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Review

Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins

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1. Summary

The retinoblastoma family of growth-inhibitory proteins act by binding and inhibiting several proteins with growth-stimulatory activity, the most prominent of which is the cellular transcription factor E2F. In higher organisms, progression through the cell division cycle is accompanied by the cyclical activation of a number of protein kinases, the cyclin-dependent kinases. Phosphorylation of retinoblastoma family proteins by these cyclin-dependent kinases leads to release of the associated growth-stimulatory proteins which in turn mediate progression through the cell division cycle.

2. Introduction

Proliferation of normal cells is controlled by multiple growth-regulatory pathways that act together to ensure proper growth regulation. To evade these controls, tumor cells have to acquire multiple genetic changes before they display a fully transformed phenotype. Cells respond to a variety of extracellular signals, including growth factors, mitogen antagonists and differentiation-inducing factors. Together, these factors dictate cellular behavior, including the decision to grow, differentiate or commit suicide by apoptosis. Cancer cells ignore many of these growth-regulatory signals due to mutations in genes that control either the growth-promoting (proto-oncogenes) or growth-inhibitory pathways (tumor-suppressor genes). Although these mutations appear to affect different classes of genes, it has become increasingly evident that they are both part of the same regulatory system designed to maintain the integrity of all tissues. The observation that proteins of both classes often influence each other's activity through direct interactions provides further proof of this intertwinement.

3. Gene expression and the cell cycle

The major positive regulators of the cell division cycle are a group of related proteins, the cyclins, first identified by virtue of their cyclical appearance during the cell cycle of marine invertebrates [1]. Cyclins are the positive regulatory subunits of a class of related protein kinases, named cyclin-dependent kinases (cdks) [2]. The mammalian genome encodes at least ten different cyclins and seven cdks that can associate in at least 15 different cyclin-cdk complexes [3,4]. Together, these cyclin-cdk complexes are the master regulators of the major cell cycle transitions. When cells emerge from quiescence (G0 phase) and enter the first phase of the cell cycle (G1), the expression of D and E type cyclins is induced. At the onset of DNA synthesis (S phase) cyclin A is first detected followed by cyclin B during the interval between S phase and mitosis (G2) followed by rapid degradation at the end of mitosis (M phase).

Apart from the cyclin genes, another group of genes, collectively known as the immediate early response genes, play a crucial role in the early phases of the cell cycle. Growth factors bind to specific cell surface receptors which trigger signaling cascades that ultimately result in the transcription of immediate early response genes. The immediate early mRNAs include *c-fos*, *c-jun* and *c-myc* which appear within minutes following mitogenic stimulation. These mRNAs turn over rapidly and consequently their encoded proteins appear only transiently. Immediate early mRNAs even appear when protein synthesis is inhibited, indicating that their induction depends solely on the post translational modification of pre-existing cellular factors. For *c-myc* it has been shown that induction of its mRNA is both necessary and sufficient for the transition from quiescence to the G1 and S phase of the cell cycle [5,6]. The importance of this group of genes in the initiation of the cell cycle is also highlighted by the fact that many of the immediate early response genes are deregulated in cancer.

The later transitions in the cell cycle are also marked by the coordinate expression of yet other groups of genes that are required for periodically occurring biochemical processes. For instance, at the G1/S transition genes required for DNA synthesis have to be activated. This group of cell cycle regulated genes include those for dihydrofolate reductase (DHFR), DNA polymerase a, the DNA polymerase ∂ subunit PCNA and thymidine kinase (TK) which are all induced at the G1/S transition [7]. An important aspect of cell cycle regulation is therefore the coordinated expression of groups of genes that act together during the specific phases of the cell cycle.

4. G1 cyclins and the G1/S phase transition

4.1. The restriction point

In the G1 phase of the cell cycle growth-stimulatory and growth-inhibitory signals determine whether cells progress through the cell cycle or whether they remain quiescent. Later processes (DNA synthesis and mitosis) are largely independent of extracellular signals but rather depend on intracellularly triggered controls. A critical moment in the cell cycle is the point after which the cell is irreversibly committed to complete the division cycle: the restriction point [8]. Before this checkpoint growth factors are required to progress through the first phases of the cell cycle. The actual onset of DNA synthesis, the G1 to S phase transition, follows one to 3 h after the restriction point. Both transitions are marked by the appearance of active kinase complexes of G1 cyclins and their associated cdks, that phosphorylate key substrates essential for the

execution of the different transitions [2,4]. The timing and activation of specific kinases indicates that the commitment to enter the cell cycle and the actual onset of S phase are separately controlled events.

4.2. D-type cyclins

The first group of cyclins that is expressed after cells are stimulated to enter the cell cycle are the D-type cyclins. The direct role of the D-type cyclins in the progression through G1 has been demonstrated in several ways. First, the D-type cyclins are expressed and form active kinase complexes in mid- and late G1 [4,9]. Second, enforced expression of cyclin D1 earlier in the cell cycle accelerates entry into S phase [10–12]. Third, the inactivation of cyclin D1 through microinjection of antisense plasmids or monoclonal antibodies against cyclin D1 prevents entry into S phase [10,13–15]. The inactivation of cyclin D1 before the restriction point blocks progression into S phase, whereas inactivation at later time points is without effect. This indicates that the point of cyclin D action is before the onset of S phase. Taken together, these data provide strong evidence for a critical role of cyclin D-cdk complexes in the decision to pass the restriction point [9,16,17]. However, induction of cyclin D1 alone is not sufficient to progress from G1 into S phase indicating that more proteins are involved in this process [12].

There are three different D-type cyclins, cyclin D1, D2 and D3, which are expressed in a cell-lineage specific manner. Because there are several D-type cyclins it is unlikely that any one is essential for cell cycle progression. Consistent with this, mice nullizygous for cyclin D1 do not manifest a dramatic phenotype, although they do have some cell type specific abnormalities [18]. The genes for the D-type cyclins are induced when cells are mitogenically stimulated as part of the delayed early response. The half life of the D-type cyclins is very short ($T_{1/2} < 25$ min) and the withdrawal of growth factors during the G1 phase of the cell cycle result is a rapid decrease of D-type cyclins. When macrophages are deprived from colony stimulating factor 1, cyclin D expression is immediately reduced. As a result, these cells can no longer pass the restriction point [19]. These data have led to the idea that D-type cyclins act as growth factor sensors. A prediction from this would be that deregulated expression of cyclin D would contribute to tumorigenesis by making cells less dependent on growth factors. Indeed, Resnizky et al. have shown that overexpression of cyclin D reduces serum requirement for cell cycle entry [12]. The finding that cyclin D1 transcription can be stimulated through increased expression of *c-myc*, provides a possible connection between the immediate early genes and cell cycle control [20,21]. A role for cyclin D1 in cell proliferation is also suggested by the finding that loss of cyclin D1 function in mice leads to reduced proliferative capacity of retina and breast epithelium [18].

Deregulated expression of cyclin D has also been strongly implicated in cancer. Cyclin D1, located at chromosome 11q13, is over-expressed due to translocation in parathyroid adenomas and centrocytic B cell lymphomas [22–25]. Cyclin D1 amplification has been found in breast-, gastric-, esophageal- and squamous cell carcinomas [26–29]. Further evidence that cyclin D1 has strong growth-stimulatory activity is provided by the observation that cyclin D1 can cooperate with a *ras* oncogene in the transformation of primary rat embryo fibroblasts and baby rat kidney cells [30,31]. Furthermore, cyclin D1 can cooperate with *c-myc* to induce B cell lymphomas in transgenic mice [32,33]. Finally, cyclin D1 under the control of the mouse mammary tumor virus long terminal repeat causes mammary hyperplasia and mammary carcinomas [34].

The D-type cyclins also play an important role in cellular differentiation. 32D myeloid cells that ectopically express cyclin D2 or D3 are prevented from differentiation in the presence of granulocyte colony stimulating factor [35]. The expression of cyclin D1 also inhibits the function of MyoD and therefore the differentiation of myocytes into myotubes [36,37]. Together these results indicate that deregulated expression of cyclin D can dramatically influence key cellular processes such as proliferation and differentiation.

