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Enhanced tumor cell growth suppression by an N-terminal truncated retinoblastoma protein

(gene transfer/tumor suppressor/gene therapy)

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ABSTRACT **The retinoblastoma (*RB*) gene encodes a nuclear phosphoprotein of 928 amino acids (pRB). Thus far, much effort in RB research has been focused on both the viral oncoprotein-binding domains and the C-terminal domain, whereas little is known about the N-terminal moiety of the protein. We report here that an N-terminal truncated RB protein of ≈94 kDa (pRB^{94}) exerts more potent cell growth suppression as compared to the full-length pRB protein in a diversity of tumor cell lines examined, including those having a normal endogenous RB gene. Tumor cells transfected with the pRB^{94}-expressing plasmids displayed multiple morphological changes frequently associated with cellular senescence and/or apoptosis. They failed to enter S phase and rapidly died. The pRB^{94} expressed in recipient tumor cells had a longer half-life than the full-length pRB protein and tended to remain in an active un- or hypophosphorylated form. Since it has also been found that N-terminal truncated RB proteins often accumulated in growth-arrested and/or differentiated tumor cells, we suggest that N-terminal truncation of pRB may be one of the cellular mechanisms modulating the RB protein function in cell-cycle control.**

The retinoblastoma (*RB*) gene, the first defined tumor suppressor gene (1, 2), was cloned in the mid-1980s (3–5) and is now known to encode a nuclear phosphoprotein of 928 amino acids (pRB) (6). The authenticity of the *RB* tumor suppressor gene has been reinforced by studies indicating that replacement of the cloned gene into RB-defective tumor cells from widely disparate types of human cancers could suppress or reduce their tumorigenicity (7–12). Even though the RB-mediated tumor suppression was often incomplete (9–12), the *RB* gene makes a potentially good target for gene therapy of RB-defective human cancers (12, 13).

Much effort in RB research has been focused so far on its C-terminal half. It is well demonstrated that both the pRB E1a/T antigen-binding domains (A/B pocket) and the C-terminal domain, extending from amino acid residue 379 to 928, are required for pRB to interact with the E2F transcription factor and are critical to the cell growth suppression function of pRB (14, 15). A protein-binding domain in the C terminus of pRB, outside of the A/B pocket, is also important for pRB to interact with nuclear c-Abl tyrosine kinase, and the latter may participate directly in regulation of transcription in S-phase cells (16). In contrast, little is known about the N-terminal moiety of pRB. Consequently, a bacterium-expressed 56-kDa RB protein segment lacking the N-terminal 393 amino acid residues was considered as a functional equivalent for the full-length pRB (17).

Nevertheless, low molecular mass proteins immunoreactive to several anti-pRB antibodies, including RB-WL-1, RB-PMG3-245, C36, and RB1-AB20, were often observed in human fibroblasts and hematopoietic cells (18–23). It initially was proposed by us that these pRB-like proteins could represent translation from the second in-frame AUG codon of the RB mRNA (18). In this regard, alternative splicing at exon 2 of the RB transcript has more recently been found in normal human placenta, various tumor cells, and rat tissues (24). The resultant RB transcript should be translated exclusively into a truncated RB protein lacking the N-terminal 112 amino acid residues (24). Of particular interest, variable N-terminal truncated RB proteins also have been shown to accumulate in growth-arrested or differentiated human leukemia cell lines after addition of retinoic acid, phorbol 12-myristate 13-acetate, or α-interferon (19, 22, 23). Therefore, studies on N-terminal truncated pRB may provide insights into regulation of the cellular function of pRB in addition to its cell growth suppression function *per se*.

In the present studies, we have compared the biochemical and biological properties of the full-length pRB and an N-terminal truncated RB protein of ≈94 kDa (pRB^{94}). Both RB proteins were produced in insect cells by using recombinant baculoviruses or were expressed directly in human tumor cells via plasmid vectors. The pRB^{94} was initiated from the second in-frame AUG codon of the RB transcript and lacked the N-terminal 112 amino acid residues of the full-length RB protein.

