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The Retinoblastoma Protein and Cell Cycle Control

Review

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pRB, the product of the retinoblastoma tumor suppressor gene, operates in the midst of the cell cycle clock apparatus. Its main role is to act as a signal transducer connecting the cell cycle clock with the transcriptional machinery. In this role, pRB allows the clock to control the expression of banks of genes that mediate advance of the cell through a critical phase of its growth cycle. Loss of pRB function deprives the clock and thus the cell of an important mechanism for braking cell proliferation through modulation of gene expression.

pRB and the G1 Restriction Point

pRB exerts most and perhaps all of its effects in a defined window of time in the first two thirds of the G1 phase of the cell cycle. As demonstrated by Pardee almost two decades ago, this is the time window during which the mammalian cell makes most of its decisions about growth versus quiescence. Cells entering G1 from mitosis require serum mitogens continuously until several hours before the onset of S phase; thereafter, they become relatively serum independent. This transition from a serum-dependent to serum-independent state is demarcated by a discrete point in time, which he termed the R (restriction) point (Pardee, 1989). By passing through the R point, the cell commits itself to traverse the remainder of its growth cycle through M, barring major misadventure such as DNA damage or metabolic disturbance. Such a commitment decision represents a transition in the life of the cell that is as important as the much-studied G0/G1 emergence of the cell from quiescence.

pRB undergoes a readily discernible alteration at a time close to and perhaps contemporaneous with the R point transition. Through the preceding hours of G1, pRB is found in an underphosphorylated form. The bulk of pRB prepared from cells during the last several hours of G1 is, in contrast, hyperphosphorylated. pRB maintains this hyperphosphorylated configuration throughout the remainder of the cell cycle, losing its multiple phosphate groups only upon emergence from M.

Several lines of evidence indicate that this phosphorylation causes the inactivation of the growth inhibitory functions of pRB. First, oncoproteins made by three classes of DNA tumor viruses eliminate pRB function by binding and sequestering hypophosphorylated pRB, ignoring hyperphosphorylated forms. Second, the hypophosphorylated form binds and ostensibly controls a number of other cellular proteins (see below); the hyperphosphorylated form appears to have lost the ability to interact with these proteins. Third, conditions that cause pRB phosphorylation favor cell proliferation (reviewed by Cobrinik et al., 1992).

The schedule of pRB phosphorylation leads to a simple and attractive functional model that is still largely unproven. A cell that has proceeded through most of G1 encounters the R point gate held shut by its guardian, pRB. Should conditions be propitious for advance into the remainder of the cell cycle, pRB will undergo phosphorylation and attendant functional inactivation, causing it to open the gate and to permit the cell to proceed into late G1. Cells that lack pRB function for a variety of reasons will proceed blithely into late G1 without undergoing the control normally imposed by pRB and, by extension, the upstream influences that regulate its phosphorylation. These upstream influences include growth-promoting signals such as mitogens as well as growth inhibitory agents such as transforming growth factor β (TGFβ) and contact inhibition. Having lost their pRB servant, these physiologic signals lose much of their voice in the decision of the cell to pass through the R point gate and proceed into late G1.

This model raises two major questions. How precisely do such physiologic signals determine whether or not pRB undergoes phosphorylation and resulting inactivation? And how does pRB, for its part, prevent or allow advance of the cell into late G1?

Control of pRB Phosphorylation

Accumulating evidence indicates that components of the cell cycle clock mediate much and perhaps all pRB phosphorylation. Phosphopeptide analysis of pRB suggests more than a dozen distinct sites of phosphorylation on either serine or threonine residues. This creates a puzzle: why are so many sites modified? Do they indicate that multiple kinases converge on pRB, using it as a substrate to integrate their various signals?

The amino acid sequences surrounding the subset of pRB phosphorylation sites that have been analyzed in detail are typical of those modified by cyclin-dependent kinases (CDKs) (Lees et al., 1991). Thus, G1 cyclins, serving as regulatory subunits of their partner CDKs, are presumed to direct these enzymes to the pRB substrate, resulting in its phosphorylation.

