

p53 Activation Domain 1 Is Essential for PUMA Upregulation and p53-Mediated Neuronal Cell Death

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The *p53* tumor suppressor gene has been implicated in the regulation of apoptosis in a number of different neuronal death paradigms. Because of the importance of *p53* in neuronal injury, we questioned the mechanism underlying *p53*-mediated apoptosis in neurons. Using adenoviral-mediated gene delivery, reconstitution experiments, and mice carrying a knock-in mutation in the endogenous *p53* gene, we show that the transactivation function of *p53* is essential to induce neuronal cell death. Although *p53* possesses two transactivation domains that can activate *p53* targets independently, we demonstrate that the first activation domain (ADI) is required to drive apoptosis after neuronal injury. Furthermore, the BH3-only proteins Noxa and PUMA exhibit differential regulation by the two transactivation domains. Here, we show that Noxa can be induced by either activation domain, whereas PUMA induction requires both activation domains to be intact. Unlike Noxa, the upregulation of PUMA alone is sufficient to induce neuronal cell death. We demonstrate, therefore, that the first transactivation domain of *p53* is indispensable for the induction of neuronal cell death.

Key words: apoptosis; Noxa; PUMA; *p53*; neuronal cell death; PERP

Introduction

The *p53* tumor suppressor gene is upregulated in multiple neuronal death paradigms including ischemia (Chopp et al., 1992; Cheng et al., 2003), hypoxia (Banasiak and Haddad, 1998; Halterman and Federoff, 1999), and excitotoxicity (Xiang et al., 1998; Cregan et al., 1999). Overexpression of *p53* alone is sufficient to trigger apoptosis in postmitotic neurons (Slack et al., 1996; Cregan et al., 1999); consistent with this, *p53* null mice exhibit reduced brain damage after excitotoxicity and stroke (Crumrine et al., 1994; Morrison et al., 1996). These studies emphasize the importance of *p53* as a key apoptotic factor after neuronal injury.

It has become clear that the apoptotic mechanisms induced by *p53* depends on the tissue type and insult (for review, see Ryan et al., 2001). *p53* can function as a transcription factor; however, its requirement for the induction of apoptosis remains controversial. *p53* contains two distinct activation domains (ADI and

ADII). Studies involving ectopic expression of *p53* proteins containing inactivating deletions or mutations within ADI have generated conflicting results. It has been shown that the transcriptional function of ADI is essential for the induction of apoptosis in certain cellular systems (Attardi et al., 1996; Jimenez et al., 2000), but not in others (Haupt et al., 1995; Chen et al., 1996; Ding et al., 2000). Furthermore, it has been shown that mutations in ADII can diminish apoptotic activity and arrest cells in the G₁ phase of the cell cycle (Zhu et al., 2000). In some systems, therefore, *p53*-mediated apoptosis requires ADI to be intact.

In addition to its role in transactivation, there is increasing evidence that *p53* is capable of transcriptional repression, further suggesting the importance of *p53* in regulating gene expression during apoptotic cell death (Seto et al., 1992; Murphy et al., 1996; Venot et al., 1998; Koumenis et al., 2001; Nakade et al., 2004). *p53* has also been shown to induce cell death in a transcriptionally independent manner (Caelles et al., 1994; Haupt et al., 1995; Bennett et al., 1998). Studies have shown that *p53* can function at the mitochondrial level (Marchenko et al., 2000; Mihara et al., 2003; Bonini et al., 2004; Chipuk et al., 2004). Differences in cell type and apoptotic stimulus may account for the variability in the required mechanism by which *p53* induces apoptosis. In summary, there are several *p53*-dependent apoptotic pathways: one requiring transactivation, a second that functions through transcriptional repression, and a third that functions exclusively at the mitochondrial level.

Despite the importance of *p53* in the regulation of neuronal apoptosis, little is known regarding the mechanisms by which *p53* mediates apoptosis in postmitotic neurons. In view of the diverse mechanisms by which *p53* functions, in this study we questioned the mechanism underlying *p53*-mediated apoptosis in neurons.

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Table 1. Primer sequences for RT-PCR gene expression analysis

Gene	Sequence	
	5'-Sense-3'	5'-Antisense-3'
NOXA	CAACGCGGGCAGAGCTACCTGA	TGGGCTTGGGCTCCTCATCTGCTC
APAF-1	GACATCAAAACATCTACATCA	CTTCGGCAGCTAACACAG
PUMA	CCTCAGCCCTCCCTGTACCAG	CCGCCCTGCTACTGCGGTTG
PERP	ATCCTGCCCTGCTGCTCTCA	GGCGGCCCAAAAGGTCATC
S12	GGAAGGCATAGTCTGTG	CCTCGATGACATCTTGG

Our results demonstrate that the transactivational function of p53 is essential to induce neuronal cell death. Although two distinct domains have been identified, ADI is critical for the induction of the BH3-only Bcl-2 family member PUMA, which is required to drive neuronal cell death.

Materials and Methods

Cell culture and recombinant adenovirus infection. Cortical and cerebellar granule neurons (CGNs) were cultured as described previously (Cregan et al., 1999; Fortin et al., 2001). The cDNA for Wtp53, p53 Δ I (Kubbutat et al., 1997), p53 Δ V, p53 Δ Pro, and p53 Δ 22/23 was a kind gift from Dr. Karen Vousden (Beatson Laboratories, Glasgow, UK). The p53 double transactivation mutant p53 Δ DM was generously provided by Dr. Xinbin Chen (University of Alabama, Birmingham, AL). To generate the mutation for the second activation domain (ADII) alone (p53 Δ 53/54), Wtp53 cDNA was digested with BsrDI, and the resulting 5' fragment was ligated to the corresponding 3' cDNA fragment from BsrDI-digested p53 Δ DM. The cDNA for Noxa and cDNA for HA-PUMA were kind gifts from Dr. Eri Oda (University of Tokyo, Tokyo, Japan) (Oda et al., 2000) and Dr. Bert Vogelstein (Howard Hughes Medical Institute/Johns Hopkins University, Baltimore, MD) (Yu et al., 2001), respectively. N-terminal 3xFlag tag was added by subcloning full-length Noxa cDNA into p3XFLAG-myc-CMV-24 (Sigma, St. Louis, MO). Recombinant adenoviral vectors carrying expression cassettes for Noxa, PUMA, wild-type, or mutant human p53 proteins were constructed, purified, and titered as described previously (Cregan et al., 2000). Adenoviral vectors were added to cell suspensions immediately before plating.

Mice and genotyping. Mice carrying a targeted null mutation for p53 were obtained from The Jackson Laboratory (Bar Harbor, ME), and p53 genotyping was done by PCR, as described previously (Fortin et al., 2001). p53^{Q5} mice expressing p53 containing point mutations at codons 25 and 26 (L25Q,W26S) were a generous gift from Dr. Geoffrey Wahl (The Salk Institute, La Jolla, CA). Genotyping of these mice was performed as described previously (Jimenez et al., 2000). Mice carrying a targeted null mutation for PUMA were a generous gift from Dr. Andreas Strasser (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia), and genotyping of these mice was performed as described previously (Villunger et al., 2003).

