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Hyperphosphorylation of the Retinoblastoma Gene Product Is Determined by Domains Outside the Simian Virus 40 Large-T-Antigen-Binding Regions

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With the murine retinoblastoma (RB) cDNA, a series of RB mutants were expressed in COS-1 cells and the pRB products were assessed for their ability (i) to bind to large T antigen (large T), (ii) to become modified by phosphorylation, and (iii) to localize in the nucleus. All point mutations and deletions introduced into regions previously defined as contributing to binding to large T abolished pRB–large T complex formation and prevented hyperphosphorylation of the RB protein. In contrast, a series of deletions 5′ to these sites did not interfere with binding to large T. While some of the 5′ deletion mutants were clearly phosphorylated in a cell cycle-dependent manner, one, ΔPvu, failed to be phosphorylated despite binding to large T. pRB with mutations created at three putative $p34^{cdc2}$ phosphorylation sites in the N-terminal region behaved similarly to wild-type pRB, whereas the construct ΔP5-6-7-8, mutated at four serine residues C terminal to the large T-binding site, failed to become hyperphosphorylated despite retaining the ability to bind large T. All of the mutants described were also found to localize in the nucleus. These results demonstrate that the domains in pRB responsible for binding to large T are distinct from those recognized by the relevant pRB-specific kinase(s) and/or those which contain cell cycle-dependent phosphorylation sites. Furthermore, these data are consistent with a model in which cell cycle-dependent phosphorylation of pRB requires complex formation with other cellular proteins.

A number of different mechanisms appear to regulate progression through specific restriction points in the eucaryotic cell cycle. For example, transition from quiescence (G_0) to the S phase requires a rapid, transient induction of the proto-oncogene-c-*myc* (27). Suppression of c-*myc* expression by the introduction of antisense oligonucleotides blocks the G_0-to-S transition (32, 35). In some cells, failure to enter the S phase because of suppression of c-*myc* expression resulted in differentiation (14, 32, 35). A restriction point which appears to be regulated by protein kinase $p34^{cdc2}$ in the context of the maturation- or mitosis-promoting factor complex also exists prior to mitosis (for a review see reference 26). In this case, a specific phosphorylation state of $p34^{cdc2}$ activates the mitosis-promoting factor complex, promoting entry into mitosis (24). Thus, in contrast to c-*myc*, with which the regulation of expression controls the G_0-to-S transition, posttranslational modification of constitutively expressed, regulatory proteins controls progression through the mitotic restriction point.

Another potential point of regulation in the cell cycle exists after mitosis, when a cell can become quiescent or progress towards the S phase. Recent evidence has suggested that another oncoprotein, the product of the retinoblastoma (RB) gene (*RB1*; 13), may influence the cell cycle at this point. The product of *RB1* (pRB) is a nuclear phosphoprotein with an apparent molecular weight of 110,000 to 116,000 (18) and a long half-life (3). The series of pRB bands observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels are the result of phosphorylation of serine and threonine residues on pRB (5), and it has been demonstrated recently that these phosphorylation states vary in a cell cycle-dependent manner (2, 3, 5, 22). Specifically, cells in a state of quiescence or blocked in the cell cycle at the G_1-S boundary contain relatively low levels of pRB, predominantly in an underphosphorylated state. When cells are allowed to progress towards the S phase, pRB quickly becomes phosphorylated (5) and, following the completion of mitosis, is apparently dephosphorylated again (21). It remains unclear whether underphosphorylated pRB is sufficient or necessary for the maintenance of G_1 or G_0 and whether it is responsible for the control of entry into or exit from these states.

In contrast to cells which display a normal proliferative phenotype, the lack of proliferative control in a number of neoplastic tissues shows a clear association with the dysfunction of *RB1*. For example, while 80% of the RB tumors studied expressed the *RB1* message, they lacked the RB protein because of sequence mutations causing premature termination during translation (6, 7, 34). In some non-RB tumors, a mutant pRB lacking many of the characteristics of apparently functional pRB was expressed (15, 28, 29, 33). One of these characteristics, although not a normal function, was the ability of pRB to associate with a number of viral proteins. Specifically, it has been clearly demonstrated that simian virus 40 (SV40) large T antigen (large T), adenovirus E1A, or papillomavirus E7 protein forms stable complexes with pRB, as determined by coimmunoprecipitation assays (4, 9, 25, 30, 31). These coimmunoprecipitation studies indicated, however, that only the underphosphorylated species of pRB associated with the viral proteins (20). With a series of large T and E1A deletion mutants, a common, discrete region on these viral proteins was found to be responsible for binding pRB (31). These regions were further

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TABLE 1. Summary of phosphorylation mutants[a]

Mutant	Amino acid change for the following residue (consensus sequence):							
	Thr-246 (RTPRR)	Thr-350 (RTPRK)	Ser-601 (LSPLR)	Ser-605 (RSPKK)	Ser-781 (RSPYK)	Ser-788 (SSPLR)	Ser-800 (ISPLK)	Ser-804 (KSPYK)
RB^K	T	T	S	S	S	S	S	SP
ΔP1-2	A	R	—	—	—	—	—	—
ΔP1-2-3-4	A	R	A	A	—	—	—	—
ΔP3-4	—	—	A	A	—	—	—	—
ΔP5-6-7-8	—	—	—	—	G	A	A	RN

[a] Six Thr or Ser residues in mouse pRB, contained in $p34^{cdc2}$ consensus sequences (23), were changed by site-directed mutagenesis. Two additional Ser residues with hydrophobic residues (Leu or Ile) at position −1, instead of basic or polar residues (Arg, Lys, or Ser), were also mutated. Mutant ΔP5-6-7-8 contains an additional mutation in which the Pro residue following Ser-804 is changed to an Asn residue. —, The relevant amino acid was not changed in that particular mutant. Thr-246, Thr-350, Ser-601, Ser-605, Ser-781, Ser-788, Ser-800, and Ser-804 correspond to residues numbered 1 to 8, respectively, in Fig. 1.

determined to be required for cellular transformation by large T or E1A (12, 19, 31). Hu et al. further defined the regions in pRB required for E1A and large T binding by using an extensive series of deletion mutants, translated in vitro and coimmunoprecipitated with large T or E1A (16). Combined with the cell cycle-dependent phosphorylation of pRB, these data suggest that progression through the cell cycle, specifically from G_0-G_1 to S, may be controlled by the phosphorylation state of pRB.

