Simian Virus 40 Large T Antigen Binds a Novel Bcl-2 Homology Domain 3-containing Proapoptosis Protein in the Cytoplasm*

(Received for publication, June 4, 1999, and in revised form, October 29, 1999)

Shih-Chong Tsaıˇ, Kishore B. S. Pasamartiˇ, Laura Pajakˇ, Michael Franklin‡, He Wang‡, William J. Henzel, John T. Stults‡, and Loren J. Field‡***

From the ‡Kranert Institute of Cardiology and Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202-5225 and the Department of Protein Chemistry, Genentech Inc., South San Francisco, California 94080-4990

A 193-kDa SV40 large T antigen (T-Ag)-binding protein, designated p193, was identified and cloned. Inspection of the deduced amino acid sequence revealed the presence of a short motif similar to the Bcl-2 homology (BH) domain 3, suggesting that p193 may be a member of a family of apoptosis promoting proteins containing only BH3 motifs. In support of this, p193 expression promoted apoptosis in NIH-3T3 cells. Deletion of the BH3 motif abolished p193 apoptosis activity. p193-induced apoptosis was antagonized by co-expression of Bcl-XL. Immune cytoplogic analysis indicated that p193 is localized to the cytoplasm of transfected cells. p193-induced apoptosis was also antagonized by co-expression of T-Ag, which resulted in the cytoplasmic localization of both proteins. The p193 binding site was mapped to an N-terminal region of T-Ag previously implicated in transforming activity. These results suggest that T-Ag possesses an antiapoptosis activity, independent of p53 sequestration, which is actuated by T-Ag/p193 binding in the cytoplasm.

Normal development is dependent upon an intricate balance between cell proliferation and programmed cell death (apoptosis). Alteration of this balance can have significant pathophysiological consequences; tumorigenesis results when cell proliferation is favored, whereas autoimmune and/or degenerative disorders result when apoptosis is favored.

In mammalian cells, apoptosis can be induced by at least two independent regulatory pathways. The first pathway relies on direct activation of the death receptors (members of the tumor necrosis factor receptor superfamily; reviewed in Ref. 1). For example, activation of the tumor necrosis factor receptor 1 or CD95 receptors initiates a signal transduction cascade primarily through Fas-associated death domain, which rapidly activates caspase 8, thereby initiating apoptosis. Apoptosis can also be regulated through the activities of Bcl-2 family members (reviewed in Ref. 2). The prototypical family member, Bcl-2, was originally identified as the product of a gene activated by chromosomal translocation in some human lymphomas (3–5). Subsequent analyses have identified a family of approximately 20 proteins that share homology to Bcl-2 at one or more domains (known as Bcl-2 homology (BH)1 domains 1–4). Functional analyses have shown that family members with the greatest homology to Bcl-2 tend to promote cell survival, whereas those more distantly related tend to promote apoptosis. The proapoptosis group is further subdivided into the Bax subfamily (which contains BH1, BH2, and BH3 domains; see Refs. 6–10) and the BH3-only subfamily (which, as the name implies, contains only BH3 domains; see Refs. 11–18). Commitment to apoptosis is governed, at least in part, by the relative levels of prosurvival and proapoptosis Bcl-2 family members, which, in turn, regulate the activity of Apaf-1 (an activator of caspase 8). Thus, the caspase family of cysteine proteases are the downstream effectors of apoptosis, regardless of the initial regulatory pathway. Once activated, the caspases effect cell death by initiating a proteolytic cascade that destroys cellular organelles, thereby giving rise to distinct morphologic changes that are diagnostic for apoptosis (reviewed in Ref. 19). These include nuclear condensation, fragmentation of DNA at nucleosomal junctions, mitochondrial disintegration, and ultimately autolysis of the cell.

The DNA tumor virus oncoproteins have provided an interesting model system with which to dissect the molecular regulation of cell growth and death. The transforming activities of these proteins (as exemplified by SV40 large T antigen and adenovirus E1A) reside largely in their ability to bind to, and thereby alter the activity of, endogenous cell cycle and cell death regulatory proteins (reviewed in Refs. 20 and 21). In the case of T antigen (T-Ag), amino acid residues 105–115 are required for binding to members of the retinoblastoma family (RB and the related proteins p107 and p130; see Refs. 22–25). T-Ag/RB binding blocks sequestration of E2F family members (which are maintained in an inactive state by binding to RB). Once released, these transcription factors activate expression of genes needed for S phase entry (reviewed in Refs. 26–28). The discontinuous region localized between T-Ag amino acid residues 350–450 and 532–625 is required for binding to p53 (29). Among other activities, p53 functions as a transcriptional co-activator of both proapoptosis and growth inhibitory genes. T-Ag/p53 binding prevents transcriptional activation of these genes and concomitantly inhibits their activities (30, 31).

