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## Identification of a Novel Antiapoptotic Functional Domain in Simian Virus 40 Large T Antigen

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**The ability of DNA tumor virus proteins to trigger apoptosis in mammalian cells is well established. For example, transgenic expression of a simian virus 40 (SV40) T-antigen N-terminal fragment (N-termTag) is known to induce apoptosis in choroid plexus epithelial cells. SV40 T-antigen-induced apoptosis has generally been considered to be a p53-dependent event because cell death in the brain is greatly diminished in a p53<sup>-/-</sup> background strain and is abrogated by expression of wild-type (p53-binding) SV40 T antigen. We now show that while N-termTags triggered apoptosis in rat embryo fibroblasts cultured in low serum, expression of full-length T antigens unable to bind p53 [mut<sup>(p53-)</sup>Tags] protected against apoptosis without causing transformation. One domain essential for blocking apoptosis by T antigen was mapped to amino acids 525 to 541. This domain has >60% homology with a domain of adenovirus type 5 E1B 19K required to prevent E1A-induced apoptosis. In the context of both wild-type T antigen and mut<sup>(p53-)</sup>Tags, mutation of two conserved amino acids in this region eliminated T antigen's antiapoptotic activity in REF-52 cells. These data suggest that SV40 T antigen contains a novel functional domain involved in preventing apoptosis independently of inactivation of p53.**

Apoptosis is a morphologically distinct form of cell death which represents an important mechanism for regulation of cellular proliferation. The discovery that the most common chromosomal translocation found in B-cell follicular lymphomas results in the overexpression of the antiapoptotic *bcl-2* gene product (3, 38) underscores the importance of apoptosis in regulating cellular growth. Presumably, Bcl-2 overexpression allows the outgrowth of abnormal cells which otherwise would be eliminated by apoptosis. More recently, a family of *bcl-2*-related viral and cellular genes which can modulate apoptosis (7, 25) has been described; additional gene families whose products also inhibit apoptosis have been identified (8, 15, 34).

Apoptosis has also been shown to be a common host cell response to a wide variety of viral infections, and many viruses, in turn, produce proteins which inhibit apoptosis. Among the small DNA tumor viruses, recent evidence indicates that expression of the adenovirus E1A protein (13), the human papillomavirus (HPV) E7 protein (17, 26), or an N-terminal fragment of the large T antigen (Tag) of simian virus 40 (SV40) (N-termTag) (35) can induce apoptosis in rodent cells of epithelial origin. All of these viral proteins are able to bind to and inactivate pRb, p107, and p130 but not affect directly the activities of p53. This discovery is consistent with the more general observation that disruption of normal cellular growth control pathways (in this case, inactivation of the pRb family of proteins) can trigger apoptosis (1, 20, 27). Interestingly, expression of adenovirus E1A, HPV E7, and SV40 N-termTag in p53<sup>-/-</sup> knockout mouse cells has been shown to cause transformation and limit apoptosis. This finding is consistent with the known antiapoptotic functions associated with those DNA tumor virus proteins known to inactivate p53 function: adenovirus E1B 55K, HPV E6, and the carboxyl half of SV40 Tag. However, the most potent antiapoptotic viral protein identified

thus far, the adenovirus E1B 19K protein, does not interact directly with p53 and is able to overcome both p53-dependent (41) and -independent (22) E1A-induced apoptosis.

The domains of SV40 Tag involved in preventing apoptosis have not been directly studied. However, based on the finding that choroid plexus epithelial cells from p53<sup>+/+</sup> (but not p53<sup>-/-</sup>) mice undergo apoptosis when an N-TermTag is expressed, protection has been assumed to involve those regions of Tag that bind to and inactivate p53 (35). Consistent with this, we previously found that three domains of Tag, the extreme N terminus (amino acids [aa] 1 to 82), the pRb family binding region (aa 105 to 113), and the p53 binding region (aa 350 to 450 and 540 to 708), are all required to immortalize primary mouse embryo fibroblasts (MEFs) (11). However, we also observed that full-length mutant Tags defective for p53 binding [mut<sup>(p53-)</sup>Tags] neither immortalized nor induced apoptosis in MEFs; rather MEFs expressing mut<sup>(p53-)</sup>Tags ceased proliferation with a characteristic senescent morphology. The differing fates of cells expressing mut<sup>(p53-)</sup>Tags versus N-termTags suggested the existence of a novel antiapoptotic activity in the full-length protein. In this study, we identified a functional region of T antigen which protects against apoptosis independently of p53 binding and inactivation. Interestingly, this region includes a short, 16-aa domain (aa 525 to 541) which has greater than 60% amino acid homology with a domain of adenovirus type 5 (Ad5) E1B 19K previously shown to be essential for preventing adenovirus E1A-induced apoptosis. In quantitative studies, expression of full-length SV40 Tags bearing changes at two conserved amino acids in this region resulted in significantly decreased viability of the rat embryo fibroblast (REF) cell line REF-52; furthermore, these cells died with a characteristic apoptotic morphology. Moreover, decreased viability was observed when these single amino acid mutations were introduced not only into mut<sup>(p53-)</sup>Tags but also in an otherwise wild-type Tag (i.e., p53 binding) background. Thus, we have identified a novel antiapoptotic domain which contributes to SV40 Tag's oncogenic function independently of p53 binding.