Cyclins of the D class (D1, D2, and D3) are most prominently implicated in the phosphorylation of pRB. These cyclins serve as regulators of the CDK4 and CDK6 kinases. Incubation of D cyclins with CDK4/CDK6 and a pRB fusion protein yields phosphorylation of pRB in vitro (Kato et al., 1993; Ewen et al., 1993a). Moreover, as described below, physiologic regulators that interfere with CDK4/CDK6 function also serve to block pRB phosphorylation in vivo. These D cyclins are unique in their ability to form physical complexes with pRB; the precise functional significance of this interaction remains to be elucidated (Dowdy et al., 1993; Ewen et al., 1993a).

A role for cyclin E in contributing to pRB phosphorylation is also strongly suggested. Ectopic expression of cyclin E in human osteosarcoma cells causes pRB phosphorylation (Hinds et al., 1992). Moreover, in most cells, the levels of cyclin E mRNA and protein rise dramatically at the time

in mid-to-late G1 when pRB undergoes phosphorylation (Lew et al., 1991; Koff et al., 1991). pRB undergoes phosphorylation in a normal fashion in DNA tumor virus-transformed cells; in these cells, viral oncoproteins occupy the site on pRB to which D cyclins normally bind (DeCaprio et al., 1989). This suggests that certain cyclin–CDK complexes that do not depend on cyclin D–pRB association can also participate in pRB phosphorylation in late G1. Taken together, these various lines of evidence converge on the notion that cyclin E–CDK2 complexes, like cyclin D–CDK4/CDK6 complexes, participate in pRB phosphorylation.

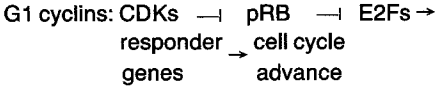
One line of experiments suggests that both classes of cyclins may be important. When ectopically expressed in Saccharomyces cerevisiae, pRB undergoes a phosphorylation that is almost indistinguishable from its modification in mammalian cells. This phosphorylation requires multiple yeast G1 cyclins, *CLN3* plus either *CLN1* or *CLN2*. The loss of *CLN3* function can be largely reversed by introduced mammalian cyclin D1; absence of *CLN1/CLN2* function can be compensated by mammalian cyclin E (Hatakeyama et al., 1994). This hints at a similar collaboration operating in mammalian cells.

The precise nature of the collaboration between the D cyclins and cyclin E in promoting pRB phosphorylation remains unclear. One simple model holds that cyclin D–CDK4/CDK6 complexes initially create hyperphosphorylated pRB; an identical spectrum of phosphorylated residues on pRB is then maintained by subsequently activated cyclin E–CDK2 complexes. Alternatively, cyclin D–CDK4/CDK6 complexes may modify pRB in a way that makes it into an attractive substrate for further, qualitatively distinct modification by cyclin E–CDK2.

Effector Functions of pRB: Control of Transcription by E2F

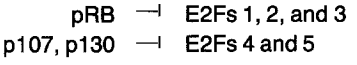
As demonstrated 4 years ago, when pRB is hypophosphorylated, it is capable of binding to the E2F transcription factor; phosphorylation causes it to lose its grip on E2F, presumably enabling the latter to proceed with the activation of a cohort of client genes whose transcription it controls (Chellappan et al., 1991).

We now realize that the term E2F subsumes a group of at least five distinct, closely related transcription factors that are all targeted to variants of the consensus nucleotide sequence TTTCGCGC. This sequence is present in the promoters of a number of genes that are known or presumed to be important for cell growth control, including most notably c-*myc*, B-*myb*, *cdc2*, dihydrofolate reductase, thymidine kinase, and the promoter of the *E2F-1* gene itself (reviewed by Nevins, 1992; LaThangue, 1994). This generates the next level of model building: by reversibly sequestering E2Fs, pRB controls the expression of genes whose products are important participants in the cell cycle program. Thus,



Not unexpectedly, this scheme, though correct in outline, has greater complexity in its details. Of the five known E2Fs, only three, E2F-1, E2F-2, and E2F-3, are known to be under the direct control of pRB; hypophosphorylated pRB appears to bind the other two only weakly. These outliers (E2F-4 and E2F-5) seem to be under the control of at least one of the cousins of pRB in the cell, p107. (The role of the other cousin, p130, remains to be reported.) p107 seems to have weaker affinities for E2F-1, E2F-2, and E2F-3 than does pRB (Ginsburg et al., 1994; Beijersbergen et al., 1994; Cao et al., 1992; Cobrinik et al., 1993; Dyson et al., 1993; Lees et al., 1993; Shirodkar et al., 1992).