Adult cardiac myocytes are terminally differentiated cells that exhibit little if any capacity to reenter the cell cycle (32). In previous studies aimed at identifying potential therapeutic targets to promote cardiomyocyte proliferation, transgenic

* Supported by the NHLBI, National Institutes of Health, and a grant from Bristol-Myers Squibb. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Current address: Eli Lilly and Company, Indianapolis, IN 46285.

¶ Current address: Bristol-Myers Squibb, Princeton, NJ 08540.

** To whom correspondence should be addressed: Herman B Wells Center for Pediatric Research, James Whitcomb Riley Hospital for Children, 702 Barnhill Dr., Rm. 2666, Indianapolis, IN 46202-5225. Tel.: 317-274-5085; Fax: 317-274-5378; E-mail: ljfield@iupui.edu.

† The abbreviations used are: BH, Bcl-2 homology; CMV, cytomegalovirus; GAL, galactosidase; HPLC, high pressure liquid chromatography; PSD, post-source decay; RB, retinoblastoma; T-Ag, T antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
mice that express the SV40 large T-Ag oncprotein in the ventricles and/or atria were generated (33, 34). T-Ag expression in the hearts of transgenic mice led to the development of myocardial tumors composed of differentiated, proliferating muscle cells. Given the extremely low frequency of primary myocardial tumors (which presumably reflects a highly redundant series of cell cycle checkpoints), we reasoned that these transgenic tumors would provide a useful reagent with which to identify novel T-Ag-binding proteins. Analysis of cell lines derived from the transgenic cardiac tumors revealed that p53 and p107 were prominent T-Ag-binding proteins (35). Two additional novel proteins of 193 and 380 kDa were also observed to bind, either indirectly or directly, to T-Ag (35).

Here we have cloned and characterized the 193-kDa SV40 T-Ag-binding protein. Sequence analysis suggested that p193 may be a new member of the BH3-only proapoptosis family. This notion was supported by the observation that p193 expression promoted a prompt apoptotic response in NIH-3T3 cells. Immune cytoligic analysis indicated that p193 is a cytoplasmic protein and that co-expression of T-Ag resulted in the cytoplasmic localization of both proteins. p193-induced apoptosis occurs in G1, and pulse-chase experiments revealed that T-Ag is also localized in the cytoplasm (albeit transiently) at the same point of the cell cycle. The data are consistent with the notion that T-Ag possesses an antiapoptosis activity, independent of p53 sequestration, that is actuated by T-Ag/p193 binding in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Isolation and Sequence Analysis of p193 Proteins**—AT-2 cardiomyocytes were homogenized in 20 ml of NET, precleared with protein A-Sepharose beads, and mixed with anti-T-Ag monoclonal antibody PAb419 (90 min at 4 °C). Immune complexes were collected with protein A-Sepharose, displayed on polyacrylamide gels, and visualized by staining with Coomassie Brilliant Blue. The region of the gel containing p193 was excised, alkylated with iodoacetamide, and digested with F-trypsin (0.2 μg of trypsin at 37 °C for 17 h) as described (36). The peptides were then extracted with 5% formic acid/50% acetonitrile and premade spot of matrix (0.5 ml of 20 mg/ml C-18 peptides were then extracted with 5% formic acid/50% acetonitrile and premade spot of matrix (0.5 ml of 20 mg/ml cross Biotechnology), or an IgG subtype-matched nonspecific antibody (anti-Myc subtype 9E10, Santa Cruz Biotechnology). Immune complex was then visualized via autoradiography (p193) or Western blotting (T-Ag) as described above.