MATERIALS AND METHODS

Manipulation of the Baculovirus/Insect Cell Expression System for pRB and pRB^{94} Expression. By site-specific mutagenesis, two *Bam*HI sites were introduced into the human *RB* cDNA (12) at nucleotides −4 and +3548 (the A of the first in-frame AUG codon in the pRB coding sequence was designated +1) for the full-length pRB or at nucleotide +322 and +3548 (the second in-frame AUG codon of pRB was located at nucleotides 337–339) for pRB^{94}. Each desired *RB* cDNA fragment was inserted into the unique *Bam*HI site of the baculovirus transfer vector pVL1393 (Invitrogen). Transfer of *RB* cDNAs from the recombinant transfer vectors to the viral genome was accomplished by cotransfection of insect Sf9 cells with a wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA (25). The recombinant viruses were subjected to plaque purification to obtain pure stocks of pRB- or pRB^{94}-expressing baculoviruses.

Purification of pRB and pRB^{94} Proteins. The RB proteins were purified from baculovirus-infected insect Sf9 cells by immunoaffinity chromatography. Briefly, insect cells were harvested 12–36 hr after the virus infection and lysed at 4°C with EBC buffer (50 mM Tris·HCl, pH 8.0/120 mM NaCl/0.5% Nonidet P-40/50 μg of aprotinin per ml). The lysates were clarified by centrifugation and the pRB- or pRB^{94}-

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Abbreviations: T antigen, large tumor antigen; CMV, cytomegalovirus.

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containing supernatants were incubated with biotinylated RB-WL-1 polyclonal anti-RB antibodies (18) at 4°C overnight. The procedure for biotinylation of rabbit IgGs using succinimide ester has been described (26). The RB protein–IgG–biotin complexes were collected on streptavidin agarose gel columns and proteins were eluted from the columns by using 100 mM glycine (pH 2.2) and neutralized immediately with 1 M phosphate (pH 8.0).

Construction of pRB and pRB^{94} Expression Plasmids and Transfection. To construct pRB and pRB^{94} expression vectors, the same RB cDNA fragments as described above were inserted into the corresponding cloning sites of three plasmid vectors—namely, pRc/CMV (Invitrogen), pHβAPr-1-neo (27), and pLLRNL (28). Tumor cells were transiently transfected with the RB expression plasmids via Lipofectin reagent (GIBCO/BRL Life Technologies) according to the manufacturer's specifications.

RESULTS

Characterization of the Full-Length and N-Terminal Truncated RB Proteins Expressed in Insect Cells. Two baculovirus transfer vectors were constructed containing *RB* cDNA fragments coding for the first and the second in-frame AUG codon-initiated RB proteins, respectively. As shown in Fig. 1, the baculovirus-produced truncated RB proteins of ≈94 kDa, which lacked the N-terminal 112 amino acids (pRB^{94}), were phosphorylated to various degrees. This phosphorylation pattern ranged from un- or hypophosphorylated to hyperphosphorylated, whereas the baculovirus-produced full-length RB proteins (pRB) were mostly present in un- and hypophosphorylated forms. The phosphorylation pattern observed for the full-length pRB was consistent with a previous report (29). However, it was also possible to obtain either the un- or hypophosphorylated form of pRB^{94} or a major hyperphosphorylated form of the full-length pRB by adjusting the multiplicity of infection (moi) and timing for harvesting the insect cells (data not shown). Therefore, we believe that the final products of the un- or hypophosphorylated pRB and pRB^{94} reflected merely an exhausting of the endogenous kinase pool in the postinfected insect cells. Moreover, the

