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Biochemistry

Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering

(nuclear binding)

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ABSTRACT **We have examined the functional consequences of mutations present in defective alleles of the retinoblastoma susceptibility gene (*RB1*) isolated from two spontaneously arising tumors. Unlike cDNA clones expressing the wild-type protein p110^{Rb}, those encoding the two mutant proteins failed to induce the appearance of senescent cells in transfected Saos-2 human osteosarcoma cells. The mutant proteins were also defective in binding to the E1A oncoprotein, were unable to become hyperphosphorylated, and failed to become tightly associated with nuclear structures. We conclude that mutations in two distinct regions of the protein concomitantly affect these four aspects of p110^{Rb} function.**

The gene responsible for susceptibility to retinoblastoma (*RB1*) has been identified and isolated by molecular cloning (1–3). Mutations of the *RB1* gene have been found in retinoblastomas (2–5), osteosarcomas (2–6), bladder carcinomas (7), small cell lung carcinomas (8–10), and a prostate carcinoma (11). It is unclear how wild-type protein p110^{Rb} regulates normal cell proliferation and why its inactivation leads to neoplastic growth. Some clues to its regulatory function may be provided by the observation that the phosphorylation state of p110^{Rb} varies through the cell cycle, with a period of maximal phosphorylation being attained in S phase and reduced phosphorylation being observed after M phase (12–14). Moreover, end-stage differentiation of cells in culture (for example HL-60 cells treated with phorbol ester or retinoic acid) and their exit from the growth cycle is accompanied by a reduction of the amount of the phosphorylated form of p110^{Rb} (15, 16). The p110^{Rb} observed in quiescent T lymphocytes becomes heavily phosphorylated after growth stimulation by mitogens (17). These observations suggest that p110^{Rb} acts in a cell cycle-specific way and that phosphorylation may be a principal means of controlling its growth-regulatory activities. Although it has been suggested that p110^{Rb} contains several "consensus" sites of phosphorylation for the cell cycle protein kinase p34^{cdc2} (18), direct proof that p34^{cdc2} normally phosphorylates p110^{Rb} has not been reported.

p110^{Rb} is able to form tight complexes with the oncoproteins encoded by the transforming genes of human adenovirus (19), simian virus 40 (SV40) (20), and human papilloma virus (21). The SV40 large tumor antigen (T antigen) (14) and the adenovirus E1A protein (D.J.T., unpublished results) bind preferentially to the underphosphorylated form of p110^{Rb}. If these viral oncoproteins interfere with the growth-inhibitory activity of the p110^{Rb} protein, as some have speculated, this would suggest that it is the underphosphorylated form of p110^{Rb} that mediates its growth-inhibitory activity. Recently, three groups have found that similar regions of p110^{Rb} synthesized *in vitro* are required for interaction with E1A (22) and T antigen (23, 24). Two noncontiguous regions of p110^{Rb} are required for binding to E1A, encompassing amino acid residues 393–572 and residues 646–772 (22). To date, all mutated forms of p110^{Rb} found in tumors have alterations in one or both of these domains.

We have investigated the function of p110^{Rb} by studying mutants of the *RB1* gene isolated from a retinoblastoma tumor and a small cell lung carcinoma. We developed a biological assay for p110^{Rb} function that demonstrated that the wild-type *RB1* allele induces senescent-appearing cells in transfected cultures of Saos-2 cells, but the mutant alleles are indeed incapable of doing so.

In addition, using vectors expressing p110^{Rb} proteins tagged with a novel epitope, we found that these two unrelated mutant alleles express proteins defective in three properties of wild-type p110^{Rb}: the ability to bind to the E1A oncoprotein, the ability to become hyperphosphorylated, and the ability to become tightly associated with nuclear structures.

