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Apoptosis Induced by the Nuclear Death Domain Protein p84N5 Is Inhibited by Association with Rb Protein

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Rb protein inhibits both cell cycle progression and apoptosis. Interaction of specific cellular proteins, including E2F1, with Rb C-terminal domains mediates cell cycle regulation. In contrast, the nuclear N5 protein associates with an Rb N-terminal domain with unknown function. The N5 protein contains a region of sequence similarity to the death domain of proteins involved in apoptotic signaling. We demonstrate here that forced N5 expression potently induces apoptosis in several tumor cell lines. Mutation of conserved residues within the death domain homology compromise N5-induced apoptosis, suggesting that it is required for normal function. Endogenous N5 protein is specifically altered in apoptotic cells treated with ionizing radiation. Furthermore, dominant interfering death domain mutants compromise cellular responses to ionizing radiation. Finally, physical association with Rb protein inhibits N5-induced apoptosis. We propose that N5 protein plays a role in the regulation of apoptosis and that Rb directly coordinates cell proliferation and apoptosis by binding specific proteins involved in each process through distinct protein binding domains.

INTRODUCTION

Coordination of cell proliferation and cell death is required for normal development and tissue homeostasis in multicellular organisms. A defect in the normal coordination of these two processes is a fundamental requirement for tumorigenesis. Progression through the cell cycle is highly regulated, requiring the transit of numerous checkpoints (for review, see Hunter, 1993). The extent of cell death is physiologically controlled by activation of a programmed suicide pathway that results in a morphologically recognizable form of death termed apoptosis (Vaux *et al.*, 1994; Jacobson *et al.*, 1997). Both extracellular signals, such as tumor necrosis factor, and intracellular signals, like DNA damage, can induce apoptotic cell death. Although many proteins involved in apoptosis or the cell cycle have been identified, the mechanisms by which these two processes are coordinated are not well understood.

Mutation of the retinoblastoma tumor suppressor gene (Rb) alone is sufficient to cause retinoblastoma in humans, suggesting that it might play a role in the normal coordination of cell proliferation and cell death (Goodrich and Lee, 1993). The retinoblastoma tumor suppressor protein (p110Rb) can inhibit progression through the G1 phase of the cell cycle (Goodrich *et al.*, 1991). This is accomplished largely by modulation of cellular transcription factors, like E2F1, through direct physical association (Bagchi *et al.*, 1991; Kaelin *et al.*, 1992; Weintraub *et al.*, 1992; Flemington *et al.*, 1993). Cyclin-dependent kinase phosphorylation of Rb protein (p110Rb) allows transit through the Rb-enforced checkpoint (Connell-Crowley *et al.*, 1997) by disrupting physical association with these cellular proteins. Several lines of evidence suggest that p110Rb may also regulate apoptosis. Ectopic expression of p110Rb inhibits apoptosis triggered by radiation (Haas-Kogan *et al.*, 1995), E2F1 (Hsieh *et al.*, 1997), p53 (Haupt *et al.*, 1995), myocyte differentiation (Wang *et al.*, 1997), or ceramide (McConkey *et al.*, 1996). Rb protein is also a target for cleavage by caspases during apoptosis (An and Dou, 1996; Janicke *et al.*, 1996). Finally, mouse embryos lacking functional Rb have inappropriately high levels of apoptosis in the CNS, the liver, the eye lens, and skeletal muscle (Zacksenhaus *et al.*, 1996). Although these findings suggest that p110Rb may regulate apoptosis, it is unclear whether this regulation is a novel function or an indirect consequence of Rb-mediated effects on the cell cycle.

The C-terminal half of p110Rb is sufficient for many of its known molecular activities, including modulation of tran-

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scription factor function and induction of cell cycle arrest. The purpose of the N-terminal half of p110Rb is undefined. Several observations suggest that this region may be important for normal function. First, some mutations causing low-penetrance retinoblastoma specifically alter the N-terminal half of the protein (Dryja *et al.*, 1993; Hogg *et al.*, 1993; Lohmann *et al.*, 1994). Second, the amino acid sequence of the N-terminal half of p110Rb is conserved between mouse, rat, chicken, frog, newt, and human. Finally, N-terminally truncated Rb transgenes are incapable of rescuing developmental defects observed in mice deficient in wild-type Rb (Riley *et al.*, 1997).

To discover a role for this region of p110Rb, the N5 gene was isolated on the basis of its ability to encode a protein that specifically associates with the N-terminal half of p110Rb (Durfee *et al.*, 1994). Three other proteins, a 70-kDa heat-shock protein (Inoue *et al.*, 1995), a kinase (Sterner *et al.*, 1996), and MCM7 (Sterner *et al.*, 1998), have been discovered to bind the N-terminal half of p110Rb. The relevance of these interactions for Rb function is not completely understood, although association of p110Rb with MCM7 does inhibit DNA replication in vitro (Sterner *et al.*, 1998). The N5 protein (p84N5) is normally localized exclusively to the nucleus during interphase and has a region of structural similarity to the death domains of several well characterized proteins involved in apoptosis, including tumor necrosis factor receptor 1 (TNFR-1) (Feinstein *et al.*, 1995). We have sought to test the hypothesis that p84N5 is involved in regulating apoptosis and that Rb may modulate this activity through physical association with p84N5. We demonstrate that forced expression of p84N5 potently induces apoptosis, that an intact death domain is required for this effect, that p84N5 is normally modified during apoptosis, that dominant interfering N5 mutants compromise cellular responses to ionizing radiation, and that physical association with p110Rb inhibits p84N5-mediated apoptosis. These findings suggest that Rb may have a direct role in the regulation of apoptosis through the inhibition of a novel nuclear death domain protein. Furthermore, inhibition of p84N5-induced apoptosis identifies a novel function requiring the N-terminal domain of p110Rb. Because p84N5 is unique among death domain proteins in being localized exclusively to the nucleus, the study of p84N5 may uncover novel apoptotic signaling mechanisms within the nucleus. We propose that Rb has a direct role in coordinating apoptosis and the cell cycle by interaction with distinct cellular proteins that affect each process.

MATERIALS AND METHODS

Cell Culture

SAOS-2, 5637, and 293 cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) in a 5% CO_2 incubator at 37°C.

Plasmids

The cDNAs encoding p84N5 were subcloned into the pCEP4 expression vector (Invitrogen, San Diego, CA) to create pCMVN5. The p35 (Clem and Miller, 1994), Bcl-2 (McDonnell *et al.*, 1990), p110Rb, and p110RbΔCdk (Leng *et al.*, 1997) cDNAs were also expressed under control of the cytomegalovirus promoter in pCDNA3.1 (p110RbΔCdk) or pCMV (p35, Bcl-2). PCR-based site-directed mutagenesis was performed as previously described (Fisher and Pei, 1995). The template for PCR mutagenesis was the complete N5 cDNA inserted into pBSK (Stratagene, La Jolla, CA). The N5-PP mutant was created using the following pair of adjacent phosphorylated primers: N5DD1.2 (5′-CT TGA TCT TGC SRG GCA ACC RSG AGC TGC TTA GC-3′) and N5A4R (5′-AG GGA GTT CAT GCA ACA CCT G-3′). The N5Δα4 deletion mutant was created with the phosphorylated primers N5A4F (5′-TCA TGT CTT CAC TGT CAC ACT-3′) and N5A4R. Mutagenesis creates an in-frame deletion of nucleotides 1843–1884 of the N5 cDNA. The Sculptor in vitro mutagenesis system (Amersham, Arlington Heights, IL) was used according to manufacturer's specifications to create the N5-R mutant. The *Hin*dIII to *Bam*HI N5 fragment from pCMVN5 was inserted into M13mp19 and served as the single-strand DNA template for mutagenesis. The N5DD1.2 oligonucleotide was used as the primer. The mutations were confirmed by sequence analysis using the Thermo Sequenase radiolabeled terminator cycle sequencing kit according to manufacturer's recommendations (Amersham).

