Collective Inhibition of pRB Family Proteins by Phosphorylation in Cells with p16INK4a Loss or Cyclin E Overexpression*

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The activity of the retinoblastoma protein pRB is regulated by phosphorylation that is mediated by G1 cyclin-associated cyclin-dependent kinases (CDKs). Since the pRB-related pocket proteins p107 and p130 share general structures and biological functions with pRB, their activity is also considered to be regulated by phosphorylation. In this work, we generated phosphorylation-resistant p107 and p130 molecules by replacing potential cyclin-CDK phosphorylation sites with non-phosphorylatable alanine residues. These phosphorylation-resistant mutants retained the ability to bind E2F and cyclin. Upon introduction into p16INK4a-deficient U2-OS osteosarcoma cells, in which cyclin D-CDK4/6 is dysregulated, the phosphorylation-resistant mutants, but not wild-type p107 or p130, were capable of inhibiting cell proliferation. Furthermore, when ectopically expressed in pRB-deficient SAOS-2 osteosarcoma cells, the wild-type as well as the phosphorylation-resistant pRB family proteins were capable of inducing large flat cells. The flat cell-inducing activity of the wild-type proteins, but not that of the phosphorylation-resistant mutants, was abolished by coexpressing cyclin E. Our results indicate that the elevated cyclin D- or cyclin E-associated kinase leads to systemic inactivation of the pRB family proteins and suggest that dysregulation of the pRB kinase provokes an aberrant cell cycle in a broader range of cell types than those induced by genetic inactivation of the RB gene.

The retinoblastoma tumor suppressor protein pRB is a nuclear phosphoprotein that is ubiquitously expressed in somatic cells. It inhibits cell proliferation when ectopically expressed and is thought to play an important role in the growth decision-making in the late G1 phase of the cell cycle (1, 2). pRB is considered to inhibit cell proliferation by physically interacting with cellular proteins, most notably with the E2F family of transcription factors (3–5). Upon complex formation with E2F, pRB inhibits transcriptional activation of those E2F-dependent genes whose products are essentially required for G1-to-S phase cell cycle transition. Furthermore, the pRB-E2F complex is capable of acting as a repressor against promoters containing E2F-binding sites, thereby actively repressing transcription in an E2F-dependent manner (4–6). Sequential phosphorylation of pRB during G1 by cyclin D-associated cyclin-dependent kinase (CDK)3/4/6 and cyclin E-CDK2 abolishes the ability of pRB to form physical complexes with cellular proteins, including E2F, and leads to G1-to-S phase cell cycle progression and subsequent cell division.

The p16INK4a-pRB pathway is perturbed in most, if not all, cancer cells (7–12). The changes include mutational inactivation of p16INK4a, cyclin D overexpression, and production of cyclin D mutants that cannot interact with p16INK4a. All of these changes lead to biochemical inactivation of pRB through phosphorylation, indicating that pRB plays a central role in preventing cellular transformation. On the other hand, genetic inactivation of a single copy of the RB gene predisposes only a limited set of malignancies such as retinoblastoma and osteosarcoma. This raises an intriguing possibility that dysregulation of the p16INK4a-pRB pathway gives rise to transformation in a broader range of cell types than those induced by RB gene inactivation.

There are two proteins, p107 and p130, that share the so-called “pocket domain” with pRB (13–16). They are also capable of inhibiting cell growth upon ectopic expression (17–19). Among the members of the pRB pocket protein family, p107 and p130 are more homologous to each other than to pRB and share unique properties allowing interaction with cyclins and CDKs (14, 15, 20–22). Through the interaction, p107 or p130 is capable of inhibiting the kinase activity of cyclin-CDK (23, 24). In addition, p107 and p130 selectively bind E2F-4 and E2F-5, whereas pRB preferentially binds E2F-1, E2F-2, and E2F-3 (25–30). Hence, each member of the pRB family is likely to perform shared as well as unique cell cycle regulatory roles in a single cell. Furthermore, we (33, 36) and others (30–32, 34, 35) have recently shown that a different member of the pRB pocket protein family may become a key regulator of the cell cycle in different cell types.

p107 and p130 are phosphorylated in a cell cycle-dependent manner. Like pRB, p107 can be a substrate for cyclin D-CDK4, and p107-induced cell cycle arrest was reportedly released by cyclin D-CDK4, but not by cyclin E-CDK2 (19, 37, 38). In the case of p130, however, both D-type cyclins and cyclin E are capable of reversing the growth suppression mediated by p130. These observations raise the idea that the function of the entire

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1 The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin.