METHODS

Construction of Expression Plasmids. p110^{Rb} expression plasmids were constructed in pSVE, a variant of the SV40 early promoter-based vector pJ3Ω (25). The p110^{Rb}–hemagglutinin epitope fusion plasmid (named pRbWT-HA/SVE) and missense mutation 567L were constructed by using single-strand oligonucleotide mutagenesis (26) and confirmed by DNA sequencing. The p110^{Rb}–hemagglutinin epitope fusion results in the addition of the amino acid sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Lys to the natural carboxyl terminus of p110^{Rb}. A ligation hybrid between pH592 [a cloned fragment generated by *in vitro* amplification of NCI-H592 mRNA spanning the boundaries of exon 22, which differs from the wild-type *RB1* sequence only in the deletion of exon 22 (27)] and pRbWT-HA/SVE generated pRbΔ22-HA/SVE. Thus, plasmids pRb567L-HA/SVE and pRbΔ22-HA/SVE differ from pRbWT-HA/SVE at codon 567 and codons 738–775, respectively.

Transfection of Saos-2 Human Osteosarcoma Cells. Saos-2 cells were provided by the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Hazelton). The day before transfection, 2×10^6 cells were seeded onto 9-cm tissue culture dishes. Cells were transfected by using the calcium-phosphate method of Chen and Okayama (28), with 1 μg of supercoiled pBabe-puro (29) and 10 μg of the indicated expression plasmids. DNA precipitates

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

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were left on the cells for 6 hr at 37°C in a 2.5% CO_2 atmosphere, following which the cultures were refed with fresh medium and the atmosphere was adjusted to 5% CO_2. The day after transfection, the cultures were split 1:4. The second day after transfection, the cultures were fed with Dulbecco's modified Eagle's medium/10% fetal bovine serum containing 1 μg puromycin (1 μg/ml; Sigma) and were refed at 3-day intervals for 6 weeks. Control cultures (without transfected DNA) showed no living cells after 3 days in this selection medium.

Immunoprecipitation of Transiently Expressed p110^{Rb}. Subconfluent 60-mm cultures of COS-1 cells were transfected by using the DEAE-dextran technique (30). Cells were labeled for 4 hr in methionine- or phosphate-deficient medium containing 100 μCi (1 Ci = 37 GBq) of Tran^{35}S-label (ICN) or [^{32}P]orthophosphate. Cultures were lysed in 1 ml of ELB [50 mM Hepes, pH 7.0/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40/aprotinin (1 μg/ml; Sigma)/leupeptin (1 μg/ml; Sigma)/phenylmethylsulfonyl fluoride (50 μg/ml)/2 mM sodium pyrophosphate/1 mM sodium orthovanadate] for 30 min at 0°C. Clarified lysates were immunoprecipitated with 2 μl of monoclonal antibody-containing ascites fluid and washed in ELB. For double precipitations, samples boiled in 2% SDS were diluted 200-fold with ELB containing 1% Nonidet P-40 and reprecipitated with anti-hemagglutinin monoclonal antibody 12CA5 and protein A-Sepharose as before. Coprecipitation of p110^{Rb} with E1A was performed by incubation of clarified lysates of COS-1 cells in ELB with 100 ng of purified E1A produced in bacteria (31), provided by Jeff Settleman (Whitehead Institute, Cambridge, MA). After 30 min on ice, the E1A-containing complexes were precipitated by using anti-E1A monoclonal antibody M73 (provided by Ed Harlow of Massachusetts General Hospital, Charlestown, MA) as described above, then reprecipitated by using 12CA5 to distinguish the p110^{Rb} encoded by the transfected plasmids from the endogenous monkey p110^{Rb}.

