom portion with anti-Grb2 antibodies. In addition, we describe protocols to examine the constitutive association of integrins with caveolin-1 and caveolin-1 with Fyn, the integrin-mediated activation of the fraction of Fyn associated with caveolin-1, and the integrin-dependent association of Fyn with Shc. Finally, we describe a glutathione 3-transferase (GST) pull-down assay for detecting the binding of tyrosine phosphorylated β4 subunit to the SH2 or PTB domain of Shc.

When examining integrin-mediated Shc signaling, it is important to choose an appropriate cell type. In most of our previous experiments we have employed untransformed (and often primary, nonimmortalized) fibroblasts, endothelial cells, and epithelial cells. There is reason to believe that integrin-mediated Shc signaling may be abnormal in neoplastic cells. In fact, caveolin-1 and at least two of the Shc-linked integrins, $\alpha_1\beta_1$ and $\alpha_5\beta_1$, are often downregulated in oncogenically transformed cells ***(8,9)***. Thus, unless one desires to compare integrin-mediated signaling in normal and neoplastic cells, it is better to examine normal, possibly primary cells.

Integrin-mediated signaling can be activated: by incubating cells in suspension with soluble anti-integrin antibodies followed by appropriate secondary antibodies (Fig. 1B); by incubating cells in suspension with polystyrene beads coated with anti-integrin antibodies (Fig. 1C); or by plating cells on dishes coated with ECM proteins or anti-integrin antibodies. Because many integrins bind to multiple ECM ligands and, conversely, the same ECM molecule is often recognized by several integrins, antibody-based methods of ligation have the advantage of allowing the examination of signaling events triggered by a specific integrin heterodimer ***(4–7,10)***. Several monoclonal antibodies to individual integrin subunits are now available commercially. In addition, the American Type Culture Collection (ATCC, Rockville, MD) distributes various hybridomas producing anti-integrin monoclonal antibodies (**Table 1**). Regardless of whether they bind to the ligand-binding pocket and inhibit adhesion or not, the majority of anti-integrin antibodies are able to cluster integrins at the cell surface. Because integrin aggregation is sufficient to stimulate signaling events such as the activation of FAK and the recruitment and tyrosine phosphorylation of Shc, most anti-integrin antibodies are suitable for studies on integrin signaling ***(4–7,10)***.

The activation of the fraction of Fyn associated with caveolin-1, the association of Fyn with Shc, the tyrosine phosphorylation of Shc, and the association of Shc with Grb2 are all events that can be examined easily after extracellular matrix ligand- or antibody-mediated ligation of integrins. In contrast, we have so far been unable to coimmunoprecipitate integrins with Shc upon plating of

Table 1
Anti-integrin and Control Antibodies

Antibody	Clone	Source
human α_1 integrin	TS 2/7	ATCC (Rockville, MD)
human α_2 integrin	P1E6	Gibco-BRL (Gaithersburg, MD)
human α_3 integrin	P1B5	Gibco-BRL
human α_5 integrin	P1D6	Gibco-BRL
mouse α_5 integrin	5H10-27	Pharmingen (San Diego, CA)
human α_6 integrin	FW-14-14-15	ATCC (Rockville, MD)
mouse α_6 integrin	GoH3	Immunotech (Westbrook, ME
human β_1 integrin	4B4	Coulter Inc. (Hialeah, FL)
human β_1 integrin	TS 2/16	ATCC (MD)
human β_2 integrin	P4H9	Gibco-BRL
human β_4 integrin	3E1	Gibco-BRL
human MHC class I	W6.32	Sigma (St. Louis, MO)

the cells on extracellular matrix ligand. The coimmunoprecipitation of β_1 and α_v integrins with Shc is by its very nature a complicated experiment because it requires the successful extraction and preservation of a quaternary complex, which includes the relevant integrin, caveolin-1, Fyn, and Shc. In addition, during physiological adhesion to the extracellular matrix integrins are ligated and thereby activated in an asynchronous manner. The association of integrins with caveolin-1 and caveolin-1 with Fyn are constitutive and quite easy to analyze.

2. Materials

2.1. Coimmunoprecipitation of Integrins with Caveolin-1 and Caveolin-1 with Fyn

1. Lysis buffer: 50 m*M* HEPES at pH 7.4, 150 m*M* NaCl, 1% Triton X-100 (Sigma Biochemical, St. Louis, MO) , 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10% glycerol (Gibco-BRL, Gaithersburgh, MD). It is convenient to store this buffer at 4°C because it has to be used cold. To avoid modification of extracted proteins by cellular proteases and phosphatases, appropriate inhibitors should be added before use.
2. Stock solutions of protease and phosphatase inhibitors:
   a. Phenylmethanesulfonyl fluoride (PMSF): Prepare 100 m*M* in isopropanol. Store at 4°C.
   b. Aprotinin: Prepare 1 mg/mL in double distilled water (DDW). Store in aliquots at –20°C.
   c. Leupeptin: Prepare 1 mg/mL in DDW and store in aliquots at –20°C.
   d. Pepstatin A: Prepare 1 mg/mL in methanol. Store in aliquots at –20°C.
   e. NaF: Prepare 0.5 *M* in DDW and store at 4°C.
   f. Na_3VO_4: Prepare 0.5 *M* in DDW and store at –20°C.
3. Complete lysis buffer: For 10 mL, add 100 µL of PMSF, aprotinin, leupeptin, and pepstatin, 40 µL of Na_3VO_4, 0.5 mL of NaF, and 44.6 mg of $Na_4P_2O_7$ to 9 mL

of lysis buffer. Complete lysis buffer contains 1 m*M* PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL of pepstatin A, 2 m*M* Na_3VO_4, 10 m*M* $Na_4P_2O_7$, and 25 m*M* NaF.

4. Mouse IgG agarose (Sigma) and Protein-G Sepharose (Pharmacia Biotechnology, Piscataway, NJ). Store at 4°C.
5. Rabbit IgG agarose (Sigma) and Protein-A Sepharose (Pharmacia). Store at 4°C.
6. Purified anti-integrin and control anti-MHC class I monoclonal antibodies can be obtained from the sources listed in **Table 1**. Store at –20°C.
7. Affinity-purified rabbit antibodies to a fusion protein comprising amino acid residues 1–97 of human caveolin-1 can be obtained from Transduction Laboratories (Lexington, KY; cat. no. C13630). These antibodies immunoprecipitate human, mouse, rat, dog, and chicken caveolin-1.
8. Control normal rabbit IgGs (Pierce, Rockford, IL).
9. Standard reagents for SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotting to nitrocellulose membranes.
10. Blocking buffer A: 5.0% BSA in 10 m*M* Tris-HCl, at pH 7.5, 100 m*M* NaCl at pH 7.4 (TBS). Readjust pH to 7.4 with NaOH after dissolving BSA.
11. Blotting wash buffer: 10 m*M* Tris-HCl, at pH 7.5, 100 m*M* sodium chloride, 0.1% Tween-20 (Sigma).
12. Affinity-purified goat antibodies to a synthetic peptide modeled after amino acid residues 28–48 of human Fyn (Fyn-3G) can be purchased from Santa Cruz Biotechnology (Santa Cruz, CA; cat. no. sc-16G). These antibodies immunoblot human, mouse, rat, and chicken c-Fyn.
13. Affinity purified antibodies to a synthetic peptide modeled after amino acid residues 2 to 20 of caveolin-1 (N-20) can be purchased from Santa Cruz Biotechnology (cat. no. 894). These antibodies blot human, mouse, and rat caveolin-1.
14. Horseradish peroxidase (HRP)-conjugated protein A and anti-goat IgGs (Amersham, Arlington Heights, IL). Store at 4°C.
15. Secondary reagent dilution buffer: 5.0% nonfat dry milk (Carnation) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (Sigma) at pH 7.4.
16. Standard reagents for enhanced chemiluminescence (ECL) (Pierce). Store at 4°C.

2.2. Integrin Ligation

1. The extracellular matrix (ECM) proteins fibronectin, collagen I, collagen IV, laminin 1, laminin 4, and vitronectin can be purchased from Gibco-BRL. The control polypeptide poly-L-lysine is available from Sigma. These proteins are dissolved in phosphate-buffered saline (PBS) and stored in aliquots at –80°C. Laminin 5 matrices are prepared according to the protocol described below (**step 1b** in **Subheading 3.2.3.**).
2. **Item 6** from **Subheading 2.1.**
3. Polystyrene beads, mean diameter approx 2.5 μm (Interfacial Dynamics Corporation, Portland, OR). Store at 4°C.
4. 100 m*M* methane-sulfonic acid ethyl ester (MES) at pH 5.5.
5. Affinity-purified rabbit anti-mouse IgGs (Amersham).

6. Dulbecco's modified Eagle's medium (without serum).
7. PBS without $CaCl_2$ and $MgCl_2$.
8. 0.5 *M* sodium orthovanadate: Dissolve in DDW and store at 4°C.
9. 0.1% BSA: dissolve in PBS and add 0.02% NaN_3 as preservative. Store at 4°C.
10. 10 m*M* EDTA: Dissolve in PBS and autoclave.

2.3. Coimmunoprecipitation of Integrins and Caveolin-1 with Shc

1. **Item 1** from **Subheading 2.1.** For the coimmunoprecipitation of $\alpha_6\beta_4$ with Shc, include 1 m*M* EDTA and eliminate 1 m*M* $CaCl_2$, which may activate calpain and induce proteolysis of the β_4 tail ***(11)***. To avoid modification of extracted proteins by proteases and phosphatases, appropriate inhibitors should be added to the lysis buffer before use as described in **Subheading 2.1.3.**
2. **Items 2**, **4**, **6**, and **9–11** from **Subheading 2.1.**
3. Purified monoclonal antibody to the N-terminal cytoplasmic tail of caveolin-1 (CO60) from Transduction Laboratories.
4. Affinity-purified rabbit anti-Shc antibodies (Upstate Biotechnology, Lake Placid, NY). Store at –20°C.
5. HRP-conjugated Protein A (Amersham). Store at 4°C.
6. **Items 14** and **15** from **Subheading 2.1.**

2.4. Kinase Assay on Caveolin-1-Associated Fyn

1. **Items 2**, **5**, and **7–9** from **Subheading 2.1.**
2. Modified RIPA buffer: 50 m*M* HEPES at pH 7.4, 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% sodium dodecyl sulphate (SDS) (Bio-Rad Laboratories, Hercules, CA), 10% glycerol (Gibco-BRL). Store at 4°C. Add protease and phosphatase inhibitors as described in **Subheading 2.1.3.**
3. Rabbit antibodies to a synthetic peptide modeled after Fyn amino acid residues 35–51 (Fyn-2) can be obtained from Upstate Biotechnology (cat. no. 06-133). These antibodies immunoprecipitate human, mouse, rat, and chicken c-Fyn.
4. Triton X-100 Wash (TXW) buffer: 25 m*M* HEPES at pH 7.4, 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1% Triton X-100 (Sigma), and 10% glycerol (Gibco-BRL).
5. Kinase wash buffer: 25 m*M* HEPES at pH 7.4, 100 m*M* NaCl, 10 m*M* $MgCl_2$. Store in 50-mL aliquots at –20°C.
6. Kinase assay buffer: 25 m*M* HEPES at pH 7.4, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 10 μ*M* p-nitrophenyl phosphate (PNPP) and 20 μ*M* cold ATP. Store in 1-mL aliquots at –20°C.
7. [γ-^{32}P]-ATP (>4500 Ci/mmol, ICN Pharmaceuticals, Costa Mesa, CA).

2.5. Coimmunoprecipitation of Fyn with Shc

1. **Items 1–4**, and **9–11** from **Subheading 2.1.**
2. Purified monoclonal antibody to amino acids 85–206 of human Fyn (MAb 15) can be purchased from Santa Cruz Biotechnology (cat. no. sc-434). Store at 4°C.

3. **Items 4** and **5** from **Subheading 2.3.**
4. **Items 15** and **16** from **Subheading 2.1.**

2.6. Coimmunoprecipitation of Tyrosine Phosphorylated Shc with Grb2

1. **Items 1–4**, **9**, and **10** from **Subheading 2.1.**
2. Monoclonal antibody to the SH2 domain of human Shc (PG-797) can be obtained from Santa Cruz Biotechnology (cat. no. sc-967).
3. Blocking buffer B: 1.0% BSA in TBS - 0.1% Tween-20 at pH 7.4.
4. HRP-conjugated recombinant PY20 anti-phosphotyrosine monoclonal antibody (RC-20) (Transduction Laboratories). Store at –20°C.
5. Affinity-purified rabbit anti-Grb2 antibodies (cat. no. sc-255) (Santa Cruz Biotechnology). Store at 4°C.
6. **Item 4** from **Subheading 2.3.**
7. **Items 15** and **16** from **Subheading 2.1.**

2.7. Gst Pull-Down Assay

1. **Items 1–3** and **9–11** from **Subheading 2.1.**
2. SDS buffer: 50 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl, 1% SDS. Store at room temperature (RT).
3. Glutathione Agarose beads (Sigma).
4. Rabbit anti-β_4 cytoplasmic peptide serum (Chemicon, Temecula, CA). Store at –20°C.
5. GST-fusion proteins encoding the SH2 or PTB domain of Shc are produced and purified according to established protocols ***(12)***.
6. **Item 4** from **Subheading 2.3.**
7. **Items 15** and **16** from **Subheading 2.1.**

3. Methods

Cells are propagated in culture by standard methods and deprived of growth factors when they reach 60–70% confluency. Starvation times depend on the cell type: primary mouse embryo fibroblasts (MEFs), human lung fibroblasts (WI-38), human umbilical vein endothelial cells (HUVECs), and human keratinocytes are starved for 36 h; Fisher rat thyroid (FRT) cells and human HaCat keratinocytes are also starved for 36 h; mouse NIH-3T3 fibroblasts for 24 h; and human embryonic kidney 293 cells for 20 h. It is important that the cells are not confluent at the end of starvation, because otherwise it may be difficult to obtain a single-cell suspension after EDTA detachment. The use of trypsin is not recommended, because some integrin dimers are at least partially sensitive to the enzyme, especially if used in combination with EDTA.

The expression of various integrin dimers at the cell surface can be examined by FACS analysis or immunoprecipitation of surface-labeled cells. Obviously, it is easier to examine Shc signaling after cross-linking with anti-β_1

integrin antibodies if the Shc-linked integrins $\alpha_1\beta_1$ and $\alpha_5\beta_1$ are expressed at levels higher than those of other β_1 integrins.

The association of integrins and caveolin-1 with Shc is examined after antibody-mediated cross-linking of integrins. Effective and simultaneous ligation of a large fraction of integrins can be obtained by incubating suspended cells with anti-integrin monoclonal antibodies followed by rabbit anti-mouse IgGs or with polystyrene beads coated with anti-integrins monoclonal antibodies. In the first case, the integrin clusters induced are smaller, but virtually all integrins ligated can be recovered by immunoprecipitation (**Fig. 1B**). In the second case, the integrin clusters obtained are much larger, but a fraction of the integrins ligated remain attached to the beads after cell lysis (**Fig. 1C**). In both cases, the integrin clusters generated contain both ligated and unligated integrins, because caveolin-1 is known to oligomerize ***(13)***.

3.1. Coimmunoprecipitation of Caveolin with Integrins and Caveolin with Fyn

We have used the following protocol to examine the constitutive association of β_1 integrins with caveolin-1 and of caveolin-1 with Fyn in normal human fibroblasts and endothelial cells. These cells possess good levels of caveolin-1 and Fyn. In addition, the monoclonal antibodies to the ectodomain of human integrins, which we have tested (*see* **Table 1**), do not interfere with the association of integrins with caveolin-1. As far as the association of caveolin-1 with Fyn, it is best to use the antibodies to Fyn here suggested because we have observed that certain other antibodies do not precipitate the complex, presumably because the corresponding epitope is masked.

1. Prepare two 15-cm diameter dishes of almost confluent cells for each coimmunoprecipitation. Place the dishes on ice, aspirate the medium, and add 1 mL of complete lysis buffer to each monolayer. After scraping cells and collecting lysates into Eppendorf tubes, incubate on ice for 30 min. Clarify the extracts by centrifugation at 10,000*g* for 30 min at 4°C and measure protein concentration by using a standard protein assay (Bio-Rad or Pierce). Use 4.0 mg of total proteins for each immunoprecipitation.
2. Incubate the lysates to be immunoprecipitated with monoclonal antibodies with 200 µL of a 50% slurry of mouse IgG agarose beads (to remove proteins that bind unspecifically to mouse IgGs and agarose). Incubate the lysates to be immunoprecipitated with rabbit polyclonal antibodies with 200 µL of a 50% slurry of rabbit IgG agarose beads (to remove proteins that bind unspecifically to rabbit IgGs and agarose). The beads should be washed three times with lysis buffer prior to being added to the lysate. After rotating the tubes for 1 h at 4°C, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C and transfer the supernatants to new tubes.
3. To coimmunoprecipitate integrins and caveolin-1, add 10 µg of purified monoclonal anti-integrin or control anti-MHC antibody and 100 µL of a 50% slurry of

Protein-G Sepharose beads. To coimmunoprecipitate caveolin-1 and Fyn, add 5 μg of affinity-purified anti-caveolin-1 rabbit antibodies C13630 or control normal rabbit IgG and 100 μL of a 50% slurry of Protein-A Sepharose beads. Rotate the tubes for 3 h at 4°C. The beads should be washed three times with lysis buffer prior to being added to the lysates. At the end of incubation, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C.

4. Wash the beads carrying the immunocomplexes five times with cold lysis buffer by centrifugating the tubes at 10,000*g* for 2 min at 4°C.
5. Separate the samples on a 10% SDS-PAGE gel. An aliquot of lysate containing 50 μg of total proteins can be run as a positive control for immunoblotting. Transfer to nitrocellulose following standard protocols.
6. To saturate the unoccupied protein-binding sites, incubate the nitrocellulose membrane in blocking buffer A for 1 h at RT and then rinse briefly twice in blotting wash buffer.
7. Incubate the blot containing the integrin immunoprecipitates and relative negative and positive controls with anti-caveolin-1 antibody N—20, and the blot containing the caveolin-1 immunoprecipitates and relative negative and positive controls with anti-Fyn antibody Fyn-3G (both at 0.5 μg/mL in TBS at pH 7.4 containing 3% BSA) for 2 h at RT.
8. Wash the membranes with blotting wash buffer 3 times for 10 min at RT.
9. Incubate the membranes with HRP-conjugated Protein-A diluted 1:3,000 in secondary reagent dilution buffer for 40 min at RT.
10. Repeat **step 8**.
11. Remove excess wash buffer and subject to ECL following standard protocols.
12. Expose to X-ray film for 1 min. Adjust exposure according to the result. The intensity of ECL signal decreases rapidly and no signal can be detected after 30 min.

3.2. Integrin Ligation

3.2.1. Crosslinking of Integrins with Soluble Antibodies

1. Detach the cells by incubation in PBS containing 10 m*M* EDTA (some integrins may be cleaved by trypsin, especially in presence of EDTA), wash them with DMEM and resuspend them at 2×10^7/150 μL in DMEM.
2. Hold the cells in suspension at RT for 30 min. This step is required to fully deactivate integrin signaling.
3. Add 20 μg of anti-integrin monoclonal antibody or control anti-MHC class I monoclonal antibody and incubate on ice for 40 min with occasional mixing.
4. Wash twice with cold DMEM by centrifugation at 1,000*g* for 5 min to remove the unbound antibodies.
5. Resuspend the cell pellet in 150 μL of DMEM containing 10 μg of affinity-purified rabbit anti-mouse IgGs.
6. Incubate the cell suspension for desired periods of time (e.g. 2, 5, 10, 20, and 30 min) at 37°C. Mix gently every few minutes.
7. At the end of the incubation, add 1 mL of cold PBS containing 0.5 m*M* Na_3VO_4 and centrifuge at 1,000*g* for 5 min at 4°C.
8. Lyse the cell pellet as required by the specific experiment.

3.2.2. Ligation of Integrins with Antibody-Coated Polystyrene Beads

1. Coating of the beads:
   a. The polystyrene beads are usually provided as a 8–10% slurry. Pipet the volume needed to obtain 50 μL packed beads per sample.
   b. Wash the beads with 1 mL of MES buffer at pH 5.5 three times by centrifugation at 10,000*g* for 2 min at RT.
   c. Resuspend the beads in 300 μL of MES buffer at pH 5.5 and add 20 μg of purified anti-integrin monoclonal antibody. Control beads are coated with the same amount of anti-MHC class I monoclonal antibody.
   d. Rock the tubes for 1 h at room temperature (RT).
   e. At the end of incubation spin the tubes at 10,000*g* for 2 min at RT and remove the supernatants.
   f. Wash the beads four times with 1 mL of PBS (without Ca^{2+} and Mg^{2+}) by centrifugation at 10,000*g* for 2 min at RT.
   g. Resuspend the beads in 150 μL of DMEM.
2. Follow **steps 1–3** from **Subheading 3.2.1.**
3. Gently mix 150 μL of cell suspension with 150 μL of anti-integrin antibody-coated beads in an Eppendorf tube.
4. Follow **steps 6–8** from **Subheading 3.2.1.**

3.2.3. Adhesion to Anti-integrin Antibody- or ECM-Coated Dishes

1. Coating of the plates:
   a. For coating with ECM proteins, incubate 10-cm diameter plates with 2 mL of PBS containing 10 μg/mL purified ECM protein or control poly-L-lysine for 2 h at RT (optimal adhesion is obtained generally with 2.5–20 μg/mL of ECM protein depending on the matrix molecule and cell type employed). Wash twice with PBS. Saturate with 0.1% BSA (heat-inactivated) in PBS for 2 h at RT. Wash three times with DMEM and maintain wet until use.
   b. Laminin 5 matrices are prepared from the murine cell line RAC-11P/SD ***(14)***. Briefly, the cells are grown to confluency, washed three times with PBS, and incubated overnight at 4°C in PBS containing 20 m*M* EDTA, 10 μg/mL leupeptin, 1 m*M* phenylmethanesulfonyl fluoride (PMSF), and 10 μg/mL soybean trypsin inhibitor. The cells are then removed from the underlying matrix by pipetting (they will detach as a single continuous layer and leave behind a matrix which consists predominantly of laminin 5). The plates are finally saturated with 0.1% BSA in PBS and either used immediately or stored at –20°C in PBS containing 10% DMSO.
   c. For antibody coating, incubate the plates with PBS containing 10 μg/mL affinity-purified rabbit anti-mouse IgGs for 2 h at RT. Wash twice with PBS. Saturate with 0.1% BSA in PBS for 2 h at RT. Incubate with PBS containing 20 μg/mL monoclonal anti-integrin antibody in PBS for 2 h at RT. Wash three times with DMEM and leave in DMEM until use.
2. Follow **steps 1** and **2** from **Subheading 3.2.1.**, but resuspend 10^7 cells in 6 mL of DMEM.

3. Plate the cells on ECM- or anti-integrin antibody-coated dishes at 37°C in a 5% CO_2 incubator for desired periods of time (e.g. 5, 15, 30, 45, 60, 90, and 120 min). As a control, the cells are plated on poly-L-lysine or anti-MHC class I antibody-coated dishes, respectively. Plating 10^7 cells per 10-cm diameter plate works well for fibroblasts, but the number of other cells plated per dish should be adjusted so that they barely touch each other during spreading.
4. At the end of each time point, gently wash the dishes with cold PBS to remove unattached cells and lyse the attached cells as required by the specific experiment.

3.3. Coimmunoprecipitation of Integrins or Caveolin-1 with Shc

1. After ligating integrins as described in **Subheadings 3.2.1.** or **3.2.2.** for various periods of time, add 1 mL of complete lysis buffer to each sample and incubate on ice for 30 min. Clarify the extracts by centrifugation at 10,000*g* for 30 min at 4°C and measure protein concentration by using a standard protein assay (Bio-Rad or Pierce). Use 4.0 mg of total proteins for each immunoprecipitation.
2. Incubate the lysates with 200 µL of 50% mouse IgG agarose beads to remove proteins that bind unspecifically to mouse IgGs and agarose. The beads should be washed three times with lysis buffer prior to being added to the lysates. After rotating the tubes for 1 h at 4°C, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C, and transfer the supernatants to new tubes.
3. Add 10 µg of purified monoclonal anti-integrin, anti-caveolin-1, or control anti-MHC antibody and 100 µL of 50% Protein-G Sepharose beads and rotate the tubes for 3 h at 4°C. The beads should be washed three times with lysis buffer prior to being added to the lysate. At the end of incubation, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C.
4. Follow **steps 4–6** from **Subheading 3.1.**
5. Incubate the blot with affinity-purified rabbit anti-Shc antibodies (at 0.5–1 µg/mL in TBS at pH 7.4 containing 3% BSA) for 2 h at RT.
6. Follow **steps 8–12** from **Subheading 3.1.**

3.4. Kinase Assay on Caveolin-1-Associated Fyn

1. After ligating integrins for various periods of time, as described in **Subheadings 3.2.1.**, **3.2.2.**, or **3.2.3.**, add 1 mL of modified RIPA buffer with protease and phosphatase inhibitors per sample and incubate on ice for 30 min. After sonication, clarify the extracts by centrifugation at 10,000*g* for 30 min at 4°C. Measure protein concentration by using a standard protein assay (Bio-Rad or Pierce) and use 2 mg of total proteins for each immunoprecipitation.
2. Incubate the lysates with 200 µL of a 50% slurry of rabbit IgG agarose beads (to remove proteins that bind unspecifically to rabbit IgGs or agarose). The beads should be washed three times with lysis buffer prior to being added to the lysate. After rotating the tubes for 1 h at 4°C, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C and transfer the supernatants to new tubes.
3. Add 5 µg of affinity-purified anti-Fyn or anti-caveolin rabbit antibodies and 40 µL of 50% Sepharose Protein-A beads (avoid using more beads to reduce background) and rotate for 2 h at 4°C.

4. Wash for 2 min at 6000*g*, twice with TXW buffer at 4°C and twice with kinase wash buffer at RT.
5. To initiate the reaction, add to the pelletted beads 30 μL of kinase assay buffer containing 20 μCi of [γ^{32}-P]ATP(>4500 μCi/mmol).
6. Shake the tubes in a mixer for 15 min at RT.
7. Pellet the beads by centrifugation at 1000*g* for 1 min and dispose appropriately of the radioactive supernatant (this step will significantly reduce the amount of free radioactive ATP loaded on the gel).
8. Resolve the immunocomplexes on a 10% SDS-PAGE gel.
9. Fix the gel in 7% acetic acid and 15% methanol for 1 h.
10. Incubate the gel in 1 *M* KOH at 60°C for 2 h to remove the bulk of radioactive phosphate transferred to serine and threonine residues (the phosphate bound to tyrosine residues is largely resistant to alkali).
11. Wash the gel with water six times for 10 min.
12. Repeat **step 9**.
13. Dry gel at 80°C for 2 h.
14. Expose it to X-ray film for 15 min at –80°C. Develop the film and adjust the exposure time to obtain desired intensity of the bands.

3.5. Coimmunoprecipitation of Fyn with Shc Following Integrin Ligation

1. After ligating integrins as described in **Subheadings 3.2.1.**, **3.2.2.**, or **3.2.3.** for various periods of time, add 1 mL of complete lysis buffer to each sample and incubate on ice for 30 min. After sonication, clarify the extracts by centrifugation at 10,000*g* for 30 min at 4°C. Measure protein concentration by using a standard protein assay (Bio-Rad or Pierce) and use 4 mg of total proteins for each immunoprecipitation.
2. Follow **step 2** from **Subheading 3.3.**
3. Add 5–10 μg of anti-Fyn (Mab 15) or control anti-MHC (W6.32) monoclonal antibody and 100 μL of 50% Protein-G Sepharose beads and rotate the tubes for 3 h at 4°C. The beads should be washed three times with lysis buffer prior to being added to the lysate. At the end of incubation, pellet the beads by centrifugation at 10,000*g* for 5 min at 4°C.
4. Follow **steps 4–6** from **Subheading 3.1.**
5. Follow **steps 5** and **6** from **Subheading 3.3.**

3.6. Tyrosine Phosphorylation of Shc and Association of Shc with Grb2

1. Follow **step 1** from **Subheading 3.5.**, but use 2 mg of total proteins for each immunoprecipitation.
2. Follow **step 2** from **Subheading 3.3.**
3. Add 5 μg of anti-Shc (PG-797) or control anti-MHC (W6.32) monoclonal antibody and 100 μL of 50% Sepharose Protein-G beads and rotate for 2 h at 4°C.
4. Follow **steps 4–6** from **Subheading 3.1.** Cut the blot at the level of 35 kDa molecular mass. The top portion is probed with anti-P-Tyr and the bottom with anti-Grb2 antibodies.

5. Incubate the top portion of the blot in blocking buffer B, and the bottom in blocking buffer A for 1 h at RT. Rinse twice with blotting wash buffer.
6. Incubate the top portion of the blot with 50–100 ng/mL HRP-conjugated recombinant anti-P-Tyr monoclonal antibody RC-20, and the bottom with 0.5 μg/mL affinity-purified rabbit anti-Grb2 antibodies, both diluted in PBS-3% BSA for 1 h at RT.
7. Follow **steps 8**, **11**, and **12** from **Subheading 3.1.** for the top portion of the blot. Follow **steps 8** through **12** from **Subheading 3.1.** for the bottom portion of the blot.

3.7. Association of Shc GST-Fusion Proteins with Tyrosine Phosphorylated β_4

1. Ligate $\alpha_6\beta_4$ by the method described in **Subheadings 3.2.1.** or **3.2.2.** and lyse the cells in SDS buffer. The lysate is then heated to 80°C for 5 min, sonicated, clarified at 10,000*g* for 10 min, and diluted with 9 vol of complete lysis buffer. Use 5 mg of total proteins for each pull-down assay.
2. Incubate gluthatione-agarose beads carrying 25–50 μg of the GST-fusion protein containing the PTB or SH2 domain of Shc with the denatured lysate for 2 h at 4°C. Control samples are incubated with glutathione beads carrying GST only.
3. Wash the beads 5 times with lysis buffer by centrifugation at 10,000*g* for 2 min at 4°C.
4. Separate the samples on a 7.5% SDS-PAGE gel and transfer to nitrocellulose according to standard protocols.
5. Follow **step 6** from **Subheading 3.1.**
6. Incubate the blot with rabbit anti-β_4 cytoplasmic peptide serum (1:500 in TBS at pH 7.4 containing 3% BSA) for 2 h at RT.
7. Follow **steps 8–12** from **Subheading 3.1.**

4. Notes

1. The three isoforms of Shc migrate at 46-, 52-, and 66-kDa molecular mass. Whereas p46 and p52 are products of two different start sites, p66 originates from a distinct mRNA generated by alternative splicing. Hemopoietic cells generally do not express p66. The major isoform recruited to activated integrins and thereby phosphorylated on tyrosine is p52.
2. The HRP-conjugated recombinant PY20 monoclonal antibody RC-20 can detect very small quantities of phosphotyrosine and outperforms all the other anti-P-Tyr reagents that we have tried. Because it is directly conjugated to HRP, one does not need to use secondary antibodies that often crossreact in blotting with the heavy or light chains of immunoglobulins used during the immunoprecipitation. This reagent, however, should not be reused as it is quite unstable.

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4

Assays for Kinase Activity

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1. Introduction

1.1. Outline of the Major Signaling Pathways Activated by Integrins

Cell adhesion to the extracellular matrix causes a number of intracellular changes resembling those induced by receptor protein tyrosine kinases, including increased tyrosine phosphorylation of a number of cellular proteins, elevation of phosphoinositide 3-OH kinase (PI3-K) products, and increase in intracellular calcium concentrations ***(1)***. Although integrins do not possess any intrinsic enzymatic activity, they influence the activity of cytoplasmic kinases either directly or indirectly. All β_1- and α_v-containing integrins can activate the tyrosine kinase focal adhesion kinase (FAK) by an incompletely understood mechanism involving the cytoplasmic domain of the β subunit. Upon activation FAK undergoes autophosphorylation at Tyr 397 and combines with the Src Homology 2 (SH2) domain of a src-family kinase, such as c-src or c-fyn. The FAK-bound src family kinase in turn phosphorylates the C-terminal domain of FAK promoting the recruitment of additional signaling molecules, such as Grb2 ***(2)***. Autophosphorylated FAK has also been shown to be able to associate with PI3-K ***(3)***.

Although there is evidence suggesting that FAK can, upon overexpression, regulate the Ras-extracellular signal-regulated kinase (ERK) signaling pathway via its interaction with Grb2, the activation of FAK is not sufficient to promote Ras-ERK signaling in normal cells ***(4)***. Recent studies have revealed that certain integrins, including $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_6\beta_4$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$, are coupled to the Ras-ERK pathway by an additional mechanism, involving the adaptor protein Shc ***(5–7)***. Ligation of these integrins sequentially induces recruitment and tyrosine phosphorylation of Shc and association

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of Shc with Grb2. Because only those integrins that recruit Shc are able to induce activation of ERK and a dominant-negative form of Shc suppresses this event, Shc appears to play a crucial role in the activation of ERK in response to cell adhesion.

Integrins can also activate the Rac-Jun N-terminal kinase (JNK) signaling pathway *(7,8)* and their ability to activate both ERK and JNK may be important for expression of immediate-early genes and progression through the G_1 phase of the cell cycle *(5,7)*. Finally, there is also evidence suggesting that integrins regulate the activity of a novel serine/threonine-specific kinase, integrin-linked kinase (ILK) *(9)*, promote the activation and translocation to the nucleus of the Abl tyrosine kinase *(10)*, and regulate $p70^{S6}$ kinase, an enzyme that appears to participate in the activation of G1 cyclin-dependent kinases *(11)*.

In this chapter we describe methods to assay the activity of Src-family kinases, FAK, PI3-K, ERK, JNK, and $p70^{S6}$ kinase. The various kinases are isolated by immunoprecipitation or other affinity methods and incubated in vitro in a buffer containing radioactive [γ-^{32}P]-ATP and, generally, an exogenous substrate. Transfer of the radioactive γ-phosphate is monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or thin layer chromatography (TLC).

1.2. Outline of the Assays

Src family kinases are immunoprecipitated from cell lysates using standard immunoprecipitation protocols and subjected to an in vitro autophosphorylation assay. Briefly, if a rabbit antibody is to be used for immunoprecipitation, the lysates are preabsorbed with normal Rabbit IgG agarose beads to remove proteins that bind unspecifically to rabbit IgGs or agarose. Conversely, for immunoprecipitation with monoclonal antibodies, the lysates are preabsorbed with normal Mouse IgG Agarose beads to remove proteins that bind unspecifically to mouse IgGs or agarose. The lysates are then incubated with specific rabbit polyclonal or mouse monoclonal antibodies and the resulting immune complexes are captured with Protein-A or Protein-G Sepharose beads, respectively. The beads carrying the immunocomplexes are then spun down, washed, and incubated with radioactive [γ-^{32}P]-ATP at 37°C in presence of manganese and/or magnesium ions. The samples are finally separated by SDS-PAGE. The gel is fixed, dried, and subjected to autoradiography.

FAK is immunoprecipitated by a standard protocol and incubated with the exogenous synthetic substrate poly(Glu-Tyr) (4:1) in presence of radioactive [γ-^{32}P]-ATP. The reaction is carried out at 30°C in the presence of manganese ions. Radioactive poly(Glu-Tyr) is separated by SDS-PAGE and visualized by autoradiography.

For PI3-K assay, the enzyme is generally isolated by immunoprecipitation. The immobilized immune complexes are incubated with radioactive [γ-^{32}P]-ATP and phosphatidyl inositol (PI) at room temperature (RT) in presence of magnesium ions. The products of the reaction are separated by TLC.

p70^{S6} kinase is isolated by immunoprecipitation and incubated in presence of radioactive [γ-^{32}P]-ATP and the ribosomal S40 subunit at 30°C. The sample is separated by SDS-PAGE and the phosphorylated ribosomal S6 protein visualized by autoradiography.

The in vitro kinase assay for ERK is similar to those described above. The most widely used in vitro substrate is myelin basic protein. After the reaction, the phosphorylated substrate is separated by SDS-PAGE and visualized by autoradiography.

The technique most widely employed to measure JNK activity relies on the ability of the enzyme to bind with high affinity to the N-terminal domain of its substrate c-Jun. A GST fusion protein comprising the N-terminal domain of c-Jun (amino acid residues 1–223), including the docking site for JNK and the two major phosphorylation sites (Ser 63 and Ser 73), is used both as a bait to isolate the enzyme from cell lysates and as a substrate for the in vitro reaction ***(12,13)***. Briefly, an expression vector encoding the GST-Jun fusion protein is transformed into XL1-Blue or BL-21 *Escherichia coli*. The bacteria are grown in appropriate medium and, after induction with isopropyl-β-D-thiogalactopyranoside (IPTG), lysed. The fusion protein is captured onto glutathione-agarose beads using established methods ***(14)***, and the agarose beads carrying the fusion protein are then used to precipitate JNK from cell lysates. After washing, the immobilized complex is incubated with radioactive [γ-^{32}P]-ATP at 30°C in the presence of magnesium ions. The phosphorylated fusion protein is separated by SDS-PAGE and visualized by autoradiography.

2. Materials

2.1. Src and Fyn Kinase Assay

1. Lysis buffer: 50 m*M* HEPES at pH 7.4, 150 m*M* NaCl, 1% Triton X-100 (Sigma Biochemical Co., St. Louis, MO), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Bio-Rad Laboratories, Hercules, CA), 10% glycerol (Gibco-BRL, Gaithersburgh, MD), 1.5 m*M* $MgCl_2$, 1 m*M* EGTA. This buffer can be stored at room temperature, but it is convenient to store it at 4°C because it has to be used cold. To avoid modification of extracted proteins by proteases and phosphatases, appropriate inhibitors should be added to the lysis buffer before use.
2. Stock solutions of protease and phosphatase inhibitors:
   a. Phenylmethanesulfonyl fluoride (PMSF): Prepare 100 m*M* in isopropanol. Store at 4°C.
   b. Aprotinin: Prepare 1 mg/mL in double-distilled water (DDW). Store in aliquots at –20°C.

   c. Leupeptin: Prepare 1 mg/mL of leupeptin in DDW and store in aliquots at –20°C.
   d. Pepstatin A: Prepare 1 mg/mL in methanol. Store in aliquots at –20°C.
   e. NaF: Prepare 0.5 *M* solution in DDW and store at 4°C.
   f. Na_3VO_4: Prepare 0.5 *M* solution in DDW and store at –20°C.
3. Complete lysis buffer. For 10 mL, add 100 µL of PMSF, aprotinin, leupeptin, and pepstatin, 20 µL of Na_3VO_4, 2 mL of NaF, and 44.6 mg of $Na_4P_2O_7$ to 7.5 mL of lysis buffer. Complete lysis buffer contains 1 m*M* PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, 1 m*M* Na_3VO_4, 10 m*M* $Na_4P_2O_7$, and 100 m*M* NaF.
4. Wash buffer: Prepare as lysis buffer without either SDS or deoxycholate.
5. HNTG buffer: 50 m*M* HEPES at pH 7.4, 150 m*M* NaCl, 0.1% Triton X-100, and 10% glycerol. Store at 4°C.
6. Kinase buffer: 20 m*M* HEPES at pH 7.4, 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, 150 m*M* NaCl. Store in aliquots at –20°C.
7. Affinity-purified rabbit antibodies to c-src (N-16) or c-fyn (FYN3) (Santa Cruz Biotechnology, Santa Cruz, CA). Store at 4°C.
8. PBS without calcium and magnesium.
9. Bradford protein assay reagent (Bio-Rad).
10. Rabbit IgG Agarose (Sigma).
11. Protein-A Sepharose (Pharmacia).
12. [γ-^{32}P]-ATP (> 4500 Ci/mmol, ICN Biomedicals, Costa Mesa, CA).
13. Standard solutions for SDS-PAGE.
14. Gel fixative: 7% acetic acid, 3% glycerol, 20% methanol. Store at RT.
15. Mixer: Eppendorf model 5432 is suitable for this and the following assays.

2.2. FAK Kinase Assay

1. Lysis buffer. 50 m*M* Tris-HCl at pH 7.5, 150 m*M* NaCl, 1% NP-40 (Sigma). This buffer can be stored at room temperature, but it is convenient to store it at 4°C because it has to be used cold.
2. To avoid modification of extracted proteins by proteases and phosphatases, add protease and phosphatase inhibitors to lysis buffer as described in **items 2** and **3** from **Subheading 2.1.**
3. Kinase buffer: 50 m*M* Tris-HCl at pH 7.4, 10 m*M* $MnCl_2$. Store in aliquots at –20°C.
4. Mouse IgG Agarose (Sigma).
5. Protein-G Sepharose (Pharmacia).
6. Purified anti-FAK Mab 2A7 (Upstate Biotechnology, Lake Placid, NY). Store at 4°C.
7. Poly(Glu-Tyr) (4:1) (Sigma). Store at –20°C.
8. **Items 8** and **9** and **12–15** from **Subheading 2.1.**

2.3. PI3-K Assay

1. Lysis buffer: PBS, 1% NP-40. Add protease and phosphatase inhibitors to lysis buffer as described in **items 2** and **3** from **Subheading 2.1.** Store at 4°C.
2. Wash buffers:
   a. Buffer A. 0.5 *M* LiCl, 0.1 *M* Tris-HCl at pH 7.6. Keep at 4°C.

   b. Buffer B. 10 m*M* Tris-HCl at pH 7.6, 100 m*M* NaCl, 1 m*M* EDTA at pH 8.0. Keep at 4°C.
   c. Buffer C. 20 m*M* HEPES at pH 7.5, 50 m*M* NaCl, 5 m*M* EDTA at pH 8.0, 0.03% NP-40. For 10 mL, add 100 µL of aprotinin and PMSF, 20 µL of Na_3VO_4, and 79.6 mg of $Na_4O_7P_2$ to 9.8 mL of Buffer C. Rotate for 1 h at 4°C. Complete Buffer C contains 30 m*M* $Na_4O_7P_2$, 200 µ*M* Na_3VO_4, 10 µg/mL aprotinin, and 1 m*M* PMSF.
3. PI from bovine liver (Avanti Polar Lipids, Alabaster, AL). Prepare 10 mg/mL in DDW, aliquot, blow N_2 gas in the tubes before closing, and freeze at –20°C.
4. Rabbit IgG Agarose (Sigma).
5. Protein A Sepharose (Pharmacia).
6. Affinity-purified rabbit antibodies to the p85α subunit of PI3-K (Z-8) (Santa Cruz Biotechnology). Store at 4°C.
7. ATP, disodium salt (Sigma). Make 10 m*M* stock in DDW and store it in aliquots at –20°C.
8. [γ-^{32}P]-ATP (> 4500 Ci/mmol, ICN).
9. Adenosine, hemisulfate salt (Sigma). Make 10 m*M* stock in DDW and store it in aliquots at –20°C.
10. Kinase buffer: 20 m*M* Tris-HCl at pH 7.6, 75 m*M* NaCl, 10 m*M* $MgCl_2$, 0.2 mg/mL PI, 10 µ*M* ATP, 100 µ*M* Adenosine. Store at –20°C.
11. 1 *N* HCl.
12. Extraction buffer: 1:1 chloroform:methanol.
13. Chromatography buffer: 50% chloroform, 39% methanol, 11% 4 *M* NH_4OH.
14. Whatman paper 3MM (Whatman International, Maidstone, England).
15. 30% Ammonium hydroxide.
16. PI-4P standard (Sigma): Disolve 1 mg of PI-4P in 0.5 mL of chloroform, 0.5 mL of methanol, and 2.5 µL of 1 *N* HCl.
17. Silica gel 60 TLC plates (VWR Scientific, Rochester, NY).
18. Iodine (Sigma).

2.4. p70^{S6} Kinase Assay

1. Lysis buffer: 50 m*M* Tris-HCl at pH 7.5, 135 m*M* NaCl, 1% NP-40, 2 m*M* EDTA, 10 m*M* $MgCl_2$. Store at 4°C.
2. Stock solutions:
   a. β-glycerophosphate: Prepare 1 *M* solution in DDW and keep at 4°C.
   b. Dithiothreitol (DTT): Prepare 1 *M* solution in DDW and store in aliquots at –20°C.
   c. Paranitrophenyl phosphate (PNPP): Prepare 250 m*M* solution in DDW and store in aliquots at –20°C.
   d. **Items 2** from **Subheading 2.1.**
3. Complete lysis buffer: For 10 mL, add 100 µL of PMSF, aprotinin, and leupeptin, 20 µL of Na_3VO_4, 250 µL of β-glycerophosphate, 20 µL of DTT, and 400 µL of NaF to 9 mL of lysis buffer. The complete lysis buffer contains 0.1 m*M* PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 m*M* Na_3VO_4, 25 m*M* β-glycerophosphate, 2 m*M* DTT, and 20 m*M* NaF.

4. Affinity-purified rabbit antibodies to $p70^{S6}$ kinase (C18) (Santa Cruz Biotechnology). Store at 4°C.
5. Kinase assay buffer: 50 m*M* MOPS at pH 7.0, 10 m*M* $MgCl_2$. Store at –20°C.
6. Complete kinase assay buffer. For 5 mL, add 10 µL of DTT and 400 µL of PNPP to 4.6 mL of kinase assay buffer. Complete buffer contains 1 m*M* DTT and 10 m*M* PNPP.
7. 40S ribosomal subunit. Prepare according to published procedure ***(15)***.
8. **Items 8–15** from **Subheading 2.1.**

2.5. ERK Kinase Assay

1. **Items 1** and **2** from **Subheading 2.2.**
2. Kinase buffer: 50 m*M* Tris-HCl at pH 7.4, 10 m*M* $MgCl_2$. Store in aliquots at –20°C.
3. Affinity-purified rabbit anti-ERK antibodies (C14) (Santa Cruz Biotechnology). Store at 4°C.
4. Myelin basic protein (Sigma). Dissolve 4 mg/mL in DDW. To avoid multiple cycles of thawing and freezing store in aliquots at –20°C.
5. **Items 8–15** from **Subheading 2.1.**

2.6. JNK Kinase Assay

1. Lysis buffer: 25 m*M* HEPES at pH 7.7, 300 m*M* NaCl, 0.1% Triton, 0.2 m*M* EDTA, 1.5 m*M* $MgCl_2$. Store at 4°C. Protease and phosphatases are added to the buffer shortly before use.
2. **Items 2** from **Subheading 2.4.**
3. Complete lysis buffer: For 10 mL, add 100 µL of PMSF, aprotinin, leupeptin, and pepstatin A, 20 µL of Na_3VO_4, 200 µL of β-glycerophosphate, and 5 µL of DTT to 9 mL of lysis buffer. Complete lysis buffer therefore contains 1 m*M* PMSF, 10 µg/mL aprotinin, leupeptin and pepstatin A, 20 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4, and 0.5 m*M* DTT.
4. Wash buffer: 20 m*M* HEPES at pH 7.5, 50 m*M* NaCl, 0.1 m*M* EDTA, 2.5 m*M* $MgCl_2$, 0.05% Triton X-100. Store at 4°C.
5. Kinase buffer: 20 m*M* HEPES at pH 7.5, 20 m*M* β-glycerophosphate, 10 m*M* PNPP, 10 m*M* $MgCl_2$, 10 m*M* DTT. To obtain 10 mL of kinase buffer, add 200 µL of 1 *M* HEPES at pH 7.5, 100 µL of 1 *M* $MgCl_2$, 200 µL of β-glycerophosphate, 100 µL of DTT, and 400 µL of PNPP to 9 mL of DDW. Prepare it immediately before use and keep it on ice.
6. GST-cJun(1–223). Prepare according to published procedure ***(13,14)***.
7. Glutathione-agarose beads (Sigma).
8. **Items 8**, **9**, and **12–15** from **Subheading 2.1.**

3. Methods

3.1. Src and Fyn Kinase Assay

1. Prepare complete lysis buffer and keep it on ice. It is sufficient to use 1 mL to lyse 10^7 cells.

2. Ligate integrins at the cell surface by using one of the methods described in Chapter 3.
3. Add lysis buffer and extract on ice for 30 min.
4. Clarify lysate at 10,000*g* for 10 min at 4°C.
5. Transfer supernatant to a fresh microcentrifuge tube.
6. Determine protein concentration using Bradford method.
7. Wash Protein-A Sepharose and rabbit IgG agarose three times with lysis buffer. It is sufficient to use 80 μL of rabbit IgG agarose and 40 μL of Protein-A Sepharose packed beads per sample.
8. Pipet the amount of lysate corresponding to 500 μg of protein into a fresh tube. Use lysis buffer to bring the volume to 500 μL.
9. Add 160 μL of 50% rabbit IgG agarose slurry and incubate for 1 h at 4°C.
10. Spin down the beads and transfer supernatant to a fresh tube.
11. Add 2.5 μg of anti-src or anti-fyn antibodies and 40 μL of 50% Protein-A Sepharose slurry per sample.
12. Rotate the tubes for 3 h at 4°C.
13. Collect beads by centrifugation at 10,000*g* for 2 min at 4°C.
14. Wash the beads once with wash buffer by brief high-speed centrifugation as above.
15. Wash twice with HNTG buffer as above.
16. Wash twice with kinase buffer as above.
17. Add 30 μL of kinase buffer containing 10 μCi of [γ-^{32}P]-ATP to each tube (*see* **Note 1**).
18. Place tubes into mixer and place it at 30°C for 20 min.
19. Terminate the kinase reaction by adding 70 μL of sample buffer into each tube.
20. Boil samples for 5 min.
21. Resolve samples in a 10% polyacrylamide gel. The molecular mass of Src and Fyn is 60 and 58 kDa, respectively (*see* **Note 2**).
22. Incubate gel in fixing solution for 1 h (*see* **Note 3**).
23. Dry gel at 80°C for 2 h.
24. Expose it to X-ray film for 15 min at –80°C. Develop the film and adjust the exposure time to obtain desired intensity of the bands.

3.2. FAK Kinase Assay

1. **Steps 1–8** from **Subheading 3.1.**
2. Add 160 μL of 50% mouse IgG agarose slurry and incubate for 1 h at 4°C.
3. **Step 10** from **Subheading 3.1.**
4. Add 2.5 μg of monoclonal anti-FAK antibody (2A7) and 80 μL of 50% Protein-G Sepharose slurry per sample.
5. **Steps 12** and **13** from **Subheading 3.1.**
6. Wash three times with lysis buffer.
7. Wash twice with kinase buffer.
8. Add 25 μL of kinase buffer containing 10 μg of poly(Glu-Tyr) and 5 μL of kinase buffer containing 10 μCi of [γ-^{32}P]ATP to each tube.
9. Place tubes into mixer and place it at 30°C for 20 min.
10. **Steps 19** and **20** from **Subheading 3.1.**

11. Separate samples on a 10% polyacrylamide gel. Since Poly(Glu-Tyr) is a synthetic random polymer it appears as a broad diffuse band with an average molecular mass of 52 kDa.
12. **Steps 22–24** from **Subheading 3.1.**

2.3. PI3-K Assay

1. **Steps 1–14** from **Subheading 3.1.** Remember to use PI3-K lysis buffer at **step 3** and 2.5 μg of anti PI3-K antibody at **step 11**.
2. Wash three times with lysis buffer by centrifugation at 10,000*g* for 2 min at 4°C.
3. Wash twice with the Buffer A as above.
4. Wash twice with the Buffer B as above.
5. Wash twice with the Buffer C as above.
6. Prepare PI solution: Each point requires 10 μg of PI. Calculate the total amount of PI needed and place the required volume of stock solution into a microfuge tube. Dry the PI solution under N_2 gas taking care not to blow it out of the tube. Resuspend the dried PI in 20 m*M* HEPES at pH 7.5 to the final concentration of 1 mg/mL and sonicate for 5 s.
7. Add 10 μL of PI to each sample.
8. Add 50 μL of kinase buffer containing 100 μCi of [γ-^{32}P]-ATP to each tube.
9. Place tubes into the mixer and keep it at room temperature for 15 min.
10. Stop the reaction with 100 μL 1 *N* HCl per sample.
11. Extract samples with 200 μL of extraction buffer.
12. Pipet the bottom layer into a new microfuge tube. Briefly, draw all of the organic bottom layer along with a small amount of aqueous layer into the pipet tip. The aqueous phase will gradually rise above the organic layer. Empty the organic layer into the new tube, taking care not to contaminate with the aqueous phase.
13. Dry samples in a Speed Vacuum Concentrator (Savant, Holbrook, NY). This takes 30 to 60 min.
14. Pour chromatography buffer into tank and cover the inside walls with Whatman 3MM paper. Close and allow to equilibrate until the buffer wets at least 70% of the height of the 3MM paper. This takes about 90 min.
15. Resuspend samples in 10 μL of PI-4P standard.
16. Mark as many origins of migration as required onto a silica gel 60 TLC plate using a soft pencil. They should be at least 2 cm apart from one another and 3 cm from the edge of the plate. Spot 2 μl of each sample, dry with a blow-drier, and repeat the procedure until the entire volume is loaded (**Fig. 1**).
17. Place plate in chromatography tank. Run until buffer front is about 1 cm from the top of the plate.
18. Remove plate and air dry for 20 min.
19. Stain with iodine vapors: Place the plate in a chamber containing a few crystals of solid iodine and incubate until standards are visible. This takes approx 15 min. The PI-4P standard migrates slower than unphosphorylated PI and the radioactive product of the reaction migrates approximately with the same speed of PI-4P (**Fig. 1**).
20. Expose to X-ray film at –70°C with intensifier screen.

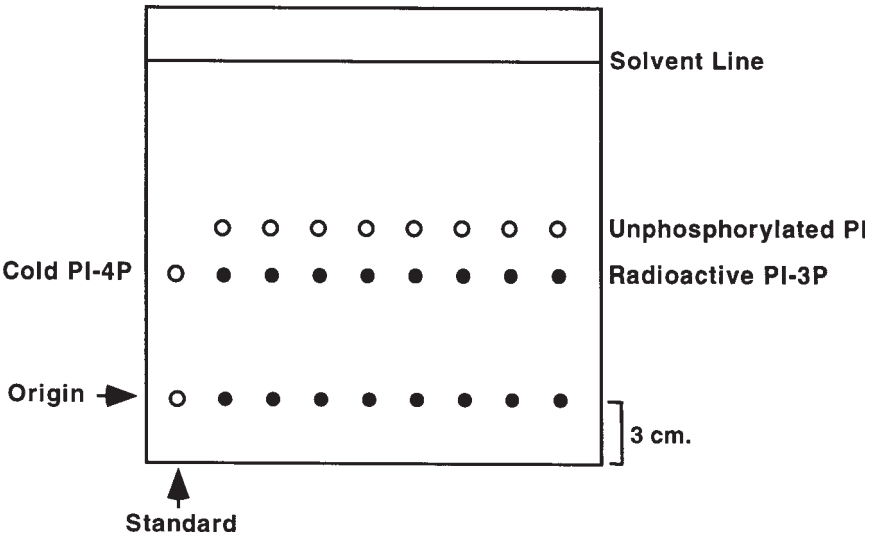

Fig. 1. Separation of PI3-K products by thin layer chromatography.

3.4. p70^{S6} Kinase Assay

1. **Steps 1–10** from **Subheading 3.1.**
2. Add 2.5 μg of anti-p70^{S6} kinase antibodies and 80 μL of 50% Protein-A Sepharose slurry per sample and incubate for 1 h at 4°C.
3. **Steps 12** and **13** from **Subheading 3.1.**
4. Wash the beads twice with lysis buffer by centrifugation at 10,000*g* for 2 min at 4°C.
5. Wash the beads twice with kinase buffer by centrifugation at 10,000*g* for 2 min at 4°C.
6. Add 25 μL of kinase buffer containing 17 μg of 40S ribosomal subunit and 5 μL of kinase buffer containing 8 μCi of [γ-^{32}P]-ATP to each tube (*see* **Note 4**).
7. Place tubes into mixer and place it at 30°C for 30 min.
8. **Steps 19** and **20** from **Subheading 3.1.**
9. Separate samples on a 14 % gel (the molecular mass of 40S ribosomal subunit is 23 kDa).
10. **Steps 22–24** from **Subheading 3.1.**

3.5. ERK Kinase Assay

1. **Steps 1–10** from **Subheading 3.1.** It is sufficient to use 200 μg of protein for immunoprecipitation.
2. Add 2.5 μg of anti-ERK antibodies and 80 μL of 50% Protein-A Sepharose slurry per sample.
3. **Steps 12** and **13** from **Subheading 3.1.**
4. Wash twice with lysis buffer.

5. Wash twice with kinase buffer.
6. Add 25 μL of kinase buffer containing 4 μg of MBP and 5 μL of kinase buffer containing 8 μCi of [γ-^{32}P]-ATP to each tube (*see* **Note 5**).
7. Place tubes into mixer and place it at 30°C for 30 min.
8. **Steps 19** and **20** from **Subheading 3.1.**
9. Separate samples on a 12% gel (molecular mass of MBP is 15 kDa).
10. **Steps 22–24** from **Subheading 3.1.**

3.6. JNK Kinase Assay

1. **Steps 1–6** from **Subheading 3.1.** Remember to use JNK lysis buffer at **step 3**.
2. **Step 8** from **Subheading 3.1.**
3. Add 10 μg of GST-cJun-GSH-agarose to the lysate (*see* **Note 6**).
4. Wash five times with wash buffer.
5. Wash twice with kinase buffer (*see* **Note 7**).
6. **Step 17** from **Subheading 3.1.**
7. Place tubes into mixer and place it at 30°C for 30 min.
8. **Steps 19–24** from **Subheading 3.1.** It may be necessary to expose gel longer, up to 5 h. Phosphorylated GST-cJun(1–223) runs at approx 53 kDa molecular mass.

4. Notes

1. Working with ^{32}P requires caution. Make sure to use Plexiglas shields while in the vicinity of radiation. Also, contact your Radioactivity Department for proper disposal procedures.
2. To reduce the radioactive waste, do not let the front dye, where unincorporated [γ-^{32}P]-ATP migrates, run out of the gel. Instead, cut it out and dispose it with the solid radioactive waste.
3. Use 3% glycerol in fixing buffer to prevent cracking of the high percentage polyacrylamide gels (e.g., those of ERK and p70^{s6} kinase assays).
4. The MBP substrate for ERK kinase assay is very susceptible to degradation. Keep it on ice and freeze it back as soon as possible.
5. In the JNK kinase assay do not use less than 10 μg of GST-Jun per sample.
6. Thorough washing of the GST-cJun(1–223)-glutathione-agarose beads at the end of JNK kinase assay is very important. Cutting on washes may result in increased background.
7. For p70^{S6} kinase assays it is possible to use intact 80S ribosomes, which are easier to obtain in good yields than 40S ribosomal subunits.
8. The activation of ERK, JNK, and p70^{S6} kinase may be detected by immunoblotting because, once activated by phosphorylation, these kinases shift in molecular mass. There are also excellent commercial antibodies that specifically recognize the activated forms of ERK and JNK and can be used in immunoblotting experiments.

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5

Immunoblotting of Integrins

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1. Introduction

1.1. The Use of SDS-PAGE/Immunoblotting for the Analysis of Integrins

SDS PAGE/immunoblotting is the method of choice for the qualitative and quantitative detection of specific integrins in a given sample. In the simplest case, the cells under investigation are lysed, the lysates separated by SDS PAGE, transferred onto a membrane, and the integrins are then identified by using specific anti-integrin antibodies. If the aim of an experiment is limited to the identification or quantification of a specific integrin subunit in a given sample SDS PAGE/immunoblotting of a cell lysate using specific antibodies yields sufficient information. The advantage of separating and blotting the protein extract onto a membrane compared to a simple detection of a protein of interest, e.g., in an ELISA assay is the additional information about the size of the protein that is detected. This is particularly important when it is not clear if the antibodies used for detection of the protein cross-react with other proteins in the cell lysate.

The known 17 α and 8 β subunits of integrins can associate to form more than 20 possible integrin heterodimers that link the cell to a wide array of extracellular matrix molecules ***(1,2)***. The identification of a single integrin chain is necessary but may not be sufficient to answer the question of which integrin heterodimer or heterodimers are engaged in a certain process. To answer this question, the integrins must first be immunoprecipitated under conditions that do not disrupt integrin heterodimers. Next, the coprecipitated α or β subunits can be identified by immunoblotting.

The analysis of integrins by SDS PAGE/immunoblotting is the method of choice if functional data are to be related to the presence of specific integrins

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on the surface of cells. However, because the integrin content of the cell lysate does not necessarily reflect the actual situation on the cell surface, the specific detection of surface-labeled integrins is essential to unequivocally correlate functional data to the cell-surface expression of an specific integrin heterodimer. By using cell-surface labeling, the immunoprecipitation of a specific integrin subunit with subsequent SDS PAGE/immunoblotting may answer the question of whether or not this particular integrin subunit is present on the cell surface. This way, a cell-membrane-impermeable biotinylation reagent may be used for cell-surface labeling, followed by detection with avidin-peroxidase conjugate.

If immunoprecipitation is performed under nondenaturing conditions, the avidin-peroxidase detection results in a complex pattern of integrin subunits. This pattern reflects the other integrin subunits that are engaged in forming the αβ heterodimers. Moreover, the pattern can be used as an "integrin-fingerprint" of a cell when comparing different cells.

1.2. Mature and Immature Forms of Integrin Subunits

Some integrin subunits exist in different forms, such as mature or immature forms, splicing or conformational variants. The analysis of cell lysates by immunoblotting has to take these facts into consideration to avoid misinterpreting results.

Many integrin α subunits are in their mature form, cleaved proteolytically in the extracellular domain close to the membrane ***(1)*** (**Table 1**). Under native conditions, the large extracellular domain is connected to the smaller transmembrane and cytoplasmatic domains via a disulfide bridge. However, under reducing conditions, the mature forms of these integrin α subunits are split into a large extracellular domain and a smaller fragment containing the transmembrane and cytoplasmatic parts of the α subunit. This reductively cleaved subdomain migrates at approx 25–30 kDa. In consequence, the large extracellular domain migrates 25–30 kDa faster than the immature full-length α subunit. It should be noted that there is always a considerable amount of immature—not proteolytically processed—α subunits in a cell lysate. This immature form is therefore not cleaved under reducing conditions and detection of these α subunits after reducing SDS-PAGE using an antibody against the extracellular domain often results in the appearance of two bands.

Among the β subunits, in many cell lines the β_1 subunit also exists in two different forms. β subunits are not processed proteolytically. In this case, the difference is that the immature form is not fully glycosylated and it migrates faster than the mature, fully glycosylated form ***(3)***. Antibodies directed against the integrin β_1 subunit usually result in the detection of a double band, independent of the use of reducing or nonreducing SDS-PAGE sample buffer. However, when investigating β_1 integrin by surface biotinylating, the slower

Table 1
Integrin α Subunits Posttranslationally Proteolytically Cleaved

yes	no
α_{IIb}	α_1
α_v	α_2
α_3	α_4
α_5	α_9
α_6	α_{10}
α_7	α_L
α_8	α_M
α_E[a]	α_X

[a]α_E is different from the other proteolytically cleaved α subunits in that the cleavage site is close to the N terminus. Under reducing conditions, the large fragment therefore in this case consists of most of the extracellular domain plus the transmembrane and cytoplasmic domains whereas the small fragment represents the extreme N-terminal extracellular part.

migrating form of the β_1 subunit usually gives a much stronger signal in the avidin-peroxidase detection. This occurs because the slower migrating, fully glycosylated form is predominant on the cell surface.

Another source of variation is the existence of alternatively spliced forms of integrins that may be abundant in certain cell lines or tissues ***(4)***. Conformationally different forms of integrin subunits that migrate differentially and can be converted into on another under certain conditions also have been reported ***(5)***.

1.3. Practical Considerations in Working with Integrins

1.3.1. Solubilization of Integrins

To investigate the integrins that are expressed by a given cell line, the first step is to lyse the cells in a buffer that will result in the solubilization of the integrins. This may be brought about by lysing the cells in a buffer containing detergents such as Triton X-100, NP-40, or octylglucoside. Integrins are solubilized as intact αβ heterodimers in these buffers ***(6)***. The addition of a strong denaturing detergent such as SDS disrupts the αβ subunit interaction and yields single integrin subunits.

1.3.2. Immunoprecipitation of Integrins

For the SDS-PAGE/immunoblotting analysis of whole-cell lysates, whether or not the integrins in the cell lysate are solubilized as intact heterodimers or as

single-chain subunits is not usually a critical question. However, in the case when integrins are immunoprecipitated before SDS PAGE/immunoblotting it is important to decide whether the integrins are solubilized as αβ heterodimers or as single α and β chain subunits. Under denaturing conditions in the presence of SDS, the immunoprecipitation by using an antibody against a β subunit, the corresponding α subunits do not precipitate and this information is lost. This will not be a problem if the aim of the study is restricted to the identification of this particular integrin subunit in a certain sample. Denaturing conditions may actually be required by some antibodies for them to bind to their epitope. This information is usually provided by the supplier of the antibody. In case of doubt it is recommended to perform the immunoprecipitation in parallel under both denaturing and nondenaturing conditions. Another reason for using harsher denaturing conditions for immunoprecipitation is that these may lead to cleaner precipitates because of the higher stringency of the immunoprecipitation and washing steps.