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pRB family of proteins is collectively regulated by phosphorylation, most likely through G1 cyclin-CDK. Although this idea has long been suspected, there is little published information as to the functional regulation of p107 and p130 by phosphorylation.

In this work, we generated p107 and p130 mutants that are resistant to cyclin-CDK-mediated phosphorylation. By expressing these phosphorylation-resistant molecules, we demonstrate that the growth-inhibitory activity as well as the cell differentiation/senesence-promoting activity of pRB, p107, and p130 are inactivated by phosphorylation. Our results indicate that elevation of the levels of pRB kinases through either p16INK4a loss or cyclin E overexpression abolishes the total activities of the pRB family proteins and, as a result, predisposes a broad spectrum of cells to a dysregulated cell cycle.

**EXPERIMENTAL PROCEDURES**

**Cells**—The human osteosarcoma line SAOS-2 was provided by Dr. Phil Hinds (Harvard Medical School, Boston). The human osteosarcoma line U2-OS was obtained from American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium with 10% (for U2-OS) or 15% (for SAOS-2) fetal bovine serum.

**Construction of Plasmids**—The phosphorylation-resistant pRB mutant, pRBAS/T-P, was described previously (33, 39). Other mutant constructs were generated by multiple rounds of oligonucleotide-mediated mutagenesis with the use of the Chameleon site-directed mutagenesis system (Stratagene) according to the manufacturer’s instructions. cDNAs encoding p107N385, p107DE, and p107L18 were gifts from Dr. Liang Zhu (Albert Einstein College of Medicine, New York) (40). The p130ΔS46–1119 and p130ΔL62–697 constructs were gifts of Dr. Peter Whyte (McMaster University, Ontario, Canada) (23). Some of the constructs were tagged with a hemagglutinin (HA) epitope at either the amino or carboxyl terminus. cDNAs encoding the wild-type and mutant pRB family proteins were inserted into mammalian expression vector pSP65-CMV (41). The pRc/CMV vector was used to express human cyclin E cDNA, and the pSV vector for adenovirus E1A (12 S and E1A-928 mutant).

**Immunoprecipitation and Immunoblotting**—Either 20 μg of the indicated pocket protein expression plasmids or 10 μg of the pocket protein expression plasmids and 10 μg of E1A (either 12 S or E1A-928 mutant) expression plasmids were transfected into 1 × 10⁵ U2-OS cells in a 100-mm plate by the calcium phosphate precipitation method as described previously (42). After 2 days of culture in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, the transfected cells were harvested and lysed in E1A lysis buffer (42). Cell lysates were first treated with anti-HA monoclonal antibody (12CA5) for 1 h, and the immune complexes were collected on protein A-Sepharose beads. After washing the beads, immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride filters, and immunooblotted with appropriate antibodies. Proteins were visualized using the enhanced chemiluminescence detection system (ECL, PerkinElmer Life Sciences). The antibodies used were anti-cyclin A (H-432, sc-75, Santa Cruz Biotechnology), anti-E2F-4 (C-108, sc-512, Santa Cruz Biotechnology), and anti-adenovirus E1A (14G9A, Pharmingen).

**Coloncy Formation Assay**—20 μg of the expression plasmid was transfected into U2-OS cells together with 2 μg of the puromycin resistance gene (pBabe-puro) (43) by the calcium phosphate precipitation method. At 16 h after the transfection, the cells were split into four 100-mm plates. The transfected cells were then selected in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in the presence of 2 μg/ml puromycin for 2 weeks. During the selection, the medium was changed twice a week. After the drug selection, the cells were stained by crystal violet. For cyclin E coexpression, 10 μg of the expression plasmid (pCMV-CD20) (40) was transfected together with 20 μg of pCMV or pCMV-cyclin E.

