Regions of the Retinoblastoma Gene Product Required for Its Interaction with the E2F Transcription Factor Are Necessary for E2 Promoter Repression and pRb-Mediated Growth Suppression

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Studies of naturally occurring mutations of the *RB1* tumor suppressor gene have indicated that the E1A/T antigen-binding domain is important for pRb function. Mutations engineered within the C-terminal 135 amino acids of pRb also abrogate its growth-suppressive function during the G₁ interval of the cell cycle. Both the pRb E1A/T antigen-binding domain and the C-terminal domain are required for interaction with the E2F transcription factor. A series of mutated pRb proteins has been used to define the C-terminal sequences which determine E2F binding, adenovirus E2 promoter inhibition, and negative growth control. Deletion of the C terminus to residue 870 allowed full pRb function, while further deletion to residue 841 inactivated pRb in each assay. Amino acid sequences immediately C-terminal to the E1A/T antigen-binding domain were absolutely required for pRb activity. Mutations which prevented pRb from interacting with E2F also eliminated pRb-mediated E2 promoter repression and inactivated the ability of pRb to suppress cell growth.

Naturally occurring mutations in the retinoblastoma susceptibility gene (*RB1*) correlate with the loss of growth control in a number of malignancies, suggesting that the *RB1* gene product (pRb) operates as a tumor suppressor (reviewed in reference 51). The identification of pRb as a target of the oncoproteins of several DNA tumor viruses, including adenovirus E1A (53), simian virus 40 T antigen (35), and human papillomavirus E7 (16), further emphasizes the importance of pRb in negative growth control. Simian virus 40 T antigen binds selectively to the underphosphorylated forms of pRb, which are present predominantly during the G₁ phase of the cell cycle (35). Phosphorylation of pRb in late G₁ is presumed to be required for cells to enter the S phase, and hyperphosphorylated forms of pRb, manifested in the S phase, persist until cells exit mitosis (6, 10, 13). The hypophosphorylated forms of pRb, but not the fully phosphorylated forms, interact with the E2F transcription factor (2, 8, 11, 25). The cell cycle timing of the formation of the E2F-pRb complex correlates inversely with pRb phosphorylation, so that E2F-pRb complexes are present during G₁ and persist into the S phase (7, 14, 48).

One potential mechanism for how pRb might be involved in negative growth control and how E1A or T antigen might ablate this suppressive function is suggested by the targeting of E2F by both pRb and E1A. E2F is a cellular transcription factor which can bind sites within the promoters of several proliferation-related genes, such as *c-myc*, *N-myc*, the dihydrofolate reductase gene (*DHFR*), the thymidine kinase gene, *cdc2*, and *RB1* itself (5, 12, 15, 22, 24, 27, 38, 50). The interaction of pRb with E2F leads to repression of transcription from promoters containing E2F-binding sites, including the adenovirus E2 gene (25, 52), *cdc2* (12), *c-myc*, and *RB1* (22). E1A disrupts this regulation by interacting with pRb, releasing E2F in an active form that presumably leads to dysregulation of these genes (1, 3, 8, 9, 25, 40, 43). This proposed mechanism is supported by mutational analysis of both pRb and E1A. *RB1* is inactivated by mutations which

affect the domains required both for binding E1A and for interacting with E2F (25, 30). Similarly, analysis of the domains of E1A required to dissociate the E2F-pRb complex suggests extensive overlap with the domains of E1A required both for pRb binding and for E1A-mediated transformation (conserved regions 1 and 2 [43]).

Although naturally occurring mutations of the pRb E1A/T antigen-binding domain indicate that these sequences are required for growth suppression, the C-terminal 135 amino acids of pRb have also been demonstrated to be essential for both E2F binding (25, 41) and inhibition of cell growth (41). Here, I show that the C-terminal pRb sequences required for its interaction with E2F are required for repression of promoters containing E2F-binding sites and for pRb-induced growth suppression.

MATERIALS AND METHODS

Cells and preparation of extracts. C33A cervical carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Saos-2 osteosarcoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. HeLa cell cultures were maintained in Joklik modified minimal essential medium supplemented with 5% bovine calf serum. Extraction of HeLa cells (54), heparin-agarose chromatography (54), and microextraction of C33A cells for reconstitution analysis (46) were performed as described previously.