Cell Fractionation. Cultures of transfected COS-1 cells labeled with Tran^{35}S-label as above were rinsed with ice-cold phosphate-buffered saline and drained well. Cells were swollen on their dishes by using 1 ml of ice-cold 10 mM Tris, pH 7.5/10 mM KCl/2 mM $MgCl_2$/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)/phenylmethylsulfonyl fluoride (50 μg/ml) for 10 min. Swollen cells were scraped from the dish and transferred to a 1-ml Dounce homogenizer and disrupted with 20 strokes using the A pestle. Cell homogenates were transferred to a microcentrifuge tube and centrifuged for 5 min at 2000 × *g*. The clarified supernate was carefully withdrawn and adjusted to 50 mM Hepes, pH 7.0/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40. The pellet, which contained nuclei and membrane fractions by microscopic examination, was resuspended in ELB by vortexing briefly. After a 30-min extraction period on ice, both fractions were clarified by centrifugation at 15,000 × g for 15 min and immunoprecipitated using anti-hemagglutinin monoclonal antibody 12CA5 (37).

Two-Dimensional Chymotryptic Peptide Mapping. Cultures of COS-1 cells (six 15-cm dishes for each plasmid) were transfected with p110^{Rb} expression plasmids as above. Cells were labeled 2 days after transfection with [^{32}P]orthophosphate (2 μCi per plate) for 4 hr, then immunoprecipitated via direct scale-up of the immunoprecipitation described above. Proteins separated by SDS gel electrophoresis were electrophoretically transferred to nitrocellulose, detected by autoradiography, and excised. Proteins were digested *in situ* with chymotrypsin B4 (Sigma) by using the method of Luo and Sefton (33), oxidized with performic acid, and subjected to two-dimensional peptide mapping using thin-layer electrophoresis followed by ascending chromatography (34).

RESULTS

We began by constructing cDNA expression vectors containing mutations found in *RB1* alleles in the retinoblastoma tumor Rb-104 and the small cell lung carcinoma NCI-H592 (27). The mutant *RB1* allele in Rb-104 contains a missense mutation directing replacement of a normally present serine with a leucine at residue 567 (9) and is designated here as 567L. A point mutation in the *RB1* allele found in NCI-H592 inactivates a splice acceptor site and results in a deletion of exon 22 (codons 738–775) from the processed *RB1* mRNA (27); a vector encoding a p110^{Rb} protein deleted in the identical codons is denoted here as Δ22.

To analyze the mutant proteins encoded by these alleles with facility and without contamination by endogenous wild-type protein, we developed an expression system that allows synthesis of mutant or wild-type p110^{Rb} tagged with an epitope of the influenza virus hemagglutinin antigen. This vector-encoded protein can be immunoprecipitated by using a monoclonal antibody (12CA5) that recognizes the hemagglutinin epitope tag (37). In preliminary experiments not shown here, we found that the epitope-tagged form of p110^{Rb} is indistinguishable from naturally occurring protein with respect to its nuclear localization, tightness of association with the nucleus, and extent of phosphorylation. Further, transfection of vectors expressing both the natural wild-type protein and the epitope-tagged variant gave rise to Saos-2 cells with aberrant morphology during cotransfection experiments (see below) to a similar extent. Thus by all presently testable criteria, the epitope-tagged molecule is functionally equivalent to the wild-type protein, and we will refer to the epitope-tagged wild-type protein as "wild type" in further discussions.

Since the 567L and Δ22 alleles appeared to confer growth advantage on the spontaneously arising tumors from which they were isolated, we presumed that these alleles specify functionally inactive p110^{Rb}. Nonetheless, we wished to demonstrate this by a direct measure of their biological activities. We initially attempted to measure the ability of p110^{Rb} expression plasmids to inhibit the growth of cultured cells, specifically the Saos-2 human osteosarcoma cells that themselves lack wild-type p110^{Rb} (35). In preliminary tests, transfection of the wild-type p110^{Rb} expression plasmids reduced the number of puromycin-resistant colonies of Saos-2 cells after cotransfection of p110^{Rb} expression plasmids with a puromycin-resistance gene (29), but the extent of this effect has been variable.