**Flow Cytometric Analysis**—U2-OS cells were transfected with 20 μg of the pRB family expression plasmid together with 2 μg of the CD20 expression plasmid (pCMV-CD20) (40) as a marker. 40 h after the transfection, cells were harvested and treated with an anti-CD20 antibody (fluorescein isothiocyanate-labeled CD20, Becton Dickinson) for 30 min. Cells were then washed with phosphate-buffered saline and fixed in 70% ethanol on ice. The cells were washed again and resuspended in 100 μl of phosphate-buffered saline containing 25 μg/ml RNase A for 20 min. Prior to flow cytometry, 700 μl of propidium iodide solution (100 μg/ml propidium iodide and 0.1% sodium citrate) was added to the cell suspension, and cells were incubated for another 15 min on ice. The intensity of propidium iodide staining was measured by a FACSCalibur (Becton Dickinson) on cell populations that were positive for CD20 expression to determine the DNA content. Cell cycle profiles of the CD20-positive cells were analyzed using CELL Quest and ModFit cell cycle analysis software (Becton Dickinson).

**RESULTS**

**Generation of Phosphorylation-resistant p107 and p130**—To address the role of cyclin-associated CDK-dependent phosphorylation in the function of p107 and p130, we generated phosphorylation-resistant mutants as described previously in a study on the function of pRB (33). Human p107 possesses 18 Ser-Pro or Thr-Pro ((Ser/Thr)-Pro) motifs that are potential phosphorylation targets for cyclin-CDK. The serine and threonine residues making up all of the (Ser/Thr)-Pro motifs were replaced by alanine to generate p107ΔS/T-P. Similarly, all of the 27 (Ser/Thr)-Pro motifs in human p130 were mutated to generate p130ΔS/T-P. In the case of p130, p130ΔCDK was also made by mutating 12 serine/threonine residues constituting the canonical CDK consensus motif (S/T/P/X/L/R) to alanine residues. All of the pocket protein mutants used in this work are summarized in Fig. 1.

**Interaction of the Phosphorylation-resistant pRB Family Proteins with E2F and Cyclin**—Since the phosphorylation-resistant mutants possess multiple point mutations, we first investigated whether or not these proteins retain sufficient structural integrity to interact with cyclins and E2F proteins in the manner of their respective wild-type proteins. To do so, cDNA encoding the wild-type or the phosphorylation-resistant mutant protein was transfected into U2-OS cells. As demonstrated in Fig. 2 (A, upper panel, lanes 2, 4, and 6; and C, upper panel, lanes 2, 4, 6, 10, 12, and 14), the wild-type pocket proteins were detected as broad bands, indicating that they were variably phosphorylated. In contrast, the phosphorylation-resistant mutants were all detected as fast migrating bands (Fig. 2, A, upper panel, lanes 3, 5, 7, and 8; and C, upper panel, lanes 3, 5, 7, 8, 11, 13, 15, and 16), indicating that they were resistant to phosphorylation in cells.

In sequential immunoprecipitation-immunoblotting analyses, endogenous E2F-4 proteins were co-immunoprecipitated with all wild-type and mutant pocket proteins, with the exception of p130ΔS/T-P (Fig. 2, A, middle panel; and B). In each case, E2F-4 molecules coprecipitated with the mutant pocket proteins were detected in amounts similar to those coprecipitated with their respective wild-type counterparts, although the expression levels of the mutant proteins were significantly less than those of the wild-type proteins. This indicates at least that the affinities of the mutant pocket proteins for binding E2F-4 are not reduced despite introduction of multiple mutations. Notably, the E2F-4 binding affinity of pRBα/pRBβS/T-P was significantly less than that of p107/p107ΔS/T-P (Fig. 2, A and B) as reported previously (18, 44). Furthermore, wild-type p107, wild-type p130, p107ΔS/T-P, and p130ΔCDK, but not p130ΔS/T-P, physically interacted with endogenous cyclin A (Fig. 2A, lower panel). Again, in each case, coprecipitated cyclin A levels were found to be similar to the levels coprecipitated with their respective wild-type counterparts. In contrast, wild-type pRB and pRBAS/T-P did not associate with cyclin A.