For Northern blots, 10 μg of total RNA was denatured by glyoxal, displayed on agarose gels, transferred to Genescreen (NEN Life Science Products), and reacted with a nick-translated full-length p193 cDNA as described (41). For mapping the p193 binding site on T-Ag, in vitro transcription/translation products from a full-length p193 cDNA clone and various T-Ag deletion constructs were mixed, and immune complex was generated with an N-terminal specific anti-T-Ag monoclonal antibody (PAb419). Immune complex was then visualized by autoradiography. The various T-Ag deletion constructs were generated via polymerase chain reaction amplification using oligonucleotide primers incorporating stop codons or base pair substitutions as indicated in the text. The full-length construct was confirmed by two rounds of serum depletion (starvation medium contained 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium was then added, and cells were processed for autoradiography and immune cytology at various points thereafter as described (44). To localize T-Ag during the cell cycle, subconfluent cultures of AT-2 cells in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum received a 40-min pulse of [3H]thymidine (26 Ci/mmol, Amersham Pharmacia Biotech) and 10% newborn calf serum in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum received a 40-min pulse of [3H]thymidine. The cells were then washed and cultured with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were then processed for autoradiography and immune cytology at various points thereafter as described (44). For the colony assay, NIH-3T3 cells transfected with CMV-null, CMV-p193 sense, or CMV-p193 antisense plasmid (anti-GST, Amersham Pharmacia Biotech), and the resulting immune complexes were subjected to Western blot analysis. 100 μg of total protein from nontransfected cells and 100 μg from transfected cells were included as controls. The blots were probed with an anti-Myc (9E10) or anti-T-Ag (PAb416) antibody, and signal was developed using the ECL method. To demonstrate p193/T-Ag binding in vivo, [35S]methionine-labeled in vitro transcription/translation (TNT kit, Promega) product obtained from a full-length p193 cDNA subcloned into pBlue-script ISK (Stratagene, La Jolla CA) was mixed with 1.2 μg of recombinant SV40 T-Ag (Molecular Biology Resource) and reacted with anti-T-Ag (PAb419) or an IgG subtype-matched nonspecific control antibody (anti-MAP kinase 12, Santa Cruz Biotechnology). Immune complex was then visualized via autoradiography (p193) or Western blotting (T-Ag) as described above.

For Northern blots, 10 μg of total RNA was denatured by glyoxal, displayed on agarose gels, transferred to Genescreen (NEN Life Science Products), and reacted with a nick-translated full-length p193 cDNA as described (41). For mapping the p193 binding site on T-Ag, in vitro transcription/translation products from a full-length p193 cDNA clone and various T-Ag deletion constructs were mixed, and immune complex was generated with an N-terminal specific anti-T-Ag monoclonal antibody (PAb419). Immune complex was then visualized by autoradiography. The various T-Ag deletion constructs were generated via polymerase chain reaction amplification using oligonucleotide primers incorporating stop codons or base pair substitutions as indicated in the text. The full-length construct was confirmed by two rounds of serum depletion (starvation medium contained 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium was then added, and cells were processed for autoradiography and immune cytology at various points thereafter as described (44). To localize T-Ag during the cell cycle, subconfluent cultures of AT-2 cells in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum received a 40-min pulse of [3H]thymidine. The cells were then washed and cultured with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were then processed for autoradiography and immune cytology at various points thereafter as described (44). For the colony assay, NIH-3T3 cells transfected with CMV-null, CMV-p193 sense, or CMV-p193 antisense plasmid (anti-GST, Amersham Pharmacia Biotech), and the resulting immune complexes were subjected to Western blot analysis. 100 μg of total protein from nontransfected cells and 100 μg from transfected cells were included as controls. The blots were probed with an anti-Myc (9E10) or anti-T-Ag (PAb416) antibody, and signal was developed using the ECL method. To demonstrate p193/T-Ag binding in vivo, [35S]methionine-labeled in vitro transcription/translation (TNT kit, Promega) product obtained from a full-length p193 cDNA subcloned into pBlue-script ISK (Stratagene, La Jolla CA) was mixed with 1.2 μg of recombinant SV40 T-Ag (Molecular Biology Resource) and reacted with anti-T-Ag (PAb419) or an IgG subtype-matched nonspecific control antibody (anti-MAP kinase 12, Santa Cruz Biotechnology). Immune complex was then visualized via autoradiography (p193) or Western blotting (T-Ag) as described above.