We observed an additional distinct response that proved especially useful in assaying the activity of transfected *RB1* alleles. As expected, we found that puromycin effectively killed cultures of Saos-2 cells within 4 days. When cultures were cotransfected with the puromycin-resistance plasmid and a plasmid expressing wild-type p110^{Rb}, as many as 10% of the cells escaped killing by puromycin. These surviving cells, as shown in Fig. 1 *B* and *E–H*, were large and flat with indistinct and occasionally multiple nuclei. These cells contained numerous vacuoles, and some cells exhibited retraction of their cytoplasm, resulting in a pseudoaxonal morphology. In all respects, these cells resembled the senescent phenotype of primary fibroblasts seen after extended passage. Importantly, such senescent cells were never seen in cultures transfected either with the puromycin-resistance gene or with p110^{Rb} expression vectors alone. Presumably, the cells are not killed by puromycin because they have stabilized the expression of the puromycin-resistance plasmid.

The frequency of the appearance of these senescent cells (5–10%) in transfected cultures was as much as 1000-fold greater than the highest frequency of stable transfection of puromycin resistance in our experiments, though transient transfection efficiencies in this range are common (28). Thus it seems likely that the induction of this senescent phenotype

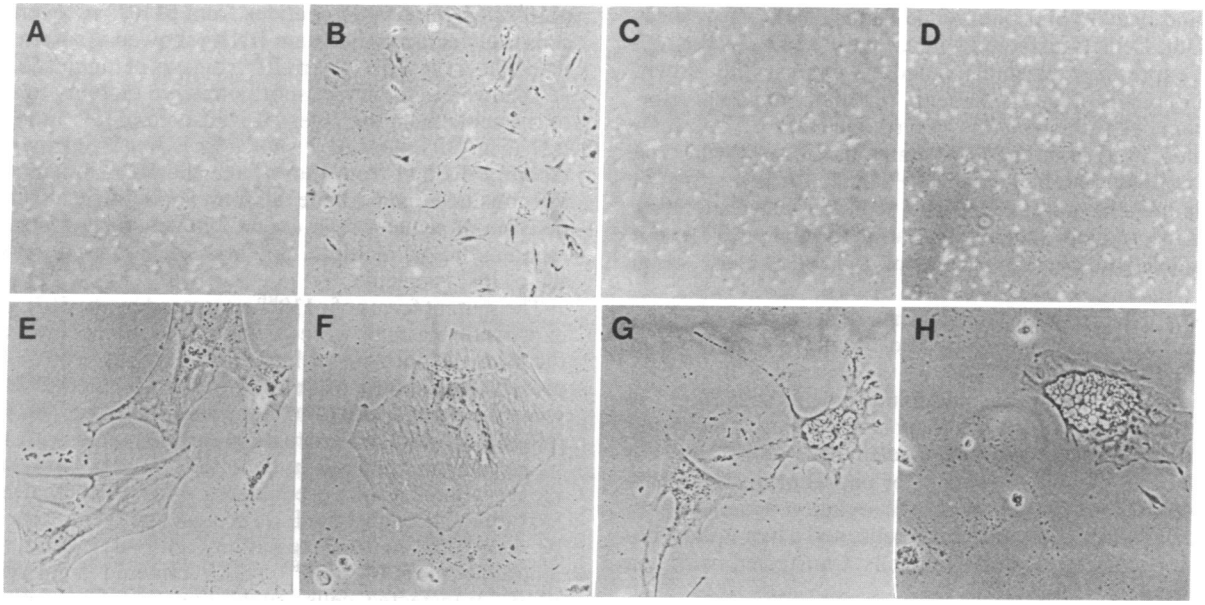

FIG. 1. Photomicrographs of Saos-2 cultures transfected with p110^{Rb} expression plasmids pSVE (*A*), pRbWT-HA/SVE (*B* and *E–G*), pRb567L-HA/SVE (*C*), or pRbΔ22-HA/SVE (*D*) and selected in growth medium containing puromycin (1 μg/ml; Sigma) for 7 days. Photomicrographs of representative fields of cultures are shown. The large, flattened cells observed in *B* and *E–G* are not observed except in cultures transfected with the wild-type p110^{Rb} expression plasmid. (*A–D*, ×20; *E–G*, ×70.)

