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APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death

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p53 is a transcriptional activator which has been implicated as a key regulator of neuronal cell death after acute injury. We have shown previously that p53-mediated neuronal cell death involves a Bax-dependent activation of caspase 3; however, the transcriptional targets involved in the regulation of this process have not been identified. In the present study, we demonstrate that p53 directly upregulates Apaf1 transcription as a critical step in the induction of neuronal cell death. Using DNA microarray analysis of total RNA isolated from neurons undergoing p53-induced apoptosis a 5–6-fold upregulation of Apaf1 mRNA was detected. Induction of neuronal cell death by camptothecin, a DNA-damaging agent that functions through a p53-dependent mechanism, resulted in increased Apaf1 mRNA in p53-positive, but not p53-deficient neurons. In both in vitro and in vivo neuronal cell death processes of p53-induced cell death, Apaf1 protein levels were increased. We addressed whether p53 directly regulates Apaf1 transcription via the two p53 consensus binding sites in the Apaf1 promoter. Electrophoretic mobility shift assays demonstrated p53–DNA binding activity at both p53 consensus binding sequences in extracts obtained from neurons undergoing p53-induced cell death, but not in healthy control cultures or when p53 or the p53 binding sites were inactivated by mutation. In transient transfections in a neuronal cell line with p53 and Apaf1 promoter–luciferase constructs, p53 directly activated the Apaf1 promoter via both p53 sites. The importance of Apaf1 as a p53 target gene in neuronal cell death was evaluated by examining p53-induced apoptotic pathways in primary cultures of Apaf1-deficient neurons. Neurons treated with camptothecin were significantly protected in the absence of Apaf1 relative to those derived from wild-type littermates. Together, these results demonstrate that Apaf1 is a key transcriptional target for p53 that plays a pivotal role in the regulation of apoptosis after neuronal injury.

Introduction

Apoptosis is a biological process that plays a crucial role in nervous system development and injury. During development, cell death is essential for the regulation of neuronal cell number as well as protection against the propagation of aberrant cells (Henderson, 1996). In the mature nervous system, inappropriate cell death is implicated as an underlying defect in many types of neurodegeneration (Portera-Cailliau et al., 1995; Smale et al., 1995), as well as in acute neurological insults (Li et al., 1995; Nitatori et al., 1995; Rink et al., 1995). Therefore, understanding the molecular events triggering apoptosis is an important step towards the development of effective treatment strategies for such neurological diseases.

The p53 tumor suppressor gene is involved in the regulation of apoptosis in several death paradigms. In oncogenesis, p53 plays an essential role in preventing the propagation of DNA-damaged cells and controlling aberrant cell cycle regulation (for review see Prives and Hall, 1999). In the mature nervous system, p53 has been implicated as a key regulatory molecule after neuronal injury (for review see Hughes et al., 1997). Enhanced expression of p53 has been observed in injured neurons before cell death induced by focal ischemia (Li et al., 1994; McGahan et al., 1998), exci-

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Table I. **Microarray analysis of gene induction during p53-mediated neuronal cell death**

Symbol	Accession No.	Fold change	D-call	Description
APAF1	AF064071	5.2	I	Apoptotic protease activating factor 1
Casp1	L28095	1.0	NC	Caspase 1, interleukin-1β converting enzyme
Casp2	NM007610	−1.1	NC	Caspase 2; apoptosis-related cysteine protease
Casp3	NM009810	−1.1	NC	Caspase 3; apoptosis-related cysteine protease
Casp6	Y13087	1.0	NC	Caspase 4; apoptosis-related cysteine protease
Casp7	U67321	1.0	NC	Caspase 7; apoptosis-related cysteine protease
Casp8	AJ007749	1.0	NC	Caspase 8; apoptosis-related cysteine protease
Casp9	AB019600	−1.2	NC	Caspase 9; apoptosis-related cysteine protease
Casp11	Y13089	1.0	NC	Caspase 11; apoptosis-related cysteine protease
Casp12	Y13090	1.0	NC	Caspase 12; apoptosis-related cysteine protease
Casp14	AF092997	1.3	NC	Caspase 14; apoptosis-related cysteine protease

Microarray analysis of RNA extracted from neurons 48 h after infection with either Ad-p53 or Ad-p53-173L. Fold induction represents the ratio of gene expression in cells transduced with Ad-p53 versus Ad-p53-173L. Accession numbers indicate the sequence used as probes in the microarray analysis. Data represents the average of two independent determinations.

totoxicity (Sakhi et al., 1996; Xiang et al., 1996), and hypoxia (Banasiak and Haddad, 1998). Furthermore, we have shown that p53 overexpression itself is sufficient to trigger apoptosis in postmitotic neuronal cultures (Slack et al., 1996; Cregan et al., 1999). Although the mechanisms by which p53 induces apoptosis in proliferating cells are becoming elucidated, those involved in the induction of p53-mediated neuronal cell death appear to be distinct and are poorly understood.