Integrin subunits must be immunoprecipitated under nondenaturing conditions if the aim of the study is the identification of the corresponding α and β subunits in the heterodimer(s). This is particularly true in the case of those integrin subunits known to assemble with more than one corresponding α or β subunit to form functional heterodimers such as the integrin subunits α_4, α_6, α_v, β_1, β_2, β_3 and β_7. In the simplest case, the integrin under investigation is immunoprecipitated and the identification of the corresponding integrin subunits is then carried out by probing the blot with antibodies against different integrin chains known to form heterodimers with the precipitated subunit. If the immunoprecipitation is carried out from a surface biotinylated cell extract the detection with avidin-peroxidase may give an idea about the other integrin subunits in the immunoprecipitate by revealing their molecular mass. Another approach for the identification of integrin subunits forming heterodimers with a particular integrin subunit is to perform an immunodepletion. For example, for integrin $\alpha_8\beta_1$: After subjecting an extract from surface-labeled cells to three rounds of immunoprecipitation with antibodies against β_1 subunit no α_8 subunit could be immunoprecipitated from this extract anymore. Therefore it was shown that the α_8 subunit forms heterodimers exclusively with the β_1 subunit ***(7)***. In other cases, certain integrin heterodimers can be identified in a sample by immunoprecipitating with an antibody that exclusively recognizes a specific heterodimer, such as the monoclonal antibody LM609 in the case of integrin $\alpha_v\beta_3$ ***(8)***.

1.3.3. SDS-PAGE and Immunoblotting of Integrins

1.3.3.1. Choice of Reducing/Nonreducing Sample Buffer

The migration of integrin subunits is affected by the presence or absence of a reducing agent in the sample buffer. As outlined above, this is particularly

important when integrin α subunits that are in their mature form cleaved proteolytically are subjected to SDS-PAGE under reducing conditions (**Table 1**). To avoid the appearance of two forms of α chains (a mature and an immature form), we recommend a nonreducing SDS-PAGE sample buffer. Moreover, under nonreducing conditions the migration difference between the α subunits and the β subunits is usually more pronounced. There are two explanations for this observation. First, the mature α subunits are present as intact chains under nonreducing conditions; second, β subunits with cysteine-rich domains migrate considerably faster under nonreducing conditions compared to the reduced state, when their disulfide loops become unfolded and bulkier. The use of nonreducing conditions is therefore helpful for the identification of integrins in case multiple α and β subunits are present in the sample because the range of molecular mass is wider. Of course, the migration difference of certain integrin α and β-subunits observed under reducing or nonreducing conditions can also be helpful for their identification.

Integrin immunoprecipitates may sometimes contain large amounts of IgG. The presence of large amounts of IgG can interfere with the migration of integrin α subunits that often migrate similar to IgG around 150 kDa. This is often the case if a nonaffinity-purified serum is used for immunoprecipitation. In case the large amounts of IgG interfere with the migration/detection of the integrin subunits it is recommended to use reducing conditions for SDS-PAGE. Because of reductive cleavage, the IgG is split into heavy chains and light chains, running at approx 50 and 30 kDa, thereby shifted far below the spectrum of 90–200 kDa where integrin subunits including the large fragments of reductively cleaved α subunits migrate.

1.3.3.2. Choice of Gel

Because the molecular weight of integrin α and β subunit ranges between 90 and 200 kDa, a low percentage acrylamide gel is recommended for separation, e.g., 7.5%. The large pores of a 7.5% gel also facilitate subsequent transfer of the integrin subunits onto the membrane. Another possibility is the use of gradient gels, e.g., 4–12% or 4–20% in which proteins can be separated over a wide range of molecular mass. This is particularly important if the cytoplasmatic domains of integrin α subunits are to be investigated under reducing conditions. Under reducing conditions, the transmembrane and cytoplasmatic domain of these α subunits migrate at approx 20–25 kDa. Thus, they can easily be lost if a low percentage acrylamide gel is used. We recommend a 4–20% gradient gel to detect the transmembrane and cytoplasmatic domains of these α chains.

1.3.3.3. Choice of Membrane

Either nitrocellulose or PVDF membranes are suitable for the transfer of integrins from the SDS-PAGE onto a membrane. PVDF membranes have the

advantage of being more resistant to mechanical stress. This is an important consideration if the blots are to be stripped and reused several times for the detection of different antigens. They also have a higher capacity for binding of proteins.

1.3.4. Detection of Integrins on a Blot

1. For the detection of proteins on a blot it is normally necessary to use antibodies that are able to detect the denatured form of the protein. Ultimately, the suitability of an antibody for immunoblotting can only be assessed by trying it out.
2. If integrin samples are run in a reducing SDS-PAGE sample buffer, special attention has to be paid when using antibodies raised against the cytoplasmatic domain of those α subunits that are proteolytically cleaved in their mature form and held together by a disulfide bridge (**Table 1**). As outlined above, the small cytoplasmatic and transmembrane domains of the mature form of those α subunits are separated from the extracellular domain and migrate at approx 20–25 kDa, a size range that is easily lost on low-percentage gels used for the separation of integrins. Therefore, when using a *reducing* SDS-PAGE sample buffer for the investigation of these α subunits the following observations can be made in the size range between 100 and 200 kDa:
   a. An antibody against the cytoplasmatic domain will only recognize the immature (mostly intracellular) form of the α subunit and result in a single band.
   b. An antibody recognizing the extracellular part of these α subunits detects two bands: a larger immature and another smaller mature form where the small cytoplasmatic and membrane domain has been proteolytically and then reductively cleaved off.
   c. It is mainly the mature, proteolytically cleaved form of these α subunits that is present on the cell surface. Therefore, when using avidin-peroxidase for the detection of surface-labeled α subunits mainly the smaller, mature form will be detected.

   It is therefore generally recommended not to use antibodies directed against the cytoplasmic domain of those integrin α subunits that are proteolytically cleaved when running reduced gels. However, these differences in the reactivity towards antibodies can also be helpful to distinguish between immature and mature forms.
3. Independently of the presence of a reducing agent, antibodies against the integrin β_1 subunit—including those against the cytoplasmic domain—will detected a double band on an immunoblot. Here, the upper band represents the fully glycosylated form of β_1 integrin whereas the faster migrating form represents the intracellular precursor of β_1 subunit that is not yet fully glycosylated. Thus, when surface biotinylated β_1 subunit is immunoprecipitated and detected with avidin-peroxidase the slower migrating, fully glycosylated form of β_1 subunit usually gives a much stronger signal than the faster migrating form.
4. Antibodies that recognize an epitope formed by both α and β subunit are not useful for immunoblotting because the denaturing conditions will disrupt such an epitope.

2. Materials

Note: Deionized, distilled water (DDW) should be used throughout this protocol.

2.1. Preparation of Cell Lysates

1. Phosphate-buffered saline (10× PBS at pH 7.4): 80.06 g NaCl, 2.01 g KCl, 14.42 g Na_2HPO_4, 2.04 g KH_2PO_4. Fill to 1 l with deionized water and autoclave (store at room temperature [RT]).
2. Sulfo-NHS-biotin (e.g., Pierce, Rockford, IL) for cell surface labeling (*see* **Note 1**).
3. 0.1 *M* glycine in PBS. The amino group of the amino acid glycine is used after the labeling reaction to quench residual NHS-groups before the cells are lysed. This prevents biotinylation of cytoplasmatic proteins during cell lysis.
4. Lysis buffer (nondenaturing): 1% NP-40, 20 m*M* Tris-HCl at pH 7.5, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 2 m*M* EGTA, 10% glycerol. This buffer can be kept at 4°C for several weeks. Instead of NP-40, other nondenaturing detergents for the solubilization of integrins can be used, e.g., 1% Triton X-100 or 50 m*M* octylglucoside ***(6)***.
5. Lysis buffer (denaturing): Add 0.1% SDS and 1% deoxycholate to the nondenaturing lysis buffer.
6. Immediately before use add the following protease inhibitors to the lysis buffer: 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.1 U/mL), leupeptin (10 µg/mL), and pepstatin A (4 µg/mL). If phosphorylation of integrins is investigated, the addition of 1 m*M* orthosodium vanadate as tyrosine phosphatase inhibitor and 50 m*M* sodium fluoride as serine/threonine phosphatase inhibitor is recommended.
   PMSF is prepared as a 100 m*M* stock in 100% EtOH and stored at –20°C (**Note 2**). Under these conditions the solution is stable for several weeks. For the preparation of the lysis buffer dilute the PMSF stock 1:100 and immediately vortex vigorously to avoid precipitation of the PMSF. Add the protease and phosphatase inhibitors immediately before use and keep the lysis buffer always on ice.
7. SDS-PAGE sample buffer (2×): 100 m*M* Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 0.001% bromophenol blue. For a reducing SDS-PAGE sample buffer add 100 m*M* DTT immediately before use (prepare a 1 *M* DTT stock solution and store it at –20°C, this solution is stable for several weeks).

2.2. Immunoprecipitation of Integrins

For the precipitation of antibody-integrin complexes use protein A or G coupled to Sepharose, e.g. from Pharmacia (Uppsala, Sweden) or Sigma (St. Louis, MO). Always check the Sepharose conjugate product description for the reactivity of the Sepharose conjugate with the antibody species you use for the immunoprecipitation of integrins, e.g., protein A does not bind monoclonal mouse antibodies but protein G does. Wash the Sepharose conjugate in lysis buffer and prepare a 1:5 (v/v) suspension of the Sepharose conjugate in cell lysis buffer.

2.3. SDS-PAGE/Immunoblotting of Integrins

Solutions for the preparation of a 7.5% acrylamide separation gel with a 4% stacking gel:

1. Gel solution: 29.2% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide in DDW (*see* **Note 2**).
2. 4× separation gel buffer (lower buffer): 1.5 *M* Tris-HCl at pH 8.8, 0.4% SDS.
3. 4× stacking gel solution (upper buffer): 0.5 *M* Tris-HCl at pH 6.8, 0.4% SDS. These three solutions can be stored at 4°C for several weeks. The acrylamide solution should be stored in the dark.
4. TEMED.
5. 10% (w/v) ammonium persulfate (AP) in DDW. This solution is stable at 4°C for up to 1 wk.
6. Acrylamide gel apparatus.
7. 10× SDS-PAGE running buffer: 0.25 *M* Tris, 1.92 *M* glycine, 1% SDS. This solution is stable at room temperature for several weeks and is diluted immediately before use 1:10 with DDW.
8. Prestained molecular mass standard (e.g., Bio-Rad, Hercules, CA).
9. Tank transfer system for transfer of proteins from the acrylamide gel onto a membrane (e.g., Bio-Rad).
10. 10× transfer buffer: 0.1 *M* CAPS at pH 11.0. Store at room temperature, stable for several weeks. For preparation of the 1× transfer buffer dilute the 10× buffer in DDW/methanol with a final methanol concentration of 10%.
11. Whatman 3 MM filter paper or equivalent.
12. 0.45-μm nitrocellulose or PVDF membrane (Millipore, Bedford, MA).
13. Methanol for prewetting the PVDF membrane (*see* **Note 2**).
14. A Pasteur pipet for rolling over the gel to remove air bubbles between gel and membrane.

2.4. Detection of Integrins on a Membrane

1. Methanol for prewetting the PVDF membrane.
2. Washing solution: 0.05% Tween 20 in TBS (150 m*M* sodium chloride in 20 m*M* Tris-HCl at pH 7.5).
3. Blocking solution: 5% nonfat dry milk and 2% BSA dissolved in washing buffer. If surface biotinylated integrins are detected with avidin-peroxidase, omit the nonfat dry milk because it contains biotin that will interfere with the avidin-peroxidase conjugate.
4. Secondary peroxidase conjugated anti-species antibody to bind and detect the primary antibody on the membrane (available e.g., from Sigma or Bio-Rad) (*see* **Note 3**).
5. Avidin-peroxidase conjugate: To detect surface-biotinylated integrins on the membrane use avidin-peroxidase conjugate (e.g., Sigma or Bio-Rad). For use, the avidin-peroxidase conjugate is diluted in blocking solution without milk.
6. The antibody/secondary antibody-peroxidase conjugate complexes on the membrane are detected by using chemiluminescence. Prepare the solution (e.g., ECL,

Amersham, Arlington Heights, IL) immediately before use. After soaking the blots in the chemiluminescence solution the solution can be stored at 4°C for several reuses (for up to 1 wk).

7. For detection of the light generated by the antibody/secondary antibody-peroxidase conjugate complexes on the membrane, a suitable film such as Biomax (Kodak) may be used. Cut the film in the dark room to a size that is sufficient to cover the blot. For proper alignment of the film with the membrane after development you can use a fluorescent marker (e.g., Stratagene, La Jolla, CA) that is fixed in the film cassette close to the membrane. This fluorescent marker will give a signal on the film that can then be used to realign the film after development with the membrane.

2.5. Stripping of Membranes

Proteins transferred on a membrane can be reprobed several times provided that after each round of probing the residual antibodies are completely removed from the membrane.

1. Use a tightly sealed box to avoid evaporation of β-mercaptoethanol. Alternatively, membranes can also be sealed in a plastic bag.
2. Stripping buffer: 2% SDS in TBS (150 m*M* sodium chloride, 20 m*M* Tris-HCl at pH 7.5).
3. Stripping solution: add 70 µL of β-mercaptoethanol (*see* **Note 2**) to 10 mL of stripping buffer immediately before use.
4. Water bath at 55°C with an insert that allows rocking of the box or the sealed plastic bag.

3. Methods

3.1. Preparation of Cell Lysates

3.1.1. Adherent Cells

1. Take a dish out of the incubator and put it on ice.
2. Remove the medium.
3. Wash the cells twice with ice-cold PBS.
4. If the aim of the study is to elucidate the cell surface expression of integrins the cells have to be surface labeled at this point, before preparation of the cell lysate
   a. Add an ice-cold solution of 0.5–1 mg/mL of sulfo-NHS-biotin in PBS on top of the cell layer (4 mL for a 10-cm dish) and incubate on ice with occasional gentle rocking to ensure that the whole cell layer is covered with the labeling solution.
   b. After 15–30 min incubation remove the labeling solution and wash the cells with ice-cold PBS (*see* **Note 4**).
   c. Add 4 mL of an ice cold solution of 0.1 *M* glycine in PBS to the cells and incubate for another 10 min.
   d. Wash with ice-cold PBS and proceed with lysis.

5. Add ice-cold cell lysis buffer including inhibitors on top of the cells (1 mL per 10-cm dish corresponding to approx 5×10^6 cells for a confluent dish) and rock on ice for 30 min.
6. Remove the solution from the dish by pipetting it into an Eppendorf tube.
7. Put the tube in a centrifuge and spin down at 15,000*g* for 10 min at 4°C to remove insoluble cell debris.

3.1.2. Suspension Cells

1. Remove sufficient cells from the culture flask (e.g., 5×10^6 cells).
2. Spin cells down gently at 300*g* and remove the medium.
3. Wash twice in ice-cold PBS.
4. Surface labeling of suspension cells:
   a. Add 1 mL of an ice-cold solution of 1 mg/mL of sulfo-NHS-biotin in PBS to the cell suspension and incubate on ice with occasional gentle rocking.
   b. After 15–30 min incubation, spin down cells gently, remove the labeling solution, and wash the cells with ice-cold PBS.
   c. Add 1 mL of an ice-cold solution of 0.1 *M* glycine in PBS to the cells and incubate for another 10 min.
   d. Spin down cells, wash with ice-cold PBS and proceed with lysis.
5. Add ice-cold cell lysis buffer including inhibitors (e.g., 1 mL) and rock for 30 min on ice.
6. Put the tube in a centrifuge and spin down at 15,000*g* for 10 min at 4°C to remove insoluble cell debris.

Under these conditions the integrins are solubilized in the resulting supernatant. It can be aliquoted and stored at –20°C for several weeks or used immediately (*see* **Note 5**).

For the analysis of the whole-cell lysate by SDS-PAGE/immunoblot take an aliquot (e.g., 10 µL, corresponding to approx 5×10^4 cells), add an equal volume of 2× SDS-PAGE sample buffer (reducing or nonreducing), boil it for 3 min, and run the sample on an SDS-PAGE (*see* **Note 6**).

3.2. Immunoprecipitation of Integrins

For removal of proteins from the cell lysates that bind nonspecifically to Sepharose beads it may be necessary to preincubate the cell lysates with these beads. This is particularly desirable when investigating cell-surface biotinylated integrins to get a clean result.

1. Preclearing of the cell lysate with Sepharose beads: Add 100 µL of a 1:5 (v/v) suspension of protein A or G coupled to Sepharose in cell lysis buffer to an eppendorf tube containing the cell lysate (e.g., 1 mL) prepared according to **Subheading 3.1.** and rotate or rock the sample for 1 h at 4°C.
2. Take the Eppendorf tube and spin down at 15,000*g* for 10 min at 4°C to remove the beads and insoluble protein complexes that may have formed during the incubation.

3. Place an aliquot of the supernatant or, if no preclearing step was performed, of the cell lysate prepared according to **Subheading 3.1.** (e.g., 100 µL, corresponding to approx 5×10^5 cells) into an Eppendorf tube.
4. Dilute the antibodies in cell lysis buffer and add 200 µL to the cell lysate to obtain a final volume of 300 µL. If the antibody is used for the first time try 1–5 µL of an antiserum or 1–5 µg of a monoclonal antibody for each immunoprecipitation. The predilution of the antibody is recommended if several samples are to be investigated, to assure equal amounts of antibody in each sample (*see* **Note 7**).
5. Rotate or rock the samples at 4°C for 2 h.
6. Add 100 µL of a 1:5 (v/v) suspension of protein A or G coupled to Sepharose in cell lysis buffer. Check Sepharose product description whether the antibody species used for the immunoprecipitation is bound by this Sepharose. Rotate or rock for another 30–45 min at 4°C.
7. Take the eppendorf caps and spin down for 30 s at 3000–5000*g* (*see* **Note 8**). Remove supernatant carefully (a gel-loading pipet tip with an elongated narrow tip is helpful for draining the pellets because the beads may not pass through). Resuspend beads in 1 mL of lysis buffer and spin down as above. Repeat so that the beads are washed 3–5 times. After removing the last wash, take the beads, add 25 µL 1× SDS-PAGE sample buffer (reducing or nonreducing) and incubate for 3 min in a boiling water bath. Spin down briefly and apply the supernatant or a fraction of it on the SDS-PAGE (again, a gel-loading pipet tip will help to remove the sample solution without too much of contamination with beads).

3.3. SDS-PAGE/Immunoblotting of Integrins

1. For the separation of integrins by SDS-PAGE, prepare a 7.5% acrylamide gel. Add 7.2 mL of DDW and 3.6 mL of the 30% acrylamide solution (29.2% acrylamide/0.8% *bis*, *see* **Note 2**) to 3.5 mL of separation gel buffer into a vacuum flask. After brief degassing, add 10 µL TEMED and 55 µL 10% AP to start the polymerization. This recipe is for two minigels but it can be adjusted to individual needs.
2. Quickly pour the solution between the glass plates (precleaned with ethanol) and carefully overlay with DDW. After 10–20 min the gel is polymerized and the overlaid water can be poured away.
3. Prepare the 4% acrylamide stacking gel by adding 3.1 mL of DDW and 0.65 mL of 30% acrylamide solution to 1.25 mL of separation gel buffer into a vacuum flask. After brief degassing add 5 µL of TEMED and 15 µL of 10% AP to start the polymerization.
4. Quickly overlay the stacking gel solution over the separation gel and insert the comb. When polymerization is complete, carefully remove the comb and insert the gel into the acrylamide gel apparatus. Fill the apparatus with 1× SDS-PAGE running buffer.
5. Load the samples and run the SDS-PAGE at 100 V until the bromophenol blue dye front has reached the bottom of the gel.
6. Prepare a sufficient amount of 1× transfer buffer according to **Subheading 2.3., step 10**. For the transfer of proteins onto the membrane use a tank transfer sys-

tem. This system is more suitable for the transfer of large proteins of 100–200 kDa than a semidry blot system because prolonged transfers are possible without buffer depletion.

7. Disassemble the gel sandwich and cut away the stacking gel and discard it. Incubate the 7.5% separation gel for 20 min in the transfer buffer.
8. Meanwhile cut a PVDF or nitrocellulose membrane to the same size as the gel plus 1–2 mm on each edge and incubate the membrane for 10 min in the transfer buffer. The PVDF membrane has to be prewetted for 5 s in methanol before it can be incubated in the transfer buffer.
9. Cut 4 Whatman 3 MM filter papers slightly larger than the gel and prewet them in transfer buffer.
10. After equilibration of the gel in transfer buffer assemble the transfer sandwich in the following order: put two prewetted filter papers on a clean even surface and put the membrane on top of it. Add a few drops of transfer buffer on top of the membrane, lay the gel on the membrane and remove residual air bubbles between membrane and gel by gently rolling with a Pasteur pipet over the gel. Put another two prewetted filter papers on top of the gel and place the sandwich between two sponges in the transfer cassette so that the membrane is kept in tight contact with the gel. Mount the transfer cassette in the tank transfer system and fill the tank with 1x transfer buffer.
11. Try to avoid heating of the transfer system caused by electrophoresis by working in the cold room and using a magnetic stirrer for the buffer reservoir. Transfer the proteins for 3 h at 50 V. Alternatively, transfer can be accomplished overnight at 40 V.
12. After finishing the transfer disassemble the sandwich and remove the membrane. Membranes can be dried and stored at 4°C for several weeks, if necessary. If a PVDF membrane is used, drying is recommended before detection of proteins because the drying enhances the stability of the interaction of the proteins with the membrane. The dry PVDF membranes must be placed in a small amount of methanol to prewet the membrane and then in distilled water before blocking or probing with antibodies.

3.4. Detection of Integrins on a Membrane

1. Preblock the membrane with the blocking solution for 30 min to 1 h at room temperature. (If surface biotinylated integrins are to be investigated, omit the milk in the blocking solution.) All incubations and washing steps should be performed on a rocking table.
2. Remove the blocking solution and incubate the membrane with the first antibody diluted in the blocking solution for 1 h at room temperature. If you have no previous experience with the antibody used for the detection of integrins you may start with a 1:1000 dilution of antiserum or IgG. If the result is not satisfactory strip and reprobe the membrane with a different concentration of the antibody.
   If surface biotinylated integrins are to be detected use a 1:5000 dilution of avidin-peroxidase in blocking solution at this point instead of the primary antibody solution. After 40 min to 1 h incubation continue with ***step 5*** *(washing).*

3. Remove the antibody solution. Wash the membrane for 5 min in the washing buffer. Remove the washing solution and repeat washing for another 2× 5 min.
4. Add the secondary peroxidase-labeled anti-species antibody diluted in blocking solution. Try a 1:5000 solution first. If signals turn out to be too weak after developing you can try a higher concentration (1:2000 or 1:1000).
5. Remove the secondary antibody solution and wash the membrane five times for 5 min in washing buffer.
6. Prepare the chemiluminescence substrate solution according to the manufacturer's recommendations. Use a small tray for the preparation of the chemiluminescence substrate solution and put the membrane in it so that it is completely covered. Incubate the membrane in the chemiluminescence substrate solution as indicated, e.g. for 30 s (*see* **Note 9**).
7. Remove the membrane from the chemiluminescence substrate solution, seal the membrane in plastic wrap and mount it in a film cassette. For the realignment of the film with the gel after development it is recommended to pin a fluorescent marker close to the membrane.
8. In a dark room, place a film onto the membrane and close the film cassette. Make sure that the film is in close contact with the membrane by using a film cassette that insures a tight fit.
9. Expose the film for a few seconds up to 1 h.
10. Slide the film into a developer machine. If the signal is too high or too low you can re-expose the film on the same membrane either immediately or after re-incubation of the membrane in chemiluminescence solution.

3.5. Stripping of Membranes

By removing the primary/secondary antibody complex the same membrane can be reused several times for the detection of proteins. Although repeated reprobing can lead to loss of signal, the following procedure generally allows for up to five reprobings (*see* **Note 10**).

1. Heat a water bath to 55°C. This water bath should contain an insert that can be moved.
2. Remove the membrane from the plastic wrap and put it into 20 mL of washing buffer for 5 min at room temperature to wash away the chemiluminescence solution.
3. Remove the washing solution. Add the stripping solution under a fume hood (contains β-mercaptoethanol, *see* **Note 2**). Use sufficient solution so that the membrane is completely covered (e.g., 10 mL for a blot from a minigel). Use a box that can be tightly sealed so that no β-mercaptoethanol can evaporate. Alternatively, membranes can also be sealed in a plastic bag.
4. Put the box in the preheated water bath and incubate for 30 min at 55°C with gentle rocking.
5. Take the box out of the water bath and go under a fume hood to remove the stripping solution in a waste container for β-mercaptoethanol containing solutions.
6. Wash the membrane 3× 5 min in washing solution. Washing solutions with β-mercaptoethanol odor must be discarded into a proper waste container. Repeat

washings until the β-mercaptoethanol odor is no longer detectable anymore in the washing solution.

7. The membrane is ready now for the next round of probing as described in **Subheading 3.4.** The membrane should be preblocked as described in **Subheading 3.4.1.** before reprobing.

4. Notes

1. Sulfo-NHS-biotin: The labeling technique for cell-surface proteins described in this protocol is labeling with sulfo-NHS-biotin. The charged sulfate group renders this biotinylation reagent highly hydrophilic and prevents it from diffusion through the cell membrane so that only cell surface molecules are labeled. The NHS group reacts with primary amino groups of lysines and forms a stable covalent bond. Make sure that no other amino groups are present during the labeling reaction, e.g., residual cell-culture medium or Tris-containing buffers. Another labeling technique frequently used for working with integrins is cell surface iodination (described in **ref. *9***). However, the biotinylation protocol is clearly much faster and user-friendly. Moreover, the known poor reactivity of some integrin subunits such as β_3 towards iodination is avoided.
2. Carcinogens or toxic chemicals: Many reagents used here such as PMSF or β-mercaptoethanol are toxic and should be treated with great caution. Contact your hazardous waste department for proper handling and disposal procedures in your area.
3. Secondary antibodies: Instead of the secondary anti-species antibody you can also use a protein A- or G-peroxidase conjugates to bind and detect the primary antibody on the membrane. They do not only bind to one species of antibody but have a broader spectrum of species (check product description for binding spectrum, e.g., Sigma or Bio-Rad). Antibodies, secondary peroxidase-conjugated antibodies and protein A- or G-peroxidase conjugates are diluted in blocking solution for use.
4. Washing adherent cells with PBS: Some cell lines tend to round up and loose matrix contact upon prolonged incubation in PBS because of absence of divalent cations. In these cases incubate for shorter times in biotinylation solution, e.g., for 10–15 min, which is usually sufficient for cell-surface labeling. Avoid moving the dish with the adherent cells too harshly. Alternatively, if cells start to detach from the matrix take them completely into suspension by collecting them gently with a cell scraper and complete the procedure as described for suspension cells.
5. Storage of cell lysates: During storage of lysates under these conditions the integrin protein chains are rather stable. This is not necessarily true, however, for posttranslational modifications, such as tyrosine or serine/threonine phosphorylation. When investigating phosphorylation of integrins it is therefore recommended to proceed with the fresh cell lysates.
6. Protein concentration in the cell lysate: Depending on the sensitivity of the antibody and the abundance of the integrin to be investigated a higher amount of total protein may have to be used. If this is the case, lyse cells in a smaller volume of

lysis buffer or, if the lysate is not used for subsequent immunoprecipitations, the cells can be lysed directly in a small volume of 2x SDS-PAGE sample buffer. In the case of adherent cells, when lysing the cells directly in a small volume of SDS-PAGE sample buffer use a cell scraper to ensure that all cells are subjected to lysis.

7. Immunoprecipitation after cell-surface labeling: When immunoprecipitating integrins after surface labeling it is recommended to include a control-immunoprecipitation of an intracellular protein (e.g., FAK, paxillin). The intracellular control protein should not be detectable with avidin-peroxidase under these conditions. If it is, then the biotinylation reagent may have passed the membrane. More likely, there has still been residual biotinylation reagent during cell lysis. In this case, make sure that the incubation step with 0.1 *M* glycine in PBS was at least 10 min.
8. Spinning down Sepharose beads: These conditions (3000–5000*g* for 30 s) are sufficient to precipitate the Sepharose beads with the immunocomplexes. Higher *g*-forces or longer spinning are not necessary and will only favor the nonspecific precipitation of protein aggregates that may have formed during the incubation steps and that lead to additional signals on the blot.
9. Handling of chemiluminescence solution: After removing the membrane from the tray with the chemiluminescence solution this solution can be kept at 4°C for up to 1 wk and reused several times, thus saving the reagent.
10. Stripping of membranes: If the second antigen to be detected on the membrane is migrating far away from the first antigen you do not necessarily need to strip the membrane but proceed immediately with washing and reprobing. Other methods for stripping of membranes are available ***(10)***.

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6

Application of Flow Cytometry in the Analysis and Sterile Sorting of Cell Populations Based on Integrin Expression

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1. Introduction

1.1. Flow Cytometry

Flow cytometry is the rapid analysis of populations of cells on a cell-by-cell basis by passing them individually through a beam of laser light, and the measurement of light emitted from the cells by scatter or fluorescence ***(1,2)***. Integrins have been studied by flow cytometry since the mid-1980s when immunologists started examining CD11 (integrins α_M, α_X, and α_L) as markers of T-cell differentiation in mixed cell populations ***(3,4)***. These analyses highlight many of the advantages and utility of flow cytometry over other forms of integrin analysis. With the appropriate antibody, flow cytometry can identify individual cells in a mixed cell population. Cell-surface expression of integrins can be differentiated from intracellular pools by flow cytometry analysis, unlike Western blotting or immunoprecipitation. Multicolor analysis can simultaneously measure the levels of two or three different integrins, enabling the researcher to determine that both an α and β subunits are present on the plasma membrane. Lastly, flow cytometry can sterilely sort viable cells on the basis of their cell-surface integrin expression and retain their viability for further growth and analysis.

1.2. Use of Flow Cytometry to Study Integrins

Integrins are very amenable to analysis by flow cytometry. They are transmembrane glycoproteins ***(5)*** and, as such, are highly accessible to antibodies. As mentioned above, flow cytometry can be a powerful technique to demon-

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strate that particular integrins are expressed on the cell surface. Cells that express a particular integrin can be isolated from other cells by sterile cell sorting. In addition to these methods, there are flow cytometric methods that can examine integrin heterodimer formation and ligand binding. There are several antibodies available that recognize a conformational epitope formed by the dimerization of specific integrin αβ heterodimers. These conformational epitopes become even more informative through the use of antibodies that recognize ligand-induced binding sites (LIBS) ***(6)***. Flow cytometric analysis using anti-LIBS antibodies can determine that a particular αβ heterodimer is present and that it is bound to ligand. Lastly, a recently described method uses flow cytometry to evaluate the contribution of a particular integrin to integrin-mediated phagocytosis ***(7)***. The protocols described in this chapter not only examine adherent epithelial cell types; they can be applied to nonadherent mesenchymal or hematopoietic cell types. These protocols will examine the use of flow cytometry to examine the expression of integrins and the use of flow cytometry to isolate populations of cells that express a particular integrin or integrin heterodimer.

1.3. Use of Flow Cytometry to Select Cells Expressing Particular Integrins

Flow cytometry can be used on a population of transfectants to isolate cells that are ectopically expressing the transfected integrin ***(8)*** or have inhibited expression of a specific integrin ***(9)***. This can greatly enrich the population of transfectants, allowing for the faster isolation of transfected cell lines. In clonal cell lines, flow cytometric analysis usually shows a Poisson distribution of integrin expression (**Fig. 1**). Any portion of this population can be selected and sterilely sorted for further study and propagation. Through this method, cells can be isolated that have either low or high expression of a particular integrin. Further growth of these selected cells creates a population with slightly higher or lower expression of integrins. Multiple sorts for higher or lower expression of an integrin can result in the isolation of a cell population with 2- to 10-fold differences in integrin expression; this is comparable to population changes effected through gene transfection methodologies ***(9)***.

1.4. Strategy for Examining and Isolating Cell Populations with Specific Integrin Expression

In the methods section, we describe how flow cytometry and sterile sorting were used to isolate a population of MDA-MB-231 breast cancer epithelial cells with sevenfold lower expression of α_2 integrin. These methods are useful for cells that are hard to transfect. In addition, these methods do not genetically alter the cell. The methods section is broken down into two main portions:

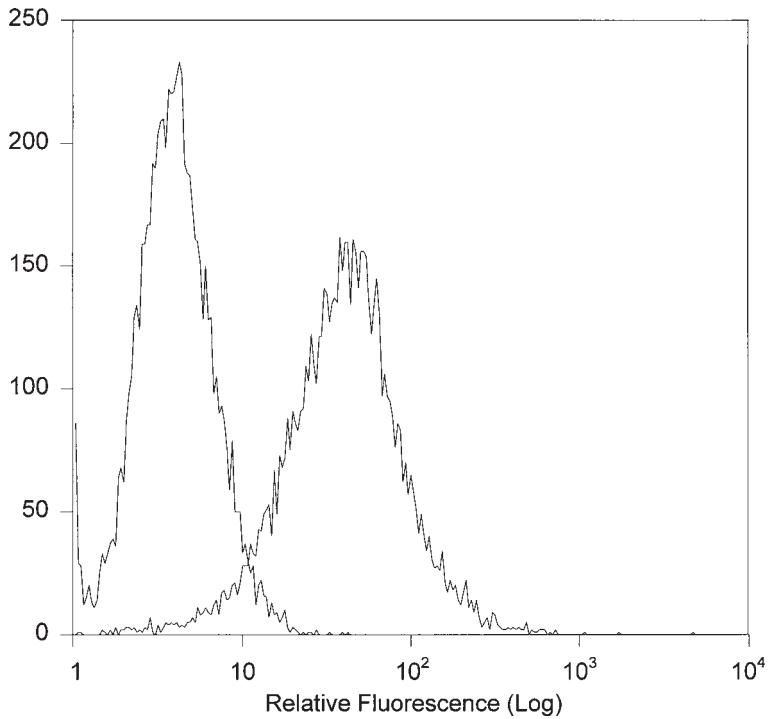

Fig. 1. Poisson distribution of expression. The rightmost plot shows the expression of α_2 integrin on MDA-MB-435 cells. The leftmost plot is the secondary antibody control.

1. In **Subheading 3.1.** flow cytometry is used to evaluate the expression of integrins on the surfaces of adherent epithelial cell lines. This method was used to assess integrin expression in a panel of breast cancer cell lines ***(10)***.
2. In **Subheading 3.2.** sterile sorting is used to select a population of MDA-MB-231 cells with low expression of α_2 integrin.

2. Materials

2.1. Flow Cytometric Analysis of Epithelial Cells

1. Primary antibodies: The most important reagents for flow cytometry are specific antibodies. There are three major types of specific antibodies for analysis of integrins. There are numerous antibodies that recognize a single α or β subunit such as GoH3 ***(11)***, which recognizes α_6 integrin. Some antibodies recognize a conformational epitope created when the integrins form a heterodimer [i.e. DATK32, $\alpha_4\beta_7$ ***(12)***; 6F1, $\alpha_2\beta_1$ ***(13)***; BHA2.1, $\alpha_2\beta_1$ ***(14)***; LM609, $\alpha_v\beta_3$ ***(15)***; and P3G2, $\alpha_v\beta_5$ ***(16)***]. These types of antibodies provide outstanding specificity for flow cytometry and may be of great use in demonstrating that both members of a heterodimeric pair are present; however, these antibodies will not work if only

one member of the heterodimer is present. A subset of conformational epitope antibodies are the LIBS antibodies. These antibodies recognize a specific epitope that is exposed only when the integrin heterodimer binds ligand ***(6)*** [i.e. LIBS1 and Ab15 recognize β_3 ***(6)***; PAC1 recognizes $\alpha_{IIb}\beta_3$ ***(17,18)***; HUTS-21 recognizes ligand bound β_1 on $\alpha_4\beta_1$ and $\alpha_5\beta_1$ ***(19,20)***]. These antibodies have been used in flow cytometry to identify ligand-bound integrin heterodimers. Lastly, some antibodies recognize the cytoplasmic tail of the integrin ***(21)***. These antibodies will be less effective for use in flow cytometry because in intact viable cells, the antibody could not cross the cell membrane to recognize the cytoplasmic epitope. If an antibody to a cytoplasmic epitope is to be used, the cell must be fixed in paraformaldehyde and permeabealized with a detergent such as Triton X-100. This will allow the antibody to cross the cell membrane; however, analysis will be of total integrin expression rather than expression on the cell surface. This method should only be used if there are no alternative antibodies available. A good reference in which to find commercially available antibodies to integrins is *Linscott's Directory of Immunological and Biological Reagents* ***(22)***. Companies that typically carry antibodies to integrins include Pharmingen (San Diego, CA) and Chemicon International (Temecula, CA). For short-term storage (<1 mo) store antibodies at 4°C. Antibodies can be damaged by freezing and thawing, therefore, for long-term storage, unless otherwise directed, aliquot the antibody and freeze at –80°C.

Another major consideration of antibodies is sterility. Flow cytometry can sterilely sort cells but will not sterilize a nonsterile cell preparation. Antisera or antibodies raised as an ascites may not be sterile and may include viruses, bacteria, fungi, mycoplasma, and so on. These contaminants are not a problem if the antibodies are being used for flow cytometric analysis of cells, but if the antibodies are being used for sterile sorting, the antibodies should be sterile. Sterile filtering of the antibodies will decrease the risk of contamination from bacteria and fungi. To minimize loss of antibody when filtering, the antibody should be filtered with a low-protein-binding filter in media with serum or bovine serum albumin (BSA) as a carrier. If possible, a sterile antibody should be purchased for sterile sorting. Lastly, some antibody preparations contain azide to inhibit bacterial growth. If possible, antibodies with azide should not be used for sterile sorting, since azide is a potent poison and the cells will be incubated with the antibodies for 1–2 h.

2. Secondary antibodies: Antibodies to integrins are not routinely available conjugated to a fluorochrome. As a result, fluorochrome-conjugated secondary antibodies must be used. These must be matched to the species (goat, rat, mouse, hamster) and isotype (IgG1, IgA, IgM) of the primary antibody. Most flow cytometers use a laser with a wavelength of 488 nm. This wavelength will excite both fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE), which emit at 520 and 575 nm, respectively. If two-color flow cytometry is to be performed, it will be necessary for the primary antibodies to be of different species, which allows two different fluorochrome-conjugated secondary antibodies to be used.

It is important to know the fluorochrome present on the secondary antibody. Fluorescein conjugated antibodies will appear yellow in white light and R-phycoerythrin conjugated antibodies will appear pink. Numerous companies sell conjugated secondary antibodies. Fluorochrome conjugated antibodies are light sensitive and should be stored wrapped in foil at 4°C.

3. Sterile polypropylene or polystyrene 12 × 75-mm snap-cap tubes (Becton Dickinson, Franklin Lakes, NJ).
4. Sterile serologic pipets (Becton Dickinson) and sterile Pasteur pipets.
5. Phosphate-buffered saline (PBS): 130 m*M* NaCl, 10 m*M* NaH_2PO_4, 10 m*M* $NaHPO_4$ at pH 7.4.
6. Solution to detach cells: Sterile trypsin EDTA solution (Gibco-BRL, Grand Island, NY), or 0.53 m*M* EDTA in PBS or Dispase (Gibco-BRL).
7. Sterile IMEM + 2% FBS: Improved minimum essential media, Eagle's (Gibco-BRL) (IMEM) + 2% serum. Media should be used that promotes the health of the cells as they will be in the media for 2–3 h and undergo the stress of centrifugation and flow cytometry. If a avidin biotin method is to be used in the flow cytometry protocol; IMEM, RPMI, Ham's F10, and Ham's F12 media should be avoided because they contain added biotin. L-15, Eagle's minimum essential media, and Dulbecco's modified Eagle's medium do not contain biotin.
8. Hemacytometer and phase-contrast microscope. Levy Hemacytometer (Reichert, Buffalo, NY).
9. Syringe (Becton Dickinson) and syringe filter (Gelman Sciences, Ann Arbor, MI).
10. Aluminum foil.
11. Forceps.
12. 100% ethanol.
13. Sterile nylon mesh for filtering cells. 35 μm nylon mesh monofilament cloth (Small Parts Inc., Miami Lakes, FL) cut into 2 × 2-cm squares and autoclaved in foil for later use. Alternatively, sterile cell strainers that feature a 40-μm mesh in a polypropylene frame can be purchased from Becton Dickinson.
14. Flow cytometer or access to a flow cytometry facility. Most flow cytometers are sold by two companies Becton Dickinson (FACScan and FACStar cell sorters) and Coulter (Coulter Elite flow cytometer, Hialeah, FL). The plots presented were obtained on a FACStar^{PLUS} flow cytometer in the Lombardi Flow Cytometry Core Facility (Becton Dickinson).

2.2. Flow Cytometric Cell Sorting for Isolating Integrin-Expressing Subpopulations

1. All items from **Subheading 2.1.**
2. Sterile IMEM + 20% FBS: Collection media for sterile sorting. Includes twice the typical concentration of serum. During sorting, this media will be diluted with cells in serum-free sheath buffer from the flow cytometer.
3. Antibiotics: If flow cytometry is to be used for sterile selection of cells, antibiotics should be added to all media. Penicillin-streptomycin, kanamycin, or gentamicin (Gibco-BRL) are all suitable for killing bacteria. Under poor sorting

conditions, an antifungal agent such as amphotericin B may need to be added to prevent fungi and yeast. Because antifungal agents can be harsh to tissue-culture cells, they should only be used when improvements in sterile technique and sample handling still can not avoid contamination.

4. Tissue culture plasticware. Sterile 25- and 75-cm^2 tissue culture flasks (Becton Dickinson).
5. Cell freezing media: Media containing 7.5% DMSO, 20 m*M* HEPES, 10% FBS, penicillin, and streptomycin (Gibco-BRL).

3. Methods

3.1. Flow Cytometric Analysis of Epithelial Cells

1. The first step in flow cytometric analysis for adhesion molecules is growing the cells to the proper density. Cells should be between 60 and 80% confluent. Cells that are highly confluent tend to be difficult to remove from the flask and may come off in multicellular clumps. This will result in poor staining of individual cells, because not all of the cell is exposed to antibody. Cell clumps can clog a flow cytometer interrupting analysis. Flow cytometry does not require many cells. A typical flow cytometry histogram contains 1×10^4 cells. Analysis of the expression of a single integrin with appropriate controls can be performed with as few as 2×10^5 cells.
2. Wash cells once with sterile PBS to remove serum and media, this will aid in removal of the cells from tissue-culture plastic. Cells will round slightly in PBS. There are several ways to remove cells from tissue culture plastic. Cells can be removed with PBS + EDTA, Trypsin EDTA, or dispase. Prior analyses in our laboratory has not observed a difference in integrin expression if the more gentle PBS + EDTA is used, compared to the more harsh trypsin EDTA. If possible use the most rigorous method available as it will reduce multicellular clumps. All three methods use the same procedure. Aspirate off the PBS and add 4–6 mL of PBS + 0.53 m*M* EDTA or trypsin EDTA or dispase and let stand for 1–2 min. Aspirate off EDTA or proteinase and let cells stand for approx 2 min. Examine the cells by phase contrast microscopy, when cells begin to peel off the flask, add 6 mL of ice-cold IMEM + 2% FBS and agitate the flask gently to remove cells. Disperse the cells by pipetting the media back and forth with a 10-mL serological pipet.
3. Sterilely remove 50 µL of cell suspension and examine it on a hemocytometer. Examine the cells to determine their total number and to determine whether there are multicellular clumps. If there are some clumps, try to break up the clumps by pipetting the cell suspension back and forth through a 10-mL pipet. This can gently break up clumps.
4. Transfer the cell suspension to labeled 12 × 75-mm snap-cap tubes. The number of tubes needed depends on the number of integrins to be examined on the cells. One tube is needed for each antibody (primary and secondary) used plus an additional negative control tube. For example: if you are examining integrins α_2 and β_1, you will need five total tubes; one negative control, one tube for each second-

ary antibody used, one tube for anti-α_2 and its secondary antibody, and one tube for anti-β_1 and its secondary antibody. Fewer cells can be placed in the negative control as only 5×10^4 cells are used for gating the cell population and setting limits on the flow cytometer.

5. Centrifuge the cells in snap-cap tubes at low speed (400*g*) for 10 min.
6. While the cells are centrifuging, prepare 100 μL of primary antibody incubation media for each antibody to be examined. Typically 2–10 μg/mL of primary antibody is sufficient to observe integrins. For an initial analysis of a cell line with an anti-integrin antibody, you should titrate the antibody. A recommended range of concentrations is 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/mL of antibody. Too little antibody will result in low staining, too much antibody will waste antibody and may increase nonspecific staining. The integrin antibody (typically 1–2 μL) is added to cold IMEM + 2% FBS and sterilized by expressing it through a syringe filter into a sterile tube.
7. After centrifugation, aspirate the media containing residual EDTA and proteases. Resuspend the cells with 4 mL cold IMEM + 2% FBS to wash out residual EDTA and proteases. Centrifuge the cells for 10 min at 400*g* and aspirate media. The cells in the negative control, and secondary antibody control tubes are resuspended in 1 mL cold IMEM + 2% FBS and placed on ice. The cells to be incubated with primary antibody (anti-integrin) are resuspended in 100 μL of ice-cold primary antibody incubation media. The cell/antibody suspension is incubated on ice for 1 h, with intermittent agitation (invert the tube every 15 min).
8. After incubation, suspend the cell/antibody suspension with 4 mL cold IMEM + 2% FBS to dilute unbound antibody. Centrifuge the cells for 10 min at 400*g*. Aspirate media and resuspend pellet in 4 mL IMEM + 2% FBS. Centrifuge both primary antibody tube(s) and the tube (s) for secondary antibody analysis 10 min at 400*g*.
9. While cells are centrifuging prepare secondary antibody media. Dilute secondary antibody in ice-cold IMEM + 2% FBS. Concentrated stock solutions of secondary antibody usually work well at a concentration of 0.05–1.0 μg/mL. For an initial analysis, the secondary antibody should be titrated. A recommended range of concentrations is 0.05, 0.1, 0.25, 0.5 and 1.0 μg/mL of antibody. As with the primary antibody, insufficient secondary antibody will result in weak staining, too much secondary antibody can result in nonspecific staining and give an artificially high background. This is why cells are segregated specifically for analysis by secondary antibody. Titration of both primary and secondary antibodies may take two or more analyses. For the first analysis, choose a higher concentration of secondary antibody (0.5 or 1.0 μg/mL) while titrating the primary antibody. Once the primary antibody has been optimized, titrate the secondary antibody to give as little nonspecific fluorescence as possible and still give a strong signal with primary.
10. Aspirate media from the cell pellets and add 100 μL of secondary antibody incubation media. Snap on caps and return all tubes to ice. Cover samples with foil to protect the fluorochrome and incubate on ice for 45 min with intermittent agitation.

11. After incubation, dilute the cell/antibody suspension in the primary and secondary antibody tubes with 4 mL cold IMEM + 2% FBS to wash out unbound secondary antibody. Centrifuge the cells for 10 min at 400*g*. Aspirate media and resuspend cell pellets in 4 mL IMEM + 2% FBS. Centrifuge cells for an additional 10 min at 400*g*.
12. After centrifugation, aspirate media and resuspend cell pellets in cold IMEM + 2% FBS. Cells should be resuspended at 1–2 × 10^6 cells/mL for flow cytometry. A lower concentration of cells will result in a long, tedious, sort. Higher concentrations of cells may result in coincidence where two cells are analyzed simultaneously.
13. If examination of the cells at **step 4** identified a large number of multicellular clumps, filter the cells through sterile nylon mesh into new, sterile 12 × 75-mm snap-cap tubes. Sterilize a pair of forceps by immersing them in 100% ethanol and passing them through the flame of a Bunsen burner. Pipet the cell suspension up into a 1000-μL pipet tip on a pipettor. Grab a sterile piece of nylon mesh with the forceps and place it on top of an open 12 × 75-mm snap-cap tube. Push the filter mesh against the inside of the tube with the 1000-μL pipet tip and express the cells through the filter mesh and down the inside wall of the snap cap tube. Remove the filter mesh and cap the tube. Repeat this process with sterile forceps, mesh, and pipet tips for each cell sample to be analyzed.
14. Analysis will consider forward scatter (FSC), side scatter (SSC) as well as fluorescence intensity. Forward scatter measures the size of the cell, whereas 90° side scatter measures the granularity of the cell caused by reflection of the light. These parameters should be used to identify a population of single cells. Multicellular clumps will have increased side scatter and somewhat increased forward scatter. Similarly, cell debris will have decreased forward scatter. The population can then be gated with both forward and side scatter to analyze the single-cell population. Analysis of this single-cell population will determine the baseline autofluorescence from the cells.
15. All samples are analyzed for fluorescence intensity. Analysis of the sample with secondary antibody only identifies how much fluorescence is caused by nonspecific association of secondary antibody with the cells. Nonspecific fluorescence or background autofluorescence should be subtracted from fluorescence obtained from cells incubated with anti-integrin primary antibody and secondary antibody to obtain the relative fluorescence as a result of the integrin antibody.

3.2. Flow Cytometric Cell Sorting for Isolating Integrin-Expressing Subpopulations

The protocols for cell sorting are essentially the same as analysis of integrin expression except that all reagents, including antibodies, must be sterile (*see* **Note 1**).

1. Cell number: For cells that are to be selected based on integrin expression, the initial number of cells needed will be determined by the number of cells you need at the end. For example: Some cell types die or grow poorly when plated at low

seeding density. To insure survival of selected cells may require a minimum of 5×10^4 cells. If the protocol selected is to isolate cells that represent the highest 1.0% of expression for a particular integrin, a minimum of $5–6 \times 10^6$ cells ($5 \times 10^4 / 0.01 = 5 \times 10^6$) will need to be analyzed.

2. Cells are washed with PBS, trypsinized, and placed in a sterile 15-mL conical tube.
3. Centrifuge the cells at low speed (400*g*) for 10 min and resuspend the cells in 4 mL of sterile cold IMEM + 2% FBS.
4. Place $3–4 \times 10^4$ cells in two 12 × 75-mm snap-cap tubes labeled as negative control and secondary antibody control.
5. Place the remaining cells to be sorted in a sterile 12 × 75-mm snap-cap tube and centrifuge the cells at 400*g* for 10 min.
6. Resuspend cells in filter sterilized primary antibody incubation media as described in **Subheading 3.1.**
7. Proceed with protocol as described in **Subheading 3.1.** to **step 12**.
8. Cells should be resuspended at $1–2 \times 10^6$ cells/mL for flow cytometry. A lower concentration of cells will result in a long, tedious, sort. Higher concentrations of cells may result in coincidence, allowing two cells being analyzed simultaneously. This is especially troublesome because coincident cells may both be sorted, diluting the population of cells with the desired expression.
9. Label a separate set of 12 × 75-mm snap-cap tubes and fill each with 2 mL of growth media containing antibiotics and twice the concentration of serum (20% FBS). These tubes are used to collect the sorted cells.
10. Cell sorting proceeds the same as analysis except that the analyzer is programmed to deflect cells with certain characteristics (fluorescence, side scatter, or forward scatter) from the flow stream to collection containers. For cell selection, the stream of cells in buffer flowing through the analyzer is charged. Droplets of buffer containing cells break off after analysis, retaining the charge. The droplet passes charged plates that can attract or repel the charged droplet. Typically, this process allows a maximum of two populations to be simultaneously sorted (droplets deflected by positive charge or by a negative charged plate) into 12 × 75-mm snap-cap tubes. If sorting untransfected cell lines, it is useful to save a population of sterilely sorted cells that have the median expression level. This will serve as a control that changes in integrin expression are not caused by the trauma of sterile sorting. The analyzer can count the number of droplets deflected giving a good approximation of the number of cells collected.
11. Immediately after sorting, place sorted cells into a 25-cm^2 tissue culture flask containing 15 mL of warm (37°C) IMEM + 10% FBS containing antibiotics.
12. Grow cells until 80% confluent. Passage cells into a 75-cm^2 tissue-culture flask. Grow cells until confluent. Passage into two or more 75-cm^2 tissue culture flasks. Grow until 80% confluent and freeze cells from one 75-cm^2 flask in 3–5 1-mL aliquots of freezing media.
13. To further enrich the selected population, repeat the sorting protocol. The results obtained in **Fig. 2** required five sorts. Selection of cell lines will be gradual; however a continuous change in integrin expression should occur in the selected direction

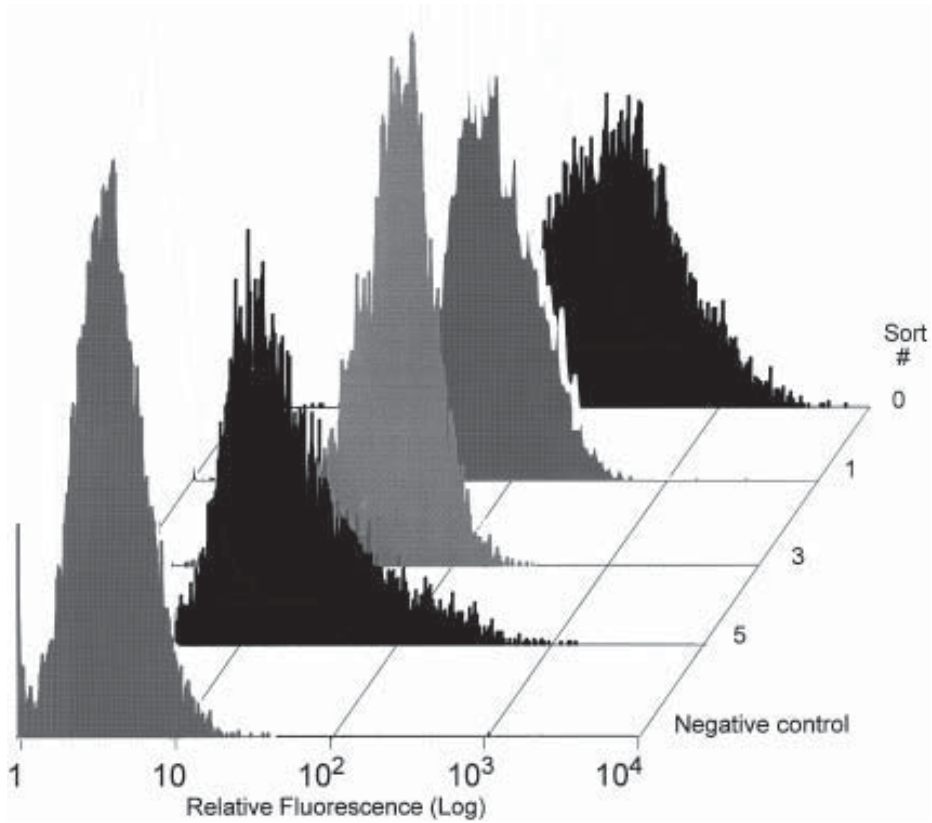

Fig. 2. Flow cytometric analysis of integrin α_2 expression on MDA-MB-231 cells. Cells were selected for lowest 2% expression in each round of selection. Median values are 212 for the initial sort, 77.7 for sort 1, 45.3 for sort 3, and 18.4 for sort 5. Negative control is MDA-MB-231 cells treated with secondary antibody only.

(either positive or negative) after each sort (*see* **Notes 2** and **3**). When possible, freeze cells after each sort so the entire process does not have to be repeated if the cells get contaminated or otherwise perish. This also allows for retrospective analyses.

4. Notes

1. Sterile sorting of cell lines for expression of a particular integrin is a gradual process, that is particularly useful when cells are difficult to transfect. A sterile sorted population may shift quickly if the loss or gain of expression confers a growth advantage (unrestricted growth or escape from apoptotic pathways ***[23]***). Similarly, a sterilely sorted population may not retain the desired expression level if it confers a growth disadvantage (more restricted growth) and may sort slowly or not at all.
2. We have found difficulty in increasing expression of an integrin in cell lines that already express large amounts of that integrin. These cells tend to grow slower

and the increased adhesion poses increased technical difficulties in obtaining single cells and avoiding multicell clumps.

3. Rapid isolation of cells with very different integrin expression patterns should be regarded with caution. On one occasion, we obtained a very rapid isolation of two cell populations from a supposedly clonal cell line. Upon further analysis, one cell population did not express the integrin at all. DNA fingerprint analysis determined that the original population was a mixed population and the sterile selection had isolated the contaminating cells. It is recommended that prior to analysis of sterilely sorted cells, the cells be characterized (i.e., DNA fingerprinting, cell marker, or Southern blot) to ensure they are descendants from the parental cell line.

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7

Expression of Integrin Transcripts in Human Cancer Cells

Wadih Arap and H.-J. Su Huang

1. Introduction

Integrins are a well-studied class of cell-surface receptors involved in cell-matrix interactions. All integrins are heterodimeric glycoproteins composed of an α and a β chain. Integrins belong to a large family of proteins. Currently over 16 α chains and 9 β chains have been reported. The particular combination of α and β chains largely determines ligand specificity ***(1,2)***.

Several lines of evidence indicate that integrins are important in a variety of cancer-related processes ***(3)***. First, integrins play a key role in the malignant behavior of cells from malignant melanoma ***(4–6)***, sarcoma ***(7)***, lymphoma ***(8)***, and cancers of breast ***(9)***, prostate ***(10)***, lung ***(11)***, and many other tumor cell types ***(3)***. Second, integrin expression alters oncogenic transformation ***(12)***, tumor progression, and metastasis ***(3)***. Overexpression of $\alpha_5\beta_1$ in Chinese hamster ovary cells ***(13)*** and of $\alpha_2\beta_1$ in human breast cancer cells ***(14)*** suppresses tumorigenesis. Heterologous mis-expression of the $\alpha_2\beta_1$ integrin in human rhabdomyosarcoma cells increases experimental pulmonary metastasis in nude mice ***(7)***. In contrast, the expression of the $\alpha_4\beta_1$ integrin in murine melanoma cells suppresses the ability of these cells to metastasize to the lung ***(15)***. Third, selective blocking of integrin function peptides which mimic integrin binding sites, such as Arg-Gly-Asp (RGD; **ref. *2***) or by a polymeric form of fibronectin leads to inhibition of tumorigenesis and metastasis in experimental systems ***(16,17)***.

Expression of integrins is also relevant in preclinical and clinical settings. Decreased integrin α_3 expression has been shown recently to correlate with a poor prognosis in patients with adenocarcinoma of the lung ***(18)***. Furthermore, upregulation of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins has been observed in the blood vessels of tumors ***(19)***, and blocking of the α_v integrins by specific antibodies

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(19,20), cyclic RGD peptides *(19–21)* or RGD peptidomimetics *(22)* can inhibit neovascularization. This specific expression pattern has allowed novel strategies to target tumor vasculature *(23–25)*. The significance of integrin expression has not been fully explored yet and methods to examine such expression will find clinical relevance.

Individual integrin chain RNAs can be quantitated by Northern blotting or by RNAase protection assays. However, these methods are time-consuming, relatively insensitive, and require microgram quantities of total RNA. Therefore, reverse transcriptase-mediated polymerase chain reaction (RT-PCR)-based methods have been developed. Some approaches use the principle of competitive PCR during the amplification step, in which a reporter gene is co-amplified along the target integrin gene. Nonetheless, we will describe only nonquantitative RT-PCR detection of integrin RNA expression.

RT-PCR is useful for detection of integrin expression in cell lines and tissues. For detection of integrin transcripts, RT-PCR is a simple, fast, and an extremely sensitive screening assay. Another advantage of RT-PCR methods is that fresh cells are not required and only a minute amount of RNA is needed. These features make RT-PCR particularly suitable for analyzing gene expression in clinical samples in which the quantity of material is limiting. Possibly, new technologies such as laser capture microdissection will add further value to RT-PCR-based approaches.

Here we present a series of primer sequences and protocols designed for rapid screening of integrin RNA expression in a panel of human cancer cell lines. This type of analysis may be used as a complement to fluorescence activated cell sorting (FACS) analysis or can be used alone if antibodies or cells are not available.

2. Materials

1. Trizol (Gibco-BRL, Gaithersburg, MD) reagent.
2. Chloroform.
3. 100% and 70% ethanol.
4. RNAase-free double-distilled water (ddH_2O) and TE buffer (*see* **Note 1**).
5. 1× PBS and EDTA/PBS (Irvine Scientific).
6. **Table 1** lists the reagents and the protocol for RT-PCR amplification. The reagents used in this chapter were purchased mainly from Perkin Elmer (Norwalk, CT), but other commercial vendors (such as Gibco-BRL; Boehringer-Mannheim Biochemicals, Indianapolis, IN; New England Biolabs, Beverly, MA; Stratagene, La Jolla, CA; Promega, Madison, WI; and so on) may be used as sources.
7. **Table 2** contains the sequence of sense and antisense oligomers used to identify individual integrin chains. Processing of primers included synthesis, desalting, and purification by reverse-phase cartridge by Genosys Biotechnologies, The Woodlands, Texas (*see* **Note 2**).

Table 1
Materials and Protocols for RT and PCR Reactions

Component	RT mix (μL)	PCR mix (μL)
Template	0.5[a]	10.0[f]
$MgCl_2$, 25 m*M*[b]	2.0	2.0
10 × PCR buffer II[b]	1.0	4.0
dd H_2O	0.5[c]	32.0
dNTP mix, 10 m*M* each[d]	4.0	–
Antisense primer, 0.1 μg/μL	1.0[e]	–
Sense primer, 0.1 μg/μL	–	1.0
RNAase inhibitor, 20 U/μL[b]	0.5	–
Reverse transcriptase[b]	0.5	–
Taq DNA polymerase[b]	–	1.0
Total	10.0	50.0

[a]Total RNA @1 μg/μL.
[b]RT-PCR kit (Perkin Elmer, cat. no. N808-0017). Other vendors may also be used (see text).
[c]DEPC-treated H_2O must be used.
[d]dNTP mix concentration may be increased (up to 25 m*M* of each dNTP) during optimization.
[e]*See* **Note 6**.
[f]RT reaction mix.

3. Methods

3.1. Total RNA Preparation

1. For attached cell lines, harvest cells at approx 75% confluency (*see* **Notes 3** and **4**).
2. Wash cells twice with 1× PBS.
3. Detach cells with EDTA/PBS (*see* **Note 5**).
4. Centrifuge cells, remove supernatant.
5. Add Trizol reagent (Gibco-BRL) and follow the manufacturer's protocol.
6. Check OD_{260}, OD_{280}, and calculate the OD_{260}/OD_{280} ratio (optimal ratio = 2) to check RNA purity. We also electrophorese an aliquot of the samples on an agarose gel to ensure that the RNA is intact.
7. For cell lines grown in suspension, pellet the cells by centrifugation, and follow **steps 4–6** (*see* **Note 4**).
8. Store RNAs at –80°C.

3.2. Reverse Transcription

Table 1 shows an example of a protocol for reverse transcription (RT) of integrin RNA transcripts.

1. Add reagents to a microtube (Perkin Elmer, cat. no. N801-0540; or equivalent) in the following order: DEPC-treated ddH_2O, 10× buffer II, $MgCl_2$, dNTP mix,

Table 2
Human Integrin Chain Primers and Amplified Products

Integrin chain	Sense/ Antisense	Primer sequence (5' and 3')	AT/GC	Primer region	Product size (bp)
α_2	Sense	CACTCGATTTGGTTCAGCAA	11/9	1701–1720	283
	Antisense	GAACCACTTGTCCAAAGGCA	10/10	1984–1965	
α_3	Sense	GCCAGCATTGGTGACATCAA	10/10	1193–1212	179
	Antisense	GAATAGCCGAAGGTGGCCAA	9/11	1371–1352	
α_4	Sense	ATGCTGCAAGATTTGGGGAA	11/9	1134–1153	265
	Antisense	GCACCAACTGCTACATCTAC	10/10	1398–1379	
α_5	Sense	CCAGGATGGCTACAATGATG	10/10	1232–1251	222
	Antisense	CCCACAATCAGATCAGGATA	11/9	1453–1434	
α_6	Sense	CAAGATGGCTACCCAGATAT	11/9	1312–1331	210
	Antisense	CTGAATCTGAGAGGGAACCA	10/10	1521–1502	
α_V	Sense	AGATCTGGACCAGGATGGTT	10/10	1174–1193	197
	Antisense	ATCTGTGGCTCCTTTCATTG	11/9	1370–1351	
α_L	Sense	GAAGAAGTCTCAGAGCTGCA	10/10	1606–1625	273
	Antisense	ATCCCCTTCAAGGTCCTTCA	10/10	1878–1859	
α_M	Sense	TGTGATGCTGTTCTCTACGG	10/10	1657–1676	300
	Antisense	TCCTACAGTCAGGTCTACCA	10/10	1969–1950	
α_X	Sense	GTGCTGTCTACCTGTTTCAC	10/10	1710–1729	158
	Antisense	AGCCAGGTCCACCAGTCCAT	8/12	1867–1848	
α_{IIb}	Sense	CTGACTGGCACACAGCTCTA	9/11	1133–1152	241
	Antisense	ATGTCTACGGCACCTCGAAG	9/11	1373–1354	
β_1	Sense	GTTACACGGCTGCTGGTGTT	9/11	911–930	264
	Antisense	CTACTGCTGACTTAGGGATC	10/10	1175–1156	
β_2	Sense	ACCTGGAGGACAACTTGTAC	10/10	933–952	206
	Antisense	TGAGATGGACCACATTGCTG	10/10	1138–1119	

β_3	Sense	ACCACTGATGCCAAGACTCA	10/10	843–862	296
	Antisense	GCATCAACAATGAGCTGGAG	10/10	1138–1119	
β_4	Sense	AACGATGAACGGTGCCACCT	9/11	859–878	222
	Antisense	CTCCACGATGTTGGACGAGT	9/11	1080–1061	
β_5	Sense	AGGATGCACTGCATTTGCTG	10/10	1136–1155	273
	Antisense	TCCACCGTTGTTCCAGGTAT	10/10	1409–1390	
β_6	Sense	GGAATGGACAGCAAACTAGC	10/10	1055–1074	243
	Antisense	GGAGTCCTTCTGAAGTAGAC	10/10	1297–1278	
β_7	Sense	TGCTGGTGTTCACTTCAGAC	10/10	999–1018	307
	Antisense	GCATCCATGATGAGCTGTAC	10/10	1305–1286	
β_8	Sense	ATGACGGAAACTGTCATCTG	11/9	1588–1607	182
	Antisense	AGCAATGGTGCCTGGCAAGA	9/11	1769–1750	

RNAase inhibitor, antisense primer, and total RNA. Oligo(dT) may be used as the antisense primer instead of the integrin-specific primer (*see* **Note 6**).
2. Incubate at 65°C for 10 min. Cool the reaction mix to 37°C.
3. Add reverse transcriptase.
4. Incubate the RT reaction at 37°C for 1 h. For RT using Superscript (Gibco-BRL), incubate at 42°C for 1 h. We find convenient to perform the RT reaction in a thermal cycler (PCR System 9600, Perkin Elmer; or equivalent).