is not dependent upon the inefficient processes of DNA integration that normally limit the establishment of stably transfected cells. Independent of the mechanism, it is clear that these senescent cells present a highly sensitive and facile assay for the functioning of the intact *RB1* allele. Accordingly, we tested the two mutant plasmids and found that neither induced this senescent phenotype (Fig. 1 *C* and *D*). We concluded that by this assay the mutant proteins lack a biological activity shown by the wild type. We speculate that loss of this function contributed to the pathogenesis of the Rb-104 and NCI-H592 tumors in which they arose.

We sought to extend our analysis of the mutant p110^{Rb} proteins by analyzing biochemically based phenotypes of the wild-type and mutant proteins. The proteins expressed by the plasmid vectors encoding both the wild-type and mutant forms of p110^{Rb} were analyzed by transfection of the plasmid DNAs into COS-1 cells followed by immunoprecipitation of cell lysates with monoclonal antibody 12CA5, which recognizes the hemagglutinin epitope on the tagged p110^{Rb} (37). Parallel cultures of COS-1 cells were transfected with control, wild-type, or mutant DNAs and labeled for 4 hr with [^{35}S]methionine or alternatively with [^{32}P]orthophosphate and precipitated with a monoclonal antibody recognizing the hemagglutinin epitope. Fig. 2*A* shows the immunoprecipitated proteins from [^{35}S]methionine-labeled cells. Cells transfected with plasmid vector expressing the wild-type p110^{Rb} expressed both a fast migrating form of p110^{Rb} and a set of slowly migrating bands, corresponding to the underphosphor-

