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Johannes Boonstra

G1 Phase Progression

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LANDES BIOSCIENCE / EUREKAH.COM
GEORGETOWN, TEXAS
U.S.A.

KLUWER ACADEMIC / PLENUM PUBLISHERS
NEW YORK, NEW YORK
U.S.A.

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Molecular Biology Intelligence Unit

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Kluwer Academic / Plenum Publishers

Kluwer Academic / Plenum Publishers, 233 Spring Street, New York, New York, U.S.A. 10013
http://www.wkap.nl/

Please address all inquiries to the Eurekah.com / Landes Bioscience:
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Phone: 512/ 863 7762; FAX: 512/ 863 0081; www.Eurekah.com; www.landesbioscience.com.

G1 Phase Progression edited by Johannes Boonstra/CRC, 6 x 9/ Landes/Kluwer dual imprint/
Landes series: Molecular Biology Intelligence Unit

ISBN: 0-306-47831-5

Library of Congress Cataloging-in-Publication Data

G1 phase progression / [edited by] Johannes Boonstra.
p. ; cm. -- (Molecular biology intelligence unit)
Includes bibliographical references and index.
ISBN 0-306-47831-5
1. Cell division. 2. Cell cycle.
[DNLM: 1. G1 Phase--physiology. QH 605 G995 2003] I. Boonstra, Johannes. II. Molecular biology intelligence unit (Unnumbered)
QH605.G123 2003
571.8'44--dc21

2003012217

Dedication

To my wife Jannie and children Wijnand, John and Hilja.

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PREFACE

One of the most intriguing properties of life concerns the ability of organisms to reproduce. Whether the organism consists of only one cell or of trillions of cells, individual cells must be able to grow and to divide. A cell reproduces by duplicating its contents during a period of growth followed by division into two identical daughter cells. This process is called the cell cycle.

Progression of cells through the cell cycle is strongly dependent upon external factors. For single cell organisms the presence of nutrients in the environment is essential for cell cycle progression, and these nutrients will activate signal transduction systems in the cell that regulate cell cycle progression. Other external factors, such as temperature, osmolarity, pH, will have specific effects on cell cycle progression. In multicellular organisms, in addition to these factors, external factors created by other cells also may have effects on cell cycle progression.

Understanding the mechanism by which single cell organisms reproduce themselves will reveal the way these organisms are able to cope in an ever-changing environment. In multicellular organisms the reproduction from, one mature organism towards a second one is even more complicated, as in multicellular organisms the cells not only have to reproduce themselves, but they have also to undergo complex differentiation steps to form a functional organism.

During the last few decades it has become clear that many problems that arise in multicellular organisms are related in many cases to modifications in the molecular machinery that underlay cell reproduction. The most apparent example in this respect concerns cancer, one of the most prominent diseases for mankind in this time. In addition, environmental hazards, such as UV irradiation and chemical pollutants in many cases exert their effects on organisms by modification of the cell reproduction machinery. During the last few decades these problems have led to an enormous interest in cell cycle research, and because of this a wealth of information has been obtained regarding the molecular mechanisms that underlay cell reproduction. However, the precise nature of the regulation of cell cycle progression is still not resolved in full detail. This is mainly due to the complex protein networks in the cell that regulate cell cycle progression and the interaction of these networks with signal transduction complexes. The composition and localization of these networks is largely dependent upon specific conditions, resulting in almost endless possibilities of interaction. So the precise knowledge of how cells regulate their cell cycle will last for many more years, although the developments in molecular biology and technology ensure a wealth of new information.

In this contribution, several specialists describe the current knowledge of the molecular networks that regulate cell cycle progression, with an emphasis on the G1 phase of the cell cycle. In the first part, the individual

molecules that form the network are described, including cyclins, cyclin-dependent kinases, inhibitors of these kinases and retinoblastoma and p53. The second part describes the signaling cascades by which external factors influence the cell cycle network, including mitogens, the extracellular matrix, nutrients and oxygen radicals. Then the effects of specific external conditions on cell cycle progression are presented, such as serum starvation and subsequent re-addition and stress conditions (heat, osmolarity). In the last part, two chapters are presented that describe the relation between cell cycle progression with cell differentiation and with apoptosis.

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Chapter 1

Restriction Points in the G1 Phase of the Mammalian Cell Cycle

Johannes Boonstra

Abstract

Progression through the cell cycle of mammalian cells is dependent upon external factors such as growth- and extracellular matrix factors. On the other hand, cell cycle progres sion can be inhibited by the addition of specific inhibitors of cell proliferation, or by stress conditions. In most cases, the cell cycle arrest occurs in the G1 phase of the cell cycle, and therefore this cell cycle phase plays a crucial role in the regulation of cell cycle progression. The restriction point R is defined as the point in the G1 phase after which the cells are independent of growth factors for progression through the remainder of the cell cycle. In this chapter a brief overview is presented on the molecular identity of R. Most evidence points to a close relationship between R and the functioning of the retinoblastoma protein. In addition, evidence is presented that in addition to R, also other arrest points are present in the G1 phase.

Introduction

The most fundamental property of living organisms appears their ability to reproduce themselves. This capacity is based upon the fact that cells are able to duplicate their DNA during a certain period of their life span followed by division into two genetically identical daughter cells. This cycle of DNA duplication followed by cell division is known as the cell cycle. Due to this process, a multicellular organism is able to grow from one single fertilized oocyte. However, multicellular organisms do not just consist of a multitude of identical cells, but in contrast are composed of an enormous amount of different cells. Some of these cells proliferate, while others are not proliferating anymore, but are differentiated into cells with a defined function like muscle and nerve cells. This means that during development, some cells of an organism are switching from a proliferative state into a differentiated, nonproliferative state, indicating that during development these cells stop their progression through the cell cycle. In addition, during development, but also in mature organisms, other cells are induced for programmed cell death, so-called apoptosis, due to developmental programs or due to external conditions causing damage to the cells. These observations indicate that decision points are present in the cell cycle at which cells may decide to continue, or in contrast, to stop progression through the cell cycle.

In virtually all cells, the cell cycle consists of four discrete phases, being the DNA synthesis phase (S phase), the M phase or mitosis when the DNA is segregated and the cell actually divides into two daughter cells, and the gap phases between the two during which the cells

G1 Phase Progression, edited by Johannes Boonstra.

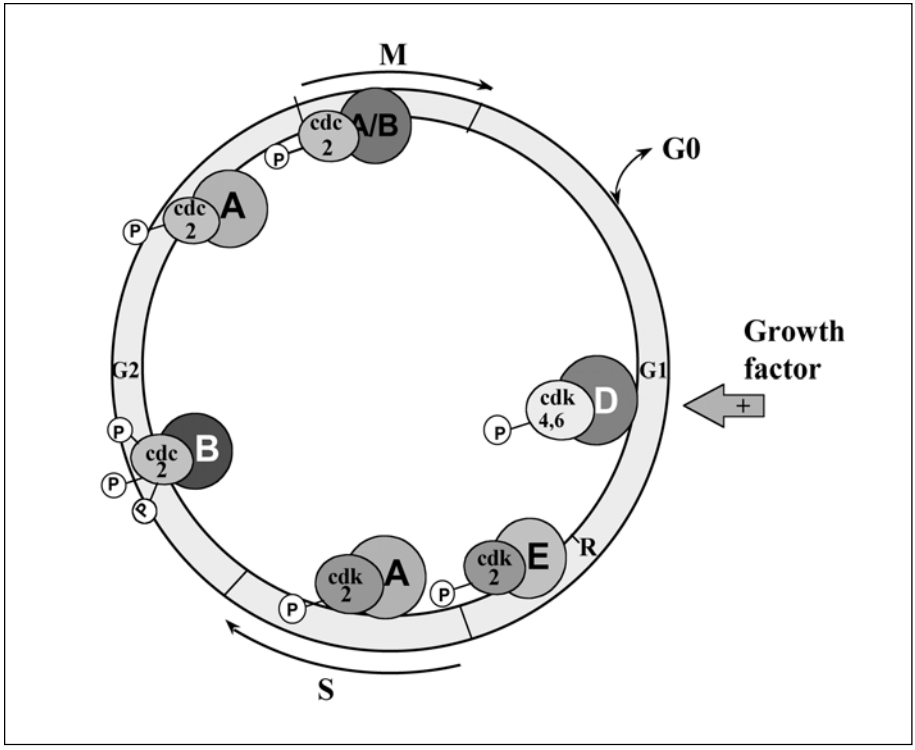

Figure 1. Overview of the cell cycle in mammalian cells. The mammalian cell cycle basically consists of four phases: first gap phase (G1), DNA synthesis (S), second gap phase (G2) and mitosis (M). The transition between the different phases is regulated by cyclin/cdk complexes. Different cyclins (A, B, D and E) are present during different cell cycle phases and interact with different CDKs. As long as growth factors are present, adherent cells will continue to proliferate. In the absence of growth factors cells will stop dividing and enter the quiescent state (G0). With permission from 102

grow in size. The G1 phase is situated between M and S phase and the G2 between the S and M phases (Fig.1). G2 phase cells contain twice the amount of DNA as the G1 phase cells. In mature organisms, most of the nonproliferating cells have been demonstrated to contain a G1 phase amount of DNA, indicating that the decision to pass through the cell cycle or to stop progressing through the cell cycle is usually situated in the G1 phase. Therefore, the G1 phase plays a prominent role in cell cycle regulation and thus in the functioning of multicellular organisms. Consequently, if the G1 phase regulation of the cell cycle is disturbed, the organism faces enormous problems, as is the case in the development of cancer when uncontrolled cell proliferation occurs, or when cells differentiate prematurely. In this chapter I will discuss the decision points present in the G1 phase of mammalian cells as well as their role in the regulation of cell cycle progression, with the emphasis on the restriction point (R).

The Restriction Point

Cell cycle research has gained enormous attention since methods were developed that allowed cell growth under tissue culture conditions, and since methods were developed that allowed synchronization of large amounts of cells in a particular phase of the cell cycle. Already in these early days it became clear that optimal culture conditions for cell proliferation required

the presence of a large number of components in the culture medium, in addition to nutrients especially polypeptide growth factors. These growth factors were usually added by using sera from fetal or newborn origin. If normal cells were depleted of these growth factors, the cells responded by arrest of proliferation and became quiescent. In this quiescent state the cells contained a G1 phase amount of DNA. Therefore it was suggested that the cells entered the quiescent state from the G1 phase, or in other words, the G1 phase contained a decision point at which the cells decided to continue progressing through the cell cycle or to arrest proliferation. As soon as this decision point was passed, the cells became independent of those external factors, as was shown already in 1971 by Temin,[1] who demonstrated that chicken cells became independent of mitogenic factors several hours before entry into S phase. These findings were supported by studies of Pardee who introduced then the term restriction point R.[2] R is defined as the point after which the cell is committed to enter S phase, more or less independent of the external conditions. Furthermore, R can be considered as different from other checkpoints in the cell cycle. These latter merely control whether the metabolic household of the cell is in order, that the genome is intact and that previous cell cycle phases have been finished properly.[3] R was proposed to represent one single point in the G1 phase at which the cells decide to continue to proliferate or to enter the quiescent state, the so-called G0 phase. Conditions that shift cells into a quiescent, nondividing state, include in addition to deprivation of growth factors, also limitation of nutrients or some amino acids, addition of certain drugs and high cell density (reviewed in 4), although it should be realized that such conditions are normally not occurring in an intact organism. Therefore, the presence of growth factors seems to represent the physiological regulator of G1 phase progression and hence is required to pass R.

Evidence in favor of a defined position of R in the G1 phase was obtained by elegant studies of Zetterberg and collaborators using time-lapse analysis of mammalian cells in culture.[5,6] Using asynchronously growing cell populations, this approach allowed the analysis of cell cycle progression of individual cells and their response to environmental changes. Thus it was demonstrated that cell cycle progression was rapidly interrupted in the early G1 phase by a short period of growth factor starvation.[6] Interestingly only cells younger than 3 hours postmitosis responded in this way, cells older than 4 hours postmitosis were not arrested but advanced through the remaining part of the cell cycle with the same speed as untreated cells. The postmitotic cells that were arrested in G1 upon growth factor starvation were indicated as G1-pm (G1-postmitosis) cells, while the remaining population of G1 cells was indicated as G1-ps (G1-pre S phase) cells. The latter were able to initiate DNA synthesis in the absence of growth factors.[6] The transition from G1-pm to G1-ps cells represents the passage of R. Detailed analysis of these cell populations demonstrated clearly that the transition period between these two G1 cell populations occurred in a narrow time frame lasting about one hour. Furthermore, cells in the G1-pm period were shown to react quickly on growth factor starvation.[6] Another interesting observation was that addition of growth factors to starved G1-pm cells did result in cell cycle progression, but the delay in total cell cycle time was considerably higher than the starvation time. Detailed analysis demonstrated that at least in normal mammalian cells the delay in cell cycle time was about 8 hours, independent of the starvation time. These findings suggested that in the G1-pm period the cells leave the cell cycle upon growth factor starvation, and that upon readdition of growth factors the cells have to recover from this treatment.[6] The strength of these observations comes from the fact that the results were obtained with nontransformed, normal cells, and that no specific treatments were required as addition of inhibitors or other drugs or application of synchronization methods.

The presence of R in the G1 phase has been widely accepted in literature, but it should be mentioned here that also an alternative view of cell cycle regulation has been developed, the so-called continuum model of cell cycle regulation (reviewed in:7). According to the continuum model, cell cycle regulation does not occur by specific decision points in the G1 phase,

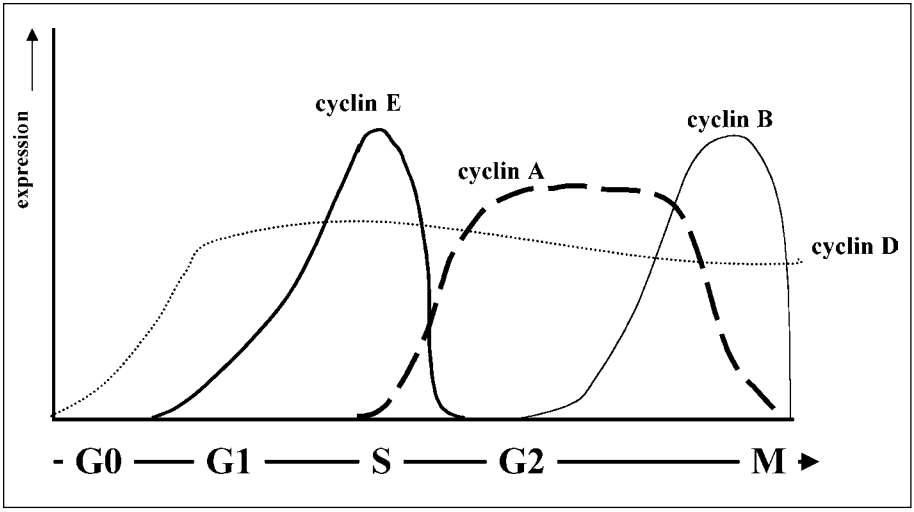

Figure 2. Expression of cyclins during the mammalian cell cycle. Upon stimulation of quiescent cells (G0) different cyclins are orderly expressed. Cyclin D is absent in quiescent cells, but rapidly accumulates after growth factor stimulation. Cyclin D is subsequently expressed constitutively throughout subsequent cell cycles while expression of cyclins E, A and B is related to a specific cell cycle phase. With permission from ref. 102.

but in contrast is the result from processes occurring in all cell cycle phases. The model proposes that the regulation of cell cycle progression is the result from mass accumulation. This mass accumulation includes specific molecules that trigger the initiation of DNA replication. Thus in the experiments described above,[6] the delay in cell cycle time of G1-pm cells was due to the delay in mass accumulation in the early cell cycle. Furthermore, it was proposed that the G1-ps cells would exhibit a cell cycle delay in the second cell cycle, as was indeed deduced from analysis of cells in the second cell cycle.[8,9] These observations and the conclusions about the presence or absence of R in the G1 phase indicate, that a definite proof about R requires a detailed knowledge of the molecular mechanisms underlying regulation of G1 phase cell cycle progression. Research during the past 20 years has yielded a wealth of information on proteins that play a significant role in cell cycle regulation, and cyclins and cyclin dependent kinases (CDKs) appear to fulfill a central role in this regulation.

Cyclins and Cyclin Dependent Kinases

The identification of the key molecules responsible for cell cycle regulation originated from the discoveries of maturation promoting factor (MPF) in frog oocytes,[10] of CDC proteins in temperature-dependent mutants of *Saccharomyces cerevisiae*,[11] and of cyclins in sea urchin oocytes.[12] These initially independent studies converged by the finding that MPF consisted of two key subunits, i.e.,the cyclin and the cyclin-dependent kinase (CDK).[13] CDKs are serine/threonine protein kinases that require binding of a cyclin in order to be ready to become activated. Mammalian cells contain multiple CDKs that are activated by multiple cyclins (reviewed amongst others in 14-17). CDK activity is regulated by several processes, including phosphorylation on threonine and tyrosine residues, some of these phosphorylation steps are stimulatory, while others are inhibitory (see also Chapter 3). In addition a large family of inhibitor proteins have been discovered which may inhibit CDK activity by either binding to CDK alone or to the complex of CDK with its cyclin (see also Chapter 4).

Different cyclin-CDK complexes are required for different cell cycle events (Fig.1). The most important mammalian cyclin-CDK complexes known so far are the mitotic cyclins A and B in association with CDC2 and the G1 cyclins, cyclins D and cyclin E, in complex with CDK4/6, and CDK2 respectively (Fig.2).[18-21] The first cyclin-CDK which is activated during the G1 phase is composed of a D-type cyclin in association with CDK4 or CDK6 depending on the cell type.[21] As cells progress through the G1 phase, cyclin E is synthesized with a peak late in G1 (Fig.2). Cyclin E associates with CDK2 and has been demonstrated to be essential for entry into S phase.[22] Once cells have entered S phase, cyclin E is degraded and CDK2 then associates with cyclin A.[23] Finally, cyclin A and the B-type cyclins associate with CDC2 (=CDK1) to promote entry into mitosis. Cyclin A binds to CDC2 with a peak of activity in G2 phase and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B-CDC2. For exit from mitosis, cyclin B destruction is required.[24]

Since the D-type cyclins and cyclin E play a prominent role in G1 phase progression, it is tempting to suggest that these cyclins with their respective CDK partners underlie the molecular mechanism of R as discussed above. Indeed, during the last decade, a wealth of information has been obtained suggesting the involvement of the D and E cyclins and their respective CDK partners in G1 phase arrest, and hence in the passage of the cells through R. This information included experiments in which it was shown that microinjection of cyclin D1 antisense plasmids or monoclonal anti-cyclin D1 antibodies resulted in an inhibition of S phase entry.[25,26] Similar experiments indicated also an important role for cyclin E and its partner CDK2[27-29] in G1 phase progression. Furthermore, mitogenic stimulation of cells was reported to be associated with induction of cyclin D1,[30-33] while inhibition of G1 phase progression by various inhibitors was associated with suppression of cyclin D expression or inhibition of cyclin D-associated CDK activity.[34-38] Finally, overexpression of cyclin D has been implicated in the development of a wide range of tumors, yielding cells with a relatively short G1 phase (reviewed in 39, 40). However, the nature of R itself is not defined by the G1 phase cyclins or their CDK partners themselves, as it was demonstrated in some studies that overexpression of cyclin D1 may result in a G1 arrest,[41,42] while other cyclin D isoforms, i.e.,cyclin D2 and D3, have been implicated to be involved in the maintenance of the nonproliferative state or in cell differentiation.[43-45] Furthermore, coexpression of cyclin D2 and Ha-Ras under low serum conditions can induce a scenescence-like phenotype.[46] These results suggest that D-type cyclins may have different roles depending on their levels of expression and cell type and therefore it is tempting to suggest that R is determined by components downstream of the cyclins D and E in the G1 phase of the cell cycle.

Downstream of Cyclin D and Cyclin E

One of the most important cyclin/CDK substrates in mammalian cells during the G1 phase is the product of the retinoblastoma tumor suppressor gene (pRB) (for details see chapter 5). pRB is phosphorylated in a cell-cycle-dependent manner and binds in the hypophosphorylated state to transcription factors, particularly members of the E2F family (Fig. 3).[47-51] E2F consists of at least five different isoforms that form heterodimers with a second group of proteins known as DP-1.[49,52,53] pRB is present in this hypophosphorylated form during early G1 and becomes phosphorylated on several residues during mid- to late G1. This phosphorylation causes the release and activation of the E2F transcription factors, allowing transcription of genes that mediate progression through S phase.[54] Initial activation of pRB is thought to occur in the G1 phase by phosphorylation by cyclin D/CDK complexes. D-type cyclins have been shown to bind directly to pRB in the absence of a kinase and thus might target the pRB to CDK4/CDK6 kinases. After the initial phosphorylation by cyclin D/CDK, cyclin E/CDK2 complexes are thought to subsequently phosphorylate pRB late in G1, thereby triggering the onset of S phase.[55,56]

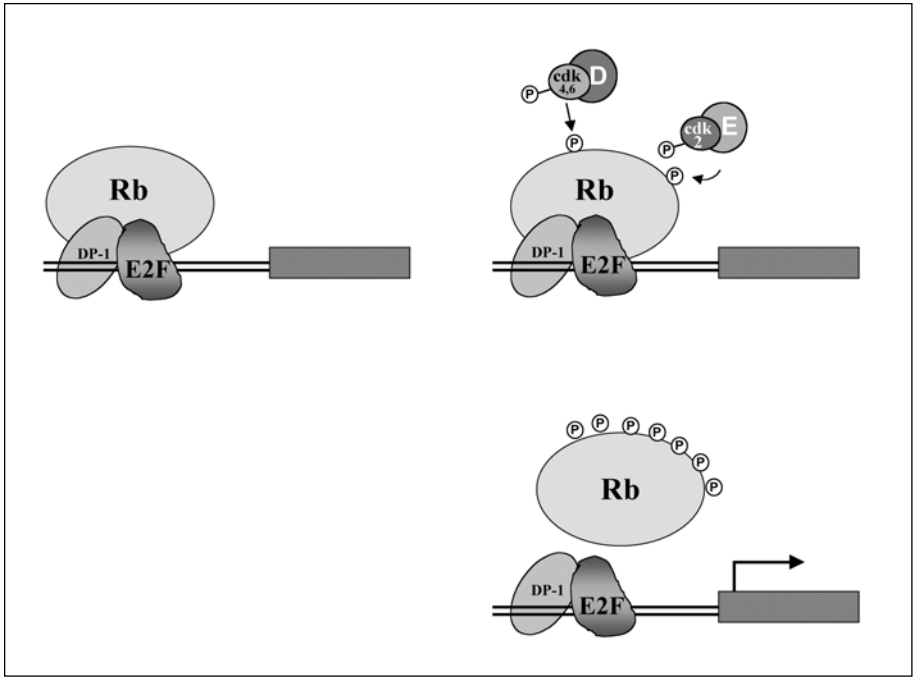

Figure 3. Phosphorylation of retinoblastoma (pRB) by cyclin D and cyclin E leads to gene expression. Nonphosphorylated retinoblastoma binds to the transcription factor complex E2F/DP-1, thereby inactivating transcriptional activity. Phosphorylation of pRB by cyclin D/CDK4,6 complexes during G1 phase, followed by phosphorylation by cyclin E/CDK2 at the end of G1 phase, results in release of the transcription complex from pRB, and consequently in the activation of the E2F complex and entrance into S phase. With permission from 102

Because of the connection between pRB functioning and activation of S-phase genes, it is tempting to suggest a coupling between pRB and R. This suggestion is supported by the observations that almost all cancers are associated with some defect in the pRB pathway.[57,58] Furthermore, pRB is targeted and inactivated by viral oncoproteins such as simian virus 40 (SV40) large T-antigen and human papillomavirus E6 and E7,[59,60] and microinjection of pRB resulted in G1 arrest in pRB-lacking cells.[61] Staurosporine, a protein kinase inhibitor, has been shown to induce G1 phase arrest in normal cells, but not in most transformed cells. Furthermore, when pRB-defective cells were infected with a pRB-expressing retrovirus, staurosporine caused G1 phase arrest.[62] These and many other observations indeed suggest that pRB constitutes at least an important component of R in mammalian cells. According to the scheme described above, both cyclin D1 and cyclin E in combination with their CDK partners are responsible for pRB phosphorylation, and hence for passage of R. The role of cyclin E in this process is however rather complicated. Cyclin E has been implicated in the phosphorylation of pRB during late G1 phase, but in addition cyclin E expression has been demonstrated to be E2F dependent.[63,64] Furthermore, it has also been shown that cyclin E regulates a rate-limiting step in entry into S phase that is distinct from pRB phosphorylation. Ectopic expression of cyclin E, but not of cyclin D1, can override G1 arrest in a phosphorylation-deficient mutant of pRB. These studies demonstrated that cyclin E induced S-phase and completion of the cell cycle can occur in the absence of E2F-mediated transactivation.[65-67] This effect of cyclin E was

demonstrated to be due to a direct activation of cyclin A promoter activity, whereas under normal conditions the cyclin A promoter is activated by pRB through the cell cycle regulatory element (CCRE).[68] Indeed, ectopic expression of cyclin A reversed RB-mediated G1 arrest.[68] A similar situation has been obtained in cells transfected with the Abelson murine leukemia virus. In these cells, vAbl blocks the serum-induced entry into S phase without interfering with cyclin D1 or cyclin E induced CDK activation and subsequent hyperphosphorylation of pRB. However, in these cells the accumulation of cyclin A mRNA and protein was demonstrated to be attenuated.[69] Altogether these observations suggest that the activation of CDK2 by cyclin E in collaboration with E2F which results in the induction of cyclin A form probably the molecular basis of R. Of interest are computer simulations that indicate two important aspects in the regulation of cyclin E/CDK2 activation underlying R.[70] These aspects include the positive feedback loop between CDC25A phosphatase activity and cyclin E/CDK2 activity and the antagonistic interaction between the CDK inhibitor p27 and cyclin E/CDK2. The role of the phosphatase and inhibitor protein is described in more detail in chapters 3 and 4 respectively. Similar studies revealed a possible role of pRB by regulating E2F activity.[71,72] However, using the time-lapse analysis, Zetterberg and collaborators demonstrated recently that cyclin E was not required for passage of R of normal human diploid fibroblasts, indicating that the molecular component of R is situated upstream of cyclin E.[73]

The observations described briefly above indicate a possible molecular target of R of the G1 phase of the cell cycle, however the real nature of R is still not clear yet. This is caused by the wealth of information obtained from tumor cells in which deregulations were observed in the molecular pathways associated with pRB function, the information from cells overexpressing parts of these pathways, or in contrast, from cells in which particular components were inactivated or knocked out. As a cell is an independent and dynamic unity, all these modifications from the normal pathways may result in deviations from this normal situation, which enable the cells to by-pass the normal regulatory pathways, and hence at this moment it seems not possible to pinpoint the molecular entity that represents R. But, as stated above, the nature of R seems to be closely related to pRB, E2F and cyclinE/CDK2 functioning at the end of the G1 phase.

Arrest in the G1 Phase

Regulation of cell proliferation in a multicellular organism is determined by external factors, including growth and extracellular matrix factors. However, under particular conditions the cells may escape from the cell cycle, regardless the presence of the proliferation inducing conditions as described above. This may occur under physiological conditions, when the cells follow a differentiation program or when they are induced for apoptosis and under nonphysiological conditions. Cells may escape from progression through the cell cycle in an organism, for example in case of extremes in temperature or after irradiation, or upon application of proliferation inhibiting drugs. In addition, tissue culture grown cells can be subjected to even other conditions that induce growth arrest, for example by depletion of growth factors or nutrients. The question arises whether under these partly physiological and partly nonphysiological conditions cell proliferation is arrested in the G1 phase at R or whether other arrest points exist in the G1 phase. This question however, is difficult to answer, since no defined molecular characteristics are associated to the restriction point, and therefore it is difficult to judge whether in vivo a cell under particular conditions is arrested at R or not. Even in vitro this question is hard to answer, since under proliferation inhibitory conditions, the cells are manipulated by external factors and these manipulations may affect the cells on other levels as well. Nevertheless many attempts have been made to define the phase in the cell cycle where cells stop the progression through the cell cycle and start a differentiation program, become nonproliferating or are induced for apoptosis.

The differentiation of cells in vivo was associated with an arrest in the G1 phase of the cell cycle as described as early as in 1968 by Baserga.[74] Early studies of Scott and collaborators suggested that prior to differentiation of proadipocytes, growth arrest had to occur at a G1 arrest state designated G_D, and this state was distinct from other G1 arrest states as induced by serum or nutrient deprivation.[75,76] Since these early studies, cell differentiation has been studied on a molecular level, especially the differentiation of pheochromocytoma cells induced by nerve growth factor (NGF) gained wide interest.[77] Addition of NGF to these cells caused arrest of cell proliferation, and the induction of a differentiated phenotype.[78] DNA content analysis of PC12 cells revealed that upon the induction of differentiation the cells were indeed arrested in the G1 phase of the cell cycle.[79] Furthermore, these cells were characterized by high levels of cyclin D1/CDK4/p21 complexes and low levels of CDC2 or CDK2 associated complexes, while the kinase activity corresponding to these complexes was significantly decreased.[80] The high levels of cyclin D1 were caused by the continuous presence of serum in these experiments. Removal of serum resulted in a decrease of cyclin D1, but had no effect on the differentiated phenotype of the cells.[80] Induction of differentiation of another cell line, i.e.,human myeloblastic leukemia cells, by transforming growth factor β1 was accompanied by loss of expression of cyclin E and CDK2 and increased expression of cyclin D1 and CDK5. In addition, cyclin D2 and cyclin D3 were expressed to a higher level in differentiating cells as compared to proliferating cells.[81] High levels of cyclin D3 expression were also observed in HL60 and other cells induced for differentiation, suggesting a general role for cyclin D3 in induction or maintenance of differentiation.[44] In addition to cyclin D3, also the retinoblastoma protein relative pRB2/p130, as well as pRB itself, have been implicated to play a role in differentiation.[82,83] pRB interacts with several differentiation-specific transcription factors, such as MyoD, and activates transcription.[84] These observations suggest that upon induction of differentiation, the cells stop progression of the cell cycle during the G1 phase at a position that may reflect R.

Another physiological situation for cells to stop progression through the cell cycle concerns the entrance of an apoptotic pathway, (for details see chapter 12). Apoptosis, or programmed cell death, enables an organism to eliminate damaged or useless cells in an orderly manner without the induction of an inflammatory reaction. Apoptosis is required to maintain tissue homeostasis and is required for several developmental processes.[85] Different studies have indicated that CDKs and cyclins participate in apoptosis, especially cyclin A/CDK complexes (for review see 86). However, during apoptosis CDK activation appears to be the consequence rather than the cause of the induction of apoptosis. Both CDK2 and CDC2 appear to play a role in apoptosis, and both CDKs act normally in S and G2 phases rather than in the G1 phase. As such, R seems not to represent the point in the cell cycle where apoptotic cells leave the cell cycle. On the other hand, induction of apoptosis by external factors such as serum deprivation, hypoxia, or conditions leading to DNA damage, has been demonstrated to involve the action of p53, and normally the activity of p53 may lead to arrest of cell cycle progression. Some pro-apoptotic proteins, Bax and IgF-Bp3 appear transcriptional targets of p53, while in addition p53 induces transcription of Fas.[87] Fas is a cell surface protein that triggers apoptosis upon ligand (FasL) binding. In addition, p53-induced apoptosis may also occur independent of its transcriptional function.[88] Also these studies indicate no specific role for R in apoptotic cells.

All other studies dealing about arrest of cells in the cell cycle are performed using nonphysiological or extreme situations, and under these conditions many cellular components and/or cellular interactions may be affected which cause subsequent arrest of cell cycle progression. For example, deprivation of cells from serum or growth factors will result in a relatively rapid degradation of cyclin D expression.[89] As a consequence so-called quiescent G0 cells have no cyclin D and all down stream effects are inhibited, including pRB phosphorylation[90] and E2F activation. Addition of inhibitors influence in many cases also cyclin D and cyclin E expression.[34,35,62,91-94] Exposure of cells to stress conditions, irradiation, heat or others, usually

results in the activation of p53 and consequently in the expression of p21-$^{cip1/waf1}$, resulting in inhibition of cell cycle progression.[95-101] In this respect some interesting results were obtained in our laboratory using synchronized CHO cells.[102] In these studies it was demonstrated that CHO cells react differently on serum withdrawal depending upon their position in the cell cycle. When growth factors are removed immediately after mitosis, the cells appear to enter a G0-like state:signal transduction cascades are not activated, the expression of cyclin D and pRB rapidly decreases and no DNA synthesis occurs. When the cells are depleted from serum later in G1, also no DNA synthesis takes place, but signal transduction cascades appear active, cyclin D is still expressed after overnight starvation. These findings suggest that in CHO cells (and possibly in other cells as well), at least two serum-dependent restriction points exist that regulate progression through the G1 phase.[102] The first restriction point is found to be located within 10 – 15 minutes after mitosis and may correlate with the point described previously[103] that leads to apoptosis.[102] The second serum-dependent point is located at the end of mid-G1 and appears to represent R.[102]

The observations described briefly above, indicate that under normal, physiological conditions when the cells leave the cell cycle to start a differentiation program, cell proliferation is arrested in the G1 phase of the cell cycle most likely at a site representing R. In contrast, physiological induction for apoptosis seems not to be related to the G1 phase, but rather to the G2 phase of the cell cycle. Interestingly, apoptotic cells resemble also morphologically to some extent mitotic cells and evidence has been obtained that apoptosis occurs directly following mitosis.[102] Other conditions that lead to cell cycle arrest, especially conditions that lead to damage to the cells (heat, irradiation), or exposure to proliferation inhibiting drugs, include in many cases an inhibition of cyclin/CDK activity by the enhanced expression of CDK inhibitor proteins. In this case the arrest may occur independently from the pRB functioning and thus not be related to R, although in some cases R may be involved.

In conclusion, evidence obtained so far indicates that cells have several arrest points in the G1 phase, one of them being R. The underlying molecular network of R seems strongly related to the functioning of pRB. However, it should be realized that most of the experiments were performed using transformed cell lines grown on an artificial substrate in the presence or absence of serum and in some cases overexpressing proteins. These conditions are far away from the in vivo situation of a normal cell, and future experiments using highly sophisticated single cell methods have to prove the validity of the current views on regulation of G1 phase progression.

Acknowledgements

I would like to thank my colleagues Arie Verkleij, Esther Hulleman, Siegfried de Laat and Jose Bijvelt for their valuable suggestions on this manuscript.

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Chapter 2

Cell Cycle Regulation during G1 Phase in Yeast:

Decisions, Decisions, Decisions

Curt Wittenberg and Karin Flick

Abstract

Coordination of cell cycle events is essential for the faithful duplication of cellular components during proliferation as well as for the adaptability of cells and organisms to varying internal and environmental conditions. In budding yeast, coordination of many of cell cycle events is imposed at START, a regulatory event during late G1 phase. As in other eukaryotes, cyclin dependent protein kinases (CDKs) are largely responsible for cell cycle regulation and for providing responsiveness to environmental signals. Consequently, understanding the mechanisms governing the activity of G1-specific forms of the CDK and the signals that modulate their activity is critical for understanding the regulation of cell proliferation. This chapter summarizes our current state of understanding of the landmark events of G1 phase of the budding yeast cell cycle with particular attention to their regulation by CDK.

Introduction

The capacity to proliferate is central to the development and propagation of biological systems. However, that capacity must be regulated with regard to the internal and extracellular conditions if those processes are to be successful. In most cells of eukaryotic systems the primary regulation of cell proliferation is imposed predominantly during the G1 phase of the cell cycle. The budding yeast, *Saccharomyces cerevisiae*, is no exception. The tractability of yeast to classical and molecular genetic approaches and the more recent advent of genome wide molecular genetic analysis has made budding yeast an excellent system for the study of the regulation of cell proliferation. Such studies, combined with the extensive body of data derived from other experimental systems, have provided our current view of the eukaryotic cell cycle and its regulation. Although it is immediately apparent that there are substantial differences between organisms in the specifics of the cell cycle machinery, there is a high degree of conservation of regulatory elements and motifs. It is in the context of conservation of function that we appreciate the malleability of the individual components of the cell cycle machinery that enables them to appropriately serve the specific needs of diverse cell types and organisms.

G1 Phase Progression, edited by Johannes Boonstra.

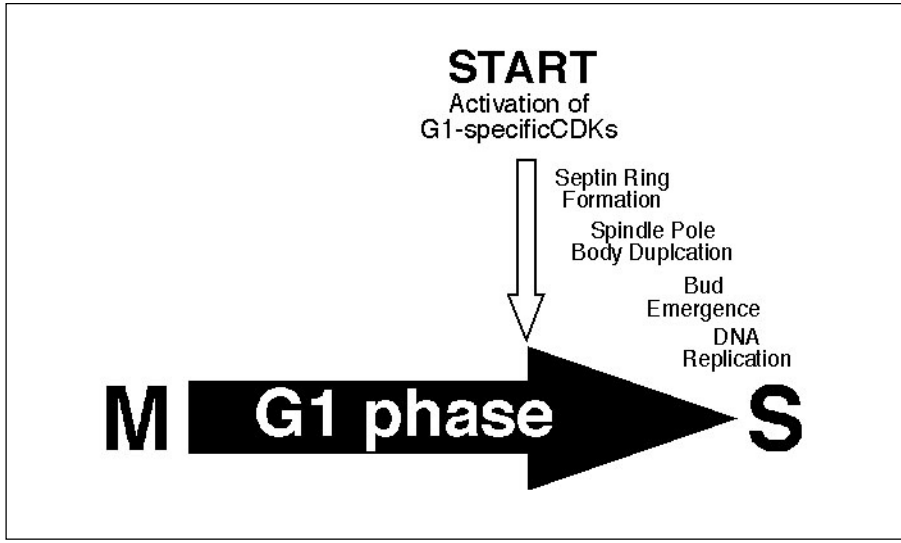

Figure 1. Relationship of START and landmark events of G1 phase. The G1 gating event START occurs during late G1 phase promoting the execution of multiple independent pathways that lead to the completion of the landmark events of G1 phase. The best molecular correlate of START is the activation of the G1 specific forms of the cyclin dependent protein kinase.

Defining G1 Phase: How Do We Know We Are There?

To understand the regulatory motifs operating during G1 phase of the cell cycle, we must first consider how G1 phase is defined and, consequently, what properties designate a cell as residing in the G1 interval. Historically, cell cycle phases were defined based upon the microscopically observable events of the nuclear cycle. Based upon that view G1 phase was defined as the interval between the completion of mitosis and the initiation of DNA replication. As such, G1 cells are characterized by the presence of a nucleus containing unreplicated chromosomal DNA. However, because we are interested in the manner in which the nuclear cycle relates to regulation of cell proliferation we must consider additional properties characteristic of cells in this interval.

Unlike the majority of other cell types, G1 cells of the budding yeast can be distinguished from nonG1 phase cells based upon their morphology. That is, during most of G1 phase cells lack a bud, whereas cells in all other phases of the cell cycle are budded (Fig. 4). This is a consequence of the existence of a rigid cell wall and the establishment of the asymmetric pattern of cell wall deposition during late G1 phase of the cell cycle. This peculiarity of budding yeast has been, in part, responsible for its particular usefulness for studies of late G1 phase events and for the recognition of a G1 regulatory event known as "START".[1]

Despite being called a "gap" phase, the primary integration of growth, developmental decisions and cell cycle progression occurs during the G1 phase of the cell cycle. In fact, several "landmark" events of the cell cycle occur during the G1 interval. These include 1) the assembly of the new bud site, 2) disassembly of the septin ring marking the site of cytokinesis in the previous cell cycle and assembly of a new septin ring at the newly selected bud site, 3) polarization of the actin-based secretory system toward the new bud site, 4) duplication of spindle pole bodies and 5) initiation of DNA replication. These events are each tightly coupled to START (Fig. 1).

Establishing the Nature of G1 Regulatory Events

What is START and how is it defined? Cells deprived of nutrients, either as a consequence of nutrient withdrawal or growth into stationary phase, accumulate during the unbudded portion of G1 phase of the cell cycle (reviewed in 2). Once a cell has committed to a new mitotic cell cycle it will complete that cycle prior to committing to another pathway despite the withdrawal of nutrients. Furthermore, either partial depletion or a decrease in the "quality" of nutrients results in a progressive lengthening of G1 phase. Together those observations suggest the existence of a nutrient sensing mechanism coupled to a gating event late in G1phase that restricts cell cycle progression. It is that gating event that is referred to as START.

However, START has relevance in addition to its role in modulating proliferation during the mitotic cell cycle. Under many starvation conditions both haploid and diploid cells arrest as unbudded G1 cells. Cells residing in the unbudded phase of the cell cycle are competent to commit to alternative developmental fates related to the sexual cycle (Fig. 2). Under appropriate conditions diploid cells arrested during G1 phase proceed through meiosis and sporulate, generating haploid progeny. Conversely, haploid cells, arrested during G1 phase in response to peptide mating pheromones secreted by cells of the opposite mating type will mate to form diploid cells. Thus, each of these alternative developmental pathways results in synchronization of cells during the unbudded G1 state in the presence of the appropriate stimulus. Together, these cellular behaviors also provide a basis for the definition of START.

A comparison of the regulatory systems discussed below and the mechanisms utilized in animal cells (see Chapter 1) will reveal many striking parallels. Among the most apparent is the presence of a gating event for cell cycle progression during late G1 phase preceding the initiation of DNA replication, START in yeast and the restriction point in animal cells (reviewed in 3). Although the complexity of the regulatory circuits and differences in the elements between the systems makes it difficult to draw one to one associations between the various elements of those circuits, the general regulatory motifs are conserved.

Cyclin Dependent Protein Kinases As the Molecular Determinants of Start

The molecular mediators of cell cycle transitions in eukaryotes are the cyclin dependent protein kinases (CDK's). All of the major cell cycle transitions occur as a consequence of modulating the activity of these heterodimeric enzymes. Regulation of entry to and exit from G1 phase in the budding yeast is no exception. In yeast, a single CDK catalytic subunit, the Cdc28 protein kinase, is sufficient for regulation of cell cycle progression (reviewed in ref. 4). It is referred simply as the CDK throughout this chapter.

Regulation of CDKs occurs largely via three mechanisms (reviewed in 4-5). First, the availability of the positive regulatory subunits, the cyclins, is regulated with respect to cell cycle position. In contrast, the level of the CDK catalytic subunit is constant. Next, CDK activity is modulated by both activating and inhibitory phosphorylation of the catalytic subunit. Although there are two relevant sites for covalent modification of CDKs, only the activating phosphorylation by CDK activating kinase (CAK) within the T-loop of the catalytic site appears to play a role during G1 in the budding yeast. Finally, the heterodimeric CDK complex is subject to inhibition by inhibitor proteins known as cyclin dependent protein kinase inhibitors (CKIs). In yeast there are nine cyclins that associate with and activate the Cdc28 CDK, six B type cyclins (Clb1-Clb6) and three G1 cyclins (Cln1-Cln3) (reviewed in 2,4). Entry into G1 phase occurs as a consequence of inactivation of B type cyclin-associated CDK and progression from G1 phase into S phase is a consequence of activation of B type cyclin-associated CDK. All of the events associated with START, including the activation of these S phase forms of the CDK, occur as a consequence of the activation of G1 cyclin-associated CDK. Thus, it is activation of the G1-specific CDK that defines the START event. The implementation of these forms of regulation during G1 phase is explored in more detail below.

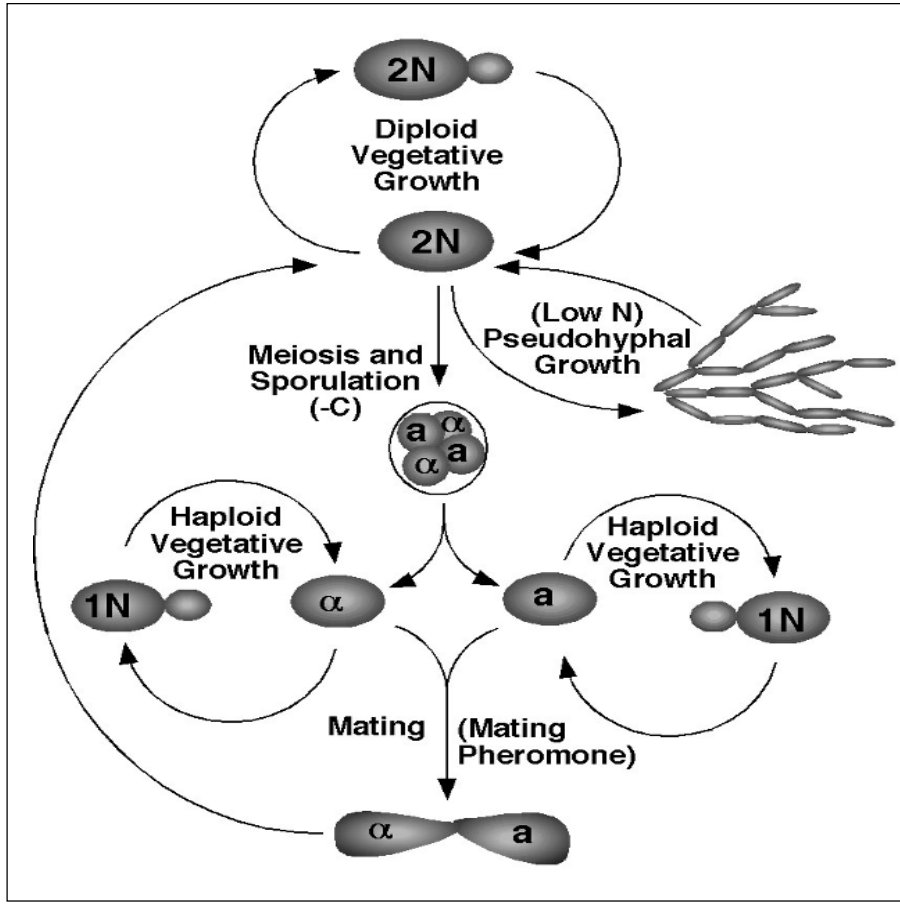

Figure 2. Life cycle of the budding yeast *S. cerevisiae.* Both diploid and haploid cells of budding yeast can proliferate vegetatively via a mitotic cell cycle. The generation of haploid progeny from diploid cells occurs when cells are deprived of glucose in the presence of nitrogen and a nonfermentable carbon source. An ascus containing four haploid progeny (ascospores), two of each mating type, is produced via meiosis and sporulation. Upon germination of the ascospores haploid vegetative cells of different mating types can differentiate into gametes and mate to form a zygote which can then proliferate mitotically to form diploid vegetative cells. When diploid vegetative cells are starved for nitrogen in the presence of a carbon source on a solid substrate they can undergo pseudohyphal differentiation. Pseudohyphal cells bud in a unipolar fashion generating chains of cells that have the capacity to invade solid substrates.

Why Restrict These Regulatory Events to G1 Phase

What is the purpose of regulating these processes via a specific G1 event or, for that matter, why should they be coordinated at all? Although there is no reason for these events to occur if cells are not embarking on a new cell cycle, it is not obvious why these events need to be coordinately executed during late G1 phase. Neither the spindle nor the bud is required until mitosis or later. In fact, in most cell types the formation of the contractile ring is delayed until mitosis when the division plane must be established. In animal cells, although centrosomes are replicated early, the spindle is not constructed until after replication is complete. This suggests that early execution of these events might be a peculiar requirement of cells that, like

budding yeast, have a closed mitosis. However, that must not be the case since in some fungi, all of which have closed mitosis, these events are also delayed. Apparently these events need not be executed coordinately. The best explanation for coordinate regulation may be that it represents an economical implementation of cell cycle regulatory networks.

Coordination of the Events of Cell Cycle Initiation during G1 Phase

Understanding the coordination of events of the cell division cycle requires a closer look at those events and their regulation by CDKs. The major cell cycle events regulated during G1 phase as a consequence of START (Fig. 3) are addressed individually below.

Budding

The most obvious developmental event during the yeast cell cycle is the emergence of the new bud, a morphological indicator of commitment to a new cell cycle. The process by which a bud is generated can be separated into several steps (reviewed in 6). First, the site for bud emergence is selected relative to the site utilized in the prior cell cycle based upon rules determined by the status of the mating type locus. MATa and MATα cells bud adjacent to the prior bud site (axial budding) whereas MATa/α cells bud opposite the site used in the prior cell cycle (bipolar budding). Bud site selection occurs during the previous cell cycle at which time the site of emergence of the prospective bud is marked by a protein complex that will nucleate the events of bud emergence and growth (reviewed in 7). Despite the difference in budding pattern, the details of events occurring at the bud site appear to be mechanistically identical in all cell types.

Bud site selection appears to be independent of CDK activation.[8,9] However, prolonging the duration of G1 phase can result in a loss of identity of that preselected bud site such that a new site is selected for use in the ensuing cell cycle. Such resetting has been observed during mating pheromone arrest, starvation or subsequent to release from a cell cycle block imposed by depletion of G1 cyclin associated forms of the CDK. It has been suggested that the molecular markers at the bud site may have a limited lifetime under specific conditions. In support of that idea there does seem to be a relationship between the maintenance of the selected site and the time cells spend in G1 phase. In fact, one model to explain the differences between budding patterns in mother and daughter diploid cells posits that because daughter cells spend more time in G1 phase prior to utilization of the bud site (see below), the cortical mark proximal to the bud site decays and a distal site is then utilized. Whatever the explanation, it appears that the influence of G1-specific cell cycle regulators upon bud site selection is a passive rather than an active one.

In contrast, both the assembly of a new septin ring[10] (see below) and the generation of the polarized growth signal (reviewed in 6,11,12) depend upon activation of the G1-specific forms of the CDK. In cells arrested by depletion of G1 cyclins, or by environmental influences that restrict the activation of Cln/CDK complexes, including pheromone treatment or starvation, the accumulation of proteins that promote polarized secretion fails to occur at the new bud site. Upon activation of Cln/CDK complexes, elements promoting polarization of the actin cytoskeleton and thereby polarized secretion accumulate and are activated at the bud site. These are among the earliest indicators of commitment to a new cell cycle. Although the bud site appears to be present prior to the formation of the new septin ring and the generation of polarized growth signals, these structures appear to be integral to maintenance and utilization of those signals as mutations in many of the elements of those structures result an inappropriate pattern of bud site selection.

Several components of the relatively large complex of proteins that accumulates at bud sites are essential for bud formation. Central to that process are the Cdc42 GTPase,[13,14] a Rho

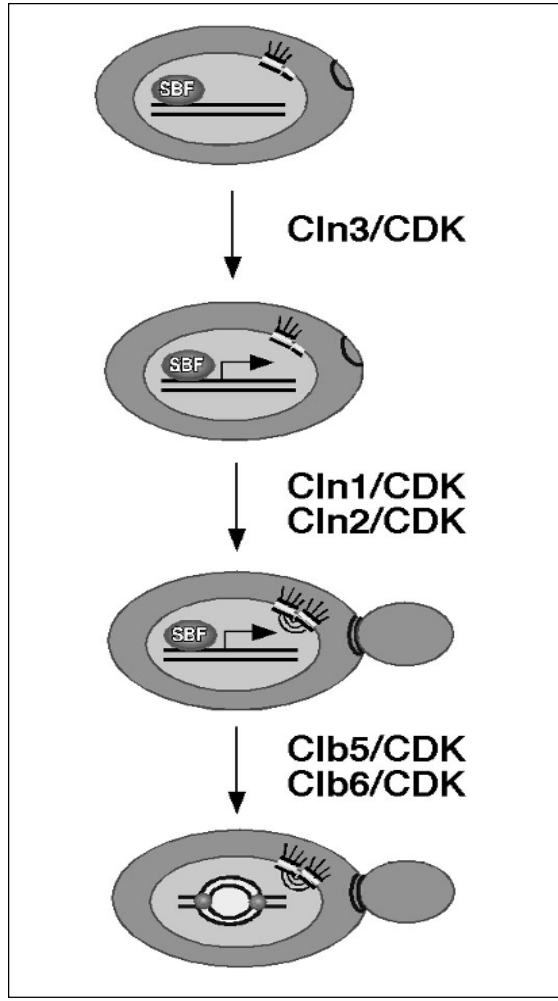

Figure 3. Cyclin/CDK dependent events during G1 phase. Distinct roles are associated with the activation of specific cyclin/CDK complexes during G1 phase. Activation of the Cln3 associated form of the CDK results in the activation of G1 specific transcription. Among the G1 specific transcripts are the G1 cyclins Cln1 and Cln2 and the S phase cyclins Clb5 and Clb6. Activation of Cln1- and Cln2-associated CDK leads to the duplication of spindle pole bodies, construction of the new septin ring and polarized growth leading to bud emergence. Clb5- and Clb6-associated CDK, which are activated in a Cln1/2-dependent manner, activate of DNA replication.

family member, and the PAK family protein kinases, Ste20 and Cla4.[8,9,15] These protein kinases interact both physically and genetically with Cdc42. Both Cdc42, which localizes to the bud site, and its guanine nucleotide exchange factor Cdc24, which must be recruited to those sites by Bem1,[16,17] are essential for budding and polarized growth.[18] Although alone neither of the PAK family kinases are essential, inactivation of both results in failure to bud or grow in a polarized manner.[15] In addition to being required for budding, Ste20 is also required for mating pheromone signal transduction. However, mutations that separate the two functions have

established independent roles in each process. Many other proteins that either accumulate at bud sites or interact genetically with Cdc42 are essential for proper bud morphogenesis although the precise roles of most of those proteins are unknown (reviewed in 11-12).

The polarization of cell growth is a consequence of localization of the secretory apparatus at the bud tip. The polarity promoting factors, like Cdc42 and the PAK kinases, are essential for the reorganization of the actomyosin cytoskeletal system upon which the secretory system relies for its organization. Thus, activation of G1-specific forms of the CDK results in a dramatic reorganization of both the filamentous cytoplasmic actin and the actin containing cortical patches such that secretory vesicles are delivered to and participate in exocytosis at the site of bud growth.[19] However, the mechanism by which Cln/CDK promotes polarization is unknown.

Establishment of the Septin and Contractile Rings

The septin ring and underlying actomyosin ring are the other prominent structures coupled to bud emergence that form as a consequence of the activation of G1-specific CDKs.[10] Although each apparently functions independently, these structures are juxtaposed to each other in the cell membrane and formation of the actomyosin contractile ring is dependent upon a templating function provided by the septin ring. The septin ring does not play an essential role in bud emergence. However, it is important for both proper bud morphogenesis, proper timing of nuclear division and it is essential for cytokinesis (reviewed in 20). Like the septin ring, the actomyosin ring plays a role in cytokinesis but, in this case, is not essential for that process. Finally, the septin ring is essential for proper bud site selection. Several components of the axial bud site selection machinery, including Bud3, Bud4 and Bud10/Axl2, associate with the septin ring, which appears to serve as physical marker of the previous bud site at least in haploid cells.

So why assemble the septin ring at START if it's primary role is in the organization of the contractile system? Despite the observation that septins are not essential for early cell cycle events, the septin ring plays important roles throughout the cell cycle. First, bud morphogenesis is aberrant in cells lacking a septin ring. This probably results from the failure to restrict the deposition of new cell wall material to a sufficiently small region of the cell surface to promote proper bud formation. Next, the septin ring is a docking site for the Hsl1/Hsl7/Swe1 complex, components of the checkpoint that monitors formation of the bud.[21-24] Formation of that complex occurs only when the portion of the bipartite septin ring structure associated bud is present, thereby restricting the Swe1 protein kinase from phosphorylating and inhibiting mitotic forms of the CDK. When the bud and its associated septin ring are absent, Swe1 is free to inhibit the B type cyclin associated forms of the CDK restricting mitosis.

Both the disassembly of the "old" septin ring (that utilized during the previous mitotic cycle) and the assembly of the new septin ring appear to be influenced by G1 cyclin associated CDK.[25] The disassembly of the "old" ring normally occurs at or around the time of the formation of the new ring and, at least in haploid cells, may play a role in localization of the new ring and, thereby, utilization of the new bud site. In fact, the septin Cdc3 is one of the few established in vivo substrates for Cln2/CDK kinase and its phosphorylation appears to be important for the timing of disassembly of the "old" septin ring. However, the basis for the Cln/CDK dependence of septin ring assembly has not been established.

Initiation of DNA Replication

DNA replication is not a landmark event of G1 phase but instead demarks the exit from that phase of the cell cycle. In fact, if mitosis is considered the culmination of the nuclear cycle then DNA replication is certainly the initiating event. Initiation of DNA replication is a consequence of the activation of G1 cyclin dependent CDK. Initiation is a multi-step process

consisting of construction of a preinitiation complex on replication origins followed by "firing" of those origins (reviewed in 26). Although only a portion of DNA replication origins is fired early during S phase preinitiation complexes are established on both "early" and "late" origins during G1 phase. In addition, licensing of origins must occur prior to replication restricting DNA replication to once per cell cycle. Cyclin dependent protein kinases catalyze a cascade of events that are critical for these regulatory processes.

The formation of the prereplication complex during early G1 phase of the cell cycle is a prerequisite for initiation of DNA replication.[27,28] Constraining origin firing to once per cell cycle is, at least in part, a consequence of limiting the accumulation and activity of several components of the prereplication complex to G1 phase via regulated gene expression, CDK dependent phosphorylation, localization and regulated proteolysis (reviewed in 29). Notably, the expression of CDC6, a member of the same transcriptional regulatory network as the gene encoding the G1 cyclin Cln3, is restricted to early G1 phase.[30] Association of Cdc6 with origin recognition complex (ORC) at replication origins is a prerequisite for the recruitment of Mcm proteins and formation of the prereplication complex.[31,32] A state permissive for the expression of Cdc6 and its recruitment to replication origins is established only after B type cyclin associated CDK is inactivated as cells exit mitosis, via the combined influences of APC dependent proteolysis and expression of the B type cyclin CDK inhibitor, Sic1 (reviewed in 33-34).

Activation of replication via the firing of replication origins is formally defined as the exit from G1 phase. Origin firing is triggered by the activation of CDK complexes containing Clb5 and Clb6, the S phase cyclins.[35,36] Although the genes encoding both of these cyclins are transcribed concurrently with those encoding the G1 cyclins, Cln1 and Cln2, these cyclins fail to activate the CDK due to the presence of the Clb/CDK inhibitor, Sic1.[37,38] Only when Sic1 is targeted for proteolysis via phosphorylation by Cln/CDKs, which are refractory to Sic1 inhibition, do the Clb-associated CDKs become active.[39,40] Thus, the events leading to origin firing are a direct consequence of the activation of G1 cyclin-associated CDKs, the event that defines the START.

It has been known for almost a decade that Clb/CDKs must be active for DNA replication to initiate. However, a target of Clb/CDKs required for the activation of origins has only recently been defined. Clb5/6/CDK must phosphorylate Sld5, an essential component of the replication complex, for DNA replication to occur.[41] Phosphorylation appears to promote the association of Sld5 with Dpb11, one of the earliest proteins recruited to activated origins of replication.[41,42] Although the functions of Sld5 and Dbp11 remain obscure, their early association with origins and the failure of other replication complex components to load in their absence suggests that they play a role in recruitment of replication factors.

CDK activity also plays a number of other roles in regulation of DNA replication. Perhaps the best characterized is its role in limiting replication to once per cell cycle. This function appears to be achieved via the phosphorylation of multiple targets including Mcm proteins, Cdc6 and ORC components.[43,44] One function of phosphorylation of Cdc6 is to target it for SCF dependent proteolysis.[45,46]

Spindle Pole Body Duplication

The fungal spindle pole body, a functional analog of the mammalian centrosome, must duplicate and separate prior to establishment of the mitotic spindle (reviewed in 47). In budding yeast spindle pole body duplication occurs late during G1 phase followed closely by separation to form the mitotic spindle (reviewed in 2). Like budding and septin ring formation, spindle pole body duplication is dependent upon activation of the G1-specific form of the cyclin dependent protein kinase. This is a multi-step process involving duplication of spindle pole components, maturation and separation. The duplication of spindle poles, like DNA

replication is limited to once, and only once, per cell cycle. Via a division of labor reminiscent of their roles in promotion and licensing of DNA replication, a complex choreography of CDK activities is involved in promoting duplication, separation and licensing of SPBs. Whereas G1-specific CDKs can promote duplication, S phase specific CDKs promote maturation, and, finally, mitotic forms of the CDK block reduplication.[48] Thus, as in DNA replication, Cln/CDK complexes and Clb5-6/CDK complexes have inductive activities and Clb1-4/CDK complexes act to inhibit reduplication. Misregulation of these events leads to reduplication and aberrant spindle formation. The targets of CDKs relevant to the regulation of spindle body duplication are unknown.

Initiation of a New Cell Cycle: When and Where

Activation of the G1-specific CDKs, the determining event in the initiation of a new cell cycle, occurs primarily as a consequence of the accumulation of the G1 cyclins, CLN1, CLN2 and CLN3 (reviewed in 2). Their abundance is regulated via the combined effects of cell cycle regulated transcription and ubiquitin dependent proteolysis. CLN3 is transcribed during the M/G1 transition due to the action of Mcm1 and at least one additional factor.[30,49] Cln3, upon binding to the CDK, activates G1-specific transcription,[50-52] thereby, promoting the accumulation of CLN1, CLN2, CLB5, CLB6 and many other G1-specific transcripts. Cln1 and Cln2-associated CDKs promote and coordinate START dependent processes. Many of the other G1-specific gene products participate in those processes including budding, cell wall synthesis, spindle pole body duplication and DNA replication.[53]

G1-Specific Genes and Their Transcriptional Activators

The transcriptional activation of G1-specific genes is attributed to two transcription factors, MBF and SBF. SBF is a heterodimer of Swi4 and Swi6, and MBF is a heterodimer of Mbp1 and Swi6. Swi4 and Mbp1 are sequence-specific DNA-binding proteins that recognize SCB and MCB elements, respectively (reviewed in 54). Whereas Cln3/CDK activates G1-specific transcription,[50-52] Clb-associated CDK is thought to repress those genes by promoting the dissociation of SBF from its target promoters. There is some controversy concerning the capacity of Clb/CDK to repress MBF-activated transcription.[53,55]

Before the availability of the yeast genome sequence and the approaches promoted by it, only few targets of the SBF and MBF transcription factors were known. But recently genome-wide microarray analysis has given us an expanded view of the G1-specific transcription program and the SBF/MBF targets.[53,56] Those studies have led to the identification of 300 genes exhibiting a G1-specific pattern of expression. Of those, 76 (as well as 46 that are not as tightly clustered) were placed in the CLN2 cluster based upon their strong cell cycle regulated expression coordinate with CLN2 during late G1 phase. Consistent with the previously established behavior of members of this family of genes, all were strongly induced by overexpression of CLN3 and repressed by overexpression of CLB2. The promoters of these genes contain at least one MCB, one SCB or a related sequence. In addition to the CLN2 cluster, 92 genes involved in cell wall synthesis, most of which have SCB elements in their promoters, were found to be coregulated, exhibiting a peak of expression of these genes occurring nearly coincident with budding and a response to CLN3 and CLB2 overexpression similar to CLN2. This study found that MCB containing genes are induced by CLN3 and, in contrast to other studies, are repressed by CLB2.

Complementing the microarray analysis is a study in which SBF and MBF target genes were identified using ChIP (chromatin immunoprecipitation) assay followed by microarray analysis of the transcription factor bound genes.[57] About 200 new targets of the two G1-specific transcription factors were identified, 163 genes bound by SBF, 98 genes bound by MBF and 43 genes bound by both. Interestingly, target genes of SBF are predominantly involved in

budding, cell-wall synthesis, mating and pseudohyphal growth whereas MBF targets are predominantly involved in DNA synthesis and repair, and probably meiosis.

Why have two transcription factors, MBF and SBF, which are so similar regulated? After all, budding and DNA replication, the two processes in which the largest number of genes in this family are involved, occur approximately simultaneously during the cell cycle. This may be explained, in part, by the requirement for distinct patterns of gene regulation during diverse developmental programs. For example, during meiosis DNA replication occurs without budding, whereas during mating synthesis of membrane and cell wall occurs without DNA replication. Segregating groups of genes based upon distinct regulatory motifs may provide for differential regulation under these circumstances.

Cln3 Dependent Activation of G1-Specific Transcription

Both SBF and MBF are bound to promoters prior to the activation of G1 transcription.[58-60] Transcriptional activation is dependent on Cln3/CDK activity.[51,52] Although either Cln1 or Cln2/CDK are capable of activating G1-specific transcription, this appears only to occur in the absence of CLN3. How Cln3/CDK promotes transcription remains unclear. However, it is not necessary for recruitment of SBF to the promoter nor is activation of transcription dependent on phosphorylation of SBF/MBF components by Cln3/CDK.[61] Despite extensive analysis, a stable interaction between Cln3 and the G1 specific transcriptional machinery has not been detected (K.F. and C.W., unpublished observations). Recently, that issue has begun to be addressed via analysis of the order of recruitment of transcriptional regulatory factors to the promoter of the HO gene, a G1-specific gene required for the initiation of mating type switching.[62,63] Although the G1-specific activation of HO depends upon SBF binding to the promoter and occurs in response to activation of Cln3/CDK, its regulation is considerably more complex than that of many G1-specific genes as a consequence of higher order regulation that restricts its expression to mother cells (reviewed in 64). Despite that complexity, SBF association with the HO promoter, like with other G1-specific genes, occurs early during G1 phase independent of the activation of Cln/CDK. That, in turn, recruits the Srb/mediator complex to the chromatin [63] or may simply reposition previously recruited complexes from a distal position to the basal promoter.[65] Whichever is the case, it is surprising that Srb/mediator is not recruited to the promoter as a component of the Pol II holoenzyme, as had been generally assumed, but instead associates independent of Pol II. In fact, it is the recruitment of Pol II that seems to depend upon CDK activity. A similar sequence of events occurs at the CLN1, CLN2 and PCL1 promoters suggesting that this process is general to G1-specific transcriptional activation.[63]

Despite significant progress in the understanding of Cln3 dependent transcription, key questions remain unanswered. We now know that recruitment of RNA polymerase II is regulated by Cln3/CDK. However, the target of that protein kinase and the events leading to RNA Pol II recruitment remain to be elucidated. For instance, it is not yet known whether recruitment of TBP and its associated factors are present at G1-specific promoters at the time of Pol II recruitment or whether its recruitment is CDK-dependent. Furthermore, it is unclear whether the regulation of G1-specific transcription is modulated in response to environmental stimuli via Cln3 dependent or independent mechanisms. Defining the role and relationship of these factors will be important for a full understanding of the mechanism of transcriptional activation of G1-specific genes.

Establishing Maintaining and Exiting the G1 State

Progression through the cell cycle is marked by phases of low and high B type cyclin associated CDK activity (Fig. 4). G1 phase is characterized by low activity, a requirement for exit from mitosis. G1 phase ends when Clb/CDK activity is reestablished promoting entry into

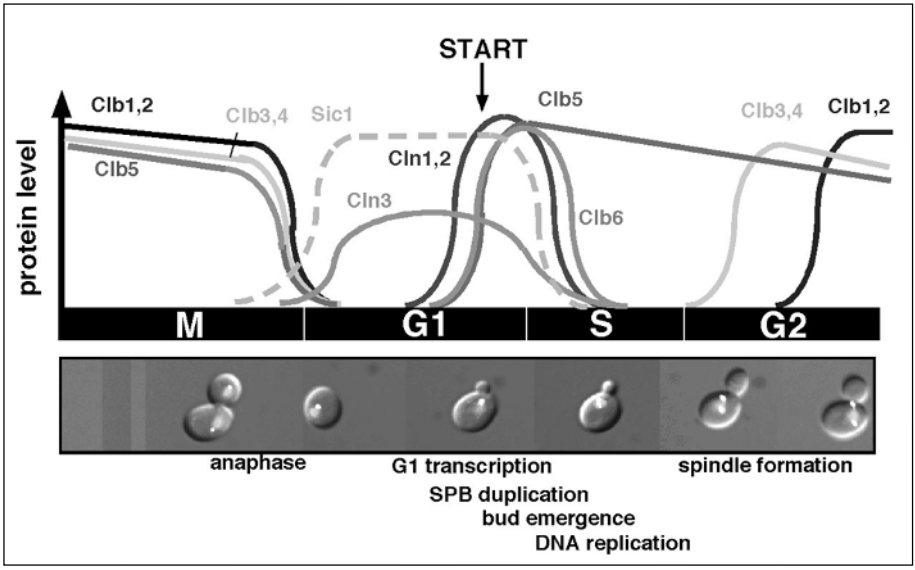

Figure 4. Relationship between cyclin periodicities and cell cycle events. The pattern of accumulation of cyclins (solid lines) and the Clb/CDK inhibitor Sic1 (broken line) during the cell cycle is depicted. The level of the CDK is constant with respect to cell cycle position. M phase cells are characterized by the presence of high levels of mitotic cyclins Clb1-5. B type cyclins are destroyed by activation of APC and inhibited by rising Sic1 level as cells exit M phase leading to the low Clb/CDK state of G1 phase. Cln3 accumulates during early G1 phase leading to activation of G1-specific transcription and, thereby, the accumulation of the G1 cyclins Cln1 and Cln2 as well as the S phase cyclins, Clb5 and Clb6, which are maintained in an inactive state via the activity of Sic1. Cln1- and Cln2-associated CDK then promote SPB duplication, bud emergence, the destruction of Sic1 and the inactivation of APC. Cln/CDK dependent phosphorylation also promotes the destruction of Cln proteins. Destruction of Sic1 results in the activation of Clb5- and Clb6-associated CDK leading to the initiation of DNA replication. Finally, mitotic cyclins reaccumulate from mid-S phase through G2 phase reestablishing the M phase state.

S-phase (reviewed in 5). The transition between these distinct states of the cyclin dependent protein kinase is accomplished via a complex interplay of transcriptional regulation, protein phosphorylation and ubiquitin dependent proteolysis.

During mitosis high levels of Clb/CDK activity are maintained by at least three separate mechanisms involving phosphorylation of proteins by the Clb/CDK. First, Cdh1, an activator of the anaphase-promoting complex (APC) required for ubiquitin dependent proteolysis of cyclin B, is inhibited by phosphorylation.[66,67] As a consequence, B type cyclins are stable. Second, the Clb-specific CDK inhibitor Sic1 is phosphorylated and, thereby, targeted for destruction via the SCF-Cdc4 ubiquitin ligase.[39,40,68,69] Finally, the transcription factor Swi5, required for transcription of the *SIC1* gene, is phosphorylated and, as a consequence, excluded from the nucleus.[70]

At the completion of anaphase the phosphatase Cdc14 is released from sequestration in the nucleolus by Net1, a component of the Mitotic Exit Network, and spreads throughout the cell where it dephosphorylates Sic1 and Swi5.[71-73] As a result, Sic1 is both more highly expressed and more stable leading to the inhibition of Clb-associated CDK. Furthermore, Cdh1 is dephosphorylated, activating APC-Cdh1, thereby, leading to ubiquitin dependent proteolysis of B type cyclins. Thus, although Cdc14 acts as a switch to set the cells to the low kinase

state, it is not necessary to maintain it. Maintenance of the low kinase state is achieved by maintenance of activated APC and the CDK inhibitor Sic1.

The low Clb/CDK state of G1 phase creates a permissive environment for a number of cell cycle events and plays a role in limiting those events with respect to cell cycle position. Two of those have already been discussed, the formation of the prereplication complex and the initiation of spindle pole body duplication. Both events are restricted to G1 phase and are antagonized by the activity of B-type cyclin-associated CDK. Although the intiation of SPB duplication is poorly understood, the formation of the prereplication complex has been well characterized. CDC6, which encodes an essential component of that complex, is coexpressed with CLN3 and SWI4 during the M/G1 transition.[30] When expressed in cells with low CDK activity, Cdc6 accumulates and associates with DNA bound origin recognition complex (ORC) creating a state that is permissive for origin firing and subsequent DNA replication.[74]

The accumulation of Cln3 during G1 phase leads to the activation of G1-specific transcription. Promoting the accumulation of Cln1 and Cln2 as well as the S phase specific B type cyclins, Clb5 and Clb6. Although Cln-associated CDKs become active upon expression of the genes, accumulation and activation of Clb5/6-associated CDK activity is restricted due to the persistence of Sic1. However, this is a short-lived state because among the targets of Cln/CDK is Sic1. Once phosphorylated, Sic1 becomes a target for ubiquitination by SCF-Cdc4 and is consequently degraded via the proteasome.[39,40,68,69] Cln/CDK also antagonizes the activity of APC-Cdh1.[66] Although the regulation of Clb6 accumulation is poorly understood the protein appears to be highly unstable and accumulates for only a short interval around the beginning of S phase (Haase S. personal communication).

G1-Specific Targets of CDKs

Activation of G1-specific forms of the CDK is known to be required for the efficient execution of a number of cell cycle regulated events. However, only a few targets with functions related to those processes have been identified. The best characterized are Sic1 and Cln2. In both cases, phosphorylation of the protein by Cln-associated CDK promotes recognition by the F box component of the E3 ubiquitin ligase SCF and ubiquitination by the associated E2 ubiquitin conjugating enzyme Cdc34. However, the specific form of SCF involved in ubiquitination differs. Phosphorylated Sic1 is specifically ubiquitinated by SCF-Cdc4 [39,40,68,69] whereas SCF-Grr1 is required for ubiquitination of phosphorylated Cln2.[39,40,68,75-78]

The basis for recognition of distinct phosphorylated targets by different SCF complexes has been studied in some detail. At least three distinct mechanisms other than phosphorylation appear to contribute to the specificity of the interaction:first, the nature of the protein-protein interaction domain in the F box component of the SCF complex;[79-81] second, the properties of the degron domain of the target protein;[82,83] third, localization of the proteins involved in the interaction.[84,85] Although each mechanism has been shown to contribute to the regulation of specific targets by their cognate SCF complex, the degree to which each involved in determining the relative stability of these targets is only beginning to be elucidated.

The relative timing of destruction of Cln2 and Sic1 is thought to be important for the proper choreography of events during the transition from G1 into S phase.[83,86] Both premature destruction and persistence of Sic1 have been associated chromosomal aberrations and alterations in cell cycle dynamics.[39,87] Although the phenotypic consequences of misregulation of G1 cyclin abundance are less clear, it is likely that G1 cyclins must persist for a sufficient period and accumulate to a sufficient level to phosphorylate their targets including Sic1. Conversely, they must be eliminated prior to S phase to prevent perturbation of subsequent cell cycle events. Expression of G1 cyclins outside of G1 disrupts the switch from polarized to isotropic growth and delays abscission of mother and daughter cells.[19]

The timing of Cln2 and Sic1 destruction is determined by their phosphorylation dependent interaction with the F box proteins, Grr1 and Cdc4, respectively. In both cases that interaction requires phosphorylation at multiple sites.[39,40,77] At least in the context of the Sic1/Cdc4 interaction, it has been shown that the phosphorylation sequences (phosphodegrons) in Sic1 are suboptimal for the interaction with Cdc4.[83] Thus, multiple copies of those phosphodegrons are required for effective interaction between those proteins. Conversely, the presence of a single optimal phosphodegron is sufficient for that interaction and promotes premature inactivation of Sic1 along with the associated phenotypic consequences. By requiring multiple phosphorylations for the recognition of each protein by the ubiquitin ligase, destruction of both Sic1 and Cln2 are delayed until the requisite Cln/CDK dependent phosphorylation events have been completed. Only when Sic1 is inactivated does Clb5/6/CDK become active allowing cells to initiate S phase.

Clearly, Cln2 and Sic1 are not the only G1-specific CDK substrates. Others include Far1, a multifunctional protein that acts, in part, as a Cln-specific CDK inhibitor,[88,89] and Ste20, a MEKK, both of which are components of the mating pheromone signaling apparatus (see below) and the cellular morphogenesis machinery.[90-92] Ste20 also plays a role in pseudohyphal differentiation. Although phosphorylation of Ste20 is thought to be associated with desensitization to mating pheromones, that has yet to be demonstrated. On the other hand, phosphorylation of Far1 by Cln/CDK is known to target it for ubiquitination by SCF-Cdc4 and proteolysis.[93] Like Far1, a putative effector of Cdc42,[94] Gic2,[95] and Cln1[78] and Cln3[96,97] are targeted for ubiquitination via Cln/CDK dependent phosphorylation. The septin Cdc3 is an established substrate.[25] However, the role of phosphorylation of Cdc3 remains unclear.

Cln Localization and Its Importance in Regulation of Cell Cycle Events

We have considered the importance of cyclin gene expression and proteolysis as well as the differential sensitivity of specific cyclin/CDK complexes to CDK inhibitors as important factors in the regulation of CDK function. Recently, attention has become focused upon the importance of subcellular localization in regulating the function of G1 cyclins. Those studies have established that Cln2 is predominantly cytoplasmic whereas Cln3 is predominantly nuclear, consistent with their recognized roles during the cell cycle.[85,98,99] Perturbation of that localization either by destroying or ectopically introducing signals for nuclear localization or nuclear exclusion (NLS or NES, respectively) substantially alter to functional specificity and regulated accumulation of those cyclins.[85,99] This suggests that localization plays an important role in regulation of those processes. Thus, cytoplasmic localization of Cln3 via ablation of its natural NLS enables it to partially complement a loss of Cln2 function. Furthermore, cytoplasmic Cln3 fails to complement a loss of Cln3 function consistent with an essential role for Cln3 in the activation of G1-specific transcription.[85,99] Although, no natural localization sequences were identified in Cln2, its localization to the nucleus via addition of an NLS, diminished its functionality. Interestingly, restriction of Cln2 to either subcellular compartment was associated with its inactivation prompting the hypothesis that it must shuttle between compartments to execute its functions.

Although Cln2 is largely localized to the cytoplasm, it is present at some level in the nucleus and can perform functions there. Nevertheless, no recognizable NLS or NES has been found in the protein. However, elimination of CDK phophorylation sites is sufficient to relocalize a substantial portion of the protein to the nucleus. The same mutations block ubiquitination and proteolysis in vivo raising the possibility that the effect of phosphorylation on stability is a consequence of its mislocalization.[85] However, several observations argue against that explanation. First, Grr1 is present and functions in both compartments.[84] Next, Cln2 becomes phosphorylated in both compartments. Finally, phosphorylation of Cln2 affects SCF-Grr1 binding

both in vivo and in vitro, arguing that the effect on ubiquitination is independent of its localization.[79,82] This does, however, raise the interesting possibility that phosphorylation at specific sites regulates localization independent of proteolysis. That possibility remains to be investigated.

In addition to being regulated at the level of the protein, Cln3 appears to be regulated at the level of the mRNA. Apparently, Whi3, an RNA binding protein identified in a screen for mutations that reduce cell size,[100] binds to CLN3 mRNA and promotes its localization to distinct cytoplasmic foci.[101] Although there is no obvious effect on Cln3 abundance or translation, it has been speculated that by restricting Cln3 synthesis to specific cytoplasmic sites, Whi3 may modulate Cln3/CDK activity. The mechanism by which this affects cell size is not yet clear. However, this observation may represent another manner by which Cln/CDK accumulation can be regulated.

Coordinating Cell Proliferation with Cell Growth and Development

To this point we have primarily discussed the importance of the G1 interval in regulation of the faithful duplication of cells. However, G1 phase is also important as a cell cycle interval for the integration of environmental and internal signals. We have already mentioned the recognition of such responses as the basis for defining START. What has not been discussed is the manner in which those signals are integrated with the cell cycle machinery. In this section we discuss several aspects of that regulation including the regulation of START by nutrients and the involvement of START in the regulation of cell differentiation (Fig. 2). The control of cell growth and development in yeast is covered in more depth in later chapters.

Regulation of Cell Size

Yeast cells show an amazing uniformity in cell size when growing under the same conditions. To achieve this characteristic cell size, cell growth must be coordinated with cell cycle progression. Growth of daughter cells is largely restricted to G1 phase. Cells must achieve a minimal critical cell size for budding. Once the cell has budded, the new growth occurs primarily in the bud. Under most growth conditions daughter cells are born below the minimum critical cell size and must grow before entering a new cell cycle, whereas mother cells will exceed that size and, consequently, will proceed without a requirement for growth.

Minimal cell size is strongly correlated with CLN3 expression. Increasing the expression of CLN3 by providing multiple copies of the CLN3 gene, by fusing the CLN3 gene to a stronger promoter or by introduction of hyperstable alleles of CLN3 leads to budding at a smaller cell size.[102-106] Conversely, decreasing Cln3 dosage or expression leads to an increase in cell size at budding. Cln3 is thought to convey its effect on cell size via activation of SBF and MBF.[51,52] But a mechanism by which Cln3 coordinates cell size and cell cycle progression has not been established. It has been suggested that nuclear to cytoplasm ratio might be the measure for cell size and that this ratio might be somehow reflected by Cln3/CDK activity. However, as of yet, there is no experimental evidence to support this hypothesis.

Minimum cell size is intrinsic to cells growing under the same conditions, but alterations in environmental factors such as nutrient quality can lead to adjustments in cell size. When glucose, the preferred fermentable carbon source, becomes available to cells growing on poorer carbon sources, they not only change the expression pattern of many genes but they also undergo an increase in cell size.[107,108] To achieve this increase in size, cells delay passage through Start by controlling the level of Cln/CDK activity.

The signal transduction pathway involved in that response has been studied in some detail. Addition of glucose to yeast cells first evokes a transient peak in intracellular cAMP level followed by a resetting to a higher basal level.[109-111] This response is mediated by the activation

of adenylate cyclase (CYR1) through Ras-GTP or activation of the G-protein coupled receptor via Gpr1/Gpa2.[112,113] The resulting cAMP, in turn, activates cAMP-dependent protein kinase (PKA, encoded in yeast by TPK1-3) (reviewed in 114).

The increase in cell size in response to glucose is achieved by reducing Cln/CDK activity. This is, at least in part, a consequence of the specific repression of CLN1.[111,115] This response can be mimicked by exogenously added cAMP. However, under those circumstances the spectrum of that response is broadened such that other G1-specific mRNAs, including CLN2 and CLB5, are repressed.[111,116] Whether the difference in response is due to a difference in the intracellular level of cAMP or whether there is a qualitative difference in the signal generated in response to these two stimuli is unclear.

The specific effect of glucose on CLN1 transcription is surprising because under most conditions CLN1 and CLN2 transcription are coordinately regulated. This differential regulation of CLN1 and CLN2 is a consequence of the composition of cis-acting promoter elements. The repressive effect of glucose is mediated via the MCB-like elements in the CLN1 promoter. Despite their MCB-like character of the relevant promoter elements, the differential effect of glucose on CLN1 and CLN2 is apparently mediated by SBF rather than MBF. Although transcriptional repression of CLN1 has been shown to be sufficient for the glucose-induced increase in size at budding, evidence suggests that post-transcriptional regulation also plays a role.[115]

Interestingly, differential regulation of CLN1 and CLN2 has been revealed under two other conditions by genome-wide expression analysis. First, CLN1 but not CLN2 mRNA is repressed in response to an increase in cell ploidy.[117] Cell size in yeast, as in most other organisms, increases with ploidy. The repression of CLN1 would be expected to lengthen G1 phase and, thereby, increase cell size. Next, stimuli inducing pseudohyphal growth appear to repress CLN1 but leave CLN2 unaffected (see below).[118] Although the RAS/cAMP pathway is important in the pseudohyphal growth response,[113,119-122] it is not clear whether the differential regulation of CLN1 and CLN2 in those cells is mediated through a similar mechanism to that involved in regulation of cell size.

The mechanism via which the glucose signal mediated by cAMP is translated into repression of CLN1 is still unknown. Inactivation of the primary targets of the cAMP/protein kinase A (PKA) pathway, the multistress response transcription factors Msn2 and Msn4,[123-125] fails to abrogate the cell size increase in response to glucose (K.F. and C.W. unpublished observation). An obvious target of that regulation is CLN3 because of its involvement in both G1-specific transcription and cell size control. However, the level of both CLN3 transcripts and Cln3 protein have been shown to increase in response to glucose and cAMP, respectively.[126] This is in contrast to the decrease in CLN1 expression observed under the same conditions and suggests that glucose must affect CLN1 expression independent of its effect on CLN3.