4.3. Cyclin E

After cyclin D1 induction, but well before the onset of S phase, cyclin E is transcriptionally induced and forms an active complex with cdk2. Cyclin E expression reaches its maximum near the G1/S boundary and is expressed periodically during the following cell cycles [38,39]. Several lines of evidence support a role for cyclin E in the onset of DNA synthesis. Entry into S phase of mammalian cells is blocked by the inhibition of cyclin E and cdk2, either by antibody microinjection or by the expression of a dominant negative cdk2 mutant [40–43], whereas overexpression of cyclin E shortens the G1 interval in mammalian cells [11,12]. Like cyclin D1, cyclin E expression only moderately accelerates entry into S phase. However, when both cyclin D and cyclin E are over-expressed simultaneously a further decrease in the time spent in G1 is observed, indicating that D and E-type cyclins regulate different processes during the G1 to S phase transition [44]. This was also suggested by the fact that cyclin E, but not cyclin D1, is essential for S phase entry in cells lacking the product of the retinoblastoma tumor-suppressor gene, pRb (see below and [43,44]). Also, in lower organisms, cyclin E is required for the initiation of S phase since *Drosophila* embryos in which cyclin E is homozygously deleted arrest in the G1 phase of cell cycle 17, the moment when normally cyclin E expression is induced [45]. Although cyclin E seems to regulate an important aspect of the G1/S transition, no strong evidence is present that deregulated cyclin E expression contributes to human cancer.

4.4. Cyclin A

Cyclin A is induced shortly after cyclin E and binds and activates cdk2 in S phase and cdc2 in G2 and M phase. Although cyclin A has been implicated in the control of mitosis, it appears that cyclin A is also involved in the regulation of S phase entry. Inactivation of cyclin A by antibody injection blocks entry into S phase [41,46]. Furthermore, the enforced expression of cyclin A early in G1, results in the acceleration of S phase entry and the over-expression of cyclin A in asynchronous cells causes a decrease of the number of cells in G1 [47]. Together these results suggest a role for cyclin A in the regulation of S phase entry. Consistent with this is the observation that cyclin A deregulation is implicated in transformation. In one hepatoma, the integration of hepatitis B virus resulted in the formation of a chimeric cyclin A protein that lacks the cyclin destruction box. As a result, the half life of the chimeric protein was prolonged significantly, causing a net increase in cyclin A protein levels in the virally infected cells [48]. One observation suggests that cyclin A expression is stimulated by signals from cell surface adhesion receptors and mediates cell growth control. A stable cell line of NRK fibroblasts expressing ectopic cyclin A is able to grow in suspension, whereas the parental cell line is anchorage dependent [49]. This could indicate that cyclin A synthesis is involved in mediating the anchorage independent growth properties of transformed cells.

5. Mitotic control by cyclin B

Mitosis is regulated by cyclin B in association with cdc2 (reviewed in [50,51]). Cyclin B is synthesized in S phase and accumulates with cdc2 towards M phase and is rapidly degraded during mitosis. The exit from mitosis depends on the abrupt ubiquitin-mediated degradation of cyclin B. In normal cells, unduplicated DNA or DNA damage prevents the activation of the cyclin B-cdc2 complex with the result that cells arrest in G2. In tumor cells this checkpoint is often defective, causing cells to become anaploid by entering M phase regardless of the replication state of the DNA [52]. The role of cyclin B in mitotic control was also illustrated in *Drosophila* cells that undergo endo-reduplication, continuous DNA synthesis without intervening mitosis. These cells do not contain mitotic cyclin B, thereby skipping M phase and as a consequence progress into G1 with double DNA content [53].

6. Regulation of cdks

As was mentioned above, the activation of a cdk requires the association with a regulatory subunit, the cyclin. The cell cycle-dependent expression of these cyclins is one mechanism by which cdk activity is controlled. However, it has become clear that additional mechanisms exist that control cdk activity.

6.1. Regulation by phosphorylation

First, the cdk component needs to be phosphorylated to acquire full activation of its kinase activity. The targets of this phosphorylation are the residues that block the protein-substrate binding site in cdks. The phosphorylation of threonine 160 in cdk2 or 161 in cdc2 makes the catalytic pocket accessible for the protein substrate [54]. The Cdk Activating Kinase (CAK) responsible for phosphorylation of these threonine residues is the MO15-cdk7 kinase, which in turn requires cyclin H for activity [55,56]. CAK can phosphorylate cyclin-cdk2, cyclin-cdc2 and cyclin D2-cdk4 complexes. In contrast to cdc2, cdk2 can be phosphorylated by CAK in the absence of bound cyclin. This could represent a cdk2-specific activation pathway, allowing the formation of phosphorylated inactive monomeric cdk2 kinases. The relevance of this observation under physiological conditions is still unclear. The identification of CAK as a cyclin-cdk complex which can function as an activator of other cyclin-kinase complexes, suggests that cyclin-cdk cascades may reflect an important regulatory pathway in cell cycle control. In addition to CAK phosphorylation, cdks are regulated by a second phosphorylation event mediated by the *wee-1/mik-1* related protein kinases [51]. The cdc2 kinase is inactive in S phase due to the phosphorylations on Tyr-15 and Thr-14. Phosphorylation of cdc2 on Thr-161 stabilizes the interaction with cyclin B and is essential for the activation of cdc2 kinase activity. The cdc25C phosphatase dephosphorylates Tyr-15 and Thr-14 at the end of G2, thereby activating cdc2 kinase activity [50].

6.2. Regulation by specific inhibitors

Recently it has been shown that a family of cyclin dependent kinase inhibitors (cdkIs) plays a major role in the negative regulation of cyclin-cdk activity (for reviews see [57–59]). These G1 cyclin inhibitors are involved in the arrest in G1 of cells in response to anti-proliferative signals. This arrest enables cells to enter processes such as terminal differentiation, cellular senescence, or to repair DNA damage. The cdkIs can be subdivided into two categories. The first class is a group of broadly-acting inhibitors that associate with a complex containing a cyclin, a cdk and the proliferating cell nuclear antigen (PCNA) [60,61]. The first cdkI of this class that was identified is $p21^{cip1}$. A molecule of $p21^{cip1}$ binds to and inhibits a wide variety of cyclin-cdk complexes including cyclin D-cdk4, cyclin E-cdk2, cyclin A-cdk2 [62–66]. However, $p21^{cip1}$ is present in most cyclin-cdk complexes in normal cycling cells. Significantly, active cyclin-cdk complexes can be immunoprecipitated with $p21^{cip1}$ antibodies, indicating that $p21^{cip1}$ can be present in active

cyclin-cdk complexes. Expression of $p21^{cip1}$ is induced when quiescent fibroblasts and T lymphocytes are mitogenically stimulated [66–69]. This probably indicates that low concentrations of $p21^{cip1}$ are synthesized in growth-stimulated cells to facilitate the assembly of active cyclin-cdk complexes, whereas higher concentrations of $p21^{cip1}$ are inhibitory.

$p21^{cip1}$ expression is in part under the control of wild type p53 [65]. DNA damage results in the increase of p53 protein levels that in turn induce $p21^{cip1}$ synthesis. This increase in $p21^{cip1}$ levels results in a further binding of $p21^{cip1}$ to cyclin-cdk complexes. This inhibits cyclin-cdk kinase activity, thereby allowing cells time to repair DNA damage before proceeding into S phase. $p21^{cip1}$ expression is also increased 10- to 20-fold in senescing fibroblasts, coincident with their loss of proliferative capacity [66]. In these cells an accumulation of inactive cyclin E-cdk2 is observed presumably as the result of $p21^{cip1}$ activation [70].

In addition to a role in cell cycle control and senescence, it is likely that $p21^{cip1}$ is also involved in regulation of differentiation. Thus, induction of $p21^{cip1}$ has been observed in cultured hematopoetic cell lines undergoing differentiation and in differentiating myoblasts [71–73]. However, mice lacking a functional $p21^{cip1}$ gene do not manifest major haematopoetic or muscle abnormalities [74]. This may indicate that $p21^{cip1}$ is redundant with other members of this gene family. At the same time, these data argue against a unique and essential role for $p21^{cip1}$ in the assembly of active cyclin-cdk complexes.