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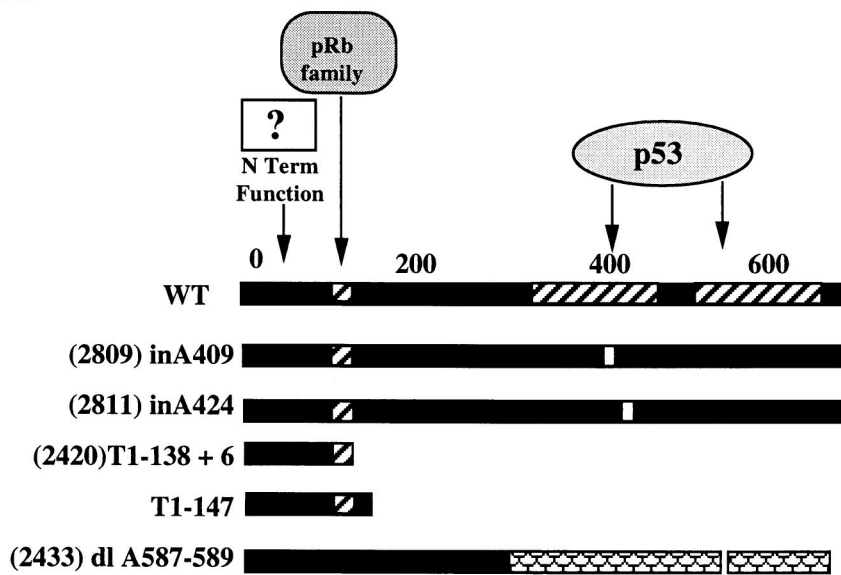
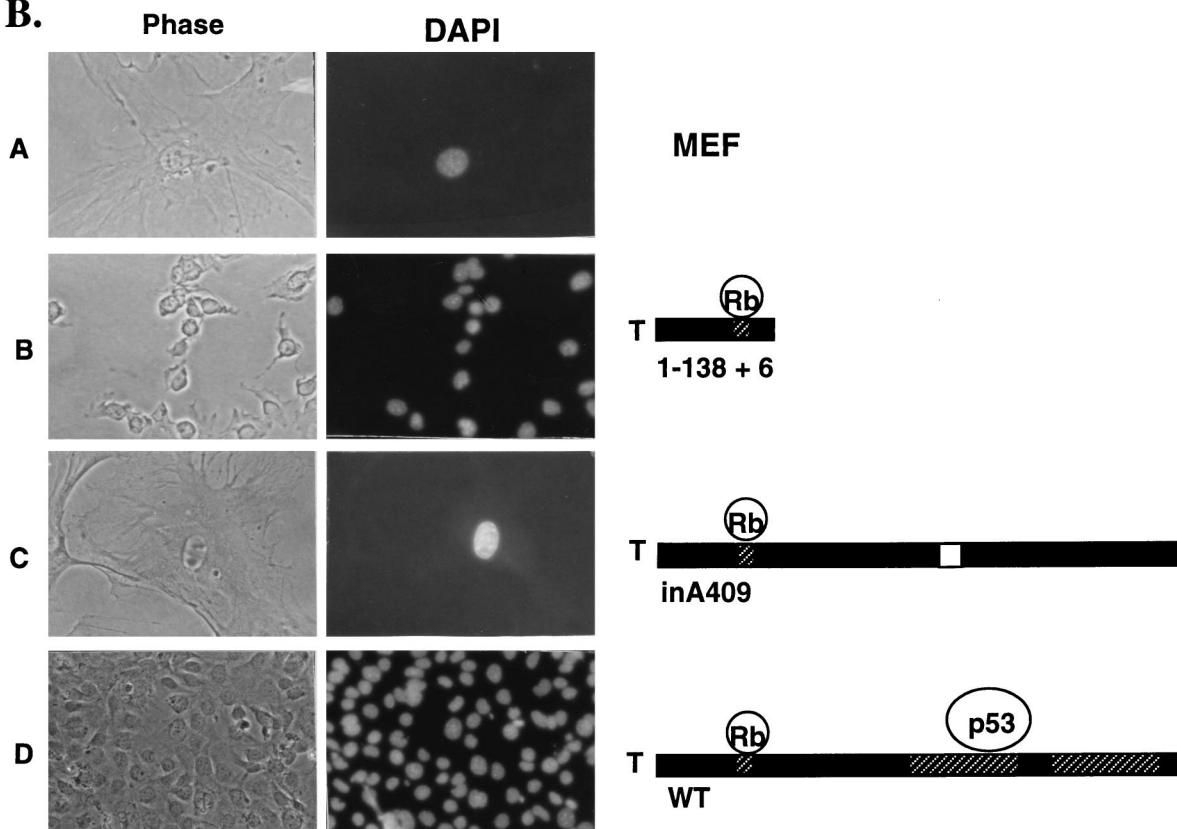
**A.****B.**

FIG. 1. (A) Functional regions of Tag. The regions of SV40 Tag required for initiation of cellular DNA synthesis and immortalization of primary MEFs are represented by hatched bars. Full-length mutant Tags which are defective for p53 binding (*inA409* and *inA424*) because of small linker insertions at aa 409 or 424 are illustrated; mutants unable to bind p53 because of a truncation upstream of the p53-binding domain (T1-138 + 6 and T1-147) or a misfolded carboxyl half (*dlA2433*, lacking aa 587 to 589) are also shown. WT, wild type. (B) Cellular morphology of control MEFs transfected with pSV2neo alone (A), cells expressing mutant Tags (B and C), and cells expressing wild-type Tag (D). Early-passage C57BL/6 MEFs were transfected at passage 0 or 1 with the neomycin resistance plasmid pSV2neo, with or without a plasmid encoding a truncated or full-length mutant of SV40 Tag or wild-type Tag. Representative fields (magnification,  $\times 40$ ) are shown in phase contrast and following DAPI staining to detect DNA morphology.