Transfection Assays

SAOS-2, 5637, or 293 cells were seeded in 100-mm dishes the day before transfection. Cells were transfected by the calcium phosphate precipitation method (Wigler *et al.*, 1979) using 6–30 μg of total DNA. For cotransfections, 6 μg of pCMVN5 and 24 μg of Bcl-2, p35, pCrmA, or pRb were used. Subsequent to transfection, attached and detached cells were collected separately at the indicated times. Cell viability was assessed by trypan blue staining in each cell population. Cell populations were then pooled and analyzed further.

β-Gal activity in transfected cells was visualized subsequent to fixation with 5% glutaraldehyde in PBS for 15 min followed by extensive washing in PBS containing 5 mM $MgCl_2$. Cells were stained in PBS containing 20 mM $K_3Fe(Cn)_6$, 20 mM $K_4Fe(Cn)_63H_2O$, 1 mM $MgCl_2$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) until a suitable color developed, usually after ~6–12 h.

Fragmented DNA was extracted from ~10^7 cells transfected with either pCMVN5 or pCMV in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% Triton X-100. The lysate was clarified by centrifugation, and the cleared lysate was treated with RNase A (50 μg/ml) for 1 h at 37°C. This was followed by proteinase K treatment (100 μg/ml) in 0.5% SDS for 2 h at 50°C. The DNA solution was extracted with phenol/chloroform, and DNA was precipitated in EtOH. DNA was dissolved in 10 mM Tris, 1 mM EDTA in preparation for electrophoresis on a 1.8% agarose gel. DNA fragmentation was also assayed after transfection with the indicated expression vectors by labeling free DNA ends with terminal deoxytransferase (TUNEL). Cells were collected by trypsin–EDTA treatment, washed two times with PBS, and stained by TUNEL using the APO-DIRECT kit (Phoenix Flow Systems, San Diego, CA) according to manufacturer's directions. Flow cytometry analysis was performed on FACSCalibur instrument (Becton Dickinson, San Jose, CA).

Clonogenicity assays were performed by transfection of 10 μg of the indicated plasmid along with 3 μg of pEGFP-C1 (Clontech, Palo Alto, CA). One day after transfection, cells were examined by fluorescence microscopy for green fluorescent protein (GFP)-positive cells to ensure that transfection had been successful. Transfection of pCMVN5 typically gave 30–50% of the GFP-positive cells that were observed with pCMV. Cultures were then incubated for an additional 2 wk in the presence of 500 μg/ml G418 (Sigma, St. Louis, MO). After G418 selection, the number of GFP-positive colonies with >20 cells was determined per 100× microscope field under fluorescence microscopy.

To assess the effects of N5 death domain mutants on sensitivity to ionizing radiation, SAOS-2 (SD8) cells, a subline of SAOS-2 cells, were transfected as above with the death domain mutant expression vectors and pEGFP-C1. One day after transfection, viable successfully transfected cells were collected by FACS (Becton Dickinson

FACS Vantage) based on GFP fluorescence, and 7000 cells were plated per well of a 96-well plate. The following day, cells were treated with 0 or 20 Gy radiation from a Nasatron ^{137}Cs irradiator (US Nuclear, Burbank, CA). Two days after irradiation, the relative number of remaining viable cells was determined by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay according to manufacturers instructions (Boehringer Mannheim, Indianapolis, IN). The data are presented as the ratio of the OD_{490} of treated versus untreated cells.

Microinjection

The N5 GST fusion protein was produced as described (Durfee *et al.*, 1994). The fusion protein was eluted in PBS plus 5 mM glutathione and then dialyzed against 25 mM Tris, pH 7.2, 25 mM KCl, 2% glycerol in preparation for injection. The p110Rb and p56Rb were produced and purified as previously described (Connell-Crowley *et al.*, 1997). The protein concentration of the injected samples is indicated by Figure 5C. Injection was performed directly on cells growing on 35-mm culture dishes using an Eppendorf micromanipulator with femtotip capillary micropipettes. The injection pressure used was between 50 and 100 hPa with an injection time of 0.3–0.5 s. Apoptotic cells were detected 90 min after injection by observation of characteristic morphological changes under phase-contrast microscopy and by staining with the DNA binding fluorochrome bis-(benzimide)-trihydrochloride (Hoechst 33342; Sigma) and fluorescent microscopy as described previously (Ormerod *et al.*, 1993).

Western Blotting

Transfected or treated cells were extracted in a buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin on ice for 10 min. Cell debris was pelleted by microcentrifugation, and the total protein concentration of the soluble extract was determined by Bradford assay according to manufacturers instructions (Bio-Rad, Hercules, CA). Total soluble protein (70 μg) for each sample was loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose, and the blot was blocked with a solution of 10% dried milk powder in TTBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The blot was incubated with primary antibody diluted in fresh TTBS for 1 h at room temperature or 4°C overnight. Primary antibody was detected using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence as described by the manufacturer (Amersham).

Immunofluorescent Staining

SAOS-2 cells were seeded onto chamber slides 2 d before irradiation. Cells were irradiated with the indicated dose of γ-irradiation, and incubation was continued for 3 d before fixation in 100% ice-cold MeOH. Fixed cells were washed in PBS and blocked with TTBS + 5% dry milk. Fixed cells were incubated with primary antibody diluted in TTBS for 1 h at room temperature. After washing, cells were incubated with FITC-conjugated secondary antibody (Vector Labs, Burlingame, CA) diluted in TTBS + 1 μg/ml Hoechst 33342. After washing, slides were mounted with Vectashield (Vector Labs) before photography under fluorescence microscopy.

RESULTS

Forced Expression of p84N5 Induces Cell Death by Apoptosis

To ascertain whether forced expression of p84N5 affected cell viability, full-length N5 cDNA was placed under the control of the human cytomegalovirus immediate early gene promoter (pCMVN5) and transfected into SAOS-2 osteosarcoma cells. Transfection of pCMVN5 reproducibly resulted in fewer surviving cells at 16 and 40 h after transfection (Figure 1A) compared with transfection with empty vector (pCMV). Cells were collected at 0 and 24 h after transfection, and cell viability was determined by trypan blue staining. Relative to SAOS-2 cells transfected with pCMV, cells transfected with pCMVN5 had increased numbers of nonviable cells and decreased numbers of viable cells (Figure 1B). The growth potential of SAOS-2 cells successfully transfected with pCMVN5, as measured by clonogenicity, was also severely compromised. The clonogenicity of cells cotransfected with pCMVN5 and the G418-selectable vector pEGFP-C1 was nearly 10-fold lower than cells cotransfected with pCMV and pEGFP-C1 (Figure 1C). Transfection into 293 embryonal kidney cells gave similar results. Cells cotransfected with pCMVN5 and pEGFP-C1 had approximately fivefold fewer colonies after G418 selection than cells transfected with pCMV and pEGFP-C1. We also observed loss of viability upon pCMVN5 transfection in 5637 bladder carcinoma cells (our unpublished results). Loss of viability upon p84N5 expression was not, therefore, cell line specific.