**RESULTS**

**Cloning of p193**—To identify the T-Ag-binding proteins in cardiomyocytes, immune complexes were generated using protein prepared from [35S]methionine-labeled AT-2 cells, a cell line derived from the transgenic heart tumors (35). Proteins with apparent molecular masses of 380, 193, and 120 kDa (see Fig. 1c) were detected in immune complex generated with either anti-T-Ag (PAb419; Fig. 1a, lane 3) or anti-p53 (PAb421...
pAb246; lanes 2 and 6, respectively) monoclonal antibodies. These proteins were not present in immune complexes generated with IgG subtype-matched nonspecific control antibodies (DYS1 (Fig. 1a, lane 1) and PAb240 (lane 5)), nor in controls lacking primary antibody (lane 4). Previous studies have shown that the 120-kDa protein is p107 (45, 46) and that the 180-kDa protein (present only in PAb421 anti-p53 immune complex) is the murine homologue of RAD50, a protein involved in the repair of dsDNA breaks in yeast (40). The 380-kDa protein has not yet been characterized.

To clone p193, large scale anti-T-Ag immune complex preparations were resolved on polyacrylamide gels and visualized by Coomassie Blue staining. The region containing p193 was excised, digested with trypsin in situ, fractionated by HPLC, and analyzed by mass spectroscopy using PSD (Fig. 1b). Information obtained from the PSD experiment was used to search a protein sequence data base using a modified version of FragFit. The search indicated that p193 was homologous to a previously identified open reading frame of unknown function isolated from a human immature myeloid cell line (47). Reverse transcriptase-polymerase chain reaction was used to generate a short cDNA clone spanning the region homologous to the largest p193 peptide. This clone was then used to screen an adult mouse heart cDNA library.

Six overlapping cDNA clones were ultimately obtained (Fig. 1c). Sequence analysis revealed an open reading frame 5067 nucleotides in length that encoded a protein of 1689 amino acid residues and with a deduced molecular mass of 192,346 Da (Fig. 2a). All of the p193 proteolytic peptides identified in the PSD experiment were present in the deduced amino acid sequence of p193. Underlined sequences correspond to the peptides identified by PSD mass spectrometry. Boldface sequence corresponds to the BH3 homology. Comparison of the BH3 domain of p193 and those of the BH3-only family members are shown in Fig. 2b.}

**Downloaded from http://www.jbc.org/ by guest on July 22, 2018**
Myc antibodies (Fig. 3a). Full-length p193Myc and T-Ag were readily detected in total protein prepared from the co-transfected cells (Fig. 3a). p193Myc was detected in anti-T-Ag immune complex, and T-Ag was detected in anti-Myc immune complex. Neither protein was present in immune complex generated with an IgG subtype-matched nonspecific control antibody. Thus, cloned p193Myc binds to T-Ag in vivo. Immune precipitation analyses of mixtures of in vitro translated p193 and recombinant T-Ag were also performed (Fig. 3b). Radiolabeled p193 was present in immune complex generated with anti-T-Ag antibody but not in immune complex generated with an IgG subtype-matched nonspecific control antibody, confirming that the 193-kDa T-Ag-binding protein was successfully cloned. Northern blots revealed a somewhat restricted pattern of p193 expression in adult mouse tissues (Fig. 3c). Relatively high levels of p193 mRNA were detected in the heart, as might be anticipated given that the protein was originally identified in cell lines derived from cardiac tumors.

p193 Binds to the N Terminal of T-Ag—To identify the region of T-Ag that binds to p193, in vitro translation products from a series of T-Ag deletion constructs were mixed with in vitro translated full-length p193, and immune complexes generated with anti-T-Ag antibody were resolved on polyacrylamide gels and visualized by autoradiography (Fig. 4). p193 was present in immune complex generated with T-Ag mutants with deletions encompassing as much as amino acid residues 147–708, indicating that the p193 binding site resides within T-Ag amino acid residues 1–147. In contrast, p193 was not present in immune complex generated with a T-Ag mutant in which amino acid residues 92–708 were deleted, indicating that the C-terminal boundary of the binding site lies within T-Ag amino acid residues 92–147. Importantly, point mutations at T-Ag amino acid residues 107 and 108, which disrupt binding of RB family members did not effect p193 binding (Fig. 4, construct 1–147 D RB). Thus, p193 binds to the N-terminal region of T-Ag distinct from the RB family member binding site.