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because they do not possess cyclin A-binding spacer sequences. These observations indicate that p107ΔS/T-P, p130ΔCDK, and pRBΔS/T-P are capable of interacting with cellular targets with affinities comparable to those of their respective wild-type molecules.

Conformation of the phosphorylation-resistant mutant as the “pocket protein” was further examined with the use of the adenovirus E1A 12 S product, which specifically binds the pocket domains and inactivates the pRB family proteins (45–47). Upon transient coexpression in U2-OS cells, the mutant pRB family molecules, except p130ΔS/T-P, formed stable complexes with E1A to levels comparable to those associated with their respective wild-type counterparts (Fig. 2C). Moreover, an E1A point mutant, E1A-928, which does not bind to wild-type pRB, did not bind pRBΔS/T-P as well (Fig. 2C, middle panel, lanes 10 and 11). The E1A mutant is also known to bind p107 and p130 less effectively. Consistently, p107ΔS/T-P and p130ΔCDK exhibited reduced affinities for E1A-928, as is the case with wild-type p107 and p130 (Fig. 2C, middle panel, compare lanes 4–7 with lanes 12–15) (48–50). These observations provide further evidence that the structural conformations of the phosphorylation-resistant pocket protein mutants pRBΔS/T-P, p107ΔS/T-P, and p130ΔCDK are indistinguishable from those of their respective wild-type molecules, pointing to the notion that these mutants function through normal biological pathways that involve their wild-type counterparts when expressed in cells. On the other hand, p130ΔS/T-P, which has 27 point mutations, lost its structural integrity as a pocket protein because of the introduced mutations.

**G1 Cell Cycle Arrest of p16INK4a-defective U2-OS Cells by the Phosphorylation-resistant pRB Family Proteins**—It has been shown that U2-OS cells are highly resistant to pRB overexpression (19). Since the U2-OS cells do not express functional p16INK4a (51), a specific inhibitor of cyclin D-CDK4/6, the cells exhibit dysregulated pRB kinase activity (52). It is therefore considered that, in U2-OS cells, the dysregulated pRB kinase instantly inactivates ectopically expressed wild-type pRB proteins. If this is the case, then the growth of U2-OS cells must be inhibited by phosphorylation-resistant pRB. To address this, a cDNA expression vector for wild-type pRB or the phosphorylation-resistant pRB mutant, pRBΔS/T-P, was cotransfected with the CD20 expression vector, and the cell cycle distribution of the CD20-positive cells at 40 h after the transfection was examined with the use of a flow cytometer. As previously reported (19), ectopic expression of pRB had very little effect on the cell cycle distribution profile of U2-OS cells (Fig. 3). On the other hand, pRBΔS/T-P caused severe G1 cell cycle arrest in U2-OS cells (Fig. 3). This indicates that, in these cells, the growth-suppressive activity of pRB is neutralized by phosphorylation, most likely through the dysregulated cyclin D-CDK kinase because of the lack of p16INK4a.

The U2-OS cells allowed us to examine whether the growth-suppressive activity of p107 or p130 is also under the control of cyclin-CDK-mediated phosphorylation. To this end, we transiently introduced wild-type p107 or p107ΔS/T-P together with CD20 into U2-OS cells (Fig. 3). Whereas the expression of wild-type p107 did not affect the cell cycle distribution significantly, phosphorylation-resistant p107 provoked strong accumulation of cells in G1 phase, indicating that the mutant molecules caused G1 cell cycle arrest in U2-OS cells. This indicates

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**FIG. 1.** Schematic drawing of pRB family proteins and their mutants used in this work. HA represents the hemagglutinin epitope.
that p107 is capable of inhibiting cell cycle progression and that the activity is under phosphorylation control in U2-OS cells (Fig. 3).