## MATERIALS AND METHODS

**Plasmids and generation of stable cell lines.** Primary REFs were harvested from day 12 to 16 C57BL/6 mouse or Fisher rat embryos as described previously (11). REF-52 cells were a kind gift of Jim Pipas (University of Pittsburgh). All cells were maintained in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Gibco/BRL, Grand Island, N.Y.). SV40 plasmids *inA2809* (insert of 4 aa at aa 409; also called *inA409* in this report), *inA2811* (insert of 4 aa at aa 424; also called *inA424* in this report) (45), *dIA2433* (deletion of aa 587 to 589), *inA2420* (expresses aa 1 to 138 plus 6 additional aa) (9), T147 (expresses aa 1 to 147) (30), Ad5 E1A (42), and pSV2neo (31) have been described previously (Fig. 1A). The new mutant plasmids WT/528MS, *inA409/528MS*, *inA424/528MS*, WT/539AD, *inA409/539AD*, and *inA424/539AD* were generated by using conditions suggested by the manufacturer of the PCR-based, site-directed mutagenesis QuikChange kit (Stratagene, La Jolla, Calif.). The upper primer used for the methionine-to-serine mutation at aa 528 was 5'-CCCTGGAATAGTCACTCGAATGAGTACAGTGTGCC-3', and the lower primer was 5'-GGCACACTGTACTATTGAGGTGACTATTCCAGGG-3'; the upper primer used for the alanine-to-aspartic acid mutation at aa 539 was 5'-GCCCTAAACACTGCAGGACAGATTGTGAAAAC-3', and the lower primer was 5'-GTTTACAAATCTGCTCTGCAGTGTGTTTAGGC-3'. Putative mutagenized plasmids were screened by restriction enzyme digestion. Mutations were confirmed by using the sequencing primers 5'-GGCTCTGCTGACATAGAAGAATGG-3' and 5'-GCCACAGGTCTGTACCAAAATTAA CATAAGAAGC-3' in the ABI PRISM Dye Terminator Cycle Sequencing method (Perkin-Elmer, Branchburg, N.J.) according to the manufacturer's instructions. Double-stranded fluorescent sequencing was performed with an ABI DNA sequencer at the Dartmouth Molecular Biology Core Facility.

For generating cells expressing various Tags or other proteins, primary rodent fibroblasts or REF-52 cells were seeded at  $10^5$  cells per 10-cm-diameter plate the day prior to calcium phosphate transfection; 48 h later, medium containing 400 µg of G418 per ml was added to cultures. Individual G418-resistant clones were selected 21 days later and either fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) to reveal DNA morphology (primary cells) or subcloned for further experiments.

**Immunofluorescence and DAPI staining.** Cells were seeded in 15-mm-diameter wells on Teflon-coated glass slides (Cel-line, Newfield, N.J.) and allowed to adhere. The following day, cells were washed twice with phosphate-buffered saline (PBS), and fresh DMEM containing 0.5 or 10% FCS was added to each well. Four days later, cells were washed twice with PBS and fixed in freshly prepared (4%, wt/vol) paraformaldehyde. After 15 min at room temperature, cells were washed with PBS and the primary antibody (Tag antigen monoclonal antibody [MAb] 419 and E1A MAb M73 [a gift of Ed Harlow, Massachusetts General Hospital Cancer Center, Boston, Mass.]) was added at a 1:10 dilution in PBS containing 10% goat serum. Slides were incubated in a humid chamber for 1 h at 37°C and then rinsed three times in PBS–10% goat serum. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G antiserum (Gibco/BRL) diluted 1:200 in PBS–10% goat serum was then added to the wells for 40 min at 37°C. The cells were washed three times with PBS and then incubated with DAPI (1 µg/ml [final concentration] in PBS) for 1 min. Cells were washed once more with PBS, and cover slips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.). Slides were examined on a Zeiss Axiophot microscope at a magnification of  $\times 400$ .

Primary cells were examined in essentially the same manner except that all staining was done in Falcon 10-cm-diameter tissue culture dishes in order to maintain colony and individual cellular morphology of cells undergoing apoptosis or senescence. Cells were photographed with a Nikon inverted microscope at a magnification of  $\times 40$ .

**In situ DNA fragmentation assay.** Apoptotic cells were detected by using an Apoptag in situ detection kit (Oncor, Gaithersburg, Md.). Briefly, cells were allowed to adhere to Teflon-coated slides in duplicate 15-mm-diameter wells. The next day, cells were washed twice with Tris-buffered saline (TBS), and DMEM containing either 10 or 0.5% FCS was added to each well. After 4 days, the assay was performed according to the manufacturer's instructions except that the cells were stained with DAPI (1 µg/ml in PBS) for 1 min prior to the final rinse. Slides were allowed to air dry, mounted with Vectashield, and observed and photographed with a Zeiss Axiophot microscope.

**Western analysis and immunoprecipitations.** For Western blot analyses, cells were washed twice with ice-cold PBS and then lysed with 1 ml of cold EBC buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.5% Nonidet P-40). Protein concentrations were determined by the Pierce colorimetric protein assay (Pierce, Rockland, Ill.), and 10 µg of total cellular protein was added to 5× sodium dodecyl sulfate loading buffer, boiled for 3 min, and electrophoresed on a 4 to 20% precast gradient gel (Bio-Rad, Hercules, Calif.). Following transfer to nitrocellulose, the blot was blocked for 1 h in 5% nonfat milk–PBS and probed with either MAb 419 (against SV40 Tag), PAb240 (against p53),  $\alpha$ -p21 (N-20)-G (Santa Cruz Biochemicals, Santa Cruz, Calif.), or  $\alpha$ -Bax (N-20)-G (against Bax) (Santa Cruz). The blots were rinsed with PBS–0.1% Tween 20 (Sigma, St. Louis, Mo.) and incubated with an appropriate secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Amersham) diluted 1:5,000 in 5%

milk–PBS. Following four additional rinses in PBS–0.1% Tween 20, blots were developed by using chemiluminescence (Amersham).

Immunoprecipitations were performed by labeling  $6.25 \times 10^5$  cells/10-cm-diameter tissue culture dish with 100 µCi of  $^{35}\text{S}$  Express protein labeling mix (NEN/Dupont) per ml for 90 min in methionine-free medium containing 2.5% dialyzed FCS (Gibco/BRL). Cells were lysed in 1 ml of ice-cold EBC, the cellular debris was removed by centrifugation, and lysates were precleared by using 40 µl of protein A-Sepharose beads (Pharmacia Biotech Inc.) suspended in a 1:1 ratio with TBS containing 10% bovine serum albumin (TBS-BSA) on a rotator at 4°C. Following centrifugation, the supernatants were transferred to clean tubes and incubated with 100 µl of MAb 419 (tissue culture supernatant) for 90 min, 40 µl of protein A-Sepharose beads (1:1 in TBS-BSA) was added, and the mixture was incubated overnight on a rotator at 4°C. The following day, the beads were collected by centrifugation, washed three times with ice-cold PBS, and resuspended in 40 µl of 1× sodium dodecyl sulfate sample buffer. Samples were boiled, subjected to electrophoresis on a 12% polyacrylamide gel, and visualized by fluorography.