To confirm that loss of cell viability was due to p84N5 overexpression, lysates prepared from transfected 293 cells were analyzed for p84N5 by Western blotting using the mouse anti-N5 monoclonal antibody 5E10 (Durfee *et al.*, 1994). The antibody recognized two proteins of 84 and 58 kDa apparent molecular mass in several cell lines. Increased expression of both proteins was observed in cells transfected with pCMVN5 relative to transfection with pCMV alone (Figure 1D). Furthermore, p84N5 was confined primarily to the nucleus as determined by immunofluorescent staining of transfected cells with the anti-N5 monoclonal antibody and expression of a near full-length GFPN5 fusion protein (our unpublished results). Because an increase in both proteins was observed upon transfection of pCMVN5, both the 84- and 58-kDa anti-N5 immunoreactive proteins were likely derived from expression of the N5 gene. Furthermore, pCMVN5 used an intron-less cDNA, indicating that the 58-kDa form of the protein was not generated as a result of alternative splicing.

To examine the morphology of transfected cells, 293 cells were cotransfected with a β-galactosidase expression vector and pCMVN5 or pCMV. Twenty-four hours after transfection, adherent cells were stained for the presence of β-galactosidase with X-Gal. As expected, the number of surviving β-galactosidase–positive cells was significantly lower in pCMVN5-transfected cells than in cells transfected with pCMV. Of the few surviving pCMVN5-transfected, β-galactosidase–positive cells, a large proportion exhibited the condensed morphology typical of apoptotic cells (Figure 2A). In contrast, transfection with pCMV did not alter the morphology of β-galactosidase–positive cells.

Because the morphology of pCMVN5-transfected cells was consistent with that of cells undergoing apoptosis, we assayed transfected cells for internucleosomal fragmentation of nuclear DNA. SAOS-2 and 5637 cells transfected with pCMVN5 or pCMV were analyzed for internucleosomal DNA fragmentation by gel electrophoresis. DNA laddering similar to that observed in cells treated with staurosporine, a well characterized apoptotic trigger, was detected in both SAOS-2 and 5637 cells transfected with pCMVN5 (Figure

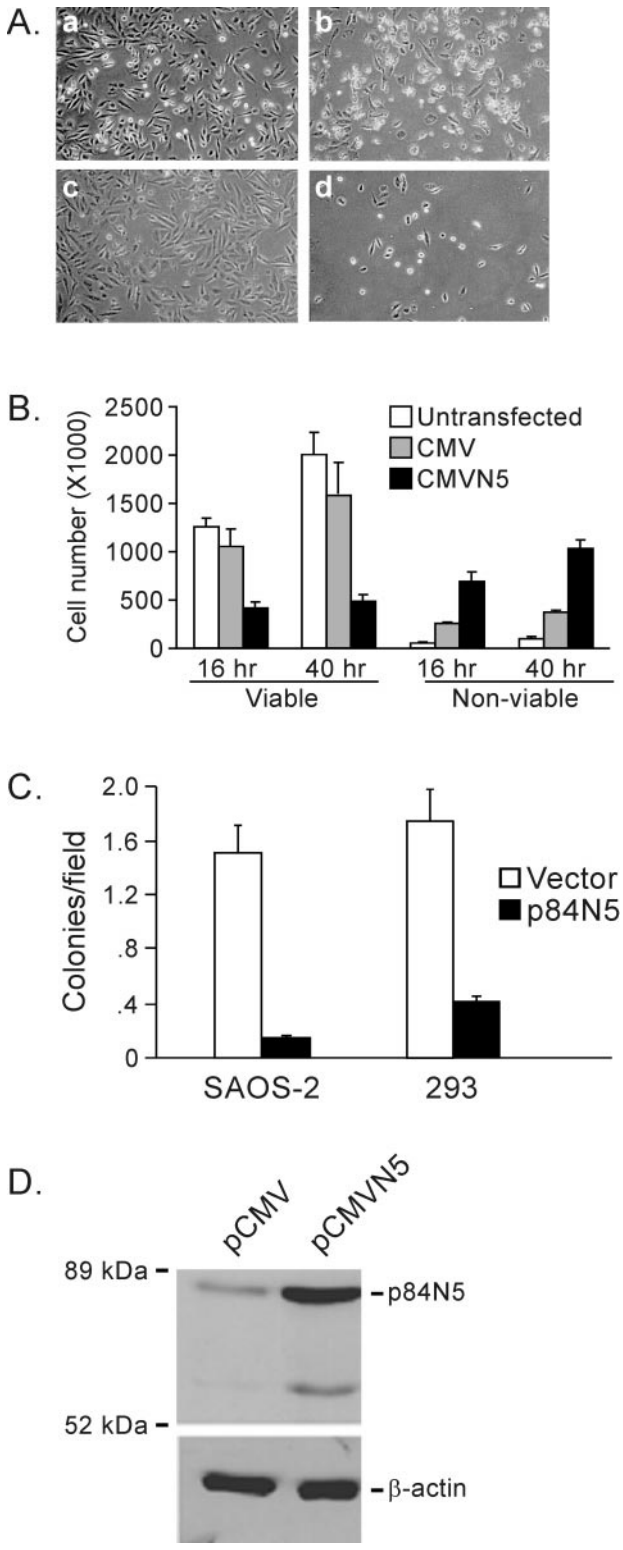

Figure 1. Expression of p84N5 decreases cell survival. (A) SAOS-2 cells were transfected with pCMVN5 (b and d) or pCMV (a and c). After 16 h (a and b) or 40 h (c and d) of transfection, cells were photographed under phase contrast at 100×. (B) Attached and detached cells transfected as above were collected at the indicated times and stained with trypan blue. Greater than 95% of attached cells were viable, and >95% of detached cells were nonviable. The mean number of total viable or nonviable cells present from three experiments is indicated. (C) The indicated cells were transfected with pCMVN5 (p84N5) or pCMV (Vector) plus pEGFP-C1 as indicated. Cells were grown in the presence of G418 for 2 wk, at which time the number of GFP-positive colonies of >20 cells was counted in at least 30 randomly selected 100× fields of view under fluorescent microscopy. Results are expressed as the mean colonies per 100× field of three independent experiments. (D) 293 cells were transfected with pCMV or pCMVN5 as shown. Extracts were prepared 24 h later, and equal amounts of total protein were analyzed by Western blotting for the protein indicated on the right. The position of molecular weight standards is shown on the left.