To characterize potential functional domains of pRB, we have produced a series of mutant RB proteins which were assessed for their ability (i) to bind to large T, (ii) to become phosphorylated to higher-molecular-weight species, and (iii) to localize in the nucleus. Our results indicate that, while all of the pRB mutants studied are translocated to the nucleus, pRB mutants which fail to bind to large T also fail to be hyperphosphorylated (i.e., phosphorylated in a cell cycle-dependent manner). Furthermore, both the N- and C-terminal portions of pRB, outside regions previously defined to be involved in complex formation with large T, clearly affect the phosphorylation of pRB. These data are consistent with a model in which phosphorylation of pRB by the relevant kinase(s) requires complex formation between pRB and either large T or the cellular protein(s) with which pRB is normally associated.

MATERIALS AND METHODS

Cell lines and DNA. COS-1 cells and the SV40 promoter-containing pECE vector were provided by T. Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada). R. Bernards (Massachusetts General Hospital, Boston) kindly provided the full-length murine cDNA.

Construction of RB deletion mutants. The sequence around the initiation codon of the murine RB cDNA was modified by replacing the first 32 bp with the double-stranded sequence GAATTCACC **ATG** GGG CCC AAA ACC CCC CGA AAA ACG (boldfacing indicates the initiation codon), selected to conform to the Kozak rules (17). This sequence resulted in modification of the Pro-2 residue to a Gly residue. The mutant, designated RB^{Kozak} or RB^K, was used to produce all subsequent mutants. The deletions mutants ΔBal, ΔKpn, and ΔPvu were produced by removing the sequence between the initiation methionine and the respective restriction sites, blunt ending with the Klenow fragment, and religating the plasmids. ΔDra was produced by cutting with *Dra*III, blunt ending the 3-bp 3′ overhang, and religating the insert. Δ22, ΔS/S, and ΔS/N mutants were produced by cutting and religating the appropriate restriction sites in the murine equivalent of exon 22 (see Fig. 1 for amino acids deleted).

PCR-directed mutagenesis. Potential phosphorylation sites in RB with the $p34^{cdc2}$ kinase consensus sequences (23) were mutated by the polymerase chain reaction (PCR) with primers containing single-base-pair mismatches in the codons of interest. Four pairs of primers were used to change six sites with exact $p34^{cdc2}$ phosphorylation consensus sequences and two additional sites with imperfect sequences (Table 1). The products of two pairs each spanned unique restriction sites in the murine RB cDNA. Following PCR, the products were individually subcloned into pGEM7 (Promega) and sequenced to exclude additional mutations introduced during PCR. Two pairs of correct sequences were ligated together by virtue of new restriction sites introduced at their junctions and subsequently ligated into the appropriate sites in the RB cDNA.

Coimmunoprecipitation of in vitro translation products. RB wild-type or mutant cDNA in either the pGEM7 or the pBluescript KS^+ (Stratagene) vector was transcribed from the T7 promoter. In vitro translations with rabbit reticulocyte lysates (35 μl; Promega) were performed in accordance with the manufacturer's instructions. In vitro translation products (10 μl) were mixed with 150 μg of a COS-1 cell lysate (typically 100 μl). NP40 buffer (25 mM Tris [pH 7.6], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) was added to a final volume of 750 μl, and the RB proteins were allowed to associate with large T for 1 h at 4°C. Immunoprecipitations with an anti-large T monoclonal antibody (PAb 419; E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) were performed as described below.

Transfections. Transfections of COS-1 or NIH 3T3 cells were performed with Lipofectin (Bethesda Research Laboratories) (11). In brief, 5 to 20 μg of DNA was mixed with 25 μl of Lipofectin reagent in 3.0 ml of OptiMEM medium (Bethesda Research Laboratories). The solution was allowed to stand for 20 min at room temperature. The solution was added to 50% confluent cells in 60-mm dishes which had been previously washed three times in phosphate-buffered saline. After incubation for 5 h at 37°C, 3.0 ml of fresh medium Dulbecco minimal essential medium plus 20% fetal calf serum) was added. On the following day, the medium was replaced with 5.0 ml of 10% fetal calf serum in Dulbecco minimal essential medium. Cells were used for immunoprecipitations 2 days later.

Nuclear localization of pRB mutants. NIH 3T3 cells, grown on cut glass slides in 60-mm dishes, were transfected as described above. After 3 days, the slides were removed and the cells were fixed in −20°C methanol for 1 min and washed carefully four times in phosphate-buffered saline. Fixed cells were probed with a mouse anti-human pRB monoclonal antibody (Mh-RB-02, 1:100 dilution; Pharmingen) for 1 h. The slides were developed with the horseradish peroxidase