**Viability assays.** Cells were seeded at subconfluent densities in six-well plates and allowed to adhere overnight. The following day, wells were washed twice with PBS, and fresh DMEM containing 0.5 or 10% FCS was added. After 2, 4, and 6 days, floating cells were removed along with medium, and adherent cells were trypsinized. Cells were pooled and then centrifuged. The pellet was resuspended in 0.4% trypan blue solution (Sigma), and the percentage of cells that did not take up the dye was determined by two observers. At least 200 cells were counted for each time point in each experiment.

## RESULTS

**Primary REFs expressing  $\text{mut}^{(p53-)}\text{Tags}$  undergo senescence, while expression of N-termTags results in apoptosis.** Previous work in our and other laboratories has demonstrated that  $\text{mut}^{(p53-)}\text{Tag}$  expression is unable to immortalize or extend the life span of primary rodent fibroblasts (11, 21, 37, 44). A diagram representing some of these immortalization-deficient full-length and truncated Tags is shown in Fig. 1A. Primary MEFs expressing a representative N-termTag, *inA2420* (T1-138 + 6), formed colonies which, at the time of control (not transfected) cell senescence, contained cells which (i) were dramatically smaller than senescing cells, (ii) often failed to survive as long as senescing cells because they rounded up and died, and (iii) exhibited nuclear morphology (shrunken nuclei with chromatin condensation) characteristic of apoptosis (Fig. 1B, panel B). In contrast, primary cells expressing a  $\text{mut}^{(p53-)}\text{Tag}$  (*inA2809/inA409*) ceased cell division with a senescent morphology identical to that seen with control cells (reference 11 and Fig. 1B, panels A and C). Wild-type Tag-expressing cells formed dense colonies of small, adherent, and rapidly dividing cells which were inevitably immortal (Fig. 1B, panel D). Similar results were obtained when primary REFs expressing  $\text{mut}^{(p53-)}\text{Tags}$  were studied, although the time (and number of population doublings) prior to control cell senescence was greater in REFs than in MEFs (data not shown).

**REF-52 cells expressing N-termTags undergo increased cell death with the morphological characteristics of apoptosis.** To study quantitatively the apoptosis induced by Tag, the REF-52 cell line was used to generate early-passage stable cell clones expressing either full-length or truncated SV40 Tags defective for p53 binding. Although immortalized, the REF-52 line has the same genetic requirements for SV40 Tag-induced transformation as primary REFs (32, 46) and has been used extensively for DNA tumor virus studies (19). REF-52 cells were transfected with N-termTag-expressing plasmid *inA2420*, (T1-138 + 6), T147dl or T147Ddl (encodes aa 1 to 147, either with or without small t antigen, respectively), or *dIA2433*, a mutant with a misfolded carboxyl half due to a deletion of aa 587 to 589 (Fig. 1A). Mutant *dIA2433* has been previously shown to encode a protein which is completely defective for several carboxyl-region T-antigen functions, including ATP binding and ATPase activity (9), oligomerization, and p53 binding (37, 44), and is not recognized by anti-Tag MAbs directed against conformationally sensitive determinants located within the car-



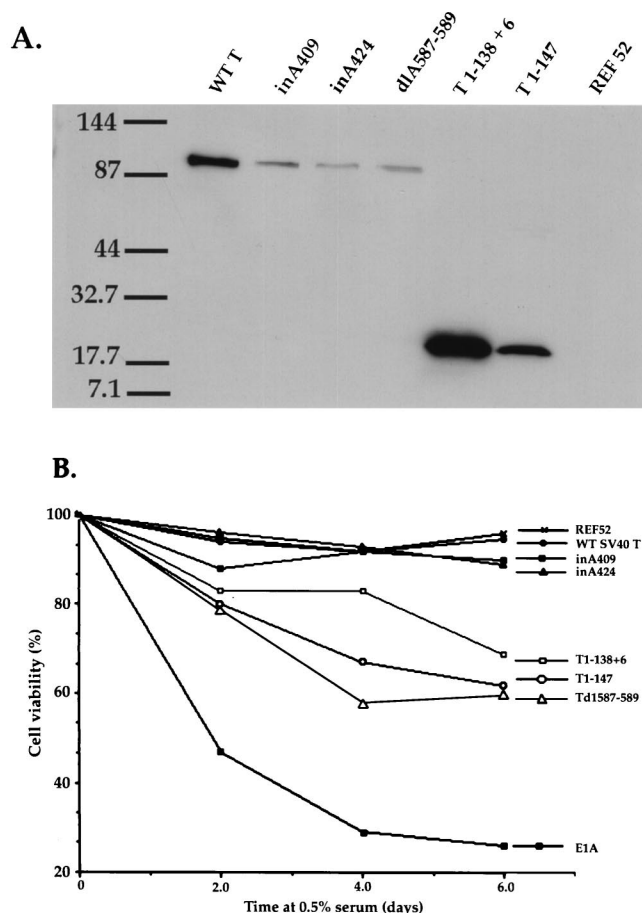


FIG. 2. (A) Western blot analysis of mutant Tags. SV40 mutant Tags expressed in early-passage REF-52 cell lines were detected by using anti-Tag MAb 419, specific for the amino terminus of Tag. WT, wild type. (B) Viability of REF-52 cells expressing truncated and full-length mutant Tags. Viability was determined for floating and adherent cells cultured in DMEM-0.5% FCS by trypan blue exclusion and is expressed as a percentage of the total number of cells counted. A minimum of 200 cells was counted for each time point, and the experiment was repeated at least three times with individually derived cell lines, with similar results.

boxyl half of Tag (10). Early-passage, stable clones expressing full-length or truncated Tags able to bind pRb, p107, and p130 but defective for p53 binding were generated and tested for appropriately sized Tag expression by Western blotting (Fig. 2A).