2B). Typically ~10% of viable SAOS-2 or 5637 cells remaining at the time of harvest were successfully transfected, so the amount of fragmented DNA was lower in transfected cells than in the cells uniformly undergoing apoptosis upon staurosporine treatment. DNA laddering was not detected in cells transfected with pCMV. DNA fragmentation was also analyzed in transfected SAOS-2 cells by TUNEL. As expected, a significant increase in the percentage of cells with labeled DNA was observed upon transfection with pCMVN5, whereas transfection of pCMV did not increase the percentage of labeled cells above untransfected controls (Figure 2C). Again, nonviable cells, as determined by propidium iodide permeability, were excluded from the analysis. Typically 10% of the remaining cells treated with pCMVN5 were successfully transfected, indicating that most of the transfected cells contained fragmented DNA.

Apoptosis is subject to both positive and negative regulation. Negative regulators include members of the Bcl-2 gene family (for review, see Kroemer, 1997) and caspase inhibitors (for review, see Kidd, 1998). We cotransfected pCMVN5 with a Bcl-2 expression plasmid to determine whether p84N5-induced cell death was sensitive to a negative apoptotic regulator. Coexpression of Bcl-2 with p84N5 significantly decreased the percentage of cells exhibiting fragmented DNA as measured by TUNEL relative to cells cotransfected with pCMVN5 and empty vector (Figure 2C). The percentage of cells containing fragmented DNA upon cotransfection of pCMVN5 and the Bcl-2 plasmid was similar to that of the negative control pCMV. To test the requirement for caspase activation in p84N5-induced cell death, we coexpressed p35, the broad-spectrum baculovirus caspase inhibitor. Cotransfection of pCMVN5 with a p35 expression plasmid also decreased the percentage of cells exhibiting fragmented DNA compared with cells expressing p84N5 alone (Figure 2C). Interestingly, DNA fragmentation induced by p84N5 was insensitive to coexpression of CrmA, a pox virus caspase inhibitor with narrower specificity. Cotransfection of pCMVN5 with the CrmA expression plasmid gave a similar percentage of cells containing fragmented DNA as transfection of pCMVN5 alone.

p84N5 Is a Death Domain-containing Protein

N5 protein has a region of statistically significant sequence similarity to the death domain of several proteins involved in apoptosis, including TNFR-1 and fas/apo1 (Figure 3A)

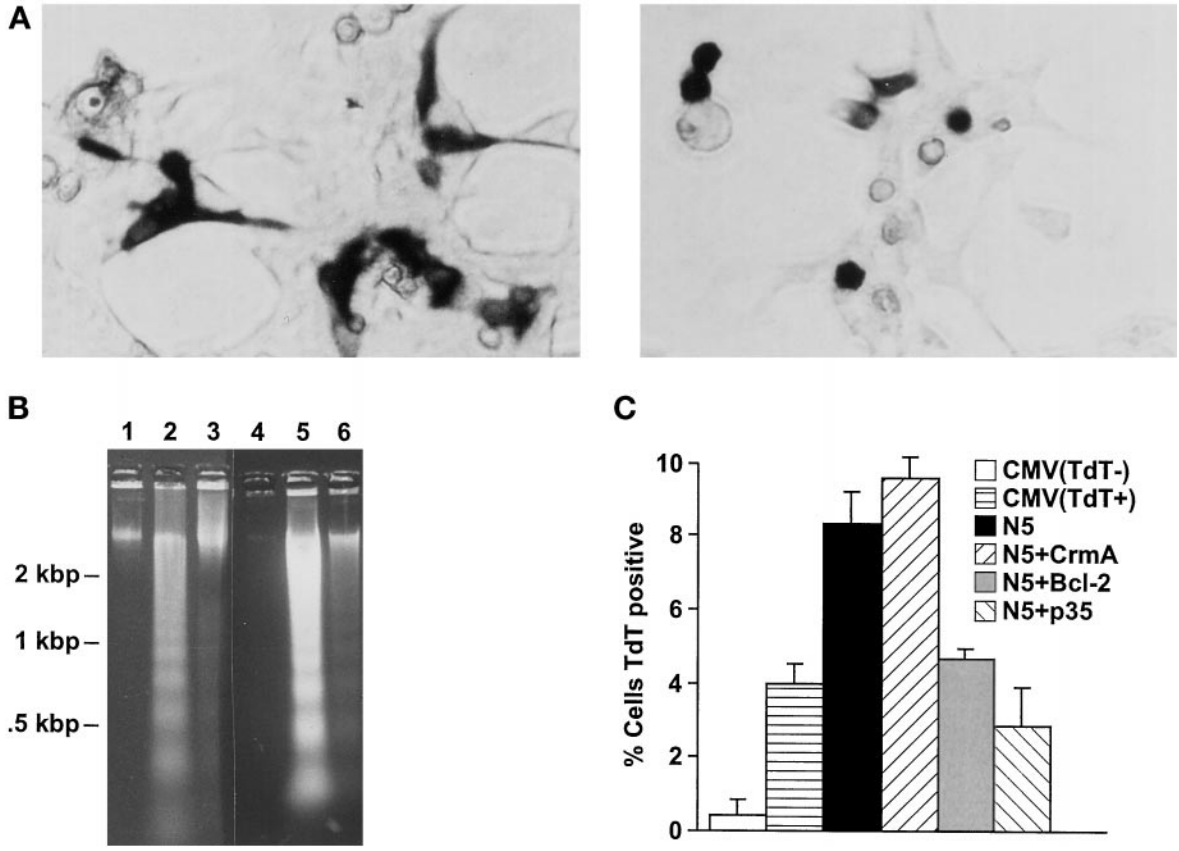

Figure 2. Forced expression of p84N5 induces apoptosis. (A) SAOS-2 cells were cotransfected with pCMVβ-Gal and pCMV (left panel) or pCMVN5 (right panel), fixed 24 h later, and stained with X-Gal. Cells were photographed under phase-contrast microscopy at 400×. Note the rounded, condensed morphology of pCMVN5-transfected cells. (B) 5637 (lanes 1–3) or SAOS-2 (lanes 4–6) cells were transfected with pCMV (lanes 1 and 4) or pCMVN5 (lanes 3 and 6), or treated with staurosporine (lanes 2 and 5). Fragmented DNA was extracted 24 h later, resolved by agarose gel electrophoresis, and stained with EtBr. Transfection efficiency for pCMVN5 was ~10% for both cell lines. DNA markers are indicated at the left. (C) SAOS-2 cells were transfected with the indicated expression vectors, and apoptosis was measured 24 h later by TUNEL assay and flow cytometry. The percentage of cells containing fragmented DNA is presented as the mean of three independent experiments. Maximum transfection efficiency for pCMVN5 under the conditions used was 10%, indicating that nearly all pCMVN5-transfected cells contained fragmented DNA.

(and see Feinstein *et al.*, 1995). For example, the p84N5 death domain has 26% amino acid identity in an optimal global alignment with the death domain of RIP, a kinase that interacts with fas/apo1 receptor. The p84N5 death domain has 24% amino acid identity to the TRADD death domain, a protein associated with the TNFR-1. The TRADD and RIP death domains have 24% identity with each other. The death domain is a protein-protein interaction motif required for apoptotic signaling by these proteins. It is composed of five sequential alpha helical regions. To our knowledge, none of the currently identified death domain-containing proteins involved in apoptosis is localized exclusively to the nucleus. Because p84N5 resides exclusively within the nucleus during interphase (Durfee *et al.*, 1994), we wished to determine whether the putative p84N5 death domain is required for function.

