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Biological Function of the Retinoblastoma Protein Requires Distinct Domains for Hyperphosphorylation and Transcription Factor Binding

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Despite the importance of the retinoblastoma susceptibility gene to tumor growth control, the structural features of its encoded protein (pRb) and their relationship to protein function have not been well explored. We constructed a panel of deletion mutants of pRb expression vectors and used a biological assay for pRb that measures growth inhibition and morphologic changes in pRb-transfected Saos-2 cells to correlate structural alterations of the pRb coding region with function. We tested the deleted proteins for the ability to bind to viral oncoprotein E1A and to the transcription factor E2F. We also measured the ability of the mutant proteins to become hyperphosphorylated in vivo and to be recognized as substrates in vitro by a cell cycle-regulatory kinase associated with cyclin A. We identified two regions of pRb that are required for E2F binding and for hyperphosphorylation. E1A binding domains partially overlap but are distinct from both of these other two regions. Biological function of pRb is dependent on retention of the integrity of both of these biochemically defined domains. These data support the model that pRb is a transducer of afferent signals (via the kinase that phosphorylates it) and efferent signals (through transcription factor binding), using distinct structural elements. Preservation of both of these features is essential for the ability of pRb to induce growth inhibition and morphologic changes upon reintroduction into transfected cells.

The retinoblastoma (Rb) tumor suppressor gene product (pRb) is a 110-kDa nuclear protein that is modified by cell cycle-regulated hyperphosphorylation (4, 8, 24). pRb is able to complex with the oncoproteins encoded by several DNA tumor viruses, including adenovirus E1A (35), and simian virus 40 (SV40) T antigen (9). Recently, pRb has been shown to be able to bind to the cellular E2F transcription factor (1, 2, 6), suggesting that transcriptional regulation of gene expression is a facet of tumor suppression by pRb.

Thorough mutational analysis of the structural and functional elements of pRb has not been reported. Mutant forms of the Rb-1 gene identified in human tumor cells all affect the regions of pRb required for E1A binding, and thus all are defective in binding to this oncoprotein (16). Additionally, these mutants lack the ability to become hyperphosphorylated and to become tightly associated with the cell nucleus (15, 20, 34). No other functional domains of pRb have been identified, although large amino-terminal deletion mutants of murine pRb encoded proteins that were poorly phosphorylated (13). At least one naturally occurring mutant form of pRb containing a mutation of the E1A-binding domain is unable to interact with E2F (2). Hiebert et al. (14) has recently shown that E2F binds to a bacterially expressed fusion protein encoding the carboxyl terminus of pRb but does not bind to a similar protein that is truncated at codon 792 or altered in the E1A-binding domain.

Analysis of the biological properties of pRb or its mutant forms has proven difficult. A retrovirus encoding pRb has been reported to reduce the tumorigenic behavior of infected cells (3, 18), but retroviral vectors expressing pRb mutants have not been reported. Goodrich et al. reported growth arrest of cells microinjected with high concentrations of a fusion protein encoding the carboxy-terminal portion of pRb or with baculovirus-expressed pRb (12). Unfortunately, neither of these observations has led to a reliable or quantifiable assay of pRb function for use in analysis of Rb gene mutants.

We have developed an assay that measures growth inhibition and morphologic changes of cells caused by pRb. In this assay, pRb induces cell cycle arrest and an aberrant flat-cell morphology after cotransfection of cells with a pRb expression vector and a puromycin resistance gene (34). A large fraction of these cotransfected cells fail to divide and demonstrate resistance to puromycin for periods of time much longer than those exhibited by cells transiently transfected with a drug resistance gene without the pRb expression vector. Transfected cells also adopt an enlarged, vacuolated morphology similar to that of cells experiencing terminal senescence. Enumeration of the aberrant flat cells (which is the phenotype of nearly all of the cells not killed by the puromycin) is a measure of the efficiency of cell cycle blockade and morphologic changes induced by pRb.

We report here the construction of a panel of deletion mutants of pRb expression vectors that span the length of the pRb coding region. We have tested each pRb mutant for biological function in the assay described above and have measured the biochemical parameters of E1A and E2F binding and of cell cycle-regulated hyperphosphorylation. We have identified distinct segments of pRb that are required for each of these activities. Retention of the ability of pRb to induce morphological changes and cell division arrest in the assay described above is dependent upon conservation of all of these biochemically defined domains.

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MATERIALS AND METHODS

Construction of mutant pRb expression plasmids. Two types of pRb expression vectors were used in this study. The first, pRbWT3HA/SVE (34), contains an SV40 early promoter as well as splicing and polyadenylation signals from small-t antigen. It was used in experiments that measure the biological effects of the pRb deletion mutants. The second vector, pRbWT3HA/TM1 (33), is a derivative of the T7-based vector pTM1 (26) that utilizes T7 RNA polymerase encoded by a recombinant vaccinia virus vector to direct synthesis of the encoded pRb mutant protein. Proteins encoded by both vectors are identical to the wild-type pRb but have an additional 10 amino acids appended to their carboxyl termini. These amino acids (YPYDVPDYAK) comprise an epitope that is recognized by the monoclonal antibody (MAb) 12CA5 (36), which is directed against the hemagglutinin (HA) antigen of influenza virus.

Mutants were constructed by using oligonucleotide-directed mutagenesis (21) in plasmid pRbWT3HA/SVE, and mutations were confirmed by DNA sequencing. Initially, *Xho*I restriction endonuclease sites were introduced into the pRb coding region. The sequence 5′-CTCGAG-3′ that comprises the *Xho*I site was substituted for two native codons of the pRb coding region. Each linker insertion mutant was named according to the location of the first of these two codons. To construct the deletion mutants described here, pairs of linker insertion mutants were digested with *Xho*I and *Sca*I (which cleaves in the vector sequences) and religated to form plasmids with deletions between two *Xho*I sites. Naming of the deletion mutants corresponds to that described above for the linker insertion mutants. For example, mutant Δ37-89 contains the sequence CTCGAG (encoding Leu-Glu) replacing the nucleotides encoding codons 37 to 90 inclusive, and mutant Δ89-140 contains CTCGAG as a replacement for codons 89 to 141. Segments of DNA spanning the mutation site were then shuttled into plasmid pRbWT3HA/TM1 to construct the identical mutant in this alternate vector.

Expression of pRb in COS-7 cells. Plasmids encoding mutant pRb forms under the control of the SV40 promoter (in plasmid pSVE) were transfected into cultures of COS-7 cells. Cultures were then labeled with ^{35}S-Translabel (ICN) and analyzed by immunoprecipitation with the anti-HA epitope antibody 12CA5 as described previously (34).