Starvation

Starvation in yeast, which usually occurs as a consequence of limiting nitrogen or carbon source, leads to cell cycle arrest at START (reviewed in 127). If an essential nutritional component is absent from the growth medium cells undergo a variety of metabolic adaptations that together comprise the quiescent state referred to as stationary phase and sometimes equated to G0 in animal cells. As in other conditions leading to G1 phase arrest, down regulation of Cln/CDK function plays a critical role. This is demonstrated by the observation that cells expressing either CLN1 from a weak but constitutively active promoter or hyperstable alleles of Cln2 fail to arrest properly in response to starvation signals.[128,129]

Repression of Cln/CDK activity occurs as a consequence of a decrease in G1-specific transcription. This appears to result from a decrease in the accumulation of Cln3 protein. Cln3

translation appears to be particularly sensitive to nitrogen starvation as a consequence of an untranslated ORF in the 5' UTR region of the CLN3 mRNA.[130] An independent mechanism appears to lead to a decrease in the stability of Cln3 under nitrogen starvation conditions.[128]

The putative phosphatidylinositol kinases, TOR1 and TOR2, have been implicated in the translational regulation of Cln3. The TOR inhibitor rapamycin induces entry into a G0 like state with a 90% reduction of the bulk translation rate. This can be prevented by overexpression of hyperstable Cln3 alleles or CLN3 with a 5'UTR that is not translationally inhibited by rapamycin (such as the 5'UTR of UBI4).[131] However, this mechanism appears to be nonspecific since, in contrast to nitrogen starvation where Cln3 translation drops despite maintenance of the overall translation rate, in the presence of rapamycin a decrease in the overall translation rate is observed.

Despite the observation that Cln3 down regulation is a component of starvation induced G1 arrest, other mechanism must exist. Simply inactivating Cln3 leads to a delay in the execution of START but not to arrest in G1 phase. Three other cyclins that contribute to progression out of G1 phase, Cln1, Cln2 and Clb5, become unstable under nitrogen starvation conditions. Furthermore, Sic1 is stabilized under those conditions.[128] Whether these effects are sufficient to explain the G1 arrest that occurs in response to starvation remains to be established.

Exit to Meiosis

When starved for nitrogen on a poor carbon source, diploid cells exit the cell cycle and progress into meiosis[132] whereas haploid cells enter a quiescent G1 or stationary phase under the same conditions. Regulatory circuits that make mitosis and meiosis incompatible govern this decision. In vegetatively growing diploid cells, G1 cyclins inhibit the meiotic progression by inhibiting the meiosis specific transcription factor Ime1, which promotes the meiotic specific program of gene expression. This is probably a consequence of repression of IME1 transcription.[133] In haploid cells, repression of IME1 is maintained by the RME1 transcription factor, thereby, preventing entry into meiosis.[134] RME1 has also been shown to promote G1 cyclin transcription by acting at the CLN2 promoter, thereby promoting the mitotic cell cycle.[135]

In contrast, when cells are starved for nitrogen and carbon source G1 cyclins are rapidly down regulated. As a consequence, the restriction of IME1 expression is lifted allowing the starvation signal and heterozygosity at the MAT locus to collaborate in promoting the activation of Ime1 and, thereby, the transcription of early meiotic genes. One of those genes, the Ime2 kinase, is responsible for phosphorylation of Sic1, targeting it for degradation.[136] That function is performed by Cln1- and Cln2-associated CDK during the mitotic cell cycle. Thus, in the meiotic cell cycle G1 cyclins are not only unnecessary, they are antagonistic.[133,137] Although the down regulation of G1 cyclin gene expression might explain why meiotic cells replicate their DNA without budding, how events such as SPB duplication are regulated during meiosis in the absence of G1 cyclins remains to be explained.

The Mating Pheromone Response

The pheromone response demarks the initiation of mating, another developmental pathway embarked upon during G1 phase required to complete the sexual cycle by restoring the diploid state (reviewed in 138) (Fig. 2). Restricting mating to G1 phase ensures the proper conditions for nuclear fusion and the stability of ploidy with each mating type contributing a single haploid set of chromosomes. To coordinate those events mating pheromone promotes cell cycle arrest during G1 phase as well as establishing the conditions appropriate for cell and nuclear fusion.

The primary effect of the mating pheromone signaling pathway on the cell cycle is exerted via the inhibition of G1 cyclin associated CDKs. Binding of peptide mating factors elaborated

by haploid cells of each mating type bind to G protein coupled seven transmembrane receptors to stimulate a MAP kinase signaling pathway that terminates with the MAP kinase Fus3 (reviewed in 139). Fus3 phosphorylates and, thereby, activates Far1. Far1 is a bifunctional protein that acts both to promote formation of the mating projections[94] and as a Cln-specific CDK inhibitor invoking cell cycle arrest during G1 phase.[140,141] These disparate functions have been attributed to separable domains of Far1 one of which binds to Cdc24, the guanine nucleotide exchange factor for Cdc42, and one of which binds to Cln/CDK.[141] Activation of Far1, in part, involves a phosphorylation dependent redistribution of Far1 from the nucleus to the cytoplasm.[142] The inhibition of Cln/CDKs by Far1 has two important consequences with regard to cell proliferation. First, it diminishes the capacity of Cln1 and Cln2 to phosphorylate targets required for cell cycle progression. Second, and perhaps more importantly, it inhibits the capacity of Cln3 to promote G1-specific transcription, limiting the accumulation of G1-specific gene products.[143]

The mechanism of Cln/CDK inhibition by Far1 is unclear. It is known that Far1 binds to Cln2/CDK complexes upon pheromone-induced activation. However, it appears that Far1/CDK complexes retain the capacity to phosphorylate Far1 and perhaps other substrates.[88,89] Paradoxically, phosphorylation of Far1 by Cln/CDK has been shown to promote ubiquitination by SCF-Cdc4 and proteolysis.[93] One explanation for this apparent contradiction is that, although efficient phosphorylation of Far1 occurs in the context of Cln/CDK/Far1complexes, most of that fraction is cytoplasmic and evades ubiquitination because SCF-Cdc4 is restricted to the nucleus.[84] However, this must not be the entire explanation since a portion of Far1 clearly remains nuclear and must function there to inhibit the capacity of Cln3/CDK responsible for the activation of G1-specific transcription.

The pattern of Far1 accumulation during the cell cycle plays an important role in restricting the pheromone response to G1 phase. Transcription of Far1 in proliferating cells is restricted to G1 phase and is induced several fold in response to mating pheromone.[89,144,145] Although the protein is stable during G1 phase, it becomes highly unstable when CDK activity is high. Consequently, the protein is unable to accumulate subsequent to activation of Cln/CDK. That alone is an important part of the cell cycle restriction of the mating pheromone response. It is conceivable that phosphorylation of Far1 by bound CDK in the cytoplasm occurs in anticipation of the desensitization of cells to mating pheromone, a prerequisite for resumption of proliferation in cells that fail to mate. In that scenario, as the pheromone signal decays and activated Fus3 diminishes, Far1 would no longer be restricted from the nucleus resulting in its destruction by SCF-Cdc4 and the reactivation of Cln/CDK. In addition, Cln/CDK dependent phosphorylation of Ste20 may reinforce that effect by further restricting the pheromone signal upstream of the MAP kinase, thereby making cells refractory to pheromone signaling.

The Role of G1 Phase in Pseudohyphal Differentiation

Limitation for nitrogen in the presence of an abundant carbon source in some strains of yeast invokes a differentiative response known as pseudohyphal growth which involves a dramatic remodeling of cell morphology and cell cycle dynamics (reviewed in 146-148) (Fig. 2). Cells become highly elongated, filamentous and acquire the capacity to invade a solid substratum. Consequently pseudohyphal growth is considered a foraging response. The cell elongation is associated with a prolonged period of polarized growth during the cell cycle and, although the cells do undergo proper cytokinesis, they fail to subsequently excise. Finally, cells adopt a unipolar budding pattern, forming a new bud at a site distal from the prior site of cytokinesis, thereby, promoting the formation of long chains of cells that emanate from the initiating mother cell unidirectionally with occasional branching.

Only a fraction of the cells in a population growing on solid substratum undergo the pseudohyphal differentiation making analysis of that response quite challenging. Consequently, the cell cycle dynamics and signal transduction responses are most often studied in cells in which a pseudohyphal state has been induced by genetic manipulation. Based upon such studies it appears that these cells experience an elongated G2 phase and a substantially diminished G1 phase.[149] This is consistent with the observation that inhibition of Clb/CDK, via manipulation of the CLB genes or effectors of the Clb/CDK, can enhance the pseudohyphal response and that hyperactivation of Cln/CDK, via overexpression of CLN genes, can enhance it.[150-152] In fact, CLN1 is strongly induced in response to activation of Tec1, one of several transcriptional activators important in the pseudohyphal growth response.[118] Conversely, inactivation of CLN1 results in a strong suppression of pseudohyphal growth whereas overexpression of B type cyclins diminishes that response.[152] Each of these alterations of CDK activity has an effect on polarized growth in vegetatively growing cells consistent with the observed effect on pseudohyphal differentiation.

There is substantial experimental support for relatively simple models in which nutrients activate one or more signal transduction pathways that lead to the modulation of CDK activity and the stimulation of Tec1/Ste12 and Flo8 dependent transcription. However, the experimental observations that need to be reconciled with these models is exceedingly complex. For example, although inactivation of CLN1 suppresses the pseudohyphal growth response, it is enhanced by inactivation of CLN3.[152] This is difficult to reconcile with our knowledge that inactivation of CLN3 represses CLN1 and CLN2 gene expression. On the other hand, inactivation of GRR1, which is known to hyperactivate CLN1 and CLN2, enhances pseudohyphal differentiation as expected. However, when CLN1 and CLN2 are inactivated along with GRR1 the enhancement of the response persists suggesting that there are other important Grr1 targets, in addition to, CLN1.[79] Neither of these observations negates the models involving Cln/CDK and Clb/CDK. However they indicate that the regulation is not likely to be explained by these simple models. Nevertheless, it is clear that pseudohyphal differentiation involves a substantial remodeling of cyclin/CDK dynamics during the cell cycle and that G1 regulatory elements are among the potential targets.

Response to Environmental Stress

In their natural environment, the conditions to which yeast are exposed can change very quickly. Nutrients may deplete, temperature, osmolarity and the acidity of surrounding might change or cells might be exposed to oxidizing conditions or radiation. Cells need to respond to those changes as quick as they appear. Consequently, cells have developed a standardized program of gene expression that is evoked under suboptimal conditions referred to as ESR (environmental stress response) as well as programs customized for specific environmental stresses. The ESR program is thought to protect essential cellular processes against potential dangers posed by stress.[153]

Whereas starvation involves a transition from active growth to cell cycle arrest, cells submitted to other stresses resume growth after adapting to the new conditions. In most cases those stresses result in a transient arrest during G1 phase followed by adaptation. These regulatory pathways are discussed in detail in a later chapter (Herrero et al, this volume).

Conclusion and Perspectives

Yeast has served as an excellent model system for understanding the eukaryotic cell cycle and its regulation. The parallels between the yeast system and animal cell systems are easily demonstrable. Certainly, all of the fundamental elements including cyclin dependent protein kinase and their regulators have paralogs in each system. Genetic complementation has

established the functional homology of many of them. However, it is also clear that there are substantial differences of the specific implementation of these regulatory systems between organisms. As a consequence it is useful to exercise caution when drawing analogies between the two systems.

The temptation is strong to draw a parallel between the regulation of START in yeast and the G1 restriction point in animal cells. In the broadest sense both regulatory events act as a gate restricting cell cycle progression in the absence of the appropriate external and internal conditions. Although the molecular basis of the restriction point and START are only loosely defined, both apparently involve large-scale alterations in gene expression influenced by cyclin dependent protein kinases. It has been pointed out that Cln3/CDK is analogous in some ways to cyclin D/CDK4 and that SBF and MBF share both structural and regulatory properties with the E2F family of transcription factors.[54] In fact, the retinoblastoma protein (pRB) expressed in yeast cells can be shown to be hyperphosphorylated by Cln3 along with either Cln1 or Cln2,[154] a function carried out by cyclins D and E in animal cells.[155] In addition, cyclin D and E can complement a deficiency of yeast G1 cyclins. On the other hand, there is no Rb / p107/p130 family member encoded by the yeast genome. Furthermore, under appropriate conditions human cyclin B can also complement a deficiency of yeast G1 cyclins.[156,157] Thus, care must be taken in interpreting these observations.

Outstanding Problems

Despite the rapid progress in understanding the cell cycle in recent years numerous important questions remain unanswered. Several questions central to furthering that understanding are raised here.

First, we must identify the relevant targets of G1 specific forms of the CDK that regulate the central events of G1 phase as well as the functional significance of those phosphorylation events. The known targets of Cln/CDKs are relatively limited considering the spectrum of processes they control. Identification of some CDK targets has been achieved via identification of interacting proteins. However, stable interactions of protein kinases with their substrates are likely to be unusual. Thus, alternative approaches are critical. The proteins remaining to be identified include such important proteins as the target(s) of the Cln3/CDK that is required for the activation of G1-specific transcription and the targets of Cln1/2/CDK that drive spindle pole body duplication, budding, and septin ring formation. As such, they represent the major regulatory events during G1 phase. Identifying these substrates will likely be a major step in broadening our understanding of the eukaryotic cell cycle.

Another significant challenge will be to understand the role of CDK phosphorylation of those substrates. Of the known substrates of G1-specific forms of the CDK, those best understood are targeted for SCF-dependent degradation as a consequence of phosphorylation. This suggests that many of targets yet to be identified will be regulated at the level of protein stability. Although this is by no means certain, it is worthy of consideration in the search for, and evaluation of, new Cln/CDK targets and may play an important role in directing that search.

Understanding the basis for cell cycle periodicity is another uncharted area. Although we consider Cln3/CDK to be the initiator of cell cycle periodicity recent evidence suggests that there is an underlying cell cycle oscillator. That is, cells lacking an cyclin dependent oscillator still maintain the periodicity of cell cycle events suggesting the existence of a free-running oscillator that sets the periodicity of cyclin/CDKs.[158] The identity and nature of the oscillator remains to be determined.

Despite these important unanswered questions, our understanding of the eukaryotic cell cycle in recent years has increased dramatically. Genetic and biochemical analysis in yeast has made substantial contributions to that understanding. Budding yeast continues to provide a

testing ground for new approaches in the post-genomic era. Genome wide approaches to determining patterns of gene expression, protein-DNA and protein-protein interactions have been successfully implemented using budding yeast. Furthermore, analysis of the phenotypic consequences of gene deficiencies on a genome wide level by robotic approaches is being perfected. These approaches promise to accelerate the process of functional analysis of genes and the definition of novel pathways that regulate the cell cycle or are regulated by it. Subjecting candidate genes identified by these approaches to careful molecular genetic analysis promises to provide a more precise understanding the cell cycle and its regulation.

Acknowledgements

We would like to thank researchers whose work we have relied upon as the basis for this review. By necessity we have cited numerous review articles but recognize the work of the many individual studies that have contributed to our understanding of these areas. We would like to thank Steve Haase and Peter Kaiser for helpful discussion and comments on the manuscript. Steve Haase also kindly contributed the material for (Fig. 4). C.W. acknowledges support from the US Public Health Service and the US National Science Foundation. K.F. acknowledges funding from an APART Fellowship of the Austrian Academy of Sciences.

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CHAPTER 3

Role of Cyclins and Cyclin-Dependent Kinases in G1 Phase Progression

R. Curtis Bird

Abstract

Cyclin and the cyclin dependent kinases (CDKs) are the principle regulators of cell cycle progression through activation of cell cycle checkpoints. These are the core of the complex that composes the mechanism which drives the irreversible transitions that must be successfully negotiated for cells to successfully navigate the cell cycle and proliferate. During G1 phase there are a series of critical criteria that must be met for cells to proceed to S phase. These criteria and those necessary for cells to reenter the cell cycle through G1 phase, are governed by a subset of the cyclin/CDKs, those specifically activated during G1 phase, that are responsible for rendering cells permissive for irreversible entry into S phase and continued proliferation or, alternatively, retirement from the cell cycle to become senescent, quiescent or to terminally differentiate.

Introduction

The process of development in multicellular animals is the result of a successful series of continuous decisions to proliferate or not and then to differentiate, ultimately terminally, into a specific tissue cell type.[1] Cells make these decisions to enter the transition from quiescence to proliferation and then to retire from the cell cycle and terminally differentiate based on integrating a large number of external and internal cues that must also be integrated with intrinsic genetic controls.[2,3] Other possible post-proliferative fates which can await cells include quiescence, senescence or even apoptotic death in response to environmental conditions, such as starvation, or stimulation by specific peptide factors.[3-7] It is the nature of the mechanisms which govern, integrate and control the subtleties of these decisions during G1 phase that are the focus of this review. The core components of these processes have proven to be a rich source for understanding both the processes which regulate cell growth and differentiation but also the evolution of life since they represent some of the most conserved processes yet described in eukaryotes. Indeed, even single cell species, such as the fission and budding yeasts, make many of these same decisions, albeit in a less complex regulatory framework, but employing many of the same mechanisms and functionally homologous proteins.[8] The period of the cell division cycle which follows mitotic division, G1 phase, is the period during which many of these decisions are made.[9] Thus it is, during G1 phase, that the function of these critically important components will be examined.

G1 Phase Progression, edited by Johannes Boonstra.

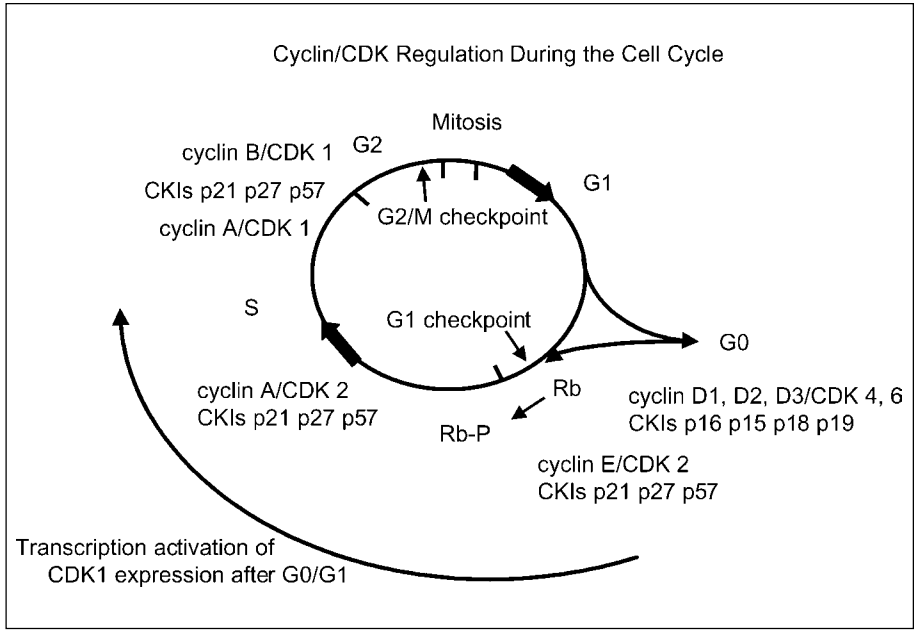

Figure 1. The eukaryotic cell cycle phases including major checkpoints, proteins and cyclin/CDK complexes active in their completion. Cyclin-dependent kinase (CDK) inhibitors (CKIs) associated with each cyclin/CDK are shown. The major functional checkpoint for RB protein phosphorylation and release is noted but further phosphorylation by CDKs activated later in the cycle can occur. G0 phase represents all of the nonproliferative cells residing outside the active cell cycle.

> "If man was to think beyond what the senses had directly given him, he must first throw some wild guess-work into the air, and then, by comparing it bit by bit with nature, improve and shape it into a truth."
> William Smith, 1859
> "We have almost unlimited time." Charles Darwin, 1858
> (both quoted from Eiseley in 10)

The construction of the modern paradigm we call the cell cycle is the work of countless scientists over more than 50 years. It was first glimpsed as a concept by Howard and Pelc[11] but the elegance of the current model is truly an egalitarian work. Though there are truly some giants among us, for example Drs. Nurse, Hartwell and Hunt honored recently by the Nobel Prize in Medicine and Physiology, 2001, it is also their students, colleagues and friendly competitors who have made the cell cycle the wonderfully complex mechanism we understand today. Since the time when it was first thoroughly reviewed as a cellular phenomenon,[9] the cell cycle has been the subject of regular and numerous reviews both global and focused. In contrast to that model proposed by Kuhn,[12] who first used the term paradigm to describe a scientific model, the cell cycle has truly been the work of many scientists adding their important section of mortar to this cellular construct. This chapter will focus on the G1 phase role of the molecules at the center of the model, the cyclin-dependent kinases and their cofactor cyclins that are thought, in no small measure, to create the cell cycle through their activities.

The Function of Cyclin/CDK Master Integration Complexes

The products of two families of genes govern progression through the cell cycle. Cyclin-dependent kinases (CDKs) and their cofactor cyclins form the core of the principle regulatory complexes that control the transition checkpoints during sequential phases of each cell cycle.[13-16] They also ensure the ordered and irreversible progression of each phase thus ensuring against premature entry into the subsequent phase.[17] Cyclin/CDKs were discovered separately in yeast and amphibian oocytes. These protein complexes were identified by virtue of their activity in inducing premature mitoses in oocytes or their mutability to temperature sensitive forms which blocked cells at a specific phase of the yeast cell cycle.[1,18-23] The presence and activity of the particular cyclin/CDK pairs present in cells are so important to the regulation of cell cycle progression and cell proliferation that their very activation state has been said to essentially define each cell cycle phase.[24,25] Additionally, this concept appears to be universally conserved throughout eukaryota.[26,27] Their discovery has been suggested as the seminal discovery at the heart of understanding cell proliferation controls.[28]

CDK1 (cdc2) is the original and archetypal CDK and is the basis for much of the effort in characterizing these molecules though its principle effects are not upon events in G1 phase. CDK1 is controlled during the continuous cell cycle principally at the level of post-translational modification and through its association with its cofactors cyclin and the p21-$^{Cip1/Waf1}$ and p27-^{KIP1} families of inhibitors to form phase-specific CDK–based integration complexes.[29,30] These complexes appear to have evolved in structure into divergent component subtypes that are responsible for the sequential transition of cells from one cell cycle phase through to the next.[31]

Much work followed that illuminated the role of cyclin/CDK pairs throughout the cell cycle but one functional theme seemed to be conserved – the concept of the cell cycle checkpoint.[32] The most important of these critical regulatory gateways occur just prior to the two most important events of the cell cycle – S phase and mitosis.[24] It is prior to these checkpoints when cells must assess the external and internal environmental cues and conditions and decide to proceed with a simple yes/no switch (Fig. 1). In continuously cycling cells at the G1 checkpoint, cells assess mass and component synthesis, cell signaling complicit with both appropriate growth factor and survival factor binding and determine that the DNA is intact and free of detectable defects prior to entry into S phase. Failure to comply with these requirements results in failure to complete the checkpoint followed by retirement or exit from the cell cycle. Retired cells must then choose to remain quiescent, become senescent, succumb to apoptotic death, terminally differentiate or reenter the cell cycle by passing the G1 checkpoint successfully.[25] The actual decision to commit to S phase, DNA replication and completion of the cell cycle appears to be made in late G1 phase.[33] Cyclin/CDK complexes bound to pRB (the retinoblastoma susceptibility gene product) phosphorylate this target and inactivate it releasing E2F transcription factors and potentiating the transition to S phase.[34] It is primarily the function first of cyclin D/CDK4 and cyclin D/CDK6 and subsequently cyclin E/CDK2 to sequentially phosphorylate the RB protein promoting its complete inactivation.[35,36] These functions are resisted by the CKIs (cyclin-dependent kinase inhibitors - see below). Overcoming this resistance and relieving the G1 checkpoint leaves the way open to S phase.

When cells are stimulated to reenter the cell cycle from quiescence/G0 phase they usually do so in response to peptide growth factor stimulation. Such stimuli invariably induce sequential expression of cyclin D and then cyclin E initiating the cell cycle signaling cascade and are likely rate-limiting events required for entry into S phase.[25,37] This is somewhat different from the regulation of cyclins that occurs later in the cell cycle as continued growth factor stimulation rather than internal controls are necessary for continued G1 cyclin expression. Other factors such as appropriate cell anchorage are also required to promote the proliferation of

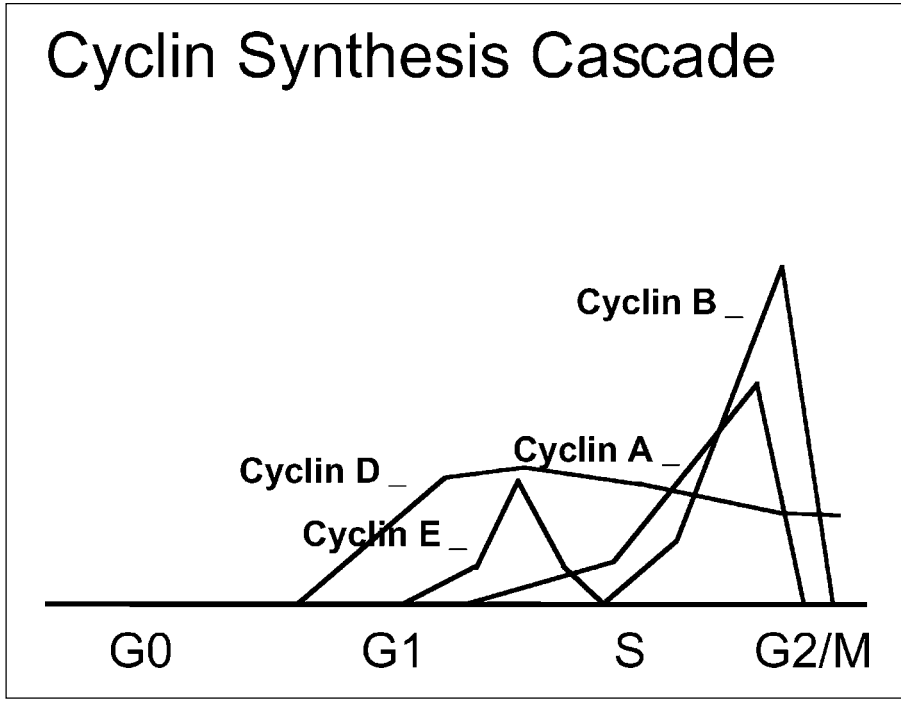

Figure 2. The cyclin synthesis cascade during a typical eukaryotic cell cycle. Expression levels of both G1 cyclins (cyclins D and E) are shown as are cyclins synthesized later in the cell cycle (cyclins A and B). Levels of expression are relative and integrated over cell cycle phase.

many cells and these processes also effect induction of cyclin D expression.[38] In most cases, positive stimulation of cells to reenter the cell cycle is channeled through activation of cyclin D expression initially. Subsequent suppression of cell cycle progression or restriction point activation to suppress proliferation is focused through the Cip/KIP and INK4 families of CDK inhibitors.[38]

The CDKs are serine/threonine protein kinases that phosphorylate a variety of known and as yet undiscovered targets.[14] Normally CDKs are held in a tightly controlled complex that suppresses their activity and arrests cell cycle progression until the moment when checkpoint suppression is relieved. When CDKs become activated checkpoint controls are functionally removed and cells can progress through the checkpoint to the next phase of the cell cycle.[38] This is the situation each time a continuously cycling cell passes through the G1 checkpoint. Signaling requirements include growth factor stimulation with subsequent stimulation of sequential downstream signal transduction components.[39] Signal transduction frequently involves growth factor receptors, a variety of protein kinases, as well as the c-ras protein, culminating in the expression of cyclin D1.[38] Paired with CDK4 or CDK6 these cyclin/CDK complexes can stimulate cells to pass the G1 checkpoint allowing them to successfully reenter the cell cycle.

All of these fates and decisions must be integrated through a common master integration complex or switch to be read out as a simple decision to proliferate or not and then, if not, to proceed through the appropriate cell fate gateway. The decision to proliferate or not is under functional control of the G1 phase cyclin/CDKs. Such integration requires that the cyclin/CDKs

interact with growth factor/mitogen and survival factor signaling systems, the products of oncogenes and tumor suppressor genes, differentiation factors and morphogens and even apoptotic factors that promote cell death. Although the ultimate fate of a cell may be affected by one or more of these other genes/systems, the decision to proliferate or retire from the cell cycle must be integrated through the G1 cyclin/CDK integration complex.

G1 Cyclin and CDK Gene Families

Currently, there are estimated to be approximately 3.0×10^9 bp encoding a minimum of approximately 35,000 genes in *Homo sapiens* and, by extension, most mammals. Of this number more than 100 genes are thought to be involved in the processes that regulate proliferation. Among these proliferation genes there are, to date, six G1 cyclin (cyclins D1, D2, D3, E1, E2, and C) and two G1 CDK (CDK4 and CDK6) genes identified as active in G1 phase.[40,41]

Cyclin Functions

Ultimately, the function of each cyclin is to bind to its appropriate CDK pair thus priming the integration complex for activation by external and internal signals. Waves of cyclin synthesis and degradation drive waves of CDK activation in a series of cascading activity inductions and suppressions.[16,42] Such tight control over active positive and negative regulation results in relatively narrow windows of time during each cell cycle phase when each CDK is active. The induction process is driven primarily by the activation of cyclin transcription followed by the suppression process which is driven by ubiquitination and degradation of cyclin.[38,42] Ubiquitination results in rapid export of D cyclins from the nucleus followed by proteolytic degradation in the cytoplasm in late G1/S phase.[43-45] Thus, upon stimulation of G0/quiescent cells to proliferate by peptide growth factors, there is a direct transcription activation of cyclin D1 driven by the signal transduction network that links growth factor receptors to the target genes in the nucleus.[46-48] Sustained G1 phase progression requires cyclin D expression supported by continued growth factor stimulation (Fig. 2). There is also evidence demonstrating extensive redundancy in the regulatory molecules that drive cell cycle progression particularly among the G1 cyclins, however, differential tissue-specific expression among the D cyclins has been observed consistent with differences in the promoter elements present in each gene.[16] Expression of cyclin D1 is dependent on the proliferation stimulatory transcription factor AP-1, composed of c-fos/c-jun heterodimers, and the same complex appears to suppress CKI p16/^{Cip1} expression.[16] There is also evidence for translational control of cyclin D1 coordinate with changes in transcription.[49]

In contrast, expression of cyclin E is more similar to that of cyclins expressed later in the cell cycle in that it follows a periodic kinetic pattern beginning to accumulate late in G1 phase followed by degradation in mid-S phase (Fig. 2). Peak expression occurs at the G1/S interface. Activation appears to be the result of E2F liberation from pRB binding and inhibition following phosphorylation of pRB by cyclin D/CDK4 and CDK6 directly stimulating cyclin E expression.[16]

CDK Functions

CDKs encode serine/threonine protein kinases that selectively phosphorylate targets that potentiate cell cycle progression in a cell cycle phase specific context. The principle CDKs associated with G1 phase cyclins are CDK4 and CDK6 in association with cyclin D1, D2 and D3 and CDK2 associated with cyclin E.[16] Much effort has been expended investigating the downstream targets of these complexes to determine the critical targets and systems stimulating the G1/S phase transition. It is clear from this work that the majority of their activity is directed towards inactivating the RB protein and subsequent liberation of the pRB bound E2F

family of transcription factors.[50-53] No other significant phosphorylation targets have been verified. Thus, though indirect, it appears that the principle output of the G1 cyclin/CDK complexes is modulation of transcription activity directed towards breaking through the restriction point threshold to S phase. All of the positive and negative signals directed towards these complexes are integrated into regulating this transition. Overexpression of the G1 cyclins, either D or E type, can have significant effects on cell proliferation. These include shortening of G1 phase, reduction in dependence on mitogen stimulation and even reductions in cell size resulting from, possibly, accelerated cell cycle times (reviewed in ref. 25).

Cyclin Ds are synthesized beginning as cells make the transition across the G0/G1 interface accompanied by binding to the associated CDK4/CDK6 activities being activated approximately at mid-G1 phase. Cyclin E is synthesized somewhat later in G1 phase with the activation of associated CDK2 as cells approach the G1/S phase transition. A single CDK activating kinase (CAK) enzyme appears to phosphorylate both of these cyclin CDK pairs on a single threonine.[54,55] Once the checkpoints regulated by these molecules have been successfully surmounted, cells rapidly degrade the G1 cyclins inactivating their associated CDKs and subsequently find themselves in S phase.

Recent evidence has expanded the complexity of this critical period when cells make decisions to retire from the cell cycle and differentiate or proceed through another proliferation cycle. Long thought to be confined to G2/M transition, CDK1 has also recently been shown to exert influence over the decision to enter S phase to guard against rereplication of DNA by suppressing the formation of prereplication complexes.[17,56] Although a principle problem only after S phase, there clearly must be a permissive state expressed for cells to enter S phase. Additionally, the timing of S phase entry appears to be under the influence of CDK1 activity as conditional mutants are unable to prevent premature entry into S phase. This provides evidence that CDK1 is involved in G1 checkpoint control through a role in ensuring against premature entry into S phase.[17] In support of this contention, the promoter for the human CDK1 gene is activated upon reentry into the cell cycle during G1 phase causing CDK1 transcription to be up-regulated more than 10-fold.[57,58] This activation is initiated as cells reenter the cell cycle through the G0/G1 phase transition.

Kinetics of Cyclin/CDK Function during G1 Phase

Cyclin was first described as a protein that appeared periodically during the cell cycle the amount of which was observed to rise and fall in a cyclic pattern every cell cycle in sea urchin embryos.[59] Now known to be cyclin A and B together, characterization of the expression of these molecules set the pattern for all subsequent cyclin molecules associated with cell cycle progression. G1 cyclins follow this paradigm as well. Cyclin D family proteins appear as cells reenter the cell cycle from quiescence and diminish about the G1/S interface while cyclin E appears slightly later vanishing once cells have entered S phase.[40] In their role as the activating cofactor of CDKs, cyclins represent the first level of activation for this complex system with cyclin regulation being exerted principally through transcription activation, initially during the accumulation phase, and then through transcription inhibition and proteolytic degradation.[28,60] The waves of each successive cyclin expression, accumulation and then degradation are the hallmark of cell cycle progression forming a sequential cascade of irreversible events that irresistibly follow the cycle through to mitosis (Fig. 2).

When cells exit the cell cycle there is generally a decline in cyclin levels.[40] However, exceptions exist. In some transformed cells, in the presence of fetal bovine serum, arrested cells will express significant levels of cyclin D1, however, these cyclins are complexed to $p21^{Cip1}$ as well as CDK4 and are inactivated.[61] Under such conditions these complexes may be acting as a sink to bind excess $p21^{Cip1}$. CDKs, in contrast, are present at essentially constitutive levels throughout

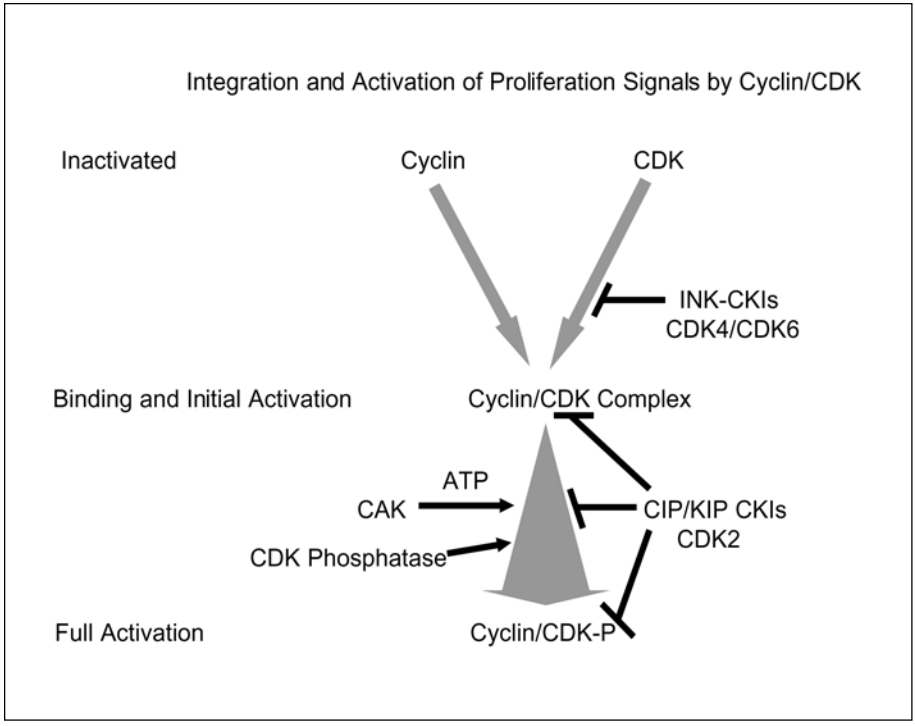

Figure 3. Cyclin/CDK complexes are master integrator complexes through which both inhibitory and stimulatory signals are passed. Principle regulators include CDK activator kinases (CAKs) and cyclin-dependent kinase inhibitors (CKIs) of the Cip/KIP and INK4 classes. Arrows indicate activating interactions while T-terminations reflect inhibitory interactions for CDK activity.

the cell cycle as long as cells are continuously dividing. However, once cells retire from the cell cycle, there is a decline in levels as quiescent cells express very little detectable CDK.[57,58,62]

Ubiquitination and Proteolysis in Cyclin/CDK Regulation

A reciprocal strategy is employed in cell cycle regulation that allows one regulatory molecule to simultaneously activate one cell cycle phase while inhibiting another. For example, one G1 cyclin CDK can activate G1/S transitions while inhibiting G2/M transitions. This strategy ensures that cell cycle progression is irreversible and irresistible. These irreversible and irresistible characteristics of successive cyclin/CDKs are also reinforced through ubiquitin-mediated proteolytic degradation of the cyclin cofactors once the checkpoint has been passed.[28] Proteolytic control of cyclin abundance is focused principally on the early (premid S phase) period of the cell cycle. There may also be differences in ubiquitination control depending upon whether CDK is bound to cyclin and whether cyclin D or E is involved.[16,63] Cyclins all encode a PEST sequence (protein motifs containing proline-P, glutamate-E, serine-S and threonine-T residues) which is recognized by the appropriate F-box protein and targets them for ubiquitination and proteolytic degradation.[3] The difficulty has been in establishing how specificity in the targeting of the ubiquitination is achieved.

Ubiquitination is a multistep process of covalent modification in which a prospective target reacts with a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and

a specificity factor (E3) that provides specificity in substrate selection. E1 is charged, through a high-energy thioester bond, with one covalently bound ubiquitin monomer. The ubiquitin is then passed to E2 by binding to a cystein residue. Multiple E2 molecules are bound to the E3 component in a complex which then specifically ligates the ubiquitin to the selected target protein. Successful addition of poly-ubiquitin through multiple reactions results in degradation of the target protein in the proteosome.[28] E3 is a multicomponent complex itself and contains the F-box protein which appears to specify the target sequence.[16] F-box proteins are transcriptionally and proteolytically regulated as well raising the complexity of control systems for degradation significantly.[28]

Proteolysis of multiple selected targets is a required activity to initiate S phase or for cells to exit G1 phase and make the transition out of the cell cycle.[60] At the end of each cyclin synthesis and CDK activation phase, there is an abrupt deactivation of CDK through proteolytic elimination of its cyclin partner.[60] In cases where a checkpoint has been successfully completed, further cell cycle progression includes proteolytic elimination of the cyclin. Degradation is managed through ubiquitination of cyclin and its subsequent proteolysis by the proteosome.[64] This is certainly true of cyclin E complexes which are subject to ubiquitination and degradation.[65] However, proteolytic control is not limited to the cyclins. To initiate S phase, the CKI p27-^{Kip1} associated with cyclin E/CDK2 complexes is also ubiquinated and subsequently degraded allowing CDK activation and phosphorylation of pRB.[33,65] This results in a low level of p27-^{Kip1} during active continuous proliferation with an accumulation of p27-^{Kip1} mass once cells retire from the cell cycle. Phosphorylation by CDK2 results in ubiquitination and proteolytic degradation of p27-^{Kip1} as cells reenter the cell cycle. In contrast, the p21-^{Cip1} protein does not appear to be regulated by ubiquitin-mediated proteolysis.[16,66-69]

Regulation of G1 Cyclin/CDK Complexes by Phosphorylation

Once synthesized and assembled, cyclin/CDK complexes are regulated by phosphorylation of the CDK component (Fig. 3). Modification through phosphorylation and dephosphorylation of the CDKs is well described particularly for CDK1 (cdc2/CDC28 homologues) though less is known regarding the other CDK molecules including those active in G1 phase.[3] In general, CDK1 is phosphorylated on a threonine residue (threonine 161 in CDK1 and threonine 160 in CDK2) by a CDK-activating kinase (CAK) to activate the enzyme.[37] Phosphorylation on a tyrosine residue (tyrosine 15 in CDK1 or tyrosine 17 in CDK4) and threonine residues (threonine 14) prior to activation suppresses kinase activity due to their location within the active CDK enzyme site. Substrate specificity is still a developing discipline among CAK and CDK phosphatases, however, some substrate specificity has been observed for different CAK enzymes. For example, CAK1p preferentially phosphorylates single CDK monomers while CAK p40-^{MO15} appears to prefer cyclin/CDK complexes.[70] p40-^{MO15} also prefers CDK6 complexes containing cyclin D3 cofactors while the CAK1p enzyme is able to phosphorylate CDK6 bound to any cyclin D molecule. This multilevel control appears designed to prevent premature activation while sufficient preparation and synthesis, particularly of the cofactor cyclin, proceeds. The cdc25C gene product dephosphorylates at least one of these sites finally activating the kinase activity within the CDK and liberating the cell from checkpoint restriction. Comparable controls appear to function in all the G1 CDKs.[3,37] The cdc25A and cdc25B phosphatases appear to be selective for G1 and S phase specific CDK activation, respectively. Transcription of the G1 phase cdc25A gene can be stimulated by the activity of oncogene products such as the myc/max complex making activation of existing CDK activity sensitive to oncogene-dependent pathways both through transcription of its cyclin cofactor and dephosphorylation of the kinase itself.[71] Reduction in cdc25A activity results in cyclin E1/CDK2 inhibition and has been correlated with cellular senescence.[6]

Other Components of G1 Cyclin/CDK Complexes

The first event in cyclin/CDK regulation following synthesis of the cyclin molecule is assembly of the cyclin/CDK complex.[16] However, this appears be more complex than previously understood. At least one report of possible assembly factors has been published.[72] There is also evidence for association of the cdc37/Hsp90 chaperonin complex with cyclin D/CDK4/CDK6 complexes facilitating assembly and CDK stability.[73,74] Cyclin/CDK complexes associate with CKIs—cyclin-dependent kinase inhibitors. CKIs come in two classes: the Cip/KIP family of molecules which are broad spectrum inhibitors (including p21-Cip1, p27-KIP1 and p57-KIP2) and the INK4 family (including p16-^{INK4A}, p15-^{INK4B}, p18-^{INK4C}, and p19-^{INK4D}) of narrow-spectrum inhibitors.[16,33] The specific sub-type of each CKI present in any cyclin/CDK complex appears to be both cell type and stimulus dependent. In general during G1 phase, INK4 CKIs bind cyclin D/CDK4/CDK6 complexes and Cip/KIP CKIs bind cyclin E/CDK2 complexes.

As stated earlier, late G1 phase progression to S phase is primarily the function first of cyclin D/CDK4 and cyclin D/CDK6 and subsequently cyclin E/CDK2 to sequentially phosphorylate the RB protein promoting complete inactivation. This results in E2F release and entry into S phase provided inhibition by bound CKIs can be relieved. These functions are resisted by the CKIs p16-^{INK4A} and Cip/KIPs p21 and p27 of which at least p16-^{INK4A} has been shown to be a tumor suppressor gene.[16,33] Evidence directly linking p27-^{KIP1} with cyclin E/CDK2 complexes also exists. Single CKI knockout mice have a variable and generally nonsevere phenotype reflecting probable redundancy in function among these genes.[33] Interestingly, p57 knockout mice show the most obvious and, after birth, lethal defects from single CKI gene deletions (reviewed in ref. 69). Other CKI knockouts result in far more subtle defects and changes.

p27 is most closely associated with cell cycle arrest and retirement from the cell cycle.[34] Levels of p27 drop dramatically, as a function of ubiquitination and proteolysis, as cells reenter the cell cycle, and rise as cells exit to quiescence.[75] Cyclin/CDKs may also contribute to p27 inactivation by sequestering the protein.[34] There is evidence that p21 and p27 may be divided in their activity between cell types.[76] For example, p21, p27 and p57 are all expressed in different tissues during mouse embryo development and in adult tissues.[69] Additionally, p21 expression may be suppressed and cyclin D1 promoter activity enhanced in response to activation by the small GTPase Rho.[77] Somewhat paradoxically, CDK activation potential has also been described for Cip/KIP inhibitors through their chaperonin-like activity perhaps promoting assembly into an active cyclin/CDK complex.[78]

The INK4 protein family, including p16-^{INK4A}, is associated with CDK4 and CDK6 complexes associated with cyclin D.[33] INK4 proteins, when bound to cyclin/CDK complexes, appear to be dominant over other activating factors until inactivated themselves.[38] Although not the principle regulators of G0/G1 transitions (this role appears to fall to p27), p16 and/or an alternatively spliced variant from the same locus, ARF, also appear to function in maintaining proliferative senescence[4,5,7,16] as is p15-^{INK4B}.[79] There is evidence that INK4 binding may destabilize existing cyclin/CDK pairs during G1 and also prevent the assembly of new cyclin/CDK pairs when it is appropriate.[80] Once cells have retired from the cell cycle and become senescent they have been demonstrated to accumulate both p16 and p21 CKIs though it is unknown if these gene products directly suppress cell proliferation under such conditions.[69]

Thus, there is a delicate interplay between factors/activities that balance cell life and death as well as proliferation and mere survival and all integrated through a single series of complexes. The cyclin/CDK integration complexes that regulate these fates in G1 phase are bound and regulated by a changing cast of CKIs and reacted upon by different CAKs and CDK phosphatases. These multiple stimulatory and inhibitory signals are all focused on regulating their association with, and phosphorylation of, RB protein leading ultimately to E2F release. Thus,

while we currently have a very detailed and growing understanding of cyclin/CDK integration complex function and regulation many additional factors including those regulating CKIs, CAKs and other stimuli remain enigmatic and await further study.

Subcellular Location of Cyclin/CDK Regulatory Complexes

Cyclins are normally synthesized in the cytoplasm like all proteins but then are rapidly transported to the nucleus where they bind their CDK partner.[37] Cyclin ubiquitination at the time of CDK inactivation results in rapid export of cyclins from the nucleus followed by proteolytic degradation in the cytoplasm.[43-45] There is also evidence for the displacement of CDK2 from the nucleus as part of the early apoptotic response.[81] Although associated with cyclin A in this study and thus an S phase function, such data points to the importance of subcellular location in understanding cyclin/CDK function. Such changes in location could directly affect substrate specificity and availability. An understanding of the subtleties of this process awaits further investigation but may reveal that location may affect CDK activity and its ultimate impact on proliferation and apoptosis. There is some indication as to where the location-specific information may reside in cyclin/CDK complexes. There are indications that, although principally involved in CDK inhibition, the Cip/KIP molecules may participate in cyclin/CDK complex assembly and perhaps in the transport/localization of these complexes in the nucleus.[78,82] Additionally, there is evidence that CDKs, especially CDK1, are not uniformly distributed within the nucleus being concentrated in localized nuclear regions in several as yet unidentified structures.[83]

Targets of Cyclin/CDK Activity

During G1/S phase transitions, the principle target of the cyclin/CDKs appears to be the RB protein which can be phosphorylated on multiple sites by G1 phase cyclin/CDKs.[3,25] Indeed, beyond pRB there is little known regarding other CDK targets during G1 phase.[16] Phosphorylation inactivates pRB liberating bound E2F transcription factors found as part of the cyclin/CDK/ pRB complex (Fig. 4). First cyclinD/CDK4 and CDK6 complexes phosphorylate pRB and then cyclinE/CDK2 is capable of further phosphorylation of this target.[37] Inactivation of pRB allows full realization of the CDK activity and release of E2F allowing the transcription of S phase-specific genes.[25] Active pRB may also help support G1 phase progression particularly in the early period of this cell cycle phase.[84]

Although only one real target of G1 CDK activity has been verified, the release of E2F transcription factors by deactivated pRB protein results in the activation of a variety of targets downstream from the G1 CDKs. These include activation of transcription of cyclin E, cyclin A, CDK1 (cdc2), CDC6, the CDK phosphatase CDC25A, the RB homologue p107, B-myb, and other E2F family members.[85-88] It is also unlikely that this is a comprehensive list as other protein targets are likely to be identified. These target genes all encode proteins with important roles to play in cell proliferation and cell cycle control downstream from the G1 cyclin/CDKs. Another important target of the E2F transcription factors is the ARF protein synthesized from an alternative open reading frame from the p16-^{INK4A} locus. The ARF protein is thought to link pRB and the p53 protein providing a plausible link between the G1 cyclin/CDKs and apoptosis.[85]

Control of transitions into and out of the cell cycle during G1 phase have implicated cyclin/CDKs and their pRB target in regulation of differentiation. There continues to be controversy regarding the actual role pRB plays in G1 progression, however. Recent evidence has identified differentiation-specific morphogens such as skeletal muscle potentiating MyoD and Id proteins as indirect targets following cyclin/CDK activation. This suggests a prominent role for pRB and its regulators such as the G1 cyclin/CDKs in G1/G0 to terminal differentiation transitions. There is also evidence suggesting cyclin E/CDK2 phosphorylation of the MyoD

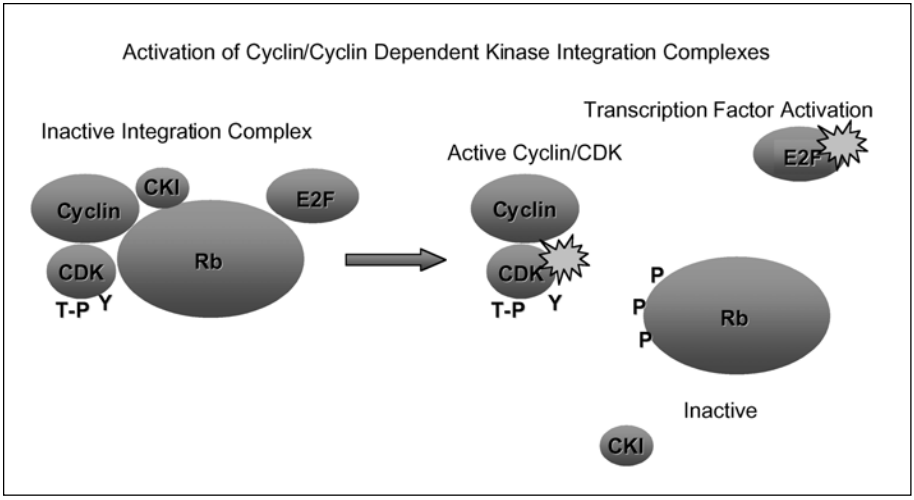

Figure 4. The best described target of the G1 cyclin/CDKs is the RB protein and its associated E2F transcription factors. Threonine (T) residues and tyrosine (Y) residues that are sites of phosphorylation (P) on CDKs are noted as are CKI inhibitors. Activation of CDK and E2F activity is noted by star-burst symbols.

muscle-specific transcription factor enhances instability.[33,89] Additionally, MyoD itself can reinforce terminal differentiation by activating p21-^{Cip1} expression reinforcing cell cycle arrest.[37] In support of this proposal, expression of p21-^{Cip1} is enhanced during muscle differentiation and induced expression of p21-^{Cip1} can enhance muscle gene expression.[69] However, p21 knockout mice are not defective in muscle development though double knockout mice that include p57 deletions are defective in this pathway. This suggests more redundancy in the regulation of these cyclin/CDK functions.[90] Late G1 events such as the activation of cyclinE/CDK2 can also affect differentiation suppressing the activity of Id protein which acts to inhibit differentiation promoted by MyoD.[52] The activity of differentiation modulators appear to be reactivated after the critical G1/S transition has occurred resetting the system in anticipation of the next cell cycle and the next G1 phase. However, muscle morphogens may participate in more generalized functions related to cell cycle exit. There is evidence that Id protein family members are necessary for G0/G1 transitions and this may reflect a need to relieve the differentiation signal prior to reentry into the cell cycle.

There is also evidence suggesting that the regulation of pRB and particularly E2F family members, possess additional subtleties that have only recently been detected. There is evidence that different members of these 2 families of proteins interact and exchange partners to create a changing population of transcription factor heterodimers that bind and release E2F responsive promoters as cells reenter the cell cycle from G0 to G1 and then into S phase.[53] As this complexity becomes better appreciated a new and more complete understanding of the interactions between these factors will be possible. This may also explain why only the original RB protein and not other members of this family, such as p130, seem to be a tumor suppressor gene target.[91]

Cancer and the Deregulation of G1 Cyclin/CDKs

Examination of cell proliferation indices in spontaneous tumor specimens reveals a higher percentage of proliferating cells than normal tissue but a cell cycle time, and thus proliferation

rate, for each cell that is relatively unchanged. This has been interpreted as evidence that mutations supporting neoplasia cause either an enhancement in the number of proliferating cells or a suppression of mechanisms resulting in cell loss.[84] Both possibilities are likely to be operative given the appropriate mutations. However, it is interesting that cell cycle period and relative control are not markedly affected. Only those processes regulating entry and exit from the cell cycle appear to be vulnerable. Thus, cyclin/CDKs active in G0/G1 transitions, and the molecules that regulate them, make likely targets for such mutations because they are principle regulators of entry and exit from the cell cycle.

Among the G1 cyclin/CDKs and their associated protein partners, only cyclin D1, cyclin E, p16 and pRB appear to be directly associated with neoplastic development.[42] Both cyclin D1 and E are frequently found to be elevated in expression level in tumor specimens and experimental overexpression of cyclin D1 in transgenic mice induced mammary hyperplasia and adenocarcinoma.[92-101] Classified as a tumor suppressor gene, p16-^{INK4A} is inactivated in up to 85% of tumor-derived cell lines.[33] Because approximately 80% of human tumors are carcinomas and these tissues depend on reinitiation of cell cycle for tissue replacement, there is strong circumstantial evidence linking cancer development with mechanisms regulating reentry into cell cycle. Not surprisingly, p16 is the second most common mutation found in cancer.[38] This suggests an important role for these proteins in G0/G1 transitions and darkly underlines their vulnerability to neoplastic mutation.

An indication of the importance of the regulatory proteins that modulate cyclin/CDK activity can be demonstrated by the frequency with which these genes are altered or deregulated in spontaneous cancers.[38] p53 mutations are the most commonly observed defects in human cancers and they generally loose their ability to arrest cell cycle because p21-^{Cip1} is a principle downstream target for transcription activation by p53. Failure to activate p21-^{Cip1} potentiates failure to arrest cell cycle progression through loss of this CKI activity.[102] DNA repair-mediated arrest of cell cycle in G1 appears to be the result of p53 activation[3] as p53 knockout mice fail to arrest in response to DNA damaging radiation.[103,104] This arrest is also mediated through p53 activation of p21-^{Cip1} transcription and consequently the inhibition of G1 cyclin/CDK activity.[105] Other proteins that interact with cyclin/CDKs can also be abnormally regulated in cancer. These include CDK4, p27 and the CDK phosphatase cdc25A (reviewed in ref. 42). In most cases an elevation in the level of expression is observed. Examples of CDK4 mutations that fail to respond to p16 suppression have also been observed.[106]

Transition through the G1 checkpoint into S phase requires activation of the E2F-family of S phase transcription factors and deregulated or excessive expression of E2F is a central defect found in many cellular models of cancer development.[107] Although not sufficient by itself, cells that overexpress E2F and that also are pRB defective, or in which the p53 checkpoint is suppressed, are unable to enforce a G1 phase checkpoint prior to entry into S phase. Because it is principally the role of the G1 cyclin/CDK complexes to regulate E2F release from pRB, and to integrate with the p53 G1 phase checkpoint, this mechanism represents a fundamental integration switch through which the decision to proliferate must be processed. These qualities also make this checkpoint system fundamentally vulnerable to neoplastic mutation

Viral Homologues of Cyclin D

Another group of terrestrial organisms have targeted the G1 cyclins, particularly cyclin D homologues, as a critical regulatory backdoor through which to wrest control of proliferation from the cell.[108-110] These include several herpesviruses of the γ-herpes family that possess cyclin D homologues that can substitute for G1 cyclins forcing pRB phosphorylation and inactivation thus releasing E2F transcription factors (reviewed in refs. 3 and 111). Such viruses as *Herpesvirus saimiri* and Human herpesvirus 8 (HHV8 or Kaposi's sarcoma herpesvirus) are both examples of viruses which utilize this strategy to drive cells towards S phase. Other

examples drive cell cycle to the brink of the G1/S phase transition and then arrest cell cycle progression in an attempt to render only viral genomes replication competent. Such observations independently underscore the importance attributed to these proteins in regulating normal proliferation and differentiation systems as well as their potential to promote neoplasia.

Conclusions

Since its discovery, the cyclin/CDK complex has come to embody the essence of cell cycle phase transitions and is now essentially the defining activity of each cell cycle phase. Although initially defined in terms of reentry into the cell cycle, G1 cyclin/CDKs are now viewed in a more global context that encompasses the regulation of cell cycle progression, egress into and out of the cell cycle to quiescence, the transition to terminal differentiation, senescence and possibly control of some apoptotic processes. Despite much progress in understanding the subtleties of these complexes and their function in different cell types, there is still much to learn regarding function and particularly those specific G1 CDK targets responsible for the transition from G0 to and from G1 and from G1 to S phase. More interacting protein targets are certain to be identified. Likewise, the subtleties controlling localization of cyclin/CDKs and their complex partners have only been preliminarily described and much more information is also likely to be discovered. As these complexes become better understood and the processes they control elucidated we will also better understand how cells control their proliferation, their differentiation and how these mechanisms become corrupted during the development of cancer. Hopefully such insights also can provide the targets and means necessary to develop effective therapeutic strategies.

Acknowledgements

For those who work in the discipline of cell cycle, there is a tendency to track our academic or scholarly lineages back through those eminent giants, the shoulders of whom, we all stand upon. My entry into this discipline began under the guidance of Dr. Arthur Zimmerman, Department of Zoology, University of Toronto. Art was a post-doctoral fellow of Dr. Daniel Mazia, UC Berkley, and I mark my scientific heritage back to the Mazia lab and its wonderful efforts to define the mitotic spindle and that part of the cell cycle concerned with mitosis. It was through Art's gentle and compassionate guidance that I learned to grow into an independent scientist. I was also fortunate to benefit from Art's close association with many other eminent cell cycle biologists. These include his long time collaborators Drs. Ivan Cameron and George Padilla as well as Dr. Arthur Forer. There also was another group present in that department that would have an important impact on cell cycle biology and genetics: Dr. Yoshio Masui and his student Manfred Lohka. Fred and I were contemporaries and his discoveries with Dr. Masui, including the first isolation of the cyclin/CDK complex, are cornerstones of the discipline. I would trace a lineage through Fred and his mentor to Dr. Clement Markert, Dr. Masui's post-doctoral mentor, and ultimately to Dr. Hans Speman who began discovery of the cell cycle by study of embryo development.

I thank Dr. Allen Dingle, McMaster University, who opened my eyes to the wonder of scientific discovery and the thrill experienced each time a well planned and executed experiment unfolds in our hands. That thrill is still there and it is the one thing – the only necessary thing—to keep a scientist active, curious and productive. I thank Dr. Bruce Sells for the post-doctoral experience in molecular biology and genetics when that discipline was in its infancy.

I gratefully acknowledge discussions with colleagues Dr. Vicky van Santen regarding viral cyclins and Drs. Robert Kemppainen and Joseph Renden for many long miles and the discussions that supported them. Lastly, I thank my collaborators, Drs. Bruce Smith, Lauren Wolfe, and Allison Church Bird for critical reading of the manuscript and discussion and thought

regarding cell cycle, cell proliferation and cancer development. Supported by Scott-Ritchey Research Center Foundation and Food Animal and Disease Research Program at Auburn University, College of Veterinary Medicine.

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Chapter 4

Roles of Cyclin Kinase Inhibitors in G1 Phase Progression

Angela L. Tyner and Andrei L. Gartel

Abstract

Cell cycle progression is tightly controlled by cyclin/Cyclin dependent kinase (CDK) complexes. Two families of cyclin kinase inhibitors have been identified based on their structures and affinities for cyclin/CDK complexes. Members of the INK 4 (Inhibitors of CDK 4) family bind and inhibit CDK 4 and CDK 6, while members of the Cip/Kip (Cdk interacting protein/Kinase inhibitory protein) family inhibit CDK2. In this chapter we discuss the roles of the INK4 and Cip/Kip families in cell cycle regulation and tumorigenesis. We describe mouse CDK inhibitor knockout models and functions of the CDK inhibitors in development. The coding sequences of the *p16* (INK4A) gene partially overlap with the coding sequences of another gene, ARF. These two products of the INK4A/ARF locus indirectly control the activities of two major tumor suppressors, RB and *p53*, and these genes encoding all of these proteins are often mutated in tumors. In addition to regulating cell proliferation, CDK inhibitors may be involved in regulating differentiation, cell migration, senescence and apoptosis, and play important roles in preventing the development of cancer.

Introduction

Cell cycle progression is tightly controlled by the regulation of expression and activity of cyclin/Cyclin dependent kinase (CDK) complexes (reviewed in 1-3). Cyclins are activating subunits that interact with specific CDKs to regulate their activity and substrate specificity. The best-characterized substrates of cyclin/CDKs are members of the retinoblastoma protein (RB) family. During G0 and early G1 phases of the cell cycle, RB proteins bind E2F transcription factors and negatively regulate gene expression required for S-phase progression. The decision to undergo DNA synthesis and subsequent mitosis is made late during the G1 phase of the cell cycle, during which time active cyclin/CDK complexes phosphorylate and inactivate members of the RB family, leading to expression of E2F responsive genes required for DNA replication.[4]

G1 progression is regulated by the D-type cyclins (D1, D2, D3), and cyclin E.[5] D-type cyclins are associated with either CDK4 or CDK6, while cyclin E is associated with CDK2. The D-type cyclins are the first to be expressed in response to growth promoting signals, and they bind and activate CDK4 or CDK6. This is followed by expression of cyclin E during mid to late G1, which activates CDK2.[6] Active complexes containing CDK4/6 or CDK2 phosphorylate RB proteins on different sites, and full phosphorylation and inhibition of pRB requires the activities of both cyclin D and E regulated CDKs (reviewed in 7,8).

G1 Phase Progression, edited by Johannes Boonstra.

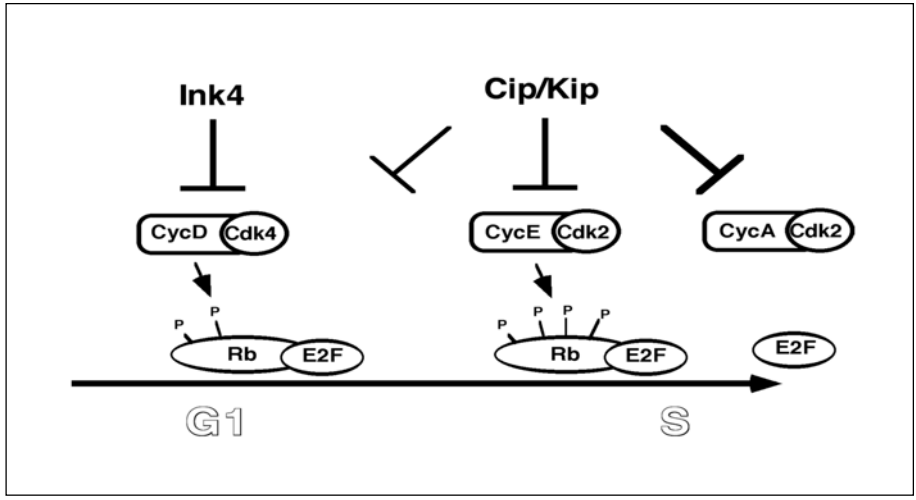

Figure 1. CKI inhibition of cyclin/CDKs. Ink 4 family CKIs bind and inhibit CDK4 and CDK6, while members of the Cip/Kip family have a broader specificity in vitro, but inhibit CDK2 containing complexes in vivo.

Two families of cyclin kinase inhibitors (CKIs) that play key roles in the regulation of cyclin/CDKs have been identified based on their structures and affinities for cyclin/CDK complexes. Members of the INK 4 (Inhibitors of CDK4) family bind and inhibit CDK4 and CDK6, while members of the Cip/Kip (CDK inhibitory protein/Kinase inhibitory protein) family have a broader specificity in vitro, but inhibit CDK2 containing complexes in vivo (Fig. 1) (reviewed in 3, 9-11).

Mitogenic stimulation promotes cell cycle progression that is accompanied by assembly of cyclin D/CDK4/6 complexes.[12] A number of studies suggest that sequestration of Cip/Kip CKIs in cyclin D containing complexes prevents them from inhibiting cyclin E/CDK2, thereby allowing cell proliferation to proceed. In cells overexpressing cyclinD/CDK, *p27* was recruited into cyclin D1/CDK4 complexes, and CDK2 containing complexes were activated.[13] In other studies Myc was found to activate cyclin E/CDK2 by in part by inducing expression of cyclin D1 and cyclin D2 which associate with and sequester *p27* and *p21* in proliferating cells.[14,15] Ectopic expression of the INK4 CKI *p16* inhibited Myc-induced dissociation of *p27* from cyclin E/CDK2.[15] CDK4, CDK6, and CDK2 activity was inhibited in U2-OS cells engineered to overexpress *p16*.[16] In these cells *p27* was redistributed from CDK4 to CDK2 containing complexes upon induction of *p16*.

Thus, in addition to inhibiting CDK4 through direct binding, INK4 CKIs may indirectly regulate CDK2 activity by displacing Cip/Kip proteins from cyclin D containing complexes. The displaced Cip/Kip proteins are then able to bind and inhibit CDK2 containing complexes (Fig. 2). The Cip/Kip CKIs may positively and negatively regulate cyclin/CDK complexes by promoting their assembly and/or stabilization, and inhibiting their activities (see below). The different functions of the two families of CKIs and roles that the individual members of each family play in the regulation of G1 progression are discussed below.

The Cip/Kip Family of Cyclin Kinase Inhibitors

The three members of the Cip/Kip family *p21*,[17-19] *p27*[20,21] and *p27*[22,23] contain a conserved region of sequence at the amino terminus that is required and sufficient for the inhibition

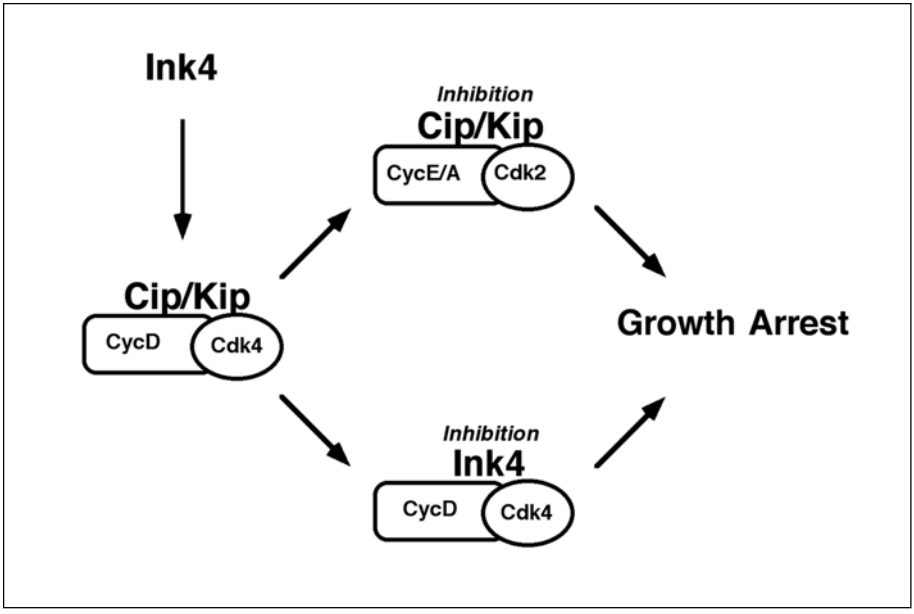

Figure 2. Regulation of Cip/Kip activity by sequestration in cyclin D containing complexes. Mitogenic signals induce cyclin D which can bind and sequester Cip/Kip CKIs. INK4 CKIs can promote growth arrest by displacing sequestered Cip/Kip CKIs from cyclin D containing complexes, freeing them to inhibit CDK2 activity.

of cyclin/CDK complexes, while the carboxy terminal regions are variable in length and function.[22,24-26] These CKIs can bind individual cyclin and CDK subunits but have a stronger affinity for cyclin/CDK complexes.[25,27,28] Analysis of the crystal structure of the 69 amino acid N-terminal domain of *p27* bound to the phosphorylated cyclinA/CDK2 complex showed that *p27* binds the complex as an extended structure that interacts with both cyclin A and CDK2. *p27* was found to insert into the catalytic cleft of CDK2 causing conformational changes and blocking ATP binding.[29] Primary structure similarities between the three Cip/Kip family members suggest that they will interact similarly with cyclin/CDK complexes.

Although isolated as inhibitors, Cip/Kip CKIs have been detected in active cyclin D/CDK4 complexes (Fig. 2).[30-33] *p21* was shown to stabilize interactions between CDK4 and cyclin D and promote the formation of active complexes in a concentration dependent manner.[33] Additional data supporting a role for Cip/Kip proteins in promoting CDK4 activity in vivo came from studies with *p21*/*p27* deficient mouse embryonic fibroblasts (MEFs). These cells contained undetectable levels of cyclin D/CDK4 complexes.[34] However the *p21*/*p27* deficient cells retained some sensitivity to INK4 CKI *p16* which specifically inhibits cyclin D/CDK4 complexes, suggesting the presence of some active cyclin D/CDK4.[34] Low amounts of active cyclin D3/CDK4 were detected in *p21*/*p27* deficient MEFs in another study,[35] also suggesting that other factors may influence assembly and activation of cyclin D/CDK4 complexes.

The three members of the Cip/Kip family have distinct and overlapping roles in regulating G1 progression and subsequent growth and differentiation. Expression of *p21* is induced following DNA damage and detected in differentiating cells both in vitro and in vivo. Its expression is induced in response to a number of mitogenic stimuli (reviewed in 36). In contrast *p27* is expressed in quiescent cells and its levels and inhibitory activities decrease upon

mitogenic stimulation (reviewed in 37-39). Replicative senescence is regulated by the INK4 CKI *p16*,[40] *p21*,[41] and in some cases *p27*.[42] Only disruption of the *p57* gene resulted in profound developmental defects in mice.[43,44] Distinct roles for the related Cip/Kip CKIs may reflect differences in their expression patterns and regulation under different growth conditions. However unique interactions have also been identified for each of the three Cip/Kip family members.

p21(Cip1)

p21 was identified independently by several groups using a number of different screening strategies. It was identified as a CDK binding protein, and was subsequently named CIP1, for CDK interacting protein 1,[18] and Cap20.[45] Micro-sequencing of a protein that interacted with CDKs led to its cloning using PCR.[19] *p21* was identified as the product of a gene activated by wild-type *p53*, and it was named WAF1 (wild-type *p53* activated factor).[17] It was also cloned using an expression screen designed to identify inhibitors of DNA synthesis from senescent fibroblasts, and it was named SDI1 (senescent cell-derived inhibitor).[46] Using subtractive hybridization the *p21* cDNA was isolated based on its increased expression in human melanoma cells that were induced to differentiate, and named MDA-6 for melanoma differentiation associated protein.[47]

p21 contains a carboxy terminal binding site for proliferating cell nuclear antigen (PCNA) and is found in quaternary complexes containing *p21*, PCNA, and cyclin/CDK in normal cells.[19,48,49] Through its direct interaction with PCNA, *p21* can also block DNA synthesis by DNA polymerase δ.[50-52] Several studies have suggested that *p21* regulates DNA repair through its interactions with PCNA, although the results have not been conclusive. Different approaches have led investigators to conclude that *p21* is required,[53,54] unnecessary,[55-57] and inhibitory[58,59] for DNA repair, with nucleotide excision repair as the general focus of these studies. Mismatch repair activity was shown to be inhibited by *p21* or a *p21* peptide that bound PCNA, and this inhibition could be reversed by increasing levels of PCNA in the reaction.[60] Recently *p21* was found to inhibit PCNA stimulated long patch base excision repair.[61]

p21 expression has been shown to be regulated largely at the transcriptional level by both *p53*-dependent and independent mechanisms. In most tissues examined except the spleen, expression of *p21* is *p53* independent.[62,63] Expression of *p21* in tissues of the adult mouse is localized to terminally differentiating cells. Highest levels of *p21* expression were found in tissues with high turnover rates, such as the skin and linings of the gastrointestinal tract.[63-65] The *p21* promoter contains two conserved *p53*-binding sites and at least one of these is required for *p53* responsiveness following DNA damage.[66] In addition a variety of transcription factors that are induced by a number of different signaling pathways activate *p21* transcription by *p53*-independent mechanisms, including Sp1, Sp3, Ap2, STATs, C/EBPα, C/EBPβ, and the bHLH proteins BETA2 and MyoD (reviewed in 67).

p21 expression may also be regulated post-transcriptionally by both ubiquitin-dependent and -independent proteasomal mediated degradation.[68-70] A degradation signal was identified in the C-terminus of *p21* that mediates interactions with the 20S proteasome, providing a mechanism for ubiquitin-independent degradation of *p21*.[70] Other factors have also been shown to regulate *p21* protein turnover, including the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα), which binds and regulates levels of *p21* protein in liver cells.[71] Recent reports suggest that glycogen synthase kinase-3 (GSK-3) phosphorylates *p21*, promoting its degradation and that AKT can inhibit this process.[72]

Disruption of the *p21* gene in the mouse did not result in gross abnormalities, although studies using *p21* deficient MEFs revealed an essential role for *p21* in inducing growth arrest following DNA damage.[73,74] In vivo, *p21* appears to play roles in regulating renewal of

keratinocytes[75] and hematopoietic cells.[76] It has been shown to play a role in the control of T-cell proliferation and female *p21* deficient mice have decreased viability and develop a syndrome similar to human lupus.[77] In the kidney, *p21* appears to regulate the balance between hyperplasia and hypoplasia, and its disruption ameliorates progression to chronic renal failure after partial renal ablation.[78] Survival of *p21*-deficient mice decreased 40% after hyperoxic lung injury suggesting that *p21* protects lung from oxidative stress.[79] *p21* induction was correlated with differentiation of muscle[62,80] and embryos lacking both *p21* and *p57* have severe defects in the formation of skeletal muscle and altered lung development, suggesting redundant functions for *p21* and *p57* during development.[81]

p21 has been shown to act as an inhibitor of both *p53*-dependent and independent apoptosis (reviewed in 82). The HCT116 human colon carcinoma cell line undergoes cell cycle arrest after treatment with adriamycin, but *p21*-null HCT116 cells undergo apoptosis after the same treatment.[83,84] Likewise, when HCT116 cells were infected with a recombinant adenovirus expressing wild-type *p53*, cells with intact *p21* and *14-3-3* genes underwent cell cycle arrest. In contrast, cells lacking either *p21* or *14-3-3* underwent cell death and double knockout cells died substantially more quickly than cells lacking only one of the two genes.[84] DNA damage and oxidative stress activate *p53*-dependent apoptosis or *p53*-dependent or independent expression of *p21* that then protects against apoptosis.[83,85] The ability of *p21* to inhibit cell proliferation may contribute to its capacity to act as a tumor suppressor. However the capacity of *p21* to induce cell cycle arrest after stress may also protect cells from undergoing stress-induced apoptosis and contribute to oncogenesis.[82]

The first evidence for a tumor suppressor function for *p21* in vivo came from studies in *p21*/*p18* double deficient mice which have increased incidence and progression of pituitary tumors, with most animals developing tumors by one year of age.[86] Aging studies in *p21* deficient mice have also demonstrated a role for *p21* in tumor suppression, albeit a much weaker one than *p53*. Mice lacking *p21* formed spontaneous tumors by 16 months of age in comparison with 20 months for wildtype animals.[87] Interestingly *p21* deficient mice were less susceptible to irradiation-induced carcinogenesis, presumably because of increased *p53*-dependent apoptosis.[87] Mice lacking *p21* have been found to be more susceptible to chemically induced skin carcinoma.[75,88] Loss of *p21* was associated with earlier development of mammary gland tumors, increased tumor multiplicity and aggressiveness in MMTV/v-Ha-ras mice.[89] *p21* was also shown to synergize with pRB and INK4A/ARF to protect against tumor progression in mice.[90,91]

p27(Kip1)

p27 was identified as a protein associated with cyclin E/CDK2 in arrested cells[20] and in a yeast two-hybrid screen designed to identify proteins that interact with cyclin D/CDK4.[21] Human *p27* contains an N-terminal CDK-inhibition domain and a C-terminal QT domain.[23,24] Unlike *p21* and *p57*, the C-terminus of *p27* has not been reported to bind PCNA.

Post-transcriptional mechanisms are largely responsible for regulation of *p27* expression. *p27* levels increase as cells become quiescent and decrease when cells are stimulated to reenter the cell cycle.[92-94] This decrease is due in a large part to a decrease in the half-life of *p27* as cells begin to progress through the cell cycle.[95] Following growth stimulation, *p27* is phosphorylated by CDK2 and targeted for degradation through an ubiquitin-dependent pathway mediated by interaction with the F-box protein SKP2.[96-98] The F-box protein SKP2 binds the extreme carboxy terminus of phosphorylated *p27* and promoting its ubiquitination and degradation. Increased expression of *p27* in G0 is also regulated at the translational level.[99,100] *p27* activity may also be regulated within the cell by sequestration in cyclin D/CDK2 containing complexes[14,15] and subcellular localization.[101]

Multiple functions for *p27* in regulation of growth and proliferation have been revealed in *p27* null mice.[102-104] *p27*-deficient mice grow 20-40% larger than wild-type littermates due to alterations in the balance between proliferation and withdrawal from the cell cycle at critical periods of development. These mice develop intermediate lobe pituitary hyperplasia and adenoma, and female homozygous $p27^{-/-}$ mice are infertile. Although ovarian follicles develop, they do not progress to form corpora lutea. *p27* has been shown to act as a safeguard against excessive cell proliferation following experimental induction of inflammatory injury in the kidneys of mice.[105] It is expressed throughout the adult liver and plays a critical role in maintaining hepatocyte quiescence.[105B] *p27*-deficient and $p27^{+/-}$ mice have been shown to be prone to tumor development in multiple tissues following γ-irradiation and other challenges.[106]

Cell culture experiments suggested that *p27* is a negative regulator of cyclin E/CDK2 that can be titrated away by cyclin D containing complexes. Studies in knockout mice have provided genetic evidence for interactions between *p27* and cyclin D1.[107,108] Disruption of the genes encoding either cyclin D1 or *p27* result in opposite phenotypes. For example, cyclin D1 deficient mice are small and often die early in life, while *p27* are larger than normal. However, mice deficient for both cyclin D1 and *p27* were of normal size. A subset of other abnormalities was also corrected in mice deficient for both cyclin D1 and *p27*, such as retinal hypoplasia in cyclin D1 deficient animals and retinal hyperplasia in *p27* deficient mice.

While the human *p27* gene maps to a region of chromosome 12p13 that is frequently altered in hematological malignancies, the gene encoding *p27* is rarely mutated in cancers. However reduced expression of *p27* has been correlated with poor survival among patients with breast, prostate, or colorectal carcinomas (reviewed in 37-39). Decreased expression of *p27* protein has also been associated with poor prognosis in hepatocellular carcinomas.[109-111] In a variety of cancers, cytoplasmic localization of *p27* protein was detected and may reflect impaired inhibitory activity in the nucleus.[112]

p57(Kip2)

p57 is the most structurally complex member of the Cip/Kip family. cDNAs encoding this CKI were isolated in a homology based screen using a *p21* cDNA as a probe,[22] and in a two-hybrid screen for cyclin D interacting proteins.[23] Human *p57* has an amino terminal CDK-interaction domain, a proline-alanine rich PAPA domain, and a carboxy terminal QT domain.[22,23] A carboxy terminal QT domain is also found in *p27*.[23] The *p57* CDK interaction domain shares structural similarity to that of *p21*.[113] The carboxy terminus of *p57* has been shown to interact with PCNA, albeit with an affinity that is 10-fold lower than that of *p21*.[114] Deletion of either the amino terminal cyclin/CDK interaction domain or the carboxy terminal PCNA binding domain compromised the ability of *p57* to inhibit transformation.[114] The amino terminus of *p57* has been shown to interact with the muscle specific transcription factor MyoD, leading to its stabilization.[115]

p57 is encoded by a paternally imprinted gene in both mice[116] and humans.[117] The human gene maps to chromosome 11*p15*.5, a region lost in many cancers and implicated in Beckwith-Wiedemann syndrome, which results in congenital defects, organomegaly and increased risk for developing childhood tumors. Mutations in the *p57* gene have been identified in a small number of patients with Beckwith-Wiedemann syndrome.[118,119] In the mouse, the *p57* gene maps to the distal region of chromosome 7 where it is linked to other imprinted genes including Igf2 and H19.[116]

p57 is expressed in a tissue specific manner[22,23] and widely expressed during embryo morphogenesis.[120] Disruption of the maternally expressed *p57* gene in the mouse caused several developmental abnormalities that are consistent with a role in Beckwith-Wiedemann syndrome including renal dysplasia and adrenal cytomegaly.[43,44] Increased proliferation, apoptosis, and

altered differentiation were also noted. However phenotypic differences were observed between *p57* null mice that were generated by different groups, probably due to differences in the genetic backgrounds of the mice used in the studies. For example *p57* was required for postnatal survival in the C57BL/6-129Sv background, but outbreeding of these animals with the CD1 mouse strain resulted in increased survival.[43] Yan and colleagues found variable penetrance of the *p57* null phenotypes and approximately 10% of their *p57* null mice survived without obvious abnormalities.[44]

Redundant roles for *p57* and *p21* in regulating the development of skeletal muscle have been demonstrated in *p57*/*p21* deficient mice.[81] The phenotype of the *p57*/*p21* deficient mice is similar to that of myogenin deficient animals, and it is proposed that these two CKIs act at the myogenin step. The Cip/Kip CKIs may also regulate other events in muscle differentiation as stability of the muscle specific transcription factor MyoD is enhanced when its phosphorylation by cyclin E/CDK2 complexes is inhibited.[121] In addition, MyoD stability is increased through direct interaction with *p57*, independent of its CDK inhibitory activity.[115]

The INK4 Family of Cyclin Kinase Inhibitors

Four members of INK4 CKI family: *p16*(INK4A),[122] *p15* (INK4B),[123] *p18* (INK4C)[124] and *p19* (INK4D)[125] share 40% homology with each other and similar structural characteristics – the presence of multiple ankyrin repeats. The third ankyrin repeat is essential for interactions of INK4s with CDK4/CDK6.[126] Usually they compete with D-type cyclins for binding to CDKs and therefore presence of pRB is crucial for growth suppression by INK4 (Fig. 1).[124,127,128] In the absence of pRB, inhibition of cyclin D-CDK4/CDK6 complexes by INK4 does not lead to cell cycle arrest, indicating that the pRB is the critical target of cyclin D-dependent kinases in the G1 phase of the cell cycle.[127,128] The INK4 and cyclin binding sites on the CDK do not overlap and INK4 blocks cyclin binding indirectly. It causes allosteric changes in the CDK that alter the cyclin binding site. Both INK4 and the cyclin have to interact with both CDK lobes for their function, but they need them in different relative orientations, and INK4 prevails any time it is present.[129]

However, if the CDK4/6-cyclinD complex is assembled first, INKs can bind this complex and inhibit it's activity.[125,130] The structure of the inactive ternary complex between CDK, the INK4, and a D-type cyclin reveals that INK4 inhibits the CDK-cyclin complex by distorting the ATP- and cyclin-binding sites, with the cyclin bound at an interface that is substantially reduced in size.[131] These observations support the notion that INK4 binding weakens the cyclin's affinity for the CDKs and may increase the rate of cyclin dissociation. Since INK4 can also prevent the assembly of new CDK4/6-cyclinD complexes,[9] INK4-CDK4/6 complexes are much more abundant than the ternary INK4-CDK4/6-cyclinD complexes in the cell.