We mutagenized the N5 cDNA to remove the fourth alpha helix or to change certain conserved amino acids within it. Analogous mutations in the TNFR-1 protein compromised its ability to signal cytotoxicity. The pCMVN5-PP mutant substituted prolines for leucine at residue 617 and for tryptophan at residue 620. Another mutant, pCMVN5-R, contained an arginine for tryptophan substitution at residue 620. An analogous mutation in TNFR-1 (W378A) compromised its ability to signal cytoxicity (Tartaglia *et al.*, 1993). The pCMVN5$\Delta\alpha$4 mutant lacked the 13 amino acids that make up helix 4 in N5. A similar deletion of helix 4 in TNFR-1 inhibited its ability to induce apoptosis. The function of these mutants was tested by analysis of the clonogenicity of transfected cells. Relative to wild-type pCMVN5, pCMVN5$\Delta\alpha$4 and pCMVN5-PP had little effect on the clonogenicity of transfected cells (Figure 3A). Cells transfected with these mutants gave seven- to eightfold more GFP-positive colonies than were observed with pCMVN5. The clonogenicity of these mutants was close to that of the empty vector pCMV. The pCMVN5-R mutant was also less effective in reducing clonogenicity, generating approximately fourfold more GFP-positive colonies than pCMVN5. We analyzed the expression of the mutant proteins to ensure that the decrease in activity was not due to loss of protein expression. Extracts prepared from 293 cells transfected with

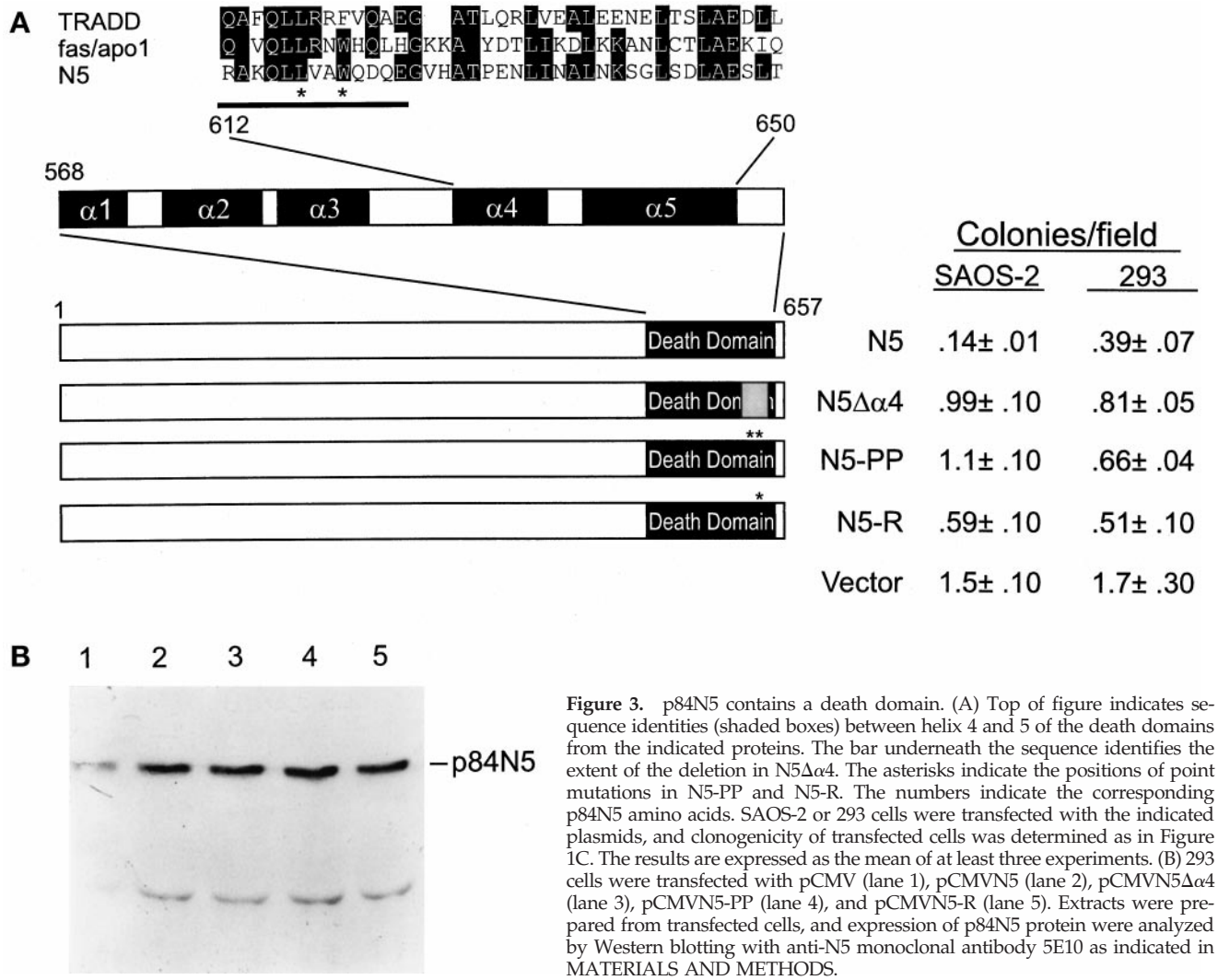

Figure 3. p84N5 contains a death domain. (A) Top of figure indicates sequence identities (shaded boxes) between helix 4 and 5 of the death domains from the indicated proteins. The bar underneath the sequence identifies the extent of the deletion in N5Δα4. The asterisks indicate the positions of point mutations in N5-PP and N5-R. The numbers indicate the corresponding p84N5 amino acids. SAOS-2 or 293 cells were transfected with the indicated plasmids, and clonogenicity of transfected cells was determined as in Figure 1C. The results are expressed as the mean of at least three experiments. (B) 293 cells were transfected with pCMV (lane 1), pCMVN5 (lane 2), pCMVN5Δα4 (lane 3), pCMVN5-PP (lane 4), and pCMVN5-R (lane 5). Extracts were prepared from transfected cells, and expression of p84N5 protein were analyzed by Western blotting with anti-N5 monoclonal antibody 5E10 as indicated in MATERIALS AND METHODS.

each of the mutants, as well as pCMVN5, revealed similar levels of protein overexpression (Figure 3B). All of the mutations created in the death domain, therefore, significantly decreased the specific activity of p84N5.

Dominant interfering mutants of proteins such as Fas or TRADD were created by subtle mutations within their death domains (Park and Baichwal, 1996; Vaishnaw *et al.* 1999). Presumably these mutations blocked transmission of the death signal but permitted other normal protein–protein interactions. We tested the ability of the N5 death domain mutants to interfere with cellular responses to ionizing radiation. Treatment of SAOS-2 cells with γ radiation caused a transient G2/M cell cycle arrest and subsequent apoptosis (Haas-Kogan *et al.*, 1995). Because expression of Rb inhibited this process, it was possible that dominant interfering N5 mutants would have similar effect. Equal numbers of cells successfully transfected with each of the mutants and a GFP expression plasmid were collected by FACS and either irradiated or left untreated. Two days later the number of remaining viable cells in treated versus untreated cells was compared by XTT assay. In vector control-transfected cells, the number of viable cells in the irradiated sample was approximately one-half of the untreated sample, which was consistent with the normal response of SAOS-2 cells to γ-radiation (Haas-Kogan *et al.*, 1995). Cells transfected with the N5 mutants, however, had a greater number of surviving cells in the irradiated sample relative to the untreated sample, ranging from 64 to 75%. Like Rb, therefore, expression of N5 death domain mutant proteins inhibited the normal response of SAOS-2 cells to radiation.