Expression of p193 Promotes Apoptosis—To determine the effects of p193 expression on cell growth, NIH-3T3 cells were transfected with either CMV-βGalMyc (an expression construct encoding β-galactosidase with a Myc-epitope tag) or CMV-p193Myc. At 48 h posttransfection, FACS analyses using a fluorescein isothiocyanate-conjugated anti-Myc antibody revealed that most cells expressing CMV-βGalMyc had a normal 2C DNA content (Fig. 5a; the inset shows a CMV-βGalMyc transfected cell; green signal is anti-Myc immune fluorescence, and blue signal is Hoechst fluorescence). In contrast the preponderance of cells expressing CMV-p193Myc exhibited hypodiploid DNA content, indicative of apoptotic cell death (Fig. 5b). Visual inspection of the cultures confirmed that the bulk of the transfected cells were dying and had markedly condensed chro-

---

**Fig. 3.** a. p193 binds to T-Ag in NIH-3T3 cells. Protein prepared from cells co-transfected with CMV-p193Myc and CMV-T-Ag was reacted with the indicated antibodies, and the resulting immune complex was analyzed by Western blotting using anti-Myc and anti-T-Ag antibodies. *Tfx.*, transfection; *Tot. Pro.*, total protein; *IP.*, immune precipitation; *Cont.*, control.

b. In vitro translated p193 binds to recombinant T-Ag. Radiolabeled in vitro translated p193 was mixed with recombinant T-Ag and then reacted with the indicated antibodies. The resulting immune complexes were displayed on a polyacrylamide gel and transferred to nylon membranes. p193 was visualized by autoradiography, and T-Ag was visualized by Western blot. c. Northern blot analysis of p193 expression in adult mice. Total RNA (10 μg) prepared from the indicated tissues was probed with a full-length p193 cDNA. The integrity of the RNA samples was confirmed by staining the Northern blots with methylene blue (lower panel).

**Fig. 4.** p193 binds to the N terminus of T-Ag. The schematic diagram depicts the T-Ag constructs used in the mapping experiments. These products were translated in vitro and mixed with in vitro translated full-length p193. Immune complex generated with anti-T-Ag antibody PAb419 was resolved on a polyacrylamide gel and visualized by autoradiography. Construct 1–92Myc encoded a Myc epitope tag at the C terminus. WT, wild type.
matin (Fig. 5b, inset; green signal is anti-Myc immune fluorescence, and blue signal is Hoechst immune fluorescence). Thus, expression of p193 can induce apoptosis.

To determine at what point in the cell cycle p193 induced cell death, serum-starved NIH-3T3 cells were transfected with CMV-p193Myc. Medium containing serum and [3H]thymidine was then added, and the cultures were processed for anti-Myc immune cytology and autoradiography at various time points thereafter. Most cells expressing CMV-p193Myc were dead by 20 h post-serum replenishment (Fig. 5c, black trace), and DNA synthesis never reinitiated in these cells (Fig. 5c, red trace). This suggests that p193-induced apoptosis occurs during G1. In contrast, the preponderance of nontransfected cells on the same chamber slide reinitiated DNA synthesis by 14 h post-serum replenishment (Fig. 5c, green trace), thus establishing the fidelity of the synchronization protocol. In control experiments, cells expressing CMV-βGALMyc reinitiated DNA synthesis at a rate comparable to the nontransfected cells (not shown), indicating that transgene expression per se did not have an impact on cell cycle progression or viability. p193Myc immune reactivity in the synchronized cultures was initially localized uniformly throughout the cytoplasm but became restricted to the perinuclear region prior to the onset of cell death (Fig. 5, d and e, respectively). Bax, a well characterized pro-apoptosis protein, undergoes a similar cytoplasmic to perinuclear redistribution during apoptosis (10). Finally, it is of interest to note that p193Myc-expressing cells are viable if cell cycle progression is blocked; >90% of p193-expressing cells were viable at 40 h posttransfection if maintained under low serum conditions. This suggests that some degree of cell cycle progression is needed to actuate apoptosis.

Cell death induction by BH3-only family members can be antagonized by co-expression of prosurvival members of the Bcl-2 family. To determine whether p193 shares this trait, NIH-3T3 cells were transfected with CMV-p193Myc alone or co-transfected with CMV-p193Myc and CMV-Bcl-XL (a construct encoding human Bcl-XL). The preponderance of cells transfected with p193 alone were dead at 68 h posttransfection, whereas co-transfection with Bcl-XL markedly antagonized p193-induced apoptosis (Fig. 5f). In control experiments, virtually no viable cells were seen following co-transfection with CMV-p193Myc and CMV-GFP (an expression construct encoding green fluorescent protein), indicating that co-expression of two CMV-driven constructs does not abate p193-induced cell death (Fig. 5f).