Reconstitution of the E2F-Rb complex. The construction of the C-terminal pRb deletions (see Fig. 2) has been described previously (29). The polymerase chain reaction (PCR) was used to amplify the region between amino acids 379 and 928 in order to produce glutathione *S*-transferase (GST)-pRb fusion proteins containing these deletions. The deletions shown in Fig. 3A were created by PCR amplification and subsequent cloning of sequences between amino acids 806 and 928, 825 and 928, and 850 and 928 into the *BsmI* and

*Eco*RI sites of the above C-terminal deletion mutants to yield the full set of mutants shown in Fig. 3A. As an example, sequences between residues 825 and 928 were cloned into pGTRb(*dl*785–909) to yield pGTRb(*dl*785–825). The PCR oligonucleotide primer includes three extra amino acids (as a result of the *Bsm*I site), Arg-Met-Gln; therefore, mutant 803 to 806 results in the substitution of R-803, M-804, and Q-805 for N-803, I-804, and V-805 rather than a simple deletion. Each of the clones was sequenced by the dideoxynucleotide-chain termination method, and no PCR-induced mutations were found.

For reconstitution of the E2F-pRb complex, 100 ng of the glutathione-Sepharose-purified proteins (49) was incubated for 10 min on ice with 5 μ g of C33A whole-cell extract or 0.5 μ g of heparin-agarose-purified HeLa cell extracts in a total volume of 5 μ l. E2F DNA-binding activity was then measured by gel retardation of a 32 P-end-labeled probe from the adenovirus type 5 E2 promoter which lacks the activating transcription factor-binding site (24). DNA-protein complexes were separated on a 4% polyacrylamide gel buffered with 0.25 $\times$ Tris-borate-EDTA.

Transient-transfection assays. Calcium phosphate precipitates of supercoiled plasmid DNA (3.0 μ g of E2-CAT plasmid, 15 μ g of *RB1* or *RB1* mutant plasmids, and 5 μ g of pBC12RSV-SEAP [4] plasmid) were added dropwise to the cell culture medium and incubated for 16 h as described previously (21). Construction of the E2-CAT DNA (34) and the wild-type *RB1* plasmid pJ3 Ω hRb (44) has been described previously. The full-length versions of the C-terminal deletion mutants shown in Fig. 2A (obtained from E. Harlow, MGH Cancer Center) were cloned from pBlueScript into the pJ3 Ω vector by using the *Asp* 718 and *Xba*I restriction sites which are unique to both vectors. The pRb C-terminal internal deletions shown in Fig. 3A were cloned into the pJ3 Ω vector by changing the *Eco*RI site of the pGEX2TRb mutant plasmid to *Xba*I and cloning the unique 0.85-kb *Mlu*I-*Xba*I restriction fragment (encoding residues 641 to 928) into the pJ3 Ω hRb(*dl*785–909) plasmid. Thus the original C-terminal deletion was replaced with sequences containing the new mutation. A plasmid expressing a secreted form of the alkaline phosphatase gene, pBC12RSV-SEAP (4), was used as an internal control for transfection efficiency. The chloramphenicol acetyltransferase (CAT) activity and secreted embryonic alkaline phosphatase (SEAP) activity produced in each transfection were measured as described previously (4, 21). The CAT activity was normalized to the SEAP activities. In general, the SEAP activities did not vary by more than 15%, and if the SEAP activity varied by more than 50% the sample was excluded from the analysis.

Biological assay of pRb function. The colony formation assay was performed as described by Qin et al. (41). To ensure high levels of pRb expression and to link a selectable marker to pRb expression, wild-type pRb and deletion mutants pRb(*dl*785–806), pRb(*dl*803–825), pRb(*dl*841–850), pRb(*dl*870–928), and pRb(*dl*803–806/870–909) were subcloned into the unique *Hind*III and *Xba*I sites of pRC-CMV-Neo (Invitrogen). Calcium phosphate precipitates of 20 μ g of each pRb-expressing plasmid were incubated with 70% confluent Saos-2 cell cultures for 4 to 6 h prior to glycerol shock (15% glycerol in 1 $\times$ N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]-buffered saline for 2 min). At 2 days after transfection, the cells were split 1:4 and selected in medium containing 400 μ g of G418 per ml. Approximately 2 weeks later, the cells were washed with Tris-buffered saline and stained with Giemsa blue. Immunofluorescence was performed with a 1:20 dilution of the anti-Rb monoclo-