The wild-type and two forms of phosphorylation-resistant p130 molecules, p130\(D_{S/T-P}\) and p130\(D_{CDK}\), were next examined for their growth regulatory activity in U2-OS cells. In contrast to pRB and p107, p130, even in its phosphorylation-resistant forms, did not significantly affect the cell cycle profile of U2-OS cells in this transient expression assay (Fig. 3). Given the observation that p130\(D_{S/T-P}\) cannot bind E2F or cyclin A (Fig. 2), this finding is consistent with the conclusion that the mutant is functionally inactive. In contrast, since p130\(D_{CDK}\) can still bind with the target molecules, the growth-inhibitory activity of p130, if it exists, may be significantly weaker than that of pRB or p107 in U2-OS cells.

**Growth Suppression of U2-OS Cells by the Phosphorylation-resistant pRB Family Proteins**—We next investigated the long-term effects of the pRB family proteins on the growth of U2-OS cells. To do so, we transfected cDNA expression vectors for the pRB family proteins together with the puromycin resistance gene. After selection of the transfected cells with puromycin, the number of puromycin-resistant colonies was counted. As shown in Fig. 4, expression of wild-type pocket proteins in U2-OS cells resulted in a weak reduction of puromycin-resistant colonies, indicating that these proteins are growth-inhibitory.

As expected from the transient assay, the phosphorylation-resistant pRB and p107 molecules reduced puromycin-resistant colonies quite strongly (Fig. 4). Furthermore, in this colony formation assay, the phosphorylation-resistant p130 mutant p130\(ΔCDK\) suppressed U2-OS colony formation more effectively than wild-type p130. This indicates that p130 is also capable of inhibiting cell proliferation and that this growth-inhibitory activity is under phosphorylation control. In contrast, p130\(D_{S/T-P}\), which lacks all 27 (Ser/Thr)-Pro motifs, was not growth-suppressive in the colony assay (Fig. 4). Accordingly, as suggested from its inability to bind E2F-4 or cyclin A, the introduction of mutations into one or several (Ser/Thr)-Pro sites present in p130\(ΔCDK\) but absent in p130\(D_{S/T-P}\) appears to provoke structural inactivation of p130. Alternatively, since p130 receives multiple phosphorylation and its function is reportedly modified depending on phosphorylation status (53), a certain degree of basal phosphorylation might be required for the activation of p130 as a growth suppressor. If this is the case, then p130\(ΔS/T-P\) may be functionally inactive because it cannot receive activating phosphorylation by the proline-directed kinases that target the (Ser/Thr)-Pro motifs. In any case, the failure of p130\(D_{S/T-P}\) to inhibit cell growth argues against the idea that cell cycle-inhibitory effects of the phosphorylation-resistant mutants are due to the overexpression and accumulation of functionally inactive pocket proteins.

![Image of E2F- and cyclin-binding abilities of the phosphorylation-resistant pRB family proteins.](http://www.jbc.org/)