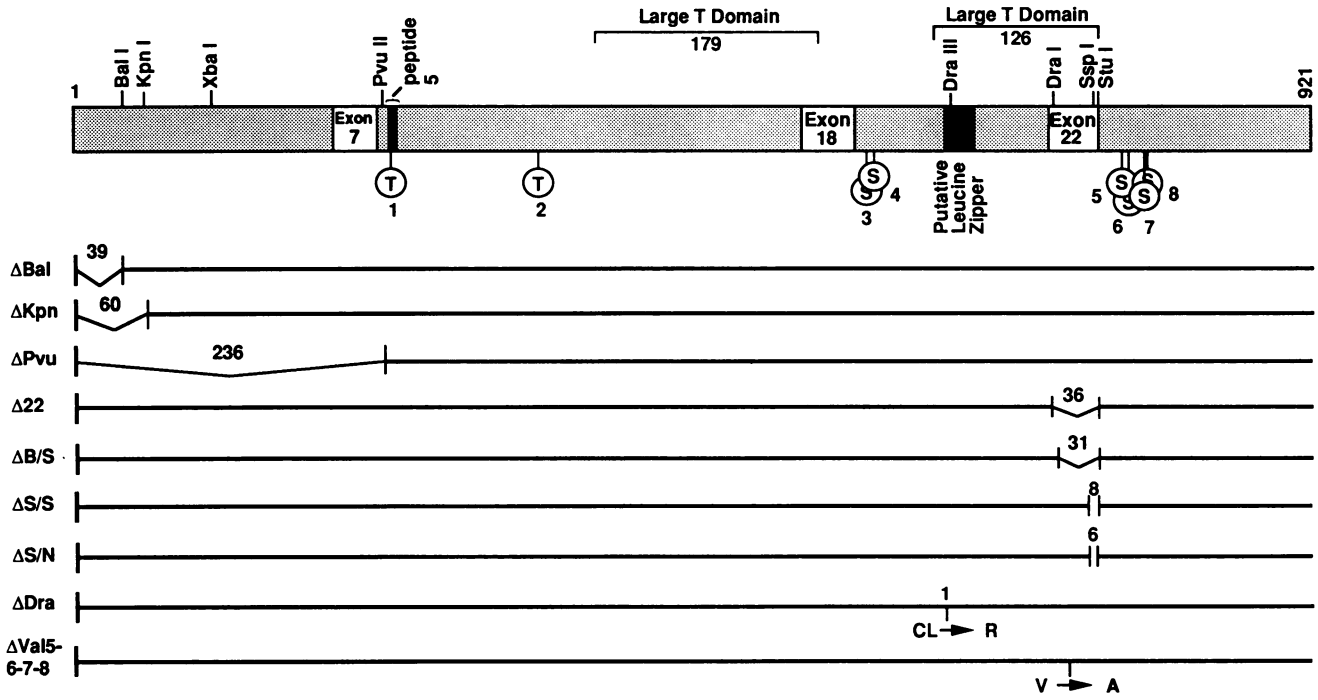

FIG. 1. Map of murine RB deletion mutants. Deletions mutants were constructed as described in Materials and Methods. Numbers above the deleted portions refer to the numbers of amino acids deleted from the transcribed RB protein. The Thr(T) and Ser(S) residues indicated are those contained within $p34^{cdc2}$ kinase consensus sequences (23) and are numbered sequentially (Table 1). Phosphorylation mutants are thus named according to the numbered Thr or Ser codons which have been modified. The large T domains are the murine analogs of those defined by Hu et al. (16) in the human RB protein. The position of the peptide from which the rabbit anti-RB sera were produced is indicated (peptide 5). Amino acid deletions in mutants were as follows: ΔBal, 2 to 39; ΔKpn, 2 to 60; ΔPvu, 2 to 236; Δ22, 733 to 768; ΔB/S, 737 to 768; ΔS/S, 761 to 768; ΔS/N, 761 to 766; ΔDra, Leu-658 to Cys-659 changed to Arg (substitution and deletion); ΔVal5-6-7-8, Val-735 to an Ala residue in exon 22.

Vectastain kit (Vector; with diaminobenzidine as the color substrate) in accordance with the manufacturer's instructions.

Immunoprecipitations. When ^{35}S- or ^{32}P-labeled lysates were required, transfected cells in 60-mm dishes were incubated in 1.5 ml of medium lacking methionine or phosphate, respectively, and containing 10% dialyzed fetal calf serum and 150 μCi of [^{35}S]methionine per ml or 800 μCi of $^{32}P_i$ per ml (Amersham), respectively, for 3 h. Dishes were placed on ice, washed three times in ice-cold phosphate-buffered saline, and lysed by the addition of 1.0 ml of lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40; Sigma). The dishes were scraped with a rubber scraper, and the lysate was collected in an Eppendorf tube and, after 20 min on ice, spun in a microcentrifuge for 15 min. The total amount of incorporated counts (labeled lysates) or total protein (assay from Bio-Rad) was determined for each sample.

Immunoprecipitations were labeled by adding a maximum of 400 μl of lysate (typically 10^6 to 10^7 cpm of ^{35}S- or ^{32}P-labeled lysates to 300 to 750 μg of total protein) and NP40 buffer together in a maximum total volume of 750 μl. A rabbit anti-RB polyclonal antibody (pep 5-X8) (10 μl) and 20 μl of a mouse anti-large T monoclonal antibody (PAb 419 or PAb 416; E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) were added, and the mixture was rocked for 1 h at 4°C. When the monoclonal antibody was used, 15 μl of a rabbit anti-mouse immunoglobulin G antibody was added after 30 min. A 10% protein A-Sepharose suspension (100 μl; washed in NP40 buffer containing 5% bovine serum albumin; Pharmacia) was added to each sample, and the mixture was rocked for 30 min. Samples were spun down and washed five times in $RIPA^+$ buffer (50 mM sodium phosphate [pH 7.6], 100 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and twice in $RIPA^-$ buffer (50 mM sodium phosphate [pH 7.6], 100 mM NaCl). SDS-PAGE samples (20 μl; 4×-concentrated SDS) were added, and the lysates were boiled for 3 min. Recovery of the samples from the beads was facilitated by cutting off the bottom of the Eppendorf tubes with a hot scalpel and making a hole in the bottom of the tubes with a 27-gauge needle, and the samples were briefly spun into a new Eppendorf tube. Samples were run on a 7.5% SDS-PAGE gel. Gels containing ^{35}S-labeled lysates were treated with En^3Hance (NEN) after being stained and destained.

Gels, Western immunoblotted on nitrocellulose, were probed with the mouse anti-human RB monoclonal antibody Mh-RB-02 and developed with the Vectastain kit in accordance with the manufacturer's instructions.

RESULTS

Coimmunoprecipitation of in vitro-translated RB. We initially tested a number of RB mutants (Fig. 1) for binding to SV40 large T by mixing in vitro-translated RB protein with an unlabeled COS-1 cell extract and immunoprecipitating the mixture with the anti-large T monoclonal antibody PAb 419 (Fig. 2). As demonstrated by others (9), the in vitro-translated RB protein appears as a series of products initiating at successive methionine codons in the RB cRNA. Poor initiation at the first Met codon appeared not to be a function of the surrounding sequence, since replacement of this se-