FIG. 1. The pRb C terminus is required for E2F inactivation. (A) Schematic diagram of the adenovirus type 5 E2 promoter-CAT construct depicting the DNA-binding motifs within the minimal promoter as described previously (34). The arrow indicates the transcriptional start site. (B) Effect of pRb expression on E2F-dependent transcription. E2F-dependent transcription, as assayed by the level of CAT activity produced in transient transfection of C33A cells, was measured in the absence or presence of the wild-type pRb protein (+Rb), a pRb protein lacking the C-terminal 135 amino acids [+Rb(*dl*792–928)], the wild-type pRb protein plus the E1A 12S product (+Rb + E1A), or the mutant pRb in the presence of 12S E1A [Rb(*dl*792–928) + E1A]. The transfection assays shown in panel B included an internal control plasmid, pBC12RSV-SEAP. Kinetic assays of the amount of alkaline phosphatase secreted into the medium did not vary by more than 15%. Assays were performed in duplicate, with identical results.

nal antibody RbAB1 (Oncogene Science) and fluorescein isothiocyanate-conjugated sheep anti-mouse Fab fragments as described previously (7).

RESULTS

The C terminus of pRb is required for E2F inactivation. The ability of pRb to repress transcription and its ability to negatively regulate cell growth, as measured by the suppression of colony formation, can be segregated on the basis of cell type. Saos-2 osteosarcoma and C33A human cervical carcinoma cell lines are both *RB1*^{–/–} and *p53*^{–/–} (45, 47); however, only Saos-2 cells are blocked in G₁ by enforced expression of pRb (20, 41) (see below and Fig. 4). Therefore, I analyzed the ability of pRb and pRb mutants to repress transcription from the adenovirus E2 promoter in C33A cells. Our previous work suggested that the C-terminal 135 amino acids of pRb was required for interaction with the E2F transcription factor (25). To determine whether this domain was also required for repression of an E2F-dependent promoter, wild-type pRb or the C-terminal pRb deletion mutant Rb(*dl*792–928) was transfected along with an E2F-dependent adenovirus type 5 E2-CAT construct (Fig. 1A) into the C33A cell line. In the absence of pRb expression, appreciable levels of E2 expression were obtained (Fig. 1B). When a plasmid encoding wild-type pRb was included in the transfection, the level of expression was reduced four- to fivefold.

FIG. 2. Mapping the distal boundary of the C-terminal domain of pRb. (A) Schematic diagram of the GST-pRb C-terminal deletion mutants. Residues 379 to 928 were PCR amplified from pRb deletion plasmids described previously (29) and cloned in frame with GST. Shaded areas indicate the A and B subdomains of the E1A/T antigen-binding domain (29, 30). Black boxes indicate deleted sequences. (B and C) Reconstitution of the E2F-Rb complex. Partially purified extracts from HeLa cells which contain human papillomavirus E7 protein and thus lack the E2F-pRb complex (B) or whole-cell extracts of C33A cells which lack functional pRb and also lack the E2F-Rb complex (C) were incubated with no added GST-Rb (lane NO RB) or with the indicated GST-pRb protein prior to gel mobility shift analysis with an E2F-specific fragment from the E2 promoter as the probe. (D) Effect of the pRb C-terminal mutation on E2 promoter repression. Transient-transfection analysis was performed with the E2-CAT construct alone, with wild-type pRb, or with the indicated pRb deletion mutant. The indicated levels of CAT activity have been corrected for variations in transfection efficiency by using an internal control plasmid, pBC12RSV-SEAP, expressing a secreted form of alkaline phosphatase. Data are the average of two independent experiments.

Inclusion of a plasmid encoding the 12S E1A protein reversed this effect, eliminating the ability of pRb to repress E2 expression. The use of the Rb(*dl792-928*) plasmid in the transfection assay had no effect on E2 expression, indicating that the deleted C-terminal domain of pRb plays a role in modulating E2F-mediated transcription.