INK4 genes are differentially expressed during mouse development. INK4C and INK4D are broadly and abundantly expressed during mouse embryogenesis and throughout adult life, while INK4A and INK4B are not expressed before birth and very weakly in aged mice (reviewed in 132). In adult human tissues the most widely expressed INK4 protein is *p18*, followed by *p15*, *p16*, and *p19* suggesting that in humans *p19* expression is more restricted and *p16* expression is more abundant.[133] Most human tissues express two or more members of INK4 family suggesting that INK4 proteins provide redundant and nonoverlapping characteristics in cell cycle control, differentiation and oncogenesis.

p16(INK4A)

p16 is a founding member of INK4 family, which was originally cloned as a CDK4 interacting protein that inhibits CDK4 kinase activity.[122] The gene is located on chromosome 9*p21* (chromosome 4 in mice). *p16* was inactivated in up 85% of studied tumor cell lines including

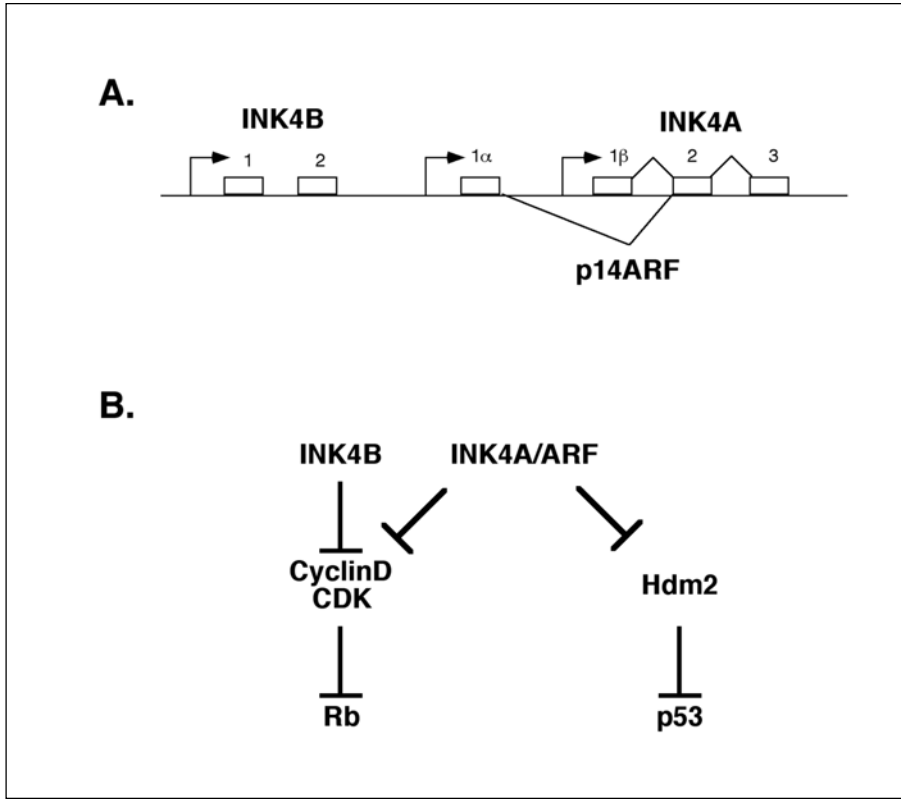

Figure 3. INK4A-ARF and INK4B human loci. (A) Schematic representation of the chromosomal location and gene structure of INK4A-ARF and INK4B loci. (B) INK4A and INK4B indirectly regulate the retinoblastoma protein (Rb), while ARF regulates *p53*.

melanoma, lung, pancreas, bladder, head and neck, breast, brain, ovarian and some others.[134,135] Later it was shown that *p16* is inactivated in a variety of primary human tumors including bladder carcinoma,[136] glioma,[137] mesothelioma,[138] T cell acute lymphoblastic leukemia,[139] prostate and ovarian adenocarcinoma,[136] sarcoma,[140] hepatocellular carcinoma[141] and renal cell carcinoma.[142] Two major mechanisms of *p16* inactivation in human tumors are represented by small homozygous deletions of the gene and inappropriate methylation of the *p16* promoter that leads to gene silencing.[143]

p16-specific germ line mutations were identified in a kindred with familial melanoma and pancreatic adenocarcinoma[135,144] pointing to *p16* loss as the earliest genetic abnormality in development of pancreatic adenocarcinoma.[145] In addition, in early melanomas the helix-loop-helix transcription factor Id1 may repress *p16*, which may represent one of the earliest mechanisms of *p16* inactivation in melanoma initiation.[146,147] The polycomb family member Bmi-1 may repress *p16* expression leading to fibroblast immortalization and in combination with H-ras to neoplastic transformation.[148] A novel potential oncogene SEI-1, cloned by interaction with *p16*, appears to antagonize *p16* function by making cyclin D1-CDK4 complex resistant to inhibition by *p16*. Expression of SEI-1 is rapidly induced after addition of serum to quiescent fibroblasts and it may facilitate the formation and activation of cyclin D-CDK complexes in the face of inhibitory levels of *p16*.[149]

All of these data suggest that *p16* is bona fide tumor suppressor, which is frequently inactivated in human cancers. *p16* can inhibit CDK4-6/cyclinD kinases and maintain pRB in its active anti-proliferative state. It appears that loss of tumor suppressors *p16* or pRB, or overexpression of proto-oncogenes cyclin D1 or CDK4 take place in large majority of human tumors implying that *p16*/cyclinD1/CDK4/ pRB signaling pathway functions in tumor surveillance[150,151] (Fig. 3). Surprisingly in addition to *p16*, the INK4a locus also encodes the p14ARF (alternative reading frame; *p19* in mouse) protein via splicing of alternative first exons (1α vs. 1β) to a common second exon sequence that is translated in two different reading frames (Fig. 3A). This results in the production of two proteins *p16* and p14ARF that do not share amino acid homology. P14ARF associates directly with MDM2 (HDM2) to inhibit MDM2-dependent degradation of tumor suppressor *p53* and causes stabilization of *p53* protein (Fig. 3B). p14ARF is induced by oncoproteins such as Myc,[152] E1A,[153] activated Ras[154] and v-abl[155] suggesting that it acts as a sensor of hyperproliferation caused by proto-oncogenes. *p53* is mutated in more than 50% of human tumors, the proto-oncogene HDM2 is overexpressed in 10% of tumors and ARF is inactivated in many others, indicating that ARF-MDM2-*p53* pathway (Fig. 3B) is disrupted in many cancers.[151] Thus, two products of INK4A/ARF locus indirectly control the activities of two major tumor suppressors pRB and *p53* (Fig. 3B).

Elimination of exons 2 and 3 of the INK4A/ARF locus (Fig. 3A) in the germ line of mice disrupted both the *p16* and ARF(*p19*) genes.[156] These *p16*/*p19*ARF knockout mice had no apparent defects and developed normally, but were prone to tumor development. They acquired predominantly sarcomas and lymphomas after about 34 weeks and tumor incidence was accelerated after treatment with DMBA.[157] Mice lacking *p19*-ARF displayed a very similar phenotype developing spontaneous sarcomas, lymphomas and carcinomas after about 38 weeks.[156] MEFs from both *p16*/ *p19*-ARF- and *p19*-ARF-null mice had an unusually rapid proliferation rate and were immortal, and susceptible to transformation by oncogenic Ras.[158,159] In contrast, "pure" *p16*-null mice[160] showed very low susceptibility to spontaneous tumor development within the first year after birth and their MEFs were resistant to transformation by oncogenic Ras.[161,162] At the same time *p16*-null mice showed higher incidence of tumors with more rapid rate after treatment with carcinogens than their wild type littermates.[162] Thus, *p16* loss is not sufficient to ensure tumor development, but it greatly sensitizes mice to carcinogenic insults (reviewed in 160).

Surprisingly, *p16*-null mice developed melanomas while *p16*/ARF-null and ARF-null strains never developed ones. In addition, when a *p16*ARF deletion on one chromosome was combined with the inactivated *p16* allele on the other, the rate of occurrence of melanomas was strongly increased, and DMBA treatment gave rise to in an even higher incident of aggressive melanomas in these mice.[161] These data indicate that ARF is haplo-insufficient in *p16*-null mice suggesting cooperation between INK4A and ARF pathways in melanoma progression. Interestingly, in melanoma kindreds with INK4A/ARF mutations, p14(ARF) was often functionally impaired suggesting the importance of p14(ARF) in human melanoma predisposition.[163] INK4A/ARF/*p21*-deficiency in primary mouse keratinocytes results in increased proliferation and increased tumorigenic susceptibility with a very aggressive and poorly differentiated phenotype upon their injection into nude mice.[91] These results indicate that *p16*ARF and *p21* genes cooperate to suppress keratinocyte tumorigenesis in vitro and in vivo.

When mammalian cells are cultured in vitro, they undergo a limited number of population doublings and then arrest in a state called replicative senescence. Both *p16* and ARF appeared to be involved in emergence of senescence, since both can induce senescence in cultured cells and both are often accumulated in senescent cells.[164-167] Antisense *p16* expression delayed onset of senescence in human diploid fibroblasts supporting the role for *p16* in replicative senescence.[108] At the same time *p16*-null fibroblasts undergo senescence, ARF-null MEFs are immortal[158,161,162] indicating that at least in mouse cells ARF, but not *p16* controls senescence in vitro.

Additional functions of *p16* include regulation of matrix-dependent cell migration[168] and regulation of anoikis.[169] *p16* inhibited $\alpha_v\beta_1$ integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of $\alpha_v\beta_1$ to focal contacts and coexpression of CDK6 reversed the cell spreading-inhibitory effect of *p16*.[168] The ability to undergo apoptosis upon loss of anchorage (anoikis) is a key feature specifically lost during malignant transformation of most epithelial cells. Stable transfection of *p16* restores anoikis in a variety of human cancer cells. Anoikis in *p16*-transfected cells was associated with suppression of anchorage-independent growth as well as complete loss of tumorigenicity and it was based to selective transcriptional upregulation of the α_5 integrin chain of the $\alpha_5\beta_1$ fibronectin receptor.[169] These data suggest that in addition to its inhibitory effects on the cell cycle, *p16* may be acting as important inhibitor of epithelial malignancies.

p15(INK4B)

The *p15* gene is closely linked to *p16* on chromosome 9*p21* (chromosome 4 in mice) and is codeleted with INK4A/ARF locus in a variety of human cancer cell lines.[123,135] Expression of *p15* is induced in response to TGF-β[120] mediating G1 arrest in epithelial cells.[170] Ectopic expression of the proto-oncogene c-Myc alleviates G1 cell cycle arrest and contributes to the development of many cancers. Overexpression of c-Myc in cell culture makes the cells resistant to antiproliferative effects of TGF-β.[171] It has been suggested that direct repression of *p15* and *p21* by c-Myc may be responsible for TGF-β resistance and this repression is based on interactions between c-Myc and Smads[172] and/or c-Myc and Sp1/Sp3.[173]

The role of *p15* in tumor suppression is not entirely clear. *p15* is transcriptionally induced in response to oncogenic Ras via Raf-Mek-Erk pathway and induction of *p15* is associated with premature G1 arrest.[174] *p15* was able to inhibit cellular transformation by Ras and a new oncogene Rgr[175] and it suppresses S-phase entry that induced by oncogenic Ras. MEFs isolated from *p15* knockout mice were susceptible to transformation by these Ras, whereas MEFs deficient for other INK4 proteins and wild type MEF are not.[174]

p15 is inactivated in leukemias and lymphomas either by specific deletions or by hypermethylation.[176] Inactivation of *p15* by hypermethylation was also described in gliomas.[177] *p15*-null mice develop hemangiosarcomas with low incidence and after long latency. Exposure of *p15*-null mice to DMBA and to ultraviolet B rays did not give rise to any tumors suggesting that loss of *p15* in mice does not confer increased sensitivity to carcinogenic exposure.[178] All these data indicate that *p15* has limited tumor-suppressing potential.

p18(INK4C)

The *p18* gene was mapped to 1p32, a chromosomal region linked to abnormalities in human tumors.[124] Induction of *p18* correlates with exit from the cell cycle in B cells programmed to differentiate[179] and in myoblasts propelled to differentiate to myotubes.[180] Mice lacking *p18* are viable and fertile, but they develop gigantism and organomegaly.[178,181] They exhibit higher proliferative rate in lymphocytes upon mitogenic stimulation and develop lymphoproliferative disorders.[178,181] It appears that *p18* plays an important role in modulating TCR-mediated T cell proliferation and it may provide a novel target to modulate T cell immunity.[182]

p18-null mice also have deregulated epithelial cell growth leading to the formation of cysts in kidneys and in mammary epithelium, and they acquire pituitary and testicular tumors that lead to the death of 40% of these animals before 18 month of age.[178] These mice also show enlarged lymph nodes and cell expansion of plasma cells in the medulla similar to *p15*-null mice phenotype. However, *p18*/*p15* double null mice do not display a more austere phenotype suggesting that *p18* and *p15* are active in different cell types or that they block CDK4/CDK6 activity on different substrates.[178]

Mice lacking both *p18* and *p27* die from pituitary adenomas by 3.5 months of age indicating that *p18* and *p27* mediate two separate pathways to suppress pituitary tumorigenesis by controlling the function of pRB.[181] *p18-p21* double null mice developed pituitary adenomas, gastric neuroendocrine cell hyperplasia, and lung bronchioalveolar tumors later in life. Hence, with the exception of pituitary tumors, the different tumor spectra of *p18-p27* and *p18-p21* double null mice indicate that functional collaboration between different CDK inhibitors suppresses tumor in mice growth in tissue-specific fashion.[86]

p19(INK4D)

Human *p19* was identified as CDK4 binding protein in yeast two hybrid screening[125] and the gene encoding this CKI maps to chromosome 19p13.[183] It is ubiquitously expressed in all mouse tissues with the strongest expression in mouse testis, spleen, thymus, and brain.[184] *p19* is strongly expressed in terminally differentiated neurons and throughout adulthood.[184] Overexpression of *p19* in a myeloid progenitor cell line induces cell differentiation in the presence of CSF-1.[130] IFN-α inhibited the growth of ANBL-6 myeloma cell line via induction of *p19* expression, while *p19* was not inducible by IFN-α in the KAS-6/1 myeloma cell line that is resistant to IFN-α treatment.[185] These data suggest that *p19*, but not other CDK inhibitors transmits specific growth inhibitory signals in some cell types.

Targeted disruption of *p19* in mice did not affect fetal or adult development and did not lead to tumor formation. Tumors did not arise in increased frequency in *p19*-null animals that were exposed to ionizing radiation or to carcinogens. MEFs and lymphoid B and T cells from these animals proliferated normally and express lineage-specific differentiation markers. *p19*-null males developed testicular atrophy associated with increased cell death and partially compromised spermatogenesis.[186] Male mice lacking both *p18* and *p19* produce low numbers of mature sperm and are infertile, while female fertility is not affected.[187] These data suggest that *p19* is not a tumor suppressor but is required for production of mature spermatozoa. *p18* and *p19* collaborate in regulating of spermatogenesis and they are essential for male fertility.

p19-null/*p27*-null mice exhibited bradykinesia, proprioceptive abnormalities and seizures, and died at about 18 days after birth. It was shown that neurons were proliferating and undergoing apoptosis in all parts of the brain of these mice, including normally quiescent cells of the hippocampus, cortex, hypothalamus, pons, and brainstem. Thus, *p19* and *p27* cooperate to maintain differentiated neurons in a quiescent state.[188]

Summary

Over the last few years a variety of roles for the INK4 and Cip/Kip CKIs in G1 phase progression have been revealed. As discussed above, the INK4 CKIs inhibit cyclin D/CDK4-CDK6, while the Cip/Kip CKIs inhibit cyclin E/CDK2. However the INK4 CKIs also appear to promote growth arrest by displacing sequestered Cip/Kip CKIs from cyclin D containing complexes, freeing them to inhibit CDK2 activity. In addition to negatively regulating growth, Cip/Kip proteins may facilitate assembly and/or promote stability of cyclin D/CDK4 complexes. Biological roles for CKIs that were not initially apparent are being revealed as various combinations of CKI genes are disrupted in mice. Reduced CKI activity, through regulation of expression or genetic mutation often contributes to the development of cancer. Agents that can restore CKI activities may prove to be useful therapeutic agents in the treatment of this disease.

Acknowledgements

Cyclin kinase inhibitor research of the authors is supported by NIH Grant R01 DK56283 (A.L.T.), and CRB and IDPH Penny Severns Breast and Cervical Cancer Grants (A.L.G.).

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Chapter 5

Role of RB/E2F in G1 Phase Progression

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Overview

The progression from G1 to S phase requires the de novo expression of genes that encode proteins and enzymes involved in DNA replication. Regulation of these S-phase genes is, therefore, an important component of the biological program during G1 progression. The coordinated regulation of S-phase genes is principally controlled by the E2F-family of transcription factors. E2F was discovered as a cellular transcription factor that binds to a specific DNA sequence (TTTCGCGC) in the adenovirus E2 promoter.[1,2] This E2F-binding sequence (generalized to TTTSSCGC, where S can be either C or G) is found in the promoters of many genes required for DNA replication (Fig. 1).[3] The E2F-binding sequence is recognized by a heterodimer of E2F and DP. A total of six E2F and three DP proteins have been identified. The E2F-regulated S-phase genes are silenced in quiescent and early G1 cells, and become activated at the G1/S transition. Interestingly, E2F contributes to both the silencing and the activation of these S-phase genes.

The function of E2F is regulated by the RB-family of proteins: pRB, p107, and p130. The founding member pRB, is encoded by the retinoblastoma susceptibility gene (*Rb*). Consistent with its ability to suppress retinoblastoma, the RB protein functions as an inhibitor of cell proliferation. The growth inhibitory function of pRB is dependent on its interaction with E2F. pRB and the related p107 and p130 can each bind to E2F without disrupting the E2F-DNA interaction. Consequently, pRB/p107/p130 can each be recruited to E2F-regulated promoters. The RB-family of proteins exerts a net negative effect on E2F-dependent transcription through two mechanisms. First, pRB/p107/p130 prevent E2F from interacting with transcription coactivators, hence, they inhibit the trans-activation function of E2F.[4] Second, pRB/p107/p130 contain additional protein binding sites, which can recruit several chromatin modifying enzymes to silence transcription.[4] Thus, pRB/p107/p130 collaborate with E2F to inhibit the expression of S-phase genes.

During G1 progression, disruption of the pRB/E2F interaction is required to convert E2F-regulated promoters from a repressed to an active conformation. Disruption of the pRB/E2F interaction is achieved through the action of Cyclin-dependent protein kinases (CDKs), which phosphorylate the RB-family of proteins to inactivate their protein-binding functions. Phosphorylation of p130 can also target it for degradation by the proteosome.[5] Hence, disruption of the pRB/E2F complex is a critical function of the CDKs during G1 progression.

G1 Phase Progression, edited by Johannes Boonstra.

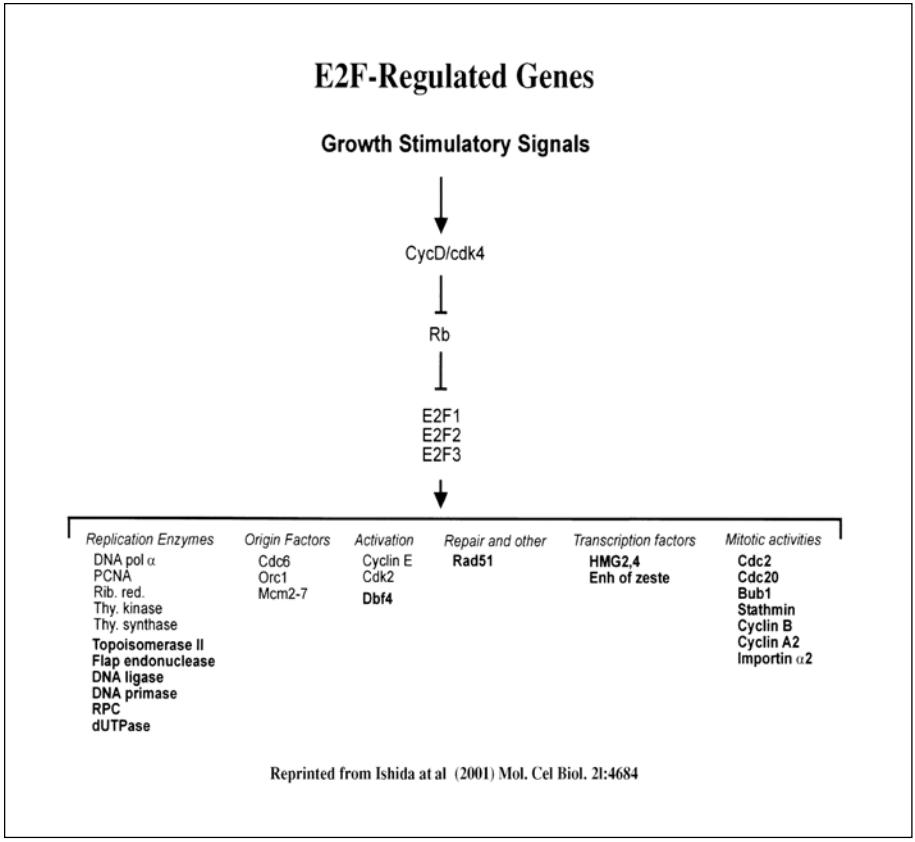

Figure 1. A partial list of E2F-regulated genes. This list of E2F-regulated genes is adopted from.[3]

The current model for the role of pRB/E2F in G1 progression has been supported by the phenotype of fibroblasts lacking either all three members of the pRB-family,[6] or those lacking E2F-1, 2, and 3.[7] The triple-knockout cells that lack pRB, p107 and p130 cannot undergo growth arrest when starved for growth factors or challenged with anti-mitogenic signals. These observations show that the RB-family proteins are required for the establishment of cell cycle arrest, and that they have redundant functions in inhibiting cell cycle progression.[6] On the other hand, the triple-knockout cells that lack E2F-1, 2 and 3 are defective in S-phase entry. This result suggests that E2F-1, 2 and 3 are required for the stimulation of S-phase gene expression and that they have redundant functions in promoting DNA synthesis.[7]

Although the current model of pRB/E2F interaction is supported by the cell cycle phenotypes in ex vivo culture, this model cannot adequately explain the phenotypes of the various knockout mice (Tables 1 and 2). Thus, there is much work to be done on the biological functions of pRB and E2F. To facilitate access to the most recent information on these proteins, the relevant NCBI links (Unigene, LocusLink, OMIM) for each one of the pRB and E2F family members are summarized in Tables 1 and 2.

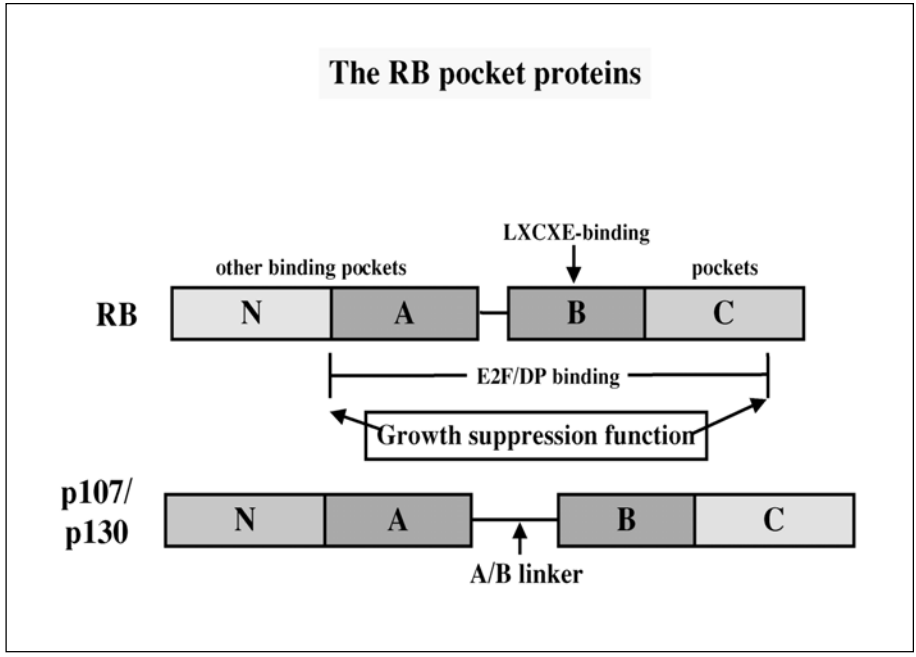

Figure. 2. Schematic diagram of RB-family of pocket proteins. The A/B domain is conserved among pRB, p107 and p130. A linker region separating A and B is conserved between p107 and p130. However, the A/B-linker of pRB is distinct from that of p107/p130. The N- and C-regions of p107 and p130 are also related, but distinct from those of pRB. The N-region is not required for the suppression of cell proliferation. The C-region is required for growth suppression and contributes to the stable association of pRB with the E2F/DP heterodimer on DNA. The binding site for the LXCXE-peptide motif is conserved and it resides entirely within the B-region of the pocket proteins.

The RB Family of Proteins

Protein Structure and Function

pRB Contains Multiple Independent Protein Binding Sites (Pockets)

The RB-family of proteins is defined by a shared A/B domain (Fig. 2). This A/B domain is conserved through evolution, and the A/B-domain proteins have been identified in plants, invertebrates and vertebrates. The crystal structure of the pRB A/B domain fragment has been determined.[8] The A and B domains each adopts a "cyclin fold" composed of five alpha helices.[8] An extensive interface exists between these two cyclin folds, explaining the interdependence of these two domains for stability.[8]

The A/B domain contains at least two distinct protein-binding sites, one for the LXCXE-motif and the other for E2F. The LXCXE-motif is found in cellular proteins, e.g., histone deacetylase, as well as viral oncoproteins such as E7 of human papilloma virus-16, E1A of adenovirus and T-antigen of SV40 virus. It should be noted that E2F does not contain the LXCXE-motif. Instead, it interacts with pRB through a C-terminal sequence (Fig. 4). The A/B domain crystal structure has revealed that the binding site for the LXCXE-peptide is entirely within the B-domain.[8] Indeed, targeted mutations in the B domain can abolish the binding of LXCXE-proteins without disrupting the pRB/E2F interaction.[9,10] In addition to the binding

of LXCXE-motif and E2F, the A/B domain may contain additional protein-binding sites, as indicated by a significant conservation of structural features throughout its surface.[8]

The A/B domain contains a variable linker region that separates the A and B sub-domains (Fig. 2). The linker region of pRB is relatively short but it cannot be deleted. However, the pRB linker can be replaced with random sequences without affecting pRB function. In p107 and p130, this linker region is large and contains sequence homology with CDK inhibitors.[11,12] Whether the A/B-linker of pRB also contains protein binding function is presently unknown. The protein binding sites in the A/B domain are commonly referred to as "pockets". Hence, pRB and related proteins are known as "pocket proteins".

The A/B domain is surrounded by N- and C-terminal extensions that are less well conserved among the pocket proteins (Fig. 2). In pRB, both the N- and C-regions contain protein-binding sites and these regions have been shown to mediate the association of pRB with many cellular proteins.[5,13] The C-region of pRB is also required for its association with the E2F/DP heterodimer on DNA. Hence, the growth suppression function of pRB requires the A/B domain and the C-region (Fig. 2). Besides the interaction with E2F/DP, the C-region of pRB contains other protein binding-sites. For example, the nuclear c-Abl tyrosine kinase binds to a C-pocket that is distinct from the E2F-contact sites in the C-terminal region.[14,15] Consequently, a stable complex containing pRB, E2F and c-Abl can be assembled on DNA.[16] The N-region of pRB is not required for its association with E2F and it is dispensable for growth suppression. However, the N-region of pRB may be important for other biological functions of pRB. Taken together, the current evidence shows that the RB-family of pocket proteins can assemble protein complexes. In other words, these pocket proteins serve a scaffolding function in the nucleus.

Pocket Proteins Assemble Transcription Repression Complexes

The multiple protein-binding sites of pRB and related pocket proteins are required for the establishment of transcription repression at E2F-regulated promoters. For example, histone deacetylase 1-3 (HDAC) contain the LXCXE-motif and bind to the B domain of pRB.[4] Because pRB can simultaneously bind E2F and the LXCXE-motif, it can recruit HDAC to E2F-regulated promoters. HDAC can catalyze the deacetylation of core histones and transcription factors at these promoters to establish an inactive chromatin conformation, thus contributing to the silencing of E2F-regulated genes.[4] It should be noted that the B-domain mutants of pRB, while unable to bind HDAC, could still block S-phase entry.[9,10] This is probably because pRB can recruit chromatin-modifying enzymes other than HDAC. Among them are the Brg complex,[4,17] histone methyltransferases,[18] and DNA methyltransferase I,[19] all of which can contribute to gene silencing.

Through the analysis of E2F complexes in cell lysates, it was found that the E2F-complexes contained predominantly E2F4 or E2F5 associated with p130 in quiescent cells (Fig. 3A). E2F4 can associate with pRB and p130, however, E2F5 associates specifically with p130.[20] The p130/E2F4 and p130/E2F5 complexes mediate transcription silencing of E2F-regulated promoters in quiescent cells (Fig. 3A). As cells enter G1, these p130/E2F complexes disappear with the degradation of p130. The E2F-promoters become occupied by complexes of pRB with E2F1, 2, or 3, which continue to impose transcription repression. At the G1/S transition, with the phosphorylation of pRB, E2F1, 2, and 3 become active and stimulate the transcription of S-phase genes (Fig. 3A).

Recently, through chromatin cross-linking and immunoprecipitation (ChIP) methods, E2F and pocket proteins have been shown to associate with some of these promoters in living cells.[21-23] At present, it is not known if the pocket-protein/E2F complexes recruit the same chromatin-modifying enzymes to all of the E2F-regulated promoters. In other words, the precise composition of the transcription complexes at each of these promoters has not been

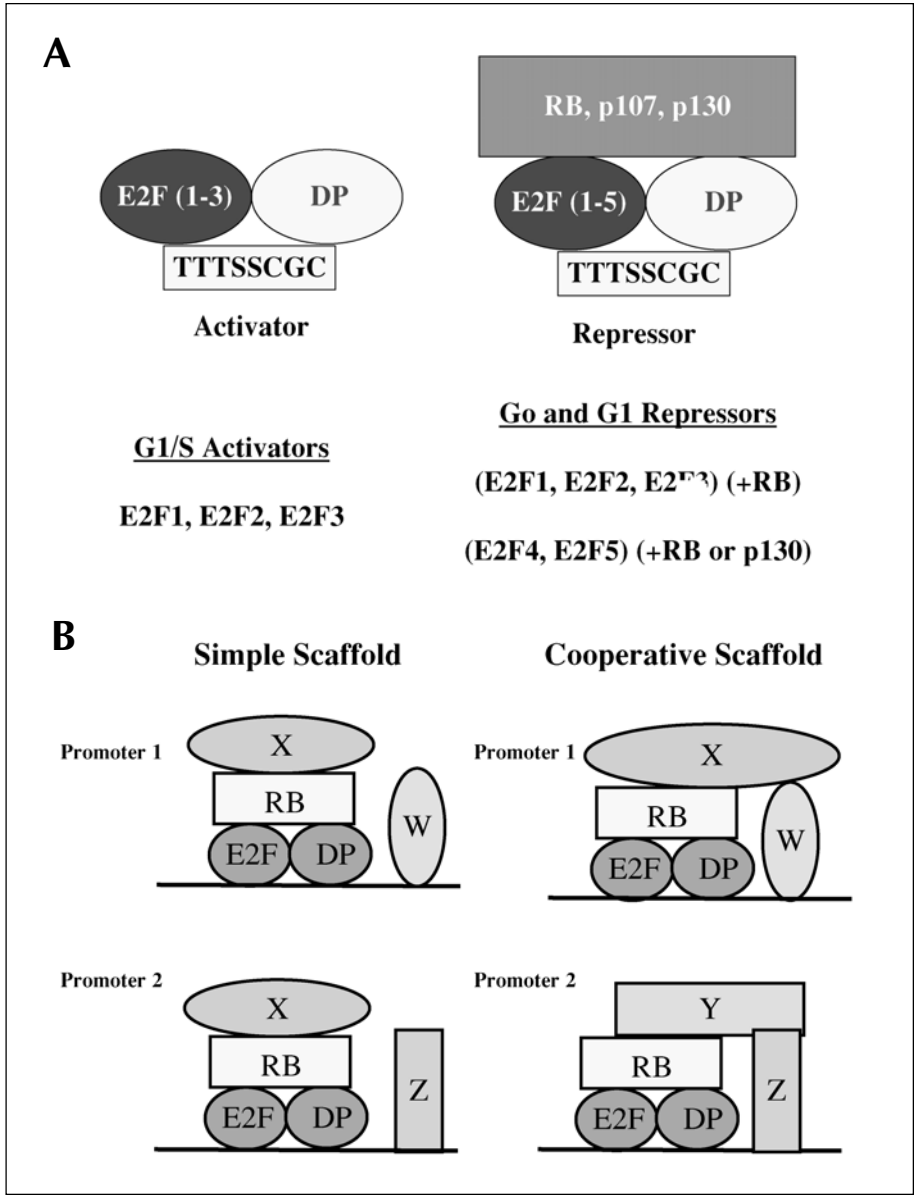

Figure 3. The pocket protein/E2F complexes. A) The predominant E2F complexes in quiescent or early G1 cells are the transcription repressor complexes composed of RB-pocket proteins and E2F/DP heterodimers. At G1/S transition, RB-pocket proteins are inactivated by phosphorylation, allowing E2F/DP to activate transcription.B) Two models for the assembly of transcription repressor complexes by the RB-pocket proteins at E2F-regulated promoters. Factor X or Y is recruited to the promoters by the protein binding pockets of pRB. Factors W and Z are other transcription factors that also bind to these E2F-regulated promoters. In the model of simple scaffolding, the composition of pRB-assembled complexes is invariable at different promoters. In the model of cooperative scaffolding, the composition of pRB-assembled complexes is variable, depending on the protein binding functions of neighboring transcription factors.

elucidated. The present model of pRB/E2F considers a vertical interaction between pRB, E2F and DNA. In its simplest form, this model would predict a defined composition for pocket protein-assembled repression complex at every E2F-regulated promoter (Fig. 3B). However, an alternative model of cooperative scaffolding might also be considered.

The current understanding of transcription regulation suggests a combinatorial specificity in controlling promoter functions. The E2F-regulated promoters must also contain binding sites for other transcription factors (Fig. 3B). Because most transcription factors can interact with chromatin-modifying enzymes, the composition of the repression complex at an E2F-regulated promoter might not be determined by pRB/E2F alone. Instead, the assembly function of the pocket protein might be influenced by other transcription factors and their protein-binding capabilities. This concept of "cooperative scaffolding" would predict a different composition of the pRB-assembled complex at each promoter (Fig. 3B). In other words, the pRB/E2F complex might recruit different proteins at different promoters. In this model, the pRB pockets would cooperate with neighboring proteins to determine which particular chromatin modifiers are stably recruited to the repressor complex. With the advance in cross-linking methods to preserve promoter complexes, it might become possible to determine the composition of pRB-assembled complexes at different promoters. Such methods will provide tools to distinguish between the simple scaffolding or the cooperative scaffolding function for the pocket proteins.

Inactivation of RB Pockets by Phosphorylation

The function of RB pockets is regulated by phosphorylation. The major kinases for the phosphorylation of RB-pocket proteins are the CDKs. The RB protein is a substrate for CDK4/6, CDK2, and CDC2. During cell cycle progression, the orderly activation of these CDKs leads to the sequential phosphorylation of pRB at sixteen phosphorylation sites. Phosphorylation inactivates the RB-pockets, causing the disruption of pRB-assembled transcription repression complexes. Therefore, phosphorylation of pRB by the CDKs results in the expression of E2F-regulated genes.

pRB is phosphorylated by CDKs at multiple sites. It has become clear that the different phosphorylation sites exert differential regulatory effect on the different pockets.[24,25] The pRB/E2F interaction can be disrupted by phosphorylation at one of seven sites in the C-terminal domain and two sites in the A/B-linker.[25] To prevent the disruption of pRB/E2F, nine phosphorylation sites need to be mutated: seven in the C-terminal region and two in the A/B-linker. A phosphorylation site mutated (PSM) pRB lacking these nine sites (PSM.RB-9I) binds E2F constitutively despite its phosphorylation at the remaining sites.[25] The interaction between pRB and the LXCXE-motif, on the other hand, is disrupted by the phosphorylation of two threonine sites (T821/826) in the C-terminal region.[24] Mutation of these two threonines (PSM.RB-2T) is sufficient to prevent the dissociation of pRB from LXCXE-proteins. With the C-pocket, phosphorylation of two serines (S807/811) is required to disrupt the pRB/c-Abl interaction. Mutation of these two serines (PSM.RB-2S) is sufficient to prevent the dissociation of pRB from c-Abl.[24] Among these phosphorylation site mutated pRBs, only PSM.RB-9I is a constitutive inhibitor of cell proliferation, supporting the requirement for pRB/E2F interaction in blocking cell cycle progression.

Taken together, the current evidence suggests that the pRB/E2F interaction is the most sensitive to inhibition by phosphorylation. This is because phosphorylation at any one of nine CDK phosphorylation sites is sufficient to disrupt pRB/E2F interaction. By contrast, the LXCXE-binding pocket and the C-pocket are each regulated by a distinct subset of the phosphorylation sites. The differential regulation of pRB pockets by the different phosphorylation sites suggests an additional complexity to the protein-assembly function of pRB. Whether this complexity is relevant to the biological functions of pRB has not been determined.

The pRB-related p130 protein is also phosphorylated at multiple sites by CDKs, in particular, by the cyclin D/CDK4 kinase that is activated early during G1 progression. Phosphorylation of p130 targets it for poly-ubiquitination and degradation by the proteosome.[5] The level of p130 is elevated in quiescent cells to mediate the silencing of E2F-regulated promoters. Phosphorylation and degradation of p130 during G1 progression, therefore, contributes to the activation of E2F-regulated genes.

Contrary to p130, the levels of pRB and p107 increase as cells progress from G1 into S phase. This is because the promoters of the *Rb* and p107 genes contain binding sites for E2F. The increased expression of pRB and p107 in proliferating cells seems to be at odds with their role as inhibitors of S-phase entry. Indeed, pRB and p107 are mostly phosphorylated and inactivated during S, G2 and M phase of the cell cycle, raising the question for why they are expressed beyond G1/S transition. Recent observations that pRB can contribute to cell cycle arrest in S or G2 phase may have provided an answer. The constitutively active PSM.RB-9I is found to inhibit DNA replication when it is expressed in S-phase cells.[26] Moreover, dephosphorylated pRB can accumulate in S-phase cells in response to DNA damage, and this causes a prolonged arrest of cells with S-phase DNA content.[27] These observations suggest that pRB and p107 may regulate cell cycle progression beyond the G1/S transition, and provides an explanation for the up-regulation of their expression.

Phenotypes of Pocket-Protein Knockouts (Table 1)

Phenotypes of Knockout Mice

The major phenotypes of *Rb*, p107 and p130 knockout mice are summarized in (Table 1) The *Rb*-knockout mice die between embryonic day 10 and 14, and exhibit the striking phenotype of apoptosis in the developing nervous systems and other tissues.[28,29] By contrast, the knockout of either p107 or p130 does not affect viability in the 129 background, although lethality was reported by one group in another strain background.[30-32] The double knockout of p107 and p130 causes neonatal lethality, which is associated with defects in bone development. The knockout phenotypes suggest p107 and p130 to have redundant biological functions; whereas pRB may have unique biological functions not shared by p107 and p130.

Table 1. Summary of pocket protein knockout phenotypes

Pocket Protein	Human Chromosomal Location	Unigene	OMIM	LocusLink	Summary of Phenotypes
RB	13q14.2	Hs.75770	180200	5925	Embryonic lethality at ~e13[28,29]
P107	20q11.2	Hs.87	116957	5933	Viable Myeloid hyperplasia dependent on mouse strain[30]
P130	16q12.2	Hs.79362	180203	5934	Viable or embryonic lethality dependent on mouse strain[31,32]

Cell Cycle Defects of *Rb*-Knockout Fibroblasts

The *Rb*-knockout cells can undergo growth arrest in response to serum withdrawal. However, the triple knockout cells (lacking *Rb*, p107 and p130) do not undergo growth arrest when starved for growth factors or challenged with growth inhibitory signals.[6] Thus, at the cellular level, pRB, p107 and p130 have redundant functions in the establishment of quiescence. Although *Rb*-knockout cells can undergo growth arrest under some conditions, they are defective in the growth arrest response to genotoxic stress. Thus, pRB has an essential function in DNA damage-induced cell cycle arrest.

The *Rb*-knockout cells do not undergo G1 arrest in response to ionizing radiation or other damage inducers.[33] The DNA damage-induced G1 arrest is triggered by p53, through the up-regulation of p21-^{Cip1}. The increased expression of p21-^{Cip1} inhibits CDK activities and leads to the accumulation of dephosphorylated pRB. The pRB-dependent transcription repression appears to be an essential component of the G1 checkpoint response to DNA damage.

The *Rb*-knockout cells can undergo G2 arrest in response to DNA damage (Naderi S, Hunton IC, Wang JYJ. Radiatum-dose dependent Maintenance of G2 arrest requires the retinoblastoma protein. Cell Cycle 2002; 1:193-200.). While pRB is not required for the initiation of the G2 checkpoint, it is required for the maintenance of G2 arrest. The maintenance of G2 arrest can be observed when mouse embryo fibroblasts (MEFs) are exposed to a high dose of ionizing radiation (IR)(> 8 Gy). Wild type MEFs cease proliferation and enter a premature senescent state when treated with IR dose at or greater than 8 Gy. MEFs can enter this senescent state with 4N DNA content, consistent with a prolongation of G2 arrest. This G2 prolongation in wild type MEFs is correlated with the accumulation of unphosphorylated pRB. Importantly, *Rb*-knockout MEFs cannot enter the senescent state, but will exit the G2 checkpoint to resume proliferation or to die, depending on the IR dose (Naderi S, Hunton IC, Wang JYJ. Radiatum-dose dependent Maintenance of G2 arrest requires the retinoblastoma protein. Cell Cycle 2002; 1:193-200.).

The *Rb*-knockout cells exhibit a third checkpoint defect in response to genotoxic stress, and this occurs in S-phase. Exposure of fibroblasts to a high dose of cisplatin causes the accumulation of dephosphorylated pRB in S-phase cells and this causes a prolonged arrest of cells with S-phase DNA content.[27] The *Rb*-knockout MEFs do not undergo this S-phase arrest.[27] Taken together, these observations suggest that pRB not only block cells in G1, but it can also block cells in S and G2 phase. Whether the S and G2 arrest also involves pRB/E2F complex and the repression of E2F-regulated genes will remain to be determined.

The E2F Family of Transcription Factors

Protein Structure and Function

The E2F-family of transcription factors contains a DNA binding domain and a transcription activation domain (Fig. 4). The DNA binding domain is classified in the winged-helix group and recognizes the core sequence (TTTSSCGC).[34] The DNA binding domain of the E2F subunit alone binds weakly to this core sequence, but a E2F/DP heterodimer binds this consensus DNA sequence with high affinity (Fig. 3). The heterodimeric interaction between E2F and DP is mediated through a hydrophobic heptad repeat region adjacent to the DNA binding domain.[35] In E2F1 through 5, the C-terminal transcription activation domain contains the binding site for the A/B domain of the pocket proteins. Thus, the A/B domain of the RB family protein can directly inhibit the transactivation function of E2F.

The E2F-family contains six members (Fig. 4). Among them, E2F1, E2F2 and E2F3 are considered as the activators of transcription, whereas E2F4, E2F5 and E2F6 are involved in

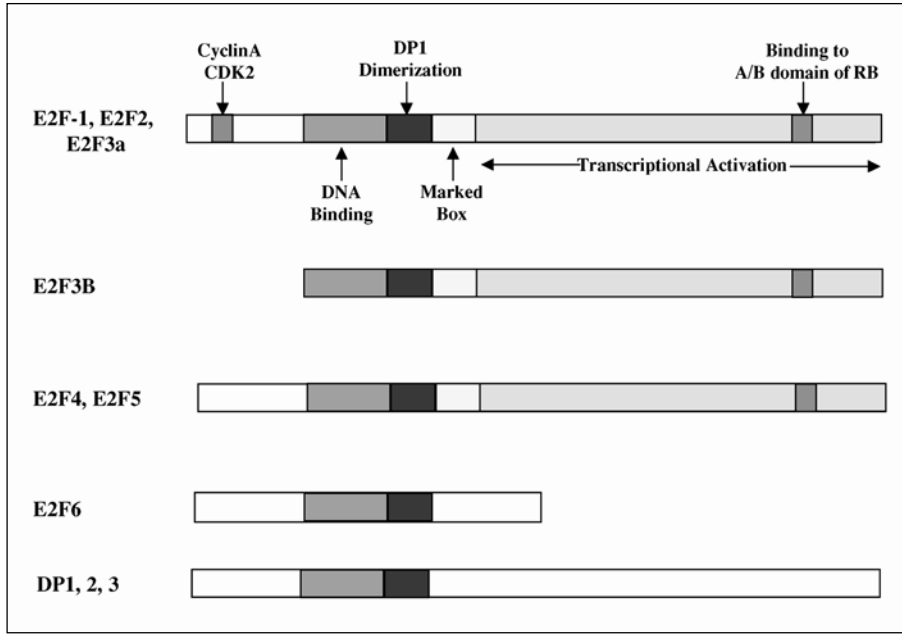

Figure. 4. Schematic diagram of E2F family members. The activating members (E2F1, E2F2, and E2F3A) represent activities that trigger activation of gene in (Fig. 1). The various functional motifs are listed. The E2F3B variant is similar to E2F3A, except that it lacks an N-terminal region that encompasses the Cyclin A/CDK2 binding site. The repressing E2Fs (E2F4 and E2F5) are similar to the activating class, except that these also lack the N-terminal Cyclin A/CDK2 binding motif. Finally, E2F6 is firmly a repressor, but lacks pRB interaction or transcription activation regions. The heterodimeric partner (DP proteins) is also depicted.

transcription repression. pRB preferentially binds E2F1, E2F2 and E2F3, although it also interacts with E2F4. The p130 protein primarily binds E2F4 and E2F5. E2F6 does not interact with any of the RB-pocket proteins. Biologically, E2F1, E2F2 and E2F3 are required for cell cycle progression, whereas E2F4 and E2F5 are required for the establishment of quiescence and terminal differentiation. Despite these general designations, there is extensive functional overlap among the E2F family members. In addition, the distinction between transcription activator or repressor depends upon the phosphorylation status of the pocket proteins. E2F1, E2F2 and E2F3A are activators of S-phase genes at the G1/S transition, following pRB phosphorylation. However, they are components of transcription repressor complexes in early G1, when the protein-assembly function of pRB is active. By contrast, E2F4 and E2F5 are mostly linked with transcriptional repression (reviewed in Trimarchi J, Lees JA. Sibling rivalry in the E2F family. Nature Reviews Mol Cell Biol 2002; 3:11-20.).

In addition to the noncovalent regulation by pocket proteins, the E2F proteins are also regulated by covalent modifications, including phosphorylation, acetylation and ubiquitination. In the N-terminal region of E2F1, E2F2 and E2F3, a binding site for cyclin A/CDK2 is found (Fig. 4). Interestingly, cyclin A/CDK2 phosphorylates E2F and DP and abolishes their DNA binding function.[36-38] Additionally, the DNA binding activity of E2F is also regulated by acetylation, mediated by the general coactivators of transcription, i.e., p300 and CBP.[39,40] E2F can be targeted for poly-ubiquitination and degradation by the proteosome.

Phenotypes of E2F Knockout Mice (Table 2)

Activator E2F (E2F1, E2F2, E2F3) Knockouts

The individual and combination knockouts of E2F1, E2F2 and E2F3 have demonstrated that these three E2F members have redundant functions in activating S-phase genes. A triple knockout of these three activating E2Fs causes early embryonic lethality in mice.[7] The triple knockout cells also show severe cell cycle defects.

By contrast to the triple knockout, the E2F1$^{-/-}$ mice are viable. Because of the redundant E2F2 and E2F3, elimination of E2F1 alone does not inhibit cell proliferation. Instead, E2F1$^{-/-}$ mice showed increased number of thymocytes and a slight increase in tumor incidence at old age.[41,42] The E2F2$^{-/-}$ mice are also viable, and exhibit the phenotype of enhanced T-cell proliferation.[43] The E2F2$^{-/-}$ T-cells are hypersensitive to the mitogenic effect triggered by the activation of the T-cell antigen receptor. This T-cell hypersensitivity may account for the resulting development of autoimmune syndromes in the E2F2$^{-/-}$ mice.[43] The E2F3$^{-/-}$ mice are also viable and apparently normal. However, E2F3-knockout cells exhibited proliferation defects that are not found with E2F1 or E2F2 single knockout cells. Thus, E2F3 appears to be the most important in stimulating S-phase entry.[44,45]

Table 2. Summary of E2F knockout phenotypes

E2F Member	Human Chromosomal Location	Unigene	OMIM	Locuslink	Summary of Knockout Mouse Phenotypes
E2F1	20q11.2	Hs.96055	189971	1869	Viable Increased Tumor Incidence[42] Increased T-cell population[41]
E2F2	1p36	Hs.121487	600426	1870	Autoimmune syndromes; hyper-reactive T-cells[43]
E2F3	6p22	Hs.1189	600427	1871	Viable Proliferation defects observed in knockout MEFs[44]
E2F4	16q21-q22	Hs.108371	600659	1874	Neonatal lethality; opportunistic infections[47] Immature hematopoietic cells; intestinal defects[46]
E2F5	8p22-q21.3	Hs.2331	600967	1875	Hydrocephalus[48]
E2F6	22q11	Hs.42287	602944	1876	Not reported
DP1	13q34	Hs.79353	189902	7027	Embryonic lethal (L. Yamasaki, personal communication)
DP2	3q23	Hs.19131	602160	7029	Not reported

See Notes on Tables 1 and 2 at the end of this chapter.

Repressor E2F (E2F4, E2F5) Knockouts

The single knockout of E2F4 or E2F5 causes defects in the differentiation of selective tissues and cell types. Two different strains of E2F4$^{-/-}$ mice have been made and they exhibited different phenotypes.[46,47] In the work from Nevins and colleagues,[46] the E2F4$^{-/-}$ mice are mostly viable, but are underweight and weak. Proliferation defects in the intestines were observed with E2F4$^{-/-}$ mice. Additionally, the bone marrow cells and a myeloid subset exhibited increased apoptosis in the absence of E2F4.[46] In the work from Lees and colleagues,[47] the E2F4$^{-/-}$ mice exhibited neonatal lethality. Treatment with antibiotics could prolong the life of these E2F4$^{-/-}$ mice, which showed chronic sinusitis. This strain of E2F4$^{-/-}$ mice also exhibited defects in erythropoiesis.[47] The two labs apparently used different ES cell lines in their experiments, but whether this is the basis of the different phenotypes remains to be determined. The unifying result in both E2F4$^{-/-}$ mice is the accumulation of undifferentiated precursor cells in the intestines or in the hematopoietic compartments.[46,47]

The E2F5 $^{-/-}$ mice had severe, but tissue-specific defects, including hydrocephalus due to accumulation of fluid in the choroid plexus, which is a central nervous system (CNS) tissue with a high level of E2F5 expression.[48] E2F5 interacts specifically with p130 and is the only E2F that is exclusively involved in quiescent cells. By comparison, E2F4 is present throughout the cell cycle and can interact with all three members of the pRB-family. The limited phenotypic defect of E2F5 knockout mice is likely due to compensation by E2F4.

The functional redundancy of E2F4 and E2F5 is demonstrated by the phenotype of the double knockout mice and of cells derived from these mice.[46] Both E2F4 and E2F5 can interact with p130, a pRB family member linked most strongly with differentiation and quiescence. It is likely that either E2F4 or E2F5 is required for differentiation, since the combination of E2F4 and E2F5 mutations resulted in neonatal lethality. While E2F4$^{-/-}$ cells can enter quiescence when deprived of growth factors, cells lacking both E2F4 and E2F5 failed to enter quiescence in response to growth factor deprivation. Moreover, these double E2F4$^{-/-}$E2F5$^{-/-}$ knockout cells are refractory to growth arrest by p16.[49] Thus, the conclusion is that there is redundancy between E2F4 and E2F5, but one member is required. When either E2F4 or E2F5 were absent, the remaining member compensates for the absence.

Spotlight on E2F3 in G1/S Control

Several lines of evidence suggest that E2F3 may be important for the control of G1 progression. Microinjection of cells with neutralizing antibodies to E2F3, but not to E2F1, hampered cell cycle progression.[50] Fibroblasts derived from E2F3$^{-/-}$ mouse embryos already exhibited defects in proliferation, with increased cell doubling time and extended S-phase kinetics. The defects in S-phase gene expression and S-phase entry could be rescued by reintroducing E2F3. The loss of E2F3 alone also affected the expression of S-phase genes including DHFR, cdc6, Mcm3, DNA Pol α.[7] In particular, the p68 subunit of the DNA polymerase α gene has been conclusively identified as a specific target gene for E2F3. The endogenous E2F3 was specifically associated with this promoter. The expression of the DNA pol α p68 gene was lost in E2F3$^{-/-}$ cells.[51] Lastly, recent experiments with the MYC protein have supported a role for the E2F3 proteins in G1 progression. An important factor in G1 progression is the induction of the Cyclin E promoter; both E2Fs and MYC can provide for gene activation of the Cyclin E promoter.[52] The G1-induction activity of MYC utilized both E2F2 and E2F3, but not E2F1.[53] Intriguingly, MYC also induces the synthesis of both E2F2 and E2F3 through the E-box elements in the respective promoters.[54] Thus, the diverse evidence supports a role for E2F3 in G1 control. Notably, E2F1, E2F2, and E2F3 are highly redundant and the roles of E2F3 in cell cycle progression have only recently been appreciated.

The E2F3 gene encodes two proteins (E2F3A and E2F3B) (Fig. 4) that differ in the N-terminal region. The N-terminal Cyclin A/CDK2 binding site that is conserved among E2F1, 2, and 3A is not found in E2F3B. The expression and the activity of these two E2F3 exhibit a complex variation through the cell cycle. Both expression and activities of E2F3A and E2F3B have very different kinetics. The DNA binding activity of E2F3A was only detected in G1, whereas E2F3B appears to bind DNA throughout the cell cycle. Similarly, the E2F3A gene is also expressed in G1. The E2F3A promoter is cell cycle regulated, and contains elements that are typical of cell cycle-regulated promoters (e.g., dual E2F and SP1 sites). The E2F3A promoter is also regulated by MYC in its G1 inductive capacity.[53] Thus, the variation of the E2F3A protein and the associated activity is likely responsible for activation of S-phase genes. However, as described below, the determining factor may be the ratio of E2F3A to E2F3B.

By contrast, E2F3B RNA and protein are constitutive and not subject to cell cycle regulation. The E2F3B DNA binding activity, RNA and protein are constitutive through the cell cycle. By contrast to the E2F3A promoter, the E2F3B promoter is constitutive and is not regulated in the cell cycle. Lastly, E2F3B specifically interacts with pRB and does not associate with p130[55,56] again suggesting a role on G1 repression. The emerging model is that the fluctuation of E2F3A reflects the transcription activation through the cell cycle. The ratio of E2F3A/E2F3B then reflects overall transcription activation or repression. The complementation of cell cycle phenotypes by reexpression of E2F3A or 3B also reflect that E2F3A is important for transcription_activation in S–phase.[7] Thus, the existing data reveals complex, but potentially important features of the E2F3 proteins.[50,53,55,56]

pRB and E2F1 in the Regulation of Apoptosis

Besides the regulation of cell cycle progression, the pRB/E2F interaction also contributes to the regulation of apoptosis. Among the E2Fs, E2F1 in particular has a pro-apoptotic function. Among the pocket proteins, pRB in particular can inhibit apoptosis.

The ectopic expression of E2F1 can induce apoptosis in cultured cells, especially under conditions when cells are deprived of survival signals.[57] Recent studies have identified E2F-binding sequences in the promoters of several pro-apoptosis genes, including caspase-3 and Apaf1.[58] Identification of E2F-regulated pro-apoptosis genes has provided a mechanistic basis for the apoptotic function of E2F1.

The role of pRB in the inhibition of apoptosis is suggested by the phenotype of *Rb* knockout mice. The developing nervous systems, both central and peripheral, of *Rb*-knockout mice exhibit ectopic mitosis coupled to apoptosis.[29] A similar apoptosis phenotype is observed with the epithelial cells in the developing lens of *Rb*-knockout mice.[29] Importantly, the double knockout of *Rb* and E2F1 can rescue some of the apoptotic phenotype.[59] In particular, the apoptosis of neurons in the CNS and of the lens epithelial cells can be suppressed in the *Rb*/E2F1-double knockout embryos.[59] The apoptosis defect in the developing CNS of *Rb*-knockout mice is also rescued by the elimination of Apaf1, which is a central component of the intrinsic apoptotic pathway.[60] Indeed, Apaf1 expression is de-regulated in *Rb*-knockout embryos, consistent with the observation that Apaf1 is an E2F-regulated gene.[58,60] The *Rb*/E2F1-double knockout mice continue to exhibit increased apoptosis of neurons in the peripheral nervous system and in skeletal muscles,[59,61] suggesting other E2Fs or E2F-independent apoptosis in the *Rb/E2F1*-double knockout mice.

The finding that pRB/E2F regulates G1 progression and apoptosis brings into view an interesting coupling between cell proliferation and cell death. It appears that pRB/E2F represses the expression of genes required for S-phase and for programmed cell death, whereas E2F activates DNA synthesis and apoptosis. The coupling of the S-phase and the apoptotic gene expression may provide an important fail-safe mechanism to control cell proliferation.

This mechanism can account for death associated with oncogenic stimulation, e.g., by c-Myc and E1A. Because of the dual function of pRB/E2F in regulating the gene expression programs for S-phase and for apoptosis, the continued proliferation of a cell would require the support of survival signaling. Exactly how the pRB/E2F-regulated apoptosis program is suppressed during each S-phase will await further investigation.

Concluding Remarks

The interaction between RB-pocket proteins and E2F is a central mechanism in the coordinated regulation of cell cycle-dependent transcription. The basic tenants for this mechanism have been elucidated. The requirement for RB-pocket proteins in the establishment of growth arrest has been confirmed. The ability of pocket proteins to assemble protein complexes and to silence E2F-regulated promoters is well documented. The requirement for E2F in the promotion of S-phase entry has also been confirmed. The association of E2F with critical S-phase genes in living cells has been shown. Thus, the role of pRB/E2F in cell cycle regulation has been elucidated.

This current understanding of pRB/E2F, however, is not sufficient to explain the various phenotypes of the knockout mice. Thus, the biological functions of RB-pocket proteins and the E2F family members have not been resolved. Clearly, pRB can interact with many cellular proteins other than E2F. Thus, the biological functions of pRB may expand beyond the repression of E2F-regulated promoters. Likewise, E2F6 is a family member that can bind to the consensus E2F-binding sequence but it does not interact with pRB, p107 or p130. Hence, E2F-regulated promoters may be controlled by mechanisms that do not involve the pocket proteins. Although it is an inclination of scientists to adopt a unifying theory in the interpretation of biological phenomena, we may not be able to explain all of the *Rb*-knockout phenotypes based on the pRB/E2F interaction and vice versa.

The current understanding of cell cycle regulation is mainly based on studies with cell lines, which proliferate indefinitely and without a purpose. However, cell proliferation in vivo is seldom continuous and is always for a defined purpose, either to regenerate a tissue, to heal a wound or to fight a foreign invader. Indeed, a majority of the normal proliferation is directed at the formation of differentiated cell types. It is therefore not surprising to find that the RB-pocket proteins and the E2Fs play important roles in the regulation of differentiation.

In differentiation, the pRB/E2F complexes are joined by a number of tissue-specific proteins to regulate growth arrest and cell cycle reentry. In myogenic differentiation, a functional interaction between pRB and the myogenic transcription factor MyoD has been demonstrated.[62] This functional interaction is required for the establishment of permanent growth arrest, one that cannot be reversed by mitogenic stimuli.[10] In myeloid differentiation, a functional interaction between pRB/E2F and METS, a myeloid-specific transcription repressor, is required for the establishment of permanent growth arrest.[63] In hepatocytes, the pocket protein/E2F complex interacts with HBP1 to maintain the quiescent status.[64,65] These cell type-specific transcription factors may dictate the target gene selection by the pRB/E2F-family of complexes during development. This might contribute to the tissue-specific phenotypes associated with the pocket protein and E2F knockouts.

The elucidation of the biological functions of RB-pocket proteins and E2F transcription factors will depend on the identification of genes that are targeted for regulation by pRB/E2F. It is important to bear in mind that the target gene specificity might be variable, depending on the cell context. The current framework of transcription repression and transcription activation through pRB/E2F interaction will serve as important guidelines for future studies. However, the precise composition of each promoter complex may ultimately determine the effect of RB-pocket proteins and E2F on gene expression.

Notes on Tables 1 and 2

In these tables, the relevant access numbers to generally accessible databases is provided for each E2F and RB family member. The Unigene, Locus Link, and OMIM databases can all be accessed from the home page of National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). The Unigene database (http://www.ncbi.nlm.nih.gov/Unigene) is an organized page of information for a given gene, such as chromosomal location, expression profiles, orthologues in other species and other useful links. The information can be accessed by using the indicated Unigene numbers. Within Unigene, there will be links to LocusLink and OMIM with the indicated numbers. OMIM denotes Online Mendelian Inheritance in Man and is a database that summarizes current findings for a given gene (http://www.ncbi.nlm.nih.gov/Omim/). Selected current references can also be found in a given OMIM page. Finally, LocusLink provides organized pages for a given gene (http://www.ncbi.nlm.nih.gov/LocusLink/). Links to Pubmed, OMIM, nucleotide and protein sequences, Unigene, and single nucleotide polymorphism locations are all provided within LocusLink. Lastly, the human chromosomal location of each gene has been provided for researchers that may be investigating the links of E2F and pRB family members to cancer and other diseases.

Acknowledgements

We acknowledge Drs. Doug Cress, Joe Nevins, Lili Yamasaki, Peggy Farnham, Rene Bernards, Tim Kowalik, Brian Dynlacht, Chris Glass and Shi Huang for providing preprints and unpublished data. Due to space limitations, we did not include all the citations that are relevant to this general topic. There is not an intentional omission of any specific work. A.S.Y. is grateful to these agencies for past and present research support: NIH (GM44634 and CA94187); the Army (BC990538); the American Heart Association (96002320); and the Center for Digestive Disease at New England Medical Center (NIH P30 DK34928). J.Y.J. Wang is the Herbert Stern Endowed Chair of Biology at UCSD, and has been supported by grants from the NIH (CA43054, CA58320 and HL57900).

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Chapter 6

Regulation of the G1 Phase Progression by Growth Factors

Esther Hulleman and Gerda S.A.T. van Rossum

Abstract

During the years it has become evident that the process of cell division in nontransformed cells is dependent on the presence of growth factors, as well as on cell attachment. When nontransformed cells are cultured in suspension or when growth factors are withdrawn from the medium, cells will stop cell cycle progression and enter the quiescent state (G0). Cells will remain in this quiescent state until extracellular conditions change and cells are stimulated to reenter the cell cycle. Upon stimulation of quiescent cells, various signal transduction cascades such as the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol 3-kinase (PI3K) signal transduction route are activated, leading to early gene expression and resumption of the cell cycle. The same signal transduction pathways are supposed to play an important role also in the control of G1 phase progression during the ongoing cell cycle, even though signal transduction that occurs when quiescent cells are stimulated to reenter the cell cycle can not really be compared with the signal transduction routes that are activated during normal cell cycle progression. For example, most early genes that are induced upon mitogenic stimulation of quiescent cells, do not show cell cycle dependent expression in an ongoing cell cycle. In this review the main signal transduction pathways involved in proliferation of fibroblast like cells will be discussed in relation to G1 progression, starting from the Ras signaling pathway.

Introduction

Signal Transduction

Signal transduction is the process in which an extracellular signal is transmitted to an intracellular target, via a cascade of protein-protein interactions and phosphorylation and dephosphorylation events. Several extracellular signaling molecules are known, such as hormones, neurotransmitters and growth factors.

Growth factors bind and activate either receptors with an intrinsic protein-tyrosine kinase activity, also called receptor tyrosine kinases (RTKs), or receptors that transmit signals to the cytoplasm by interacting with GTP-binding proteins (G-proteins). Together, these receptors form the most important groups of receptors for the regulation of cell proliferation.

RTK-type growth factor receptors are characterized by an extracellular ligand-binding domain, a single transmembrane region and a large intracellular catalytic domain (reviewed in ref. 1). Upon binding of growth factors like epidermal growth factor (EGF), platelet-derived

G1 Phase Progression, edited by Johannes Boonstra.

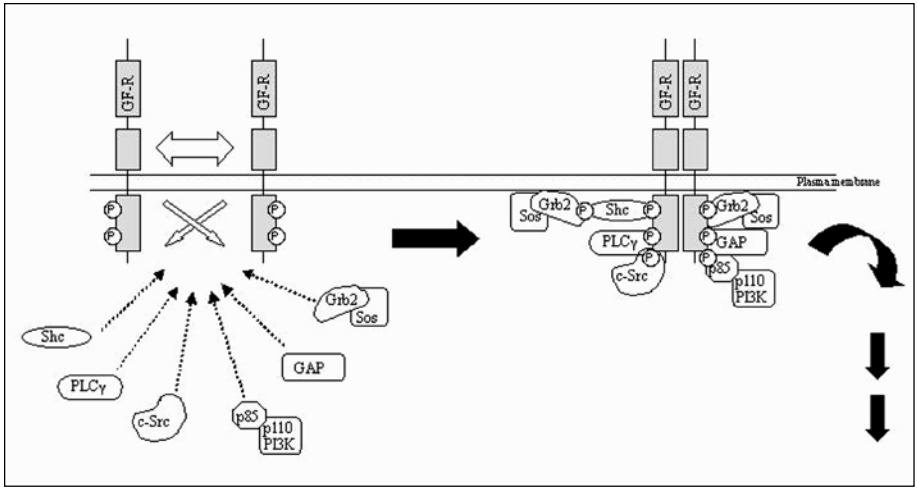

Figure 1. Signaling through receptor tyrosine kinases (RTKs). Upon binding of growth factors RTKs dimerize, which leads to activation and autophosphorylation of the receptors. This phosphorylation triggers the recruitment of a number of target proteins to the receptor, leading to the induction of downstream signaling cascades.

growth factor (PDGF) or fibroblast growth factor (FGF), these receptors dimerize which leads to activation and autophosphorylation of the receptor on tyrosine residues in the intracellular domain. This phosphorylation triggers the recruitment of a number of target proteins to the receptor, like for example phosphoinositide-specific phospholipase Cγ (PLCγ), the p85 kDa subunit of phosphatidylinositol 3-kinase (PI3-kinase), GTPase-activating protein (GAP), growth factor receptor binding protein 2 (Grb2) and members of the Src family of cytoplasmic tyrosine kinases. In some instances, receptor binding results in the tyrosine phosphorylation and a direct change in the activity of the target molecule (e.g., PLCγ). In other cases, however, proteins without any enzymatic activity are bound, such as Grb2 and p85. These proteins serve as adaptor proteins to couple the activated receptor to other intermediates. All protein interactions consequently lead to modification (for example phosphorylation or dephosphorylation) and activation of other target proteins, thus creating a signal transduction cascade that finally results in activation of nuclear transcription factors and induction of gene expression (Fig. 1). In addition, growth factor receptors like FGFR and EGFR have been found to translocate to the nucleus and have been suggested to physically act as transcription factors as well.[2-4]

G protein-coupled receptors (GPCRs) can be activated by different external stimuli such as growth factors, hormones and neurotransmitters (reviewed in ref. 5). These receptors mostly consist of seven hydrophobic transmembrane helices with a large hydrophobic tail at the C-terminus[6] that interacts with GTP-binding proteins (G-proteins). G-proteins are heterotrimers composed of an α subunit and a βγ dimer, that can bind GTP and hydrolyze GTP to GDP (reviewed in ref. 7). Upon binding of GPCRs to hormones or other ligands, the G-proteins are activated, leading to conversion of G_α from the inactive GDP bound state into the active GTP-bound state and dissociation of the $G_{\alpha\beta\gamma}$ complex. Thereupon, both the GTP-bound G_α as well as free βγ subunits can activate several effector enzymes, such as PLC species or adenylyl cyclases. Activation of adenylyl cyclases leads to the generation of cyclic AMP (cAMP) from ATP, which can subsequently induce activation of protein kinase A (PKA).[8] Activation of PLC by the receptor-ligand complex on the cell surface results in hydrolysis of the plasmamembrane

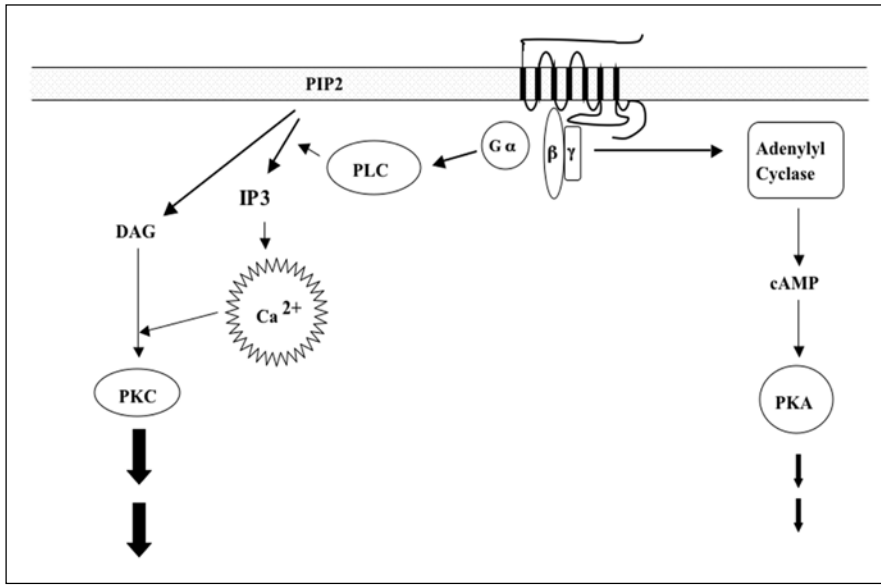

Figure 2. Signal transduction by G-protein coupled receptors (GPCRs). Upon binding of hormones or other ligands to GPCRs, the G-proteins that are coupled to the receptor are activated. This activation leads to a conversion of G_α from the inactive GDP-bound state into the active GTP-bound state and dissociation of the $G_{\alpha\beta\gamma}$ complex. Thereupon, both the G_α and the free $G_{\beta\gamma}$ subunits can activate several effector proteins, leading to the induction of downstream signal transduction cascades (for details, see text).

phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2). This generates two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 induces the release of Ca^{2+} from internal stores and DAG can, together with Ca^{2+}, activate protein kinase C (PKC). PKC, in turn, phosphorylates several proteins to modify their activity[9] (Fig. 2). Together, activation of different substrates by RTKs, GPCRs and other pathways controls specific cellular responses such as differentiation, proliferation, cell migration and survival.

Ras Pathways to Cell Cycle Control and Cell Transformation

Ras is a central protein in different signal transduction pathways that can be induced by different extracellular signals that stimulate cell surface receptors (reviewed in ref. 10). *Ras* genes are activated in up to 30% of human tumors and are in general found to play an important role in integrating mitogenic signals with cell cycle progression. Ras proteins bind guanine nucleotides (GDP and GTP) with high affinity and possess intrinsic GTPase activity. Ras biological activity is controlled by a catalyzed GDP/GTP cycle. Most Ras molecules in the cell exist in their inactive state, characterized by a state that allows binding to GDP. The exchange of GDP by GTP is followed by a conformational change of the Ras protein to its activated state. The activated Ras-GTP complex can subsequently transmit signals downstream the pathway by interacting with target proteins. In the last few years several proteins have been shown to interact with the activated form of Ras, such as Raf proteins, PI3 kinase, PKC zeta, MEKK1, members of the RalGDS family, Rin1 and others[10] (Fig. 3). We will focus on the most common signal transduction pathways that have been shown to regulate G1 progression in fibroblasts.

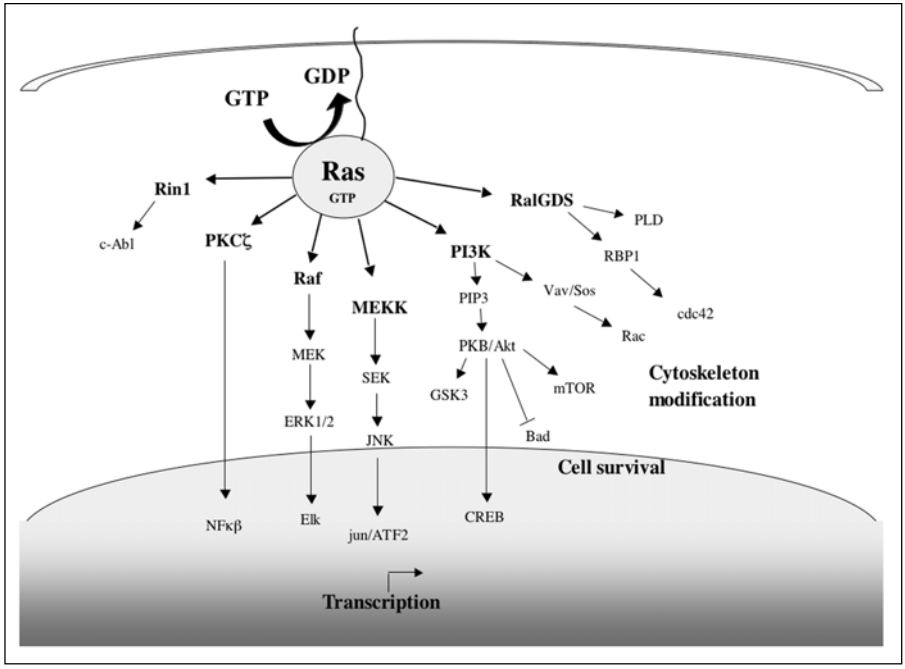

Figure 3. Ras induced signaling cascades. Upon stimulation of RTKs or GPCRs membrane bound Ras is activated by GDP/GTP transfer. Several downstream signal transduction cascades are subsequently activated, leading to different cellular responses.

The MAP Kinase Pathway

The mitogen-activated protein kinase (MAPK) pathway can be activated by different extracellular stimuli and can have different intracellular substrates, depending on the MAPK isoform involved. In mammalian cells different MAPK isoforms have been described, based on sequence homology and function, such as c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPKs), p38 MAP kinases and extracellular regulated kinases (ERKs). In general, the MAPK isoforms are activated by phosphorylation on regulatory threonine and tyrosine residues by dual specificity protein kinases, also referred to as MAP kinase kinases (MAPKKs). The most important MAPK pathway that is activated upon stimulation of RTKs is the signal transduction pathway that leads to phosphorylation of $p44^{MAPK}$ and $p42^{MAPK}$ (also called ERK1 and ERK2 respectively) and their translocation to the nucleus. When growth factor receptors are activated, the adaptor protein Grb2 is bound to the receptor, together with the guanine-nucleotide exchange factor Sos. Binding of Sos, leads to the activation of Ras, which subsequently recruits Raf-1 to the plasmamembrane. Thereupon, Raf-1 is activated and can, in turn, activate the MAP kinase kinase MEK (MAPK- or ERK Kinase), which finally phosphorylates $p44/p42^{MAPK}$ (ERK1/2) on Thr-183 and Tyr-185 (TEY motif). Upon activation, ERK can phosphorylate targets in the cytoplasm, such as $p90\text{-}^{RSK}$, cytoskeletal elements, cytosolic phospholipase A_2 ($cPLA_2$) and others,[11-13] or translocate to the nucleus where it may phosphorylate and activate several transcription factors, such as c-myc, c-jun, $p62\text{-}^{TCF}$/Elk-1, c-Ets-1 and c-Ets-2[14-16] (Fig. 4). The importance of the MAPK signal transduction pathway as a target of Ras is underscored by the ability of constitutively active MEK protein kinase to mimic the effects of Ras activation in some cell types.[17,18]

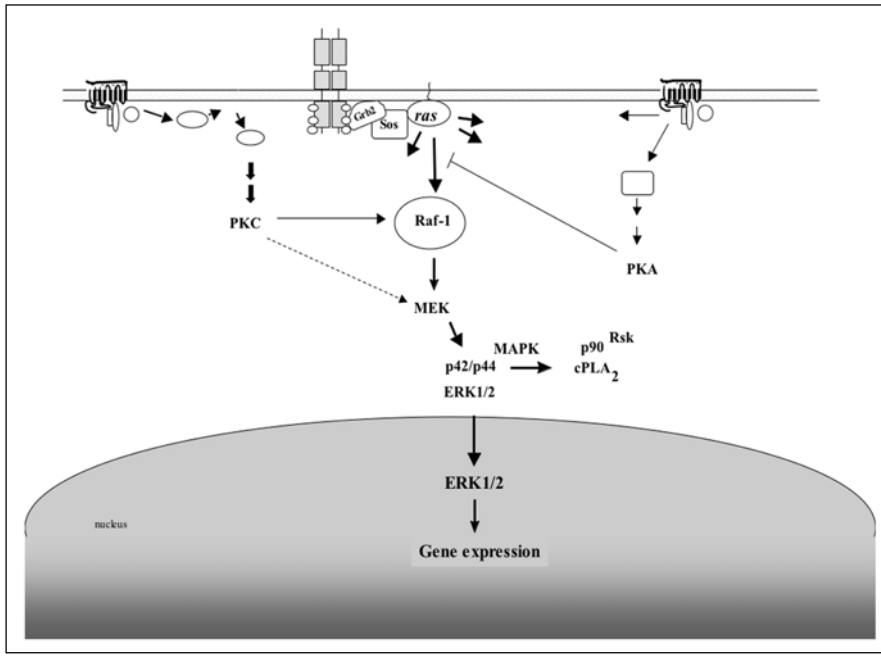

Figure 4. Overview of the p42/44-^{MAPK} (ERK1/2) signal transduction pathway. Upon activation of Ras by RTKs or GPCRs the MAP kinase kinase kinase raf-1 is activated, leading to the phosphorylation of the MAP kinase kinase MEK. Thereupon, the MAP kinases ERK1/2 are activated. Activated p42/44-^{MAPK} (ERK1/2) can subsequently translocate into the nucleus to phosphorylate and activate transcription factors or phosphorylate different proteins in the cytoplasm (for details, see text).

MAP Kinase during the Cell Cycle in Mammalian Cells

Most research done on the role of MAPK during the cell cycle in mammalian cells has focused on the stimulation of quiescent cells to reenter the cell cycle (G0/G1). Upon activation, MAPK translocates to the nucleus, where it phosphorylates transcription factors and induces early gene transcription.[19] Activation of the MAPK pathway at the G0/G1 transition has been shown to induce expression of cyclin D,[20,21] presumably through activation of the AP-1 transcription factor complex.[22] At the same time the cyclin dependent kinase inhibitor (CKI) p27^{KIP1} was found to decrease,[23] a process that is inhibited by overexpression of a dominant negative form of ERK or by the use of a MEK inhibitor.[24] MAPK has been shown to phosphorylate p27^{KIP1} in vitro[24] and this may trigger the degradation by the ubiquitin pathway.[25] However, specific activation of the MAPK signal transduction pathway on its own was found not to be sufficient for degradation of p27^{KIP1}.[26] Thus, although the induction of cyclin D is dependent only on the MAPK pathway, degradation of p27^{KIP1} seems to require additional events.

Since the activation of the MAPK signal transduction pathway is thought to regulate the exit from G0, both by downregulating the CKI p27^{KIP1} and by inducing the expression of cyclin D[27,28] the same process has been suggested to occur at the G1/S transition in mammalian cells.[26,29,30] Downregulation of p27^{KIP1} and induction of cyclin D would lead to the activation of the cyclin D/CDK4 complex, resulting in the phosphorylation of the RB protein and passage of the restriction point (R). Indeed, an activation of p42^{MAPK}/p44^{MAPK} is observed at the G1/S transition in Chinese Hamster Ovary (CHO) cells[31] and a sustained activation

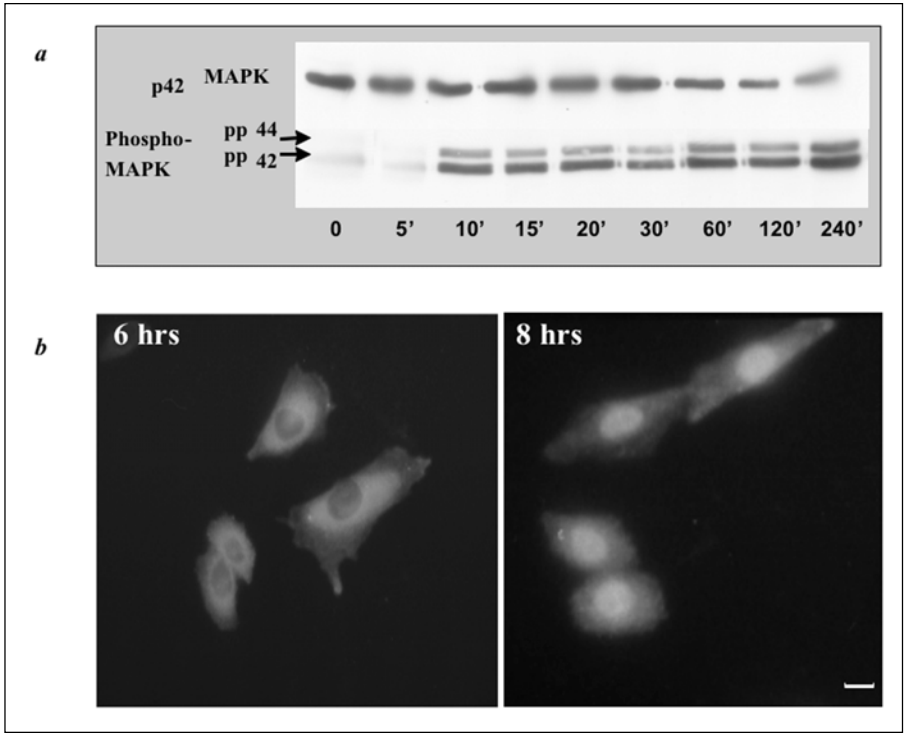

Figure 5. Effects of the presence of growth factors on MAPK activation and cell cycle progression. In continuously growing CHO cells synchronized by mitotic shake-off, p42/44-^{MAPK} (ERK1/2) are phosphorylated immediately after mitosis (panel A) and ERK2 translocates to the nucleus at the end of mid G1.[34] This translocation is independent of the presence of growth factors, although the initial activation of MAP kinase appears to be required. If serum is removed after the initial phosphorylation of p42^{MAPK}, ERK2 still translocates to the nucleus (panel B), but no ^{3}H-thymidine incorporation is observed (panel C, following image). If serum is removed before the phosphorylation of p42^{MAPK}, no nuclear translocation of MAPK is observed (data not shown) and no ^{3}H-thymidine incorporation occurs, indicating that cells enter S phase only if growth factors are present both in early and late G1 phase.

of MAP kinase was found to be necessary for fibroblasts to pass the restriction point; inhibition of the p42^{MAPK}/p44^{MAPK} cascade by antisense constructs, overexpression of kinase inactive mutants or inactivation by MAP kinase phosphatase (MKP-1) blocks DNA synthesis and cell proliferation.[32,33] In addition, a nuclear translocation of p42^{MAPK} at the end of mid G1 has been described in continuously cycling CHO cells grown in the continuous presence of growth factors.[34] This translocation of MAP kinase was found to be required for progression through G1/S; inhibition of this nuclear translocation by the use of the MEK inhibitor PD098059 blocked cell cycle progression.[34] However, although the nuclear translocation of p42^{MAPK} was required for progression through R, it was not sufficient for entry into S phase. Experiments in which serum was withdrawn at different points during the cell cycle showed that MAP kinase is phosphorylated immediately after mitosis (Fig. 5A), but at a growth factor signal later in G1 is needed for progression through R. If serum is removed after the initial phosphorylation of p42^{MAPK}, ERK2 still translocates to the nucleus, but no DNA synthesis takes place (Fig. 5B, C). However, if serum is removed immediately after mitosis (before the initial phosphorylation

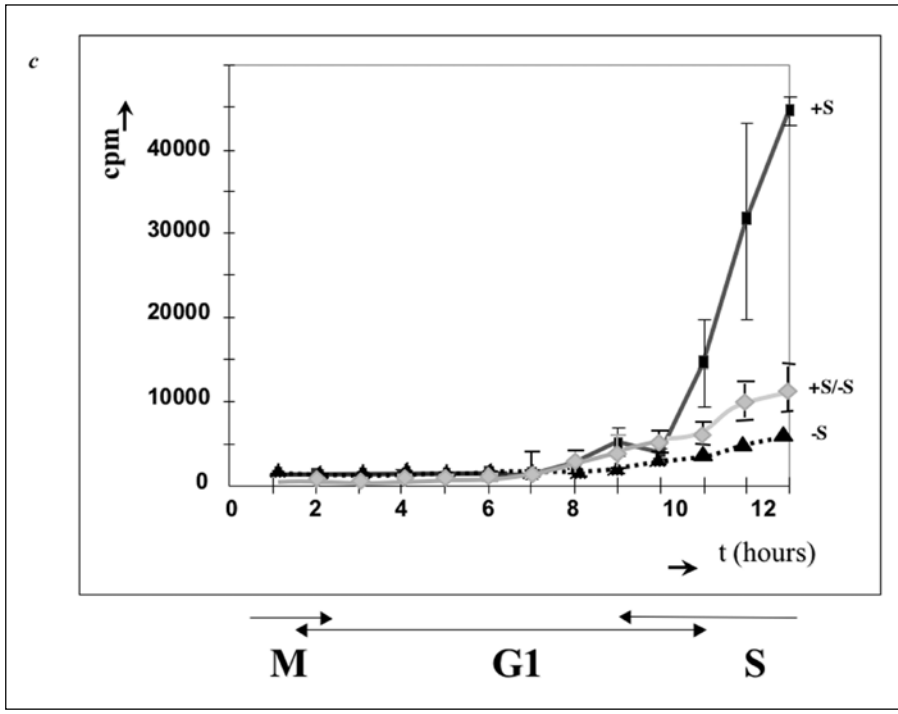

Figure 5C.

of $p42^{MAPK}$) no nuclear translocation of ERK2 is observed. Thus, the actual trigger for the translocation of $p42^{MAPK}$ at the end of mid G1 appears to function independently of the presence of growth factors, although serum seems to be required during the early times after mitosis. For progression through R, however, the presence of growth factors and a sustained activation of MAP kinase do seem to be important.