Biological assays. Saos-2 cells (American Type Culture Collection) were grown in medium containing 10% fetal bovine serum and seeded into individual wells of a 24-well tissue culture plate at a density of 10^5 cells per well. The cells were transfected with the BES-buffered saline (BBS; BES is *N,N*-bis[2-hydroxyethyl]-2-amino ethanesulfonic acid)-$CaCl_2$ method of Chen and Okayama (7), using 1 μg of the indicated pRb expression plasmid and 20 ng of pBABEpuro (25) for each well. pBABEpuro is a plasmid vector that encodes the puromycin resistance gene in the context of a retroviral vector plasmid. While it can be used to generate recombinant retroviruses, we used it simply as a source of the puromycin resistance gene via plasmid transfection. We have obtained similar results (33a) with use of the G418 resistance plasmid pSV2-neo, which contains no retroviral sequences. We have used pBABEpuro routinely for this assay since puromycin is more rapid than G418 at killing untransfected cells, and results of the assay become clear earlier.

On the day after transfection, 20% of the cells in each well were divided into the wells of a 96-well plate. Two days after transfection, the cells were fed with medium containing puromycin (1 μg/ml), and they were fed every 3 days with the same medium. After 10 days of drug selection, cultures were fixed in methanol and stained. Wells were photographed with a dissecting photomicroscope, and individual cells were counted on the photographic prints.

Expression of pRb mutants in CV-1 cells and detection of pRb. About 2 × 10^6 CV-1 cells grown on 9-cm-diameter dishes were infected with vaccinia virus vector vTF7.3 (26), which encodes the phage T7 RNA polymerase, and then lipofected (29) with 5 μg of pTM1 (T7-based) vector DNAs encoding the HA epitope-tagged forms of pRb deletion mutants as described previously (33).

For Fig. 4A, transfected cultures were lysed in MLB (25 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40 [NP-40], 1 mM dithiothreitol [DTT], 2 mM sodium pyrophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg of aprotinin [Sigma] per ml, 1 μg of leupeptin [Sigma] per ml, 50 μg of phenylmethylsulfonyl fluoride [PMSF] per ml) and clarified by centrifugation. One percent of each cell extract was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (6% polyacrylamide gel), transfer to Immobilon filters (Millipore), and detection by Western immunoblotting with antibody 12CA5, which recognizes the carboxy-terminal epitope.

For Fig. 4B, transfected cells were labeled with ^{35}S-Translabel (ICN) (100 μCi/ml per plate) for 4 h before lysis in MLB and clarification. Twenty percent of each extract was mixed with 1 μg of bacterially produced E1A protein, incubated on ice for 1 h, and subjected to immunoprecipitation with anti-E1A MAb M73 (generously provided by Ed Harlow) before analysis on an SDS–6% polyacrylamide gel. ^{35}S-labeled proteins were detected after fluorography by using diphenyloxazole. Gels were exposed for 16 h.

For Fig. 4C, cultures transfected identically to those described above were labeled with 100 μCi of $^{32}P_i$ per ml for 4 h, then lysed in MLB, and immunoprecipitated as described above with 20 μl of MAb 12CA5. Half of each sample was analyzed by SDS-PAGE (6% polyacrylamide gel), and radiolabeled proteins were detected by direct autoradiography. Exposure time was 6 h. The second half of each sample was used for phosphopeptide mapping.

For Fig. 4D, cultures transfected identically to those described above were not radiolabeled but were immunoprecipitated as described for Fig. 4C. Additionally, two plates of vTF7.5-infected CV-1 cells were transfected with pCycANHA/TM1, which contains an epitope-tagged version of the human cyclin A gene (obtained from Jon Pines). The HA-tagged cyclin A was purified along with its associated kinase(s) by an identical immunoprecipitation in MLB. The immune complexes were washed once in 50 mM Tris · Cl (pH 7.4)–1 mM DTT, and the immunoprecipitated proteins were eluted in 25 μl (per plate) of the same buffer containing 40 μg of the peptide cognate to MAb 12CA5 (YPYDVPDYA) per ml. Eighty percent of the each eluted pRb mutant protein was mixed with 5% of the eluted cyclin A in a final reaction volume of 40 μl containing 50 mM Tris · Cl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 500 μCi of [γ-^{32}P]ATP (>3,000 Ci/mmol; ICN) per ml. Half of each sample was analyzed by SDS-PAGE (6% polyacrylamide gel) and direct autoradiography. Exposure time was 3 h.

E2F-binding assays. A method of preparation of whole-cell extracts (28) was adapted to immunoprecipitation as follows. One 9-cm plate of CCL64 cells (from Seong-Jin Kim, National Institutes of Health) was infected with vTF7.5 and transfected with pTM1 plasmids encoding the indicated

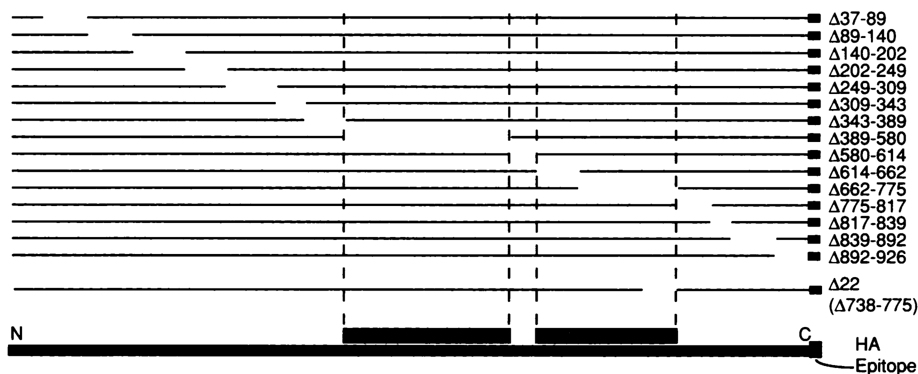

FIG. 1. Schematic representation of deletion mutants of pRb expression vectors used in this study. The coding region of pRb is represented by the heavy line at the bottom, locations of E1A-binding domains are represented by shaded boxes, and the synthetic epitope tag is represented by the box at the carboxyl terminus. Each mutant is depicted as a gapped line, with the size and location of the gap proportional to the site of the mutation. Each mutant is designated by the location of the first of the two codons that were converted into an *XhoI* restriction site (CTCGAG) prior to deletion of sequences between adjacent *XhoI* mutant sites (see Materials and Methods). For example, mutant Δ37-89 contains the sequence CTCGAG replacing the nucleotides encoding codons 37 to 90 inclusive, and mutant Δ89-140 contains CTCGAG as a replacement for codons 89 to 141.