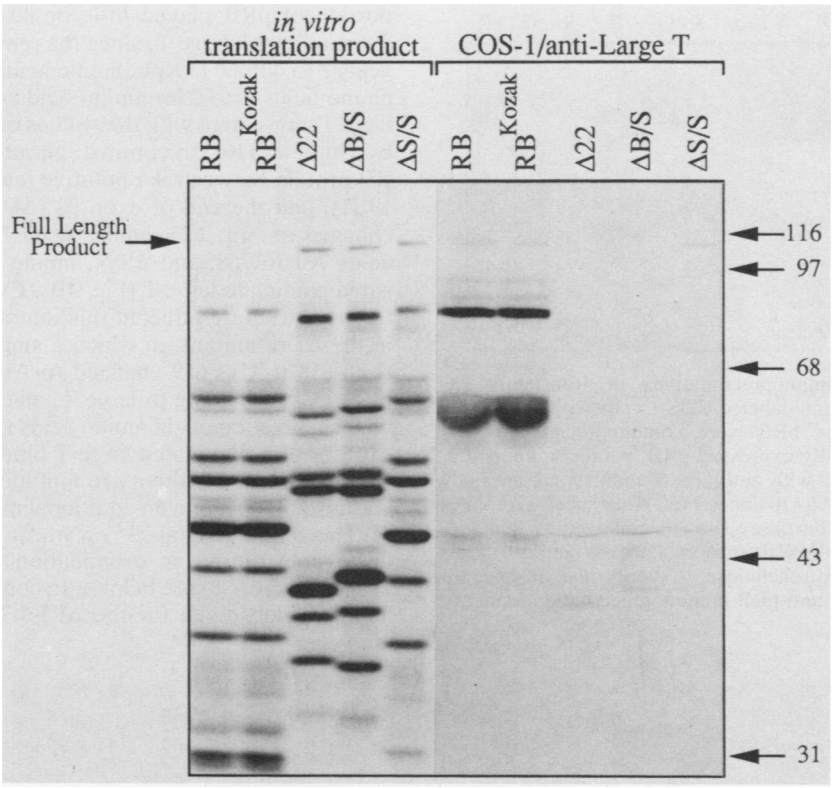

FIG. 2. In vitro translation of pRB mutants. pRB mutants were transcribed from the T7 promoter in pBluescript (RB and RB^{Kozak}) or pGEM7 (Δ22, ΔB/S, and ΔS/S) and translated in a rabbit reticulocyte lysate. The [^{35}S]methionine-labeled products were mixed with an unlabeled COS-1 cell lysate, immunoprecipitated with the anti-large T monoclonal antibody PAb 419, and run on a 12% SDS-PAGE gel. Numbers at right indicate molecular masses in kilodaltons.

quence (GCGCGTC **ATG** CCG) with one conforming to the Kozak rules (GAATTCACC **ATG** GGG) (17) produced the same pattern and intensity of products (compare RB to RB^{Kozak}). More efficient translation from the first Met codon was in fact seen in transcripts generated from the T7 promoter in pGEM7 (RB mutants Δ22, ΔB/S, and ΔS/S) than from the T7 promoter in pBluescript (RB and RB^{Kozak}), suggesting that the distance from the promoter to the initiation codon (45 bp in pGEM7 versus 75 bp in pBluescript) may influence initiation.

When the mixed unlabeled COS-1 cell extract and [^{35}S]methionine-labeled in vitro translation lysates were immunoprecipitated with anti-large T, wild-type products (RB and RB^{Kozak}) initiating down to about 60 kDa were coprecipitated very efficiently, as previously demonstrated (9). In contrast, all of the exon 22 mutants, with deletions of 36 (Δ22), 31 (ΔB/S), or 8 (ΔS/S) amino acids, failed to strongly coprecipitate with large T. Interestingly, in contrast to the relatively abundant 88-kDa fragment of the ΔS/S mutant (eight-amino-acid deletion), which failed to coimmunoprecipitate with large T, the more weakly expressed full-length product of ΔS/S bound to large T. While this result initially suggested that the N-terminus of pRB plays a role in binding to large T, we have been unable to observe even weak binding of ΔS/S to large T expressed in COS-1 cells (see below). A number of smaller, strongly expressed bands in the in vitro-translated wild-type pRB and mutant pRB were also observed to bind relatively weakly to large T, although we have been unable to demonstrate specificity for these interactions.

Association of RB mutants with large T in COS-1 cells. Since posttranslational modification of pRB is apparently an important aspect of its function, further analyses of additional pRB mutants were pursued by transient expression from the SV40 promoter in the pECE vector following transfection into COS-1 cells. The murine RB protein migrates faster on SDS-PAGE gels than do the monkey and human RB proteins, thus allowing identification of the highly expressed murine RB protein against the monkey background. Wild-type pRB (Fig. 3, lane 2) was expressed at considerably higher levels than was the endogenous monkey background (Fig. 3, lane 1). During this transient expression of pRB in COS-1 cells, a large proportion of pRB appeared not to be complexed with large T. This result was determined by exhaustive immunoprecipitation of large T with either of two anti-large T monoclonalantibodies, PAb 419 or PAb 416 (lanes 3, 4, and 5 and lanes 7, 8, and 9, respectively) followed by immunoprecipitation with anti-pRB (lanes 6 and 10). That the transiently expressed pRB was in excess of large T under these conditions was evident by the lack of large T in the final anti-pRB lanes, as determined by autoradiography (lanes 6 and 10) or by Western blot analysis (data not shown). Combined with the fact that large T is also involved in interactions with a number of other cellular proteins (8, 10), it is clear that the stoichiometry between pRB and large T should be different in immunoprecipitations with anti-pRB versus anti-large T.

Immunoprecipitations of wild-type pRB and mutant pRB with anti-pRB are shown in Fig. 4A, overexposed to reveal the more weakly expressed mutant proteins, and those with anti-large T are shown in Fig. 4B. Immunoprecipitations of the mutant pRB proteins indicated that the N-terminal

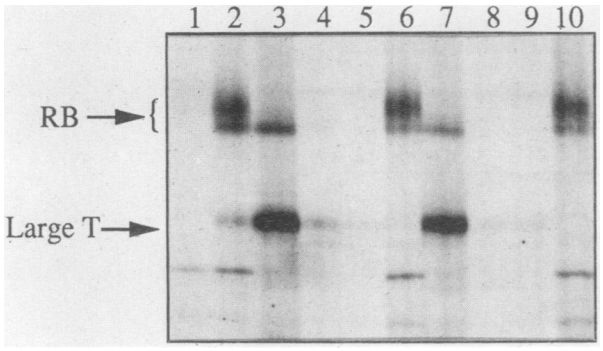

FIG. 3. Exhaustive immunoprecipitations of transiently expressed pRB. [^{35}S]methionine-labeled COS-1 cells without (lane 1) or with (lane 2) wild-type pRB were immunoprecipitated with anti-pRB. For the transiently expressed pRB, parallel immunoprecipitations were performed with anti-large T monoclonal antibody PAb 419 (lanes 3 to 5) or PAb 416 (lanes 7 to 9) four successive times (only the first, third, and fourth are shown), followed by immunoprecipitations with the anti-pRB antisera (lanes 6 and 10). Gels were also blotted onto nitrocellulose to verify the absence of residual large T in the last anti-pRB immunoprecipitation (data not shown).

portion of pRB played little or no direct role in complex formation with large T, since the removal of 39 (ΔBal, amino acids 2 to 40), 60 (ΔKpn, amino acids 2 to 60), or 236 (ΔPvu, amino acids 2 to 236) amino acids did not limit binding to large T, consistent with the studies of in vitro-translated pRB by Hu et al. (16). In contrast, all mutations produced in the RB protein between the putative leucine zipper motif (exon 19 [1]) and the end of exon 22 (ΔDra, Leu-658 to Cys-659 changed to Arg; Δ22, amino acids 733 to 768; ΔS/S, amino acids 761 to 768; and ΔS/N, amino acids 761 to 766) abrogated binding to large T (Fig. 4B). Even small changes in the tertiary structure affected this interaction, as demonstrated in the ΔDra mutant, in which a single mutation or deletion (Leu-658 to Cys-659 changed to Arg) resulted in the complete loss of binding to large T, and in the ΔS/N mutant, in which a deletion of six amino acids (761 to 766) at the end of exon 22 also eliminated large T binding.