3.3. PCR Amplification

Table 1 shows an example of protocol for PCR amplification of integrin cDNAs.

1. Add the reagents to the RT reaction mix in the following order: ddH_2O, 10× buffer, $MgCl_2$, sense primer, and *Taq* DNA polymerase.
2. Amplify the DNA in a thermal cycler (PCR System 9600, Perkin Elmer; or equivalent) using the following cycle profile (*see* **Note 7**):

First cycle	95°C × 3 min (initial denaturation)
	95°C × 1 min (denaturation)
35–40 main cycles	TM × 1 min (annealing)
	72°C × 1 min (extension)
Final cycle	72°C × 10 min (long extension)

3. Store samples short term at 4°C or freeze them at –20°C.

3.4. Gel Electrophoresis of the RT-PCR Products

1. The diagnostic bands are between 158 and 307 bp (**Table 2**).
2. For optimal detection, we use a minigel containing a mix of 3% Nusieve agarose and 1% Seakem agarose (both from FMC) in 1× TAE, with 5 μg/mL of ethidium bromide.
3. We recommend running an aliquot of 10–20 μL of the sample with standard DNA loading buffer.
4. Run appropriate size markers. We use either the 100-bp DNA ladder or the 1-Kb DNA ladder (Gibco-BRL). Examples of typical results are shown in **Fig. 1**.

3.5. Selection of Control Cells for Detection of Integrin Expression

A panel of human cell lines that can be used to assess integrin expression by RT-PCR is shown in **Table 3** (*see* **Note 8**). These cell lines serve as both positive and negative controls (*see* **Notes 9** and **10**). Any human cancer cell with an unknown integrin chain expression profile can be tested using this RT-PCR protocol.

4. Notes

1. RNAase-free buffers can be obtained from commercial vendors or, alternatively, ddH_2O may be DEPC-treated. Make all containers and materials RNAase-free.
2. The primers must be designed so that they flank an intron. This design will prevent or minimize amplification of contaminant genomic DNA. As an additional control, the total RNA template may be treated with RNAase-free DNAase. Despite

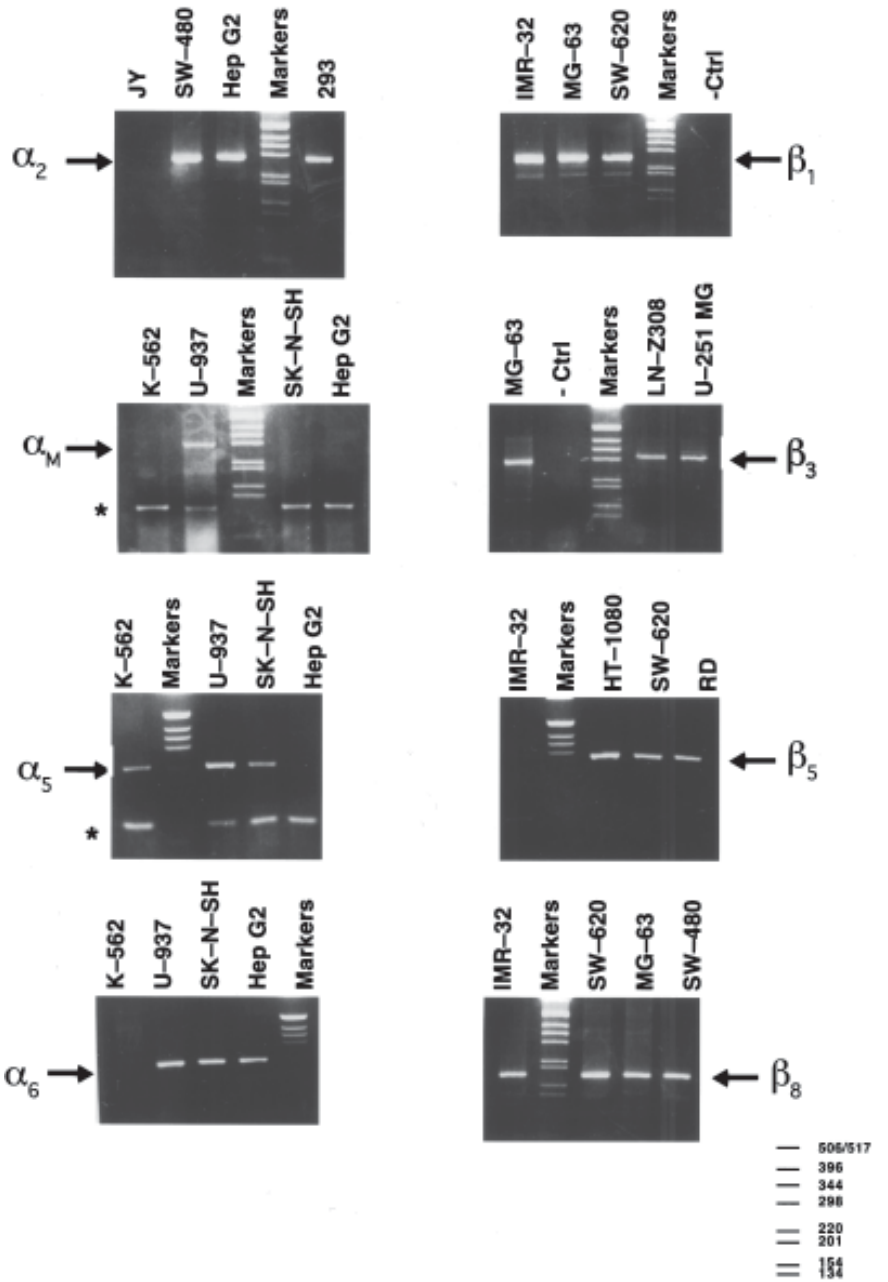

Fig. 1. Integrin Expression in Cancer Cells Determined by RT-PCR. α_2, α_5, α_6, α_M, β_1, β_3, β_5, and β_8 chains are shown as examples. Bands corresponding to integrin transcripts are shown by arrows; (*) indicates nonspecific bands. PCR reactions without corresponding RT reactions (no reverse transcriptase added) are used as the negative controls for β_1 and β_3. Agarose gel electrophoresis was performed as described (**Subheading 3.4.**). A schematic representation of the DNA ladder used as a size marker (bp) is shown on the lower right of the figure.

Table 3
RT-PCR Expression of Integrin Chains in Human Cancer Cell Lines

Integrin chain/ Cell line	α_2	α_3	α_4	α_5	α_6	α_V	α_L	α_M	α_X	α_{IIb}	β_1	β_2	β_3	β_4	β_5	β_6	β_7	β_8
DLD-1	+	+	–	+	+	+	–	–	–	+	+	–	+	+	+	+	+/–	+
D-247MG	+	+	+	+	+	+	–	–	–	+	+	–	+	+/–	+	–	+/–	+
HT-1080	+	+	–	+	+	+	–	–	–	+	+	–	+	+	+	–	+/–	+
IMR-32	+	–	+	–	+	+	–	–	–	+	+	–	+	–	–	+	–	+
JY	–	–	+	–	–	+/–	+	+	+	+	+	+	+	+/–	+	–	+	+
K-562	+/–	–	–	+	–	+	–	–	–	+	+	–	+	–	+	–	+/–	–
MG-63	+	+	+	+	+	+	–	–	–	+	+	–	+	–	+	–	+/–	+
PEER	–	–	+	+	+	–	+	–	–	–	+	+	+	–	–	–	+	–
RD	–	+/–	–	+	+	+	–	–	–	+	+	–	+	–	+	+	–	–
SK-N-SH	+	+	+/–	+	+	+	–	–	–	+	+	–	+	+/–	+	–	+/–	+
SW-620	+	+	–	+	+	+	–	–	–	+	+	–	+	+	+	+	+	+
U-251MG	+	+	+	+	+	+	–	–	–	+	+	–	+	+	+	–	–	+
U-937	–	–	+	+	+	+	+	+	+/–	–	+/–	+	+	+/–	+	–	+	–

(+), positive; (–), negative; (+/–), ambiguous. DLD-1, colorectal carcinoma; D-247MG, gliosarcoma; HT-1080, fibrosarcoma; IMR-32, neuroblastoma; JY, B-cell leukemia; K-562, erythroleukemia; MG-63, osteosarcoma; PEER, T-cell leukemia; RD, rhabdomyosarcoma; SK-N-SH, neuroblastoma; SW-620, colorectal carcinoma; U-251, glioblastoma multiforme; U-937, monocytic leukemia.

these precautions, nonspecific bands occur occasionally and may represent contamination by genomic DNA, imperfect annealing of primers to other known or unidentified integrin chains, or differential integrin gene splicing (more than 18 splicing variants have been identified).

3. We tested our protocol using both RNA or poly(A) RNA and found that in most cases, the integrin expression results were similar. Therefore, there is little need for laborious preparation of poly(A) RNA.
4. Expression of integrin RNA is optimal if cultured cells are maintained properly. We change the media the night before RNA isolation and harvest the cells at 75–80% confluency. For cells growing in suspension, ensure that they are in log phase by verifying the color of the media and the presence of cells undergoing mitosis upon microscopic examination.
5. We avoid the use of trypsin because it may increase release of RNAases. Instead, we use EDTA/PBS to detach cells. If trypsin mixtures are used they must be quickly neutralized.
6. Generally, comparable results were obtained when oligo(dT) or a gene-specific downstream primer (integrin antisense oligomers, **Table 2**) was used for the RT reaction. However, if oligo(dT) is used, the resulting cDNAs can serve as templates for amplifying multiple integrin chains.
7. We calculate the theoretical annealing temperature based on DNA melting temperature (TM). TMs are calculated by the equation TM = 2(A + T) + 4(G + C) and/or by the nearest neighbor method (Geneworks or equivalent software will have these features). Such calculations provide a starting point and optimal annealing conditions must be determined empirically. The primers shown in **Table 2** are mostly 20-mers with AT/CG ratios of approx 1, and their TMs are in the optimal range of approx 60–65°C.
8. Many tumor cell lines can be used for positive or negative controls in addition to the ones presented in **Table 3**. Examples include cell lines derived from carcinomas (lung, UCLA-P3; liver, HepG2; breast, MCF7; bladder, J82; cervix, HeLa; colorectal, MIP-101; epidermoid, A431), melanomas (M21, SKMEL, LOX), gliomas (U-87MG, A172), lymphoid T cells (JURKAT, HPB/ALL) or B-cells (C1R, WT6, RAMOS, B14, CC13, D475), and myeloid cells (HL-60, KG-1).
9. Given the amplification step of RT-PCR and the possibility of posttranscriptional regulation, the expression level of integrin RNA may not reflect the level of cell-surface protein. Moreover, certain integrin chains may exist as an intracellular pool unable to be expressed at the cell surface without the presence of another integrin chain. Thus, we recommend confirming of the expression results by using a nonamplification, protein-based assay. FACS analysis is the most frequently used method.
10. The expression of the α_{IIb} integrin chain has been reported to be restricted to cells of the megakaryocytic lineage, such as platelets. Nevertheless, examples of expression of this integrin in several tumor cell lines, including multiple carcinomas ***(26,27)*** and melanomas ***(28)***, have been reported recently. We repeated our α_{IIb} RT-PCR detection experiments with another independent set of human α_{IIb} primers (sense: 5'-CTCCACTGTATATGGAGAGC-3'; antisense: 5'-GTCTG

GGTATCCGTTGTCAT-3') and observed similar expression as shown in **Table 3**. However, we have not confirmed the expression of $\alpha_{IIb}\beta_3$ by FACS analysis.

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8

Functional Analysis of the β_2 Integrins

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1. Introduction

1.1. The β_2 Integrins

The interaction of integrins with their ligands is an essential step in regulating many cellular functions ***(1)***. The β_2 integrins, which are exclusively expressed on leukocytes, are of critical importance for leukocyte functions ***(2–5)***. The discovery of an inherited defect in leukocyte adhesion (called leukocyte adhesion deficiency, LAD), caused by the lack of expression of β_2 integrins on the cell surface, underscored the important biological role of these receptors in the inflammatory and immune responses ***(6)***.

The β_2 integrins (CD11/CD18) are four noncovalently linked glycoprotein heterodimers, each consisting of a distinct α subunit (CD11a, b, c, or d) noncovalently linked to a common β_2 subunit (CD18) ***(2,3,5)***. Each of the two subunits of the receptor spans the plasma membrane once and has a short cytoplasmic tail. The N-terminal half of the α subunits contains seven tandem repeats, 60 amino acids each. Three of the repeats contain EF-like calcium binding sites. In all four β_2 integrins as well as $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_E\beta_7$ integrins, an additional domain (called A or I domain), approx 200 amino acids long, is found between the second and third repeats. Structure prediction algorithms suggest that the seven repeats adopt a β-propeller fold, with the A-domain tethered by a hinge to the top of the β-propeller ***(7)***. The N-terminal region of β_2 subunit contains a predicted A-like domain ***(8)***, with the C-terminal half comprised of four cysteine-rich repeats which are characteristic of the integrin β subunits ***(9)***. Recent studies have shown that the α subunit A-domain is responsible for the binding of β_2 integrins to many of their protein ligands ***(10–15)***, with perhaps a contribution from the A-like domain of the β_2 subunit for some receptors ***(9)***. The β_2 A-like domain is also involved in formation of the heterodimer

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(6). The crystal structure of the CD11b and CD11a A-domains reveals that this domain assumes an α/β fold, with a solvent accessible metal ion-dependent adhesion site (MIDAS) on the top of the structure *(8,16)*.

The predominant cellular ligands for the β_2 integrins are the intercellular adhesion molecules ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50), which are members of the immunoglobulin (Ig) superfamily *(5)*. Soluble ligands, which mainly interact with CD11b/CD18 (also called the complement receptor 3, CR3, Mac-1, Mo1), include the complement fragment iC3b, coagulation factors fibrinogen (Fg) and factor X, and the hookworm-derived neutrophil inhibitory factor (NIF) *(2,13)*.

The activity of integrins is tightly regulated, and least pathologic adhesion should take place. Quantitative as well as qualitative changes in these receptors have been described, contributing to agonist-induced alterations in leukocyte adhesive properties. Receptor translocation from intracellular pools to the plasma membrane *(17–19)*, and redistribution of the receptors in the plane of the plasma membrane (clustering) *(20,21)*, contribute to the increased avidity of β_2 integrins in activated cells. In addition, conformational switching of individual receptor, which increases the affinity of β_2 integrins, also facilitates adhesion *(22)*. These alterations can be induced by treatment of leukocytes with phorbol esters or inflammatory mediators, or by cross-linking of different leukocyte surface glycoproteins *(23)*. Integrin-ligand interactions are metal ion-dependent. Recent studies have found that the metal ions also play an important role in regulating integrin activity *(24,25)*.

1.2. Binding Studies for β_2 Integrins: General Consideration

The generation of monoclonal antibodies *(26)* has provided powerful reagents to dissect the molecular basis of cell adhesion. Molecular characterization of the surface of leukocytes with monoclonal antibodies yielded a rich harvest of adhesion receptors, including the β_2 integrins. Functional studies at both the protein and cellular levels using monoclonal antibodies are important in defining ligands for the integrins, and mapping ligand-binding sites on these receptors. In addition, cell-based binding studies have been used to examine the role of integrins in specific adhesion-dependent tasks, and the effect of cell activation on the functional state of the receptors.

Binding studies are usually carried out using soluble or cell-associated integrins and ligands. When the two components are soluble, one (usually the integrin) is immobilized by adherence to a surface such as glass or plastic, and the free ligand is added for an appropriate period of time to allow the interacting equilibrium to be reached. After the unbound ligand is removed by washing, the bound ligand can be detected by various methods depending on how the ligand is labeled. Direct detection can be performed when the ligand is

labeled with such reagents as alkaline phosphatase, horseradish peroxidase, or radioactive iodine. A second binding step is required when the ligand is labeled with biotin, or when a labeled secondary reagent is used ***(27)***.

Cell-based binding studies are performed by incubating the cell-associated integrin or ligand with the immobilized ligand or integrin respectively, for an appropriate period of time. The unbound cells are removed by gentle washing, which is a crucial step for cell-adhesion assays. The bound cells can be detected by counting them directly under a microscope, or by employing a cellular acid phosphatase-based detection system. Alternatively, cells can be labeled directly with biotin or a radioactive tracer and detected. Cell aggregation is another type of cell adhesion assays, in which both integrin- and ligand-expressing cells are allowed to interact in the fluid phase, and the interaction can be quantified visually under a microscope or using a FACS (fluorescent-activated cell sorter) machine ***(28)***.

Like many other integrins, the β_2 integrins mediate a variety of adhesion functions in leukocytes and necessarily interact with a number of different ligands. β_2 integrins mediate firm adhesion and extravasation of leukocytes during their trafficking to inflamed sites ***(29)***. In mediating leukocyte-endothelium adhesion, β_2 integrins interact with cellular ligands such as CD54 and CD102 expressed on vascular endothelial cells, as well as with fibrinogen-immobilized on endothelium ***(30)***. The endothelial expression of CD54 can be upregulated by inflammatory cytokines such as IFN-γ and TNF-α ***(31,32)***. In contrast, CD102 is constitutively expressed on endothelial cells, and its expression is refractory to several inflammatory cytokines ***(33,34)***. The different styles of CD54 and CD102 expression indicate that CD54 may have an important role in leukocyte extravasation during inflammatory responses, whereas CD102 may be responsible for normal recirculation of lymphocytes through tissue endothelium. A soluble form of CD54 is found in human serum ***(35)***, but the affinity of soluble CD54 for CD11a/CD18 and CD11b/CD18 is too low to be detected ***(36,37)***. Recently, several studies have found that the majority of cell-surface CD54 molecules form homodimers, which show an enhanced avidity for binding to β_2 integrins ***(38,39)***. In this chapter, we will describe the binding of CD54 and CD102-bearing endothelial cells to purified CD11b/CD18 ***(40)***.

In addition to a shared role for β_2 integrins in leukocyte trafficking, each member plays distinct roles in leukocyte adhesive events ***(22)***. CD11b/CD18 has one of the broadest ligand repertoires. It is essential in promoting adhesion and phagocytosis of particles coated with the complement fragment iC3b ***(41,42)***, which only binds to CD11b/CD18 in its active state. Rosetting assays of granulocytes, monocytes, or macrophages with sheep erythrocytes coated with iC3b (EiC3b) have been described elsewhere ***(28,43)***. In this chapter, binding of EiC3b to purified CD11b/CD18 or its recombinant A-domain will be

described ***(11)***. To determine the binding properties of CD11b/CD18, a soluble, monomeric form of iC3b conjugated with alkaline phosphatase (AP) is used ***(44)***. Binding assays using iC3b-AP and purified CD11b/CD18 avoid such problems as pinocytic uptake and degradation of ligand that are inherent in cell-based assays. They also allow quantitative binding isotherms to be derived, an analysis not possible in cell-based assays in which multimerization of ligand prevents accurate determination of receptor affinity. By using this binding system, the stoichiometry of binding of iC3b to CD11b/CD18 has been shown to be 1:1, and this interaction is of high affinity (K_d approx 12.5 n*M*) ***(44)***. Monomeric iC3b also binds to the recombinant CD11b A-domain, but with lower affinity (K_d approx 300 n*M*) ***(45)***. The methods for defining the affinity of CD11b/CD18 and CD11b A-domain for iC3b are provided below.

The Hookworm-derived neutrophil inhibitory factor NIF has been defined recently as a CD11b/CD18 antagonist ***(13,15)***. NIF binds to the CD11b A-domain and partially overlaps the binding site of human iC3b in the A-domain. The interaction of NIF with CD11b/CD18 in resting and activated neutrophils revealed similar affinity (K_d approx 1 n*M*), suggesting that the NIF binding site in the receptor is activation-independent. The binding of NIF to purified CD11b A-domain will be described ***(13)***.

By using synthetic peptides, the fibrinogen γ chain region Gly^{190}–Val^{202} and the CD102 region Gly^{21}–Ser^{42} from the first Ig-like domain have been shown to function as minimal recognition sequences for CD11b/CD18 ***(46,47)***. The same peptide region from CD102 also interacts with CD11a/CD18 ***(48)***. Binding of the CD102 peptide P1 to purified CD11b/CD18 will be described ***(47)***.

Binding of integrin-expressing cells to purified ligands on an inert surface is an ideal system to study regulation of integrin avidity. In this situation, only the cell-surface receptor for the purified ligand is involved in binding, allowing regulation of its avidity to be measured in isolation from other adhesion mechanism. This system has been used successfully for defining the modulation of CD11a/CD18 avidity by cross-linking T-cell receptor ***(23)***, as well as the regulation of CD11b/CD18 and CD11c/CD18 avidity by ligating the integrins themselves ***(47)***. The protocol for the latter experiment is given below.

The closely related protocols that are required to perform these functional binding studies, such as purification of CD11b/CD18, expression and purification of the recombinant CD11b A-domain, generation of EiC3b and iC3b-AP, will also be described.

2. Materials

2.1. Purification of CD11b/CD18

1. Human buffy coat cells.
2. Phosphate-buffered saline (PBS): 0.01 *M* sodium phosphate at pH 7.4, 0.15 *M* NaCl.

3. Red cell lysing buffer: 0.01 *M* sodium phosphate at pH 7.4, 0.8% NH_4Cl.
4. Leukocyte lysing buffer: PBS at pH 7.4, 2 m*M* $MgCl_2$, 1% Triton X-100, protease inhibitors (50 m*M* NaF, 1 m*M* PMSF, 1 m*M* benzamidine, 5 μg/mL leupeptin, 2 μg/mL aprotinin). Use freshly prepared protease inhibitors.
5. Anti-CD11b mAb 44 (American Type Culture Collection, cat. no. HB 249) or LM2/1 (ATCC cat. no. HB 204).
6. Cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden, cat. no. 17-0430-01).
7. BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, cat. no. 23225).

2.2. Expression and Purification of the Recombinant CD11b A-Domain

1. LB plate: Dissolve 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, and 15 g of bacto-agar in 1 L deionized H_2O, and autoclave for 20 min. After it cools to 50°C, add ampicillin to 100 μg/mL, and pour into 100 × 15-mm dishes (Fisher, Pittsburgh, PA, cat. no. 08-757-13). After the medium becomes solid, store at 4°C for up to 3 mo.
2. LB medium: Dissolve 10 g tryptone, 10 g of NaCl, and 5 g of yeast extract in 1 L deionized H_2O, and autoclave for 20 min.
3. Isopropyl Thio-β-D-galactoside (IPTG) (Fisher cat. no. BP1755-10). Make 100 m*M* stock solution, filter it though 0.2-μm filter, and store in aliquots at –20°C.
4. Phosphate-buffered saline (PBS): 0.01 *M* sodium phosphate at pH 7.4, 0.15 *M* NaCl.
5. *Escherichia coli* lysing buffer: 50 m*M* Tris-HCl at pH 8.0, 1% Triton X-100, 1 m*M* EDTA, 10 m*M* 2-mercaptoethonal (Sigma, St. Louis, MO, cat. no. M-6250).
6. 15 mL Falcon tube (Becton Dickinson, Rutherford, NJ, cat. no. 2054).
7. Glutathione Sepharose 4B (Pharmacia Biotech cat. no. 17-0756-01).
8. Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, cat. no. 500-0006).
9. Glutathione (Sigma cat. no. G-4251).
10. BCA protein assay reagent kit (Pierce cat. no. 23225).
11. Thrombin protease (Pharmacia Biotech cat. no. 27-0846-01).
12. Bio-Gel P-6DG column (Econo-Pac 10DG column) (Bio-Rad cat. no. 732-2010).
13. Empty Econo-Pac chromatography column (Bio-Rad cat. no. 732-1010).
14. Mono S HR5/5 column (Pharmacia Bioteck cat. no. 52-1623-00-05).
15. POROS HS/P column (Boehringer Mannheim, Indianapolis, IN, cat. no. 1769-570).

2.3. Generation of Biotinylated EiC3b

1. Sheep blood (Colorado Serum, Denver, CO, cat. no. CS1112).
2. Veronal buffered saline (VB saline): 5 m*M* barbital sodium (Fisher Scientific cat. no. B22-500) at pH 7.4, 150 m*M* NaCl.
3. 15 mL Falcon tube (Becton Dickinson cat. no. 2054).
4. Veronal-buffered sucrose (VB sucrose): 5 m*M* barbital sodium at pH 7.4, 1.42 *M* sucrose.
5. VBSG++: VB saline, 2 m*M* $MgCl_2$, 0.3 m*M* $CaCl_2$, 0.1% gelatin.
6. Anti-sheep blood cell stroma fractionated antiserum (Hemolysin) (Sigma cat. no. S-1389).

7. Sulfo-NHS-Biotin (Pierce cat. no. 21217), Make stock solution 100 mg/mL in DMSO, store at –20°C.
8. C5-deficient human serum (Sigma cat. no. C-1163), store at –80°C.
9. 60% buffer: 40% VB saline/ 60% VB sucrose, 2 m*M* $MgCl_2$, 0.3 m*M* $CaCl_2$, 0.1% gelatin.
10. Soybean trypsin inhibitor (STI) (Worthington Biochemical cat. no. 3571).

2.4. Binding of EiC3b to purified CD11b/CD18 or CD11b A-domain

1. 96-well flat-bottomed polystyrene plate (Costar, Cambridge, MA, cat. no. 3590).
2. Coating buffer: 25 m*M* Tris-HCl at pH 8.0, 150 m*M* NaCl, 2 m*M* $MgCl_2$.
3. Blocking buffer: 1% BSA in coating buffer.
4. Binding buffer: VBSG++: VB saline, 2 m*M* $MgCl_2$, 0.3 m*M* $CaCl_2$, 0.1% gelatin.
5. Bio-EiC3b 1.5×10^8/mL.
6. Streptavidin-alkaline phosphatase conjugate (Sigma cat. no. S-2890).
7. *p*-nitrophenol phosphate (Sigma cat. no. N-9389).

2.5. Generation of Alkaline Phosphatase-Conjugated iC3b

1. Fresh human blood (100 mL).
2. Flint glass culture tubes (18 × 150 mm) (Fisher cat. no. 14-958J).
3. Iodoacetamide (Sigma cat. no. I-6125).
4. Veronal-buffered saline (VB saline): 5 m*M* barbital sodium (Fisher cat. no. B22-500) at pH 7.4, 150 m*M* NaCl.
5. Activated thiol-Sepharose (ATS) (Sigma cat. no. T-8512).
6. Bio-Rad protein assay kit (Bio-Rad cat. no. 500-0006).
7. L-cysteine (Sigma cat. no. C-7755).
8. Bio-Gel P-6DG column (Econo-Pac 10DG column) (Bio-Rad cat. no. 732-2010).
9. Mono Q HR5/5 column (Pharmacia Bioteck cat. no. 52-1622-00).
10. Alkaline phosphatase (Sigma cat. no. P-6774).
11. PBS: 0.01 *M* sodium phosphate at pH 7.4, 0.15 *M* NaCl.
12. N-Succinimidyl-3-(2-pyridyldithio) Propionate (SPDP) (Pierce cat. no. 21857).
13. NAP-25 desalting column (Pharmacia cat. no. 17-0852-01).

2.6. Measure the Affinity of Purified CD11b/CD18 or CD11b A-Domain for iC3b

1. 96-well flat-bottomed polystyrene plate (Costar cat. no. 3590).
2. Coating buffer: 25 m*M* Tris-HCl at pH 8.0, 150 m*M* NaCl, 2 m*M* $MgCl_2$.
3. Blocking buffer: 1% BSA in coating buffer.
4. Binding buffer: 25 m*M* Tris-HCl at pH 7.4, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% gelatin.
5. iC3b-AP.
6. *p*-nitrophenol phosphate (Sigma cat. no. N-9389).

2.7. Binding of NIF to CD11b A-domain

1. 96-well flat-bottomed polystyrene plate (Costar cat. no. 3590).
2. Coating buffer: 25 m*M* Tris-HCl at pH 8.0, 150 m*M* NaCl, 2 m*M* $MgCl_2$.

3. Blocking buffer: 1% BSA in coating buffer.
4. Biotinylated NIF.
5. $MgCl_2$ stock solution: 50 m*M* in deionized H_2O. Store at room temperature.
6. $CaCl_2$ stock solution: 50 m*M* in deionized H_2O. Store at room temperature.
7. $MnCl_2$ stock solution: 50 m*M* in deionized H_2O. Store at room temperature.
8. EDTA stock solution: 100 m*M* in deionized H_2O. Store at room temperature.
9. EGTA stock solution: 100 m*M* in deionized H_2O. Store at room temperature.
10. Streptavidin-alkaline phosphatase conjugate (Sigma cat. no. S-2890).
11. *p*-nitrophenol phosphate (Sigma cat. no. N-9389).

2.8. Binding of CD102 Peptide P1 to purified CD11b/CD18

1. 96-well flat-bottomed polystyrene plate (Costar cat. no. 3590).
2. Coating buffer: 25 m*M* Tris-HCl at pH 8.0, 150 m*M* NaCl, 2 m*M* $MgCl_2$.
3. Blocking buffer: 1% BSA in coating buffer.
4. Binding buffer: 25 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl, 2 m*M* $MgCl_2$, 0.5% BSA.
5. Purified CD11b/CD18.
6. Synthetic P1 peptide (GSLEVNCSTTCNQPEVGGLETSY) 2 mg/mL in PBS at pH 7.4. A tyrosine is added to the C-terminal of the peptide for iodination.
7. ^{125}I-labeled P1 with specific activity approx 3 μCi/μg.

2.9. Binding of Endothelial Cell Line Eahy 926 Cells to Purified CD11b/CD18

1. 96-well flat-bottomed polystyrene plate (Costar cat. no. 3590).
2. Coating buffer: 25 m*M* Tris-HCl at pH 8.0, 150 m*M* NaCl, 2 m*M* $MgCl_2$.
3. Blocking buffer: 1% BSA in coating buffer.
4. Binding medium: Dulbecco's hypoxanthione/aminopterin/thymidine medium, 40 m*M* HEPES at pH 7.4, 2 m*M* $MgCl_2$.
5. Purified CD11b/CD18.
6. Endothelial cell line Eahy 926 (ATCC cat. no. CRL-7678) is cultured in Dulbecco's hypoxanthione/aminopterin/thymidine medium containing 10% fetal calf serum. Eahy 926 is induced to express CD54 by incubating the cells with 10 ng/mL TNF-α overnight.

2.10. Binding of Monocytic Cell Line THP-1 Cells to Purified Fibrinogen

The monocytic cell line THP-1 cells (ATCC cat. no. TIB-202) are cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 m*M* L-glutamine, 10 m*M* HEPES at pH 7.4, 50 μ*M* 2-mercaptoethanol. They mainly express CD11b/CD18 and CD11c/CD18 ***(47)***.

3. Methods

3.1. Purification of CD11b/CD18

CD11b/CD18 is purified from human buffy coat cell lysates by affinity chromatography using anti-CD11b monoclonal antibodies (MAbs) ***(40,49)***. The

same method has also been used to purify the other members of β_2 integrins CD11a/CD18 and CD11c/CD18 ***(23,47)***. CD11b/CD18 purified in this way remains heterodimeric, and maintains its ligand binding activity ***(40,49)***. On the other hand, it has been reported that CD11b/CD18 purified by affinity chromatography using the ligand iC3b lost its ligand-binding activity ***(44,50)***. However, treatment of the purified inactive CD11b/CD18 with MAb KIM-127 caused a shift of this receptor from the inactive to an active state ***(45)***. Purification of CD11b/CD18 using iC3b was described previously ***(28)***. Here we describe the protocol of isolating CD11b/CD18 by antibody affinity chromatography with anti-CD11b mAb 44 ***(41)*** or LM2/1 ***(51)***.

3.1.1. Preparation Human Buffy Coat Cell Lysates

1. 200 units of human buffy coat cells (approx 2 L) are centrifuged in a Sorvall RC5C centrifuge at 2500*g* for 5 min at 4°C. Collect the leukocytes on top of the red blood cell pellet. Centrifuge again at 3200*g* for 10 min at 4°C. Collect leukocytes.
2. Hypotonic lysis of the remaining red cells. Add 2 vol of red cell lysing buffer to 1 vol of the leukocyte pellet, and incubate for 30 min at 37°C. Centrifuge at 3200*g* for 10 min at room temperature. Repeat the above lysing procedure if necessary.
3. Wash the leukocytes twice with ice-cold PBS.
4. Lysis of the leukocytes. Add 2 vol of leukocyte lysing buffer to 1 vol of the leukocyte pellet, and incubate for 20 min on ice, mix the sample during incubation.
5. Centrifuge the sample at 6500*g* for 30 min at 4°C. Collect supernatant and further centrifuge in a Beckman ultracentrifuge at 50,000*g* for 1 h at 4°C. Collect the supernatant. The leukocyte lysates can be stored at –80°C.

3.1.2. Antibody Affinity Chromatography

1. Couple MAb 44 or LM2/1 to 10 mL cyanogen bromide-activated Sepharose 4B. Wash the column extensively with 0.1% Triton X-100 in PBS (*see* **Note 1**).
2. Pass the leukocyte lysates over the column.
3. Wash the column extensively with 0.1% Triton X-100 in PBS; 20 m*M* glycine/NaOH at pH 9.5, 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1% n-octyl glucoside.
4. Elute CD11b/CD18 with 50 m*M* diethylamine at pH 11.5, 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1% n-octyl glucoside into tubes containing neutralizing buffer (10% by volume of 1 *M* Tris-HCl at pH 6.8).
5. Lyophilize 100 μL of sample from each fraction, and analyze the samples on 8% SDS-polyacrylamide gel.
6. Pool the peak fractions and measure the protein concentration by using BCA protein assay reagent kit with the standard protocol. The protocol is described in the instructions attached to the kit.
7. Store the purified CD11b/CD18 in aliquots at –80°C. The activity can be maintained in this way for up to 6 mo. The usual yield of CD11b/CD18 is 1–2 mg.

3.2. Expression and Purification of the Recombinant CD11b A-Domain

Recent studies have shown that the isolated integrin A-domain is responsible for the binding of several protein ligands ***(11–14)***, establishing this domain as an independent structural and functional unit. A recombinant form of the CD11b A-domain expressed in *E. coli* was found to bind directly and specifically to iC3b and NIF in a metal ion-dependent manner ***(10–13)***. Interestingly, the binding of iC3b to plastic-immobilized CD11b A-domain was found to be temperature-independent, in contrast to that of membrane-bound or purified CD11b/CD18 heterodimer ***(50)***. This difference suggests that the recombinant A-domain may assume a functionally active conformation, which is expressed in the membrane-bound holoreceptors only after cells are activated by agonists. Recent crystal structure studies revealed two different conformations of the recombinant CD11b A-domain, one with a ligand mimic glutamic acid coordinated in MIDAS, whereas the other one without ***(8,52)***. Whether these two conformations represent the high-affinity and low-affinity states of the A-domain, and whether switching between these two conformations is the mechanism for regulating integrin activity are currently under investigation.

3.2.1. Protein Expression

1. Transform *E. coli* (JM109) with plasmid pGEX-2T that encodes a GST-CD11b A-domain fusion protein. Grow the transformed *E. coli* on LB plate overnight at 37°C. The colony containing plate can be used within 1 wk.
2. Pick up a single colony from the plate, and put it in 50 mL LB medium (Amp. 100 μg/mL). Shake overnight at 37°C.
3. Add the 50 mL *E. coli* culture to 950 mL LB medium (Amp. 50 μg/mL), and shake for approx 3–4 h at 37°C.
4. Check OD (600 nm). When OD = 0.45–0.55, start IPTG induction.
5. Add IPTG to the 1 L *E. coli* culture, make the final concentration of IPTG = 0.2 m*M*. Shake for 3–4 h at 37°C.
6. Check OD (600 nm). When OD reach 1.00, harvest *E. coli* by centrifugation at 6000g for 30 min at 4°C (Sorvall [Newtown, CT] RC3C, Rotor H-6000A code 24).
7. Wash the *E. coli* pellet once with PBS. Spin at 10,000g for 10 min at 4°C (Sorvall RC5C, Rotor GSA code 10). Store the pellet at –20°C.

3.2.2. Lysis of the E. coli

1. Keep the sample on ice. Lyse the *E. coli* by adding 10 mL *E. coli* lysing buffer to *E. coli* pellet from a 1 L culture. Transfer it to 15-mL Falcon tube (8–9 mL sample per 15-mL tube).
2. Sonicate the sample with Ultrasonic Liquid Processor XL2020 (Misonix Inc., Farmingdale, NY). Sonicate for 10–15 s at 4°C, put on ice for 45–50 s. Sonicate 10–15 times.

3. End-to-end shake the sample for 30 min at 4°C.
4. Centrifuge at 20,000g for 30 min at 4°C (Sorvall RC5C, Rotor SS34 code 05). Collect the supernatant.
5. Run a small amount of supernatant on 12% SDS-PAGE to estimate the amount of fusion protein and its solubility (*see* **Note 2**).

3.2.3. Purification of the GST-A-Domain Fusion Protein

1. Mix the supernatant with the prewashed glutathione Sepharose 4B beads (use 1-mL beads for the supernatant from a 1 L *E. coli* culture), and incubate for 1 h at 4°C with shaking. Pour the sample into an empty Econo-Pac chromatography column. Keep the drop through portion, it may still contain some amount of fusion protein.
2. Wash the column extensively with 50 m*M* Tris-HCl at pH 8.0; 50 m*M* Tris-HCl at pH 8.0, 500 m*M* NaCl, 1% Triton X-100; 50 m*M* Tris-HCl at pH 8.0; until no protein is detected in the wash (checked by Bio-Rad protein assay kit).
3. Elute the GST-A-domain fusion protein with 50 m*M* Tris-HCl at pH 8.0, 20 m*M* glutathione (freshly prepared). Check with Bio-Rad protein assay kit until no more protein is eluted, and pool the peak fractions.
4. Run the supernatant, the drop through and the fusion protein on 12% SDS-PAGE.
5. Measure the fusion protein concentration by using BCA protein assay reagent kit with the standard protocol. The protocol is described in the instructions attached to the kit. Adjust the protein concentration to about 1 mg/mL with 50 m*M* Tris-HCl at pH 8.0. Keep a small amount of fusion protein for SDS-PAGE.

3.2.4. Fusion Protein Cleavage and A-Domain Purification

1. Cleave the fusion protein with thrombin, use 5 units of thrombin per mg of fusion protein, shake the sample gently overnight at 4°C or leave the sample on ice over two nights (*see* **Note 3**).
2. Run the fusion protein and the cleaved protein on 12% SDS-PAGE to check the cleavage efficiency (*see* **Notes 4** and **5**).
3. Run the sample through Bio-Gel P-6DG column (Econo-Pac 10DG column) to remove glutathione. Elute with 20 m*M* Tris-HCl at pH 8.0.
4. Purify the CD11b A-domain with FPLC using a Mono S HR5/5 column or POROS HS/P column, and elute with a gradient (0–0.5 *M*) NaCl in 20 m*M* Tris-HCl at pH 8.0 (*see* **Note 5**).
5. Check the purity of CD11b A-domain with 12% SDS-PAGE, and measure the protein concentration. Usually, at least 2 mg of purified CD11b A-domain can be obtained from 1 L *E. coli* culture.
6. Add EDTA to a final concentration of 10 m*M* to preserve the protein, store in aliquots at 4°C. The activity of CD11b A-domain can be maintained in this way for up to 6 mo.

3.3. Generation of Biotinylated EiC3b

3.3.1. Wash Sheep Red Blood Cells (RBC)

1. To 400 μL sheep blood, add 900 μL VB saline/10 m*M* EDTA (in 15-mL Falcon tube), and incubate for 15 min at 37°C.

2. Wash RBC twice with VB saline/10 m*M* EDTA, and twice with VBSG++. Pellet RBC by spinning at 8000g for 5 min in a Sorvall RT6000B centrifuge. Resuspend RBC in 1 mL VBSG++.
3. To 40 μL RBC suspension, add 960 μL H_2O (1:25 dilution), and check OD (541 nm). When OD (541 nm) (1:25) = 0.210, the RBC concentration is 5×10^8/mL. Adjust RBC concentration to 5×10^8/mL using VBSG++.

3.3.2. Sensitize Sheep RBC

1. Add 1 vol of anti-sheep blood cell stroma fractionated antiserum (hemolysin) to 240 vol of RBC suspension. Gentle shake for 30 min at 37°C, and leave on ice for 30 min.
2. Wash cells once with VBSG++. Resuspend cells in VBSG++ to 5×10^8/mL.

3.3.3. Biotinylate Sensitized Sheep RBC

1. Add Sulfo-NHS-Biotin to cell suspension to a final Sulfo-NHS-Biotin concentration of 0.5 mg/mL. Incubate for 30 min at 4°C.
2. Wash twice with VBSG++. Resuspend in VBSG++ to 5×10^8/mL.

3.3.4. Coat Sheep RBC with iC3b

1. Add C5-deficient human serum in 1:10 dilution to cell suspension. Incubate for 1 h at 37°C with gentle shake.
2. Wash twice with VBSG++, and twice with 60% buffer/1 mg/mL soybean trypsin inhibitor (STI). Resuspend in 60% buffer/1 mg/mL STI.
3. Check OD (412 nm) (1:25). Adjust to OD 0.560, so that Bio-EiC3b concentration is 1.5×10^8/mL. They can be used within 2 wk when kept on ice and prepared with fresh sheep blood.

3.4. Binding of EiC3b to Purified CD11b/CD18 or CD11b A-Domain

1. Immobilize purified CD11b/CD18 or CD11b A-domain on 96-well polystyrene plate. Pipet 1 μg of purified CD11b/CD18 or 2 μg of purified CD11b A-domain in each well of the 96-well plate. Add coating buffer to the wells to make the final volume 50 μL/well. Make triplicate wells. Seal the wells with parafilm. Incubate for 16 h at 4°C to allow the protein to attach the plastic surface. Control wells are prepared by only adding coating buffer.
2. Saturate nonspecific binding sites on the plate surface. Remove the solution from each well. Wash the wells once with 100 μL/well blocking buffer, aspirate and discard. Add 100 μL of blocking buffer to each well. Incubate for 1 h at room temperature.
3. Add Bio-EiC3b to CD11b/CD18- or CD11b A-domain-coated plate. Wash the wells twice with 100 μL/well binding buffer, aspirate and discard. Add 3×10^6 Bio-EiC3b cells diluted in 50 μL binding buffer to each well, and gently spin down the cells, and incubate for 15 min at 37°C. For checking the effects of monoclonal antibodies, specific CD11b/CD18 MAbs and control MAbs are included during the binding incubation at a final concentration of 50 μg/mL (*see* **Note 6**).

4. Remove the unbound cell. Shake the plate gently and aspirate the free cells in suspention (*see* **Note 7**).
5. Fix the bound Bio-EiC3b cells. Add 50 μL 1% glutaraldehyde in PBS to each well, and incubate for at least 2 h at 37°C. Neutralize excess glutaraldehyde with 1% BSA for 2 h at 37°C.
6. Quantitate the bound Bio-EiC3b cells. Add 50 μL of streptavidin-alkaline phosphatase conjugate solution (5 μg/mL) to each well, and incubate for 30 min at 37°C. After washing, add 50 μL/well of *p*-nitrophenol phosphate solution (2 mg/mL), and incubate for 30 min at 37°C. Determine the absorbance at 405 nm.

3.5. Generation of Alkaline Phosphatase-Conjugated iC3b

3.5.1. Prepare Serum

1. Place 100 mL of fresh human blood in flint glass culture tubes (18 × 150-mm), incubate for 30 min at room temperature.
2. Stir the clot with Pasteur pipet, put the tubes on ice for 1.5 h.
3. Centrifuge at 1900g for 30 min at 4°C in a Sorvall RT6000B centrifuge.
4. Collect the serum (approx 50 mL).

3.5.2. Purify iC3b

1. To 50 mL of fresh human serum, add iodoacetamide to a final concentration of 20 m*M*, and incubate for 1 h at 37°C, to block all free -SH groups.
2. Dialyze three times against 4 L of VB saline at pH 7.4, at 4°C, change buffer every 4 h or overnight.
3. Add $MgCl_2$ and EGTA to the serum to a final concentration of 5 m*M* of $MgCl_2$ and 7 m*M* of EGTA.
4. Swell 3 g of activated thiol-Sepharose (ATS) with distilled H_2O (1 g swells to approx 4 mL). Wash and equilibrate the gel with VB saline at pH 7.4, 5 m*M* $MgCl_2$, 7 m*M* EGTA (VBS/Mg/EGTA), use 200 mL for 1 g of Sepharose powder.
5. Add the ATS gel (approx 12 mL) to the serum, stir at 37°C for 2 h.
6. Cool the serum/ATS slurry to 4°C. Pour into a 1.5 × 30-cm column.
7. Wash the ATS beads with >10× bed vol of VBS/Mg/EGTA buffer, >10 bed vol of 1 *M* NaCl, and >5 bed vol of 20 m*M* Tris-HCl at pH 7.4. Check by Bio-Rad protein assay kit until no more protein coming out in the wash.
8. Elute iC3b with 10 m*M* L-cysteine in 20 m*M* Tris-HCl at pH 7.4. Collect 3-mL fractions. Check by Bio-Rad protein assay kit.
9. Run 8% SDS-PAGE (reduced) (*see* **Note 8**). Pool the peak fractions
10. Run the sample through Econo-Pac 10DG column, elute with 20 m*M* Tris-HCl at pH 7.4, 5 m*M* EDTA, 0.02% NaN_3. This removes L-cysteine and changes buffer.
11. Further purify iC3b (180 kDa) with Mono Q HR5/5 column through FPLC, elute with a gradient (0–0.5 *M*) of NaCl in 20 m*M* Tris-HCl at pH 7.4, 5 m*M* EDTA, 0.02% NaN_3 (*see* **Note 9**).

3.5.3. Make 2-Pyridyldisulfide-AP

1. To 10,000 units of AP (140–160 kDa) (equal 5 mg in 0.5 mL), add 0.5 mL PBS and 25 μL of SPDP stock (20 m*M* in DMSO). Incubate for 30 min at 37°C.

2. Equilibrate a NAP-25 desalting column with 25 mL PBS, 1 m*M* EDTA.
3. Load the sample onto the column, elute with PBS, 1 m*M* EDTA, collect 1-mL fractions and pool peak fractions. This removes excess cross-linker SPDP.

3.5.4. Couple AP to iC3b

1. Put equimolar 2-pyridyldisulfide-AP and purified iC3b together, shake gently for 24 h at room temperature.
2. Run the sample through Econo-Pac 10DG column, elute with 20 m*M* Tris-HCl at pH 7.4, 0.02% NaN_3.
3. Conjugated and free proteins are separated with a Mono Q HR5/5 column through FPLC, elute with a gradient (0–0.5 *M*) of NaCl in 20 m*M* Tris-HCl at pH 7.4, 0.02% NaN_3.
4. Run a 6% native gel to check the purification results. Pool the peak fractions, and measure the protein concentration (*see* **Note 10**).

3.6. Measure the Affinity of Purified CD11b/CD18 or CD11b A-Domain for iC3b

1. Pipet 1 μg of purified CD11b/CD18 or 2 μg of purified CD11b A-domain in each well of the 96-well plate. Add coating buffer to the wells to make the final volume 50 μL/well. Make triplicates. Seal the wells with parafilm. Incubate for 16 h at 4°C to allow the protein to attach the plastic surface.
2. Wash the wells once with 100 μL/well blocking buffer, aspirate and discard. Add 100 μL of blocking buffer to each well. Incubate for 1 h at room temperature.
3. Wash the wells twice with 100 μL/well binding buffer. Add to each well increasing amounts (5–200 n*M*) of iC3b-AP diluted with binding buffer in the absence or presence of 5 m*M* EDTA, and incubate for 1 h at 37°C to allow the interacting equilibrium to be reached.
4. Wash the wells three times with 100 μL/well binding buffer.
5. Add 50 μL/well of *p*-nitrophenol phosphate solution (2 mg/mL), and incubate overnight at 4°C. Determine the absorbance at 405 nm.
6. Make a dose-response binding curve. Specific iC3b-AP binding is obtained by subtracting background binding (in the presence of 5 m*M* EDTA) from total binding (in the absence of 5 mM EDTA). The amount of iC3b-AP that occupies 50% of the CD11b/CD18 or CD11b A-domain binding sites is the apparent K_d value, when expressed as molarity.
7. For estimation of K_d and the total amount of CD11b/CD18 or CD11b A-domain immobilized on plastic, the dose-response binding curve can be linear transformed by double-reciprocal plot method or Scatchard plot method ***(53)***.

3.7. Binding of NIF to CD11b A-Domain

1. Pipet 1 μg of purified CD11b A-domain in each well of the 96-well plate. Add coating buffer to the wells to make the final volume 50 μL/well. Make triplicate wells. Seal the wells with parafilm. Incubate for 16 h at 4°C to allow the protein to attach the plastic surface.

2. Wash the wells once with 100 μL/well blocking buffer, aspirate and discard. Add 100 μL of blocking buffer to each well. Incubate for 1 h at room temperature.
3. Wash the wells twice with 100 μL/well 5 m*M* EDTA in 25 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl to remove the protein bound metals, and then wash twice with 25 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl, 0.1% gelatin. Pipet 2 μL of 50 m*M* stock solution of $MgCl_2$, $CaCl_2$, or $MnCl_2$, or pipet 2.5 μL of 100 m*M* stock solution of EDTA or EGTA to each well. Then add 2.5 ng Bio-NIF diluted with 25 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl, 0.1% gelatin to a final volume of 50 μL to each well. The final concentration of $MgCl_2$, $CaCl_2$, or $MnCl_2$ is 2 m*M*, and that of EDTA or EGTA is 5 m*M*. Leave one set of wells without adding either metals or metal chelate. Incubate for 1 h at 37°C.
4. Wash the wells three times with 100 μL/well 25 m*M* Tris-HCl at pH 7.4, 150 m*M* NaCl, 0.1% Gelatin.
5. Add 50 μL of alkaline phosphatase-conjugated streptavidin solution (5 μg/mL) to each well, and incubate for 30 min at 37°C. After washing, add 50 μL/well of *p*-nitrophenol phosphate solution (2 mg/mL), and incubate for 30 min at 37°C. Determine the absorbance at 405 nm.

3.8. Binding of CD102 Peptide P1 to Purified CD11b/CD18

1. Dilute the purified CD11b/CD18 with coating buffer to 20 μg/mL, and pipet 50 μL in each well. Seal the wells with parafilm. Incubate for 16 h at 4°C.
2. Wash the wells once with 100 μL/well blocking buffer, aspirate, and discard. Add 100 μL of blocking buffer to each well. Incubate for 1 h at room temperature.
3. Wash the wells twice with 100 μL/well binding buffer, aspirate, and discard. Add 0.05 μg of ^{125}I-labeled P1 diluted with binding buffer to a final volume of 50 μL to each well in the presence or absence of 100-fold cold P1 (5 μg). Incubate for 1 h at 37°C.
4. Wash the wells three times with 100 μL/well binding buffer.
5. Add 50 μL 1% SDS to each well, and incubate for 5 min at room temperature.
6. Suspend and take 40 μL of the 1% SDS solution from each well, and check the radioactivity using a gamma-counter.

3.9. Binding of Endothelial Cell Line Eahy 926 Cells to Purified CD11b/CD18

1. Immobilize purified CD11b/CD18 on 96-well polystyrene plate. Pipet 1 μg of purified CD11b/CD18 in each well of the 96-well plate. Add coating buffer to the wells to make the final volume 50 μL/well. Make triplicate wells. Seal the wells with parafilm. Incubate for 16 h at 4°C to allow the protein to attach the plastic surface.
2. Saturate nonspecific binding sites on the plate surface. Remove the solution from each well. Wash the wells once with 100 μL/well blocking buffer, aspirate, and discard. Add 100 μL of blocking buffer to each well. Incubate for 1 h at room temperature.
3. Add endothelial cells to CD11b/CD18-coated plate. Wash the wells twice with 100 μL/well binding medium, aspirate, and discard. TNF-α-stimulated or

unstimulated Eahy 926 cells are removed from the tissue culture flasks with 5 mM EDTA in PBS, washed and resuspend in binding medium to 1×10^6/mL. Add 100 µL to each well, and incubate for 1 h at 37°C. Monoclonal antibodies to CD11b/CD18, CD54, and CD102 can be used to define the binding specificity. For TNF-α-stimulated Eahy 926 cells, CD54 plays a major role in the interaction, while for unstimulated Eahy 926 cells, CD102 is the major ligand in the interaction ***(40,48)***.

4. Remove the unbound cell. Gently wash three times with binding medium.
5. Quantitate the bound cells. Count the bound cells under a microscope.

3.10. Binding of Monocytic Cell Line THP-1 Cells to Purified Fibrinogen

96-well microtiter plates are coated with purified fibrinogen (Fg) (30 µg/mL in PBS) for 16 h at 4°C. Nonspecific binding sites are blocked with 1% BSA in PBS for 2 h at room temperature. 100-µL aliquots of CD102 peptide P1 treated THP-1 cells at 2×10^6/mL in RPMI 1640 medium supplemented with 40 m*M* HEPES at pH 7.4, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$ are added to each well, and incubated for 30 min at room temperature. For antibody blocking assays, the P1-treated THP-1 cells are pretreated with different MAbs for 10 min at room temperature before added to Fg-coated wells. Nonadherent cells were removed by three washes with the binding medium. The binding is quantitated by scoring the number of attached cells with 200x magnification of four randomly chosen fields from each well.

3.11. Cell-Aggregation Assay

PMNs or T lymphocytes isolated from human peripheral blood cells are washed with RPMI 1640 medium and resuspended to a concentration of 1×10^6 cells/mL. Aliquots of 100 µL are added to each well of flat-bottomed 96-well microtiter plates in the absence or presence of phorbol esters, and incubated at 37°C for 1 h. For inhibition of the phorbol ester-induced cell aggregation, cells are preincubated with different monoclonal antibodies or inhibitors for 15 min at room temperature before being treated with the agonists. For quantitative measurement of cell aggregation, the free cells of four randomly chosen areas (2.5 mm^2) per well are counted. The amount of aggregated cells is expressed as: percent aggregation = 100 × [1–(number of free cells)/(total number of cells)] ***(28,54)***.

4. Notes

1. A column of cyanogen bromide-activated Sepharose 4B without MAb can be prepared and used before the MAb-coupled column, to preclear any nonspecific binding to the Sepharose.
2. The CD11b A-domain-GST fusion protein is approx 50 kDa. If little protein is detected by SDS-PAGE from the *E. coli* supernatant and pellet, optimize the

fusion protein expression conditions, such as cell strain, medium composition, incubation temperature, and induction conditions. Exact conditions will vary for each fusion protein expressed. If the majority of the protein is found in the postsonicate pellet, it may indicate that the sonication is not sufficient. Alternatively, the fusion protein may be insoluble. Fusion protein solubility can be dramatically increased by lowering the growth temperature (20–30°C), decreasing IPTG concentration (< 0.1 m*M*), or increasing aeration during incubation.

3. A small amount of precipitation may be seen after thrombin-cleavage incubation. Remove it by centrifugation. To decrease the possibility of forming precipitation, make sure that the fusion protein concentration is not more than 2 mg/mL for the wild-type CD11b A-domain during cleavage.
4. The molecular mass of GST is 26 kDa, that of CD11b A-domain 24 kDa. Make sure that all the fusion protein has been cleaved before moving to the next step. If the cleavage is not complete, it is difficult to separate the fusion protein from the cleaved protein by ion-exchange chromatography later on, as the pI value of the fusion and cleaved proteins is similar.
5. Mono S HR5/5 and POROS HS/P columns are strong cation exchange columns. The pI of CD11b A-domain is 10-11, and that of GST 4-5. In a buffer of neutral pH, A-domain is positively charged and GST negatively charged. Therefore, they can be well separated by ion-exchange chromatography.
6. If the epitopes of monoclonal antibodies used are known, the ligand binding site(s) in the integrin can be mapped.
7. The way to remove the unbound cells is usually a crucial step for success of the binding assays. For EiC3b binding assay, gentle shaking of the plate will be enough to remove the free cells. For some stronger binding cells, washing the wells with binding buffer may be necessary to get rid of the free cells.
8. iC3b is a heterodimeric glycoprotein of 180 kDa. In 8% reduced SDS-PAGE, three bands are expected: β chain 70 kDa, and α chain fragments 68 and 42 kDa.
9. The pI of iC3b is 4–5, and it can be purified by Mono Q HR5/5 column, which is a strong ion-exchange column.
10. The iC3b-AP coupling efficiency of this protocol is approx 40%.

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9

Expression of Heterologous Integrin Genes

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1. Introduction

Cell-extracellular matrix interactions are mediated predominantly by integrins, cell-surface receptors that exist as heterodimers of noncovalently associated α and β subunits ***(1)***. Integrins have been implicated in organ and tissue development, normal and aberrant cellular growth, and modulation of intracellular signal-transduction mechanisms ***(2–7)***. The expression of individual integrin subunits in mammalian systems has been instrumental in studies aimed at examining the structure and function of integrins and in analyzing the ability of integrins to modulate cellular functions and intracellular signaling (for review, *see* **ref. *8***).

High levels of expression of integrin heterodimers have been obtained in several cell types under the control of either CMV or SRα or SV40 or the mouse metallothioneine-inducible promoter. More recently, inducible systems based on components of the tetracycline-resistance operon or on nonmammalian steroid hormones have also been generated to modulate integrin gene activity ***(9,10)***; these systems have the remarkable advantage of a tighter control of expression versus previously used inducible systems.

Several strategies have emerged to introduce DNA into eukaryotic cells; however, an ideal procedure valid in all cases is not available. This chapter will describe two approaches successfully used in our laboratory to transfect integrin subunit cDNAs into mammalian cells: electroporation, that utilizes short bursts of high-voltage electricity ***(11)***, and lipofection, that utilizes lipid-DNA complexes. It should be noted that the efficacy of each method may vary in different applications, thus their use should be tested for the investigator's

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specific needs; low-expression levels of the desired integrin could be a reflection of protein instability or difficulties with transfection. The latter should be tested by a readily assayable indicator gene, such as β-galactosidase or luciferase integrin. It should also be noted that because integrins are heterodimeric complexes, an exogenously expressed subunit must pair with an endogenous subunit to be correctly processed and transported to the cell surface. If endogenous subunits are not available to heterodimerize with the transfected subunit, cotransfection of both α and β subunit cDNAs should be performed.

Although we will selectively discuss protocols for transient transfection of Chinese hamster ovary (CHO) cells, the same procedures can be used to generate either stable or transient transfectants of various cell types. An immunological and a functional approach for the detection of the successfully expressed integrin heterodimer will also be described in this chapter.

2. Materials

2.1. Transient Transfection of CHO Cells

2.1.1. Electroporation

1. Subconfluent (70%) CHO cells, approx 1×10^7 cells/electroporation.
2. CHO growth medium: 500 mL DMEM supplemented with high glucose (Life Technologies, Gaithersburg, MD), 5 mL of 200 m*M* L-glutamine (Gemini BioProducts, Calabasas, CA), 5 mL penicillin-G (10,000 U/mL) Streptomycin (10,000 mg/mL) (Gemini BioProducts), 5 mL of 200 m*M* nonessential amino acids (Life Technologies) and 10% heat-inactivated fetal bovine serum (Gemini BioProducts). Store growth medium at 4°C.
3. Phosphate-buffered saline (PBS) ***(12)***. Sterilize and store at 4°C.
4. Trypsin-EDTA (0.05% trypsin and 0.53 m*M* EDTA) (Life Technologies). Store at 4°C.
5. 0.4% Trypan blue stain (Life Technologies) diluted 1:2 in PBS.
6. Hemocytometer (Reichert-Jung, Horsham, PA).
7. Electroporation buffer: 10 m*M* sodium phosphate and 150 m*M* sodium chloride at pH 7.4. Sterilize and store at 4°C.
8. Salmon sperm DNA (Sigma, St. Louis, MO) that has been sonicated and resuspended at 10 mg/mL ***(12)***. Store at –20°C.
9. Integrin cDNA subcloned in a mammalian expression vector and purified with either commercially available Maxi-prep kits or by cesium-chloride purification ***(12)***. Cesium-chloride-purified DNA must be dialyzed against sterile-deionized water. Visualize DNA with ethidium bromide. Store at –20°C (*see* **Note 1**).
10. 150-mm tissue-culture plates (Falcon, Franklin Lakes, NJ).
11. 15-mL conical tubes (Corning, Corning, NY).
12. Electroporation cuvets, 0.4-cm electrode gap (Bio-Rad, Hercules, CA).
13. Gene Pulser II, electroporator with capacitance extender (Bio-Rad).

2.1.2. Lipofection

1. Mammalian expression vector DNA of interest, purified as described in **Subheading 2.1.1.**, **step 9**.
2. Approximately 40–70% subconfluent CHO cells in 60-mm tissue culture plates.
3. CHO growth medium (refer to **Subheading 2.1.1.**, **step 2**).
4. Lipofectin (Life Technologies).
5. OptiMEM (Life Technologies).
6. Sterile 1.5-mL microcentrifuge tubes (USA/Scientific, Ocala, FL).
7. 60-mm tissue culture plates (Corning).

2.2. Detection of Exogenous Integrins at the Cell Surface

2.2.1. Cell Staining for Flow Cytofluorometric Analysis

1. CHO growth medium (refer to **Subheading 2.1.1.**, **step 2**).
2. PBS (refer to **Subheading 2.1.1.**, **step 3**).
3. Trypsin-EDTA (refer to **Subheading 2.1.1.**, **step 4**).
4. Trypan blue (refer to **Subheading 2.1.1.**, **step 5**).
5. Hemocytometer.
6. 5-mL round-bottom tubes (Falcon).
7. Species-specific antibody (Ab) that recognizes the extracellular domain of the exogenously expressed integrin (primary Ab, 1° Ab) and that does not cross-react with the hamster protein.
8. Negative control Ab. Isotype matched Abs either against a cytoplasmic protein, or against a surface protein that is not expressed by CHO cells or nonimmune IgG can be used.
9. Secondary Ab (2° Ab) conjugated to a fluorophore (e.g., fluorescein isothiocyanate, FITC).
10. 3% Paraformaldehyde (Sigma) stock solution in PBS. Working solution is diluted 1:50 (*see* **Note 2**).

2.2.2. Surface Iodination and Immunoprecipitation

1. Approximately 2×10^7/mL CHO cells.
2. Sterile PBS (refer to **Subheading 2.1.1.**, **step 3**).
3. Sterile 100 m*M* $CaCl_2$ (J.T. Baker, Phillipsburg, NJ).
4. Sterile 100 m*M* $MgCl_2$ (J.T. Baker).
5. Lactoperoxidase (Sigma) at 3 mg/mL in PBS. Store in aliquots at –20°C.
6. 30% stock solution of hydrogen peroxide (H_2O_2). The working solution is prepared fresh and diluted to 0.24% in PBS. Store on ice (*see* **Note 3**).
7. 1 mCi sodium iodine-125 ($Na^{125}I$). Store behind lead shielding at room temperature (*see* **Note 4**).
8. CHO growth medium (refer to **Subheading 2.2.1.**, **step 2**).
9. Trypan blue (refer to **Subheading 2.2.1.**, **step 5**).
10. Hemocytometer.
11. 15-mL conical tube (Corning).

12. Lysis buffer: 20 m*M* Tris-HCl at pH 8.0 (American Bioanalytical, Natick, MA), 1% Triton-X 100 (Sigma), 10% glycerol (J.T. Baker), 150 m*M* NaCl (J.T. Baker), 1 m*M* PMSF (Life Technologies), 10 μg/mL aprotinin (Sigma), and 10 μg/mL leupeptin (Calbiochem, La Jolla, CA).
13. 1.5-mL microcentrifuge tubes.
14. Nonimmune Ab.
15. Ab specific for the exogenously expressed integrin.
16. Protein A sepharose (Sigma).
17. Rotating platform.
18. SDS sample buffer: 50 m*M* Tris-HCl at pH 6.8 (American Bioanalytical), 2% SDS (American Bioanalytical), 10% glycerol, and 0.1 *M* dithiothreitol (Bio-Rad) and 0.1% bromophenol blue (Bio-Rad).
19. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus.
20. Coomassie stain solution: 40% methanol, 10% acetic acid, and 0.02% Coomassie blue.
21. Gel dryer.
22. Autoradiography cassette with intensifying screens and film.

2.3. Assaying for Functional Integrin Expression

2.3.1. Cell Adhesion Assay

1. CHO Growth medium (refer to **Subheading 2.1.1.**, **step 2**).
2. PBS (refer to **Subheading 2.1.1.**, **step 3**).
3. Trypsin-EDTA (refer to **Subheading 2.1.1.**, **step 4**).
4. Trypan blue (refer to **Subheading 2.1.1.**, **step 5**).
5. Hemocytometer.
6. Linbro®/Titertek® microtitration 96-well plate (ICN Biomedicals, Aurora, OH).
7. Assay Buffer: DMEM (refer to **Subheading 2.1.1.**, **step 2**) supplemented with 1% bovine serum albumin (BSA) (Sigma).
8. 3% Paraformaldehyde (refer to **Subheading 2.1.1.**, **step 10**).
9. 0.5% Crystal violet (Sigma) dissolved in deionized water and filtered through filter #1 paper (Whatman, Fairfield, NJ) prior to using.
10. 96-well plate reader with 630-nm wavelength filter.