pRb forms, as described for Fig. 4. Twenty-four hours after the beginning of transfection, the cells were rinsed with cold *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered saline, scraped from the dish, and transferred to a microcentrifuge tube. The cells were washed twice more in HEPES-buffered saline, then resuspended in 100 μl of buffer A (100 mM KCl, 10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.4 mM NaF, 0.4 mM Na_3VO_4, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF), and stored on ice for 20 min. The total lysate volume was measured (approximately 150 μl), 1.67 volumes of buffer B (1.6 M KCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.4 mM NaF, 0.4 mM Na_3VO_4, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, 20% [vol/vol] glycerol) was added, and the tubes were mixed gently at 4°C for 30 min. Nuclei were pelleted by centrifugation in a refrigerated microcentrifuge at 13,000 rpm for 30 min, and the supernatant was transferred to a multiple dialysis unit (Bethesda Research Laboratories) equilibrated with buffer C (40 mM KCl, 20 mM HEPES [pH 7.9], 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 0.4 mM NaF, 0.4 mM Na_3VO_4, 0.5 mM DTT, 0.1 mM PMSF) for 90 min at 4°C. Dialyzed samples were recentrifuged at 13,000 rpm for 10 min, and the epitope-tagged pRb was immunoprecipitated with 20 μl of 12CA5 ascites fluid and 20 μl of protein A-Sepharose, washing the antibody-bound beads three times with buffer D (40 mM KCl, 20 mM HEPES [pH 7.9], 1.0 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 3 mg of bovine serum albumin per ml). Half of the washed beads were transferred to a fresh tube, drained well, and treated with 20 μl of buffer D containing 0.5% deoxycholate. After 5 min at room temperature, the supernatant was withdrawn, and 2.2 μl of 10% NP-40 was added. These samples were tested for E2F activity by mobility shift assay on 5% acrylamide gels run in Tris-acetate buffer, using ^{32}P radiolabeled duplex DNA (5'-GAGAAAGGGGCGCGAAACTAGT) derived from the adenovirus type 5 E2 promoter, (containing a single E2F-binding site [underlined]). In this experiment, expression and integrity of the mutant pRb forms were verified by radiolabeling of each culture with [^{35}S]methionine as described above and analysis of half of the immunoprecipitates by SDS-PAGE and fluorography (data not shown). ^{32}P radiolabel was distinguished from ^{35}S radiolabel by exposure of the dried mobility shift assay gel through one layer of heavy aluminum foil and the use of an intensifying screen.

Proteolytic phosphopeptide mapping of pRb. The method used was provided by Kunxin Luo and Bart Sefton (Salk Institute). An aliquot of mutant pRb proteins labeled in vivo with ^{32}P (the other half of each of the samples analyzed in the experiment shown in Fig. 4C) was separated on an SDS-6% polyacrylamide gel and electroblotted onto nitrocellulose. Phosphoproteins bound to nitrocellulose fragments were cleaved with 50 mg of CNBr per ml in 70% formic acid (predominantly at methionine residues) and analyzed on 0.4-mm-thick SDS-25% polyacrylamide gels (40 cm in length). All of each sample was analyzed, the gel was dried without fixation, and radioactive phosphopeptides were detected by using an intensifying screen.

The conditions used for digestion are nearly specific for cleavage of proteins at methionine residues (32), but under oxidizing conditions, cleavage at cysteine residues can result from CNBr proteolysis. To reduce oxidation, proteins for mapping were exposed continuously to 1 mM DTT during purification and were fully reduced by boiling in 50 mM DTT prior to electrophoresis. While we cannot exclude the possibility of a partial digest, we note that methionine cleavage by CNBr is usually rapid and complete. Contrariwise, trypsin proteolytic mapping of $p34^{cdc2}$ phosphorylation sites can be complicated by partial digestion (30) since the $p34^{cdc2}$ consensus sequences contains basic residues that are also the recognition site for trypsin. Phosphorylation of these residues can lead to partial digestion with trypsin, but such a problem would not be expected with CNBr cleavage. The gel shown in Fig. 5 was exposed for 8 days with an intensifying screen.

RESULTS

pRb deletion expression vectors. To analyze the relationship between the structure of pRb and its biochemical and biological properties, we constructed a series of small deletions of the pRb coding region that span nearly the entire length of the protein. These mutants and the predicted alterations of the pRb coding region are shown in Fig. 1. Each of the mutant coding regions was constructed in the mammalian SV40-based pRb expression vector

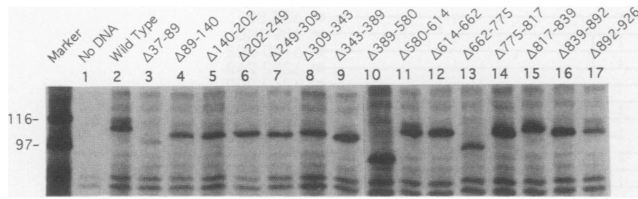

FIG. 2. Expression of pRb mutants by using the SV40-based expression vector pSVE. Plasmid preparations used for bioassay of pRb mutants (Fig. 3 and Table 1) were used to transfect COS-7 cells to verify pRb expression from the mutant plasmid vectors. pRb was detected by immunoprecipitation from ^{35}S-labeled cultures, using the anti-HA epitope antibody 12CA5. Numbers at the left indicate molecular masses (in kilodaltons) of ^{14}C-labeled marker proteins (Sigma).

pRbWT3HA/SVE (34) for biological assay and also transferred into the mammalian T7-based pRb expression vector pRbWT3HA/TM1 (26, 33) for analysis of the biochemical properties of the expressed protein. We used an epitope-tagged form of pRb (34) so that all of the deletion mutants could be analyzed by using the same MAb and to distinguish the plasmid-encoded pRb mutants from the normal pRb present in the transfected cells. Also, the MAb directed against this carboxy-terminal epitope should be less likely to interfere with pRb-associated proteins or to be disrupted by posttranslational modification than are antibodies directed against native pRb epitopes.

Function of each of the pRb mutant vectors was confirmed before further testing. The pSVE-based mutants (used for biological assay; see below) were tested for expression of the appropriate-size mutant protein in COS-1 cells (Fig. 2). All of the deletion mutants produced an appropriate-size protein at approximately the same level of expression (based on radiolabeling with [^{35}S]methionine) except for mutant Δ37-89, which was expressed about half as abundantly as the other mutant or wild-type proteins. The TM1-based vectors were tested as described below for Fig. 4.