The BH3 Motif Is Required for p193-induced Apoptosis—To establish the importance of the BH3 domain in p193-induced apoptosis, the CMV-p193Myc expression construct was modified such that amino acid residues 1563–1576 were deleted (VRILKAHGDEGLHV). This modification resulted in the deletion of the BH3 motif (amino acid residues 1566–1572; see Fig. 2), and the resulting construct was designated CMV-p193ΔBH. NIH-3T3 cells transfected with CMV-p193ΔBH were viable (Fig. 5f). Indeed, the survival was similar to that obtained with cells co-transfected with CMV-p193 plus CMV-Bcl-XL. The level of TUNEL positivity was also monitored. NIH-3T3 cells were transfected with CMV-βGALMyc, CMV-p193Myc, or CMV-p193ΔBH. 48 h later, the cells were processed for anti-Myc immune reactivity (see Fig. 6) (rhodamine secondary antibody, red signal), TUNEL positivity (fluorescein isothiocyanate-conjugated probe, green signal), and DNA content (Hoechst staining, blue signal). In agreement with the flow cytometric data presented in Fig. 5b, the preponderance of cells expressing the CMV-p193Myc construct were TUNEL-positive (Fig. 6c). In contrast, only low levels of TUNEL positivity were detected in cells expressing the control CMV-βGALMyc construct or the CMV-p193ΔBH construct. Representative images of cells transfected with these constructs are presented in Fig. 6b. These data indicate that the BH3 domain at amino acid residues 1566–1572 is required for p193-induced cell death.

T-Ag Is Transiently Localized in the Cytoplasm during M and G1—Given that p193 was originally identified as a T-Ag binding protein and that T-Ag is a nuclear oncoprotein, the cytoplasmic/perinuclear localization of p193Myc was somewhat surprising. To further address this paradox, NIH-3T3 cells co-transfected with CMV-p193Myc and CMV-T-Ag were examined. Survival was greatly enhanced in cells co-expressing p193 and T-Ag (Fig. 5f), indicating that, like Bcl-XL, T-Ag can antagonize p193-induced apoptosis. Moreover, immune cytologic analysis indicated that p193Myc and T-Ag co-localized to the cytoplasm in the majority (approximately 63%) of the co-transfected cells (Fig. 7, a and b, green and red signals, respectively). In contrast, co-transfection with CMV-βGALMyc and CMV-T-Ag did not result in prominent cytoplasmic T-Ag immune reactivity (not shown). These results raised the possibility that p193-T-Ag binding might normally occur in the cytoplasm.

Cytoplasmic T-Ag immune reactivity has previously been noted in mitotic cells (49, 50). A pulse-chase experiment was...
thymidine-positive cells exhibited cytoplasmic T-Ag immune reactivity from 4 h through 10 h post-S phase (Fig. 7c). In contrast, \[^{3}H\]thymidine-positive cells with mitotic figures were only observed at 4 and 6 h post-S phase. Thus, cytoplasmic T-Ag localization persisted through cytokinesis and well into G1. This point is further illustrated by the presence of \[^{3}H\]thymidine-positive daughter cells with cytoplasmic T-Ag immune reactivity at 8–12 h post-S phase (Fig. 7, d and e). Thus, T-Ag is transiently located in the cytoplasm at the same point of the cell cycle (G1) when p193-induced cell death occurs.

**Loss of p193 Activity Promotes Proliferation**—The data presented above indicate that forced expression of p193 promotes apoptosis prior to the onset of S phase. A colony growth assay employing a p193 antisense construct (CMV-p193as) was used to determine the consequences of diminished p193 expression. Transfection of NIH-3T3 cells with CMV-p193as resulted in markedly increased colony size as compared with transfection with CMV-null (a control expression vector lacking insert; see Fig. 8). Quantitative reverse transcription-polymerase chain reaction analyses indicated that expression of the CMV-p193as construct diminished expression of the endogenous p193 gene (data not shown). As expected, transfection with CMV-p193 s (an expression vector encoding p193 in the sense orientation) yielded no visible colonies (Fig. 8), consistent with the proapoptotic activity of p193 noted above. These results were reproduced in four independent experiments using three independent DNA preparations.