The extreme sensitivity to mutation of the exon 22 region was also observed in an additional mutant, ΔVal5-6-7-8 (Fig. 5). This mutation (Val-735 on an Ala residue in exon 22) was discovered during an examination of the phosphorylation mutant ΔP5-6-7-8 (see below). In contrast to the normal large T binding observed for the ΔP5-6-7-8 mutant, ΔVal5-6-7-8

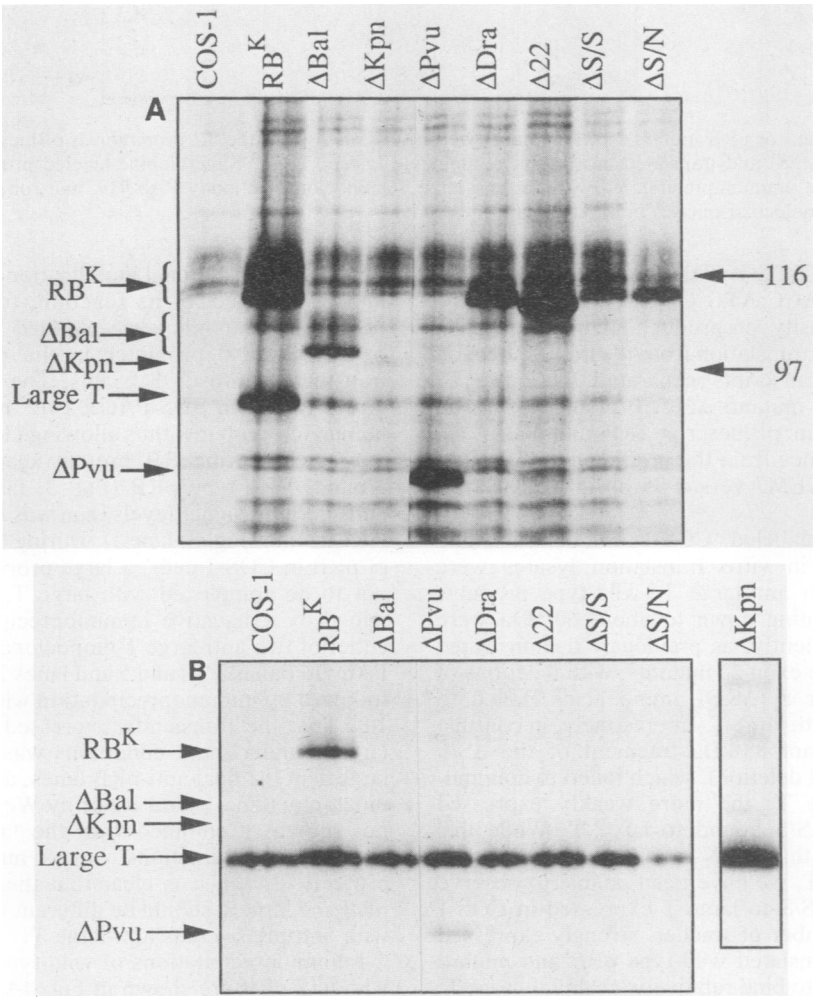

FIG. 4. Immunoprecipitations of transiently expressed RB mutants in COS-1 cells. Mutants expressed from the SV40 promoter in [^{35}S]methionine-labeled COS-1 cells were immunoprecipitated with either rabbit anti-pRB sera (A) or anti-large T monoclonal antibody PAb 419 (B). Samples were separated on a 7.5% SDS-PAGE gel. Numbers at right in panel A indicate molecular masses in kilodaltons.

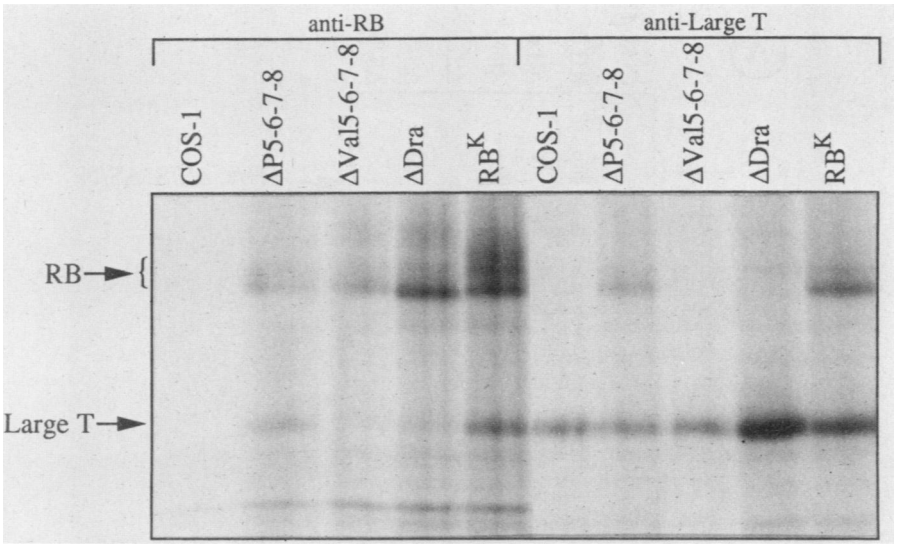

FIG. 5. Immunoprecipitations of the ΔVal5-6-7-8 mutant. Immunoprecipitations of mutants expressed in [^{35}S]methionine-labeled COS-1 cells were performed as described in the legend to Fig. 4. Mutant ΔVal5-6-7-8 contains the same mutations as mutant ΔP5-6-7-8 but has an additional mutation at Val-735 (exon 22) resulting in the presence of an Ala residue.

did not coprecipitate with large T. The very subtle nature of the ΔVal5-6-7-8 mutation suggests that this portion of pRB may interact directly with large T, as opposed to contributing to the "scaffold" of this domain.