In REF-52 cells and many other cell lines, low-serum conditions have been shown to induce apoptosis in cells receiving apoptotic stimuli, whereas cells maintained in high serum (10%) do not display significant levels of apoptosis (18, 19, 28). To quantitatively examine the viability of early-passage REF-52 cell lines expressing N-termTags, trypan blue dye exclusion assays were performed on cells grown in medium containing either 10 or 0.5% FCS. Figure 2B shows that the viability of REF-52 cells expressing N-termTags was significantly diminished in 0.5% FCS; however, in cells expressing  $\text{mut}^{(p53-)}\text{Tag}$  (inA2409/inA409 or inA2411/inA424), viability was similar to that of cells expressing wild-type Tag or to the G418-resistant parental REF-52 cell line. Moreover, in REF-52 cells expressing N-termTags, DAPI staining 48 h following serum depletion revealed shrunken nuclei and chromatin condensation characteristic of apoptosis; this morphology

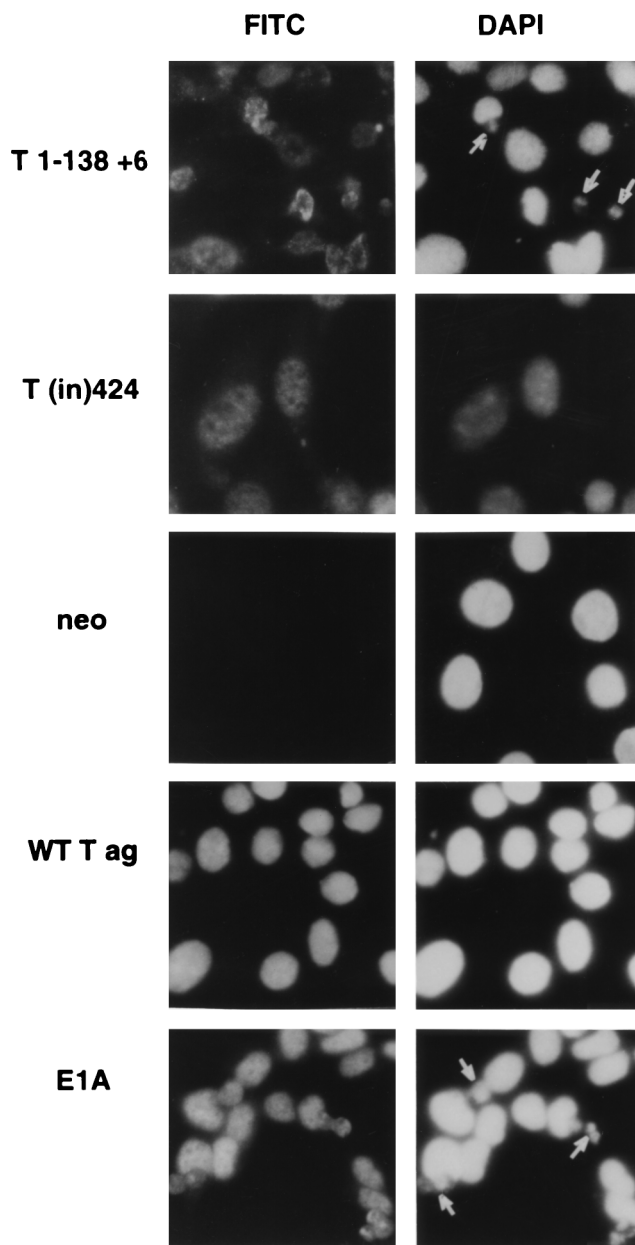


FIG. 3. Indirect immunofluorescence of Tag expression and nuclear morphology of REF-52 cells expressing truncated or full-length mutant Tags. Early-passage REF-52 cells expressing either truncated (top row of each panel) or full-length (second row) mutant SV40 Tags were grown in DMEM-0.5% FCS for 4 days. Neomycin-resistant REF-52 cells (third row) and wild-type (WT) Tag-expressing cells (fourth row) and are shown as negative controls for apoptosis. Cells expressing adenovirus E1A (bottom row) are shown as a positive control for induction of apoptosis. Cells were fixed and then stained with MAb 419 and FITC-labeled goat anti-mouse immunoglobulin G to detect Tag or M73 to detect E1A and subsequently stained with DAPI to detect DNA. Arrows indicate apoptotic cells in T1-138 + 6- and E1A-expressing cells. The same field of cells was photographed with appropriate filters to permit detection of FITC or DAPI.

was not seen in REF-52 cells expressing  $\text{mut}^{(p53-)}\text{Tags}$ , the parental REF-52 cell line, or wild-type Tag (Fig. 3). Expression of small t antigen did not affect viability or nuclear morphology of cells expressing N-termTag (data not shown). In sum, the N terminus of Tag was able to induce apoptosis when this region was expressed alone in REF-52 cells deprived of serum. In

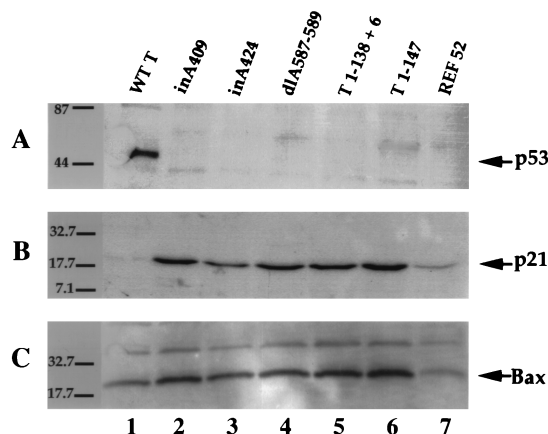


FIG. 4. Expression p53, p21<sup>Waf1/Cip1</sup>, and Bax protein levels in REF-52 cells expressing truncated or full-length mutant Tags. The level of p53 expression was determined by Western blot analysis using MAb 240; p21<sup>Waf1/Cip1</sup> and Bax levels were detected by using polyclonal anti-rabbit antisera against amino-terminal peptides of each protein. WT, wild type.

contrast, this activity appeared to be suppressed when the N terminus was part of a full-length Tag, even in linker insertion mutants unable to bind p53.