Semiquantitative reverse transcription-PCR analysis. Total RNA was isolated from cells using Trizol isolation reagent according to the manufacturer's instructions (Invitrogen, San Diego, CA). Pilot experiments were performed to determine the linear range of amplification with respect to quantity of starting template and PCR cycles. The primers used for the detection of p53-inducible genes are shown in Table 1. Twenty-five to 100 ng of total RNA were used for cDNA synthesis and targeted gene amplification using the SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis was performed at 48°C for 45 min, followed by a 2 min initial denaturation step at 94°C. This was followed by 35 cycles (Noxa), 30 cycles (APAF-1, PUMA, PERP), or 25 cycles (S12) at 94°C for 30 sec, 55–62°C for 30 sec, and 72°C for 1 min. The resulting products were sequenced to confirm identity.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed on total protein extracts as described (Fortin et al., 2001), with the following modifications. Cells were harvested, centrifuged, extracted in lysis buffer (100 mM HEPES, pH 7.4, 5 mM MgCl₂, 2.5 mM EDTA, 20% glycerol, 0.5 M KCl, 0.5 mM PMSF, 0.1% NP-40, 5 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 20 μ M sodium orthovanadate), and assayed for protein concentration by the method of

Bradford (protein assay reagent; Bio-Rad, Hercules, CA). Protein extract (10–20 μ g) was incubated with an excess of indicated ³²P-labeled double-stranded DNA probes (60,000 cpm/0.2 ng of DNA). The oligonucleotides used included 5'-ATGGAGGCACGTCCCCAGCGACAGCAGGCTC-3' (APAF1) and 5'-CCTGCCTTGCCTGGACTTGC-3' (P21) corresponding to the p53 consensus binding sequences within the Apaf-1 (Fortin et al., 2001) and p21 (Macleod et al., 1996) promoters, respectively. The binding reaction (25 μ l) was performed at room temperature for 20 min in binding buffer (50% glycerol, 250 mM KCl, 100 mM HEPES, pH 7.4, 5 mM DTT, 5 mg/ml BSA, and 0.5% Triton X-100) with 0.1 μ g of sonicated herring sperm DNA, and 1 μ l of p53 Pab421 monoclonal antibody was added to the binding buffer (Ab-1; Oncogene Research Products, Uniondale, NY). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction and incubated for 20 min before the addition of labeled probe. Complexes were resolved on a 5% polyacrylamide, 1 \times Tris–glycine gel, dried, and visualized by autoradiography.

Western blot analysis and immunostaining. Cells were fixed and permeabilized in ice-cold methanol for 20 min, rehydrated in three changes of PBS, and incubated for 2 hr with primary antibody to p53 (CM1; Novacastra Laboratories, Newcastle, UK) or Cox IV (Molecular Probes, Eugene, OR). Cells were washed with PBS and then incubated for 1 hr with Alexa 488- and/or Alexa 546-conjugated secondary antibodies (Molecular Probes). Cells were again washed in PBS and counterstained with Hoechst. Western blot analysis was performed as described previously (Cregan et al., 1999), with antibodies against p53 (CM1; Novacastra Laboratories), Flag (Sigma), hemagglutinin (HA) (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (SC-1616; Santa Cruz Biotechnology) as a loading control.

Cell viability and caspase assays. Cell survival was measured by three methods: LIVE/DEAD, terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL), and MTT assays. At the times indicated, neuronal viability was determined using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) following the manufacturer's instructions. TUNEL was used to visualize cells with fragmented DNA. At the indicated times after treatment, cells were fixed in 4% paraformaldehyde for 20 min, washed in three changes of PBS, and then incubated for 1 hr at 37°C with 75 μ l of a mixture (Roche Diagnostics, Laval, Quebec, Canada) consisting of 0.5 μ l of terminal transferase, 0.95 μ l of biotin-16-dUTP, 6.0 μ l of CoCl₂, 15.0 μ l of 5 \times TdT buffer, and 52.55 μ l of distilled water. The reaction was stopped by incubation in 4 \times SSC buffer, followed by three washes in PBS. Cells were then labeled with a streptavidin–cyanine 2 secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature and counterstained with Hoechst 33258 (1 μ g/ μ l) for 5 min. Images were captured using a Zeiss (Oberkochen, Germany) Axiovert 100 equipped with a Sony (Tokyo, Japan) HAD 3CCD power color video camera and analyzed using Northern Eclipse software. The fraction of TUNEL-positive cells as a percentage of total cell number was determined. For both the LIVE/DEAD and TUNEL assays, a minimum of 500 cells was scored for each treatment, and the data represent the mean and SD from a minimum of three independent experiments. In certain experiments, viability was assessed using the colorimetric MTT survival assay (Cell Titer kit; Promega, Madison, WI) that measures the mitochondrial conversion of the tetrazolium salt to a blue formazan salt, used as described previously (Slack et al., 1996).

For the caspase activity assay, cells were harvested and extracted in caspase lysis buffer (1 mM KCl, 10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 10% glycerol) for 15 min on ice, and 10 μ g of protein was used to determine caspase activity as described previously (Cregan et al., 1999). Caspase activity is reported as the ratio of fluorescence output in treated samples relative to corresponding untreated controls.

Results

Transcriptional activation is essential for p53-mediated neuronal cell death

P53 is a multifunctional protein, the mechanism of action of which depends on the cell type or the death stimulus. To under-

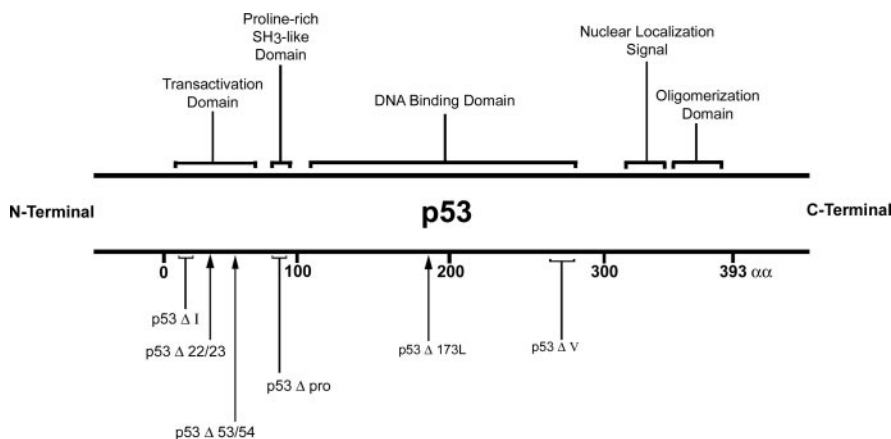


Figure 1. Schematic representation of p53 constructs. Various p53 constructs were used to determine the importance of each functional domain for neuronal apoptosis. The p53 Δ I construct contains a deletion of the conserved box 1 sequence, which abolishes MDM2 binding without affecting DNA-binding activity. The p53 Δ 22/23 construct contains two inactivating point mutations within the first transactivation domain (ADI); residues 22 and 23 were mutated from Leu and Trp to Glu and Ser, respectively. p53 Δ 53/54 has mutations at residues 53 and 54 from Trp and Glu to Phe and Ser, respectively, which inactivates the second transactivation domain (ADII). The p53 Δ DM contains both transactivation domain mutations (p53- Δ 22/23 and p53- Δ 53/54). p53 Δ Pro is a deletion of the proline-rich region of the p53 protein, the p53- Δ 173L mutant has a point mutation at residue 173 to Leu, and p53 Δ V is a deletion of the conserved box V sequence of p53, resulting in inactivation of DNA binding.

stand the mechanism by which p53 participates in injury-induced neuronal death, we determined which functional domains were required for the induction of apoptosis. We constructed a panel of adenoviral vectors carrying wild-type and specific deletion/point mutants of p53 (Fig. 1). To facilitate a functional comparison between these constructs, we first verified that all p53 mutants (1) were expressed at comparable levels in CGNs by Western blot analysis and immunohistochemistry, (2) retained the correct conformation by performing EMSAs, and (3) were localized to the appropriate cellular compartment by immunofluorescence and confocal microscopy.