Native p84N5 Is Modified during Apoptosis

Because p84N5 is constitutively expressed in many cell lines without loss of cell viability, we analyzed whether p84N5 is altered during apoptosis to account for its apparent activation. We examined endogenous p84N5 during apoptosis induced by ionizing radiation or treatment with staurosporine. Expression of endogenous p84N5 was monitored subsequent to treatment by Western blotting an equal quantity of total protein extracted from viable, adherent cells or from nonadherent cells. As above, the anti-p84N5 monoclonal

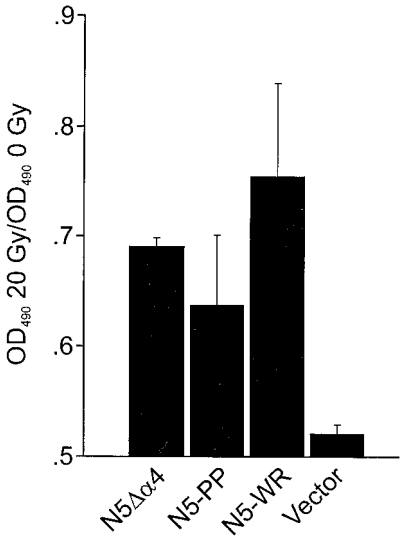

Figure 4. Expression of N5 death domain mutants interferes with the response of SAOS-2 cells to ionizing radiation. The indicated N5 death domain mutants were transfected into SAOS-2 cells, and successfully transfected cells were treated with 0 or 20 Gy of γ-radiation. The relative number of viable cells remaining 2 d later was determined by XTT assay. The results are expressed as the ratio of the OD_{490} of irradiated cells divided by the OD_{490} of untreated cells. The results for each sample are the mean ± SD of two independent transfections performed in triplicate.

antiserum specifically recognized 84-and 58-kDa immunoreactive proteins in lysates prepared from untreated cells (0 Gy) (Figure 4A.). In adherent cells treated with 20 Gy, the relative level of the full length 84-kDa protein declined significantly. This was accompanied by the appearance of novel immunoreactive protein migrating with an apparent molecular mass of ~62 kDa. In adherent cells treated with 10 Gy, an increase in the 62-kDa protein is detected without a major change in the p84N5 level. Adherent cells treated with 5 Gy showed little change in N5 protein compared with untreated cells. In nonadherent cells the 62-kDa form made up the majority of N5 protein, with nearly complete loss of the 84-kDa form at each dose of radiation; however, the 58-kDa form of the protein was still present in these cells. Appearance of the 62-kDa immunoreactive protein was also detected in cells treated with staurosporine or in cells undergoing spontaneous apoptosis (Figure 4A, lane S, and detached, untreated cells). To ensure equal loading of total cell protein, blots were reprobed for β-actin. The β-actin protein was intact and present in approximately equal concentration in each of the samples. These results indicated that expression of endogenous p84N5 was altered specifically during apoptosis and was accompanied by the appearance of novel forms of N5-related protein with smaller apparent molecular mass. The continued presence of the intact 58-kDa anti-N5 immunoreactive protein, as well as β-actin, within treated cells demonstrated that loss of p84N5 and was not due to nonspecific proteolysis that typically occurs during necrosis or the later stages of apoptosis.

The N5 protein normally has a specific subnuclear localization that gives a characteristic punctate nuclear staining pattern (Durfee *et al.*, 1994). We have examined the localization of p84N5 3 d after irradiation in an attempt to determine whether this localization changes during apoptosis. Irradiated cells are fixed and stained for N5 protein and counterstained for DNA with Hoechst 33342. In some apoptotic cells with pyknotic nuclear morphology, N5 protein staining is no longer confined exclusively to the nucleus and can be detected throughout the cell. In most cells without overt apoptotic morphology, N5 staining is still confined to the nucleus but is more homogeneous than in the unirradiated controls (Figure 4B). In none of the unirradiated cells examined can N5 protein staining be detected outside the nucleus. These results suggest that the alteration in p84N5 observed by Western blotting is accompanied by a change in subnuclear localization. As apoptosis proceeds with attendant changes in gross nuclear morphology, N5 protein can be detected outside of the nucleus.

Association with Rb Protein Inhibits p84N5-induced Apoptosis

Previous studies demonstrated that p84N5 binds to hypophosphorylated forms of p110Rb both in vivo and in vitro (Durfee *et al.*, 1994). A domain within the N-terminal half of p110Rb was necessary and sufficient for efficient binding. To test whether p110Rb influences p84N5-induced apoptosis, an N5 GST (GSTN5) fusion protein was mixed with purified p110Rb and microinjected into SAOS-2 cells (Figure 5C). The GSTN5 protein contained the C-terminal half of p84N5, including residues that are both necessary and sufficient for binding p110Rb in vitro (Durfee *et al.*, 1994). Apoptosis was measured 90 min after injection by observation of apoptotic morphology and an increase in permeability to low concentrations of the DNA-binding fluorochrome Hoechst 33342 (Ormerod *et al.*, 1993). Injection of GSTN5 results in a large increase in cells with condensed, brightly stained nuclei relative to cells injected with GST (Figure 5A). Increased permeability to Hoechst 33342 and altered morphology are characteristic of the early stages of apoptosis. Coinjection of p110Rb with GSTN5 reduced the percentage of cells with bright nuclear staining to that of the negative control, GST (Figure 5B). Mixing GSTN5 with GST had no effect on the percentage of apoptotic cells typically seen upon microinjection of GSTN5 alone.

Rb protein may inhibit GSTN5 directly or it may influence GSTN5 indirectly through Rb-mediated changes in the cell cycle or transcription. To distinguish between these two possibilities, GSTN5 was coinjected with an N-terminal–truncated form of Rb protein (p56Rb). The p56Rb lacks residues required for p84N5 binding (Durfee *et al.*, 1994) yet is capable of regulating cell cycle progression (Goodrich *et al.*, 1991) and binding most of the other cellular proteins with which p110Rb normally interacts. Mixing p56Rb with GSTN5 had no effect on the percentage of brightly stained cells typically observed upon injection of GSTN5 alone or with GST (Figure 5B).