The possible existence of two parallel pathways



raises many questions. It is unclear whether all the E2Fs target a common constituency of responder genes or, alternatively, whether each is specialized to regulate its own private clientele of transcriptional promoters. There is also suggestive evidence that each of these E2Fs forms complexes with pRB and its cousins in defined periods of the cell cycle, hinting that each may be specialized to control genes in a defined period of G1 or S. Beyond this, the rationale for their multiplicity is unclear.

The precise mechanisms by which pRB (and its cousins) regulate gene function may be more subtle than simple alternating cycles of sequestration and liberation of E2Fs. Gel retardation analyses indicate that complexes of pRB and E2Fs can bind to the consensus E2F target DNA sequence in a number of promoters (Nevins, 1992; LaThangue, 1994). This indicates that pRB binding does not preclude simultaneous association of an E2F with DNA. Thus, pRB (or its cousins) may act in situ at transcriptional promoters, using already bound E2Fs as docking sites. Indeed, much evidence suggests that pRB may actively repress transcription of promoters on which this complex is sitting. Hence, removal of an E2F-binding site may liberate a promoter from pRB-mediated transcriptional repression (Weintraub et al., 1992; Lam and Watson, 1993; Dynlacht et al., 1994; Qin et al., 1995).

Alternative Effector Pathways Regulated by pRB

pRB may regulate a number of downstream effectors besides the much-studied E2Fs. pRB is a relatively abundant nuclear protein, being present in molar amounts that are as much as two orders of magnitude above the levels of the E2Fs. Accordingly, it has substantial capacity to control the activities of yet other nuclear proteins.

Genes encoding other pRB-binding proteins have been reported over the past several years. Included among these are the Elf-1, MyoD, PU.1, ATF-2, and c-Abl proteins (reviewed by Wang et al., 1994). The effector functions of most of these remain unknown. Most intriguing of these is the nuclear tyrosine kinase encoded by the cellular *abl* proto-oncogene. Hypophosphorylated pRB is reported to bind directly to the active catalytic domain of the c-Abl

kinase, blocking its function. As expected, hyperphosphorylated pRB loses this binding ability (Welch and Wang, 1993, 1995). By binding multiple effectors such as E2Fs and Abl, pRB may be able to modulate simultaneously the activity of a number of downstream growth-controlling pathways.

The various pRB-regulated downstream effector pathways suggested by these associations would seem a priori to contribute equally to the ability of pRB to control cell proliferation. But two lines of evidence indicate that the initially discovered E2F-regulated pathway represents the pRB function of preeminent importance. Ectopic expression of E2F-1 enables a cell to move from quiescence into S phase, thereby traversing the entirety of G1 (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994). Moreover, introduction of an E2F-1 expression plasmid into human osteosarcoma cells can override a pRB-imposed block of the growth of these cells (Qin et al., 1994). Taken together, these functional tests suggest that E2Fs are dominant determinants of G1 progression and may even be the only critical rate-limiting effectors of pRB action.

One observation suggests that pRB and, by extension, the E2F transcription factors under its immediate control play a more central role in controlling cell cycle progression than do its cousins p107, p130, and their respective effectors: many human tumor cell genomes have been found to have lost pRB function through chromosomal gene mutation, while a comparable loss of a p107 or a p130 allele has never been reported (reviewed by Weinberg, 1992). But this hardly proves the unique importance of pRB in cell cycle regulation. Equally weighty roles of p107 and p130 may well be masked by a functional redundancy that they have with one another. Such redundancy would drastically reduce the likelihood of their being eliminated from tumor cell genomes during tumor progression.

pRB as an Integrator of Positive Signals

The evidence cited above indicates that the pRB transducer sits at a critical node in the signaling circuitry of the cell, acting as a recipient of several afferent signals that converge on it and responding to these in turn by regulating a number of downstream effector pathways such as those involving E2Fs and Abl. The richness and complexity of the afferent signaling pathways have only recently been appreciated.