Western blot analysis revealed that all p53 mutant proteins were expressed, in CGNs, at levels comparable with wild-type p53 (Fig. 2A), and immunohistochemistry demonstrated that all the adenoviral constructs demonstrate comparable infectivity (Fig. 2B). To ensure that the protein conformation was not disrupted by specific deletions or site-directed mutagenesis, p53 sequence-specific DNA-binding activity was analyzed by EMSA. All p53 mutants, except the DNA-binding mutant Ad-p53 Δ V, could effectively bind DNA exhibiting complex formation comparable with that seen with wild-type p53 (Fig. 2C). Finally, because none of these mutants disrupted the nuclear localization domain, they localized appropriately to the nucleus (Fig. 3A). These experiments demonstrate that the p53 mutants used in this study are equally stable and maintain an appropriate protein conformation.

Recently, studies have revealed that p53 could translocate to the mitochondria and was capable of inducing apoptosis exclusively at this site (Marchenko et al., 2000; Sansome et al., 2001; Mihara et al., 2003; Bonini et al., 2004). To determine whether wild-type p53 or any of the mutant p53 proteins were targeted to the mitochondria, neurons were labeled with antibodies directed against p53 and a mitochondrial marker, α -CoxIV. Analysis of p53 immunostaining by confocal microscopy revealed p53 localization exclusively within the nucleus. No colocalization with the mitochondria was found with overexpressed wild-type p53, even when nuclear condensation was evident, nor was there colocalization with the double transactivation mutant Ad-p53 Δ DM

(Fig. 3B). These studies show that, in neuronal cells, p53 does not localize to the mitochondria and functions primarily in the nucleus. The following results demonstrating the requirement of an intact transactivation domain further support this conclusion.

To determine which functional domains of p53 are required for the induction of neuronal cell death, CGNs were transduced with adenoviral vectors expressing wild-type or mutant p53 proteins and assessed for apoptosis. After direct expression of p53, cells were assayed for caspase activation, by *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (DEVD-AFC) cleavage, and for survival, using two independent assays, LIVE/DEAD staining and TUNEL (Fig. 4A,B,D). Caspase activity closely paralleled cell death assays (Fig. 4C). The most efficient constructs for inducing caspase activation and apoptosis were Ad-p53wt and Ad-p53 Δ I. This was expected because Ad-p53 Δ I lacks the mouse double minute 2 (MDM2)-binding

domain, which should enhance protein stability and thereby promote apoptotic activity. Loss of the proline-rich motif impaired apoptotic activity, revealing only partial caspase activation and TUNEL staining. Because p53 has two transactivation domains, we examined the importance of these domains in the induction of apoptosis. Inactivation of either domain resulted in a significant decrease in p53-induced apoptosis. However, inhibition of AD1 appeared to have a more profound effect on the ability of p53 to induce cell death (Fig. 4A). Cells expressing p53 Δ 22/23 exhibited only 23% apoptosis at 72 hr relative to wild-type p53, which led to >63% cell death by 72 hr. In contrast, mutation of the second transactivation domain, Ad-p53 Δ 53/54, resulted in a more modest reduction in apoptotic activity with ~34% of cells dying by 72 hr. When both transactivation domains were inactivated (Ad-p53 Δ DM), there was no detectable increase in caspase activity or apoptotic death relative to control cells expressing Ad-GFP (green fluorescent protein). Similarly, the DNA-binding domain mutant p53 Δ V was apoptotically inert. In fact, the p53 Δ DM and p53 Δ V mutants were unable to induce caspase activation or cell death even when the time course was extended to 96 hr. In conclusion, the results of these studies demonstrate that neuronal apoptosis induced by direct expression of p53 requires DNA-binding activity and an intact transcriptional activation domain. Furthermore, the results suggest that ADI may play a more prominent role in the transcriptional induction of proapoptotic genes.

Reconstitution of responsiveness to DNA damage-induced apoptosis in p53-deficient neurons

It is well known that p53 function can be modulated by post-translational modifications or the coactivation of other death signals (Morris et al., 2001; Xu, 2003). We therefore examined the function of wild-type and mutant p53 proteins in response to camptothecin, a DNA-damaging agent that is known to induce cell death through a p53-dependent mechanism (Xiang et al., 1998; Morris et al., 2001). Wild-type and mutant p53 proteins were expressed in p53-deficient neurons to determine whether they could reestablish sensitivity to DNA damage-induced apoptosis. CGNs were transduced with adenoviral vectors expressing

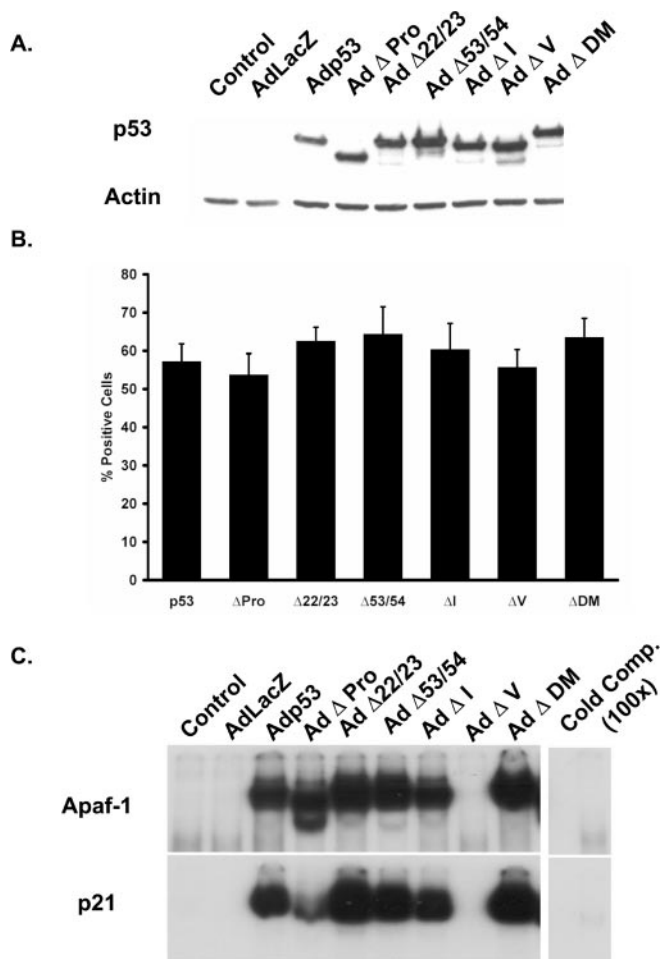


Figure 2. p53 mutant proteins are stable, expressed at comparable levels, and retain the appropriate protein conformation. *A*, Western blot showing comparable expression levels of p53 mutant proteins. CGNs were infected at 25 MOI with the indicated Ad-p53 construct, and protein lysates were collected at 36 hr after infection (30 μ g protein/lane). *B*, CGNs were infected with the indicated Ad-p53 construct at 25 MOI. After 36 hr, neurons were fixed and immunostained for p53 and counterstained with Hoechst. Data represent the mean and SD of three independent experiments. *C*, EMSA. Protein was extracted from CGNs 36 hr after infection with the indicated Ad-p53 constructs. p53-binding activity to the APAF1 and p21 p53 response elements was assayed by EMSA. Binding reactions were performed with neuronal extracts (10–20 μ g protein) and the indicated oligonucleotides in the presence of p53 antibody (Ab1). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction and incubated for 20 min before the addition of labeled probe. All p53 constructs tested efficiently bound DNA, with the exception of Δ V, which inactivates DNA binding ($n = 3$).