Other members of the $p21^{cip1}$ family of cdkIs include $p27^{kip1}$ and $p57^{kip2}$. Both appear to have a similar substrate specificity as $p21^{cip1}$ but probably allow the cell to respond to different growth-regulatory signals. Thus, $p27^{kip1}$ is lost from cyclin D-cdk4 complexes following stimulation of T cells with IL-2, whereas inducers of cAMP increase $p27^{kip1}$ levels in macrophages [69,75].

A second group of cdkIs is more restricted in its ability to inhibit cdk activity. $p16^{INK4a}$ was the first member of this family of cdkIs that now consists of at least four members, including, apart from $p16^{INK4a}$, $p15^{INK4b}$, p18, and p19 [76–80]. These cdkIs act as competitive inhibitors of D-type cyclins by forming specific complexes with the D-type cyclin partners cdk4 and cdk6 [76,79,80]. When overexpressed in pRb positive cells, all four members of this family can cause a G1 arrest, indicating that they are potent inhibitors of cell cycle progression. Consistent with this, loss of $p16^{INK4a}$ has been observed in a variety of human cancers and germ line mutations in $p16^{INK4a}$ have been found in familial melanoma [81–83]. In normal cells these inhibitors probably also act to mediate growth-inhibitory signals to the cell cycle machinery. Thus, treatment of human keratinocytes with TGFβ leads to a rapid induction of $p15^{INK4b}$, causing the cells to arrest in G1, whereas in a different cell system, Ewen et al. found that TGFβ induces a G1 arrest by reducing the expression of cdk4 [80,84]. The enforced expression of cdk4 is able to overcome a TGFβ-induced cell cycle block [84]. This indicates that a TGFβ-induced growth arrest depends on the efficient inhibition of D-type cyclin-associated kinase activity.

7. Substrates for cyclin-cdk complexes

The direct involvement of the sequentially activated cyclin-cdk complexes in cell cycle progression supports a model in which each successive cyclin-cdk complex phosphorylates a unique set of substrates that is essential for each transition. Thus, S phase cyclin-cdk complexes phosphorylate and activate proteins essential for DNA synthesis, whereas M phase cyclin-cdk complexes phosphorylate proteins involved in mitosis and cytokinesis. The targets of the D-type cyclins would then control the proliferation-differentiation switch.

Although many substrates for cyclin-cdk complexes have been identified in vitro, relatively few proteins are known whose phosphorylation is relevant to cell cycle progression. The best examples come from the study of cyclin B-cdc2 in mitotic events. For example, phosphorylation of lamins by cyclin B-cdc2 plays a major role in the disassembly of the karyoskeletal system. Similarly, chromosome condensation, occurring in M phase, is also accompanied by extensive phosphorylation of histone H1 on cdk sites (reviewed in [85]).

Recently, major attention has been focused on the phosphorylation of growth regulators in the G1 phase of the cell cycle. A key substrate for G1 cyclin-cdk complexes is the product of the retinoblastoma gene, pRb. Over the past decade it has become increasingly clear that pRb is a negative growth regulator that acts in the G1 phase of the cell cycle. In man, inactivation of one allele of *RB* predisposes to retinoblastoma. In these tumors the second allele is also inactivated, indicating that the loss of *RB* is an essential step in tumorigenesis. Somatic mutations that inactivate *RB* are also found in other human tumors such as osteosarcomas, indicating that loss of *RB* also contributes to other types of human cancer [86]. Reintroduction of a wild type *RB* gene in certain *RB* negative tumor cell lines causes a reversion of the transformed phenotype, supporting the role of pRb in regulation of cell proliferation [87,88]. pRb acts as a negative regulator in the G1 phase of the cell cycle because over-expression of pRb arrests most cells in G1 and the introduction of pRb after the restriction point is without effect [89,90]. Adenovirus and SV40 can induce quiescent cells to enter S phase, most likely to enforce the expression of host cellular genes required for viral DNA replication. The adenovirus E1A, HPV E7 and SV40 large T proteins bind to pRb and thereby inactivate pRb's ability to restrain cell division [91–93]. For all the viral oncoproteins it was demonstrated that the region involved in the interaction with pRb is also required for their transforming activity [94].

pRb is phosphorylated in a cell cycle-dependent manner [95–97]. In G0 and early G1 phases of the cell cycle pRb is found in the hypophosphorylated state. During the progression through G1, pRb undergoes additional phosphorylations resulting in a hyperphosphorylated form that persists through S, G2 and most of M phase. Several lines of evidence indicate that the growth-suppressive activity of pRb is regulated by phosphorylation. First, the phosphorylation of pRb is stimulated by signals that favor cell growth, whereas growth inhibitory signals prevent the phosphorylation of pRb. Moreover, the transforming T antigen of SV40 binds and thereby inactivates preferentially the hypophosphorylated form of pRb, suggesting that only the hypophosphorylated form is active in growth inhibition. Furthermore, the hypophosphorylated form of pRb binds several cellular proteins, among which the transcription factor E2F. pRb phosphorylation or binding to viral proteins of pRb, results in the release of these cellular proteins thereby allowing cell cycle progression (see below). All these data suggest that pRb in its hypophosphorylated state can prevent progression through the cell cycle. In mid G1, pRb is phosphorylated and thereby inactivated as brake on the cell cycle. The lack of pRb or inactivation by viral proteins will remove the pRb constraint on cell cycle control with the consequence of deregulated cell proliferation.

Accumulating evidence indicates that phosphorylation of pRb is controlled by cyclin-cdk complexes. The sites in the pRb protein that are phosphorylated are cdk consensus sites [98]. Because of the timing of pRb phosphorylation, it seems likely that G1 cyclin-cdk complexes mediate these phosphorylations. The connection between D-type cyclin-associated kinase activity and pRb phosphorylation is strengthened by a large body of evidence. First, the timing of pRb phosphorylation coincides with the appearance of active cyclin D1-cdk4 complexes. Also, when pRb protein was incubated with lysates of insect Sf9 cells engineered to express both cyclins and cdks, pRb could be phosphorylated by cyclin D1 (and D2 or D3) together with cdk4 or cyclin D2 or D3 in combination with cdk2 [99,100]. Furthermore, premature induction of D-type cyclin expression after serum stimulation in fibroblasts results in phosphorylation of pRb at an earlier time point [12,44]. Interestingly, cells that lack a functional pRb do not appear to require cyclin D-associated kinase activity to enter S phase suggesting that pRb is a critical target of cyclin D-associated kinase activity [15,101]. Significantly, the ability of the cdk4 inhibitors, $p16^{INK4a}$ and p18, to induce a cell cycle arrest is also dependent on the presence of a functional pRb [77,102–104]. The interplay between pRb and cyclin D1 is also apparent from studies with tumor cells lacking pRb function. Loss of pRb function, either through mutation or viral inactivation, correlates with a decrease in cyclin D expression, suggesting strongly that cyclin D is only required in pRb positive cells [15,105,106]. Although compelling evidence has been obtained that D-type cyclin-cdk complexes are primarily responsible for pRb phosphorylation, there are indications that other cyclin-cdks can also contribute to pRb phosphorylation. When pRb is over-expressed in the osteosarcoma cell line SAOS-2 cells, it causes an arrest in the G1 phase of the cell cycle. Ectopic expression of either cyclin E or cyclin A rescues the cell arrest and causes phosphorylation of pRb [99,107,108]. Although cyclin A is able to release the pRb-mediated growth arrest, it seems unlikely that cyclin A-associated kinase activity is responsible for the early phosphorylation of pRb in mid G1. Rather it would seem possible that cyclin A-cdk2 contributes to subsequent additional phosphorylation of pRb in the S and G2 phases of the cell cycle.

In contrast, cyclin E expression increases at the time of pRb phosphorylation [109]. Also, in the cervical carcinoma cell line C33A, transfected pRb is readily phosphorylated in the absence of apparent cyclin D-associated kinase activity [105,107]. Although premature cyclin E expression in G1 results in a earlier entry into S phase, this is not accompanied by the earlier onset of pRb phosphorylation [44]. It is possible that under normal conditions, phosphorylation of pRb by D-type cyclins must occur before it can function as a substrate for cyclin E-cdk2 complexes.