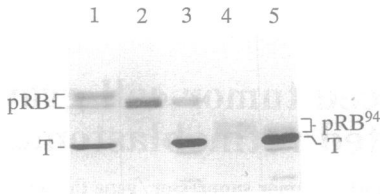

FIG. 1. Complex formation of the baculovirus-expressed full-length pRB and the N-terminal truncated pRB^{94} with simian virus 40 T antigen. Immunoaffinity chromatography-purified pRB or pRB^{94} was mixed with T antigen and aliquots of the mixture were immunoprecipitated with the anti-T antibody PAB419 (Oncogene Science) followed by Western blotting as described (12). The blot was sequentially incubated with MAb-1 anti-RB antibody (CIBA–Corning Diagnostics, Triton Products) and PAB419. Lanes: 1, lysate from the simian virus 40 immortalized human fibroblast cell line WI38VA13 (ATCC CCL75.1) (control); 2, purified full-length pRB; 3, coprecipitate of T antigen and pRB; 4, purified pRB^{94}; 5, coprecipitate of T antigen and pRB^{94}.

baculovirus-produced un- and hypophosphorylated pRB^{94} proteins resembled the full-length pRB in that both were capable of forming protein–protein complexes with the simian virus 40 large tumor antigen (T antigen) (Fig. 1). In addition, by immunocytochemical staining, both the recombinant pRB^{94} and full-length pRB were exclusively found in the nuclei of insect cells up to 24 hr postinfection (moi = 1; data not shown). Thus, pRB^{94} apparently shares major biochemical and biological properties with the full-length pRB in terms of phosphorylation, nuclear localization, and viral oncoprotein binding.

Enhanced Tumor Cell Growth Suppression by pRB^{94}. We next tested to determine whether pRB^{94} expression could also suppress tumor cell growth. To compare the effects of pRB^{94} and full-length pRB on tumor cells, three types of RB plasmid vectors were constructed. The vectors contained pRB^{94} or the full-length pRB coding sequences whose expression was driven, respectively, by the β-actin gene promoter (27), the cytomegalovirus (CMV) promoter (30), and the long terminal repeats of Moloney murine leukemia virus (28). A combined technique was used involving immunocy-

Table 1. DNA synthesis in various RB-defective tumor cells expressing exogenous pRB or pRB^{94} proteins

Recipient cells	Tumor origin	Promoter in vector	% cells incorporating [^{3}H]thymidine			
			pRB^{94}		Full-length pRB	
			RB$^+$	RB$^-$	RB$^+$	RB$^-$
5637	Bladder carcinoma	I	2	43	34	45
		II	2	41	21	39
		III	3	39	25	32
MDA-MB-468	Breast carcinoma	II	0*	39	14	40
		III	1	31	16	28
H2009	Lung carcinoma	I	0*	19	19	26
DU145	Prostate carcinoma	II	1	33	23	33
Hs913T	Fibrosarcoma	II	1	36	18	34
Saos2	Osteosarcoma	II	1	35	19	32

Tumor cells ≈24 hr after transfection with pRB^{94} or the full-length pRB expression vectors containing a β-actin, CMV, or long terminal repeat promoter were labeled with [^{3}H]thymidine for 2 hr and then fixed and immunostained with MAb-1 anti-RB antibody. Stained slides were subsequently coated with autoradiographic emulsion and exposed for 5–7 days. Detailed methods have been described (31). About 400 to 1600 pRB^{94}- or the full-length pRB-expressing tumor cells and 600 RB$^-$ tumor cells in the same slides were assessed for each entry of [^{3}H]thymidine uptake. Lack of cellular DNA synthesis as determined by failure of the vast majority of the tumor cells to incorporate thymidine implies the indicated tumor cell population tended not to progress through the cell cycle (30). Differences in % tumor cells incorporating [^{3}H]thymidine were statistically significant between pRB^{94}- and pRB-expressing tumor cells (two-tailed t test, $P < 0.0001$), whereas no such differences were observed between the RB$^-$ tumor cells ($P = 0.57$ by t test). Promoters in vector: I, β-actin gene promoter; II, CMV promoter/enhancer; III, long terminal repeat of Moloney murine leukemia virus.
*<0.5%.