While the processes described above suggest that a sustained MAP kinase activity is essential for cell proliferation in fibroblasts, activation of this same MAPK signal transduction pathway during G1 can also lead to cell cycle arrest.[35-37] This stop in cell cycle progression is correlated with the up-regulation of $p21^{CIP1}$ and concomitant inhibition of cyclin/CDK activity and seems to depend on the level of activation of the raf/MAPK pathway. Low level-activation of raf promotes cell proliferation, whereas high level-activation of the raf/MAPK signal transduction pathway causes cell cycle arrest. This expression of $p21^{CIP1}$ is induced by the MAPK signaling pathway in a p53 independent manner[38,39] and may also be involved in the differentiation of rat pheochromocytoma (PC12) cells.

Actions of $cPLA_2$ during the Cell Cycle

One of the targets of the MAPK signal transduction pathway is cytosolic phospholipase A_2 ($cPLA_2$). $cPLA_2$ is partially activated through phosphorylation by ERK1/2 and becomes fully activated when it is translocated to membranes by calcium.[40] However, it is now becoming clear that ERK1 and 2 are not the only MAPK family members involved in phosphorylation and activation of $cPLA_2$. Also c-Jun NH_2-terminal kinase (JNK), $p38^{MAPK}$ and MAP kinase interaction protein kinase 1 (Mnk1) are able to phosphorylate $cPLA_2$[41-43] depending on

the cell type and the stimuli. In fibroblasts, however, $cPLA_2$ has been shown to be activated by ERK1/2.[44,45] In those cells, other pathways have been identified (besides the "traditional" raf-MEK-ERK1/2 signal transduction pathway) that can lead to activation of $p44/p42^{MAPK}$. These pathways include PKC/PI3K-MEK-ERK1/2 or MEK-independent signaling through PKC or PI3K, depending on the type and duration of the stimulus.[44,46,47]

Activation of $cPLA_2$ results in the release of arachidonic acid, which is the rate-limiting step in the biosynthesis of eicosanoids (i.e., prostaglandins, thromboxanes, leukotrienes, lipoxins, epoxyeicosatrienoic acid). These eicosanoids are formed via the cyclooxygenase, lipoxygenase or cytochrome p450 epoxygenase pathways, depending on the cell type, and are important regulators of many physiological responses in the cell, such as modulation and release of neurotransmitters, blood vessel tone and cell proliferation.[48-51] The important role of $cPLA_2$ in the control of cell proliferation is emphasized by the findings that most tumor cells produce elevated levels of eicosanoids, resulting in induced tumor growth, invasiveness and metastatic activity of the tumor cells.[52] Accordingly, $cPLA_2$ was found to be overexpressed in oncogenic Ras-transformed non small lung cancer cells and is thought to play a role in oncogenic Ras-transformation of rat-2 fibroblasts.[53,54]

The activity of $cPLA_2$ was recently shown to be cell cycle dependent in continuously growing neuroblastoma and CHO cells.[55] $cPLA_2$ activity was found to be high in mitosis, during mid/late G1 and following the G1/S phase transition. These changes in $cPLA_2$ activity were due to an increased phosphorylation of $cPLA_2$ rather than an increase in protein expression, since no correlation was observed between $cPLA_2$ protein expression and $cPLA_2$ activity. However, phosphatase treatment of $cPLA_2$, prevention of ERK1/2 activation by removal of growth factors or inhibition of the upstream activator MEK reduced the activity of $cPLA_2$ significantly, showing that $cPLA_2$ activity during the ongoing cell cycle is regulated by phosphorylation through ERK1/2 (Fig. 6). Subsequently, it was shown by the use of different PLA_2 inhibitors that the activity of $cPLA_2$ in mid/late G1 is required for cell cycle progression to S phase. Inhibition of $cPLA_2$ activity during early G1 markedly reduced DNA synthesis.[56] However, an inhibition of $cPLA_2$ activity for 24 hours did not result in cell cycle arrest, suggesting that other PLA_2 family members can take over the function of $cPLA_2$. These effects of $cPLA_2$ on cell cycle progression were mediated by lipoxygenase rather than cyclooxygenase products, since G1/S progression was inhibited only when lipoxygenase activity was prevented. Treatment of cells early in G1 with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) completely blocked DNA synthesis and remarkably reduced the expression of cyclin A, suggesting an arrest in G1 phase. Moreover, NDGA treatment in early S phase also blocked DNA synthesis and cyclin A expression, indicating that lipoxygenase is also required for S phase progression. Similar results were described in lympholeukemic cells, in which NDGA treatment suppressed cell proliferation by blocking progression through G1/S.[50] More evidence is arising now, showing that eicosanoids can influence cell cycle progression. Cyclin D1 expression and S phase entry were recently shown to be induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in Swiss 3T3 fibroblasts[57] and prostaglandin A_2 (PGA_2) and δ^{12}-prostaglandin J_2 (δ^{12}-PGJ_2) are able to arrest cells at the G2/M phase of the cell cycle.[58] Furthermore, the 15-lipoxygenase product, 15S-HETE has been shown to decrease the percentage of cells in S phase, concomitant with an increase in the numbers of cells in G0/G1, in prostate carcinoma (PC3) cells.[59] This effect on the cell cycle in PC3 cells is thought to occur through induction of gene transcription via activation of the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors. Although the exact functions and mechanisms by which different eicosanoids regulate cell cycle progression are in general not yet well understood, these results show that not only the activation of $p42/p44^{MAPK}$, but also the actions of components further downstream the MAPK signaling pathway are important regulators of the cell cycle.

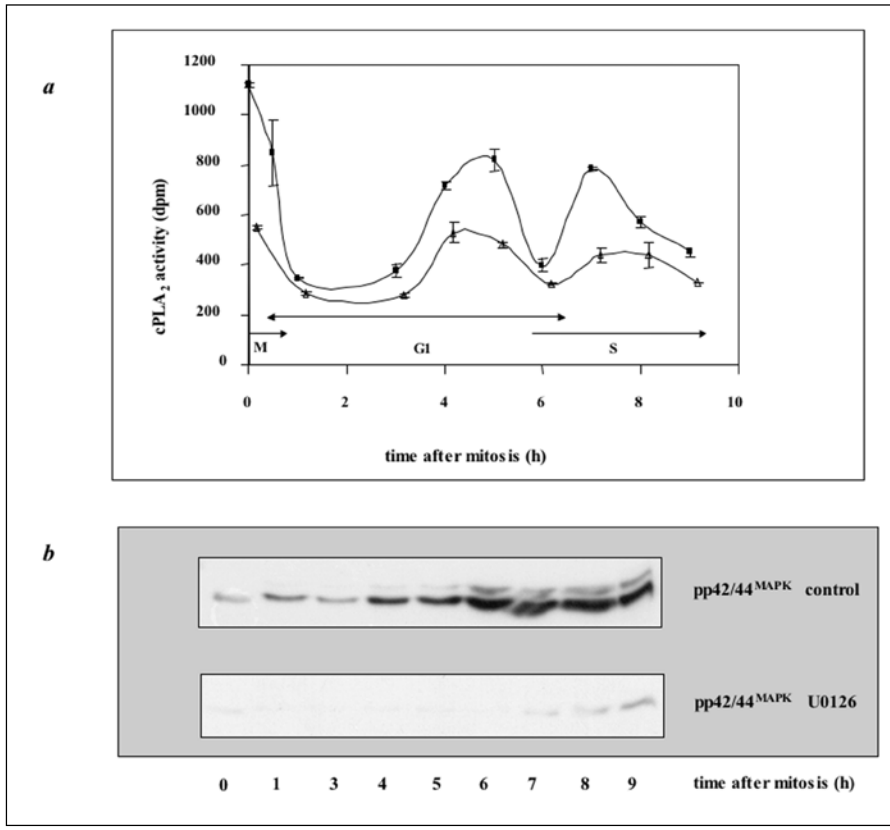

Figure 6. Influence of the MEK inhibitor U0126 on $cPLA_2$ activity and p42/44-^{MAPK} phosphorylation. N2A cells synchronized by mitotic shake-off were harvested at the indicated time points without U0126 treatment (●) or were then treated for 10 min with the specific MEK inhibitor U0126 (Δ) and thereafter harvested A) $cPLA_2$ activity during the ongoing cell cycle. $cPLA_2$ activity was measured as described in 55. B) Western blot showing the phosphorylation of p42/44-^{MAPK} at different times after mitosis with and without MEK inhibitor.

The Phosphatidylinositol 3-Kinase Pathway

In addition to the MAPK pathway, other signal transduction routes such as the phosphatidylinositol 3-kinase (PI3K) pathway are also controlled by Ras. PI3K is a heterodimer consisting of two subunits, catalytic and regulatory, with the molecular weights of 110kD (p110) and 85kD (p85) respectively,[60,61] which is thought to possess both protein kinase and lipid kinase activity.[62] PI3K can phosphorylate the 3'-OH group of the inositol ring in inositol phospholipids, leading to the production of three different lipid products: phosphatidylinosititol 3-phosphate (PtdIns(3)P), phosphatidylinosititol 3,4-diphosphate (PtdIns(3,4)P_2) and phosphatidylinosititol 3,4,5-triphosphate (PtdIns(3,4,5)P_3). These phosphoinositides function as second messengers and activate downstream molecules such as p70-^{S6kinase} and members of the Rho family GTPases. Three classes of PI3K have been described, mainly differing in their substrate specificity (reviewed in ref. 63). Class I PI3Ks are the best characterized class of PI3 kinases and are found to be activated by different extracellular stimuli.

Currently, four isoforms of the catalytic p110 subunit have been described (α, β, γ and δ) and seven adaptor proteins, encoded by three genes: p85α, p85β and p55γ. The p110αβ and δsubunits can be activated by RTKs; upon activation of the receptor, the p110 subunit of PI3 kinase is recruited to the RTK by the p85 adapter subunit, leading to activation of p110. At the plasmamembrane, p110 can subsequently phosphorylate its main substrate PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3. The p110γ subunit can be activated by heterotrimeric G-proteins in GPCRs and does not interact with p85. In addition, activated Ras has been shown to bind directly to different p110 catalytic subunits, leading to the activation of PI3 kinase.[64,65]

Activated PI3K is thought to interact directly with some cellular signal proteins[61,62,66] and to regulate endocytosis and degradation of activated growth factor receptors.[67-69] However, the action of the phosphoinositide products of PI3K seems to be more important for cell signaling. Phosphoinositides can interact with proteins containing lipid-binding protein domains (pleckstrin homology (PH) domains), such as phosphoinositide-dependent kinases (PDKs) or protein kinase B (PKB, also named Akt).[70-74] PtdIns(4,5)P_2 and/or PtdIns(3,4,5)P_3 is thought to stimulate PKB directly by binding to the PH domain of PKB, causing conformational changes and its translocation to the plasma membrane. At the membrane, PKB is subsequently phosphorylated by PDK1 and PDK2 on Thr308 and Ser473 respectively, leading to the phosphorylation of different downstream cellular PKB-targets such as the forkhead transcription factor (FKHR), glycogen synthase kinase-3 (GSK-3) and Bad (Bcl-2/Bcl-XL-antagonist, causing cell death). At the same time PDK1 is also important for the regulation of other kinases, such as PKC[72,75] and the ribosomal p70-^{S6kinase} (Fig. 7).[76,77] Other important targets for the products of PI3 kinases are the GTPases Rac and Rho. These GTPases cocoordinately regulate the dynamic organization of the actin cytoskeleton and the assembly of associated integrin structure (see also chapter 7).

PI3K Signaling during the Cell Cycle

PI3K has been found to be an essential component of signaling pathways involved in the control of many different cellular processes. However, so far its actual role in the control of cell division seems to be rather secondary; its role in the control of cell survival appears to be more important. PI3K acts on cell survival through PKB, which exerts anti-apoptotic effects by phosphorylating a number of substrates involved in death regulation. Among the PKB targets are the pro-apoptotic Bcl-2 family member Bad, the cAMP response element binding protein (CREB) and proteases of the caspase family (reviewed 78,79). PKB also phosphorylates and inactivates GSK-3, an enzyme that was initially identified as a regulator of glycogen metabolism but now also has been implicated in the induction of programmed cell death.[80,81] Furthermore, PKB has been found to phosphorylate several members of the forkhead family of transcription factors, FKHR, FKHRL and AFX.[82-85] This phosphorylation induces the export of these transcription factors from the nucleus to the cytoplasm, leading to a down-regulation of the cell death receptor Fas. Another down-regulated gene whose expression is regulated by forkhead transcription factors is the CKI p27^{KIP1}, providing a more direct link with cell cycle regulation.[86] Other proteins involved in cell cycle control are affected by activation of PKB as well; the CKI p21$^{CIP1/WAF1}$ has been reported to be a direct target of PKB,[87] whereas cyclin D1 appears to be indirectly regulated.[88,89] In addition, the E2F transcription factor activity was found to be induced in a PKB-dependent manner.[90] It has been suggested that activation of PKB can bypass a specific checkpoint in the cell cycle,[79] since activation of PKB can overcome the effects of the tumor-suppressor PTEN (for phosphatase and tensin homologue deleted from chromosome 10), a phosphatase that dephosphorylates PtdIns(3,4,5)P_3. Over-expression of PTEN can either induce apoptosis or elicit cell cycle arrest. Activation of PKB can overcome these effects of PTEN.[91,92] Moreover, PTEN is frequently mutated or deleted in a variety of

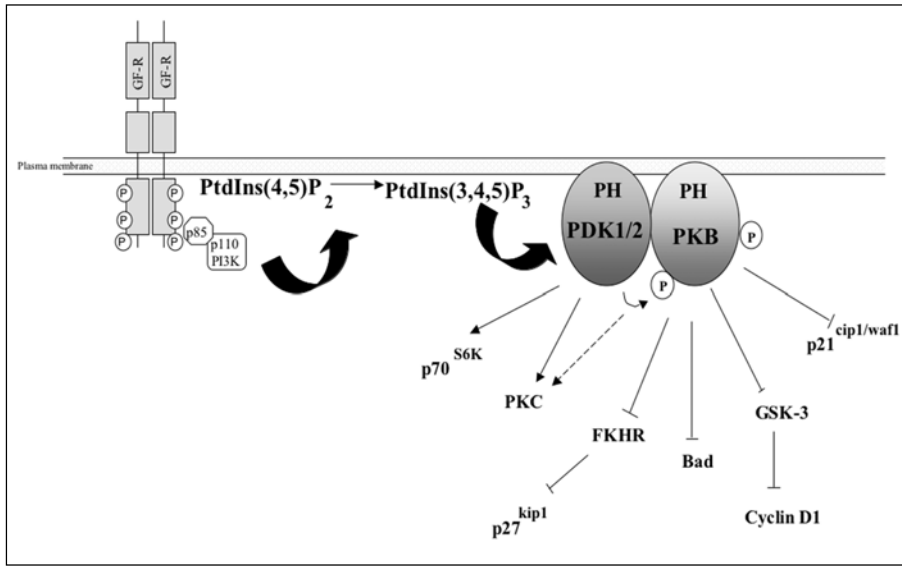

Figure 7. Overview of the PI3 kinase signaling pathway. Upon activation of growth factor receptors, the p85 subunit of PI3 kinase is recruited to the receptor, which subsequently binds and activates the p110 subunit of PI3K. As a consequence, p110 can phosphorylate PtdIns(4,5)P_2 at the plasmamembrane to generate PtdIns(3,4,5)P_3. Thereupon, PDK and PKB translocate to the plasmamembrane. PDK then phosphorylates different substrates including PKB, leading to the phosphorylation of other downstream targets (for details, see text).

different human tumors (reviewed in ref. 93) and cells lacking PTEN are found to have elevated levels of PKB kinase activity.

Currently, none of the processes described above has been investigated during an ongoing cell cycle. However, upon stimulation of quiescent cells, two waves of PI3K activity have been described,[94] suggesting a role for PI3K in late G1 phase, analogous to the MAPK signaling pathway.

Continuous Cell Cycle Progression vs. Growth Factor Stimulation of Quiescent Cells

Several studies have demonstrated that growth factor stimulation of quiescent cells induces two waves of signal transduction activity (also see chapter 11). The first one leads to exit from G0, whereas the second one is required for progression through G1/S. For Ras, also two waves of activity have been described after growth factor stimulation.[95] Interestingly, the second activation that occurs late in mid-G1 phase, at about five hours after serum addition, is much stronger than the first activation and is thought to be regulated by a mechanism intrinsic to cell cycle progression.[95] Only the early phase of Ras activation appears to correlate with activation of the MAP kinase pathway and may be directly linked to expression of cyclin D while the late Ras activity appears to be required for PI3K-dependent processes.[94] However, both signaling pathways are thought to cooperate in different cellular processes. Thus, the induction of MAPK activity at the G0/G1 transition triggers expression of cyclin D at the transcriptional level, but at the same time the PI3K pathway is activated which might cooperate in cyclin D transcription. In addition, PI3K is thought to regulate the translation of the

cyclin D1 protein and to inhibit its phosphorylation.[88] At the second wave of Ras activation PI3 kinase seems to be the more important effector protein. Activation of the PI3K-PKB pathway would lead to the down-regulation of $p27^{KIP1}$ expression by forkhead transcription factors and MAPK might play a secondary role in this downregulation by phosphorylating $p27^{KIP1}$.

As mentioned before, however, the situation might be quite different during an ongoing cell cycle. The second wave of Ras activity, for example, does not seem to be present in continuously growing cells. Time-lapse experiments using microinjection of different antibodies showed that an anti-Ras antibody could block entry into S phase only when introduced several hours before the beginning of DNA synthesis.[96] In fact, since $p27^{KIP1}$ levels are very low in continuously cycling cells one could imagine that the down-regulation that is observed at the end of G1 phase in growth factor stimulated cells is not necessary when cells do not exit from G0 (Fig. 8).

For the expression of cyclin D, however, only the timing of induction seems to differ in continuously cycling cells as compared to cells stimulated with growth factors. Microinjection experiments showed that during the ongoing cell cycle Ras activation was necessary for the expression of cyclin D only at the end of G2 phase of the preceding cell cycle. In addition, in continuously cycling cells synchronized by the mitotic shake-off method, $p42^{MAPK}$ (ERK2) was found to be phosphorylated immediately after mitosis.[97] Although this MAPK phosphorylation was much less than observed with growth factor stimulation, the mechanism that is needed for the expression of cyclin D could be similar to that described in growth factor stimulated cells. Ras could activate the MAP kinase pathway at the G2/M transition, resulting in the expression of cyclin D in early G1. If Ras function is inhibited at the end of G2, before MAPK is phosphorylated, no expression of cyclin D will be observed, whereas an inhibition of Ras function after the MAPK phosphorylation (immediately after mitosis) will have no effect on the expression of cyclin D.

Conclusions

Summarizing we can conclude that we are only at the beginning of understanding how signal transduction processes during the ongoing cell cycle are linked to cell cycle progression. Most signal transduction processes that are observed at the G0/G1 transition upon the stimulation of quiescent cells might not necessarily reflect the actual situation in continuously growing cells, although it seems clear that experiments using growth factor induced cells might give some indications about cellular processes and possible protein-protein interactions. So far, only relatively little is known about signal transduction events during the ongoing cell cycle but Ras signaling to cyclin D seems to be a major event. The shortening of G1 phase detected in Ras transformed cells can be associated to an increased expression of cyclin D and can be abrogated by cyclin D antisense RNA. Moreover, in continuously cycling cells the induction of cyclin D1 can overcome, to some extent, the requirement for cellular Ras.[96] Other important targets of Ras may be $p27^{KIP1}$ and $p21^{CIP1/WAF1}$, mainly regulated by the MAPK and PI3K pathways, although Ras might have additional downstream targets such as cyclin E, cyclin A, cdc25 and the family of E2F transcription factors as well. The same cell cycle regulators, cyclin D, $p27^{KIP1}$ and $p21^{CIP1/WAF1}$, are thought to be regulated by PKC (reviewed 98,99). Like PI3K and MAPK, PKC has been shown to be activated at two distinct times after growth factor stimulation.[100] Thus, the network of signal transduction acting on components of the cell cycle machinery seems to be even more complex. Different levels of protein activity during different sub phases of the cell cycle will probably contribute to correct timing of the localization and activity of many players in cell cycle control. Although both the basic cell cycle machinery and the different signal transduction pathways are reasonably well understood, determining the precise connection between the two will still be a challenge for the coming years.

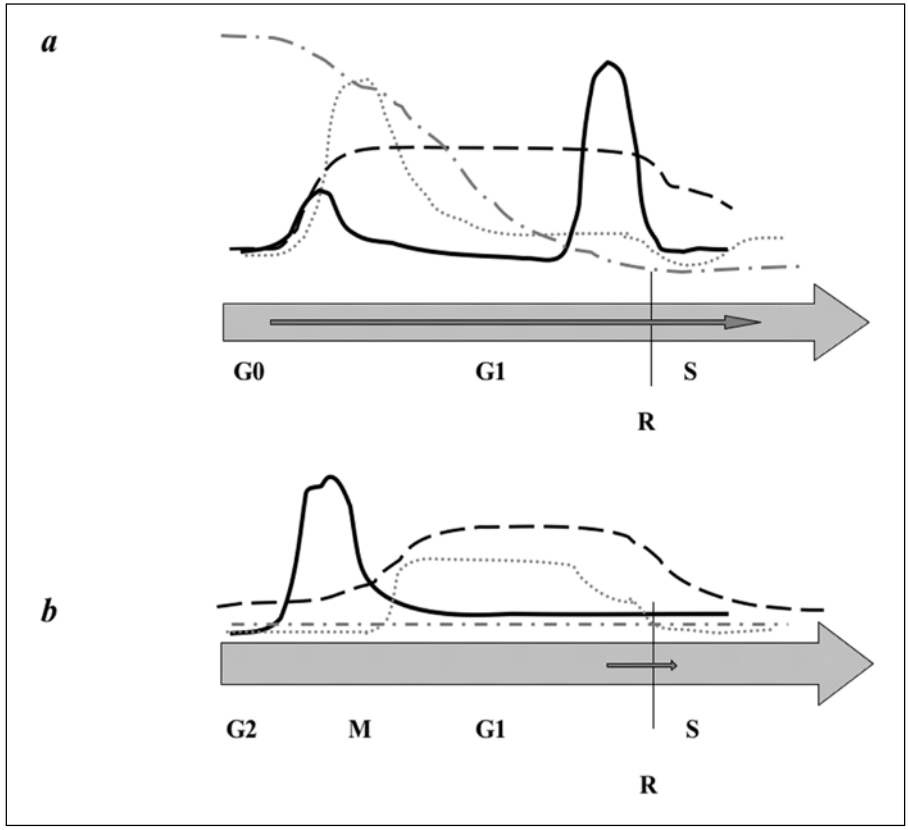

Figure 8. Model representing cellular processes during continuous cell cycle progression vs growth factor stimulation of quiescent cells. In growth factor induced cells (*a*) two waves of signal transduction activity are observed; one at the G0/G1 transition and one at G1/S. Ras activity peaks at the second wave (—), whereas MAP kinase is mainly activated at the first wave (• • •), leading to an increase in cyclin D expression (- - -) and a decrease in $p27^{KIP1}$ expression (−•−•). In continuously growing cells (*b*) Ras activation is observed only at the G2/M transition (—), leading to MAP kinase activation early after mitosis (• • •). Hardly any changes occur in the expression of $p27^{KIP1}$ (−•−•), while cyclin D is induced after mitosis following MAPK activation (- - -). Horizontal arrows represent a translocation of $p42^{MAPK}$ to the nucleus.

Acknowledgement

We would like to thank Robert Doornbos for useful discussions.

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CHAPTER 7

Regulation of Signaling and the Cell Cycle by Cell Interactions with the Extracellular Matrix

R.L. Juliano

Abstract

Adhesive interactions with the extracellular matrix, mediated primarily by integrins and transmembrane proteoglycans, play a key role in regulating the G1 phase of the cell cycle. A primary aspect of this is adhesion regulation of important signaling pathways, particularly the Erk/MAP Kinase cascade. The regulation exerted by matrix is quite complex; and takes place at several different levels of the signaling pathway. While some aspects of adhesion regulation of signaling seem to function ubiquitously, encompassing many different cell types and different matrix molecules, other aspects are influenced by the precise composition of the matrix and the transmembrane proteins interacting with matrix. Since there are over twenty distinct mammalian integrins, it seems likely that we are just beginning to understand the more specific aspects of integrin and matrix regulation of mitogenic signaling.

Introduction

Adhesive interactions between cells and macromolecular components of the extracellular matrix (ECM) can have profound effects on cell survival, growth, and differentiation. This chapter addresses the role of cell-ECM interactions, primarily mediated by integrins, in regulation of signal transduction and consequences for the G1 phase of the cell cycle. While several aspects of integrin-mediated regulation of cell cycle traverse have been identified, a primary link between the ECM and G1 phase regulation concerns the Erk/MAP Kinase pathway.

Basic Aspects of Integrin-ECM Interactions

The ECM

The extracellular matrix is comprised of a network of proteins and proteoglycans that provide both structure and information to cells. Some of the major protein components of the ECM include fibronectin, vitronectin, various laminins, and many members of the collagen family.[1] In addition to arising from a multiplicity of genes, many ECM proteins are the products of alternatively spliced messages, thus adding further complexity. Various ECM proteins are expressed in a developmentally regulated and tissue specific manner. However, certain

G1 Phase Progression, edited by Johannes Boonstra.

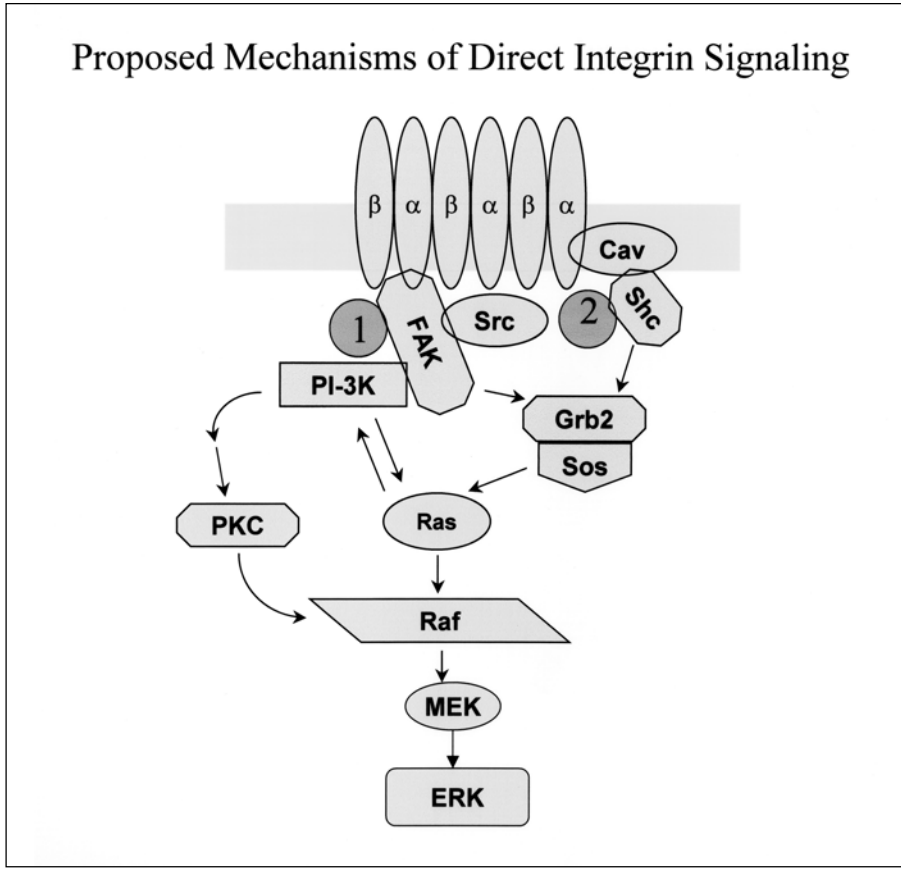

Figure 1. Direct Signaling by Integrins. Integrin αβ heterodimers interact with ECM proteins and cluster in the membrane. This leads to two possible scenarios for direct activation of signaling. (1) FAK is activated, recruiting Src as well as PI-3-Kinase. This can lead to recruitment of the Grb2-Sos complex, activation of Ras and subsequent activation of the Raf/Mek/Erk kinase cascade. (2) Integrin clustering leads to formation of a complex with caveolin and a Src family kinase (probably Fyn). This activates Grb2-Sos and the downstream cascade. PKCs can also contribute to the activation of the Raf/Mek/Erk cascade.

components such as fibronectin and some common collagens are nearly ubiquitous. In addition to proteins, a large number of polysaccharides and proteoglycans contribute to the mechanical and biological properties of the ECM.[2] Cell interactions with the ECM are primarily mediated by the integrin family of cell adhesion receptors;[3] however, membrane proteoglycans also make important contributions.[4] The coupling of integrins and proteoglycans to ECM components leads to the formation within the cell of specialized multi-protein structures termed focal adhesions.[5] These structures provide both a mechanical linkage between cell membrane receptors and the actin cytoskeleton, as well as a locus for the assembly of signaling components.

Integrins

Members of the integrin family of cell-surface glycoproteins provide a key interface between cell and the ECM. Integrins are heterodimers having an α and a β subunit; with each

subunit having a large extracellular domain, a single membrane spanning region, and a short cytoplasmic domain (except for the β4 subunit).[6,7] The vertebrate integrin family includes at least 18 distinct α subunits and 8 β subunits, leading to the formation of approximately two dozen distinct heterodimers. Integrins undergo dynamic changes during the ligand binding process[8,9] and can exist in various affinity states for their ligands. Integrin affinity states can be regulated either by extracellular factors such as divalent cations, or by complex intracellular processes that involve the R-Ras and Rap1 small GTPases.[10,11] Both the α and β subunit cytoplasmic domains are important contributors to various aspects of overall integrin function including linkage to cytoskeletal proteins, signal transduction, and regulation of integrin affinity. A large number of cytoskeletal, adaptor and signaling proteins interact with integrin cytoplasmic domains and contribute to integrin-mediated functions.[7]

Direct Signaling by Integrins

A concept that has emerged over the last several years is that integrins can directly activate intracellular signaling processes. Thus integrin engagement with ligand, accompanied by integrin clustering, can recruit a number of signaling components and lead to activation of important signal transduction pathways, especially the activation of MAP Kinases. The abundant literature on direct integrin signaling has been comprehensively reviewed;[7] here we will sketch out some of the basic ideas.

An early insight into the possibility of integrin signaling was the observation that integrin-mediated adhesion could lead to enhanced tyrosine phosphorylation.[12] It became apparent that adhesion was activating a nonreceptor tyrosine kinase now known as FAK (focal adhesion kinase).[13,14] This protein consists of a central kinase domain flanked by large amino-terminal and carboxy terminal segments. A region of the c-terminal called the 'focal adhesion targeting' (FAT) sequence is responsible for recruiting FAK to integrin-rich adhesion structures. FAK is capable of binding to a number of other signaling and structural proteins including c-Src, PI-3-Kinase, GRAF (a Rho-GAP), paxillin, talin and p130 Cas; some of these interactions depend on the phosphorylation status of FAK. Tyrosine phosphorylation and activation of FAK accompanies integrin-mediated adhesion, and de-phosphorylation promptly occurs when cells are detached.[15,16]

An intriguing aspect of integrin signaling relates to activation of the Erk and Jnk MAP Kinase pathways. Several different mechanisms have been proposed to account for this phenomenon (Fig. 1). One putative mechanism is that FAK acts similarly to a receptor tyrosine kinase in activating the Ras-Erk cascade. Thus, upon integrin mediated cell-ECM adhesion, FAK is autophosphorylated at Y397. This provides a recognition site for the c-Src SH2 domain and recruits Src, which then phosphorylates FAK at additional sites. One site, Y925, allows binding of the SH2 domain of the adaptor protein Grb-2 and its partner, Sos, an exchange factor for Ras. This sets the stage for activation of Ras followed by activation of the downstream kinase cascade comprised of Raf-1, MEK, and Erk[17] Another possible model for Erk activation by integrins involves the transmembrane protein caveolin-1, the Src-family kinase Fyn, and the adaptor protein Shc. In this model, a sub-set of integrin a subunits is able to activate Fyn thus causing tyrosine phosphorylation of Shc and subsequent recruitment of the Grb-2/Sos complex. This then triggers Ras and the downstream kinase cascade leading to Erk activation.[18] In both of the above models, Ras plays a key role in propagation of the signal from integrins to Erk. However, there is also evidence for Ras-independent mechanisms, possibly involving protein kinase C.[19] The existence of several models for integrin-mediated Erk activation may be a reflection of the fact that signaling processes are often highly cell-context dependent. Integrin engagement has also been reported to directly activate other arms of the MAP Kinase pathway including c-Jun Kinase (JNK) and p38.[20,21]

Integrins and Rho-Family Proteins

There is a link between direct signaling through integrins and activation of Rho-family GTPases, particularly Rho A, Rac1, and CDC42. Rho-family GTPases influence many key cellular processes, but are particularly important in regulation of the actin cytoskeleton.[22] Thus Rho A promotes the formation of stress fibers, while Rac and CDC 42 regulate cortical actin structures such as lamellipodia and filopodia. Studies have shown that integrin engagement activates several Rho family GTPases.[23-25] The mechanism(s) of integrin modulation of Rho family GTPases have yet to be worked out in detail; presumably exchange factors and/or GTPase activating proteins are important. For example, RhoA undergoes a complex response, first dipping in activity and then displaying increased activity as integrins are engaged. The initial dip in RhoA activity has been linked to Src activity leading to tyrosine phosphorylation and activation of p190 RhoGAP.[25] This may serve to decrease local actinomyosin contractility and thus promote cell spreading on the ECM at initial sites of cell adhesion. In any case, it seems likely that integrin modulation of Rho GTPases plays a significant role in cytoskeletal organization, cell motility, and signaling.

Integrin Regulation of the Receptor Tyrosine Kinase-Ras-Erk Pathway

Although direct signaling by integrins may be implicated in certain contexts (such as localized control of cell movement), the ability of integrins and their associated cytoskeletal partners to regulate other signaling cascades seems likely to be of greater fundamental importance. Thus normal cells require integrin-mediated anchorage to the ECM in order to traverse through the cell cycle. Recently it has become clear that a key aspect of this anchorage regulation of the cell cycle relates to anchorage control of signaling cascades, particularly the Erk/MAP kinase pathway.[7,26]

There are multiple interconnections between integrins, cytoskeletal components, and the Receptor Tyrosine Kinase/Ras/MAP Kinase cascade. Regulation occurs at least at three different levels. The first is at the level of activation of the RTK. The second concerns coupling between upstream and downstream events in the pathway. The third involves the transmission of the signal between the cytoplasm and nucleus.

That integrins influence the efficient activation of RTKs has been demonstrated in many cell systems. Early work [7,27] showed that integrin aggregation and occupancy were required for growth factor triggering of tyrosine phosphorylation of epidermal growth factor (EGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors. Further, there are at least two examples of integrin activation of RTKs that take place in the absence of growth factors.[28,29] Some insights are beginning to emerge concerning the underlying molecular mechanisms for RTK/integrin collaboration. It seems obvious that formation of direct or indirect complexes between the RTKs and the integrins could lead to enhanced opportunities for RTK dimerization and cross-phosphorylation. In the case of EGFR, there is a direct physical association with $\beta 1$ integrins that may lead to EGFR clustering. Recently evidence has emerged indicating that integrin association with cytoskeletal components may also be very important. For example, FAK can link indirectly to integrins via its carboxy domain, which can bind paxillin and talin; in turn, paxillin binds the $\alpha 4$ integrin subunit, while talin can bind various integrin β subunit cytoplasmic tails.[7] The amino terminal domain of FAK seems to be able to associate with activated EGFR or PDGFR, although the interaction may be indirect.[30] This is a very interesting model that could account for some of the reported integrin/RTK synergisms.

A second locus of integrin regulation of the RTK/Ras/Erk cascade involves the coupling between upstream and downstream signaling elements in the pathway. Thus, in 3T3 cells, the loss of integrin-mediated cell anchorage blocks the propagation of the signal from Ras to Raf-1. In this system growth factor mediated activation of RTKs and subsequent GTP loading of Ras occur normally in cells in suspension. However, the activation of Raf-1 and of the downstream

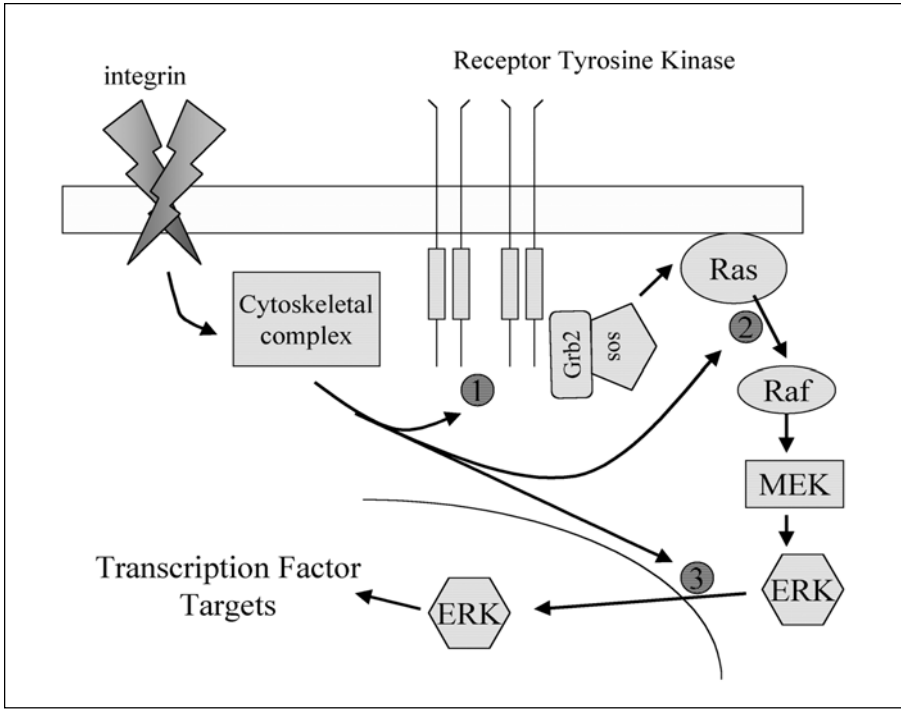

Figure 2. Integrin Modulation of Growth Factor Signaling. The formation of integrin clusters and cytoskeletal complexes influences the RTK-Ras-Raf-Mek-Erk pathway at three distinct loci. (1) Integrin-ECM interactions can enhance RTK clustering and autophosphorylation; this tends to occur if the RTK is relatively abundant in the membrane. (2) Integrin-dependent cytoskeletal complexes influence the coupling between Ras and Raf. (3) Integrin-dependent cytoskeletal complexes influence the trafficking of Erk between the cytoplasm and the nucleus.

kinases Mek and Erk are drastically reduced.[31] Thus there seems to be an anchorage dependent step between Ras and Raf in the signaling cascade triggered by peptide mitogens. Similar results were observed by another group,[32] with the exception that the locus of anchorage regulation was placed between Raf and Mek. Anchorage regulation of Erk activation seems to clearly involve the actin cytoskeleton. In particular, it is cortical actin filaments rather than focal contacts and stress fibers that are important.[33] Consistent with this notion, ectopic expression of active CDC42, which promotes cortical actin assembly, partially rescued Erk activation in suspended cells.[33]

A final locus of anchorage regulation of the MAP Kinase cascade concerns the transmission of the signal from cytoplasm to nucleus. Clues to the existence of this aspect of regulation came from studies showing that forced activation of Erk is insufficient to drive cells into the cell cycle.[34] The basic picture that emerges is that inactive Erk is held in the cytoplasm by virtue of its association with Mek. Upon activation, Erk is dually phosphorylated, dissociates from Mek and enters the nucleus, possibly as a dimer.[35] Upon dephosphorylation, inactive nuclear Erk reassociates with Mek and is exported from the nucleus.[36] However, there is also a level of regulation by integrins and the actin cytoskeleton. Thus, in suspension cells, or in cells treated with cytochalasin D, the normal trafficking of active Erk to the nucleus is disrupted.[37] The

mechanism underlying this actin-based modulation of Erk trafficking is completely undefined at this point.

In summary, integrins and their associated cytoskeletal components regulate the Erk/MAP Kinase pathway at several different levels (Fig. 2). This seems to play a key role in anchorage regulation of cell cycle traverse, as discussed below.

ECM Regulation of G1: The Key Role of Erk Activation

Current views of how cell anchorage to the ECM regulates the G1 phase of the cell cycle center on the roles of Erk, cyclin D1, and the CDK inhibitors $p21^{cip1}$ and $p27^{kip1}$.[38] In particular, a protracted activation of Erk is thought to be essential.[39] Some of the evidence leading to these conclusions is summarized below.

The key event of hyperphosphorylation of the RB protein requires both soluble mitogens and cell anchorage; this is observed in human and rodent fibroblasts.[40,41] The cyclin-CDK complexes most important for regulating pRB phosphorylation in response to adhesion seem to be CDK 4, 6 which bind cyclin D1. Thus two groups have found that the expression of cyclin D1 mRNA and protein is strongly adhesion-dependent.[42-44] This emphasis is supported by the observation that ectopic expression of cyclin D1 can relieve anchorage dependence of cell cycle traverse in rat fibroblasts.[43] Several groups have found an anchorage dependence of the activity of cyclin E-CDK2 complexes due to changes in the levels of associated CKIs. In nonanchored cells, the expression of $p21^{cip1}$ is increased and the turnover of $p27^{kip1}$ is decreased.[40,45] This may lead to an increase in the amount of $p21^{cip1}$ and $p27^{kip1}$ associated with cyclin E-CDK2 complexes and thus a reduction in activity.[47] In situations where there is reduced expression of cyclin D-CDK4, 6 complexes, this also can lead to a redistribution of $p21^{cip1}$ and $p27^{kip1}$ to cyclin E-CDK2 complexes, thus furthering the inhibitory process.[38]

Cyclin A expression and consequent S phase transition are also affected by cell anchorage in fibroblasts.[39,42,46] In NIH3T3 cells, the expression of cyclin A is transcriptionally regulated through an E2F site in the cyclin A promoter. The effects of loss of anchorage in these cells could be reversed by over-expression of cyclin D1. The underlying mechanisms here likely relate to the activity of cyclin D and cyclin E dependent kinase complexes and their ability to phosphorylate pRB and p107 and thus release E2F family transcription factors.

Returning to G1 regulation, several studies have emphasized the importance of sustained Erk activity in the G1 transition. Thus Erk is known to be critical for the induction of cyclin D1 expression.[47,48] It also seems that, while only moderate activation of Erk is required for cyclin D1 induction, this active state must be sustained.[38,39] Finally, it has been shown that integrin mediated adhesion to the ECM protein fibronectin is essential for sustained activation of Erk.[49] This seems to be a critical feature of ECM influence on cell cycle traverse. However, although sustained Erk activation is critical for cyclin D1 induction and G1 traverse, it is not sufficient. Thus, forced activation of Erk in suspended cells is unable to drive cells through G1.[50] A possible explanation for this is the recently described anchorage-dependence of Erk trafficking to the nucleus.[37]

In addition to Erk, it has very recently become clear that Rho family GTPases play a critical role in regulating the timing of cyclin D1 expression and thus G1 traverse. One mechanism for this may involve the effects of Rac on regulating the translation of cyclin D1.[51] In addition a key role for Rho has recently been defined. Thus Rho seems to act as a switch that permits sustained activation of Erk and mid-G1 expression of cyclin D1; however, in addition, Rho inhibits early G1 expression of cyclin D1 by repressing effects of Rac/CDC42.[52] Since both Rac and Rho can be regulated by integrin mediated adhesion, this provides another strong linkage between the ECM and cell cycle control. A summary of current concepts on ECM regulation of G1 events is depicted in Figure 3.

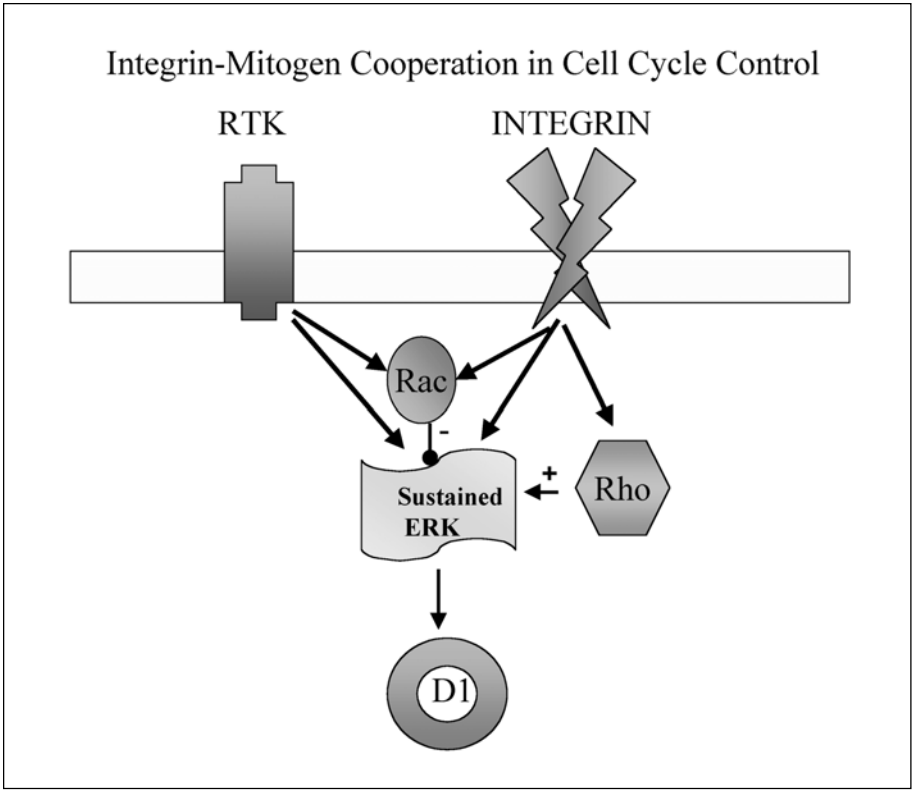

Figure 3. Integrin-Mitogen Collaboration in G1 Cell Cycle Control. The stimulation of the RTK-Ras-Raf-Mek-Erk pathway by growth factors leads to Erk activation. However, integrin mediated cell anchorage and cytoskeletal assembly is required for the strong and sustained Erk activation needed to induce cyclin D1. This process is antagonized by active Rac and enhanced by active Rho, both of which are regulated by integrin-mediated adhesion.

Cell Cycle Effects Specific for Individual Integrins and ECM

While the overall mechanisms of integrin signaling are similar within this protein family, there are also a number of examples of integrin-specific effects that impact on cell cycle regulation. For example, in myoblasts $\alpha5\beta1$ has been associated with cell cycle traverse while $\alpha6\beta1$ has been linked to withdrawal from the cell cycle and promotion of differentiation.[53] In endothelial cells differences have been observed between $\alpha1\beta1$ and $\alpha2\beta1$, two integrins that both bind to collagens; thus $\alpha1\beta1$ promotes cell proliferation while $\alpha2\beta1$ does not.[54] By contrast, in another cell system, mammary epithelial cells, the reverse seems true and $\alpha2\beta1$ promotes cell proliferation better than $\alpha1\beta1$.[55] Observations of this sort reinforce the notion that cell signaling processes are highly context dependent. There are also a number of examples of specific effects of matrix proteins. Thus, in vascular smooth muscle cells the state of organization of collagen influences cell cycle.[56] In this system cells plated on organized collagen fibrils arrest in G1 while cells plated on monomeric collagen continue to proliferate. There are also several reports indicating that different subdomains of fibronectin and/or different levels of organization of fibronectin fibrils can modulate cell cycle progression.[57,58] Finally there have been

several reports from Ingber and colleagues that connect the degree of cell spreading and cytoskeletal organization with the ability to traverse the cell cycle.[59,60]

Thus, while a number of examples of specialized regulation by integrins exist, for the most part integrin mediated cell-ECM interactions regulate cell cycle by acting through Erk and Rho GTPases to influence cyclin D1 and CDKI levels.

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Chapter 8

Impact of Nutrients on the Cell Cycle of *Saccharomyces cerevisiae*

C. Theo Verrips

Abstract

The length of G1 phase of *S. cerevisiae* is mainly determined by the flux of nutrients. However this relation is not linear, as below a growth limiting C-flux of about 20 fmol.cell^{-1}.h^{-1}, this C-flux is not longer mainly directed to energy production and synthesis of building blocks for biopolymers, but to the synthesis of di- and polysaccharides. The length of the first part of the G1 phase is determined by the synthesis of building blocks and this length can be described quite well with Monod kinetics. Subsequently the translation of CLN3 is the rate limiting step in which the concentration of loaded tRNA, ribosomes and cAMP, all closely related to nutrient fluxes, determine its length. After reaching a threshold value for these parameters, the Cln3/CDK is formed.

In the following part of the G1 phase, the active Cln3/CDK, in cross talk with the Wsc1/PKC pathway, determines the rate of Cln1,2/CDK formation. This complex determines the rate of the last part of the G1-phase before START, as this complex directs the transcription of late G1 phase genes, the phosphorylation of Sic1p and its own degradation. Most likely the phosphorylation of Sic1p and its subsequent degradation determines the exact location of START.

Introduction

Progression through the cell cycle of *Saccharomy ces cer evisiae* is highly determined by the availability of nutrients in the environment. The regulation of cell cycle progression by nutrients is occurring primarily in the G1 phase of the cell cycle. In this chapter an attempt will be given to explain the observations that after passing START, nutrient limitation is no longer a determinant factor for completion of the cell cycle,[1] and that the length of the G1 phase is determined by nutrient limitation (Table 1).

Evidence for the role of nutrients in the determination of the length of the G1 phase was obtained in fed-batch cultures of cells synchronized early in the G1 phase by elutriation.[2] The cells were cultured under C-limitation, and the C-source was added to the cells at various but constant rates.[2,3] The results of these studies demonstrated that the relationship between the flux of the growth rate determining nutrient and the length of the G1 phase was bi-phasic (Fig. 1). These observations indicate that large changes in the length of the cell cycle are due to relatively small changes in C-nutrient fluxes. It is remarkable that the inflection point coincides with the accumulation of reserve carbohydrates, this in spite of the low flux of the C-source into the cells under these conditions. Apparently, cells monitor the nutritional conditions of their environment and measure their internal metabolic status and, as a consequence, determine

G1 Phase Progression, edited by Johannes Boonstra.

Table 1. Expression of key cell cycle control genes as function of the galactose consumption rate[2,3]

Length of the Total G1-Phase	Expressed Gene	Time of Expression During G_1 Phase (Minutes)
65 min	*CLN3*	0
	CLN1	50
	CLN2	50
	SWI4	25-50
	SWI6	0 and 50
225 min	*CLN3*	0 and 140
	CLN1	200
	CLN2	200
	SWI4	160
	SWI6	180
580 min	*CLN3*	0 and 200
	CLN1	500-550
	CLN2	550
	SWI4	200-500
	SWI6	250 and 450

whether or not the restriction point START will be passed. The key proteins in this decision process will be discussed below. This discussion is restricted to aspects that are nutrient or energy state (including phosphorylation) dependent. Finally, a model will be formulated describing the rise and fall in expression level of several key genes during progression through the G1 phase.

In addition to the key proteins of the cell cycle, the emphasis in this chapter will also be on a number of metabolites that play a key role in the regulation of cell cycle progression, in particular ATP (but also the ratio's [ATP:ADP] and [ADP:AMP]), GTP and cAMP. Biosynthesis of ATP is tightly controlled,[4] which makes sense as ATP is involved in many biosynthetic processes and is in equilibrium with GTP. ATP is required for many phosphorylation processes that play crucial roles in cell cycle regulation. GTP is directly involved in the regulation of many proteins involved in cell cycle progression, cell integrity and cell growth mainly via G proteins.[5] Finally ATP is the precursor of cAMP, a key factor in the regulation of expression of cell cycle correlated genes. The α-ketoglutarate/glutamate ratio and the concentration of glutamate play a role in the coordination between available energy, the availability of the intracellular building blocks such as amino acids and purines and ribosomal activity.

Many aspects of the G1 phase of the cell cycle, like bud appearance and spindle body duplication are not discussed here as they are reviewed excellently in Chapter 2, whereas the effect of stress on the G1 phase is described in Chapter 10.

Nutrient Conditions and Utilization

The description of the G1 phase of the cell cycle as function of nutrient conditions, requires a definition of these conditions and their consequences. To understand the various events during the cell cycle in a quantitative way, it is necessary to use synchronised cells growing under well-defined conditions. Therefore a cultivation method was developed using elutriated cells, which were subsequently grown in a fed-batch fermentor, in which the rate of addition of the growth limiting nutrient determined the rate of progression through the cell cycle.[2,3]

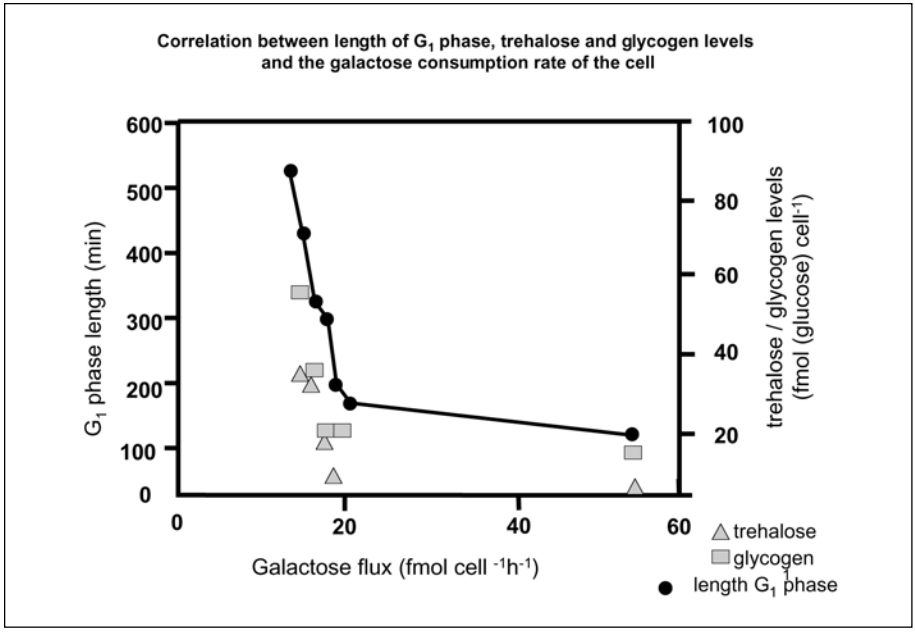

Figure 1. Correlation between the length of the G1 phase, trehalose and glycogen levels and the carbon (galactose or glucose) consumption rate of the cell

Most often either glucose or galactose were used as the rate limiting nutrients, although N-, P- and S-limited cells can also be cultivated in this way. Under glucose-limited conditions the high affinity glucose uptake system is active. This system is regulated by Snf3p, often described as the extracellular (glucose) sensor. Snf3p differs from other Hexose transporters (Hxt's) because it has a long C-terminal extension with several casein kinase boxes and nucleotide binding sites[6] located in the cytoplasm. Therefore, it may be expected that Snf3p has both in- and extracellular sensing functions. Ammonia uptake occurs via Mep1,3p and is regulated by Mep2p.[7] Galactose is taken up via Gal2p, which as the other genes of the galactose catabolism, are under tight control.[8] A potential third sensor, the G-protein coupled receptor Grr1p,[9] will be discussed in the context of the Skp1p/Cdc34p F-box-dependent ubiquitination complex (SCF).

The C- and N-nutrient pathways converge in the central nitrogen metabolism (Fig. 2). The central nitrogen metabolism and the biosynthesis of building blocks derived from glutamate and glutamine is tightly regulated. As demonstrated recently with micro-array analysis,[10] the transcription factor Gcn4p regulates at least 539 genes involved in the central nitrogen metabolism. In addition to genes encoding enzymes involved in amino acid, nucleotide and vitamin synthesis, a number of other genes playing a role in cell growth are regulated by Gcn4p, in particular 45 genes encoding enzymes or regulatory proteins involved in energy generation, including *GLG1, GSY1,2* and *GLC3* of the glycogen biosynthetic pathway.

Several intracellular sensors for amino acids and related components have been identified as well. These include Gcn2p, the well characterised primary sensor of the general control of amino acid biosynthesis.[11] Gcn2p, the translation initiator factor 2α (eIF2α) kinase, mediates derepression of *GCN4* mRNA translation under nutrient limited conditions. The mechanism of activation of Gcn2p is coupled to the level of uncharged tRNA, as Gcn2p has a histidyl-tRNA synthase like site, which can bind uncharged tRNA's. Gcn2p has also a site that enables interaction with ribosomes. Recently it has been found that glucose limitation results in upregulation

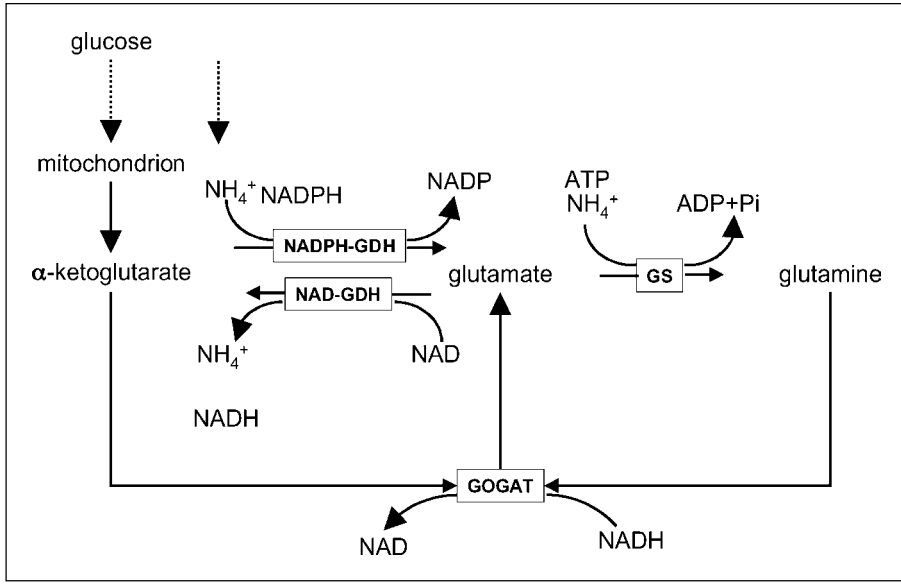

Figure 2. Integration of the C- and N-fluxes in the synthesis of building blocks in the Central Nitrogen Metabolism of *S. cerevisiae*, grown on ammonia as a sole N-source.

of *GCN4* mRNA translation via eIF2α kinase.[12] Just as for amino acid limitation, this requires the tRNA binding site of eIF2α kinase, but not its ribosome binding site. Via activation of *GCN4* translation, eIF2α kinase also contributes to the synthesis of glycogen under glucose-limited conditions. The role of eIF2α kinase in both amino acid and glucose limitation is another mechanism to ensure the correct balance between N- and C-compounds, as well as the correct balance between preparation for a new cell cycle or maintenance of the physiological state of the cell.

It is important to mention that Gcn4p contains a nearly optimal phosphorylation site for recognition by the SCF-CDC4 ubiquitination complex and may therefore be degraded by the proteasome.[13] Using an overexpression screen, 119 genes were identified that confer arrest in cell cycle progression.[14] *GCN4* proved to be one of the most potent inhibitors. Therefore elimination of Gcn4p after it has performed its function may be important for cell cycle progression. It is not known whether Gcn4p is phosphorylated by Cdc28p/Cln1,2p at the end of the G1 phase.

The common metabolites/signalling molecules between the C- and N-routes and progression through the cell cycle, notably cAMP, ATP and related molecules like GTP, the [ATP/ADP] or [ATP/AMP] ratio's and important metabolites with regulatory or branchpoint properties, like Glu-6-P, Glu-1-P, UDP-Glu and trehalose, will be discussed in detail below.

cAMP

Synthesis of cAMP in *S. cerevisiae* is catalyzed by adenylate cyclase, encoded by *CDC35,* which converts ATP into cAMP. It depends for this conversion on the presence of either of two membrane bound Ras proteins encoded by *RAS1* and *RAS2* respectively. The Ras proteins require the membrane bound guanidine-nucleotide exchange protein Cdc25p for their activation. This protein activates Ras by catalysing exchange of bound GDP for GTP. There is evidence of a physical interaction between Ras2p and adenylate cyclase, indicating that the latter is also located at the plasma membrane. Ira1,2p are negative regulators of Ras1,2p, as

they stimulate the intrinsic GTPase activity of Ras1,2p, thereby returning Ras1,2p in their inactive GDP bound state.[15] Addition of glucose results in a transient peak in cAMP.[16,17]

The levels of cAMP in the cell are influenced by the rate of turnover, which is catalyzed by low and high affinity cAMP phosphodiesterases, encoded by *PDE1* and *PDE2* respectively. It is important to realise that both synthesis of cAMP and its conversion by Pde1,2p occur at the plasma membrane. Therefore the concentration of cAMP close to the membrane will be higher than in the nucleus, where cAMP plays an important role as well.

The effects of the nutrient/Ras/cAMP pathway on growth are mediated by at least three different ways, but predominantly through Protein Kinase A (PKA). This enzyme is a heterotetramer composed of two regulatory subunits encoded by *BCY1* and two catalytic subunits encoded redundantly by three genes *TPK1, 2* and *TPK3*. The other ways include:(a):Increase of transcription and translation. The increase in transcription of genes involved in translation is regulated by cAMP through the transcription factor Rap1p.[18] In addition to the ribosomal proteins,[19] also other proteins are upregulated in this way; (b):It is well established that overexpression of *CLN3* is sufficient to bypass the essential requirement for cAMP, but the mechanism of this bypass has been elucidated only recently. The level of Cln3p translated from normal mRNA is considerably higher than when Cln3p is translated from a messenger encoding Cln3p but with an untranslated region originating from a heterologous gene. This increase proved to be cAMP dependent (Fig. 3). It is still unknown whether this is a unique feature of the *CLN3* messenger or that the translation of other messengers is regulated in a similar way. It is important to mention that studies have established that Cln3p is not a direct target for phosphorylation by PKA. Recently the cAMP level was determined as a function of cell cycle progression in a fed batch controlled growth experiment and found a peak close to the point of *CLN3* translation.[20]

ATP and Related Components

The synthesis of ATP and GTP is well studied and has turned out to be one of the best feed-back regulated biosynthetic pathways in *S. cerevisiae*. The yeast cell senses carefully whether extracellular sources of adenine, hypoxanthine or guanine are present. If not, these molecules are synthesized by the cell.[4] ADP and ATP synthesis from adenine leads to feedback inhibition of Ade4p, which is the controlling enzyme of the pathway. This and other loops guarantee that when there is a high demand for ATP, e.g., when DNA has to be synthesised, no feedback inhibition takes place, whereas if the demand is low, no energy is wasted in this highly energy requiring process.

Unfortunately, exact data are lacking on the fluctuation of ATP levels and other nucleotide triphosphates as a function of the progression through the cell cycle. In continuous cultures under aerobic conditions the ATP level of nonsynchronised cells depends on the growth rate.[17,21] The ATP:ADP ratio under these conditions is below 6.0 for low growth rate and > 9.0 for rapid growth;[17] whereas the amount of ATP is between 5-9 μmol/g wet weight. Based on these experiments, it is assumed that the absolute level of NTP's as well as the ratio [ATP:ADP] decrease when the growth rate decreases. As phosphorylation of a number of key proteins in the cell cycle is essential for their functioning cq. elimination, these reactions may slow down at lower ATP levels. The ratios [ATP:ADP] or [ATP:AMP] are important in the regulation of Snf1p, a key kinase in glucose repression.[22] It is not clear whether Snf1p is directly activated by AMP, but its activity correlates remarkably well with the [ATP:AMP] ratio, which rapidly decreases more than 200-fold upon glucose removal.[23]

Changes in the ratio [ATP:ADP] results in a proportional change in the ratio [GTP:GDP] and as such this effects the activity of G-proteins. The most important GTP coupled processes for cell cycle progression described here are related to Ras1,2p and Rho1p. Ras1,2p activates

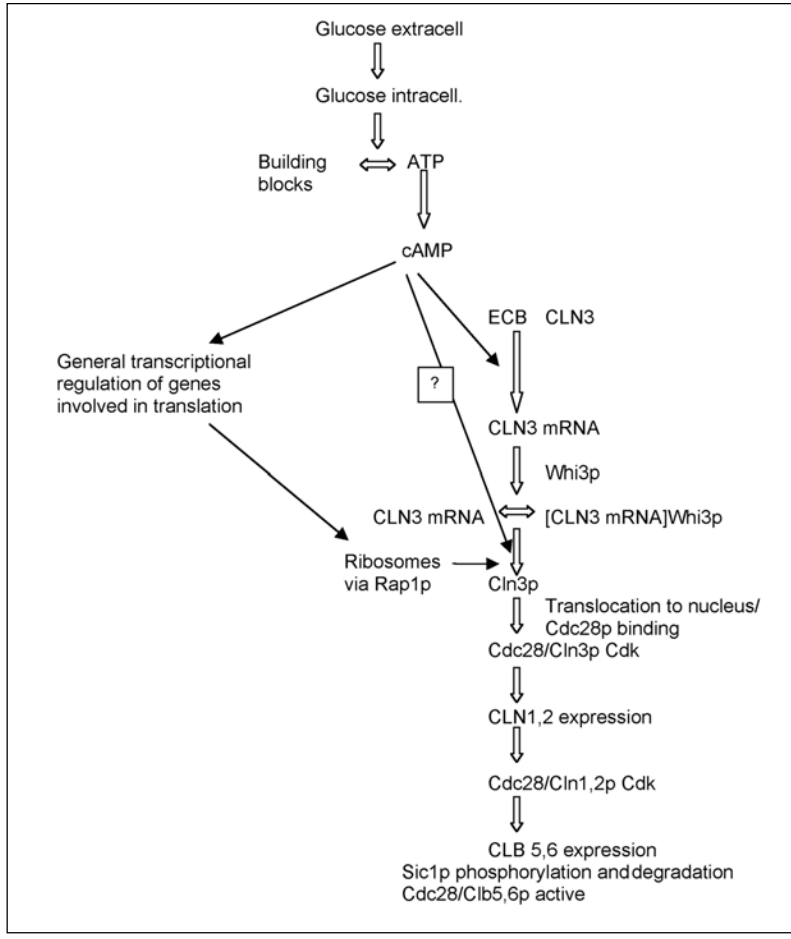

Figure 3. Relation between the C-flux (glucose or galactose), cAMP level, ribosome synthesis and the subsequent translation of *CLN3* and the formation of the active Cln3/CDK complex

adenylate cyclase (Cdc35p) which synthesizes cAMP as described above. Rho1p is involved in maintaining cell integrity (see below).

Glu-6-P, Glu-1-P and UDP-Glu

In continuous cultures the level of Glu-6-P increases slightly as the growth rate decreases, the increase in Glu-1-P is more significant as it increases nearly ten-fold if the growth rate drops from $\mu = 0.2$ to $\mu = 0.05$ h^{-1}. The Fructose1,6-P level decreases about 2.5 fold under these conditions.[17] Therefore it is clear that there is a rerouting of hexose from respiration/ energy generation and building block formation to synthesis of either reserve compounds and/ or cell wall components. When cells grow on galactose, the uptake of galactose is ensured by Gal2p. Galactose is converted into UDP-Gal and subsequently to UDP-Glu, which is a key component in synthesis of cell walls, reserve components, and Glu-6-P[24] (Fig. 4). UDP-Glu is also synthesised in cells growing on glucose/galactose and can be considered as a key metabolite involved in the balance between energy generation and growth or arrest in the G1 phase.

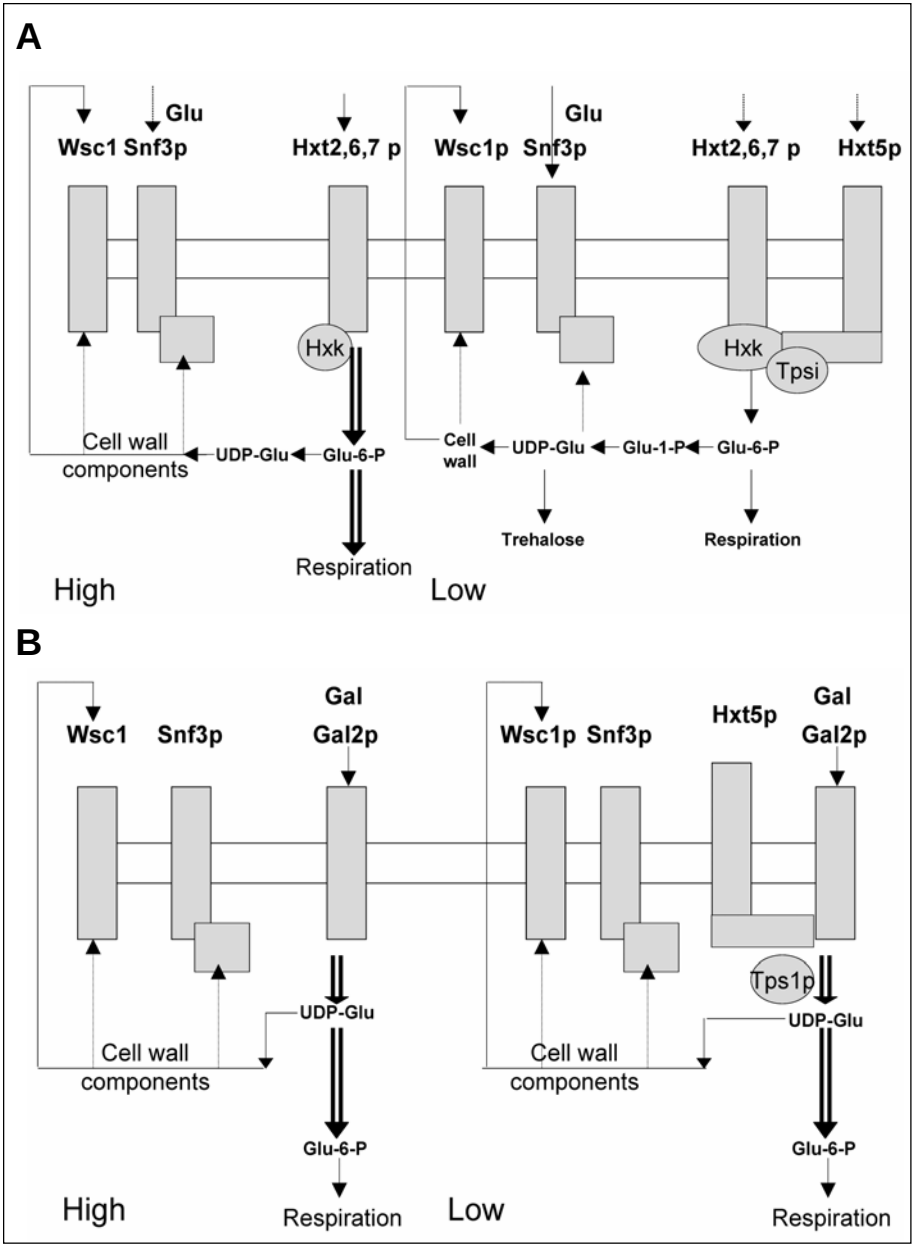

Figure 4. Sensing and uptake of C-sources (glucose (A) and galactose (B)) as function of the growth rate and the redirection of the C-sources from energy production and synthesis of building blocks to the synthesis of storage di- and polysaccharides at low growth rates (compare Fig. 1 and Table 1).

The importance of the regulation of the UDP-Glu synthesis has been shown recently. PAS kinases, as function of nutrient levels, down regulate the synthesis of UDP-Glu; Psk1,2p via Ugp1p (UDP-Glu pyrophosphorylase) and Gsy2p respectively.[25]

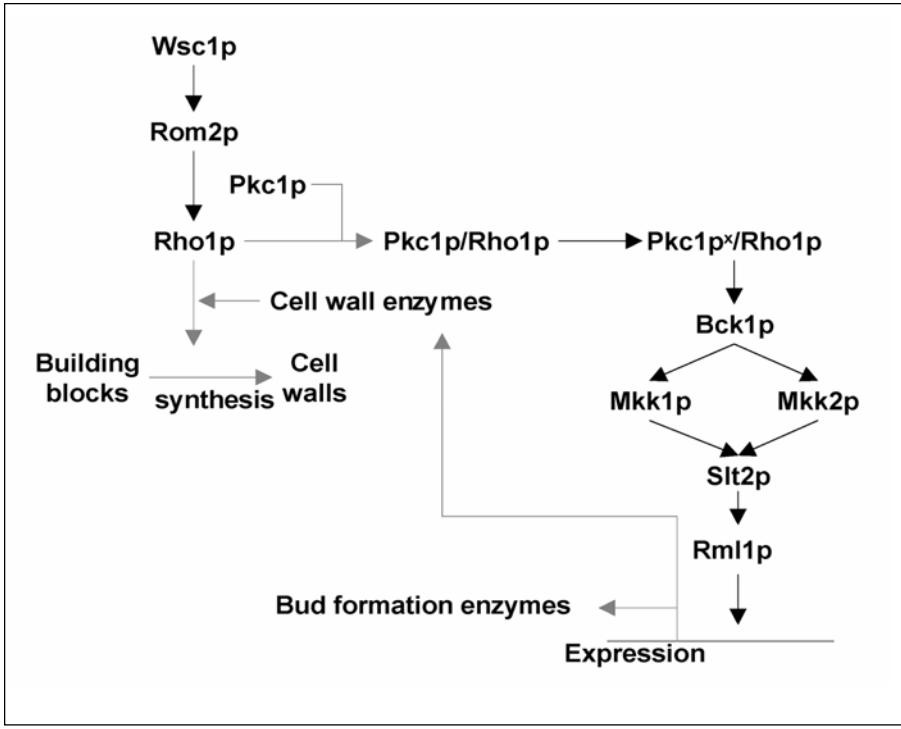

Figure 5. Sensing the cell integrity by the Wsc1p/Rho1p pathway that regulates activation of the Pkc1p/Slt2p/Rml1p signal transduction pathways resulting in expression of genes and the synthesis of building blocks and cell wall components.

Trehalose, Glycogen and Cell Integrity

Trehalose and glycogen have a dual role in the cell. On the one hand they serve as energy reserves and on the other hand, trehalose is an effective intracellular protectant for the cell[26,27] and as such they contribute to the integrity of the cell. At a high growth rate, which means high flux through glycolysis, no significant level of trehalose and only a moderate level of glycogen are accumulated. However, at low growth rates the biosynthesis of these compounds is higher than their break down leading to build up of these components at low growth rates (Fig. 1). The onset of trehalose synthesis at a low flux rate coincides with two other events:at low growth rate *HXT5* and Hxt5p are expressed[28] and the mitogen activated protein kinase (MAPK) Slt2p is not phosphorylated[3] and consequently the PKC pathway involved in cell integrity does not function fully (Fig. 5) and therefore cell growth is affected.

The *HXT5* promoter is different from the promoters of the other HXT's. It contains two STRE elements, two HAP2/3/4/5p binding sites and one post-diauxic shift (PDS) element and is under control of Msn2,4p transcription factors[29] and other factors, one of which is nutrient limitation (low growth rate). Remarkably, the *HXT5* promoter is very similar to the *GSY2* promoter that is also under Gcn4p- and stress control.[10,30] Hxt5p differs from the other Hxt's in that it has a long N-terminus, located at the cytoplasmic side of the membrane. In analogy with other C- or N-terminal extensions it can be expected that this cytoplasmic domain is involved in regulatory or synthetic processes to accumulate trehalose and glycogen. Trehalose-P-synthase (Tps1p) a key regulator in trehalose synthesis, is one of the candidates for

this regulation, because when bound to Hxt5p it may form the start of a vectorial process resulting in the generation of these carbohydrates.[31] It is likely that close to the inflection point of Figure 1, an important redirection of the hexose flux occurs from respiration/energy generation and synthesis of building blocks for protein and nucleotide synthesis towards synthesis of reserve carbohydrates. This redirection causes a significant extension of the G1 phase. Glycogen synthesis is not only regulated at the transcriptional level. Glc7p and Snf1p stimulate Gsy2p, however Snf1p may also stimulate another component of glycogen synthesis. The Cdk, Pho85p, when complexed with cyclins Pcl8p or Pcl10p phosphorylates Gsy2p at Ser654 and Thr 667 and consequently Gsy2p is inactivated.[32] This inactivation can be circumvented by Glucose-6-P upon interaction with Gsy2p-P, demonstrating another direct interaction between metabolic rate and biosynthesis of storage carbohydrates and G1 phase duration (Figs. 1, 4). There is also some evidence that cAMP activated PKA inactivates Gsy2p in vivo.[32,33] It is of interest to mention that Pho85p has also a direct, but still not clearly understood role in cell cycle progression.[33]

In conclusion, limited nutrient conditions result in a relative low level of ATP, with the likely consequences for the phosphorylation of key proteins, lower ratio [ATP:AMP] and/or [ATP:ADP], and a slow increase of intracellular building blocks for protein and DNA synthesis. The important balance between the accumulation of internal building blocks and constituents of the cell wall is regulated by the relative fluxes through Glu-6-P, Glu-1-P and UDP-Glu for high nutrient fluxes and the fluxes through Glu-6-P, Glu-1-P, UDP-Glu and trehalose-P at low growth rates. At these low growth rates Hxt5p appears as a key player in the uptake of glucose and redirects Glu-1-P to reserve components on both glucose and galactose as growth rate limiting nutrients. At higher growth rates Hxt2,6,7p have the role to optimize respiration, whereas Hxt1,3p are transporters active under high glucose/low oxygen conditions (Fig. 4).

Of all the metabolites/regulatory molecules ATP, GTP, cAMP and UDP-Glu seem to be the most important, but the intracellular concentration of the amino acid status measured by the ratio loaded:unloaded tRNA, is crucial for both the synthesis of Cln3p and for the decision to pass START as shown below.

Key Proteins

A large number of cell cycle regulated genes were identified by microarray hybridisation.[34,35] In the processing of these data a nearly complete list of genes was produced which were activated at the M⇒G_1 transition, the G1 phase and the G1⇒S phase transition.[34,35] In total the expression of more than 300 genes is restricted to the G1 phase. As will discussed later, two boxes, SCB and MCB in the promoters of these genes, are involved in the G1-dependent regulation. In the promoter of about 60% of these genes a Swi4,6 dependent Cell cycle Box (SCB) has been determined, in 30% a Mlu dependent Cell cycle Box (MCB) and in more than 10% both boxes are present. A few are regulated via the Early Cell cycle Box (ECB). The main results of these and additional studies are summarised in Table 2. From this list, proteins that play a key role in cell cycle progression are selected which are discussed in some detail below (in chronological order of there functionality in the G1 phase). Many important genes and their products are not described in detail in this chapter, because data are lacking to construct a model that encompasses all these players.

Cyclin 3

Cln3p functions upstream of all other G1-cyclins. Although Cln3p is not essential as such, it is necessary for a punctional execution of the progress through the G1 phase and START as function of environmental conditions, in particular of the nutrient conditions.[2,3,36]

Table 2. Cell cycle related genes expressed at the M/G1 phase transition, the G1 phase and the G1/S phase transition[31-34]

Functions	Phases in the Cell Cycle				
	M/G1 Transition		G1 Phase		G1/S Transition
Cell cycle control	**CLN 3**		**CLN1**	**CLN2**	**CLB5**
	PLC 9		HSL1		**CLB6**
	SWI4		PLC1	PLC2	
	SWI5		RME1		
	SIC1		SWE1		
Nutrition	AUA1	CIT2	BAT2		
	GLK1	GLK1	PHO8		
	HXT1	INH1			
	HXT2	QCR9			
	HXT4	GSY2			
	HXT5	PIG1			
	HXT7	COX8			
	COQ5	COX7			
Budding	RGA1	TIP1	BNI4	MCD1	
	GYP6	YGP1	BUD9	MSB8	
	CHS1	EGT2	CDC10	RSR1	
	GFA1	BEM1	GIC2	SPH1	
	SKT5		GIN4	SRO4	
			CWH41	TUB4	
			EXG1	CSI2	
			FKS1	CTS1	
			GAS1	RNR1	
Mitosis	**CDC6**		CNM87	NUF1	
	CDC46		SPC42	SPC97	
	CDC47		SCP98	TUB4	
	MCM3		BIM1	BUB1	
			IPL1	PDS5	
			SLK19	SMC1	
			SMC3	MCD1	
			PDS1		

Bold are the gene/gene products discussed in this chapter in some detail.

Transcription of CLN3

CLN3 mRNA levels fluctuate during the cell cycle, with a peak at the M⇒G1 phase transition[2,34-38] (see also Table 1). An ECB has been identified in the promoter of *CLN3*[38,39] and Mcm1p alone or in an still unknown complex binds to this box to ensure that *CLN3* mRNA peaks at M⇒G1 phase transition. The ECB regulated transcription of *CLN3* contributes to the proper timing of the G_1⇒S-phase transition.[39]

A direct regulation of *CLN3* transcription by low levels of glucose has been described and the results are included in the model presented in Fig. 3. Depending on the rate of progression through the G1 phase, the level of *CLN3* mRNA decreases or peaks just before budding once again (Table 1).

Translation of CLN3 mRNA

The *CLN3* mRNA is translated into a protein of 580 amino acids and the Codon Bias Index (CBI) of this messenger is 0.264. Cln3p is present at a lower level than the G1 cyclins but the level of *CLN3* mRNA level is comparable to that of *CLN1,2*, so the low amount of Cln3p is not caused by poor transcription of the gene or by instability of the mRNA.

A factor contributing to the low amount of Cln3p may be the binding of Whi3p to *CLN3* mRNA.[40] Whi3p contains a RNA-recognition motif that specifically binds the *CLN3* messenger and localises this messenger into discrete cytoplasmic foci, thereby inhibiting the translation of *CLN3* mRNA. Further proof for this came from studies with the *whi3* mutant in which the expression of genes controlled by SBF (composed of Swi4p and Swi6p) and MBF (composed of Mbp1p and Swi6p) is accelerated, whereas overexpression of *WHI3* caused G1 phase arrest, but this arrest can be circumvented by constitutive *CLN2* expression.[40] These results suggest that Whi3p reduces the translation of Cln3p in an early stage of the G1-phase. Another factor regulating the translation of *CLN3* mRNA is a short upstream open reading frame (uORF) of its 5' leader sequence. This region was defined as a translational control element.[41] This control element is critical for the growth dependent regulation of Cln3p synthesis, because it specifically represses *CLN3* expression during conditions of diminished protein synthesis or low growth rate. This leaky scanning mechanism implies that the uORF represses Cln3p synthesis simply by decreasing the numbers of scanning ribosomes reaching the AUG of Cln3p. Cln3p synthesis may therefore be directly proportional with the number of ribosomes, which is dependent of the growth rate and therefore to the Monod equation:

$N_{rib} = (N_{rib})_{max} * S /(K_s + S)$ [in which S is the concentration of the rate limiting nutrient and K_s the affinity constant of the uptake system]. This approach enables the development of a mathematical description for the first subphase of the G1 phase. Besides the increase in translation capacity the cAMP level increases during the first subphase, also resulting in an increase in *CLN3* translation.[17] Whether this is a direct effect of cAMP or indirect via the above mentioned increase in ribosomes, has not been determined, although the latter seems more likely as it has been established that activation of Cln3p synthesis is not dependent on PKA.

An important observation on the control of nutrients on Cln3p was that its translation was repressed and its degradation was enhanced during nitrogen deprivation.[42]

Functions of Cln3p

Whereas transcription of *CLN3* is not directly nutrient dependent, its translation is directly dependent on both the extra- and intracellular nutritional conditions. Making the decision to commit to cell division or not is such a crucial factor in the evolution of eukaryotes that it was expected that nutrients or derivatives thereof must have a direct influence on this decision, which is executed via Cln3p. On its own, Cln3p seems not to have any function. Only in combination with Cdc28p an active cyclin dependent kinase complex is formed.