wild-type or mutant p53 proteins and then challenged with the DNA-damaging agent camptothecin. Cell death was monitored within 24 hr of drug treatment to minimize the contribution of p53 expression alone to the death response. Consistent with previous studies, 76% of wild-type neurons treated with camptothecin underwent apoptosis after 24 hr (Fig. 5). Uninfected or Ad-GFP-infected p53-deficient cells exhibited only 10–15% cell death (Fig. 5). Cells reconstituted with wild-type p53 (Ad-p53wt) or a p53 mutant lacking the MDM2-binding domain (Ad-p53 Δ I) were rescued in their apoptotic response revealing 64 and 57% cell death, respectively. Cells expressing p53 with a mutation in the proline-rich motif (Ad-p53 Δ Pro) revealed an intermediate apoptotic response of 37% cell death. Interestingly, mutants lacking either one of the transactivation domains (Ad-p53 Δ 22/23, Ad-p53 Δ 53/54) also exhibited intermediate levels of apoptosis

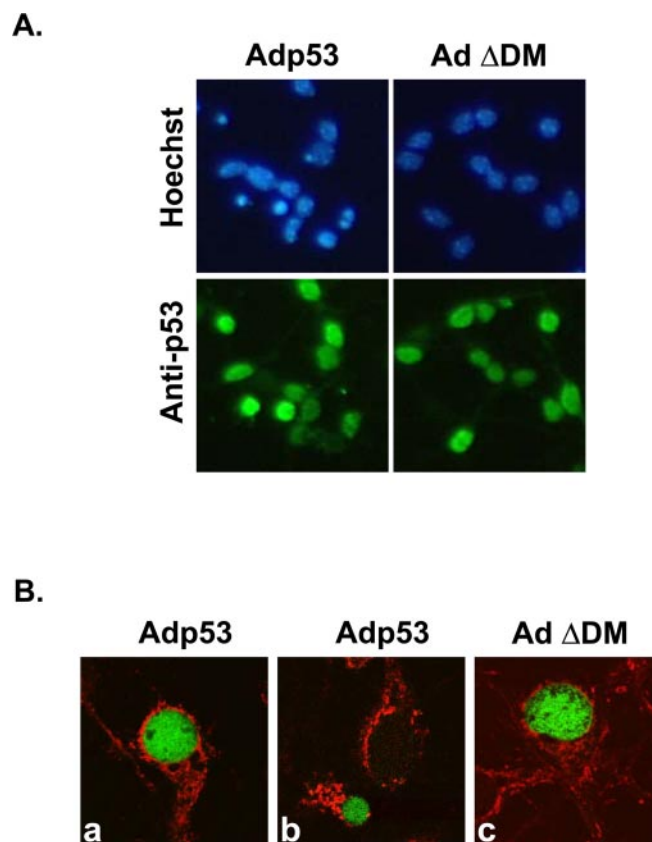


Figure 3. p53 mutant proteins localize to the nucleus. Cortical neurons were infected with Ad-p53WT or Ad-p53DM at 25 MOI. After 48 hr, neurons were fixed and immunostained for p53 (*A*, *B*) and/or mitochondrial-specific CoxIV (*B*) or counterstained with Hoechst (*A*). p53 remained localized to the nucleus even under conditions of cell death, as denoted by condensed nuclei (*b*).

(Fig. 5). Consistent with our results obtained by direct overexpression, mutation of the first transactivation domain, p53 Δ 22/23, severely impaired the ability to rescue apoptosis, exhibiting only a slightly higher kill than p53-deficient neurons (19% cell death). In contrast, cells expressing the mutation in the second transactivation domain, p53 Δ 53–54, resulted in a partial rescue of the death response such that 32% of neurons had undergone cell death (Fig. 5). This corresponded to approximately half the activity obtained after reconstitution with wild-type p53. In contrast, expression of p53 proteins either lacking function of both transactivation domains (p53DM), or DNA-binding activity (p53 Δ V) could not restore the apoptotic response to camptothecin. Consistent with results obtained by direct expression of the p53 mutants in neurons, reconstitution experiments revealed that the transcriptional activity of p53 was essential for the induction of neuronal cell death after DNA damage. Although p53 contains two distinct transactivation domains, there appears to be a difference in the ability of these domains to induce apoptosis. The finding that the ADI mutant exhibited a significantly greater defect than ADII in the rescue of the apoptotic response suggests that ADI may play a more prominent role in the induction of proapoptotic genes. We therefore asked whether these p53 mutants could induce known p53 transcriptional players effectively.

Transactivation mutants of p53 activate common and distinct proapoptotic targets

The results presented above suggest that the transactivation function is essential for p53-mediated neuronal cell death; however,

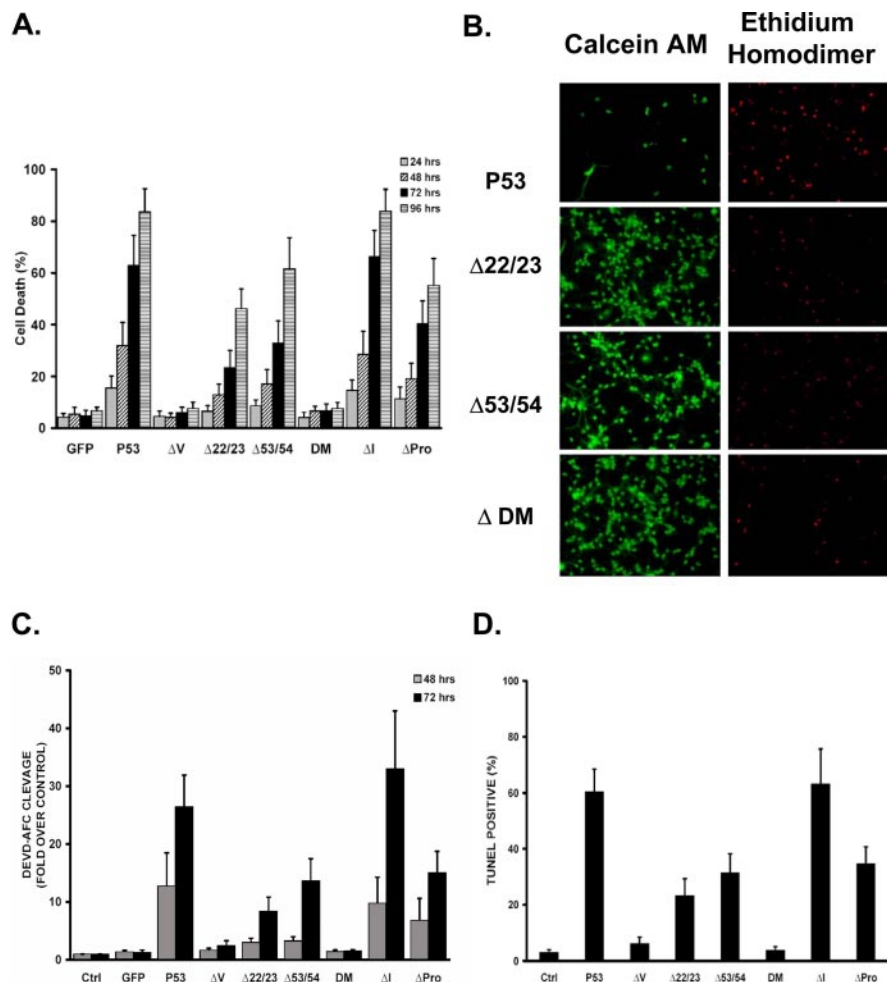


Figure 4. Transactivation domain 1 of p53 is essential for the induction of neuronal cell death. CGNs were infected with wild-type Ad-p53 or p53 mutant constructs at 25 MOI. *A*, LIVE/DEAD viability/cytotoxicity assay (Molecular Probes) was performed at 24, 48, 72, and 96 hr after infection. *B*, Photomicrographs of LIVE/DEAD cell assay. Scale bar, 100 μ m. *C*, Caspase-3 activity was measured at 48 and 72 hr by DEVD-AFC cleavage. *D*, The fraction of TUNEL-positive cells was measured 72 hr after infection. Data represent the mean and SD of five independent experiments ($n = 5$).