**Fig. 2.** E2F- and cyclin-binding abilities of the phosphorylation-resistant pRB family proteins. A, extracts were prepared from U2-OS cells transfected with the indicated constructs, and lysates were immunoprecipitated (IP) with anti-HA monoclonal antibody (12CA5). The anti-HA immunoprecipitates were then subjected to 7% SDS-polyacrylamide gel electrophoresis, followed by anti-HA immunoblotting (upper panel). The same filter was also immunoblotted with anti-E2F-4 (middle panel) or anti-cyclin A (α-CycA; lower panel) antibody. B, shown is a longer exposure of the anti-E2F-4 immunoblotting (lanes 1–3 of the middle panel in A). C, U2-OS cells were cotransfected with indicated constructs and adenovirus E1A (12 S or E1A-928 mutant) expression plasmids, and then the lysates were subjected to immunoprecipitation (anti-HA antibody) and immunoblotting (anti-HA antibody (upper panel) and anti-E1A antibody (middle panel)). The whole cell lysates were also immunoblotted with anti-E1A antibody (lower panel). It should be noted that pRB\(ΔS/T-P\), which is derived from mouse \(RB\) cDNA, migrated a bit faster than the hypophosphorylated form of the wild-type pRB protein, which is derived from human \(RB\) cDNA, \(SRa\), empty vector as a negative control.
The pRB-induced flat cells express bone differentiation markers as well as cell senescence markers such as β-galactosidase (57). Hence, pRB not only inhibits the cell cycle, but also promotes differentiation and senescence in SAOS-2 cells. Despite several trials, however, p107 and p130 failed to effectively induce flat cells in SAOS-2 cells (19, 57). Since our cDNA expression system employs the powerful SRα promoter, we wondered whether higher levels of p107 or p130 in SAOS-2 cells might induce flat cells. We therefore introduced the p107 or p130 expression vector into SAOS-2 cells together with the puromycin resistance gene. After 2 weeks of puromycin selection, cells were stained, and flat cell formation was examined. As demonstrated in Fig. 5, p107 caused a strong flat cell formation comparable to that by pRB. p107ΔS/T-P also effectively induced flat cells. Furthermore, wild-type p130 and p130ΔCDK, but not p130ΔS/T-P, were capable of inducing flat cells, although the efficiency was significantly lower than that of the induction by p107 or pRB. It should also be noted that the flat cells induced by p130 were more rounded than those induced by pRB or p107.

We next employed several deletion mutants of p107 (see Fig. 1) to delineate the regions responsible for the flat cell formation. The p107N385 mutant, which lacks the amino-terminal one-third of p107, was capable of inducing flat cells. In contrast, deletion of the central one-third (p107DE) or truncation of the C-terminal region (p107L19) resulted in a total loss of the ability to induce flat cells (Fig. 5). This indicates that, as in pRB, the pocket function is involved in the flat cell induction by p107. The conclusion was further supported by a series of p130 deletion mutants (see also Fig. 1). In these mutants, deletion of
the spacer region (p130Δ620–697) did not affect the flat cell-inducing activity, whereas deletion of the B box and the C terminus (p130Δ846–1119), which specifically inactivates the p130 pocket function, resulted in the loss of flat cell-inducing activity (Fig. 5).

By employing the flat cell assay, we were also able to determine the (Ser/Thr)-Pro motifs whose mutations lead to functional inactivation of p130. To this end, we generated two additional mutants, p130ΔCDK2 and p130ΔCDK3 (see Fig. 1). Like p130ΔCDK, p130ΔCDK2 was capable of inducing flat cells when expressed in SAOS-2 cells, whereas p130ΔCDK3 failed to do so (Fig. 5). Taken together with the finding that p130Δ620–697 (which lacks Ser-639, Ser-642, Ser-662, Ser-672, Ser-688, and Ser-694) is still active in inducing flat cells, this indicates that mutations within the (Ser/Thr)-Pro clusters adjacent to the amino terminus of the A box of p130 may be involved in the functional inactivation of p130 and suggests the importance of this region in the structural integrity of p130.

**Effect of Cyclin E on p107/p130-mediated Flat Cell Formation**—The ability of p107 and p130 to induce SAOS-2 flat cells gave us another opportunity to examine whether or not the function of these pRB homologs is regulated by cyclin-CDK-mediated phosphorylation. In the SAOS-2 flat cell assay with pRB, coexpression of cyclin E together with pRB has been shown to promote pRB hyperphosphorylation by cyclin E-CDK and, as a result, to abolish the ability of pRB to induce flat cells (42). With this observation in mind, we wondered whether the flat cell-inducing activity of p107 or p130 might also be controlled by the cyclin E-associated kinase. We thus cotransfected the p107 or p130 expression vector together with the cyclin E expression vector into SAOS-2 cells and examined p107- or p130-mediated flat cell induction in the presence or absence of ectopic cyclin E (Fig. 6). In the case of wild-type p107 or p130, the number of flat cells generated was severely reduced by coexpression with cyclin E. In striking contrast, both p107ΔS/T-P and p130ΔCDK induced flat cells irrespective of cyclin E coexpression. We also examined the role of D-type cyclins in the flat cell formation by p107 or p130 in SAOS-2 cells. However, in contrast to cyclin E, ectopic expression of cyclin D was extremely toxic to SAOS-2 cells, and we were not able to successfully coexpress cyclin D (D1–D3) together with the pocket proteins under our experimental conditions. We concluded from the observation that the flat cell-inducing activity of both p107 and p130 was again under the control of phosphorylation and that cyclin E-associated kinase is capable of inactivating p107 and p130 activities through phosphorylation.