Definition of the distal boundary of the pRb C-terminal domain. The C-terminal boundary of pRb sequences required for E2F binding was mapped by using a series of internal C-terminal deletions (Fig. 2A) (29). The ability of

each of these mutant pRb proteins to interact with E2F was assayed by in vitro reconstitution of the E2F-Rb DNA-binding complex. The region of pRb including residues 379 to 928 was amplified by PCR, and the products were cloned in frame with GST, as shown in Fig. 2A. Equivalent amounts of the bacterially produced fusion proteins, purified on glutathione-Sepharose, were incubated with either partially purified E2F from HeLa cells (Fig. 2B) or whole-cell extracts from C33A cells (Fig. 2C), and the E2F-containing complexes were analyzed by gel mobility shift. Compared with

the wild-type pGTRb(379-928) protein, the GST-pRb mutants pGTRb(dl893-909) and pGTRb(dl870-909) retained the ability to interact with E2F and formed complexes with E2F from both cell extracts, whereas further deletion of residues between 841 and 870 eliminated sequences required for complex formation.

To correlate the binding of E2F in vitro with the in vivo function of pRb transcriptional repression, the C-terminal deletion mutations were subcloned into a mammalian expression vector for use in transient-transfection analyses. For mammalian expression, full-length, non-NH₂-terminal truncated pRb proteins were used. Expression of wild-type pRb, as well as pRb(dl893-909) and pRb(dl870-909), caused a reduction in transcription from the E2 promoter, whereas pRb(dl841-909), pRb(dl803-909), and pRb(dl785-909), which showed no interaction with E2F in vitro, showed little or no repression in vivo (Fig. 2D). These data show a correlation between the ability of pRb to bind to E2F in vitro and its ability to exert transcriptional effects in vivo.

FIG. 3. Mapping the proximal boundary of the pRb C-terminal domain. (A) Schematic diagram of the GST-pRb internal deletion mutants. Internal deletion mutants were constructed by filling in the deletions depicted in Fig. 2A by PCR amplification as described in detail in Materials and Methods. Shaded areas indicate the A and B subdomains of the E1A/T antigen-binding domain (29, 30). Black boxes indicate deleted sequences. (B) Reconstitution of the E2F-pRb complex. Whole-cell extracts of C33A cells were incubated with no added GST-pRb (lane NO RB) or with the indicated GST-pRb protein prior to gel mobility shift analysis with an E2F-specific fragment from the E2 promoter as the probe. (C) Effect of pRb internal mutations on E2 promoter repression. Transient-transfection analysis was performed with the E2-CAT construct alone, with wild-type pRb, or with the indicated pRb deletion mutant. The indicated levels of CAT activity have been corrected for variations in transfection efficiency by using an internal control plasmid, pBC12RSV-SEAP, expressing a secreted form of alkaline phosphatase. Data are the averages of two independent experiments.

Definition of the proximal boundary of the pRb C-terminal domain. A set of internal deletion mutations beginning with residue 785, 803, or 841 and extending toward the C terminus was created by PCR, yielding the GST-pRb internal deletions shown in Fig. 3A. By using the pRb(dl870-909) mutation as the template for PCR, it was possible to create double mutations containing, for example, deletion of residues 803 to 806 and 870 to 909. This method of construction requires the insertion of linker sequences encoding three amino acids included in the 5' oligonucleotide used for PCR. Thus the pRb(dl803-806) mutation results in a substitution of R-M-Q for N-I-V rather than a deletion of three amino acids. To rule out any effect of residues 909 to 928 (contained in all of the above mutations), a stop codon was inserted after residue 870, creating one further C-terminal deletion of residues 870 to 928.

The bacterially produced GST-pRb proteins were tested by reconstitution of the E2F-pRb complex in vitro. The purified GST-pRb proteins were incubated with extracts of C33A cells and analyzed by gel mobility shift (Fig. 3B). Only the wild type and the extreme C-terminal truncation mutant, pRb(dl870-928), retained the ability to interact with E2F. All other mutations between residues 785 and 870 inhibited

FIG. 4. Colony suppression assay of transfected Saos-2 or C33A cells. Cells were transfected with 20 μ g of the empty pRC-CMV-neo, pRC-CMV-neo-RB1 wild-type, or pRC-CMV-neo-RB1 mutant plasmid and grown in the presence of G418 for 2 to 3 weeks before being stained with Giemsa blue. Panels A to D contain Saos-2 cells; panels E and F contain C33A cells. (A) pRC-CMV-neo vector (no pRb); (B) wild-type pRb; (C) Rb(*dl*785–806); (D) Rb(*dl*803–825); (E) pRC-CMV-neo vector (no Rb); (F) wild-type pRb.