Recently, a further element in the fine regulation of Cln3/CDK has been elucidated. In contrast to Cln2p that is randomly distributed in the cell (at least when it is not bound and phosphorylated by Cdc28p), Cln3p resides predominantly in the nucleus, because the last 22 amino acids of Cln3p contain a potential bipartite nuclear localisation site (NLS). Deletion of the last 22 amino acids of Cln3p resulted in a primarily cytoplasmic localisation.[43] Binding to Cdc28p is not necessary for translocation of Cln3p, however it is still unclear whether Cln3p binds to Cdc28p in the cytoplasm followed by translocation to the nucleus or that this complex is formed in the nucleus, although the latter is more likely.

Although the kinase activity of Cln3/CDK is 200 times lower than that of Cln1,2/CDK, Cln3/CDK is active enough to stimulate the Swi4/Swi6p complex that is already bound to the SCB of a large number of promoter regions. In spite of many studies, the exact nature of the

activation of transcription by Cln3/CDK is still not known, but it is proposed that polymerase II is activated.[44] This activation results in the transcription of many genes including *CLN1,2* (Table 2).

Degradation of Cln3p

As many other proteins that coregulate the cell cycle, Cln3p is relatively unstable, with a reported half life ($t_{1/2}$) of 10 minutes. The instability is mainly caused by a short amino acid sequence, called PEST sequence, at which the process of proteasome mediated degradation is started.[45] If the PEST sequence is removed, the $t_{1/2}$ increases to about 2 hours. Cln3p truncated in this way can replace Cln1,2p completely in the G1 phase of the cell cycle.

It is clear that Cln3p is regulated in a number of ways as a function of the nutrient status of the cell. Figure 6 summarizes the present knowledge on the regulation of Cln3p and is mainly based on the model presented by Hall et al.[18]

SWI4

Transcription

The *SWI4* promoter region contains an ECB, just as the promoter region of *CLN3* and Mcm1p directs transcription of this gene. Consequently *SWI4* mRNA is present during the late M- and G1 phase. The availability of a carbon source does not change its expression pattern dramatically.[46]

Translation of SWI4 mRNA

The translation of *SWI4* mRNA (CBI 0.065) results in a protein of 1093 amino acids. It contains ankyrin repeats that mediate interaction with the Cln2/CDK complex. Its DNA binding domain lies between amino acids 36-168, whereas 149 amino acids of its C-terminus are required for binding to Swi6p.

There are no data available of any regulation of *SWI4* mRNA translation and, as the protein is relatively stable, it is expected to be present at a more or less constant level during M⇒G1 phase transition and during G1 phase. Swi4p contains a NLS, which is an indication that Swi4p is predominantly present in the nucleus.

Functions of Swi4p

The permanent presence of Swi4p in the nucleus does not mean a permanent functioning of Swi4p there. Two aspects are crucial for its functionality. In the absence or presence of low amounts of Swi6p in the nucleus, Swi4p is inactive, as its C-terminal domain forms an intramolecular complex with its N-terminal domain, which contains the site that recognises the SCB sequence. Swi6p is present in the cytoplasm in the S-, G2- and early M phases, because phosphorylation of Ser160 blocks the adjacent NLS. In the G1 phase this phosphate group is removed and Swi6 relocalizes to the nucleus and its C-terminus forms an intermolecular complex with the C-terminus of Swi4p, thereby liberating the N-terminus of Swi4p. Subsequently this N-terminal domain binds in a helix-turn-helix manner to the sequence CACGAA of the SCB, similar to the binding of Mbp1 to MCB sequence.[47] However, even then the Swi4p/Swi6p complex is unable to drive expression of genes such as *CLN1,2*. This is only possible after activation of this complex via Cln3/CDK activity, although the exact nature of this activation is still unknown.

The roles of Cln3p and Swi4p are reasonably well described by the scheme presented in (Fig. 6). Alternatively Swi4p can also function independently of Swi6p. In that case Slt2p kinase activity is essential.[48] This pathway may serve two purposes. It may function in addition

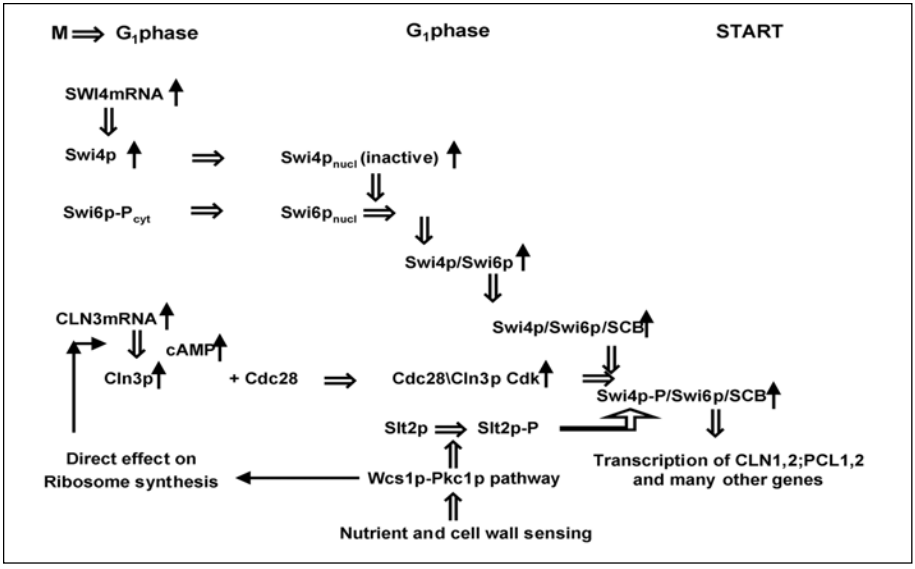

Figure 6. Activation of *CLN1,2* expression by Cln3/CDK and Slt2p. The increase in cAMP, ribosomes, loaded tRNA's results in increased translation of Cln3p and consequently Cln3/CDK activity. In a concerted action with positive signals related to cell integrity via Wsc1p/Pkc1p/Slt2p signal transduction this stimulates transcription of *CLN1,2* and many other genes through the activation of the Swi4p/Swi6p complex (Table 2).

to the Swi4p-P/Swi6p activity to maintain cell integrity during normal cell growth and bud formation, but it may also increase the transcription of factors essential during stresses by activation of *PLC1,2*. It is important to mention that overexpression of *PLC1,2* can suppress cell lysis defects in *slt2* mutants, whereas overexpression of *CLN1,2* is incapable of restoring these defects.

Degradation of Swi4p

Swi4p has no PEST sequence and seems to be quite stable. However this is certainly an issue that has to be analysed in much more detail to enable to quantify the scheme proposed in Figure 6.

Cyclin 1,2

Cyclin 1 and 2 are redundant proteins that play a crucial role in the cell cycle. Only overexpression of *CNL3* or improvement of the stability of Cln3p e.g., by removal of its PEST sequence, eliminates the essentiality of these cyclins for the viability of *S. cerevisae*.

Transcription

CLN1,2 mRNA is undetectable in S- and M phases which is consistent with the fact that the transcription of *CLN1* and *CLN2* depends on the activation of SBF by the active Cln3/CDK complex. As the formation of an active Cln3/CDK complex is dependent of the nutritional status of the cell, also the length of time before transcription of *CLN1,2* depends on the nutritional status (Table 1). An important observation is that *CLN1* is repressed by glucose via

cAMP, whereas transcription of *CLN2* may be stimulated via the cAMP pathway,[49] is rapid and does not require protein synthesis. The maximum level of *CLN1,2* mRNA is comparable to that of *CLN3*. The mRNA's of *CLN1,2* are relatively unstable.

Translation

The CBI's for *CLN1* is 0.124 and for *CLN2* 0.181. Just as *CLN3* mRNA, *CLN2* (and *CLN1*) mRNA has a ribosomal recognition motif (RRM) which enables Whi3p to bind to this messenger, although with a lower efficiency than to the *CLN3* mRNA.[40] Translation of *CLN1,2* mRNA seems not to be dependent on additional control mechanisms linked to the nutritional status, as the 5' untranslated regions of these mRNA's are different from that of *CLN3*. The increase in Cln1,2p is due to the increase in mRNA and the increase in the translation capacity of the cell and as such there is a link to nutrient status of the cell. Cln2p consists of 545 amino acids and contains a PEST sequence, however phosphorylation by CDK of Ser396 and Ser427, both located outside the PEST sequence, mainly determines the half life of Cln2p.[50] In contrast to Cln3p, Cln2p does not have a NLS or nuclear export signal (NES).

Functions of Cln1p and Cln2p

Cln1,2 and 3p are often considered as cyclins having essentially the same function, but appear at different times during G1. Cln3p is less abundant than Cln1,2p.

Cln1p and Cln2p are quite similar and, as most detailed studies have been carried out on *CLN2*/Cln2p, only Cln2p will be discussed in detail here. One of the most intriguing aspects of Cln2p concerns its targets in vivo. Cln2p, in contrast to Cln3p, is not located predominantly in the nucleus. Nuclear accumulation of hypophosphorylated Cln2/CDK occurs by a Ran GTPase-independent process, but is energy dependent, which is again a link to the nutrient status of the cell. Cdc28p binds to Cln2p, phosphorylates Cln2p at a number of the 7 potential Cdc28p phosphorylation sites. When the C-terminus of Cln2p is fully phosphorylated the Cln2/CDK complex is efficiently excluded from the nucleus. When phosphorylated Cln2p is in the cytoplasm, it is relatively unstable.[50]

Besides the transcription of *CLB5,6* (see below) the active Cln1,2/CDK complexes directly or indirectly trigger DNA replication and bud initiation and most likely also SPB duplication. In spite of many efforts, all the targets of Cln1,2/CDK are still unknown, one of the exceptions is the septin Cdc3p, an essential protein in septin rings.[52] A very important role of Cln1,2/CDK is the phosphorylation of the Clb-inhibitor Sic1p. A six-fold phosphorylation of Sic1p is necessary for its degradation (see below). Such a high level of phosphorylation is only possible if the capacity of CDK is above a certain level, probably reached only after CDK has activated the MBF complex, thereby initiating the synthesis of mRNA encoding for proteins essential for the S-phase, e.g., Clb1,5,6p and Cdc7,21,45p.[47]

Degradation of Cln1,2p

Cln2p is an unstable protein with a $t_{1/2}$ of about 8 minutes. Rapid degradation of Cln2p is induced by Cdc28p dependent phosphorylation. This phosphorylation is required for SCF-dependent ubiquitination and subsequent degradation. *CDC34* encodes the ubiquitin-conjugating component of SCF, and Grr1p is a SCF component that binds specifically to phosphorylated Cln2p and this binding is required for SCF-dependent ubiquitination of Cln2p. The link between degradation of Cln2p by SCF-Grr1 is interesting as Grr1p was originally linked to the glucose metabolism.[53] The instability of *CLN1,2,3* mRNA and Cln1,2,3p provides the cell with an efficient mechanism to react very rapidly to environmental changes and to direct its resources for essential processes to cope with these changes.

SIC1

Sic1p is an important protein in the regulation of the cell cycle, as it blocks the Clb/CDK-complexes during end M- to end G1 phase.

Transcription

SIC1 transcription occurs during late M- and early G1 phase, but it has no ECB in its promoter. The transcription depends on the transcription factor Swi5p. The *SWI5* gene is transcribed as a result of Clb1,2 activity at the end of the G2 phase.[54] However as Swi5p is phosphorylated by Clb/CDK complexes, it is unable to enter the nucleus until inactivation of Cdc28p at the end of anaphase. The promoter elements responsible for periodic transcription of *SWI5* were identified, and Mcm1 was shown to be a critical component of the transcription complex.[54]

Translation

SIC1 mRNA has a CBI of 0.034 and encodes a protein of 284 amino acids. No typical features of its translation have been described.

Functions of Sic1p

Sic1p is necessary to inactivate Clb/CDK complexes during M-G1 phase transition and G1 phase but it does not inhibit Cln/CDK complexes. The property of Sic1p to block Clb/CDK while leaving Cln/CDK unaffected is of great importance in cell cycle regulation. In this way all the tasks of the G1 phase can be performed. Only when all these tasks are completed and Cln/CDK has reached its threshold value, Sic1p will be phosphorylated.[55,56] The importance of Sic1p is demonstrated by *SIC1* deletion or overexpression. Deletion of *SIC1* causes uncontrolled and premature DNA synthesis, which resulted in massive rate of chromosome loss,[56,57] whereas overexpression results in arrest in G1 phase. There are no data available to show that Sic1p activity depends on nutrients or nutrient regulated signal transduction systems apart from the fact that its stability is dependent on its degree of phosphorylation and as such linked to the physiological state of the cell.

Degradation of Sic1p

At its N-terminus Sic1p contains 9 Ser- or Thr phosphorylation sites. It has been established that at least 6 of these sites have to be phosphorylated before Sic1p-P_6 is efficiently recognized by SCF-Cdc4, which catalyses ubiquitination. Most probably Cln/CDK is the kinase involved in these phosphorylations, whereas Cdc14p can dephosphorylate Sic1p-P_x, at least in vitro. This controlled regulation of Sic1p level is of importance for the transition of START. In the early G1 phase the phosphorylation capacity of Cln3/CDK is insufficient to outpreform Cdc14p or other phosphatases like Ppz1, which results in G1 arrest when overexpressed.[14] Therefore during that part of the G1 phase, in which Cln3/CDK is active, Sic1p remains un- or under-phosphorylated and therefore able to block Clb containing CDK's. Only when sufficient Cln1,2/CDK activity is present (close for START) phosphorylation occurs rapidly and the crucial number of 6 phosphorylations will be reached, resulting in a rapid degradation of Sic1p-P and therefore creating the opportunity for Clb5,6p to replace Cln1,2p in the CDK complex. As described above Cln1,2p are also degraded after phosphorylation by Cln1,2/CDK activity, but obviously after this CDK has done its work in G1 phase progression. The phosphorylation of Sic1p by active Cln1,2/CDK offers an excellent opportunity to develop a mathematical model for passing START.[56,58]

CLB 5,6

Transcription

The *CLB5,6* promoters contain MCB sequences and consequently their transcription depends of the active Mbp1/Swi6 (MBF) complex. Swi6p is only present in its nonphosphorylated state in the nucleus, which is in the G1 phase. At least one of the active Cln1,2/CDK complexes is necessary for the activation of the MBF complex, therefore *CLB5,6* transcription only occurs after *CLN1,2* expression, late in G1 phase.

Translation

The CBI's of *CLB5,6* are around 0.07. The mRNAs encode proteins of 380 amino acids and the homology between Clb5p and Clb6p is 48%. There is no evidence of a special regulation of *CLB5,6* mRNA at the translational level, which in fact makes sense as the decision to pass START has already been taken. A fast and not overregulated progression through the final phase of G1- and S phases may provide evolutionary advantages.

Functions of Clb5,6p

Clb5,6p are typical S phase cyclins. They are functionally redundant with the closely related Clb1,2,3,4p cyclins. They form S phase promoting factors (SPF) probably consisting also of Cdc7p and Cdc28p. They are involved in spindle body separation and reduplication, are essential to maintain sister chromatid cohesion during the metaphase arrest resulting from DNA damage-induced checkpoint response.[59] They do not have a role in S phase checkpoint. Surprisingly Clb5,6p, but not Clb3,4p, are required together with Cln3p for recovery after heat-shock induced G1 phase arrest.

Degradation

Clb5p is more sensitive to degradation during the G1 phase ($t_{1/2}$ = 10 minutes) than during the S phase or $G_2 \Rightarrow$ M phase transition, where its $t_{1/2}$ is about 20 minutes. The mechanism of this difference is not understood.

CDC6

Transcription

Cdc6p is an ATP/GTPase that controls S phase initiation.[60,61] Microarray analysis[34,35] shows clearly that *CDC6* transcription peaks at M⇒G1 phase transition, which is consistent with the presence of an ECB in the promoter of *CDC6,* which is recognised by the Mcm1 transcription factor[38] that coordinates not only transcription of *CDC6* but also that of *CLN3, SWI4, CDC46 and CDC47* . The level of *CDC6* mRNA fluctuates about 20-fold throughout the cell cycle.

Translation

Translation of *CDC6* mRNA (CBI = 0.114) results in a protein of 513 amino acids. Three consensus motifs for GTPase activity are located in its N-terminus. The first 47 amino acids are sufficient for DNA-binding and interaction with Cdc28p in vitro. One of the Cdc4p interaction domains is also located in that region, the other is located between amino acids 341-390.

Function of Cdc6p

Cdc6p is a protein that plays an important role in the duplication of DNA later in the cell cycle, and is required for entry into S phase. Cdc6p can only be incorporated into the

prereplication complex (PreRC) in a very small window during the cell cycle,[62,63] notably after activation of *CLN3* and before activation of Clb5,6p. Later in the cell cycle, Cdc6p has various roles, one of which is the conformational change in the structure of the origin of replication complex (ORC), a reaction that most likely requires ATP and increases binding of ORC to DNA.[61] In this respect it is noteworthy that Cdc6p has both ATP- and GTP-ase activity which is necessary to control initiation of DNA replication.

Degradation of Cdc6p

Cdc6p is a labile protein and depends for its degradation on phosphorylation and consequently recognition of its phosphorylated form by SCF-CDC34. This phosphorylation takes place in the final part of G1 phase,[13] whereas Cdc6p plays an important role in the S phase. A plausible hypothesis to cope with this dilemma is that only Cdc6p, which is not part of the preRC, is phosphorylated. Cdc28 regulates this process.[64]

PKC1

Pkc1p is an essential serine-threonine specific protein kinase. It consists of a number of domains:Hr1 repeats (amino acids 5-77 & 118-193), a C2 domain (a.a. 211-288), diacylglycerol domains (a.a.415-461 & 482-531), a protein kinase domain (a.a. 824-1083) and the Protein kinase C-terminus (a.a. 1084-1149).

Transcription

DNA-micro-array studies of several groups[34,35] showed that *PKC1* transcription is not regulated during the cell cycle, although Pkc1p may play an important, but still unknown role in morphogenesis during the cell cycle.[65,66]

Translation

Pkc1p is a large protein of 1151 amino acids and is essential. The codon bias is 0.135. Its localization changes as a function of the cell cycle:at the G1⇒S phase transition it is localized to the prebud site. This localization is dependent on the actin cytoskeleton. Pkc1p can interact with many other proteins, including Rho1p and Mkk1p.[67,68]

Functions of Pkc1p.

Pkc1p has many functions, most of which are related to maintaining cell integrity. These functions include phosphorylation of CTP synthase and activation of plasma membrane H^+ ATPase (Pma1p) in response to glucose availability. There is no hard evidence of direct involvement of Pkc1p in phosphorylation of Cln1,2p. The two main functions of Pck1p are activation of the mitogen activated protein kinase (MAPK) pathway and a MAPK pathway independent function, which is nevertheless essential for the yeast. This second pathway has been proven by deletion of several components of the MAPK pathway that all result in loss of cell integrity but not in loss of viability, whereas deletion of *PKC1* is lethal. The exact nature of this pathway remains to be elucidated. The Pkc1p kinase pathway can be activated by diacylglycerol, the liberation of which from phospholipids can be induced by Cdc28p activity, or by phospholipids directly and probably also by Ca^{2+}. Moreover Pkc1p can be activated probably by Tor2p, also via Rho1p.[68-70]

The main activation of Pkc1p seems to occur via Wsc1p. Wsc1p is a transmembrane protein with a small cytoplasmic domain, a single transmembrane domain and a large, Ser and Thr rich extracellular domain. These Ser and Thr residues are O-mannosylated and linked to the cell wall. The latter property makes Wsc1p an excellent sensor of the state of the cell wall,

including the synthesis and properly coordinated translocation of cell wall proteins and cell membrane constituents. Impairment of these translocation processes results in a drastic Pkc1p mediated response, notably the reduction of the synthesis of ribosomal RNA and proteins.[71] As described above, both Cln3p and Cln1,2p are dependent on the capacity of the translational machinery, and consequently the imbalance of the cell membrane and cell wall constituents result in a delay or even arrest of the cell cycle in the G1 phase. The most likely mechanism for this is that Wsc1p transfers its message of this imbalance to the guanine nucleotide exchange factors Rom1,2p. Subsequently Rom1,2p transfers this message to Rho1p/GDP resulting in an exchange of GDP for GTP after which Rho1p/GTP interacts with Pkc1p and activates the MAPK cascade.[5] This cascade starts with the activation of the MEKK Bck1p, subsequently the activation of the redundant MEKs Mkk1p and Mkk2p and finally the MAPK Slt2p.

In the context of the nutrient driven cell cycle progression, it is important to discuss Slt2p. Slt2p kinase is activated under various conditions. Cell cycle driven conditions that activate Slt2p are actin perturbation and bud formation. Slt2p can also activate Swi4p in the absence of Swi6p, thereby activating the transcription of a number of genes.[46] Interestingly one of these genes is *PCL1*, although Slt2p can not replace SBF activation by Cln3/CDK.

Recent studies have shown that the phosphorylation of Slt2p depends on the growth rate of the cells and is therefore nutrient driven. Although hard evidence is still lacking, there are indications that either Slt2p assists Cln3p in its functions or that activated Slt2p independently stimulates SBF. Recent studies showed that overexpression of *CLN3* resulted in a clear increase of the phosphorylation of Slt2p.[3] Deletion of *SLT2* resulted in a significant reduction of proportion of cells that passed START within 17 hr. The cells that passed START did that after a much longer time.[3]

The Wsc1p/Rho1p/Pkc1p/Slt2p/Rlm1p pathway is summarised in Figures 5 and 6, including the potential contribution of Slt2p to activation of SBF.[5,67,68]

Model

Based on the present data a mathematical model can be designed, that links nutrient fluxes to the length of the G1 phase and describes the passage of START. The model has 4 subphases:

In the first subphase amino acids, nucleotides are synthesized and used to synthesize a sufficient number of ribosomes and loaded tRNA's to ensure efficient and consistent protein synthesis. French and collaborators quantified the synthesis of rRNA and ribosomes and found that after starvation (in our approach after elutriation) the rate of synthesis of rRNA and ribosomes is dependent on nutrient supply and loading of rRNA genes and not on polymerase I level.[72] The rate of these syntheses is related to nutrient fluxes according to Monod equations. Consequently the length of this subphase is completely determined by the nutrient fluxes and whether the C-flux is mainly directed to energy generation and building block formation or to the synthesis of storage di- or polysaccharides (Fig. 1).

In the second subphase *CLN3* mRNA is translated and Cln3p forms with Cdc28 an active Cln3/CDK complex. *CLN3* mRNA is present in the first subphase, but not or hardly translated because Whi3p is bound to a special site of *CLN3* mRNA. Moreover in the first part of this subphase the cAMP level is quite low, which slows down translation. Cln3/CDK activates transcription of many genes via SBF and MBF (see Table 2). Important are the transcription of the *CLN1,2* genes via SBF. In the model a straight forward phosphorylation of SBF by Cln3/CDK is considered to drive this transcription but this is a considerable oversimplification of reality. The second subphase can be described mathematically with a limited number of linear differential equations.

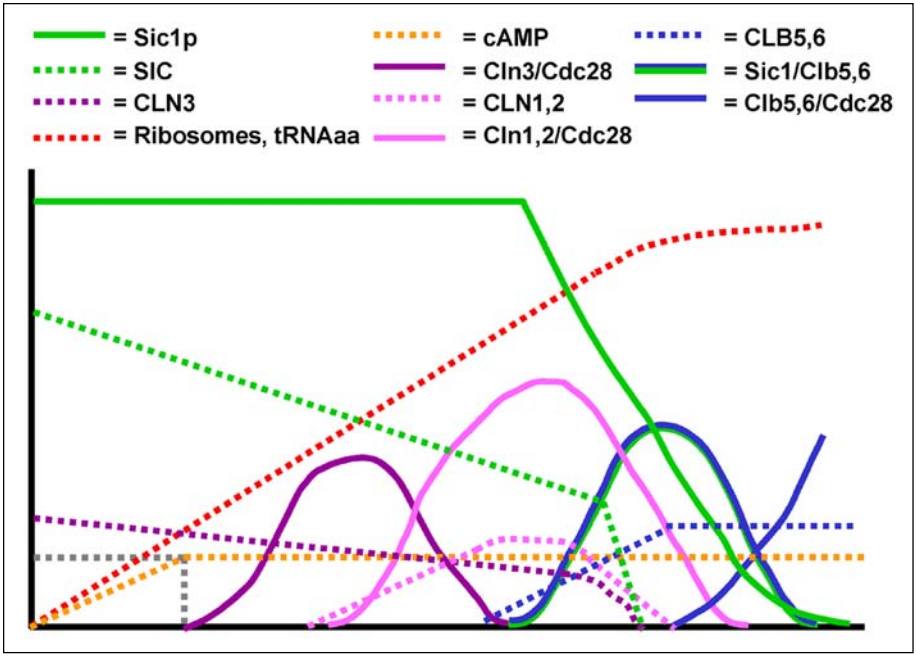

Figure 7. A schematic representation of the subphases of the G1 phase (for details see text)

In the third subphase, Cln1,2/CDK is formed and a large number of genes involved in bud formation and DNA duplication and segregation are transcribed, amongst these are *CLB5,6* (Table 2). Although the capacity of basic energy generating and biosynthetic processes still increases, these increases are assumed to be proportional with the increase in cell size. It is also assumed that in addition to its function to drive transcription, Cln1,2/CDK performs two other crucial functions:direct or indirect phosphorylation of Sic1p and inactivation of the Cln1,2/CDK complex. Also this phase can be described by a limited set of linear differential equations.

In the fourth subphase the phosphorylation of Sic1p outperforms the action of phosphatases and this results in at least a 6-fold phosphorylation of Sic1p. This multiphosphorylation creates a molecular switch, which can be described according the equations used by Ferrell.[58] In the six or even higher phosphorylation state, Sic1p is ubiquitinated and subsequently degraded via the proteosome. In the absence of Sic1p, active Clb5,6/CDK is formed and the passage through START is a fact.

A schematic representation of the results of the model is given in Figure 7, whereas on http://www.bio.uu.nl/mcb/CellCycle.htm , the model is presented in more detail. At present the parameters of the model are determined and subsequently in an iterative process of experiments and calculations the model will be improved.

Conclusions

In this chapter many aspects of qualitative interrelations between nutrient concentrations and fluxes on one hand and the cell cycle of *S. cerevisiae* on the other have been given. It is also possible to provide a nutrient (time) dependent view of the most important events of the cell cycle. These events can be verified and quantified by experiments using DNA- and protein

arrays, special antibodies to determine phosphorylations and metabolomics. The main events in G1-phase before START are:

Synthesis of building blocks. Starting with daughter cells obtained from the elutriator, the first processes in these cells include the synthesis of amino acids and subsequently nucleotides to ensure that protein synthesis will become efficient because of levels of loaded tRNA's and ribosomes above threshold values. Most likely also sufficient cAMP is synthesized to ensure efficient translation of *CLN3* into Cln3p.

In fact the Cln3p level reflects a check of nutrient status as the level is the result of synthesis and degradation of Cln3p. Only if sufficient Cln3p is present formation of active Cln3/CDK will occur. This drives activation of SBF and subsequent transcription of *CLN1,2* and other genes under control of SBF and translation of these mRNA's. Thereafter replacement of Cln3p by Cln1,2p on Cdc28 takes place. Active Cln1,2/CDK results in the transcription of a number of late G1- and S-phase messengers a.o. *CLB5,6*. More or less simultaneously incorperation of Cdc6p into the Origen of Replication Complex and multifold phosphorylation of Sic1p occurs. A six- or more fold phosphorylated Sic1p is needed before degradation of Sic1p by the proteosome system starts. Auto-phosphorylation results in degradation of Cln1,2p and they are replaced in the Cdc28 complex by Clb5,6p. These events take place in a small time window, known as START.

After quantification of these processes with a rather simple mathematical model, the G1 phase can be modelled as function of the nutrient supply to the cell [56,73,74] (see http://www.bio.uu.nl/mcb/CellCycle.htm). When that has been achieved the role of yeast in cancer research[75] will even become bigger.

Acknowledgements

I would like to thank Herman Silljé, Hans Paalman and René Verwaal for their valuable contributions and discussions, Natal van Riel for development of the mathematical description of G1 phase progression, and John Chapman for correcting the manuscript.

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CHAPTER 9

The Role of Reactive Oxygen Species in G1 Phase Progression

Cristina Martínez Muñoz and Jan Andries Post

Abstract

Reactive oxygen species (ROS) are produced during pathological conditions by phagocytes. The function of these produced ROS is to kill microbes and neoplastic cells. At first sight it seems unlikely that ROS would play a role on cell cycle progression. However, nonphagocytic cells also generate ROS, be it at lower levels, after ligand-activation of a variety of receptors, such as cytokines, peptide growth factors or G-protein coupled receptors. These nonphagocytic cells have a ROS generating system that is cytokine- or growth factor-activated and which is structurally and genetically distinct from, but functionally similar to, the NADPH oxidase system of phagocytes. This system is responsible for the production of superoxide and subsequent hydrogen peroxide. Therefore, a certain ROS concentration is considered physiological. ROS are able to increase cytosolic Ca^{2+}, to activate protein kinases, such as mitogen-activated protein kinases and protein kinase C, to inhibit phosphatases, to stimulate phospholipases A_2 and D, and to regulate transcription factors such as nuclear factor-κB, and activator protein 1. Furthermore, different groups have shown that ROS can stimulate growth and growth responses in a variety of mammalian cell types. Taken together, ROS could serve as second messengers and redox modulation could play an important role in regulation of mitogenic signal transduction cascades, which are essential for G1 phase regulation and subsequent cell cycle progression.

Introduction

Reactive oxygen species (ROS) are commonly thought to be toxic compounds resulting in damage of the building blocks of the cells: DNA, proteins and lipids. In this way, ROS will clearly result in impairment of cell function or even in cell death. However, evidence is accumulating that ROS might also play a role as signaling molecules and as such they might be involved in cell cycle progression. In this chapter, dealing with the effect of ROS on cell cycle progression, we give a short introduction on ROS, discuss plasma-membrane receptor-activated ROS production, the effect of endogenous and exogenous produced ROS on signal transduction cascades, which are of importance in mitogenic signaling and finally discuss experimental data linking ROS and cell cycle progression.

Reactive Oxygen Species

ROS are produced in normal and pathological cell metabolism. ROS are or can lead to free radicals, which are molecules with one or more unpaired electrons able to react with

G1 Phase Progression, edited by Johannes Boonstra.

nonradicals resulting in formation of new radicals, and the start of a chain reaction.[1] The sources of ROS in biological systems are either exogenous or endogenous. Exogenous sources are pollutants, tobacco smoke, which leads to a potent mixture of primarily nitrogen oxides ($NO^{\bullet}$) and the hydroxyl radical ($OH^{\bullet}$),[2,3] iron salts,[4] diets containing plant food with large amounts of natural phenolic compounds,[5] ultraviolet radiation,[6] and ionizing radiation, which produces the $OH^{\bullet}$.[7] An important endogenous ROS source is the inflammatory response. Phagocytic cells such as neutrophils and macrophages have a unique superoxide ($O_2^{-\bullet}$) producing system resulting in a respiratory burst that is activated in acute or chronic inflammation. The system producing $O_2^{-\bullet}$ is the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex and the ROS produced play a crucial role in the killing of microbes and neoplastic cells.[8,9] Other intracellular sources of ROS are peroxisomes, which are organelles responsible for metabolism of molecules, as fatty acids, and produce hydrogen peroxide (H_2O_2) as a byproduct.[9] Another important source of endogenous production of ROS is through the mitochondrial respiratory chain.

In aerobic life, organisms depend on mitochondrial respiration for the generation of energy (adenosine 5'-triphosphate (ATP)). During this process the oxygen molecule (O_2) is reduced to water (H_2O), however ROS will be generated.[10] A single electron transfer onto O_2 results in the formation of $O_2^{-\bullet}$. Superoxide dismutases (SOD) convert $O_2^{-\bullet}$ into H_2O_2, a less reactive ROS which can subsequently be converted to H_2O and O_2 spontaneously or by catalase[9] or glutathione peroxidase (GPX).[11] However, in the presence of transition metal ions such as iron, copper or nickel, H_2O_2 gives rise to the highly reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton or Haber/Weiss reaction. $OH^{\bullet}$, being a very reactive radical, can induce lipid peroxidation, DNA damage, and protein oxidation and is considered the most important radical in the oxygen free radical-related cell damage.[12]

Cells have developed two main types of antioxidant (AOX) defense mechanisms: AOX enzymes like SOD, catalase and GPX, which are the backbone of the cellular AOX defense system.[13] The reaction of SOD and catalase are already mentioned above. GPX reduces both H_2O_2 and organic hydroperoxides using reduced glutathione (GSH) as the electron donor. Nonenzymatic AOXs are a variety of lipophilic (such as bilirubin, carotenoids) and hydrophilic (such as GSH, ascorbic acid) molecules or protein components (sulphydryl groups on cysteine) that act as free radical scavengers.[13] Oxidative stress implies an excess of ROS, arising from the overproduction of ROS or from the deficiency of AOX defense that results in reversible or irreversible damage of DNA, RNA, protein and lipids.[14] A third line of defense is formed by repair systems, which recognize and repair or remove damaged biomolecules.

ROS are also generated as a result of ligand-receptor interactions as described below more accurately. In this chapter we will focus on this induced and thus controlled endogenous production of ROS, which is of great importance for the regulation of cell growth.

Redox-Dependent Signal Transduction

ROS are generally considered cytotoxic, because of the oxidative damage they may cause to cellular components. However, at low concentrations, ROS may function also as physiological mediators of cellular responses. For example, high levels of $NO^{\bullet}$ are produced by macrophages and are involved in the immunological response, killing microbes and neoplastic cells.[15-17] In lower amounts, $NO^{\bullet}$ has a clear function in signal transduction in neuronal and vascular tissue.[18] This concentration dependence between the role of ROS in immune response and signal transduction is likely to be applicable to other ROS. $O_2^{-\bullet}$ and H_2O_2 are produced in large amounts by cells of the immune system. Other cell types, including vascular smooth muscle cells, chondrocytes and fibroblasts appear to produce lower amounts of these molecules,[18] again implying a different physiological function of the ROS produced.

In the last several years it has become increasingly evident that ligand-stimulated ROS generation plays a role in the complex world of signal transduction.[18-20] This phenomenon has been observed in a wide variety of cell types and is stimulated by a diverse collection of receptor-ligand complexes, including cytokines, peptide growth factors acting though tyrosine kinases and agonists of receptors with seven transmembrane domains.[21]

Cytokines

The cytokines interleukin-1α (IL-1) and tumor necrosis factor-α (TNF-α) are secretory products of activated monocytes and macrophages during inflammatory responses.[22] IL-1 exhibits a variety of stimulatory activities on maturation, differentiation and growth of many cell types involved in development and inflammation, such as fibroblasts, synovial cells, endothelial and epithelial cells, bone marrow cells, and T- and B-lymphocytes.[23-26] TNF-α was first described for its anti-tumor activity,[27] but it is also important in nontumor cells, since it increases the interferon-γ-mediated expression of class II major histocompatibility antigens necessary to induce an immune response.[28,29] H_2O_2 is also produced following interferon-γ stimulation.[30,31] Also after cytokine stimulation, a variety of noninflammatory cells produce and release ROS. Using ROS sensitive dyes, it is shown that human skin fibroblasts release ROS, mainly $O_2^{-\bullet}$, in response to IL-1 and TNF-α.[32] Interferon and interleukine stimulate the release of $O_2^{-\bullet}$ in endothelial cells[33] and transforming growth factor β1 is required to activate a plasma membrane H_2O_2 -generating NADH-oxidase in human lung fibroblasts.[34]

Peptide Growth Factors

In A431 human epidermoid carcinoma cells, a transient increase in the intracellular concentration of ROS was measured after epidermal growth factor (EGF) treatment. One of the ROS formed by this process appeared to be H_2O_2, because the EGF induced ROS production was abolished by incorporation of catalase into cells by electroporation. Furthermore, this resulted in an inhibition of the EGF induced tyrosine phosphorylation of various cellular proteins including the EGF receptor (EGFR) and phospholipase C-γ1,[35] indicating that the ROS formed upon activation of the EGFR play an important role in downstream processes. In rat vascular smooth muscle cells, an increase in the intracellular concentration of H_2O_2 was also observed after stimulation with platelet-derived growth factor (PDGF). This increase could be blunted by increasing the intracellular concentration of the scavenging enzyme catalase or by the chemical AOX N-acetylcysteine, a precursor of GSH. Also in this cell type, the response to PDGF, which includes tyrosine phosphorylation, mitogen-activated protein kinase (MAPK) activation, DNA synthesis and chemotaxis was inhibited when the growth factor–stimulated rise in H_2O_2 concentration was blocked.[36] Other peptide growth factors, such as the basic fibroblast growth factor,[37] insulin[38] and granulocyte-macrophage colony-stimulating factor,[39] also trigger the rapid production of ROS.

G-Protein Coupled Receptors

Also activation of certain G-protein coupled receptors lead to the production of ROS. In vascular smooth muscle cells, treatment with angiotensin II (Ang II) increase the intracellular production of $O_2^{-\bullet}$, resulting from the activation of both the NADPH and NADH oxidases.[40] In these cells Ang II causes a rapid, sustained activation of phospholipase D-mediated phosphatidylcholine hydrolysis, resulting in the formation of phosphatidic acid (PA) and indirectly diacylglycerol (DAG).[41,42] PA has also been shown to stimulate NADPH oxidase activity in neutrophils.[43-45] At the moment there is much debate whether PA is the molecule that activates the NADPH oxidase or whether subsequent conversion of PA to DAG is required for the stimulation of the oxidase.[40,45-47] Furthermore, thrombin,[48] thyrotropin,[31,49] parathyroid hormone,[31]

lysophosphatidic acid,[50] sphingosine 1-phosphate,[51] mechanical shear stress[52] and phorbol esters[53] induce H_2O_2 in various cell types.

Taken together, the fact that ROS are generated upon ligand-receptor interactions suggests that ROS serve as second messengers for receptor-mediated cell signaling. One of the questions to be addressed concerns the mechanism by which ROS is produced upon these ligand-receptor interactions

ROS Generation by NADPH Oxidase

The pathway by which ROS are generated in a controlled fashion is poorly characterized except in certain specialized cell types. The receptor-mediated generation of H_2O_2 has been studied extensively in phagocytic cells. Activation of phagocytic cells by a host of different agonists leads to the assembly of a multicomponent NADPH-oxidase complex.[54] The NADPH-oxidase complex generates large quantities of $O_2^{-\bullet}$, utilizing electrons derived from NADPH to reduce molecular oxygen to $O_2^{-\bullet}$.[55,56] The $O_2^{-\bullet}$ generated is then dismutated spontaneously or enzymatically to H_2O_2 and can lead to the formation of $OH^{\bullet}$ and hypochlorous acid, all of which are effective killing agents.[54]

The NADPH oxidase complex consists of two cytosolic components ($p47^{phox}$ and $p67^{phox}$), two transmembrane flavocytochrome b components ($gp91^{phox}$ and $p22^{phox}$)[54] and the GTP-binding protein Rac. The Rac1 GTPase belongs to the Rho family of small GTP binding proteins, and its role in the production of ROS in phagocytic cells such as neutrophils is well established.[57] In human neutrophils, the activity of the NADPH oxidase system is regulated by the small GTP-binding protein Rac2,[58] whereas in guinea pig macrophages, the NADPH oxidase appears to be regulated by Rac1.[59] Activation of this system is absolutely dependent on GTP.[60-62]

Biochemical evidence suggests the existence of a cytokine- or growth factor-activated ROS generating system in nonphagocytic cells, that is structurally and genetically distinct from, but functionally similar to, the NADPH oxidase system of phagocytes.[63] Several components of the phagocytic NADPH oxidase appear to be present in other nonphagocytic cells,[64] such as a homolog of $gp91^{phox}$.[65] Overproduction of Rac1 in fibroblasts was associated with increased production of H_2O_2,[66,67] indicating that Rac proteins, in particular Rac1, serve a similar function in nonphagocytic cells as in phagocytic cells.[68] It has been demonstrated that the increase in ROS after EGF, PDGF, TNF-α or IL-1 stimulation is Rac1 dependent, since expression of a dominant negative allele of Rac1 inhibits the rise in ROS observed after Ras expression or after stimulation by the above mentioned growth factors or cytokines.[66]

ROS and Signal Transduction

Some reports suggested that low levels of ROS modulate signal transduction pathways in mammalian cells,[69] which is in line with the observations that blocking or reducing ROS levels upon receptor activation blunted the activation of the subsequent signaling cascade. As will be discussed below, ROS are involved in the regulation of intracellular Ca^{2+} concentration, protein phosphorylation, and transcription factors.

Stimulation of Ca^{2+}-Signaling

ROS affect Ca^{2+}-signaling in both endothelium and smooth muscle cells.[70] Exposure to the $O_2^{-\bullet}$ generating system, xanthine oxidase/hypoxanthine (XO/HX) and exogenously supplied H_2O_2 affect Ca^{2+}-signaling. The cytosolic Ca^{2+} concentration is increased by inhibition of the activity of an ATP-dependent Ca^{2+}-pump of the sarcoplasmic reticulum of smooth muscle cells.[71-73] H_2O_2 also affects Ca^{2+}-transport mechanisms that are associated with the plasma membrane.[71] In endothelial cells, $O_2^{-\bullet}$ derived from exposure to the XO/HX reaction,

stimulated the entry of extracellular Ca^{2+}.[74] In contrast, to the enhancing effects of H_2O_2 in smooth muscle cells, in endothelial cells H_2O_2 only induces intracellular Ca^{2+} oscillations at low concentrations.[75,76] At high concentrations it attenuates the Ca^{2+}-signaling, both by inhibiting intracellular Ca^{2+} release and by impairing Ca^{2+} influx.[75-77] This suggests a physiological role of ROS in the regulation of Ca^{2+}- signaling. However, a clear link between physiological generated ROS and Ca^{2+} homeostasis remains to be established. Stimulation of Ca^{2+}-signaling by ROS may lead to the induction of signal transduction cascades in other cell types, resulting in activation of different proteins, such as protein kinase C (PKC) and transcription factors.

Protein Phosphorylation

Protein phosphorylation clearly plays a role in several signal transduction pathways important in cell cycle regulation. The phosphorylation state of proteins is determined by the activity of protein kinases and protein phosphatases. Administration of ROS was found to stimulate phosphorylation of proteins involved in signal transduction pathways, amonst others PKC,[78,79] protein kinase B via an EGFR/Phosphatidylinositol 3 (PI 3)-kinase pathway,[80] phospholipase A_2,[81,82], phospholipase D,[83,84] Src kinases,[85,86] EGFR,[87,88] PDGF receptor,[89] and MAPK.[90-92]

In particular the effects of H_2O_2 on the EGF-induced signal transduction pathway have been studied in detail.[87] It has been demonstrated that administration of H_2O_2 increases the phosphorylation of the EGFR,[93] and H_2O_2-induced tyrosine phosphorylated EGFR forms a complex with SHC-Grb2-SOS followed by the activation of Ras and MAPKs.[87] ROS also induces the phosphorylation and activation of MAPKs independently of the activation of the EGFR.[36,50] We have reported that activation of p42/p44-^{MAPK} by $ONOO^-$ depends on the activation of a calcium dependent PKC, but is MAPK kinase (MEK)-independent [94,95] Activation of p42/p44-^{MAPK} by H_2O_2, on the other hand, has been shown to be mediated by the activation of the PKC, Raf-1 and MEK,[78] indicating that different ROS and reactive nitrogen species might have different targets. Moreover, we have demonstrated that H_2O_2 causes an inhibition of the EGFR internalization in fibroblasts,[96] which could lead to a constitutive downstream signaling towards cell cycle progression. There are some indications that the mechanism involved in the increased phosphorylation of the above mentioned proteins is due to inhibition of phosphatases.[97]

Tyrosine phosphatases are especially sensitive to redox regulation and are easily inactivated by oxidation of a critical cysteine residue located in the catalytic site.[98,99] They therefore represent a target for both exogenous and endogenous derived ROS. H_2O_2 inactivates the low molecular weight phosphotyrosine-protein phosphatases by the oxidation of two cysteines localized into the active site region forming a disulfide bond.[100] Since these phosphatases down-regulate the function of PDGF and insulin receptor,[101,102] the inactivation could result in a prolonged activation of these receptors after ligand stimulation. Tyrosine phosphatase protein 1B is also rapidly and transiently inactivated by ROS after EGFR triggering,[103] and under physiological conditions regulation of phosphatase activities by the redox status was observed.[104]

Transcription Factors

In response to ROS, changes in the expression patterns of a number of "early response" genes, such as *c-myc, c-fos, c-jun* and *egr-1* have been reported.[105,106] These genes encode for transcription factors involved in the regulation of cell cycle progression. Oxidative stress has been shown to increase the activity of transcription factors such as activator protein 1 (AP-1), nuclear factor-κB (NF-κB), Gadd153/CHOP, and STAT3.[37,107-117] This increase in activity is due to an increased phosphorylation induced by oxidant-activated upstream kinases[109] or by

modulation of the redox status of the critical cysteine residues in transcription factors that regulate their DNA-binding activity.[110-115] The following is an example of a pathway regulated by endogenous generation of ROS: activation of Rac-1, by interference of the integrin-mediated cell adhesion, generated ROS, which was essential for NF-κB transcriptional regulation of IL-1. The latter induced collagenase-1 gene expression, resulting in a change in cell morphology.[118]

ROS and Cell Cycle Progression

Cell Proliferation

Production of ROS upon activation of cells by polypeptide growth factors, such as PDGF and EGF, and the effect of exogenous added ROS on signal transduction cascades normally involved in cell cycle control, suggest that endogenous produced ROS play a role in cell proliferation.

$O_2^{-\bullet}$ generation has been frequently implicated in the control of normal cell growth and the promotion of malignant transformation.[68,119-121] Relatively high concentrations of ROS will lead to apoptosis or necrosis, however low concentrations of $O_2^{-\bullet}$ and H_2O_2 can stimulate proliferation in a variety of mammalian cell types (for a review see ref. 122). Low concentrations of $O_2^{-\bullet}$, generated from exogenous applied HX/XO, resulted in increased fibroblast proliferation.[123] Furthermore, removal of endogenous produced ROS by SOD or catalase resulted in inhibition of fibroblast proliferation.[123] $O_2^{-\bullet}$ also stimulated growth responses in hamster fibroblasts (BHK-21),[124] human fibroblasts, Balb/3T3,[125,126] human amnion cells,[127] and human histocytic leukemia cells (U937).[125] Growth responses are also elicited by hydrogen peroxide in BHK-21,[124] and rat fibroblasts (208F),[128] as well as in mouse osteoblastic cells[129] and Balb/3T3 cells, where H_2O_2 enhanced the insulin induced DNA synthesis.[130] An inhibitory effect on the proliferation of rabbit kidney epithelial cells, human melanoma cells, rat fibroblast and BHK-21 cells was observed after removal of the endogenous produced ROS by the addition of exogenous SOD or catalase to the medium.[124] Such observations have led to the suggestion that $O_2^{-\bullet}$ and H_2O_2 might function as mitogenic stimuli, which could be involved in growth promoting processes.

Moreover, not only SOD and catalase have been shown to have an inhibitory effect on cell cycle progression, also other AOXs have a negative effect, confirming that cell cycle regulation is regulated by ROS. Epigallocatechin-3-gallate (EGCG), an AOX present in tea, was shown to induce G1 cell cycle arrest in human breast carcinoma (MCF-7) cells, due to an inhibition of cyclin dependent kinases, CDK-2 and CDK-4 activities as well as induction of $p21^{waf1/cip1}$ and p27, resulting in a hypophosphorylated retinoblastoma tumour suppressor protein (pRB).[131] EGCG also inhibited the proliferation of EGF-stimulated breast epithelial cells (MCF10A).[132] EGCG induced $p21^{waf1/cip1}$, resulting in an increased association of $p21^{waf1/cip1}$ with cyclin D, leading to an inhibition of the cyclin D1-CDK activity and subsequently in a reduced phosphorylation of the pRB.[132]

Rac-induced $O_2^{-\bullet}$ production plays a critical role in mitogenic signaling. An effector site of Rac1 was identified that is necessary for mitogenic signaling and implicated $O_2^{-\bullet}$ generation as a candidate effector pathway of Rac1-dependent cell growth.[67] Rac1 regulates cell growth, migration and cellular transformation by controlling the intracellular production of ROS.[68,133] Furthermore, in Swiss 3T3 cells, a mutant form of activated Rac stimulated the transition from G1 to the S-phase of the cell cycle.[134]

When ROS are involved in cell proliferation an inhibition of cell growth might be mediated by reduction of ROS levels. As described above AOXs such as EGCG, induce cell cycle arrest. A comparable correlation has been observed during cell density induced growth arrest. This has been shown to coincide with a decrease in the steady-state levels of intracellular ROS

and a consequent increase in protein tyrosine phosphatase activity leading to an impairment of mitogenic signaling. Addition of a low concentration of H_2O_2 significantly increased thymidine incorporation in confluent cultures, suggesting a causal link between redox changes and growth control by cell density.[135]

Taken together, these investigations suggest that ROS, namely $O_2^{-\bullet}$ and H_2O_2, play an important role in mitogenic signaling leading to regulation of cell cycle progression. However, this could be dependent on the cell type as well as on the concentration of ROS added, as we will discuss next.

ROS and Cell Cycle Regulators

There is not much known on the effect of ROS on the cell cycle regulators. As already described above, after H_2O_2 treatment, proteins involved in the signal transduction pathways become activated. Examples are PKC,[78,79] EGFR,[87,88] PDGFR,[89] and MAPKs.[90-92]

Among others, activation of MAPK is essential for cyclin D1 expression and provides a link between mitogenic signaling and cell cycle progression (see Chapter 6). We found a sustained expression of cyclin D1 and D2 when Her 14 fibroblasts were incubated with 3 mM or higher H_2O_2 concentrations. However, this effect was not due to the MAPK pathway, since MEK inhibitors did not influence cyclin D expression. Furthermore, cyclin D1 and D2 levels remained constant even after addition of a protein synthesis inhibitor, indicating that the effect of H_2O_2 was not due to an induction of protein synthesis. Our results indicate that H_2O_2 reversibly inhibits the ubiquitin-proteasome dependent degradation of cyclin D1 and D2, probably by transiently inhibiting the ubiquitination and/or the proteasome.[136] These observations suggest that H_2O_2 may inhibit the degradation of other cyclins responsible for the regulation of cell cycle progression. For example, since cyclin B degradation is critical for cell division, the inhibition of the degradation of this cyclin by H_2O_2 would inhibit mitosis.

ROS and Cell Cycle Checkpoints

Under normal circumstances the cell cycle proceeds without interruptions. However, in response to agents that cause damage, most normal cells have the capacity to arrest cell cycle progression and resume progression after the damage is repaired. These surveillance control mechanisms that check the proper completion of earlier events are referred to as checkpoints. Contrary to the above mentioned studies, others have shown cell cycle arrest after ROS induction instead of a stimulation of the cell proliferation. The following are effects of ROS on the G1 phase: Ataxia-telangiectasia (AT)-deficient cells appear to lack many critical cell cycle checkpoint responses after oxidative stress induced by t-butyl hydroperoxide treatment, in comparison with normal human fibroblasts (NHFs). Unlike NHFs, AT-deficient fibroblasts failed to induce p53 or to show G1 and G2 phase checkpoint functions, as measured by inhibition of p33-CDK2/cyclin E-associated in vitro kinase activity, and p34-CDC2/cyclin B histone H1 in vitro kinase activity in response to t-butyl hydroperoxide treatment. Treatment of NHFs with t-butyl hydroperoxide stimulated pAT-associated kinase activity. These results indicate that pAT is involved in responding to certain aspects of oxidative damage and in signaling this information to downstream effectors of the cell cycle checkpoint functions.[137] The RAD9 gene product has also been found to be necessary for checkpoint arrest in response to peroxide exposure.[138] In human fibroblasts, an increase in endogenous ROS, caused by reduction of GSH by diethylmaleate (DEM), induced the expression $p21^{waf1/cip1}$, independently of p53, and subsequent G1 arrest. Furthermore, in these cells N-acetylcysteine prevented the gene induction by DEM.[139] In T47D-H3 cells, which contain mutated p53, an S-phase arrest was observed after hyperoxia (95% O_2, 40-64 h), associated with acute inhibition of CDK2 activity and DNA synthesis.[140] In plant cells, low concentrations of menadion, a quinone which

results in the formation of $O_2^{-\bullet}$, resulted into a cell cycle block at the G1/S transition, reduction in DNA replication and delayed entry into mitosis. This cell cycle arrest coincided with the inhibition of CDK activities.[141] In human diploid fibroblasts (HDFs) a senescent-like growth arrest is observed after addition of sub-lethal concentrations of H_2O_2. A transient elevation of p53, a sustained elevation of p21 and a sustained pRB hypophosphorylation was observed.[142] Higher concentrations of H_2O_2 caused detachment of a fraction of HDFs, 16-32 h after treatment, showing an activation of caspase-3 and typical morphological changes associated with apoptosis. Interestingly, pretreatment of these cells with low-doses of H_2O_2 induced G1 arrest and prohibited induction of apoptosis by a subsequent H_2O_2 challenge.[143]

The above findings indicate that the concentration of ROS and the cell type is of importance for the effect of ROS. Since those studies showed a G1 arrest, there is a redox imbalance leading towards oxidative stress, which could lead to DNA damage and therefore to G1 arrest.

ROS and Apoptosis

ROS is required in many instances for the execution of the apoptotic program,[11] such as in response to γ-irradiation[144] and TNF.[29] Furthermore, ROS, such as H_2O_2[145-147] and $O_2^{-\bullet}$,[148] can trigger apoptosis in various systems. For example, H_2O_2 was able to induce apoptosis in cells of oligodendroglia origin.[147] Apoptosis was also induced in a human fibroblast cell line W138 after treatment with 1mM HX and 0.05 U/ml XO, this induction was hindered by catalase. Cell cycle analysis revealed an induction of cells in the S/G2 phase 24 and 48 h after stimulation, suggesting that ROS induced a G1 arrest in proliferating fibroblasts. This was supported by accumulation of p53 and $p21^{waf1/cip1}$. Reactive nitrogen species are also able to induce apoptosis. For instance, Tempol, a nitroxide free radical, induced apoptosis in a human leukemia cell line (HL60). Furthermore, direct injection of neurons with SOD delayed cell death after nerve growth factor deprivation[149] and growth factor withdrawal-induced apoptosis in a lymphocyte cell line did not occur when GPX was overexpressed in those cells.[150] On the other hand, several reports suggest that necrosis rather than apoptosis is mainly induced after ROS treatment.[151,152]

Models of Cell Cycle Regulation by H_2O_2

Combining experimental data obtained from both endogenous and exogenous ROS, the following model on the role of ROS on growth factor induced cell cycle progression is proposed.

As shown in Fig. 1, after ligand stimulation the tyrosine kinase receptor will be autophosphorylated, leading to the activation of Rac, a regulator of the NADPH-oxidase complex, resulting in generation of large quantities of $O_2^{-\bullet}$. The $O_2^{-\bullet}$ generated is then rapidly dismutated spontaneously or enzymatically by SOD to H_2O_2. H_2O_2 rapidly and transiently inactivates phosphatases by oxidation of cysteines in their active center. Therefore, the phosphorylation of the receptor will be prolonged, leading to activation of the mitogenic signaling cascade, where kinases are able to phosphorylate other kinases or proteins involved in this signaling. Since the phosphorylation state of proteins is determined by the activity of protein kinases and protein phosphatases and the latter are transiently inhibited, the cascade towards the mitogenic signaling will be favored. Subsequently, the degradation of H_2O_2 by catalase or GPX will reduce the inhibition of the phosphatases, resulting in repression of the mitogenic signaling. Thus, after ligand binding the balance between ROS and AOXs and the subcellular localization of such events will be responsible for the regulation of signal transduction cascades involved in among others regulation of the G1 phase. Moreover, when this balance is disturbed due to an excess of ROS or a lack of AOXs, resulting in oxidative stress, other effects such as protein damage, lipid peroxidation or DNA damage will occur, leading to an arrest of the cell

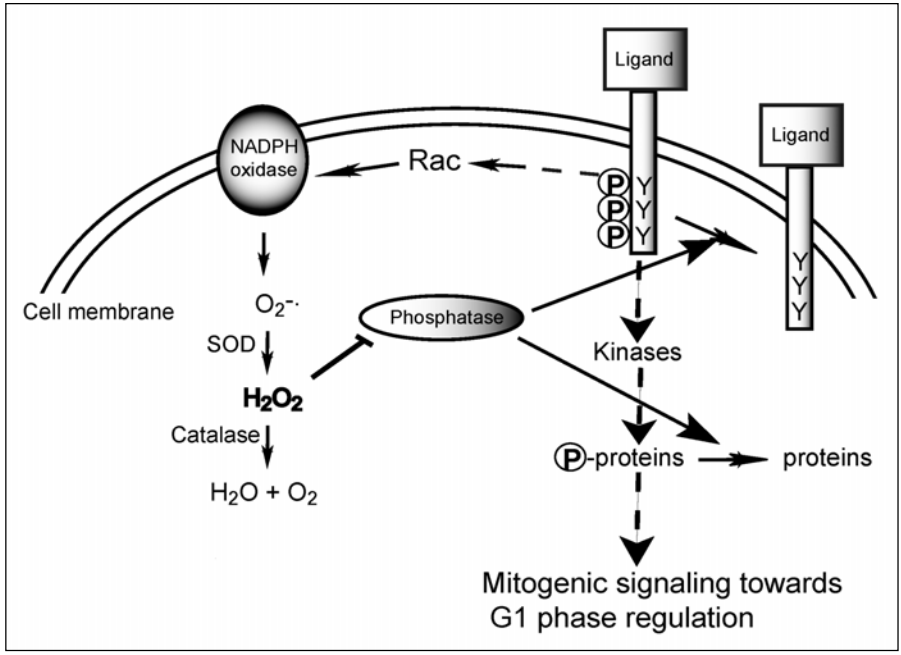

Figure 1. Proposed model for the role of ROS in growth factor-induced cell cycle progression. Upon activation of the receptor a rise in ROS occurs, which subsequently transiently inhibits phosphatases, resulting in favoring the mitogenic signaling (for details see text).

cycle, allowing the cells to repair this damage. However, if the damage is irreversible, the cell will undergo apoptosis or necrosis.

Acknowledgments

C. Martínez Muñoz, one of the authors, is supported by Unilever, Vlaardingen, the Netherlands, the Technology foundation STW, the Applied Science division of NWO, and the technology program of the ministry of Economic Affairs, the Netherlands (Grand No. UBI 4443).

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CHAPTER 10

Regulation of G1 Phase of Yeast Cells by Stress

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Abstract

Different environmental stresses, among them heat, hyperosmotic and oxidative ones, cause yeast cells to arrest at G1. The duration of the arrest is proportional to the intensity of the stress, and is concomitant to downregulation of *CLN1*/*CLN2* expression. In certain cases it has been shown that the stress signal operates by interfering with Cln3 activity. Cln3 is the upstream activator of other G1 and S cyclins in the normal cell cycle and, therefore, inactivation of Cln3/Cdc28 kinase complexes leads to immediate cell cycle arrest at G1. Transcriptional regulators respond to some or all of the above stresses through a number of signal transduction pathways, although these have not been demonstrated to operate directly on the G1 cell cycle machinery. Only the Skn7 transcriptional activator, which participates in the oxidative stress response, has been functionally related to the cell cycle machinery acting at G1. The stress-induced G1 arrest does not result from the activation of checkpoint mechanisms that detect several kinds of cellular damage and arrest yeast cells at other cell cycle stages different from G1. However, Rad53 (a component of several checkpoint pathways) could be important for the G1 arrest induced by DNA-damaging agents.

Introduction

The yeast *Saccharomyces cerevisiae* is a unicellular eukaryotic microorganism that lives naturally in a changing environment. In order to survive and proliferate in every changing situation, *S. cerevisiae* is capable of modifying its chemical and physical cellular state in response to variations in environmental parameters such as temperature, osmolarity, redox state or nutrient availability. These responses to external stresses[1] may also be important for defending against constitutive (or metabolic) stresses caused by the proliferation of the yeast cell itself, i.e., through the production of reactive oxygen species that can cause oxidative damage to different cellular structures.[2] The mechanisms underlying the different stress responses in budding yeast can be reproduced in laboratory conditions and have been subject of a growing number of studies[1] that have demonstrated that some of these mechanisms are shared by higher eukaryotic cells. Since genetic and biochemical studies in the latter are more complicated, yeasts (particularly *S. cerevisiae*) represent a perfect model for studying the mechanisms by which eukaryotic cells transiently or permanently respond to a number of different stresses.

Recent analyses of the whole transcriptome in *S. cerevisiae* have shown the existence of a common set of genes that exhibit similar responses (by inducing or repressing expression) to a series of different stresses.[3-7] The term "common environmental response" has been coined to describe this expression pattern,[6] which indicates that different environmental changes (or metabolic aggressions) may cause similar alterations in the cell and therefore induce common

G1 Phase Progression, edited by Johannes Boonstra.

response pathways. Furthermore, specific subsets of genes may also be important for particular stress responses.[3-7] The general stress response transcription factor Msn2/4[8-9] is essential for regulating the expression of both common and specific stress genes.[5,6] Although changes in gene expression are probably the single most important feature in stress responses, such responses may also involve post-transcriptional effects (for instance on protein compartmentalization or stability).[10,11]

A number of signal transduction pathways are important for yeast responses to external stresses. In *S. cerevisiae*, four pathways mediated by mitogen-activated protein kinases (MAPK) are activated by external stresses.[12,13] The alpha factor pathway responds to sexual pheromones and is mediated by the Fus3 MAPK. The hyperosmolarity-response pathway is driven by the Hog1 MAPK. The cell integrity pathway is activated by alterations at the cell surface (due to heat shock or hyposmolarity stress among other causes) and is mediated by protein kinase C (Pkc1) and the Mpk1/Slt2 MAPK. Nutrient starvation activates the Kss1 MAPK pathway, which induces filamentous growth. Some of these MAPK pathways share common elements, and so there exists a cross talk among them that must be finely regulated to assure the correct response (such as the activation of a specific subset of genes) against each particular stress.[12-16] Other transduction pathways, not mediated by MAPKs, may also sense some of the above mentioned external stresses in yeast. Thus, the Ras-mediated pathway responds to changes in the external carbon source[17] and modulates Msn2/4 activity by regulating its nuclear compartimentalization.[18] The Tor1/2 kinase-mediated pathway responds to nutrient deprivation by modifying protein biosynthesis rate, amino acid transport and cytoskeleton organization.[19] Although the intermediate components of these pathways are mostly known, knowledge of the target genes and/or specific functions modulated by each pathway remains limited in most cases.

One of the most immediate effects of environmental stresses upon yeast is the alteration of cell proliferation. Inhibition of cell proliferation may be temporary or permanent depending on the intensity of the stress. Temporary growth arrest is a common result of various mild to medium intensity stresses, and is followed by a resumption of cell growth at division rates that may even be similar to those of unstressed cells. In the subsequent sections we will describe a number of studies that demonstrate that stress-induced growth arrest occurs mostly at the G1 stage of the cell cycle, and is an active process that can be modulated by altering the cell cycle machinery operating at G1 (described in detail in Chapter 2). It should also be emphasized at this point that, when mechanistically analyzing stress effects at G1, a distinction has to be made between those functions required for cell cycle arrest and those needed for growth resumption. Consequently, the particular conditions and intensities of the stresses applied in each case must be taken into consideration when comparing experiments from different authors and before coming to any conclusions; different short term responses (growth arrest or resumption) may occur in different ways depending on the experimental conditions. We will focus our attention on heat shock and osmotic and oxidative stresses. Nutritional stress is considered in a separate chapter.

The stress-induced growth lag could be envisaged as a period for adaptation to new environmental conditions, during which the cell finely adapts its metabolism to the new situation. *S. cerevisiae* is able to adapt to damage-induced alterations of the cell cycle events (arrest of DNA replication origins, chemical or radiation damage to DNA, mitotic spindle alteration, inhibition of bud formation) by inducing checkpoint mechanisms (described in Chapter 2) that transiently arrest the cell cycle at different stages. The possible relationship between checkpoint genes and stress-induced G1 arrest will be discussed here. We will also consider the hypothetical mechanisms (which may be general or particular according to each type of stress) that may transduce external signals to the cell cycle machinery operating at G1.

Effect of Heat Shock on the G1 Phase of the Cell Cycle

Heat Shock and the Cell Cycle Machinery

Increasing the temperature of exponentially growing *S. cerevisiae* cultures from 25 to 37-39°C elicits a heat shock response characterized by acquisition of thermotolerance through the protective molecule trehalose, and up-regulation of heat shock proteins.[20,21] Some of the latter act as chaperones that help in refolding proteins damaged by heat shock. Heat shock also implies reprogramming the expression of a large set of genes.[5,6] Apart from the genes required for heat shock protein synthesis, other genes involved in respiration and alternative carbon source utilization are also up-regulated upon heat shock, including the genes responsible for the protein degradation machinery. On the contrary, heat shock (like other forms of stresses) causes transient down-regulation of genes for translation and protein synthesis.[5,6]

Moderate heat shock also leads to transient growth arrest and the accumulation of un-budded cells at the G1 phase of the cell cycle.[22,23] Pioneering studies by Rowley et al[24] demonstrated that this resulted from a specific effect of heat stress on the cell cycle machinery. In fact, heat shock transiently down-regulated the expression of G1 cyclin genes *CLN1* and *CLN2* but not that of *CLN3*. This down-regulation was parallel to the transient accumulation of un-budded cells. Growth arrest was suppressed by ectopic over-expression of *CLN2* and by a hyperstable *CLN2-1* allele, which was opposed to other heat shock physiological effects. Transient growth arrest also occurred in a *Δcln1 Δcln2* mutant where Cln3 was the only functional G1 cyclin, and was suppressed by the hyperstable Cln3-1 cyclin. Given the hierarchically primary role of Cln3 function as the inductor of *CLN1* and *CLN2* expression through activation of the SBF (Swi4/Swi6) factor (see chapter 2), the above results could be interpreted at that time as heat shock transiently inhibiting Cln3 activity. The effect on other G1 transcript levels would be secondary to this one. Our group has shown that *CLB5* mRNA levels [which depend on the activation of the MBF (Mbp1/Swi6) factor by Cln3] are also transiently down-regulated when a heat shock is applied on asynchronously growing yeast cultures.[25]

The essential role of Cln3 in the G1 response to heat shock has been confirmed in more recent studies by Li and Cai,[26] who worked with alpha factor-synchronized populations of yeast cells at G1 that were subjected to moderate heat shock before being relieved of the pheromone-induced arrest. Under these conditions, cells that had been heat-shocked for a short period before pheromone release, recovered from the START arrest later than nonshocked cells. This confirmed results from asynchronously growing cultures, and this growth delay was parallel to a delay in the expression of *CLN1*, *CLN2* and *CLB5*. We have also observed a delay in recovery from pheromone-induced G1 arrest when heat shock was continuously applied throughout the whole period during which cells were relieved from alpha factor treatment.[25] Taken together, the two studies with synchronous cultures[25,26] demonstrate that heat shock is acting on cell cycle processes occurring no later than START, although effects at other stages of the cell cycle should not be discarded in asynchronous populations. Importantly, a null *cln3* mutant was unable to recover from alpha factor arrest when heat shocked, and over-expression of *CLN3* and *CLB5* efficiently eliminated G1 heat-induced growth inhibition.[26]

Two conclusions can be drawn from these results. First, Cln3 is a central element in recovery from G1 arrest caused by heat shock. Regulation of Cln3 must occur at a post-transcriptional level, since the levels of *CLN3* mRNA are not significantly affected by the stress.[26] Second, at least under stress conditions Clb5 (and Clb6) can act as activators for *CLN1*/*CLN2* expression even in the absence of Cln3, and contribute to stress recovery.[26] This situation contrasts with what occurs in undisturbed growing cells, in which Clb5/Clb6 cyclins are important for the initiation of DNA replication but not for Cln1/Cln2 activity nor cell budding.[27]

Sensing and Transducing the Heat Shock Signal for G1 Growth Arrest

How heat stress is sensed by the yeast cell, and how the signal is subsequently transduced to the cell cycle machinery, is poorly understood, although sphingolipids seem to play a role in this process. Initial studies demonstrated the accumulation of sphingolipids in response to heat shock,[28,29] whereas the addition of external sphingolipid molecules to yeast cells resulted in the activation of a *lacZ* reporter gene controlled by the general stress-sensitive STRE promoter element.[30] More recently, it has been shown that sphingolipid-deficient yeast strains lack transient heat-induced G1 arrest.[31] This study used mutants at different stages of the sphingolipid biosynthetic and degradation pathways, and demonstrated that sphingoid bases are important for G1 arrest. Moreover, addition of exogenous phytosphingosine induced transient G1 (and also G2/M) arrest, which was reversed by a hyperactive Cln3-1 cyclin.[31] These results support an active role of sphingolipids as heat stress sensors upstream of Cln3. Whether this merely reflects the importance of membrane plasticity in heat stress response, or whether sphingolipids carry out an active role as mediators of the signalling pathway, remains unknown. The second possibility would be similar to the situation in mammalian cells, where the role of sphingolipids and ceramides as mediators of stress responses in processes such as apoptosis, senescence, differentiation or immune response has been well established.[32-34]

The work of Raboy et al[35] points to the existence of an intermediate step involving protein degradation in recovery from heat-induced G1 arrest. Mutants in *RAD6/UBC2* (coding for a ubiquitin-conjugating enzyme) arrest upon a mild heat shock applied to exponentially growing cells, but (contrary to wild type cells) they remain permanently arrested. This is accompanied by deficient recovery of *CLN2* mRNA and Cln2 protein levels at high temperature. Defective recovery in *rad6* mutants is suppressed by over-expression of *WSC2,*[35] which is a member of a gene family encoding plasma membrane proteins (Wsc1/2/3/4) thought to signal changes in cell wall integrity to the Pkc1 pathway[36] (see below).

A priori, the cell integrity pathway mediated by Pkc1 and the Mpk1 MAPK (Fig. 1) would be an obvious candidate for transducing the heat shock signal to the cell cycle machinery, since heat shock (along with other stresses) activates this pathway by modifying cell wall integrity.[13] A number of observations connect the Pkc1 pathway with the cell cycle. Thus, the activity of Mpk1 (the MAPK of the pathway, which acts downstream of Pkc1) is modulated in a cell cycle-dependent manner that peaks at the G1/S transition. This has been associated with the need for de novo cell wall construction during bud emergence.[37] Fluctuations in Mpk1 activity throughout the cell cycle could be a consequence of its regulation by cyclin/Cdc28 complexes. In fact, it has been proposed that Cdc28 kinase may play a role as activator (stimulating diacylglycerol production) of Pkc1 at START.[38] This constitutive basal activity of the cell integrity pathway would be required even at low temperatures, independently of the comparatively much higher activity needed upon heat shock.[39]

Another fact that associates the Pkc1 pathway with cell cycle events is the involvement of the pathway [often in a cell cycle (and SBF)-dependent fashion] in the expression of genes coding for proteins involved in cell wall synthesis and structure.[40] Madden et al[41] have also shown that heat shock induces Mpk1-dependent phosphorylation of Swi6 (a SBF transcription factor component), although how this relates to cell wall gene expression remains unknown. In fact, the elimination of Mpk1 activity reduces cell wall gene expression levels, but does not eliminate their fluctuating character,[40] while Swi6 is probably subject to a complex set of phosphorylation events throughout the cell cycle.[42] Finally, the cell cycle and the cell integrity pathway could also be related through Sit4. This is a serine/threonine phosphatase member of the PPP family of phosphatases which plays a role in transcription control of *CLN1/CLN2* and *SWI4.*[43,44] How Sit4 regulates cyclin expression at the molecular level is not clear, and this phosphatase may have multiple roles in the yeast cell.[45] We have recently shown that Sit4 is also a negative modulator of the cell integrity pathway which lies upstream of Pkc1[46] (Fig. 1). Thus,

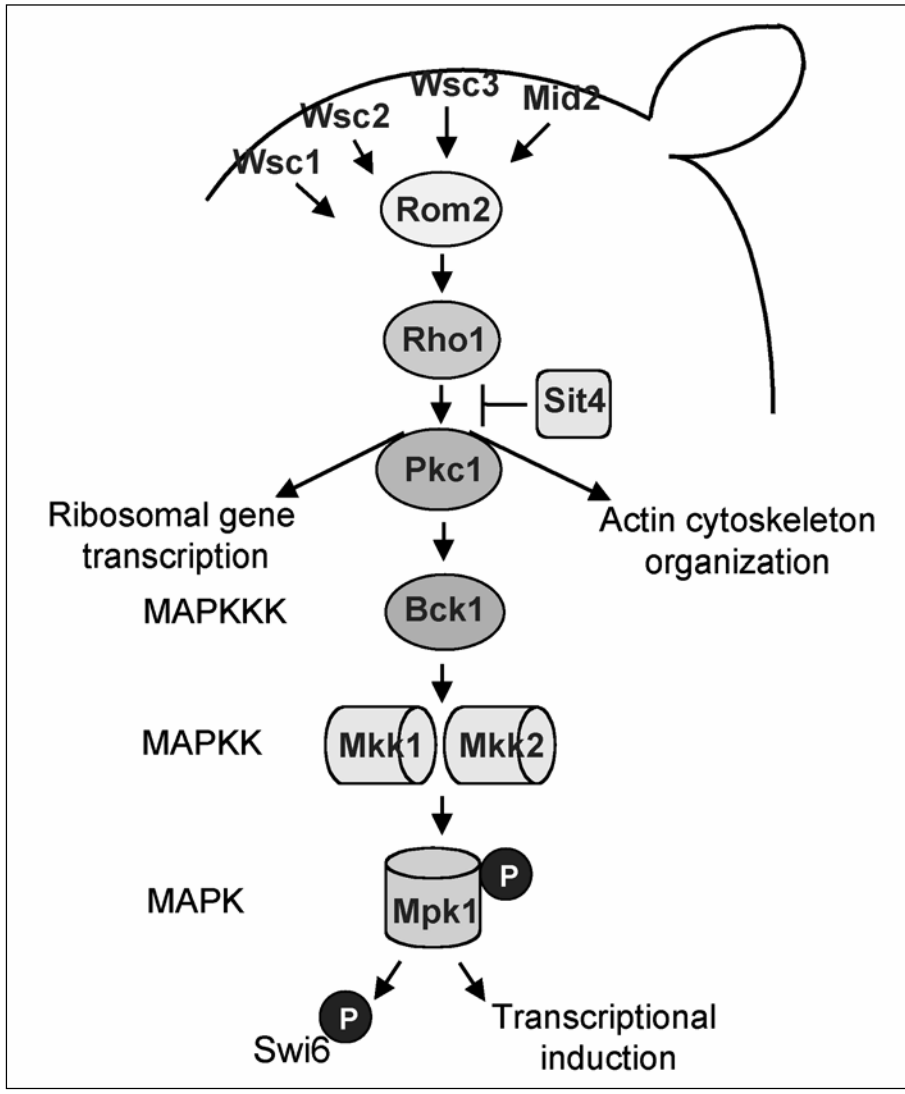

Figure 1. Cellular responses regulated by the cell integrity pathway in budding yeast. Wsc proteins and Mid2 are located at the plasma membrane, and detect alterations in the cell envelope. Sit4 negatively modulates the pathway at the Rho1/Pkc1 level. Abbreviations employed: MAPK: mitogen-activated protein (MAP) kinase; MAPKK: MAP kinase; MAPKKK: MAPKK kinase.

Sit4 modulates both Mpk1-dependent functions (such as cell wall gene expression) and Pkc1-dependent Mpk1-independent ones (such as the reorganization of the cytoskeleton upon heat shock and the expression of ribosomal genes upon disturbance of the secretory pathway). Whether this function and Sit4 cell cycle regulatory role are related remains unknown.

The hypothetical relationship between the Pkc1 pathway and transitory G1 arrest upon mild heat shock is difficult to prove due to the fact that Pkc1 pathway-defective mutants become temperature-sensitive as a result of cell wall structure defects. This may even result in cell

lysis (with the intensity of the effect depending on the genetic background and position of the particular affected step in the pathway). However, we have not observed any clear correlation between temporal cell cycle arrest and the heat shock-induced Mpk1-dependent phosphorylation of Swi6, which persists at high levels for longer than G1 arrest at 37-39 ºC.[46] Jung and Levin[47] have carried out a whole-genome analysis of genes dependent on Mpk1 activity (using either an up-regulated *MPK1* allele or heat shock). Besides *MPK1* itself, genes involved in cell wall biogenesis were also observed to be up-regulated by Mpk1 activity in a manner dependent on the transcriptional regulator Rlm1. None of the genes (whether activated or repressed) involved in the cell cycle machinery were shown to depend on Mpk1. This study suggests that basal and heat shock-related expression of cell cycle genes in yeast is not controlled by Mpk1 activity. However, the possible existence of subtle effects of the cell integrity pathway on expression of cell cycle genes (and also effects dependent on Pkc1 but not on Mpk1) could not be entirely discarded.

Another mechanism that might explain temporary G1 arrest upon heat shock is the morphogenetic checkpoint that operates from late G1 to the end of S phase (see section on Checkpoints and G1 phase). This checkpoint temporary arrests cells in G2/M in response to alterations in the cytoskeleton and disruption of the septin ring assembly.[48-51] Growth arrest under these conditions is due to inhibitory phosphorylation of Clb/Cdc28 complexes by Swe1 kinase. Heat stress causes temporary depolarization of the actin cytoskeleton in *S. cerevisiae*[52] and consequent activation of the Swe1 kinase-dependent morphogenetic checkpoint mechanism that arrests cells before mitosis.[48] Heat shock-induced G1 arrest does not, however, seem to operate through Swe1: *swe1* null mutants still display a growth response similar to wild type cells.[53] This is compatible with the idea that the morphogenetic checkpoint acts at cell cycle stages beyond START.

Recent experiments have demonstrated that the amino acid analogue azetidine 2-carboxylic acid (that causes protein unfolding after incorporating into proteins in competition with proline) induces a similar G1 arrest to that caused by heat shock, by activating the Hsf1 heat shock factor.[54] Targets for Hsf1 in this process still remain uncharacterized. The study discards this pathway as the transmitter of G1 arrest signal, since the induced protein unfolding does not activate the Pkc1 pathway.[54] It also suggests that the structural de-organization of cell proteins (or of a particular protein subset) as a consequence of the heat stress could be the signal transmitted to the cell cycle machinery.

Effect of Osmotic Stress on the G1 Phase of the Cell Cycle

Transcriptional Changes as a Consequence of Osmotic Stress

As shown in whole-transcriptome analyzes,[3-7] osmotically stressed *S. cerevisiae* cells modify the expression of more than 300 genes. A significant number of the induced genes encode proteins involved in glycerol, trehalose or glycogen metabolism, and proteins that protect against other types of stress, particularly oxidative stress. Glycerol accumulation in the cytoplasm occurs rapidly after stress, and it is a way for compensating the increase in external osmolytes.[13,55] On the other hand, osmotic stress-repressed genes include those involved in amino acid, nucleotide or mRNA metabolism, and also genes involved in translation.[3,4]

In many cases of induced or repressed genes, changes are dependent on the Hog1 MAPK. The Hog1 pathway is essential for protection against hyperosmotic stress caused by salts or other osmolytes such as sorbitol[13,56] (Fig. 2). Hog1 can be activated by two upstream branches, which are respectively regulated by the Sho1 sensor and the Sln1/Ypd1/Ssk1 phosphorelay system. Upon activation by phosphorylation, Hog1 kinase molecules translocate to the nucleus,[57,58] where they stimulate transcription of specific genes. At least three transcriptional regulators seem to depend on Hog1 for their activity, with each acting on a specific subset of

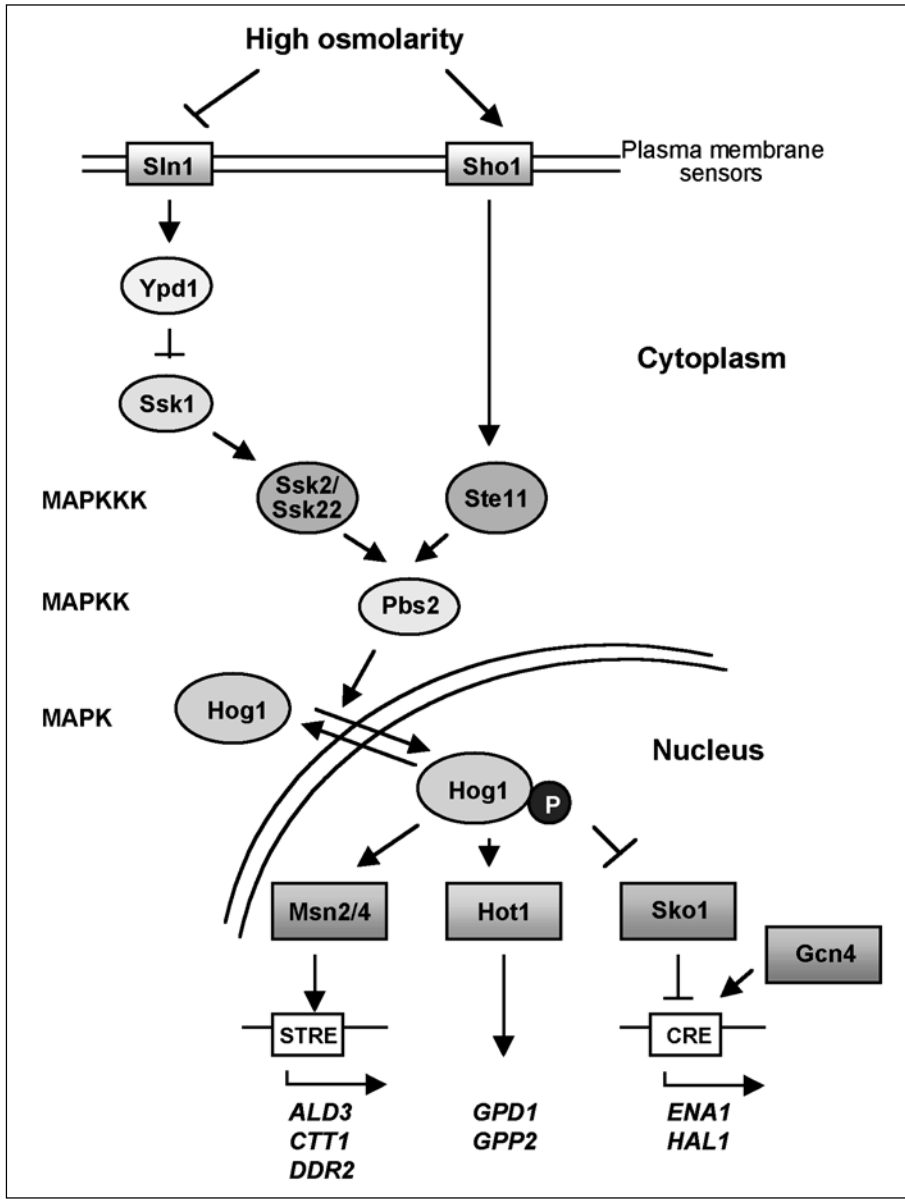

Figure 2. The Hog1-mediated pathway. See Ref. 13 for more details on the reactions leading to activation of the MAPK Hog1. Activated Hog1 enters the nucleus, where it upregulates the transcriptional factors Msn2/4 and Hot1, and inactivates Sko1. The latter competes with Gcn4 for recognition of CRE-binding sites present in a number of promoters. The two factors Msn2/Msn4 recognize promoter elements. The list of genes whose expression depends on the three Hog1-regulated factors is not exhaustive.

genes (Fig. 2). It has been suggested that the general stress regulator Msn2/4 acts downstream of Hog1 and modulates a group of genes. This proposition is based on whole-transcriptome studies[3] that reveal a number of genes (at least 46, including *ALD3*, *CTT1* and *DDR2*) whose

transcription is reduced in both *hog1* and *msn2/4* mutants. Although Msn2/4 also shuttles between cytosol and the nucleus as Hog1, that compartmentalization is a protein kinase A-regulated process.[18] Furthermore, not all Msn2/4-dependent genes are Hog1-dependent.[3,8] These facts reveal the complexity of the stress response involving the Msn2/4 transcription factor, which could also receive stimuli other than osmotic stress in a Hog1-independent manner. Hot1 is the second transcriptional regulator acting with Hog1 on the expression of another subset of genes in response to osmotic shock.[59] Induction of the Hog1-dependent genes *GPD1* and *GPP2*, both of which are involved in glycerol biosynthesis, is greatly diminished in *hot1* mutants.[59] A third transcriptional regulator whose activity depends on Hog1 is Sko1. This is a repressor of the bZIP family that recognizes cAMP response element (CRE)-like sequences present in the promoters of *ENA1* and *HAL1*, both of which are implicated in Na^+/K^+ ion homeostasis.[60] Sko1 carries out its repressing activity by recruiting the general corepressor complex Ssn6/Tup1. This interaction is disrupted upon direct phosphorylation of Sko1 by osmotic stress-activated Hog1, and this relieves expression of the target genes.[61] At least in the case of *HAL1*, Sko1 acts antagonistically with respect to Gcn4, a bZIP transcriptional activator that also binds CRE sequences.[62] Recent work indicates that Sko1 may play a complex role in osmotic stress-regulated Hog1-mediated transcription.[63] Thus, upon osmotic shock Hog1 would relieve the repressing role of Sko1 while inducing the function of an activator acting on CRE sites present in the promoters of five oxidoreductase genes. This would also help to explain the overlap between osmotic and oxidative stress in gene expression induction.