3. Methods

3.1. Transient Transfection of CHO Cells

The following methods: electroporation ***(13)*** and lipofection have been used to transiently transfect CHO cells with an integrin cDNA, subcloned into a mammalian expression vector. In our laboratory, the best results have been achieved using plasmids that have been purified by cesium-chloride gradients. However, commercially available maxi-prep kits (e.g., Promega, Madison, WI or Qiagen, Chatsworth, CA) can also be used to purify the plasmid.

3.1.1. Electroporation

1. Grow CHO cells to subconfluent levels (50–70%) and subculture the cells 24 h before electroporation. Approximately 1×10^7 cells will be needed for each electroporation.
2. The following day, wash cells once with sterile PBS.
3. Detach cells by incubating with trypsin-EDTA for 3–5 min.
4. Neutralize trypsin-EDTA with an equal amount of CHO growth medium and transfer the cells into a 15-mL conical tube.
5. Remove 10 μL of cells and dilute into 90 μL 0.2% trypan blue. Count the cells using a hemocytometer, and determine the total number of cells.
6. Centrifuge the remaining cells for 4 min at 300*g*.
7. Wash the cells once with sterile PBS and place on ice.
8. Add 2–100 μg plasmid DNA to sterile electroporation cuvet (*see* **Note 5**).
9. Add 50 μg salmon sperm DNA to each cuvet.
10. Equilibrate the volume of each cuvet using sterile water.
11. Resuspend 1×10^7 cells/cuvet in electroporation buffer, so that the final volume of each cuvet does not exceed 500 μL (*see* **Note 6**).
12. Add cells to the cuvet and incubate on ice for 15 min.
13. Dry the outside of the cuvet thoroughly (*see* **Note 7**).
14. Set the electoporator to 350 V and 950 μF.
15. Electroporate the cells.
16. Allow the cells to recover on ice for 15 min.
17. Plate the cells into a 150-mm tissue-culture dish with CHO growth medium and incubate at 37°C with 5% CO_2 in a humidified incubator (**Notes 8** and **9**).
18. Replace the medium 24 h later and incubate the cells with 5% CO_2 in a humidified incubator until the cells are to be analyzed for integrin expression. (*See* **Notes 10** and **11**.)

3.1.2. Lipofection

1. Place 0.5–2 μg purified plasmid DNA into a 1.5-mL microcentrifuge tube and bring the final volume to 100 μL with OptiMEM and gently mix by pipetting up and down several times (*see* **Note 5**).
2. Place 0.5–10 μg Lipofectin in a 1.5-mL microcentrifuge tube and bring the volume to 100 μL with OptiMEM and gently mix by pipetting up and down several times (*see* **Note 12**).
3. Incubate both 1.5-mL microcentrifuge tubes for 30 min at room temperature.
4. Combine the contents of both tubes (gently mixing) and incubate for 10 min at room temperature.
5. Wash the CHO cells 2× with OptiMEM (*see* **Notes 13** and **14**).
6. Add 800 μL OptiMEM to the 1.5-mL microcentrifuge tube that contains both the DNA and lipofectin and gently mix.
7. Add the solution from **step 6** drop wise to the CHO cells.
8. Incubate the cells for 7–14 h at 37°C with 5% CO_2 in a humidified incubator (*see* **Note 15**).

9. Replace transfection solution with CHO growth medium and incubate as in **Subheading 3.1.1.**, **step 18**.

3.2. Detection of Exogenous Integrin at the Cell Surface

Integrin expression can be analysed using Ab5 specific to the heterologous integrin by flow cytofluorometric analysis (**Subheading 3.2.1**) or by immunoprecipitation of surface-iodinated transfected cells (**Subheading 3.2.2**). Adhesion assays should also be performed to confirm that the expressed integrin is functionally active (**Subheading 3.3.1**). In our transient transfection system, maximal exogenous integrin expression is observed at 48 h after the transfection, however, it is suggested to determine the optimal time for each cell type and integrin of interest.

3.2.1. Flow Cytofluorometric Analysis

1. Remove medium from the CHO cells and wash once with sterile PBS.
2. Detach the cells from the tissue-culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
3. Neutralize the trypsin with an equal volume of CHO growth medium.
4. Remove a 10-µL aliquot of cell suspension and add it to 90 µL of 0.2% trypan blue.
5. Count the cells using a hemocytometer and determine the total number of cells.
6. Centrifuge the cells at 300*g* for 4 min.
7. Resuspend the cells in CHO growth medium, and then place 5×10^5 cells, each, into two separate 5-mL round-bottom tubes (*see* **Note 16**).
8. Repeat **step 6**.
9. Aspirate the supernatant.
10. Resuspend the cells in 50 µL of CHO growth medium containing the 1° Ab (*see* **Note 17**).
11. Incubate the cells for 30 min on ice.
12. Add 1 mL of CHO growth medium to each tube, and repeat **step 6**.
13. Wash the cells 2× with 1 mL CHO growth medium.
14. Resuspend the cells in 50 µL of medium containing the 2° Ab (*see* **Notes 18** and **19**).
15. Repeat **steps 11** and **12**.
16. Wash the cells once with CHO growth medium and once with sterile PBS.
17. Resuspend the cells in 0.06% paraformaldehyde in PBS and perform flow cytofluorometric analysis.

3.2.2. Surface Iodination and Immunoprecipitation

1. Remove medium from the CHO cells and wash once with sterile PBS.
2. Detach the cells from the tissue-culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
3. Neutralize the trypsin with an equal volume of CHO growth medium.
4. Remove a 10 µL aliquot of cell suspension and add it to 90 µL 0.2% trypan blue.
5. Count the cells using a hemocytometer and determine the total number of cells.
6. Centrifuge the cells at 300*g* for 4 min.

7. Wash the cells 3X with PBS.
8. Resuspend the cells in PBS, containing 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, at a final concentration of 2×10^7 cells/mL in a 15-mL conical tube.
9. Add 200 µL of lactoperoxidase to the cells.
10. Add 1 mCi $Na^{125}I$ to the cells (*see* **Notes 4**, **20**, and **21**).
11. Add 40 µL 0.24% H_2O_2 to the cells.
12. Incubate cells on ice for 5 min, with gentle vortexing after each minute.
13. Add 40 µL of 0.24% H_2O_2, and repeat **step 12**.
14. Add 10 mL PBS containing 0.5 mg/mL tyrosine to bind all of the remaining free $Na^{125}I$ (*see* **Note 22**).
15. Incubate the cells for 5 min at room temperature (*see* **Note 23**).
16. Wash cells 3× with 10 mL of PBS.
17. Determine the volume of the cell pellet, and lyse with an equal volume of lysis buffer at 4°C for 30 min.
18. Centrifuge at 14,000*g* for 30 min, and transfer the supernatant to a fresh tube.
19. Count an aliquot of the lysate in a gamma counter to determine the amount of radioactive material in the starting lysate. Use counts per minute, cpm.
20. Incubate lysate with nonimmune serum followed by Protein A-sepharose for 30 min each, at 4°C (*see* **Note 24**).
21. Centrifuge the Protein A-sepharose beads at 5000*g* for 5 min.
22. Repeat **steps 20** and **21**.
23. Repeat step **19**.
24. Remove an equal amount of cpms, for each immunoprecipitation, from the supernatant and incubate it with an Ab that is specific for the transfected integrin (*see* **Notes 25** and **26**). Using equal cpms is an important control when comparing two different immunoprecipitations to each other.
25. Incubate this reaction overnight at 4°C (*see* **Note 24**).
26. Add an equal volume of protein A Sepharose as in **step 20**, and incubate for 2–4 h at 4°C (*see* **Note 24**).
27. Wash the immunocomplexes 5X with lysis buffer (*see* **Note 27**).
28. Resuspend the immunocomplexes in SDS sample buffer.
29. Heat samples to 95°C for 3–5 min.
30. Separate proteins on a 7.5% SDS-PAGE.
31. Coomassie stain and dry the SDS-PAGE gel to fix and visualize the molecular weight markers if they were not prestained or radiolabeled.
32. Visualize the labeled proteins by autoradiography (*see* **Note 28**).

3.3. Assaying for Functional Integrin Expression

3.3.1. Cell Adhesion

1. Coat Linbro® 96-well plates as described ***(14)***, with 100 µL per well of the testing substrate in PBS at 4°C overnight, or at 37°C for 1 h (*see* **Note 29**).
2. Discard the substrate and rinse the plates 3× with 200 µL PBS per well.
3. Block plates with 100 µL of blocking buffer per well, and incubate at 37°C for 1 h.
4. Remove medium from the CHO cells and wash once with sterile PBS.

5. Detach the cells from the tissue culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
6. Neutralize the trypsin with an equal volume of CHO growth medium, and transfer the cells to a 15-mL conical tube.
7. Remove a 10-μL aliquot of cell suspension and dilute into 90 μL 0.2% trypan blue.
8. Count the cells using a hemocytometer and determine the total number of cells.
9. Centrifuge the cells for 4 minutes at 300*g*.
10. Wash the cells once with sterile PBS.
11. Wash the cells 3× with assay buffer and resuspend the cells in the appropriate volume of assay buffer.
12. Rinse the coated and blocked plates 3× with 200 μL of PBS per well.
13. Place a 100-μL aliquot of cell suspension into the wells and incubate at 37°C and 5% CO2 in a humidified incubator (*see* **Note 30**).
14. Gently wash the wells 2× with 200 μL of PBS.
15. Fix the adherent cells with 100 μL of 3% paraformaldehyde at 4°C for 30 min (*see* **Note 2**).
16. Gently wash the wells 2× with 200 μL of PBS.
17. Stain the cells with 100 μL of 0.5% crystal violet, and incubate at room temperature for 2 h or overnight.
18. Aspirate the crystal violet, wash the wells 2× with 200 μL of PBS, and aspirate any remaining liquid from the wells.
19. Read OD_{630nm}.

4. Notes

1. Use appropriate safety precautions when using ethidium bromide.
2. When working with paraformaldehyde, gloves should be used at all times.
3. 30% Hydrogen peroxide can cause burns, so proper precautions should be taken when working with concentrated peroxides.
4. ^{125}I is a volatile compound that will readily incorporate into the thyroid. Consult your institutions and Nuclear Regulatory Commission guidelines for working with this material.
5. Concentrations of purified integrin cDNA necessary for successful transfection will have to be determined in each case.
6. Each electroporation must contain 1×10^7 cells, and the final volume of each cuvette should not exceed 500 μL, therefore, the volume of DNA and water must be taken into consideration prior to resuspending the cells.
7. To avoid electrical shocks, the outside of the cuvet must be dried thoroughly.
8. Cellular debris after the electroporation will be observed.
9. It is essential to dilute the cells into at least 6 mL of growth medium.
10. The medium needs to be changed 24 h after the electroporation to remove the cellular debris and dead cells.
11. Surface expression of the transfected integrin is easily detectable at 48 h after electroporation, however, the optimal time of expression for each integrin may vary for each cell type and integrin.

12. The volume of lipofectin needed must be determined for the cell type and integrin of interest.
13. Cells remain attached to the tissue-culture plate.
14. Cells should be subconfluent in a 60-mm dish (approx 40–70%), however the density should be adjusted in a cell-type-dependent manner.
15. Incubation time is dependent upon cell type.
16. Two separate tubes of cells will be needed for the staining. In one tube, the cells will be stained with an isotype-matched control 1° Ab (**Subheading 2.2.1.**, **step 8**), and the other tube will be stained with a 1° Ab that specifically recognizes the transfected integrin (Subheading **2.2.1.**, **step 7**).
17. The specific 1° Ab must recognize the extracellular domain of the exogenously expressed integrin. Either monoclonal or polyclonal Abs can be used and the concentration of the Ab will vary accordingly. It is important to use a 1° Ab that will not cross-react with endogenous integrins. It is also important to determine the saturating concentration of both the 1° and 2° Ab to be used.
18. Select a 2° Ab for its species specificity against the 1° Ab. This Ab must be conjugated to a fluorophore such as FITC. The use of a 1° Ab that is directly conjugated to a fluorophore is possible.
19. Restrict the samples' exposure to light because of the sensitivity of fluorophores.
20. You must work in a properly authorized fume hood and behind protective lead shielding when working with an opened vial containing free $Na^{125}I$.
21. All waste, both liquid and solid, must be disposed of as radioactive waste throughout the remainder of the experiment.
22. Iodination reaction can also be stopped with 10 mL of PBS containing 0.02% sodium azide to inhibit lactoperoxidase activity, but this will not bind the free $Na^{125}I$.
23. After quenching the free $Na^{125}I$, it is safe to work outside the hood, but always following the proper precautions when working with radioactive material.
24. All incubations during the immunoprecipitation should be done with mild agitation on a rotating platform. Samples should be shielded with lead foil.
25. If using a mouse Ab it may be necessary to use either an intermediate incubation with rabbit anti-mouse IgG.
26. A species-specific Ab for the transfected integrin should be used. Avoid the use of Abs that will cross-react with endogenous integrins.
27. For immunoprecipitations using polyclonal Abs, the first three washes should be performed with lysis buffer, supplemented with 350 m*M* NaCl, followed by two washes using lysis buffer containing 150 m*M* NaCl. Immunoprecipitations using MAbs should be washed 5X with the lysis buffer as described in the material section.
28. Dried gel should be exposed to film in the presence of intensifying screens at –70°C. The length of the exposure must be determined for each experiment.
29. Duplicate wells for the adhesion assay should be coated with an extracellular matrix molecule (ECM) that specifically binds for the transfected integrin or an Ab that will recognize the exogenous integrin. The concentration of ECM used for coating will have to be determined for each cell type.

30. Cell number per well will vary according to cell type, as will the length of time needed for cell attachment.

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10

Preparing a Polyclonal Antibody to Mouse β_1 Integrin with Function-Blocking Activity

Gwynneth M. Edwards and Charles H. Streuli

1. Introduction

Integrins are an important family of cell-surface receptors that mediate adhesive interactions. These transmembrane glycoproteins are composed of noncovalently associated α- and β-subunit heterodimers. Twelve different α-chains and eight different β-subunits combine to give more than 20 distinct integrins and alternative splicing of the α- and β-chains increases the complexity of their interactions ***(1)***. Integrins have been shown to mediate cell adhesion to at least 12 separate matrix components and are also involved in cell–cell contacts via interactions with members of the immunoglobulin and cadherin families ***(2)***. Whereas cellular adhesion to specific components of the extracellular matrix (ECM) is an important function of integrin receptors, signals initiated by ligation of these receptors also modulate cell spreading, migration, proliferation, differentiation, and survival (reviewed in **refs. *1*** and ***3***). Specific function-blocking anti-integrin antibodies are invaluable tools with which to study the role of these receptors in mediating the effects of ECM on cellular processes.

Antibodies generated against integrin peptides or recombinant portions of integrins are not usually function-blocking although they are often useful reagents for techniques such as western blotting, immunoprecipitation, or immunofluorescence. Production of polyclonal function-blocking anti-integrin antibodies has been most successful when the integrin used as immunogen has been purified under nondenaturing conditions so that the protein is as close to its native conformation as possible. Here we describe a method that yielded a β_1 integrin antibody with function-blocking activity.

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1.1. Background

Cell-matrix interactions are critical for regulating the phenotype of many cells. In mammary gland, epithelial secretory cells in pregnant and lactating animals contact a specialized ECM known as basement membrane and this interaction in conjunction with lactogenic hormones is essential for tissue-specific milk protein gene expression *(4)*. Laminin-1 has been identified as the critical component in basement membrane necessary for differentiation *(5)*. Evidence that functional β_1 integrin receptors on the mammary epithelial cell surface are also required for the expression of milk proteins came from experiments that used an antibody raised in goat against a purified membrane fraction subsequently shown to contain anti-β_1 integrin activity *(6,7)*. This antibody was able to block hormone-induced milk protein expression in single mammary epithelial cells embedded in basement membrane extract *(8)*. However, the supply of antibody has since been depleted and there are virtually no other function-blocking anti-mouse β_1 integrin antibodies available. Thus, to further investigate the role of β_1 integrin in milk protein expression in mouse mammary gland, a new reagent had to be generated.

1.2. Strategy

The strategy employed to produce a new function-blocking anti-β_1 integrin antibody was based on purifying embryonic mouse $\alpha_4\beta_1$ integrin then making antibodies to this complex in rabbits. This approach depended on the lack of α_4 integrin in mammary epithelium because expression of this integrin is normally confined to lymphoid and myeloid cells *(9)*. By covalently immobilizing a rat monoclonal antibody specific for mouse α_4 integrin to sepharose, $\alpha_4\beta_1$ integrin from mouse embryos was purified by affinity chromatography (based on the protocol in Knudsen et al. **ref.** *10*). Antibodies to the purified $\alpha_4\beta_1$ integrin complex were then generated in rabbits. $\alpha_4\beta_7$ integrin would also have been purified by the anti-α_4 integrin affinity column but expression of β_7 integrin is thought to be confined to the cells of the Peyer's patch *(11)* and antibodies generated against this protein should therefore have no affect on the function of mammary epithelial cells. Indeed, in β_7 knockout mice, the only phenotype seen affects the Peyer's patch cells *(12)*. In the context of mammary gland, only the antibodies made against β_1 integrin would be relevant. Purified IgG was then prepared from antisera and tested for its specificity and effectiveness in western blotting, immunoprecipitation, and immunofluorescence as well as its ability to prevent adhesion to physiological substrata and to block hormone-induced milk protein expression in mouse mammary epithelial cells.

1.3. Outcome

The newly generated antibody was specific for β_1 integrin and was able to immunoprecipitate β_1 integrins from murine mammary cells and immunostain

β_1 integrin on the basal surface of murine mammary gland alveoli but had limited effectiveness in Western blotting. Its capacity to affect biological processes was also investigated. The anti-β_1 integrin antibody blocked adhesion of murine fibroblasts (NIH3T3) and murine mammary epithelial cells (CID9 and secondaries) but not quail fibroblasts (QT6) to physiological substrata. Further studies have indicated that the antibody recognizes β_1 integrins in rat cells ***(13)***. Moreover, this antibody inhibited ECM-dependent milk protein expression in mammary epithelial cells, blocked ECM-mediated survival in mammary epithelial cells ***(14,15)***, prevented the migration of oligodendrocyte precursors on astrocyte-derived ECM ***(16)*** and inhibited the development of ductal end buds in mouse mammary gland in vivo ***(17)***. Thus, a function-blocking antibody to β_1 integrin has been generated by the method described here. In principle, such a procedure should adapt itself to any integrin and yield antibodies that enable investigation into the effects of ECM/integrin interactions on cell function.

2. Materials

2.1. Preparation of Integrin-Sepharose Column

1. 5 mg of monoclonal antibody (*see* **Note 1**).
2. Dialysis tubing, e.g., Spectro/por (Pierce Warriner, Chester, UK) 12,000–14,000 kDa cut-off.
3. Coupling buffer: 0.2 *M* Na_2CO_3 at pH 9.0, 0.5 *M* NaCl. Store at room temperature.
4. Centricon 30 concentrators from Amicon (Dancers, MA).
5. Cyanogen bromide (CNBr)-activated sepharose from, for example, Sigma (St. Louis, MO, cat. no C 9142). Note that CNBr-Sepharose has a limited shelf life and should be used soon after purchase.
6. 170 mL 1 m*M* HCl in a 20-mL conical flask.
7. Small scintered-glass filter funnel, 250-mL buchner funnel, vacuum source, spatula.
8. 2 × 15-mL Falcon screw-top tubes.
9. Low speed centrifuge.
10. Blocking buffer: 1 *M* Tris-HCl at pH 8.0, 1 *M* NaCl, store at room temperature.
11. Washing buffer: 0.1 *M* sodium acetate at pH 4.0, 0.5 *M* NaCl, store at room temperature.
12. 10 x stock solution of PBS: 80 g/L NaCl, 2 g/L KCl, 2 g/L KH_2PO_4, 11.25 g/L Na_2HPO_4.
13. Storage buffer: PBS, 0.02% sodium azide.

2.2. Extraction of Mouse Embryonic Integrins

All buffers and equipment used in this procedure should be precooled and kept at 4°C or on ice.

1. This method uses 50 g mouse embryos (E14.5–16.5) that have been collected and stored in liquid nitrogen or have been freshly harvested (*see* **Note 2**).
2. 4 × 50-mL Falcon plastic tubes.

3. Protease inhibitor stock solutions: 1.5 m*M* pepstatin in methanol (1 mg/mL, 1000×, Sigma cat. no. P 4265), 20 m*M* leupeptin in ethanol (10 mg/mL, 1000×, Sigma cat. no. L 2023), 200 m*M* phenylmethylsulphonylfluoride (PMSF) in isopropanol (35 mg/mL, 400×, Sigma cat. no. P 7626) and 100 m*M* phenanthroline (19.8 mg/mL, 100×, Sigma cat. no. P 9375) all stored at –20°C.
4. Integrin buffer: 10 m*M* Tris-acetate at pH 7.8, 150 m*M* NaCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$. Store at 4°C. Add 1× protease inhibitors from stock solutions to the required volume immediately prior to use and keep on ice.
5. Nonidet P-40 (NP-40) is equivalent to the detergent, IGEPAL CA630, available from Sigma cat. no. I 3021.
6. Extraction buffer: 170 mL integrin buffer containing 0.5% v/v NP-40.
7. Tissue homogenizer, e.g., the Ultra-Turrax T25 model from Janke and Kunkel.
8. Ultracentrifuge with a swing-out rotor. For example, the Beckman (High Wycombe, UK) Model L8-70M ultracentrifuge, the SW28 rotor and six ultracentrifuge tubes.
9. 10 *M* acetic acid, reagent grade.
10. 1 *M* Tris base, reagent grade.

2.3. Purification of Embryonic Integrin

1. Small column (for example, Sigma cat. no. C 3669) containing the anti-integrin-Sepharose. This is connected downstream of a 20-mL precolumn (Sigma cat. no. C 6169) containing Sepharose CL-4B (Sigma cat. no. CL-4B-200) by the appropriate tubing.
2. Column buffer 1: integrin buffer containing 0.02% v/v NP-40 at 4°C (*see* **Subheading 2.2., step 5**).
3. Column buffer 2: integrin buffer containing 0.02% v/v NP-40 and 1 *M* NaCl at 4°C.
4. Integrin elution buffer: 50 m*M* diethylamine at pH 11.5. Prepare just before use by adding 52 μL diethylamine free base (Sigma cat. no. D 3131) to 10 mL H_2O and keep on ice.
5. Integrin neutralizing buffer: 0.5 *M* sodium acetate at pH 4.0 at 4°C.
6. 1.5-mL Eppendorf tubes.
7. Column storage buffer: 100 m*M* Tris at pH 8.0, 0.02% sodium azide.

2.4. Preparation of Integrin Antigen for Immunization

1. Dialysis buffer: PBS. Note, sodium azide should not be added to this buffer as this will have an adverse effect on the rabbits used for immunization.
2. Dialysis tubing, e.g., Spectro/por 12,000–14,000 kDa cut-off.
3. Centricon 30 concentrators.
4. Freund's complete adjuvant (Sigma cat. no. F 5881).
5. Freund's incomplete adjuvant (Sigma cat. no. F 5506).
6. Syringes and needles.

2.5. Purification of IgG from Serum

1. 5 mL of serum.
2. 4 mL Protein A-Sepharose. (Pharmacia cat. no. 17-0963-03) that has a capacity of 20 mg of human IgG/mL Sepharose.
3. Column with a 5–10 mL capacity (e.g., Sigma cat. no. C 3794) and tubing.

4. 1 *M* Tris-Cl at pH 8.0.
5. Column buffer A: 100 m*M* Tris-Cl at pH 8.0.
6. Column buffer B: 10 m*M* Tris-Cl at pH 8.0.
7. IgG elution buffer: 100 m*M* glycine at pH 3.0.
8. IgG neutralizing buffer: 1 *M* Tris-Cl at pH 8.0.
9. 1.5-mL Eppendorf tubes.
10. Dialysis buffer: PBS.
11. Centricon 30 concentrator.
12. Regeneration buffer 1: 2 *M* urea, freshly made.
13. Regeneration buffer 2: 1 *M* $LiCl_2$.
14. Regeneration buffer 3: 100 m*M* glycine at pH 2.5.
15. Column storage buffer: 100 m*M* Tris at pH 8.0, 0.02% sodium azide.

2.6. Testing for Anti-Integrin Activity

2.6.1. Labeling Cells with ^{35}S-Methionine

1. Appropriate cell line(s) and cell-culture facilities.
2. 50-mm culture dishes and small cell scrapers.
3. Labeling medium such as DMEM/F12 base (Sigma cat. no. D9785) that contains no methionine. This medium requires the following to be added: 30 m*M* $NaHCO_3$, 2 m*M* glutamine, 400 μ*M* leucine, 400 μ*M* lysine, a few drops of 0.5% phenol red, 0.5 m*M* Na pyruvate, 0.5× nonessential amino acids (NEAA), 0.3 m*M* $MgCl_2$, 0.4 m*M* $MgSO_4$, 300 μ*M* $CaCl_2$, 30 μ*M* methionine.
4. Express [^{35}S] protein labeling mix from NEN Life Science Products, Boston, MA cat. no. NEG-072.
5. Perspex box (home made) with a lid containing an open dish of activated charcoal (to absorb radioactive gases; Sigma cat. no. C 4386) and one of water (to provide humidity).
6. Cell lysis buffer: 50 m*M* Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$. Add 1× protease inhibitors (*see* **Subheading 2.2.**, **step 3**) just prior to use. Keep on ice.
7. Tray to hold ice and cell-culture dishes.

2.6.2. TCA Precipitations

1. Carrier buffer: 20 m*M* L-methionine, 0.02% BSA, 0.05% sodium azide in PBS. Store at 4°C
2. 30% TCA, on ice.
3. Whatman (Clifton, NJ) GF/C filters.
4. Millipore (Bedford, MA) filter unit.
5. Vacuum source.
6. 15% TCA, on ice.
7. 95% ethanol

2.6.3. Immunoprecipitating Labeled Integrins

1. ^{35}S-methionine-labeled cell lysates (*see* **Subheading 2.6.1.** above).
2. Cell lysis buffer (*see* **Subheading 2.6.1.** above).

3. Equipment and solutions for SDS-PAGE using a 30%:0.8% acrylamide:bis-acrylamide solution and 2× SDS sample buffer containing 2% SDS, 100 m*M* Tris-HCl at pH 6.8, 20% glycerol, 0.04% bromophenol blue, +/– 2% β-mercaptoethanol.
4. Crude antisera or purified IgG.
5. Protein A-Sepharose (Zymed Labs Inc., South San Francisco, cat. no. 10-1041).
6. 10 m*M* Tris at pH 6.8.
7. SDS gel fix: 10% acetic acid, 50% isopropanol.
8. Amplify (Amersham Arlington Heights, IL, cat. no. NAMP 100).
9. Gel dryer, e.g., by Bio-Rad (Richmond, CA).
10. Equipment for autoradiography.

2.7. Adhesion Assays

1. Stock solutions of physiological substrata stored at –70°C (*see* **Note 3**).
2. Dilution buffer: PBS (*see* **Subheading 2.1.**), 0.5 m*M* $CaCl_2$, 0.9 m*M* $MgCl_2$, on ice.
3. Multichannel pipettors.
4. Maxisorp Nunc-Immuno 96-well plates (Nunc, Wiesbaden-Biebrich, Germany, cat. no. 442404).
5. Adhesion medium: 5% bovine serum albumin (BSA) in DMEM/F12 medium (Life Technologies, Bethesda, MD, cat. no. 074-02500A).
6. Appropriate cell lines.
7. 85- and 140-mm tissue-culture dishes.
8. HBSS calcium-free medium (Sigma cat. no. H 4891) containing 4.17 m*M* $NaHCO_3$ at pH 7.4.
9. HBSS/EDTA made by adding 0.2% w/v EDTA in HBSS medium at pH 7.4.
10. HBSS/Trypsin-EDTA made by adding 0.5 mg/mL porcine trypsin (Sigma cat. no. T 0646) to HBSS/EDTA at pH 7.4. Note that HBSS is a very mild buffer. After each addition, its pH needs readjusting.
11. Turkey egg white trypsin inhibitor (Sigma cat. no. T 4385) at 250 mg/mL in PBS.
12. Purified anti-integrin IgG at 1 mg/mL in PBS.
13. Purified preimmune IgG at 1 mg/mL in PBS.
14. 8% paraformaldehyde. Make an 8% w/v solution of paraformaldehyde by heating to 50°C (do not heat above this temperature) for 30 min. Carefully, add 2 *M* NaOH dropwise until the solution just clears (too much NaOH will infringe on the buffering capacity of the PBS used later to make a 1% paraformaldehyde solution). Cool, aliquot, and store at –20°C.
15. 1% paraformaldehyde. Thaw an aliquot of 8% paraformaldehyde and dilute 1:1 in 2× PBS. Dilute 1:4 in 1× PBS.
16. 2% crystal violet—stir in water for 30 min, filter through a Whatman filter # 1 then a 0.45-μm filter.
17. Cell stain:1% paraformaldehyde, 0.02% crystal violet.
18 2 *M* guanidine hydrochloride.
19. Microtiter plate shaker.
20. Microtiter plate reader.

3. Methods

3.1. Preparation of Integrin-Sepharose Column

1. Dialyze a known volume and concentration of monoclonal antibody (5 mg total) against 2 L coupling buffer overnight at 4°C.
2. Concentrate the monoclonal to give 1 mL using a Centricon 30. Assuming a 100% recovery from the dialysis and concentration steps, this yields a 5 mg/mL protein solution. However, it is advisable to retain a small quantity of the concentrated protein for subsequent analysis if required.
3 Weigh out enough CNBr-activated Sepharose to produce 1 mL swollen gel (*see* manufacturers directions) and add this to a 250-mL flask containing 170 mL 1 m*M* HCl. Gently swirl the contents of the flask periodically over a 20 min period to swell the gel.
4. Collect the CNBr-activated sepharose by pouring the contents of the flask through a small scintered-glass funnel under a gentle vacuum. Use extra 1 m*M* HCl to collect any remaining sepharose. Drain off most of the 1 m*M* HCl (do not allow to dry out).
5. Wash the sepharose with 5 mL of coupling buffer and drain under gentle vacuum until the Sepharose is firm (do not allow to dry out).
6. Using a metal weighing spatula, scoop the sepharose out of the funnel and place it in a 15-mL screw-top tube. Add 1 mL of coupling buffer to the tube and mix gently. Add the 1 mL of 5 mg/mL monoclonal antibody to the tube and gently mix. A few extra drops of buffer may be required to ensure the mixture is fluid.
7. Close the tube and allow the coupling reaction between the monoclonal antibody and CNBr-activated Sepharose to proceed at room temperature for 2.5 h while rocking the tube gently on its side.
8. Centrifuge the contents of the tube at low speed (approx 400*g*) for 5 min and, using a Pasteur pipet, syphon off the supernatant and save it in case the monoclonal was not bound by the CNBr-activated sepharose. This can be quickly checked later by reading the absorbance at 280 nm or analyzing in a protein assay.
9. Transfer the contents of the tube to the scintered-glass funnel and wash with 5 mL of coupling buffer. (First ensure the buchner flask is rinsed and empty and collect this initial wash for the reason given above).
10. Wash the sepharose with a further 50 mL of coupling buffer and then transfer it to a new 15-mL screw-top tube. Add 2 mL of blocking buffer (to ensure all the activated sites on the Sepharose are reacted) and rock gently overnight at 4°C.
11. Wash the anti-integrin Sepharose in the scintered-glass funnel alternatively with 20 mL of washing buffer and coupling buffer, repeating this three times.
12. Finally, if the anti-integrin-Sepharose is to be used immediately, wash with PBS in the scintered-glass funnel, make a slurry in PBS, and pour into a small column (*see* **Subheading 3.3.**). If the Sepharose is to be stored then wash with PBS containing 0.02% sodium azide to prevent microbial growth. Note that the sepharose must subsequently be washed extensively to remove the sodium azide before it is used to purify integrins.

3.2. Extraction of Mouse Embryonic Integrins

The whole procedure is carried out keeping the tissue on ice and using pre-cooled equipment, containers, and buffers.

1. Evenly divide 50 g of embryos between the four 50-mL Falcon tubes and then distribute 170 mL of cold extraction buffer on top of the embryos. If the embryos were frozen, allow them to thaw slightly.
2. Using a medium setting, disrupt the tissue in each tube sequentially with the tissue homogenizer for 30 s, storing on ice afterwards. Repeat for a total of four times. Reduce the speed of the homogenizer if the extract foams excessively.
3. Incubate the extract on ice for a further 15 min before distributing it between the 6 ultracentrifuge tubes taking care to balance pairs of tubes. Centrifuge at 113,000*g* (27,000 rpm for the Beckman SW28 rotor) for 20 min.
4. Decant off the supernatant and measure its volume. Calculate the volume of 10 *M* acetic acid required to acidify the supernatant—i.e., 2 μL of 10 *M* acetic acid per milliliter of supernatant. Slowly add the acetic acid dropwise while stirring well. Allow the mixture to sit on ice for 45 min before centrifuging at 113,000*g* for 20 min.
5. Decant off the supernatant and calculate the volume of 1 *M* Tris base required to neutralize it, i.e., 10 times the volume of the 10 *M* acetic acid used in **step 4**. Add the 1 *M* Tris base dropwise while stirring. The extract is now ready to load on to the anti-integrin column.

3.3. Purification of Embryonic Integrin

All steps are performed at 4°C. Remember to save a small sample at each step in the procedure.

1. Pour the anti-integrin-Sepharose made in **Subheading 3.1.** into the small column and connect this downstream of a precolumn containing 20 mL sepharose CL-4B. Under gravity, equilibrate the columns in column buffer 1.
2. Load the precolumn with the neutralized embryo extract made in **Subheading 3.2.** until the last of the extract just enters the column. Begin washing the columns with column buffer 1 until the extract has visually cleared the precolumn.
3. Disconnect the precolumn and continue washing the anti-integrin-Sepharose column under gravity until the absorbance at 280 nm of the flow through is <0.1 (at least 100 mL). Change to column buffer 2 and again wash the column until the absorbance is < 0.1 (at least 50 mL). Re-equilibrate in column buffer 1 (at least 25 mL).
4. To elute the bound integrin, wash the column in 10 mL of integrin elution buffer. Collect 0.9-mL fractions in Eppendorf tubes that already contain 0.1 mL of integrin-neutralizing buffer and mix the contents of the tube as soon as each fraction has been collected.
5. Read the absorbance of each tube at 280 nm and pool those tubes containing protein. The yield of integrin can be estimated by assuming that A280nm = 1 is the equivalent of 1 mg/mL protein. Alternatively, protein concentration can be assessed using a protein assay such as that marketed by Bio-Rad (*see* **Note 4**).

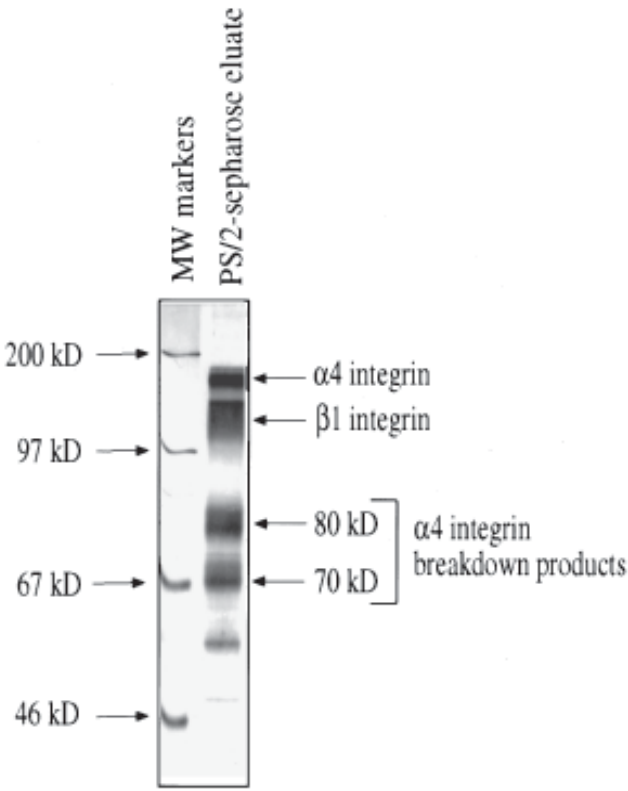

Fig. 1. Purified $\alpha_4\beta_1$ integrin protein. 9 μg of purified $\alpha_4\beta_1$ integrin was separated by 7.5% nonreducing SDS-PAGE and visualized by silver staining. The α_4 integrin band migrates at approx 150 kDa and the β_1 integrin band at approx 130 kDa. Breakdown products of α_4 integrin migrate at approx 70 and 80 kDa.

6. As soon as possible, re-equilibrate the anti-integrin column in integrin buffer (*see* **Note 5**). For storage at 4°C, wash the column in column storage buffer.
7. Analyze the purified integrin by 7.5% nonreducing (*see* **Note 6**) SDS-PAGE followed by silver staining. For example, *see* **Fig. 1**.

3.4. Immunizing Rabbits with Purified Integrin

1. Dialyze the purified integrin against 2 L PBS overnight at 4°C and then concentrate the protein using a centricon 30 to bring it to an appropriate volume for immunizing rabbits (100 μg/mL).
2. After prebleeding, rabbits are injected with a 1:1 mix of 50 μg of purified integrin and 0.5 mL of Freund's complete adjuvant mix at four sites/animal (250 μL/site).
3. Animals are boosted after 12 wk with the same amount of integrin in Freund's incomplete adjuvant mix and blood taken at biweekly intervals to test for production of antibody (*see* **Note 7**).

3.5. Purification of Polyclonal Anti-Integrin IgG from Serum

1. Pour a 4-mL Protein A-Sepharose column and equilibrate it in column buffer A.
2. Adjust 5 mL of antiserum with 0.5 mL 1 *M* Tris to pH 8.0 and clear by centrifugation at 20,000g for 20 min in a microfuge.
3. Load the serum onto the Protein A-Sepharose column, then wash with 40 mL (10 column vol) of column buffer A followed by 40 mL of column buffer B.
4. Elute bound IgG with 12 mL of IgG elution buffer collecting 0.9-mL fractions in eppendorf tubes containing 0.1 mL of IgG-neutralizing buffer.
5. Read absorbance at 280 nm and pool tubes containing >0.1 OD units.
6. To estimate the yield of purified IgG, use the following equation:

$$\text{Concentration of IgG in mg/mL} = A_{280nm} \times 0.8.$$

7. Dialyze the purified polyclonal anti-integrin IgG against 2 L PBS (*see* **Note 8**).
8. Concentrate the IgG as required. Aliquot and store at –70°C.
9. To regenerate the Protein A-Sepharose column, wash sequentially in 5-column vol each of the regeneration buffers 1, 2, and 3. Finally re-equilibrate and store in column storage buffer containing 0.02% sodium azide. Note that the column will need extensive washing in column buffer A to remove the sodium azide before reuse.

3.6. Testing for Anti-Integrin Activity

It is not feasible to test for anti-integrin antibody production by the ELISA method as the yield of purified integrin antigen is low. Therefore, anti-integrin activity and specificity can be determined and titered by immunoprecipitating integrins from ^{35}S-methionine metabolically labeled cells (**Fig. 2**). Because the cell type used in immunoprecipitations will be determined individually for each particular integrin used to produce antibodies, what follows is a description of ^{35}S-methionine labeling for the murine mammary epithelial cell line, CID-9, and the melanoma cell line, K1735-M2 (**Fig. 3**), although this method may also suffice for other cell types (*see* **Note 9**).

3.6.1. Labeling Cells with ^{35}S-Methionine

1. Culture cells to approx 80–90% confluency on 50-mm dishes.
2. Wash the cells twice in warm labeling medium and then incubate in this medium for 30 min at 37°C. Replace with 1 mL of 200 μCi/mL ^{35}S-methionine in fresh medium and place the culture dishes in the perspex box with open dishes of activated charcoal and water. Close the lid and place the box in the tissue-culture incubator overnight. Some cell types are sensitive to the lack of serum and therefore 2–5% fetal calf serum that has been dialyzed against the labeling medium should be included in the labeling of these cells.
3. The next day, wash the cells twice in warm labeling medium taking care to dispose of the radioactive waste appropriately. Place the culture dishes on ice.
4. Scrape the cells into cell lysis buffer. This is done by first adding 0.3 mL of ice-cold lysis buffer to the washed cells, scraping the cells then carefully removing

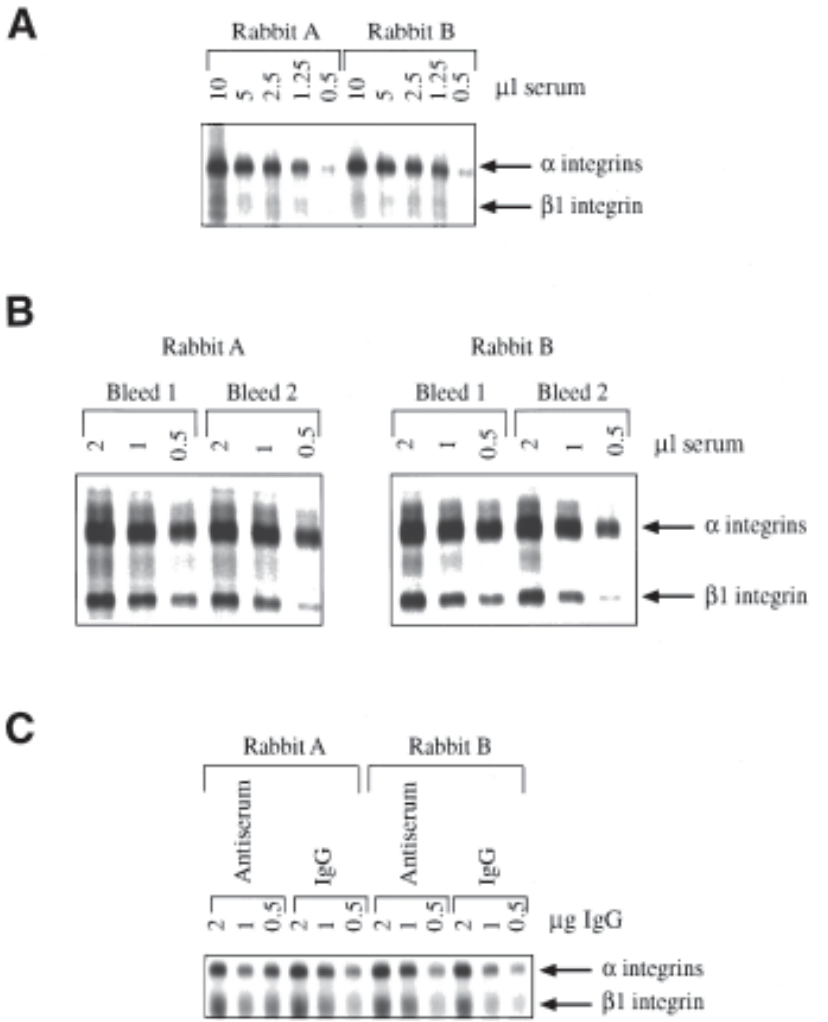

Fig. 2. Anti-$\alpha_4\beta_1$ integrin antibody titrations. 1×10^7 cpm of ^{35}S-methionine labeled CID9 cell lysate was immunoprecipitated with increasing amounts of anti-$\alpha_4\beta_1$ antibody. The precipitated proteins were separated by 6.25% nonreducing SDS-PAGE, the gels dried and the α and β_1 integrin bands visualized by autoradiography. **(A)** The antisera produced by the two rabbits (A and B) were compared by immunoprecipitating labeled lysate with the indicated volumes (µL) of serum. **(B)** Successive bleeds of the rabbits taken 4 mo apart were compared. **(C)** After IgG purification on Protein-A-Sepharose, the relative titer of antibody was compared to the starting antiserum.

the lysate to an Eppendorf tube. Repeat this with a second aliquot of lysis buffer and add to the Eppendorf tube.

5. Lyse the cells by high-speed shaking at 4°C for 30 min.

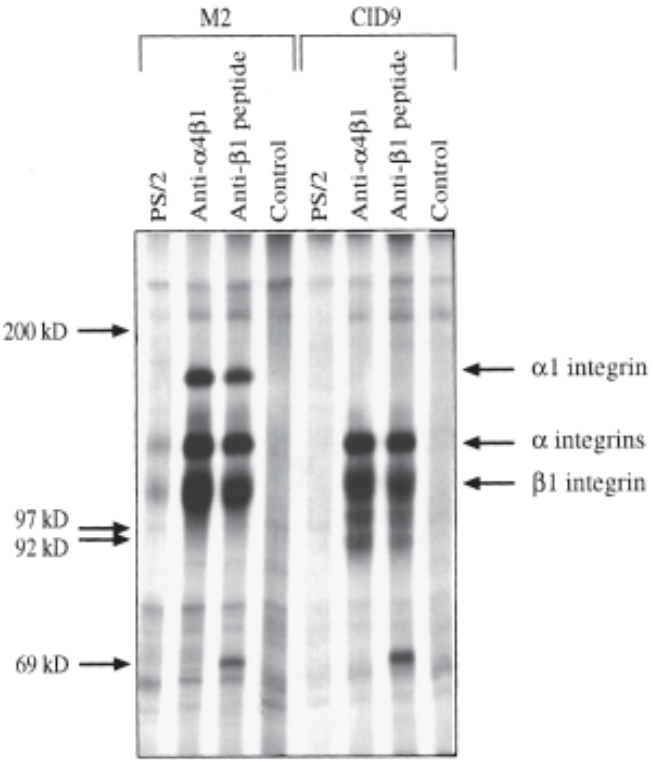

Fig. 3. Specificity of anti-$\alpha_4\beta_1$ integrin antibody. ^{35}S-methionine labeled M2 and CID9 cell lysates (5×10^6 cpm/lysate) were immunoprecipitated with 10 μg PS/2, anti-$\alpha_4\beta_1$ integrin, anti β_1 integrin peptide or control rabbit IgG. After separating precipitated proteins by 6.25% nonreducing SDS-PAGE, the gel was dried and labeled proteins visualized by autoradiography. α integrins migrate at approx 150 kDa and β_1 integrins at approx 130 kDa. The band at approx 180 kDa precipitated by the anti-$\alpha_1\beta_4$ and anti β_1 peptide antibodies is α_1 integrin that is present in M2 but not CID9 cells ***(18)***.

6. Clear the lysates by centrifugation (top speed in microfuge) and determine the radioactivity incorporated into cell proteins by TCA precipitation.
7. Store the lysates at –70°C.

3.6.2. TCA Precipitation

1. In triplicate, add 10 μL of radioactive lysate to 490 μL of carrier buffer. Mix well and then add 0.5 mL of 30% TCA, mix, and leave on ice for 30 min.
2. Remove the contents of each tube and individually filter through a Whatman GF/C filter using the Millipore (Bedford, MA) filter unit.
3. Rinse the tube with 1 mL of 15% TCA and wash each filter five times in 15% TCA then twice in 95% ethanol.

4. Dry the filters in air. Place in scintillation tubes and cover with scintillation fluid.
5. Count the filters and estimate cpm/mL lysate.

3.6.3. Immunoprecipitation of Integrins

1. Within a set of samples, use equal amounts of radioactivity for each immunoprecipitation. Generally, 5×10^6 cpm/immunoprecipitation is adequate to detect labeled integrin protein bands easily.
2. Thaw the labelled lysates on ice, vortex, and centrifuge at top speed in the microfuge before transferring the required volume of lysate to an Eppendorf tube. Bring the volume of each lysate to 1 mL by adding lysis buffer. Remove a 10-µL aliquot from each tube and add it to 10 µL 2x SDS sample buffer. These samples will then be run on 7.5% SDS-polyacrylamide gel to visually confirm that equal counts had been used within an experiment.
3. Volumes between 1 and 100 µL of crude antisera or purified IgG are then added to the lysate and the mixture incubated for 1 h at 4°C.
4. 50 µL of a 1:1 slurry of Protein A-Sepharose in lysis buffer is added to the tubes and the mixture agitated for a further 40 min at 4°C.
5. The Sepharose beads are recovered by a short spin in the microfuge and the supernatant removed with a Pasteur pipet.
6. The beads are washed five times in lysis buffer by adding 1 mL of lysis buffer, spinning down, and removing the supernatant each time.
7. Finally wash the beads in 10 m*M* Tris at pH 6.8.
8. Resuspend the beads in 20–50 µL SDS sample buffer, boil for 3 min, and chill on ice.
9. Immunoprecipitated proteins are then separated by 7.5% nonreducing SDS-PAGE (*see* **Note 10**).
10. Fix the gel for 30 min in 10% acetic acid, 50% isopropanol. Wash the gel for 30 min in water with two changes followed by 30 min in Amplify.
11. Dry the gel down on filter paper, e.g., using a Bio-Rad gel dryer. Radioactive bands are visualized by autoradiography at –70°C (for example, *see* **Figs. 2** and **3**).

3.7. Adhesion Assays

Adhesion assays are useful for determining whether the newly generated anti-integrin antibody is function blocking (**Fig. 4**). As integrins often have overlapping adhesive interactions with physiological substrata, it is important to test the effects of the anti-integrin antibody in more than one cell line on a range of physiological substrata. Before any anti-adhesive effects of the antibody can be assessed, initial experiments must be performed to determine the lowest density of substrata that permits the highest amount of cell attachment for each cell line.

3.7.1. Preparation of 96-Well Plates

1. Stock solutions of physiological substrata stored at –70°C are thawed according to the supplier's instructions (e.g., mouse laminin and collagen IV are thawed on

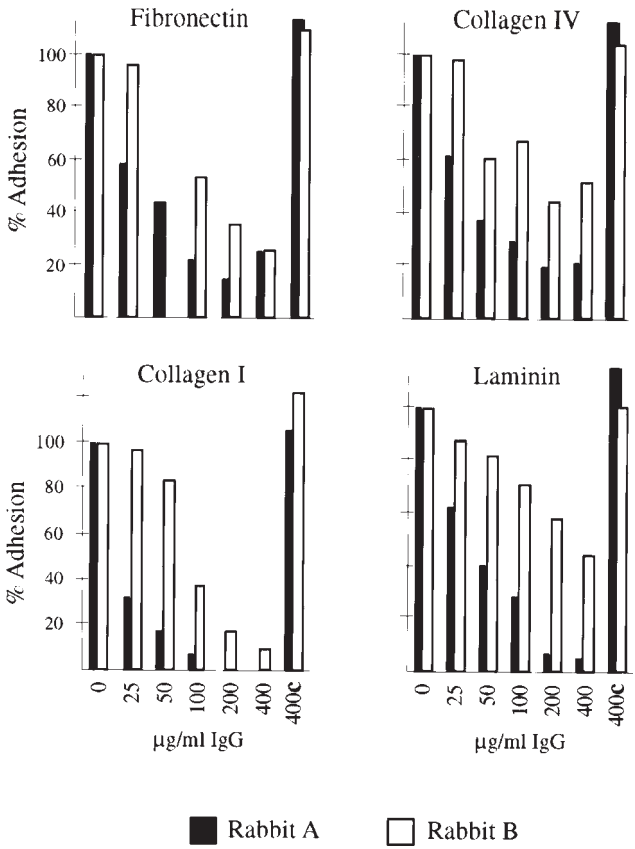

Fig. 4. Adhesion-blocking capability of antisera from two different rabbits. CID9 cells (3×10^4 cells/well) were incubated for 60 minutes in the presence of the indicated concentrations of IgG purified from the antisera from two rabbits (A and B) immunized with $\alpha_4\beta_1$ integrin protein or with control rabbit IgG (400c) in 96-well dishes coated with physiological substrata. After washing away unattached cells, adhered cells were fixed, stained with crystal violet then solubilized in 2 *M* guanidine hydrochloride before reading the absorbance at 595 nm. Adhesion is plotted as a percentage of adhesion in the absence of IgG. Note that the adhesion-blocking titer of antibody from rabbit A is significantly greater than that from rabbit B, even though their titers in immunoprecipitation assays were indistinguishable (*see* **Fig. 2**).

ice, whereas fibronectin is warmed to 37°C) before dilution into ice-cold dilution buffer. For initial experiments to determine the optimal substrata density, a range of concentrations of substrata will be required, typically in the range of 1–20 µg/mL, but it is advisable to check the literature for cell-specific requirements (*see* **Note 3**).

2. Using a multichannel pippettor, aliquot 100 μL/well of diluted substrata in triplicate into chilled 96-well plates and leave the wells to coat overnight at 4°C. Remember to leave two sets of control wells: one to control for cell background (i.e., cells will be added to wells with no substrata) and the other as a general background control (i.e., wells with no substrata or cells). Additional control wells containing 100 μL of poly-D-lysine can also be included for maximal cell adhesion.
3. Remove the unbound substrata by flicking the plate upside down into a sink and then patting the upturned plate onto paper towels.
4. Wash the wells twice with 200 μL of PBS.
5. Block any remaining reactive sites in the wells by adding 200 μL/well adhesion medium at 37°C for at least 1 h.

3.7.2. Cell Preparation

1. Culture cells on 85-mm dishes almost to confluency.
2. Passage the cells and replate them onto 140-mm dishes so that they are approx 70–80% confluent within a further 24 h culture (*see* **Note 11**).
3. Wash the cells three times in HBSS/EDTA and then incubate in this medium (4 mL/140-mm dish) until the cells have completely rounded up at 37°C (*see* **Note 12**).
4. Add 1 mL of HBSS/EDTA-trypsin to the HBSS/EDTA already on the plate for 2 min at 37°C. The cells should detach rapidly.
5. Stop the trypsin action by adding turkey egg white trypsin inhibitor (20 μL of 250 mg/mL solution or 5 mg/dish).
6. Gently triturate up and down, remove the cells and wash them in adhesion medium by centrifugation in a 15 mL Falcon tube at 160g for 4 min.
7. Bring the cells up to typically 5–10 mL in adhesion medium and count them (*see* **Note 13**).
8. If the cells are to be used in initial experiments to determine the optimal substrata density, dilute directly to 3×10^5 cells/mL in adhesion medium.
   or
   If the cells are to be used in an adhesion assay to test the anti-integrin antibody, dilute to 6 x 10^5 cells/mL in adhesion medium and then mix 1:1 with a 2x solution of antibody diluted into medium. The final concentration of cells will therefore be 3×10^5 cells/mL and the final concentrations of antibody should range from 0 to 500 μg/mL. As a control, preimmune IgG should be included in the assay at the highest concentration tested for the anti-integrin IgG.

3.7.3. Adhesion Assay Procedure

1. Remove the adhesion medium from the wells and add 100 μL cells per well using a multichannel pipettor or fill wells individually (3×10^4 cells/well). Mix the cells in the tube every 30 s to prevent them settling to the bottom of the tube but do not shake or swirl the plates.
2. Carefully place the 96-well dish in the humidified tissue-culture incubator and allow the cells to adhere for 30–120 min at 37°C depending on the cell type. This can be monitored under the microscope.

3. At the end of the incubation period, remove any unattached cells by inverting the 96-well dish onto a paper towel and patting gently.
4. Wash the wells gently three times in adhesion medium then once in PBS (200 μL/well).
5. Incubate attached cells in 100 μL/well cell stain at 37°C for 1 h.
6. Wash the 96-well dishes carefully five times by submerging the 96-well plates in a tray of H_2O then inverting the dish onto paper towels. Photographs can be taken at this time to record any effects on cell spreading.
7. Adhered cells are then solubilized by adding 100 μl/well 2 *M* guanidine hydrochloride and shaking for 5 min on a microtiter plate shaker at medium speed.
8. Read the absorbance of the wells at 595 nm on a microtiter plate reader.

4. Notes

1. The monoclonal we used to make our anti-α_4 integrin column (a rat monoclonal to mouse α_4 integrin known as PS/2) is available from Serotec (Oxford, UK, cat. no. MCA 1230) and the PS/2 producing cells from ECACC (Oxford, UK, cat. no. 93091302).
2. 50 g of embryo is the maximum amount of material that can be processed at one time using an SW28 rotor.
3. The substrata used in an adhesion assay will reflect the cell type, the integrin under investigation, as well as the species involved.
4. This procedure yielded only very small quantities of purified integrin. Typically 100 μg purified $\alpha_4\beta_1$ was recovered from 50 g of embryo. However, 200 μg of purified integrin was adequate to immunize and boost two rabbits.
5. The PS/2 monoclonal antibody was extremely sensitive to the elution buffer and therefore we made a new batch of anti-integrin sepharose for each purification. Given the differences in the stability of monoclonals, each one will have to be assessed for its ability to be used more than once in the integrin-purification procedure.
6. In general, SDS-PAGE analysis of the purified integrin intended for use as immunogen should reveal two wide protein bands corresponding to α and β integrins. We also observed two breakdown products of α_4 integrin (**Fig. 1**).
7. More than one animal should be used to produce antibodies as the immune response can vary from animal to animal. We encountered a marked difference in adhesion-blocking ability between anti-sera from two animals (**Fig. 4**) although no difference was seen in their ability to specifically immunoprecipitate integrin from ^{35}S-labeled mammary epithelial cell lysates (**Fig. 2**).
8. Although it is important to prevent microbial growth in protein solutions, we found that if sodium azide were used in the preservation of our function-blocking antibody, it could not be removed subsequently (by dialysis or by gel filtration) to a sufficient degree that did not cause toxicity in cells. Therefore, it is best to aliquot and store the antibody at –70°C and thaw shortly before use.
9. We metabolically labeled CID-9 cells and M2 cells with ^{35}S-methionine then checked the specificity of our newly generated anti-β_1 integrin antibody against the monoclonal used as immunogen (PS/2) and a polyclonal against the β_1 integrin C terminus (**Fig. 3**).

10. It is sometimes useful to analyze both reduced and nonreduced immunoprecipitated integrins but these must be run on separate SDS gels because of the very sensitive nature of integrins to reducing agents.
11. The number of cells required to achieve 70–80% confluency in 24 h will have to be predetermined and will depend largely on the cell type.
12. The amount of time required for rounding of cells in HBSS/EDTA will vary depending on the cell type. For example, the murine mammary epithelial cell line, CID-9, takes up to 45 min, whereas NIH3T3 fibroblast cells rounded up in only 20 min.
13. Some epithelial cells cannot be fully disaggregated by trypsinization and the cell suspension should therefore be passed through a 20-μm nylon mesh before counting.

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11

Integrin Gene Targeting

Jan F. Talts*, Cord Brakebusch*, and Reinhard Fässler

1. Introduction

1.1. Knockout Technology

Gene ablation, also known as knockout, is a powerful method to analyze gene function in vivo. Many integrin genes have been disrupted already, confirming but also contradicting previous results ***(1)***. In addition, new functions have been revealed, significantly increasing our understanding of the biological roles of integrins. Knockout of the remaining integrins will similarly elucidate their function during development. In addition, knockins of subtle mutations ***(2,3)*** and conditional knockouts ***(4,5)***, resulting in tissue-specific or temporally restricted gene ablation, will allow us to assess structure-function relationships of integrins in vivo and to investigate the function of integrins in a specific cell type at a specific time point in development.

Two important achievements allowed generation of mice with specific mutations in the genome. First, the establishment of stable totipotent mouse embryonic stem (ES) cell lines derived from the inner cell mass (ICM) of blastocysts ***(6,7)*** and second, the finding that transfected DNA can recombine with its homologous counterpart in the genome of mammalian cells ***(7,8)***. Also important was the introduction of positively selectable marker genes allowing to select for stably transfected cells ***(9)***. Establishment of negatively selectable marker genes allowed further to select against heterologous integration of the targeting construct in the genome ***(10)***.

1.2. Generation of a Knockout Mouse

The first step in generating knockout mice is the construction of a targeting vector (**Fig. 1**). The classical targeting vector contains an antibiotic resistance

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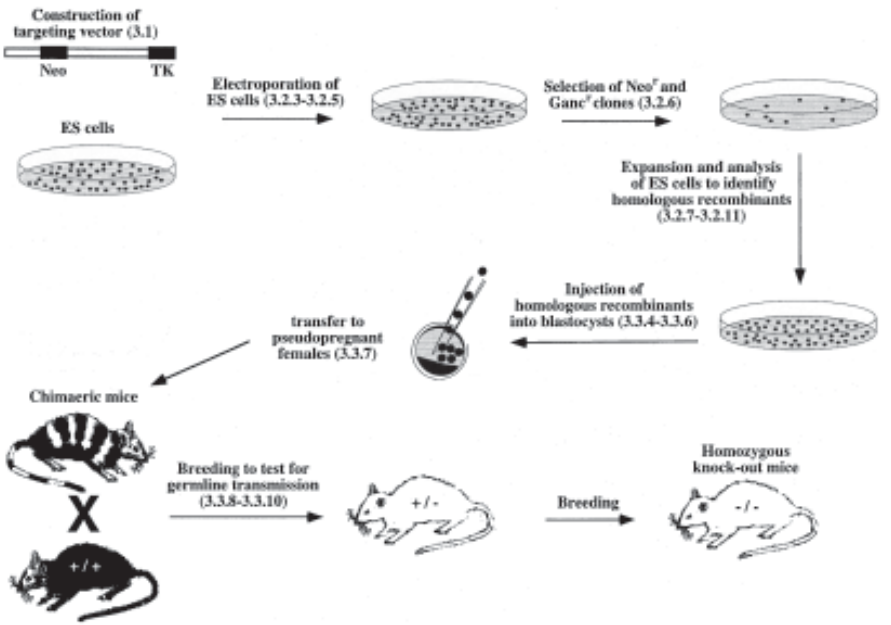


Fig. 1. Generation of knockout mice. The numbers in brackets indicate the corresponding sections in the text.

gene that serves as a positive selection marker (e.g., a neomycin resistance gene, *see* **Note 1**) flanked on both sides by homologous DNA of the gene of interest. The flanking DNA facilitates homologous recombination that replaces the endogenous DNA by the targeting construct ***(11)***. The selection marker is inserted into an exon leading to a disruption of the coding sequence. Recombined mutant ES cells express the antibiotic resistance gene and can easily be selected.

However, homologous recombination is a rare event in mammalian cells ***(12)***. Thus, in most cases, ES cells will integrate the targeting vector randomly into the genome (heterologous recombination). Several strategies have been devised to increase the rate of homologous recombination. First, the length of flanking homologous DNA can be increased ***(11)***. Second, a negative selectable marker, such as the herpes simplex virus (HSV) thymidine kinase gene, can be added to one end of the construct ***(10)***. This marker will be lost upon homologous recombination, but will incorporate randomly into the genome upon heterologous recombination. ES cells lacking this marker will be selected for, increasing the percentage of cells with homologous recombination by approx 2- to 10-fold ***(13)***. Other methods used to ensure homologous recombination are promotor trapping ***(14)*** or polyadenylation trapping ***(15)***. Here the

selection marker gene lacks a promotor or a terminating signal, respectively, and is therefore only active if the missing functions are provided upon homologous recombination.

The targeting vector is electroporated into ES cells, which are subsequently grown in the presence of antibiotics to select for cells with stable transfection of the targeting vector. Only cells expressing the drug resistance gene survive. Resultant colonies are isolated, expanded, and further analyzed. Because homologous recombination occurs only in a few cells, ES cell colonies are subjected to polymerase chain reaction (PCR) or Southern analysis to identify the new gene polymorphism present in knockout clones. Next, such clones are injected into blastocysts in which the ES cells intermingle with inner cell mass cells. The injected blastocysts are transferred into the uterus of foster mice where they develop into chimeric mice. The mutated ES cells contribute to the development of germ as well as somatic cells (for review, *see* **ref. *16***).

The contribution of ES cells to the chimeric mice is assessed easily if the coat color genes of the ES cells and the blastocysts used for injection are different. For example, if ES cells are derived from agouti 129/Sv mice and the blastocyst from black C57Bl/6J mice, the percentage of agouti in the coat color of the chimera roughly indicates the contribution of ES cells. Germ-line transmission can be recognized by mating this chimera to C57Bl/6J mice. ES-cell-derived germ cells will result in agouti animals, since agouti is dominant over black.

Embryonic lethality, as observed for some integrin-null mutants, makes it difficult to study development and function of integrins in adult animals. Conditional knockout, i.e., time-and tissue-restricted gene disruption, circumvents this problem as it allows a local gene ablation in the adult animal. This can be achieved with the Cre-loxP system ***(4,5)***. Short loxP sequences are inserted upstream and downstream of an essential part of the gene, creating a "floxed" gene. The loxP sites should not influence expression of the gene and mice carrying homozygously a floxed gene should be normal. Such mice are crossed with mice expressing the viral Cre recombinase under the control of a cell-specific or inducible promoter. The Cre recombinase binds to loxP sites and cleaves out the intervening DNA sequence, thus introducing a null mutation into floxed genes. Therefore, offspring of such a mating protocol acquire gene ablation only in Cre-expressing cells and a wild-type phenotype in all other cells.

Replacement of a protein by a mutant form using homologous recombination (knockin) allows the study of structure-function relationships in vivo. Inactivation of potential phosphorylation sites and mutation of subdomains of known or unknown function will help to elucidate the function of specific parts or amino acid residues of integrins. In vivo expression of the mutant proteins, in the same cells and at the same level as in wild-type mice, will yield information about signal-transduction processes and protein–protein interactions in the

living animal. The knockin technique lacks various problems associated with conventional techniques. Possible problems encountered using such techniques (expressing mutant proteins in tissue culture or transgenic animals) are misplaced expression, wrong expression levels, and background of wild-type proteins.