Apart from pRb, two pRb-related proteins, p107 and p130, share many features with pRb. Both p107 and p130 were identified as proteins that bind to the region of E1A required for transformation and share significant homology with pRb [110–113], indicating that pRb belongs to a small family of structurally and functionally related proteins. Indeed, like pRb, both p107 and p130 can form stable complexes with the cellular transcription factor E2F (see below and [114–117]). The finding that both p107 and p130 are bound by viral transforming proteins raised the possibility that p107 and 130 are also endowed with growth-inhibitory activity. Indeed, when transiently transfected, both proteins are able to induce a G1 arrest in certain cell types [118–120]. One idiosyncrasy of p107 and p130, not shared by pRb, is that they contain a domain within the region required for viral protein binding, called the spacer, with which they can form complexes with cyclin E-cdk2 or cyclin A-cdk2 [113,121,122]. Although cyclin E-cdk2 and cyclin A-cdk2 are able to phosphorylate p107 in vitro, it is unlikely that these cyclin-cdk complexes control the growth-inhibitory activity of p107 and p130. First, the presence of stable higher order complexes between p107, E2F and cyclin A-cdk2 or cyclin E-cdk2 indicates that cyclin A or cyclin E do not disrupt the E2F-p107 complexes. Furthermore, both cyclin A and E are unable to rescue a p107-induced growth arrest, suggesting that these cyclins are not involved in the functional inactivation of p107 [107]. Rather it seems that p107 may act to bind and inactivate cyclin A and cyclin E kinase complexes, in a similar fashion as the $p21^{CIP1}$ family of cdkIs. Indeed comparison of the structure of p107 and $p21^{CIP1}$ reveals a short region of homology between the

two proteins that is responsible for the cyclin-cdk interaction. Consistent with a p21^{CIP1}-like role for p107, Zhu et al. have shown that overexpression of p107 can inhibit pRb phosphorylation mediated by cyclin E or cyclin A complexes [123]. Thus, p107 can cause a G1 arrest by one of two mechanisms. One involving the binding and inactivation of cyclin-cdk complexes and another mechanism involving the binding and inactivating of cellular growth-promoting factors like E2F [124].

Apart from being able to bind cyclins through the spacer element, p107 can interact with D-type cyclins through the pocket structure. Indeed, Li et al. have demonstrated that p107 is associated with D-type cyclins in vivo and a cyclin D-cdk4 complex is able to phosphorylate p107 in an in vitro kinase assay [113]. p107 is hypophosphorylated in the G0 and early G1 phase of the cell cycle. Phosphorylation of p107 occurs in mid G1, coincident with the appearance of cyclin D expression [125]. In contrast to pRb, hypophosphorylated p107 reappears at the beginning of S phase. This may be explained by the observation that p107 abundance strongly increases at the G1/S transition. Most likely, the newly synthesized p107 cannot be efficiently phosphorylated by the declining cyclin D1-associated kinase activity, causing the reappearance of hypophosphorylated p107 in early S phase [114,125]. Phosphorylation of p107 in mid G1 can be mimicked by the expression of cyclin D1 in combination with cdk4, but not by cyclin A or E in combination with cdk2. Overexpression of a kinase-inactive mutant of cdk4 abolishes the phosphorylation of p107 in vivo, both indicating that in vivo cyclin D-associated kinase activity is responsible for p107 phosphorylation. The functional significance of this phosphorylation was illustrated by the finding that a p107-mediated G1 arrest could be overcome by co-expression of cyclin D1-cdk4, but not by the overexpression of cyclin E or cyclin A [107,125].

The pRb-related p130 is also regulated through phosphorylation. In quiescent cells p130 is in the hypophosphorylated state. With progression through G1, phosphorylation of p130 is induced. The moment at which p130 is phosphorylated coincides with the phosphorylation of p107 [126]. Thus, efficient entry into the cell cycle is accompanied by the phosphorylation of all three pRb family members. The moment of p130 phosphorylation indicates that it is too mediated through the activity of D-type cyclin associated kinase activity. That p130 is inactivated by cyclin D-cdk4-mediated phosphorylation is substantiated by the finding that a p130-induced cell cycle arrest can be rescued by cyclin D1-cdk4. Wolf et al. show that the p130 protein can be efficiently phosphorylated by cdk2 in vitro [119]. However the timing of the phosphorylation of p130 during the cell cycle suggests an earlier time point for the onset of p130 phosphorylation. Also the existence of higher order complexes containing E2F, p130 and cyclin E/cdk2, as was found for p107, makes it unlikely that p130 is functionally inactivated by cyclin E-cdk2 complexes [114]. Whether cyclin E-cdk2 can contribute to the subsequent phosphorylation of p130 later in the cell cycle remains unclear.

8. Targets of pRb and pRb related proteins: Regulation of the transcription factor E2F

Compelling evidence has been obtained that pRb regulates the activity of transcription factors that in turn regulate the expression of division-promoting factors. Several lines of evidence indicate that the cellular transcription factor E2F is an important target for pRb-mediated growth control. First, the interaction between pRb and E2F is dependent on the pocket region of pRb. Mutations in pRb found in human tumors frequently involve the pocket domain and abolish the interaction with E2F [127–129]. Second, the binding of viral proteins to pRb prevents the interaction between pRb and E2F [130]. The activation of the viral E2 promoter region, which carries two E2F DNA binding sites, depends on the interaction between E1A and pRb. The binding of pRb by viral proteins results in the appearance of free E2F that can activate the E2 promoter. Third, E2F associates only with the hypophosphorylated, growth-inhibitory, species of pRb [95–97,128]. Finally, E2F binding sites are present in a number of genes that are regulated in a cell cycle-dependent manner and the presence of these E2F sites contributes to the cell cycle-regulated expression of genes such as *c-myc*, *b-myb*, thymidine kinase, dihydrofolate reductase, DNA polymerase a and E2F itself [131,132]. Together these data suggest a model in which hypophosphorylated pRb inhibits the transcription factor E2F through direct binding, thereby preventing the expression of genes whose products mediate cell cycle progression. The phosphorylation of pRb by cyclin-cdk complexes abolishes pRb-mediated inhibition of E2F indicating the functional interplay between the cell cycle clock and cell cycle-regulated gene expression. Loss of pRb function, either through mutation, viral inactivation or phosphorylation results in the loss of control of the E2F transcription factor. Apart from pRb, the related proteins p107 and p130 are also found in complex with E2F. These interactions are also disrupted upon viral infection and phosphorylation, suggesting a more complicated network in cell cycle control by pocket proteins and E2F.

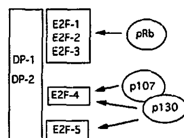

Fig. 1. The E2F family of transcription factors and their pocket protein partners.

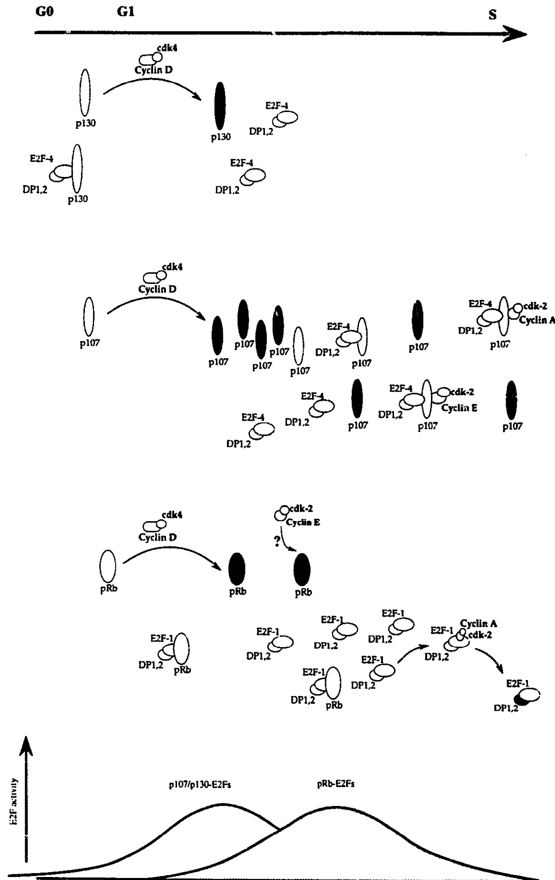

Fig. 2. Model for the regulation of E2F mediated transactivation and repression in the cell cycle. The hypophosphorylated species of p130, p107 and pRb are depicted in white, phosphorylated p130, p107 and pRb are indicated in black. The amount of E2F and E2F-pocket protein complexes are a reflection of the phosphorylation status of their respective pocket protein partner and the expression levels of the different E2Fs and pocket proteins. The complex formation of E2F-4-p130 and E2F-4-p107 and E2F-1-pRb are depicted. In S phase, cyclin A-cdk2 phosphorylates DP1 and DP-2, as indicated in black, reducing the DNA binding affinity of the E2F-DP complex. The lower panel represents the activity of the different E2Fs. The p107/p130 E2Fs resemble the activity associated with E2F-4 and E2F-5, whereas pRb-E2Fs are compromising E2F-1, E2F-2 and E2F-3.