**Induction of apoptosis by N-termTags does not alter the expression of p53, p21<sup>Waf1/Cip1</sup>, or Bax.** By analogy with adenovirus E1A expression, in which apoptosis is accompanied by an increase in p53 protein levels, induction of apoptosis by N-termTags but not by mut<sup>(p53-)</sup>Tags suggested a possible difference in the abilities of the two classes of mutants to stabilize p53. Induction of apoptosis has also been associated with alterations in the levels of two other potentially relevant apoptosis-related proteins, p21<sup>Waf1/Cip1</sup> and Bax. Alternatively, if apoptosis induced by an N-termTag is independent of p53 activity, one might not see a difference in p53 protein levels or the levels of p21<sup>Waf1/Cip1</sup> and Bax. Figure 4A shows that while p53 levels were stabilized in wild-type Tag-expressing cells (lane 1), p53 levels remained barely detectable in REF-52 cells expressing either mut<sup>(p53-)</sup>Tags (lanes 2 and 3) or N-termTags (lanes 4 to 6), as determined by Western analysis. These findings suggest that there was no appreciable change in p53 protein levels compared with that of the parental cell line. [<sup>35</sup>S]methionine pulse-chase labeling of cell lines followed by immunoprecipitation with an anti-p53 antibody confirmed a lack of change in p53 half-life as a consequence of expression of mut<sup>(p53-)</sup>Tags (data not shown). This result suggests that intracellular p53 protein levels do not modulate SV40 Tag-induced apoptosis.

Since the absence of a detectable increase in p53 protein levels does not rule out a change in p53 transcriptional activity (2), we examined the protein products of two p53 transcriptional target genes: the cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> and the death-promoting Bcl-2 homolog Bax. p21<sup>Waf1/Cip1</sup> levels were greatly reduced following expression of wild-type Tag (Fig. 4B, lane 1), presumably due to Tag's sequestration of p53 and inactivation of its transcriptional activity (Fig. 4B, lane 1). In contrast, the level of p21<sup>Waf1/Cip1</sup> was clearly greater than the level found in parental REF-52 cells following expression of either the mut<sup>(p53-)</sup>Tags (Fig. 4B, lanes 2 and 3) or N-termTags (Fig. 4B, lanes 4 to 6). This result suggests that p21<sup>Waf1/Cip1</sup> induction may be occurring in a p53-dependent manner, presumably in response to a perturbation of normal cell cycle control mechanisms following inactivation of pRb family members by these mutant Tags. How-

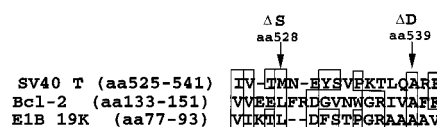


FIG. 5. Amino acid sequence homology of SV40 large Tag, human Bcl-2, and Ad5 E1B 19K. The Gene Inspector program (Textco) was used to perform a dot matrix analysis of SV40 Tag and Ad5 E1B 19K for amino acid sequence similarity. The region shown had by far the greatest homology and corresponds to the same region of E1B 19K which shows significant homology to the BH-1 domain of human Bcl-2. Boxed amino acids represent amino acids that are identical or conserved among the three proteins.

ever, despite the uniform induction of increased p21<sup>Waf1/Cip1</sup> protein levels in cells expressing either N-termTags or mut<sup>(p53-)</sup>Tags inA2409/inA409 and inA2411/inA424, apoptosis was seen only in cells expressing the Tag fragments, suggesting that induction of p21<sup>Waf1/Cip1</sup> does necessarily accompany the apoptotic response. This finding is consistent with a recent report that p21<sup>Waf1/Cip1</sup> induction can affect cell cycle arrest but does not induce apoptosis (6).

To investigate another potentially relevant protein encoded by a p53-inducible gene (16), the levels of cellular Bax were examined (Fig. 4C). Bax is a Bcl-2 homolog and heterodimerization partner known to promote cell death (25); in some cell

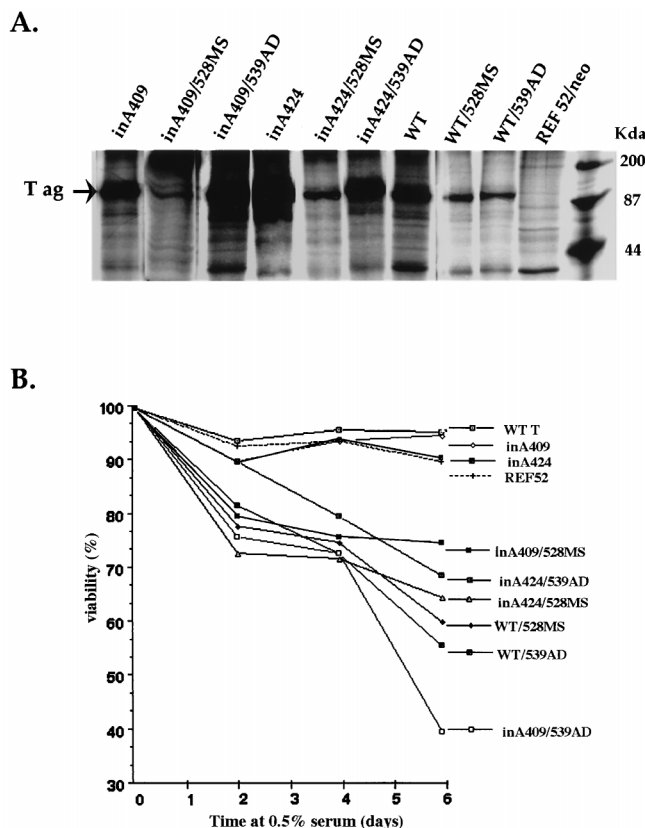


FIG. 6. (A) Immunoprecipitation of <sup>35</sup>S-labeled cell extracts from early-passage REF-52 cell lines expressing wild-type (WT) or mutant Tags containing changes at aa 528 or 539. Cells were labeled with [<sup>35</sup>S]methionine, and Tag was immunoprecipitated with MAb 419. (B) Viability of early-passage REF-52 cells expressing inA409 (inA2409) or inA424 (inA2411) mutant Tags with additional mutations at aa 528 or 539. Viability was determined for adherent and floating cells by trypan blue exclusion. The experiment was repeated with multiple individually derived REF-52 cell lines for each mutant, with similar results.