Before constructing a targeting vector, one has to consider carefully how the resulting knockout mouse will be analyzed. If a lethal phenotype is expected, it is helpful to establish double-knockout ES cells from the beginning, to analyze differentiation and function of null cells in chimeric mice ***(17–19)***, teratomas ***(20)***, or embryoid bodies ***(17,21,22)***. To distinguish knockout from wild-type cells in these models, it is useful to combine the knockout with the knockin of a reporter gene, such as *lacZ* or green fluorescent protein.

2. Materials

2.1. Construction of Targeting Vector

Chemicals, where no specific supplier is mentioned, can be bought from any major supplier, such as Sigma (St. Louis, MO).

2.1.1. Isolation of a Genomic DNA Clone from a 129/Sv Library

1. Cosmid library derived from 129/Sv mouse tissue.
2. Kanamycin or ampicillin (cosmid dependent).
3. Nylon membranes, for example Hybond N+ (Amersham, Buckinghamshire, England).
4. Whatman (Clifton, NJ) 3MM papers.
5. LB: 10 g of bactotryptone (Difco, Detroit, MI), 5 g of bacto-yeast extract (Difco), 10 g of NaCl. Fill to 1 L with deionized water, adjust the pH to 7.0, and autoclave. LB can be stored for a very long time at room temperature (RT). Because no antibiotics are added, it will become apparent immediately if the LB is contaminated.
6. LB agar: Add 15 g of bacto agar (Difco) to LB, autoclave, and allow medium to cool to 50°C before adding antibiotics and pouring. Plates can be stored for 1 mo at 4°C. When plates are fresh they will exude moisture when incubated at 37°C. This increases the risk of cross contamination and is avoided by drying the plates in a laminar flow hood for 20 min before use.
7. Denaturation solution: 1.5 *M* NaCl, 0.5 *M* NaOH (can be stored at RT).
8. Neutralization solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl at pH 7.2 (can be stored at RT).
9. 1 *M* NaPi solution: 684 mL 1 *M* Na_2HPO_4, 316 mL 1 *M* NaH_2PO_4 at pH 7.2 (can be stored at RT).
10. Church buffer: 500 mL 1 *M* NaPi, 350 mL 20% SDS, 1 mL 0.5 *M* EDTA, 10 mg/mL sheared salmon sperm DNA, 10 g of bovine serum albumin (BSA). Fill to 1 L with deionized water (can be stored at RT).
11. α-^{32}P-dCTP (Amersham) The half life of ^{32}P is approx 14.3 d. It is a radioactive isotope and as such is dangerous. Normal precautions for radioactive work should be taken. Radioactive contamination is easily detectable with a Geiger counter, which should always be in the room designated for radioactive work.

12. A random priming labeling kit for DNA. For example Amershams Prime-It labeling kit.
13. 20× SSC: 175.3 g of NaCl, 88.2 g of Na-citrate, adjust the pH to 7.0, autoclave (can be stored at RT).
14. Wash solution 1: 2× SSC, 1% SDS (can be stored at RT).
15. Wash solution 2: 0.4× SSC, 1% SDS (can be stored at RT).
16. Midi or maxi Qiagen plasmid purification kit (Qiagen, Santa Clarita, CA).

2.1.2. Analysis of Genomic DNA and Construction of External Probe

1. Restriction enzymes and buffers (store at –20°C).
2. Taq DNA polymerase and buffer (Boehringer Mannheim, Mannheim, Germany) (store at –20°C).
3. dATP, dCTP, dGTP, dTTP (Boehringer Mannheim) (store at –20°C).

2.1.3. Construction of Targeting Construct

1. pMC1 Neo poly A vector (Stratagene, La Jolla, CA) containing a promoter, a *neomycin resistance* gene, and a polyadenylation signal or pNeo plasmid (Pharmacia, Uppsala, Sweden) containing only the *neomycin resistance* gene.
2. Restriction enzymes and buffers (from **Subheading 2.1.2.**).
3. T4 DNA ligase and ligation buffer (Boehringer Mannheim).
4. PCR reagents (from **Subheading 2.1.2.**).

2.2. Production of Mutated Embryonic Stem Cell Clones

2.2.1. Preparation of Feeder Cell Lines

1. Mice expressing a neomycin (or hygromycin or puromycin) resistance gene (*see* **Note 3**).
2. 70% ethanol.
3. Sterile dissecting equipment.
4. 10× phosphate-buffered saline (10× PBS): 80.06 g of NaCl, 2.01 g of KCl, 14.42 g of $Na_2HPO_4 \times 2\ H_2O$, 2.04 g of KH_2PO_4. Fill to 1 L with deionized water and autoclave (store at RT).
5. Trypsin/EDTA solution: 10× stock (Gibco, Paisley, Scotland) (store at –20°C) made 1× with 1× PBS (aliquot and store at –20°C, store the presently used aliqout at 4°C).
6. EF medium: DMEM high glucose + Na-pyruvate (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 2 m*M* L-glutamine (Gibco).
7. Dimethylsulfoxide (DMSO).

2.2.2. Handling of Embryonic Stem Cells

1. Everything except the dissecting equipment from **Subheading 2.2.1.**
2. ES medium: DMEM high glucose + Na-pyruvate (Gibco) supplemented with 15–20% FBS (FBS needs to be tested for ES cell use, or can be bought from

Gibco already tested), 2 m*M* L-glutamine (Gibco), 0.1 m*M* 2-mercaptoethanol, 1× nonessential amino acids of 100× stock solution (Gibco), and 1000 U/mL leukemia inhibitory factor (LIF) (ESGRO from Gibco).

2.2.3. Preparation of DNA for Electroporation of Embryonic Stem Cells

1. Restriction enzymes and buffers (from **Subheading 2.1.2.**).
2. Agarose.
3. 50× TAE-buffer: 242 g of Tris-base, 57.1 mL of glacial acetic acid, 100 mL 0.5 *M* EDTA at pH 8.0, fill to 1 L with deionized water (can be stored at RT).
4. Ethidium bromide.
5. Phenol (equilibrated with 10 m*M* Tris-HCl, 1 m*M* EDTA at pH 8.0).
6. Chloroform.
7. Isoamylalcohol.
8. 3 *M* Na-acetate at pH 5.2, adjust pH with glacial acetic acid (can be stored at RT).
9. 70% ethanol.
10. Absolute ethanol.
11. 1× PBS (*see* **Subheading 2.2.1.**).

2.2.4. Preparation of ES Cells for Electroporation

1. ES cells (from **Subheading 3.2.2.**).
2. 1× PBS (*see* **Subheading 2.2.1.**).
3. Trypsin-EDTA (*see* **Subheading 2.2.1.**).

2.2.5. Electroporation of Embryonic Stem Cells

1. ES medium (from **Subheading 2.2.2.**).
2. Feeder cells (from **Subheading 3.2.1.**).
3. Electroporation cuvet for eukaryotic cells (Bio-Rad, Richmond, CA).
4. Gene Pulser (Bio-Rad).

2.2.6. Selection of Positive Clones

1. ES medium (from **Subheading 2.2.2.**).
2. G418 (Geneticin) (Gibco or Sigma).
3. Gancyclovir (Syntex, Palo Alto, CA).

2.2.7. Seeding Feeder Cells for Picking Embryonic Stem Cell Clones

1. Feeder cell ampoules (from **Subheading 3.2.1.**).
2. Everything from **Subheading 2.2.6.**
3. EF medium (from **Subheading 2.2.1.**).
4. 24-well plates (Costar, Cambridge, MA).

2.2.8. Picking G418-Resistant Clones

1. Trypsin-EDTA solution (from **Subheading 2.2.1.**).
2. ES medium (from **Subheading 2.2.2.**).

3. G418 (Geneticin) (Gibco or Sigma).
4. 24-well plates (Costar).
5. 96-well plates (Costar).

2.2.9. Freezing Clones

1. Trypsin-EDTA solution (from **Subheading 2.2.1.**).
2. Freeze medium: 70% DMEM, 20% FBS, and 10% DMSO.
3. ES medium (from **Subheading 2.2.2.**).
4. G418 (geneticin; Gibco or Sigma).

2.2.10. DNA Preparation from Embryonic Stem Cell Clones

1. Lysis buffer: 100 m*M* Tris-HCl at pH 8.5, 5 m*M* EDTA, 0.2% SDS, 200 m*M* NaCl, 100 µg/mL proteinase K. The proteinase K stock solution (10 mg/mL) should be kept at –20°C and always be added freshly.
2. Isopropanol.

2.2.11. Analysis of Genomic DNA

1. Restriction enzyme and buffer (from **Subheading 2.1.2.**).
2. BSA.
3. Agarose and ethidium bromide (from **Subheading 2.2.3.**).
4. 5× TBE buffer: 54 g of Tris-base, 27.5 g of boric acid, 20 mL 0.5 *M* EDTA at pH 8.0, adjust to 1 L with deionized water (can be stored at RT).
5. Nylon membrane: for example Hybond N+ (Amersham).
6. Church buffer, labeling kit, wash solution 1, and wash solution 2 (from **Subheading 2.1.1.**).

2.3. Production of Chimeric Animals

2.3.1. Preparation of Microinjection Pipets

1. HCl.
2. HNO_3.
3. Glass capillaries (Drummond Scientific, Broomall, PA).
4. Flaming/Brown micropipet puller (Sutter Instrument, Novato, CA).
5. Microscope (Zeiss, Jena, Germany).
6. EG-40 Micropipet grinder (Narishige, Tokyo, Japan).
7. MF-900 Microforge (Narishige).
8. 10% Hydrofluoric acid.
9. Deionized water.

2.3.2. Preparation of Vasectomized Males

1. Avertin 100% stock: Dissolve 10 g of 2,2,2-tribromoethyl alcohol in 10 mL *tert*-amyl alcohol. For use dilute the stock solution to 2.5% in PBS. Store both stock and use solutions at 4°C wrapped in aluminum foil to protect them from light.
2. Sterile dissecting equipment.

3. 70% ethanol.
4. H8× Dexon surgical thread (Braun Dexon, Spangenberg, Germany).

2.3.3. Preparing Pipets for Blastocyst Handling

1. Glass capillaries (Drummond Scientific).
2. MF-900 Microforge (Narishige).

2.3.4. Isolation of Blastocysts

1. C57Bl/6J mice.
2. Sterile dissecting equipment.
3. 70% ethanol.
4. 0.60 × 30-mm syringe needle (Braun Melsungen, Melsungen, Germany).
5. Microscopic depression slides, clear (Fisher Scientific, Nidderau, Germany).
6. Flush medium: High glucose DMEM without $NaHCO_3$, buffered with 20 m*M* HEPES at pH 7.4, supplemented with 10% FBS.
7. ES medium from **Subheading 2.2.2.**

2.3.5. Preparation of ES Cells for Blastocyst Injection

1. The same as in **Subheading 2.2.2.**

2.3.6. Microinjection of Embryonic Stem Cells into C57Bl/6J Blastocysts

1. Petroleum jelly without any additives.
2. Dichlor-dimethyl-silane.
3. Flush medium (from **Subheading 2.3.4.**).
4. Dimethyl polysiloxan.
5. Microscopic depression slides, clear (Fisher).
6. ES medium (from **Subheading 2.2.2.**).
7. Zeiss Axiovert 100.
8. Narishige micromanipulators.

2.3.7. Uterus Transfer of Injected Blastocysts

1. Female BDF1 (F1 from C57Bl/6J × DBA cross).
2. Vasectomized FVB mice (from **Subheading 3.3.3.**).
3. Avertin (from **Subheading 2.3.3.**).
4. Sterile dissecting equipment.
5. 70% ethanol.
6. 0.45 × 25-mm syringe needle (Braun Melsungen).
7. H8 × Dexon surgical thread (Braun Dexon).
8. Clay Adams MikRon Autoclip 9-mm wound clips (Becton Dickinson, Rutherford, NJ).

2.3.8. Breeding Schemes

1. C57Bl/6J mice.
2. 129/Sv mice.
3. Chimeric mice.

2.3.9. Identifying Mutant Animals by Southern Blotting

1. Numbered ear clips (National Band and Tag, Newport, KY).
2. Lysis buffer: 100 m*M* Tris-HCl at pH 8.5, 5 m*M* EDTA, 0.2% SDS, 200 m*M* NaCl, 100 μg/mL proteinase K. The proteinase K stock solution (10 mg/mL) should be kept at –20°C and always be added freshly.
3. Reagents (from **Subheading 2.2.11.**).
4. Phenol (equilibrated with 10 m*M* Tris-HCl, 1 m*M* EDTA at pH 8.0).
5. Chloroform.
6. Isopropanol.

2.3.10. Identifying Mutant Animals by PCR

1. Numbered ear clips (National Band and Tag).
2. PCR reagents (from **Subheading 2.1.2.**).
3. Agarose and 50× TAE from **Subheading 2.2.3.**

2.4. Specialized Knockout Technologies

2.4.1. Use of a Reporter Gene

2.4.2. Generation of Homozygous Knockouts in ES Cells

2.4.3. Analysis of Homozygous Knockout ES Cells via Embryoid Bodies

1. Wild-type ES cells.
2. Homozygous knockout ES cells.
3. DMEM high glucose + Na-pyruvate (Gibco) supplemented with 2 m*M* L-glutamine (Gibco) and 20% FBS (Gibco).
4. 1× PBS (from **Subheading 2.2.1.**).
5. Trypsin-EDTA (from **Subheading 2.2.1.**).
6. Bacteriological Petri dishes.
7. Lab-Tek chamber slides (Nalge Nunc, Naperville, IL).

2.4.4. Analysis of Homozygous Knockout ES Cells via Teratoma Formation

1. Wild-type ES cells.
2. Homozygous knockout ES cells.
3. DMEM high glucose + Na-pyruvate (Gibco) supplemented with 2 m*M* L-glutamine (Gibco) and 20% FBS (Gibco).
4. 1× PBS (from **Subheading 2.2.1.**).
5. Trypsin-EDTA (from **Subheading 2.2.1.**).
6. 129/Sv male mice.
7. 5-Bromo-2'-deoxy-uridine (BrdU) (Boehringer Mannheim).

2.4.5. Analysis of Homozygous Knockout ES Cells in Chimeric Mice

1. Wild-type ES cells.
2. Homozygous knockout ES cells.
3. ES medium (from **Subheading 2.2.2.**).

2.4.6. Conditional Knockout

2.4.7. Subtle Mutation Assay(s)

3. Methods

3.1. Construction of Targeting Vector

3.1.1. Isolation of a Genomic DNA Clone from a 129/Sv Library

To obtain homologous DNA for the targeting construct, a genomic clone containing part of the gene has to be isolated. As frequency of homologous recombination is higher with isogenic DNA ***(11,23)***, the genomic library and the ES cells should be from the same mouse strain. We routinely use cosmid libraries made of genomic DNA from D3 or J1 ES cells and electroporate our constructs into R1 ES cells ***(24)***. Other commonly used ES cell lines derived from 129/Sv mice are D3, J1, E14, CC1.2, CCE, and AB1.

1. Plate cosmid library derived from 129/Sv cells or mice. Grow overnight at 37°C. The density of the colonies should be approx $3–5 \times 10^4$ colonies/plate.
2. Make replica filters from each master plate.
3. Place the replica filters, with the colonies on top, on Whatmann 3MM filters soaked in denaturation solution and incubate for 5 min.
4. Transfer the membranes, with the colonies on top, to Whatmann 3MM filters soaked in neutralization solution and incubate for 5 min. Repeat this procedure to ensure complete neutralization.
5. Wash membranes in 0.04 *M* NaPi solution and fix DNA to membranes by UV crosslinking.
6. To remove bacterial debris, rinse filters in 0.04 *M* NaPi solution.
7. Prehybridize the filters in Church buffer for 1 h at 65°C.
8. Label probe to detect the DNA of interest using a commercial labeling kit. These kits usually provide good radioactive labeling of DNA probes.
9. Hybridize filters at 65°C overnight in Church buffer containing $0.5–1 \times 10^6$ cpm/mL activity of the ^{32}P-labeled cDNA probe. Take care that filters stay wet during the whole incubation period.
10. Wash filters twice at 65°C for 30 min in approx 10 hybridization vol of wash solutions 1 and 2, respectively.
11. Cover filters with nylon wrap. Place them in an autoradiography cassette and expose to an autoradiography film overnight at –70°C.
12. Develop film and identify positive clones.
13. Pick clones corresponding to specific signals with a yellow tip and transfer to 1 mL of LB medium containing 25 μg/mL ampicillin (or other antibiotics depending on cosmid backbone). Grow aliquot of bacteria overnight at 37°C on LB agar plates containing 25 μg/mL ampicillin (or other antibiotics depending on cosmid backbone).
14. Repeat the screening from **steps 2–11** to ensure the isolation of individual bacterial clones.

15. Pick single positive clones for DNA isolation. Large-scale DNA isolations can be performed with the midi or maxi Qiagen plasmid purification kit following the instructions of the manufacturer.

3.1.2. Analysis of Genomic DNA and Construction of External Probe

The gene is first analyzed by restriction enzyme analysis. Intron–exon borders and intron sizes are determined by PCR. Knowledge of the transcription start site is often helpful when insertions are planned into the 5' noncoding region. Based on this analysis the targeting construct and the detection of homologous recombinants by restriction digest and Southern blot are planned. Preferred enzymes for this screening are *Bam*HI, *Bgl*II, *Eco*RI, and *Eco*RV because of their effective cutting of DNA, insensitivity to methylation of recognition sites, and low costs.

Homologous recombinants are recognized by digestion of genomic DNA of ES cell clones with restriction enzymes cutting within and upstream or downstream of the targeting construct. Hybridization of the Southern blot with an "external" probe outside of the targeting construct will result in a band corresponding to the wild-type locus and, in case of homologous recombination, a second band of readily discernible size corresponding to the targeted locus. Before use, the probe has to be checked on a genomic Southern blot for the absence of repetitive sequences. The size of the probe can be 0.4–3 kb.

3.1.3. Construction of Targeting Construct

The classical targeting vector contains an expression cassette of a positive selection marker, e.g., a *neomycin resistance* gene (*see* **Note 1**) flanked by homologous DNA of the gene of interest. To avoid expression of partial gene products, the mutation should be made close to the 5' end of the gene. For the same reason it is advisable to delete the transcription start, the starting codon, and—if existing—the signal sequence.

The frequency of homologous recombination correlates with the length of homologous DNA in the targeting construct ***(11)***. Each arm flanking the neocassette should be at least 2 kb in length and the total amount of homologous DNA should be between 8 and 10 kb.

If PCR is used for screening for homologous recombinants, one primer should reside within the resistance gene and the other outside of the targeting construct. In that case one of the flanking arms has to be shorter (1 kb) to allow efficient amplification.

A widely used expression cassette for the resistance marker contains the neomycin gene under the control of the mouse *phosphoglycerate kinase (PGK)* promoter, followed by the PGK polyadenylation signal. This cassette enables

strong expression of the *neomycin resistance* gene independent of the integration site of the targeting construct. Usual targeting rates range from 1:10 to 1:40, depending on the construct (length of homologous DNA, source of genomic DNA, and so on) and the place of integration (presence of silencers or enhancers, and so on).

Homologous recombination is a rare event in mammalian cells. To increase the percentage of homologous recombinants among the resistant ES cell clones, several strategies can be employed. Use of long sequences of flanking homologous DNA has been mentioned already as one possibility. Another is double selection, i.e., the additional use of a negatively selectable marker gene at the end of the construct, such as the HSV *thymidine kinase (TK)* gene, which is lost upon homologous recombination. In case of heterologous recombination the *TK* gene is often incorporated and the resulting clones are therefore sensitive to gancyclovir. The shorter the distance between the two markers, the more efficient the double selection will work. On the other hand, reduction of the length of intervening homologous DNA will decrease the chance of homologous recombination.

Other methods used to enrich for homologous recombination are promoter trapping or polyadenylation trapping. This means that the selection marker gene lacks a promoter or a terminating signal, respectively, and is therefore only active if the missing functions are provided by the integration site. However, promoter trapping can only be used when the targeted gene is expressed in ES cells.

3.2. Production of Mutated Embryonic Stem Cell Clones

After a targeting vector is constructed, it is next inserted into the genome of ES cells by homologous recombination. It is of crucial importance that mutant ES cells contribute to the germ-cell lineage of chimeric mice. Therefore, they should be cloned and checked for germ-line capability when passaged more than 30 times. This is done by injecting ES cells into blastocysts of different coat color following the protocols in **Subheading 3.3.** If ES cells have germ-line capability, resulting chimeras will be able to father agouti offspring.

To keep the ES cells totipotent and undifferentiated, they are cultured together with feeder cells in ES medium supplemented with LIF. The feeder cells need to be neomycin resistant when neomycin is used to select for DNA uptake. Feeder cells are good for approx 10 d and can be seeded 1 d before plating ES cells or together with ES cells. As a rough time schedule, resistant clones can be picked 7 d after electroporation. About 4 d later, half of the cells are frozen and the other half is grown another 5–7 d and used for preparation of genomic DNA.

3.2.1. Preparation of Feeder Cell Lines

Coculture of ES cells with feeder cells prevents ES cells from differentiating. Feeder cells produce two forms of LIF ***(25)*** and should be used only for approx 10 d. They can be stored frozen.

1. Mate mice expressing a *neomycin resistance* gene.
2. Kill pregnant mouse at day 13.5 or 14.5 counting the day of the plug as day 0.5. Embryos at day 14.5 give more cells.
3. Place dead mouse on multiple layers of paper tissue in a sterile hood. Wet belly of mouse with 70% ethanol for 2 min. Open belly of the mouse with sterile scissors and forceps. Take care not to damage the gut to avoid contamination.
4. Hold uterus where both horns meet and cut it free. Cut along the horns to remove mesometrium and cut at the ends of the uteri. Put the uteri containing embryos in a 6 cm Petri dish filled with 2 mL of PBS.
5. Cut through the uterus by making a small incision, then insert the closed scissors and open them. Push the embryos through the holes, remove membranes, cut umbilical cord, and transfer them to a new 6-cm Petri dish containing 2 mL of PBS.
6. Cut off heads of embryos and transfer remnants to a new 6-cm Petri dish filled with 2 mL PBS.
7. Open bellies of embryos and remove intestine, liver, kidneys, and spleen with forceps. Transfer remaining material to a new 6-cm Petri dish filled with 2 mL of PBS.
8. Cut embryos into small pieces. Incubate in trypsin/EDTA (1 mL/embryo) and incubate 10 min at 37°C.
9. Break tissue pieces with a 5 mL pipet and incubate 10 min at 37°C.
10. Break tissue pieces with a 2 mL pipet and add 4 mL EF medium/embryo.
11. Add 2.5 mL of the cell suspension to 20 mL of EF medium in a 750-mL tissue-culture flask (cells derived from one embryo are seeded in two flasks).
12. Incubate cells, without changing medium, at 37°C, 5% CO_2, checking them daily for confluency. When they have reached confluency, let cells grow for an additional 3 d.
13. Wash cells with 15 mL of PBS/750 mL flask, then add 2.5 mL of trypsin/EDTA and incubate for 10 min at 37°C.
14. Add 2.5 mL of EF medium/750-mL flask and resuspend thoroughly. Reseed 0.5 mL of cell suspension/750-mL flask.
15. Pellet the rest of the cells in 50-mL tubes by centrifugation at 500g for 10 min.
16. Resuspend cells in 50 mL PBS and γ-irradiate the cell pellet with 40 gray to mitotically inactivate feeder cells.
17. Freeze cells (1 mL/vial, three vials/750-mL flask) in DMEM with 10% DMSO and 20% FCS.
18. Wash the tube that contained the feeder cells with 3 mL of medium and transfer to a 6-cm tissue-culture dish. Culture this plate to control sterility.
19. Grow reseeded cells until they have reached confluency and then an additional 3 d. Repeat the freezing procedure starting with **step 13**, omitting the reseeding.

3.2.2. Handling of Embryonic Stem Cells

To assure totipotency and germ-line transmission of ES cells, great care in the culturing of ES cells is of utmost importance. Use feeder cells and supplementation with LIF to the ES medium to prevent differentiation. In fact, LIF and feeder cells fulfill the same function. We use both simultaneously to guarantee optimal conditions. We do not add antibiotics to the medium as they might mask mycoplasma infections, which accompany bacterial infections but are resistant to antibiotics and not detectable under light microscope. We keep the passage number low and freeze a large stock of ES cells with high frequency of germ-line transmission.

If the cultures are too dense the ES cells will start to differentiate. Therefore cultures have to be split. Differentiated colonies can be distinguished by their brownish color. If they differentiate into endoderm they will acquire a cobblestone-like appearance.

1. Thaw ES cells quickly at 37°C.
2. Wash ES cells with ES medium and centrifuge for 5 min at 500g.
3. Resuspend ES cells in ES medium.
4. Add the ES cells on a layer of subconfluent feeder cells. Alternatively, feeder cells can be seeded together with the ES cells.
5. Change medium every day and 2 h before trypsinizing the cells.
6. Split the cells before the ES cell colonies become brownish in their middle. If they do, the ES cells will start to differentiate. Well growing cultures should be passaged every other day. Dense cultures can be split 1:5 to 1:7.

3.2.3. Preparation of DNA for Electroporation of Embryonic Stem Cells

When electroporating ES cells it is important that the DNA of the construct is pure and 100% linearized.

1. Linearize the construct in the multiple cloning site of the vector by cutting 60 μg of DNA in 100 μL of restriction enzyme buffer with 100 U of the appropriate restriction enzyme at 37°C for several hours.
2. Check 1 μL of restriction digest for complete digestion on a 0.7% agarose gel in 1× TAE and separate undigested construct as control.
3. Extract the DNA with 1 vol phenol/chloroform. Transfer the upper aqueous phase to a new tube and extract with 1 vol of chloroform/isoamylalcohol.
4. Transfer the upper aqueous phase to a new tube and add 0.1 vol 3 *M* Na-acetate at pH 5.2. Mix and then add 2.5 vol absolute ethanol. Mix by vigorous inversion. White, coiled DNA will precipitate. Transfer the DNA with a plastic rod to a 1.5-mL screw-cap tube with 1 mL 70% ethanol. Invert several times. The DNA can be stored at –20°C until electroporation.
5. Carefully remove supernatant with a blue tip and air dry the DNA in the sterile hood at RT for 5–10 min. Resuspend the pellet in 700 μL PBS. Incubate the DNA solution at 37°C until the pellet is completely dissolved.

3.2.4. Preparation of ES Cells for Electroporation

We routinely electroporate with 60 μg of linearized targeting construct/4 × 10^7 ES cells.

1. Wash ES cells twice with PBS.
2. Add Trypsin-EDTA and incubate for 5–10 min at 37°C. Resuspend the ES cells thoroughly in 10 mL of ES medium.
3. Transfer the cells to a 15-mL tube with pointed bottom. Take 50 μL of the cell suspension and count the ES cells in a Bürkner cell-counting chamber.
4. Centrifuge for 5 min at 500g and resuspend the cell pellet in 10 mL of PBS. Repeat this step twice.
5. Decant the supernatant, resuspend the ES cells by flicking the tube with a finger and resuspend 4 × 10^7 cells in 700 μL PBS/DNA solution from **Subheading 3.2.3.**

3.2.5. Electroporation of Embryonic Stem Cells

Electroporate 60 μg of linearized DNA into 4 × 10^7 ES cells. Distribute the electroporated cells on eight 10 cm dishes with feeder cells.

1. Transfer the ES-cell/DNA solution from **Subheading 3.2.4.** to a sterile electrocuvet using a Pasteur pipet.
2. Electroporate using the Bio-Rad Gene Pulser with a setting of 0.8 kV and 3 μF at RT. This setting will result in 30–50% cell death. The time constant should be 0.04 ms.
3. Carefully suck the cells out of the cuvet with a Pasteur pipet filled with 1 mL ES medium.
4. To 8 cell-culture dishes (diameter 10 cm) containing subconfluent layers of feeder cells (1 vial/10 cm plate; from **Subheading 3.2.1.**) add aliquots of electroporated cells to a final volume of 8 mL. Shake the plates crosswise to distribute the electroporated ES cells evenly and incubate the plates at 37°C, 5% CO_2.

3.2.6. Selection of Positive Clones

Stably transfected ES cells are selected by addition of antibiotics to the growth medium.

1. Twenty to twenty-four hours after the electroporation, start selection with growth medium containing 500 μg/mL G418.
2. If negative selection is used, start negative selection 48 h after the electroporation by supplementing the growth medium with 2 μ*M* gancyclovir.
3. Change medium every 24 h. Before changing, shake the plates gently to detach dead cells. Change medium twice at peak of death, which occurs at approx 3–4 d after electroporation.
4. Pick clones clearly visible by eye 6–8 d after beginning the selection.

3.2.7. Seeding Feeder Cells for Picking Embryonic Stem Cell Clones

1. Thaw feeder cell ampoules (from **Subheading 3.2.1.**).
2. Transfer 10 feeder vials to 20 mL of EF medium (1 feeder vial/24-well plate).

3. Centrifuge feeder cells at 500g for 5 min. Decant the supernatant and resuspend the cells in 10 mL of EF medium.
4. Add 48 mL of EF medium to 2 mL of resuspended feeder cells and mix thoroughly.
5. Seed 1 mL of cell suspension per well in a 24-well tissue-culture plate and shake plate crosswise to distribute the cells.
6. Before picking of ES cell colonies, change EF medium to ES medium containing 500 μg/mL G418 and in case of negative selection 2 μ*M* gancyclovir.

3.2.8. Picking G418-Resistant Clones

Pick clones using a stereomicroscope in a laminar flow hood. Approximately 48 clones can be picked per hour. We routinely pick 240–360 colonies. At least two homologous recombinants are needed to prove that a phenotype is not caused by a clone-specific secondary alteration of the genome.

1. Add 150 μL of trypsin-EDTA solution to each well in a sterile 96-well microtiterplate. Keep at 37°C until use.
2. Take a 20-μL pipet with a sterile tip. Put the tip close to an ES cell colony. Remove the colony from the feeder cell layer with a short scratch, suck it into the tip of the pipet, and transfer it to the trypsin-EDTA in the microtiter plate. Transfer 24 colonies and then continue to **step 3**.
3. Check under the microscope that all wells contain colonies.
4. Add 150 μL of ES medium to a well using a 200-μL pipet and resuspend thoroughly by pipetting several times up and down. Take care to avoid foam formation.
5. Transfer the cell suspension to a well in a 24-well plate containing feeder cells and 1 mL of ES medium with G418. Wash the microtiter well with 150 μL of medium to be sure that all cells are transferred.
6. Repeat **steps 4** and **5** with the next well and so forth.
7. Incubate at 37°C, 5% CO_2 and change medium the next day.

3.2.9. Freezing Clones

Freeze cells when the medium just starts to turn yellow, approx 3–5 d after picking. Freeze all clones on one 24-well plate at the same time even if some are lagging behind in growth.

1. Aspirate the medium and wash each well with 2 mL of PBS.
2. Add approx 120 μL trypsin-EDTA solution to each well (approx 3 drops from a 5-mL plastic pipet) and incubate at 37°C for 5 min.
3. Add 1 mL of ice-cold freeze medium to each well of the 24-well plate.
4. Resuspend thoroughly and freeze 0.5 mL. Transfer freeze vials immediately into a carton box standing on and surrounded by dry ice. Transfer the full box immediately to the –80°C freezer.
5. Add at least 1.5 mL of ES medium without G418 to each well and incubate at 37°C for 6–16 h. Then replace the medium by 1 mL of new ES medium to prohibit the toxic effect of residual DMSO.

3.2.10. DNA Preparation from Embryonic Stem Cell Clones

1. After freezing the G418-resistant clones, incubate the remaining cells until medium turns yellow.
2. Suck off medium and add 0.5 mL of lysis buffer to each well.
3. Incubate for at least 8 h in a humidified 37°C incubator. This incubation can be extended up to 8 d without decreasing yield or quality of genomic DNA.
4. When all samples are lysed, add 0.5 mL of isopropanol to each well and shake on an orbital shaker for at least 8 h at RT. DNA should become visible as a white weblike structure.
5. Place the 24-well plate on a black surface (this makes DNA more easily visible) and spool the DNA on a bacterial inoculation loop cut. The DNA should stick closely to the fork. Big DNA chunks are difficult to resuspend.
6. Resuspend DNA in 200 µL of water in 1.5-mL test tubes. Close the tubes tightly and incubate for 24 h in a 55°C oven. The DNA can then be stored at RT.

3.2.11. Analysis of Genomic DNA

The genomic DNA is analyzed by restriction digest and Southern blotting. Check both sides of the construct for homologous recombination by hybridizing the blot with an external probe, which is not contained in the targeting construct. This probe will detect homologous recombination, but not random integration into the genome. Check for additional heterologous integrations by hybridization with an internal probe, preferably the selection marker. This probe will detect all integrations of the targeting construct into the genome.

1. Cut 20 µL of DNA solution (from **Subheading 3.2.10.**) in a total volume of 30 µL restriction enzyme buffer with 40 U of the desired enzyme in the presence of 100 µg/mL BSA for 8–20 h at 37°C. It is essential that the DNA solution is carefully mixed with the restriction enzyme by pipetting the digestion mix up and down several times.
2. Apply digestion reactions on a 0.7% agarose gel containing 1× TBE and 1 µg/mL ethidium bromide. Separate for 6–8 h at 3 V/cm.
3. Photograph gel on an UV table together with a ruler.
4. Blot the digested DNA onto a nylon membrane according to the instructions of the manufacturer.
5. Mark the position of the wells on the nylon membrane with a pencil and fix the DNA onto the membrane by UV crosslinking or baking.
6. Prehybridize the filters in Church buffer for 1 h at 65°C. Radioactively label the probe by random priming following the instructions provided in the kit. Hybridize filters overnight at 65°C in Church buffer containing 0.5–1 × 10^6 cpm/mL activity of the ^{32}P-labeled cDNA probe. The filters should be just covered with hybridization solution.
7. Wash the filter twice in 10 hybridization vol of solutions 1 and 2 for 30 min each.
8. Expose with an autoradiography film or a phosphor imager plate until clear bands are visible.

3.3. Production of Chimeric Animals

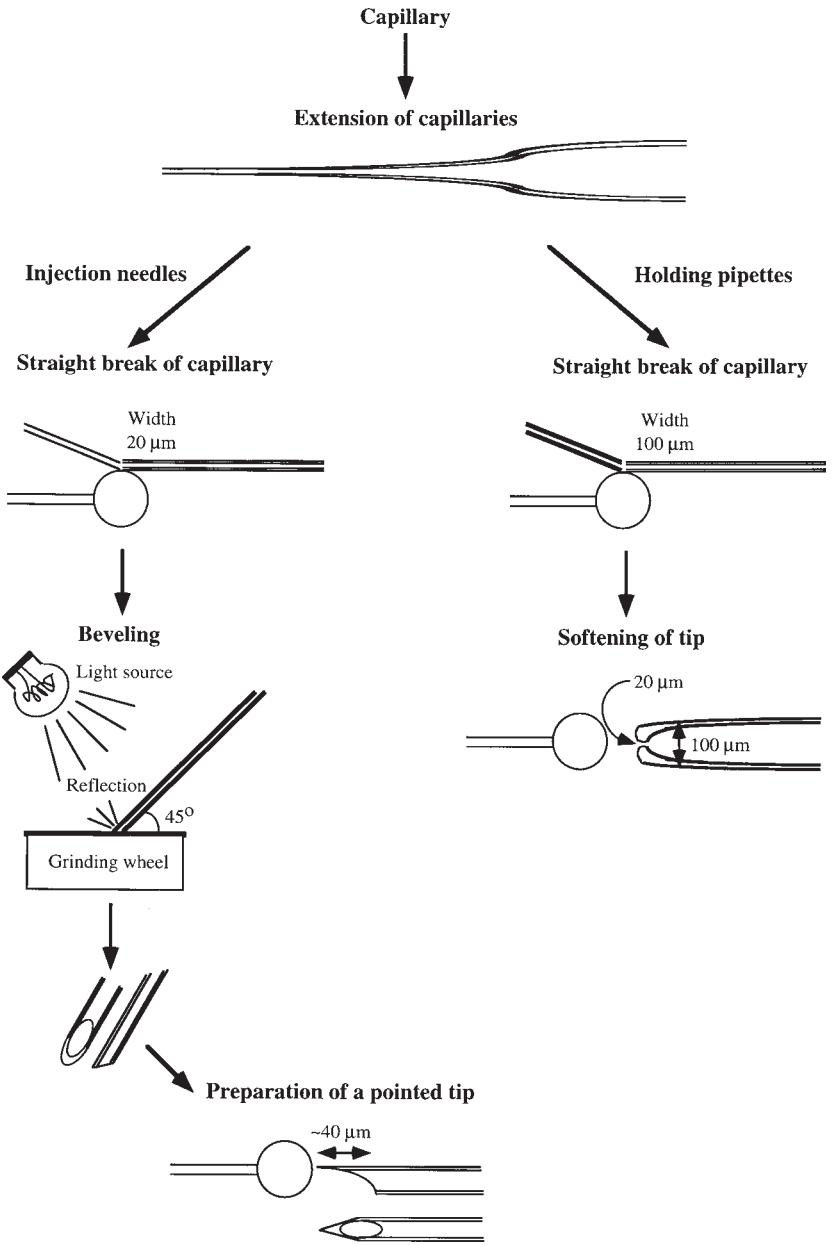
When ES cells with the desired mutation have been identified, they are injected into the cavity of blastocysts that are then transferred into the uterus of pseudopregnant foster mice. There they will develop into chimeric mice containing blastocyst and ES-cell-derived cells.

3.3.1. Preparation of Microinjection Pipets

One pipet is needed to hold the blastocysts (holding pipet) and another to inject the ES cells (injection needle). Both can be made from the same type of glass capillaries with an external diameter of 1 mm and an internal diameter of 0.85 mm. It is possible to make an injection needle by elongating the glass over a flame and breaking the tip with a razor blade. Afterward, the tip has to be examined under a microscope to judge its suitability for blastocyst injection. We, however, do not recommend this method as injection needles obtained in this way are usually of poor quality. To make high-quality needles, the capillaries have to be heated, pulled, beveled, and bent using specialized equipment. We use a puller from Sutter instruments, a de Fonbrune-type microforge from Narishige and a grinding machine from Narishige. The different steps of this process are depicted in **Fig. 2**.

1. Soak glass capillaries overnight in 82% HCl, 18% HNO_3 in a 100-mL measuring cylinder. Then wash the capillaries several times in water until the pH is neutral. This procedure prohibits ES cells from sticking to the inner surface of glass capillaries.
2. Pull the capillaries using the puller. The capillaries should be reduced to a diameter of 1 μm over a distance of approx 1.5 cm.
3. Measure the diameter of the capillaries in a microscope. The ideal diameter for an injection needle is 20–30 μm and 70–100 μm for a holding pipet. For breaking the capillaries at the appropriate point, the capillary tip is placed on the glass bead of the microforge at the site where it has the correct diameter. Heat the bead so that it just fuses with the capillary. After fusion, switch off immediately. Rapid contraction of the cooling bead results in a vertical break of the capillary.

Fig. 2. *(continued on next page)* Preparation of injection needles and holding pipets. Glass capillaries are extended in a puller. Glass capillaries are broken in a microforge to get a vertical break. The capillary is placed at the appropriate diameter over the glass bead of the microforge. The glass bead is heated. Heating is switched off when the capillary just fuses with the bead, resulting in cooling and contraction of the bead, and breakage of the needle. For preparation of injection needles, capillaries (20-μm diameter) are beveled at an angle of 45° on a grinding wheel. When the tip touches the grinding wheel, a light reflection is visible. Finally, tips are sharpened in the microforge. The glass bead is heated so that it just starts to glow. The capillary is lowered until it touches the surface of the bead and quickly moved back, resulting in a



pointed extension of the tip. For holding pipettes, capillaries are broken at a diameter of 100 µm on a microforge. The sharp edges of the opening are then softened by partly melting them. The hot glass bead of the microforge is placed close to the opening without touching it. Partial melting will soften the edges and reduce the diameter of the opening.

4. Use a grinding wheel to bevel the capillaries. Lower the capillary at 45° angle until it touches the grinding wheel. This can be controlled by using a light source that will reflect light from the slightly bent capillary tip after it touches the grinding stone.
5. Wash the capillary using a needle holder connected via a tygon tube to a 50-mL plastic syringe. Suck in 10% HF for 10 s and blow out for another 10 s. Wash 10 times with distilled water and 5 times with 100% ethanol.
6. Check the capillary for correct diameter (20–30 μm) under the microscope.
7. Create a sharp pointed tip using the microforge. Heat the glass bead so that it just starts to glow. Lower the capillary until it touches the surface of the bead and quickly move it back. This creates a fine sharp tip.
8. To make the needle parallel to the bottom of the injection chamber, a bend is introduced. Adjust the needle on the microforge so that the opening is facing the operator. Then heat the glass bead to orange glowing. Bring the needle at the bending point close to it without touching. While the needle is bending, move the bulb up slowly until an angle of approx 20–30° is achieved.
9. For holding pipets follow **steps 1–3**. Check the pipet for correct diameter (70–100 μm), and soften the edges of the tip using the microforge. Heat the glass bead so that it just starts to glow. Lower the capillary until it nearly touches the surface of the bead. When the tip starts to melt, quickly move it back. Finally, follow **step 8** to create an angle on the pipet.

3.3.2. Preparation of Vasectomized Males

Vasectomized male mice are necessary to identify female foster mice that are in oestrus cycle.

1. Anesthetize 4–8-wk-old male FVB mice by intraperitoneal injection of 0.7–0.9 mL 2.5% Avertin, depending on the size of the mouse (0.014–0.018 mL/g). The exact dose may vary with different preparations of Avertin.
2. Wet lower part of belly with 70% ethanol.
3. Make a short cut (3–5 mm) along the median of belly at the level of the top of the hind legs. When lifting the muscle wall at the incision, fat pads should be visible at the left and the right side.
4. Grasp a fat pad with a forceps and pull it out slowly. The testes will appear, connected to the fat pad. The vas deferens is a U-shaped tube leaving the testes and is accompanied by a red blood vessel.
5. Tie off the vas deferens at two sites by two knots with surgical thread. The distance between the knots should be approx 4 mm. Cut the intervening part.
6. Gently push testes and fat pad back into the mouse. Repeat the procedure with the other testes.
7. Sew the muscle wall with one or two stitches (depending on the length of the incision) with surgical thread. Sew the skin together with three stitches.
8. Put the mouse back into the cage under a heating lamp or covered by shavings to prevent excessive cooling. The male mice become less aggressive if they wake up in the presence of a female mouse (not anesthetized).

9. Ten days after the operation the mice can be used for matings. It is advisable to check sterility before using them to generate pseudopregnant foster mothers.

3.3.3. Preparing Pipets for Blastocyst Handling

For precision pipetting it is preferable to use a mouth pipet when handling blastocysts.

1. Heat a glass capillary used for injection needles in a little flame. When the glass becomes soft, remove the capillary from the flame and pull both ends to produce a fine extension.
2. At an internal diameter of 200 μm cut the capillary with the help of a diamond-point pencil.
3. Soften the sharp edges of the tip by moving it quickly through the flame.
4. Connect the pipet to tubing long enough to allow convenient mouth-controlled handling.

3.3.4. Isolation of Blastocysts

1. Mate C57Bl/6J mice.
2. Kill a 3.5-d pregnant mouse (counting the day of the plug as day 0.5) by cervical dislocation. Lay the mouse with its back on a sheet of paper towels and disinfect with 70% ethanol.
3. Lift the skin between the hind legs with a forceps and make a small incision through the body wall without hurting the intestines. Make a big cut on both sides up to the chest.
4. Gently push the gut aside until the two uterus horns are fully visible. Push bladder and surrounding fat pads to the direction of the tail until the cervix is clearly visible.
5. Using a stereomicroscope, grasp the uterus with forceps above the cervix and cut cervix close to the uterus. Stretch the mesenterium and cut it with bent scissors as close to the uterus as possible. Cut uterus at the border to the oviduct and split the beginning of the uterus by a short incision to facilitate flushing.
6. Transfer uterus to flush medium. Several uteri can be collected in the same dish.
7. Transfer uterus to an empty microscopic depression slide (diameter approx 2 cm). Flush both uterus horns with approx 0.5 mL flush medium using a 1-mL syringe with a 0.60 × 30-mm needle inserted at the end of the uterus. The uterus horns should swell during flushing.
8. Collect blastocysts with a mouth pipet, transfer them to a microscopic depression slide with prewarmed ES medium and incubate for 1–4 h at 37°C, 5% CO_2.

3.3.5. Preparation of ES Cells for Blastocyst Injection

1. Seed 2–5 × 10^6 ES cells with feeder cells (1 vial from **Subheading 3.2.9.**) into one well of a 6-well plate and incubate for 1–2 d. If cells are grown for two or more days, medium should be changed daily.
2. Change the medium at the day of injection 1–2 h prior to trypsinization.

3. Wash cells twice with 4 mL of PBS, add 0.4 mL of trypsin-EDTA and incubate for 5 min at 37°C. Resuspend cells thoroughly in 10 mL ES medium.
4. Transfer cells to a 15-mL conical bottom tube. Centrifuge for 5 min at 500g and resuspend the cell pellet in 5 mL of ES medium.
5. Take 500 µL of the cell suspension to a 15-mL tube with pointed bottom, add 10 mL of ES medium and centrifuge for 5 min at 500g.
6. Decant supernatant and resuspend the ES cells in the remaining 100–200 µL medium by flicking the tube with a finger.

3.3.6. Microinjection of Embryonic Stem Cells into C57Bl/6J Blastocysts

A number of different injection systems are available. The procedure requires an inverted microscope with interference- or phase-contrast objectives. The magnification range required is 50× to 200×. The injection microscope should be equipped with a cooling device for the microinjection chamber, as blastocysts survive the injection procedure better when kept at 10°C and become more rigid, which makes needle penetration easier. The microinjection chamber is a metal slide with a glass slide bottom glued together with vaseline (**Fig. 3**). Two micromanipulators are required, one for the microinjection needle and one for the holding pipet. We use an air-filled system. The flow in these pipets is regulated by hand using syringes.

1. Silanize cover slides (42 × 16 mm) with dichlor-dimethyl-silane.
2. Wash silanized cover slides with soap and water and dry gently.
3. Glue the cover slide to the microinjection chamber using pure petroleum jelly without any additives. Make sure that the petroleum jelly seals completely.
4. Prepare a rectangular field of flush medium on the cover slip. Leave some space to the rim of the metal slide.
5. Transfer the ES cells from **Subheading 3.3.5.** to the medium, leaving the upper left corner free.
6. Transfer the blastocysts to the upper left corner.
7. Mount the slide with blastocysts and ES cells on the object tray, which has been cooled to 10°C.
8. Install the holding and the injection needles. The needles should be parallel. Lower them into the medium.
9. Cover the medium carefully with dimethyl polysiloxan.
10. Collect 100–200 healthy-looking ES cells (medium size, round, smooth surface) with the injection needle (**Fig. 4.1**).
11. Immobilize a blastocyst with the holding pipet using gentle suction. Lift it from the bottom and move the holding pipet to a region free of ES cells and blastocysts. Preferred blastocysts are those who have expanded fully, but not yet hatched from the zona pellucida.
12. Use the injection needle to turn the blastocyst to a position in which the inner cell mass is clearly visible. Either on the opposite side to the injection site (**Fig. 4.2**) or to either side of the blastocyst equator. Adjust the injection needle in extension

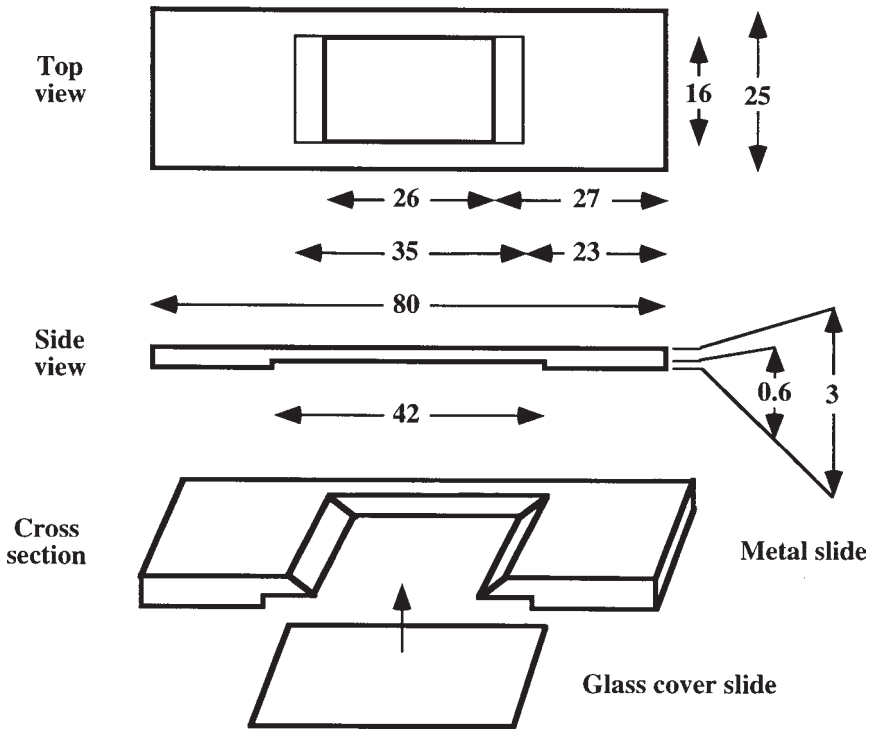


Fig. 3. Microinjection chamber. The microinjection chamber is prepared by gluing a glass cover slide to the recess at the bottom of a specially designed metal slide, using petroleum jelly as a glue. The heat conducting metal allows cooling of the microinjection chamber. (Dimensions in mm).

of the holding pipet, preferably at a border between two trophoblast cells. Apply some extra suction with the holding pipet to make sure that the blastocyst is secured in position. Control that some ES cells are at the tip of the injection needle (**Fig. 4.2**).

13. With a short swift movement, push the injection needle into the blastocyst (**Fig. 4.3**) and inject about 15 ES cells into the blastocoel (**Fig. 4.4**). Pull out the injection needle (**Fig. 4.5**). The blastocyst may collapse now.
14. Deposit injected blastocysts at lower left corner (**Fig. 4.6**).
15. After injecting all blastocysts, transfer them to a microscopic depression slide filled with ES medium and incubate them for 1–4 h at 37°C, 5% CO_2.

3.3.7. Uterus Transfer of Injected Blastocysts

1. Mate 6-wk-old BDF1 mice with vasectomized FVB mice.
2. Anesthetize a day 2.5 pseudopregnant mouse, counting the day of the plug as day 0.5, by intraperitoneal injection of 0.7–0.9 mL 2.5% Avertin, depending on the size of the mouse.

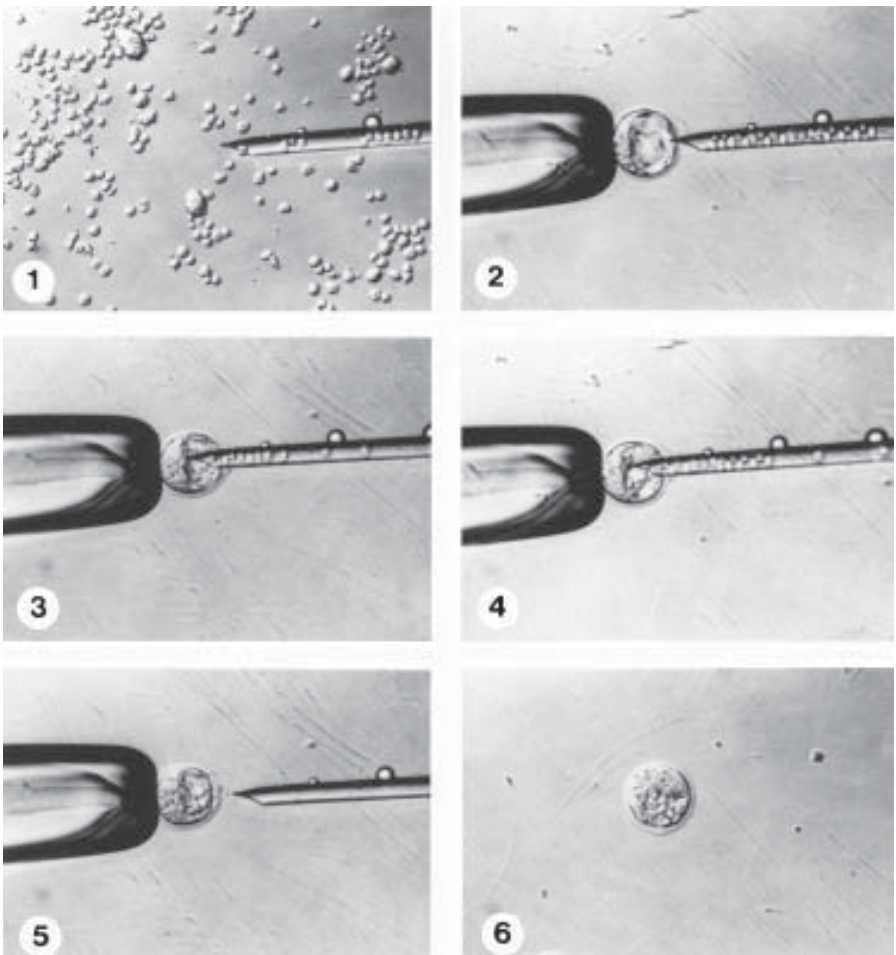


Fig. 4. Microinjection of ES cells into blastocysts. (1) ES cells are collected into the injection needle. (2) A blastocyst is immobilized by the holding pipet so that the ICM is distant from the site of injection. The injection needle is positioned in extension of the holding pipet, pointing to the border between two trophectodermal cells. ES cells are moved to the tip of the needle. (3) The injection needle is pushed into the cavity of the blastocyst. (4) Approximately 15 ES cells are injected into the blastocoel. (5) The injection needle is withdrawn. (6) The injected blastocyst is deposited in a corner of the microinjection chamber. It is incubated for 1–4 h in ES medium and then transferred to the uterus of pseudopregnant mice.

3. Lay the mouse on its belly on a stack of paper towels as support, with its tail pointing towards yourself. Disinfect with 70% ethanol. Pull the hairs aside on a spot halfway between the last rib and the thigh and halfway between the spine and the side of the mouse.

4. Make a vertical incision, using straight scissors, through the skin. Enlarge the opening by tearing with the outside of the scissors. The white fat pad above the orange ovary should be visible under the peritoneum. If not, gently move the skin incision until it is visible. Cut through the muscle wall and enlarge the opening in a similar way. Take care not to damage the red blood vessels or white nerves.
5. Grasp the fat pad with a forceps and pull out the ovarium carefully. Take hold of the uterus at the top on the side opposite to the blood vessels and pull it out.
6. Make sure to have an air bubble in the capillary needle and suck up 4–7 blastocysts (from **Subheading 3.3.6.**). The air bubble makes sure that a minimal volume is used and is also necessary as a visual control for the injection process.
7. Hold the uterus close to the oviduct and pierce a hole in the upper part of the uterus with a 0.40 × 19-mm needle.
8. Insert the capillary containing the blastocysts through the hole into the uterus and inject the blastocysts. The air bubble above the blastocysts should go down to the tip of the pipet.
9. After transfer, blow the transfer capillary pipet out into ES medium. Check under the microscope that no blastocysts were sucked back into the capillary.
10. Gently push the uterus back into the mouse using a closed forceps. Touch only the fat pad. Lift the skin and shake gently so that the organs go back to their right places.
11. Sew the muscle wall with 1 stitch. Clip the skin together with a wound clip.
12. Repeat the procedure for the other uterus horn.
13. Put the mouse back into the cage under a heating lamp or covered by shavings to prevent excessive cooling.
14. The mouse should give birth after 16–18 d. It is important not to change the cage 4 d before to 4 d after the birth of the pups. This will reduce cannibalizing of the offspring by the foster mother.

3.3.8. Breeding Schemes

Using ES cells derived from 129/Sv mice, chimeric animals are recognized by agouti stripes. To test for germ-line contribution of the ES cells these chimeras should be mated to C57Bl/6J mice. Because agouti coat color is dominant over black coat color, mice developing from ES-cell-derived germ cells will be agouti. If the targeted mutation is heterozygous viable, 50% of the agouti pups will carry the targeted allele. These heterozygous agouti offspring are then bred with each other to obtain outbred homozygous animals. An inbred line can be established by mating the chimeras to 129/Sv mice. Heterozygous animals will then have a pure 129/Sv background. We routinely establish an outbred and an inbred line of two independent ES cell clones to assure that the observed phenotype is not caused by a spontaneous mutation and to test whether the phenotype is independent of genetic background.

3.3.9. Identifying Mutant Animals by Southern Blotting

1. Mark mice by clipping ears with a numbered marker or by any other means at 2–3 wk of age. At the same time, clip 5 mm of tail of the mice for genotyping and put it in a 1.5-mL test tube.
2. Add 500 μL of DNA lysis buffer and rotate at 55°C overnight.
3. Add 250 μL of phenol and 250 μL of chloroform. Shake vigorously for 1 min. Separate phases by centrifugation at 10,000*g* for 5 min.
4. Transfer the upper aqueous phase to a new tube and add 500 μL of chloroform. Shake vigorously for 1 min. Separate phases by centrifugation at 10,000*g* for 5 min.
5. Transfer the upper aqueous phase to a new tube and add 500 μL of isopropanol. Invert several times. A white precipitate should form.
6. Pellet DNA by 2 min centrifugation at 3000*g*. Aspirate the isopropanol, spin down again, and remove remaining isopropanol.
7. Add 100 μL of deionized water and incubate for several hours at 55°C to dissolve the DNA.
8. Analyze DNA by Southern blot following the procedure in **Subheading 3.2.11.**

3.3.10. Identifying Mutant Animals by PCR

PCR is a fast nonradioactive alternative to screening by Southern blots. In a three-primer reaction, one primer pair amplifies a short fragment specific for the wild-type gene, the other a short fragment of different size specific for the mutated gene.

1. Mark mice by clipping ears with a numbered marker or by any other means at 2–3 wk of age. At the same time, clip approx 1 mm of tail of the mice and put it in a 1.5-mL test tube.
2. Add 80 μL of 1× PCR buffer containing 100 μg/mL proteinase K and incubate on a mechanical shaker at 60°C overnight. If tail tips are mashed after 1 h with a yellow pipet tip incubation time can be reduced to 2 hours.
3. Incubate at 95°C for 5 min to inactivate the proteinase K.
4. Spin tubes at top speed in a table-top centrifuge for 5 min.
5. Take 60 μL of the supernatant and add 540 μL of water.
6. Prepare a PCR master mix containing per reaction 0.4 μL of 10 m*M* dNTP, 2 μL of 10× PCR buffer, 1 μL of each of the three primers at 20 pmol/μL, 0.4 μL of 1 U/μL *taq* DNA polymerase and 12.2 μL water.
7. Add 18 μL of PCR master mix to each PCR tube.
8. Add 2 μL of diluted DNA to each tube. Do not forget to have positive and negative control samples.
9. Mix on vortex, overlay with mineral oil, put the reactions in the PCR machine at 95°C, and start the PCR reaction using the following touchdown protocol. The final annealing temperature is of course dependent on the chosen primer pairs.
   a. 30 s at 95°C (denaturation).
   b. 30 s at 65°C (annealing).

c. 30 s at 72°C (elongation). Cycle back to a. 10 times, decreasing each time the annealing temperature by 1°C.
d. 30 s at 95°C (denaturation).
e. 30 s at 55°C (annealing).
f. 30 s at 72°C (elongation). Cycle back to d. 35 times.
g. Pause at 4°C.

10. Analyze PCR products on a 2% agarose gel.

3.4. Specialized Knockout Technologies

In addition to the simple knockout of a gene, knockouts can be combined with knockins of reporter genes, such as *lacZ* or *green fluorescent protein*. Conditional knockouts allow a tissue- and time-specific gene ablation. Replacement of a wild-type gene by another gene carrying subtle mutations is achieved by knockins. If a lethal phenotype is expected double knockout ES cells can be established for generating chimeric mice, teratomas, or embryoid bodies.

3.4.1. Use of a Reporter Gene

The analysis of knockout animals is often facilitated when the targeted gene disruption is combined with the activation of a reporter gene, for example *lacZ*. This is especially helpful when homozygous null ES cells are used to create chimeric mice. Mutant cells can then easily be distinguished from normal cells by the activity of the reporter gene. The *lacZ* gene codes for β-galactosidase, an enzyme easily detectable by a color reaction. It should be kept in mind that endogenous β-galactosidase activity is present in some organs such as the small intestine of young animals, kidney, testis, and so on ***(26)***. In that case, chimeric mice have to be assessed by in situ hybridization or immunohistochemistry to detect knockout cells.

3.4.2. Generation of Homozygous Knockouts in ES Cells

Generation of ES cells with homozygously targeted alleles can be accomplished either by consecutive gene targeting using two different positive selectable markers ***(27,28)*** or with a single targeting construct by growing the neomycin-resistant heterozygous mutant ES cells in high concentrations of G418 ***(29)***. A high concentration of G418 (1–3 mg/mL) will select for cells with more than one insertion of the *neomycin resistance* gene. It is recommended to use a mutant form of the *neomycin resistance* gene ***(30)*** under the control of a weak promoter (e.g., HSV *TK* promoter).

Homozygously targeted ES cells can be used in vivo to make chimeric animals (**Subheading 3.4.5.**, **Fig. 5**), teratomas (**Subheading 3.4.4.**), and for several in vitro systems (**Subheading 3.4.3.**, **Fig. 6**).

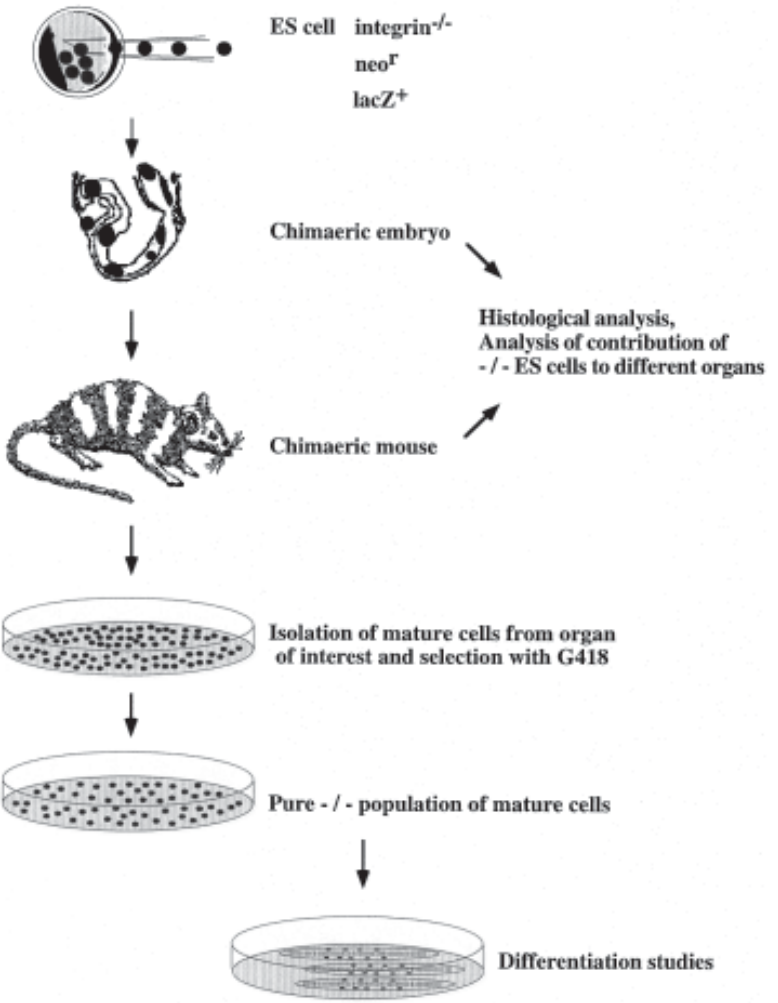


Fig. 5. Analysis of knockouts with an embryonic lethal phenotype using chimeric mice. Homozygous mutant cells are injected into blastocysts. Developing chimeric embryos and chimeric mice can be analyzed for the contribution of mutant cells to different organs. If the knockout was coupled to the knockin of *lacZ*, whole-mount in situs of embryos and *lacZ* stainings of tissue sections can be done to identify mutant cells. Mutant cells from different tissues can be isolated by organ cultures in the presence of antibiotics, corresponding to the resistance introduced along with the null-mutation. As an example, the isolation of β_1 integrin-null myoblasts from β_1 integrin-null chimeric mice is shown. After killing wild-type myoblasts, the β_1 integrin-null myoblasts can be differentiated in vitro giving rise to fully functional myotubes.