E2F-pRb complex formation. Thus, it appears that even a small disruption of the proximal C-terminal domain such as the substitution of three amino acids at residues 803 to 806 is sufficient to ablate the E2F-Rb interaction.

The ability of these mutant pRb proteins to interact with E2F in vivo was tested by determining the effects of pRb mutant expression on E2F-dependent transcription. For transient expression in mammalian cells, full-length pRb proteins containing representative deletion mutations spanning the entire C-terminal domain were rebuilt. In comparison with wild-type pRb or the C-terminal deletion mutant pRb(*dl*870–928), all other mutant proteins which failed to interact with E2F also failed to repress transcription from the E2 promoter (Fig. 3C). This correlation between E2F binding in vitro and the ability of pRb to repress E2 transcription in vivo suggests that the latter effect is a direct consequence of pRb binding to E2F.

C-terminal sequences necessary for negative growth control. The C-terminal sequences from residues 792 to 928 of pRb appear to be required for growth suppression as assayed by inhibition of colony formation after reintroduction of pRb into the pRb-deficient osteosarcoma cell line, Saos-2 (41). To determine the precise C-terminal sequence requirements for negative growth control and to correlate these requirements with the ability of pRb to interact with E2F, selected pRb mutants were tested in this colony assay. Here, the pRb mutants were driven by the strong cytomegalovirus (CMV) immediate-early promoter to ensure high levels of expression. As expected, wild-type pRb inhibited the formation of G418-resistant colonies to a greater extent than the parental CMV-neo vector alone (Fig. 4A and B). Likewise, the

pRb(*dl*870–928) mutant also inhibited G418-resistant colony formation (Fig. 4D). However, deletion of residues 785 to 806, 803 to 806, 803 to 825, and 841 to 850 yielded pRb proteins which failed to significantly inhibit colony formation (Fig. 4C and 5). Thus, the ability to interact with E2F and repress transcription correlates with the ability of pRb to negatively regulate cell growth.

The stability and nuclear localization of the large C-terminal pRb deletion mutant pRb(*dl*792–928) have been determined previously (41). To confirm that these small C-terminal deletion mutants were expressed at equivalent levels and properly localized to the nucleus, Saos-2 cells were transiently transfected with the above CMV-pRb constructs and the expressed pRb mutant proteins were detected by immunofluorescence with a pRb-specific monoclonal antibody. Each of these proteins was expressed at similar levels and localized to the nucleus (Fig. 5).

The negative growth function of pRb as manifested by inhibition of colony formation appears to be limited to the slowly cycling Saos-2 cell line. This may be due to a lack of pRb-specific kinase activity in Saos-2 cells, which allows the accumulation of large quantities of underphosphorylated pRb (41). By contrast, C33A cells cycle rapidly and, as shown in Fig. 4E and F, are refractory to exogenously added wild-type pRb. Introduction of the wild-type pRb plasmid had no effect on the formation of G418-resistant colonies. Thus in C33A cells, the pRb-mediated repression of the E2 promoter (as shown above) is probably the direct effect of an interaction between pRb and E2F and not an indirect consequence of pRb blocking the cell cycle at an interval when E2F is inactive.