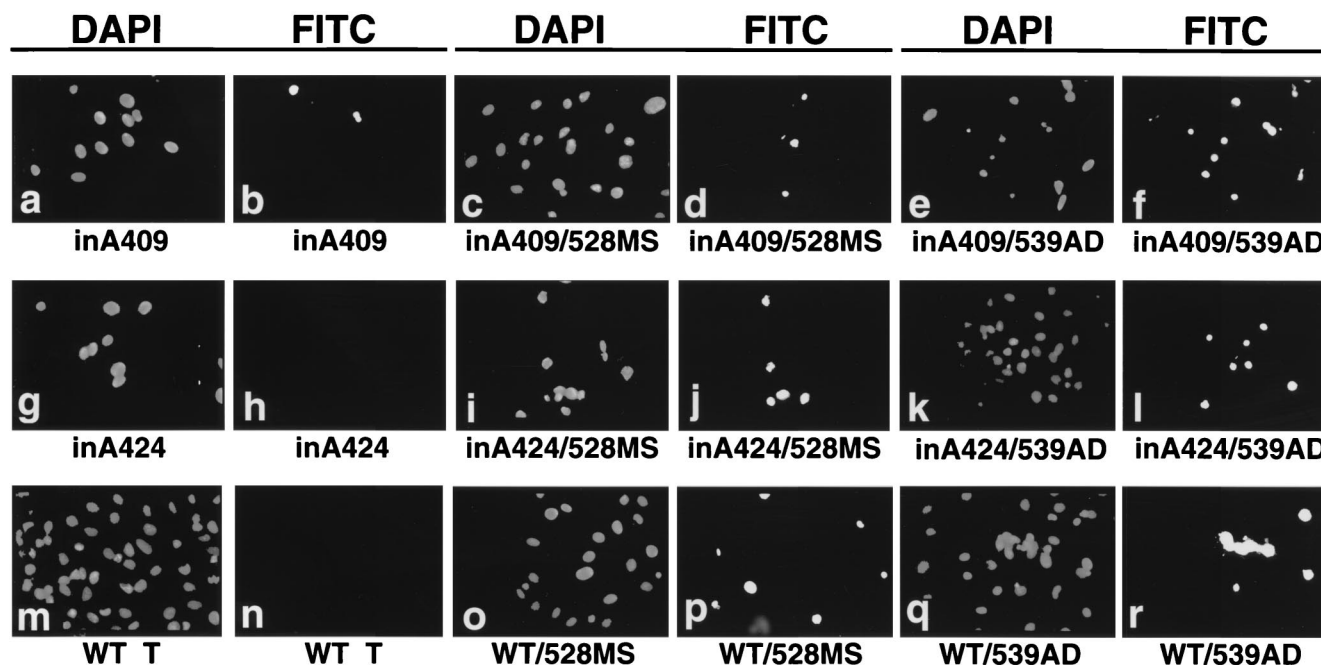


FIG. 7. In situ detection of apoptosis in REF-52 cell lines stably expressing Tags containing mutations altering aa 528 or 539. Early-passage REF-52 cell lines stably expressing truncated or full-length mutant Tags with or without additional mutations at aa 528 or 539 are shown after incubation in DMEM-0.5% FCS for 4 days. DAPI staining shows nuclear morphology of apoptotic cells. For the in situ detection of DNA fragmentation (TUNEL assay), FITC-conjugated antidigoxigenin antibody was used to detect DNA fragments end labeled with digoxigenin-dUTP (magnification,  $\times 400$ ).

types, it is expressed at higher levels during p53-induced apoptosis (16, 29). However, in our studies, we saw no significant difference in Bax levels in REF-52 clones expressing either wild-type Tag (Fig. 4C, lane 1),  $\text{mut}^{(p53-)}\text{Tags}$  (lanes 2 and 3), or N-termTags (lanes 4 to 6). Taken together, the results to examine expression of p53, p21<sup>Waf1/Cip1</sup>, and Bax suggest that a p53-independent mechanism may play a role in SV40 Tag-induced apoptosis.

**Amino acids 528 and 539 of Tag are critical for preventing apoptosis induced by N-termTags.** Alignment of SV40 large and small T antigens with the Ad5 E1B 19K amino acid sequences was performed in order to identify sequences in SV40 large T or small t antigen which might be homologous to critical sequences in E1B 19K and might possess antiapoptotic activity. Dot matrix analyses using the Gene Inspector program (Textco, Inc., West Lebanon, N.H.) revealed that the region with the greatest sequence similarity between SV40 antigens and the Ad5 E1B 19K protein corresponds to aa 525 to 541 of SV40 Tag and aa 77 to 93 of Ad5 E1B 19K (Fig. 5). Interestingly, this region of Ad5 E1B 19K has been previously identified as homologous to the Bcl-2 family's BH-1 domain; both regions appear essential for efficient protection from apoptosis (4). Although the conserved glycine of BH-1 is not present in SV40 Tag, flanking amino acids show >60% homology with E1B 19K and Bcl-2. Since aa 525 to 541 are not required for p53 binding and do not affect the transformation efficiency of Tag (12, 21, 44), we reasoned that the conserved residues may be critical for preventing apoptosis by a mechanism independent of direct sequestration of p53. To examine the role of this region in inhibiting apoptosis, site-directed mutagenesis was performed to change the methionine at aa 528 of SV40 Tag to a serine (528MS) and the alanine at aa 539 to aspartic acid (539AD) (Fig. 5); similar mutations in the corresponding positions in E1B 19K have been shown previously to render this protein defective for its antiapoptotic function (7). These mu-

tations were introduced into both wild-type Tag and the p53 nonbinding Tags *inA2809/inA409* and *inA2811/inA424*.