**DISCUSSION**

In this work, we provide evidence that the activities of the entire pRB family of proteins are collectively regulated by phosphorylation through cyclin D- or cyclin E-associated CDK with the use of the phosphorylation-resistant pRB family proteins. Cells lacking p16INK4a are highly resistant to ectopic overexpression of wild-type pRB during their growth stage (19). Since p16INK4a acts as a specific inhibitor of cyclin D-CDK4/6, dysregulated cyclin D-CDK4/6 is suspected to phosphorylate and inactivate pRB in cells with p16INK4a loss (52, 58). This idea is confirmed by our current work, in which phosphorylation-resistant pRB inhibited G1-to-S phase cell cycle progression of the p16INK4a-defective U2-OS cells (51), whereas wild-type pRB failed to do so. A similar result has been recently reported with the expression of a distinct phosphorylation-resistant pRB family protein, p107ΔS/T-P.
resistant pRB mutant in which 10 out of 16 potential CDK phosphorylation sites have been replaced by alanine residues. As is the case of our transient expression experiment, U2-OS cells in which expression of phosphorylation-resistant pRB was induced arrested in G₁ within 48 h. Intriguingly, however, the G₁-arrested cells gradually entered into S phase after 48 h and underwent endoreplication between 4 and 6 days (59).

We demonstrate in this work that phosphorylation-resistant p107 and p130 are also capable of inhibiting U2-OS cell growth. Again in these cases, the wild-type molecules had much weaker effects on the growth of U2-OS cells. This indicates that the U2-OS cell cycle is principally sensitive not only to pRB, but also to p107 and p130. However, like that of pRB, the growth-inhibitory activities of wild-type p107 and p130 are neutralized via phosphorylation in U2-OS cells. This in turn suggests that, to initiate cell cycle progression, the cells need to inactivate all of the pRB family pocket proteins that are functioning as cell cycle brakes.

Since cyclin D-associated kinase is capable of phosphorylating p107 (30, 37, 38), it is logical to speculate that cyclin D-CDK4/6, which is deregulated in p16INK4a-defective U2-OS cells, is involved in the inactivation of p107 and p130. This conclusion is consistent with the previous observation that cell growth inhibition by p107 or p130 is ameliorated by ectopic coexpression of cyclin D (19, 37, 38, 60).

We further demonstrate in this work that p107 and p130 share with pRB the biological activity of inducing flat SAOS-2 cells. Previous reports have demonstrated that these pRB homologs, particularly p130, shows little induction of flat cells in the SAOS-2 cell assay (19, 57). We suspect that the difference is due to the protein expression levels. In our system, the cDNA-directed proteins were expressed with the use of the SRα promoter (41), which is significantly stronger than the cytomegalovirus promoter used in the previous works. Since the flat cells exhibit differentiation phenotypes (57), all of the pRB family proteins appear to be capable of promoting differentiation programs via the shared pocket functions.

One intriguing result of this study is that the flat cell-inducing activity of wild-type p107, p130, and pRB is abolished by cyclin E coexpression. In contrast, cyclin E had little effect on the flat cell induction by phosphorylation-resistant pRB, p107, or p130. Although cyclin E is considered to be a critical downstream effector of “pRB family-E2F interplay” for entry into the S phase (61, 62), our observation clearly points to the notion that cyclin E acts as an upstream regulator of the pRB family proteins and that elevated cyclin E-CDK2 inactivates the flat cell-inducing activity of pRB, p107, or p130 through phosphorylation. This study thus demonstrates that the two established pRB kinases, cyclin D-CDK4/6 and cyclin E-CDK2, are capable of phosphorylating and functionally inactivating the pRB homologs p107 and p130 as well. In other words, the total activity of the pRB family proteins is under the control of the pRB kinases.