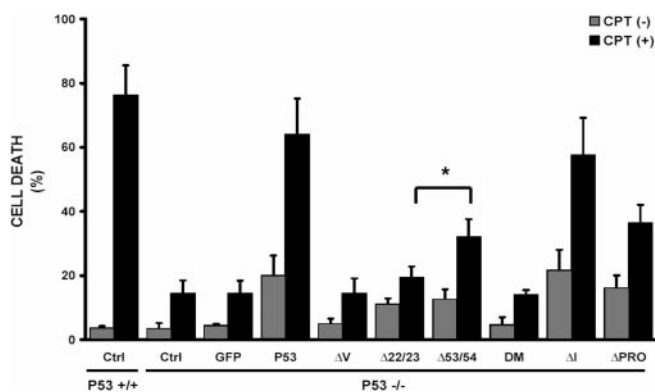


Figure 5. Reconstitution of responsiveness to DNA damage-induced apoptosis in p53-deficient neurons. Cortical neurons obtained from p53-deficient mice or wild-type littermates were infected with Ad-p53 or Ad-p53 mutant constructs and 16 hr later were challenged with 10 μ M camptothecin. The reconstitution of apoptotic cell death was determined by LIVE/DEAD assay 24 hr after treatment. * $p < 0.05$ by two-way ANOVA compared with wild type, followed by t test. Data represent the mean and SD of three independent experiments ($n = 3$). Ctrl, Control.

the two transactivation domains exhibit slightly differential apoptotic effects. We therefore asked whether there might be a difference in the target genes activated by these two domains. The induction of a series of proapoptotic p53 target genes by each of the p53 mutants was examined and compared using semiquantitative RT-PCR. The proapoptotic genes examined were *Noxa* (Oda et al., 2000), *Apaf-1* (Fortin et al., 2001), *PUMA* (Han et al., 2001; Nakano and Voudsen, 2001; Yu et al., 2001), and *Perp* (Attardi et al., 2000). Consistent with caspase activation and survival assays, maximal activation of proapoptotic genes was found with wild-type p53 and the MDM2-binding mutant (Ad-p53 Δ I) (Fig. 6). In contrast, no induction over GFP controls was found with the DNA-binding mutant (Ad-p53 Δ V) or Ad-p53 Δ DM lacking both transactivation domains. Cells expressing p53 lacking the proline-rich motif Ad-p53 Δ Pro exhibited target-specific differences in gene induction relative to GFP controls. Some of the transcriptional targets for p53 could be equally induced by either one of the transactivation domains. For example, the targets *Noxa*, *Apaf-1*, and *Perp* could be efficiently induced by either one of the two transactivation domains. In contrast, there was a significant loss of activity in the induction of *PUMA* when either one of the transactivation domains were lost. Indeed *PUMA* mRNA levels were close to control levels, in cells expressing either the ADI or the ADII mutant.

Apoptotic response in neurons carrying a targeted mutation in activation domain 1

Based on caspase assays, apoptotic activity, and reconstitution assays, it appears that for *Puma*-mediated induction *in vitro*, both activation domains are important for the initiation of apoptosis in neuronal cells. We next asked whether a mutation in one of the existing transactivation domains in the endogenous p53 gene is sufficient to abrogate the p53-mediated apoptotic response as seen in the overexpression and reconstitution experiments above. To answer this question, we used p53QS knock-in mice that express a p53 protein containing a double point mutation of amino acid residues 25 (Leu-Gln) and 26 (Trp-Ser) analogous to the 22/23 mutation in the human p53 protein (Jimenez et al., 2000). Primary cortical neurons were cultured from p53QS mice along with their heterozygous littermates. As a basis for comparison, cells were also cultured from p53 knock-out and wild-type mice of the same genetic background. Cell survival was assessed at 24 or 48 hr after camptothecin treatment to determine whether p53QS neurons were affected in their response to DNA damage (Fig. 7A). Although 86% of wild-type cells had undergone apoptosis by 48 hr, only 13% of p53 null cells were apoptotic. Neurons homozygous for the QS mutation exhibited only 16% cell death, a response similar to p53-deficient neurons. Mice heterozygous for the QS mutation exhibited 83% cell death similar

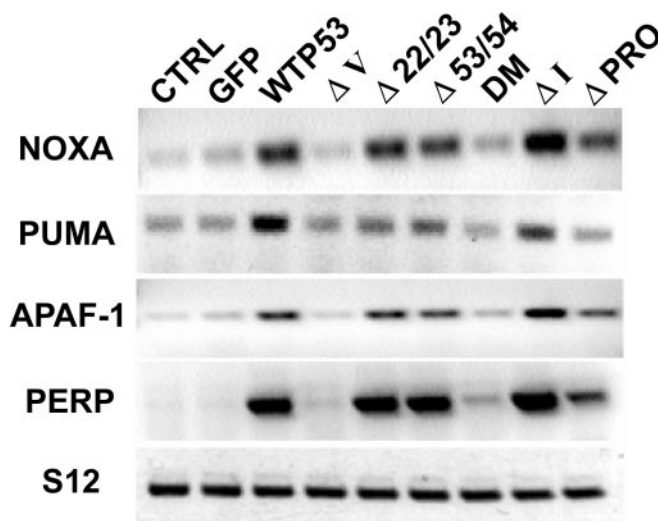


Figure 6. p53 mutants cause differential upregulation of target genes in CGNs. Total RNA was extracted from CGNs 40 hr after infection with Ad-p53 or Ad-p53 mutant constructs as indicated and analyzed for Noxa, Apaf-1, PUMA, and Perp or S12 expression using semiquantitative RT-PCR. CTRL, Control.

to that observed in wild-type cells. This suggests that AD1 is essential for p53 induction of apoptosis in postmitotic neurons.

We then examined the ability of the p53QS protein to induce expression of p53 target genes after camptothecin treatment. We specifically examined the two p53 target genes, *PUMA* and *Noxa*, because of their differential induction profiles in response to adenoviral-mediated expression of p53. In wild-type mice, both *Noxa* and *PUMA* are upregulated in response to camptothecin treatment (Fig. 7*B*). Although camptothecin-induced expression of *Noxa* mRNA was essentially unaffected in p53QS neurons relative to wild-type neurons, upregulation of *PUMA* mRNA was completely abrogated (Fig. 7*C*). These results suggest that, similar to the response seen with ectopically expressed p53 mutants, the endogenous mutant p53QS is unable to activate expression of *PUMA*. AD1, therefore, is essential for the transcriptional induction of *PUMA* and neuronal apoptosis in response to DNA damage-induced injury.