Phosphorylation of pRB deletion mutants. Immunoprecipitations from COS-1 cells metabolically labeled with [^{35}S]methionine suggested that the ability of pRB to become highly phosphorylated correlated with complex formation with large T. For example, the wild-type protein (Fig. 4A, RB^K) and the 5′ deletion mutant ΔBal, both of which bound to large T, appeared to be present as both underphosphorylated and higher-molecular-weight, phosphorylated species. The only exception was the ΔPvu mutant, which appeared as a single band (Fig. 4A) yet bound efficiently to large T (Fig. 4B). In contrast, single bands were observed for all mutants which failed to bind to large T (ΔDra, Δ22, ΔS/S, and ΔS/N), indicating that such mutants also failed to be recognized by the cell cycle-dependent kinase(s). To directly examine the phosphorylation state of the pRB mutants, we transiently expressed them in COS-1 cells, metabolically labeled them with $^{32}P_i$, and immunoprecipitated them with anti-pRB (Fig. 6A to C). While the monkey pRB background was considerably stronger in this analysis than in the ^{35}S analysis, the murine pRB could be clearly identified on the Western blot (Fig. 6A) and the ^{32}P autoradiogram (Fig. 6B). The results demonstrated that all of the RB protein bands exhibited some degree of phosphorylation. However, all mutants which failed to bind to large T (ΔDra, Δ22, ΔS/S, and ΔS/N) appeared only as underphosphorylated species, the higher-molecular-weight, phosphorylated bands in these lanes (Fig. 6A and B) being due to the COS-1 endogenous pRB background (compare the hyperphosphorylated bands of RB^K with the ΔDra, Δ22, ΔS/S, and ΔS/N bands). In contrast, the wild type (RB^K) and the ΔBal mutant, both of which bound to large T, existed as under- and hyperphosphorylated proteins. It was clear, however, that the pRB interaction with large T was a necessary characteristic for hyperphosphorylation of pRB but was not sufficient. This result was demonstrated with the ΔPvu mutant, which bound to large T but was present only in the underphosphorylated form (Fig. 6C). Phosphorylation of the ΔKpn mutant could not be assessed because of the low levels of expression of this mutant.

Nuclear localization of pRB mutants. The RB protein is actively transported into the nucleus (18). Immunohistochemical studies of actively dividing cells show that the RB protein is present throughout the cytoplasm when the nuclear membrane breaks down during mitosis and reaccumulates in the nucleus following cell division (P. Hamel, unpublished observations). To determine whether the pRB mutants exhibited this characteristic, we transiently expressed them in NIH 3T3 cells, which have undetectable levels of endogenous RB protein after immunohistostaining, and probed them with an anti-pRB monoclonal antibody. With the exception of ΔDra and Δ22, all of the mutants showed exclusive nuclear localization (data not shown) (summary in Table 2). ΔDra and Δ22 were unique in that

TABLE 2. Summary of characteristics of pRB mutants[a]

Mutant	Large T binding	Hyperphosphorylation	Localization
RB^K	+	+	N
ΔBal	+	+	N
ΔKpn	+	ND	N
ΔPvu	+	−	N
ΔDra	−	−	N/C
Δ22	−	−	N/C
ΔB/S	−	−	N
ΔS/S	−	−	N
ΔS/N	−	−	N
ΔP1-2	+	+	N
ΔP1-2-3-4	+	+	N
ΔP3-4	+	+	N
ΔP5-6-7-8	+	−	N
ΔVal5-6-7-8	−	−	N

[a]Binding to large T by pRB mutants was determined by coprecipitation assays in COS-1 cells or by mixing labeled, in vitro-translated pRB mutants with unlabeled COS-1 extracts. pRB mutants were also assessed for their ability to become highly phosphorylated by use of [^{35}S]methionine- and/or $^{32}P_i$-labeled lysates. The cellular localization of pRB mutants was determined by transient expression of the mutants in NIH 3T3 cells (data not shown). ND, Not determined. N, Nuclear; N/C, nuclear and cytoplasmic.

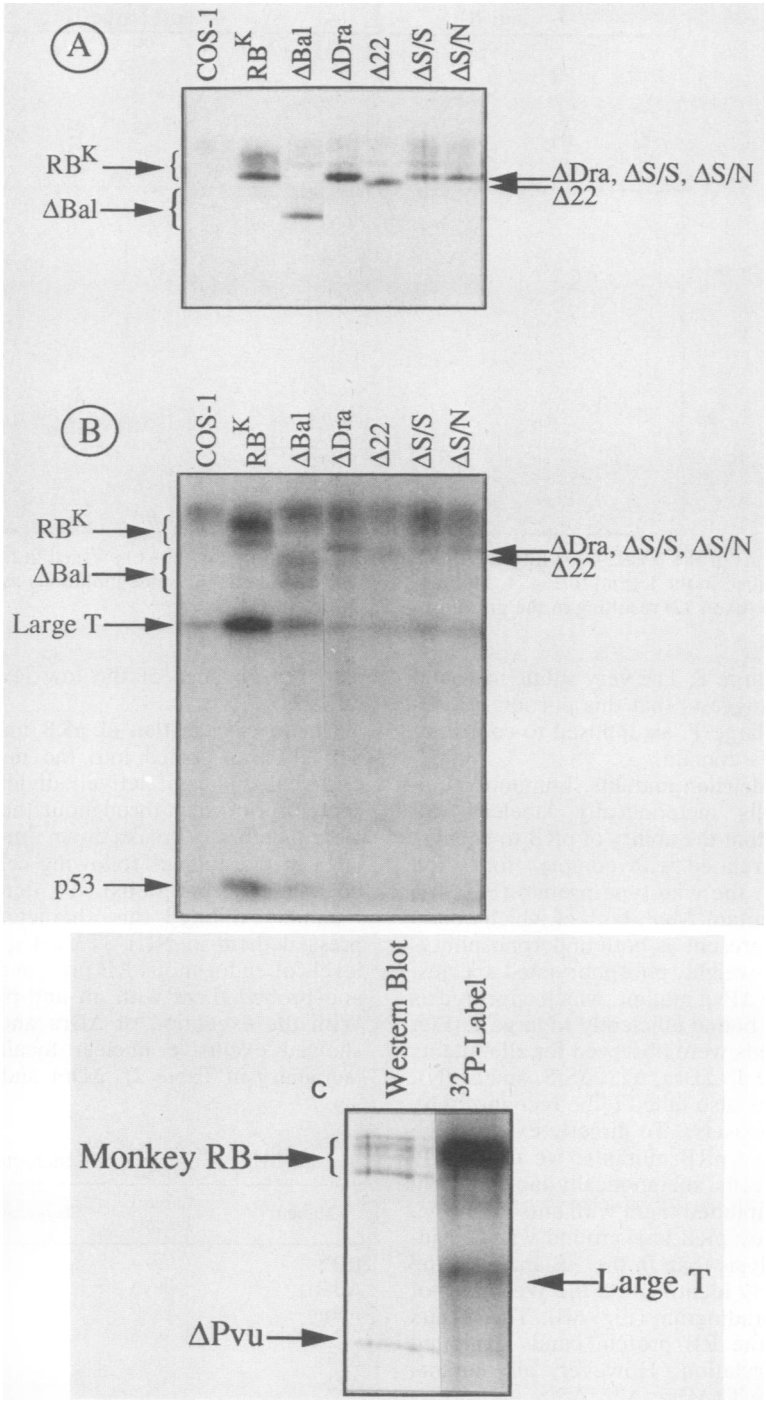

FIG. 6. Immunoprecipitations of $^{32}P_i$-labeled pRB mutants. pRB mutants expressed in $^{32}P_i$-labeled COS-1 cells were immunoprecipitated with anti-pRB sera and run on a 7.5% SDS-PAGE gel. Samples were electroblotted onto nitrocellulose and developed with an anti-pRB monoclonal antibody (A), and the ^{32}P-labeled proteins were determined by autoradiography (B). Mutant ΔPvu (C) was treated in the same manner, with the exception that immunoprecipitations were performed with the mouse anti-pRB monoclonal antibody Mh-RB-02. Coprecipitation of the phosphorylated large T and p53 proteins is indicated.

there were significant amounts of RB protein in the cytoplasm, although a strong nuclear component was also evident.