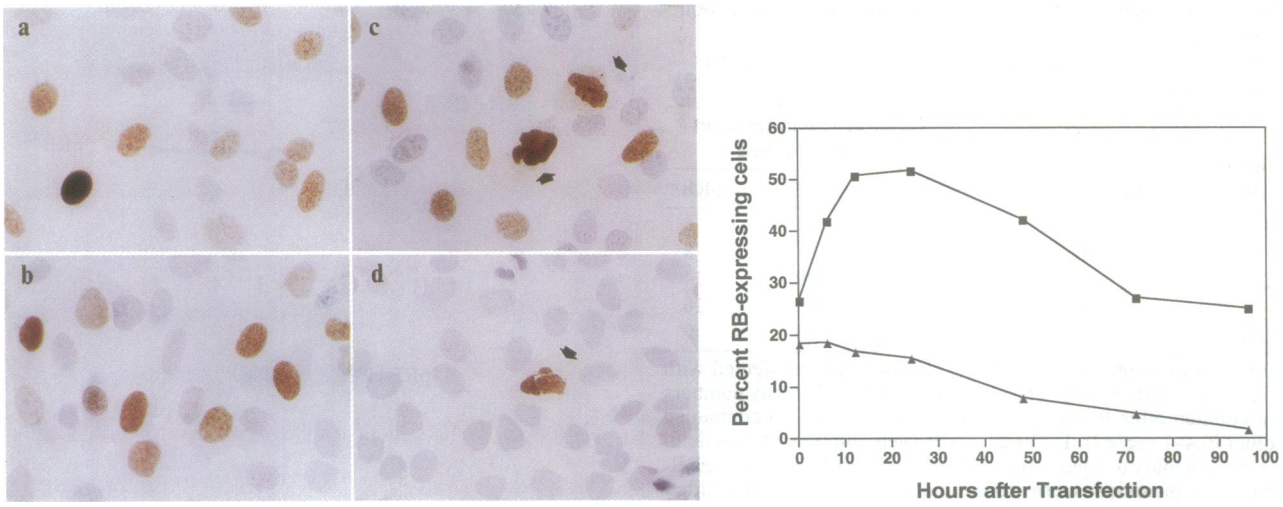

FIG. 2. Time course analysis of the full-length pRB- or pRB^{94}-expressing tumor cells in transiently transfected cell cultures. A RB-defective bladder carcinoma cell line, 5637 (ATCC HTB9), was transfected with the pRB or pRB^{94} expression plasmids containing a CMV promoter. At each indicated time point after transfection, tumor cells were harvested and immunocytochemically stained with the anti-RB monoclonal antibody MAb-1. (*Left*) Examples of the RB staining pattern, showing pRB (*a* and *b*) and pRB^{94} (*c* and *d*) expressing tumor cells (brown) in the mass cultures. Tumor cells were stained 12 hr (*a* and *c*) and 96 hr (*b* and *d*) after transfection. Arrows indicate pRB^{94}-expressing tumor cells with apoptotic-like morphological changes. (×500.) (*Right*) Time courses of the full-length pRB-expressing (■) and pRB^{94}-expressing (▲) tumor cells. For each entry, at least 1000 cells were counted from three independent experiments. Almost all tumor cells expressing pRB^{94} had died by 96 hr after transfection, while the majority of tumor cells expressing the full-length pRB survived.

tochemical staining and [^{3}H]thymidine *in situ* labeling of the tumor cells after transfection with the pRB^{94} or the full-length pRB-encoding plasmid vectors. A series of RB$^-$ tumor cell lines with diverse tissue/organ origins were examined. The data are summarized in Table 1. The studies demonstrated that the RB-defective tumor cells expressing exogenous pRB^{94} did not progress through the cell cycle, as evidenced by their failure to incorporate [^{3}H]thymidine into DNA. However, the percentage of tumor cells undergoing DNA replication was only slightly lower in cells producing the exogenous full-length pRB than in cells that were RB$^-$ (Table 1).