**DISCUSSION**

We have shown that p193, a T-Ag-binding protein present in the AT-2 cardiomyocyte tumor cell line, is a new member of the BH3-only proapoptosis gene family. Like other BH3-only proteins, p193-induced apoptosis can be antagonized by co-expression of prosurvival Bcl-2 family members (in our case, Bcl-X\(_L\)) (14). Co-expression of T-Ag antagonizes p193-induced apoptosis, the precise molecular mechanism by which this occurs remains to be established. p193 differs markedly in size as compared with other BH3-only family members; the next largest family member, BID, is only 21.95 kDa (14).

Indeed, previous mutational analyses have identified transforming copy number losses in AT-2 cardiomyocytes. Subconfluent cultures received a 40-min pulse of \[^{3}H\]thymidine (to mark cells in S-phase) followed by a chase with radiolabeled-iodo-free medium. The cultures were processed for anti-T-Ag immune cytology and autoradiography at various time points thereafter. Significant percentages of the

used to determine how long T-Ag persists in the cytoplasm of AT-2 cardiomyocytes. Subconfluent cultures received a 40-min pulse of \[^{3}H\]thymidine (to mark cells in S-phase) followed by a chase with radiolabeled-iodo-free medium. The cultures were processed for anti-T-Ag immune cytology and autoradiography at various time points thereafter. Significant percentages of the
1–108 were required to effectively transform B2–1 cells and that an approximately 185-kDa protein bound to this region of T-Ag. Moreover, binding between T-Ag and the 185-kDa protein was not disrupted by point mutations abrogating the binding of RB family members. Given the similarity in molecular mass and binding specificity, p193 may be the same protein as p185.

Other studies have demonstrated that mutations at T-Ag amino acid residues 1–82 (52), 3–35 (53), and 17–27 (54) all have an impact upon transforming activity in selected cell types. Some of these mutants are thought to disrupt the N-terminal J domain, a sequence motif that functions as a DnaJ molecular chaperone (54, 55). DnaJ binds to members of the 70-kDa heat shock protein family, and this complex facilitates correct protein folding, formation of multiprotein complexes, and protein transport across intracellular membranes (56). Although our data indicate that the C-terminal boundary of the p193 binding site resides between T-Ag amino acids 92–147, the N-terminal boundary of the binding site is not yet mapped. Mutations encompassing residues upstream of amino acid 92 could alter p193/T-Ag binding, by direct disruption of the binding domain or by altering the tertiary structure of T-Ag. Confirmation of the importance of p193 binding for T-Ag transforming activity requires precise mapping of the binding site followed by assessment of transforming activity with appropriately mutated T-Ag expression constructs. Finally, given the relative proximity of the p193 and RB family member binding sites, it will be of interest to determine whether RB, p107, and/or p193 sterically compete for T-Ag binding. Such a mechanism could account for the absence of RB in anti-T-Ag immune precipitates from the myocardial cell lines, despite the presence of hypophosphorylated RB in total protein prepared from these cells (Fig. 1; see also Ref. 45).

p193 also appears to be unique among the BH3-only family members with respect to its ability to bind to T-Ag. However, it is of interest to note that the BH3-only proteins Bik and BNIP-3 (as well as Bax and Bak, proapoptosis proteins containing BH1, BH2, and BH3 domains) are able to bind to adenoviral E1B 19K protein (9, 11, 12, 57). It is thought that the antiapoptotic activity of the E1B 19K protein is due at least in part to binding with proapoptosis Bcl-2 family members (58). Previous studies have identified a T-Ag antiapoptotic activity at amino acid residues 525–541 that appeared to act independently of p53 sequestration (59). These authors noted a significant degree of sequence homology between this region of T-Ag and amino acid residues 77–93 in E1B 19K as well as Bcl-2 amino acid residues 133–151. Although these observations suggest that the binding activity at T-Ag amino acid residues 525–542 might be functionally similar to E1B 19K protein sequestration of proapoptosis proteins, experiments aimed at establishing direct binding of T-Ag to Bax were unsuccessful (59).