Although we do not know the exact molecular mechanisms underlying differentiation by the pRB family, pocket-binding molecules other than E2F proteins may be involved in this process since pRB mutants that cannot interact with E2F still retain the ability to induce flat cells (57). The idea is indeed supported by our current observation that cyclin E cannot abolish flat cell formation by the phosphorylation-resistant pRB family molecules because the cyclin E gene is a critical downstream target of E2F (63, 64). Since pRB could induce flat cells at a lower level, but p107/p130 only at higher levels, the affinity of p107 and p130 for such a differentiation regulator may be significantly weaker than that of pRB. In striking contrast, induction of G₁ arrest by transient expression of pRB requires its ability to bind to E2F (65–67). Accordingly, as has been reported by a number of groups (59, 62, 68, 69), acute G₁ arrest by phosphorylation-resistant pRB may be bypassed by ectopic overexpression of cyclin E, a major downstream effector of E2F.

The cell cycle of U2-OS or SAOS-2 cells is sensitive to any of the three pRB family proteins because it is halted by the wild-type or phosphorylation-resistant mutant proteins. This results in a marked difference in the case of certain hematopoietic cells such as Ba/F3 lymphoid cells and 32D myeloid progenitor cells (33, 36). In the latter cases, the cells are totally resistant to pRB, even in its phosphorylation-resistant form, but are sensitive to p130. This indicates that inactivation of pRB is not an absolute prerequisite for cell cycle progression in all somatic cells. The conclusion was further supported by a recent work with another cell line, C33A (35). Furthermore, p130 inhibits proliferation of the glioblastoma cell line T98G, which is resistant to the growth-suppressive effects of p107 and pRB, in colony formation assay (17). This differential sensitivity of cells to each of the pRB family proteins appears to reflect, at least in part, differential expression of pRB-regulated E2F proteins (E2F-1, E2F-2, and E2F-3) and p107/p130-regulated E2F expression.
E2F proteins (E2F-4 and E2F-5) (33, 36). In cells wherein pRB-regulated E2F proteins act as central transcriptional regulators of the cell cycle, loss of pRB directly leads to dysregulation of the cell cycle. Conversely, in cells in which p107/p130-regulated E2F proteins play a major role in the cell cycle control, pRB inactivation per se has nothing to do with cell growth. On the other hand, elevation of cyclin D-CDK and/or cyclin E-CDK activities gives rise to the functional inactivation of the entire pRB protein family, promoting cell cycle progression irrespective of the cellular sensitivity to the pRB family proteins.

Our results point to a substantial difference between dysregulation of pRB kinase and genetic inactivation of the RB gene in the context of cell type-specific transformation and indicate that dysregulated pRB kinase provokes an aberrant cell cycle in a broader range of cell types than those induced by a simple loss of pRB. Obviously, there are cells whose cell cycle is strongly dependent on pRB. In such cells, p16INK4a is ineffective in suppressing proliferation in the absence of pRB (70–72). In many cell types, however, growth decision-making may involve various combinations of the pRB family proteins. Furthermore, the relative contribution of each pRB family member to the decision-making may also vary among different cell types. Hence, in those cells, inactivation of the whole pRB protein family may be an obligatory prerequisite in proceeding to the cell cycle. This in turn indicates that, during multistep carcinogenesis, such a situation could be most easily provided and fixed by genetically modifying components that constitute the p16ink4a-pRB pathway or by cyclin E overexpression, as has been frequently shown in breast cancer (73). Finally, given the importance of the functional loss of the pRB family proteins in cellular transformation, the phosphorylation-resistant mutants generated in this work will become powerful therapeutic tools in gene therapies against a broad range of human cancers.

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