8.1. E2F complexes in the cell cycle

The complexes between E2F and pRb family of proteins are subject to cell cycle regulation (Fig. 2). At the G1/S phase transition, an increase in free E2F is observed, whereas quiescent cells contain predominantly E2F in complex with pocket proteins [117,133,134]. However, most cycling cells contain these higher order complexes as well. In general, E2F in complex with pRb is found in the G1 phase of the cell cycle. Although phosphorylation of pRb takes place before the G1 to S phase transition, the E2F-pRb complex persists well into S and G2 phases of the cell cycle [117]. This probably indicates that the appearance of free E2F at the G1/S transition is not primarily due to the dissociation of pre-existing E2F-pRb complexes but rather the result of the new synthesis of E2F [135,136]. This newly synthesized E2F is no longer bound by the phosphorylated 'free' pRb. This mechanism allows the generation of free E2F in the presence of E2F-pRb complexes. Mouse fibroblasts that are homozygously deleted for the *RB* gene, appear not to differ in their composition of higher order E2F complexes in the G0 state of the cell cycle [114]. This indicates that pRb is not a major component of higher order E2F complexes in quiescent mouse fibroblasts. In most quiescent cells, such as fibroblasts and T lymphocytes, E2F forms a complex with p130 [114,117,121,137]. However, in certain cell types, complexes containing E2F-p107 and E2F-pRb can also be observed in G0 cells [114]. That some cell types differ in the presence of G0 E2F complexes could reflect a difference in their capacity to become quiescent. The E2F-p130 complexes disappear when fibroblasts are stimulated to enter the cell cycle [114,119]. In mouse fibroblasts, the disappearance of the E2F-p130 complex coincides with the phosphorylation of p130 by cyclin D-cdk4 [126]. Because p130 contains a spacer element that can interact with cyclin-cdk complexes, higher order complexes containing E2F-p130 and cyclin-cdk complexes are also observed. In vitro reconstitution experiments have demonstrated that p130-E2F complexes can associate with cyclin E-cdk2 and cyclin A-cdk2. The E2F-p130 cyclin E-cdk2 complexes have been observed in fibroblasts in the late G1 phase of the cell cycle [114].

The complexes between p107 and E2F also show a complex pattern of appearance during the cell cycle. In late G1, DNA binding complexes have been observed that contain E2F, p107, cyclin E and cdk2. In S phase cyclin E is no longer found in these complexes, instead E2F is found associated with p107, cyclin A and cdk2 [117,121]. Upon entry into mitosis the cyclin A component is degraded and at the beginning of the subsequent G1 phase E2F is again found in complex with p107 alone. When quiescent cells are stimulated to enter the cell cycle, the p107-E2F complex only becomes apparent in late G1 and persists in S phase [134]. The late G1 complex consists of E2F-p107 and cyclin E-cdk2. Although in mouse fibroblasts hypophosphorylated p107 is observed in G0 cells, it does not result in complex formation with E2F. It is likely that the p130 out-competes p107 in early G1 for binding to E2F indicating that p130 has a higher affinity for E2F-4. In late G1 p107 expression is strongly induced and newly synthesized hypophosphorylated p107 reappears when cyclin D-associated kinase activity declines. This results in the emergence of E2F-p107 complexes. In contrast, expression of p130 does not increase or in some cell types even decreases in late G1. As a result of this, E2F-p130 complexes are not found in S phase [119,126]. Taken together, the formation of the different E2F complexes largely depends on the availability and relative affinity of the different components.

The changes in the multi-protein complexes do not only occur during the cell cycle, but also following the induction of differentiation or senescence. For instance, E2F complexes undergo dramatic changes during embryo carcinoma cell differentiation: The proportion of free E2F declines and the amount of E2F complexes containing pocket proteins increases [138,139]. Similar changes occur during *Xenopus* development. Philpot and Friend have shown that free E2F is present in *Xenopus* oocytes and early embryos [140]. E2F-pRb complexes are first detected in the mid-blastula phase and become more prominent at later stages of development. In mammals, E2F complexes can be modulated by a variety of signals, including cytokines [141]. Stimulation of Burkitt's lymphoma cells and myeloblastic cells with either interleukin-6 or interferon α or β gave an immediate reduction of E2F DNA binding complexes. This decrease was correlated with a decrease in *c-myc* expression and the induction of a growth arrest, suggesting that it may reflect an important event in cytokine signaling. The mechanism by which cytokines reduce E2F DNA binding is unclear, but the restoration of E2F binding activity in cell extracts by EDTA suggests that cytokines regulate DNA binding activity by post-translational modifications. Finally, upon muscle differentiation, multiple changes in E2F complexes occur [142]. The p130-E2F complex is only present in fully differentiated myotubes. The formation of p130-E2F complexes did not occur in a differentiation-defective myoblast cell line, although other higher order E2F complexes were readily detected. Thus, the formation of the p130-E2F complex seems a necessary event in the onset of differentiation.

8.2. The E2F family of transcription factors

The DNA binding complex named E2F is a heterodimeric complex consisting of an E2F component and a dimerization partner, the DP component. The binding of the E2F and DP component to DNA is synergistic. Consequently, E2F site-dependent transactivation by E2F and DP proteins is also highly interdependent [143–146]. The interaction with the DP component is also essential for the high affinity interaction of E2F with pRb and p107 [146–

148]. The E2F component is encoded by at least five different genes, E2F-1 through E2F-5. For the DP component two different genes have been isolated, DP-1 and DP-2 [147,149–154]. All the different E2Fs are structurally related and have several regions that share a high degree of homology. These regions include the DNA binding domain, the DP dimerization domain and the transactivation/pocket protein binding region [135]. The two DP proteins share limited homology with the E2Fs except for the region that corresponds to the DNA binding and dimerization region of these proteins [152,155,156]. Although the E2Fs are highly homologous in their pocket protein interaction domains, they display specificity for pocket protein binding [151,157]. Of the five known E2Fs only E2F-1, E2F-2 and E2F-3 are found associated with pRb in vivo [151,152]. E2F-4 and E2F-5 appear not to be under pRb control but rather found associated with the pocket proteins p107 and/or p130 (Fig. 1) [120,135,147,153,158]. All E2Fs can interact with both DP-1 and DP-2 in vivo, and each complex is capable of activating transcription of reporter genes that have an E2F consensus DNA binding sites in their promotors. This also suggests that the pocket binding specificity is not determined by the DP component but is mediated by the E2F subunit [152].The complexity of the multiple E2Fs and DPs in mammals is not found in *Drosophila*. At this moment only a single homologue of E2F, DP and the *RB* gene have been identified [159,160] (N. Dyson, pers. comm.). Significantly, all E2F DNA binding activity in *Drosophila* can be accounted for by these three proteins, indicating that it is unlikely that additional family members exist in *Drosophila*. The functional significance of the *Drosophila* E2F homologue dE2F was demonstrated by disruption of the single dE2F gene [161]. Embryos homozygous for null mutations of dE2F can no longer induce DNA synthesis after cycle 17, when maternally provided dE2F is no longer present. Mutant embryos also lack the coordinated transcription of genes essential for replication. This suggests that dE2F, in most cells, is essential for the G1 to S phase transition.