Stable early-passage clones of REF-52 cells which expressed these new mutant Tags were generated. Expression of Tags of the expected size in individual cell clones was confirmed by both Western analysis (data not shown) and immunoprecipitation of <sup>35</sup>S-labeled cell extracts, using the anti-Tag MA b 419 (Fig. 6A). Mutant proteins were consistently expressed at levels that were equal to or at least one-third of that of wild-type Tag. Figure 6B shows the results of a trypan blue dye exclusion assay of cellular viability of these cell lines grown in low serum (0.5%) and demonstrates that mutation of either aa 528 or aa 539, in the context of a full-length Tag which cannot bind p53, substantially decreased cell viability. Surprisingly, we found that mutations at the same sites but in a wild-type Tag background (producing proteins which are able to bind p53 and morphologically transform cells) also significantly decreased the percentage of viable cells when cultured in low serum (Fig. 6B). Since the WT/528AS and WT/539AD mutant Tags contain all three regions necessary for immortalization and transformation (including the p53 binding region), expression of these mutants is likely to result in a high percentage of abnormally cycling cells. It is probable that this population of aberrantly cycling cells is vulnerable to p53-independent apoptosis, accounting for the low viability of WT/528AS- and WT/539AD-expressing cells in low serum.

To confirm that the decreased viability of REF-52 cells was due to apoptotic cell death, cells expressing these mutant Tags were evaluated by using DAPI staining (to assess nuclear morphology) and by in situ dUTP-FITC incorporation, or TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin end labeling) assay (to estimate the extent of cellular DNA fragmentation). DAPI and TUNEL analysis showed few apoptotic cells in REF-52 cells expressing *inA409*, *inA424*, and wild-type Tag (Fig. 7a, b, g, h, m, and n), while



cells expressing the mutation at aa 528 (Fig. 7c, d, i, j, o, and p) or 539 (Fig. 7e, f, k, l, q, and r) consistently showed more apoptosis by evaluation of both nuclear morphology and TUNEL staining.

## DISCUSSION

Viruses appear to have evolved mechanisms to prevent or delay cell death in infected cells, thus ensuring an adequate time frame for viral DNA replication and production of progeny virions (40). Both p53-dependent and p53-independent forms of apoptosis appear to function in many systems. For example, when cells are infected by human adenoviruses, p53 is required to mediate apoptosis in response to expression of E1A in certain cell types (see reference 40 for a review); however, it is becoming increasingly clear that p53-independent mechanisms of adenovirus-induced apoptosis also exist (33, 36). The physiological relevance of p53 mutation or deletion in protecting mammalian cells from apoptosis and in tumor development has been confirmed in several systems by either functional inactivation of p53 or the genetically engineered absence of p53 (17, 23, 35). However, in addition to functional inactivation of p53 through the formation of complexes between p53 and either the adenovirus E1B 55K (4) or the E4orf6 protein (14), adenoviruses also express a powerful antiapoptotic E1B 19K gene product which may work through both p53-dependent and p53-independent mechanisms (40). In fact, in some cell types, including primary baby rat kidney epithelial cells, sequestration of p53 by E1B 55K alone does not prevent apoptosis in the absence of E1B 19K expression (28). Similarly, E1B 55K is unable to block p53-independent E1A-induced apoptosis, while E1B 19K significantly overcomes this process (22). Thus, E1B 19K function may complement p53 sequestration and thereby enhance the antiapoptotic defenses of the adenoviruses.

In this work, we have identified an antiapoptotic region of Tag which prevents apoptosis in the context of wild type WT Tag and therefore appears to function independently of sequestration of p53 by Tag. Furthermore, the fact that mutations at aa 528 or 539 permitted apoptosis even in a fully transforming wild-type Tag background suggests that escape from apoptosis is not necessarily required for development of a transformed phenotype. Indeed, we found that the frequency of focus-forming colonies was approximately the same for wild-type Tag and mutants WT/528MS and WT/537AD (10), yet the cell lines expressing these mutant Tags exhibited diminished viability in low serum. Analogous observations have been made with E1A/Ras-transformed (18) and c-Myc-transformed MEFs (39), which, in low serum, undergo dramatic apoptosis even though surviving cells maintain a transformed phenotype. Similarly, primary baby rat kidney cells expressing E1A and E1B 55K (which binds to p53) without E1B 19K form dense foci characteristic of transformation but at low serum concentrations also exhibit a loss of viability associated with the hallmark changes of apoptosis (28). Thus, oncogene expression and/or tumor suppressor inactivation sufficient for the morphological transformation of cells does not necessarily preclude significant apoptosis. Conversely, we have shown by expression of  $\text{mut}^{(p53-)}$ Tags in primary rodent cells and REF-52 cells that protection from apoptosis does not necessarily result in transformation. Thus, transformation and protection from apoptosis are likely to involve overlapping but distinct pathways of oncogene activity and tumor suppressor gene inactivation.

SV40 Tag's antiapoptotic region also appears to prevent E1A-induced apoptosis in primary cells. We stably coexpressed a  $\text{mut}^{(p53-)}$ Tag (mutant *inA2811*) with E1A in primary MEFs

and found that colony regression and apoptosis are prevented (10). Protection from E1A-induced apoptosis was not seen with N-termTags or if the antiapoptotic region was mutated. This finding suggests that the antiapoptotic domain of SV40 Tag may work through a generalized p53-independent pathway.

The newly identified antiapoptotic region of SV40 Tag, although it does not contain the conserved glycine of BH-1, has >60% homology with the BH1 region of the Bcl-2 family. BH-1 is necessary, but not sufficient, for Bax interaction (43). Using nonstringent immunoprecipitation conditions, we have been unable to demonstrate an *in vivo* interaction between Tag and Bax (10), consistent with the requirement for the conserved glycine for Bax interaction (5). Thus, the mechanism by which SV40 Tag is able to prevent apoptosis independently of p53 binding remains to be determined. However, the relevance of SV40 Tag-induced apoptosis in the presence of wild-type p53 and transformation is underscored by the recent discovery of high levels of apoptosis in the early stages of a transgenic SV40 Tag-induced pancreatic carcinoma model (24). In this model, Tag-induced apoptosis appears to be independent of p53 expression since similar levels of cell death were observed in a  $p53^{-/-}$  background and a  $p53^{+/+}$  strain. The modulation of apoptosis may instead involve the changing interactions of SV40 Tag with cellular proteins involved in triggering or protecting cells from apoptosis. Whether the region that we have identified is involved in regulating the levels of apoptosis seen in early and late tumors in the transgenic pancreatic carcinoma model system remains to be determined. However, the molecular characterization of SV40 Tag's interactions with cellular antiapoptotic proteins may yield clues to the complex mechanisms modulating oncogene-induced apoptosis and transformation.

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