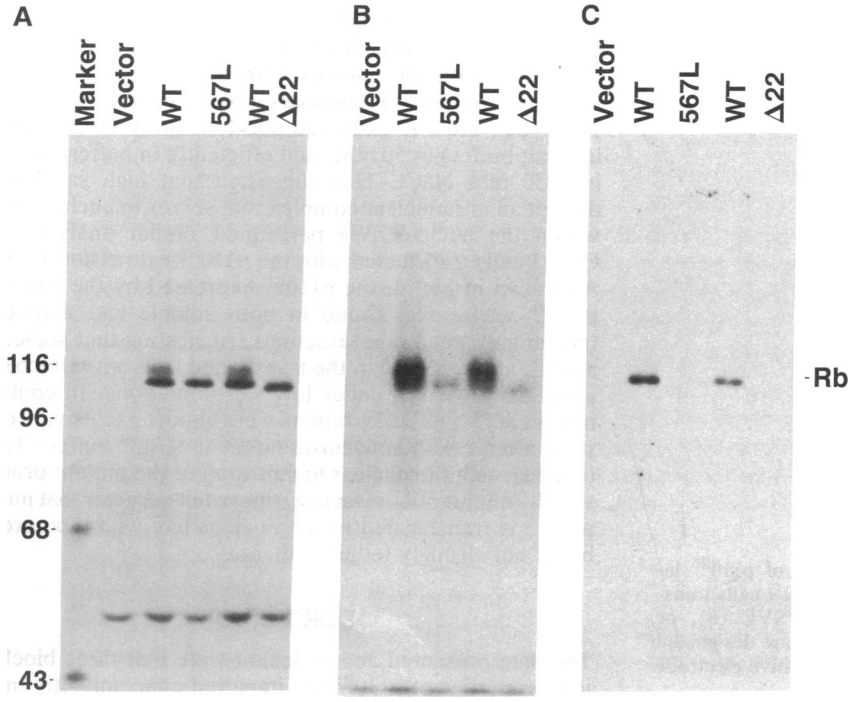

FIG. 2. Analysis of p110^{Rb} expressed in COS-1 cells transfected with pSVE (Vector), pRbWT-HA/SVE (WT), pRb567L-HA/SVE (567L), or pRbΔ22-HA/SVE (Δ22). (*A*) Cultures labeled with Tran^{35}S-label were analyzed by immunoprecipitation using monoclonal antibody 12CA5, which recognizes the hemagglutinin epitope. (*B*) Cultures labeled with [^{32}P]orthophosphate were analyzed by double immune precipitation using first 21C9 anti-p110^{Rb} antibody (provided by Jonathan Horowitz of Duke University, Durham, NC) followed by reprecipitation using 12CA5. (*C*) p110^{Rb} expressed by the transfected plasmids was analyzed for its ability to complex the E1A protein by double precipitation first using purified E1A protein and monoclonal antibody M73 recognizing E1A and then reprecipitated using 12CA5 antibody. ^{14}C-labeled molecular size markers (Sigma) and their respective molecular masses (in kDa) are shown in *A*. Rb, p110^{Rb}.

ylated and heavily phosphorylated forms of $p110^{Rb}$, respectively (36). Cells transfected with either the 567L or the Δ22 $p110^{Rb}$ expression plasmids failed to express the slowly migrating bands. Fig. 2*B* presents proteins immunoprecipitated from cells labeled with [^{32}P]orthophosphate. This analysis demonstrates that the slowly migrating form of wild-type $p110^{Rb}$ contains the bulk of the ^{32}P label. A less intense ^{32}P signal is present that is associated with the faster migrating form. Thus, the more rapidly migrating form of $p110^{Rb}$ is not unphosphorylated but rather phosphorylated at a low, basal level.

Cells transfected with plasmids encoding the mutant DNAs 567L and Δ22 expressed only the basally phosphorylated form of $p110^{Rb}$, as shown both by the absence of the slowly migrating $p110^{Rb}$ from [^{35}S]methionine labeled cells (Fig. 2*A*) and by the absence of the intensely labeled, slowly migrating form of $p110^{Rb}$ from the ^{32}P-labeled cells (Fig. 2*B*). We conclude that two distinct types of phosphorylation events modify $p110^{Rb}$: a low, basal phosphorylation occurs in both mutant and wild-type transfected cells, and a hyperphosphorylation occurs exclusively in cells transfected with the wild-type $p110^{Rb}$ plasmid.

To assess further the differences between the modifications of the hyperphosphorylated wild-type versus the basally phosphorylated mutant $p110^{Rb}$ molecules, we performed two-dimensional mapping of chymotryptic digests of proteins immunoprecipitated from COS-1 cells transfected with $p110^{Rb}$ expression vectors. As shown in Fig. 3*A*, 10 heavily ^{32}P-labeled spots and at least 10 other less intensely labeled spots are identified on the map of the wild-type

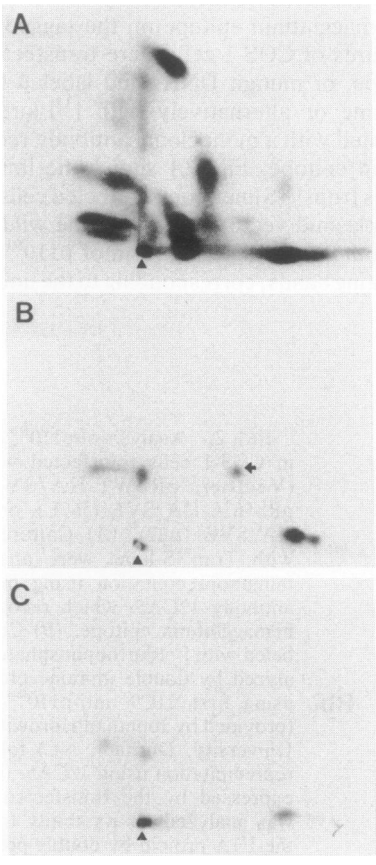

FIG. 3. Two-dimensional chymotryptic analysis of $p110^{Rb}$ labeled with [^{32}P]orthophosphate from cultures of COS-1 cells transfected with pRbWT-HA/SVE (*A*), pRbΔ22-HA/SVE (*B*), or pRb567L-HA/SVE (*C*). The locations of the origins are designated by triangles, and the maps are arranged with the positive electrode at the right.