The relevance of the existence of many different E2Fs in higher organisms is still unclear. One possibility is that, although they can all recognize the same E2F consensus sequence, they differ subtly in DNA binding specificity. As a result, they may control different sets of genes. Consistent with this, the promoters of the thymidine kinase and *b-myb* genes contain E2F sites that interact preferentially with E2F-p107 complexes [162–164]. Furthermore, infection of rat fibroblasts with a recombinant adenovirus that mediates expression of E2F-1, leads to transcriptional activation of only a subset of E2F-site containing promoters [165]. In addition, the specific pattern of appearance of the E2F-pocket protein complexes in the cell cycle indicates that the various E2Fs are active at different points in the cell cycle. Moreover, the different E2Fs show a unique pattern of expression during the cell cycle. Vairo et al. have shown that the predominant E2F present in unstimulated T cells is E2F-4. E2F-4 is expressed throughout the cell cycle of re-stimulated quiescent fibroblasts and human keratinocytes [120,153]. This in contrast to the pRb interacting E2F-1 whose expression is very low or absent in quiescent cells and is induced only 8–10 h after serum stimulation [135,136,150]. The strictly-timed activation strongly argues for specific functions of the E2F family of transcription factors.

8.3. E2F regulated genes

The regulation of E2F-pocket protein complexes by G1 cyclins and the targeting of these complexes by viral transforming proteins indicates that E2F is a key regulator of gene expression during the cell cycle. Indeed, the list of promoters containing E2F binding sites includes genes that encode cell cycle regulators such as *c-myc*, *N-myc*, *cdc2*, *b-myb*, *E2F-1* and *cyclin A*, as well as genes encoding proteins for cell cycle-regulated biochemical processes such as DNA polymerase α, thymidine synthetase, thymidine kinase and dihydrofolate reductase (DHFR). Many of these genes are induced in quiescent cells following DNA tumor virus infection, concomitant with the induction of S phase. The ability of these viruses to activate these genes is dependent on their ability to bind and inactivate the retinoblastoma protein and its relatives. This emphasizes the important role of E2F in the regulation of these genes. Recent analyses have indicated that the E2F binding sites are critical for the growth-regulated activity of these promoters [164,166–168]. The DHFR promoter contains two inverted and overlapping E2F sites located at the transcription initiation site. Transcription from a truncated DHFR gene promoter increases more than 10-fold at the G1/S phase transition, which was lost following mutation of the E2F sites [169]. Furthermore, introduction of the DHFR-E2F sites upstream of a heterologous promoter causes a strong transcriptional increase at the G1/S boundary [170]. Together these data indicate that the E2F sites are essential elements for the cell cycle-regulated expression of the DHFR gene.

One puzzling aspect of the different genes containing E2F sites is that their transcriptional activation does not occur at the same time in the cell cycle. *c-myc* and *N-myc* are immediate early genes, whose expression is induced within minutes after serum stimulation. In contrast, the genes encoding products involved in DNA replication are first expressed at the G1/S transition. Since both appear to depend on E2F, additional mechanisms must exist to account for the distinct expression patterns. One hypothesis to explain this difference is that other transcription factors act in concert with E2F to mediate promoter activity. Elements that contain binding sites for Sp1 and CCAAT-box binding factors have been implicated to cooperate with E2F in growth-regulated transcription [136,170]. However, the existence of different E2Fs, that display a distinct

pattern of expression and complex formation with their inhibitory pocket proteins in the cell cycle, may also allow differential regulation of promoters that contain E2F sites.

8.4. Regulation of E2F activity

The ability to activate transcription of all five members of the E2F gene family is strongly inhibited upon binding of their respective pocket protein partners [120,147,153,171–175]. Mutant forms of pRb that fail to bind E2F also fail to inhibit E2F transactivation. Conversely, mutants of E2F that can no longer bind pRb, but still transactivate, are resistant to pRb inhibition [176]. Furthermore, in vitro, E2F-1 mediated transcription can also be suppressed by purified pRb [177]. It is therefore most likely that inhibition of E2F transactivation by pocket proteins is due to direct binding of the pocket proteins to the E2F transactivation domain. The binding of pRb, p107 and p130 to the transactivation domains of E2Fs, suggest that these pocket proteins interfere with the interactions of E2F with the basal transcription machinery. Phosphorylation of pRb, p107 and p130 decreases the affinity for E2F and therefore result in the accumulation of 'free' active E2F transcription factors.

This model may turn out to be an oversimplification as the presence of cyclin-cdk complexes in the E2F-p107 and E2F-p130 complexes may modify the activity of these complexes. In agreement with this view, Li et al. have shown that the binding of an E2F-p107-cyclin A complex to the thymidine kinase promoter is greatly enhanced when the cells enter S phase [164]. This coincides with an increase in thymidine kinase mRNA levels and DNA replication. These results suggest a mechanism by which the E2F, p107, cyclin A complex, in association with the cdk2, can mediate the S phase-activated transcription of the thymidine kinase promoter in growth-stimulated cells.

In addition to pocket protein binding, the activity of E2F transcription factors is controlled by phosphorylation. Mudryj et al. showed that in the *c-myc* promoter, an increase in E2F DNA binding activity is observed within 4 h after serum stimulation. This increase does not result from the disappearance of higher order complexes and also occurs in the absence of protein synthesis [178]. This would suggest that E2F DNA binding affinity is increased rather than an increase in E2F expression or the release of E2F from pocket proteins. Furthermore, adenovirus E1A can stimulate E2F DNA binding activity in cell extracts by an ATP-dependent process [179]. Phosphorylation of E2F can also contribute to the activation of E2F-mediated transcription. E2F-1 can be phosphorylated on two serines (amino acids 332 and 337) in late G1. These phosphorylations occur at the time that cyclin E-associated kinase activity is induced [180]. The phosphorylation of E2F-1 inhibits the interaction with pRb, thereby relieving its inhibition by pRb.

In addition to positive regulation, evidence also exists that E2F is subject to negative regulation by phosphorylation. In S phase, the E2F-1-DP-1 heterodimer is found in a quarternary complex with cyclin A and cdk2. Cyclin A-cdk-2 complexes can directly interact with the amino terminus of E2F-1 in vivo [181]. The association of E2F-1-DP-1 dimer with cyclin A-cdk2 leads to the phosphorylation of DP-1 and loss of DNA binding affinity [177,181,182]. The phosphorylation of DP-1, which can be observed in late S phase when cyclin A expression peaks, can only occur when DP-1 is bound to E2F-1 [181]. The region of E2F-1 involved in the interaction with cyclin A-cdk2 is conserved between the pRb-interacting E2Fs. In contrast, the N-terminal cyclin A binding domain is absent in the p107- and p130-interacting E2F-4 and E2F-5, suggesting that these E2F subtypes escape this type of negative control. This is also suggested by the observation that in S phase DNA binding complexes containing E2F and p107 and cyclin A are readily observed. Downregulation of E2F activity by cyclin A-cdk2 during S phase may explain why many of the genes that are transcriptionally induced at the G1/S transition decrease in expression during S phase. Recent data by Resnitzky et al. cast doubt on this model. They show that enforced expression of cyclin A enhances S phase entry rather than inhibit it by the presumed down modulation of E2F activity [47].

Recently, yet another level of E2F-1 regulation has been described. The proto-oncogene MDM2 interacts with the activation domain of E2F-1, thereby stimulating the activity of the E2F-1-DP-1 complex [183].

8.5. Transcriptional repression mediated by E2F complexes

Whereas most E2F sites in cellular promoters act as positive elements that confer cell cycle-regulated activation, in some cases E2F sites have been shown to act primarily as negative elements. For instance, the *b-myb* promoter is repressed in G0 and G1 by binding of an E2F-p107 complex to an upstream E2F site. Mutation of this E2F site was sufficient to relieve transcriptional repression in G0, resulting in a promoter with constitutively high activity that was equal to the G1/S levels seen with the wild type promoter [162]. This indicates that p107-E2F complexes can act as active transcriptional repressors in G0 and early G1. The disruption of these complexes in mid to late G1 leads to de-repression and activation by other regulatory elements in the same promoter. Similar mechanisms have been observed for the promoter of the insulin-like growth factor-1 (IGF-1), *cdc-2*, *c-myc* and *E2F-1* genes [136,171,172,184,185]. Consistent with this, Weintraub et al. showed that a synthetic promoter construct in which E2F sites were placed upstream of a strong promoter, the E2F sites act as negative elements [186]. The transcriptional repression is most likely caused by the recruitment of pRb to the promoter because co-expression of E1A (that disrupts E2F-pRb complexes) relieves this

repression. Furthermore, the E2F sites did not act as negative elements in pRb$^{-/-}$ cells, suggesting that only E2F-pRb complexes were active repressor complexes [186]. When fused to a DNA binding domain of GAL 4, both pRb or p107 repress transcription of promoters that contain GAL 4 DNA binding sites, indicating that transcriptional repression is a universal property of the retinoblastoma protein family [187,188].