Biological function of pRb deletion mutants. We tested the ability of each of the mutant pRb expression vectors to regulate growth, as measured by the ability to induce growth cessation and morphologic changes in transfected Saos-2 cells as described previously (34). This assay stems from our observation that after cotransfection of cultures of Saos-2 cells with pRb expression vectors together with a drug resistance plasmid, between 1 and 5% of the cells in the transfected culture remain resistant to the drug selection for several weeks. Transfection with the drug resistance plasmid alone results in one to five stable drug-resistant colonies per million transfected cells visible after several weeks of culture. All of the remaining cells in puromycin-containing medium die.

Since the number of drug-resistant single cells is over 1,000-fold greater than the number of stable drug-resistant colonies that arise from transfection of these plasmids (but is similar to the rate of transient gene expression obtained with this transfection method [7]), we hypothesized that the resistant cells result from prolonged expression of the drug resistance gene in cells that were transiently transfected. In the absence of the pRb expression plasmid, all but a few of these cells would lose expression of the transfected gene, probably during DNA replication and chromosome segregation. Because of this, and since pRb is capable of preventing entry into S phase (12), we presume that cells cotransfected with pRb expression plasmids and drug resistance plasmids have a stable phenotype because they cannot divide and segregate the unintegrated puromycin resistance gene.

This proposed mechanism and the role of pRb in it are supported by the following observations. (i) Surviving puromycin-resistant cells are seen in cultures cotransfected with a pRb expression plasmid and pBABEpuro (Fig. 3A) but not in cultures transfected with pBABEpuro alone (Fig. 3B) or with a pRb expression plasmid alone (not shown). (ii) In cultures cotransfected with pBABEpuro and pRb expression plasmids, the surviving single cells adopt an enlarged, vacuolated phenotype with elongated processes (compare the cells in Fig. 3A with Saos-2 cells of the normal phenotype in Fig. 3C), similar to that demonstrated by cells infected with a pRb-expressing retrovirus (18). Additionally, no mitotic cells are identifiable, and nuclear labeling with [^{3}H]thymidine is undetectable (data not shown). (iii) Cotransfection of cells with E1A-expressing plasmids along with pRb expression plasmids and pBABEpuro results in no aberrant cells being formed (26a). (iv) Cell cycle arrest resulting from use of the DNA synthesis inhibitor hydroxyurea also results in prolongation of puromycin resistance in pBABEpuro-transfected cells (26a) without the morphologic alterations characteristic of pRb-transfected cells. (v) The observed changes in transfected cell morphology are independent of treatment with puromycin; these morphologic changes are also seen in cells transfected with bifunctional pRb and β-galactosidase expression vectors subsequently visualized by staining for β-galactosidase enzymatic activity (26a).

To test the biological activities of the pRb deletion mutants, small cultures of Saos-2 cells were cotransfected with a puromycin resistance gene together with each of the mutant pRb expression vectors (by using the SV40-based expression vector pSVE [34]) and then treated with puromycin. Ten days after transfection, cultures were examined microscopically for the number of cells surviving under puromycin treatment and for the accompanying morphologic changes. The number of cells that survive in puromycin-containing medium after cotransfection with the panel of mutant vectors was determined in several experiments; all but a few displayed the aberrant flat-cell morphology. The results of a typical experiment performed in quadruplicate are presented in Table 1. Most of the deletion mutants produced aberrant cells at a frequency less than 5% of the level of the wild-type plasmid. Three mutants, Δ343-389, Δ580-614, and Δ892-926, displayed slightly increased activity in this assay. In repeated experiments, these three mutants always produced slightly more aberrant cells than did the wild-type plasmid. Mutants Δ202-249 and Δ249-309 displayed a reduced number of surviving cells.

FIG. 3. Photographs of cultures used for assessment of pRb biological activity. Cultures of Saos-2 cells were transfected with various plasmids as described in Materials and Methods and incubated for 10 days under various conditions. Magnification for all panels, ×100. (A) Saos-2 cells transfected with pRbWT3HA/SVE (1 μg) and pBABEpuro (20 ng) and incubated in puromycin-containing medium. (B) Saos-2 cells transfected with pSVE (1 μg) and pBABEpuro (20 ng) and incubated in puromycin-containing medium. No viable cells were found in this culture. (C) Untransfected Saos-2 cells grown in normal medium.