To ensure that the effects of Rb protein observed were not specific for the truncated N5 fusion protein used or for the microinjection assay, we tested the effects of cotransfection of Rb expression plasmids on pCMVN5-induced apoptosis. Coexpression of wild-type p110Rb with p84N5 had a small effect on the proportion of cells undergoing DNA fragmentation that is observed upon expression of p84N5 alone

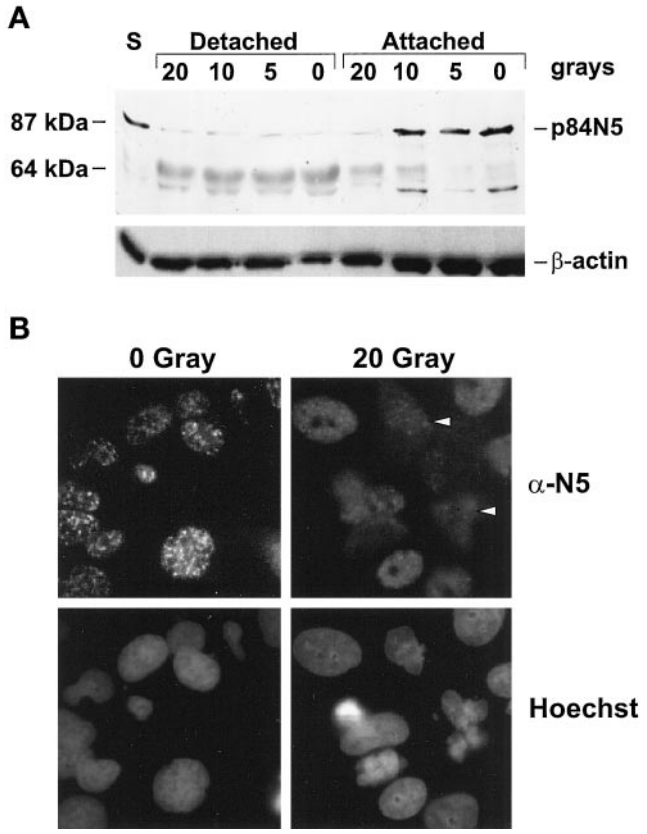

Figure 5. The structure and subcellular localization of endogenous p84N5 is altered during apoptosis. (A) SAOS-2 cells were treated with the indicated dose of ionizing radiation or staurosporine (S). Attached or detached cells were harvested separately 72 h (irradiated) or 12 h (staurosporine) later, and p84N5 expression was analyzed in each sample by Western blotting as in Figure 1D. The positions of p84N5, β-actin, or a 64-kDa molecular weight standard are indicated on the left. (B) SAOS-2 cells were irradiated with the indicated dose of γ-radiation. Three days later, cells were processed for immunofluorescent staining using the α-N5 5E10 monoclonal antibody as described in MATERIALS AND METHODS. Cells were photographed at 630×. The arrows indicate two examples of cytoplasmic N5 staining in cells with pyknotic nuclear morphology. Note the more homogeneous α-N5 staining in the irradiated sample.

(Figure 6D); however, a form of p110Rb containing alanine substitutions at 14 consensus cyclin-dependent kinase phosphorylation sites significantly reduced the percentage of cells containing fragmented DNA. This mutant p110Rb is constitutively active because it is resistant to negative regulation by phosphorylation. Consistent with our microinjection experiments, expression of a phosphorylation-resistant form of p56Rb did not affect the percentage of cells exhibiting p84N5-induced DNA fragmentation.

DISCUSSION

The results presented in this study demonstrate that forced expression of p84N5 induces apoptotic cell death. This conclusion is based on the observation that loss of cell viability upon expression of p84N5 is accompanied by changes in cellular morphology and internucleosomal DNA fragmentation that are characteristic of apoptotic cells. Furthermore, we demonstrate that some, but not all, naturally occurring inhibitors of apoptosis suppress p84N5-induced apoptosis. Apoptosis induced by p84N5 does not require p53 as indicated by the fact that p53-negative SAOS-2 cells (Chandar *et al.*, 1992) are susceptible to p84N5-induced death.

Although the physiological role of p84N5 is unknown, several observations suggest that it may normally function in the regulation of apoptosis. For example, the N5 cDNA contains a region with statistically significant sequence similarity to the death domain of several proteins involved in apoptotic signaling (Feinstein *et al.*, 1995). Because several proteins with no known role in cell death also contain death domains, this fact alone is not sufficient to suggest apoptotic function; however, we demonstrate that expression of p84N5 can induce apoptosis and that this function is dependent on an intact death domain. Analogous mutations in helix 4 of the p84N5 and the TNFR-1 death domains (W620P or R for N5; W378A for TNFR-1) compromise the ability of these proteins to induce apoptotic cell death. N5 protein, therefore, likely has a death domain that may function like those in other well characterized death domain proteins. In addition, we demonstrate that expression of endogenous p84N5 is altered during apoptosis triggered by ionizing radiation. Loss of p84N5 is accompanied by accumulation of N5 protein, with an increased electrophoretic mobility. This alteration is coincident with a change in the subnuclear localization of N5 protein. Although further work is required to determine the mechanism of p84N5 alteration, our observation is reminiscent of caspase-mediated proteolysis of proteins, such as PARP (Lazebnik *et al.*, 1994) or DFF (Liu *et al.*, 1997), that occur specifically during apoptosis. Based on caspase substrate specificity as determined by Talanian *et al.* (1997), several potential caspase cleavage sites exist within p84N5. For example, $DVLD^{102}$ of p84N5 is a near-optimum caspase 2, 3, or 7 substrate. We also demonstrate that some apoptotic inhibitors (Bcl-2 and p35) inhibit p84N5-induced apoptosis, but not others (CrmA). This observation demonstrates that p84N5 expression does not result in nonspecific cell toxicity but rather activates a specific apoptotic pathway. Other physiological triggers of apoptosis activate similar pathways because they show a similar sensitivity profile to inhibitors. For example, apoptosis triggered by ionizing radiation is sensitive to Bcl-2 and p35 but not to CrmA (Datta *et al.*, 1997). Finally, we show that expression of death domain mutants of N5 interfere with the normal response of SAOS-2 cells to ionizing radiation. This suggests that subtle alteration of the N5 death domain creates dominant interfering mutants. Similar effects have been observed in other death domain proteins such as Fas or TRADD (Park and Baichwal, 1996; Vaishnaw *et al.* 1999). All of these findings suggest that p84N5 normally plays a role in the regulation of apoptosis. Additional experiments will be required to confirm this hypothesis and establish the mechanism used by p84N5.