Recently Weintraub et al. have suggested a mechanism for the active transcriptional repression by pRb [189]. They suggest that pRb is recruited to promoters through E2F, where pRb binds and inactivates neighboring transcription factors. The promoter-localized pRb is able to repress transactivators like Elf-1, PU-1 and c-Myc, but has no effect on VP-16, SP-1 and CTF. One explanation for this difference is the observation that promotor localized pRb binds to Elf-1, PU-1 and c-Myc but not to the insensitive transcription factors like VP-16, SP-1 and CTF [189]. Furthermore, in vitro data suggest that the binding of these transactivators by pRb prevents their interaction with the transcriptional machinery [189].

The in vivo significance of pocket protein-mediated trans-repression remains unknown. Obviously, it would provide a mechanism to silence S phase-specific gene expression in other periods of the cell cycle. In addition, transrepression might be required for the induction of differentiation. In differentiated cells the predominant E2F complex is E2F-p130. It is therefore well possible that the E2F-p130 complex in these cells functions to actively suppress proliferation-associated genes.

8.6. *E2F and cell biology*

The results discussed above place the different E2Fs in a central position in a regulatory network that controls growth and differentiation. The observation that E2Fs are under the control of the retinoblastoma family of growth-inhibitory proteins suggests that the inactivation of E2Fs plays an important role in the cell cycle arrest imposed on cells by the pocket proteins. That E2F indeed plays an important role in the control of the transition from G1 to S is suggested by several observations. Ectopic expression of E2F-1 can prevent cells from entering quiescence and can induce S phase entry in quiescent fibroblasts in the absence of serum [190,191]. These effects depend on the ability of E2F-1 to bind DNA and activate transcription. The ability to stimulate cell cycle progression is not limited to the pRb-interacting E2Fs. Introduction of E2F-4 together with its dimerization partner DP-1 in the osteosarcoma cell line SAOS-2, reduces the number of cells in G1, indicating that E2F-4 activation stimulates cell cycle progression [147]. Moreover, most members of the E2F gene family, including E2F-1, 2, 3, 4 and DP-1 proteins, are able to transform cells. Deregulated expression of E2F-1, 2 or 3 can lead to transformation of a rat embryo fibroblast cell line [192,193], whereas the overexpression of E2F-1 or E2F-4 together with activated *ras* can transform primary rat embryo fibroblasts [147,194]. Furthermore, a chimeric E2F-1 protein in which the transactivation domain is replaced by the activation domain of herpes virus VP16 exhibits increased transformation ability. This suggests that the activation of E2F target genes is involved in the observed transformation [194]. The role of DP-1 and DP-2 proteins in transformation is still unclear. Although these proteins cooperate with an activated *ras* oncogene in the transformation of rat embryo fibroblasts, this does not seem to depend on the interaction with E2F. This suggests an E2F-independent effector function for the DP proteins in cell growth control [195].

The expression of the viral E1A protein in quiescent cells can induce S phase entry. This effect is mediated by the binding of pRb and pRb-related proteins p107 and p130. That the major consequence of this binding, release of E2F transcription factors, is essential in S phase entry was shown by the co-expression of an E2F dominant negative mutant. This mutant was able to block E1A-induced cell cycle progression, indicating that activation of E2F is essential for E1A-induced cell cycle progression [196]. The hypothesis that members of the pocket protein family can induce a cell arrest by inhibition of E2Fs was further substantiated by the observation that pocket protein-induced cell cycle blocks could be overcome by overexpression of their E2F partners. The pRb-induced cell cycle block could be rescued by the overexpression of E2F-1 and to a much lesser extend by E2F-4 [107,120,191]. In contrast, a p107- or p130-mediated growth arrest could efficiently be rescued by overexpression of E2F-4 and not by E2F-1 [118,120]. The observation that both cyclin A and cyclin E are transcriptionally induced by E2F-1, suggest a potential inactivation of pRb through E2F-1-mediated transcriptional activation [165]. Together these results indicate that the different E2Fs are involved in cell cycle control and that the different pocket proteins regulate cell cycle progression via distinct pathways involving different members of the E2F transcription factor family.

8.7. *E2F and apoptosis*

Overexpression of E2F-1 can induce S phase entry in the absence of serum. However, these cells do not complete a cell cycle but rather undergo programmed cell death [197–200]. Also the generation of stable cell lines that overexpress E2Fs has been unsuccessful in several different cell types, indicating that high levels of E2F are not tolerated in most cells (R. Kerkhoven and R.L.B, unpublished data). One explanation for the induction of apoptosis by E2F is that E2F stimulates the expression of proteins that are able to induce apoptosis. For example an E2F target such as *c-myc* has been shown to induce apoptosis [201]. It is also possible that the untimely entry into S phase is responsible for apoptosis after the induction of E2F in low serum. The ability of E2F to induce apoptosis provides an explanation for the induction of apoptosis by the functional inactivation of pRb, either by

viral inactivation or mutation. White et al. have shown that E1A expression can induce apoptosis in primary cells [202]. Furthermore, the loss of both pRb alleles in mice causes degeneration of a number of tissues, most likely due to apoptosis [203–205]. Recent data have implicated p53 in the E2F-induced apoptosis. E2F-1-mediated apoptosis is suppressed by co-expression of pRb or a transdominant negative mutant of p53 [198,200]. It has also been demonstrated that p53 is important in the apoptosis that results from loss of pRb function. In the absence of p53, loss of pRb results in uncontrolled proliferation and the inactivation of one *RB* allele in a p53 nullizygous mouse gives rise to a higher tumor incidence [206]. Taken together, this could indicate that the loss of pRb results in the activation of E2F that causes p53-dependent apoptosis. Although E2F-1 mediated apoptosis appears to be p53-dependent, the inability to generate stable E2F overexpressing cell lines that lack p53 function, indicates that p53-dependent and p53-independent pathways are involved.

8.8. Other targets of pRb family members

Apart from the E2Fs, the pocket proteins regulate a number of other pathways involved in cell cycle regulation. pRb influences the activities of other transcriptional regulators by direct interaction, including Elf-1, MyoD, ATF-2, PU-1, UBF, BRG-1, MDM2 and c-Abl [207–210]. Elf-1 is a member of the ETS family of transcription factors that regulates gene expression during T cell development. The mechanism by which pRb regulates Elf-1 is analogous to E2F: hypophosphorylated pRb interacts with the transcriptional activation domain of Elf-1 and thereby inactivating Elf-1 in resting T-cells. Activation of T cells results in phosphorylation of pRb and activation of transcription mediated by Elf-1. In addition to the repression of transcription, it is possible that pRb activates genes that are involved in suppression of cell growth. Indeed positive regulation of transcription by pRb has been reported in the case of the ATF-2 transcription factor, which mediates the activation of the TGFβ2 promoter by pRb [207]. The TGFβ proteins induce a G1 arrest in many cell types, so that the activation of expression of these factors provides a means to constrain cell proliferation. Although a direct interaction between ATF-2 and pRb has been demonstrated in vitro, the exact mechanism by which pRb affects ATF-2 dependent transcription is still unclear. Transcriptional activation can also be mediated through the pRb control element (RCE). This element is present in the promoters of *TGFβ1*, and *c-Jun*. The pRb-specific effect on RCEs is probably mediated by SP1. The SP1 protein can bind to the RCE element and pRb can enhance both the DNA binding and transactivational activity of SP1 [211,212]. This activation is most likely mediated by the sequestering of an inhibitor of SP1 by pRb, named SP1-I, which in the absence of pRb prevents SP1 from binding to DNA and to activate transcription [212].