Moreover, we also repeatedly observed that tumor cells expressing the pRB^{94} protein often became flat and large, showing marked convolution of the cellular surface and a high incidence of pseudo- or true multinucleated cells, which represent a series of morphological changes frequently associated with cellular senescence and/or apoptosis. These RB$^+$ senescent-like or apoptotic-like tumor cells exclusively lacked [^{3}H]thymidine incorporation. Such multiple morphological changes were also found in tumor cells transfected with the full-length pRB expression vectors, but with much lower frequency. In fact, when we kept track of these pRB^{94}- and pRB-expressing tumor cells by time course analysis (Fig. 2), it became even more evident that the pRB^{94} was a much more potent cell growth suppressor than the full-length pRB. For instance, as shown in Fig. 2, at 12 hr after transfection, most of the 5637 tumor cells expressing either the full-length pRB or pRB^{94} proteins had normal viable morphology compared to their parental RB$^-$ 5637 cells within the same microscopic fields, although some apoptotic-like tumor cells did occur in tumor cell cultures transfected with the pRB^{94} vectors (arrows in Fig. 2 *Left*). Soon afterwards, the incidence of such apoptotic-like tumor cells increased along with dead cells. All such cells showed intense pRB^{94+} staining. At ≈4 days after transfection, almost all tumor cells expressing pRB^{94} had died, while the majority of the 5637 tumor cells treated with the pRB vectors survived and retained pRB expression (Fig. 2). By time course analysis, identical results were also obtained with the RB-defective breast carcinoma cell line MDA-MB-468, except that multinucleated cells rather than apoptotic-like cells were more frequently seen in this breast tumor cell line after transfection with the pRB^{94} vectors (Fig. 3).

An assay described earlier by Baker *et al.* (30) was also used to determine whether transfection of the full-length pRB and pRB^{94} plasmids would suppress the recipient tumor cells in forming geneticin-resistant colonies. We found that, although introduction of the full-length pRB plasmid vectors considerably suppressed the formation of geneticin-resistant colonies in the RB$^-$ bladder carcinoma cell line 5637, tumor cells treated with pRB^{94} plasmid vectors formed even fewer (≈4-fold less) colonies (Table 2). Furthermore, in those colonies that did form after transfection with the pRB^{94} plasmids, pRB^{94} expression was no longer observed as eval-

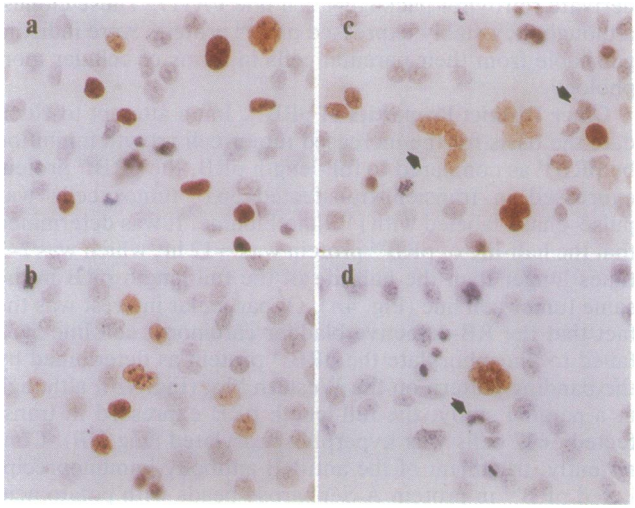

FIG. 3. Morphological changes and RB staining in the pRB- or pRB^{94}-expressing tumor cells after transfection of mass cultures of the RB-defective breast carcinoma cell line MDA-MB-468 (ATCC HTB132). Examples of the RB staining pattern, showing pRB-expressing (*a* and *b*) and pRB^{94}-expressing (*c* and *d*) tumor cells (brown) in the mass cultures. Tumor cells were stained 12 hr (*a* and *c*) and 48 hr (*b* and *d*) after transfection. Arrows indicate multinucleated tumor cells expressing pRB^{94} protein. (×500.)