RB mutations at $p34^{cdc2}$ consensus sequence sites. The protein kinase $p34^{cdc2}$ has been implicated in the control of entry into mitosis and regulation of the G_1-to-S transition in *Saccharomyces cerevisiae* (for a review, see reference 26), although only the former activity is well characterized in larger eucaryotes (e.g., HeLa cells). Since pRB exists in different phosphorylation states during the cell cycle and contains six $p34^{cdc2}$ consensus sequences (23), a series of mutations were generated in the relevant Thr or Ser codons

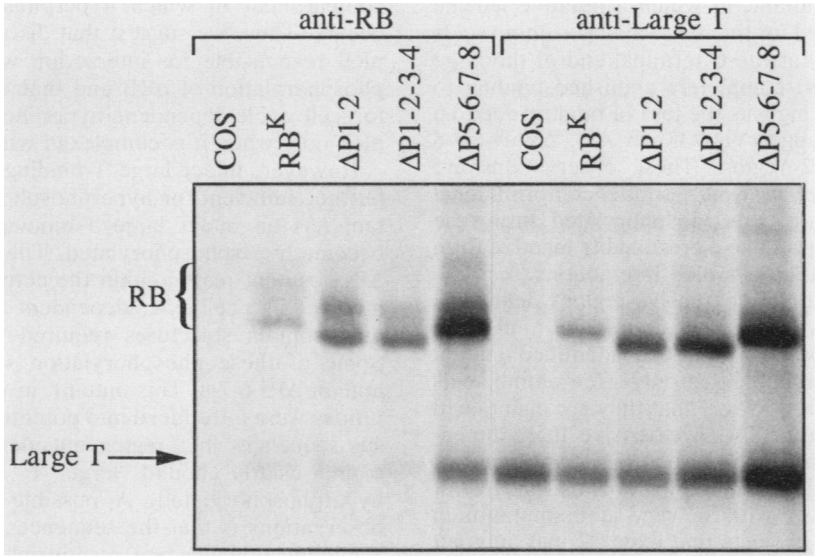

FIG. 7. Immunoprecipitations of [^{35}S]methionine-labeled pRB phosphorylation mutants. The wild type or phosphorylation mutants were expressed in [^{35}S]methionine-labeled COS-1 cells and immunoprecipitated with either anti-RB or anti-large T. The numbering of the phosphorylation mutants refers to the modified Thr or Ser residues contained within p34^{cdc2} kinase consensus sequences in pRB (Fig. 1 and Table 1).

(Table 1). Figure 7 shows the [^{35}S]methionine-labeled mutants immunoprecipitated with anti-pRB and anti-large T. All of the mutations introduced into these sites resulted in proteins able to bind to large T. Interestingly, the mutations introduced into the first two p34^{cdc2} sites (mutants ΔP1-2 and ΔP1-2-3-4) resulted in a protein product which migrated considerably faster than did the wild-type murine protein but maintained its ability to be modified to the higher-molecular-weight, phosphorylated species. Mutant ΔP3-4 behaved identically to the wild type (RB^K) with respect to electrophoretic mobility (data not shown). In contrast, the mutations introduced into four serine residues in exon 23 (ΔP5-6-7-8) resulted in a protein which migrated with the wild-type, underphosphorylated protein but did not exhibit phosphorylation to the higher-molecular-weight bands. That pRB mutant ΔP5-6-7-8 was relatively under phosphorylated was verified by immunoprecipitations of the $^{32}P_i$-labeled cells (Fig. 8A and B). Hyperphosphorylation of the wild type and the mutants ΔP1-2, ΔP1-2-3-4, and ΔP3 could be clearly seen against the COS-1 background. In contrast, ΔP5-6-7-8 did not exhibit the strong signal associated with hyperphosphorylated pRB. This analysis does not rule out the possibility that ΔP5-6-7-8 has minor hyperphosphorylated forms; more detailed analyses such as tryptic digests are in progress.

DISCUSSION

Recently, Hu et al. defined two independent domains on the human RB protein which are required for complex formation with large T and E1A (homologous to murine amino acids 387 to 566 and 639 to 766) (16). Deletions in any part of these domains abrogated binding to the viral proteins. Similarly, we show that all mutations introduced into these regions (ΔDra, Δ22, ΔS/S, ΔS/N, and ΔVal5-6-7-8; Fig. 4B and 5) prevented the pRB mutants from interacting with large T in COS-1 cells. It is also clear that the large T-E1A-binding site on pRB is particularly sensitive to perturbation. For example, the small conformational change

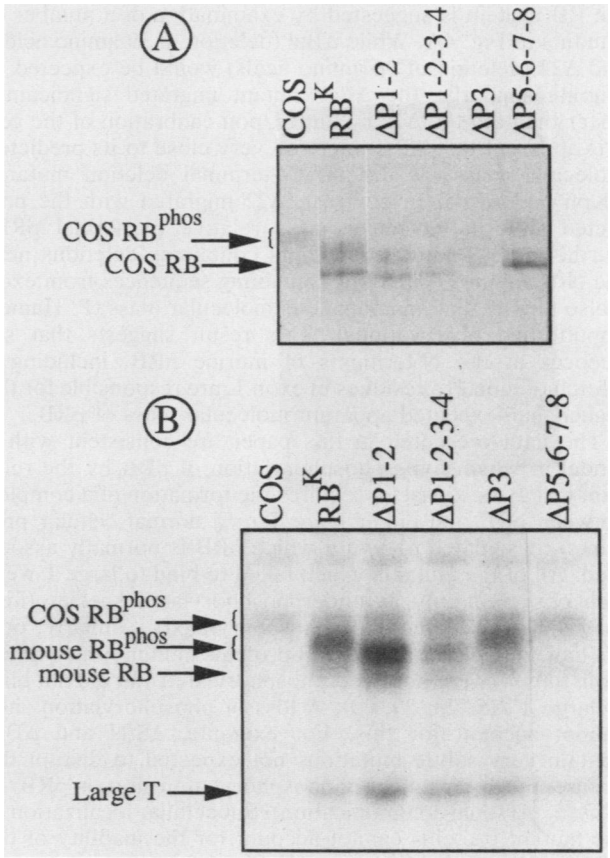

FIG. 8. Immunoprecipitations of $^{32}P_i$-labeled pRB phosphorylation mutants. $^{32}P_i$-labeled COS-1 cells were immunoprecipitated with anti-pRB sera and run on 7.5% SDS-PAGE gels. (A) Western blot. (B) Autoradiogram.