Recently, interactions between pRb and the cellular proteins c-Abl, BRG-1, MDM2 and UBF have been reported that seem to play different roles in the pRb-mediated cell cycle arrest. The nuclear tyrosine kinase c-Abl is found associated with pRb in vivo. Only the hypophosphorylated form binds to the catalytic domain of c-Abl thereby inactivating the kinase activity of c-Abl. Although phosphorylation of pRb regulates the interaction with c-Abl, its binding to pRb is not mediated by the E2F binding domain of pRb. Welch and Wang have suggested that the assembly of multiprotein complexes containing both E2F, pRb and c-Abl are essential to control of the activity of a number of genes involved in cell cycle proliferation [213,214]. pRb also associates with BRG1 and hBRM1 [215,216]. Both BRG1 and hBRM1 share extensive sequence similarity to the *Drosophila* gene *Brahma*, an activator of homeotic gene expression and the yeast transcriptional activator SNF2/SWI2. *Brahma* does not bind to specific DNA sequences but seems to function by inducing alterations in chromatin structure. Dunaief et al. show that BRG1 associates with the hypophosphorylated form of pRb and binding is abolished in pocket mutant pRb from human tumor cell lines [215]. Viral oncoproteins block the pRb-BRG-1 interactions and BRG-1 cooperates with pRb in the formation of flat, growth-arrested cells. Interestingly, the cervical carcinoma cell line C33A has no detectable level of BRG-1 expression and is not sensitive to a pRb-induced cell cycle arrest, suggesting that BRG-1 and pRb may cooperate to induce growth arrest [107]. This is also substantiated by the finding that the BRG-1 homologue hBRM cooperates with pRb in flat cell induction in SAOS-2 cells [215]. The interaction of pRb and hBRM1 is involved in the stimulation of transcription. The presence of both proteins upregulates the glucocorticoid receptor-mediated transcription, pointing to hBRM1 as a target for pRb-mediated transcriptional activation [206].

Recently, an interaction between pRb and MDM2 has also been described [202]. The cellular oncoprotein MDM2 was originally identified as a protein that is amplified in certain tumors. MDM2 also binds to and inhibits transactivation by the p53 tumor suppressor gene. Xiao et al. show that MDM2 interacts with pRb and, as with p53, inhibits pRb growth-inhibitory function [210]. MDM2 binds to the C-terminus of pRb, but binding of MDM2 appears to block the interaction with E2F, thus providing a possible explanation for the observed rescue by MDM2 of pRb growth suppressive activity. On the other hand, Martin et al. have demonstrated that MDM2 can also directly bind to and activate E2F-1 [183]. Together these results indicate that MDM2 can stimulate E2F mediated transactivation via two ways, first by releasing pRb and second through the activation of E2F itself.

The interaction between pRb and the RNA polymerase I transcription factor UBF gives another dimension to the role of pRb in the regulation of transcription. Actively growing cells require the ongoing synthesis of ribosomal RNA. Cavanaugh et al. show that the pocket of pRb is

required for the interaction between pRb and UBF in cell extracts [217]. The activity of UBF is inhibited by the addition of pRb in vitro. The nature of this interaction would suggest a general role for pRb in the modulation of gene expression.

The myogenic helix-loop-helix protein MyoD promotes skeletal muscle specific gene expression and induces a cell cycle arrest. The members of the retinoblastoma gene family appear to play an essential role in the withdrawal of the cell cycle and phenotypic differentiation. Although an interaction between myoD and pRb has been observed [208], the mechanism by which myoD induces differentiation is still unclear. Recent data indicate that myoD may induce terminal cell cycle arrest by increasing the expression of the cdk inhibitor p21 [73]. As a result of this pRb remains in the hypophosphorylated state and is able to induce a G0 arrest. Consistent with a role for pRb in myogenesis, inactivation of pRb by mutation or binding to viral proteins interferes with myogenesis. In contrast, myogenic differentiation in pRb-deficient mice seems normal. One explanation for this apparent discrepancy is the observation that muscle differentiation in $pRb^{-/-}$ cells correlates with the increased expression of p107 [218]. However, the phenotype of these differentiated muscle cells is different from their wild type counterparts because they are able to re-enter the cell cycle after serum stimulation. Apparently, p107 is not able to fully replace pRb in the induction of myocyte differentiation.

Apart from the interaction with E2F-4, we and others have shown that p107 forms a complex with the c-Myc oncoprotein [219,220]. p107 interacts with the transactivation domain of c-Myc resulting in the inhibition of c-Myc-mediated transactivation. This interaction is of particular interest because of the interaction between a proto-oncogene and a growth-inhibitory protein could reflect a direct communication between proteins that regulate cell growth and differentiation. Furthermore, naturally occurring mutants of the *c-myc* proto oncogene that are found in Burkitt's lymphoma, escape the p107-mediated suppression, providing a possible explanation for the increased growth rate of these cells [220]. It will be worthwhile to investigate whether similar mutations, that allow the escape from pocket protein inhibition, are also present in E2F genes in human tumors.

9. Functional differences between pRb family members

Although pRb, p107 and p130 all have strong growth suppressive activity, only pRb has been found mutated in human cancer. Furthermore, only loss of one allele of *RB* in mice leads to predisposition to cancer ([203–205,221], D. Cobrinik, personal communication). It is possible that p107 and p130 are functionally redundant and that only loss of both genes in the same cell causes growth deregulation. In support of this redundancy argument is the fact that p107 and p130 share the ability to interact with E2F-4 and cyclin A or E-cdk complexes.

Several lines of evidence indicate that pRb is more important in growth inhibition than p107 and p130. For instance, p16, which prevents phosphorylation of all three members of the pRb family, requires only the presence of a functional pRb to induce a G1 arrest [77,102–104]. Furthermore, cyclin D-associated kinase activity is only required for cell cycle progression in pRb positive cells. At first glance, this may seem surprising, as the downstream targets of p107 and p130 (E2F-4 and c-Myc) have strong growth-promoting activity [147,219]. That p107 and p130, in addition to pRb, contribute to cell cycle control is substantiated by several observations. First, all three pocket proteins are phosphorylated and inactivated at mid G1 by D-type cyclin-associated kinase activity. Second, the transforming proteins of several DNA tumor viruses bind to and inactivate all three members of the pRb family. This may indicate that for the efficient induction of S phase, all three family members have to be inactivated. Consistent with this view, efficient induction of a cell cycle block by X-ray irradiation requires the presence of all three pRb family members [222]. The loss of pocket function is tolerated by most cell types with respect to their capacity to complete cell division. It is therefore unlikely that pocket proteins are an integral component of the cell cycle clock. Rather, it would seem more likely that the primary role of pRb family proteins is in the regulation of differentiation. In agreement with this, the functional inactivation of both RB alleles in mice leads to embryonic lethality between day 13 and 15 of gestation as a result of defects in erythropoiesis and neural development [204,205]. On the other hand, many important differentiation and proliferation decisions can be taken in the developing embryo in the absence of pRb. It is well possible that in these $pRb^{-/-}$ embryo's p107 and p130 can compensate for loss of pRb. As was discussed above, in differentiating myoblasts, p107 can indeed partially replace pRb as a cofactor in differentiation. Conversely, loss of p107 or p130 may be compensated by pRb. That this is indeed the case is supported by the recent finding that mice that carry only one functional *RB* allele and two inactivated *p107* alleles have reduced body weight and viability, whereas both $pRb^{-/+}$ and $p107^{-/-}$ mice show no major developmental abnormalities (M-H Lee, personal communication).

10. Concluding remarks

All of our knowledge concerning the mechanism of action of the retinoblastoma family of growth-inhibitory proteins stems from the last decade [223]. Although many, if not most, of the basics of pRb action are now understood in some detail, several issues remain to be clarified. In particular, the recent finding that the fruit fly carries only one E2F-like gene and one pRb-like gene, whereas the mammalian genome carry at least 5 E2F genes and en-

codes three retinoblastoma family members is intriguing. It most likely indicates that for proper differentiation and growth control of the multitude of specialized cell types in the mammalian body, the network of growth stimulatory and growth-inhibitory proteins had to be expanded. Indeed, the initial studies using mice that carry targeted disruptions of these genes seems to support the notion that the three pRb family members differ subtly in their role in differentiation and proliferation. At the same time this would suggest that the five E2Fs also differ with respect to their ability to control proliferation and differentiation. It will probably be a lot less than ten years before we get the answers to these questions.

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