DISCUSSION

The *RB1* gene product is composed of at least two functional domains. The region required for interactions with DNA tumor virus antigens (29, 30) consists of two discontinuous elements (the A and B subdomains) separated by a spacer sequence. Although the latter element is required for E1A/T antigen binding, it can be replaced by entirely foreign amino acid sequences (29). The A and B subdomains are targets for mutations which both disrupt E1A/T antigen binding and inactivate pRb function. More recent reports have suggested that the C-terminal 135 amino acids are important for the association of pRb with E2F and for negative growth control (20, 25, 41). As shown above, sequences extending from the end of the E1A/T antigen-binding domain (amino acid 792) through residues 841 to 870 are required for E2F binding, indicating that the interaction of pRb with a cellular target protein requires a more extended pRb segment. However, E2F binding is apparently inhibited by oncoproteins that interact with only a portion of this region (29, 30, 43). At high concentrations the C-terminal pRb deletion mutants, including pGTRb(*dl*792–928) (41), can interact with E2F in extracts from Saos-2 cells, but at least 10-fold more protein is required, suggesting that the affinity of these mutant proteins for E2F is low. Furthermore, the pRb C-terminal mutants have no appreciable effect in vivo in either the repression of E2 transcription or Saos-2 colony suppression (41) (Fig. 1 and 4). The most important feature of the analysis presented here is the correlation of the abilities of pRb to interact with E2F, to repress transcription of the E2 promoter, and to negatively regulate cell growth. Indeed, the inability to separate E2F binding from growth inhibition highlights the physiological role of E2F as a target of pRb action.

FIG. 5. Summary tabulation of pRb mutants and their function in E2F reconstitution, E2-promoter repression, and growth suppression. N.D., not determined.

The growth of pRb-negative C33A cells, unlike Saos-2 cells, is not inhibited by pRb, illustrating that the pRb-mediated repression of the E2 promoter, at least in C33A cells, is probably a consequence of a direct interaction of pRb and E2F and not an indirect effect of pRb growth suppression. Other changes besides the loss of pRb or p53 must account for the differential effects of wild-type pRb expression in these cell lines, with the deficiency in endogenous pRb kinase activity likely to be the critical factor (41).

Previous results indicated that highly purified E2F failed to interact with bacterially produced GST-pRb protein, suggesting a requirement for one or more accessory factors for high-affinity interaction (25, 42). Because I used crude preparations of E2F which probably contain such factors, the experiments here may not directly define the molecular contacts between E2F and pRb. It is therefore possible that the C-terminal domain of pRb is required for interaction not with E2F but with an accessory factor. Although a murine E2F-pRb complex capable of binding DNA has yet to be identified, a 60-kDa pRb-binding protein (RBP60) which allows highly purified E2F to interact with pRb has been identified in murine cells (42). The determination of the molecular contacts between E2F, pRb, and other associated factors and the timing of expression of the individual components should shed light on the regulation of this complex during the cell cycle.

Because only the underphosphorylated forms of pRb interact with E2F (2, 8, 11, 23, 31), one level of regulation of the complex is likely to involve the state of pRb phosphorylation. In Saos-2 cells, the negative regulatory effects of pRb can be overcome by the coexpression of D2-, D3-, E-, and

A-type cyclins, which may induce one or more cyclin-dependent kinases (cdks) to phosphorylate pRb (16a, 28). The mapping of the specific G₁ cyclin-cdk phosphorylation sites on pRb should prove informative in determining the regulatory domains of pRb. In particular, it is possible that different G₁ cyclin-cdk complexes target different domains and hence different functions of pRb. In this regard, a baculovirus-expressed pRb protein phosphorylated by cdk4-cyclin D complexes fails to reconstitute an E2F-Rb complex in vitro (32a).

The E1A/T antigen domain of pRb is homologous to a related protein, p107, which is also a target of DNA virus tumor antigens (18) and binds E2F (17, 19, 37, 38, 48). However, the complexes formed between E2F and p107 differ from the E2F-pRb complex both in cell cycle timing and in protein composition. The E2F-pRb complex is present during G₁, and although the timing of disruption of the complex is difficult to ascertain because of G₁ contamination of S-phase preparations of cells, it appears that the E2F-pRb complex persists into the S phase (7, 14, 48). Meanwhile, the E2F-p107 complex exists in at least two forms, one containing cyclin A and cdk2 and present only in the S phase and a second containing cyclin E and cdk2 and present in the late G₁ and early S phases (17, 19, 33, 37-39, 48). Both p107-containing complexes contain specific DNA-binding activity and kinase activity. To date, no cyclins or cdks have been found to associate directly with the E2F-pRb complex, although recent reports suggest that cyclins D2 and D3 interact directly with pRb (16a, 32a, 36). Like E2F, the D2 and D3 G₁ cyclins require the C-terminal domain of pRb for their binding (16a). The regions of homology between

pRb and p107 are limited to the A and B segments of the E1A/T antigen domain (18), suggesting that the C-terminal domain of pRb may encode functions specific to *RB1*.

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