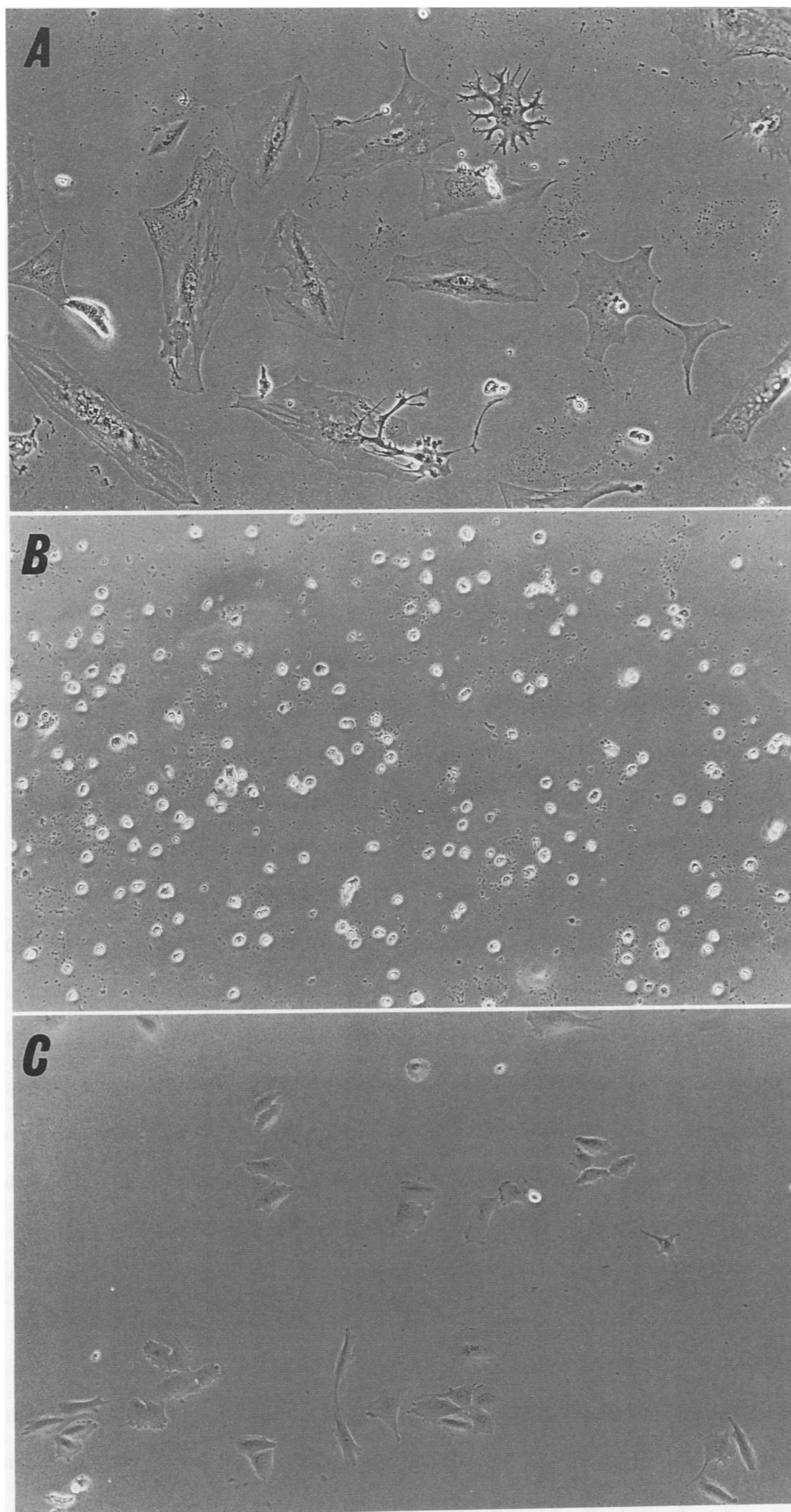
A
B
C

TABLE 1. Biological activities of pRb mutant expression vectors[a]

pRb plasmid	No. of cells (mean ± SD)[b]	% of wild-type level (avg ± SD)[c]
WTpRb/SVE	1,109 ± 146	100 ± 13
Δ37-89	0 ± 1	0.0 ± 0.1
Δ89-140	28 ± 2	2.5 ± 0.2
Δ140-202	39 ± 13	3.5 ± 1.2
Δ202-249	435 ± 40	39.2 ± 3.6
Δ249-309	204 ± 31	18.4 ± 2.8
Δ309-343	39 ± 8	3.5 ± 0.7
Δ343-389	1,380 ± 96	124 ± 8.6
Δ389-580	6 ± 6	0.5 ± 0.5
Δ580-614	1,474 ± 256	133 ± 23
Δ614-662	6 ± 3	0.5 ± 0.3
Δ662-775	11 ± 7	1.0 ± 0.6
Δ775-817	11 ± 3	1.0 ± 0.3
Δ817-839	72 ± 34	6.5 ± 3.1
Δ839-892	39 ± 6	3.5 ± 0.5
Δ892-926	1,481 ± 151	134 ± 14
pSVE vector	0 ± 0	0.0 ± 0

[a] Measured as the frequency of induction of morphologic changes accompanied by growth inhibition. The number of aberrant flat cells retaining puromycin resistance after transfection of 2×10^4 Saos-2 cells with a mixture of the indicated pRb pSVE expression vector and pBABEpuro 10 days after drug selection was determined.

[b] Determined for four independently seeded wells.

[c] Normalized to the number of cells resulting from WTpRb/SVE transfection. The empty pSVE vector produced no surviving cells in this assay.

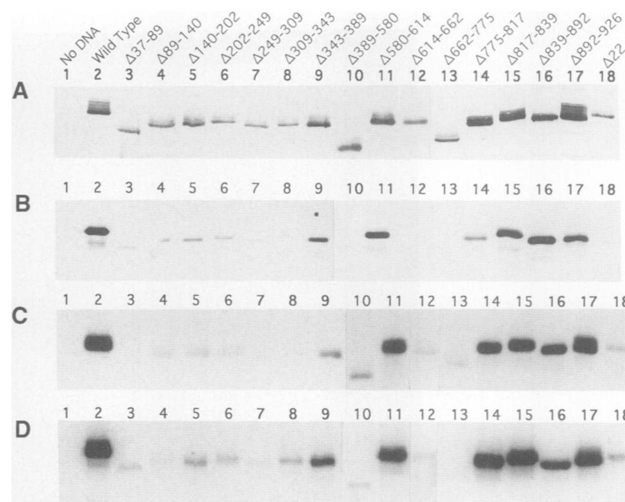

FIG. 4. Characterization of proteins expressed by pRb mutant expression vectors. (A) Detection of mutant pRb by Western blotting with antiepitope MAb 12CA5; (B) precipitation of mutant pRb proteins with purified E1A and MAb M73 recognizing E1A; (C) immunoprecipitation of in vivo-^{32}P-labeled mutant pRb with antiepitope MAb 12CA5; (D) in vitro phosphorylation of immunopurified mutant pRb proteins with cyclin A-associated kinase.

Biochemical properties of pRb deletion mutants. We next sought to determine whether the mutant proteins were altered in the biochemical properties associated with wild-type pRb. We expressed each of the mutants in CV-1 cells, using the vector pTM1, and analyzed the encoded proteins by Western blotting with MAb 12CA5 (36), which recognizes the synthetic carboxyl-terminal epitope (Fig. 4A). Each mutant expressed an appropriately migrating protein. This analysis revealed additional differences between mutant proteins reflected by the presence or absence of slowly migrating hyperphosphorylated forms of the proteins, as will be discussed below.

E1A binding of pRb mutants. We tested our panel of deletion mutants for the ability to coprecipitate with purified E1A protein synthesized in bacteria (11) (Fig. 4B). Only the three deletion mutants that affect the previously determined (16, 19) domains sufficient for E1A binding were completely unable to form this complex (in addition to the naturally occurring deletion Δ22 (Fig. 4B, lane 18), which affects the more C-terminal E1A-binding domain, as described earlier [34]). Some of the amino-terminal mutants bound with less efficiency than did the wild-type protein (note the faint bands present in lanes 7 and 8 [mutants Δ249-309 and Δ309-343]).

Hyperphosphorylation of pRb mutants. All naturally occurring mutants of pRb have been found to be underphosphorylated in cells. This observation leads to the hypothesis that the ability to be hyperphosphorylated reflects an important correlate to the growth-regulatory activity of pRb. Analysis of the Western blot results discussed above (Fig. 4A) revealed that wild-type pRb expressed in this system exists both in a fast-migrating form that is underphosphorylated and in at least four slower-migrating bands that represent hyperphosphorylated pRb, as has been shown previously (33). Presumably, each band represents a class of pRb with distinct phosphorylation sites or combination of sites. Mutant Δ22, which contains a deletion of the distal E1A-binding domain, does not express the hyperphosphorylated form(s) seen in the wild-type protein. We have shown previously that Δ22 contains some basally phosphorylated residues, on four chymotryptic peptides resolved by two-dimensional peptide analysis (34). Mutant Δ22 is found by Western blot analysis (Fig. 4, lanes 18) to be predominantly in a fast-migrating form, with a single slightly retarded band that probably represents the basally phosphorylated form. The presence of this basal phosphorylation, since it is preserved in the tumor-derived, nonfunctional pRb mutants, does not correlate with the function of pRb as a tumor suppressor.