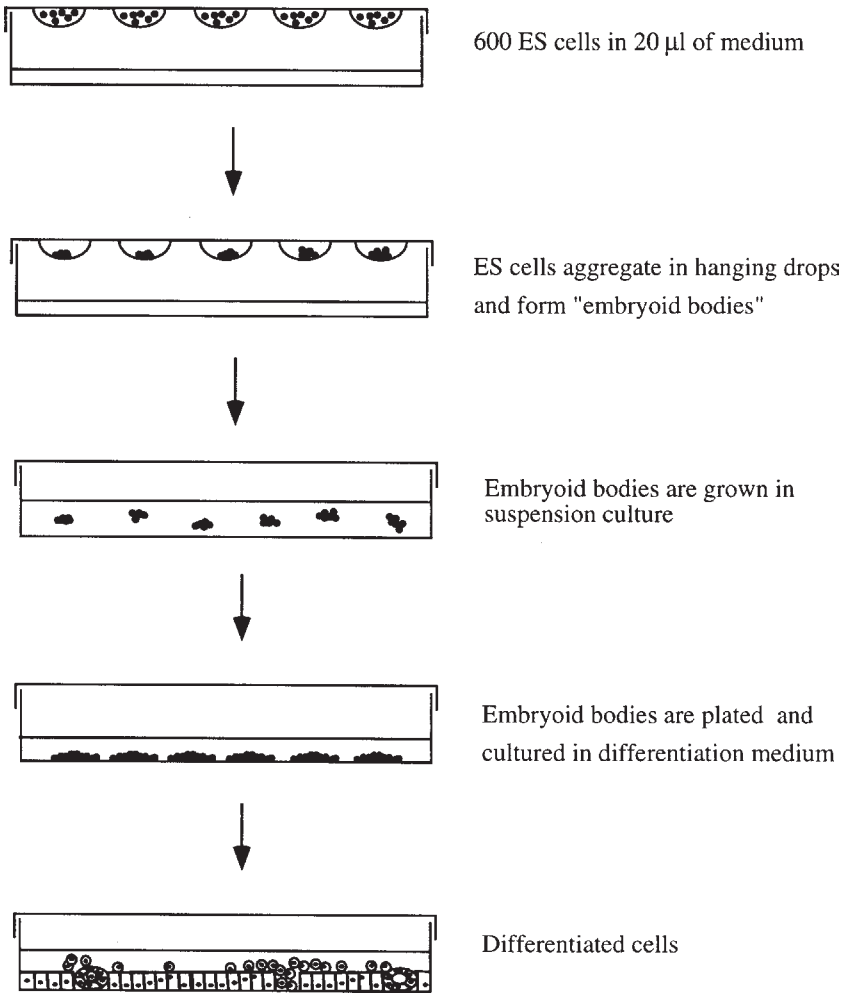


Fig. 6. Embryoid body formation. Approximately 600 ES cells are seeded in drops hanging under the lid of a cell-culture dish. The lid is placed on a cell-culture dish containing PBS. The cells are allowed to aggregate for 2 d in hanging drops and for a further 3–7 d in suspension culture and then plated on chamber slides where they differentiate.

3.4.3. Analysis of Homozygous Knockout ES Cells via Embryoid Bodies

Embryoid bodies form when ES cells are allowed to differentiate in vitro (**Fig. 6**) ***(21)***. ES cells differentiate into a wide variety of cell types derived from all primitive germ layers. Comparing embryoid bodies derived from normal and mutant ES cells, allows to study the effect of the mutation on the

differentiation potential of ES cells in vitro. Especially in knockouts with an early lethal phenotype, as for example for β_1 integrin, this in vitro technique is highly valuable to study for instance muscle development ***(17)***, keratinocyte differentiation ***(22)*** and angiogenesis ***(20)***.

1. Wash ES cells twice with PBS, trypsinize for 5–10 min and resuspend thoroughly. Add 10 mL of DMEM supplemented with 20% FBS to the cells.
2. Take 50 μL of the cell suspension and count the cells in a Bürkner cell counting chamber.
3. Resuspend the ES cells in DMEM supplemented with 20% FBS at a concentration of 3×10^4 cells/mL.
4. Pipet droplets (20 μL) containing approx 600 ES cells to the inverted lid of a cell-culture dish. Put the lid back to the cell culture dish containing 5 mL of PBS and incubate the cells in "hanging drops" for 2 d. This will allow the formation of cell aggregates (embryoid bodies).
5. Embryoid bodies form after different time periods depending on if they are normal or have a mutant phenotype affecting adhesion. When aggregates have formed, they can be transferred to bacteriological dishes and grown in suspension until they are 5-d old, counting the start of "hanging drop" culture as day 0.
6. Five-day-old aggregates are plated on chamber slides and can be incubated for up to 30 d. Specimens are analyzed after time intervals, fixed, sectioned, and stained with antibodies, to study the effect of the targeted gene on cell proliferation, blood vessel formation, or any other aspect of interest.

3.4.4. Analysis of Homozygous Knockout ES Cells via Teratoma Formation

Teratomas are benign tumors that form after ectopic injection of ES cells into syngeneic male mice ***(31)***. Syngeneic male mice are used to inhibit an immune response of the host. The teratomas contain cells derived from all primitive germ layers. Comparison of teratomas of normal and mutant ES cells may demonstrate the importance of the knockedout protein for teratoma formation. Analysis of the teratoma structure may give hints for the in vivo function of the protein. Analysis of β_1-null teratomas for example revealed an important role of β_1 integrin in angiogenesis and in the formation of basement membranes ***(20)***.

1. Wash ES cells twice with PBS, trypsinize for 5–10 min and resuspend thoroughly. Add 10 mL of DMEM supplemented with 20% FBS to the cells.
2. Take 50 μL of the cell suspension and count the cells in a Bürkner cell counting chamber.
3. Wash the cells twice in PBS. Do the last wash in a 15-mL tube.
4. Decant the PBS and resuspend the ES cells in PBS at a concentration of 1×10^8 cells/mL.

5. Inject 100 µL of the ES cell suspension (1×10^7 cells) subcutaneously into the back of syngeneic 129/Sv male mice.
6. When the normal or mutant teratomas have reached approx 1 g (for normal teratomas after approx 20 d) tumors are excised and frozen or fixed for immunohistochemistry or histology. To assess cell proliferation in teratomas mice are injected with 25 mg BrdU/100 g mouse intraperitoneally 2 h before sacrifice. Incorporated BrdU is visualized by fluorescent light after incubating sections with fluorescence-labeled anti-BrdU antibodies.

3.4.5. Analysis of Homozygous Knockout ES Cells in Chimeric Mice

Blastocyst injection of homozygous mutant ES cells results in chimeric animals partly composed of null cells. Contribution of the null cells to various tissues reflects the importance of the targeted gene for differentiation and migration during development ***(17–19)***. Detection of mutant cells in the chimeric mice is facilitated if the knockout is combined with a knockin of a reporter gene.

Mature cells can be isolated from organs of interest and then selected for neomycin resistance resulting in a pure population of homozygous knockout cells, which can be used for further studies.

3.4.6. Conditional Knockout

A constitutive knockout of a protein can lead to an embryonic lethal phenotype, which prevents the analysis of protein function in the adult animal and in tissues not yet developed at the time of death. Constitutive knockouts might also result in adaptational processes during development, by which the mouse functionally compensates for the missing protein. Time and tissue restricted, "conditional," knockout, circumvents both problems, as it allows gene inactivation in the adult animal restricted to the tissue of interest or restricted to a certain time.

Successful conditional knockouts in mice were reported using the Cre-loxP system ***(4,5)***. In this system, essential parts of the gene are flanked by loxP sequences (short directed sequences of 34 bp) using homologous recombination. Although the loxP sites are relatively small, it has to be assessed that mice carrying such a "floxed" gene show a normal expression pattern and are normal.

The mice with a floxed gene are then mated to mice that express Cre only in certain tissues, at certain stages of development, or in a regulatable fashion. The viral Cre recombinase binds to the loxP sites and deletes DNA sequences intervening loxP sites of identical orientation. Time- and tissue-restricted expression of the Cre enzyme results in a likewise restricted knockout of the gene that was flanked by loxP sites. Cre-expressing mice can be transgenic animals where the Cre enzyme is expressed under the control of a tissue-specific promoter or a promoter regulated by specific drugs (e.g., tetracycline).

Because the expression pattern in transgenic mice is highly dependent on the place of integration into the genome, a more efficient strategy is the knockin of the *Cre* cDNA into a tightly regulated gene. To assure that this by itself does not result in any phenotype the *Cre* can be targeted together with an *IRES (internal ribosomal entry site)* sequence ***(32,33)*** to the 3' non coding region of the gene. Recently, regulatable forms of the Cre enzyme were reported ***(34,35)***. Fusion proteins of Cre with one or two hormone-binding domains of steroid receptors are inactive, but become activated in the presence of steroids binding to the hormone-binding domain ***(34,35)***. Only when the appropriate steroids are supplied, preferably by injection, Cre becomes active and inactivates the *loxP*-flanked gene.

Restricted expression of Cre should be tested by mating Cre mice with mice carrying silent reporter constructs that become activated by Cre. Such a construct can be the *lacZ* gene inactivated by insertion of a DNA fragment flanked by loxP sites. Cre activity deletes this insertion resulting in an active *lacZ* gene and expression of β-galactosidase. β-galactosidase activity will therefore mark all Cre expressing cells.

A conditional knockout is never complete and will result in a more or less chimeric phenotype, i.e., a mixture of wild-type and knockout cells in the Cre-expressing target tissue. Therefore, it is essential to distinguish normal and null cells in the target tissues, e.g., by in situ hybridization or immunohistochemistry. A convenient way to detect null cells is to couple the Cre-induced inactivation of a gene with the activation of a reporter gene such as *lacZ*.

3.4.7. Subtle Mutation Assay(s)

Knockins should be carried out with a minimum of changes in the targeted gene, as changes may effect the transcription of the gene. One strategy is the use of a floxed *neo–TK* cassette (a *neo–TK* cassette flanked by loxP sites) targeted to an intron or a noncoding region.

After G418 selection for homologous recombination events, positive clones are transfected transiently with a Cre-expressing construct. The Cre recombinase cleaves out the selection markers, leaving just a single loxP site behind, and recombined cells can be selected for loss of *TK* by gancyclovir treatment. If multiple changes are intended, the introduction of cDNA sequences into the genome is advisable. Wild-type cDNA should be knocked in as a control to prove that the changes in the organization of the gene locus do not result in a phenotype.

4. Notes

1. The *neomycin resistance* gene is the most commonly used selection marker. Selection is carried out by supplementing the medium with G418. Other selection markers such as hygromycin or puromycin resistance genes can also be used.

2. Different ES cell lines vary enormously in their germ-line transmission. Frequencies of transmission of the ES cell genome by chimeras range from 0 to 70% ***(36)***. Injection of ES cells derived from 129/Sv mice into blastocysts derived from C57Bl/6J results in high frequencies of germ-line transmission ***(37)***.
3. Feeder cells have to be resistant to the selection marker when cocultured with ES cells to select for stably transfected ES cells. Gene-targeted mice, without a fibroblast phenotype, can be used as a source of feeder cells. To facilitate feeder cell preparation, mice with four resistance genes have been produced. These mice have resistance genes for neomycin, 6-thioguanine, hygromycin, and puromycin ***(38)***.

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12

Determination of Positive and Negative Regulatory Elements

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1. Introduction

Transcription is the first step in the process of gene expression. Controlling where and when a gene is transcribed requires the interaction of *cis*-acting DNA control elements located near the gene, with *trans*-acting regulators, such as transcription factors and RNA polymerase. Identification of these *cis* regulating sequences called promoters and enhancers is a major key to understanding transcriptional regulation at the molecular level.

Integrins form a large and heterogeneous family of receptors that show a tightly regulated expression pattern during differentiation and morphogenesis. To understand the regulation mechanisms underlying integrin expression, several integrin gene promoters have been cloned and analyzed. These studies have shown that most integrin gene promoters lack both TATA and CCAAT boxes but are highly rich in C and G nucleotides. Besides these general features, numerous binding sites for developmental and cell type specific transcription factors, including AP-1, AP-2, GATA, Ets, and myogenic basic helix-loop-helix proteins have been described. Testing the ability of these sequences to drive transcription in cultured cells and in transgenic mice provided a new detailed insight of positive and negative regulating elements that control spatial and temporal receptor distribution ***(1–21)***.

1.2. Putative Promoter Sequences Can Be Studied in vitro

Isolation and sequence analysis of genomic DNA fragments allow the identification of potential transcription factor binding sites. As a first step in under-

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standing the mechanism responsible for the stimulation of gene expression, it is necessary to define transcription start sites and the minimal upstream sequence required to direct correct promoter activity.

To identify both positive and negative regulatory elements within the promoter sequence, different deletions must be tested for transcriptional activity. Here we describe the principles and the methodology of this approach by means of transient transfection assays and transgenic mice.

1.3. Promoters Can Be Characterized in Transfected Cells

Transient DNA-mediated gene transfer in tissue-culture cells has proved to be a rapid functional assay for the regulatory activity of DNA sequences. Transfections are carried out using putative promoter regions ligated upstream of a reporter gene. In the literature many different reporter genes have been described and all lack a mammalian homolog. Therefore, the measure of their expression level in transfected cells directly indicates the strength of the promoter tested.

The ability of a regulatory sequence to drive tissue-specific transcription can be characterized by transfecting the constructs in different cell types in which the corresponding endogenous promoter is either active or repressed. A similar approach using deletion mutants can provide a clear picture of the organization of positive and negative control elements on the analyzed sequence.

1.4. Promoters Can Be Analyzed in Transgenic Mice

To determine if the promoter sequences contain the proper regulatory elements for correct temporal and spatial expression, transgenic mice can be generated using different portions of the regulatory region linked to a reporter gene. This allows investigation of the mechanisms of gene regulation throughout the entire developmental program and in many different cell types, including those that cannot be transfected or cultured.

1.5. Strategies For Studying the β_1 Integrin Promoter

Using these approaches we characterized the 5' flanking region of the human *β_1 integrin* gene ***(4,9,22)***. This DNA fragment contains two tandemly located promoters (the distal and the proximal promoter relatively to the exon containing the ATG codon) that drive the expression of the unique β_1 gene giving rise to two mRNAs (**Fig. 1**). These molecules share the same coding sequences but diverge only in the 5' untranslated region. Sequence analysis indicated that the two regulatory regions lack TATA and CCAAT boxes, are both highly rich (75%) in C and G nucleotides and contain several Sp1-binding sequences. In addition, 1.1 kb of the distal promoter carries three AP1-binding sites, three

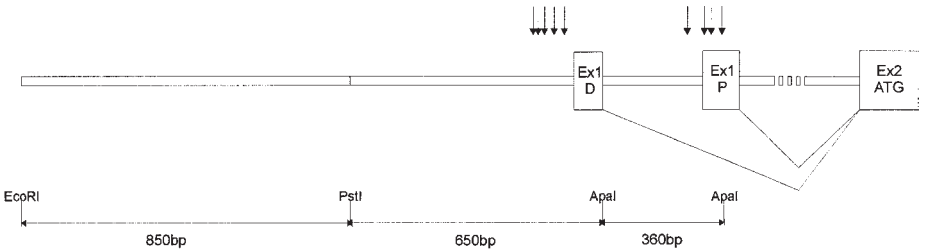

Fig. 1. Structure of the 5' flanking sequence of the human β_1 *integrin* gene. The genomic clone contains the distal and proximal promoters and the upstream sequence. The broken segment represents the first intron. Boxes D, P, and ATG are exons corresponding to 5' untranslated sequences and to the ATG-containing exon, respectively. Size and restriction enzyme sites are listed below. Downward vertical arrows denote transcription start sites mapped by primer extension analysis.

consensus sequences for the binding of NF1 factor, and an octamer. As expected for TATAless promoters, primer extension analysis indicated multiple initiation sites for both transcripts (**Fig. 2**).

By transient transfection of specific DNA constructs, we showed that the minimal sequence required to drive correct expression is a 630- and a 360-bp fragment, for the distal and the proximal promoter, respectively (**Fig. 3**) ***(4)***. In transfected cells, the distal promoter was found to be at least threefold more active than the other (**Fig. 3**) and Northern blot analysis indicated that the endogenous promoters is modulated similarly.

The activity of β_1 regulatory regions during development was studied using transgenic mice carrying constructs of a reporter gene linked downstream to the proximal and the distal promoter, respectively. Whereas mice transgenic for the construct containing the proximal promoter showed a very low level of expression, mice carrying the distal promoter highly expressed the reporter gene throughout embryonic development (**Fig. 4**). The expression pattern observed, consistently paralleled β_1 integrin subunit expression determined by immunohistochemistry ***(9,22)***. Altogether these results confirmed the observations on transfected cells and showed that the distal is the major responsible for high-level tissue-specific transcription regulation.

2. Materials

2.1. Primer Extension

1. Annealing buffer: 80% formamide 40 m*M* PIPES at pH 6.4, 400 m*M* NaCl, and 1 m*M* EDTA.
2. T4 polynucleotide kinase (Boehringer, Mannheim, Germany).
3. Moloney murine leukemia virus reverse transcriptase (Boehringer).

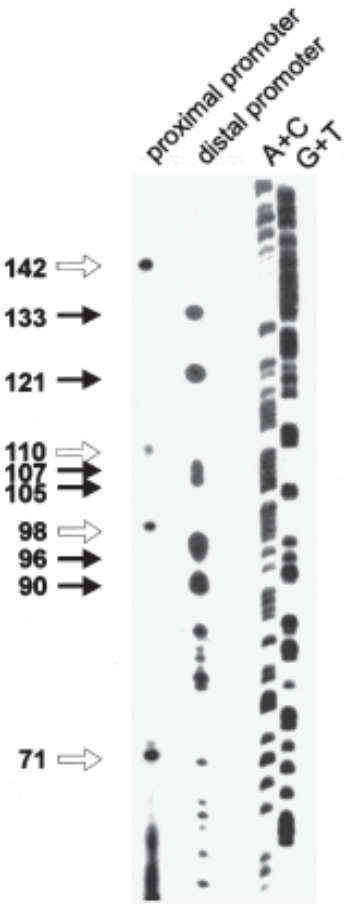

Fig. 2. Mapping of the transcription start sites of the two β_1 *integrin* transcripts by primer extension. Open and black arrows indicate position of primer extended products corresponding to exon 1P (proximal promoter) and exon 1D (distal promoter), respectively. The numbers correspond to the length in nucleotides of the extended products. Only prominent bands observed in repeated experiments are indicated in the figure. The molecular weight marker on the right is a mixture of the G+T and A+C sequencing reaction of m13mp18 single-strand phage DNA performed with the m13 universal primer.

4. Actinomycin D (Sigma, St. Louis, MO) (*see* **Note 1**).
5. Phenol/chloroform solution (1:1, Fluka, Buchs, Switzerland).
6. Formamide loading buffer: 80% formamide (Fluka). 10 m*M* EDTA (pH 8.0), 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol.
7. X-AR5 films (Kodak).

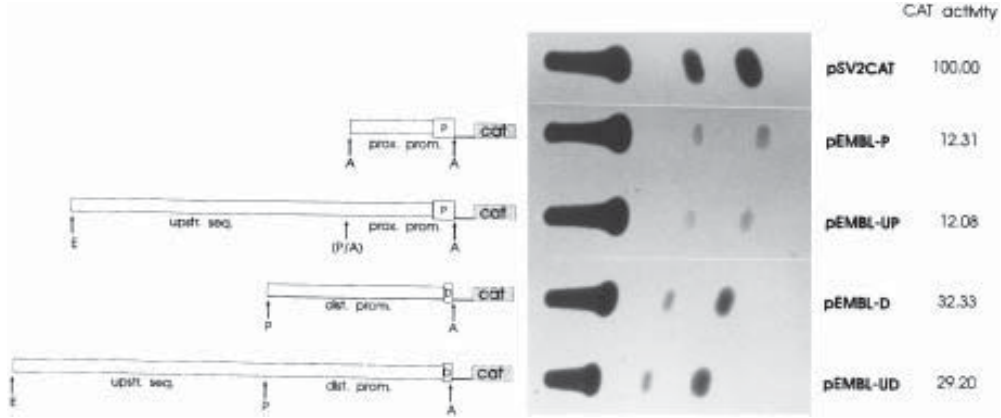

Fig. 3. CAT activity in transfected MG-63 cells. The structures of the four DNA constructs tested for promoter activity are described on the left. Autoradiography of the CAT assay is shown in the center. Measures of relative CAT activity, expressed in percentage as described in methods, **Subheading 3.3.**, are shown on the right. A, *Apa*I; E, *Eco*RI; P, *Pst*I.

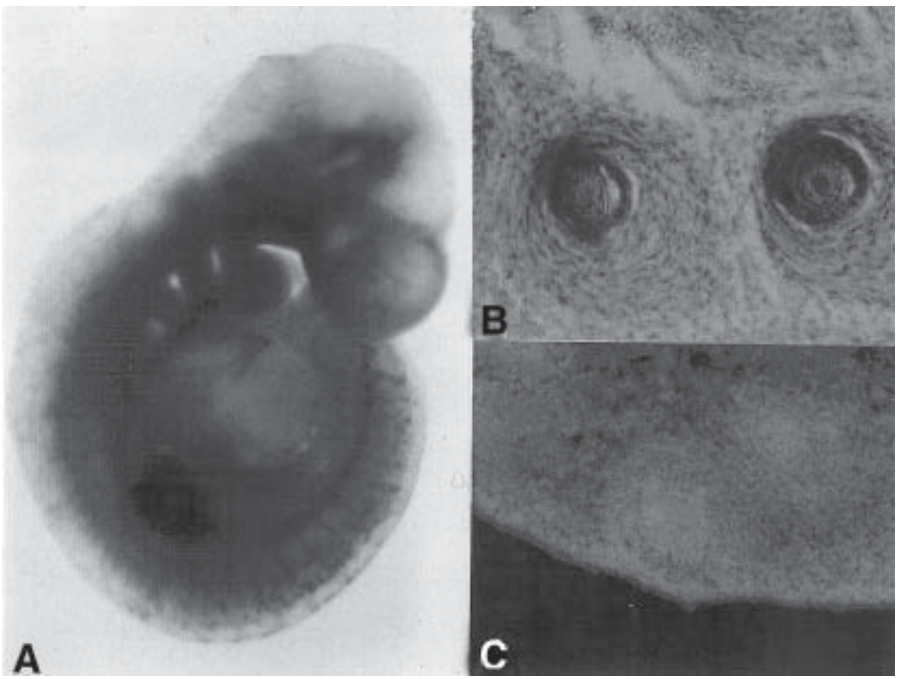

Fig. 4. Reporter gene expression driven by the β_1 *integrin* distal promoter. **(A)** *lacZ* gene expression in a whole-mount embryo at day 10.5 of gestation. (**B** and **C**) Sagittal sections of follicles of vibrissae from 14.5-d-old transgenic embryos carrying *lacZ* and *GFP* reporter gene, respectively. Note that X-gal staining **(B)** reveals the same expression pattern observed by fluorescent microscopy **(C)**.

2.2. Constructs for Transfections

1. Restriction enzymes from any suppliers.
2. Quiaquick gel extraction kit (Qiagen, Hilden, Germany) or GeneClean gel extraction kit (Bio-101, La Jolla, CA).

2.3. Transient Transfection

1. Human osteogenic sarcoma MG-63 cells (ATCC, Manassas, VA, cat. no. CRL 1427).
2. Bovine serum albumin (BSA).
3. Tranfectam reagent (Promega, Madison, WI).

2.4. CAT Assay

1. CAT reaction mixture: 50 µL of 1 *M* Tris-HCl at pH 7.8, 10 µL of ^{14}C-labeled chloramphenicol (60 mCi/mmol diluted in water to 0.1 mCi/mL), and 20 µL of acetyl coenzyme A (prepared each time at a concentration of 3.5 mg/mL in water).
2. 20-cm thin-layer chromatography (TLC) silica gel (Merck, Rahway, NJ).
3. Scintillation liquid (e.g., Insta-gel, Packard, Dowhers Grove, IL).

2.5. Preparation of Microinjectable DNA Solution

1. Quiaquick gel extraction kit (Qiagen).
2. TE: 10 m*M* Tris-HCl at pH 8.0, 0.1 m*M* EDTA at pH 8.0. Sterile filter through a 0.2-μm filter, aliquot, and store at 4°C.

2.6. Generation of Transgenic Mice

1. Mice for vasectomy: 8 or more wk-old FVB males.
2. Mice for zygote production: 8-wk-old DBA×C57 F1 or FVB females and males.
3. Mice for embryo transfer: 8 or more wk-old CBA×C57 F1 females.
4. Avertin: stock solution: 2 mg/mL 2,2,2-tribromoethyl alcohol (Sigma) in tert-amyl alcohol (2-methyl-2-butanol, Fluka). Keep at 4°C in the dark. Working solution: 300 μL of stock a in 4 mL of PBS ***(24)***. Keep at RT no longer than 1 wk.
5. Surgical equipment: fine dissection scissors; two pairs of watchmaker #5 forceps (sometimes manually sharpened); blunt fine curved forceps; serrefine clamp (1.5 in or smaller); surgical silk or catgut suture with curved needle (e.g., size 10), 1-mL syringes with 26-gage hypodermic needle.
6. Hormones for superovulation: PMS (pregnant mare serum, Sigma). HCG (human chorionic gonadotropin, Sigma). Hormones are dissolved at 500 u/mL in PBS and kept at –20°C in 100-μL aliquots. Aliquots are diluted with 900 μL of PBS before injection.
7. Stereomicroscope (Wild M8, Heerbrug, Switzerland, or equivalent).
8. M2 and M16 medium ***(23)***.
9. Hyaluronidase type IV-s (Sigma).
10. Transfer glass pipet. The pipet is prepared by manually pulling the thin end of a Pasteur pipet on a Bunsen's burner. Only pipets with 2–3 cm in length and approx 0.2 mm in diameter (not much larger than a 1-cell zygote) can be used. The tip opening is cut blunt with a diamond glass cutter. Connect it with a flexible thin tube to a mouth-piece.
11. Petri dishes for bacteriology, 6-cm diameter.
12. Injection glass needles: with (Narishige cat. no. GD-1) and without (Narishige cat. no. G-1) internal filament. Needle puller (e.g., Narishige). Microforge with 0.22-mm thick platinum wire, 10×, 20× objectives, eyepiece with graticule (Narishige).
13. Microinjection set-up: microscope with Hoffman or Nomarski optics (e.g. Olympus, or equivalent). Left and right, water-driven micromanipulators (Narishige). Gas driven microinjector (Eppendorf, Hamburg, Germany. e.g., Microinjector 5242 or newer). Holding pipet controlling syringe with teflon tubing and metal pipet holder (Narishige).
14. Embryo-tested light paraffin oil (Sigma), stored at RT.
15. Optic fibers illuminator.

2.7. Identification of Transgenic Mice

1. Tail Buffer: 100 m*M* Tris-HCl at pH 8.0, 5 m*M* EDTA, 0.2% SDS, 200 m*M* NaCl.
2. PK (100× stock): Proteinase K (Sigma), 10 mg/mL in double-distilled water (DDW). Aliquot and store at –20°C.

3. Bench centrifuge (Eppendorf cat. no. 5415C, or equivalent).
4. Phenol-chloroform. Thaw crystalline phenol (molecular biology grade, Sigma) at 37°C. Working in a fume hood (phenol and chloroform are toxic, waste should process accordingly to local laws), extract 100 mL of phenol with equal volume of 100 m*M* Tris HCl at pH 8.0 until the pH of the phenol phase reaches 8.0. Add an equal volume of chloroform (Fluka). Add 20 mL of 10 m*M* Tris HCl at pH 8.0. Store in dark glass bottles at 4°C for up 1 mo.
5. BSA: 10 mg/mL bovine serum albumin (New England BioLabs, Beverly, MA).
6. Lysis Buffer: 1X PCR Buffer (Gibco-BRL, Gaithersburg, MD), 1× proteinase K (**item 2**).
7. Thermomixer (Eppendorf cat. no. 5436).
8. Taq polymerase (Gibco BRL) and thermal cycler (Hybaid, Teddington, UK).

2.8. Analysis of lacZ Expression in Whole Mount Preparations

1. Buffered paraformaldehyde: 4% paraformaldehyde (Sigma; store at 4°C) in PBS ***(24)***. Weigh the powder and dissolve it in DDW. Warm the suspension at 60°C. Add few drops of 1 *N* NaOH until the solution clears. Add the appropriate amount of 10× PBS stock to make the final solution 1×. Store aliquots at –20°C.
2. X-Gal solution: 1 mg/mL X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, Sigma), 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$, 2 m*M* $MgCl_2$, 0,001% deoxycholate (Sigma), 0.01% NP-40 (Sigma, USA).
3. 100% ethanol. All ethanol dilution are considered in water.
4. Xylol (Fluka).
5. Paraffin pearls, 56–58°C melting temperature (e.g., Paraplast, Fisher, Pittsburgh, PA).
6. 37 and 60°C oven. 37°C water bath.
7. Glass slides and cover slips.
8. Microtome (Reichert and Jung, Nussloch, Germany).
9. Nuclear Fast Red (Sigma). Make a 2% solution in water, add a drop of acetic acid. Filter and store at room temperature in the dark.
10. DPX mounting medium (Fluka).

2.9. Analysis of lacZ Expression on Cryostat Sections

1. From **Subheading 2.4.**, **items 1**, **2**, **7**, **9**, and **10**.
2. Isopenthane and acetone (Fluka); liquid nitrogen.
3. OCT compound (Miles, New Haven, CT).
4. TESPA-treated slides: 2% TESPA (triethoxysilylpropylamine, Sigma) in acetone. 100 mL of this solution is sufficient to treat 50–100 slides. Submerge clean dry slides for 5 min in 100% acetone, for 1 min in acetone-TESPA, for 5 min in 100% acetone. Air dry. Store in a clean box at room temperature.

2.10. Analysis of Murine β_1 Integrin Expression on Cryostat Sections

1. From **Subheading 2.3.**, **items 9** and **10**. From **Subheading 2.4.**, **items 2**, **3**, **4**.
2. Acetone, chloroform, 30% H_2O_2 in water (Fluka).
3. 1% BSA (Sigma) in PBS ***(24)***.

4. Rabbit anti β_1 polyclonal antibody ***(9)***.
5. Anti rabbit IgG affinity-purified, peroxidase conjugate (The Binding Site, Birmingham, UK).
6. Quenching solution: 3% H_2O_2.
7. DAB: stock a: 3,3'-diaminobenzidine tetrahydrochloride (Sigma; handle with care, *see* **Note 15**) 0.2%; aliquot and store at –20°C. Working solution: dilute stock 1:4 with 50 m*M* Tris-HCl at pH 7.6, add 30% H_2O_2 to a final 0.3% concentration.
8. To visualize GFP, an Olympus BH2-RFCA microscope should be equipped for epifluorescence with a BP 490-FY 455 filter set.
9. To estimate GFP concentration in tissues of transgenic mice, measure the fluorescence in tissues extract at 490 nm excitation and at 509 nm emission using the Perkin-Elmer LS 3B Fluorescence Spectrometer (Norwalk, CT).

3. Methods

3.1. Mapping of the Transcription Initiation Sites of the Human β_1 Integrin Gene 5' Flanking Region

Primer extension experiments are performed to map transcription start sites. The protocol is essentially as described by Sambrook et al. ***(24)***.

1. Design a single-stranded DNA primer of 20 nucleotides complementary to the 5' end of the specific mRNA, within the first 100 nucleotides.
2. Radiolabel the oligonucleotide by phosphorylating its 5'-end with T4 polynucleotide kinase as described ***(24)***.
3. Anneal 5×10^5 cpm of the primer to 25 μg of total RNA at 30°C for 16 h, in the annealing solution.
4. Primer is extended for 2 h at 37°C with 20 units of Moloney murine leukemia virus reverse transcriptase according to manufacturer's recommendations in the presence of 200 μg/mL actinomycin D. Actinomycin D inhibits synthesis of double-stranded DNA by reverse transcriptase.
5. After phenol/chloroform extraction and ethanol precipitation, resuspend the products of the extension reaction in formamide loading buffer
6. Carry on sequencing reactions of a plasmid DNA as molecular weight marker
7. Load the samples on a 6% polyacrylamide, 8 *M* urea sequencing gel as described ***(24)***.
8. Expose the gel to X-AR5 films at –75°C with intensifying screen.

3.2. Construction of Plasmids for Transient Transfection

The 4.5-kb genomic fragment was digested to generate the following fragments containing different sequences of both promoters (**Fig. 1**):

1. A 360-bp *Apa*I fragment of the proximal promoter.
2. A 630-bp *Pst*I/*Apa*I fragment of the distal promoter.
3. A 842-bp *Eco*RI/*Pst*I fragment 5' of the distal promoter that was ligated upstream of both previous fragments.

All restriction fragments were resolved on agarose gel and extracted using Quiaquick Gel extraction columns or GeneClean. After blunt ending they were ligated into the *Sma*I site in the polylinker of plasmid pEMBL8CAT to give the following DNA constructs suitable for transient transfection analysis:

1. pEMBL-P
2. pEMBL-D

3a. pEMBL-UP
3b. pEMBL-UD

pEMBL8CAT carries a promoterless *cat* gene encoding bacterial chloramfenicol acetyltransferase, a protein whose activity can be detected by an enzymatic assay (*see* **Subheading 3.4.**). Putative promoter sequences can be cloned upstream of the *cat* reporter gene and transiently transfected into an appropriate cell line to test for the ability to drive transcription in an in vitro system.

3.3. Transient Transfections

To normalize transfection efficiency, cells are cotransfected with DNA from each construct together with the pRSLV plasmid as an internal control. This plasmid contains the Rous sarcoma virus LTR sequence that drives the *luciferase* gene expression (*see* **Note 2**).

To normalize CAT activity, cells are transfected with a positive control SV2CAT plasmid, containing the *cat* gene driven by the *SV40 early* gene promoter. The relative percent CAT activity of each construct is calculated considering the positive control as 100%. Empty pEMBL8CAT vector is used as negative control.

1. Purify all plasmid DNAs by cesium chloride density gradient.
2. Cotransfect 0.5 pmol of each construct along with 0.5 pmol of pRSLV.
3. Seed cells at 5×10^5/60-mm dish in the appropriate medium with serum
4. After 1 h put cells in a serum-free medium supplemented with 1 mg/mL BSA and incubate further for 1 h.
5. Mix DNA in Tranfectam reagent and incubate with cells for 3 h.
6. Remove the medium and incubate cells in complete medium.
7. 36–48 h after transfection, harvest cells and lyse by three cycles of freezing and thawing.
8. Centrifuge the lysed cell suspension at 12,000*g* for 5 min at 4°C.
9. The extracts are ready to be assayed for CAT activity.
10. One-tenth of each extract can be assayed for luciferase activity.

3.4. CAT Assay

1. To 50 µL of each cell extract to be tested, add 80 µL of CAT reaction mixture and incubate at 37°C for 1 h (*see* **Note 3**).

2. Add 1 mL of ethyl acetate to each sample and mix by vortexing for three times, 10 s each.
3. Spin at 12,000*g* for 5 min at room temperature.
4. Take 900 μL from the upper phase where the acetylated form of chloramphenicol is present and evaporate the solvent under vacuum.
5. Redissolve the pellet in 25 μL of ethyl acetate.
6. The samples can be then chromatographed on a silica gel that resolves the mono- and di-acetylated derivatives of chloramphenicol from the unmodified compound.
7. Amounts of cellular extracts used for CAT assays, normalized according to luciferase activity, are spotted at the origin of a 20-cm silica gel plate for thin-layer chromatography (TLC).
8. Apply 5 μL of each sample at a time and dry with a hair dryer after each application.
9. Place the TLC plate in a chamber containing 200 mL of chloroform:methanol (95:5).
10. After complete separation, expose the plate to X-AR5 film for several hours.
11. Scrape from plates the radioactive spots corresponding to the chloramphenicol monoacetylated forms and measure their radioactivity by counting in scintillation liquid.
12. Calculate relative CAT activities for each construct as percentage of pSV2CAT and pEMBL8CAT measurements.
13. To obtain significant results, repeat all transfections at least three times, using two different DNA preparations.

3.5. Design of Constructs for Promoter Analysis in Transgenic Mice

The first step in studying promoter activity in vivo in transgenic mice is to generate a DNA construct containing the sequences under analysis linked to a reporter gene cassette (*see* **Note 4**). To ease detection of the reporter gene product it is advisable to use the bacterial *lacZ* gene encoding β-galactosidase, an enzyme that can be histochemically detected in both whole-mount preparations of organs and in tissue. The β_1 integrin promoter regions were inserted upstream of a *lacZ* cassette that contained at its 3' end the polyadenylation signal of SV40. Satisfactory results were obtained linking promoter regions to the Aequorea Victoria jelly fish *GFP* gene. Its encoded protein shows an intrinsic fluorescence and therefore can be tracked by fluorescence microscopy on tissue sections.

3.6. Generation of Transgenic Mice

Transgenic mice are generated by microinjection of the diluted DNA construct into the pronucleus of a fertilized oocyte. To establish the technique, an animal care facility must be available and treatment of mice must proceed in agreement to local laws regulating in vivo experimentation.

3.6.1. Preparation of Microinjectable DNA Solution

1. Cut out of vector sequences the DNA fragment used for generation of transgenic with appropriate restriction enzymes and purify it by agarose gel electrophoresis.

DNA is extracted from gel using either the Quiagen Quiaquick spun columns or the Bio101 Gene Clean kit (*see* **Note 5**).

2. Evaluate purity and concentration of the fragment by comparing on an agarose gel a sample quantity with serial dilutions of DNA marker of known concentration. The stock solution should be adjusted to 100 ng/µL with sterile DDW. DNA treated in such way is stable at –20°C.
3. Working solution should contain approx 500 molecules per pL. Given N the number of base pairs of the DNA fragment, the concentration in ng/µL of such working solution is calculated by the following formula: X ng/µL = $5.5 \times 10^4 \times N$. Dilution should be performed using sterile filtered TE.

3.6.2. Vasectomy

1. Anesthetize a male mouse by intraperitoneal injection of 0.5 mL of diluted Avertin solution. Wash skin of lower abdomen (at the level of the top of the legs) with 75% ethanol and make a 1 cm cut with sharp scissors (1.5 cm large). Cut similarly the muscle of body wall, avoiding the fat pad surrounding genitals.
2. With bended blunt forceps gently push the scrotum to move the right testicle into the abdominal cavity (until the white testicular fat pad appears at the edge of the incision). Expose the testicle by pulling the white fat pad. Note that around the testicle, the white coiled epididymus prolongs in a wider tube: the vas deferens.
3. Pierce with the tip of the forceps the thin membrane linking the vas deferens to the testicle and blood vessel. Apply two stitches around the freed tube, at a distance of about 5 mm from each other. Cut with scissors the vas deferens between the two stitches. By gently grasping the fat with forceps, reposition the testicle inside the abdomen. The same procedure is repeated for the left testicle.
4. Separately stitch two or three times muscle and skin, then put the mouse alone back into a fresh cage. Vasectomized mice can be used 15–30 d after surgery. Testing for residual breeding capacity is advisable.

3.6.3. Generation of Superovulated and Pseudopregnant Females

Normally, enough embryos (100–200) are obtained from six superovulated females. Enough pseudopregnant females are obtained from 15 vasectomized males.

1. In an animal house with 5 AM to 7 PM light cycle, 8-wk-old females are injected intraperitoneally with 5 U (100 µL of diluted stock) of PMS at 12 AM.
2. 46 h later, inject the same mice with 5 U (100 µL of diluted stock) of HCG and put each with a breeder normal male.
3. At the same time, put two or more untreated females with each vasectomized mice to obtain pseudopregnant animals in which manipulated embryos could be transferred.

3.6.4. Collection of Zygotes

1. Early in the morning of the day following **Subheading 3.2.**, check females with a blunted, ethanol washed Pasteur pipet for the presence of vaginal plugs. This

whitish solid residue indicates that the mouse happened to mate the night before. Females showing to have mated with vasectomized mice are kept in a separate cage until transfer of embryos is possible.

2. The next step consists of isolation of zygotes from females mated with normal mice. Open the abdomen, after killing mice by cervical dislocation, and expose the "v" shaped uterus underlying bowels. Pierce the thin membrane of the mesometrium with closed sharp-ended forceps at the distal end (near the ovary) and gently remove it from the uterus (until oviductal tubes slightly detach from the ovary). Grasp the membrane that surrounds ovary and oviduct (bursa) with sharp-ended forceps. Hold the oviduct in this way and cut the utero-tubal junction with small sharp scissors (*see* **Note 9**). Pull the forceps, tear the rest of the bursa and put the oviduct in a drop of M2. This procedure is repeated for the other uterine horn.
3. Put the oviduct under the stereomicroscope in glass slide containing 100 μL of hyaluronidase. With sharp-ended forceps, tear the ampulla, a swelling of the tube in which embryos collect. Push outside embryos together with cells of the cumulus with the help of the tip of the forceps. Free zygotes from cumulus by suction and ejection from the mouth controlled transfer glass pipet. Collect them with the glass pipet and transfer them into a Petri dish containing M16. Store embryos in a cell culture 37°C, 5% CO_2 incubator.

3.6.5. Microinjection

3.6.5.1. Setting of the Equipment

Pronucleus injection is performed under a dedicated inverted microscope providing Hoffman or Nomarski optics. Zygotes are manipulated with the aid of two capillaries that can be manually moved by two micromanipulator sets. The glass needle positioned on the right holds the embryo and the one on the left penetrates the nucleus and injects DNA solution. Injection capillaries are prepared by pulling filament containing needles (*see* **Note 7**). They must be filled with a freshly prepared DNA working dilution by immersion of their bottom into the solution; the fluid reaches the tip by capillary force. Holding needles are made with filament-free needles that are pulled in the same way as above. The drawn-out side is then cut with a diamond glass cutter to give an approx 0.1 mm diameter. The tip of the needle is checked under the microforge for a perfectly flushed opening and then polished near the resistance until it narrows to approx 1/5–1/10 of the initial diameter. The holding pipet is connected to the controlling syringe and filled with paraffin oil.

3.6.5.2. Manipulation of Zygotes

After at least 2 h of recovery in the incubator, approx 20 zygotes are transferred to a drop of M2 on a glass slide. To prevent evaporation the drop is overlaid with paraffin oil. The injection chamber set is then put on the micro-

scope table and capillaries are brought in the middle of the optic field at the same focus plane of embryos. Zygotes are then moved to the top of the field, so that injected embryos can be put to the bottom. One by one, fertilized oocytes are gently sucked with the holding pipet maneuvering the connected syringe. Either one of the two pronuclei is focused, then the injection needle is brought inside the nucleus by a smooth and firm movement. DNA solution is injected until a clear swelling is noticed (*see* **Note 8**). After injection zygotes are transferred back to a new M16-containing Petri dish and put into the incubator.

3.6.6. Transfer of Embryos to a Pseudopregnant Female

1. After 1 or more hours in the incubator, discard dark looking lysed embryos and transfer about 20 healthy zygotes to the ampulla of a pseudopregnant female that showed the plug the same day of injection (*see* **Note 6**). Gently suck embryos, minimizing the liquid in-between, into the transfer pipet previously filled with a series of small air bubbles followed by M2 medium. Store the transfer pipet undisturbed nearby the stereomicroscope.
2. Put an anesthetized (anesthesia as described in **Subheading 3.1.**) female with the head facing 12 o'clock under the stereomicroscope and enlighten it with the aid of an optic fibers illuminator. Disinfect the skin of the back and cut with scissors at approx 1–2 cm above the hind-leg.
3. Detach the cut skin from the underlying muscle and displace it around to localize the underneath ovary (a reddish ball surrounded by a white fat pad). Expose the ovary by cutting the above body wall muscle and by pulling outside the fat with blunt-ended curved forceps. Hold the ovary outside, perpendicular to the vertebral column, by grasping the fat with the serrefine.
4. Using two extremely sharp forceps, a small laceration is made on the bursa, the transparent membrane surrounding oviduct and ovary (*see* **Note 10**). The opening of the infundibulum that should appear towards the operator and below coils of oviduct is probed with the tip of the forceps. Once the opening is clearly located, insert the mouth pipet and keep it in place with forceps holding the tube. Gently blow embryos until an air bubble reaches the ampulla.
5. Detach the serrefine and put back ovary and uterus inside the abdomen. After applying few stitches to the muscle and to the skin, transfer the mouse to a fresh cage and leave it to recover overnight in a warm place.

3.7. Identification of Transgenic Mice

Newborn mice are delivered 19–20 d after embryo-transfer (*see* **Note 11**). Approximately 20–30% of the litter should be transgenic. DNA obtained from a tail biopsy is used to detect the presence of the transgene either by Southern blot hybridization or by PCR. Whereas Southern hybridization is highly reliable, PCR analysis is more prone to contamination artifacts, but faster and much less labor intensive.

3.7.1. DNA Preparation for Southern Blot Hybridization

1. Label 20- 30-d-old mice by applying a numbered ear clips. Mark number, sex, and mouse coat color on a notebook for further reference.
2. Cut the tail with sharp strong scissors at approx 1 cm from the tip and collect the tissue sample in a labeled sterile 1.5-mL tube. Tail tissue is dissolved by incubation on a rotary wheel with 0.5 mL of tail buffer-PK over night at 56°C.
3. Add 0.5 mL of phenol-chloroform. Incubate samples for 10 min on the rotary wheel at RT, then spin them at maximum speed on a bench centrifuge for 5 min.
4. Collect the DNA-containing supernatant, avoiding precipitates at the interface, with a 1 mL disposable tip (cut to make the opening wider; *see* **Note 12**). Add the supernatant to a fresh tube containing 0.5 mL of chloroform. Incubate samples again on the rotary wheel for 10 min, then spin them for 5 min at maximum speed.
5. Collect the DNA containing supernatant as in **step 4** and add it to a fresh tube containing 0.5 mL of isopropyl alcohol. Invert the tube for few times and pellet the white DNA precipitate to the bottom by a short spin. Carefully eliminate the alcohol and air-dry the DNA for a couple of minutes.
6. Add to the DNA pellet 100 µL of sterile DDW and resuspend it overnight at 56°C. Store the DNA solution then at 4°C.
7. Digest DNA (10–15 µL) in a final volume of 30 µL over night at 37°C, with 40 U of appropriate enzyme (*see* **Note 13**), buffer and 0.1 mg/mL BSA.
8. Perform gel electrophoresis, Southern blotting, and hybridization following standard methods ***(24)***. Use the same injected DNA to make the probe.

3.7.2. DNA Preparation for PCR Analysis

1. Design a couple of oligonucleotides amplifying a short (<500 bp) diagnostic fragment that spans the 3' region of the promoter and the 5' region of the reporter gene. Oligos should be 20-meres and have at least a 50% GC content.
2. Label mice as in **Subheading 3.3.1.**, **step 1**. Collected no longer than 2–3 mm of the tip of the tail in an 1.5-mL tube containing 50 µL of lysis buffer.
3. Incubated samples at 60°C shaking in the Thermomixer for 2–3 h. Smash remnants of tail using a fresh yellow tip for each tube. Inactivate proteinase K by a 6-min incubation at 95°C. Spin at maximum speed for 5 min. Dilute 5 µL of the supernatant into 10 µL of sterile DDW.
4. Set up a PCR reaction following standard procedures ***(24)*** using 5 µL of the diluted sample in a final volume of 50 µL.
5. Amplify using the following cycle profile.

10 cycles	95°C, 30s
	65°C-1°C each cycle, 30s
	72°C, 30s
25 cycles	94°C, 30s
	55°C, 30s
	72°C, 30s

6. Check the reaction on agarose gel: samples showing the expected band correspond to transgenic mice.

3.8. Analysis of lacZ Reporter Gene Expression

To study whether different segments of the human β_1 *integrin* promoter could correctly drive *lacZ* reporter gene expression in time and space, embryonic and adult organs were stained for β-galactosidase activity. The resulting pattern was then compared with the distribution of the endogenous β_1 *integrin* gene. Embryos until day 12.5 and organs (as big as an adult brain) were stained for lacZ activity in toto. In all other cases staining was performed on frozen sections. Expression of murine β_1 *integrin* gene was detected by immunocytochemistry on cryostat sections.

3.8.1. Analysis of lacZ Expression on Whole-Mount Preparations

1. Dissect organs or embryos and quickly fix them in cold (4°C) buffered paraformaldehyde. The fixation time varies with the size of the specimens: from 30 min (e.g., a 10-d-old embryo) to overnight (e.g., a newborn).
2. Wash 2–3 times in PBS.
3. Stain for *lacZ* activity overnight at 30–37°C in 50 mL of X-Gal solution.
4. Store stained embryos in formalin or 70% ethanol.
5. Stained specimens can be photographed as whole-mount preparations or dehydrated, paraffin-embedded, and sectioned.
6. Dehydration and embedding: incubate samples for 30 min in each of these solutions 80%, 90%, 95% 100%, 100% ethanol. Incubate in xylol until specimens appear cleared. Repeat xylol incubation (*see* **Note 14**). Incubate for 30 min in paraffin at 60°C. Repeat incubation two times in fresh paraffin. Embed in a small box and let solidify at 4°C.
7. Cut 10–15 μm sections with microtome.
8. Collect sections on the surface of a 37°C water bath and push them onto glass slides. Leave slides to dry overnight at 37°C.
9. Rehydrate sections incubating 5 min in xylol (repeat two times), 5 min in ethanol 100% (repeat two times), 5 min in 80% ethanol, 5 min in 70% ethanol, 5 min in DDW.
10. Counter-stain 5 min with nuclear fast red.
11. Air dry and mount with cover slip using a drop of DPX. If staining is faint it is better to mount coverslips using glycerin (DPX contains xylol, *see* **Note 14**).

3.8.2. Analysis of lacZ Expression on Cryostat Sections

1. Proceed as in **Subheading 3.4.1.**, **step 1**.
2. Freeze specimens in a solid isopenthane in a bath of liquid nitrogen.
3. Fix samples on the cryostat holder with a drop of cold but still liquid OCT mounting media.
4. Cut 10- to 15-μm sections and collect them onto TESPA-treated slides.
5. Air dry sections for 1 h then stain overnight in X-Gal solution.
6. Counterstain and mount cover slips as described in **Subheading 3.4.1.**, **steps 9** and **10**.

3.8.3. Analysis of β_1 Integrin *Expression by Immunocytochemistry*

1. Freeze tissue samples as in **Subheading 3.4.2.**, **step 2**.
2. Cut sections as in **Subheading 3.4.2.**, **step 4** without fixation.
3. Fix sections in cold acetone/chloroform 10 min at –20°C.
4. Next quench endogenous peroxidase activity by an incubation in 3% H_2O_2 for 5 min at room temperature.
5. Wash in PBS and incubate overnight in 10 μg/mL anti-β_1 integrin antibody ***(9)*** in PBS/BSA at 4° in a sealed box containing a wet napkin.
6. Wash three times in PBS for 5 min.
7. Incubate for 30 min at room temperature with peroxidase conjugated anti-rabbit IgG.
8. Wash three times in PBS for 5 min.
9. Develop the immunolabeling with DAB solution under the microscope until staining reaches an acceptable level.
10. Counterstain and mount cover slips as described in **Subheading 3.4.1.**, **steps 9** and **10**.

4. Notes

1. Actinomycin D is a teratogen and a carcinogen drug. Manipulate the powder and the solution carefully. The solution is also light sensitive.
2. The enzymes CAT and luciferase are the most widely used reporter genes in transiently transfected mammalian cells because: the enzymatic activity encoded by the reporter gene is readily distinguishable from any endogenous activity of the mammalian cells; and the assay is rapid, sensitive and reliable.
3. Incubation time for CAT assay depends on the strength of the promoter used and the cell type. Usually incubations for 60 min to 3 h are performed.
4. To obtain full expression in target organs the reporter gene cassette must include all sequences enabling generation of a stable mRNA. This implies the reporter gene to be followed by a polyadenylation signal (pA). We often found convenient to add a short intron sequence upstream to the pA. A cassette suitable for this purpose contains part of a noncoding exon followed by an intron, another exon and the pA of the human β-globin gene ***(25)***.
5. Prolonged exposure to UV light of ethidium-bromide-stained DNA can result in damage to the DNA itself. Therefore it is strongly recommended to use low-energy UV lamps for the minimum time required.
6. Dissection can be helped by working under a stereomicroscope.
7. The temperature of the puller must be found empirically in a way that tips are as small as possible and with a bottle-like shape. In addition, needles must still be able to fill themselves by capillary force.
8. The movements of the needle must be firm to avoid breakage of the plasma membrane. Sometimes it is useful to make a small break to the tip of the injection needle by gently crashing it onto the holding pipet. The same trick is necessary in case of clogging. Whenever the tip gets too broad or hard to unclog, it is important to change the needle. To avoid clogging and dilution of the DNA solution with M2 medium, a low permanent flux through the needle tip can help.

9. In case no pseudopregnant females are available the same day of injection, zygotes can be kept overnight in the incubator. They can be transferred at the two-cell stage to a freshly plugged female following the same procedure outlined in **Subheading 3.5.**
10. It is important to avoid blood vessels. Bleeding can obscure the field and hinder all subsequent operations.
11. If only one or two embryos develop to term, delivery might be retarded. In this case newborns get too big to be born naturally. When a lactating female is available, it is possible to recover the litter by cesarean section and fostering.
12. High-molecular-weight genomic DNA is easily broken by shearing forces. It is therefore important to use cut tips with a wide opening.
13. The transgene almost always inserts into the genome in an array of multiple copies oriented head-to-tail. Enzymes that cut only once in the transgene are preferable because they produce fragments corresponding to the exact size of the injected DNA.
14. Xylol is toxic. Work under fume hood. Xylol tends to dissolve X-Gal precipitates: consider cryostat sections instead of paraffin embedding in case of faint staining.
15. DAB is a carcinogen. It is readily inactivated in bleach.

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A Cell-Based Adhesion Assay for the Characterization of Integrin $\alpha_v\beta_3$ Antagonists

James M. Wu, Mary P. Rosser, Anthony R. Howlett, and Richard I. Feldman

1. Introduction

1.1. Therapeutic Utility of $\alpha_v\beta_3$ Inhibitors

Integrins are heterodimeric cell-adhesion receptors that comprise a family of over 20 different combinations of α-and β-subunits that function in diverse cellular processes, including development, growth, motility, metastasis, apoptosis, inflammation, hemostasis, and bone resorption (for review, *see* **refs.** ***1,2***). $\alpha_v\beta_3$ is a member of a subclass of integrins that show binding specificities to peptide sequences encoding an RGD (-Arg-Gly-Asp-) structural motif. Recent interest has focused on $\alpha_v\beta_3$, which has been shown to have important roles in tumor angiogenesis and metastasis ***(3,4)***, hyperplasia leading to restenosis ***(5)***, osteoclast-mediated bone resorption ***(6,7)***, and retinal neovascularization ***(8)***. Both in vitro and in vivo studies, employing specific antibodies to $\alpha_v\beta_3$, as well as short RGD-containing peptide sequences, have been used to demonstrate therapeutic utility in blocking the function of this receptor in animal models.

1.2. Cell-Based Screens for $\alpha_v\beta_3$ Inhibitors

Assays that measure the binding of cell-matrix proteins to detergent-solubilized, purified integrins (solid-phase binding assays), have been used extensively to screen for and characterize compounds that inhibit integrin function (e.g., RGD peptides and mimetics). In this chapter, we describe a whole-cell assay that measures cell adhesion occurring specifically through the integrin $\alpha_v\beta_3$. This assay is able to detect direct binding-site inhibitors (such as RGD mimetics), as well as less direct blockers of the integrin function, such as modu-

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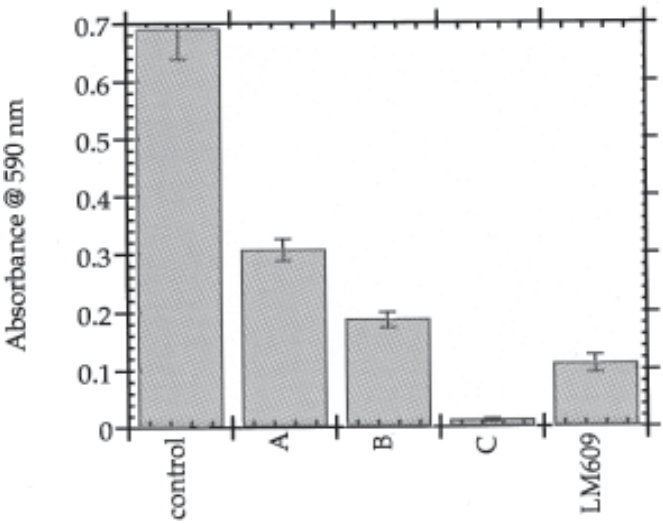

Fig. 1. Inhibition of WM-115 cell adhesion to fibrinogen by various treatments. Cells were allowed to bind to fibrinogen-coated plates for 30 min at 30°C, washed, stained, and solubilized as described in **Subheading 3.** Nonpeptidics BX-1 (A) and BX-2 (B) and the cyclic peptide GpenRGDSPCA (C) were tested at 10 μ*M*. LM-609 (8 μg/mL) was incubated with cells for 1 h prior to the assay. Background staining of cell binding to BSA coated plates (A_{590} = 0.086) was subtracted. Shown are the averages of three points +/– standard error.

lators of integrin-signaling pathways, that would not be detected in a solid-phase binding assay using purified $\alpha_v\beta_3$.

To promote cell adhesion through $\alpha_v\beta_3$, we used cells expressing relatively high levels of $\alpha_v\beta_3$. We also used fibrinogen as the cell matrix protein, because most other integrins do not bind this protein. Under our assay conditions, cell adhesion could be blocked with the specific antibody, LM609, demonstrating that adhesion occurs through $\alpha_v\beta_3$ (**Fig. 1**). LM609 controls are routinely included in the assay. The cyclic RGD peptide, GpenGRGDSPCA, inhibited cell adhesion with an IC_{50} of 165 n*M* (**Fig. 2**), which is two to three orders of magnitude less potent than in the solid phase assay (data not shown).

To increase the assay throughput, it is run in a 96-well plate format, making it amenable to semiautomated or automated pipettors and washing devices. Furthermore, the assay was sufficiently tolerant of organic solvents such as DMSO or ethanol to allow assaying of small molecule compounds that are stored in nonaqueous stock solutions (**Fig. 1**).

2. Materials

1. Cell lines: Human melanoma cells, known to express $\alpha_v\beta_3$ ***(9)***, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and char-

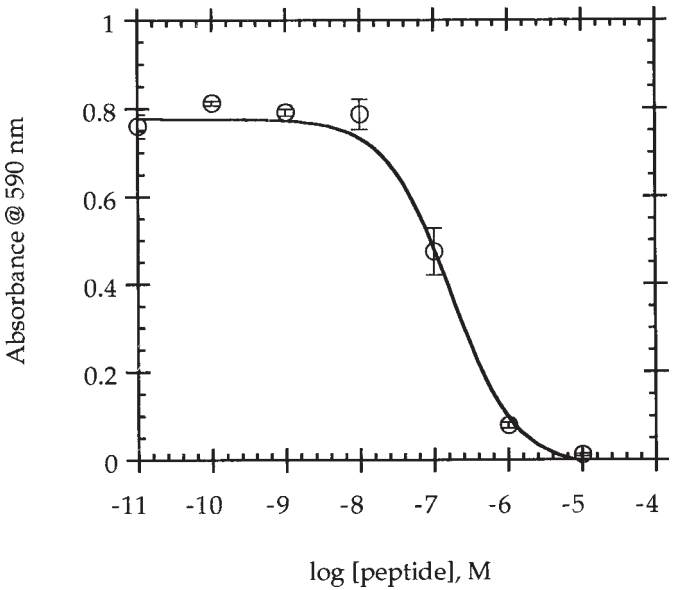

Fig. 2. Dose response of GpenGRGDSPCA in the cell adhesion assay. Increasing concentrations of the peptide, in the presence of 0.3% final DMSO, were added to wells with WM-115 cells, and the cells were allowed to incubate as described in **Subheading 3.** Data, with the backgrounds subtracted, represent the average of three points +/– standard error, and the curve is fit to a four-parameter logistic equation as described in **Subheading 4.**

acterized for expression of $\alpha_v\beta_3$ using fluorescence activated cell sorting (FACS) analysis. A highly expressing cell line, WM-115 (ATCC cat. no. CRL-1675), was chosen for development of the assay.

2. Cell growth medium: Earle's minimal essential medium (E-MEM), supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, and 2 m*M* glutamine. Store at 4°C for up to 1 mo.
3. Phosphate-buffered saline (PBS), without calcium or magnesium, is used for washing adherent cells and for dilutions of matrix proteins.
4. PBS supplemented with 2 m*M* EDTA and 1% (w/v) glucose is used to remove cells from flasks prior to assay.
5. Assay plates: 96-well EIA/RIA plates from Corning Costar (Corning, NY).
6. Fibrinogen, purchased from Calbiochem (La Jolla, CA), cat. no. 341578, is dissolved in Milli-Q or distilled water at a stock concentration of 15 mg/mL. Small aliquots (20–40 µL) are frozen and stored at –80°C. Aliquots are stable for at least 1 yr and should not be thawed and refrozen.
7. Bovine serum albumin (BSA), fatty acid-free, (Sigma cat. no. A-7030) stock solution is made 100 mg/mL water and stored at –20°C.
8. Assay medium: E-MEM, 20 m*M* HEPES at pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.25 m*M* $MnCl_2$, 0.1% BSA, sterile filtered, and stored at 4°C for up to 1 mo.

9. Antibody to $\alpha_v\beta_3$ integrin, LM609, is purchased from Chemicon (Temecula, CA), cat. no. MAB1976, as a 1 mg/mL solution and stored at 4°C. The antibody should *not* be frozen.
10. GPenGRGDSPCA, an RGD-containing peptide, purchased from Life Technologies (Gaithersburg, MD), cat. no. 12148-011, is dissolved in water at a concentration of 1 m*M* and stored in aliquots at –80°C. Aliquots are stable for at least 1 yr and should not be thawed and refrozen.
11. DMSO, cell-culture grade, is used for compound dilutions and vehicle controls.
12. Ethanol, 95%, is diluted, with water, into a 70% (v/v) working stock solution for fixation of cells and preparation of crystal violet staining solutions.
13. Crystal violet, purchased from Aldrich (Milwaukee, WI), cat. no. 22,928-8, is made as a stock solution of 15% (w/v) in 70% ethanol. The working stock is 0.2% in .75 *M* borate buffer at pH 6.0.
14. Sodium docdecyl sulphate (SDS) stock solution of 1% (w/v) in water is used for solubilization of crystal violet from stained nuclei.

3. Methods

3.1. Culture Conditions for WM-115 Melanoma Cells

1. WM-115 cells are cultured in growth medium at 37°C in a humidified atmosphere containing 5% CO_2 and are fed fresh medium every 2–3 d.
2. Cells are subcultured at a ratio of 1:6 every 5–6 d in Costar T-225 flasks. Confluent flasks contain between 5–10 × 10^6 cells per flask. Cells were passaged over 4 mo (25–30 passages) with no detectable loss in assay performance.

3.2. Preparation of Fibrinogen-Coated Plates

1. Costar 96-well EIA/RIA plates are coated, per well, with 0.1 mL × 5 μg/mL fibrinogen or 10 mg/mL BSA in PBS overnight at 4°C (*see* **Note 1**).
2. Aspirate plates and block with 0.15 mL/well × 10 mg/mL BSA in PBS, 1 h at 37°C. Wash plates 3× in PBS just prior to addition of treatments (**Subheading 3.4.**, **step 3**).

3.3. Harvesting of Cells and Preincubation with Antibody Controls

1. Confluent cells are washed twice in PBS and incubated in 2 m*M* EDTA/1% glucose/PBS to detach the cells from the flask surface.
2. Cells are removed by shaking/slapping the flasks, transferring to centrifuge tubes, and centrifuging at 800*g* for 5 min at room temperature (*see* **Note 2**).
3. The cell pellet is washed twice in assay medium, resuspended in assay medium, and counted in a hemacytometer. The final concentration of cells is adjusted to 330,000 cells/mL. The cells are held in an ice bucket and mechanically rotated to prevent cell settling and/or clumping.
4. Antibody LM609, or an IgG1 control, is added to a small volume of cells (0.3 mL for triplicate measurements on each 96-well assay plate) at a concentration of 8–25 μg/mL, and the cells are held on ice with the untreated cells for 1 h (*see* **Note 3**).

3.4. Dilution of Compounds and Addition to Plates

1. Peptide standards and compounds are made up at 10× concentrations and are added to plate wells in a volume of 10 μL/well. WM-115 cells can tolerate a maximum of 0.3% (v/v) DMSO in the assay, so the 10X stock solutions may contain a maximum concentration of 3% DMSO. Vehicle controls (10 μL × 3% DMSO) are included in several wells on each 96-well plate for both control cell adhesion and the LM609-treated cells.
2. Compound dilutions are made either manually, in polypropylene 1.7-mL microfuge tubes, or in round-bottomed 96-well plates, using an automated pipettor.
3. Washed fibrinogen plates (from **Subheading 3.2.**, **step 2**) are tapped dry on paper towels and placed on a bed of ice for the addition of peptides/compounds (10 μL/well) and, later, cells (90 μL/well).

3.5. Assay Incubation and Termination

1. Detached cells, 0.09 mL × 330,000/mL, are added to fibrinogen-coated plates containing test compounds, to attain a final assay volume of 0.1 mL/well, or approximately 30,000 cells/well. The cells are added using a multichannel pipettor, and the cells and treatments are pipetted up and down three times, to ensure adequate mixing and dispersal of cells.
2. Completed plates are transferred to a shallow water bath set at 30°C (*see* **Note 4**). The incubation is timed for 30 min. Multiple plate assays are handled in a staggered fashion.
3. Plates are removed from the water bath and set in an automatic plate washer (Bio-Tek EL404) and washed 3 × 0.2 mL PBS (*see* **Notes 5** and **6**).

3.6. Cell Fixation, Staining, and Detection

1. Washed plates are fixed in 0.1 mL/well 70% ethanol for 30–60 min at room temperature (*see* **Note 7**).
2. Cells are stained in 0.1 mL/well 0.2% (w/v) crystal violet in .75 *M* borate at pH 6.0 buffer for 30 min ***(10)***.
3. Plates are washed three times with 0.2 mL/well deionized water, and the remaining bound dye is solubilized in 0.1 mL 1% SDS for 1 h. Absorbance is read at 590 nm in an optical plate reader and analyzed using computer software (*see* **Notes 8** and **9**).