The mechanisms that cells use to generate and transduce apoptotic signals within the nucleus are not well characterized. Other nuclear proteins such as PML and the CAG repeat proteins (Huntingtin, Ataxin-1, etc.) initiate apoptotic cell death by novel mechanisms (Quignon *et al.*, 1998; Sau-

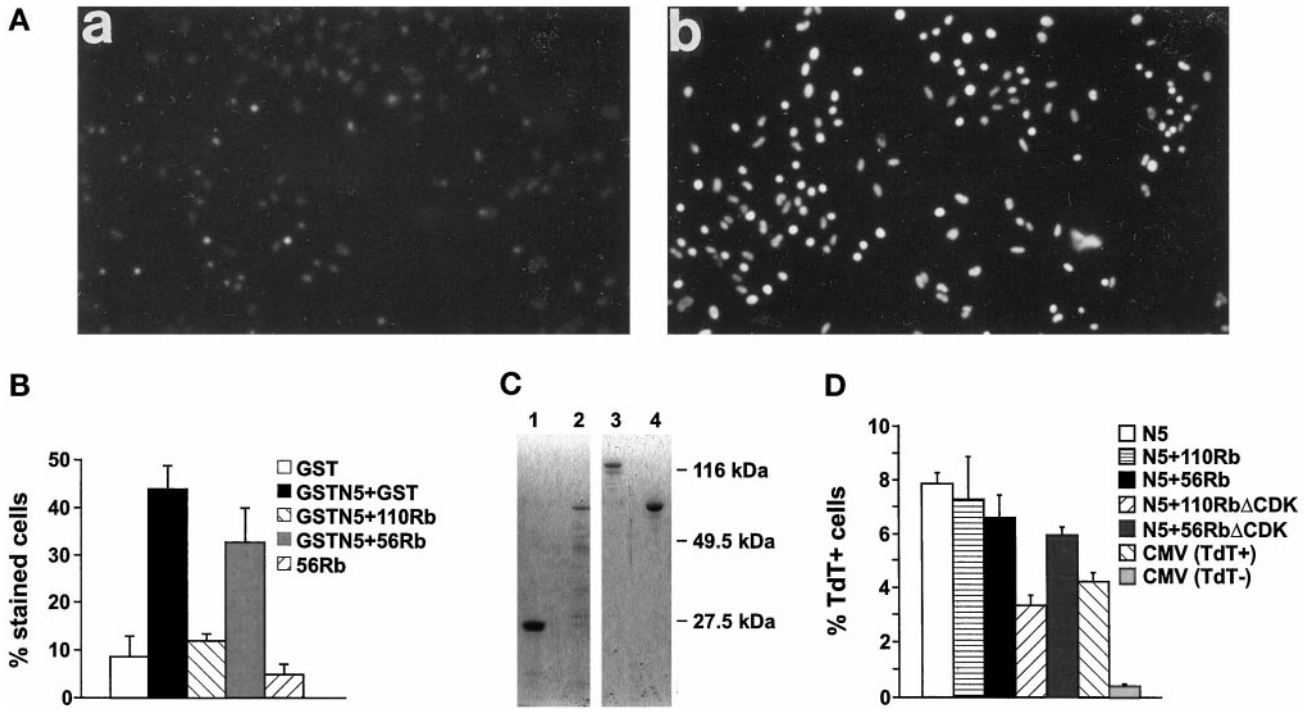

Figure 6. Association with Rb inhibits p84N5-induced apoptosis. (A) SAOS-2 cells were injected with purified GST (a) or GSTN5 (b). Ninety minutes later, Hoechst dye was added to the media at 1 μg/ml for 10 min before photography under fluorescence microscopy at 100×. All cells within the field of view have been injected. (B) Cells were injected and analyzed as above with the indicated proteins. The percentage of injected cells with apoptotic morphology and bright Hoechst staining was then counted. The results are the mean of at least three experiments. (C) Aliquots (5 μl) of GST (lane 1), GSTN5 (lane 2), p110Rb (lane 3), or p56Rb (lane 4) used for microinjection were resolved by SDS-PAGE and stained with coomassie blue. The position of molecular weight standards is indicated at the right. (D) SAOS-2 cells were transfected with the indicated expression plasmids and analyzed for apoptosis by TUNEL and flow cytometry as in Figure 2C. The percentage of cells containing fragmented DNA is shown as the mean of three experiments. The maximum transfection efficiency under the conditions used was ~10%, indicating that most of the N5-transfected cells contained fragmented DNA.

dou *et al.*, 1998). How the apoptotic signals initiated by these proteins are transduced to the apoptotic machinery is unknown. N5 protein is unique among proteins that trigger apoptosis from within the nucleus in containing a death domain. Hence, N5 could provide a physical link between apoptotic signals generated within the nucleus and the apoptotic machinery if its death domain functions like other death domain proteins involved in apoptosis. By analogy to TNF or Fas ligand signaling, N5 could potentially recruit death domain adaptor molecules to a complex that ultimately leads to caspase activation.

The N5 protein is expressed constitutively in several cell lines that have been analyzed. Constitutive expression of p84N5 at first glance is paradoxical, given our finding that forced p84N5 expression induces apoptotic cell death. Several proteins important for the signaling and execution of apoptosis, however, are also constitutively expressed in cells. These proteins are typically activated by posttranslational modification during apoptosis. For example, caspases are constitutively expressed as relatively inactive proenzymes that are activated by proteolysis (for review, see Kidd, 1998). Numerous other important mediators of apoptosis are also activated by proteolysis, including DFF (Liu *et al.*, 1997), Bid (Luo *et al.*, 1998), and sterol regulatory element binding proteins (Wang *et al.*, 1996), among others. We propose that p84N5 is also activated by posttranslational modification. Like forced expression of caspases, forced p84N5 expression would increase the amount of activated protein above a threshold necessary for triggering apoptosis. We suspect that the N5-related proteins with altered electrophoretic mobility generated during transfection or irradiation may represent activated forms of N5 protein.

Rb protein associates with >60 different cellular proteins, and association with various subsets of these proteins mediates its functions. Most of these proteins use domains within the C-terminal half of p110Rb, collectively termed the "large pocket," for binding. The large pocket is sufficient to mediate many of the well studied functions of p110Rb, including regulation of the cell cycle or transcription. The Rb domains required for inhibition of apoptosis have not been defined. N-terminal domains of p110Rb are required to rescue the excessive apoptosis observed upon genetic loss of Rb in the mouse (Riley *et al.*, 1997), suggesting that this domain may be required for inhibition of apoptosis in the affected tissues. A related question is whether the effect of Rb on apoptosis is an indirect consequence of its other established effects on the cell cycle. Our data suggest that one mechanism used by Rb to influence apoptosis is through functional association with p84N5. Coexpression of p110Rb inhibits p84N5-induced apoptosis. In addition, inhibition of p84N5-

induced apoptosis correlates with the ability of p110Rb to bind p84N5. N-terminally truncated forms of Rb lacking sequences required for p84N5 binding, yet containing an intact large pocket, do not effectively inhibit p84N5-induced apoptosis. Furthermore, phosphorylation-resistant forms of p110Rb are more effective in inhibiting p84N5 than phosphorylation-sensitive forms. Phosphorylation also inhibits p84N5/p110Rb complex formation (Durfee *et al.*, 1994). These findings are consistent with the hypothesis that Rb has a direct effect on apoptosis that is independent of its ability to regulate the cell cycle. In addition, these observations identify a novel function for the N-terminal half of p110Rb because it is required for physical association with p84N5. This conclusion may explain, at least in part, the requirement for N-terminal p110Rb domains for rescuing apoptotic defects in mouse embryos lacking wild-type Rb. None of the other proteins that require the N-terminal half of p110Rb for binding has an established role in regulating apoptosis.

Because both cell proliferation and cell death must be deregulated for tumorigenesis, genes with a role in coordinating these two processes may be expected to be important in cancer. Rb and p53 are the most frequently mutated genes in human cancer. The p53 gene has a well characterized role in regulating the cell cycle and apoptosis in response to DNA damage. On the basis of the evidence presented here, we propose that Rb may also have a direct role in the coordination of cell proliferation and cell death by association with proteins that modulate each process.

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