Physiologic signals that favor cell proliferation should encourage pRB phosphorylation. Most of these signals originate with mitogens that impinge on cell surface receptors and in turn activate cytoplasmic signal transduction pathways that convey growth stimulatory signals to the nucleus. These signals must then act in one way or another to stimulate the forward progress of the cell cycle clock. At present, the connections between these cytoplasmic mitogenic signaling pathways and the nuclear clock are poorly understood.

One clear connection, however, is revealed by the behavior of cyclin D1. As described above, it contributes importantly to pRB phosphorylation. Like the Myc protein, it turns over rapidly with a lifetime of about 15 min, and its steady-state levels rapidly decline upon removal of extracellular mitogen (Matsushime et al., 1991; Sherr, 1993). This would suggest that at the time in mid-to-late G1 when the cell is anticipating pRB phosphorylation, the cell cycle clock can gauge the extracellular mitogen environment by determining the level of cyclin D1. Levels of cyclin D above a certain threshold will thus favor pRB phosphorylation; subthreshold levels will make this step difficult.

The activity of the CDK enzymes is controlled both by their association with cyclins and by phosphorylation of the CDK polypeptide itself. For example, CDK2 is phosphorylated on threonine residue 160, a modification that is essential for its catalytic activity (Desai et al., 1992; Solomon et al., 1992). These phosphorylations are mediated by an enzyme complex termed CDK-activating kinase (CAK), which itself is composed of a CDK (termed variously MO15 or CDK7) and a cyclin (cyclin H) (Fisher and Morgan, 1994; Mäkalä et al., 1994). Since CDK7 is also dependent upon an activating phosphorylation event by yet another kinase, this suggests the possibility of positive control by an extended kinase cascade, a notion that at present lacks direct experimental support.

Mitogens must also encourage in some fashion the mid-to-late induction of cyclin E mRNA and the increase in cyclin E protein. How mitogenic signals converge on cyclin E activation and in turn on the phosphorylation of pRB is also poorly understood at present.

pRB as an Integrator of Negative Signals

We now know that a variety of physiologic growth inhibitory signals prevent pRB phosphorylation and in this way block advance of the cell through the R point and into late G1 (reviewed by Wang et al., 1994). These growth inhibitory mechanisms do not seem to impinge on pRB directly but operate instead by modulating the activities of the CDKs that are responsible for pRB phosphorylation. To date, three well-studied physiologic signals have been shown to block pRB phosphorylation in this manner. These are TGFβ, cyclic AMP (cAMP), and contact inhibition. In each instance, a negative growth signals mobilizes a CDK inhibitor (CDKI) protein that associates either with a CDK or with a cyclin–CDK complex and blocks its function.

At least three molecular mechanisms have been proposed to explain the ability of TGFβ to obstruct pRB phosphorylation. The first of these involves $p27^{Kip1}$, a CDKI that interacts with CDK2, CDK4, and CDK6 and prevents their functional activation at the hands of CAK (Polyak et al., 1994; Slingerland et al., 1994; Toyoshima and Hunter, 1994). While levels of $p27^{Kip1}$ are not affected by TGFβ treatment, the CDK2 of TGFβ-treated cells has been found to be associated with $p27^{Kip1}$, suggesting some type of posttranslational regulation of its activity (Ewen et al., 1993b; Polyak et al., 1994).

An alternative mechanism is suggested by the recent discovery that TGFβ can induce expression of $p15^{INK4B}$, another CDKI, by as much as a factor of 30. p15 targets CDK4/CDK6, competing with D cyclins for binding to CDK4/CDK6 (Hannon and Beach, 1994). Recent reports provide evidence that p15 (and its close cousin, $p16^{INK4A}$)

can only inhibit cell proliferation in cells possessing functional pRB (Guan et al., 1994; Serrano et al., 1995). If extended and solidified, this would indicate that the only physiologically important target of cyclin D–CDK4/CDK6 is pRB and, hence, that this TGFβ →p15⊣CDK4/CDK6 pathway has pRB as its sole end point.