$p110^{Rb}$. Chymotryptic peptides from $p110^{Rb}$ recovered from cells transfected with mutant DNAs showed a pattern simpler than that of the wild-type $p110^{Rb}$; digests of mutant 567L (Fig. 3*C*) showed four distinct spots similar in mobility to a subset of the spots from the digested wild-type $p110^{Rb}$, and mutant Δ22 (Fig. 3*B*) showed 5 spots, including 1 (arrow) with a mobility distinct from those from the 567L mutant $p110^{Rb}$. We conclude that both the missense mutation at 567L and the deletion of sequences in exon 22 have global effects on the modification of multiple, potential phosphorylation sites in the $p110^{Rb}$ molecule.

The mutant forms of $p110^{Rb}$ expressed by the 567L and Δ22 expression plasmids were also unable to form complexes with the E1A oncoprotein of human adenovirus type 5. Fig. 2*C* presents the results of an experiment in which COS-1 cells were transfected with $p110^{Rb}$ expression vectors and [^{35}S]methionine-labeled cell extracts were incubated with purified E1A protein expressed in bacteria (37). The proteins complexed with E1A were precipitated with monoclonal antibody M73 directed against the E1A protein and then reprecipitated by using the anti-hemagglutinin antibody. Only the fast migrating form of $p110^{Rb}$ was recovered from wild-type $p110^{Rb}$-transfected cells, suggesting that E1A forms complexes preferentially with the less phosphorylated form of $p110^{Rb}$, similar to the preference exhibited by the SV40 T antigen shown in other studies (36). No proteins precipitable with E1A and anti-hemagglutinin were recovered from extracts of cells transfected with the control vector alone or with the 567L or Δ22 mutant DNAs. These results are in agreement with the identification of E1A binding domains of $p110^{Rb}$ (22–24) and with the observed lack of E1A or T-antigen binding to other altered forms of $p110^{Rb}$ arising in tumors (32, 35).

Since the activity of $p110^{Rb}$ presumably requires correct intracellular localization, we performed immunofluorescence analysis to determine if the mutant $p110^{Rb}$ forms were translocated to the nucleus of transfected COS-1 cells (data not shown). We found that both mutant and wild-type proteins encoded by our expression vector were predominantly localized to the nucleus, showing a mottled pattern that was excluded from the nucleolus. Immunofluorescent patterns of mutant forms of the protein were indistinguishable from the wild-type pattern with the exception that roughly 20% of the cells expressing the Δ22 $p110^{Rb}$ demonstrated weak fluorescent staining of the cytoplasm in addition to nuclear staining.

We next tested whether these mutant forms of $p110^{Rb}$ are associated with the nucleus with the same affinity as the wild-type protein. Preliminary experiments showed that wild-type $p110^{Rb}$ is extracted poorly from isolated nuclei in low salt buffers (<50 mM) and efficiently in buffers containing 250 mM NaCl. This suggested that high salt buffers disrupt an intranuclear complex that serves to anchor $p110^{Rb}$ within the nucleus. We performed similar analyses with COS-1 cells transfected with the $p110^{Rb}$ expression vectors. As shown in Fig. 4, the $p110^{Rb}$ expressed by the wild-type $p110^{Rb}$ vector was found in both soluble and particulate (nuclei and membrane) fractions, suggesting that some, but not all, of the $p110^{Rb}$ in the transfected cells was attached to a nuclear structure under low salt conditions. In contrast, mutant 567L and Δ22 proteins were almost exclusively cytosolic after cell homogenization in low salt buffer. Taken together with the nuclear localization of the mutant proteins seen by immunofluorescence, this result suggests that mutant $p110^{Rb}$ is translocated to the nucleus like wild-type protein but is not strongly tethered there.