Similar to mutant Δ22, several of the mutant pRb proteins lack the hyperphosphorylated bands and retain only the fast-migrating form and the single basally phosphorylated form. Specifically, mutants deleted of any part of pRb from amino acids 37 to 343, and mutants Δ389-580, Δ614-662, and Δ662-775 (all deleted of segments of the E1A-binding domain), lack bands identifiable as the hyperphosphorylated form. Mutant Δ839-892 is expressed as a single band that is not clearly identifiable as hyperphosphorylated. Because of the phosphopeptide cleavage pattern obtained from this mutant protein (discussed below), we believe that it is hyperphosphorylated but that the deletion prevents the aberrant electrophoretic pattern associated with the hyperphosphorylated form of pRb.

To confirm the lack of hyperphosphorylation found in the pRb mutants by Western blot analysis, we tested each of the deletion mutants for the ability to be labeled with radioactive phosphate both in vitro and in vivo.

We first radiolabeled cells with ^{32}P and analyzed the mutant pRbs by immunoprecipitation with the anti-HA antibody. As shown in Fig. 4C, wild-type pRb was labeled both on the fast-migrating (underphosphorylated) form and on a set of more slowly migrating (hyperphosphorylated) forms. The nonfunctional tumor-derived mutant Δ22 was labeled only on the basally phosphorylated (underphosphorylated) form, as reported previously (34). Deletions affecting the E1A-binding domains were also underphosphorylated. Sim-

ilarly, mutants of the amino-terminal region of pRb (between codons 37 and 343) were phosphorylated to a low level and demonstrated a single band of labeled proteins.

Each of the remaining mutants retained a level of phosphorylation and a migration pattern characteristic of the hyperphosphorylated form of wild-type pRb. Mutant Δ839-892 migrated as a single, heavily labeled band, suggesting that this mutant is indeed hyperphosphorylated but that the aberrant migration associated with hyperphosphorylated pRb is dependent on the deleted sequences. One additional mutant (Δ343-389) consistently demonstrated an intermediate level of phosphorylation. As shown by phosphopeptide mapping (see below), this mutant lacks at least two of the residues phosphorylated in wild-type pRb (see also Discussion). Thus, the low level of ^{32}P incorporation in this mutant appears to result from a loss of phosphorylation sites rather than an inability to be recognized by the pRb kinase.

We also tested the ability of each of the mutant proteins to serve as a phosphate acceptor in an in vitro phosphorylation reaction. Some experimental evidence (23) suggests that the cell cycle-regulatory kinase $p34^{cdc2}$ is the enzyme responsible for hyperphosphorylation of pRb. Since $p34^{cdc2}$ functions as a complex with one or more cyclin proteins, we found it useful to prepare $p34^{cdc2}$ by coisolation with an epitope-tagged form of the human cyclin A protein expressed in CV-1 cells, using the T7 plasmid vector pTM1. In preliminary experiments, we found that pRb could be efficiently phosphorylated with this kinase preparation and also with a similar preparation of cyclin B-associated kinase. Phosphopeptide maps of the resulting cyclin A- or cyclin B-phosphorylated pRb were indistinguishable and nearly identical to maps of endogenous pRb obtained from in vivo-labeled HL60 cells (33a).

Figure 4D demonstrates pRb proteins labeled with cyclin A-associated kinase. Wild-type pRb is efficiently phosphorylated with this kinase preparation (lane 2), and the electrophoretic migration of this protein corresponds to the slowly migrating bands that correlate with the hyperphosphorylated form seen by Western blot (data not shown). No phosphorylation of proteins similar in size to pRb are detected when the kinase preparation without the pRb substrate is reacted. Mutants that are underphosphorylated in vivo are similarly underphosphorylated in this in vitro reaction. Thus, amino-terminal portions of pRb are important for pRb to be recognized as a substrate for the cell cycle-dependent kinase in vivo or to be phosphorylated in vitro with the cyclin A-associated kinase.

Proteolytic phosphopeptide mapping of pRb deletion mutants. To analyze the nature of the phosphorylation events that modify the mutant pRb proteins more specifically, we analyzed proteolytic fragments (resulting from CNBr treatment) of the mutant pRbs labeled in vivo (an aliquot of the samples analyzed in Fig. 4C). Digestion of wild-type pRb resulted in the resolution of at least seven phosphate-labeled bands in this gel (Fig. 5), consistent with the multiple phosphorylation events reported previously for pRb (22). Examination of the protein sequence of pRb reveals that complete cleavage of the epitope-tagged pRb by CNBr at methionine residues would result in 21 peptides ranging in size from 0.98 to 12.4 kDa. The five phosphorylation sites of pRb that have been identified (22) lie within CNBr-cleaved peptides of 9.1 (T-373), 7.8 (S-249 and T-252), and 7.1 (S-807 and S-811) kDa. Other $p34^{cdc2}$ consensus sites of phosphorylation lie within peptides of 12.4 and 10.5 kDa. These predicted sizes are consistent with the electrophoretic mi-

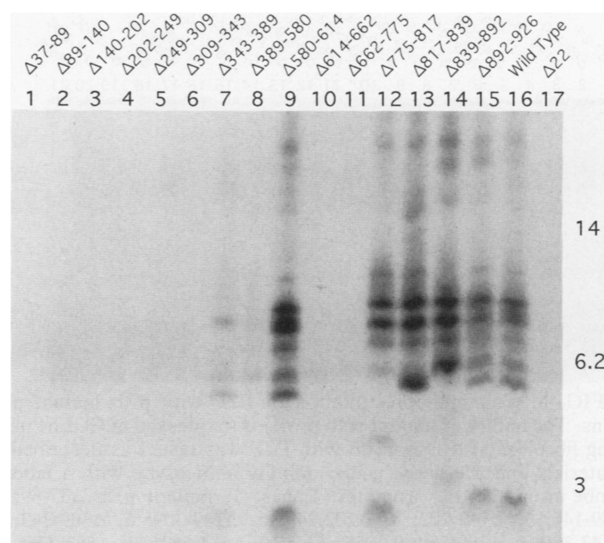

FIG. 5. Analysis of CNBr-generated phosphopeptides of in vivo-^{32}P-labeled mutant pRb proteins by SDS-PAGE (25% polyacrylamide gel). Numbers at the right indicate locations of prestained low-molecular-weight markers (Bethesda Research Laboratories), expressed in kilodaltons.

gration of the proteolytic peptides that we observe, although we cannot identify each band with certainty.