TGFβ also acts to reduce dramatically the levels of CDK4 in certain responsive cell types (Ewen et al., 1993b). This is also an effective means of preventing pRB phosphorylation in that, as mentioned above, the cyclin D–CDK4/CDK6 complexes play a critical role in the phosphorylation of pRB. The TGFβ-induced decrease in CDK4 levels may result in the liberation of substantial amounts of $p27^{Kip1}$, which in turn may proceed to antagonize the functioning of yet other CDKs in the cell. Contact inhibition and cAMP also appear to act through their ability to mobilize $p27^{Kip1}$ (Polyak et al., 1994; Kato et al., 1994). The end results of these various growth inhibitory mechanisms are the same: they all converge on pRB, blocking its phosphorylation and preventing opening of the R point gate.

Radiation and other DNA-damaging agents also block pRB phosphorylation through use of another CDKI, termed variously p21, Waf1, and Cip1 (El-Deiry et al., 1993; Harper et al., 1993; Dulic et al., 1994). The physiologic rationale here is simple: should the cell sustain genetic damage while it is in G2, M, or much of G1, a pRB-imposed cell cycle block in G1 will enable the DNA repair apparatus to go about its work of restoring genomic integrity without the threat of imminent advance of replication forks and resulting inadvertent replication of unrepaired DNA sequences. Once DNA lesions are erased, then the pRB-imposed block may be lifted and the cell permitted to advance into S and replicate its now-restored genome. Alternatively, in the face of irreparable lesions, the cell may choose to commit itself to an apoptotic death.

p53 acts as an intermediary here. Normally a highly unstable protein with a lifetime of 15 min or so, its steady-state levels increase rapidly upon DNA damage. p53 in turn activates expression of the p21 CDKI, and p21 then blocks the activity of both CDK2 and CDK4/CDK6 (Kastan et al., 1991; Hall et al., 1993). As with the other antiproliferative signals, this mechanism prevents phosphorylation of pRB and passage through the R point gate.

pRB and Regulation of the Clock

The interplay of cyclins, CDKs, CDKIs, and pRB enables the cell cycle clock to control its own forward progress. At least two types of regulatory circuits utilizing these clock components have been proposed to date. Others will surely follow.

The first of these has been suggested to operate in mid-G1, serving as a timing device that triggers activation of cyclin D–CDK4/CDK6 and the subsequent R point transition 6–10 hr after the onset of G1 (Kato et al., 1994). This model postulates that $p27^{Kip1}$ CDKI is expressed at rather constant levels throughout G1. Early in G1, any cyclin D–CDK4/CDK6 that is present will associate with and be inactivated by $p27^{Kip1}$, which is initially present in molar excess and acts as a stoichiometric inhibitor of cyclin D–CDK4/CDK6 activation. The levels of cyclin D–CDK4/CDK6 will increase gradually as cells progress through mid-G1. Eventually, cyclin D–CDK4/CDK6 levels will exceed a threshold beyond which they out-titer the limited amount of $p27^{Kip1}$ that is present. These excess cyclin D–CDK4/CDK6 complexes can now be phosphorylated by CAK and, thus empowered, can initiate pRB phosphorylation and trigger the R point transition.

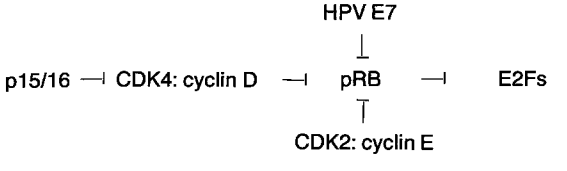
The second of these proposed regulatory circuits involves $p16^{INK4}$, the CDKI that operates to bind CDK4/CDK6 and to preempt their association with D cyclins. Some of its discoverers noted that p16 is overexpressed in cells that have been transformed by DNA tumor viruses and thus carry a sequestered, inactive pRB (Serrano et al., 1993). Others have found that pRB acts to regulate negatively the promoter of the p16 gene (Li et al., 1994). Moreover, p16 expression increases strongly toward the end of G1 after pRB undergoes inactivation through phosphorylation (Tam et al., 1994). pRB thus seems to antagonize p16 expression.