Osmotic Stress and the G1 Cell Cycle Machinery

None of the transcriptional regulators that act downstream of Hog1 have been reported to control expression of cell cycle genes. However, several whole-transcriptome analyzes[4-6] have revealed modest transitory down-regulation of *CLN1/2* expression upon moderate osmotic shock with salt or sorbitol, as occurs upon heat shock. More directed studies in asynchronously growing *S. cerevisiae* cells treated with 0.5 M KCl have demonstrated a transitory accumulation of un-budded cells that is concomitant with down-regulation of *CLN1/2* and *CLB5* expression, although not of *CLN3*.[53] In the same study, recovery from alpha-factor induced arrest at G1 was also delayed upon moderate osmotic shock, an effect that was again parallel to the physiological effects of heat shock. Two observations point to Cln3 being the target for osmotic stress and being responsible for the G1 delay:

i. the ectopic expression of CLN2 from a non Cln3-dependent promoter suppresses the accumulation of un-budded cells after shock, and
ii. the activity of immunoprecipitable Cln3/Cdc28 complexes is transiently reduced after shock.[53]

HOG1-deficient cells still undergo arrest after osmotic stress, although growth recovery is significantly delayed. Therefore, G1 arrest could be a direct result of the inhibitory effect of an increase in internal osmolarity on Cln3/Cdc28 complexes, although subsequent recovery would require the Hog1 pathway to remain intact.[53]

As occurs during heat shock, osmotic stress depolarizes the actin cytoskeleton in both *S. cerevisiae*[52] and *Schizosaccharomyces pombe*.[64] The Swe1-mediated morphogenetic checkpoint activated by stress is not responsible for the G1 arrest, since at this stage of the cell cycle *swe1* mutants exhibit the same response as wild type cells.[53] On the other hand, a temporary arrest also occurs at G2 after moderate osmotic shock on yeast cells.[65] In this case, Swe1 and Hog1 seem to cooperate in causing the arrest, though this does not occur exactly in the same manner as during the morphogenetic checkpoint. The molecular mechanism responsible for the G2 arrest has yet to be elucidated.

Effect of Oxidative Stress on the G1 Phase of the Cell Cycle

Cell Cycle Arrest As a Consequence of a Moderate Oxidative Stress

Aerobic cells are subjected to the metabolic generation of a number of reactive oxygen species, such as the superoxide anion radical, hydrogen peroxide and the hydroxyl radical. These can alter the redox status of the cell and, directly or indirectly, cause damage to nucleic acids, lipids and proteins.[66] Yeast cells have developed a number of protection mechanisms against oxidative stress.[2] These include detoxifying enzymes (such as catalase and superoxide dismutase), enzymes that maintain an appropriate redox state for protein activity (such as the thioredoxin and glutaredoxin systems) and free radical scavengers (including glutathione and ascorbic acid).

Cultures of *S. cerevisiae* subjected to 100% O_2 transitorily arrest at G1 as un-budded cells, concomitantly to temporary down-regulation of *CLN1* and *CLN2* expression.[67] These effects are exacerbated in mutants lacking the cytosolic Sod1 Cu,Zn-dependent superoxide dismutase, in which arrest is irreversible. Therefore, oxidative stress causes more or less prolonged cell cycle arrest at G1 according to the intensity of the stress. The fact that this stress has a specific effect on the cell cycle machinery was demonstrated by the fact that a hyperactive Cln3-2 form suppressed G1 arrest under hyperoxia conditions.[67] As with other kinds of stresses, these studies again suggested Cln3 as the main target for oxidative stress. They also confirmed previous observations in studies that used paraquat (a generator of superoxide anion radicals) as an external oxidant.[68] Later work[69] revealed a more complex situation: menadione arrested cells at G1 while hydrogen peroxide did so at G2/M. Thus, there may be more than one arrest point in the cell cycle following oxidative stress. Since both menadione (another generator of superoxide anion) and hydrogen peroxide cause macromolecular damage by generating hydroxyl radicals,[70] the above observations raise the possibility that each of the two oxidants directly triggers specific signals that are transduced to different elements of the cell cycle machinery. Previous physiological studies[71-73] had indicated that different oxidants applied in moderate doses were capable of pre-adapting cells for more intense oxidative stress doses in a specific oxidant-dependent manner. However, no relationship has been established between these studies and the observed cell cycle effects.

The effects of oxidative stress on the cell cycle are also observed when stress is metabolically generated in the absence of externally added oxidants. Wanke et al[74] have shown that stress resulting from diethylmaleate-induced glutathione depletion arrests yeast cells at G1, in a transitory or permanent manner depending on the diethylmaleate concentration. In this same work, experiments with a temperature-sensitive *cdc25* mutant that is defective in the Ras/protein kinase pathway indicated that oxidative stress operates on the Cdc25-mediated step at pre-START. This led the authors to hypothesize that oxidative stress would arrest cells by inactivating the Ras pathway and consequently down-regulating *CLN* expression (see section on The Ras Pathway and G1 Phase). Arrest would thus occur prior to the G1 stage as defined by alpha factor inhibition of Cln/Cdc28 complexes. However, other studies suggest that menadione may act at the START point in G1.[69] We have also observed that mutants that show constitutive protein oxidation due to the absence of Grx5 glutaredoxin[75] have problems in recovery from alpha factor arrest even in the absence of externally-added oxidants.[76] These data again reinforce the idea that oxidative stress targets some protein(s) required to execute START. These apparently contradictory results might be due to parallel heat stress caused when using thermo-sensitive *cdc25* mutants.

High concentrations of iron cause a stress at the yeast cell that *a priori* might be comparable with that caused by reactive oxygen species, because ferric iron catalyses oxygen radicals formation through the Fenton reaction.[70] Thus, yeast cells treated with iron in greater than

physiological concentrations experience a G1 block.[77] This is especially intense in mutants where the Aft1 transactivator-mediated high affinity iron uptake system is constitutively upregulated.[77] In these mutants, the G1 arrest is parallel to translational down-regulation of Cln1/2 synthesis. As with menadione-induced G1 block, this does not seem to be the result of checkpoint arrest triggered by DNA lesions, as the iron-induced block is independent of *RAD9*.[77] In contrast with experiments conducted with menadione or hydrogen peroxide, lower iron concentrations, which might lead to a transitory G1 arrest, were not employed in the latter study. This makes it difficult to compare menadione and iron stresses and their respective effects on *CLN* mRNA synthesis and translation. Nevertheless, the above results point to the existence of Cln3-mediated *CLN1/2* transcriptional regulation by menadione stress and *CLN1/2* translational regulation by iron stress.

The question arises as to whether cell cycle response to oxidative stress can be explained by the activity of known stress response regulators. Proteomic studies using 2-D gels[78] have shown that synthesis of at least 115 proteins from *S. cerevisiae* was stimulated upon hydrogen peroxide treatment, whereas about 52 were down-regulated under the same conditions. Up-regulated proteins fall into several categories: (i) antioxidant proteins, (ii) chaperones and heat shock proteins, (iii) proteins implicated in the proteolytic machinery, and (iv) enzymes, that redirect carbohydrate metabolism towards trehalose synthesis and the regeneration of NADPH, which is required in antioxidant defence reactions. An increase in NADPH levels is attained through activation of the pentose phosphate pathway and inhibition of glycolytic enzymes. Together with these, proteins required for the protein synthetic machinery were also down-regulated. Results from genome-wide transcriptome analyzes[5,6,79] roughly coincide with those quoted above, which indicates that yeast cells regulate their oxidative stress response at a transcriptional level. Importantly, many genes up-regulated by oxidative stress are also up-regulated by heat and/or osmotic shock. These include genes coding for heat shock proteins and for the thioredoxin system. None of these studies has pointed to cell cycle regulators as being among the major up-regulated or down-regulated proteins, although a modest down-regulation of *CLN* genes has been observed upon oxidative shock in whole-genome analyzes.[5,6]

Signalling Oxidative Stress to the Cell Cycle Machinery

Besides the general stress factor Msn2/4, two other transcriptional factors are particularly involved in the oxidative stress response: Yap1 and Skn7. Yap1 is a DNA binding protein that contains a leucine zipper domain characteristic of the AP-1 family of transcriptional regulators in higher eukaryotes. It recognizes the sequence T(T/G)ACTAA present in the promoter region of many genes regulated by oxidative stress.[80-82] Mutants lacking the *YAP1* gene are hypersensitive to oxidative stress and to metals such as cadmium. A cysteine-rich C-terminal region of Yap1 is important for mediating the nuclear location of the protein in response to oxidants, and thus regulating the activity of Yap1 as a transcriptional factor.[83] Recent large-scale analysis[79] of the role of Yap1 on the yeast transcriptome suggests a complex role for this regulator as activator or repressor of specific sets of genes both in unstressed and oxidatively-stressed cells. Thus, *RPI1* (a repressor of the Ras-cAMP pathway) is down-regulated by Yap1 in exponential cells but up-regulated in stationary cells and during oxidative stress.

Skn7 participates in a phosphorelay system in conjunction with the histidine kinase Sln1. The latter is the sensor in the two-component system and displays homology to bacterial sensors.[84-86] The Skn7/Sln1 pair regulates expression of a number of genes (including the thioredoxin *TRX2* gene) in response to osmotic stress.[87] The role of Skn7 in the osmotic stress response is independent of Hog1.[87] On the other hand, in the oxidative stress response Skn7 functions independently from the Sln1-mediated phosphorelay system,[88] and partially overlaps with Yap1[89] (Fig. 3). Of the Yap1-dependent genes, transcription of one subset (those implicated in scavenging reactive oxygen species) is also dependent on Skn7, while another subset (mainly in-

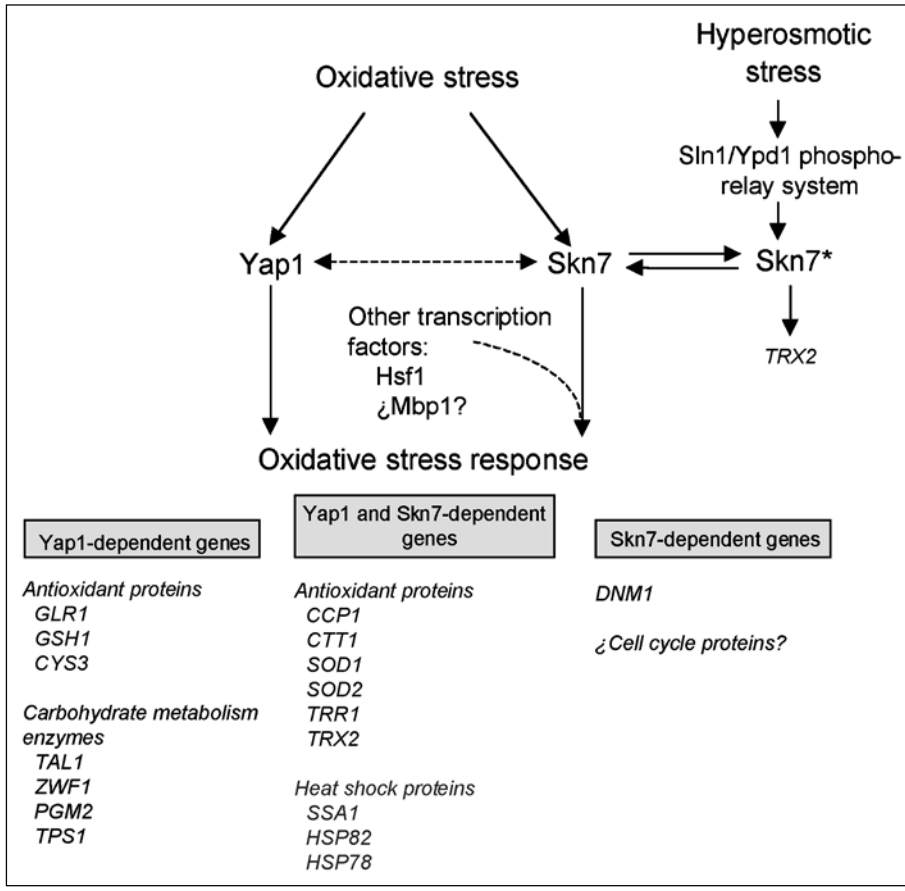

Figure 3. Role of Yap1 and Skn7 in the oxidative stress response. The scheme is based on data from ref. 87 to 91. Skn7 and Skn7* denote the two hypothetical forms on the Skn7 protein as suggested in ref. 87. Dashed lines indicate cooperation between Skn7 and other transcription factors. Only some of the genes regulated by Yap1 or Skn7 are listed (for more details see ref. 89).

cluding genes that redirect the metabolic flux towards NADPH generation through the pentose phosphate pathway) is exclusively dependent on Yap1.[89] Like other stress response regulators, Skn7 has DNA binding activity in the N-terminal region. The DNA binding domain of Skn7 is homologous with the DNA binding region of heat shock factor Hsf1,[90] though the exact DNA binding target of Skn7 is not known. Skn7 and Hsf1 physically interact and both are probably required for full induction of heat shock genes (such as *HSP12*) upon oxidative stress.[90] All of these observations situate Skn7 at the centre of a complex pattern of interactions involving different stresses and transcriptional regulators (Fig. 3).

The complexity of Skn7 roles is further illustrated by its relationship with cell cycle regulators. *SKN7* was isolated as a suppressor when over-expressed for the need of SBF and MBF for G1 cyclin expression.[85] However, Skn7 does not directly stimulate *CLN* transcription through binding to SCB or MCB elements.[85] More recent studies[91] have demonstrated the genetic and physical interaction between Skn7 and Mbp1, which is the DNA binding component of MBF. Thus, *mbp1* mutations suppress the lethality of *SKN7* over-expression, while *skn7* mutations

suppress that of *MBP1* over-expression. This suggests that Mbp1 and Skn7 may share common partners in multi-protein complexes. The same study demonstrated physical interaction between Mbp1 and Skn7 both in two-hybrid and in vitro pull-down assays. Skn7 is not a MBF component, but its DNA binding domain is needed for the interaction with Mbp1. The importance of Mbp1/Skn7 interactions for G1 cyclin expression in physiological conditions remains unclear, since *skn7* and *mbp1* single mutants display normal *CLN* expression.[85,91] Perhaps the Mbp1/Skn7 transcription factor is only necessary for *CLN* expression under particular conditions, such as those occurring during oxidative stress.[91] It would be interesting to trace the pattern of *CLN* expression and the physiological behavior of *skn7* mutants following moderate oxidative stress.

No MAPK cascade or other characterized signal transduction pathways are known to participate in the oxidative stress response in *S. cerevisiae*. Thus, the upstream effectors of Yap1 are unknown, and the integration of Skn7 in transduction pathways related to oxidative stress also remains to be elucidated.

Contrary to what occurs in budding yeast, in *S. pombe* a single MAPK, Sty1, controls the transcriptional response to several stresses (oxidative and osmotic stresses, heat shock, DNA-damaging agents, physical stress).[13,92] Specific responses to each of these stresses are a result from specific regulators that act upstream of Sty1, and/or by cofactors that are needed in conjunction with Sty1-activated targets. The oxidative stress response acts through a phosphorelay system that involves the Mak family of histidine kinases structurally related to Sln1.[93,94] This system transmits the oxidation signal to Sty1. One of the targets for Sty1 is Pap1, a transcriptional factor of the c-Jun/AP-1 family (like Yap1) that is translocated to the nucleus upon oxidative stress and regulates expression of genes involved in defences against oxidation.[92,95]

Is there a specific signal for oxidative stress damage in budding yeast that is transduced to the cell cycle machinery? The lipid peroxidation product 4-hydroxynonenal causes a G1 delay in yeast.[96] More recently, linoleic acid hydroperoxide (LoaOOH) has been used as a model for studying the effect of lipid peroxidation on the cell cycle.[97] The addition of LoaOOH (in concentrations that do not affect cell viability) to cultures synchronized at G1 with alpha-factor causes a delay in recovery from the alpha-factor arrest. This delay does not occur in mutants in *OCA1*, a gene coding for a putative tyrosine phosphatase. Viability of null *oca1* mutants is compromised by the continuous presence of LoaOOH in concentrations that do not affect wild type cells. This indicates that *OCA1*-mediated G1 temporary arrest could be important for the adaptive response to sub-lethal concentrations of lipid hydroperoxides. Interestingly, the Oca1 protein is homologous with Siw14, another putative tyrosine phosphatase whose absence causes arrest failure at G1 after nutrient starvation.[98] These observations suggest that:

i. metabolites resulting from lipid oxidation could act by signalling an adaptive response to oxidative stress, and
ii. a signal transduction pathway involving tyrosine phosphatases (among other intermediates) could act upstream of the transcriptional response regulators.

The effect of LoaOOH and other lipid-oxidized metabolites on Cln/Cdc28 complexes and on *CLN* transcription still remains to be determined.

Checkpoints and G1 Phase

Checkpoints are cellular down-regulatory mechanisms that induce transient cell cycle arrest in response to failures in a gene function that is required for the following step to take place. *S. cerevisiae* DNA-damage checkpoints are signal transduction pathways that sense any kind of damage to DNA (it has been proposed that one single double strand break is enough to trigger this response),[99] and transduce the signal (via protein kinases) to a number of targets in a variety of cellular functions, causing: G1, S and G2 cell cycle arrest and the transcriptional induction of DNA-repair genes.[100-106]

Little is known about the molecular mechanisms underlying DNA-damage checkpoints and cell cycle regulation in budding yeast. DNA-damage G1 arrest is mediated by a number of checkpoint genes: *RAD9*, *RAD24*, *RAD17*, *MEC3*, *RAD53*, and *MEC1* (see Chapter 2). Mutants in these genes are defective not only in G1 but also in S and G2 transitory blockages.[101,107,108] CDKs are the protein kinases that together with their associated cyclins are responsible for each of the cell cycle transitions in all the eukaryotic cells (see Chapters 3 and 4). Whereas in *S. pombe* the role of the CDK1 cdc2 in the DNA-damage cell cycle arrest in G2 has been well documented,[109] in *S. cerevisiae* there have been a few reports describing genetic interactions involving *cdc28* mutants in DNA-damage mediated G2 arrest.[110,111] However, little is known about the G1 regulation to date. Mec1 is a member of the phosphatidylinositol 3 kinase family which is involved in all known cell cycle arrests dependent on DNA-damage.[112-114] Recently, *mec1* mutants have been isolated that are defective in the G1 and S delays that normally occur when DNA is damaged. Interestingly, these mutants still keep an intact G2/M checkpoint.[115] This suggests that DNA-damage checkpoints signal different targets in G1/S than in the G2/M transition, and could be a first step to discern the cell cycle regulatory mechanisms linked to DNA damage.

MEC1 functionally interacts with *CLN1* and *CLN2.*[116] Deletion of either *CLN1* or *CLN2* suppresses the *mec1* lethality phenotype, apparently due to the fact that both cyclins reduce expression of *RNR1* (a gene whose transcription is induced depending on the DNA-damage checkpoint and which encodes for a subunit of the ribonucleotide reductase) at the G1 to S transition.

Rad53 is a protein kinase also involved in all cell cycle arrests that are induced by DNA damage.[104,117] Rad53 participates in recovery from G1 arrest after treatment with methyl methanesulfonate, an alkylating agent that damages DNA.[118] The same authors have also demonstrated that Swi6 is an in vitro substrate for Rad53. Thus, the model proposed by Sidorova and Breeden[118] relating Rad53 with G1 arrest is as follows: once cells have been subjected to DNA damage there is a transient G1 blockage (which correlates with a drop in *CLN1* and *CLN2* transcription) that is not dependent on the DNA-damage checkpoint. Rad53 would be required at a later stage in order to maintain the cells arrested while DNA damage becomes repaired. This would be achieved by transiently repressing the expression of both *CLN1* and *CLN2* through Rad53-mediated phosphorylation of Swi6, which in turn would result in inactivation of the Swi6/Swi4 complex.

Another mechanism, the morphogenesis checkpoint, controls the process of bud formation. When this is abnormal, it induces a Swe1-mediated G2 arrest.[48,50,119] Selection of cell polarity, the switch from hyperpolar to isotropic bud growth, and regulation of septation to allow separation between mother and daughter cells, are all functions mediated by septins.[120,121] Mutants in septins have elongated buds, a phenotype that has been explained as a consequence of Swe1 activation.[51] It has also been reported that G1 cyclins/Cdc28 complexes are involved in septin assembly,[122] but the molecular basis of this role is unclear.

As described earlier in this chapter, hydrogen peroxide causes both G1 and G2 delays in budding yeast.[69] The G2 arrest is dependent on *RAD9* and is therefore mediated by the DNA-damage checkpoint. Hydrogen peroxide itself does not damage DNA, but DNA breaks are generated when hydroxyl radicals are present in the nucleus. The superoxide-generating agent menadione also elicits blockage in G1. In both cases, G1 arrest seems to be independent of *RAD9,*[69] which gives rise to the question of what kind of checkpoint senses the oxidative state of the cell in G1. Recent data have demonstrated that the base excision repair function does not participate in triggering the DNA-damage checkpoint at G1 following oxidative stress.[123]

The Ras Pathway and G1 Phase

The Ras-cyclic AMP (cAMP) pathway plays a role in sensing and transducing the nutrient status in yeast cells. Part of this signal also connects with the cell cycle machinery. In *S. cerevisiae*, glucose activates the Ras proteins to produce cAMP and activate protein kinase A (PKA), a process for which Cdc25 (the guanine nucleotide exchange factor for Ras) is required (see Ref. 124 for a review).

Nitrogen deprivation causes a rapid G1 arrest (see chapter 8). This arrest requires a regulated Ras/cAMP pathway, since cells with hyper-activated Ras proteins fail to stop properly at G1 when nitrogen-deprived. This occurs because they cannot accumulate nutrients enough to complete a cell cycle in order to arrest at the next G1 period of the cycle.[125]

When yeast cells grow in a poor carbon source and are returned to a rich medium containing glucose, the critical cell size required to traverse START becomes bigger. Under these conditions, the expression of *CLN1* and *CLN2* is transiently repressed as a consequence of Ras/cAMP activation.[126,127] In contrast, since activity of the Ras/cAMP pathway is needed for cells nutritionally arrested to return growth, this is associated with an increase in *CLN1* and *CLN2* transcription.[128] The conclusion is that upon glucose addition to nutritionally deprived cells, a transient decrease in *CLN1* and *CLN2* transcription occurs, followed by an induction of both transcripts when cells are committed to resume growth. Strikingly, both processes appear to be mediated by Ras/cAMP activity. Moreover, *CLN3* transcription is positively regulated by glucose but not by Ras.[128] Nevertheless, cAMP contributes to stimulate Cln3/Cdc28 kinase activity and also to increase Cln3 protein levels at a post-transcriptional level, maybe through changes in the rate of Cln3 protein synthesis.[129]

Stationary phase is another situation in which cells become nutritionally deprived. This is a physiological state associated to cell acquisition of thermo-tolerance, starvation resistance and also to G1 arrest. Rim15 is a kinase necessary for the proper entry into stationary phase. The cAMP-dependent PKA negatively regulates Rim15 kinase activity by phosphorylation.[130]

Adaptation to stress is another process in which involvement of the Ras/cAMP pathway has been reported. Ssa1 and Ssa2 are two chaperones that become overproduced when cells are exposed to any kind of stress.[131] Since Ssa1 interacts with Cdc25, it has been proposed[132] that, in response to stress, a subset of chaperones (Ssa1 among others) plays a role in the refolding of denatured proteins. In these conditions, Ssa1 would positively control the Ras pathway through Cdc25. Interestingly, other authors[74] have reported a partial role for the Ras pathway in the G1 arrest provoked by glutathione depletion. Strikingly, whereas in budding yeast the Ras/cAMP pathway is inhibited by oxidative stress, in mammals the equivalent pathway becomes activated.[133]

In summary, the Ras/cAMP pathway is involved somehow in the stress responses in budding yeast (Fig. 4), serving as a connector with the cell cycle machinery, although other unknown Ras-independent pathways signalled by different stresses partially contribute to the cell cycle arrest.

Is There a Common Down-Regulator of G1 Cyclins after an External Stress?

As shown in previous sections, G1 arrest in response to an environmental stress on yeast cells is probably a consequence of the down-regulation of G1 cyclin expression. Negative regulators of *CLN3*, *CLN1* and *CLN2* expression are obvious candidates for mediators of such a response. Though much is known on activators of G1 expression at the G1/S boundary (reviewed in Chapter 2), Xbp1 is the only proposed G1 cyclin transcriptional repressor in *S. cerevisiae*.

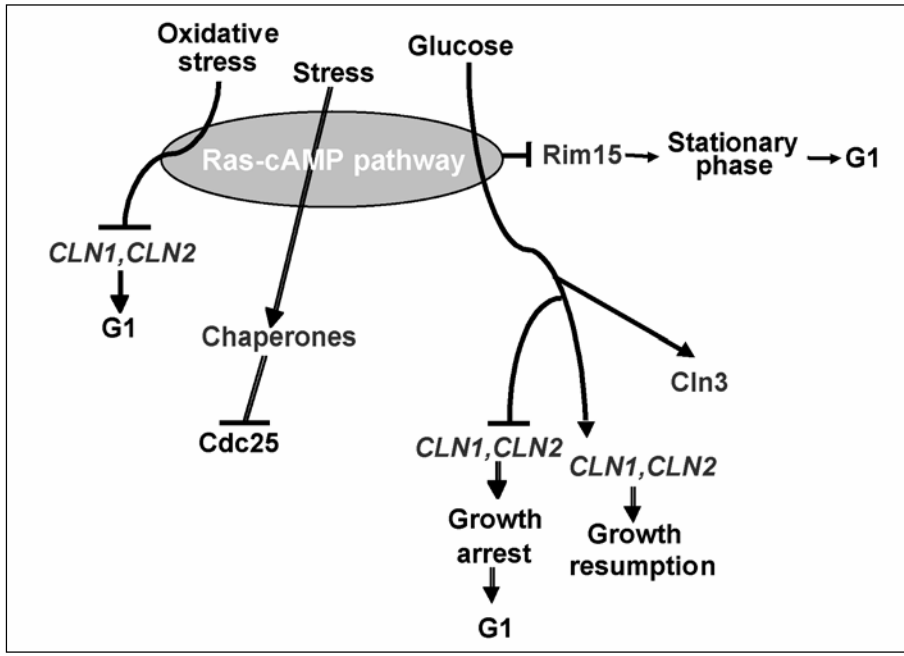

Figure 4. Possible roles of the Ras-cAMP pathway in signalling stress to the G1 cell cycle machinery.

XBP1 was initially identified as a gene displaying sequence similarity to the DNA-binding domain of Swi4 and Mbp1, the two DNA-binding subunits of the transcription factors SBF and MBF involved in the expression of G1 and S cyclins.[134] However, Xbp1 was actually shown to function as a transcriptional repressor.[134] Following determination of the consensus DNA sequence bound by Xbp1, two potential binding sites were shown to be present in the *CLN1* promoter, what suggested that Xbp1 could negatively modulate *CLN1* cyclin expression. In more recent work, the same authors employed differential display to look for the most sensitive transcripts to *XBP1* over-expression. Three out of the five found targets were cyclins: *CLN1*, *CLN3* and the *CLB2* mitotic cyclin,[135] thus relating most of *XBP1* functions to cell cycle progression. *XBP1* is regulated at the transcriptional level, and its expression is induced by several types of stresses, including heat shock, glucose starvation, osmotic and oxidative stresses.[134] Despite being stress-inducible, there is no evidence that Xbp1 has a role in *CLN* repression in such situations: *XBP1* up-regulation closely parallels a drop in *CLN1* transcription following a stress, but a *xbp1* null mutant fails to overcome the down-regulation in G1 cyclin expression when the same stress is applied.[134] However, *XBP1* is able to down-regulate G1 cyclin expression when over-expressed and, probably due to the low levels of *CLN* transcripts reached, its over-expression lengthens the G1 phase of the cell cycle. The latter observations suggest that there might be other stimuli that can activate Xbp1 to arrest the cell cycle.

Upon nutrient deprivation, yeast cells transiently arrest at G1 (see Chapter 8) and switch specific developmental programs: haploid yeast cells are able to invade the solid growing media, while diploid yeast cells switch to a pseudo-hyphal growth pattern when nutrient-deprived. Diploid cells can also induce the meiotic program, leading to the formation of spores (reviewed in Ref. 136). Sporulation only occurs in the presence of a nonfermentable carbon source and upon limitation for some other nutrient, especially nitrogen; the nutrient starvation requirements

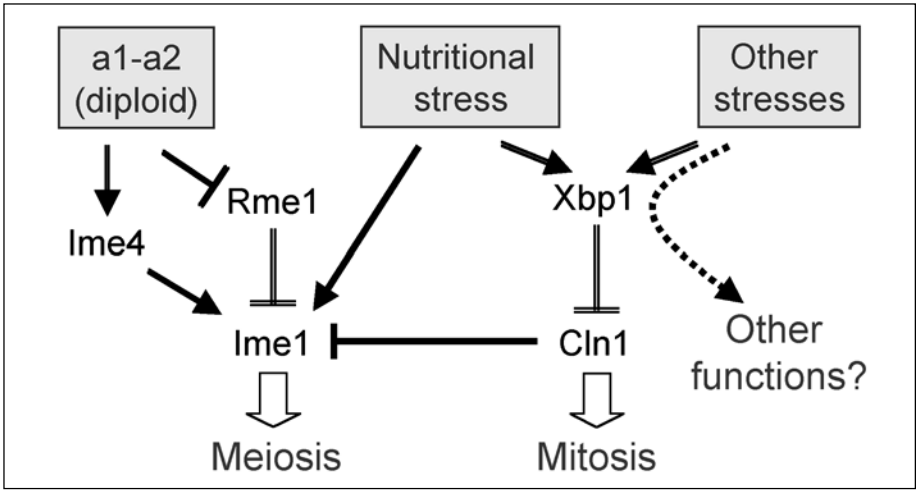

Figure 5. Scheme of the involvement of Xbp1, Cln1 and Ime1 in the developmental program (mitosis vs. meiosis) response. Open lines indicate a transcriptional control, while filled lines indicate post-transcriptional regulation.

are not only needed to initiate the process of sporulation, but also to complete it. At the molecular level, the key events in early meiosis have been elucidated: the union of the two opposite mating type haploid nuclei after conjugation allows diploid cells to express the heterodimeric repressor a1-α2, whose main role is to inhibit expression of *RME1* (an inhibitor of meiosis) and to stimulate expression of *IME4* (a positive regulator of meiosis). The effects of the Rme1 and Ime4 proteins are exerted on expression of a meiotic inducer, the Ime1 transcription factor. Besides its regulation by mating type loci, *IME1* expression is de-repressed by nitrogen and glucose deprivation, and its role as a transcriptional activator is regulated by nutrient sources. Recent studies have provided a link between G1 cyclin and repression of the meiotic program.[137] The first evidence for such a relationship came from studies on *RME1*, which besides its role as a negative regulator of meiosis, is also a positive activator of G1 cyclin expression.[138] G1 cyclins are thought to prevent activation of the *IME1* pathway by preventing Ime1 accumulation in the nucleus,[137] where it exerts its function as a transcriptional activator. As G1 cyclin expression is repressed upon nutrient deprivation and *CLN* over-expression prevents entry into meiosis, it has been proposed that G1 cyclins would act by making mitosis and meiosis incompatible.

The Xbp1 repressor is not only induced by stress but also during meiosis. Its induction may be necessary for an efficient sporulation, since *xbp1* null mutant cells are delayed in ascus formation.[135] The observation that *CLN1* expression is de-repressed in *xbp1* cells entering meiosis, suggests that *XBP1*-mediated repression of *CLN1* is necessary in the later stages of gametogenesis.[135] However, in the same study the authors showed that *cln1* mutant cells are as equally delayed in ascus formation as *xbp1* cells. Thus, the real Xbp1 target in regulating meiosis remains unknown, although a model relating Xbp1 with meiosis and stress-induced programs can be proposed (Fig. 5). Recently, a new role for *XBP1* in relation to nutrient deprivation has been described: *XBP1* is needed for the morphologic shift to an elongated phenotype when grown on nitrogen-limited medium.[139] In the latter, however, it has been proposed that Xbp1 would act through repression of *CLB2* mitotic cyclin expression, suggesting that *XBP1* roles may be not limited to the regulation of the G1 phase of the cell cycle. In summary,

although Xbp1 may be important for the response to a number of stresses, its direct role as a repressor of *CLN* expression in stress conditions should be excluded.

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CHAPTER 11

Progression from G0 through G1 and into S on Two Waves of Growth Factor-Driven Signaling

Steven M. Jones and Andrius Kazlauskas

Abstract

A widely used model system to investigate cell proliferation is stimulation of serum-arrested cells with growth factors. Recent data suggest that there are two waves of growth factor-dependent signaling events required for a proliferative response. One is an acute burst of signaling, which occurs immediately after growth factor stimulation and lasts for 30 – 60 min. The other occurs in a different time frame (8-12 hrs post stimulation), and involves activation of cyclin dependent kinases (CDKs). In addition to a general overview of growth factor-dependent signaling, we present our "two wave" hypothesis for how signaling and cell cycle progression are linked.

Three Steps in Growth Factor-Dependent Signaling

The First Step: Binding of Growth Factor to the Transmembrane Receptor

A recurring theme for growth factor receptors is ligand-induced dimerization of the receptor. There are a variety of ways in which this occurs. Some ligands, such as platelet-derived growth factor (PDGF) are dimeric, and their receptors encode a single ligand-binding domain. As a result two receptors bind to each monomer of the ligand resulting in dimerization of the receptors. The PDGF ligand is a member of a family of cysteine knot-containing proteins, and many other growth factors that share this structural feature appear to interact with their receptors in a comparable fashion.[1] Other growth factors are monomeric and manage dimerization of their cognate receptors by a different strategy. Members of the fibroblast growth factor (FGF) family have two binding sites for the receptor within a single molecule of FGF. Resolution of the crystal structure and FGF ligand-receptor complex indicated that each receptor molecule also has two binding sites for the ligand.[2-4] Hence the ligand-receptor complex consists of two molecules of receptor and two molecules of FGF. An additional feature of the FGF family is that high affinity binding of FGF to its receptor requires heparan sulfate proteoglycans, which spatially organize the ligands to functionally associate with the receptor. These are only two examples of how growth factors dimerize their receptors; a number of reviews cover additional well studied cases.[5,6]

G1 Phase Progression, edited by Johannes Boonstra.

The theme of ligand-induced dimerization of receptors is not universal. The insulin receptor is a dimer even before binding of insulin, which is a monomer. In addition, polymerized collagen is a ligand for the discoidin receptor tyrosine kinases, [7,8] and it is not obvious whether this sort of ligand is able to induce dimerization or oligomerization of its receptor. In summary, many although not all receptor tyrosine kinases are dimerized in response to binding of ligand, and there are a variety of mechanisms by which this event proceeds.

The Second Step: Activation of the Receptor's Kinase Activity

Binding of ligand results in an elevation of the receptor's kinase activity. Activation of a kinase involves a reconfiguration of various domains, including the activation loop, as well as the orientation of the upper and lower lobes of the kinase. [9] Tyrosine kinases are often phosphorylated in the activation loop, and this event probably contributes to the conformational changes leading to activation of the kinase. Recent crystallographic studies with the EphB2 receptor tyrosine kinase indicate the phosphorylation of tyrosine residues in the juxtamembrane domain may also contribute to activation of the receptor's kinase activity. The unphosphorylated juxtamembrane domain alters the conformation of the kinase to a catalytically unfavorable one. Phosphorylation of tyrosine residues within the juxtamembrane domain is predicted to relieve this autoinhibition, and hence contribute to activation of the kinase.[10] For receptor tyrosine kinases, ligand-induced dimerization brings the kinase domains into close proximity, and facilitates transphosphorylation at these residues that regulate.[5,11] Whether ligand binding promotes additional events that contribute to activation of the kinase is still not fully understood.

While the ligand promotes activation of a receptor tyrosine kinase, there are also factors that suppress the receptor's kinase activity. Cells express many types of phosphotyrosine phosphatases (PTPs) that have the potential to functionally repress the receptor's kinase activity. For instance, addition of inhibitors of PTPs leads to a rapid increase in the phosphorylation of receptor tyrosine kinases in the apparent absence of ligand. In addition, growth factors trigger a burst of hydrogen peroxide production, which has the potential to inhibit PTPs. In some cell types inhibition of peroxide production blunts PDGF-dependent tyrosine phosphorylation of the PDGFR,[12] whereas in others it does not.[13] Finally, there are proteins that prevent activation of receptor tyrosine kinases. Kek-1 is a transmembrane protein that physically interacts with the drosophila EGFR and antagonizes the effect of EGFR.[14] Whether functionally similar proteins exists in mammalian systems, as well as what step of the activation mechanism they impact remain open questions. In summary, the balance between positive and negative factors determines the activity of a receptor tyrosine kinase.

The Third Step: Recruitment and Activation of Signaling Enzymes

One of the consequences of tyrosine phosphorylation of the receptor is to enable the receptor to recruit a variety of signaling enzymes.[15,16] The interaction of signaling enzymes with the activated receptor is dependent on tyrosine phosphorylation of the receptor (i.e., conditional). This interaction is also specific, and the specificity is determined by both the receptor and the signaling enzyme.[15,16] The amino acid context surrounding the tyrosine phosphorylation site is the receptor's contribution to specificity, whereas the PTB or SH2 domain of the signaling enzymes has an intrinsic preference for binding partners, and hence makes a contribution to the specificity of the interaction. There is a long and growing list of proteins that associate with the βPDGFR,[15,16] and these same signaling enzymes can associate with many other activated receptor tyrosine kinases as well. In this review we will focus on several well-studied examples, and refer the interested reader to other reviews for additional information.[15,16]

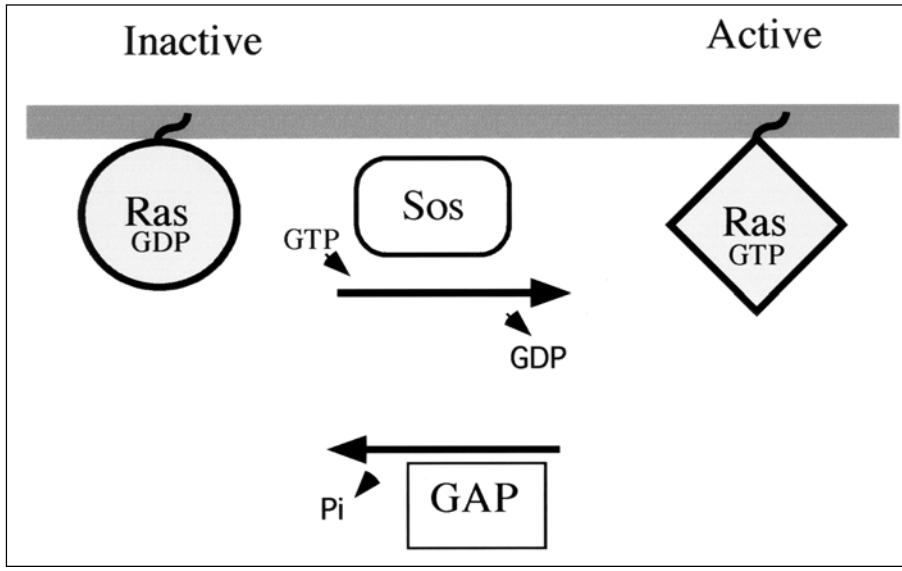

Figure 1. Activation of Ras. Activation of Ras is catalyzed by the nucleotide exchange factor Sos, which promotes the release of GDP. Although Ras has intrinsic GTPase activity, the hydrolysis of GTP to GDP is enhanced by GAP, and this event converts Ras back to its inactive state.

One of the signaling proteins activated in a growth factor-stimulated cell is Ras.[17] Activated Ras functions as a cofactor for a variety of signaling enzymes.[18] The nucleotide exchange factor, Sos promotes the exchange of GDP for GTP on Ras, which converts Ras to its active state (Fig. 1). Inactivation of Ras proceeds by a distinct chemical reaction, i.e., hydrolysis of the GTP to GDP, and is promoted by GTP-ase activating proteins called GAPs. Sos is a constitutively active cytoplasmic enzyme, whereas Ras is anchored to the membrane. Consequently, activation of Ras requires translocation of Sos from the cytoplasm to the membrane, which is mediated by adapter proteins. There are several ways by which Ras can be activated in growth factor-stimulated cells, and they all appear to involve a change in the subcellular localization of the Grb2/Sos complex. Grb2 is an SH3-SH2-SH3 adapter protein that mediates binding of Sos to activated receptors. The Grb2/Sos complex is constitutive, and in resting cells, is primarily cytoplasmic. Tyrosine phosphorylation of the receptor enables the SH2 domain of Grb2 to stably associate with receptor tyrosine kinase receptors. This relocalizes Sos to the membrane, the cellular compartment in which its substrate, Ras resides (Fig. 2). Alternative scenarios include association of the Grb2/Sos complex with other adapter proteins such as Shc. Many growth factors induce tyrosine phosphorylation of Shc, leading to its association with Grb2/Sos. This trimeric complex can relocate to the membrane via Shc's SH2 or PTB/PH domains. The functional consequence of these changes is the same as when Grb2/Sos associates with a tyrosine phosphorylated growth factor receptor: Sos gains access to Ras and activates it.

Phosphoinositide 3 kinase (PI3K) is one of the SH2 domain-containing signaling enzymes that are activated in growth factor-stimulated cells. The p85 subunit of PI3K contains a number of domains, and it is via the SH2 domains that p85, and its tightly associated p110 catalytic subunit, are recruited to activated receptors. Unlike the Grb2/Sos complex, localizing the PI3K holoenzyme to the membrane by its association with the PDGFR is not sufficient to activate the enzyme.[19] Ras must be activated, which directly binds to the catalytic subunit of PI3K,[20,21] and provides the necessary second input for activation in growth factor-stimulated

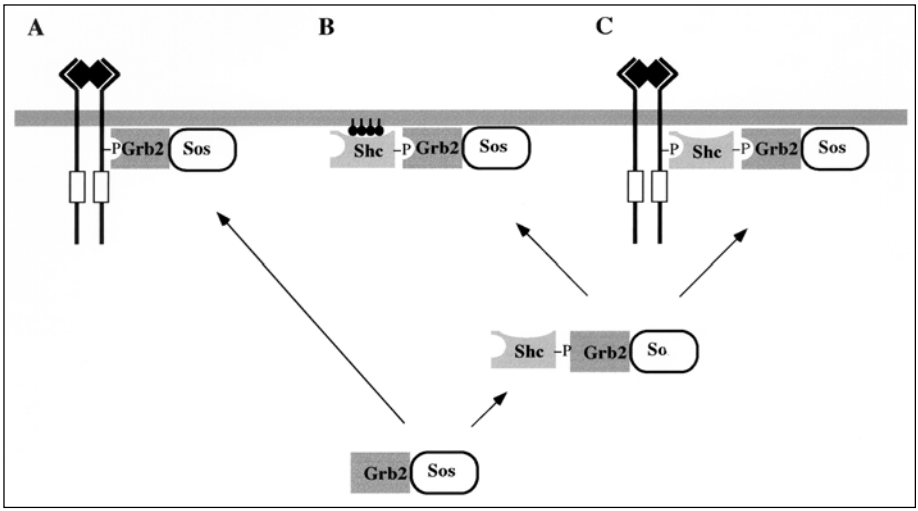

Figure 2. There are multiple ways to recruit Sos to the membrane. Grb2 constitutively associates with Sos and mediates its recruitment to the membrane in one of three ways. (A) The SH2 domain of Grb2 binds directly to a tyrosine phosphorylated receptor. (B/C) The growth factor promotes tyrosine phosphorylation of Shc and hence it association with Grb2 via the SH2 domain of Grb2. The resulting trimer is recruited to the membrane in one of two ways. The SH2 domain of Shc mediates binding to a tyrosine phosphorylated receptor (C). Alternatively, the PTB/PH domain of Shc interacts with lipids in the membrane and hence translocates the Shc/Grb2/Sos complex to the membrane. These membrane lipids include products of PI3K, which is activated in response to growth factor stimulation. The three recruitment scenarios are not mutually exclusive, and may be occurring simultaneously in growth factor-stimulated cells. As noted in Figure 1, the functional consequence of recruitment of Sos to the membrane is activation of Ras.

cells (Fig. 3). PI3K is being increasingly appreciated as a family of enzymes instead of a single entity, and the individual members of the PI3K family may have unique functions in promoting growth factor-dependent responses.[22,23]

Tyrosine phosphorylation of signaling enzymes is an additional component of their activation in growth factor-stimulated cells. While some of these proteins are direct substrates of the receptor, they may also be phosphorylated by the kinases activated by the receptor. For instance, Src family kinases (SFKs) are activated in PDGF-stimulated cells,[24-26] and they phosphorylate many proteins, including phospholipase Cγ (PLCγ).[27] PDGF-dependent tyrosine phosphorylation of PLCγ is dramatically compromised in cells expressing PDGFRs that fail to activate SFKs.[28,29] In contrast, comparable PDGF-dependent tyrosine phosphorylation of PLCγ was observed in cells that lacked SFKs versus cells in which Src had been reexpressed.[30] Tyrosine phosphorylation of signaling enzymes may proceed via more than one PDGF-activated kinase.

Converting Enzymology into a Cellular Response

In comparison to the first three steps of growth factor-dependent signaling, our understanding of how these steps culminate in cellular responses is very limited. The eventual consequence of exposing cells to growth factors such as PDGF can be cell movement, proliferation, differentiation or protection from death. How the cell selects amongst these possible responses, and the alteration in the signaling cascades necessary to mediate the appropriate response remains poorly understood. However, significant progress has been made in identifying which of

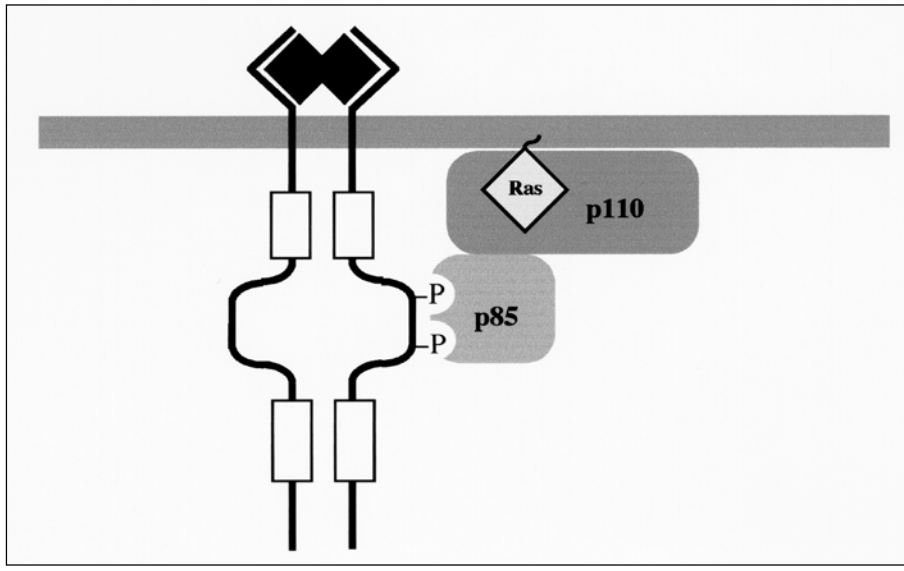

Figure 3. Activation of PI3K in PDGF-stimulated cells. Two events are required for activation of PI3K in response to PDGF. One is translocation of PI3K from the cytosol to the membrane. Autophosphorylation of the receptor's kinase insert creates binding sites for PI3K, and the SH2 domains of the p85 subunit of PI3K stably associate with the receptor. The second event is accumulation of activated Ras, which is described in Figures 1 and 2. Active PI3K phosphorylates PI-4-P and PI-4,5-P2 to generate PI-3,4-P2 and PI-3,4,5-P3, respectively, which are potent second messengers that engage a variety of signaling cascades. The figure depicts the complex that results from these events.

the many signaling enzymes are required for cellular responses such as mitogenesis. Using a variety of approaches, most investigators conclude that PI3K and to a lesser extent PLCγ are contributing to PDGF-dependent mitogenesis.[15,16,31,32] Furthermore, these two enzymes appear to be functionally redundant, as PDGF-dependent DNA synthesis can be rescued in a mitogenically incompetent receptor mutant when either the PI3K or PLCγ/PKC pathway is activated.[33] Such findings suggest the existence of a common mitogenic signaling cascade that can be accessed by various signaling enzymes. This issue will be further addressed below.

p53

p53 is a multifunctional transcription factor that is mutated in over 50% of all human tumors. It makes a major contribution to regulating cell cycle progression, apoptosis and senescence.[34-37]

Growth factor-dependent transition through G1 appears to involve inactivation of p53 activity. Many ways control p53, one of them includes Arf, which negatively regulates Mdm2, which in turn negatively regulates p53.[38] How signaling cascades interface with this layer of p53 regulation is being actively investigated. For instance, growth factors elevate Mdm 2 levels,[39,40] which would be predicted to decrease p53 activity. A series of "genetic" studies have implicated Src and c-Myc as conduits to p53. In cells that have been microinjected with neutralizing Src antibodies, PDGF fails to promote the G0 to S transition.[41] If the cells lack p53, however, neutralizing Src activity no longer blocks PDGF-dependent cell cycle progression.[42] Consequently, Src may function to depress p53 activity. This pathway may also involve c-Abl, as recent studies indicate that Src activates c-Abl in PDGF stimulated cells.[43]

Finally, expression of c-Myc has been shown to rescue PDGF dependent G0 to S transition in cells that have been injected with neutralizing Src antibodies.[44] This finding has led to the idea that Src functions to activate c-Myc,[45] and this may be somehow related to p53. However, the c-Myc rescue may not be specific to the p53 pathway, since c-Myc rescues PDGF-dependent DNA synthesis in cells expressing dominant negative Stat 3,[46] or overexpressing c-Cbl.[47] In summary, the pathways by which growth factors modulate p53 activity are being investigated, and some of the likely players have been identified. The total number of pathways that lead to p53, the potential cross-talk between them, and the identity of their members will require additional investigation.

Growth Factors Are Not the Only Extracellular Cue Needed for Cell Proliferation

Integrins

Integrins are a family of cell surface receptors consisting of heterodimers between α and β subunits that mediate attachment of cells to extracellular matrix (ECM) proteins such as fibronectin and collagen.[48,49] The intracellular domains of integrin molecules not only physically link the plasma membrane to the cytoskeleton, but also initiate signaling cascades. Evidence that integrins synergize with growth factors to drive a mitogenic response includes the observations that integrins and activated receptor tyrosine kinase coprecipitate.[50] In addition, the nature of the ECM onto which cells are plated (and hence the types of integrins that are engaged) can enhance or diminish the mitogenic potency of growth factors.[50,51] Finally, compromising the contribution of integrins by suspending a population of adherent cells alters some of the signaling pathways triggered by growth factors, and prevents cell cycle progression.[48,49,52] Additional studies have identified Rho and Rac as potential intracellular effectors by which integrins modulate cell cycle progression.[53,54] Thus the combined inputs of integrins and growth factors are required for cell cycle progression.

Cell Shape

Cell cycle progression is also controlled by cell shape and cytoskeletal tension.[55] Under conditions where the growth factor and total cell-ECM contact is kept constant, cell shape determines whether the cells do or do not move into S phase.[56,57] These studies showed that if cells were prevented from spreading, they failed to make the G1/S transition, despite engagement of integrins and the presence of growth factors. The following types of observations support the idea that cytoskeletal tension is also a regulator of cell cycle progression. Cell proliferation within a tissue is localized to regions where the ECM of the basement membrane is thinner than the surrounding regions, and the change in ECM content alters the tensional force on cells.[56-59] In addition, pharmacological disruption of the cytoskeleton (and hence the tractional force) prevents cells from moving into S phase. Finally, activation of Rho A, a small GTPase protein that modulates the integrity of the cytoskeleton, promotes degradation of $p27^{Kip1}$ and eliminates the anchorage requirement for S phase entry.[60,61] These findings demonstrate that cell proliferation is not only regulated by growth factors and the ECM. The shape of the cell and cytoskeletal tension are also important variables, and $p27^{Kip1}$ is at least one of the points at which they interface with the cell cycle program. The cells shape and tractional force variables may be particularly relevant to in vivo settings as compared with tissue culture system that are usually used to study regulation of cell proliferation.[55]

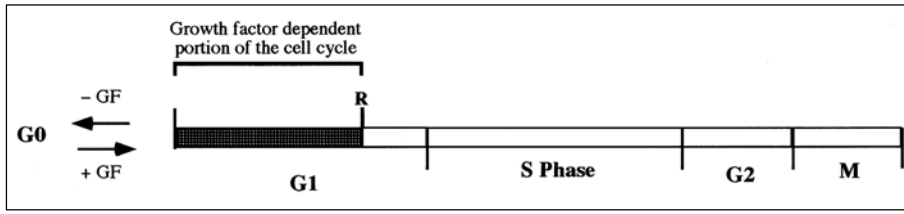

Figure 4. Only a small portion of the cell cycle is regulated by growth factors. Cells that have been deprived of serum or growth factors exit the cell cycle and enter into the G0 state. Growth factors promote exit from G0, and cells will commit to one round of the cell cycle if growth factors are present up to R. An important component of R is phosphorylation of pRB, which is further outlined in Figure 5. Once past R, most cells will continue through the other stages of the cell cycle, even if the growth factor is removed from the culture medium.

The Cell Cycle

The G0 to S Interval Is the Only Portion of the Cell Cycle That Is Regulated by Growth Factors

When plated at low cell density in serum-containing medium, cultured cells move through 4 phases of the cell cycle: G1, S, G2 and M (Fig. 4). Each of these phases is regulated by the coordinated action of kinases and proteases.[62,63] When deprived of serum, cells continue to cycle until they complete mitosis, whereupon they exit into the G0 state.[64,65] These cells can be reintroduced into the cell cycle by the re-addition of serum or purified growth factor. The mitogen must be present until R, which is several hrs prior to the transition between G1 and S.[64,66] Thus in serum-deprived cells, all of the growth factor-stimulated events that are necessary for completion of one round of the cell cycle occur before R (Fig. 4). Furthermore, growth factors are not needed at later times to complete the other stages of the cell cycle.

Figure 5 outlines the cell cycle events that constitute the G1 cell cycle program. Phosphorylation of the retinoblastoma (pRB) protein is the current molecular definition of R.[66] At least two classes of G1 cyclin-dependent kinases (CDKs) collaborate to fully phosphorylate pRB, which results in the release of the E2F family of transcription factors.[67] This class of transcription factors initiates subsequent events necessary for transition through the other phase of the cell cycle, even in the absence of serum. Consequently, the mitogenic side of growth factors is intrinsic to their ability to promote phosphorylation of pRB.

Elements of the Cell Cycle Program That Are Regulated by Signaling Enzymes

Growth factors promote phosphorylation of pRB by regulating the activity of the G1 CDKs. This involves promoting the synthesis and stability of cyclin subunits, as well as decreasing the levels of CDK inhibitors (Fig. 5B). For instance, growth factor-dependent activation of the Ras/Erk pathway increases cyclin D1 mRNA.[68-74] Furthermore, the PI3K/Akt pathway stabilizes the cyclin D1 proteins. At least in some cell types, activation of Akt inhibits glycogen synthase kinase 3 β (GSK3β)-dependent phosphorylation of cyclin D1, and thereby prevents its degradation via the proteasomal pathway.[75-78] Others have also implicated PI3K/Akt in cyclin D1 accumulation, although the mechanism of action does not appear to be in stabilization of the cyclin D1 protein, and appears to involve transcriptional activation of cyclin D1.[79] Accumulation of cyclin D1 results in the assembly of cyclin D1/CDK4,6 complexes.[70] Growth factor-dependent elimination of CDK inhibitors such as $p27^{Kip1}$ proceeds through a PI3K dependent pathway and is essential for transition through G1 and into S phase.[79-81]

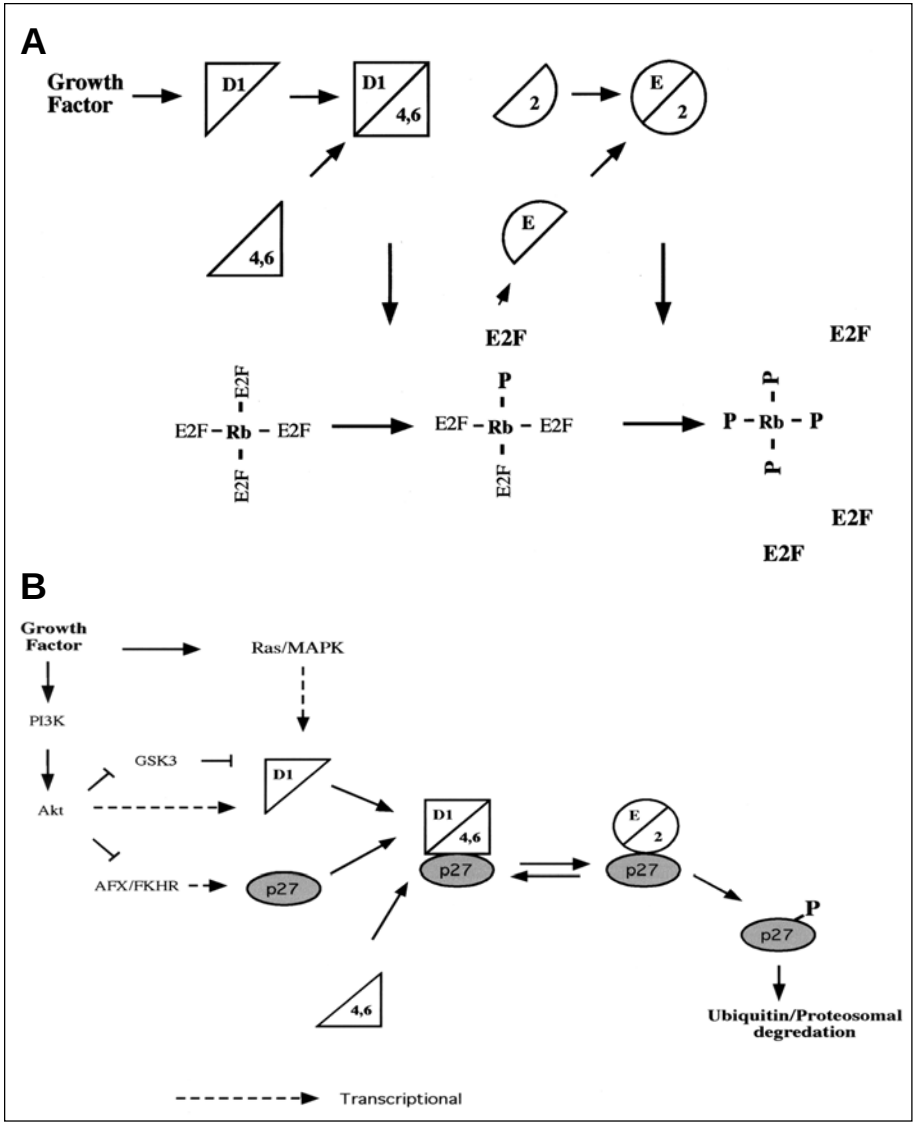

Figure 5. The G1 cell cycle program. A. Full phosphorylation of pRB requires the coordinated action of two CDKs, and results in the release of the E2F family of transcription factors.Growth factors promote the accumulation of cyclin D1, which forms a complex with either CDK4 or 6. The cyclin D1/CDK4,6 complex phosphorylates pRB releasing a small amount of E2F, which in turn drives the formation of cyclin E. The cyclin E/CDK2 complex further phosphorylates pRB, releasing more E2F. The E2F family of transcription factors promotes transcription of genes that initiate the transition into S phase.
B. Role of $p27^{Kip1}$ in the cell cycle program. $p27^{Kip1}$ and $p21^{Cip1}$ (not shown) promote assembly of the cyclin D/CDK4,6 complex. $p27^{Kip1}$ inhibits the kinase activity of both of the cyclin/CDK complexes, and appears to be more potent towards cyclin E/CDK2. There are at least three ways by which $p27^{Kip1}$ is neutralized. Growth factors suppress the synthesis of $p27^{Kip1}$ protein; cyclin E/CDK2 phosphorylates $p27^{Kip1}$ and targets it for degradation; cyclin D/CDK4,6 sequester $p27^{Kip1}$. GSK3 is glycogen synthase kinase 3 β; AFX/FKHR is the forkhead transcription factor.

Recent studies indicate that Akt acts downstream of PI3K to phosphorylate members of the forkhead family of transcription factors such as AFX/FKHR.[82-86] When phosphorylated these transcription factors move out of the nucleus and thereby ceases driving transcription of $p27^{Kip1}$.[87] Hence growth factors promote CDK activity by increasing levels of cyclins and decreasing the levels of CDK inhibitors.

Active cyclin D1/CDK4,6 partially phosphorylates pRB, which begins to release the E2F family members (Fig. 5A). Free E2F promotes the transcription and consequent accumulation of a second cyclin, cyclin E, which couples with the CDK2 kinases. The appearance of cyclin E/CDK2 has at least three functional consequences (Fig. 5B). Firstly, it acts in collaboration with cyclin D1/CDK4,6 to titrate $p27^{Kip1}$ levels. Secondly, cyclin E/CDK2 phosphorylates $p27^{Kip1}$ and hence targets it for ubiquitination and degradation via the proteasome.[88,89] Thirdly, cyclin E/CDK2 further phosphorylates pRB, which fully activates the E2F family (Fig. 5A).

The role of $p27^{Kip1}$ and a second CDK inhibitor, $p21^{Cip1}$, has become more complicated with the appreciation of an additional function for these proteins. They not only block CDK activity, but $p27^{Kip1}$ and $p21^{Cip1}$ are also instrumental in the assembly of the cyclin D1/CDK complexes [90-91] (Fig. 5). Furthermore, $p21^{Cip1}$ and $p27^{Kip1}$ are components of the active cyclin D1/CDK4,6 enzyme.[90] Finally, there appears to be a difference between cyclin D1/CDK4,6 and cyclin E/CDK2 in the way they are regulated by $p27^{Kip1}$. In contrast to the readily detectable kinase activity of the cyclin D1/CDK4,6/ $p27^{Kip1}$ complex, when $p27^{Kip1}$ joins the cyclin E/CDK2 complex, it extinguishes kinase activity.[92,93] Thus the CDK inhibitors promote activation of cyclin D1/CDK4,6, and inhibit the activity of cyclin E/CDK2. Further studies will be required to resolve the apparent paradox regarding how the CDKs both promote and inhibit cyclin D1/CDK4,6 activity.[94]

In summary, activation of the Ras/Erk or PI3K/Akt pathways results in an increase in cyclin D1 mRNA, and some investigators find that the PI3K/Akt pathway stabilizes the cyclin D1 protein. In addition, growth factors suppress $p27^{Kip1}$ levels, in a pathway that also involves PI3K. Thus cyclin D1 and $p27^{Kip1}$ are two points in cell cycle program at which growth factor-stimulated signaling makes input. Since full phosphorylation of pRB, i.e., passing R, requires the coordinated input of several distinct components of the cell cycle program, there may be additional points of the cell cycle program at which growth factor-dependent signaling make other essential contributions.

Most of the Well-Studied Growth Factor-Initiated Signaling Events Occur Many Hours Before the Cell Cycle Program

Growth Factor-Stimulated Signaling Is Transient

In acutely-stimulated cells there are two themes to the signaling events: phosphorylation/dephosphorylation and changes in the subcellular location. For instance, tyrosine phosphorylation of the PDGFR at the appropriate tyrosine residues enables stable association with PI3K.[16,95] While this relocates PI3K from the cytoplasm to a juxtamembrane location, the location of its lipid substrate, accumulation of active Ras is also needed for activation of PI3K [19-21] (Fig. 3). PI3K generates second messengers (PI-3,4-P2 and PI-3,4,5-P3), which are the activators for downstream signaling enzymes such as Akt and PKC family members.[22,96] These ser/thr kinases are some of the enzymes capable of relaying the mitogenic signal along a cascade that appears to be part of the network that integrates signals, which emanate from integrins and the sensors of cell shape and cytoskeletal integrity.

While the exact nature of this overall signaling network is far from understood, it is clear that the initial phase of growth factor-stimulated signaling events does not persist much longer than 60 minutes. For instance, PI3K products accumulate within minutes of PDGF-stimulation,

and then return to near basal levels by 30 min.[97-99] There appears to be a variety of reasons why signaling subsides, one of which relates to the half-life of the growth factor receptor. One of the proteins that is phosphorylated in response to growth factors is c-Cbl, a protein that promotes internalization and/or degradation of growth factor receptors.[100] Enzymes such as PTEN, a phosphatase capable of dephosphorylating and hence metabolizing the PI3K lipid products, may also contribute to the decline of cellular PI3K lipid products.[101] Other well-characterized mechanisms to extinguish signaling include the rapid expression of new genes that counteract the signaling enzymes. MKP-1 is a phosphatase that dephosphorylates and hence inactivates Erk family members.[102] In resting cells MKP-1 levels are low, and then rise quickly following mitogenic stimulation.

In summary, growth factors trigger a rapid burst of signaling events that subsides even in the continuous presence of growth factor. Receptor internalization and degradation, as well as the appearance of enzymes, which antagonize the signaling enzymes, are some of the ways in which the cell silences the growth factor-initiated signaling cascade.

How Do Growth Factor-Stimulated Signaling Events Engage the Cell Cycle Program?

If the first wave of growth factor-dependent signaling is complete within 60 min, then what triggers the cell cycle program, which begins roughly 9 hrs after exposure to PDGF? Since the early signaling events induce the expression of many new genes, including those that are involved in cell proliferation, perhaps the products of these genes are responsible for engaging the cell cycle program. If this were indeed the case, then exposure to growth factor for 1-2 hrs, which is sufficient to induce the immediate early genes, would also be sufficient to drive cells into S phase. However, fibroblasts require 8-10 hrs of continuous exposure to growth factor to get past R.[64,103,104] Hence the early burst of signaling is insufficient for cell cycle progression, and there must be additional inputs that the growth factor makes at latter time points. Insight into this long-standing question has come from a number of labs demonstrating that there are requirements for signaling enzymes and/or signaling events well beyond the well-studied early burst of signaling.

Growth Factor-Stimulated Signaling Beyond the First 60 Minutes

Microinjection Studies Indicate that Signaling Enzymes Are Needed Well Beyond the First 60 Minutes

One approach to investigate the importance of a signaling enzyme for growth factor-dependent mitogenesis is to eliminate it by microinjection of a neutralizing antibody directed against the signaling enzyme, and assay the effect on S phase entry. The Stacey lab used this approach, and learned that activated Ras is required for entry into S phase.[105] These studies were performed by pre-injecting cells with the antibody, and then stimulating with the mitogen. By injecting the antibody after exposing cells to the growth factor it has been possible to assess when the signaling enzyme is contributing to mitogenic signaling. Blocking Ras, SHP-2 or PI3K hours after the initial wave of growth factor-dependent signaling had occurred, prevented growth factor-dependent entry into S phase.[31,32,106-108] These studies strongly suggested that signaling enzymes are important for mitogenic signaling at times beyond the initial burst of signaling.

Two Waves of Signaling in Cells Treated Continuously with Growth Factors

To directly investigate the idea that signaling is occurring at later time points, a number of investigators began to monitor signaling events in cells that had been treated with growth

factors for longer times. For instance, several groups have reported biphasic activation of Ras in serum stimulated NIH 3T3 cells.[79,109] The level of active Ras peaks within 10 or 30 min, recedes, and then peaks again 2-6 or 2-4 hrs later. Similarly, PDGF triggers two waves of PI3K and PKC activity in HepG2 cells, an early and then a late phase; the late phase being 3-7 hrs after the addition of growth factor.[99,110] These studies demonstrate that there are two waves of activity for a number of signaling systems, and raise a number of interesting questions.

For instance, how are these two waves of activity regulated? The first wave of activity has been studied at length, as it is the one observed in acutely stimulated cells. As outlined in the sections above, there is a wealth of information regarding the mechanism by which signaling enzymes such as Ras and PI3K are activated in acutely stimulated cells. In contrast, virtually nothing is known regarding the molecular events by which the second wave of activity appears. Whether the mechanisms by which the signaling enzymes are activated during the first and second wave of signaling are comparable await further investigation.

The Second Wave of Signaling Is Required for Cell Proliferation

An additional question that arises from the observation that there are two waves of enzymatic activity is the relative contribution of each wave to growth factor-driven mitogenesis. The second wave of signaling is required for S phase entry, at least in the case of Ras and PI3K, since injecting neutralizing antibodies directed against these proteins blocked cell cycle progression. Additional approaches have also found that the second wave of PI3K, PKC and Ras activity is essential for cells to respond mitogenically to growth factors.[81,99,107,110] These findings indicate that the second wave of signaling is essential for growth factor-dependent mitogenesis.

For some of the signaling enzymes the first and second waves of signaling make unequal contributions to the mitogenic response. In the case of PI3K, and certain PKC family members, only the second wave of activity was required for PDGF-dependent entry into S phase.[99,110] The addition of pharmacological inhibitors at times corresponding to the second wave of activity attenuated PDGF-dependent DNA synthesis. The inhibitors had no effect if they were used to block only the first wave of PI3K or PKC activity. Similarly, adding synthetic PI3K lipid products or diacylglycerol (DAG), an activator of certain PKC family members, rescued PDGF-dependent DNA synthesis, but only when they were added at times corresponding to the second wave of activity. Adding the PI3K lipid products or DAG simultaneously with PDGF failed to promote PDGF-dependent DNA synthesis in this system. Hence, although PI3K and PKCs are activated during the first wave of PDGF-induced signaling, their activation at this time is dispensable for the DNA synthesis response. It is likely that they are contributing to other PDGF-dependent cellular responses such as chemotaxis and survival.[111-113]

The "Two Wave" Hypothesis for How Signaling and Cell Cycle Progression Are Linked

Growth Factor-Dependent Signaling Is Not Needed Continuously during the Interval between G0 and R

Because the initial wave of signaling occurs so much in advance (7 hrs) of even the first element of the cell cycle program, these early signaling events do not appear to be directly responsible for engaging components of the cell cycle program. In contrast, the second wave of signaling appears to overlap with the cell cycle program, and hence may be directly triggering the cell cycle program. This hypothesis has been difficult to test because the second wave of signaling requires prolonged exposure to PDGF, which probably triggers events other than those required for cell cycle progression. We have recently employed a discontinuous stimulation

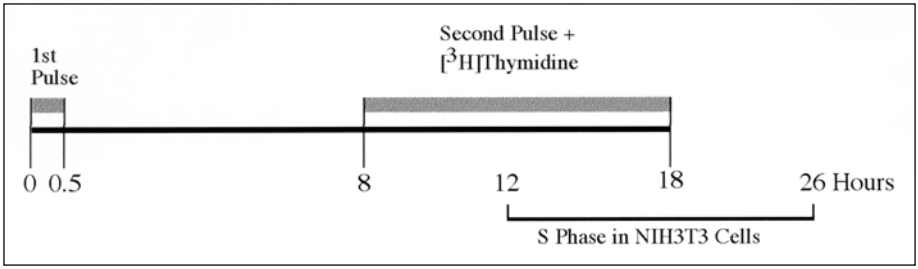

Figure 6. The discontinuous stimulation assay. Serum-arrested NIH 3T3 cells were pulsed with PDGF for 30 min, the cells were then acid washed and placed into medium containing 0.1% FBS. 7.5 hrs later PDGF and [^{3}H]thymidine were added and the cells were harvested at the 18 hr time point. S phase, as measured by an increase in the incorporation of [^{3}H]thymidine is between 12 and 26 hrs in these cells.

assay (Fig. 6) to evaluate the possibility that a late phase of signaling is responsible for engaging the cell cycle program.[114]

Two 30 min pulses of PDGF are sufficient to drive NIH 3T3 cells into S phase and through the rest of the cell cycle. Furthermore, the kinetics of S phase entry as well as events of the cell cycle program proceeded comparably in cells treated continuously or discontinuously with PDGF. The first pulse defined the start of the experiment, and the ideal time for the second pulse was 8 hrs. Importantly, the first pulse was insufficient to initiate the cell cycle program, whereas that second pulse of growth factor rapidly engaged the cell cycle program, i.e., cyclin D1 protein was detectably elevated within one hr of the second pulse. Hence the early burst of signaling made the cells able to engage the cell cycle program, which was triggered by the second pulse of PDGF. As illustrated in Figure 7, we are proposing the terms early G1 ($G1_E$), and late G1 ($G1_L$) for these portions of G1.

Different Sets of Signaling Enzymes Mediate Progression through $G1_E$ and $G1_L$

The subdivision of the G0 to S interval is also supported by the finding that different sets of signaling enzymes mediate transition through $G1_E$ and $G1_L$.[114] Of the many signaling events triggered by the first pulse of PDGF, activation of MEK and elevation of c-Myc were sufficient for transition through $G1_E$. In contrast, synthetic PI3K lipid products failed to drive cells through $G1_E$, but were sufficient for transition through $G1_L$. These findings indicate that traversing the two segments of the G0 to S interval requires nonidentical sets of signaling enzymes. Finally, transition through $G1_E$ is a prerequisite for engaging the cell cycle program, which is the consequence of subsequent exposure to growth factor.

A Common Signaling Cascade Is Used by Many Mitogens

Many agents are mitogenic, and while they interact with specific and unique cell surface receptors, it is possible that they eventually engage a common cascade to promote cell cycle progression. This idea has been investigated with the discontinuous stimulation assay described above. Six mitogens were tested for their ability to substitute for PDGF during the first or second pulse, i.e., to drive cells through $G1_E$ or $G1_L$.[114] Four of the six agents (fetal bovine serum [FBS], FGF, PDGF and lysophosphatidic acid [LPA]) were completely interchangeable. Any of the four agents given at the first pulse, followed by any one of the four in the second pulse drove cells into S phase. These findings imply that there is a common signaling cascade that can be accessed by a variety of receptor tyrosine kinases, as well as G protein coupled receptors.

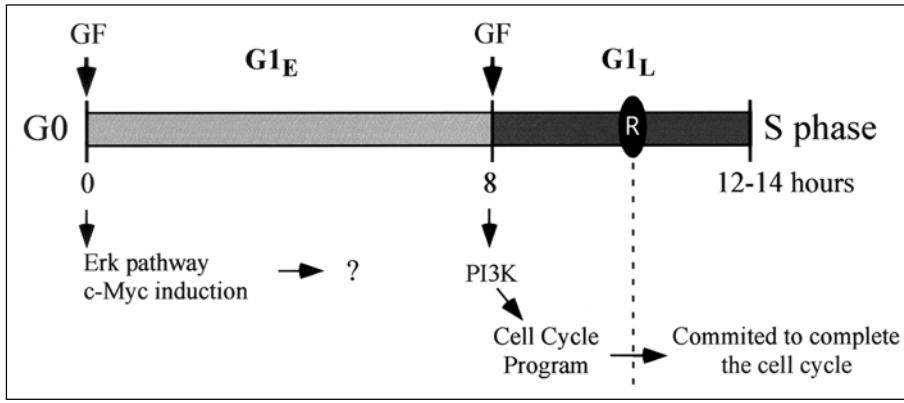

Figure 7. The two-wave hypothesis for how signaling and cell cycle progression are linked. Exposure of quiescent (G0) cells to growth factors initiates many signaling events. Of these, activation of Erk and elevation of c-Myc are sufficient to drive cells out of G0 and through the early portion of G1 ($G1_E$). Further progression through G1 requires a second input of growth factor. The timing of this requirement overlaps with the second wave of signaling, and initiates the cell cycle program. This occurs in the late phase of G1, and is termed $G1_L$. PI3K is one of the signaling enzymes that are activated at this later time and capable of engaging the cell cycle program. As outlined in Figure 5, the cell cycle program results in phosphorylation of pRB, transition past R and commitment to one round of the cell cycle. At least some of the events that occur in $G1_L$ have been well defined, i.e., the cell cycle program. In contrast, the molecular events that are necessary for transition through $G1_E$ are just beginning to be identified.

Unlike the four mitogens described above, EGF and insulin failed to drive cells through $G1_E$. However, these agents were biologically active, as EGF or insulin promoted progression through $G1_L$. Cells that had been brought through $G1_E$ by a pulse of FBS, bFGF, LPA or PDGF were driven into S phase when EGF or insulin were used for the second pulse. Hence the cells have receptors for EGF and insulin, and these receptors access the necessary events to engage the cell cycle program and propel the cells through $G1_L$ and into S phase. The failure of EGF and insulin to promote transition through $G1_E$ could be because a 30 min pulse of these two growth factors triggers a much less robust activation of Erk and elevation of c-Myc, as compared with the four agents that drive cells through $G1_E$. These findings indicate that there is a common signaling pathway that is utilized by many different agents. Furthermore, we predict that any agent capable of activating Erk and elevating c-Myc during the first wave of signaling, followed by an elevation of PI3K products 8 hrs later will be sufficient to drive NIH 3T3 cells into S phase.

Several lines of evidence indicate that the signaling pathways discussed above are not the only ones that are capable of engaging the mitogenic cascade. Cells that are nullizygous for *c-myc* are viable, although they proliferate more slowly than control cells.[115] Similarly, DAG is as effective as PI3K lipid products in rescuing PDGF-dependent DNA synthesis when added to cells at times that appear to correspond to $G1_L$.[99,110] Hence it is likely that there will be additional enzymes identified that are capable of accessing this common mitogenic cascade.

Revisiting Competence and Progression

Using subsaturating concentrations of growth factors, Pledger, Stiles, Antoniades, and Sherr demonstrated that in Balb/c 3T3s cell cycle progression required the input of two different types of factors.[103,104,116,117] Growth factors such as PDGF or FGF made the cells competent, but did not drive them into S phase. A second class of growth factors was required for

progression of the competent cell into S phase. Once competent, the cells remained in this state for many hours, and such a cell entered S phase 12-14 hrs after the addition of a progression factor.[64] There are several differences between the competence/progression system and the discontinuous stimulation assay. Firstly, saturating concentrations of growth factor were used in the discontinuous stimulation assay. Under these conditions, PDGF alone is fully capable of driving the NIH 3T3 cells into S phase. Secondly, cells that have received the first pulse of PDGF and have completed $G1_E$ have a relatively narrow window (less than four hrs) during which they must receive the second pulse of PDGF in order to continue through G1. Finally, cells enter S phase 4-6 hrs after the second pulse of PDGF. We speculate that competence is a subset of those events that drive cells through $G1_E$ and that a progression factor is required for completion of $G1_E$ and progression through $G1_L$. If this interpretation is correct, the availability of these two systems will help to further define the events that must take place in order for a cell to exit G0, enter $G1_E$ and approach $G1_L$.

Summary

There are three central ideas contained within this review. Firstly, growth factor-stimulated signaling is not restricted to a 30-60 min window, but occurs at much later time as well. Secondly, the second wave of signaling overlaps temporally with the cell cycle program and may be directly responsible for engaging it. Thirdly, the G1 to S interval appears to encompass two distinct phases of the cell cycle, during which the coordinated activation of distinct sets of signaling enzymes drive cell cycle progression. Each of these concepts is likely to initiate new investigation and hence provide additional insight into the fundamental question of how growth factors drive cell proliferation.

Acknowledgements

We would like to thank Ashley Seifert, Egle Balciunaite, Mark Nickas and Yasushi Ikuno for providing critical input.

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Chapter 12

G1 Phase Progression and Apoptosis

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Abstract

Proliferation and programmed cell death (apoptosis) exert a concerted action in modelling the organism during normal development and in maintaining tissue homeostasis. Both cell cycle progression and apoptosis biochemistry and molecular biology have been widely studied and characterized during the last ten years. Now, it is evident that each cell is able to integrate both extra- and intracellular survival and death signals thereby controlling its own growth rate or, when harmful signals prevail, inducing its self-destruction. Mainly, this is achieved because of multiple interactions between the pRB (retinoblastoma family proteins) pathway, whose main function is the control of G1 to S progression, and the p53 pathway, which guards against genomic instability by inducing both arrest of the cell cycle and apoptosis. Moreover, it has been recently shown that E2F1, the main target of pRb/p105 growth suppressive function, plays a dual role, on the one hand by inducing S-promoting genes transcription and on the other by directly influencing apoptosis execution.

Deregulated cell proliferation, together with the compensatory suppression of apoptosis needed to support it, are the common and mandatory conditions for neoplastic progression. Therefore, the key proteins controlling these two processes are elective targets for cancer therapy.

Introduction

Individual cells face three choices: to divide (proliferation), to specialize (differentiation) or to commit suicide (apoptosis). The stability of the body is maintained by signals that control the life and death of each cell. Control of life and death is vital during development and in complex multicellular networks such as the immune system and the nervous system, where communication between cells is crucial. Although the process of cell renewal and cell death appears to be opposing and mutually contradictory, substantial evidence now indicates that the two are linked. In multicellular organisms, cell proliferation and death must be regulated to maintain tissue homeostasis. Many observations suggest that this regulation may be achieved, in part, by coupling the process of cell cycle progression and programmed cell death by using and controlling a shared set of factors. An argument in favor of the link between the cell cycle and apoptosis arises from the accumulating evidence that manipulation of the cell cycle may either prevent or induce an apoptotic response. This linkage has been recognized for tumor suppressor genes such as p53 and pRb/p105, the dominant oncogene c-Myc and several cyclin-dependent kinases and their regulators. These proteins that function in proliferative pathways may also act to sensitize cells to apoptosis. Indeed, unregulated cell proliferation can result in pathologic conditions, including neoplasias, if it is not countered by the appropriate cell death.

G1 Phase Progression, edited by Johannes Boonstra.

Apoptosis: An Overview

Apoptosis or programmed cell death (PCD) is an evolutionally conserved process that removes damaged or unwanted cells. The ability to ablate cells, both during animal ontogeny and for maintenance of tissue architecture, is as essential as are the abilities to replicate and differentiate them. Many human diseases have been associated with either increased apoptosis (such as AIDS and neurodegenerative disorders) or decreased apoptosis (such as cancer and autoimmune disorders). Eukaryotic cells that die and are removed in a programmed way or in response to harmful stimuli undergo a stereotypical series of biochemical and morphological changes that ultimately lead to their clearance from the body without any inflammatory response. Any change in equilibrium from the inside (DNA damage, metabolic or cell cycle aberrations) or the outside (signals and receptors) irreversibly activates suicide. The overall of these signals ultimately converge onto mitochondria whose damage gives rise to the activation of proteins, such as the caspases, that can kill the cell. The cell, in turn, protects itself by producing antidotes such as caspase inhibitors and anti-apoptotic proteins.

Role of Apoptosis in Physiological Conditions

It is now clear that physiological cell death is an essential component of animal development, important for establishment and, in vertebrate at least, maintenance of tissue architecture. A general "modus operandi" of methazoan development is the over-production of excess cells followed by an apoptotic culling during later stages of development to match the relative number of cells of different types to achieve proper organ function. Thus, during animal development, numerous structures are formed that are later removed by apoptosis.

Apoptosis has been recognized as a prominent event during the development of the vertebrate nervous system. During embryogenesis, cell death has a morphogenetic function at various stages of the formation of the CNS: during the closure of the neural tube;[1] during the development of the mesencephalic region;[2] and in the process of negative selection of certain progenitor cells from inappropriate regions.[3] Later in the development, neurons generated in some areas of the nervous system may die as a result of limited availability of trophic factors or lack of synaptic inputs necessary to suppress the endogenous genetic death program (see for review ref. 4).