Digestion of the weakly phosphorylated proteins with amino-terminal deletions (affecting codons 37 to 343), or of proteins with mutations of the E1A-binding domains (codons 389 to 580 and 614 to 775), or of the nonfunctional mutant Δ22 protein resulted in phosphopeptides that were unresolved in this system. This finding supports the premise that the low level of ^{32}P incorporated into these mutant proteins represents not hyperphosphorylation but rather the basal-level phosphorylation previously detected in nonfunctional pRb mutant forms. Since deletion of several different segments of this part of pRb results in a lack of hyperphosphorylation, these mutants do not lack the residues that become phosphorylated but rather lack sequences that are otherwise necessary for pRb to become hyperphosphorylated.

Mutants Δ343-389 and Δ775-817 lack some of the hyperphosphorylated peptides, consistent with the sites of phosphorylation identified in this part of the protein (T-373, S-807, and S-811) (22). Mutants Δ817-839 and Δ839-892 both express proteins that produce a new phosphopeptide, conceivably, because of deletion of peptide sequences contiguous to a site of phosphorylation but not of the site itself.

Transcription factor binding of pRb deletion mutants. Recently, pRb has been shown to interact with a factor (E2F) that binds to DNA sequences of the E2 promoter of adenovirus (1, 2, 6). We confirmed this observation in a very direct manner: we expressed pRb in tissue culture cells and immunopurified the plasmid-derived pRb (under conditions similar to those for the preparation of whole-cell extracts that contain E2F DNA-binding activity), using MAb 12CA5 directed against the synthetic carboxyl-terminal epitope. We then disrupted proteins loosely associated with the immune complexes by using the ionic detergent deoxycholate and tested the released protein(s) for the presence of E2 DNA-binding activity. Figure 6 demonstrates that the exogenous wild-type pRb is associated with E2F (lane 17) and that this pRb-associated E2 DNA-binding factor has the same mobil-

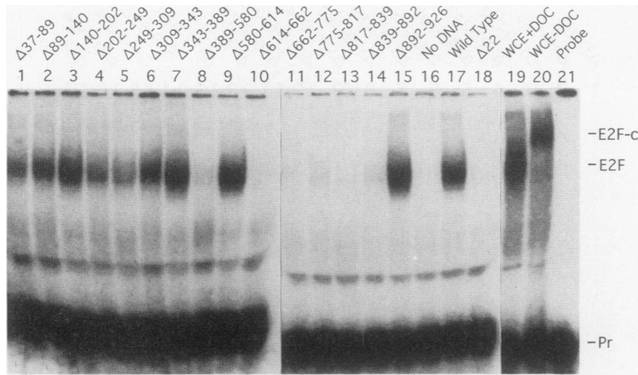

FIG. 6. Coimmunoprecipitation of E2F with pRb mutant proteins. The ability of mutant pRb proteins (expressed in CCL64 mink lung fibroblasts) to associate with E2F was tested as described in Materials and Methods, using mobility shift assay with a labeled probe from the E2 promoter. Lanes: 1, mutant pRb Δ37-89; 2, Δ89-140; 3, Δ140-202; 4, Δ202-249; 5, Δ249-309; 6, Δ309-343; 7, Δ343-389; 8, Δ389-580; 9, Δ580-614; 10, Δ614-662; 11, Δ662-775; 12, Δ775-817; 13, Δ817-839; 14, Δ839-892; 15, Δ892-926; 16, no-plasmid DNA control; 17, wild-type pRb; 18, Δ22; 19, CCL64 whole-cell extract, deoxycholate treated; 20, CCL64 whole-cell extract, without deoxycholate treatment; 21, probe only. E2F-c, position of E2F complex; E2F, position of E2F after disruption of protein-protein complexes with deoxycholate; Pr, position of labeled probe.

ity as does the E2F present in deoxycholate-treated whole-cell extract (lane 19). Immunoprecipitates from untransfected cells (lane 16) or from cells expressing the natural mutant pRb protein Δ22 (lane 18) do not contain E2F.

Figure 6 also demonstrates E2 DNA-binding activities present in immunoprecipitations of each of the deletion mutants. Each of the amino-terminal mutants (deleted of codons 37 to 389) retains the ability to bind E2F, as does mutant Δ580-614, which removes the spacer between the two halves of the E1A-binding domain. Mutations within the E1A-binding domains (Δ389-580, Δ614-662, and Δ662-775) ablate the ability of the mutant proteins to bind to E2F, as does the Δ22 mutation. Plasmids with mutations distal to the E1A-binding domains, including mutations of codons 775 to 892, also fail to associate with E2F. The plasmid with the most distal mutation, Δ892-926, that is deleted of the sequences of the natural carboxyl terminus of pRb (but retains the synthetic epitope), is able to associate with E2F as does wild-type pRb. Thus, E2F-binding function of pRb requires the E1A-binding domains and the following 117 codons but not the amino-terminal 389 amino acids, the 34 amino acids that lie between the E1A-binding domains, or the 36 carboxyl-terminal amino acids.

DISCUSSION

Summary of sequences required for pRb function. Figure 7 summarizes the biological activities of the pRb deletion mutants and the tested biochemical properties of the protein. By comparison of the biochemically defined domains of pRb with the sequences required for retention of biological activity, it is apparent that proper function of pRb as measured by our assay is dependent on both the E2F-binding domain and the amino-terminal region of pRb required for phosphorylation.

It should be noted that our finding that amino-terminal domains of pRb are required for function is in disagreement with results of two other groups who use different measures of pRb activity. Goodrich et al. (12) reported that a bacterially expressed fusion protein encoding the carboxyl terminus of pRb is able to inhibit growth of microinjected cells. Qin et al. (27) have recently reported that a carboxy-terminal fragment of pRb expressed by the strong cytomegalovirus promoter is sufficient to reduce colony formation in transfected Saos-2 cells. Their group identified no requirement for the amino-terminal portions of pRb to induce growth inhibition. One explanation for the differences in our observations might be the level of pRb introduced into the cell; the damage to pRb function by amino-terminal mutations might be compensated for by high-level expression of the mutant pRb under control of a strong promoter.

Another apparent difference between our assay and the colony inhibition assay of Qin et al. (27) is that their group achieved a much higher rate of stable DNA transformation and colony formation rate in Saos-2 cells than we did, possibly because of subtle differences in culture conditions. In lieu of colonies, their pRb-transfected cultures showed enlarged cells that failed to grow, similar to the cells that we see in our assay. Thus, it seems that apparent differences between our assay and that of Qin et al. may reflect differences not in the cellular effects of pRb but rather in the efficiency of stable DNA integration.