These observations, taken together, suggest the operation of a negative feedback loop which operates as follows. First, cyclin D, operating together with a partner CDK4/CDK6, triggers pRB phosphorylation as described above. Second, pRB, now functionally inactivated, releases a transcription factor that causes induction of p16 expression. Third, p16 proceeds to bind all CDK4/CDK6, evicting cyclin D from its CDK4/CDK6 association. Fourth, cyclin D, no longer protected by association with its CDK4/CDK6 partner, is degraded, resulting in termination of its activities toward the end of G1. (Tam et al., 1994). This mechanism would serve to limit the participation of cyclin D in cell cycle regulation to a defined window of time that begins just before the R point transition and ends at the G1/S transition.

These mechanisms may help to solve an intriguing puzzle. Some have noted that human tumor cells that lack pRB express CDK4/CDK6 but do not show cyclin D–CDK4/CDK6 complexes (Bates et al., 1994). These tumor cells have now been found to express high levels of p16, which, by binding to CDK4/CDK6, blocks D cyclins from complexing with these CDKs, resulting in the apparent degradation of the uncomplexed cyclins (Parry et al., 1995).

pRB and Cancer

A diverse body of evidence now indicates that pRB stands in the midst of a regulatory pathway that suffers disruption during the pathogenesis of very many human tumors. Indeed, it is possible that this pathway may one day be found to be deregulated in all human malignancies. To reprise the above discussions:



In retinoblastomas, in small cell lung carcinomas, and in many sarcomas and bladder carcinomas, pRB function

is lost through mutations of the pRB gene (Horowitz et al., 1990). In the great majority of cervical carcinomas, this end is achieved through the actions of the human papillomavirus E7 oncoprotein (Dyson et al., 1989; zur Hausen, 1991). In many esophageal, breast, and squamous cell carcinomas, the cyclin D gene is amplified (Jiang et al., 1992; Lammie et al., 1991); cyclin D1 overexpression is achieved in B cell lymphomas through chromosomal translocation (Motokura et al., 1991). Herpesvirus saimiri, a potently oncogenic virus in certain primate hosts, causes expression of its own virus-encoded D-type cyclin (Jung et al., 1994). The *CDK4* gene is amplified in many glioblastomas and some gliomas (He et al., 1994; Schmidt et al., 1994). p15, p16, or both may be deleted in many esophageal squamous cell carcinomas, glioblastomas, lung, bladder, and pancreatic carcinomas (Schmidt et al., 1994; Kamb et al., 1994a; Nobori et al., 1994; Mori et al., 1994; Caldas et al., 1994; Zhou et al., 1994; Jen et al., 1994) or may be present in mutant form in the germline of families with familial melanoma (Hussussian et al., 1994; Kamb et al., 1994b).

The centrality of this control pathway and the importance of its deregulation during tumor progression are further highlighted by a study of 55 small cell lung carcinomas. Of these, 48 lacked normal pRB expression but showed the wild-type $p16^{INK4}$ gene product; six of the remaining seven tumors lacked $p16^{INK4}$ protein but expressed pRB at normal levels (Otterson et al., 1994).

The various changes catalogued above all converge on the same end point of depriving the cell of the services of pRB, either through its functional inactivation (via sequestration or deregulated phosphorylation) or through its genetic inactivation (via chromosomal mutation). As a direct consequence, E2Fs are liberated from pRB control and the progression of cells into late G1 and S becomes unconstrained.

pRB and Cell Biology

The logic of the nuclear circuitry governing pRB phosphorylation, as described above, provides little insight into the role of pRB and the R point transition in normal cell physiology. As described earlier, pRB implements the decision by the cell to pass through this checkpoint in mid-to-late G1. When pRB fails to undergo phosphorylation, the progress of the cell through its active growth cycle is blocked at this point.