PUMA is a potent inducer of neuronal cell death

Because *PUMA* upregulation correlated closely with the ability to induce apoptosis, whereas *Noxa* induction did not, we directly tested the apoptotic activity of each of these BH3-only proteins in postmitotic neurons. To test this, adenoviral vectors were constructed carrying *Noxa* and *PUMA* expression cassettes. Western blots were performed to confirm the efficient expression of these proteins (Fig. 8*A,B*). CGNs expressed *Noxa* as early as 24 hr after infection, with high expression levels present at 48 hr. In contrast, *PUMA* expression was not readily detectable in neurons (data not shown), likely because of its ability to rapidly induce cell death (Fig. 9*C*). Consistent with this, *PUMA* could be efficiently expressed in HEK 293 cells, which are deficient in BAX function because of the presence of E1B (Graham et al., 1977) (Fig. 8*B*). Because of the strong proapoptotic affect of *Puma*, immunohistochemistry of both *Noxa* and *Puma* was performed in Bax-deficient neurons to verify that both BH3-only proteins were equally well expressed. Immunohistochemistry revealed efficient transduction levels of 65–70% for both adenoviral constructs (Fig. 8*C*). To examine the ability of these BH3-only proteins to induce neuronal apoptosis, primary neurons were infected with

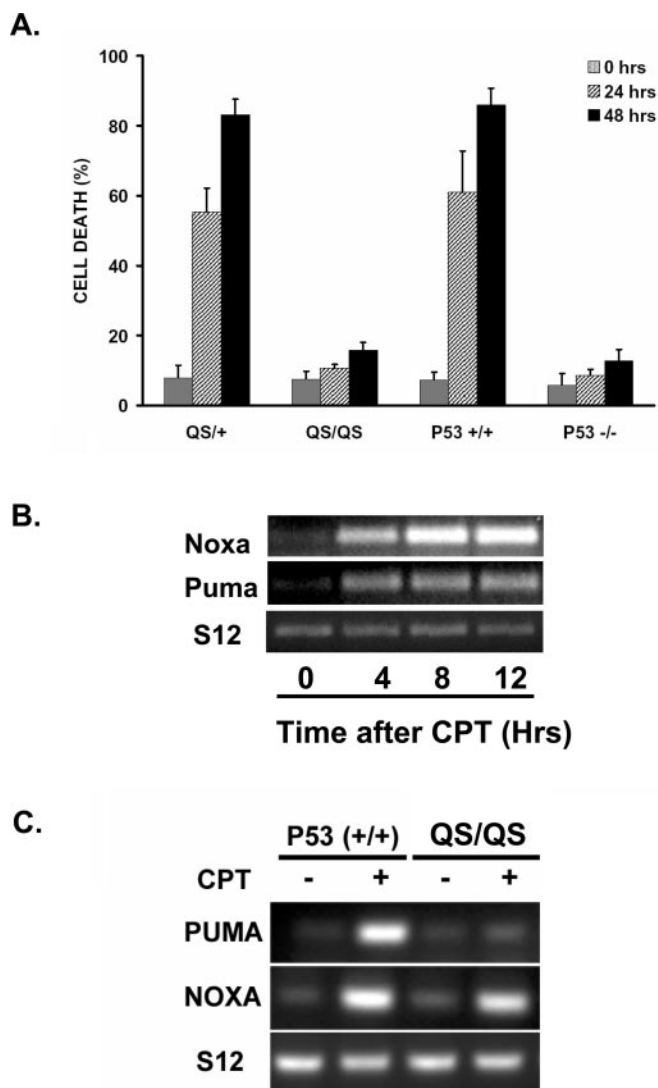


Figure 7. Transactivation domain 1 of p53 is essential for the induction of neuronal cell death *in vivo*. *A*, Cortical neurons obtained from QS mice, p53-deficient mice, or wild-type littermates were treated with 10 μ M camptothecin, and cell survival was determined by LIVE/DEAD assay at the indicated times. Cell death is reported as a percentage of corresponding untreated control cultures. Data represent the mean and SD from three independent experiments ($n = 3$). *B*, Total RNA from cortical neurons from CD1 mice treated with camptothecin was collected and analyzed at the indicated times for *Noxa*, *PUMA*, and *S12* expression using semiquantitative RT-PCR. *C*, Cortical neurons from QS mice, p53-deficient mice, or wild-type littermates were treated with 10 μ M camptothecin, and after 9 hr, total RNA was collected and analyzed for *Noxa*, *PUMA*, and *S12* expression using semiquantitative RT-PCR.

Noxa or *PUMA* vectors at varying doses to generate a dose-response curve (Fig. 9*A,B*). Our survival assays clearly showed that *PUMA* induced a rapid apoptotic response, such that at 12.5 multiplicities of infection (MOI), >50% of cells were dead at 24 hr. In contrast, *Noxa* failed to induce significant apoptosis at 24 hr even at 200 MOI. To determine whether *Noxa* induced a delayed apoptotic response, a time course survival assay was performed with CGNs infected at 50 MOI with *Noxa*, *PUMA*, or a GFP control vector (Fig. 9*C*). Again, our results demonstrated that cultures expressing *PUMA* exhibited a rapid apoptotic response such that >80% of cells were dead by 48 hr. Cells expressing *Noxa*, however, exhibited minimal cell death even at 96 hr after infection. In conclusion, these results demonstrate that the p53 target gene *PUMA* is a potent inducer of apoptosis, whereas *Noxa* on its own is inefficient at triggering cell death.