Assay of biological function of pRb. We have used a biological assay of the mutant expression plasmids that we propose measures the induction of puromycin-resistant cells by virtue of growth inhibition by pRb and a resultant prolonged expression of the cotransfected puromycin resis-

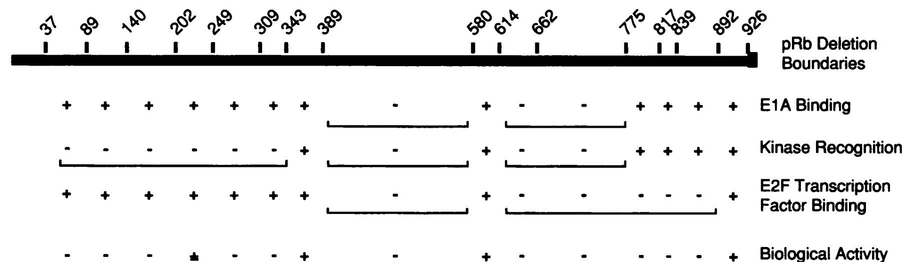

FIG. 7. Comparison of biochemical properties of pRb deletion mutants with biological activities. The boundaries of each of the deletion mutants are drawn to scale on a linear representation of the pRb coding region. The ability of each mutant to bind to E1A or E2F or to be hyperphosphorylated is indicated by a + in the relevant column. Brackets indicate regions of pRb necessary for each of the indicated activities. Biological activity is represented by +, −, or ± from the data in Table 1. From these data, it is evident that mutants deficient in either kinase recognition or E2F binding are inactive (or greatly reduced) in the ability of pRb to induce morphologic changes and growth inhibition in Saos-2 cells.

tance gene. We have concluded that this assay reflects cell growth inhibition, since the aberrant, puromycin-resistant cells fail to divide; no mitotic cells are seen, [^{3}H]thymidine labeling of nuclei is not observed, and the cell number remains relatively stable (declining slightly) over a period of weeks.

We are not unmindful that this bioassay may reflect a phenotype not directly related to the role of pRb as a tumor suppressor. However the loss of this function correlates closely with loss of the biochemical properties described above, suggesting that the bioassay measures a relevant aspect of pRb physiology.

While we have presented the results of our flat-cell assay in a quantitative manner in Table 1, most of the mutants produced fewer aberrant cells than did the wild-type pRb plasmid. Two exceptions that resulted in intermediate numbers of flat cells are mutant Δ202-249, which demonstrated 40% of the activity of the wild-type pRb plasmid, and mutant Δ249-309, which showed 20% of the wild-type activity. Three mutants (Δ343-389, Δ580-614, and Δ892-926) repeatedly produced a slightly greater number of flat cells than did the wild-type plasmid.

E1A and E2F binding. Although E1A-binding domains of pRb have been defined previously by using in vitro-transcribed pRb, we thought it useful to confirm these observations by using our pRb mutant panel in the context of pRb with cellular proteins. We found that only those mutants that were deleted of segments of pRb previously defined as the minimal sequences required for binding to E1A or T antigen (mutants Δ389-580, Δ614-662, and Δ662-775) were unable to bind to purified E1A.

Both the E1A-binding sequences of pRb and more distal sequences (involving mutants Δ389-580, Δ614-662, Δ662-775, Δ775-817, Δ817-839, and Δ839-892) are required for E2F binding. Stated in contrast, all mutants that fail to bind E1A also fail to bind E2F, but not all mutants that fail to bind E2F fail to bind E1A. Our identification of pRb sequences required for E2F binding are in agreement with those of Hiebert et al. (14), who showed recently that E2F binds to a bacterially produced C-terminal fragment of pRb but not to a smaller fragment truncated at codon 792.

Phosphorylation of pRb mutants. Mutants of the E1A-binding domains, as well as mutants deleted of sequences between codons 37 and 343, are unable to be hyperphosphorylated in vivo. These mutants are also unable to be phosphorylated in vitro by a kinase (probably p34^{cdc2}, p33^{cdk2}, or a related cyclin-dependent kinase) immunopurified by association with cyclin A. It should be emphasized that we are not claiming that a cyclin A-associated kinase is responsible for phosphorylation of pRb in vivo, although such evidence has been reported by others (23). pRb has also been shown to form relatively stable complexes with p34^{cdc2} or a related molecule, possibly as a result of a stable enzyme-substrate intermediate (17). We are unsure whether the mutant proteins that we have characterized here are unable to form this complex with p34^{cdc2} or whether they are possibly bound by this kinase but not recognized as a substrate.

By analysis of the specific sites of phosphorylation (by proteolytic phosphopeptide mapping), we found that radiophosphate associated with the mutant proteins capable of being hyperphosphorylated was resolved into at least seven phosphopeptides. Two mutants (Δ343-389 and Δ775-817) lack several proteolytic phosphopeptides that are present in the wild-type hyperphosphorylated pRb. These findings confirm the observation that several of the major sites of phosphorylation of pRb (e.g., T-373, S-807, and S-811) lie within these boundaries.

The two mutants containing deletions of the phosphorylated residues of pRb flank the boundaries of the E1A-binding domains. Since the binding of T antigen and E1A is largely specific for the underphosphorylated form of pRb (24) (Fig. 4B), as is the form of pRb that forms a tight association with the cell nucleus (33), it is provocative to speculate that phosphorylation of these domains serves to deactivate the protein-binding activity of the contiguous E1A-binding domain.

pRb as signal transducer. Our results suggest that the biological activity of pRb is dependent on the interaction of pRb with at least two distinct factors. These factors may represent both afferent (exogenous) signals impinging upon pRb and efferent signals emanating from pRb to the cell growth-regulatory machinery. The phosphorylation of pRb by its kinase is likely to be an afferent signal, since phosphorylation appears to negatively regulate pRb function (4, 24). The interaction of pRb with E2F (and probably other transcription factors) is most probably an efferent signal, as it suggests a signal transmitted downstream to the transcriptional machinery.

Thus, rather than being viewed as a proximate effector of growth control per se, the function of pRb might best be viewed as a transducer of regulatory signals from (several?) protein kinases to (several?) transcription factors. Regulation of transcription factor function by phosphorylation of the pRb transducer (rather than, or in addition to, phosphorylation of the transcription factor itself) might be advantageous to the cell in that this scheme could provide control of a greater diversity of concordantly regulated target proteins or stricter temporal regulation of cell cycle events. At least one protein (p107) homologous to pRb can also bind to transcription factors (5, 10, 31). Thus, pRb and its homologs may represent a plexus of transducers, through which growth-regulatory signals are processed and disseminated to the ultimate growth control machinery.

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