The subsequent fate of the cell is unclear, however, if only because several alternative fates exist. Most obvious is a return by the cell to G0, from which it may reemerge on some future occasion. Recently acquired evidence indicates that a pRB-imposed block on cell cycle progression may be a prerequisite for other, less reversible types of exit from the cell cycle leading to differentiation or senescence.

Our current understanding of the connections between the R point blockade and the decisions governing the long-term fate of the cell is rudimentary. Importantly, any attempts at ascribing critical roles to pRB and the R point must be tempered by the results obtained from targeted inactivation of the *Rb* gene in the mouse germline. Homozygous mutant embryos are essentially normal until day 12–13 of gestation, when they die with defects in both hepatic erythropoiesis and neuronal development (Clarke et al., 1992; Donehower et al., 1992; Jacks et al., 1992; Lee et al., 1994). By this time, the vast majority of morphogenetic decisions have already been made properly. They have occurred in the absence of pRB, a protein that is expressed widely and is purportedly controlling the proliferation of all cells in the developing embryo.

Standing in apparent opposition to these in vivo observations are several lines of evidence indicating that pRB plays an important role in the differentiation of a variety of cell types. For example, pRB levels strongly increase upon differentiation of basal keratinocytes and colonic crypt cells (Szekely et al., 1992). Moreover, in one in vitro model, CDK4, whose only substrate of consequence appears to be pRB (see above), strongly decreases upon induced differentiation of murine erythroleukemia cells. This loss of CDK4 would deprive the cell of its means of initiating pRB phosphorylation and, indeed, an accumulation of hypophosphorylated pRB is observed in these cells as they differentiate. This differentiation is prevented in cells engineered to express CDK4 constitutively (Kiyokawa et al., 1994). Hence, stopping these cells in mid-to-late G1 appears to be a prerequisite to their subsequent egress from the cell cycle into a more differentiated state.

Another in vitro model involves myeloid stem cells that can be induced to differentiate into neutrophils. Here, down-regulation of D cyclins and CDK4 ceases as cell enter into a state of differentiation. Ectopic, constitutive expression of cyclins D2 and D3 prevents this differentiation (Kato et al., 1993). As before, the prevention of pRB phosphorylation and the resulting proliferation block imposed by pRB appear to be essential for subsequent cell differentiation.

The body appears to have developed mechanisms to protect itself from the chaos that ensues when cells are called upon to differentiate but are unable to respond appropriately because they lack functional pRB (Lee et al., 1994). Without such functional pRB, certain cell types are susceptible to apoptosis. This apoptosis depends upon the p53 protein (Morgenbesser et al., 1994) and represents a means by which tissues can eliminate individual cells that have lost pRB function. Such a protective mechanism also has implications for the process of cell transformation. In particular, cell clones that have lost pRB may survive and gain further advantage through the subsequent loss of p53 function. This may explain why a variety of DNA tumor viruses (papovaviruses, adenoviruses) have evolved the capability of simultaneously inactivating both of these tumor suppressor proteins. Moreover, it may explain why certain neuronal tissues in Rb^{-} mice undergo apoptosis during development (Jacks et al., 1992).

pRB also plays an important role in the process of cell senescence. While initially discovered as a phenomenon of cells grown in culture (Hayflick, 1965), it appears increasingly likely that senescence represents an antineoplastic mechanism designed to limit the proliferative potential of cell clones in the body. Studies of senescence as it occurs in vitro indicate that it can be partially circumvented by viral oncoproteins that inactivate pRB (Shay et

al., 1991). By extension, one presumes that in vivo the loss of functional pRB through a variety of mechanisms permits evolving cell clones to gain replicative advantage and eventually to undergo immortalization.

These observations only hint at the possible roles of pRB in normal cell physiology. Unfortunately, they have provided little help in resolving the major paradoxes surrounding pRB. How can most of mouse development proceed in an essentially normal fashion in its absence? Why does pRB loss in humans lead specifically to the unusual retinal tumors and not to malignancies in the other tissues of young children? Why do humans, unlike all other mammals, suffer these eye tumors upon pRB loss? We may need to wait a long time before the answers are forthcoming.

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