Table 2. Geneticin-resistant colony formation after introduction of pRB^{94} or pRB expression in the RB$^-$ bladder carcinoma cell line 5637, the RB$^+$ fibrosarcoma cell line HT1080, and the RB$^+$ cervical carcinoma cell line HeLa

Recipient cells	Vector type	No. of geneticin-resistant colonies formed		
		Vector	pRB	pRB^{94}
5637	I	280	24	6
	II	322	33	8
HT1080	I	94	129	14
	II	88	122	16
HeLa	I	25	24	9

For each experiment, three 25-cm^2 dishes were transfected with the indicated pRB or pRB^{94} plasmid vector, and total colony numbers were counted after 10–14 days of selection in geneticin-containing medium (0.4–1 mg/ml). Differences between numbers of geneticin-resistant colonies formed after transfection with pRB^{94}- and pRB-expressing plasmids were statistically significant (one-tailed Wilcoxon test; $P = 0.03$). Vector types: I, pRc/CMV; II, pHβAPr-1-neo.

uated by Western blotting and immunocytochemical staining (data not shown). Failure to isolate long-term cultures expressing pRB^{94} in transfected tumor cells again shows that pRB^{94} did dramatically suppress tumor cell growth. In contrast, 9 of 57 cell clones (≈15%) derived from tumor cells treated with the full-length pRB plasmids were found to express the exogenous pRB proteins (12). Perhaps even more striking was that pRB^{94} expression also significantly reduced geneticin-resistant colony formation in both RB$^+$ tumor cell lines examined—namely, the fibrosarcoma cell line HT1080 and the cervical carcinoma cell line HeLa. In contrast, no such effects have been observed when an additional full-length pRB-coding gene(s) was introduced by transfection using plasmid vectors (Table 2 and ref. 32) or by microcell fusion (33).

On the other hand, pRB^{94} expression appears to have little effect on normal cells. For instance, after transfection with the pRB^{94} retroviral plasmid vectors, we were able to isolate stable, long-term single cell clones from the normal (nontumorigenic) mouse fibroblast cell line NIH 3T3 and their derivate retroviral packaging cell line PA317. These clones, although they stably expressed pRB^{94} protein, were indistinguishable from their parental cells in terms of cellular morphology.

Other Distinct Properties of pRB^{94}. In an attempt to elucidate the basis for the increased tumor cell growth inhibition by pRB^{94} as compared to full-length pRB, the pRB^{94} protein expressed in transient-transfected 5637 tumor cells was pulse–chase-labeled with [^{35}S]methionine. It was determined that the half-life of pRB^{94} protein was ≈12 hr, which was 2–3 times longer than the half-life of the full-length pRB in the same tumor cell line (Fig. 4*A*). Of particular interest was the fact that the RB-defective bladder carcinoma cell line 5637 failed to phosphorylate the pRB^{94} protein as determined by the banding pattern on the Western blot (Fig. 4*B*), although in a parallel study, the full-length pRB expressed in transfected 5637 cells was hyperphosphorylated (Fig. 4*B*). Consistently, treatment of the anti-RB antibody-immunoprecipitated pRB^{94} in protein A-Sepharose beads with potato acid phosphatase did not affect its migration on SDS/polyacrylamide gel, while identical treatment reduced the full-length pRB from its phosphorylated forms to the unphosphorylated form (data not shown). In addition, using an *in vitro* kinase reaction (34), we have also found that un- and hypophosphorylated pRB^{94} obtained from insect cells was a less effective substrate for the human cdc2 kinase when compared to the full-length pRB (data not shown).