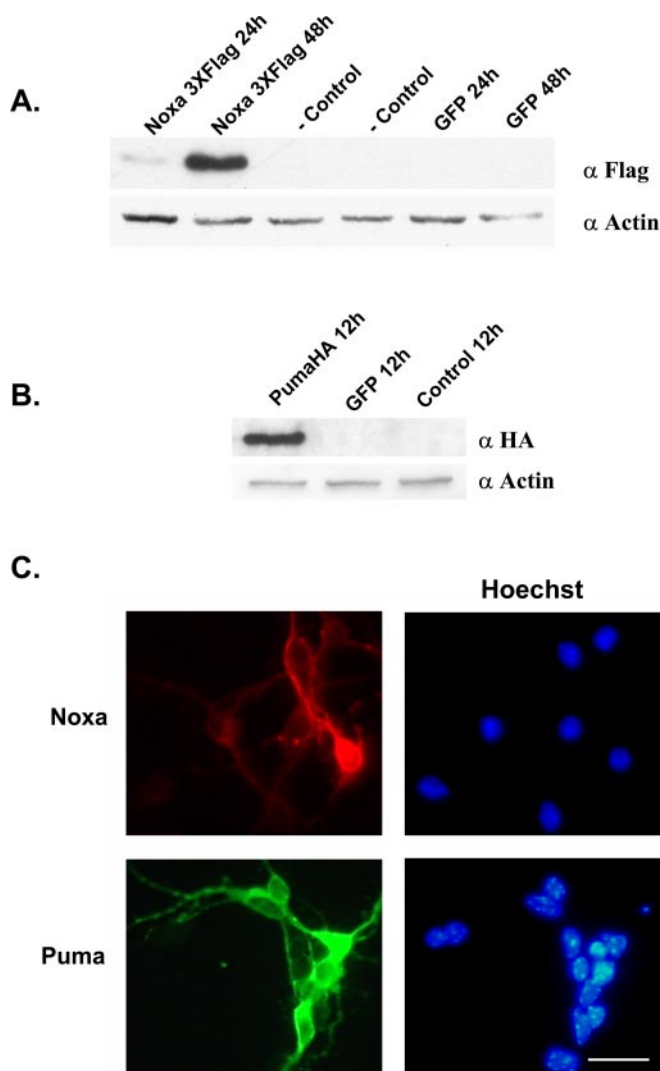


Figure 8. Upregulation of Noxa and PUMA mRNA in p53-mediated neuronal cell death. Western blots showing efficient transduction of Ad-Noxa3XFlag and Ad-PumaHA are shown. *A*, CGNs were infected with Ad-Noxa3XFlag or Ad-GFP at 50 MOI. Protein lysates were collected 48 hr after infection and were blotted for Noxa expression with an antibody against Flag or for actin as a loading control. *B*, HEK 293 cells were infected with Ad-PumaHA or Ad-GFP at 25 MOI. Protein lysates were collected 12 hr after infection and were blotted for PUMA expression with an antibody against HA or for actin as a loading control. *C*, Neurons were infected with Ad-Noxa-Flag or Ad-PumaHA at 50 MOI. After 24 hr, neurons were fixed and immunostained for Flag or HA and counterstained with Hoechst. Scale bar, 25 μ m. Figures are a representative of three independent experiments ($n = 3$).

PUMA is required for p53-mediated neuronal cell death

We have shown that the upregulation of PUMA by p53 requires the presence of both transactivation domains and that enforced expression of PUMA is sufficient to induce neuronal cell death. To determine whether PUMA is essential for p53-mediated apoptosis, we infected CGNs derived from PUMA-deficient mice or wild-type littermates with adenovirus expressing either p53, p53 Δ DM, or GFP. In Figure 10 we demonstrate that PUMA-deficient neurons are significantly more resistant to p53-induced cell death at 48, 72, and 96 hr, relative to littermate controls. These results demonstrate that the upregulation of PUMA is required for p53-induced neuronal apoptosis.

In summary, we have shown that ADI of p53, within residues 1–42 (Unger et al., 1993; Lin et al., 1994; Chang et al., 1995), is essential to induce neuronal cell death because of its efficacy at

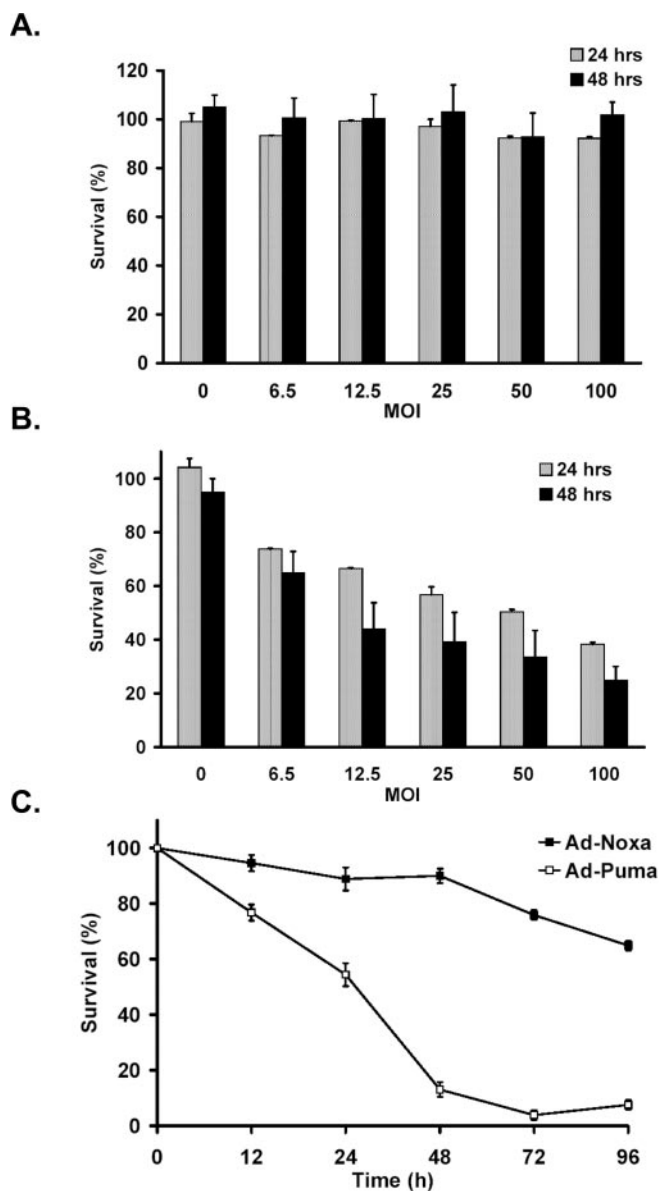


Figure 9. Forced expression of PUMA, but not Noxa, is sufficient to induce neuronal apoptosis. CGNs were infected with Ad-Noxa3XFlag (*A*), Ad-PumaHA (*B*), or Ad-GFP control at the indicated MOI. Neuronal survival was determined at the indicated times by MTT assay. Survival is measured as a percentage of Ad-GFP-treated control cells. *C*, CGNs were infected with Ad-Noxa3XFlag or Ad-PumaHA at 50 MOI. Neuronal survival was determined at the indicated times by MTT assay. Survival was measured as a percentage of Ad-GFP-treated control cells. Data represent the mean and SD from three independent experiments ($n = 3$).

inducing required proapoptotic target genes such as *PUMA*. The importance of PUMA in p53-mediated neuronal cell death is underscored by the fact that PUMA-deficient cells are resistant to apoptosis induced by p53. Thus, we have shown that PUMA is essential for the downstream propagation of the apoptotic cascade initiated by the transactivation function of p53.

Discussion

The involvement of the p53 tumor suppressor has been demonstrated in neuronal cell death induced by acute injury and neurodegenerative diseases (Chopp et al., 1992; de la Monte et al., 1997, 1998; Banasiak and Haddad, 1998; McGahan et al., 1998; Halterman and Federoff, 1999; Watanabe et al., 1999; Cheng et al., 2003). Understanding the underlying apoptotic pathways is

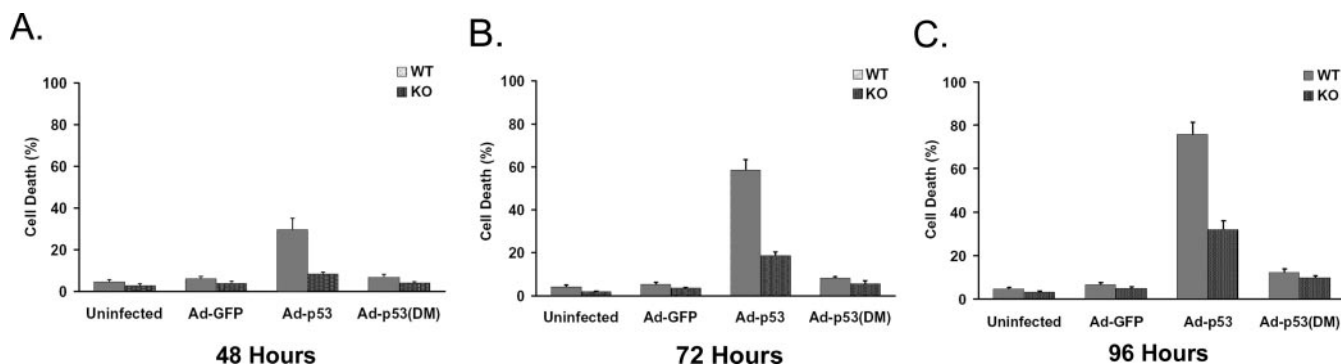


Figure 10. Upregulation of PUMA is essential for the induction of p53-mediated neuronal cell death. CGNs obtained from PUMA-deficient mice or control littermates were infected with wild-type Ad-p53, p53 Δ DM, or GFP constructs at 15 MOI. LIVE/DEAD viability/cytotoxicity assay (Molecular Probes) was performed at 48 hr (A), 72 hr (B), and 96 hr (C) after infection. Data represent the mean and SD of three independent experiments ($n = 3$).