Apoptosis is the most common form of death in cells of the immune system. One of the unique features of the immune system is its specificity. The repertoire of T and B lymphocytes, initially built from randomly selected antibody and variable region genes of T-cell antigen receptors, is shaped by selection to cope, on the one hand, with the vast universe of antigens and, on the other hand, with the danger of autoimmunity.[5] Another distinct feature is its homeostatic control: after a clonal expansion phase, antigen-reactive lymphocytes must be titrated back until the pool of lymphoid cells reaches the baseline level again.[6] This is achieved by balanced fine-tuning between growth/expansion and death by apoptosis: generally, the immune system produces more cells than finally needed, and extra cells are eliminated by apoptosis. It is astounding how many different pathways immune cells can choose from to die. In principle, death can be by neglect when the antigen-specific receptors of the lymphoid cells are not stimulated or the lymphocytes are deprived of trophic cytokines. In a more active form, death can involve the death-receptor/death-ligand system.[7-9]

Cardinal Morphological Features of Apoptosis and Comparison with Necrosis

During recent years, it has become evident that there is a continuum, rather than a clear-cut difference between apoptosis and necrosis. Some features that were initially regarded to be specific for one form of cell death have been recognized to be common in both. Furthermore, cells can switch from one mode to another in response to varying intensities of the same insult and depending on the availability of energy substrates.[10]

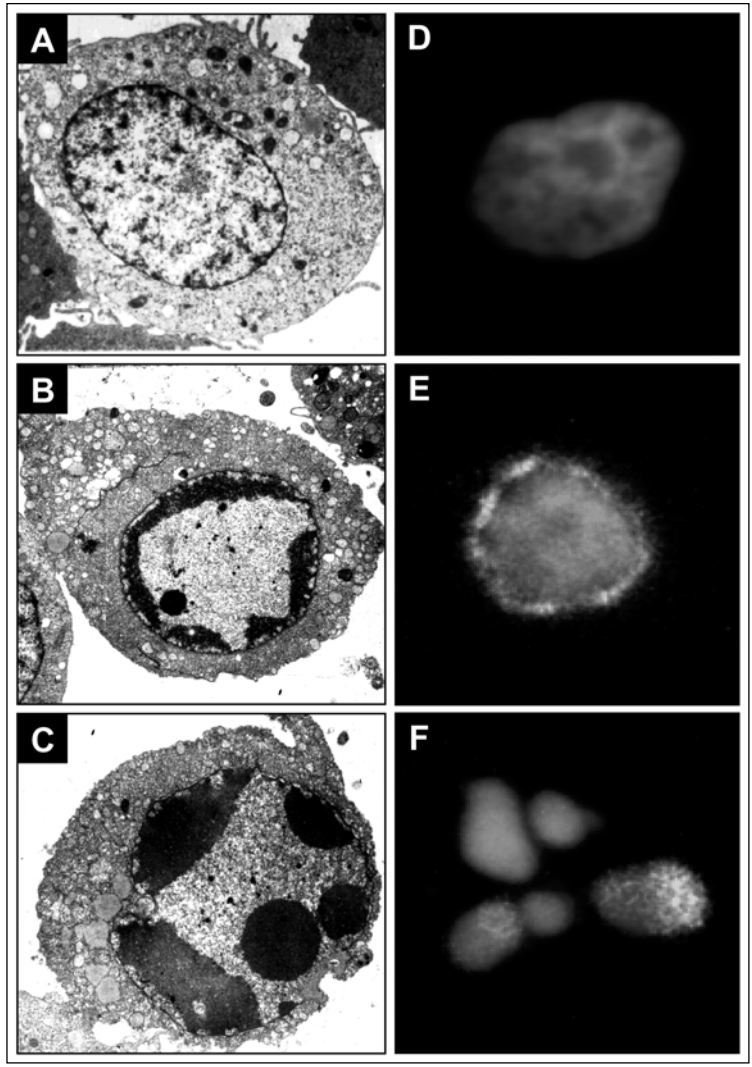

Figure 1. Ultrastructural (A,B,C) and confocal laser scanning microscopy (D,E,F) images of cycling and apoptotic cells. A and D: normal growing cells; B and E : early apoptosis; C and F: late apoptosis. Kindly provided by D. La Sala.

To exemplify it is possible to distinguish, at least, four death patterns:

Apoptosis is typically defined by stereotypic morphological changes, especially evident in the nucleus where the chromatin condenses to compact and apparently simple, globular, crescent-shaped figures.[11] Other typical features include phosphatidylserine exposure, cytoplasmic shrinkage, zeiosis and the formation of apoptotic bodies within the nucleus. The earliest definitive changes in apoptosis that have been detected by electron microscopy are compaction of the nuclear chromatin into sharply circumscribed, uniformly dense masses that abut on the nuclear envelope and condensation of the cytoplasm (Fig. 1B, E.). Continuation of condensation is accompanied by convolution of the nuclear and cellular outlines, and nucleus often breaks up

at this stage to produce discrete fragments. The surface protuberances then separate with sealing of the plasma membrane, converting the cell into a number of membrane-bounded apoptotic bodies of varying size in which the closely packed organelles appear intact; some of these bodies lack a nuclear component, whereas others contain one or more nuclear fragments in which compacted chromatin is distributed either in peripheral crescents or throughout cross-sectional area (Fig. 1C, F.). In tissues, apoptotic bodies are rapidly taken up by adjacent cells and degraded within lysosomes without any inflammatory response.

Apoptosis-like PCD is used to describe forms of PCD with chromatin condensation that is less compact/complete than in apoptosis and with the display of phagocytosis-recognition molecules before lysis of the plasma membrane. Most published forms of caspases-independent apoptosis fall into this class.[12]

Necrosis-like PCD is used to define PCD in the absence of chromatin clustering to speckles.[13] Varying degrees of other apoptosis-like features, including externalization of phosphatidylserine, might occur before lysis. Necrotic PCD usually involves specialized caspases-independent signaling pathways.

Accidental necrosis/cell lysis is the conceptual counterpart to PCD, as it is prevented only by removal of the stimulus. It occurs after exposure to high concentration of detergents, oxidants, ionophores or high intensity of pathologic insult. Necrosis is often associated with cellular organelle swelling and devoid of zeiosis. The necrotic tissue morphology is, in large part, due to events occurring after lysis of the plasma membrane.[10]

Apoptosis Triggering and Execution

Most of the morphological changes that were observed by Kerr et al [11] are caused by a set of cysteine proteases, which are activated specifically in apoptotic cells. These death proteases are homologous to each other and are part of a large protein family known as caspases. Caspases are present constitutively in all cells in an inactivated form. Caspase activation is the last and common consequence of molecular cascades signaling potentially harmful events from outside or inside the cell and it results from prevalence of pro-death onto pro-survival intracellular signaling with Bcl-2 protein family as arbiters.[14]

Extrinsic Signaling Pathways: Sensing Death Stimuli at Plasma Membrane

Two major kinds of external signals may activate the apoptotic machinery: 1) binding of specific ligands to the so-called "death-receptors"; 2) withdrawal of survival factors that would maintain the apoptotic machinery silenced by inducing the expression of pro-survival factors. Binding of death ligands to death-receptors can activate death caspases within seconds and cause demise of the cell within hours. Death-receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is defined by similar, cysteine-rich extracellular domains.[15] The death-receptors contain in addition a homologous cytoplasmic sequence termed the "death-domain" [16] that enables death-receptors to engage the cell's apoptotic machinery. The best characterized among death-receptors are CD95 (also called Fas or Apo-1) and TNFR1.[15,17]

CD95 and its ligand (CD95L) play an important role mainly in three types of physiological apoptosis: i) peripheral deletion of activated mature T cells at the end of the immune response; ii) killing of targets such as virus-infected cells or cancer cells by cytotoxic T cells and by natural killer cells; iii) killing of inflammatory cells at "immune-privileged" sites such as the eye.[17] The oligomerization, most probably the trimerization, of CD95 is required for transduction of the apoptotic signal. A complex of proteins associates with activated CD95.[8,18] This death-inducing signaling complex (DISC) forms within seconds of receptor engagement. First, the adaptor FADD (Fas associated death domain protein) binds via its own death domain to the death domain in CD95. FADD also carries a so-called death-effector domain (DED) and

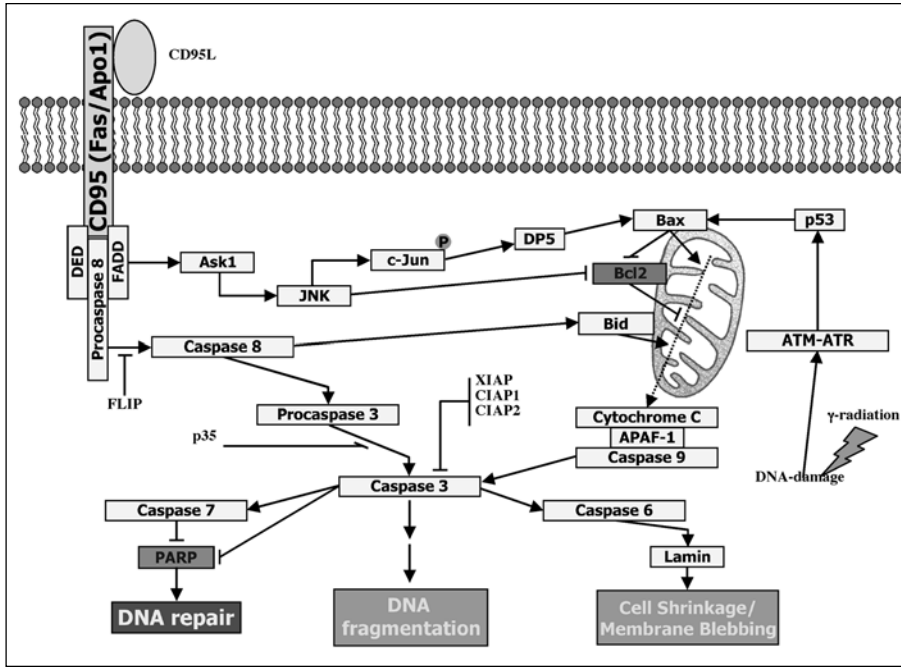

Figure 2A. CD95/ Fas-APO1 death receptor signaling. The FasL binding induces the CD95 receptor trimerization and recruits initiator caspase-8 via the adapter protein FADD. Activated caspase-8 stimulates apoptosis via two parallel cascades: it directly cleaves and activates caspase-3 and it cleaves Bid, a bcl-2 family protein. Truncated Bid translocates to mitochondria, inducing cytochrome c release, which sequentially activates caspase-9 and 3.

recruits the DED-containing procaspase-8 into the DISC. Next, procaspase-8 is activated proteolytically and active caspase-8 is released from the DISC into the cytoplasm in the form of a heterotetramer of two small subunits and two large subunits.[19] Active caspase-8 cleaves various proteins in the cell including procaspase-3, which results in its activation and the completion of the cell death program (Fig. 2A.).

TNF is a cytokine produced mainly by activated macrophages and T cells in response to infection.[20] TNF exerts its effects by binding to TNFR1 receptors, which are present on the surface of many different target cells. When occupied, these receptors recruit a cohort of intracellular 'adaptor' proteins, which cope the receptor to intracellular signaling pathways.[21] Pivotal in determining whether cells die or live in response to TNF, are the so-called INK and NF-κB signaling pathways.[22] Unlike CD95L, TNF rarely triggers apoptosis unless protein synthesis is blocked, which suggests the preexistence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressive proteins probably is controlled through NF-κB and JNK/AP-1, as inhibition of either pathway sensitizes cells to apoptosis induction by TNF.[23,24] The survival pathway that is recruited by TNF requires transcription factors of the NF-κB/Rel family. In resting cells, NF-κB is held captive outside the nucleus, and therefore away from its target genes, by proteins of the IκB family. Binding of TNF to its receptors initiates a signaling pathway that culminates in the phosphorylation of the IκB by the trimeric IκB kinase (IKK) complex. Phosphorylation marks IκB for degradation; this liberates NF-κB, which then heads for the nucleus to activate a program of gene expression.[25] NF-κB changes cell survival by switching on genes which damper pro-apoptotic

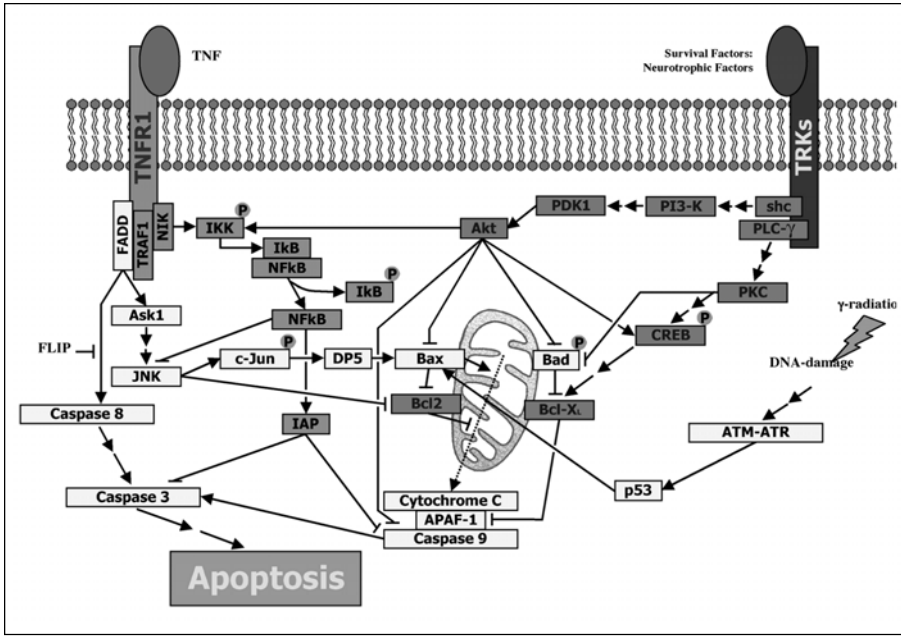

Figure 2B. TNFR pro- or anti-apototic signals and survival signal of TRK receptors. TNFR receptor promote apoptosis via the adaptor proteins TRAF1/FADD and the activation of caspase-8. Alternatively, apoptosis is inhibited via an adaptor protein NIK which activates NF-κB and induces survival genes including IAP. The survival signal requires the active inhibition of apoptosis, which is accomplished either by inhibiting caspases or by preventing their activation. The PI3K pathway, activated by many survival factors which bind to TRKs receptors, leads to activation to Akt, an important player in survival signaling. Activated Akt inhibits the pro-apoptotic bcl-2 family member Bad and directly inhibits caspase-9.

signals.[26] One of the ways in which NF-κB protects cells from apoptosis is by substantially blunting the JNK pathway (Fig. 2.).[27,28]

Interestingly, neurotrophic factors promote the survival of developing immature neurons, at least in part, by sharing the same pathway. Neurotrophins generally activate and ligate the Trk receptors (TrkA, TrkB and TrkC), which are cell-surface receptors with intrinsic tyrosine kinase activity. NGF induces the autophosphorylation of TrkA [29] that provides docking sites for signal transduction molecules such as phospholipase Cγ, phosphoinositide 3-kinase (PI3K) and the adaptor protein Shc. Activated PI3K induces the activation of Akt through 3'-phosphorylated phosphatidyl-inositol as well as phosphoinositide-dependent kinase (PDK), which in turn phosphorylates and activates Akt, a key component of cell survival.[30] The serine-threonine kinase Akt exerts its anti-apoptotic effects through downstream targets, including apoptosis regulators (Bcl-2 family members: Bax and Bad, and caspase 9) and transcription factors (IKK and cyclic AMP response element-binding protein, CREB). Akt inhibits a conformational change in the pro-apoptotic Bax protein and its translocation to mitochondria, thus preventing its redistribution to the mitochondrial membrane and caspase-3 activation.[31] Moreover, Akt phosphorylates Bad, thus preventing its binding to the anti-apoptotic factor Bcl-X_L and promoting cell survival. On the other hand, activated Akt induces the phosphorylation of CREB and IKK, which stimulate the transcription factors IκB and NF-κB and thereby the transcription of pro-survival target genes. Neurotrophic factor withdrawal induces PCD in neurons by activating JNK that phosphorylates c-Jun and p53, which in turn induce the expression

of cell-death genes.[4,32] Phosphorylated c-jun induces the expression of DP5, a pro-apoptotic member of the Bcl-2 family. DP5 is required to help Bax movement from its location in the cytosol to the mitochondria from where it induces the release of cytochrome c (Fig. 2B.).

Intrinsic Signaling Pathways: Sensing DNA Damage

The survival of organisms depends on the accurate transmission of genetic information from one cell to its daughters. Such faithful transmission requires not only extreme accuracy in replication of DNA and precision in chromosome distribution, but also the ability to survive spontaneous and induced DNA damage while minimizing the number of heritable mutations. To achieve this fidelity, cells have evolved surveillance mechanisms that monitor the structure of chromosomes and coordinate DNA repair and cell cycle progression. On the other hand, odd genetic mutations can be a healthy event particularly in germ cells. Such mutations complement genetic recombination in providing limited genomic plasticity necessary for the process of evolution to select favorable traits for future generations. The threat of expressive genetic change needs constant attention as DNA becomes damaged by inherent errors in processes such as DNA replication, as well as through genotoxic stress from reactive cellular metabolites and exogenous stimuli (ionizing radiation, chemotherapeutic drugs).

Cells differ hugely in the responses to DNA damage. For example, whereas splenic lymphocytes in fetus readily initiate apoptosis after exposure to ionizing radiation (IR), apoptosis forms no part of the response of cardiac myocytes to radiation at any stage of the development.[33] The eukaryotic strategy to deal with DNA damage can split into three components: the recognition of injured DNA, a period of damage assessment (enforced by checkpoints), and the implementation of the appropriate response (DNA repair or cell death).[34] DNA-damage checkpoints have been recently shown to control not only cell cycle arrest but also: i) the activation of DNA repairing pathways;[35,36] ii) the composition of telomeric chromatin and the movement of DNA repair proteins to the sites of the DNA damage;[37] iii) the activation of transcriptional programs;[38] iv) telomere length [39] and v) induction of cell death by apoptosis.[40-42]

The proteins that initially sense the aberrant DNA structure and initiate the signaling responses are currently unknown. Owing to their ability to bind and be activated by DNA strand breaks, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) have long been proposed as DNA damage sensors. However, genetic evidence indicates that these proteins are not activators of the global DNA damage response.[43,44] In contrast to our knowledge of damage sensors, our understanding of signal transducers is more advanced. ATM (Ataxia-teleangectasia-mutated) is one of the remarkable group of PI3K-related kinases that also includes DNA-PKcs (the catalytic subunit of DNA-PK) and ATR (Ataxia-teleangectasia-Rad3 related).[45] These proteins are all crucial in detecting the most lethal type of DNA damage, the double-strand breaks (DSBs) induced by exogenous agents such as IR, chemotherapeutic drugs and chemicals or by endogenous agents such as oxidative damage, programmed rearrangements and meiotic double-strand breaks.[46,47] The presence of DSBs is recognized by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade which can induce either cell-cycle arrest and DNA repair responses or, if the damage is irreparable, cell death. Although structurally related to the PI3K family members, ATM and ATR are protein kinases, which play a central role in the cellular response to DSBs. ATM and ATR have overlapping substrate specificities and ATR over-expression can complement the radio-resistant DNA-synthesis phenotype of cells lacking ATM. Despite the apparent overlap between the functions of ATM and ATR, it is clear that ATM mediates the IR-induced damage whereas ATR is responsible of ultraviolet-induced damage. AT patients have mutations in ATM and are defective in several responses to IR including G1 arrest,[48] reduction in DNA synthesis [49] and G2 arrest.[50] ATM plays an important part in the response to IR controlling the initial phosphorylation of several key proteins involved in the control of

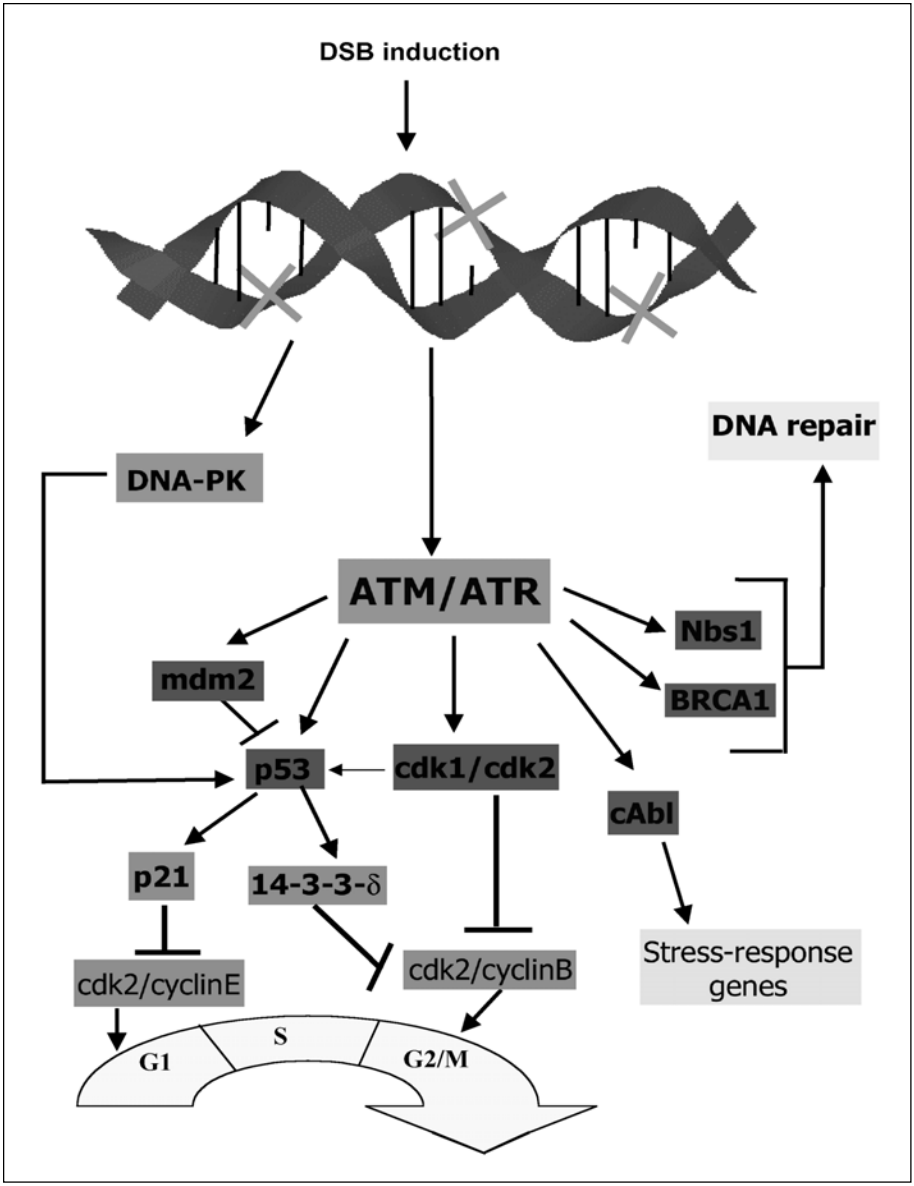

Figure 3. A central role for ATM in cellular response to double-strand breack (DSB). ATM is activated in response to DSB by unknown mechanism. Activated ATM signals the presence of DNA damage by phosphorylating targets involved in cell-cycle arrest, DNA repair and stress response.

G1 arrest (p53, MDM2), G2 arrest (Chk2 and Chk1) and DNA repair (BRCA-1 and Nbs1), and in the activation of stress responsive genes (c-Abl) [14] (Fig. 3.). ATM regulates the G1/S checkpoint, at least in part, by controlling the activation and stabilization of p53, which in turn activates two downstream effectors, $p21^{Waf1}$ and 14-3-3-σ. These downstream effectors inhibit the activity of CDK2/cyclinE and CDC2/cyclin B affecting cell-cycle arrest in G1 and

G2 phases, respectively. Other downstream ATM targets of particular relevance to the G2/M checkpoint include the checkpoint protein kinases Chk1 and Chk2 that are structurally unrelated but share some overlapping substrate specificity.[14] These kinases phosphorylate p53 at Ser 20, a residue known to be critical for p53 stabilization and function after DNA damage induced by γ- and UV-radiations.[51] Moreover, Chk1 and Chk2 phosphorylate a conserved site on protein phosphatase Cdc25, which, as a consequence, is inactivated and bound by the 14-3-3-σ protein. The inactive Cdc25 is then incapable of removing an inhibitory phosphate group on Tyr-15 of CDC2, thus preventing entry into mitosis. ATM control on DNA repair is exerted through Nbs1 and BRCA1 (DNA repair proteins have also linked to DNA-damage response ATM-dependent). In particular, BRCA1 acts as a protein scaffold that orchestrates the repair by complexes with other DNA mismatch repair proteins as Nbs1. BRCA1 is also a component of RNA polymerase II holo-enzyme complex providing the possible explanation for the reported role of BRCA1 in transcription-coupled repair of DNA damage. Furthermore, phosphorylation of c-Abl by ATM activates stress-activated protein kinase (SAPK), which is involved in transcriptional regulation of the stress-response genes.

Apoptotic Effectors: Apoptosome Assembly and Caspases

Most if not all the apoptotic signaling pathways ultimately lead to the activation of members of the caspase family of proteases, which act as signal transducers and death effectors.[52,53] There are two well-described pathways by which caspases, that function as signal transducers (known as apical, upstream or initiator caspases), become activated. In one pathway initiating at the plasma membrane, ligand binding to a death receptor (see previous section on Extracellular Signaling) results in recruitment of procaspase-8 into a multi-protein complex in which caspase auto-activation and trans-activation occurs.[21] In a second pathway, cellular stress of various sorts (see previous section on Intracellular Signaling) causes the release of mitochondrial cytochrome c. This, in association with a cytoplasmatic protein known as Apaf-1, recruits procaspase-9, forming the so-called apoptosome, and leads to caspase-9 activation.[54] Initiator caspases are activated through protein-protein interactions whereas effector caspases are usually activated by an upstream caspase. Executioner caspases are thought to have a major role in cleaving cellular substrates that determines apoptotic cell morphology and lead to cell death (see Table. 1.).

Mitochondria are the key organelles in the apoptotic response. In fact numerous pro-apoptotic signal transduction and damage pathways converge on mitochondrial membranes to induce their permeabilization. Mitochondrial membrane permeabilization differentially affects the outer membrane, which becomes protein permeable, and the inner membrane, which continues to retain matrix proteins, yet can dissipate the mitochondrial transmembrane potential. Mitochondrial membrane permeabilization induces the release of proteins that are normally strictly confined to the mitochondrial intermembrane space which in turn trigger the activation of caspases and nucleases. In particular, cytochrome c stimulates the cytosolic assembly of the apoptosome and apoptosis inducing factor (AIF) activates a DNase located in the nucleus (see for review ref. 55). Among the numerous proteins, which translocate to, reside in, act on or are released by mitochondria, Bcl-2 family members deserve particular interest. Bcl-2 family includes both pro- and anti-apoptotic members which can heterodimerize and titrate one another's function, suggesting that their relative concentration may act as a rheostat for the suicide program.[56] Anti-apoptotic members of the Bcl-2 family (such as Bcl-2 and Bcl-X_L) reside mainly, but not exclusively, in mitochondrial membranes where they locally inhibit mitochondrial membrane permeabilization.[57] Expression of Bcl-2 and Bcl-X_L prevents the redistribution of cytochrome c in response to multiple death-inducing stimuli.[58,59] Pro-apoptotic members of the Bcl-2 family such as Bax can translocate from other cellular localizations to mitochondria while undergoing a conformational change, they then oligomerize within

Table 1. Properties of main mammalian caspases

Group/ Function	Caspase	Cellular Substrates / Effect	Inhibitors
Initiators	Caspase-2		
	Caspase-8	Procaspase-3 / caspase-3 activation p21-activated kinase 2 / constitutively active kinase Bid / generates pro-apoptotic fragment $FLIP_L$ / unknown	FLIP
	Caspase-9	Procaspase-3 / caspase-3 activation PARP/ reduced poly(ADP-ribose) synthesis	X-IAP, c-IAP1 and c-IAP2
	Caspase-10	$FLIP_L$ / unknown	FLIP, X-IAP
Executioners	Caspase-3	Fodrin / plasma membrane blebbing β-Catenin / reduced α-Catenin binding and cell-cell contact NuMa / nuclear shape changes PARP / reduced poly(ADP-ribose) synthesis DNA-PKcs / reduced activity PKC δ and τ, PKN, p21-activated kinase 2, MEK K-1 / constitutively active kinases $p21^{waf1}$ / Loss of N-terminal cdk inhibitory domain from nucleus $p27^{kip1}$ / reduced p27 in cyclin E-cdk complexes pRb/p105 / unopposed E2 F1 action Bcl-2, Bcl-X_L, Bid / generates pro-apoptotic fragment	X-IAP, c-IAP1 and c-IAP2
	Caspase-6	$FLIP_L$ / unknown	X-IAP, c-IAP1 and c-IAP2
	Caspase-7	Lamins A and B / nuclear lamina disassembly NuMa / nuclear shape changes PARP / reduced poly(ADP-ribose) synthesis $p21^{waf1}$/ Loss of N-terminal cdk inhibitory dom ain from nucleus $p27^{kip1}$ / reduced p27 in cyclin E-cdk complexes	X-IAP

mitochondrial membranes and facilitate mitochondrial membrane permeabilization. This translocation / oligomerization / permeabilization reaction is inhibited by anti-apoptotic members of the Bcl-2 family and is stimulated by pro-apoptotic BH3-only members of the Bcl-2 family, such as Bid. The Bax homologue Bak constitutively resides in the outer mitochondrial membrane and also undergoes a Bid-stimulated allosteric activation leading to its oligomerization within the membrane.[60] Certain members of Bcl-2 family, such as Bcl-2 and Bcl-X_L, are cleaved by caspases-3, a process that converts their anti-apoptotic activity into a Bax-like pro-apoptotic activity. Caspase-8 is able to cleave the pro-apoptotic Bcl-2 family member, Bid, resulting in its translocation from the cytosol to mitochondria. This leads to mitochondrial aggregation around the nucleus, loss of mitochondrial membrane potential and apoptosis. Thus, caspase-8 cleavage of Bid links the death receptor pathway with mitochondrial activation of caspases, thereby amplifying the caspase signaling cascade.[61] The pro-survival proteins also seem to maintain organelle integrity, Bcl-2 directly or indirectly prevents the release from mitochondria of cytochrome c, which, along with ATP, may facilitate a change in Apaf-1 structure to allow procaspase-9 recruitment and processing.[54,57] The result is the activation of caspase-9, which then

processes and activates either caspase-3-dependent or –independent pathways to orchestrate the biochemical execution of cells. Activation of caspase-9 is further regulated by its phosphorylation state. The AKT kinase, important in the transmission of cell survival signals, acts in part to regulate apoptosis by phosphorylating procaspase-9. Moreover, the cytochrome c release from mitochondria could be induced by the TNF receptor family member Fas, in which cytochrome c release is prevented by inhibition of caspase-8 recruited to the cytosolic domain of ligated Fas. Nevertheless, cytochrome c release can sometimes contribute to Fas-mediated apoptosis by amplifying the effects of caspase-8 on activation of downstream caspases. Executioner caspases cleave several specific structural proteins and enzymes that contribute to the progression and the morphological changes observed during apoptosis. Cellular disassembly is induced by effector caspases cleavage of molecules such as nuclear lamins, which leads to chromatin condensation. They have also indirect effects on cell structures through the cleavage-induced deregulation of cytoskeletal regulators such as focal adhesion kinase and p21-activated kinase-2. Effector caspases also deregulate DNA repair (DNA-PK_{cs}) and replication and mRNA splicing. Endonuclease activity is also induced by caspase-3. Caspase-3 cleaves the caspases-activated DNAse endonuclease inhibitor, resulting in the DNA fragmentation characteristic of apoptosis.[62]

The fact that procaspases are caspase substrates insures rapid and complete conversion of a pool of proenzymes, even if only a few molecules were initially activated.[52] To avoid that even the smallest perturbation would eventually lead to full activation and apoptotic death of the cell, the presence of buffers or dampeners is needed. Among the most important regulators of the caspases are inhibitors of apoptosis proteins (IAPs). Members of the IAP family, originally identified in Baculovirus, contain one or more modules called the baculoviral IAP repeat (BIR).[63] At least one BIR motif is essential for antiapoptotic activity of members of IAP family, but not all BIRs and BIR-containing proteins are IAPs.[64] Up till now, five different mammalian IAPs (X-IAP, c-IAP1, C-IAP2, NAIP and survivin) exhibiting antiapoptotic activity in cell culture have been identified.[65] The spectrum of apoptotic stimuli that are blocked by mammalian IAPs is broad and includes ligands and transducers of the TNF family of receptors, pro-apoptotic members of the Bcl-2 family, cytochrome c and chemotherapeutic agents.[66] X-IAP appears to have the broadest and strongest anti-apoptotic activity. X-IAP, c-IAP1 and C-IAP2 are direct caspase inhibitors and they all bind to and inhibit active caspase-3 and -7 and also procaspase-9 but not caspase-1, -6, -8 or –10.[65] Like the baculoviral IAPs, the primary effect of expressing these mammalian IAPs in vivo is inhibition of the processing of caspases.[65] Control of caspase activity can be accomplished also at the receptor level by interfering with the activation of initiator caspases. This is the case of FLIP, a FLICE-caspase-8 inhibitory protein, predominantly expressed in muscle and lymphoid tissues. Upon stimulation of CD95, FLIP is recruited into the DISC together with FADD and procaspase-8 and prevents partial procaspase-8 proteolytical processing. Additional regulation is provided by Smac (second mitochondria-derived activator of caspases) or its murine homolog DIABLO (direct IAP-binding protein with low pI), which binds to IAPs and abrogate caspases inhibition.[67,68] If a cell is committed to apoptotic death such that it releases its mitochondrial contents, then Smac/DIABLO will sequester the IAP proteins and insure that they do not attempt to stop the program in its tracks.

Genes in Cell-Cycle Control and Apoptosis

Although now and then the odd genetic mutation can be a healthy event, particularly in germ cells, excessive genetic change needs constant attention as DNA becomes damaged by inherent errors in process such as DNA replication, as well as through genotoxic stress from reactive cellular metabolites and exogenous stimuli. The so-called cell-cycle checkpoints are biochemically signalling pathways that sense various types of structural defects in DNA, or in chromosome function, and induce a multifaceted cellular response that activates DNA repair

and delays cell-cycle progression.[69] When DNA damage is irreparable, checkpoints eliminate such potentially hazardous cells by permanent cell-cycle arrest or cell death. A number of observations suggest that signalling between the proliferation and cell death machinery occurs: these include the observation that mutations that promote inappropriate entry into the cell cycle often also promote apoptosis and that overexpression of anti-apoptotic members of the bcl-2 family of proteins can suppress proliferation and promote entry into G0.[70]

G1 phase is a period when cells make critical decision about their fate including the optional commitment to replicate DNA and complete the cell division cycle. Provided mitogens are available and the cellular environment is favourable for proliferation, a decision to enter S phase is made at the so-called 'restriction point' R in middle-late G1.[71] In unstressed cells, this commitment to replicate DNA and divide seems irreversible until the next G1 phase. Available data suggest that the restriction point switch, from the growth factor dependent early G1 to the subsequent mitogen-independent phases, reflects the induction of broad transcriptional programmes regulated by the parallel retinoblastoma proteins (pRBs) and Myc pathways, which regulate genes critical for G1/S transition and coordination of S-G2-M progression.[72,73] Within the pRB pathway, the molecular switch appears to be the phosphorylation of pRB/p105 by cyclin D-CDK4(6) kinases,[74] resulting in the de-repression of E2F1-3 transcription factors.[75] E2Fs and Myc jointly activate the key target gene cyclin E whose product activates the CDK2 kinase necessary for the actual initiation of DNA replication.[76] Consequently, the cyclin E protein becomes detectable and accumulates only in late G1, a few hours after the passage through R.[77] Both its position at the convergence of pRB and Myc pathways, and its essential and rate-limiting function in G1/S transition, makes cyclin E-CDK2 activity an ideal candidate for DNA damage checkpoint target. In principle progression through G1 could be blocked by preventing pRB phosphorylation or by silencing cyclin E-CDK2 activity. Both CDK2 [78] and pRBs [79] are indeed targeted by the DNA damage checkpoints, yet through distinct mechanisms corresponding to induction and maintenance of the G1 checkpoint, respectively.

Up to now, the protein identified as the heart of stress response pathways, is the transcription factor p53 that prevents the growth and survival of potentially malignant cells. Upon diverse stress stimuli, cellular p53 becomes post-transcriptionally modified, stabilised and competent to induce the expression of genes required to halt cell-cycle progression or trigger programmed cell death (see below). Among these genes, the CDK inhibitor $p21^{WAF1/Cip1}$ plays a key role in cell-cycle arrest by silencing CDKs essential for entry into S phase.[74] Moreover, p53 has been recently shown to participate in DNA repair through the activation of a ribonucleotide reductase named P53R2.[80] Although no single predominant effector of p53-induced apoptosis has been identified, there is abundant in vivo evidence that p53 pro-apoptotic function, rather than its function in cell-cycle arrest, is crucial in tumor suppression. Actually, p53 induces the expression of proteins targeting both the mitochondrial and the death-receptor-induced apoptotic pathways.[81]

Role of pRB Family Proteins and E2F Transcription Factors in Apoptosis

Recent evidence suggests that intracellular signals involved in regulating cell proliferation and cell-cycle progression also mediate apoptosis.[82] The three members of the retinoblastoma family proteins (pRBs: pRB/p105, pRB2/p130 and p107) have been shown to play important roles in the process of cell proliferation and the G1 to S transition (see for review ref. 74). The pRBs function in cell-cycle progression, is exerted by controlling the E2F transcription factor family (E2F1-6).[83] The hypothesis that pRB/p105 itself acts as a negative regulator of apoptosis is supported by in vitro studies on SAOS-2 cells, which lack pRB/p105 and p53 expression. These cells are able to undergo apoptosis after IR and become resistant to apoptosis when wild-type pRB/p105 expression is restored exogenously.[84]

Loss of pRBs oncosuppressive function, by mutation or hyperphosphorylation, triggers the p53 apoptotic pathway in the attempt to eliminate de-regulated cells (see below). Tumors with wild-type p53 contain many cells undergoing apoptosis, and thus loss of pRBs may create a survival pressure for the cell to acquire mutations in this apoptotic pathway. A first possible link between pRB/p105 and p53 appears to be the free E2F1, which is released when pRB/p105 function is lost. This possibility is supported by results obtained in transgenic mice with pRB/p105 inactivation, which developed slowly growing tumors of the choroid plexus with high apoptotic rates. However, the additional inactivation of p53 led to rapidly growing tumors at least in part because a marked reduction in apoptosis.[85] On the other hand, concomitant pRB/p105 inactivation and E2F1 deletion led to an 80% reduction in p53-dependent apoptosis.[86] In addition, in vivo studies showed that pRB/p105 $^{-/-}$ mice died in mid-gestation and revealed increased cell death in the developing central nervous system, whereas embryos mutant for both pRB/p105 and E2F1 showed significant suppression of apoptosis as well as down-regulation of the p53 pathway.[87] Moreover, it has been shown that E2F1 overexpression is capable of inducing apoptosis in mouse embryo fibroblasts after passage through S phase, and that E2F1 homozygous null mice show reduced levels of apoptosis.[88] The overall of these data strongly suggest that the mechanism by which pRB/p105 suppresses apoptosis is through regulation of E2F1 activity. This is also indirectly supported by the lack of data showing similar pro-apoptotic function of the two pRB/p105-related proteins, pRB2/p130 and p107 notwithstanding a wide overlap between them in the control of proliferation. A possible explanation is that the three members of retinoblastoma family control different E2Fs, with a much higher affinity of pRB/p105 for E2F1. Actually, there was only one report indicating an overlap between pRB/p105 and p107 in inducing apoptosis in mouse liver and central nervous system.[89]

The level and timing of pRBs progressive phosphorylation, and their consequent inactivation, are controlled by cyclin/CDK complexes whose activity are limited by CDK inhibitors such as p21 and p27 (see for review ref. 74). Several lines of evidence suggest the involvement of cyclin/CDK complexes in the apoptotic response. Increased levels of cyclin D in neurons and endothelial cells enhance apoptosis.[90,91] Other data suggest the involvement of CDK-related kinase PITSLRE in inducing apoptosis in Chinese hamster ovary (CHO) cells.[92]

Apoptosis occurs in two physiological stages, commitment and execution. The apoptotic execution is initiated by activation of specific proteases of the caspase family (see section: Apoptosis an overview). It has been suggested that, in mammalian cells, cell-cycle checkpoint regulators could be involved in apoptotic commitment. Interestingly, it has been reported that, at an early stage of the apoptotic response, pRB/p105 becomes first de-phosphorylated and immediately cleaved (see for review ref. 93). Cleavage reduces de-phosphorylated pRB/p105 into 68 kDa and 48 kDa fragments. Notwithstanding p68/RB contains the A/B pocket region, responsible for active E2Fs repression, and is found only in the nuclear fraction of the apoptotic cells, p68 fragment loses the capability to bind E2F1.[94] In addition to de-phosphorylation and interior cleavage, pRB/p105 has also been found to be cleaved in its C terminus during apoptosis and the induction of C-terminal truncated pRB/p105 is tightly associated with inhibition of apoptosis (see for review ref. 93). The C-terminal 42 aminoacid peptide of pRB/p105 binds cyclin D3 and inhibits E2F1 transcriptional activity but it fails to bind to MDM2, a regulatory protein implicated in apoptosis (see below).[95] The overall of these results suggest that de-phosphorylation and cleavage of pRB/p105 could be an early event in programmed cell death, possibly related to cell commitment to apoptosis. As a consequence, failure to induce the interior cleavage of pRB/p105 should be associated with drug resistance to anti-cancer treatments. Actually, it has been reported that activation of the pRB/p105 cleavage enzyme, a caspase protease, is required for overcome drug resistance in HL-60 cell line.[96]

E2F Target Genes Involved in Apoptosis

In addition to inducing proliferation, de-regulated E2F activity can trigger apoptosis.[83] This finding is supported by data showing that the loss of E2F1 expression suppresses apoptosis and induces hyper-proliferation in pRB/p105-deficient mouse embryos.[87] Both over-expression experiments and mutant mouse models indicate that E2F1 can induce death through either p53-dependent or p53-independent mechanisms.[97,98]

A few E2F target genes have been described that could mediate E2F-induced apoptosis.[72] Most prominent among these are p14/p19/ARF and p73. Although ARF seems to have a role in potentiating E2F-induced apoptosis through stabilization of p53, ectopic expression of ARF results in cell cycle arrest rather than apoptosis, suggesting that other genes involved in apoptosis are regulated by E2F.[99,100] E2F1 is also able to induce transcription of p73, a member of the p53 family protein, which shares sequence homology and functional similarity with p53.[101] Unlike ARF, expression of p73 induces apoptosis, and a dominant-negative p73 mutant can suppress E2F1-induced apoptosis in cells lacking p53. Moreover, induction of apoptosis by E2F1 is severely impaired in p73 $^{-/-}$ cells, and this effect is pronounced in cells lacking both p53 and p73.

The direct link between E2F1-induced apoptosis and Apaf-1 up-regulation recently shown enlightened a new pathway by which E2Fs can participate to cell death execution.[102] Apaf-1 is an essential element of the apoptosome (see Section: Apoptosis an overview) and a low-abundance protein,[103] suggesting that it can be a limiting factor in apoptosis signaling, and it has a central role in stress and oncogene-induced apoptosis.[104] Apaf-1 levels increase in pRB/p105 $^{-/-}$ embryos, particularly in the nervous system, and apoptosis in Apaf1$^{-/-}$ cells is impaired in response to increased E2F1 activity. E2F1 and, to a lesser extent, E2F2 and E2F3 have been shown to transactivate Apaf-1. Moreover, Apaf-1 is required for E2F1-induced apoptosis. Interestingly, Apaf-1 is also a direct target of p53, suggesting that p53 might sensitize cells to apoptosis by increasing Apaf-1 levels. However, E2F1 can regulate Apaf-1 expression independently of p53 and is a stronger transactivator of the Apaf-1 promoter than p53.[102]

Taken together the above results suggest that the increase in free E2F1 due to de-regulation of pRB/p105 repressional activity would result in triggering at least three proapoptotic pathways: a p53-dependent pathway, through p14/p19/ARF and/or p53 stabilization; a p73-dependent pathway, possibly parallel to the p53 one; and a p53-independent pathway, through Apaf-1 up-regulation (Fig. 4.).

c-Myc Sensitization of Cells to Apoptotic Triggers

The c-Myc proto-oncogene has been studied extensively and its product is the best characterized member of Myc family of short-lived nuclear phosphoproteins. It encodes a protein that functions as a transcription factor and stimulates cell proliferation by reducing G1 duration and promoting S phase entry. Conversely, down-regulation of c-Myc expression has an antiproliferative effect. c-Myc activity is also known to affect apoptosis and differentiation, favoring an initial commitment from proliferation to differentiation. However, c-Myc levels are down-regulated during terminal differentiation and enforced c-Myc expression usually inhibits terminal differentiation, possibly interfering with cell cycle exit. c-Myc facilitates G1 exit by positively modulating cyclin/CDK complexes, by negatively modulating the CDK inhibitors p27 and p21, and by interfering with pRB/E2F activity. In particular, c-Myc induces Id2 to overcome the pRB block on cell-cycle progression by promoting its physical association with the active, hypo-phosphorylated forms of RB pocket proteins. Id2 induction by c-Myc may represent a physiologically relevant component of the circuit connecting c-Myc and pRBs, as two phenotypic hallmarks of c-Myc activation, the ability to promote cell cycle reentry in the absence of growth factors and the ability to cooperate with Ras to transform fibroblasts, are dependent on the presence of Id2 (Fig. 5.).

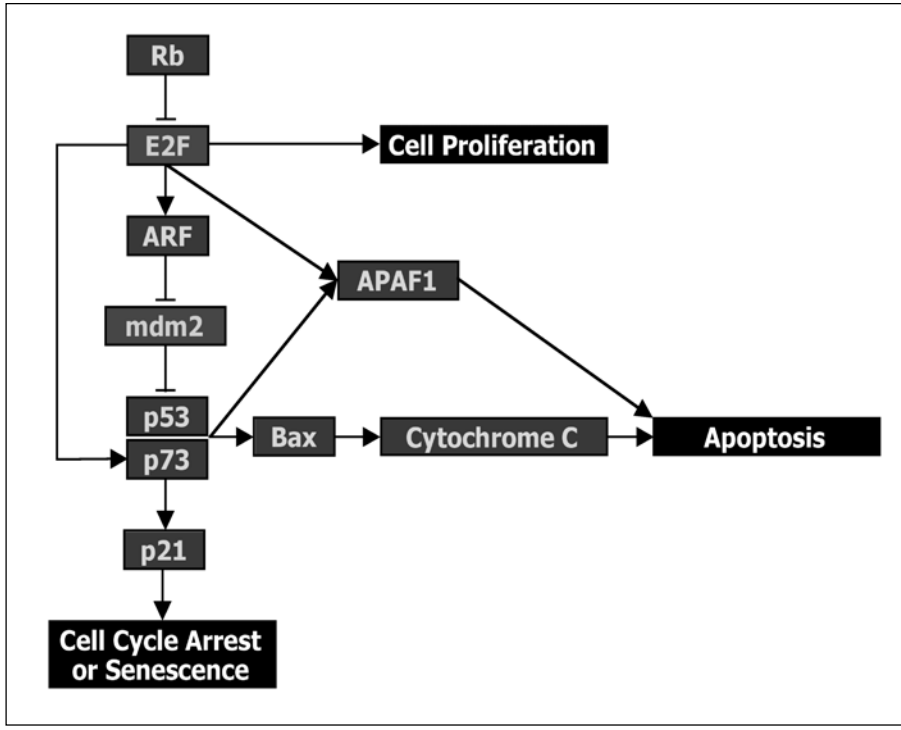

Figure 4. pRB controls proliferation and apoptosis through active repression of E2F-dependent promoters. Loss of pRB results in free-E2F which regulates ARF and Apaf1 expression to induce apoptosis and/or cell-cycle arrest. Modified from Moroni MC et al, Nat Cell Biol 2001.

The available evidence suggests that c-Myc sensitizes cells to a variety of apoptotic triggers rather than directly inducing apoptosis by itself. This biological activity is mediated through cytochrome c release but requires other apoptotic signals such as those depending on CD95/Fas and p53. The ability of c-Myc to concomitantly induce proliferation and apoptosis in certain tissues and conditions can be considered a fail-safe mechanism against the unrestrained growth of a cell carrying even a single lesion in its proliferation pathways. c-Myc over-expression promotes apoptosis under certain conditions such as during serum deprivation or hypoxia.[105] Moreover, anti-apoptotic genes, such as Bcl-2, suppress c-Myc-induced apoptosis.[106] Its ability to affect such different aspects of cell behavior can be in part related to the finding that c-Myc participates in a network of interacting proteins (Myc, Max and Mad family members) all containing a basic helix-loop-helix-zipper motif involved in dimerization and DNA binding. Over-expression of c-Myc/Max dimers induces proliferation or apoptosis while Max/Mad complexes cause cell growth arrest or differentiation.[107,108] The equilibrium among the various dimers is mainly controlled through extracellular signal-induced modifications in c-Myc or Mad expression levels.

Several models have been proposed to explain the apparently contradictory roles of c-Myc in both proliferation and apoptosis. It is tempting to speculate that inappropriate c-Myc expression pushes cells into a cell-cycle during serum deprivation or hypoxia for which they are not prepared, thereby sensitizing cells to apoptosis. However, c-Myc-induced apoptosis is independent of cell cycle position and so this explanation is not satisfying. The "dual signal" model

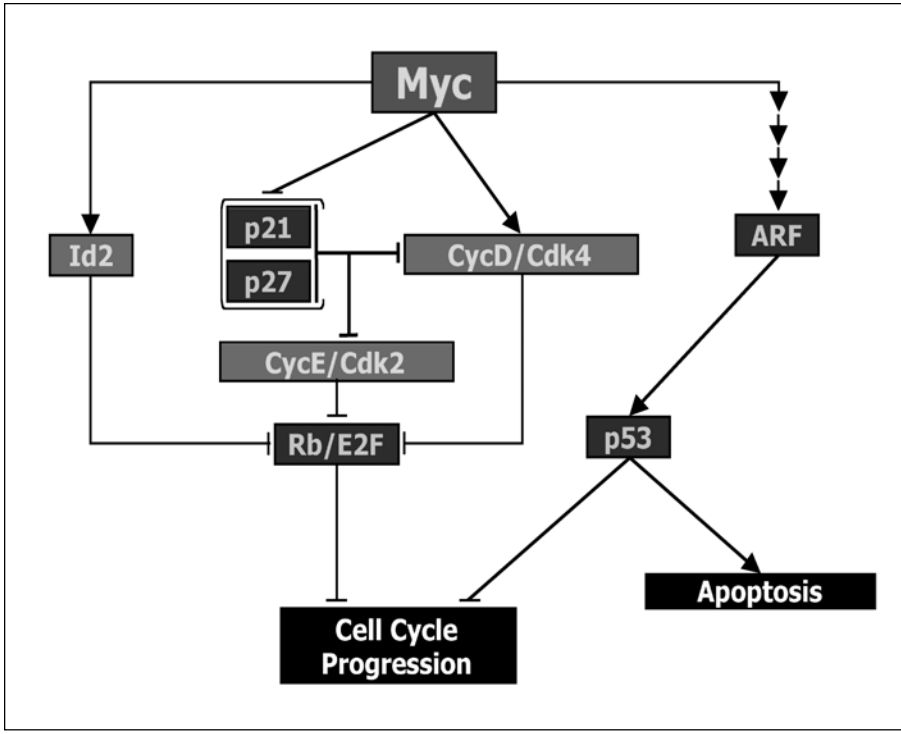

Figure 5. Schematic representation of Myc-activated and –repressed pathways affecting cell-cycle progression and apoptosis.

has been postulated in which c-Myc activates genes involved in both proliferation and apoptotic pathways.[109] Mitogens would stimulate c-Myc's proliferation pathway, while anti-apoptotic factors, such as Bcl-2, may shut down c-Myc's apoptotic pathway. The fact that c-Myc-induced apoptosis, but not proliferation, is inhibited by Bcl-2 suggests that there are two distinct sets of genes involved in these pathways that can be modulated by different signals.

c-Myc Induces Apoptosis under Certain Conditions

c-Myc is sufficient and necessary for apoptosis under certain conditions in IL-3-dependent murine myeloid 32D cells, primary and established rat fibroblasts (Rat1), and T cell hybridomas. Constitutive expression of c-Myc markedly accelerates apoptosis of 32D cells denied IL-3. Death is not restricted to a particular phase of the cell cycle but occurred in all phases, and c-Myc continues to drive S phase entry of cells, which do not immediately commit suicide.[110] The observation that in 32D cells through the influence of c-Myc, apoptosis occurrs in a stochastic fashion such as that some cells in the population die while other cells continue to proliferate, suggests that a conflict of growth and arrest signals in cells might be the cause of apoptosis rather that a direct function of c-Myc.[111] Notably, additional growth limiting treatments such as amino acid deprivation are similarly capable of eliciting apoptosis by c-Myc.

Cell death by c-Myc in the Rat1 system has been shown to be associated with the activation of certain Jun kinases (JNKs) and caspase-3, which is crucial to produce the associated chromatin collapse and nucleosomal DNA degradation.[112,113] A necessary role for c-Myc in

apoptosis was reported by Shi et al,[114] who used antisense oligonucleotides to reduce c-Myc expression in murine T hybridomas and showed that c-Myc is required for apoptosis induced by T cell receptor activation. Later studies established that c-Myc is a critical determinant of apoptosis induced by TNF-α[115] and of the magnitude of the response to ligation of the CD95/Fas death receptor.[116] However, it now appears that c-Myc is required for efficient response to a variety of apoptotic stimuli, including transcription and translation inhibitors, hypoxia, glucose deprivation, heat shock, chemotoxins, DNA damage and cancer chemotherapeutics. c-Myc does not act as a death effector in all these instances but instead acts to sensitize cells to a variety of apoptotic triggers.[117] In any case, its role in death induced by so many stimuli supports the hypothesis that c-Myc has intrinsic functions related to cell death.

c-Myc Target Genes and Apoptosis

The precise mechanism by which c-Myc induces apoptosis has not been demonstrated even if some of its transcriptional targets have been well established. Several genes that promote cell-cycle transit have been identified as c-Myc targets and two of these, cyclin A and Cdc25A, have been suggested to participate in c-Myc-induced apoptosis.[118] Enforced expression of cyclin A in Rat1 cells is sufficient to confer anchorage-independent growth capacity and susceptibility to apoptosis by serum deprivation. Cdc25A is a phosphatase responsible for activating CDC2. Cdc25A has been reported to be both necessary and sufficient for apoptosis by c-Myc in serum-deprived fibroblasts.[119] Three other cell cycle regulators that are not genetic targets of c-Myc, CDK2, CDK3 and cyclin D3, have also been reported to enhance apoptosis by c-Myc.[120]

Using c-Myc null cells to assess the serum response of the best-studied target genes, it was found that only the Cad and Gadd45 genes are misregulated in the absence of c-Myc. Cad is a housekeeping gene that participates in pyrimidine biosynthesis, which is activated by c-Myc, but it has not been assigned any role in apoptosis to date. Gadd45 (growth arrested and DNA damage-inducible gene45) is functionally undefined and repressed by c-Myc.[121] Ornithine decarboxylase (ODC) is perhaps the best studied target of c-Myc.[122,123] ODC is a housekeeping enzyme involved in polyamine synthesis and is necessary for cell proliferation. How it impacts apoptosis is unclear, although one possibility is that excessive polyamine catabolism generates reactive oxygen species, which promote mitochondria-dependent apoptosis.[124] Significantly, ODC has been shown to be necessary and sufficient for apoptosis by c-Myc in myeloid cells. ODC does not drive cell death as efficiently as c-Myc but, in support of its role, it has been reported as an important participant with c-Myc in chemotoxin-induced cell death.[125] The metabolic enzyme lactate dehydrogenase A is a c-Myc target gene recently shown to sensitize cells to a novel glucose-dependent apoptotic pathway.[126]

Regulatory Connections between c-Myc and Death Receptors

Several studies revealed regulatory circuits linking c-Myc to death receptors members of both the TNF and CD95 (Fas/APO1) transmembrane receptor family expressed on the surface of many mesenchymal and epithelial cells whose complex roles, that extend beyond death signaling including possible roles in proliferation, has been previously described (see section: Apoptosis an overview). A connection between c-Myc and death receptors has been suggested by the finding that c-Myc is a crucial determinant of the cytotoxic response to TNF-α.[127] Using a conditional Rat1 expression system, it has been shown that the induction of c-Myc impairs the ability of TNF-α to activate NF-κB and JNK. Notably, c-Myc did not block TNF-α-dependent elevation of p53, indicating that c-Myc only affects particular signaling pathway(s) not involved in the activation of TNF-α target genes.[128] c-Myc may act both upstream and downstream of the TNF-R family member CD95/Fas.[116] Similar to the case with TNF-α, Rat1 fibroblasts are not susceptible to killing by CD95/Fas ligand unless c-Myc is expressed

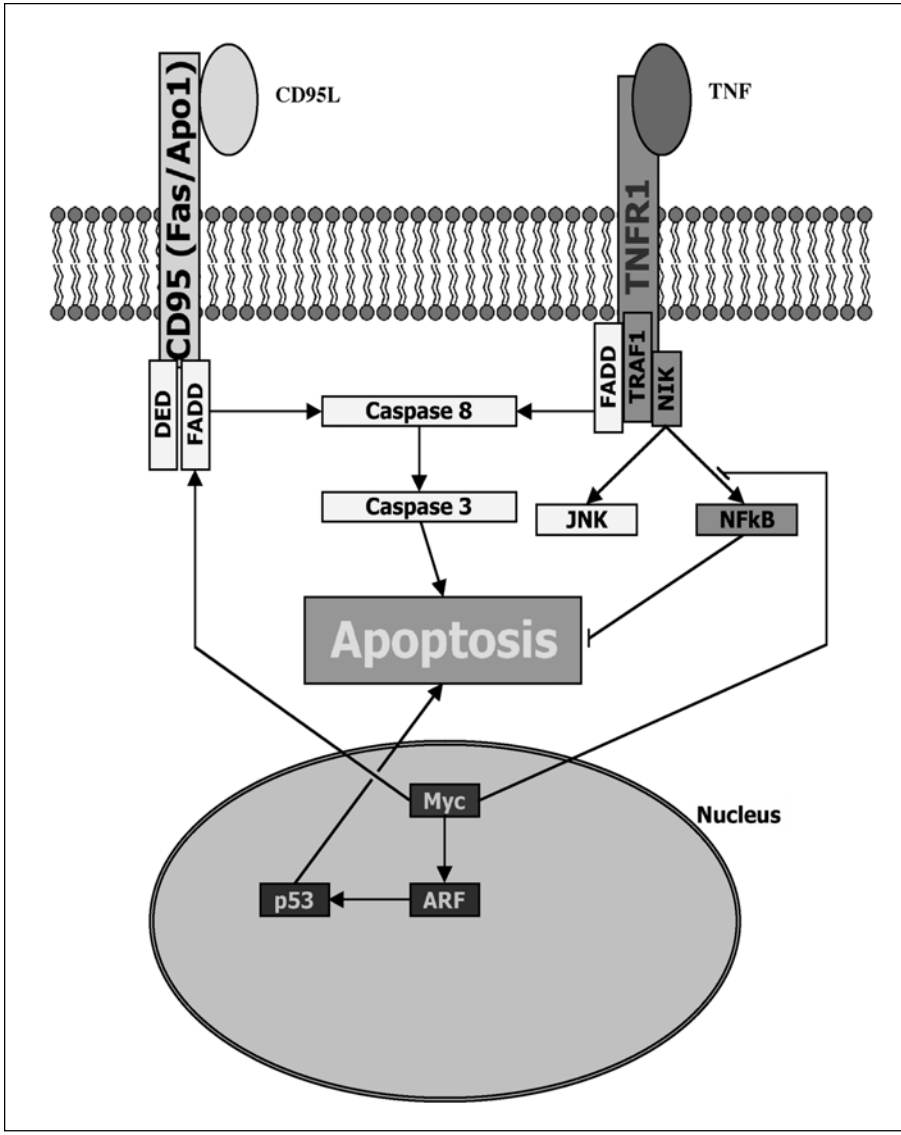

Figure 6. Regulatory links between c-Myc and CD95/Fas-APO1 and/or TNF death receptors.

(Fig. 6.). This indicates that c-Myc is necessary or perhaps that it sensitizes cells to this factor.[117] Sensitization by c-Myc in Rat1 fibroblasts is not due to up-regulation of CD95/Fas or CD95/Fas ligand, although recent evidence suggests that CD95/Fas ligand may be a target for activation by c-Myc in other cell types.[129] In any case, the biological data indicate that c-Myc can sensitize cells to CD95/Fas ligand by acting at some point down-stream of the receptor. Evidence has been as well presented in support of the hypothesis that CD95/Fas is necessary for apoptosis by c-Myc elicited by serum deprivation.[116]

p53 Family Proteins: The Crossroad between Life and Death

The p53 tumor suppressor protein is a nuclear DNA-binding phosphoprotein and is a crucial component of cellular mechanisms that are inhibited by a variety of cellular stresses. The p53 network is normally "off". It is activated only when cells are stressed or damaged. Such cells pose a threat to the organism: they are more likely than undamaged cells to contain mutations and exhibit abnormal cell-cycle control, and present a greater risk of becoming cancerous. p53 protein shuts down the multiplication of stressed cells inhibiting progress through the cell cycle. In many cases it even causes the programmed death of the cells, in a desperate attempt to constrain the damage and protect the organism.[130] Therefore, p53 protein provides a critical brake on tumor development, explaining why it is mutated and thereby inactivated in about 50% of tumors. Under normal conditions, levels of p53 are low owing to the short half-life of the protein; however both intracellular and extracellular stress signals can induce the stabilization and activation of p53. DNA damaging agents stabilize and activate p53 by covalent modifications involving phosphorylation of the transactivation domain, and both acetylation and phosphorylation of the basic allosteric control region (see for review ref. 131). In addition to post-translational modifications, protein-protein interactions and sub-cellular relocalization also have a role in the activation of p53.[132] The activation of p53 leads to the transcription of several genes whose products trigger different biological outcomes such as cell-cycle arrest, DNA repair, replication, apoptosis, senescence or differentiation.

Two p53 related genes, p73 and p63, have been recent discovered revealing additional levels of complexity in the network of p53 functions. p73 and p63, also reported as p40, ket, p51B, KET, genes encode proteins showing structural similarity to p53 [133] but with significant functional differences. Both p73 and p63 genes produce different isoforms in the cells, resulting from alternative mechanisms of splicing and from initiation of transcription at different promoters. Moreover, p73 and p63 show functional resemblance to p53, as they are able to activate some endogenous targets of p53 involved in cell-cycle arrest or apoptosis, such as $p21^{WAF1/Cip1}$, bax, MDM2, cyclin G, IGFBP3 and Gadd45.[134] Although each p53 family member shows a degree of promoter specificity distinct from the response mediated by p53.[135] Furthermore, the role of p63 and p73 in tumor suppression remains uncertain, as both of them appear not mutated in tumors and are not induced by some of the cellular stresses that cause DNA damage and activate p53, rather they seem to be involved in development and differentiation.[136]

MDM2: Keeping p53 Family Proteins under Control

The cellular level of p53 increases as a response to DNA damage or to other types of stresses, mainly through a significant increase of the protein half-life. The key player in the regulation of p53 stability is the product of the MDM2 gene.[137,138] MDM2 is the product of one of p53-inducible genes and binds the transcription domain of p53, negatively regulating p53 stability and activity. MDM2 inhibits p53 ability to function as transcription factor and shuttles p53 out of the nucleus and targets it for degradation through ubiquitin-mediated proteolysis, by a mechanism of auto-inhibitor feedback loop.[139] Through a series of steps, several copies of a small peptide (ubiquitin) are attached to the protein to be degraded. This ubiquitin chain acts as a "flag", enabling p53 to be detected by the protein-degrading machinery. The MDM2 protein is one of the enzymes involved in labelling p53 with ubiquitin. The control of MDM2 over p53 is disrupted after DNA damage. DNA damage induces the phosphorylation of human p53 at N-terminus serine residues thus producing conformational changes. Phosphorylation at the amino terminus of p53 does not affect its DNA-binding abilities, but does affect its affinity for MDM2 and its subsequent degradation.[140] Some of the residues of the p53 transcriptional domain, involved in MDM2 binding, are conserved in both p73 and

p63. This conservation suggests that both p73 and p63 could interact with MDM2. Indeed it has been demonstrated that MDM2 is transcriptionally activated by p73. In contrast to p53, MDM2 does not reduce the protein level of p73, as it does not target p73 for degradation through ubiquitin-mediated proteolysis, but represses p73 activity by disrupting the interaction between p73 and the N-terminus of the transcription adaptors/coactivators p300/CBP. The interaction between p73 and p300/CBP can enhance the p73-dependent transcription and apoptosis.[141] On the other hand, p53 transcriptional activity is affected by the binding of p300/CBP to the p53 carboxyl-terminus region. By acetylating p53, p300/CBP stimulates its ability to bind to DNA in a sequence-specific fashion.[142] Recently it has been demonstrated that, through transactivation of MDM2, p73 can lead to a reduction of the ectopically expressed p53 or of the endogenous p53 induced by adriamycin or UV-mediated damage through MDM2-mediated proteolysis. This suggests the possibility that p73 and MDM2 could cooperate to regulate the level of p53 in response to DNA damage.[143] In contrast to p53 and p73, MDM2 does not interact with p63 and does not inhibit its transcriptional activity. Given that p53, p63 and p73 can activate similar promoters, the different regulation of these proteins by MDM2 may provide a mechanism to regulate the p53-responsive elements.

Response of p53 Family Members to DNA Damage: Growth Arrest or Apoptosis

Recent research has confirmed the existence of at least three independent pathways by which the p53 network can be activated leading to PCD. One pathway is indeed triggered by DNA damage, such as that caused by IR. Here the activation of the network is dependent on two protein kinases, ATM and CDK2. ATM is stimulated by double-strand breaks and CDK2 is in turn, stimulated by ATM (for details, see section: Apoptosis an overview). The second pathway is triggered by aberrant growth signals, such as those resulting from the expression of the oncogenes Ras or Myc. In this case, activation of p53 network in human depends on p14/p19ARF protein.[144] The activation of these same signals is also required for normal cell proliferation and several mechanisms that attenuate the p53 response to allow normal cell division have been described.[145,146] The third pathway is induced by a wide range of chemotherapeutic drugs, ultraviolet light, and protein-kinase inhibitors. All three pathways inhibit the degradation of p53 protein thus stabilizing p53 at a high concentration. The increased concentration of p53 allows the protein to carry out its major function: to bind to particular DNA sequences and activate the transcription (expression) of adjacent genes (Fig. 7.). The relative contribution of these genes to the full apoptotic response, and the role of other functions of p53 that do not depend on activation of gene expression, remains to be determined. ATM, CDK1 and CDK2, activated by DNA damage, phosphorylate p53 at the amino-terminal sites that are close to the MDM-binding region of the protein, thereby blocking p53 interaction with MDM2 and leading to stabilization of p53. Moreover, p53 activation leads to cell cycle arrest within G1 phase by inducing the expression of cyclin-dependent kinase inhibitory protein, $p21^{WAF1/Cip1}$, and the consequent inhibition of cyclin D/CDKs. In these conditions, pRBs are not phosphorylated and cells do not progress through the G1-to-S- phase transition. Both p73 and p63 can induce $p21^{WAF1/Cip1}$ as well but their ability appears to be lower with respect to p53. A role in DNA repair seems to be peculiar of p53 as, up to now, there is no indication of a similar function for p63 or p73. When the genetic material is damaged, a delay in cell cycle progression facilitates DNA repair, thereby avoiding the replication and subsequent propagation of potentially hazardous mutations. The ability of the cell cycle checkpoints, signaling pathways that monitor the integrity and replication status of genome, to inhibit entry into S phase, is intimately associated with the function of the p53 tumor suppressor. DNA repair consists of an intricate network of repair systems that each targets a specific subset of lesions. Much of DNA repair is constitutive, but a number of regulatory connections between the DNA damage response pathways and DNA repair have been identified. The regulatory connections include

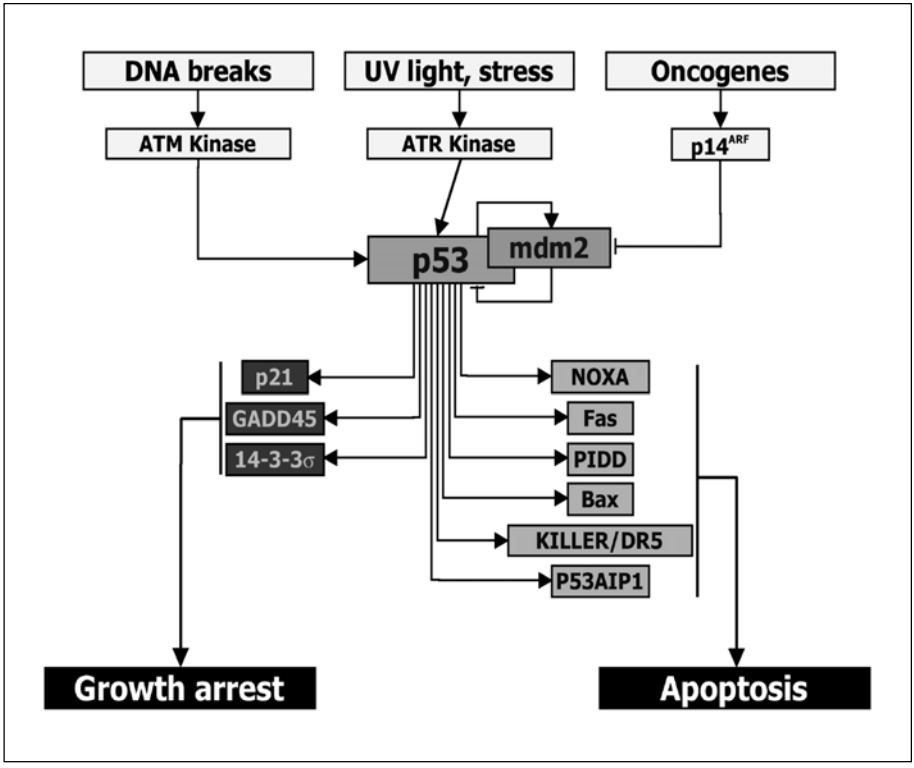

Figure 7. p53 network. Activation of the network by stress such as DNA damage, ultraviolet light and oncogenes stimulates p53 activity and its negative regulator, Mdm2. The activated p53 binds to regulatory regions of several target genes involved in inhibition of cell cycle progression and in apoptotic response. Modified from Vogelstein B et al, Nature 2000.

transcriptional up-regulation of repair proteins such as p48 and ribonucleotide reductase (p53R2), and phosphorylation of repair proteins such as BRCA1, Nbs1 and Rad55 after DNA damage. The p48 gene is induced by DNA damage in a p53-dependent fashion possibly explaining p53's role in excision repair. Also the nuclear localized subunit of p53R2 is found to be induced by p53 in response to DNA damage. Blocking p53R2 expression increases cell killing by a variety of DNA damaging agents, supporting a functional role for p53R2 in DNA repair. Regulation of ribonucleotide reductase through the DNA damage response pathways represents a conserved strategy employed by DNA damage response kinases to facilitate repair.[38] Together these results challenge the long-held notion that p53 functions mainly to induce apoptosis and suggest that p53 also promotes cell survival in response to DNA damage under certain circumstances.

However, the role of p53 in the apoptotic response remains beyond any doubt. The pro-apoptotic proteins, bax and IgF-Bp3, are transcriptional targets of p53. More recently, the NOXA and p53AIP1 genes have been discovered to be directly activated by p53.[147] Like bax, NOXA and p53AIP1 are located in mitochondria. When over-expressed these proteins induce apoptosis. Other potential mediators of p53-induced apoptosis include proteins with similarities to the classic "death-signal" receptors, the TNF receptor and Fas. It is shown that p53 trans-activates Fas/APO-1 transcription.[148] As described before, Fas is a cell surface protein that triggers apoptosis upon ligand (FasL) binding. Fas belongs to the TNFR family of genes

coding for membrane receptors, and is involved in the regulation of cell proliferation. In the presence of the DNA damage, p53 trans-activates also the KILLER/DR5 gene.[149] DR5 is another member of TNFR family. Like Fas, the binding of the ligand (TRAIL) to DR5 activates caspase-dependent apoptosis. The induction of Fas and DR5 transcription by p53 indicates that the p53 transcriptional function may assist in modulating the apoptotic response triggered by certain stimuli. Another effector recently discovered of p53-dependent apoptosis is called PIDD. Over-expression of PIDD inhibits cell growth in a p53-like manner by inducing apoptosis.[150] Finally, p53 may cause death by directly stimulating mitochondria to produce an excess of highly toxic reactive oxygen species. Moreover, p53 represses the transcription of specific genes that inhibit its capability to induce apoptosis such as Bcl-2.

After stimulation, p53 may induce cell death or cell cycle arrest. The different outcome depends upon a variety of variables. The genetic background of the cell can be important. For instance, p21-null cells do not arrest in response to DNA damage but proceed to apoptosis in a p53-dependent manner. Impaired cross-talk between p53 and pRB/p105 can lead to the same result. The anti-apoptotic effect of Bcl-2 and adenoviral E1B can prevent p53-mediated apoptosis. The extent of DNA damage and p53 protein levels are also factors that contribute to making the choice between life and death. It may be that during p53-induced cell cycle arrest, the cell attempts to repair damage, perhaps with the assistance of its enhanced repair capability from p53 induction of Gadd45. If the damage is too extensive to be repaired, the cell then is committed to die. It has been shown the p53 can be regulated by oncogenic and hyperproliferative stimuli. CKI p14/p19ARF is the main player in this mechanism. Mitogenic signals derived by oncogenes, such as Myc, E1A or E2F, are able to induce p14/p19ARF synthesis. Subsequently, p14/p19ARF binds MDM2 inhibiting its capability to induce p53 degradation.[100] Indirect stabilization of p53 by p14/p19ARF results in cell death.

p73 also participates to the induction of apoptosis in response to DNA damage, but in a different way with respect to p53, as ultraviolet light and hypoxia do not induce the accumulation of p73 in several cell types. In cells exposed to IR, p73 contributes to the apoptotic response to DNA damage by interacting with the nuclear enzyme c-Abl tyrosine kinase, which phosphorylates p73 at tyrosine residues. IR activates c-Abl through activation of the ATM kinase. On the contrary, the disruption of the interaction between p73 and c-Abl produces a failure of IR-induced apoptosis.[151,152] Moreover, cisplatin, a cancer chemotherapeutic agent which cross-links DNA, seems to increase the cellular amount of p73 and induces apoptosis. This induction requires the activation of c-Abl tyrosine kinase and functional MHL1 gene, which encodes for mis-match repair protein. Mouse embryo fibroblasts deficient in c-Abl and in mis-match repair do not up-regulate p73 and are more resistant to cell death induced by cisplatin.[153] These data support the existence of a p53-independent signalling pathway for apoptosis, which involves p73 and c-Abl as mediators of apoptosis.

Cancer: Deregulated Cell Cycle and Evasion of Apoptosis

Evolution of Cancer

Cancer is a disease in which unremitting clonal expansion of somatic cells kills by invading, subverting and eroding normal tissues. The processes governing the genesis and progression of cancers are evolutionary ones by which natural selection acts upon the inherent or acquired diversity of various somatic clones, fostering their outgrowth with some form of proliferative advantage. The evolutionary imperative of vertebrates has been to find a way to allow cell proliferation when needed, while at the same time efficiently suppressing the genesis of mutated cells leading to deregulated growth. All eukaryotes have evolved a plethora of mechanisms to minimize DNA damage so that the cells cope with the required monitoring and maintenance of genomic integrity by means of a complex network of DNA repair pathways

and cell-cycle checkpoints. When such of these measures fail, cancer is the inevitable consequence. Tumors are diverse and heterogeneous, but all share the ability to proliferate beyond the constraints limiting growth in normal tissue. Aberrations in the regulation of a restricted number of key pathways that control cell proliferation and cell survival are mandatory for establishment of all tumors. Deregulated cell proliferation together with suppressed apoptosis, constitutes the minimal common platform upon which all neoplastic evolution occurs.

An inability to respond properly to, or to repair, DNA damage leads to genetic instability, which in turn may enhance the rate of cancer development. Indeed, it is becoming increasingly clear that deficiencies in DNA-damage signaling and repair pathways are fundamental to the etiology of most, if not all, human cancers. Genetic instability is one of the hallmarks of cancer and its links to aberrations in DNA repair machinery and the cell-cycle checkpoint pathways is well documented. Evidence to support this notion continues to accumulate and here we will briefly review the available knowledge and the recently identified cancer-associated defects of G1/S checkpoint components (Table. 2.).

Except for the ATR, whose lack causes early embryonic lethality in mice and whose somatic defects might result in cell death, all the major G1/S checkpoint transducers and effectors qualify as either tumor suppressors or proto-oncogenes and their loss-of-function, mutations or over-expression have been identified in many types of human malignancies. In addition, when mouse models that mimic such defects are available, the resulting phenotypes generally support the putative roles of these checkpoint regulators and effectors in guarding against genomic destabilization and tumor development. Homozygous inactivation of ATR in mice results in early embryonic lethality associated with chromosomal fragmentation, extensive apoptosis and loss of cellular proliferative potential.[154,155] Although the reason of this lethality is not clear, it seems that, at least for rapid dividing embryonic cells, ATR is crucial for progression through the normal unperturbed cell cycle. Consistent with this, no ATR mutations have so far been documented in human disease. Hereditary mutations in at least ATM, Chk2, BRCA1, Mre11, NBS1, p53, p16 and pRB are known to cause familial cancer and /or clinical syndromes that are cancer prone.[156] In particular, deficiency of ATM has been described in patients with neurodegenerative syndrome and ataxia-telangiectasia (AT). Cancer constitutes a major cause of death in these patients, which are at particularly high risk of developing lymphoreticular malignancy (lymphomas and leukemias) and breast cancer. Cells from ATM homozygous mutants show chromosomal instability with gaps, breaks, translocations involving the T-cell receptor locus, telomere-telomere fusions and increased rates of intrachromosomal recombination as well as defective stabilization of p53.[157]

The biological and patho-physiological relevance of the checkpoint pathways is supported by their evolutionary conservation and it is evident from the consequences of checkpoint failure. Checkpoint malfunction leads to accumulation of mutations and chromosomal aberrations, which in turn increase the probability of developmental malformations or genetic syndromes and diseases, including cancer. The consistent nature of the chromosomal aberrations in specific tumor types and subtypes has strengthened the view that the tumor phenotype is established by the creation of chromosomal abnormality. These tumor specific chromosomal rearrangements have two principal consequences: gene activation or gene fusion. Gene activation is generally the consequence of a presumed oncogenic translocation of a potential gene into the same chromosomal context in proximity of genes, which are normal transcribed so that this oncogene is constitutively activated. Although gene activation is the result of chromosomal abnormality in many tumors, the most common consequence of chromosome translocation in human cancer (leukemia and solid tumors) is gene fusion. In this second category of chromosome abnormalities, the chromosome breakpoints split the genes on both partner chromosomes and lead to juxtaposition of part of each gene in the joint segments and to the creation of a composite gene. Because the breakpoints disrupt each gene within an intron, coding

Table 2. Aberrations of the G1/S checkpoint components in human tumors

Tumor Suppressor Gene/Protein	Molecular Defects	Human Cancer	Hereditary Syndrome/ Cancer Prone
ATM	Truncation, missense mutations, deletions, reduced expression	Breast carcinomas	Ataxia-telangiectasia
Chk1	Frameshift mutations	Colorectal and endometrial carcinomas	Not reported
Chk2	Truncation, missense mutations, reduced expression	Breast carcinomas, lung, colon, urinarybladder, testicular tumors	Li-Fraumeni syndrome
Brca1	Mutations, deletions, reduced expression	Ovarian and breast carcinomas	Familiar breast and ovarian carcinomas
Mre1	Missense and frameshift mutations, truncations	Breast carcinomas, lymphoid tumors	Ataxia-telangiectasia-like disorder
p53	Missence mutations, deletions, HPV-E6-mediated degradetion	Many types of cancer	Li-Fraumeni syndrome
p16	Deletions, promoter silencing, missense mutations	Many types of cancer	Familiar melanoma
RB	Deletions, promoter silencing, missense mutations, HPV-E7-mediated inactivation	Many types of cancer	Familiar retinoblastoma
(Proto)-Oncogene Gene/Protein	**Molecular Defects**	**Human Cancer**	**Hereditary Syndrome/ Cancer Prone**
Cyclin D1	Gene amplification, translocation, overexpression	Many types of cancer	Not reported
Cyclin E	Gene amplification, overexpression	Ovarian and breast carcinomas	Not reported

sequences of each gene are maintained in the same reading frame, and the resulting chimeric transcripts usually encode hybrid proteins (see for review ref. 158).

Most of these mutations produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function.[159] Accordingly with this view, malignancy would be promoted by activation of oncogenes and/or inactivation of tumor suppressor genes which, in normal conditions, directly modulate cell growth and death. A large number of tumor suppressor genes have been identified, including p53 and retinoblastoma genes family (pRB/p105, pRB2/p130 and p107) and are known to prevent cancer through direct control of cell growth. Actually, the reintroduction of the tumor suppressor genes into cancer cells restores the missing function of these genes leading to the suppression of neoplastic growth.[160-163] Alterations in at least two of several regulatory circuits is essential for transformation of normal cells in malignant cells: i) the inactivation of antigrowth signals, which in normal cells exert an active block over proliferation either transiently, maintaining the cell in a quiescent (G0) state, or permanently, in the case of post-mitotic differentiating cells; ii) resistance toward apoptosis and, therefore, loss of the major control and repairing machinery preserving the cell

against abnormalities in intracellular and extracellular environment and deregulation of the apoptotic signaling pathways.

The anti-proliferative signals are concerted, at least in part, by the retinoblastoma family proteins that, in their active hypo-phosphorylated form, are able to block proliferation by sequestering the E2F transcription factors responsible for the expression of genes essential in the progression from G1 to S phase.[74] DNA damage, signaling imbalance caused by oncogene action, survival factor insufficiency, hypoxia [117] as well as abrogation of cell-matrix and cell-cell adherence-based survival signals [164] are by default stimuli for the induction of programmed cell death. A number of sensors [21] and effectors [52,137] ensure the efficiency of normal cells in preventing the effects of potentially dangerous events. Cancer cells evade apoptosis through a variety of strategies, the most common of which is inactivation of p53. Not surprisingly mutations of p53 gene have been detected in at least 50% of human tumors. Recent works also point at pRBs and CDKs as other molecules that may have dual role as integral component of cell cycle machinery and regulators of apoptosis.[88] These molecules are generally found mutated or functionally inactivated in several tumors.

Regulation of apoptosis seems to play a key role in the oncogenic effect of proteins activated or created by chromosomal abnormalities. Insight into the genetic control of apoptosis has been demonstrated by the bcl-2 gene which was first described in association with the t(14;18) chromosomal translocation. The relative overproduction of intact bcl-2 protein is not in itself transforming but can work in synergy with c-Myc to produce clonal outgrowth in vitro and in vivo. The available data strongly argue that bcl-2 exerts its effect by inhibiting apoptosis in a cell population that normally is destined to die. On the other hand, bcl-2 is not itself malignant but increases susceptibility to subsequent transformation events such as c-Myc activation. Therefore, two signals, both from an oncoprotein and confirmatory growth/survival factors, appear to be needed to elicit malignant hyperproliferation. There are two alternative responses to oncogene expression when the second signal is absent or incomplete: prolonged quiescence or apoptosis. Prolonged quiescence or apoptosis of oncogene-expressing cells in the absence of further signals indicates that at least two signals are needed to initiate proliferation of fully differentiated cells, thereby minimizing the possibility that mere up-regulation of an oncogene could elicit hyperproliferation or growth expansion. An emerging common theme in cellular transformation by translocation-associated proteins is that these proteins seem to disrupt the normal development of tumor-target cells by altering the control of PCD. This end is illustrated by the chimeric BRC-ABL p190 and BRC-ABLp210 proteins which need to inhibit apoptosis to develop their malignant phenotype, since this modulation of cell survival is mediated by bcl-2, through a Ras-dependent signaling pathway. A functional BCR-ABL oncogene induces proliferation by inhibiting the apoptotic pathway mediated by c-Myc. Therefore, cellular transformation by BRC-ABL results after activation of both bcl-2 and c-Myc, indicating that transformation results not only from cooperation between oncogenes or the loss of a tumor suppressor and activation of a oncogene, but may also occur as a result of the activation/generation of a single oncogene (see for review ref. 158).

The link between checkpoint failure, genome destabilization and cancer inspires exploration of more rational therapies based on pharmacological or gene therapies with rate limiting events in checkpoint pathways and apoptosis.

Cancer Chemotherapy and Apoptosis

As deregulated proliferation and inhibition of apoptosis lie at the heart of all tumor development, they represent two obvious targets for therapeutic intervention in all cancers. Although most existing cancer drugs are anti-mitotic, they do not act by targeting the specific lesions responsible for deregulated tumor growth, but rather they interfere with the basic machinery of DNA synthesis and cell division. Ideally, chemotherapeutic drugs should specifically

target only neoplastic cells and should decrease tumor expansion by inducing cytotoxic and/or cytostatic effects with minimal collateral damage to normal cells. In reality, the effectiveness of chemotherapy has suffered from a range of confounding factors including systemic toxicity due to a lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance. Given the adaptability of tumor cells, it seems likely that drug resistance will continue to be an important clinical problem, even in the age of targeted therapeutics and tailored treatment regime. Although proteins that interfere with either drug accumulation or stability can contribute to clinical drug resistance, other factors acting downstream of the initial drug-induced insult can also play an important role.