4. Notes

1. $\alpha_v\beta_3$ integrin binds to a number of different substrates including: vitronectin, fibrinogen, von Willebrand factor, osteopontin, and fibronectin ***(2)***. Fibrinogen is used as the fixed substrate, because other integrins expressed in WM-115 cells do not bind this protein. BSA-coated wells are included to measure the amount of nonspecific adhesion of cells to the plates.
2. Although the adhesion assay can be performed on the benchtop under nonsterile conditions, WM-115 cells are considered to be a biohazard, and unfixed cells, washes, reservoirs, and pipet tips should be disposed of accordingly.

3. Assay conditions were optimized to achieve cell adhesion that was dependent upon $\alpha_v\beta_3$ (i.e., adhesion was inhibitable by the specific antibody LM609). Inhibition by the antibody required a 1 h preincubation with the cells on ice. LM609 inhibition of cell adhesion ranged between 80 and 100% (84% inhibition in **Fig. 1**), using our conditions, and could be reversed by incubating at 30°C for longer periods of time or with increasing levels of matrix protein coating the wells. We also found that decreasing the concentration of $MnCl_2$ from 1 to .25 m*M* increased the assay sensitivity to LM609. LM609 controls should be run with every assay and, with the exception of the preincubation step, under identical conditions as the peptide controls or compound treatments (i.e., containing 0.3% DMSO).
4. Assay incubations are performed in a shallow water bath, as opposed to a humidified incubator, to achieve more efficient temperature equilibration over the relatively short incubation time.
5. During the assay, and particularly during the washing steps, the plate wells can be visually monitored by microscope to get an idea of the real-time assay performance and whether certain steps need modification (e.g., more or fewer washes, different incubation times for subsequent staggered plates, and so on).
6. Automated plate washers are useful for their speed and convenience in the handling of multiple assay plates. However, certain precautions should be noted: particularly with the use of Ca/Mg-free PBS as the wash solution, the soak times should be minimized, or loss of cell adhesion will begin to occur in the absence of divalent cations. In addition, the pressure and vacuum settings on the plate washer must be set empirically so that the wash conditions are not too harsh. Because we adjusted our assay conditions to be relatively sensitive (i.e., easily inhibitable), adherent cells were usually found to be "pushed" to the outer periphery of the well by the force of even a gentle wash. One should also verify that the plate washer gives even performance across the entire plate.
7. Other fixation and staining methods have been tested. For example, cells can be fixed in 1–2% formaldehyde or methanol, but these chemicals have special disposal requirements. Cell nuclei have also been stained with the fluorescent dye Hoechst 33258 (data not shown); however, this technique requires the use of a fluorescent plate reader ***(11)***.
8. Typical absorbance signals from crystal violet staining range from 0.6 to 0.8 AU, with nonspecific background readings of around 0.1 AU. This background signal represents the level of crystal violet staining to wells coated with BSA protein, to which WM-115 cells do not adhere. A saturating concentration of the GpenGRGDSPCA peptide (1 μ*M*) was able to inhibit cell attachment completely, also producing a background signal of around 0.1 AU. As shown in **Fig. 1**, the assay was suitable for measuring the effect of small molecule compounds, where there was a final concentration of 0.3% DMSO.
9. Data analysis and IC_{50} estimations were performed by curve-fitting the dose response data using a four parameter logistic fit in a software package such as Kaleidagraph (Synergy Software, Reading, PA). The fit equation was:

$$y = m1+([m2 - m1]/[1+(10^{(x - m3)})])$$

where x = the log [M] of the treatment concentration, y = the absorbance signal at 590 nm, and the estimated constants are *m1* = basal signal (nonspecific binding), *m2* = maximal signal (control cell adhesion), and *m3* = log [M] of the IC_{50} value.

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Integrins in Cell Migration

Klaus von der Mark, Stephan Schöber, and Simon L. Goodman

1. Introduction

Cell-migration events play a key role in embryonic development and during physiological and pathological tissue reorganization, for example in wound healing, angiogenesis, inflammation, and tumor metastasis. In embryonic development cells proliferate from germ layers and early organ anlagen such as neural folds or somites and migrate to distinct targets along defined tracks to invade other organs and to build up new tissues. In the adult organism, cells migrate to repair tissue defects, to provide new microvasculature, to support immune defense in inflammatory regions, and to metastasize as a result of malign transformation ***(1,2)***. The precision of cell migration, the mechanism of locomotion, the control of time, speed, direction, and target of the migrating cells has been receiving increasing interest in cell biology and molecular medicine ***(3,4)***.

One of the major tissue-specific regulators of cell migration is the extracellular matrix (ECM) which supports locomotion along defined tracks and elicits specific receptor-mediated transmembrane signals controlling cell proliferation, cytoskeleton, cell shape, gene expression, or apoptosis (for reviews, *see* **refs.** ***1,3,5,6***). Although the role of the ECM in the control of cell migration was recognized some time ago ***(7–10)***, it was the discovery of the integrins as a dominant family of matrix receptors that provided new insight into the mechanism of ECM-controlled cell migration events such as actin polymerization, formation of focal contacts, and expression of protease genes (for reviews, *see* **refs.** ***1,11–14***).

A prerequisite for the discovery of the integrins and elucidation of their specific interactions with ECM ligands was the laborious and comprehensive biochemical and genetic dissection of the complex ECM into the numerous

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individual matrix molecules that build tissue-specific matrices. Despite our knowledge of about 20 different collagen types so far ***(15)***, 13 laminin isoforms ***(16)***, numerous fibronectin-splice variants, and more than 30 glycoproteins and proteoglycans ***(17)***, there is still a discouraging deficit in our knowledge on the specific interactions of the 24 identified integrins with distinct matrix proteins and their isoforms, and the mechanism and physiological relevance of these interactions. Even for some of the best-studied components such as fibronectin, vitronectin, type I collagen, or laminin-1, complex and partially conflicting data exist on the ligand-specificity of the corresponding integrins. One reason for this is that specificity and avidity of integrin-ligand interactions depends not only on the experimental conditions, but also on the cellular origin and the type of biological response analyzed ***(11)***. For example, $\alpha_2\beta_1$ integrin isolated from Lox melanoma cells binds both to collagen and laminin, whereas $\alpha_2\beta_1$ isolated from platelets binds only collagen ***(18)***. The fibronectin receptor $\alpha_5\beta_1$ supports adhesion and focal contact formation on fibronectin in many cell types, but only certain tumor cells are stimulated by fibronectin via $\alpha_5\beta_1$ to produce collagenase ***(19)***. In vitro, the ligand affinity of integrins can be modified by the type of divalent cations (Ca^{2+}, Mg^{2+}, or Mn^{2+}) ***(20)***, by ligand-derived peptides (e.g., RGD-peptides; **ref.** ***21***), by phospholipids, by intracellular regulatory mechanisms, or by activating or inhibiting antibodies ***(14)***. Another complexity in understanding integrin-mediated cell migration is introduced by the co-ordinate effect of growth factors in integrin signaling. For example, both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ allow adhesion of melanoma cells to vitronectin, but only $\alpha_v\beta_3$ supports melanoma cell migration, whereas $\alpha_v\beta_5$ does this only in co-ordination with growth factor stimulation ***(22)***. In addition, species differences between cellular responses to ECM molecules are often neglected. Thus, skeletal myoblasts from mouse or rat muscle migrate and differentiate rapidly on laminin, but not on fibronectin ***(23,24)***, whereas avian myoblasts migrate and differentiate on fibronectin ***(25)***.

Certain integrins have been shown to mediate cell migration on ECM more than others. For example, $\alpha_v\beta_3$ ***(22)*** and $\alpha_3\beta_1$ ***(26)*** have been shown to be involved in melanoma cell migration on vitronectin ***(22)*** as well as on laminin, fibronectin, or collagen type IV ***(26)***. The laminin-binding integrin $\alpha_7\beta_1$ mediates locomotion of skeletal myoblasts on laminin ***(27,28)***, and confers motility on cells as varied as human embryonic kidney cells (HEK293), human melanoma cells 530 ***(29)***, or MCF7 breast carcinoma cells ***(30)***.

Thus, the role of extracellular matrix molecules in the migration of various cell types, the involvement of integrins and the mechanism and specificity of integrin-mediated transmembrane signaling leading to cell migration are major questions being addressed currently in the rapidly expanding field of cell migration. The answers to these questions depend to a large extent on the

experimental system used to study cell migration. Most importantly, care should be taken to compare results obtained from in vitro cell migration studies using cell lines on purified matrix components to the in vivo situation where cells migrate within a complex three-dimensional extracellular matrix, along or through basement membranes and over cell surfaces. Therefore, it is now essential to define and distinguish various terms used in this context before describing the experimental set up for cell migration.

Cell migration is the general term used to describe movement of a cell in an organ or a tissue culture system from site A to B. In this chapter we restrict ourselves to uniform *substrate-dependent* migration, also termed *haptokinesis* ***(31)***, excluding migration of bacteria driven by flagella or ameboid migration. The term *migration* summarizes a number of events involved in this process, including *adhesion* to the substrate and *locomotion*, which involves the extension of filopodia, *contraction* of the actin-myosin microfilament system and *retraction* of the cell body at the rear. Cell migration of tumor cells and inflammatory cells may include *invasion* of other tissues and organs, which requires the production of proteases, and finally termination of migration and adhesion in the target tissue.

In vivo, most cell migration is directed, caused by chemotactic gradients and extracellular matrix guidance (contact guidance) and haptotactic (adhesive) gradients ***(32)***; in vitro, directed migration up or down soluble gradients *(chemotaxis)* should be clearly distinguished from *nondirected* locomotion resulting from enhanced motility (chemokinesis). Migration along matrix surfaces can be directed (by contact guidance or haptotaxis) or nondirected (haptokinesis). In two-dimensional in vitro assays of cell migration, two parameters of locomotion can be measured: *speed* as a measure of cell motility, e.g., the total track length *l* ***(2,24)*** measured over a certain time interval (dl/dt), and *persistence length*, i.e., the net migration distance of a cell from the starting point after a certain time interval which is identical to the radius *(r)* in the wind-rose plot of migrating cells (*see* **Fig. 1**). The *persistence* of migration can be described by the quotient *r/l*. Thus, the persistence quotient, *p*, of cells can theoretically be at the most *1*, when cells do not change direction. Because most cells change their direction frequently, they show only a low persistence quotient, it is important to realize that cell motility determined by track analysis in two-dimensional systems can give refined dynamic information at the level of a small but complete population of individual cells, whilst the frequently used Boyden chamber or transwell assay only gives highly selective end-point information on the net migration of unknown members of a cell population after a time interval; its advantage over the track-analysis assay is that it is fast and relatively economical, and therefore well suited to measure the effect of chemotactic agents on cell migration.

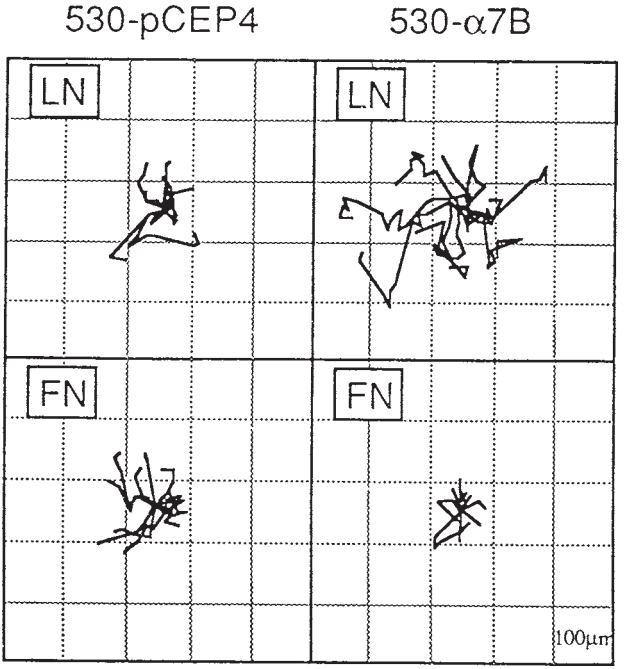

Fig. 1. Windrose plot of cell-migration tracks recorded by videomicroscopy of two different cell lines (mock-transfected and $\alpha_7\beta$-integrin-transfected 530 human melanoma cells) after 15 h of locomotion on laminin-1 (LN) and fibronectin (FN) substrates. The tracks of 10 cells were plotted with the migration start point in the middle of the graph. Despite the variations between the total track lengths of individual cells, the radius of the Windrose gives an estimate of the average net migration distance on different substrates. The figure shows that α_7 transfection confers enhanced motility on laminin-1, but not on fibonectin. Data from Echtermeyer et al. **ref. *29***); Reproduced with permission from American Society of Biochemistry and Molecular Biology.

Another important aspect in the experimental analysis of cell migration that is often neglected concerns the reciprocal interdependence of adhesion and migration. In exemplary studies Lauffenburger and colleagues ***(4,33)*** have documented that maximum speed of a cell is achieved at intermediate adhesion forces. Briefly, when adhesive forces are too high, cells cannot migrate, and when adhesion is too low, cells find no grip and also cannot move either. Adhesive forces that are in the range of 10^{-7} to 10^{-9} *N* depend on the affinity of integrin-matrix ligand binding, the number of integrin receptors per cells, and on the coating concentration of matrix proteins ***(33)***. That means, for a given cell type with a defined integrin expression rate and defined conditions for integrin-ligand binding, cells locomote fastest on intermediate substrate con-

centrations, which have to be determined for each cell type and each matrix component. They are usually in the range between 2–10 μg/mL coating solution. Here, another caveat should be noted: not all matrix proteins coat with the same efficiency, therefore, the coating efficiency for each matrix protein should be determined, in particular when the motility of a cell type on different matrix proteins is to be compared. Unfortunately, this fact is ignored in many published studies.

In conclusion, quantitative aspects will play an increasing role when analyzing and comparing specificity, affinity, and physiological role of integrin-mediated cell migration on defined ECM molecules. This applies not only to the coating concentration, but also to the actual affinity status of the integrins involved: This can be influenced by cations, lipids, and antibodies ***(11,14)*** and the expression level of integrins on the cell surface, in particular in the case of recombinantly expressed integrins, as well as by the actual cellular regulatory machinery. Cells expressing high levels of a certain integrin require lower substrate concentration for maximum motility than cells with low expression levels ***(33)***.

1.1. Methods to Analyze Cell Motility

A number of methods have been published to describe and analyze cell motility on matrix protein substrates in qualitative or quantitative manners:

1. Stripe assay ***(8,24,34)***: Cells are seeded at one end of a field consisting of 1-mm stripes coated with different matrix proteins and then allowed to migrate into these tracks. Under the phase-contrast microscope the relative persistence length of cell migration on different substrates can be compared, and the migration track of individual cells can be followed by videomicroscopy (*see* **Subheading 3.1.3.**).
2. Agarose drop assay ***(35)***: Cells are packed at high density in a small drop of agarose placed onto a matrix-protein-coated surface. Migratory properties of cells are evaluated by the ability to migrate out of the agarose drop.
3. Wound-healing assay ***(36,37)***: Cells are cultured to confluency on a matrix-coated dish. A wound is set by carefully scraping away a stripe of cells within the monolayer. Similar to the agarose drop assay, migratory properties of cells are evaluated by the ability of cells to migrate out of the wound edge into the cell-free area.
4. Colloidal-gold-track assay ***(31)***: Cells are seeded at low density in colloidal-gold-coated dishes. The colloidal gold can be coated with the matrix molecule of choice. Migrating cells clear tracks in the gold layer, leaving a permanent record of their movement.
5. Two-dimensional-track assay and videomicroscopy ***(2,24,29,33,39)***: Cells are seeded at low density in matrix protein-coated tissue-culture dishes or flasks. Single migrating cells are monitored by time-lapse video microscopy and migratory paths are measured.
6. Boyden chamber assay ***(26–30,40–43)***: Two compartments of the device are separated by a polycarbonate filter with a defined pore size. The filters are coated

with matrix proteins and cells are added to the upper compartment and those migrating through the filter are counted on the underside. By adding a chemoattractant to the lower compartment, chemotactic motility can be measured.

Here, we focus on determining cell motility by video microscopy and the Boyden chamber assay.

2. Materials

1. Cells: For each assay 2–5 × 10^4 freshly suspended cells in culture medium containing 10 m*M* HEPES are required.
2. Matrix proteins for coating: Solution of 10 μg/mL (2–25 μg/mL) human plasma fibronectin, vitronectin, laminin-1, type I collagen, (commercially available form Gibco-BRL, Gaithersburg, MD; Collaborative Research, Bedford, MA; Paesel & Lorei, Frankfurt, Germany) in sterile phosphate-buffered saline (PBS), or for type I collagen, 0.1% acetic acid.
3. Blocking solution: 2% bovine serum albumin (BSA, Sigma, St. Louis, MO, cat. no. A-7030) in PBS, sterile filtered and heat inactivated for 20 min at 60°C.
4. DAPI solution: 1 mg/mL of 4'-6 diamidine 2'-phenylindole-dihydrochloride (Boehringer, Mannheim, Germany, cat. no. 236276) in water.
5. Culture media: Dulbecco's modified Eagle's medium (DMEM) or any culture medium suitable for the cell type, containing 10 m*M* HEPES, if possible without serum.
6. Culture flasks: 25-cm^2 tissue-culture flasks for the two-dimensional-track assay; Transwell dishes (Costar, Cambridge, MA), or Boyden chambers; Nucleopore filters (Costar, cat. no. 110413 with 5-μm pore size or 150446 with 8-μm pore size), sterile.
7. Inverted microscope equipped with a heating facility, a video camera connected to a time-lapse videorecorder with a variable time-lapse range and a computer with a suitable software allowing cell tracking and data analysis.

3. Methods

3.1. Videomicroscopy

3.1.1. Coating with Matrix Proteins

Dilute stock solutions of matrix protein (native or denatured collagens, fibronectin, laminin, vitronectin) to 1–25 μg/mL in PBS (laminin, fibronectin vitronectin) or 0.01% acetic acid (fibrillar collagens). Coat tissue-culture flasks (e.g., Falcon, Los Angeles, CA, cat. no. 3015) for 1 h at 37°C, wash 2× with PBS and block for 1 h at 37°C with a 2% heat-treated bovine serum albumin (BSA) solution in PBS.

3.1.2. Seeding of Cells

Cells are trypsinized as usual and resuspended in medium containing 10 m*M* HEPES. Cells should be seeded at low density (2 × 10^3 cells/cm^2) to allow cell tracking of single cells ***(24,36)***. The flasks are equilibrated in a 5% CO_2 atmosphere at 37°C for 1–4 h to allow attachment of the cells. Then the cap is

screwed tightly, and the flask is placed under the heated microscope chamber of an inverted microscope, equipped with a videocamera.

3.1.3. Video Microscopy

Cells are viewed with a 10× phase-contrast lens. For each analysis, a field containing at least 10 single cells is chosen and filmed for 12 to 15 h, usually with a time lapse set at one picture every 2 min.

3.1.4. Data Analysis

The tracks of at least 10 cells per field documented in the time-lapse film are measured. Tracks are traced in real-time on a transparent digitizing tablet (SummaSketch II, Summagraphics, Seymour) placed over the screen. Individual tracks may be imported into a graphics program by hand (e.g., VideoMonitor, Apple Macintosh) or using a frame-grabber card and drawing lines indicating the steps the cells have taken. For graphic presentation, it is often useful to align the tracks of all cells starting with one end in the centre of a graph (Windrose plot, *see* **Fig. 1**) to illustrate the average persistence length. More exact analyses can be done with a suitable software, e.g., NIH Image for Apple Macintosh. Care should be taken with cells leaving the field, dividing cells, and cells touching each other. Data processing (determination of average and standard deviation of path length of different cells in the analysis, data presentation) can be accomplished with suitable programs such as MSExcel.

From this track analysis, the following parameters can be determined: The cell speed, which is the distance along the cell path divided through total migration time. The velocity of locomotion, which is the distance from the start point divided by time. Persistence length.

3.2. Boyden Chamber Assay

3.2.1. Coating of Filters

Transwell (Costar) multichamber plates with polycarbonate filters with a defined pore size of 5 or 8 μm. Alternatively: Boyden chambers ***(26,40)*** equipped with 5- or 8-μm pore Nucleopore filters.

3.2.2. Coating of Filters

For coating, filters are floated on a solution of matrix protein (for example 10 μg/mL) in PBS for 1 h at 37°C. After washing with PBS, both sides are blocked with 2% BSA (Sigma cat. no. A7030) in PBS overnight at 4°C.

3.2.3. Assembling the Boyden Chamber

The lower compartment is filled with degassed medium supplemented with a chemoattractant (e.g., FGF, fMLP, IGF-I, depending on the cell type) if

needed. The filter is placed air-bubble-free on top of the medium and the device is screwed together. The cells (10^4) are seeded into the upper compartment in a suitable volume (200 μL) of serum-free medium. The loaded chamber is placed at 37°C in CO_2 atmosphere.

3.2.3. Migration Assay

Cells are allowed to migrate through the pores for 6 h. Motile cells are those that migrate through the pores to the lower chamber and thus appear on the lower side of the filter. Cells remaining on the upper side are wiped away with a cotton stick. Cells on the lower side are fixed for 5 min in methanol (–20°C), washed three times with PBS, and the nuclei are stained with the fluorescent dye DAPI (1 μg/mL in PBS) for 30 min at room temperature. Filters are mounted with permaflour on slides, and nuclei are counted under the fluorescence microscope under UV excitation. Alternatively, cells appearing at the lower side of the filter may be stained with crystal violet or Giemsa stain.

3.2.5. Data Analysis and Interpretation

Using a suitable magnification, cells in randomly chosen fields are counted and data expressed as cells per field or cells per mm^2.

4. Notes

1. Cells: Using videorecorded track analysis, a minimum of 10^3–10^4 cells per assay/per well is sufficient. To obtain a cell suspension for inoculation of the matrix-coated dishes, as little trypsin as possible should be used to detach and dissociate the cells from the mass culture. For the Boyden chamber (Transwell) assay, inoculate 1–3 × 10^4 cells/well.

   Even when using cell lines, there is always a rather high degree of variability in adhesion and migration behavior of cells between experiments. Reasons for such variabilities may be inconsistencies in the cell trypsinization procedure and the culture time, passage number, and age of cells used for the migration assay. An even higher degree of variability is observed with primary cells, even when well controlled conditions are used. Therefore it is important to perform all necessary control experiments and comparative studies with the same cell populations.
2. Culture media: Cell migration assays may be carried out in any kind of culture media to which the cells to be tested are adapted. The culture media should be buffered with 10 m*M* HEPES or MOPS in addition to the HCO_3/CO_2 buffer system to allow culturing in a heated, non CO_2-regulated chamber mounted on the inverted microscope. If possible, serum or other nonspecified additives should be avoided during the migration assay.
3. Culture dishes: Time lapse videomicroscopy is done best in normal, small tissue-culture flasks, which removes the need for elaborate environmental chambers, or dishes placed in a heated, gassed, and humidified chamber under the inverted

microscope. Boyden chamber assays are done either in Transwell dishes (Costar), or in single, reusable Boyden chambers. Cell migration can also be filmed in three-dimensional collagen gels or fibrin gels ***(2)***, but here the microscopy is much more demanding owing to depth-of-focus problems.

4. Matrix proteins: Much of the confusion and discrepancies published on the specificity and activity of cells migrating on various matrix constituents result from the use of ill-defined, impure, or degraded matrix contents. Commercially available matrix proteins as well as those obtained from otherwise reputable research laboratories may vary considerably. Thus, it is important to control purity and integrity of every batch of collagen, fibronectin, tenascin, vitronectin, laminins, and so on by SDS-PAGE to avoid unnecessary conflicts and lack of reproducibility. Often it is advisable and certainly much less expensive to prepare one's own batch of collagen, fibronectin, or vitronectin according to established procedures. Commercially available matrix protein solutions are usually sterile. For a 1–10 h cell-migration assay, however, minor contaminations usually do not present any problem. If sterile coating solutions are required, sterilization of matrix proteins before coating can be achieved either by ultrafiltration through 0.45-μm filters, by antibiotics, or by ultracentrifugation. Ultrafiltration is often not possible for native collagens which tend to clog filter pores rapidly.
5. Coating with Matrix Proteins: For coating of tissue culture flasks, microwell dishes, or Nucleopore filters for the Boyden chamber system, matrix proteins should be dissolved at fairly high concentrations (1–2 mg/mL or higher) in a buffer best suited for solubilization and stored at 4°C or frozen at that concentration. For coating, matrix proteins should be diluted to concentrations between 1 and 25 μg/mL possibly with PBS or other nontoxic buffers. For quantitative comparison of the adhesive or migratory properties of matrix proteins, coating efficiencies should be controlled, as these can vary by several orders of magnitude. This can be done most conveniently by ELISA with specific antibodies. To prepare a standard curve, ELISA plates are coated with 50 μL each of serial dilutions of a defined stock solution (e.g., 50 μg/mL) of the matrix protein in 0.1 *M* acetic acid; the aliquots are allowed to dry down to approach 100% coating efficiency for the standard curve. (This method can also be used for cell-migration assays, but many matrix proteins, e.g., laminins, specifically lose activities when dried down on a cell-culture dish). Alternatively, to measure coating efficiency, a defined amount of the same, ^{125}I-labeled matrix protein can be added to the coating solution. After 1 h, the plate is washed as usual with PBS, and then the bound radioactivity is either removed with 3% SDS and counted by liquid scintillation counting or with a phosphorImager.

   Because cell motility is highest at intermediate matrix density (*see* **Subheading 1**), the optimal coating concentration should be determined by a cell-adhesion assay. Usually the coating concentration allowing 60–70% of the maximum adhesion efficiency within 1 h is a useful concentration to measure cell motility.

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15

Models for Studying Cellular Invasion of Basement Membranes

John T. Price and Erik W. Thompson

1. Introduction

1.1. Invasion Models and Their Relevance

Invasion of extracellular matrices is crucial to a number of physiological and pathophysiological states, including tumor cell metastasis, arthritis, embryo implantation, wound healing, and early development. To isolate invasion from the additional complexities of these scenarios a number of in vitro invasion assays have been developed over the years. Early studies employed intact tissues, like denuded amniotic membrane ***(1)*** or embryonic chick heart fragments ***(2)***, however recently, purified matrix components or complex matrix extracts have been used to provide more uniform and often more rapid analyses (for examples, see the following integrin studies). Of course, the more holistic view of invasion offered in the earlier assays is valuable and cannot be fully reproduced in these more rapid assays, but advantages of reproducibility among replicates, ease of preparation and analysis, and overall high throughput favor the newer assays. In this chapter, we will focus on providing detailed protocols for Matrigel-based assays (Matrigel = reconstituted basement membrane; reviewed in **ref.** ***(3)***). Matrigel is an extract from the transplantable Engelbreth-Holm-Swarm murine sarcoma that deposits a multilammelar basement membrane. Matrigel is available commercially (Becton Dickinson, Bedford, MA), and can be manipulated as a liquid at 4°C into a variety of different formats. Alternatively, cell culture inserts precoated with Matrigel can be purchased for even greater simplicity. We will provide detailed coverage here for the Boyden chamber chemoinvasion assay ***(15)***. As a compliment to the Boyden chamber analysis, the Matrigel outgrowth assay can be used and

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is also described in reasonable detail in Bae et al. ***(4)***. It should be noted that the principles detailed for these assays can be applied to various systems, and the emphasis here is on choosing the appropriate extracellular matrix and subsequent titration of each system in terms of amount of Matrigel or alternative extracellular matrix (ECM), and/or time of assay. These Matrigel-based assays are relatively short term, and in relatively low volumes, and thus can be adapted easily for the use of blocking antibodies, transient transfections, limited supplies of compounds, and even relatively small numbers of in vitro selected cells or those isolated from tissues. Indeed, many studies have employed such strategies to determine the role of particular integrins in the invasion process. For example, Seftor et al. have employed the Matrigel-based membrane invasion culture system (MICS) assay system to demonstrate an involvement of the $\alpha_v\beta_3$ ***(5)*** and $\alpha_5\beta_1$ ***(6)*** integrins for in vitro invasiveness using blocking antibodies in melanoma cells, Newton et al. have implicated the $\alpha_5\beta_1$ integrin in breast carcinoma cell invasion in the Boyden chamber chemoinvasion assay ***(7)***, and Chao et al. ***(8)*** documented increased invasiveness after transfection of $\alpha_6\beta_4$ into colon carcinoma cells. Also, synthetic integrin antagonists, such as derivatives of the integrin-binding RGD motif, have been shown to inhibit invasiveness in these assays ***(7,9)***. Whereas these studies emphasize a positive relationship between expression of specific integrins and invasiveness, others show an inverse correlation between the expression of other integrins and invasiveness. Specifically, Qian et al. ***(10)*** and Zutter et al. ***(11)*** showed that re-expression of the $\alpha_4\beta_1$ and $\alpha_2\beta_1$ integrins inhibited the invasiveness and overall malignancy of melanoma and breast cancer cells, respectively.

1.2. Invasion: An Integral Process

Invasion itself is a multistep process ***(12)***, and can de delineated into attachment to the extracellular matrix, degradation of the structural components, and migration through the extracellular matrix. These individual components can also be examined in isolation, as seen in other chapters in this volume. The importance of examining the overall invasion process, especially with respect to integrin involvement can be reflected in our growing awareness of the complex profile of signaling responses from integrins. Responses seen in isolation may be negated by additional effects on other components of the invasion assay. Ultimately, the more holistic assays mentioned above, or even studies in vivo are required to confirm the importance of any particular integrin for invasion and/or metastasis. The Boyden chamber chemoinvasion assay also lends itself easily to the use of other extracellular matrices, and the choice should be geared towards the study system. For example, fibrosarcoma cell invasion assays may better be done with an interstitial collagen gel barrier, and indeed even carcinoma cell invasion of interstitial collagen gels is meaningful

and potentially different in terms of integrin usage and protease involvement than the basement membrane invasion analysis.

2. Materials

1. Cells to be tested (*see* **Note 5** for appropriate controls).
2. Fibroblasts (if fibroblast-conditioned medium will be employed as chemoattractant, *see* **Note 1**).
3. Unsupplemented and fetal calf serum (FCS)-supplemented culture medium appropriate to your cells.
4. Bovine serum albumin (BSA): cell-culture grade, endotoxin-free. Aliquots of a 20% stock solution allows for uniform supplementation of each solution in each assay and uniformity over many assays.
5. Individual blind well modified Boyden chambers or equivalent (*see* **Note 2**).
6. PVP-free, 13-mm diameter, 8- or 12-μm polycarbonate filters or equivalent (*see* **Note 2**). It is important to order sufficient filters from the same lot number for the entire study, for uniformity.
7. Ballpoint pen with ink not dissolved by the methanol fixative for numbering filters (not needed for multiwell filters or disposable inserts).
8. Bacto- or cell-culture dishes for numbering and coating filters (not needed for multiwell filters or disposable inserts).
9. 150 mm bacto- or cell-culture plates containing paraffin wax (approx 0.5 cm deep, usually available from the pathology department). These are used to pin the filters for staining.
10. 25-gage needles or smaller to pin the filters
11. Diff-Quik staining dyes, or equivalent (*see* **Note 3**).
12. Glass microscope slides for mounting filters. Note that if the rectangular multiwell filters are used, large format glass slides are recommended.
13. Mounting medium: we use Permamount (Fisher, Philadelphia, PA).
14. Microscope and hand-held counter for manual counting, preferably with gridded eyepiece or equivalent.
15. Matrigel (Becton Dickinson) or extracellular matrix of choice (*see* **Note 4**). Always thaw carefully since it will gel once it warms up, and keep Matrigel on ice while you work with it. It should be stored at –20°C, and can be repeatedly thawed but aliquotting will prolong its working life.
16. Curved nose, fine forceps, approx 2.5 in long, for filter handling.
17. Invasion medium: The medium you usually grow your cells in supplemented with 0.1% BSA and sterile filtered.

3. Assay Methods: Boyden Chamber Chemoinvasion Assay

*3.1. Assay Preparation Overview (*see Note 5*)*

A convenient schedule, once one is experienced, is to coat the filters with Matrigel, and prepare the cells while they are drying. They should be ready around the same time to be combined into the assay and incubated. This proto-

col is written in this way, but those performing the assay for the first time may prefer to prepare the filters one day in advance, or otherwise separate the assay preparation for simplicity, and recombine them once they feel comfortable. The assay detailed below is the complete preparation for the reusable single blind well Boyden chambers, and should be adapted for other assay vessels.

*3.2. Preparing the Chemoattractant (***see Note 1***)*

3.2.1. Fibroblast-Conditioned Medium

3.2.1.1. Culture Fibroblasts

If fibroblast-conditioned medium (Fb-CM) is to be used, this is prepared from near confluent monolayers of NIH-3T3 cells, MRC-5 human embryonic lung fibroblasts, or primary fibroblasts. It may be better to be species-conservative with the choice of fibroblasts, but this has not been clearly established.

3.2.1.2. Incubation for Conditioning Media

The near confluent monolayers should be rinsed three times with either Dulbecco's Ca^{2+}/Mg^{2+}-containing PBS (if your cells don't lift off the dish) or unsupplemented medium, and then incubated for 24 h with unsupplemented medium containing 50 μg/mL ascorbic acid (prepared fresh daily from powder, protect from light), to promote collagen secretion and deposition. The medium again should be the same one you plan to use for your assays.

3.2.1.3. Harvesting Fb-CM

After 24 h, the Fb-CM should be harvested under sterile conditions, and centrifuged in the bench top centrifuge at full speed for 20 min at 4°C to remove debris. The clarified supernatant should be decanted to fresh tubes and stored frozen. Typically, we would generate a number of batches like this, pool them, and store them in frozen aliquots, as this would allow us to use a uniform batch for longer. Repeated freezing and thawing should be avoided, for uniformity, and the Fb-CM should be supplemented with the same BSA at 0.1% as is used in the invasion medium.

3.2.1.4. Reuse of Fibroblasts

The fibroblasts can be reused for subsequent generation of Fb-CM, but should be passaged and expanded at least once in FCS-containing medium between each batch.

3.2.2. Fetal Calf Serum

If FCS will be used, it should simply be a uniform batch to what the cells are growing in, and again should be aliquotted and not refrozen and rethawed for

uniformity among a series of assays. As with Fb-CM, the FCS/medium should be supplemented with 0.1% BSA prior to the assay.

*3.3. Dilution of Matrigel (***see Note 4***)*

Matrigel is supplied frozen at approx 10 mg/mL. It should be thawed on ice, mixed well, and aliquotted for storage at –70°C. Sufficient Matrigel for one assay should be diluted by adding into the appropriate volume of sterile cold water, and mixed well by pipetting. This should then be kept on ice. We use a clear polystyrene 10 × 75-mm culture tube so that dissolution of the Matrigel stream from the pipet can be visualized. We have occasionally found that the Matrigel becomes resistant to dissolution with aging and repeated thawing/freezing. As discussed in **Note 4**, Matrigel should be diluted between approx 1:10 (50 μg/50 μL/13-mm filter) and 1:25 (20 μg/50 μL/13-mm filter). It is important to make extra, as one may often need to redo a filter or two during coating, and you want to be sure you have sufficient Matrigel from the same dilution. It is possible to predilute Matrigel and store that as frozen aliquots, although we tend not to do this so as to retain the flexibility of dilution ratio for different experiments.

*3.4. Coating Filters (***see Note 4***)*

3.4.1. Place Filters in Dish

The clear polycarbonate filters have a shiny side and a dull side, and although they usually come packaged all in the same orientation, this is not always the case, so one has to check this as each filter is removed from the package. Filters are placed in the lid or bottom of a plastic Petri dish or culture dish shiny side up, the theory being that the "duller" side, placed down, will be more amenable to the thin coating of Matrigel that comes through the pores.

3.4.2. Number Filters

The filters must then be numbered with a methanol-fast, ball-point ink in the perimeter outside the inside diameter of the chamber, and each number should be followed by a "dash" so that it is always easy to tell whether the filter is upside down or not (e.g., 1-, 2-, 3-, and so on).

3.4.3. Coat Filters with Matrigel

Filters can be coated in advance and stored after drying at 4°C, but ideally they should be coated fresh before preparing the cells as described in **Subheading 3.1.**, above. We routinely coat the filters on the bench top for the shorter assays (6 h or less), but would coat the filters under the laminar flow hood for longer assays. Each 13-mm filter is coated with 50 μL of the Matrigel solution, and the larger filters should be scaled up accordingly. There are a

number of ways to do this, but the goal is to end up with a uniform layer of Matrigel which remains on the top filter surface. For the 13 mm filters, our preference is to circumscribe the coating initially with a circle of Matrigel solution, not to the very edge, but approx 11 mm diameter, as the chamber inner diameter is approx 9 mm, and avoiding the edge avoids overflow. Another method is to dilute the same amount of Matrigel further to 100 μL rather than 50, and simply pipet this onto the center of the filter. This then provides a circle of Matrigel of sufficient diameter. With either method, it is important to discard any filter where the Matrigel solution flows through the filter such that the filter ends up swimming in a pool of Matrigel. This will not give a uniform coating, especially as you want the amount of Matrigel above the filter to be as uniform as possible. The uniformity of the coating can be examined occasionally by protein staining like amido black.

3.4.4. Dry the Coated Filters

Once coated, the filters are left in the Petri dish, and transferred to the laminar flow hood. The filters should dry in approx 1 h, after which they can be immediately used or stored covered at 4°C until use. We have used filters up to 1-wk-old, but prefer to use freshly prepared filters.

3.5. Preparation of the Cells

3.5.1. Harvesting of Cells

These cells should the passaged uniformly prior to the assay to ensure comparable logarithmic growth in each cell type to be compared. If a single cell type will be tested, it should also be in logarithmic growth phase at the time of harvest. Cells should be lifted with either EDTA or trypsin as per usual passaging. Usually we stop the trypsin with 10 mL of FCS-supplemented medium.

3.5.2. Washing into Invasion Medium

To best respond to the FCS chemotactic gradient, or to remove serum from the system if Fb-CM will be employed, the cells are washed free of serum. After stopping the trypsin, we rinse the cells twice with 10 mL of invasion medium with standard resuspension and centrifugation. In some protocols, the trypsin is stopped with the invasion media, and less rinses are needed, but the danger of over-trypsinization is greater. Also, less rinses are necessary if the cells are harvested with EDTA.

3.5.3. Counting and Verifying Cell Viability

During the second wash, the cells are stained with trypan blue and counted, and then resuspended in invasion medium at 1×10^6 viable cells/mL. Only populations with greater than 95% viability should be used. These cells are

now ready for assay, and should be stored on ice if a protracted period (> 30 min) will elapse before you use them, and then allowed to equilibrate to room temperature before use.

3.5.4. Treatment of Cells

As described above, some assays will require prior and/or continued treatment of the cells with your agents of choice, and this must be factored into the protocol. In the case of neutralizing antibodies for integrins, it is appropriate to pretreat the cells after washing, counting, and viability checking with the antibody for up to 1 h at 37°C. Agents that may regulate the integrin profile of a cell may need to be introduced into the cultures prior to the assay, and may or may not need to be added to the cells during the assay period, depending on the cells, the agent, and the time of assay. Should one need to add the agent to the cells, again this should be done in a balanced way such that the BSA concentration and cell number remain constant and consistent with the other parts of the assay. As described below (**Subheading 3.6.7.** and **Subheading 3.6.7.**), we often add agents only in the top chamber after adding the cells, but this is only appropriate for some agents and you have to adjust this according to your knowledge of the projected mechanism of action of your agent.

*3.6. Assembling the Chamber (***see Note 2***)*

3.6.1. Preparation of Components

By now the cells are harvested, checked for viability, counted, and resuspended at 1×10^6/mL, and the filters have dried in the laminar flow hood. The chambers will have been cleaned and dried. You will need on hand the chemoattractant (**Subheading 3.2.** and **Note 1**), and it should be thawed on the bench to room temperature. The invasion medium and cells should also be at room temperature, so that there is no temperature differential in the chamber, as this can lead to bubbles below the filter, especially if the chemoattractant is colder.

3.6.2. Chambers

The chambers are laid out in racks, again on the bench top for shorter assays (6 h or less) or in the hood for longer assays. Neuroprobe (Gaithersburg, MD) supplies precut racks which hold 24 individual Boyden chambers, and these can be conveniently labeled with paper tape to assist in loading the assay.

3.6.3. Add Chemoattractant

It is important that the chambers are thoroughly dry before adding the chemoattractant into the lower chamber, because surface tension is required to keep the chemoattractant welled up in the lower chamber such that bubbles can be avoided when adding the filter. Remember the chemoattractant should be

supplemented with BSA (0.1% final) to make it consistent with the upper chamber. Also, in some cases in which agents are being tested, one may wish or need to include the agent in the lower chamber also, and this should be done prior to adding the chemoattractant to the lower chamber. Usually it would be done in Eppendorfs, and care must be taken to standardize the dilution of chemoattractant in each well with appropriate controls. For 1-mL chambers, use 215 μL of chemoattractant, and use 36 μL for the smaller 0.2-mL chambers. This should look like just a little too much for each well, but as mentioned just above, should sit like a cherry in the blind well, and will help avoid bubbles in the next step. As with the Matrigel coating of filters, a small amount of additional chemoattractant should be kept on hand in case some chambers need to be redone. If surface tension is lost, you must prepare a fresh chamber in its place.

3.6.4. Rehydrate Filters

Matrigel-coated filters need to be rehydrated before removing them from the dish, and this is to protect the small amount of Matrigel that has diffused through the pores to the lower filter surface. For the cell-migration assay we do not routinely rehydrate filters coated only with small amounts of collagen or Matrigel. We rehydrate the invasion assay filters with the invasion media. Rehydration is affected with approx 0.2 mL of the invasion media per filter. So as not to have all your filters swimming in the dish, it is helpful to loosen each filter separately and then remove it.

3.6.5. Load Filters into Chambers

Each filter is then lifted from the dish with curved nose, small forceps, and placed *right side-up* with a sideways sweeping motion on top of the chemoattractant into the lower chamber. The motion is important to avoid bubbles. One has to pull the leading filter edge into contact with the chemoattractant on one side of the well, and drag it across the chemoattractant to the other side in a smooth sweeping motion. Then the filter is released and should seat itself flush onto the flat surface cut into the chamber, and no bubbles should be seen beneath the filter. If bubbles are seen, the filter should be removed, and placed into a new chamber with new chemoattractant. Do not try to reuse that chamber, but you can still use the same filter. Once the filter has been loaded, the chamber top is screwed into the chamber bottom, and tightened to create a seal. Loosely tightened chambers often leak during the assay, and cannot be included in the analysis, so care is required to hand tighten maximally to avoid this. If for some reason you finish this step and the cells are not ready, cover the filter-loaded chambers with plastic wrap to protect from drying, but ideally the cells should be ready to add immediately.

3.6.6. Add Cells

The major concerns here are to avoid puncturing the filter with the pipetteman used to deliver the cells, and to ensure that a uniform number of cells are added to each chamber. Thus, the cells should be added gently to the side wall of the chamber, and the cell suspension should be thoroughly mixed between each chamber. We use 300,000 cells in the 1-mL chambers (0.3 mL), and 70,000 cells in the smaller chambers (0.07 mL). If additional agents are to be added to the chamber, we add them separately into the top chamber (*see* **Subheading 3.6.7.**). However, if no additional agents are to be added, for example if one is comparing the constitutive invasiveness of integrin-transfected clones, or cells treated differently in culture prior to the assay, the cells can be diluted further in the invasion medium so as to avoid the need for the additional step. In this case, one would dilute the cells to 0.3×10^6/0.8 mL (0.375×10^6/mL) or for the larger chambers, and 0.07×10^6/0.2 mL or 0.35×10^6 cells/mL for the smaller chamber, and add 0.8 and 0.2 mL, respectively, with the same precautions of mixing and filter avoidance as mentioned above.

3.6.7. Top-up Chambers and/or Add Additional Agents

The remainder of the assay volume provides the opportunity to add the agents that you may wish to test, e.g., anti-integrin antibodies or experimental therapeutics. These can be prediluted taking into consideration the overall volume of the cells and lower chamber. In some cases, it is important to add the agent to the lower chamber as well, and this should be done earlier, as described above. Larger chambers will be topped-up with 500 μL, and smaller chambers with 170 μL. Note that this exceeds the capacity of the chamber, and actually should cause a dome-shaped extrusion of medium above the bore of the chamber, but it should not spill sideways onto the chamber top. This is done to counteract the loss of medium that occurs during the incubation step.

3.6.8. Incubation of the Chambers

The rack(s) of chambers are then placed directly into the cell-culture incubator, and the time noted. Usually it is not necessary to cover the chambers, although some workers prefer to position lids from large culture dishes over the chambers to reduce evaporation, and effect better incubator hygiene. As described in **Note 5**, the timing of the assay varies to the application, but we typically use 6 h. Individual blind-well Boyden chambers offer the flexibility to remove triplicate wells at different time points, and/or employ different Matrigel amounts in the same experiment, to establish an assay kinetic.

3.6.9. Harvesting the Assay

At the appropriate time, the racks are removed to the bench for processing. Usually the medium of the top of each chamber is discarded by shaking it

directly into a waste beaker in a downward motion, but sometimes it is important to sample this for protein analysis, in which case an aliquot should be removed carefully to avoid disturbing the filter, clarified by centrifugation (usually in an Eppendorf tube), decanted, and processed accordingly. Once the upper chamber media is removed from all the chambers, each chamber is opened individually and its lid dropped into a beaker containing water for washing. The filter is removed carefully and pinned upside down (use the dashes after the numbers to ensure you have them upside down) into a wax plate containing a small amount of the fixative solution. It is convenient to pin each filter close to the number, and this allows you to check that you indeed have the filter upside down. This is important as we are not concerned with abrasion of cells from the upper filter surface (i.e., the cells that did not invade), but want to avoid abrasive removal of the cells from the lower surface before fixation (i.e., the ones that have invaded and we wish to count). Each chamber is processed in this way until each filter is pinned upside down into the wax dish, usually in rows. The fixation time is 15 s to 2 min, but longer periods do not adversely affect the result.

*3.6.10. Staining the Cells (*see **Note 3***)*

The fixative is decanted from the wax dish into a beaker, in case any filters cut loose, although this usually does not happen. Then perform staining. In our case, we follow the manufacturer instruction for Diff-Quik, which is 15 s to 2 min in the eosin solution, followed by 15 s to 2 min in the buffered thiazole. Each of these staining solutions can be reused, but we do not routinely reuse the fixative. After the last staining, the filters are rinsed with tap water until all the free blue dye is removed. Then each filter is removed with the forceps and carefully placed right side-up (again, use the dashes after the numbers to ensure you have them right side up) on microscope slides, usually in groups of three for each triplicate on a given slide. Each slide is labeled with the pertinent information. While still wet, or with remoistening, each filter is then wiped quite firmly with cotton swabs until all the cells from the upper filter surface are removed. Usually this will require two or three different swabs per filter to leave the filter clean, with only the stained cells on the lower surface, now trapped between the filter and the slide, remaining. The filters are then allowed to dry before being mounted with Permamount (Fisher), and cover slipped.

3.6.11. Quantitation

As described in the **Note 3**, a number of options exist for counting the cells that have invaded each filter. Suffice it to say that you should count either manually or mechanically at least five fields on each filter, and these should be randomly selected. Be aware that some assays may contain debris, and so image

analysis programs need to be constructed to deal with that on a size, shape, or optical density exclusion. Manual counting is laborious, but is often the only failsafe method. Crystal violet staining and colorimetric analysis is useful in motility assays, but the Matrigel provides a high background. Radiolabeled or fluorescently tagged cells have also been employed by some ***(1)***.

3.6.12. Washing the Chambers

Neuroprobe supplies instructions for the washing of chambers, and this can be more or less rigorous depending on the circumstances. Their recommendation is to use an enzymatic detergent (e.g., Tergozyme) for routine care of the chambers. If this is not available, a mild detergent should be substituted and if a sonic water bath is available, this should be used with the detergent. Extensive rinsing is required, first in tap water, and then deionized water (we usually employ five solid rinses in each) before leaving the chambers to air dry. In cases of urgent need of the chambers (they do arise), they can be dried faster in the laminar flow hood, or even with a hair dryer on cold settings. It is important to avoid heat, and sterilization of chambers for assays longer than 6 h or when one wishes to harvest the cells from the lower chamber (**Subheading 3.6.13.**), is effected by soaking the lower chambers in 1 *M* NaOH for 2–3 h at 67°C, and the tops in 0.1% chlorine bleach, for 2–3 h. Again thorough rinsing as described above is required. Once dry, the chambers and tops should be stored in covered containers. Avoid leaving them soaking in the sink in beakers where waste solutions and stains can be splashed in!

3.6.13. Harvesting Cells from the Lower Filter Surface

One useful adaptation of the individual Boyden chambers, which may be especially useful in integrin assays, is the ability to harvest cells from the lower filter surface for expansion in culture and analysis (e.g., integrin expression), reselection through multiple rounds in the Boyden chamber, or both. The larger-bore chambers should be used after sterilization (**Subheading 3.6.12.**), all steps should be performed under the laminar flow hood, and the chambers should be covered in the incubator and in transit to and from the hood. We have found glass immunohistochemistry chambers a useful vessel. Usually only a small number of chambers are required for selection (e.g., six), and a similar number may be run as usual to provide the associated result with respect to the number of cells invading at the time of harvest. One should take care to optimize the assay time, such that the cell population most enriched in the invasive traits will be obtained. Essentially the assay is performed as above for all steps through **Subheading 3.6.8.**, but after discarding the upper chamber medium in **Subheading 3.6.9.**, one should trypsinize the chamber such that the cells on the upper filter can be removed and the cells on the lower filter surface fall to

the bottom of the chamber. Standard trypsin solution (0.1 mL) is added to the chamber, and incubated for approx 15 min in the cell-culture incubator. The trypsin solution is then carefully removed and examined under the microscope where one should see many released cells, indicating the trypsin has been effective. If not, add an additional aliquot of trypsin, and continue to monitor for cells. Once you are confident that all the cells have been released from the upper filter surface, it is most likely that those from the lower filter surface have also been released, and you can proceed. Rinse the upper chamber thoroughly with FCS-containing medium to stop the trypsin in the lower chamber, and to remove all cells from the upper compartment. It is fortunate that these Boyden chambers fit snugly into the standard 50 Blue-Max Falcon tubes, and they can then be centrifuged in a standard cell culture centrifuge as you would spin your cells. The chambers are removed from the tubes under the hood, the tops are tossed, the filters very carefully removed, and the cell pellet from each chamber resuspended in growth medium and plated into an individual 96 well or chamber slide, depending on the application. Complete removal of the cells from the filter can be determined with staining as described above (**Subheading 3.6.10.**) for the conventional assay.

4. Assay Methods: Matrigel Outgrowth Assay

This is an additional assay which is essentially more qualitative, and relies on altered morphological responses of cells with different levels of invasiveness to a solid gel of Matrigel. Originally described by Albini for prostate carcinoma cell lines, it has been used subsequently for breast cancer cell lines (***13***; reviewed in Bae et al., **ref. *4***). The materials one needs are quite simple and are included already in **Subheading 2.**, Materials.

4.1. Culture, Treat, Harvest, Count, and Validate Cell Population for Viability

As described above for **Subheading 3.5.**

4.2. Embed Cells in Liquid Matrigel

The Matrigel must be kept on ice, and is used undiluted for this assay, which can get expensive. For this reason, we try to use the smallest vessel possible, and have had success with 150 μL of Matrigel in a 48-well chamber (96-well chambers are simply too small to allow proper viewing of the cells, and when sufficient Matrigel is available, we prefer to use 250 μL of Matrigel in a 24-well vessel). There are three formats, and we have found equivalent results with all three: The cells can be dispersed in the Matrigel layer (**Subheading 4.2.1.**), plated on top of the Matrigel layer (**Subheading 4.2.2.**), or sandwiched between two Matrigel layers (**Subheading 4.2.3.**).

4.2.1. Dispersed

First coat the well with either 100 μL (48 well) or 150 μL (24 well) of cold Matrigel, and allow to set in the incubator. This is the lower foundation layer, and serves to avoid contact of the cells with the plastic vessel surface, as many cells appear to thrive at this interface and it can give artifactual results. While the foundation layer is setting, aliquot 2×10^4 cells into sterile 4-mL (10 mm × 75-mm) culture tubes or equivalent, and centrifuge as usual for cells. Remove the supernatant, keeping in mind this pellet is effectively invisible. Resuspend the cell pellet with cold Matrigel, either 50 μL (48 well) or 100 μL (24 well), being cautious to avoid bubbles, and transfer the cell/Matrigel mixture onto the top of the now preset foundation layer, and place the culture vessel in the incubator. This should set quickly, and you should be able to see even dispersion of the cells in the upper layer. Once the Matrigel is well set, you should supplement with the standard amount of normal growth medium supplemented with FCS as is standard for your cells, although we have found that some cells will grow in the absence of serum under these conditions. These cultures should be fed as you would usually, and can be visualized and photographed with phase-contrast microscopy over 1–2 wk, although better contrast can be obtained with Hofmann optics if they are available. These plugs can be fixed and subjected to immunocytochemistry, or extracted with dispase to liberate the cells for biological and/or biochemical analyses. In breast cancer cell model systems ***(13)***, we have found a close association between metastatic capacity and stellate outgrowth rather than spheroidal patters, and similar patterns are seen with prostate carcinoma cell lines. We have not yet seen a colon-carcinoma-derived cell that did not adopt a spheroidal pattern. Our experience is that some mammary cell types enjoy this environment and form very stable spheroidal structures that persist for months if forgotten in the incubator!

4.2.2. Top

The foundation Matrigel layer is prepared as described above, and 1×10^4 cells are directly plated on top of the foundation layer, allowed to attach, and then supplemented with growth medium. These cultures are maintained and monitored as described for the dispersed cells (**Subheading 4.2.1.**). Endothelial cells form capillary-like tubes on top of Matrigel, and integrin involvement in angiogenesis can also be monitored in this type of assay, as well as the Boyden chamber assay for that matter.

4.2.3. Sandwich

Cells (1×10^4) are plated on top of the foundation layers as in **Subheading 4.2.2.**, but once they have attached, the medium they were plated in is removed, and the cells are covered with another layer of Matrigel equivalent to that used

in the dispersed assay (50 μL for 48 well, 100 μL for the 24 well). Cultures are monitored and maintained as above.

5. Notes

1. Chemoattractants: These assays are usually driven by chemotactic gradients. The chemoattractant is placed in the lower chamber only, and a diffusible concentration gradient is established relatively quickly. Also, the chemoattractant tends to bind to the lower filter surface, and thus sets up a haptotactic gradient on the filter, with more chemoattractant on the exposed lower filter surface than in the pores or upper filter surface, respectively. Although the chemotactic gradient probably neutralizes in a number of hours, the haptotactic gradient probably remains much longer, and thus longer assays are possible. Traditionally, these assays were driven by Fb-CM, usually from NIH-3T3 cells or MRC-5 human embryonic lung fibroblasts, or else primary human or mouse fibroblast cultures. Although the molecules in this complex mixture directly responsible for the chemoattraction have not been carefully resolved, it was always assumed that extracellular matrix molecules and fragments and soluble growth factors were most responsible.

   In recent years, we have used 10% FCS as the chemoattractant. This is readily available, and in most cases provides for potent general chemoattraction similar to that seen with Fb-CM. Again, this should be supplemented with the 0.1% BSA to correspond with the medium in the top chamber. In assays where more than one cell type will be compared, a single medium choice has to be made. As these are relatively short term assays, the medium may not be so critical.

   In some cases, purified growth factors (e.g., PDGF, EGF, NGFs, and so on) can be employed and although these individual factors usually do not usually drive as much migration of the cells, in certain cases are more relevant. Similarly, individual extracellular matrix proteins like fibronectin, collagen, laminin, vitronectin, or specific fragments and peptides thereof, also give lower levels of chemoattraction than that seen with FCS or Fb-CM, but could be more relevant to certain studies. For leukocyte invasion studies, the f-Met-Leu-Phe bacterially derived chemoattractant is most appropriate ***(14)***. Moreover, some cancer cells, and perhaps some normal cells like trophoblasts, show a high level of constitutive random motility (chemokinesis) and these cells may not need a chemoattractant. Although some cells (e.g., MDA-MB-231 HBC cells) do show high constitutive motility in the absence of chemoattractants, our experience has been that the invasion assay of most cell types benefits from the chemotactic gradient, and we routinely employ 10% FCS.
2. Boyden chambers/filters: There are a number of choices for the chamber. Historically, the assay was performed in reusable single, blind-well modified Boyden chambers, and also the reusable 24- or 48-well multiwell apparatus ***(13,15)***. These clear plastic chambers and apparatii are robust, long-lasting, and can be sterilized with bleach and NaOH, and are supplied by Neuroprobe. An institution with a good engineering shop may be able to have them constructed at a lower cost.

This protocol is written for the individual, blind-well Boyden chambers, of which two different sizes are available in terms of bore size, the larger 1-mL chamber, and the smaller 0.2-mL chamber. Conveniently, each uses the same filter size (13-mm diameter), but the number of cells and amount of chemoattractant and any agent one wishes to test is proportionately reduced in the smaller chamber, and this can be useful and economical at times. If possible, it is better to use the larger chambers, because one obtains a more robust result with the larger filter surface as more fields can be counted. Filter-coating procedures and chamber assembly procedures are otherwise identical. Neuroprobe and Poretics (Livermore, NY) sell filters for use in these reusable chambers. We typically use 8-μm pore for fibroblasts and sarcomatous cells, and 12-μm for epithelial cells and carcinoma cells although we have obtained useful results with each cell type on each filter pore size.

More recently available and quite popular are the disposable plastic inserts marketed by Becton-Dickinson (Bedford, MA; Bio-Coat Wells), Corning (Acton, MA; Transwells), Millipore (Bedford, MA; Millicell Inserts) and Nunc (Naperville, IL; Nunc TC inserts). These are inserts which fit into different sized culture wells, usually 24-well cell-culture dishes, which provide the assay vessel. The insert contains the porous filter that is either precoated or needs to be coated by the user. Any filter insert with appropriately sized pores (see paragraph above) can be coated with the extracellular matrix of choice. The choice of system depends on the scope of the work. For example, if you want to look at something quickly for Matrigel invasion, you may find the precoated inserts the most practical, or simply coat the raw insert (e.g., **ref. *10***) use 1.2 μg/mm^2 in the 6.5 mm^2 Transwell inserts). If you wish to examine invasion in many systems over many years, the reusable Boyden chambers and multiwell chambers are more economical, and offer greater flexibility in terms of the type and amount of coating.

3. Staining and quantitating the invaded cells: Essentially a hematoxylin and eosin (H&E) stain is performed routinely in our lab, and stained cells on the lower filter surface are either counted manually on the microscope or quantified by image analysis. Diff-Quik is formulated optimally for rapid processing in pathology labs, and works well but is expensive. It is eosin and buffered thiazole, with a green methanol fixative. Any formulation of H&E-like staining is probably suitable, depending on the method of quantification. If manually counting on the microscope, more flexibility exists and good cytoplasmic and nuclear definition is aimed for. Image analysis-assisted quantitation requires uniform nuclear staining, and nuclear size is often used to exclude debris and determine clumps, and so on, so cytoplasmic staining is less important so long as it does not interfere. Crystal violet can be used also for colorimetric quantification ***(16)***, although the background staining with Matrigel tends to be high, and it is better suited to migration assays employing less matrix on the filter. There are many other options for quantitation, and this depends on the equipment available, the specialization of the lab, and the number of assays one plans to do. It may be easy enough to devise an appropriate macro for an existing image analysis system hooked up to

a microscope, but for just a few assays, it is not practical to invest in a whole system. The insert assays in commercial disposable chambers, especially those employing more significant barriers and longer time points (e.g., Qian et al., **ref. *10*** employ 24 h), are quantified by counting the number of colonies of cells that arise in the lower chamber (a 24-well culture vessel) one wk after removal of the filter insert. Gehlsen et al. ***(1)***, when using the MICS assay system, radiolabeled the cells prior to assay and sampled from the upper and lower chamber at different time points. Similarly, fluorescent labeling of the cells can be performed and analyzed similarly in a fluorescent reader, and this lends itself easily to a higher throughput analysis in a 96-well format. The goal is to accurately, and with appropriate sampling, determine the number of cells which have traversed the Matrigel barrier, either onto the lower filter surface, or into the lower chamber, or both, and this should be adapted to your lab. We have supplied our basic protocol.

4. Matrigel coating: It is important to point out that each new assay system requires titration in terms of Matrigel concentration and/or time of assay. Typically between 25 and 50 μg Matrigel per 13-mm diameter filter provides a barrier, such that cells with similar motilities over thin layers of Matrigel (2–10 μg per filter ***[16]***) but different metastatic potentials, will show differential invasion with 25–50 μg/filter. This can be observed, for example, with the K1735 mouse melanoma series. Cells from different backgrounds will require shorter or longer periods to traverse this barrier. For example, MCF-7 human mammary carcinoma cell invasive responses to estrogenic compounds required a minimum of 9 h in assay, and were better examined after 16 or 24 h ***(17)***. However, the more motile and invasive human breast cancer cell lines, typified by MDA-MB-231 or MDA-MB-435 cells, require significantly less time to assay, and studies with these cell lines, i.e., for inhibitors, can be completed in 4–6 h. Prostate carcinoma cell lines offer the same spectrum of activities as seen with the breast cancer cell lines, again with better differentiated lines like LNCaP requiring longer times than the more motile, less-differentiated PC-3, and TSU-Pr1 cells, and intermediate activity with the DU-145 cells. Colon carcinoma cell lines in general are poorly motile, and with the exception of DLD-2 cells, require even longer times to traverse a 25-μg coated filter.

   In general, longer times with a more stringent barrier is desirable. The MICS assay system employed by Mary Hendrix and coworkers (uses a substantial coating of Matrigel and analyzes cell penetration after 72 h). These assay chambers approximate those commercialized by Corning (Transwells), and longer assay times are appropriate with these systems. The individual Boyden chambers offer flexibility in coating and assay time that allow for more rigorous analysis, although assay times greater than 24 h should be regarded with caution, and difficulties with contamination can plague assays longer than 6–9 h.

5. Planning your assay: The limiting factors are the number of cells you have, the number of chambers and/or combinations you want, the size of assay you are comfortable with, and the mode of quantitation you will use. For the single-blind well Boyden chamber assays, initially one should not use more than 12 or 24 wells

in pilot studies to titrate the system. Larger assays, up to 72 chambers, can be performed once the operator is experienced. The Neuroprobe multiwell chambers come in 24- or 48-well configurations, and the transwells come in 24-well plate configurations. Neuroprobe has recently developed a 96-well format to be precoated with Matrigel, and to be analyzed in a fluorescent plate reader. It is crucial to use triplicate wells for each point, because of the inherent variation in these assays. Additionally, known controls should be incorporated into your system. For example, to determine the role of specific integrins in invasiveness of a human breast cancer (HBC) cell line, comparison to published standards like the MCF-7 and MDA-MB-231 should be made. Observing the reported differential between MCF-7 and MDA-MB-231 gives an indication that the assay system is working, and also provides a confident reference point for whatever HBC cell line you use. Optimization of the system for your own lab conditions should be performed. Thus varying either the Matrigel concentration, the assay time or both with the known controls will provide ideal conditions for your assay. To again use the example of the new HBC cell line, the first step would be to titrate MCF-7 and MDA-MB-231 cells in your lab and find the best differential. Using small assays for manageability (24 wells, 12 per cell line, 4 points in triplicate), you may wish to use 25, 30, 35, 40 μg of Matrigel per filter, and assay for 6 h. Depending on what you expected in the new cell line, you may want to emphasize the less invasive aspect with a longer assay and/or less Matrigel, or the more invasive with a shorter time point and/or more Matrigel. The combination giving the biggest differential should work best for all types of assay in the model system.

Another important distinction, which is incorporated into the detailed protocol below where possible, is the different type of assay. Typically one might either compare different cell clones, strains, lines, and so on, or examine the effects of some agent(s) on the invasive behavior of one or more cell types. Technically, the latter are more complicated assays, and should not be attempted without establishing the baseline as mentioned in the preceding paragraph. In examining the effect of additional agents, one has to consider the nature of the agent in deciding when and where to add the agent. Some agents will require pretreatment of the cells in the culture period up to a week prior to assay, and continued exposure in the assay chamber (e.g., effects of estrogen ***[18]***). In other cases, one should preincubate the cells with anti-integrin antibodies up to 1 h prior to adding them to the assay, and then continue the presence of these agents of the assays. Some agents may need to be included in the lower as well as upper chamber, whereas it is sufficient and much simpler to add others, particularly small molecules that will diffuse quickly and equilibrate in the chamber, only to the upper chamber. Where possible, allowances are made in the detailed protocols below for these options, but the overall consideration, as indicated here, should be a guide for the user.

In using the single-blind well chambers, we have often taken advantage of being able to simultaneously perform a migration assay with the invasion assay.

This allows us to interpret the contribution of motility to overall invasiveness, and is especially useful in examining different antagonists. For example, one may expect antagonists of certain integrins to affect invasiveness by reducing motility, whereas others may interrupt signals that enhance matrix degradation machinery, and would not affect motility. In general, one may wish to show that a nonmotility-targeted, anti-invasive agent would not affect the motility, but would inhibit the invasiveness. Motility assays are described in detail in Chapter 14, but in our case we simply substitute a thin layer of Matrigel (10 μg/13-mm filter) or type IV collagen (5 μg/13-mm filter) for the Matrigel layer on top of the filter. Everything else is identical. This flexibility is not offered in the multichamber apparatus, but can be incorporated with the culture well inserts (*see* **Note 5**).