expected in the ΔDra mutant, in which a putative leucine zipper motif was disrupted, or the removal of six amino acids (amino acids 761 to 766) at the C-terminal end of the large T-binding domain (ΔS/N) completely abolished binding to large T. Even more striking was the loss of binding due to a single, conservative change (Val-735) to Ala; ΔVal5-6-7-8 mutant) in the exon 22 region. These observations are consistent with a model in which subtle conformational changes, expected when pRB is phosphorylated during the course of the cell cycle, produce a presumably inactive form of pRB and also prevent large T-pRB interactions.

In contrast to the relatively extensive regions defined in pRB for interactions with large T and E1A, a discrete peptide which interacts with pRB has been identified on viral proteins (31). It seems probable that only a few amino acids in pRB bind directly to large T and that they are maintained in the correct conformation by an extensive three-dimensional scaffold, analogous to the structural arrangement of the antigen-binding domains of immunoglobulins. The loss of binding to large T observed with the very subtle mutation in the ΔVal5-6-7-8 mutant suggests that exon 22 may interact directly with large T, while the expected structural change in the potential alpha-helix caused by the ΔDra mutation may block large T binding through an alteration of the scaffold.

It has been previously noted that the RB protein migrates on SDS-PAGE gels with a molecular mass greater than that predicted by its amino acid sequence (1). This anomaly can be seen in Fig. 4A, in which the mouse RB protein migrated at 110 kDa despite a predicted molecular mass of only 105 kDa. The source of the higher apparent molecular mass of the RB protein is suggested by examination of a number of mutants in Fig. 4A. While ΔBal (deletion of 39 amino acids) and Δ22 (deletion of 36 amino acids) would be expected to migrate similarly, the ΔBal mutant migrated significantly faster than did the Δ22 mutant. Upon calibration of the gel, it is apparent that ΔBal migrated very close to its predicted molecular mass, as did the N-terminal deletion mutants ΔKpn and ΔPvu. In contrast, Δ22 migrated with the predicted shift in molecular mass relative to normal pRB. Furthermore, additional mutants containing deletions near the N-terminus of pRB but containing sequences from exon 1 also migrated at an anomalous molecular mass (P. Hamel, unpublished observations). This result suggests that sequences at the N-terminus of murine pRB, including a stretch of nine Pro residues in exon 1, are responsible for the higher-than-expected apparent molecular mass of pRB.

The data presented in this paper are consistent with a model in which hyperphosphorylation of pRB by the relevant cell cycle kinase(s) requires the formation of a complex between pRB and either large T or a normal cellular protein(s) (designated pX) with which pRB is normally associated. All of the mutants which failed to bind to large T were also observed only as underphosphorylated species (i.e., they did not become hyperphosphorylated). Similarly, others have noted that mutant RB proteins immunoprecipitated from tumors were not hyperphosphorylated and did not bind to large T (15, 28, 29, 33). A loss of phosphorylation sites cannot account for this. For example, ΔS/N and ΔDra contain very subtle mutations not expected to disrupt the apparently large number of phosphorylation sites on pRB (P. Whyte, personal communication). Subcellular localization of the mutant proteins cannot account for the inability of the ΔDra, Δ22, ΔS/S, ΔS/N, and ΔVal5-6-7-8 mutants to associate with large T or become hyperphosphorylated. Transient expression in NIH 3T3 cells demonstrated that all pRB mutants localized to the nucleus (Table 2), the cellular compartment in which hyperphosphorylation presumably occurs. Thus, we suggest that disruption of the domains of pRB responsible for interaction with pX prevents hyperphosphorylation of pRB and that the kinase(s) responsible for cell cycle-dependent hyperphosphorylation recognizes pRB only when it is complexed with pX.

However, intact large T-binding domains are necessary but not sufficient for hyperphosphorylation. The ΔPvu mutant has an intact large T-binding domain yet does not become hyperphosphorylated. The region deleted from the ΔPvu mutant may contain the actual phosphorylation sites modified in a cell cycle-dependent manner or, alternatively, may contain structures required for complex formation. Some of these phosphorylation sites may be deleted in mutant ΔP5-6-7-8. This mutant, in which amino acid substitutions were introduced into potential $p34^{cdc2}$ kinase consensus sequences in a region outside the large T-binding domain, clearly bound large T but failed to become hyperphosphorylated. A possible interpretation of these observations is that the sequences N terminal to the large T-binding domains are responsible for kinase recognition and that the sequences C terminal to the large T-binding domains (i.e., exon 23) are the actual sites of cell cycle-dependent phosphorylation.

In all tumors in which mutant pRB is detected, pRB fails to bind to large T and is, presumably, inactive (15, 28, 29). Surveying the *RB1* mutations which have been characterized, Hu et al. (16) have noted that these mutations usually disrupt or delete regions defined as necessary for pRB-large T interactions. Mutant RB proteins detected in non-RB tumors are probably nonfunctional and have the same consequence as the absence of pRB in RB tumors. In contrast to the loss of proliferative control resulting from inactivation of the pRB protein, mutations in regions outside the large T-binding domain might be expected to cause a rather different phenotype. Specifically, phosphorylation mutants, arising because of the inability of the kinase to bind to pRB or because of the loss of phosphorylation sites, could act in a dominant-negative manner by blocking the binding of wild-type RB protein to a complex but could be unable to be hyperphosphorylated to an inactive form. Such dominant-negative mutants might lock cells in G_1 or G_0 and block cellular proliferation. Introduction of the ΔPvu or ΔP5-6-7-8 mutations into a normal cell line may constitutively suppress proliferation through competition with the wild-type pRB, yet these mutants may be unable to be modified to the inactive, hyperphosphorylated form. Alternatively, such mutant proteins may sequester the complex in which pRB is normally associated, rendering it inactive because of disruption of functional domains in mutant pRB. Such action would result in a lack of proliferative control, preventing the cell from entering quiescence (G_0) or remaining in G_1. Loss of proliferative control by this mechanism has yet to be observed in naturally occurring tumors.

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