Chemotherapeutic agents can induce a series of cellular responses that impact on tumor cell proliferation and survival. Perhaps the best studied of these cellular responses is apoptosis, an event by which various drugs can kill tumor cells by activating common apoptotic pathways. Thus, single mutations that impair apoptosis can produce multi-drug resistance. The fact that defects in apoptosis can promote drug resistance downstream of the drug-target interaction raises the possibility that genotoxic agents may induce further genetic mutations owing to damage without death. Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. The resistance to apoptosis can be acquired by cancer cells through a variety of strategies since it exists a variety of regulatory and effector components which are present in a redundant form. This redundancy holds important implications for the development of novel types of anti-tumoral therapy, since tumor cells that have lost pro-apoptotic components are likely to retain other similar ones. The most commonly occurring loss of pro-apoptotic regulator through mutation involves the p53 tumor suppressor gene, which is functionally inactivated in greater than 50% of human cancer. The tumor suppressor gene p53 and its downstream effector genes $p21^{WAF1/Cip1}$, MDM2, bax and Gadd45 seem to be important in the cellular response to genotoxic drug sensitivity.

Drug-induced apoptosis can be described as a balance between intrinsic and extrinsic survival signals and drug-induced death signals. Pro- and anti-apoptotic signals impact upon pro-apoptotic members of Bcl-2 family of proteins, which ultimately control the cellular fate. The intrinsic sensitivity or resistance to anti-cancer therapy can be modified by external survival signals provided by soluble cytokines and growth factors, cell-extracellular matrix interactions and cell-cell contact. These signals can be enacted at a cellular level by members of the bcl-2 family proteins, which act at the cytoplasmic surface of the outer mitochondrial membrane determining the cell survival threshold. The pro- and anti-apoptotic members of the bcl-2 family are able to homo- and heterodimerize, and a conceptual apoptosome has been invoked as the physical point at which disparate damage signals are integrated with survival signal and coupled to the release of mitochondrial cytochrome c and the activation of caspases (see section: Apoptosis an overview).[165] Many mechanisms of drug resistance have been described in which drug-target interactions are modified.

The first and best studied gene involved in multi-drug resistance is MDR1. This gene encodes for P-Glycoprotein (PGP), a 170KDa ATP-dependent transmembrane transporter which works as an energy-dependent efflux pump for a diverse group of lipophilic compounds and confers resistance to a variety of structurally unrelated, clinically important antineoplastic agents.[166,167] This pump decreases the total intracellular retention of drugs or redistributes intracellular accumulation of drugs away from target organelles. Over-expression of the MDR1 gene products has been implicated as a primary mechanism of tumor drug resistance and its expression is modulated by several regulatory proteins.[168,169] The transcription of MDR1 gene is negatively regulated by wild-type p53 (wt-p53) and significant increases in MDR1 mRNA levels are seen in p53 inactivated or mutated cell lines.[170,171] The c-Jun NH2-terminal protein kinase (JNK), a member of a mitogen activating protein kinase cascade is activated in human

carcinoma cells by treatment with a number of different anticancer drugs and this activation correlates with increased MDR expression.[172] Transformation of rat liver cells with v-H-ras or v-raf oncogenes causes an induction MDR1/PGP expression.[173] Wild-type p53 mediates the induction of the rat MDR1 gene by the anticancer drug daunorubicin;[174] p53 mutation and/or deletions have been linked to drug resistance in neuroblastoma, acute lymphoblastic leukemia, melanoma, osteosarcoma, breast, ovarian and testicular cancer.[175-181]

Modulation of the efficiency of drug-induced damage to the activation of apoptosis is being recognized as a key mechanism of drug resistance. Signaling pathways exist between cell-surface receptors and Bcl-2 family proteins. Trks receptor ligation leads to the phosphorylation of Akt and subsequent inactivation of the Bad pro-apototic Bcl-2 family protein which prevents it from binding to the Bcl-X_L anti-apoptotic Bcl-2 family protein and thus free Bcl-X_L to block apoptosis.[31] Pro-apoptotic proteins Bax and Bak are important for modulation of drug-induced apoptosis. In fact it is shown that mouse embryonic fibroblast lacking both Bax and Bak are completely resistant to etoposide- and staurosporine-induced apoptosis as well as loss of Bax alone, in colon carcinoma cells, results in an increase in resistance to the 5-fluorouracil chemotherapeutic agent. It is known that Bax transcription is regulated by p53 oncosuppressor gene, which is activated by a variety of DNA damage signaling. Several pathways regulate the apoptotic function of Bax through its conformational change and sub-cellular localization. Conformational changes and subsequent mitochondrial translocation of Bax have been also induced by Bcl-2 family protein Bid which is activated either through signaling from the plasma membrane death receptors Fas and TNF or through DNA damage.[182,183] Other Bid-independent mechanisms such as the p38 MAP kinase activation by nitric oxide are able to activate Bax. Extrinsic integrin-mediated survival signals, such as IL-4, IL-7 and vascular cell-adhesion molecule integrin ligand (VCAM-1), prevent drug-induced changes in the N-terminus of Bax.[184,185]

There are multiple points at which the activation of drug-induced apoptosis can be abrogated so that different cancer cells develop different resistance mechanisms. For this reason, understanding the mechanisms by which each tumor develops resistance to drug-induced damage could be useful to design target therapies. For example, breast cancer cells are deficient in caspase-3, which renders them insensitive to apoptosis induced by conventional chemotherapeutic agents. The reconstitution of caspase-3 renders the cells sensitive to etoposide and doxorubicin. Different strategies targeting survival signaling, ceramide metabolism, cyclin-dependent kinase inhibitors, receptor tyrosine kinases, could be used to induce apoptosis in cancer cells.

Targeting Survival Signaling

The extracellular signal-related kinase/MAP (ERK/MAP) kinase family is important in a large number of signaling pathways and consists of three kinases whose cascades play different roles in regulating cell death. In the ERK1/2 cascade, growth-factor-derived extracellular signals are translated to Raf-1 activation, which leads to the phosphorylation of MEK1 and MEK2, which in turn phosphorylate and activate ERK1 and ERK2. Constitutive ERK1/2 activity is detected in colon carcinoma cells which show high rate of survival. On the other hand, the other two MAP kinase members, JNK and p38MAP kinases, share effects opposing to ERK and are activated by a wide range of pro-apoptotic stimuli such as UV light, osmotic shock, inflammatory cytokine and chemotherapic drugs.[186] UV light and osmotic shock cell death signals activate MEKK1 and then MKK4 and MKK7, which phosphorylate JNK. p38MAP kinase is also activated by stress and inflammatory cytokines such as TNF and IL-1, MEKK1 and MKK3/4. Although these pathways share many similarities, they are clearly independent: MEK1/2 do not phosphorylate JNK or p38, and MKK3/4/7 do not phosphorylate ERK1/2.[187] A widely accepted model is that balance between growth-factor-activated ERK and stress-activated

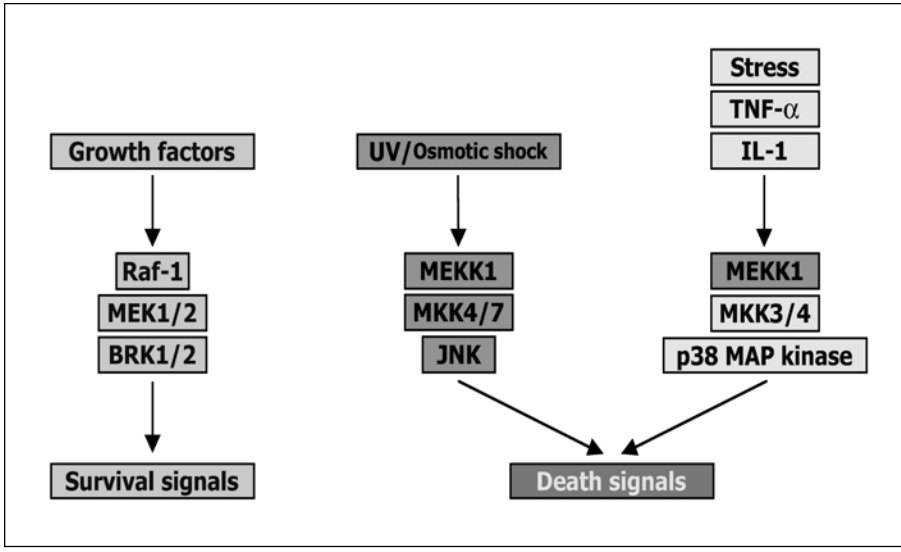

Figure 8. Different members of the ERK/MAP kinase family play different roles in the regulation of cell death. Growth-factors-derived extracellular signals result in Raf-1 activation, which triggers the activation of ERK (extracellular signal-regulated kinase), whereas death signals such as chemotherapeutic drugs and UV result in the activation of JNK and p38 mitogen-activated protein (MAP) kinase. Modified from Makin G, Dive C. Trends Cell Biol 2001.

JNK and p38 pathways determines whether the cell lives or die (Fig. 8.). In the rat pheochromocytoma cell line (PC-12), withdrawal of NGF leads to inhibition of ERK activity and to sustained JNK and p38MAP kinase activity with consequent sustained apoptosis, which can be prevented by transfection of constitutively active MEK1 mutants. The widespread involvement of these kinase cascades in death and survival signaling makes them potentially useful candidates for therapeutic modulation. A good example of their potential as targets for modulation of drug resistance was recently demonstrated by using paclitaxel, an anticancer agent that is active against a wide range of solid tumors. Exposure of breast, lung and ovarian carcinoma cell lines to paclitaxel results in increases in both JNK and ERK1/2 activity. Specific inhibition of survival signaling from MEK1/2 activity with a small molecule MEK inhibitor resulted in a significant enhancement of paclitaxel-induced apoptosis. Small-molecule inhibitors specifically targeting p38 and MEK1/2 MAP kinases (i.e. SB203580, PD098059, PD184352, U0126) have been produced and have been shown to be active against tumor growth in vivo, so their clinical use as modulators of drug-induced apoptosis appears to be promising.

Targeting Ceramide Metabolism

Ceramide is a lipid second messenger produced by the hydrolysis of sphingomyelin in response to a wide range of stimuli, including a long list of chemotherapeutic agents, growth factor deprivation, IR, heat shock and various environmental factors such as stress. Ceramide-mediated signalling can lead to apoptosis, which is mediated via two pathways (see for review ref. 188). One pathway is transcriptionally dependent and the other is transcriptionally independent. The transcriptionally dependent pathway is mediated by the activation of TNF and CD95/APO-1/Fas family receptors, which leads to ceramide production. In particular, activation of CD95/APO-1/Fas signaling by ceramide has been shown to mediate doxorubicin-

induced apoptosis. In the CD95 receptor pathway, ceramide is generated by acid sphingomyelinase in a complex series of steps. The transcription-independent pathways are characterized by the direct activation of acid sphingomyelinase by environmental stresses such as IR and oxidative damage. The subsequent production of ceramide, in turn, activates the SAPK/JNK apoptotic pathway. In addition the transcriptionally independent formation of ceramide also may affect apoptosis-related proteins of the Bcl-2 family. Moreover, drugs can impact ceramide metabolism by promoting ceramide synthesis de novo. The drugs are of a diverse nature and include the anthracyclines doxorubicin (Adriamycin) and daunorubicin, the vinca alkaloids vincristine and vinblastine, antiestrogens such as tamoxifen, the novel synthetic retinoid N-(4-hydroxiphenyl)retinamide, and the taxane paclitaxel (Taxol).

Novel Therapeutic Agents

Abrogation of survival signaling to enhance the pro-apoptotic ability of conventional chemotherapeutic agents is a powerful strategy towards overcoming drug resistance. An alternative approach is to identify molecular abnormalities in tumor cells that decrease intrinsic sensitivity to apoptosis and to target these with novel agents. Provided that these abnormalities are widespread among different tumor types, or that they are seen frequently in a common malignancy, this represents a viable strategy for new drug development. Two areas in which this approach has been taken recently is targeting the oncoproteins involved in the G1/S and G2 checkpoints deregulation and gene therapy which restore the functional tumor suppressor genes frequently inactivated in a variety of tumors.

Although conventional agents were not designed to induce apoptosis, the fact that they do so indirectly demonstrates that apoptosis can be an effective mechanism for eliminating tumor cells. Agents that induce apoptosis directly would overcome many of the problems observed with existing drugs such as cytostasis and necrosis, which produce inflammation or damage to the surrounding normal tissue and mutagenic effect. The new approach to therapy is to restore apoptosis through genetic methods. The direct relationship between p53, apoptosis and drug activation implies that restoring p53 activity in p53 null tumors, or activating pathways that are directly downstream of p53, would have clinical benefits. Reintroduction of wild-type p53 into p53 null tumors can directly induce apoptosis and restore sensitivity to the chemotherapeutic drugs, while adenoviral gene transfer of Bax activates apoptosis downstream p53 promoting tumor regression in vivo.

In certain circumstances, apoptosis in cancer can be inactivated by gene silencing rather than by mutations. Silencing of ARF, capsase-8 and Apaf1 by DNA methylation has been linked to drug resistance.[189] By using demethylating agents and/or histone deacetylase inhibitors it is possible to reactive these latent killers. However, these agents lack specificity, since normal gene expression can be controlled by similar mechanisms. Antisense approaches to decrease expression of anti-apoptotic genes including bcl-2, Ras and MDM2 are in various stages of preclinical development.[190] Specific small molecules inhibitors of Bcl-2, which block Bcl-2 homodimerization and heterodimerization leading to cytochrome c release, have now been developed.[191]

Other promising therapeutic targets include components of the pro-survival signal transduction pathways involving Ras, Akt or NF-κB that contribute to intrinsic or inducible drug resistance. For example, inactivation of NF-κB by overexpression of I-κB can restore sensitivity of tumor cells to chemotherapy.[192] In addition, small molecule inhibitors of PI-3 kinase/Akt and farnysyltransferases, necessary for the activity of Ras, can induce apoptosis, are relatively nontoxic to normal cells and mediate tumor regression.

While some degree of apoptosis inhibition must accompany tumorigenesis, it is clear that tumor cell survival reflects a fine balance between hyperproliferative, pro- and anti-apoptotic factors. Thus, the addition of yet another apoptotic stimulus in the form of a chemotherapeutic

drug may tip the balance in favor of apoptosis, at least in those cells containing a partially functional apoptotic program. In contrast, normal cells may be less sensitive to these signaling alterations. This concept has been put into practice by designing novel drugs that target only those cells containing a specific tumorigenic lesion. Rather than attempting to reactivate the intrinsic apoptotic pathway, an alternative approach would be to engage a fundamentally different apoptotic program to kill tumor cells. Mutations in the death receptor pathway are not as frequent as those in the intrinsic apoptotic pathway, such that this program might remain available to trigger an anti-tumor response. Recently, a recombinant form of TRAIL has been tested as anti-neoplastic agent. TRAIL induces the death receptor pathway and is not affected by over-expression of Bcl-2 or Bcl-X_L.[193] TRAIL is particularly attractive because it specifically targets tumor cells and is relatively nontoxic to untransformed cells.

Loss of the normal regulation of the cell cycle is a common finding in tumor cells. The progression from one phase of the cell cycle to the next is controlled by the activation of a family of serine/threonine kinases, the CDKs. Activity of CDKs is controlled by degradation of their activating cyclins. The transition from G1 to S phase involves the phosphorylation and inactivation of the retinoblastoma family proteins (pRBs) by cyclinD/CDK4, cyclinA/CDK2 and cyclinE/CDK2 complexes. Phosphorylated pRBs release the E2F transcription factors, which leads to the transcription of genes needed for S phase induction and progression. CDK activity is regulated by CDK inhibitors. This pathway is abnormal in the vast majority of human cancer, leading to constitutively inactive pRB gatekeeper proteins and to the loss of G1/S checkpoint. pRB oncosuppressor proteins can be inactivated in each specific tumor via deregulated phosphorylation as well as mutations or viral oncoprotein binding. Apoptosis is readily induced in tumor cells with inactivated pRBs but not in normal cells, demonstrating the rationale for designing novel agents. The cyclin-pRB-E2F pathways described above are potentially amenable to pharmacological manipulation. For example, it might be possible to develop small molecules that specifically inhibit certain cyclin/CDK complexes or that could affect E2F-dependent transcription, as well as peptides that can mimic the functional regions of RB proteins. Novel small-molecule inhibitors of CDKs have been used clinically to attempt to restore the functionality of pRBs. Flavopiridol is a competitive inhibitor of CDK2 and CDK4. Flavopiridol is a potent inducer of apoptosis in hematopoeitic cell lines and is active against leukaemia and lymphoma in xenograft models.[194] Another possibility is the use of gene therapy to restore the function of individual components of the pRBs pathway. In this regard, there are in vitro and in vivo experiments using the retroviral delivery approach that demonstrate the ability of pRB2/p130 to induce the repression of tumor mass.[162,195] To date, gene therapy does not give yet an efficient aid to conventional medicine, even if recent advances in newly designed strategies have been proposed. New targets remain under investigation and oncosuppressor genes such as p53 and RB proteins could be considered targets for new strategy in gene therapy.

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CHAPTER 13

G1 Phase Control and Cell Differentiation

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Abstract

Cell differentiation is usually accompanied by irreversible cell cycle exit. The G1 regulatory molecules have been shown to be exquisitely regulated during the differentiation process and in many models they have been shown to play a pivotal role in differentiation. The cell cycle exit concomitant with the onset of differentiation occurs in G1 phase and it is mediated and maintained by (i) up-regulation of CDK inhibitor proteins, (ii) activation of the RB protein family (pRB, p107 and p130) and (iii) subsequent inactivation of E2F proteins. Among these G1 regulatory molecules, $p21^{WAF1}$, $p27^{KIP1}$, p130 and E2F4 have been most predominantly involved as differentiation inducers. Studies in cell culture models as well as in vivo models through transgenic and knockout mice demonstrate that $p21^{WAF1}$ and $p27^{KIP1}$ play important but distinct roles in differentiation and that the cell cycle arrest and differentiation inducing functions can be genetically separated. Also, p130, rather than pRB, functions more frequently as the pocket protein regulating cell cycle exit during differentiation. Despite these broad generalizations, there is a large variation in the roles of these regulators depending on the model under study. Therefore, we have reviewed separately the regulation and functions of G1 phase regulatory proteins in the main differentiation models.

Introduction: The Switch Differentiation-Proliferation

With a few exceptions, cell differentiation involves exit from cell cycle and an irreversible proliferative arrest. During differentiation, each committed cell triggers the expression of number of tissue-specific genes coordinately with cell cycle exit. It is conceivable that the high expression levels of many tissue-specific genes required to bring about the tissue functions are incompatible with the transient switch-off of transcription that occurs during mitosis.

Thus, the cell committed to differentiation must take two decisions:the decision to irreversibly arrest cell cycle progression and enter the G0 state and the decision to trigger the expression of differentiation transcription factors and tissue-specific genes. The maintenance of the state of irreversible cell cycle arrest is a common feature to mature of cells from very different tissues and the general hypothesis is that common mechanisms for cell cycle arrest operate during or prior differentiation.

The data gathered over the last years aims towards the idea that the cell cycle exit concomitant with the onset of differentiation or differentiation commitment occurs in G1 phase and it is mediated and maintained by (i) up-regulation of CDK inhibitor (CKIs) proteins, particularly $p21^{WAF1}$ and $p27^{KIP1}$; (ii) activation of the RB protein family (pRB, p107 and p130) and subsequent inactivation of E2F proteins.

G1 Phase Progression, edited by Johannes Boonstra.

Although it is clear that CKIs and RB proteins are involved in the cell cycle exit associated to differentiation, there are two questions to be answered:i) Is the cell cycle arrest provoked by these proteins sufficient to trigger differentiation? ii) Do these proteins have a role in differentiation control independent from the cell cycle arrest function?

Given the redundancy of regulatory circuits that control G1 progression, and the intricate network of interactions between the regulatory proteins, it has been difficult to dissect the contribution of individual proteins to differentiation. Moreover, the data indicate that the roles of each protein may differ depending on the cell type.

CKIs in Differentiation

As a general rule, the expression of $p21^{WAF1}$ and $p27^{KIP1}$ increases during differentiation. The up-regulation of other CKIs as p57-^{KIP2}, $p16^{INKa}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ has also been reported in the differentiation response, although the involvement of these CKIs has been much less studied. In models where the issue has been studied in detail, it has been found that the onset of the up-regulation varies with the CKI in each particular model and in many cases the up-regulation of $p21^{WAF1}$ is rapid and occurs later and is transient while that of $p27^{KIP1}$ is more maintained. This is the case, for example, in intestinal cells,[1,2] keratinocytes,[3] preadipocytes,[4] muscle cells,[5,6] and myeloid cells (ref. 7 and Muñoz-Alonso and León, unpublished) it has been found that the induction of $p21^{WAF1}$ is rapid and transient while induction of $p27^{KIP1}$ occurs later and it is maintained. However, there are exceptions to this general rule, as in P19 neuronal differentiation.[8] In the models where it has been studied in detail, it has been found that $p21^{WAF1}$ up-regulation occurs at the transcription levels and depends on the Sp1 and Sp3 binding sites in the proximal $p21^{WAF1}$ promoter, as in keratinocytic,[9] neuronal[10] and intestinal cell[11] differentiation.

While it is clear that differentiation is usually accompanied by up-regulation of CKIs, it is less clear whether CKI up-regulation is a consequence or, on the contrary, triggers the differentiation process. In many models, the forced expression of $p21^{WAF1}$ and $p27^{KIP1}$ genes after transfection or viral infection is sufficient to induce differentiation, but this result is not always found. Finally, in the cases where enforced expression of CKI results in differentiation, it is not clear whether this is a result of the cell cycle arrest brought about by the CKI or whether the CKI has a pro-differentiating function unrelated to cell cycle arrest.

However, as a general conclusion it can be stated that $p21^{WAF1}$ and $p27^{KIP1}$ play nonequivalent roles in differentiation. In several models, as intestinal cell differentiation, $p27^{KIP1}$ is a more efficient differentiation inducer than $p21^{WAF1}$, while in neuronal differentiation $p27^{KIP1}$ serves to arrest cell cycle without differentiation induction (see below). Mice deficient in $p21^{WAF1}$ show abnormal keratinocytic differentiation (reviewed in 12). In the case of $p27^{KIP1}$ in glial differentiation of Xenopus retinal cells, investigators have been able to separate the differentiation-promoting region, in a N-terminal domain different from that required for its cell-cycle inhibitory function.[13]

A similar conclusion can be drawn from CKI-deficient mice models. $p21^{WAF1}$, $p27^{KIP1}$ and p57-^{KIP2} deficient mice are viable. Mice deficient in $p21^{WAF1}$ develop normally and harbor no detectable abnormalities in all their organs.[14] Mice deficient in $p27^{KIP1}$ show higher growth rate and are 20-30% larger than wild-type animals without other developmental abnormalities.[15-17] Mice lacking p57-^{KIP2} show growth retardation and defects in chondrocyte, muscle and kidney differentiation, without major effects in proliferation, and most animals die within a few hours after birth.[18-20]

Mirroring their differences in differentiation and development, $p21^{WAF1}$ and $p27^{KIP1}$ are differently involved in carcinogenesis. There are no consistent and significant changes in expression and mutations of $p21^{WAF1}$ in human cancer. In contrast, low levels of $p27^{KIP1}$ are

frequent in some tumor types and associated to malignant progression in epithelial derived tumors (breast, prostate, stomach and particularly colorectal cancer).[21-24] Moreover, $p27^{KIP1}$ nullizygous and heterozygous mice are more predisposed to radiation- or chemically-induced tumors than $p21^{WAF1}$-deficient mice. The cancer-related phenotypes of CKI- and RB-deficient mice have been reviewed elsewhere.[25,26]

Finally, there is an important variability in the regulation of cyclins among the differentiation models under study, but the over-expression of cyclin D3 emerges as a common feature in some of them, as muscular,[27,28] hematopoietic[29,30] and adipocytic[4] differentiation.

pRB and E2F in Differentiation

The elucidation of the role of E2F and pRB in differentiation have been hampered by the existence of several proteins composing both the RB family and the E2F family. pRB, p107 and p130 constitute the "pocket protein family ". pRB was the first described and best known member of the family, and is one of the tumor suppressor genes most frequently inactivated in human cancer.[31] The three pocket proteins are structurally very similar, although p107 and p130 are more closely related to one another than they are to pRB. The three proteins associate with members of the E2F family and can be phosphorylated by CDKs, although they differ on the E2F partner and phosphorylation kinetics. pRB and p107 show similar phosphorylation patterns, being hyperphosphorylated during G1 progression. In contrast, complexes of p130-E2F are predominant in G0 phase.[32] p130 is already phosphorylated in G0 cells, although it undergoes additional phosphorylation in other sites upon mitogenic stimulation of the cell.[33-35] As described below, different pocket protein-E2F complexes are formed depending on the particular differentiation model or differentiation lineage.

Disruption of both Rb alleles results in embryonic lethality. In contrast, mice deficient in p107 or p130 develop normally. However, embryos deficient in pRB/p107 or pRB/p130 show a similar phenotype than RB-deficient embryos but they die two days earlier, thus revealing some functional overlap between the pocket proteins. Interestingly, in a different mouse strain (Balb/c) deficiency in p130 results in embryonic lethality and deficiency in p107 results in severe postnatal growth impairment.[35-38]

The involvement of pRB in differentiation has been demonstrated in several differentiation models as keratinocytes, adipocytes and particularly muscle cells (recent reviews include:34,35,37-39).

In general, p130 is highly expressed in quiescent [32] and in differentiated cells. This is found in several lineages, such as muscular,[40-45] keratinocytic,[46] intestinal,[47] neuronal[48] and hematopoietic[49] lineages. Interestingly, embryonic stem cells deficient in the three pocket proteins (pRB, p107 and p130), show a deregulated G1 and impaired differentiation, as assayed by limited capacity to form differentiated teratocarcinomas.[50]

pRB -deficient mice show defects in differentiation of neuronal, lens and erythroid precursor cells,[51-53] whereas muscular differentiation cannot be properly evaluated because of the embryonic lethality of these mice. In contrast, mice deficient for p107 or p130 show no clear defects. Mice defective for both pocket proteins show neonatal lethality with deregulated chondrocyte growth and impaired bone development.[40,54] Thus these genetic disruption experiments demonstrate nonredundant roles of pocket proteins during development, and the same conclusion can be drawn from the differentiation models. The characterization of a pRB mutant that retains the ability to induce differentiation of Saos-2 cells but cannot bind E2Fs and induce G1 arrest demonstrates a critical role for pRB in regulating differentiation.[55] The ability to regulate differentiation may explain why pRB has tumor-suppressor properties lacking in p107 and p130 (reviewed in 39).

E2F is a six-member family,[56,57] although E2F5 and 6 have not been studied in relation with differentiation. Recent studies with oligonucleotide microarrays have identified a number of genes related to differentiation and cell fate, including homeobox genes and genes related to signal transduction by factors from the transforming growth factor (TGF) family. The study also revealed differences in expression among E2F members.[58] E2F1 is a paradoxical gene as it can function as an oncogene or tumor suppressor gene depending on the tissue type.[39] Induction of apoptosis by E2F1 is a fundamental property not shared by its siblings E2F2, 3, 4, or 5. The involvement of E2Fs in differentiation has been addressed in several studies, analyzing the formation of complexes of these transcription factors with pocket proteins. In most models, the E2F protein usually found in the complexes is E2F4. E2F4 is involved in keratinocyte[59] and neuronal[60] differentiation, whereas E2F1[38,39] and E2F3[61] function has been related to cell cycle progression rather than to differentiation.

Although there are a few common facts to differentiation models, summarized above, it is clear that each tissue differentiates with its own molecular peculiarities. Actually, an important conclusion that emerges from the published studies is the variety of pathways controlling the G1 arrest in differentiation depending on the cell type. Moreover, it has recently been shown that some transcription factors that induce differentiation of particular tissues control not only tissue-specific proteins but also cell cycle regulatory proteins. The clearest example is the muscular differentiation transcription factor MyoD, which up-regulates $p21^{WAF1}$, cooperate with pRB to arrest growth and binds CDK4 (reviewed in 62,63). Given the number of G1 regulatory proteins involved (CKIs, CDKs, pocket proteins and E2Fs) and their interrelations it is difficult to obtain a complete picture of their expression in a particular model, and dissect which change determines differentiation or is a consequence of differentiation. Although cell culture differentiation models may be not physiological, it allows the study of molecular changes during differentiation under controlled conditions, and a lot of information on the molecular biology of differentiation has been generated by these models.

We summarize below the involvement of G1 phase control proteins in the major differentiation models. We will first review the differentiation of epithelial-derived cell types (epidermal, neuronal, intestinal) and secondly the differentiation of mesenchymal-derived cell types (muscular and hematopoietic). The most relevant data on models of nonhematopoietic differentiation are summarized in (Tables 1 and 2).

Keratinocytic Differentiation

Up-regulation of CKIs has been shown in several models of keratinocyte differentiation. Normal human epidermal keratinocytes undergo differentiation by suspension culture, with concomitant up-regulation of $p21^{WAF1}$,[64,65,46] $p27^{KIP1}$ and $p16^{INK4a}$.[46] Primary mouse keratinocytes are induced to differentiate in response to raised calcium concentration in the medium. In this model, differentiation and withdrawal from the cell cycle also correlates with induction of $p21^{WAF1}$, $p27^{KIP1}$ and p57-^{KIP2} and their association with CDK2.[66-68] It has been demonstrated that $p21^{WAF1}$ binds to calmodulin in a calcium-dependent manner[69] thus providing a direct link between the differentiation agent and the effector molecule for growth arrest. In a different model, $p21^{WAF1}$ and $p27^{KIP1}$ are induced concomitantly with differentiation of hair follicle cells in rats.[70]

The available data on $p21^{WAF1}$ involvement in keratinocyte differentiation are somewhat contradictory. Mice deficient in $p21^{WAF1}$ have no alterations in epidermis,[59,71] but primary keratinocytes derived from these mice show impaired calcium-induced differentiation in culture, with a drastic down-regulation of differentiation markers linked with late stages of keratinocyte differentiation.[66] In contrast, keratinocytes derived from $p27^{KIP1}$ deficient mice differentiate normally.[66] Mice deficient in $p21^{WAF1}$ show a reduced self-renewal potential of

Table 1. Regulation and effects of G1 regulatory proteins in some non-hematopoietic differentiation models. It is also indicated when the lack of effect of a particular protein was found

Cell Model (Species)	Differentiation	Cell Cycle Regulator Expression	Differentiation Induced or Increased by	References
Prim. keratinocytes (hum)	Keratinocytic	↑p21 ↑p27 ↑p16	p16 (not p21, p27)	46,64,65
Prim. keratynocytes (mouse)	Keratinocytic	↑p21 ↑p27	(Inhibited by p21)	3
HaCAT (hum)	Keratinocytic	↑p21 ↑p27	E2F4	59
HIEC6 (human)	Enterocytic	↑p21 ↑p27	p21, p27 (not p16)	78
TsFHI (human)	Enterocytic	↑p21 ↑p27	p27	1,78
HT-29 (human)	Enterocytic	↑p21 ↑p27	p27	2,78
CaCo-2 (human)	Enterocytic	↑p21 ↑p27 ↑p130/E2F4		1,47
Oligodendrocytes (mouse)	Neuronal	↑p21 ↑p27 (not p16)	p21	93-96
Retinoblasts (Xenopus)	Glial	↑p27	p27	13
Ectodermal cells (Xenopus)	Neuronal	↑p27	p27	103
NTera (human)	Neuronal	↑p21	p21	80-82
N1E-115 neuroblastoma (mouse)	Neuronal	p27	p27	105
NT2/D1 embryonal carcinoma (human)	Neuronal	↑p27 (not p21)	p27 (not p21)	8
PC12 (rat)	Neuronal	↑p21 ↑E2F4	p21, E2F4	60,80-82
P19 (mouse)	Neuronal	↑p21 ↑p27 ↑p130 ↓E2F1, ↓E2F2, ↓E2F4		8,85
Retina photoreceptors (mouse)	Neuronal	↑p27 ↑p57	Not p27	98
Corti neurons (mouse)	Neuronal	↑27	Not p27	102
TSU-Pr1 (hum)	Neuronal		p21	104
ATDC5 (mouse)	Chondrocytic	↑p21 ↑p27. (not p15, p18, p19)		227
C2C12 (mouse)	Muscular	↑p18 ↑p21 ↑p27 ↑p57 (not p19) ↑p130	p16, p21, p57	41,116,117,127
C2 (mouse)	Muscular	↑D3 ↑p130		43,128
L6 (rat)	Muscular	↑p130 ↓p107		44,127
Saos-2 (human)	Osteocytic		Rb	55
3T3-L1 (mouse)	Adipocytic	↑p21 ↑p27 ↑p18 ↑p130	Rb	230,233

keratinocyte stem cell populations[72] and double $p21^{WAF1}/p16^{INKa}$ null mice show more profound alterations.[59]

In murine epidermal differentiation, maximal expression of $p21^{WAF1}$ occurs in postmitotic cells, while it is low in proliferating stem cells and mature mouse keratinocytes. Murine

keratinocytes can be differentiated in culture by high calcium concentrations. This process is accompanied by a rapid and transient up-regulation of $p21^{WAF1}$, which returns to basal levels after 24 h.[3] Enforced expression of $p21^{WAF1}$, but not of $p16^{INKa}$, in post-mitotic cells (using adenoviral vectors) inhibits differentiation.[3] This differentiation inhibition is not reproduced in human keratinocytes induced to differentiate in suspension culture. In this system, differentiation is associated to elevation of $p21^{WAF1}$, $p27^{KIP1}$ and $p16^{INKa}$, but enforced expression of $p21^{WAF1}$ does not induce differentiation. Overexpression of $p16^{INKa}$ and $p27^{KIP1}$ also fails to induce differentiation.[46] However, expression of antisense $p27^{KIP1}$ prevents the differentiation of primary mouse keratinocytes induced by suspension culture, but did not prevent growth arrest.[73] Thus, involvement of $p21^{WAF1}$ and $p27^{KIP1}$ in this differentiation system is still under discussion.

The involvement of E2Fs in keratinocyte differentiation has also been studied. Squamous differentiation of keratinocytes is associated with down-regulation of E2F1, and it is upregulated in squamous cell carcinomas with respect to healthy epidermis. Consistently, E2F1 overexpression inhibits differentiation, although suppression of E2F1 activity (with dominant negative mutants) does not induce differentiation.[74] In another study, E2F1 expression is constitutive during differentiation of human epidermal keratinocytes induced in culture, whereas E2F4 is predominantly expressed at the onset of differentiation. HaCaT cells transfected with E2F1 are unable to differentiate, but cells transfected with E2F4 show an increased differentiation rate compared to vector-transfected cells.[59] So, E2F1 and E2F4 appear to have opposite functions in human keratinocyte differentiation. Finally, it has been reported recently that pRB up-regulates $p21^{WAF1}$ in epithelial cells, but not in fibroblasts. This effect is transcriptional, and depends on Sp1 and Sp3 binding sites in the proximal $p21^{WAF1}$ promoter.[9]

Enterocytic Differentiation

$p21^{WAF1}$, $p27^{KIP1}$ and p57-^{KIP2} are accumulated, as determined by immunohistochemistry, during differentiation of human enterocytes in intestinal crypts.[75] During spontaneous differentiation of the human intestinal cell line CaCo2 cells, a well-known model of human colon cell differentiation, there is an up-regulation of $p27^{KIP1}$ and a rapid and transient elevation of $p21^{WAF1}$ levels.[65,76,77] This differentiation is also accompanied by increased expression of p107 and p130. The predominant complex that accumulated during differentiation was p130/E2F4.[47]

Differentiation of human HT-29 colon cancer cells by sodium butyrate is accompanied by transient up-regulation of $p21^{WAF1}$ and more sustained elevation of $p27^{KIP1}$. Ectopic expression of $p27^{KIP1}$ in human colon cancer derived cells increases the sensitivity of the cells to induction of differentiation, whereas enforced expression of $p21^{WAF1}$ shows the opposite result.[2,78]

The other cell line that has provided important information on the role of CKIs in intestinal cell differentiation is the human intestinal cell line tsFHI. While CaCo2 and HT-29 are tumor-derived cell lines, tsFHI cells are conditionally immortalized by the thermosensitive SV40 T-antigen mutant. At the permissive temperature (e.g., 32°C), tsFHI cells proliferate displaying crypt cell markers. When shifted to the nonpermissive temperature (39°C) the cells undergo irreversible growth arrest and differentiation into brush border cells. With differentiation, $p21^{WAF1}$ and $p27^{KIP1}$ were strongly induced, but with different kinetics:the $p21^{WAF1}$ increase was rapid but transient and the $p27^{KIP1}$ increase was delayed but sustained, i.e., a similar pattern than colon cancer cell lines reviewed above.[1] In this cell line, forced expression of $p21^{WAF1}$ and $p27^{KIP1}$ led the cells to expression of differentiation markers. This differentiation was temporally dissociated from inhibition of pRB phosphorylation, and $p27^{KIP1}$ was more efficient inducing differentiation than $p21^{WAF1}$. A striking result is that $p27^{KIP1}$ failed to complex with cyclins and CDKs, despite its fivefold increase in differentiating cells.[1] Thus, in

this cell line $p21^{WAF1}$ is the main CKI involved in irreversible growth arrest during the early stages of cell differentiation, whereas $p27^{KIP1}$ may induce or stabilize expression of differentiated traits, in a function independent from cell cycle arrest. A similar result has been reported for HT29 intestinal cells (see above). Therefore $p27^{KIP1}$ seems to be a critical protein in intestinal cell differentiation. Also, ectopic expression of $p21^{WAF1}$ and $p27^{KIP1}$ (but not $p16^{INKa}$) induce differentiation in a normal human intestinal cell line (HIEC6) and the authors suggest that $p21^{WAF1}$ may act indirectly by elevating $p27^{KIP1}$ levels.[78] A similar regulatory cross-talk between $p21^{WAF1}$ and $p27^{KIP1}$ has been reported recently for K562 myeloid leukemia cells[79] (see below).

It is noteworthy that low expression of $p27^{KIP1}$ has been linked to poor prognosis in colorectal cancer.[21,23] Thus it is conceivable that the inhibition of differentiation in tumor cells devoid of $p27^{KIP1}$ is one of the mechanisms contributing to malignant progression in these tumors.[2]

Neuronal Differentiation

Up-regulation of CKIs has been reported during differentiation induced in several neuronal cell lines. One of the most broadly used is the rat pheochromocytoma PC12, which undergoes neuronal differentiation (with neurite formation) in response to nerve growth factor (NGF). PC12 cell differentiation induced by NGF is accompanied by $p21^{WAF1}$ induction, and enforced expression of $p21^{WAF1}$ induces growth arrest and increased sensitivity to the differentiation induced by NGF, but it does not directly lead to a differentiated phenotype (i.e., neurite extensions).[80-82] Unexpectedly, cyclin D1 also increases with PC12 differentiation.[80] In the PC12 model it has been shown that up-regulation of $p21^{WAF1}$ after NGF addition is dependent on a Sp1/Sp3 binding site next to the TATA box.[10] A similar result was found in the differentiation of the intestinal cell line CaCo2.[11] It is noteworthy that a low $p21^{WAF1}$ expression in embryonic brain and spinal cord was observed during mouse embryogenesis.[83]

Murine P19 cells treated with retinoic acid differentiate into neuroectoderm, with mixed populations of postmitotic neurons, astrocyte-like cells and oligodendrocyte-like cells (reviewed in 84). Neuronal differentiation of P19 cells occurs with concomitant up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$, but $p27^{KIP1}$ induction is rapid while $p21^{WAF1}$ expression remains low until neurites are formed. Consistently, inhibition of $p27^{KIP1}$ expression by antisense oligonucleotides results in differentiation inhibition.[8] Increase in $p27^{KIP1}$ is also detected in P19 cells differentiated by ectopic expression of neuroD2 or MASH1 genes.[85] Interestingly, differentiated P19 cells display high levels of cyclins D1 (as in PC12 cells, see above) and D2[86] and consistently endogenous CDK4 activity is high while CDK2 activity is low during neural differentiation of P19 cells.[48,86,87]

Neuronal differentiation of other cell lines such human embryonal carcinoma NT2/D1 cells,[8] neuroblastoma N2aβ cells differentiated by T3 hormone [88] or NTera2[8] are associated by elevation of $p27^{KIP1}$. Differentiation of NTera2 is also accompanied with $p15^{INK4b}$ and $p16^{INKa}$ induction.[89]

Differentiation of neurons during embryogenesis is a highly regulated process in which neuronal precursor cells exit the cell cycle and differentiate in a tightly coupled process. Involvement of pRB and E2F in neurogenesis has been recently reviewed.[84,90] Several in vivo models of neuronal differentiation have been studied. Differentiation of oligodendrocytes has provided interesting clues on the role of CKIs in differentiation. Oligodendrocytes originate from multipotent cells in proliferative ventricular areas of the brain. Primary cultures of progenitors can be isolated and induced to differentiate by serum deprivation.[91] During this differentiation, both $p21^{WAF1}$ and $p27^{KIP1}$ are induced, while $p16^{INKa}$ expression is maintained.[92,93] Ectopic expression of $p27^{KIP1}$ efficiently inhibits cell cycle progression, but is not sufficient to induce rodent oligodendrocyte differentiation, as assessed by expression of differentia-

tion markers.[94,95] Consistently, oligodendrocyte precursors derived from $p21^{WAF1}$- and $p27^{KIP1}$-deficient mice continue to proliferate and show delayed differentiation, demonstrating that both CKIs are required for proper oligodendrocyte differentiation.[93,96] Interestingly, work with these mice models show that $p27^{KIP1}$ is required for withdrawal from the cell cycle while $p21^{WAF1}$ is not. Instead, $p21^{WAF1}$ is required for the establishment of the differentiation program of oligodendrocyte progenitors following growth arrest. Also, $p21^{WAF1}$-deficient mice display delayed myelinization, which is consistent with the impaired differentiation of oligodendrocytes (which are myelinating cells of the central nervous system). Thus, the two CKIs serve nonredundant roles in this program of differentiation, with $p27^{KIP1}$ being responsible for arrest and $p21^{WAF1}$ having a function independent of its ability to control cell cycle exit.[96]

Development of retina photoreceptor cells constitutes an useful model of neuronal differentiation (photoreceptor cells) in vivo. Interestingly, $p27^{KIP1}$ and p57-^{KIP2} are expressed in different subpopulations of retinal precursor cells. $p27^{KIP1}$ is up-regulated in a pattern coincident with the onset of differentiation of most retinal cells in the mouse developing eye.[97] Mice deficient for $p27^{KIP1}$ have an increased fraction of mitotic cells through retina development as well as extensive apoptosis. Enforced expression of $p27^{KIP1}$ (by adenovirus) led to premature cell cycle exit but had no dramatic effects on differentiation.[98] Interestingly, the concomitant deficiency in cyclin D1 rescues the low cellularity of the retina of $p27^{KIP1}$ deficient mice.[99] In the developing retina model, it has also been shown that p57-^{KIP2} plays distinct roles, acting first as a cell cycle inhibitor in mitotic progenitor cells and then controlling differentiation of a subpopulation of postmitotic neuronal cells (amacrine cells) during postnatal development of the retina. Interestingly, in retina of p57-^{KIP2} –deficient mice apoptosis compensates for increased cell division.[100] It has also been proposed that an increase in CDK activity mediated by CKI depletion is not sufficient to cause cell-cycle defects but it is nevertheless able to perturb differentiation.[101]

A parallel example is the differentiation of hair cells in the developing organ of Corti of the inner ear. These cells undergo their terminal division at embryonic day 13-14 with concomitant induction of $p27^{KIP1}$ expression. In $p27^{KIP1}$ null mice, cell proliferation continues after day 14.[102] Thus, in sensory neurons from retina and ear, $p27^{KIP1}$ maintains the cell in a quiescent state and allows differentiation.

Another in vivo model is the neuroectodermal differentiation of vertebrates. During gastrulation of X*enopus*, parts of early ectodermic cells differentiate into neuroectodermic cells, and $p27^{KIP1}$ expression is restricted to postmitotic cells from neural plate. In ectodermic cells, enforced expression of $p27^{KIP1}$ arrests cell cycle progression but did not induce neural differentiation.[103] Retinoblasts in *Xenopus* differentiate into glial cells. In these cells $p27^{KIP1}$ induces both growth and differentiation.[13] Overall, from these in vivo models it can be concluded that, in neural differentiation, growth arrest mediated by $p27^{KIP1}$ is not sufficient to trigger differentiation.

Human prostate cancer cell line TSU-Pr1 can differentiate into microglia-like cells by phorbol ester (TPA) treatment, with $p21^{WAF1}$ induction, and enforced expression of $p21^{WAF1}$ results in differentiation.[104] In contrast, differentiation of mouse N1E-115 neuroblastoma cells can be induced by overexpression of $p27^{KIP1}$ or pRB.[105]

Members of the pRB family are differentially expressed during development of nervous system. In the central nervous system, p107 expression was restricted to proliferating cells (i.e., cells of ventricular zone of developing mammalian neocortex), while pRB was expressed in areas of both proliferating and differentiating cells. In contrast to pRB and p107, expression of p130 was low throughout embryogenesis.[90,106] The involvement of pRB in neuronal differentiation has been studied in pRB -deficient mice. These mice are embryonic lethal, and analysis of both central and peripheral nervous systems in $Rb^{-/+}$ revealed numerous abnormalities, particularly in hindbrain, dicencephalon, spinal cord and dorsal root ganglia. These abnormalities

include ectopic mitoses, decreased neuronal cell survival and neurite outgrowth, and were accompanied by decreased expression of the neurotrophins receptors TrkA and TrkB.[51-53,107] However, despite the severe neuronal phenotype of pRB deficient mice, dorsal root ganglia and cortical progenitor cells from E12.5 Rb null embryos were able to differentiate in culture.[53,108] pRB expression also increases dramatically during neuronal differentiation of P19 cells, while no such increase occurs in mutant cells that fail to respond to retinoic acid.[48,86,87] PC12 differentiation is associated with accumulation of hypophosphorylated pRB, and microinjection of a monoclonal antibody specific for the hypophosphorylated form of pRB blocked the neurite outgrowth initiated by NGF.[109] Thus, pRB hypophosphorylation plays a crucial role in PC12 neuronal differentiation.

In developing brain, E2F1 and E2F2 expression is high in proliferating cells (ventricular zone) and suppressed in postmitotic neurons from the marginal zone, and the opposite regulation is observed for p130.[110] E2F4 is up-regulated during PC12 neuronal differentiation elicited by NGF, while E2F1, E2F3 and E2F5 are down-regulated. Moreover, ectopic expression of E2F4 enhanced the NGF-mediated differentiation of PC12 and lowered the rate at which cells lost their neuronal phenotype following NGF removal. Consistent with this role in the PC12 model, E2F4 expression also increases in the developing rat cerebral cortex and cerebellum, concomitantly with the onset of neuronal terminal differentiation.[60] Furthermore, retinoic acid-induced differentiation of P19 cells is associated with loss of expression of E2F1, E2F3 and E2F4, while E2F2 remains high. pRB and p130 also increases with differentiation.[48] E2F1 levels are very low in undifferentiated cells and increase upon RA-mediated differentiation.[111]

In other in vivo models, E2F1 is down-regulated in the developing quail neural retina between embryonic days E8-E10, just after the arrest of neuroretina division.[112] In the mouse retina, cyclin D1 protein decreases as photoreceptor matures, and transgenic mice with enforced expression of cyclin D1 disrupt photoreceptor differentiation and retina development.[113]

Muscular Differentiation

Muscular differentiation is probably the differentiation phenotype where the involvement of CKIs and pocket proteins has been studied in more detail. During differentiation, skeletal muscle cells withdraw from the cell cycle and fuse into multinucleated myotubes. This process can be reproduced in cell culture with some skeletal muscle-derived cell lines. Two of the most used cell lines are C2 and its derivative C2C12. These cells can differentiate with concomitant increase of $p21^{WAF1}$, $p27^{KIP1}$, $p18^{INK4c}$ and $p57^{KIP2}$. $p21^{WAF1}$ undergoes an initial increase but decreases when the cells become terminally differentiated. In contrast, $p27^{KIP1}$ and $p18^{INK4c}$ gradually increase, being $p18^{INK4c}$ the CKI that undergoes the greatest induction.[6,114,115] Actually, all of the CDK6 and half of the CDK4 are complexed with $p18^{INK4c}$ in differentiated C2C12 cells.[116] Interestingly, there is a switch of $p18^{INK4c}$ transcripts so the large mRNA predominant in proliferating cells disappears during differentiation while a smaller $p18^{INK4c}$ mRNA is predominant in differentiated cells. In contrast, expression of $p19^{INK4d}$ decreases with differentiation.[4,116] Enforced expression of $p21^{WAF1}$, $p16^{INKa}$ and $p57^{KIP2}$,[6,117] stimulates muscular differentiation in C2C12 cells as well as in 10T1/2, another cell line with muscular differentiation potential.

Cyclins D1, E and A are down-regulated during C2 or C2C12 muscular differentiation whereas cyclin D3 is greatly induced.[27,28,118] Cyclin D3 mediates the interaction of CDK4 and $p21^{WAF1}$ with pRB in differentiated C2 cells and critically contributes to the irreversible exit from the cell cycle.[118] Ectopic expression of cyclin D1 blocks C2C12 differentiation and this can be reversed by coexpression of $p21^{WAF1}$.[6,119] Consistently, expression of cyclin D1, but not cyclins A, B, D3 and pRB, inhibits the expression of MyoD.[28]

pRB is induced during muscular differentiation of murine myoblasts and ectopic expression of pRB induces differentiation or restores differentiation capability of murine myoblasts.[120-123] Also, repression of pRB by antisense RNA[122] or inactivation of pRB by SV40 large T antigen[120,124] inhibits differentiation. Moreover, pRB is required for muscle development (see below). As $p21^{WAF1}$ is induced during differentiation, it is expected that the the growth suppressor function of pRB is activated. The adenovirus E1A protein binds and inactivates both pRB and $p21^{WAF1}$, and is able to reactivate DNA synthesis in differentiated muscle cells. However, this reactivation is abolished by a mutant E1A gene that binds pRB, but not $p21^{WAF1}$, indicating that $p21^{WAF1}$ function is dominant over pRB in maintaining the cell cycle arrest of C2C12 differentiated cells.[125] Conversely to vertebrate myotubes, pRB is expressed in newt myotubes, an observation that can be related with the regeneration capability of urodele limbs, which requires cell cycle reentry and local reversal of differentiation.[126]

In proliferating murine C2 and C2C12 and rat L6 myoblasts, p107 is the predominant pocket protein and its levels decrease during differentiation. In contrast, p130 increases with differentiation[41,43,127] and during differentiation of C2C12 there is an early accumulation of p130/E2F4 complexes.[41,42] Additional data using differentiation-defective cell lines indicate that p130/E2F accumulation is a necessary event in terminal differentiation of C2 cells, but not for cell growth arrest.[43] The involvement of p130 in muscular differentiation is stressed by findings in myoblasts acting as reserve cells that renew the muscular tissue in adult animals. In these cells p130 but neither pRB nor p107 accumulates during muscle differentiation.[45] In cultured myoblasts, hyperphosphorylated and hypophosphorylated forms of p107 are down-regulated to the same extent, while most of the p130 that is up-regulated during differentiation corresponds to the hyperphosphorylated form.[127]

Another example of the role of pocket proteins concerns the muscular differentiation of L6 cells. In these cells, p107 levels are down-regulated during differentiation, while p130 protein levels are up-regulated. Despite both p107 and p130 become phosphorylated during myogenesis, the E2F-site DNA binding complexes containing p107 detected in undifferentiated growing cells, are replaced in myotubes with complexes containing only p130.[27]

Data obtained with cells in vitro and with knockout mice (see below) indicate that proper regulation of E2F and pocket proteins is crucial for the coupling between cell cycle arrest and differentiation onset in skeletal myocytes. For example, C2 myoblasts transfected with E2F1 are still able to fuse into myotubes, express muscle specific proteins and up-regulate $p21^{WAF1}$. However, unlike wild-type cells, these E2F1-differentiated myocytes did not switch off proliferation, indicating that the primary role of E2F1 in these cells is to maintain proliferation.[128]

MyoD is a HLH transcription factor that induces muscular differentiation (for a recent review see 62). There is cross-talk between MyoD and G1-controlling proteins as demonstrated by a number of findings.[62,63]

a. MyoD induces $p21^{WAF1}$ expression during differentiation of murine C2C12 or 10T1/2 myocytes and prevents reassociation of CDK2-cyclin A to E2F4. By these mechanisms MyoD maintains cell cycle arrest during differentiation.[42,83,115,129,130] However, $p21^{WAF1}$ is expressed in myogenic cells of MyoD- or myogenin-deficient mice,[83] demonstrating that $p21^{WAF1}$ expression is not strictly dependent on these transcription factors.
b. $p57^{KIP2}$ increases MyoD expression by stabilization of the protein in C2C12 cells. This depends on a direct interaction between the basic region of the bHLH region of MyoD and the N-terminal domain of $p57^{KIP2}$. However, there is no MyoD/$p16^{INKa}$ interaction despite that $p16^{INKa}$ also up-regulates MyoD.[114,117]
c. Myo D activates the expression of cyclin D3 in the absence of new protein synthesis in differentiating C2 cells.[118] As mentioned above, D3 is the only cyclin upregulated during myogenic differentiation.[27,131]

d. MyoD induces the expression of Rb activating its promoter.[132] Activation of Rb (as well as $p21^{WAF1}$ and cyclin D3 genes) by MyoD occurs in the absence of new protein synthesis.[118] Interestingly, MyoD also up-regulates E2F1 at early stages of differentiation. This presumably contributes to increase the concentration of pRB -E2F1 transcription-repressing complexes in differentiating myocytes.[133]
e. MyoD cooperates with pRB to activate MEF2 (a muscle-specific bHLH transcription factor that cooperates with MyoD for myogenic differentiation).[134] A direct interaction between pRB and MyoD has been described in vitro[124] but has not been reproduced in vivo.[135] In fibroblasts lacking pRB, MyoD induces an aberrant skeletal muscle differentiation program, with normal expression of early differentiation markers such as myogenin and $p21^{WAF1}$, but attenuated expression of late differentiation markers such as myosin heavy chain. Similar defects were not observed in cells lacking either p107 or p130, indicating that the defect is specific to the loss of pRB.[136]
f. Overexpression of cyclin D1 inhibits myogenesis and Myo D transcriptional activity.[6,28,119] This inhibition correlates with phosphorylation of MyoD.[137]
g. CDK4 binds, phosphorylates and inhibits MyoD in 10T1/2 cells, thus explaining the effects of cyclin D1 described above.[138] MyoD interacts with CDK4 through a conserved 15 amino acid domain in C-terminus of MyoD.[135,138] In contrast to cyclin D1 and CDK4, overexpression of cyclin E and CDK2 in differentiated myotubes cannot reactivate DNA synthesis, despite pRB phosphorylation.[139]
h. MyoD is down-regulated in G1 phase after phosphorylation on Ser200 and subsequent degradation. This phosphorylation is carried out by CDK2-cyclin E, a process reminiscent of the phosphorylation/degradation of $p27^{KIP1}$.[140]

Muscular Differentiation in Vivo

$p57^{-KIP2}$ deficient mice show defects in many tissues (bones, lens, kidney) but exhibits normal muscle development.[18,20] Also, mice deficient in $p21^{WAF1}$ show no alteration of muscle development.[14,141] Consistently, $p57^{-KIP2}$ is the only CKI expressed in adult skeletal muscle,[142] although $p27^{KIP1}$ is expressed transiently in developing myotomes of the mouse embryo.[143,144] However, double knockout mice lacking $p21^{WAF1}$ and $p57^{-KIP2}$ fail to develop myotubes, and show increased proliferation and endoreplication.[130] This phenotype resembles that of myogenin-null mice,[145] but myogenin is expressed in the $p21^{WAF1}$/ $p57^{-KIP2}$ double mutant mice.

Disruption of both Rb alleles results in embryonic lethality but partially rescued Rb mutant fetuses (with a Rb minigene that allows low expression of pRB, $mgRb:Rb^{-/-}$) survive birth. These animals express the transgenic pRB in brain but not in muscles or other tissues,[121] and the muscular differentiation is severely impaired, with increased apoptosis, shorter myotubes, giant nuclei, endoreduplication and failure to express late muscle markers. The importance of pRB in muscle differentiation is underlined by the fact that mice deficient in both pRB and Id2 (an antagonist of pRB antiproliferative activity) survive to term with minimal defects in neurogenesis and hematopoiesis (see below), but they died from severe reduction of muscle tissue.[146] In composite mutant fetus $mgRb:Rb^{-/-}/p21^{-/-}$ these defects are further augmented, demonstrating that $p21^{WAF1}$ also contributes to myogenesis in vivo. In contrast, E2F1 and p53 are dispensable during aberrant myogenesis in Rb-deficient fetuses.[147] The muscular phenotype of mice with low or no expression of pRB in muscle precursors is explained because pRB is required for expression of muscle-differentiation markers. Interestingly, development of the myogenic phenotype in $Rb^{-/-}$ cells correlated with increased expression of p107. However, these cells were induced by serum to reenter the cell cycle, demonstrating that p107 cannot maintain the terminally differentiated state in $Rb^{-/-}$ myotubes.[148]

Table 2. Regulation and effects of G1 regulatory proteins in some hematopoietic differentiation models

Hematopoietic Cell Models (Species)	Hematopoietic Lineage (Inducer-Agent)	Cell-Cycle Regulator Expression	Differentiaton Induced or Increased by	References
$CD34^+$ (human)	Myeloide (SCF,IL-6,GM-CSF)	↑p21,↑p15,↑D1		7,29,158,180,197,239
	Monocyte (FL, IL-3)	↑pRb		187
	Megakaryocyte (PPP,Tpo)	↑p21,↑D3,↑p15, ↑p27↑p16,↑E		29,180,198,202
	Erythrocyte (IL-3,SCF,IL-6, Epo)	↑p21,↑p27		29,197
Progenitor cells (human)	Erythrocyte (GM-CSF, IL-3, Epo)	↑pRb		188
FVA erythroblast (murine)	Erythrocyte (Epo)	↑p21,↑p27		214
Bone marrow macrophages (murine)	Osteoclasts (ODF/RANKL,TNFa)	↑p21,↑p27		178
B cells (human)	Activated mature B cells	↓p27,↑p18,↑pRb		218
T cells and thymocytes (human)	Activated mature T cells	↓p27		223,224
HL-60 (human)	Monocyte/macrophage (TPA, $1,25(OH)_2D_3$, butyrate)	↑p21,↑p27,↑D1,↓A, ↓E,↓B,↓cdk2	p21, p27	156-158,160,184-186
	Granulocyte (DMSO, RA)	↑p21,↑p27,↓D1,↓D2		157,158,160,186
U937 (human)	Monocyte/macrophage (TPA, $1,25(OH)_2D_3$, butyrate)	↑p21,↑p27, ↑p15,↑p16,↑p18,↓A	p21, p27	156,157,169 158,163,168,170,174
M1 (murine)	Monocyte/macrophage (IL-6)	↑p21,↑p19,↓D1, ↓E2F-1		158,181,195
32Dcl3 (murine)	Granulocyte (G-CSF)	↑p27,↑p130	p19, p130	165,182,193
NB4 (human)	Granulocyte (ATRA)	↑p15		180
UF-1 (human)	Granulocyte ($1,25(OH)_2D_3$)	↑p21,↑p27	p27	164
K562 (human)	Megakaryocyte (TPA)	↑p21,↑p27,↑D1,↓B, ↓cdc2,↓A,↓E	p21	158,171,200,205,212, Muñoz and León
CMK (human)	Megakaryocyte (Tpo)	↑p21,↑D1	p21, p27	184,199
HEL (human)	Megakaryocyte (TPA)	↑D1,↓E,↓A		184,205
MEG-01 (human)	Megakaryocyte (TPA)	↑p21,↑p27		201
MegT (murine)	Megakaryocyte	↓B		209
Dami (human)	Megakaryocyte (TPA)	↑D1	D1	205
UT-7 (human)	Megakaryocyte (TPA)	↑p21	p21	198
F-36P-*mpl* (human)	Megakaryocyte (Tpo)	↑D1,↑D2 ↑D3,↓A,↓B	D+↓cdc2 activity	207
HB60-5 (murine)	Erythrocyte (Epo)	↑p27		215
MEL (murine)	Erythrocyte (HMBA)	↑p21,↑p27,↑p15, ↑p18,↓CDK6	p21	216,217

Table 3. Hematopoiesis-related phenotypes of knockout mice or transgenic mice for G1-regulatory proteins

In Vivo Model	Hematopoietic Phenotype	References
$Rb^{-/-}$ mice	Impaired erythropoiesis	51,52,107
$p107^{-/-}$ mice (Balb/cJ background)	Myeloproliferative disorders	36
E2F-$4^{-/-}$ mice	Abnormalities in hematopoietic lineage development	149,150
C/EBP$\alpha^{-/-}$ mice (E2F repression-deficient)	Dysplasia of neutrophil granulocytes	196
$p15^{-/-}$ mice	Lymphoproliferative disorders	240
$p16^{-/-}$/p19Arf $^{-/-}$ mice	Abnormal extramedullary hematopoiesis Increased proliferation of myeloid progenitors	241 154
$p16^{-/-}$ mice	Extended life span of bone marrow macrophages Enhanced mitogenic responsiveness of T cells	242 243
$p18^{-/-}$ mice	Lymphoproliferative disorders	240
$p27^{-/-}$ mice progenitor cells	Increased proliferation of myeloid progenitors and reduced differentiation in response G-CSF	165
$p21^{-/-}$ mice	Increased proliferation and impaired self-renewal of hematopoietic stem cells, Decreased myeloid colony formation, Increased proliferation of T lymphocytes	152 244 245
E2F-1-transgenic megakaryocytes	Blocked terminal differentiation, severe thrombocytopenia	213
D3-transgenic megakaryocytes	Enhanced ploidy, increased differentiation	204
D1-transgenic megakaryocytes	Enhanced ploidy, not increased differentiation	20
p27-transgenic T cells	Impairment development and function of T cells	225

Hematopoietic Differentiation

The study of the molecular mechanisms of hematopoietic differentiation is particularly challenging because this is multilineage differentiation, in which a single population of stem cells generates at least nine distinct mature cell types, with functions ranging from immune response to oxygen transport. The involvement of the molecules controlling G1 phase in hematopoietic cell differentiation is summarized in Tables 2 and 3.

Analyses of mice lacking selectively one of G1-phase cell-cycle regulator genes have suggested that most of them may be dispensable for hematopoietic development. One exception is pRB, whose disruption causes embryonic lethality and the mutant embryos exhibit a marked increase of immature nucleated erythrocytes.[51,52,107] Recently, it has been indicated that the $Rb^{-/-}$ phenotype is a to the consequence of uncontrolled Id2 functions, as Id2-Rb double knockout mice survive to term with no defects in hematopoiesis.[146] Also, loss of other cell-cycle

regulator genes provokes altered hematopoiesis to a lesser extent. In this way, for example, targeted inactivation of E2F4 leads to a deficiency of various mature hematopoietic cell types together with an increased number of immature cells in several lineages.[149,150]

However, the functions of many cell-cycle regulators are partially redundant and, alternatively, family members are able to substitute for one another during development when one of them is target inactivated.[37] Indeed, in various instances the combined loss of two functionally similar regulators has given rise to failure of hematopoiesis. Thus, for example, E2F1 and E2F2 double-knockout mice display impaired B-cell differentiation, reduced threshold for antigen activation of T cells and, in general, increased hematopoietic progenitor proliferation.[151] Moreover, in spite of the absence of abnormality in the hematopoietic phenotype of knockout mice for one individual cell-cycle regulator, studies carried out on cells derived from such animals have demonstrated that some of these proteins play essential functions in hematopoietic differentiation. For example, the absence of $p21^{WAF1}$ promotes the entry of hematopoietic stem cells into the cell cycle, which lead to stem cell exhaustion,[152] whereas loss of $p27^{KIP1}$ or $p16^{INKa}$ induces the increase of lineage committed progenitor proliferation, indicating a dominance of $p21^{WAF1}$ in bone marrow stem cell self-renewal and of $p27^{KIP1}$ and $p16^{INKa}$ in progenitor cell kinetics.[153,154] In addition, extensive studies in a great variety of hematopoietic model systems and the fact that the expression of some cell-cycle regulators, such as $p15^{INK4b}$ and $p16^{INKa}$, is frequently lost in leukemogenesis (reviewed in 155), have proposed an involvement of these proteins in the regulation of hematopoiesis, as reviewed below.

Myeloid Differentiation

During normal myeloid differentiation, $p21^{WAF1}$ and $p27^{KIP1}$ are expressed, but with different kinetics and subcellular localization. In $CD34^+$ cells differentiating towards myeloid lineage, the expression of $p21^{WAF1}$ is nuclear and transient, and, interestingly, concurrent with cellular proliferation, suggesting that the primary role for $p21^{WAF1}$ could be in coordinating the transition into differentiation rather than in maintaining the differentiated state. In contrast, the $p27^{KIP1}$ protein level is relatively constant but its subcellular localization changes from nuclear to cytoplasmatic at progressive stages of differentiation, indicating that $p27^{KIP1}$ might serve different functions at stages-specific of myeloid maturation.[7]

Likewise, expression of both proteins are increased along myeloid lineage by multiple differentiation-inducing agents in a variety of hematopoietic cell lines, such as HL-60,[156-162] U937,[156,158,163] K562 (Muñoz-Alonso and León, unpublished), M1,[158] UF-1[164] and 32D.[165] The up-regulation of $p21^{WAF1}$ is an immediate early response to differentiation-inducers and precedes terminal differentiation, indicating that induction of this protein is a primary mediator of differentiation rather that a consequence of growth suppression.[157,158,160] In this line, in retinoic acid-induced differentiation of acute promyelocytic leukemia cells, $p21^{WAF1}$ has been shown to play a crucial role during commitment to differentiation, independently of CDK inhibition and cell cycle arrest.[166] On the other hand, it has been suggested that $p27^{KIP1}$ leads to differentiation by causing cell cycle arrest. Consistent with this idea, several reports have shown that differentiation is accompanied with an increase of CDK2-bound $p27^{KIP1}$, concomitant with CDK2 inactivation, in ML-1 and U937 cells.[167,168] Furthermore, a direct stabilization of $p27^{KIP1}$ by $p21^{WAF1}$ in myeloid derived K562 cells has been observed recently, which might help to coordinate the differentiation-specific functions of these CKIs.[79]

Ectopic overexpression of $p21^{WAF1}$ and/or $p27^{KIP1}$ in U937 cells, in the absence of hormone, results in an induction of the expression of monocyte/macrophage-specific markers[163,169,170] and in HL-60 both proteins accelerate the differentiation triggered by TPA.[160] Also, exogenous $p21^{WAF1}$ initiates differentiation of K562 cells[171] and exogenous $p27^{KIP1}$ enhances maturation of UF-1 cells[164] (Table 2).

Moreover, suppression of $p21^{WAF1}$ by antisense techniques results in decreased expression of maturation markers by differentiation agents in HL-60 and U937 cells[166,172-175] and increases sensitivity to induced apoptosis, presumably by facilitating activation of the apoptotic protease cascade.[173,174,176] In line with this last observation, in U937 promyelocytic cells, $p21^{WAF1}$ confers cell survival from monocyte/macrophage differentiation-induced cell death.[175,177] Likewise, in ODF/RANKL-treated macrophages from mouse bone marrow cells, a mixture of $p21^{WAF1}$ and $p27^{KIP1}$ antisense oligonucleotides inhibits osteoclast differentiation.[178]

$p15^{INK4b}$ expression is up-regulated during differentiation into myeloid lineage of normal $CD34^+$ progenitor, of blasts isolated from patients of acute promyelocytic leukemia and of the NB4 promyelocytic cell line.[29,179,180] Interestingly, blasts from patients developing ATRA syndrome display high levels of $p15^{INK4b}$ and ATRA treatment does not increase or even downmodulate this protein, providing new insights into understanding the pathogenesis of this syndrome.[180] Also, $p19^{INK4d}$ is induced during macrophage differentiation of M1 cells[181] and its overexpression, and the resulting inhibition of cyclin D-dependent kinase activity, leads to this lineage in 32Dcl3 cells.[182] Cyclin-dependent kinases could act during G1 phase to interfere with differentiation-specific programs, which might be executed in noncycling cells. In agreement with this concept, it has been shown that during granulocytic maturation of ML-1 cells there is an increase in CDK4-bound $p18^{INK4c}$ and CDK2-bound $p27^{KIP1}$, as well as a decrease in CDK6-bound cyclin D3, showing a complex regulation of CDKs during differentiation.[167] Furthermore, the overexpression of cyclin D2 or D3 in 32Dcl3 myeloid cells prevents their ability to differentiate to granulocytes in response a G-CSF.[183] Interestingly, cyclin D1 is up-regulated during TPA-induced macrophage differentiation of HL-60 cell line,[184-186] whereas it is down-regulated in DMSO-induced granulocytic pathway of these cells,[186] and in IL-6-induced macrophage differentiation of M1 cells.[181] In addition, overexpression of cyclin D1 inhibits induced macrophagic differentiation in M1 cells[181] but no granulocytic maturation in 32Dcl3 cells,[183] suggesting that this cyclin plays an additional role other than regulation of cell cycle progression.

Among the pRB family proteins, p130 seems be responsible for maintaining cells in G0 state of the cell cycle. In $CD34^+$ cells, p130/E2F4/DP-1 complex predominates and when the cells proliferate in response to cytokines, p130 is phosphorylated and replaced by p107 as the main E2F binding partner.[49] Similar to other systems, when hematopoietic stem cells undergo differentiation and exit the cell cycle, levels of p107 decline, while p130 increases. Likewise, changes of pRB have also observed along myeloid lineage commitment of hematopoietic progenitor cells, but whereas levels of hypophosphorylated pRB are upregulated during monocytic maturation, they are low during granulocytic pathway, and only the monocytic differentiation is inhibited by antisense Rb oligonucleotides.[187,188] In addition, induced monocytic differentiation of leukemia cell lines has been correlated with activation of pRB by hypophosphorylation[189,190] and both suppression of this protein by antisense techniques and its overexpression reduces induction of differentiation in U937 cells, but no G1-accumulation.[191] Therefore, it has been suggested that pRB plays a critical role in the monocytic lineage pathway by mechanisms that are not strictly related to control of the cell cycle. In fact, it has been proposed that pRB may promote cell differentiation through its interaction with transcription factors different from E2F. For example, during induced differentiation of U937 cells, pRB interacts with and activates the transcription factor NF-IL6.[192] However, p130 has been shown to play an important role in granulocytic differentiation,[127,193] as its enforced expression, but not of pRB, inhibits induced maturation of 32Dcl3.[193]

Also, E2F family proteins are involved in myeloid differentiation. Deregulated E2F1, in conjunction with ectopic expression of Bcl-2 to delay apoptosis, prevents granulocytic differentiation of 32Dcl3, whereas E2F3 has no effect.[194] Also, overexpression of E2F1 blocks the

induced differentiation of the M1 myeloblastic cell line by promoting cell cycle progression, but surprisingly it does prevent the induction of $p16^{INKa}$ and $p15^{INK4b}$ inhibition of CDK4 activity, and subsequent hypophosphorylation of pRB, indicating that deregulated E2F1 uncouples p15/p16- pRB pathway from growth arrest.[195] Recently, a study has demonstrated in vivo that E2F repression by C/EBFα is required for granulopoiesis, as mice harboring E2F repression-deficient C/EBFα alleles exhibit dysplasia of neutrophil granulocytes.[196]

Megakaryocytic Differentiation

The terminal process of megakaryocytic differentiation is different from that of other hematopoietic lineages, as the cells undergo endomitosis during the late phase of maturation, which causes polyploidization. Although several groups have identified a number of cell-cycle regulators implicated in endomitosis, the results of these investigations are contradictory and the precise roles of these molecules in megakaryocytopoiesis are not fully understood.

Human megakaryocytes derived from $CD34^+$ cells display high levels of $p21^{WAF1}$ and $p27^{KIP1}$. $p21^{WAF1}$ is expressed in cycling megakaryoblasts whereas $p27^{KIP1}$ is only detected in cell-cycle arrested megakaryocytes.[197] The expression of $p21^{WAF1}$ is an early event and precedes polyploidization, suggesting that it might be implicated in this process.[198] Indeed, $p21^{WAF1}$ is upregulated during induced megakaryocytic differentiation in some hematopoietic cell lines, such as CMK,[199] UT-7,[198] K562[200] (Muñoz-Alonso and León, unpublished), and MEG-01,[201] and its overexpression in two cell lines with a megakaryocytic phenotype leads to nucleus polylobulation.[198,199] In addition, thrombopoietin, the hematopoietic factor that regulates megakaryocytic differentiation and platelet production, increases $p21^{WAF1}$ transcription by the transcription factor STAT5.[199]

However, megakaryocytopoiesis in $p21^{WAF1}$ deficient mice is normal and overexpression of $p21^{WAF1}$ in $p21^{-/-}$ or normal megakaryocytes inhibits ploidization, suggesting that $p21^{WAF1}$ is not essential for the determination of the ploidy profile, but probably plays an important role in the exit from the endomitotic cell cycle.[202]

Also, $p27^{KIP1}$ is up-regulated in induced megakaryocytic differentiation of K562 (Muñoz-Alonso and León, unpublished) and of MEG-01 cells.[201] Ectopic expression of $p27^{KIP1}$ lead to megakaryocytic differentiation of CMK cells,[199] but, like $p21^{WAF1}$, its overexpression in normal megakaryocytes also induces an endomitotic cell-cycle arrest.[202] Moreover, high levels of other CKIs, $p16^{-INK4a}$ and $p15^{INK4b}$, are found in megakaryocytic lineage, associated to hypophosphorylated pRB.[180,202] Recently it has demonstrated that $p15^{INK4b}$ mediates, at least in part, the stimulation of megakaryocytic differentiation by autocrine TGF-β1.[203]

D-type cyclins are critically important for cell cycle progression and, because their expressions are high in megakaryocytes and the endomitotic process requires DNA replication, have been supposed to participate in polyploid formation. Indeed, cyclin D3 is upregulated in $CD34^+$ undergoing megakaryocytic lineage and the treatment of these cells with cyclin D3 antisense oligonucleotide inhibits their maturation, while abrogation of cyclin D1 or cyclin D2 have little effect.[29,30] Moreover, transgenic mice overexpressing cyclin D3 have megakaryocytes of higher ploidy than the control animals and exhibit an increased number of differentiated cells of this lineage,[204] demonstrating that this cyclin is involved in polyploidization. Cyclin D1 is increased in induced megakaryocytic differentiation of several human cell lines as Dami, K562 and HEL,[200,205,206] although regulation of cyclin D3 in these systems has not been reported. The overexpression of cyclin D1 alone induces growth arrest but fails to increase ploidy in Dami megakaryocytic cell line, and it enhances polyploidization during TPA-induced differentiation.[205] A recent study has shown that transgenic mice in which cyclin D1 is overexpressed in megakaryocytes display a moderate increased ploidy in these cells, with no increase in the number of differentiated cells, suggesting that this cyclin also may promote polyploidization.[206]

However, a different study shows that in F-36P-*mpl* cells overexpression of D-type cyclins alone does not induce differentiation, but together with the expression of a dominant negative form of CDC2 includes megakaryocytic maturation, indicating that decreased CDC2 activity may contribute to endomitosis.[207] This observation is consistent with reports demonstrating that endomitosis is accompanied by low CDC2 activity due to down-regulation of CDC25C phosphatase or the decreased expression of cyclin B.[208-210] As well, cyclin E has been shown to be actively complexed with CDK2 during polyploidization of HEL cells[211] and the maintenance of cyclin E in G2/M cells determines cyclin A expression and the entrance of K562 cells into re-replication cycles.[212] Similar to its role in myeloid differentiation, deregulated E2F1 also affects megakaryocytopoiesis, as overexpression of this protein blocks terminal differentiation and causes proliferation in transgenic megakaryocytes.[213]

Erythroid Differentiation

During erythropoietin-dependent terminal erythroid differentiation of primary erythroblasts from spleens of mice infected with the anemia-inducing strain of Friend virus, both $p21^{WAF1}$ and $p27^{KIP1}$ are induced, but only $p27^{KIP1}$ associates with the G1 CDKs (CDK4, CDK6 and CDK2). Binding of $p27^{KIP1}$ to CDK2 (but not CDK4 or CDK6) correlates with pRB hypophosphorylation and growth arrest.[214] In the HB60-5 cell line $p27^{KIP1}$ is also up-regulated during erythroid differentiation, with inhibition of CDK2 activity.[215] However, although $p27^{KIP1}$ overexpression causes G1 arrest, it does not promote terminal erythroid differentiation.[215,216] Studies carried out on murine erythroleukemia (MEL) cells have provided important information on the complex regulation of CDKs by CKIs during differentiation program.[216,217] In this model, terminal cell division is mediated by induction of $p15^{INK4b}$, $p18^{INK4c}$, $p21^{WAF1}$ and $p27^{KIP1}$, thereby leading to sequential inhibition of the G1 CDKs. A specific order of the combined inactivation of CDK2 and CDK6 is essential to trigger differentiation, the inhibition of CDK2 being required first. Among the CKIs, only $p21^{WAF1}$ is able to inhibit both CDKs (CDK2 and 6) and its ectopic expression induces cell differentiation, but not overexpression of other CKIs. Importantly, these investigations have also shown that differentiation decisions occur only in the G1 phase and that CDK4 and CDK6 play different roles at different stages of differentiation. On the other hand, and consistently with the phenotype of Rb-null mice, pRB phosphorylation is induced and sustained during erythroid maturation and the suppression of this protein by antisense techniques inhibits erythropoiesis in stimulated differentiation of human hematopoietic progenitors cells and leukemic cell lines.[188,191]

Lymphoid Differentiation

In resting B cells, $p27^{KIP1}$ is strongly expressed and its expression decreases during activation leading to final differentiation of normal B cells into Ig-secreting cells.[218-220] Conversely, $p18^{INK4c}$ is upregulated in this process, concomitant with inhibition of pRB phosphorylation by cyclin D3/CDK6, indicating that $p18^{INK4c}$ is involved in the subsequent early G1 arrest necessary for terminal B lymphocyte differentiation.[218,221] Also, the expression of p57-^{KIP2} varies as a function of the stage of B-cell differentiation, nonetheless the role of p57-^{KIP2} in this hematopoietic differentiation has not been examined.[222] Similarly, in the T cell lineage, $p27^{KIP1}$ is also abundant in thymocytes and peripheral T lymphocytes and its expression is down-regulated both during development when $CD4^-$ $CD8^-$ thymocytes differentiate into $CD4^+$ $CD8^+$ cells, as well as on mitogenic stimulation of peripheral T lymphocytes.[223,224] Recently, it has been demonstrated that enforced expression of $p27^{KIP1}$ in transgenic T cells resulted in differentiation arrest of these cells and impairment of T cell-dependent immune response, indicating that down-regulation of $p27^{KIP1}$ is required for the normal development of T cells.[225] In addition, p130 seems be the principal member of pRB family proteins responsible for the lymphoid cell

cycle control, as ectopic expression of p130 blocks the growth of F7 pro-B cell line, but not the high levels of phosphorylated pRB or phosphorylation-resistant pRB mutants.[226]

Other Differentiation Lineages

Chondrocytic and Osteogenic Differentiation

Chondrogenic differentiation of mouse ATDC5 prechondrocytes is accompanied by $p21^{WAF1}$ and $p27^{KIP1}$ up-regulation, while $p15^{INK4b}$, $p18^{-INKc}$ and $p19^{INK4d}$ did not change. Consistently, reduction of endogenous $p21^{WAF1}$ by antisense RNA inhibits early differentiation.[227]

Vitamin D3 or vitamin K induces differentiation in MG-63 osteosarcoma cells, accompanied by $p21^{WAF1}$ up-regulation. Ectopic expression (by adenoviral infection) of $p21^{WAF1}$ results in differentiation.[228] In another Rb-null osteosarcoma cell line, Saos-2, ectopic expression of pRB induces differentiation,[55] suggesting that pRB may be required for osteoblast differentiation. This hypothesis has been confirmed in knockout mice. As mentioned above in the muscular differentiation paragraph, mgRb:$Rb^{-/-}$ mice (i.e., mice with lower than normal expression of pRB) show shortened and brittle bones, although the authors warn that these defects may be an indirect consequence of muscle degeneration, as they are also observed in myogenin deficient mice.[147] Mice deficient for both p107 and p130 display deregulated chondrocyte growth with increased chondrocyte density, defective bone development, shortened limbs and neonatal lethality. Thus these pocket proteins play an important role in limb development through their abilities to control chondrocyte proliferation.[40] In contrast, although $p27^{KIP1}$ is induced in osteoblast differentiation and osteoblasts from $p27^{KIP1}$ null mice proliferate faster, they retain competence for proliferation.[229]

Adipocytic Differentiation

The murine cell line 3T3-L1 undergoes adipocytic differentiation in response to hormonal stimulation (insulin, dexamethasone and isobutylmethylxanthine). During induced adipocytic differentiation of 3T3-L1 cells there is an initial up-regulation of $p18^{INK4c}$, $p21^{WAF1}$ and $p27^{KIP1}$, and later, concomitant with irreversible growth arrest and terminal differentiation, the level of $p21^{WAF1}$ declines with a concomitant increase of $p18^{INK4c}$.[4,230] During 3T3-L1 differentiation, cyclin D1 expression is repressed, cyclin D2 levels are transiently elevated and cyclin D3 is highly and persistently up-regulated. Moreover, differentiated cells contain active CDK4-cyclin D3 complexes.[4] Thus, the pattern of $p18^{INK4c}$ and cyclin D3 is similar compared to muscular differentiation (see above).

pRB is required for adipocytic differentiation of fibroblasts. Fibroblasts derived from pRB -deficient mice cannot differentiate into adipocytes, and ectopic expression of wild-type pRB (but not mutant pRB) enabled $Rb^{-/-}$ fibroblasts to differentiate.[231] The transcription factors C/EBPα and C/EBPβ, are induced during adipocytic differentiation and are required for differentiation. pRB induces adipocyte differentiation through direct interaction with c/EBPα that stimulates its activity.[231,232]

In sharp contrast, fibroblasts derived from deficient for p107 and p130 can differentiate into adipocytes. Moreover, over-expression of pRB in wild-type cells promotes differentiation whereas over-expression of p107 antagonizes differentiation.[233] This difference can be in part explained for the requirement of pRB in maintaining cell cycle exit as well as potentiating the activity of the differentiation-associated transcription factor C/EBPα, as p107 does not affect C/EBPα transcriptional activity.[233] Terminal differentiated of 3T3-L1 cells contain high levels of p130 and low p107,[234,235] although shortly after the addition of the differentiation inducers there is a DNA synthesis burst accompanied by elevation of p107, that is later repressed.[234]

Interestingly, in the 3T3-L1 model there is a reciprocal effect of C/EBPα, in differentiation. C/EBPα mediates disruption of E2F/p107 complexes and induces formation of p130/E2F complexes.[235]

Lens Cells Differentiation

Ocular lens arises from a sphere of epithelial cells, and it is already formed by mouse embryonic day 11.5. pRB deficient mice display impaired lens development, with inappropriate apoptosis in lens fiber cells.[236] Lens develops normally in p57-^{KIP2} deficient and p27^{KIP1} deficient mice, but lens of mice deficient in both p27^{KIP1} and p57-^{KIP2} are grossly abnormal.[20,237]

Luteal Cell Differentiation

p27^{KIP1}-deficient mice, besides hypercellularity in many tissues, display female infertility because luteal cells fail to withdraw from the cell cycle after hormonal stimulation, although the cells complete the differentiation program. Thus, the absence of p27^{KIP1} uncouples differentiation and growth arrest during the hormone-induced differentiation of granulosa into luteal cells.[238] Interestingly, absence of cyclin D1 does not rescue the p27^{KIP1} null phenotype.[99] This model constitutes another example of p27^{KIP1} exerting a differentiation function independent from cell cycle arrest.

Acknowledgements

Work in the laboratory of the authors has been supported by grants PM98-0109 and SAF02-4193 from Spanish Ministerio de Ciencia y Tecnología. M.J. Muñoz-Alonso is recipient of a postdoctoral fellowship from the Fundación Marqués de Valdecilla. We apologize to colleagues whose work has not cited due to space limitations or unintentional oversight

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