The results from the antisense transfection experiment indicated that loss of p193 activity is associated with marked growth enhancement in NIH-3T3 cells. The increase in growth rate is in excess of that which we would anticipate from simple inhibition of apoptosis in the NIH-3T3 cells, which occurs somewhat infrequently under the growth conditions employed. In support of this, preliminary experiments have shown that cells expressing the CMV-p193as construct exhibit higher DNA synthesis labeling indices as compared with cells expressing control constructs. This observation is consistent with the notion that p193 may function at a cell cycle checkpoint and that transit through the checkpoint is accelerated in the absence of p193 activity. This hypothesis is supported in part by the serum starvation experiment described above, which indicated that at least a limited degree of cell cycle progression is required for actuation of the p193-mediated cell death program. Thus, p193 is only able to trigger cell death after transit through a specific point in G1 (i.e. the presumed cell cycle checkpoint), and accumulation of the protein in itself is not harmful to the cell. The observation that cytoplasmic T-Ag localization occurs during the same point of the cell cycle lends additional credence to this notion. Further insight into the molecular pathway of p193 must await the generation of additional loss of function models.

Our efforts to characterize p193 were motivated in part by the hope of identifying potential therapeutic targets with which to engender regenerative growth in diseased hearts. In this regard, it is of interest to note that transfection of primary cardiomyocyte cultures with E1A or E2F-1 results in a prompt apoptotic response that is only partially abated by co-expression of E1B or Bcl-2 or abrogation of p53 activity (60–64). In contrast, transfection of primary cardiomyocyte cultures with T-Ag does not elicit apoptosis (65, 66). This observation suggests that, in cardiomyocytes, T-Ag possesses an antiapoptotic activity that is lacking in E1A and E2F-1. Given that p193 is a proapoptotic T-Ag-binding protein and that T-Ag expression does not elicit an apoptotic response in cardiomyocytes, it will be of interest to determine whether abrogation of p193 activity can antagonize E1A and/or E2F-1 induced cardiomyocyte apoptosis.

Abrogation of p193 activity may also have a cardioprotective effect under pathophysiological conditions that promote cardiomyocyte apoptosis. Numerous descriptive studies have established the presence of apoptotic cardiomyocytes in a variety of cardiovascular diseases, including dilated cardiomyopathy, ischaemic cardiomyopathy, arrhythmogenic right ventricular dysplasia, acute myocardial infarction, myocarditis, allograft rejection, and preexcitation syndromes (reviewed in Ref. 67). In particular, apoptosis and resulting cardiac remodeling may contribute to the onset of dilated cardiomyopathy and heart failure (reviewed in Ref. 68). Studies in transgenic mice have implicated a number of signal transduction pathways, including the IL-6 cytokine family/gp130/LIF receptor (69), the tumor necrosis factor-a/tumor necrosis factor receptor 1 (70, 71), catheolamine/Gs-α (72), and cAMP/CAMP-response element-binding protein (73) cascades. The role in which p193 may participate in these processes remains to be established.

In summary, the data presented here indicate that p193 is a new member of the BH3-only proapoptosis gene family. p193 promotes cell death during G1, prior to the onset of DNA synthesis. T-Ag is localized in the cytoplasm during the same phase of the cell cycle, and co-expression of T-Ag antagonizes p193-induced cell death and results in the cytoplasmic localization of both proteins. p193 binds to the N terminus of T-Ag in a region that contributes to transforming activity in some cell types. Collectively, these results suggest that T-Ag possesses an antiapoptosis activity, independent of p53 sequestration, that is actuated by T-Ag/p193 binding in the cytoplasm.

Acknowledgments—We thank D. Field for excellent technical assistance; Drs. H. Nakajima and H. O. Nakajima (Indiana University School of Medicine) for assistance with the confocal microscopy; Dr. J. Leiden (University of Chicago) for the Bcl-XL clone; and Drs. H. Nakajima, H. O. Nakajima, and M. Sooqua for comments on the manuscript.

REFERENCES
1. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
2. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
3. Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., and Croce, C. M. (1984) Science 226, 1097–1099
4. Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985) Cell 41, 899–906

S.-C. Tsai, unpublished results.
Simian Virus 40 Large T Antigen Binds a Novel Bcl-2 Homology Domain 3-containing Proapoptosis Protein in the Cytoplasm
Shih-Chong Tsai, Kishore B. S. Pasumarthi, Laura Pajak, Michael Franklin, Brian Patton, He Wang, William J. Henzel, John T. Stults and Loren J. Field

J. Biol. Chem. 2000, 275:3239-3246.
doi: 10.1074/jbc.275.5.3239

Access the most updated version of this article at http://www.jbc.org/content/275/5/3239

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 70 references, 32 of which can be accessed free at http://www.jbc.org/content/275/5/3239.full.html#ref-list-1