essential for the development of effective neuroprotective therapies. Despite the importance of p53 in neuronal apoptosis, little is known regarding the mechanisms by which p53 induces cell death. The results of our studies support a number of conclusions. First, we showed that p53-mediated transactivation is essential for the induction of neuronal cell death. Second, we demonstrated that ADI of p53 is essential for the induction of target genes involved in neuronal apoptosis. Third, we showed that the induction of the proapoptotic BH3-only protein PUMA requires the presence of both activation domains to trigger neuronal apoptosis. Fourth, we showed that PUMA is required for p53-mediated neuronal cell death. These studies suggest that PUMA may provide a therapeutic target for the treatment of acute neuronal injury.

Recent studies have revealed that p53 is a multifunctional protein and can induce apoptosis by diverse mechanisms. Most notably, p53 has recently been shown to induce apoptosis exclusively at the mitochondria (Mihara et al., 2003; Bonini et al., 2004). In these studies, p53 mutants were constructed that failed to translocate to the nucleus and to activate the transcription of known p53 target genes. These p53 mutants could, however, translocate to the mitochondria where they were shown to sequester antiapoptotic BCL family protein and induce apoptosis (Mihara et al., 2003). Also, when glial cells were treated with pifithrin- α , a compound that has been reported to protect neurons from ischemic or excitotoxic insult by specifically inhibiting p53 DNA-binding ability (Culmsee et al., 2001a,b), p53 was still able to induce apoptosis through direct activity at the mitochondria (Bonini et al., 2004). Although the mitochondrial-mediated mechanism appears to be important in certain cell types and perhaps in response to specific stimuli, our studies demonstrate that this is not essential for the induction of neuronal apoptosis. Examination of double-stained neuronal cells by confocal microscopy did not reveal any colocalization of p53 with the mitochondria, even when undergoing cell death, as determined by condensed nuclei. Consistent with the absence of direct p53-mitochondrial apoptotic activity, inactivation of ADI resulted in a loss of apoptotic activity when the mutant p53 Δ 22/23 was overexpressed. Furthermore, mutants lacking the first activation domain failed to reconstitute p53 responsiveness in p53-deficient cells exposed to camptothecin. Mutation of ADI in the endogenous gene p53 QS leads to a complete loss of apoptotic activity in response to DNA damage. It should be noted, however, that the experiments presented here were limited to apoptosis induced by enforced p53 expression or DNA damage but cannot rule out the possibility that different types of p53-mediated death stimuli may

recruit other transcription-independent mechanisms. These results highlight the fact that p53-induced apoptotic mechanisms are cell type specific and that p53 transactivation function is critical for neuronal apoptosis.

p53 contains two distinct transcription activation domains, and the importance of these domains in the induction of apoptosis appears to be dependent on the cell type examined. Although ADI has been shown to be important for cell death in primary thymocytes (Chao et al., 2000; Jimenez et al., 2000), studies using human lung carcinoma cells revealed that ADI was dispensable for apoptosis. In this instance, ADII was shown to be required (Zhu et al., 1998).

Recently, studies have shown that p53-mediated transcriptional activity correlated directly with the amount of p53 interaction with p300/CREB-binding protein (p300/CBP) (Liu et al., 2003). Furthermore, both activation domains, and to a lesser extent the proline-rich domain of p53, are necessary for interaction with p300/CBP and for histone acetylation of the proximal p21 promoter required for p21 transactivation (Liu et al., 2003). The loss of p300/CBP-binding activity could account for the decreased levels of apoptotic activity observed in the ADI and Δ Pro mutants. Although the proline-rich region has been shown previously to be necessary for apoptosis (Zhu et al., 1999; Baptiste et al., 2002), other data suggest that its deletion may influence function by altering local protein structure (Edwards et al., 2003). This alteration of protein structure leads to differential promoter interactions and therefore may actually be dispensable for apoptotic activity (Edwards et al., 2003). The importance of ADI and ADII may depend on the subset of proapoptotic genes induced, and the requirement of p53 target genes may be cell type specific. Using site-directed p53 mutants, we have shown that each transactivation domain has independent effects on the induction of proapoptotic p53 target genes. Most notably, *Noxa* is readily induced by mutants carrying a mutation in ADI or ADII, whereas *PUMA* can only be induced when both transactivation domains are present. Based on these findings, we examined the proapoptotic activity of BH3-only proteins Noxa and PUMA in postmitotic neurons.

The BH3-only members of the Bcl-2 family of proteins are a unique group that only share a common nine amino acid BH3 domain with the other family members (Huang and Strasser, 2000; Puthalakath and Strasser, 2002). This BH3 domain has been shown to be required for the interaction with other Bcl-2 family members in the induction of apoptosis (Wang et al., 1996; Cheng et al., 2001; Zong et al., 2001). Previous studies have shown that Noxa and PUMA, two members of the BH3-only

family, are upregulated under conditions of p53-mediated cell death in mouse embryonic fibroblasts (Oda et al., 2000) and colon cancer cell lines (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001, 2003). Studies with mice carrying a null mutation for PUMA have shown that it is a key player in the apoptotic pathway. PUMA deficiency confers similar protection against apoptotic stimulus as seen with p53 deficiency (Jeffers et al., 2003; Villunger et al., 2003). Work done in cell lines has also shown that PUMA may be regulated by factors other than p53, such as E2F-1 (Hershko and Ginsberg, 2003) and p73 (Melino et al., 2003), emphasizing its importance in the apoptotic pathway. The primary function of the Noxa protein seems to be at the mitochondrial level (Seo et al., 2003). Recent work with Noxa-deficient mice suggests that it may play a minor role in the apoptotic pathway in fibroblasts but that it is dispensable in other tissues (Villunger et al., 2003). Several results in the present study suggest that Noxa is not likely to play an essential role in p53-mediated neuronal cell death. First, Noxa was efficiently induced by ectopic expression of p53 mutants that are defective in the induction of cell death. Second, Noxa was still induced after camptothecin treatment in p53^{QS} neurons, yet these cells did not undergo cell death. Finally, enforced expression of Noxa was inefficient at triggering neuronal apoptosis. In contrast, p53-mediated induction of PUMA strongly correlated with apoptosis, and ectopic expression of PUMA itself was sufficient to induce apoptosis. Furthermore, PUMA-deficient CGNs were found to be substantially more resistant to cell death induced by p53 overexpression than littermate controls even at time points as late as 96 hr after infection. Thus, our studies demonstrate that PUMA, unlike Noxa, is a potent inducer of neuronal apoptosis and plays a key role in neuronal injury-induced cell death.

The results demonstrate that p53 requires a functional transcriptional activation domain, specifically AD1, to induce the death of postmitotic neurons. Although multiple p53 target genes are induced in neurons, the proapoptotic BH3-only protein PUMA is a potent inducer of apoptosis in neuronal injury. Our results show that upregulation of PUMA is a key determinant in p53-mediated neuronal cell death and suggest that PUMA may serve as a key therapeutic target for the treatment of acute brain injury.

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