DISCUSSION

The data presented above demonstrate that three biochemical properties of $p110^{Rb}$ are impaired concomitantly in two

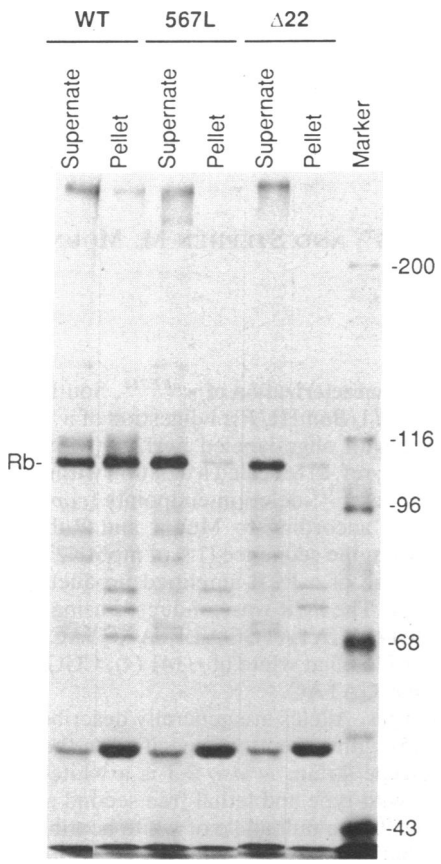

FIG. 4. Cell fractionation of COS-1 cells transfected with pRbWT-HA/SVE (WT), pRb567L-HA/SVE (567L), or pRbΔ22-HA/SVE (Δ22) and fractionated in low salt buffer into cytosolic and particulate fractions by centrifugation. $p110^{Rb}$ expressed by the transfected plasmids was recovered by immunoprecipitation using antibody 12CA5, which recognizes the hemagglutinin epitope. Radiolabeled proteins recovered from the soluble (supernate) or particulate (pellet) fractions are indicated, as well as the respective molecular masses (in kDa) of ^{14}C-labeled molecular size markers. Rb, $p110^{Rb}$.

different nonfunctional mutants: (*i*) the ability to become hyperphosphorylated, (*ii*) the ability to associate with the E1A protein, and (*iii*) the nuclear affinity measured in disrupted cells. The fact that these altered properties are shared by distinct mutant proteins suggests that these activities are functionally linked.

We have shown here that two naturally occurring mutant proteins altered in a region found by others to be critical for E1A and T-antigen binding (22–24) also failed to bind these oncoproteins. Taken together with evidence that other naturally occurring mutations impair oncoprotein binding, our observations suggest that these viral oncoproteins mimic the structure of some cellular protein(s), having $p110^{Rb}$-binding domains similar to that of E1A. We propose that the binding of such cellular protein(s) to $p110^{Rb}$ is critical to the growth-regulating activity of $p110^{Rb}$ and to the tethering of $p110^{Rb}$ to a subnuclear structure. Moreover, since the mutant proteins are underphosphorylated, we suggest that it is a complex of $p110^{Rb}$ and a cellular E1A analogue that is a requisite substrate for phosphorylation of $p110^{Rb}$. Finally, to the extent that the basal phosphorylation is shown to be physiologically modulated, our data indicate that at least two distinct signals impinge upon $p110^{Rb}$: one potentially cell cycle independent, responsible for basal phosphorylation, and the other leading to a cell-cycle dependent hyperphosphorylation that is abrogated in the mutant forms of $p110^{Rb}$ described here.

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