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16

Methods for Studying Anoikis

Steven M. Frisch

1. Introduction

We have found that integrins control apoptosis in epithelial cells, and have named this general phenomenon "anoikis," the ancient Greek word meaning "homelessness" ***(1)***. Epithelial cells that have lost interaction with their extracellular matrix—or that interact with matrix through inappropriate integrin types ***(2)*** or other molecules—cannot colonize elsewhere, because of anoikis. This process is developmentally critical, and its loss can contribute significantly to tumor malignancy (reviewed in **ref. *3***).

Indeed, several oncogenes abrogate anoikis, and a tumor suppressor gene that programs tumor cells to become epithelial cells appears to work by conferring anoikis sensitivity ***(1)***. Thus, it is important to have reliable assays for the effects of compounds or genes on anoikis. Several types of assays will be described here. The most time-consuming and conventional is stable expression of new genes in epithelial cells and assaying the resulting cell lines for anoikis relative to the parental cells. We used this assay to show that anoikis is suppressed by focal adhesion kinase (FAK) and is promoted by the kinase MEKK-1 ***(4,5)***, perhaps through the Jun-N-terminal kinase pathway. This chapter will specify the gene transfer and apoptosis assay methods that work especially well for epithelial cells. In addition, we have developed methods for assaying the effects of genes upon anoikis in transient assays that, although fairly new, appear to be highly reliable and are certainly faster.

1.2. Assaying the Effects of Genes on Anoikis in Stable-Expression Experiments

1.2.1. Generating Stable Expressors

We have focused on the cell line MDCK because of its sensitivity to anoikis and its authentic epithelial cell behavior. It has two drawbacks, though. First,

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being a canine cell line limits the choice of antibodies and nucleic acid probes, a problem that we usually solve by testing several anitbodies or generating canine homologs of genes by PCR. The second is the low transfectability by calcium phosphate and liposome-based reagents. This latter feature has prompted us to use retroviral vectors, which yield extremely efficient and stable expression.

Chiefly, we use the vector pBABE ***(6)***. This vector drives expression of the insert through the viral long-terminal-repeat (LTR) enhancer and has an internal SV40 promoter to drive the expression of a puromycin-resistance gene for selection. Following subcloning of the gene of interest into pBABE (usually in a FLAG-, myc- or HA-epitope-tagged form), the retrovirus vector is transfected by the standard calcium phosphate method into the amphotropic packaging cell line ϕNX ***(7)***, which, being based on 293 cells, is much more transfectable than its 3T3-based predecessors. Two to three days after transfection, the viral supernatant is removed, cleared by centrifugation (3000*g* for 10 min), polybrene is added to give 4 μg/mL final concentration, and it is applied to a subconfluent monolayer of MDCK cells. The MDCK cells can either be on tissue-culture plastic, or, for higher efficiency, on permeable cell culture inserts of 25-mm diameter and 3.0-μ pore size (Falcon, Los Angeles, CA), which fit into 35-mm wells. For the latter, the viral supernatant is placed on the cells from which all media have been removed, and then allowed to flow through the filter by gravity, which takes approx 1 min per mL of supernatant. The infected cells are washed briefly with medium and incubated for 1 d, followed by being trypsinized and replated on tissue culture dishes at various low (i.e., colony) densities. After cell attachment (6–8 h), puromycin is then added to give 1.5 μg/mL, and colonies are selected with refeeding every 3 d, for a total elapsed time of approx 2 wk. Colonies are then ring-isolated, transferred to 10-mm diameter wells, expanded, and lysates are tested for expression of the transgene by Western blotting using anti-epitope antibody. The total elapsed time for subcloning the gene into pBABE through obtaining positive cell lines is approx 1 mo.

2. Materials

1. MDCK cells: American Type Culture Collection (ATCC, Rockville, MD).
2. Tissue culture medium: DME-high glucose (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD) and 1× glutamine-penicillin-sreptomycin (from 100× stock, Gibco-BRL).
3. 2.4-cm cell-culture inserts (Falcon, Los Angeles, CA, cat. no. 3090)
4. Trypsin (0.25%)-EDTA: Gibco-BRL.
5. Eppendorf model 5414 microfuge.
6. PBS: 1× (PBS) (minus calcium and magnesium): Gibco-BRL.
7. Apoptosis lysis buffer (ALB): 10 m*M* Tris-HCl at pH 8.0, 10 m*M* EDTA, 0.5% Triton X100.

8. Sodium acetate: 2.5 *M* NaOAc at pH 7.3.
9. Glycogen (nuclease-free, Boehringer-Mannheim, Mannheim, Germany).
10. Ethanol (100%).
11. TE master mix: 50 m*M* NaCl in TE containing 10 μL RNase (Boehringer, Dnase-free) per mL.
12. Ficoll loading buffer: 10% Ficoll (MW 400,000; Sigma, St. Louis, MO)/20 m*M* EDTA/0.05% bromphenol blue.
13. 1.5% agarose gel in 1× TBE (5× TBE = 54 g Tris, 27.5 g of boric acid, 20 mL of 0.5 *M* EDTA per liter).
14. Cytomix: 120 m*M* KCl, 0.15 m*M* Ca chloride, 10 mM potassium phosphate at pH 7.6, 25 m*M* HEPES at pH 7.6, 2 m*M* EGTA, 5 m*M* magnesium chloride. Adjust to pH 7.6. Before use, take out a 50-mL aliquot, and add ATP to give 2 m*M* and glutathione to give 5 m*M*, readjust the pH to 7.6 on pH meter and filter sterilize.
15. 0.4-cm gap electroporation cuvets (Bio-Rad, Richmond, CA).
16. Gene Pulser electroporator (Bio-Rad).
17. ImaGene Green reagent (Molecular Probes, Eugene, OR).
18. DAPI (Sigma).
19. PBS containing 2 mg/mL bovine serum albumin (BSA) (Sigma).
20. SDS-PAGE sample buffer: 62.5 m*M* Tris at pH 6.8, 0.4% SDS, 10% glycerol, 0.05% bromphenol blue.
21. 14% Tris-glycine minigels (Novex, San Diego, CA).
22. Immobilon transfer membranes (Millipore, Bedford, MA).
23. Anti-myc epitope antibody Ab-1 (Oncogene Science/Calbiochem, La Jolla, CA).
24. Super-signal chemiluminescent detection kit (Pierce, Rockford, IL).
25. Phosphorimager (Bio-Rad).

3. Methods

3.1. Assaying Stable Expressors for Anoikis

The following is our optimized anoikis-assay protocol for MDCK cells, which generates DNA fragmentation ladders. Note that MDCK cells must be grown to confluence to be sensitive to anoikis. Also, note that the cells are extracted with Triton rather than SDS, thus leaving behind intact genomic DNA. Nonapoptotic cells will produce blank lanes on the gel as a result.

1. Plate out MDCK cells on 2.4-cm cell-culture inserts (Falcon cat. no. 3090), grow to confluence. Continue growing one additional day.
2. Trypsinize cells, spin down, resuspend in 2.0 mL of medium and count cells. Transfer 5×10^5 cells to a 2.0-mL microfuge tube and fill the tube to the top with medium.
3. Place on wheel in 37°C incubator for 2.5–3.5 h.
4. Microfuge cells for 8 s at 5220g (in an Eppendorf model 5414 microfuge), suction off supn., wash with 600 μL of PBS by inverting, respin in microfuge.
5. Resuspend pellets in 600 μL of ALB by pipetting up and down with a P1000. Transfer to a microfuge tube.

6. Vortex 20 s. Spin out debris in cold microfuge 10 min at maximum speed.
7. Transfer supernatant to new microfuge tube. Phenol-chloroform extract three times with 550 μL of phenol-chloroform each time.
8. Transfer final aqueous phase (380 μL) to a microfuge tube and add 40 μL of 2.5 *M* NaAc at pH 7.3, 1.5 μL of 20 mg/mL glycogen and 1 mL cold ethanol. Precipitate overnight.
9. Spin in cold microfuge 14 min. Take off supernatant using P1000.
10. Wash with 300 μL cold 70% ethanol. Respin 4 min, take off supernatant with P200, respin briefly, take off as much of remainder as possible with P20.
11. Redissolve pellets in 25 μL of 50 m*M* NaCl in TE containing 1 μL of RNase. (Boehringer, DNase-free) (make a master mix for all samples); incubate at 37°C for 20 min.
12. Add 3.3 μL of Ficoll loading buffer, load onto a 1.5% agarose gel in TBE (can put 10 μL of ethidium bromide in 200 mL gel before pouring.) Run blue dye half way to end of gel, photograph gel.

3.2. Assaying the Effects of Genes on Anoikis in Transient Expression Experiments

Two methods are presented. In the first, a vector containing the *Eschericia coli* β-galactosidase gene under control of a mammalian promoter is used to express the gene of interest. Following transfection, cells are placed in suspension for various periods of time and then doubly stained for nuclear morphology with the fluorescent nuclear stain DAPI and for β-galactosidase activity using the viable stain Imagene Green. The β-galactosidase-positive cells are then scored for apoptotic nuclear morphology.

The second method is technically easier to perform as it requires no microscopy. It is based on the principle that, in epithelial cells undergoing apoptosis, effector caspases specifically cleave the keratin 18 protein to yield two discrete products ***(8)***. We have taken advantage of this observation to develop a new assay for anoikis. The keratin 18 gene was N-terminally tagged with a myc epitope and substituted for the *neomycin-resistance* gene in pcDNA3.1-. The gene of interest (i.e., the candidate anoikis-regulator) is subcloned into the polylinker downstream of the cytomegalovirus (CMV) promoter. The resulting plasmid is then transfected into epithelial cells. Following expression, the cells are placed in suspension, and total cell lysates are Western blotted, using myc epitope antibody. The ratio of cleavage product to intact K18 is a measure of the degree of anoikis. Note that the assay only scores transfected cells, thus providing a high degree of sensitivity and selectivity. We also find it relatively simple to perform. Also, the transfection frequency may vary from one sample to the next but this does not affect the final results, as they are automatically normalized against intact, myc-K18.

3.2.1. β-Galactosidase/DAPI Double-Staining Method

3.2.1.1. Construction of Expression/Reporter Plasmid

We have modified the expression plasmid pHook2 (Invitrogen, San Diego, CA) by inserting a β-galactosidase coding sequence in the unique *Eco*RV site upstream of the signal peptide of the Hook epitope gene, thus placing it under the control of the RSV promoter. This leaves most of the cloning sites in the polylinker downstream of the CMV available for inserting the candidate anoikis-regulating gene. (The complete sequence of pHook2 can be found on Invitrogen's home page, www.invitrogen. com).

3.2.1.2. Electroporation of MDCK Cells

MDCK cells are electroporated as follows:

1. Ethanol precipitate 120 μg of plasmid DNA and redissolve it in 0.6 mL of cytomix.
2. Trypsinize a 50% confluent 150-mm dish of MDCK cells and spin down.
3. Wash cells once with 5.0 mL of sterile PBS and respin.
4. Resuspend one-fifth of the cells in the DNA solution and transfer to a 0.4-cm gap Bio-Rad cuvet, keep on ice.
5. Electroporate at 0.3 kV, 960 μF (Bio-Rad gene pulser), put cuvet back on ice, and keep on ice for 10 min.
6. Resuspend cells and transfer to a tube containing 5.0 mL of complete medium, spin down cells.
7. Resuspend cells and plate onto one 25-mm cell-culture insert (0.2 or 1.0 μ) for transient anoikis assay.
8. Next day, wash dead cells off the top of the filter and refeed.
9. Cells should be confluent enough the day after that (48 h postelectroporation) to perform anoikis assay.

The cells are trypsinized and placed in suspension as described for the stable transfection protocol above. During the final 15 min of suspension, both stains are added: ImaGene Green is added to give 33 μ*M* final concentration, and DAPI to give 1 μ*M*. The cells are then centrifuged for 8 s at 5220g in an Eppendorf 5414 microfuge, washed twice with PBS+ 2mg/mL BSA, resuspended in 30 μL of the latter and transferred to a microscope slide. The doubly stained cells are viewed on a fluorescence microscope, using an FITC filter to find transfected cells and a UV filter to assess normal vs apoptotic nuclear morphology.

3.2.2. Keratin 18-Cleavage Transient Assay for Effects of Transgenes on Anoikis

We have N-terminally tagged the human K18 coding sequence with a myc epitope tag and substituted it for the *neomycin resistance* gene by insertion in

the *Sma*I–*Bst*BI site of pcDNA3.1- (Invitrogen; the complete sequence of the parental plasmid is on the homepage www.invitrogen.com).

Sequences encoding candidate anoikis-regulators are most conveniently blunt-end ligated into the *Pme*I site of the plasmid, resulting in their being driven by the CMV promoter.

Cells are electroporated and subjected to suspension conditions as described above. Following suspension for various times (generally, 0, 1, 2, and 3 h provides a good time course for MDCK cells), cells are washed twice with PBS and lysed by boiling in 300 μL of SDS-PAGE sample buffer per time point (four time points can be obtained from one 25-mm diameter cell-culture insert of cells). Western blots are run on 14% minigels (Novex), electroblotted onto PVDF filters, and probed with anti-myc epitope (clone 9E10) monoclonal antibody (Oncogene Science). Following development using enzyme-linked chemiluminescence (Pierce) we scan the blot on a Bio-Rad phosphorImager and quantitate the band intensities so as reveal relative degrees of apoptosis, as judged by percentage of myc-K18 cleaved.

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17

Use of the 10-Day-Old Chick Embryo Model for Studying Angiogenesis

Peter C. Brooks, Anthony M. P. Montgomery, and David A. Cheresh

1. Introduction

1.1. Angiogenesis and its Physiological Significance

The development of new vessels plays a critical role in a number of physiological and pathological events such as embryonic development, wound healing, arthritis, and tumor growth and metastasis ***(1–4)***. One area that has received considerable attention is determining the role that integrins play in angiogenesis. Recently, we demonstrated that integrin $\alpha_v\beta_3$ was highly expressed on angiogenic blood vessels associated with a variety of solid tumors and during granulation tissue formation ***(5,6)***. Importantly, little if any $\alpha_v\beta_3$ was detected on quiescent vessels ***(5,6)***. In addition, antagonists of $\alpha_v\beta_3$ were shown to specifically inhibit angiogenesis in vivo. These findings suggest an important role for integrin $\alpha_v\beta_3$ during neovascularization. Thus, a great deal of attention has been focused on understanding the cellular and molecular mechanisms by which integrins regulate angiogenesis. Angiogenesis can be defined as the process by which new blood vessels form from pre-existing vessels. This process is similar to, but not identical with vasculogenesis, in which new blood vessels arise from blood islands and precursor cells called angioblasts ***(1–3)***. Thus, an in-depth understanding of the molecules that regulate this complex biological process is of fundamental importance in both basic and clinical research. In fact, recent advances in our understanding of the molecular and biochemical events that facilitate angiogenesis have provided insight into cellular invasion in general as well as the development of novel strategies for the treatment of neovascular diseases ***(5,7–11)***.

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1.2. In Vitro and In Vivo Models of Angiogenesis

Angiogenesis requires the coordinated function of a variety of molecules including, proteases, growth factors, cell adhesion molecules, and extracellular matrix components. Thus, the establishment of models to study this process would greatly facilitate basic research in this area. To this end, a number of in vivo and in vitro models have been developed, facilitating the study of angiogenesis. Angiogenesis involves cellular events such as adhesion, proteolysis, migration, invasion, and proliferation *(12–15)*. However, recapitulation of all these processes in one comprehensive in vitro model has not been accomplished. A variety of in vitro models have been established that may mimic certain cellular and biochemical events during the angiogenic cascade. Some of these models include the Matrigel tube-forming assay *(16,17)*, the fibrin and collagen gel-cord-forming assays *(18,19)*, the aortic ring model *(20)*, the human placental blood vessel out growth model *(21)*, and a variety of endothelial cell proliferation assays *(22)*. However, the cellular events contributing to angiogenesis do not occur in isolation, but rather are coordinately and spatially regulated, these in vitro models can provide only limited information on the angiogenic cascade as a whole.

To gain a more detailed understanding of angiogenesis as it occurs in tissues, development of a number of in vivo models have allowed researchers to study the angiogenic cascade in the context of a true physiological microenvironment. Some of these models include the rat, mouse, and rabbit corneal pocket assays *(23–25)*, the primate iris neovascularization model *(26)*, the human/mouse chimeric angiogenesis assay *(27)*, a murine Matrigel plug assay *(28)*, and the chick embryo chorioallantoic membrane (CAM) assay *(6,29)*. Whereas many of these in vivo models can be used to screen for potential antagonists as well as agonists of neovascularization, some disadvantages include, the length of time required for the assay, and the complexity and expense. Furthermore, most of these assays require the antagonists to be incorporated into a solid support or hydron pellet. Thus, the physiological effects of these antagonists are generally monitored locally, with little information available as to their systemic effects. Furthermore, in a number of these in vivo models, the microenvironments in which angiogenesis is studied is not necessarily an environment in which angiogenesis normally occurs. Therefore, an in vivo model that is relatively inexpensive, rapid, that utilizes a microenvironment in which angiogenesis naturally occurs, and allows evaluation of systemically administered antagonists would be of great benefit. To this end, we describe a modified version of the traditional chick embryo CAM assay, that encompasses all of the advantages described above. The 10-d-old chick embryo CAM assay provides the investigator with the unique flexibility to study the effects of systemically administered or locally applied antagonists of angio-

genesis in a tissue in which angiogenesis naturally occurs. Finally, an important advantage of this system is that it allows one to select inhibitors of angiogenesis that interfere with new blood vessel development without affecting pre-existing vessels.

1.3. The Chick CAM Assay as a Method to Study Angiogenesis

The CAM of the chick embryo is composed of two separate membranes—the chorion and the allantois—that fuse early (days 4 to 5) during embryonic development ***(30)***. The CAM is the major respiratory structure for the exchanges of gases and nutrients during embryonic development and thus becomes highly vascularized. Development of the blood vessels within the CAM begin in the center and migrate out to the periphery ***(31–33)***. Studies indicate that the vascular development within the CAM begins to slow and is essentially completed by day 11–14 of development ***(31–33)***. Therefore, the chick CAM provides a tissue composed of numerous mature blood vessels. In this regard, the CAM provides an ideal microenvironment in which to induce new blood vessel development from pre-existing vessels. However, as there are pre-existing vessels, quantification of angiogenesis can be complicated, and several methods have been suggested to simplify quantification ***(28)***. In this report, we provide a detailed description of a modified version of the traditional chick CAM assay.

The chick CAM assay is a relatively rapid and inexpensive method with which to study angiogenesis. In the method described below, a filter disc containing angiogenic cytokines can be placed on the CAMs of the 10-d-old chick embryos (**Fig. 1**). Following induction of angiogenesis, various antagonists can be administered either intravenously or topically and evaluated for their effects. Since new blood vessel development is initiated from pre-existing vessels, the effects of the antagonists on true angiogenesis can be evaluated. This angiogenic response can be quantified with the use of a stereomicroscope by counting the number of blood vessel branch points within the CAM directly beneath the filter disc. Thus, the chick CAM assay allow a rapid and reproducible method to study angiogenesis in the context of a physiologically relevant tissue.

2. Materials

1. 10-d-old chick embryos (McIntyre Poultry, Lakeside, CA).
2. 48-place table-top egg incubator (Lyon Electric, Chula Vista, CA).
3. Curved-tip forceps.
4. Straight-tip forceps.
5. Small dissecting scissors.
6. Styrofoam egg holder.
7. Model drill (Dremel, Emerson Electric Company, Racine, WI).
8. Model drill bit and cutting wheel.
9. Sterile cotton swabs.

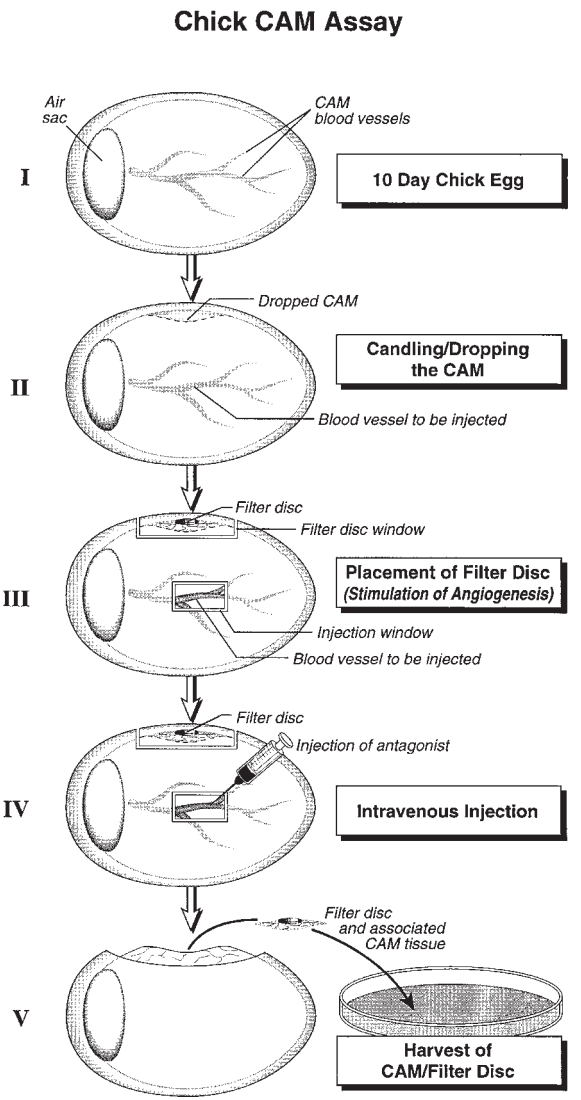

Fig. 1. *(continued on next page)* Chick CAM assay: (I) Ten-day-old chick embryos with pre-existing blood vessels are utilized for the CAM angiogenesis assay. (II) The 10-d-old embryos are candled to determine the position of prominent blood vessels for systemic administration of antagonists. The CAM is next separated from the shell, creating a false air sac. (III) A small window is cut directly over the false air sac to provide access to the underling CAM. A filter disc saturated with an angiogenic stimulator is place on the dropped CAM. The window is next sealed with sterile tape and the embryo returned to the incubator. (IV) Twelve to twenty four hours later, an injection window is cut directly over the blood vessel chosen for injection. The injection win-

10. Standard office paper punch.
11. 35-mm Petri dishes.
12. Absolute ethanol.
13. Cortisone acetate (Sigma).
14. Whatman (Clifton, NJ) type I filter paper.
15. Fibroblast basal medium (FBM) (GIBCO, Grand Island, NY).
16. Cytokines of interest: (bFGF)/(VEGF) (Genzyme, Cambridge, MA).
17. Egg candle (Lyon Electric).
18. Paraffin oil (Sigma).
19. 30-gage needles and 1-mL syringes.
20. Mab LM609 (Chemicon, Temecula, CA).

3. Methods

3.1. Preparation of Filter Discs

To prepare the filter disc for placement on the chick CAM, the paper punch used to cut the discs should be sterilized and dipped in 70% ethanol and air dried. Use the sterile paper punch to prepare the appropriate number of filter disc (6 mm in diameter) for the experiment. In general, 10 filter discs are used per experimental condition. Place these discs in a 35-mm dish and prepare 1 mL of a 3.0 mg/mL solution of cortisone acetate in absolute ethanol. The use of the cortisone acetate helps reduce inflammation associated with placement of the filter disc. Add the cortisone acetate solution to the filter discs (50 discs/1 mL) and allow the saturated disc to air dry in a sterile laminar flow hood (45–60 min) uncovered. The discs will become coated with the cortisone acetate and are then ready to be used.

3.2. Candling the Eggs

When the 10-d-old chick embryos arrive, immediately place them in the humidified incubator maintained at approximately 99.5°F and 51% relative humidity (*see* **Note 1**). The embryos should be maintained in these conditions for the duration of the experiment. The process of candling the embryos is done to locate the optimal position for the placement of the filter disc on the CAM as well as to identify potential blood vessels for intravenous injections (**Fig. 1**; *see* **Note 2**). If the experiment to be carried out requires intravenous administration of a potential angiogenesis antagonist, the selection of blood vessels for intravenous injection is the first priority. Thus, the selection of the

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dow is removed and the compound to be tested is injected in a final volume of 50–100 μL. The injection window is sealed with sterile tape and the embryos are incubated for 48 h. (V) The filter disc window is removed and the filter disc and associated CAM tissue is harvested and placed in a Petri dish for quantification.

area of the CAM for placement of the filter disc is dictated by the position of the blood vessel that will be injected. For topical application of antagonists, candling the embryo is required for determining the optimal position of the filter disc on the CAM, in an area with the least number of pre-existing vessels. If it is not necessary to select a blood vessel for injection, there is more flexibility in positioning the filter discs.

3.3. Candling Egg for Intravenous Injection

To candle the embryos, use an enclosed area such as a laminar flow hood and turn off over head lights to facilitate illumination of the embryo by the candle light. Place the broad end of the egg up to the candle light and determine the position of the air sac. Mark the position of the air sac with a soft lead pencil. To prepare the egg for intravenous injection, rotate the egg close to the light to determine the position of prominent blood vessels on the lateral sides of the egg that are well anchored and close to the surface. In general, the vessel selected for injection should be as straight as possible and from medium to small in size. Large vessels should not be used because upon injection, excessive bleeding may result. The vessel should also be branching in a direction opposite from the air sac (*see* **Fig. 1**) to ensure the proper direction of blood flow. Mark a small box around the vessel to be injected (approx 5 × 10 mm) with a soft lead pencil. Indicate the position of the vessel within the box with a straight line indicating direction of blood flow. Since the embryo will be injected intravenously, the selection of the area of the CAM for the placement of the filter disc is dictated by the position of the vessel to be injected. Thus, while keeping the blood vessel window in a horizontal position, mark a dot on the top side of the egg in an area with the least number of blood vessels. This dot will indicate the position at which you will place the filter disc on the CAM.

3.4. Dropping the CAM

To separate the CAM from the shell membrane, swab with 70% ethanol both the broad end of the egg where the air sac is located, and the top area of the shell were the dot was marked to indicate the selected position of the filter disc. Drill a small hole through the broad end of the egg were the air sac is located. Next, at the dot indicating the selected position for the filter disc, carefully drill a small hole through the egg shell only, being careful not to penetrate the white egg shell membrane. Because the CAM is closely associated to the egg shell membrane at this site, penetration of the shell membrane may cause bleeding (*see* **Note 3**). Holding the egg with the broad side toward the light, gently place the curved-tip forceps through the hole just under the shell and carefully push down on the shell membrane, being careful not to perforate the membrane. This gentle pressure begins to separate the CAM from

the shell and allows air from the air sac to be displaced resulting in the CAM dropping away from the shell. To complete the process, apply a gentle suction to the hole at the broad end of the egg which helps create the false air sac directly over the CAM. This procedure should be done in an enclosed area such as a laminar flow hood with the overhead lights turned down to facilitate illumination of the embryos by the candle light.

To create a window through the egg shell immediately over the dropped CAM, draw a 1 × 1-cm box directly over the newly created false air sac. Carefully, cut through the egg shell with the model cutting wheel, being careful not to perforate the egg shell membrane as this helps prevent egg shell fragments from dropping on the CAM beneath the shell. Leave the four corners of the egg shell window intact. Leave the shell window in place and return the embryos to the incubator until you are ready to place the filter discs on the CAMs (*see* **Note 4**).

3.5. Cytokine Stimulation of Angiogenesis

To initiate angiogenesis, the cytokine of interest (bFGF) can be diluted in FBM to the desired concentration (1.0–5.0 μg/mL) and 10 to 12 μL of the solution can be applied to each of the filter discs. Immediately remove the precut shell windows from eggs and gently place the cytokine-saturated filter disc onto the CAM in an area of the CAM that has the lowest density of pre-existing vessels. When selecting a position on the CAM, try to avoid placing the disc directly over the yolk sac, areas of high microvessel density, or towards the periphery of the CAM, because this may make quantification more difficult. Seal the window with sterile transparent tape. Carefully place the embryos back in the incubator being careful not to disturb the filter discs because moving the embryo may result in a change in the original position of the disc.

3.6. Administration of Angiogenesis Antagonists

Previous reports have demonstrated that chick angiogenic blood vessels express high levels of integrin $\alpha_v\beta_3$ ***(5,6)***. Therefore, the chick CAM model is a useful model with which to study the role of integrin $\alpha_v\beta_3$ in angiogenesis. In fact, both cyclic RGD peptides as well as specific monoclonal antibodies directed to integrin $\alpha_v\beta_3$ have been administered both topically and intravenously in this model. Moreover, administration of these antagonists resulted in significant reduction of both tumor-induced and cytokine induced angiogenesis, demonstrating the utility of this in vivo model ***(5,6,24)***.

To begin the procedure of administration of angiogenesis antagonists, allow the embryos to incubate undisturbed for 12 to 24 h to facilitate adherence of the filter discs to the CAM tissue. This is important because both topical and intravenous administration of the antagonists requires movement of the eggs that could cause the filter disc to shift from its original position on the CAM.

For intravenous administration of the antagonists, gently position the embryo in the egg holder and orient the light such that it is shining down from the top through the taped window. The position of the selected vessel for injection should be rechecked to make sure it did not move significantly from its original position previously marked on the egg (*see* **Note 5**). Once the position of the vessel is established, carefully cut along the margins of the box that was previously outlined during the candling procedure. Use extreme care when cutting this window as the embryonic membranes are directly associated with the egg shell and shell membrane at this site. Cut only through the shell itself being careful not to penetrate the white shell membrane.

Once the borders of the window are cut, place a drop of paraffin oil around the cut edge of the window, which helps to loosen the shell from the shell membrane. With the fine-tip, curved forceps gently remove the shell window from the egg. The white opaque shell membrane should be visible at this point. Place a drop of paraffin oil on the shell membrane as this will cause the shell membrane to turn transparent, facilitating visualization of the blood vessel to be injected (*see* **Note 6**). With a 30-gage needle, give one injection of no more than 100 μL total volume of the antagonist (**Fig. 1**). For example, a single injection of MAb LM609 (Chemicon) directed to integrin $\alpha_v\beta_3$, at a concentration of 1.0 mg/mL can be given. With a sterile Kimwipe, gently stop any residual bleeding and seal the window with sterile tape. Return the embryos to the incubator and allow them to incubate for 48 h.

For topical addition, the antagonists can be diluted in FBM and 15–25 μL applied to the original filter disc through the top window. The window can be resealed with sterile tape and the embryos placed back in the incubator. With topical addition, the antagonist may be applied each day for the duration of the experiment.

3.7. Quantification of Angiogenesis

To quantify angiogenesis within the chick CAMs, the embryo is removed from the incubator and placed in a 100-mm Petri dish with the window through which the filter disc was placed facing up. With a pair of dissecting scissors, carefully cut through the shell immediately surrounding the window and remove the shell to expose the underlying filter disc. Next, with a pair of forceps carefully grasp the CAM just to the side of the filter disc and cut a ring around the filter disc to remove the disc and surrounding CAM tissue. Immediately place the filter disc and surrounding CAM tissue in a 35-mm Petri dish in the same orientation as it was removed from the embryo. Wash the tissue with 1 mL of sterile PBS by gently swirling the tissue in the PBS until the CAM tissue is unfolded and lies flat against the bottom of the Petri dish. Remove the excess fluid and repeat if necessary.

Quantification of angiogenesis can be accomplished by counting the number of blood vessel branch points directly beneath the applied filter disc with the aid of a stereomicroscope (**Fig. 2**). The relative number of blood vessel branch points is indicative of the number of new blood vessel sprouts arising from pre-existing blood vessels (**Fig. 2**). Quantification of the number of branch points should be done in a double-blind fashion. The mean number of branch points from each experimental conditions can then be compared to buffer controls. Angiogenesis can be reported as the angiogenic index, which is defined as the mean number of branch points from the experimental conditions minus the mean number of branch points from buffer controls (no angiogenic stimulator). Thus, the use of the filter disc provides the unique ability to quantify angiogenesis stimulation and inhibition in a confined area within a physiologically relevant tissue microenvironment.

4. Notes

1. Chick embryos from different venders can vary significantly in their degree of vascularization and developmental status. Thus, consistent use of the same vender can decrease variability from experiment to experiment. Furthermore, proper incubation conditions including temperature and humidity are of critical importance for proper vascularization and survival of the embryos. The humidity and temperature adjustments may vary depending on the type of incubator used.
2. If an experimental protocol requires embryos of younger age than day 10, a shell-less culture system should be considered. Furthermore, it should be noted that in embryos that are 12 or more days old, the CAM adheres to the shell much tighter and therefore dropping the CAM becomes a difficult task.
3. To increase the success rate for efficiently dropping the CAMs, drill holes in only 5–6 eggs at a time than drop the CAM of these eggs before starting the next group. Drilling the hole in the egg shell, then leaving them for more than 30 min results in excessive drying and increased adherence of the CAM to the shell. This excessive drying can preventing proper separation of the CAM from the shells.
4. After dropping the CAMs and cutting the top window an incubation time of between 30 and 90 min before application of the filter disc maybe helpful in preventing the filter disc from moving around during transport back to the incubator. This incubation time helps in draining the excess embryonic fluid away from the top of the CAM. This helps increase the adherence of the filter disc to the CAM tissue.
5. During the candling process and intravenous injections, the candle light can result in excessive heating of the embryo if left in close contact for an extended period of time. The increased heating can have deleterious effects of the embryo resulting in reduced viability. Thus, minimizing the time the candle light is in close association with the eggs is important.
6. When intravenous injections are required, small-to-medium sized well-anchored surface blood vessels are the best for injection. Injection of large blood vessels

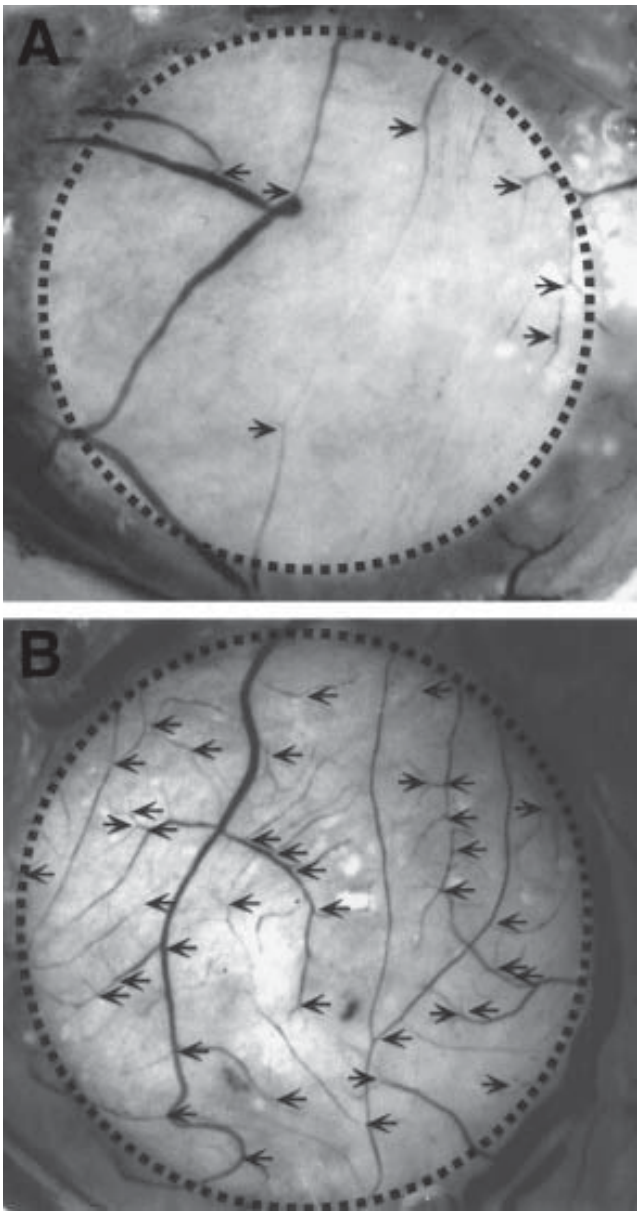

Fig. 2. Quantification of angiogenesis within the chick CAM. The filter disc and associated CAM tissue is washed with PBS such that it lies flat against the bottom of the Petri dish. Angiogenesis is quantified with the use of a stereomicroscope maintained at one focal plane. The number of blood vessel branch points (arrows) within the confined region of the filter disc (dashed circle) is determined. The number of blood vessel branch points is relative to the number of newly sprouting angiogenic vessels. **(A)** CAM tissue stimulated with buffer only. Note that the pre-existing vessels tend to be larger and less branched. **(B)** CAM tissue stimulated with bFGF.

may cause excessive bleeding. In addition, after injecting the sample volume, allow the blood flow to return in the vessel then slowly remove the needle. This procedure can help to reduce the bleeding following injection.

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18

Cell Shape and Integrin Signaling Regulate the Differentiation State of Mammary Epithelial Cells

Aruna Somasiri and Calvin D. Roskelley

1. Introduction

When they are cultured on a reconstituted basement membrane gel, mammary epithelial cells round-up, aggregate, form cell–cell junctions, and undergo an apical/basal polarization that ultimately produces a central lumen. The resulting three-dimensional 'mammospheres' closely resemble lactational alveoli in vivo and they fully differentiate when they are maintained in an appropriate mileau of lactogenic hormones. Specifically, the cells express milk protein genes, package the products in secretory vesicles, transport the vesicles to the apical cell surface, and release the products into the central lumen. Therefore, dynamic and reciprocal interactions between epithelial cells and the surrounding basement membrane are key regulators of phenotypic change in the developing mammary gland ***(1–4)***.

We have developed specialized culture models to examine individual basement membrane-dependent differentiative events that occur prior to full alveolar morphogenesis ***(5)***. For example, when basement membrane proteins in solution are overlaid upon nonfunctional mammary epithelial monolayers, the cells round-up and partially differentiate ***(6)***. Cell rounding alone, which can be mimicked in the absence of basement membrane proteins, induces expression of the lactoferrin milk protein gene ***(7)***. In vivo, lactoferrin is first expressed early in pregnancy. Therefore, cell rounding initiates an early differentiative state in mammary epithelial cells. When these naked rounded cells are then overlaid with basement membrane, lactoferrin expression continues and a second milk protein gene, β-casein, is rapidly induced ***(8)***. In vivo, β-casein is first expressed in mid-pregnancy. Therefore, the basement mem-

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brane induces an intermediate differentiative state in rounded mammary epithelial cells. It appears that cell rounding primes the cells to respond rapidly to signal transduction pathways activated by the basement membrane ***(9)***. These signals are initiated by interactions of laminin in the basement membrane with cell-surface integrins ***(10,11)***.

This review will describe in detail the three specialized culture models described above. These include: basement membrane overlay of monolayer cultures; cell rounding in the absence of exogenously added basement membrane; and basement membrane or purified laminin overlay of prerounded cultures. The differentiation state achieved in each model is monitored by assaying lactoferrin and β-casein expression by Northern blotting, Western blotting, and immunofluorescence. The function of laminin-binding α_6 integrins in each model can be assessed using a function-blocking antibody. This antibody has no effect on basement membrane-mediated cell rounding or lactoferrin expression; however, β-casein induction is abrogated both in monolayers and prerounded cells that are overlaid with laminin. Therefore, the morphological and integrin-mediated effects of laminin can be examined in isolation using these models. Given the fact that the differentiation state of many, if not all, epithelial tissues is profoundly influenced by interactions with the basement membrane, these protocols will also serve as a useful starting point for investigators working in other systems who wish to manipulate cell shape and integrin signaling.

The cell lines that can be used in these studies are numerous. We routinely utilize a functional, homogeneous mouse mammary epithelial cell line that cannot deposit an endogenous basement membrane. Therefore, the effects of exogenously added extracellular matrix proteins can be assayed with certainty. This line, designated scp2, was isolated by limited dilution cloning ***(6)*** from the functional, but heterogeneous, cell line CID-9 ***(12)***. The CID-9 line was originally isolated by differential trypsinization of the COMMA-1D line ***(13)***. The HC-11 ***(14)*** and the IM-2 ***(15)*** mouse mammary epithelial cell lines can also be used, particularly when assaying for β-casein induction. Alternatively, primary mammary epithelial cell cultures derived from mid-pregnant mice ***(2)*** can also be used. The cell lines offer the advantage that they can be readily transfected with reporter gene studies to study milk protein gene promoter activation ***(12,16)***, although primary cultures from transgenic mice can also be used in this manner ***(17)***. The antibodies and probes used to assay lactoferrin and β-casein expression have been published ***(7,8)***.

2. Materials

2.1. Basic Cell Culture

1. Mouse mammary epithelial cultures or the functional epithelial cell lines: Primary cells, Scp2, CID 9, HC-11 and IM-2.

2. Growth media: Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1, Sigma, St. Louis, MO) containing 5% fetal Bovine serum (FBS, Hyclone, Logan, UT), insulin (Sigma, 5 μg/mL) and gentamycin sulphate (Sigma, 50 μg/mL). Store the media at 4°C.
3. Trypsin solution: Dilute 0.25% trypsin in HBSS media (Sigma) to get 0.05% trypsin in HBSS media and with 0.05% final EDTA.

2.2. Basement Membrane or Purified Laminin Overlay of Monolayer Cultures

1. Matrigel (Collaborative Research, Bedford, MA), a commercially available basement membrane extracellular matrix equivalent. Thaw overnight on ice, dilute 1:1 with DMEM/F12 medium, aliquot and store at –70°C, 100 × stock (**Note 1**).
2. Laminin (Sigma), store at –70°C, dilute the 1 mg/mL stock solution 1:20 in differentiation media (*see* **Subheading 2.2.**, **step 7** below) just prior to use.
3. Insulin (Sigma) 5 mg/mL in 5 m*M* HCl, 1000× stock solution. Store at –70°C.
4. Hydrocortisone (Sigma) 1 mg/mL in 100% EtOH, 1000× stock solution. Store at –70°C.
5. Leutotropic hormone (prolactin, Sigma) 3 mg/mL in 10 m*M* NaOH, 1000× stock solution. Store at –70°C.
6. Plating media: DMEM/F12 media supplemented with 1% FBS, insulin (5 μg/mL), hydrocortisone (1 μg/mL), prolactin (3 μg/mL), and gentamycin sulphate (50 μg/mL).
7. Differentiation media: Same as plating media without FBS.

2.3. Cell Shape Manipulation

1. Poly 2-hydroxyethylmethacrylate (polyHEMA) (Sigma): 100× stock solution; 50 mg/mL in 95% ethanol. Heat the stock solution in a 37°C water bath overnight to dissolve completely. Store at room temperature.
2. Plating media: DMEM/F12 medium containing 1% FBS, 50 μg/mL gentamycin sulphate, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 3 μg/mL prolactin.
3. Differentiation media: Same as plating media without FBS.

2.4. Laminin Overlay of Prerounded Cultures

1. polyHEMA (*see* **Subheading 2.3.**, **step 1** above).
2. Laminin (*see* **Subheading 2.2.**, **step 2** above).
3. Plating media: DMEM/F12 media supplemented with 1% FBS, insulin (5 μg/mL), hydrocortisone (1 μg/mL), prolactin (3 μg/mL), and gentamycin sulphate (50 μg/mL).
4. Differentiation media: Same as plating media without FBS.

2.5. α_6 Integrin Function Blocking of Basement Membrane Overlaid Monolayers

1. Basement membrane Matrigel (*see* **Subheading 2.2.**, **step 1** above).
2. Differentiation media (*see* **Subheading 2.4.**, **step 4** above).
3. GoH3, a rat IgG that blocks α_6 integrin function (Pharmingen, Torey Pines, CA). Store at 4°C.
4. Rat IgG control antibody (Jackson ImmunoResearch, West Grove, PA). Store at –20°C.

2.6. α_6 Integrin Function Blocking of Laminin Overlaid Clusters

1. polyHEMA (*see* **Subheading 2.3.**, **step 1** above).
2. Laminin (*see* **Subheading 2.2.**, **step 2** above).
3. Differentiation media (*see* **Subheading 2.4.**, **step 4** above).
4. GoH3, a rat IgG that blocks α_6 integrin function (Pharmingen). Store at 4°C.
5. Rat IgG control antibody (Jackson ImmunoResearch). Store at –20°C.

2.7. Northern Blotting

When working with mRNA it is very important to keep all solutions and equipment RNAse free; therefore, glassware, spatulas, forceps, and so on should be baked overnight in an oven at 200°C. All plasticware used should be new and sterile. Polymer tubes should be treated with 3% H_2O_2 for 30 min and then rinsed with DEPC treated water. Always use DEPC treated autoclaved ddH_2O.

1. GuSCN solution: 4 *M* GuSCN, 25 m*M* NaCitrate (pH 7.0), and 0.5% sarkosyl. Filter through Whatman #1 and store at room temperature. Add 7 μL/mL of β-mercaptoethanol just prior to use.
2. 2 *M* sodium acetate at pH 6.0.
3. Phenol saturated with 0.5 *M* Tris/50 m*M* EDTA at pH 7.0.
4. Chloroform/ isoamylalcohol (49:1).
5. 75% EtOH.
6. 0.5% SDS solution.
7. Isopropanol.
8. 10× MOPS: 40 m*M* MOPS, 100 m*M* sodium acetate, 10 m*M* EDTA (Fisher, Pittsburgh, PA).
9. 37% formaldehyde at pH > 4.0 (Fisher).
10. Deionized formamide: Stir 50 mL of formamide with 5 g of Bio-Rad (Richmond, CA) AG501-X8 resin until pH is 7.0 and filter through Whatman #1. Store at –20°C.
11. Formaldehyde loading buffer: 1 m*M* EDTA (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol.
12. 10 mg/mL ethidium bromide (Sigma).
13. 20× SSC: 3 *M* NaCl, 0.3 *M* sodium citrate and adjust to pH 7.0 with 1 *M* HCl.
14. Hybond nitrocellulose membrane (Amersham, Arlington Heights, IL).
15. Whatman 3MM filter paper (Fisher).
16. Northern prehybridization solution: 6 × SSC, 5 × Denhardt's buffer, 25 m*M* sodium phosphate, 1.5 m*M* sodium pyrophosphate, 50% formamide, and add 0.1% SDS last.
17. 50× Denhardt's buffer: Ficoll 400 5 g, BSA (DNAse/RNAse free, 5 g), polyvinal pyrolidone 5 g. Make up to 500 mL with DEPC treated water.
18. ^{32}P random-primed, labeled probes for β-casein and lactoferrin.
19. Hybridization buffer is same as prehybridization buffer with the probe.
20. Hybridization oven.
21. X-ray film (Kodak, X-OMAT).

2.8. Western Blot Analysis for β-casein and Lactoferrin

1. Dispase (Collaborative Research).
2. RIPA lysis buffer: 150 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.4), 5 m*M* EDTA, 5% NP-40, 1% DOC, and 0.1% SDS. Store at 4°C.
3. Protease inhibitors: PMSF (Sigma) 10 m*M* in isopropanol, 250× stock solution; aprotinin (Sigma) 10 mg/mL in 0.01 *M* HEPES (pH 8.0), 200× stock solution; leupeptin (Sigma) 10 mg/mL in water, 1000× stock solution. Store all at –20°C.
4. $RIPA^+$: RIPA lysis buffer with protease inhibitors.
5. Lowry assay modified for SDS containing solutions (Bio-Rad).
6. Vertical PAGE gel and transfer apparatus (Bio-Rad).
7. 2× Sample buffer: 10% glycerol, 50 m*M* Tris-HCl (pH 6.8), 2% SDS, 0.02% bromophenol blue, 5% mercaptoethanol.
8. Kaleidoscope prestained protein standards (Bio-Rad).
9. Gel running buffer: 0.25 *M* Tris base, 1.92 *M* glycine, and 1% SDS. Buffer should be at pH 8.3.
10. PVDF nylon transfer membrane (Bio-Rad).
11. Western transfer buffer: 0.22 *M* glycine, 50 m*M* Tris (pH 8.3), 0.5% SDS, 20% MeOH.
12. Blocking solution: 4% BSA, 5% FBS in TBS-T (**Note 2**).
13. Mouse anti-β-casein and rabbit anti-lactoferrin antibodies.
14. TBS: 50 m*M* Tris-HCl (pH 7.5) and 150 m*M* NaCl.
15. TBS-T: 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, and 0.1% Tween-20 (Fisher).
16. Goat anti-mouse and goat anti-rabbit antibodies labeled with horse radish peroxidase (HRP, Jackson ImmunoResearch).
17. Enhanced chemiluminescence solutions (ECL, Amersham).
18. X-ray film (Kodak, X-OMAT).

2.9. Immunofluorescence

1. 18-mm glass cover slips (Fisher).
2. PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4 and 1.4 m*M* KH_2PO_4 at final pH approx 7.3.
3. 3.7% paraformaldehyde: Heat to 60°C in a volume of water slightly less than 2/3 of the final volume of fixative. Weigh out the paraformaldehyde and add to the water with a stir bar. Transfer to a fume hood and maintain the temperature at 60°C while stirring. Add one drop of 2 *M* NaOH. Solution should become clear fairly rapidly. However, some undissolved fine particles may remain. Remove from heat and add 1/3 volume of 3× PBS. Bring the pH of the solution to 7.2 with HCl, add water to the final volume, and filter using a .22-μm filter. Cool to room temperature, or to 4°C on ice.
4. Permeabilizing solution: 0.5% Triton X-100 in PBS.
5. Blocking solution: 1% BSA and 10% normal goat serum in PBS.
6. Mouse anti-β-casein and rabbit anti-lactoferrin antibodies.
7. Fluorochrome conjugated anti-mouse and anti-rabbit second antibodies (Jackson ImmunoResearch.).

8. Anti-fade solution: DABCO (Sigma) in 90% glycerol.
9. Microscopic glass slides (Fisher).
10. Nail polish.
11. Fluorescent microscope.

3. Methods

3.1. Routine Cell Culture Procedures

Mammary epithelial cells are routinely maintained in monolayer culture containing serum for growth and insulin to protect against apoptosis. These cells should be subcultured at subconfluence to prevent loss of function (i.e., milk protein production). Culture media and other reagents are sterilized before use by filtering through 0.2-μm pore size sterile filters and cell culture operations are carried out under aseptic conditions. Cell morphology should be monitored regularly using an inverted, phase-contrast microscope. The following protocol outlines the method we use to subculture the mammary epithelial cells (**Note 3**).

1. Remove and discard the culture media.
2. Wash the cells with 10 mL of Ca^{2+} free DMEM/F12 media 3 × 5 min each.
3. Add 2 mL of 0.05% trypsin solution and after 2 min at room temperature aspirate off.
4. Add 2 mL of fresh 0.05% trypsin solution and incubate at 37°C for 5 min.
5. Observe the cells with a inverted phase-contrast microscope: look for free-floating cells and attached cells that have detached from each other and are rounding up. If most of the cells do not appear to be detached return the dish back to the incubator.
6. After a few more minutes repeat **step 5**. If there are more cells still attached, tap the side of the dish gently so that the loosely attached cells will detach.
7. Add 10 mL of growth media to quench trypsin and transfer the cells into a sterile centrifuge tube. Spin at 150g for 5 min in a clinical bench-top centrifuge.
8. Aspirate the media and gently resuspend the pellet in 40 mL of growth media.
9. Plate 10 mL each in 4 × 100-mm tissue culture dishes (1:4 split ratio, **Note 4**).
10. Change the growth media every second day. Cultures are normally subconfluent in 4–6 d. All the cell cultures should be maintained at 37°C in a humidified incubator with 5% CO_2, 95% air.

3.2. Basement Membrane Overlay of Monolayer Cultures

1. Trypsinize a 90% confluent 100-mm dish of mammary epithelial cells and resuspend the cells in 10 mL of plating media.
2. Plate mammary epithelial cells 2×10^5 cells per 35-mm tissue-culture dish (or 5×10^5/60-mm dish, 1×10^6/100-mm dish) in plating media.
3. Allow the cells to attach overnight and then change media to serum free DMEM/F12 media containing lactogenic hormones (differentiation media) and leave for 24 h to remove growth factors. This ensures that the cells form a flat monolayer and are not proliferating when the basement membrane overlay is added.

4. Dilute basement membrane Matrigel (1:100 of stock, final concentration approx 100 μg/mL total protein) or purified laminin (1:20 of stock, final concentration 50 μg/mL) in cold DMEM/F12 medium containing lactogenic hormones. Warm at 37°C in a water bath, vortex briefly, further mix by pipetting, and add the overlay containing medium to the cells immediately (**Note 5**).
5. Change differentiation media containing either the basement membrane or laminin overlay every 48 h.
6. Cells in the monolayer will round and cluster after 2–3 d in response to the overlay. Lactoferrin and β-casein induction occurs soon thereafter, usually between 3 and 5 d. Expression of both milk proteins can be assessed by Northern blotting (*see* **Subheading 3.7.**, below), Western blotting (*see* **Subheading 3.8.**, below) or immunofluorescence (*see* **Subheading 3.9.**, below).

3.3. Cell Shape Manipulation

1. Dilute the stock polyHEMA solution 1:100 in 95% ethanol (final concentration 0.5 mg/mL) and coat regular tissue-culture dishes with 125 μL/cm^2 surface area (i.e., 1.0 mL/35-mm dish, 2.5 mL/60-mm dish, 5.0 mL/100-mm dish)
2. Evaporate the ethanol overnight in a dry, sterile incubator at 37°C. This will leave a clear polyHEMA coating on the surface of the dish. Use only completely dried dishes to plate cells.
3. Plate mammary epithelial cells on polyHEMA coated plates at 5×10^4 cells/cm^2 surface area in DMEM/F12 medium containing 1% FBS, gentamycin sulphate, insulin, hydrocortisone, and prolactin.
4. Allow cells to attach overnight and then carefully change to serum-free DMEM/F12 medium containing lactogenic hormones (differentiation media). The cells will attach to this substratum of a low concentration of polyHEMA but they will not spread; therefore, they will form rounded cell clusters.
5. The cells will express lactoferrin after 48 h. This can be assessed by Northern blotting (*see* **Subheading 3.7.**, below), Western blotting (*see* **Subheading 3.8.**, below) or immunofluorescence (*see* **Subheading 3.9.**, below). These naked cell clusters do not express β-casein.

3.4. Basement Membrane or Laminin Overlay of Prerounded Cells

1. Plate mammary epithelial cells on polyHEMA-coated plates (*see* **Subheading 3.3.**, **step 1** above).
2. Allow cells to attach overnight and then change to serum-free DMEM/F12 medium containing lactogenic hormones (differentiation media). The cells will be rounded and clustered.
3. After 48 h add a basement membrane or laminin overlay (*see* **Subheading 3.3.**, **step 4** above).
4. In 24 h cells can be collected and assayed for continued lactoferrin expression and induced β-casein expression by Northern blotting (*see* **Subheading 3.7.** below), Western blotting (*see* **Subheading 3.8.** below) or immunofluorescence (*see* **Subheading 3.9.** below).

3.5. α_6 Integrin Function Blocking of Basement Membrane Overlaid Monlayers

1. Pretreat flat cell monolayers (*see* **Subheading 3.2.**, **step 2**) with the α_6 integrin function-blocking antibody GoH3 (5 μg/mL final concentration), or control rat IgG (5 μg/mL final concentration) in differentiation medium (*see* **Subheading 2.2.**, **step 7**) for 1 h.
2. Add the basement membrane overlay (*see* **Subheading 3.2.**, **step 4**) containing GoH3 (5 μg/mL) in differentiation medium. Medium change with all supplements every second day. The GoH3 antibody will have no effect on basement membrane-mediated cell rounding and clustering.
3. On day 5, assess lactoferrin and β-casein expression by Northern blotting, Western blotting or immunofluorescence (*see* **Subheading 3.7.–3.9.**). GoH3 will have no effect on lactoferrin induction, but β-casein induction will be significantly inhibited.

3.6. α_6 Integrin Function Blocking of Laminin Overlaid Cell Clusters

1. Pretreat naked cell clusters that have been maintained on polyHEMA for 48 h in the presence of differentiation medium (*see* **Subheading 2.2.**, **step 7**) with the α_6 integrin function blocking antibody GoH3 (5 μg/mL final concentration), or control rat IgG (5 μg/mL final concentration) for 1 h.
2. Add laminin overlay (*see* **Subheading 3.2.**, **step 4**) containing GoH3 or rat IgG in differentiation medium.
3. After 24 h assess lactoferrin and β-casein expression by Northern blotting (*see* **Subheading 3.7.**), Western blotting (*see* **Subheading 3.8.**) or immunofluorescence (*see* **Subheading 3.9.**). GoH3 will have no effect on lactoferrin induction, but β-casein induction will be significantly inhibited.

3.7. Northern Blotting

3.7.1. Isolation of mRNA from Cultured Cells

1. Remove the culture medium from the dish, add 2 mL of lysis solution, scrape off all the cells and pass through a 22-gage syringe 10 times. Transfer the lysate to a 15-mL polypropylene round-bottomed tube.
2. Add 200 μL of 2 *M* sodium acetate, vortex; add 2 mL of buffer-saturated phenol at pH 7.0, vortex; add 400 μL of chloroform/isoamylalcohol, vortex, and incubate on ice for 30 min.
3. Centrifuge at 6000*g* for 20 min at 4°C. Transfer the upper aqueous phase to a new tube; do not remove the interphase. If phases do not separate well add 1 mL of GuSCN, mix, and spin again.
4. Add equal volume of isopropyl alcohol and leave for 2 h to overnight at –20°C.
5. Centrifuge at 6000*g* for 20 min at 4°C; discard the supernatant and add 300 μL of GuSCN to the pellet. Transfer the pellet in to a microfuge tube, add 300 μL of isopropanol and leave for 2 h to overnight at –20°C.
6. Centrifuge at 14,000*g* for 10 min at 4°C; discard the supernatant and wash the pellet with 75% EtOH (pellet will not go into solution). Discard the EtOH and dry the pellet for 5 min in a speed-vac at 40°C.

7. Resuspend the pellet in 100 μL of 0.5% SDS and store at –70°C. When thawing do short cycles of heat at 60°C, vortex and cool on ice until all the mRNA is completely in solution. Add 1 μL of mRNA to 99 μL of ddH_2O, measure OD_{260} and calculate mg/μL as $OD_{260} \times 4$.

3.7.2. Probing and Detecting Labeled Probes

1. Boil 4.2 g of agarose in 304.5 mL of water. Cool to 60°C and add 35 mL of 10× MOPS running buffer and 10.5 mL of 37% formaldehyde. Cast and prerun the gel for 1 h at 80 V.
2. Prepare a premix with 5 μL of 10× MOPS running buffer, 8.75 μL of 37% formaldehyde, and 25 μL formamide. Add 10 μg of mRNA and bring the volume to 50 μL with ddH_2O. Vortex and incubate at 55°C for 15 min. Cool to room temperature, add 10 μL of formaldehyde loading buffer and 3 μL of ethidium bromide, load on to the gel, and run the gel at 60 V for approx 3 h or until the lower dye front is approx 3/4 down the length of the gel.
3. Place the gel on a UV source, trim away excess agarose at the line of the sample wells at the sides and approx 2 cm beneath the lower dye front. Take a photograph for records. The mRNA will appear as a faint smear down the length of the lanes indicating species of varying sizes.
4. Soak the gel in 10× SSC for 30 min.
5. Cut four equally sized pieces of 3M filter paper slightly larger than the gel and a piece of Hybond nylon membrane (Amersham, Arlington Heights, IL) the exact size of the gel. Also cut a 4-in stack of paper towel the same size as the filter paper.
6. Soak a domestic sponge with 10× SSC in a sandwich box to approx 1/3 of the depth of the box. Soak two pieces of 3M paper on the sponge and roll out air bubbles. Place the gel on top and cover the whole box with a piece of plastic wrap. On the 3M paper cut the plastic wrap the exact size of the gel, remove, and place the gel down on 3M paper. Place the Hybond membrane on the gel, roll out air bubbles, and place the last two pieces of 3M onto the membrane, roll out air bubbles, and add the paper towels. Finally place a glass plate over the whole assembly and add a counterweight to apply moderate pressure. Leave overnight to transfer.
7. Disassemble the apparatus but before removing the membrane mark the sample wells, the lanes, the orientation, and the mRNA side. Place the membrane on a piece of 3M paper and bake for 2 h at 80°C.
8. Prehybridize in 6–10 mL of prehybridization solution overnight at 42°C in a sealable bag.
9. Boil 5,000,000 cpm of the probe/mL of Northern hybridization solution and add 6–10 mL hybridization solution.
10. Remove prehybridization solution, add the probe/hybridization solution, and hybridize overnight at 42°C.
11. Rinse the blot and wash 2× with 1× SSC/0.1% SDS for 15 min and 2× with 0.25× SSC/0.1% SDS for 30 min.
12. Expose filter to X-ray film.

3.8. Western Blot Analysis for Lactoferrin and β-casein Expression

1. Add 1 mL of dispase to each 35-mm dish and incubate at 37°C for 1 h to digest the basement membrane.
2. Transfer cell clusters to microfuge tubes and centrifuge the cell suspension at 300g in a microfuge at room temperature for 4 min, remove the supernatant, and wash the cells 3× with DMEM/F12. Make sure to resuspend the pellet each time. It is important to remove any remaining ECM proteins and all of the Dispase from the cell pellet prior to lysis.
3. Add 75 mL of RIPA$^+$ lysis buffer to the pellet, stand 15 min on ice, vortex for 10 s, and spin in a microfuge at maximum speed for 30 min at 4°C (**Note 6**).
4. Collect the supernatant and discard the pellet. Supernatant can be stored at –70°C and should be thawed on ice before use.
5. Measure the protein concentration using a Lowry assay modified for SDS containing solutions. Dilute 10 μg of protein in an equal volume of 2× sample buffer. Boil the samples at 100°C for 10 min.
6. Separate proteins on a 13% polyacrylamide gel under reducing conditions.
7. Wet transfer the proteins on to PVDF membrane.
8. Block at 4°C for 12 h with blocking solution.
9. Wash blots 3 × 5 min each with TBS-T.
10. Incubate overnight at 4°C with rabbit anti lactoferrin or mouse anti-β-casein antibodies (**Notes 7** and **8**).
11. Wash 3 × 5 min each and 2 × 10 min each with TBS-T.
12. Incubate for 1 h at room temperature with anti-rabbit or anti-mouse HRP in 1% BSA/TBS-T (1:5000).
13. Wash 3 × 5 min each and 2 × 10 min each with TBS-T.
14. Wash 3 × 5 min each and 2 × 10 min each with TBS only (**Note 9**).
15. Dab blot on tissue paper protein side up, agitate hard in ECL solutions protein side down, and develop for ECL.

3.9. Immunofluorescence

Immunofluorescence is carried out on cells cultured on glass cover slips. This is accomplished by placing 18-mm cover slips in 35-mm culture dishes prior to the treatment of the dishes with polyHEMA and/or the plating of the cells.

1. Aspirate off the media from the cover slips and rinse once with cold PBS.
2. To fix cells, completely submerge the cover slip with freshly prepared 3.6% paraformaldehyde and let it stand at room temperature for 20 min. After fixing, cells can be stored at 4°C in 1% paraformaldehyde/ PBS for approx 1 wk.
3. Rinse 3 × 5 min each with cold PBS.
4. Permeabilize 5 min with 0.5% Triton X-100 in PBS and wash 3 × 5 min each with PBS.
5. Block with 10% normal goat serum for 20 min to decrease nonspecific staining.
6. Incubate with mouse anti-β-casein antibody or rabbit anti-lactoferrin antibody for 1 h at room temperature. To minimize the use of antibody, place the coverslip

cell side down on a 75-μL drop of the antibody solution placed on parafilm, and incubate in a humid chamber.
7. Rinse 4 × 5 min each with cold PBS.
8. Incubate with FITC conjugated anti-mouse or anti-rabbit antibody (1:100) for 1 h as in **step 5**.
9. Rinse 4 ×5 min each with PBS.
10. Mount cover slips cell-side down on clean microscope slides with a drop of anti-fade solution in glycerol. Seal the cover slip to the slide with nail polish to avoid drying out.
11. Observe the staining using a fluorescent microscope with a 460-nm filter setting.

4. Notes

1. When working with Matrigel it is very important to prevent gel formation prior to use as a soluble overlay. Therefore, store the Matrigel stocks in aliquots at –70°C and when needed thaw slowly overnight on ice in a 4°C refrigerator. Always keep Matrigel on ice while working.
2. When making 4% BSA add BSA to TBS-Tween and let stand overnight at 4°C. Do not stir.
3. All the solution and media volumes are set for 100-mm tissue-culture dishes. Change the volumes according the surface area of the dish (i.e., 1/2 for 60 mm and 1/4 for 35-mm).
4. Maintaining a pure epithelial cell population is important for differentiation studies. With subculturing, scp2 cells can drift from an epithelial cell population to an epithelial-fibroblastic mixed cell population. The fibroblasts can be removed by differential adhesion. After resuspending the cell pellet in 10 mL of growth media, plate all the cells in a 100-mm dish. After 30 min, carefully collect all the unattached cell and split 1:4 in 100-mm dishes. This suspension contains epithelial cells while fibroblastic cells are left behind in the original dish.
5. Always use plastic pipets when working with ECM-containing solutions to prevent absorption to glass.
6. Lyse the cells on ice with ice cold lysis buffer. Prior to the addition of 2× sample buffer, always keep the samples on ice to prevent protein degradation.
7. Primary antibodies can be reused many times if 0.02% sodium azide is added to the antibody solution.
8. To avoid confusion with nonspecific antibody binding, always have an extra blot for mouse IgG or normal rabbit serum control.
9. Tween-20 can interfere with the ECL reaction; therefore, it is very important to wash off all the Tween-20 from the blots.

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