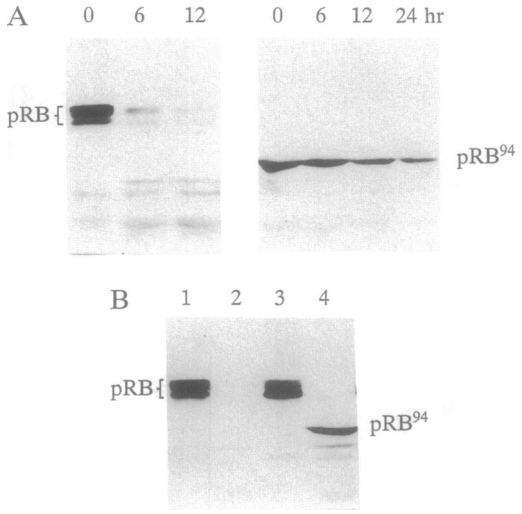

FIG. 4. Distinct biological properties of pRB^{94} in transiently transfected tumor cells. Bladder carcinoma cell line 5637 was transfected with pRB or pRB^{94} expression plasmids. (*A*) Half-life analysis of pRB and pRB^{94} in RB-reconstituted 5637 bladder tumor cells. Immediately after transfection, tumor cells were metabolically labeled with [^{35}S]methionine for 2 hr and chased with excess unlabeled methionine for 0, 6, 12, and 24 hr, respectively. pRB and pRB^{94} were determined by immunoprecipitation (18): (*Left*) (0–12 hr) half-life of pRB was <6 hr; (*Right*) (0–24 hr) half-life of pRB^{94} was ≈12 hr. (*B*) Phosphorylation status of exogenous RB proteins expressed in tumor cells. Tumor cells were harvested 12 hr after transfection and subjected to Western blotting analysis. Lanes: 1, normal human fibroblast cell line WI-38 (control); 2, parental RB$^-$ 5637 tumor cells; 3 and 4, 5637 tumor cells transfected with pRB and pRB^{94} plasmids, respectively. Only unphosphorylated pRB^{94} was detected in lane 4.

DISCUSSION

We have demonstrated here an N-terminal truncated RB protein of ≈94 kDa (pRB^{94}) that exerts enhanced tumor cell growth suppression. The pRB^{94} protein expressed in host tumor cells had a slower turnover and a tendency to remain in an unphosphorylated form. Since it is well known that only the un- or hypophosphorylated form of RB protein is able to interact with transcription factors such as E2F and is responsible for the repression of cellular proliferation (35–37), accumulation of mostly the un- or hypophosphorylated pRB^{94} in cells may account for the failure of transfected 5637 and other tumor cells to enter S phase, and this may eventually cause tumor cell death. Moreover, since N-terminal truncated RB proteins with variable size have been shown to accumulate *in vivo* in growth-arrested and/or differentiated tumor cells (19, 22, 23), as well as in a human progeria fibroblast strain at late passages (unpublished data), our results imply that N-terminal truncation of the RB protein could contribute to the cellular mechanisms modulating RB function in cell growth control and perhaps even in programmed cell death/survival control (38). The latter was intimated by the fact that pRB^{94} expression in normal mouse cells appeared not to cause cell death.

Based on the studies described above, we also postulate that the N terminus of RB protein could contain a crucial functional domain(s) for regulation of the overall reactivity of RB protein in cell growth/differentiation control. The activities regulated by the N-terminal domains might include posttranslational modification (for instance, phosphorylation and dephosphorylation), stability, and steady-state levels, as well as other autoregulatory mechanisms of the RB protein. Independent of the presence or absence of the second in-frame AUG codon-initiated RB protein(s) *in vivo* (18–21, 23, 24), the N-terminal domains of pRB may be masked or unmasked during cell growth, differentiation, and tissue-

specific development by interaction with other cellular regulatory proteins, by alternative splicing of the RB transcripts (24), or by posttranslational modification. A comparable autoregulation-like domain(s) may also exist in other tumor suppressor genes. In this connection, we have recently identified a conservative N-terminal homologous segment between p53 and RB (unpublished data).

Finally, wild-type RB-mediated tumor suppression in RB-defective tumor cells is often incomplete (9–12, 32). The potential of RB-reconstituted tumor cells to still form tumors in nude mice may have resulted from some cells in a given tumor cell population inheriting or acquiring the ability simply to inactivate the RB gene products by hyperphosphorylation or to surmount the complex cellular growth regulatory pathway in which the RB gene normally functions. This phenomenon has recently been referred to by us as tumor suppressor resistance, which may pose an obstacle to RB gene therapy of human cancers (12). Therefore, from a practical standpoint, our findings should increase the potential of RB as a therapeutic target, especially since the pRB^{94} appears to inhibit the growth of not only the RB-deficient tumor cells but also those with normal *RB* allele(s).

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