The signaling cascade induced by p53 is complex and likely differs depending on the type of tissue examined (for review see Prives and Hall, 1999). In postmitotic neurons, we and others (Xiang et al., 1998; Cregan et al., 1999; Keramaris et al., 2000) have demonstrated that p53-induced cell death involves a Bax-dependent caspase 3 activation, suggesting that these molecules are important determinants in neuronal cell death after injury. However, although Bax was shown to play a crucial role in p53-induced apoptosis, there was little or no induction of Bax protein during neuronal cell death (Johnson et al., 1998; Xiang et al., 1998; Cregan et al., 1999). Presently, little is known regarding the transcriptional targets for p53 that are important in the regulation of neuronal cell death.

Recent studies have demonstrated the involvement of caspase activation in the regulation of neuronal cell death both during development and after injury. For example, the absence of Apaf1, caspase 9, or caspase 3 results in severe craniofacial malformations with dramatically enhanced neuronal cell numbers in developing mouse embryos (Kuida et al., 1996, 1998; Cecconi et al., 1998; Yoshida et al., 1998). These gross developmental defects were attributed to failed apoptosis in the developing nervous system. Furthermore, caspases have been implicated in neuronal cell death induced by acute injury (Gillardon et al., 1997; Hara et al., 1997; Cheng et al., 1998; Endres et al., 1998; Ni et al., 1998). Since our previous studies (Cregan et al., 1999; Keramaris et al., 2000), as well as those of others, have shown that caspase activation is a key determinant in the molecular cascade by which p53 induces neuronal cell death, we examined the role of Apaf1 in this pathway. In this study, we show that Apaf1 is a direct target induced by p53 and that Apaf1 plays a pivotal role in the regulation of neuronal apoptosis after injury.

Results

Apaf1 is induced during p53-mediated neuronal cell death

We have shown previously that adenoviral-mediated delivery of p53 can induce apoptosis in postmitotic neurons (Slack et al., 1996) through a Bax-dependent mechanism (Cregan et al., 1999). However, unlike with other cell types (Miyashita and Reed, 1995), little upregulation of the putative transcriptional target, Bax, was observed (Johnson et al., 1998; Xiang et al., 1998; Cregan et al., 1999). In the present study, we used DNA microarray analysis to identify potential transcriptional targets for p53 involved in the regulation of neuronal cell death. Cortical neurons were infected at a multiplicity of infection (MOI) of 20 with recombinant adenovirus vectors carrying an expression cassette for either full length human p53 (Ad-p53) or a DNA-binding mutant (Ad-p53-173L), which was shown previously to be transcriptionally inactive (Rowan et al., 1996) and ineffective at inducing neuronal cell death (unpublished data). Microarray analysis of RNA extracted at 48 h postinfection revealed a 5–6-fold increase in Apaf1 transcript levels in cells expressing wild-type p53 relative to controls (Table I). In contrast, transcript levels of various members of the caspase family did not change (Table I).

To confirm the microarray data, we analyzed Apaf1 mRNA levels by reverse transcription (RT)*-PCR. As shown in Fig. 1 A, neurons transduced with Ad-p53 exhibited a significant increase in Apaf1 mRNA levels in comparison with uninfected cells or cells infected with the inactive DNA-binding mutant, Ad-p53-173L. This increase in Apaf1 mRNA levels was evident within 36 h of Ad-p53 infection and remained elevated at 48 h. To determine whether Apaf1 could be upregulated in response to endogenous p53 activity, we treated neurons with the DNA-damaging agent camptothecin, which has been shown previously to induce p53-dependent neuronal cell death (Xiang et al., 1998). As shown in Fig. 1 B, camptothecin induced a time-dependent increase in Apaf1 mRNA levels beginning ~8 h after treatment. To confirm that the induction of Apaf1 expression

*Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; MOI, multiplicity of infection; RT, reverse transcription.

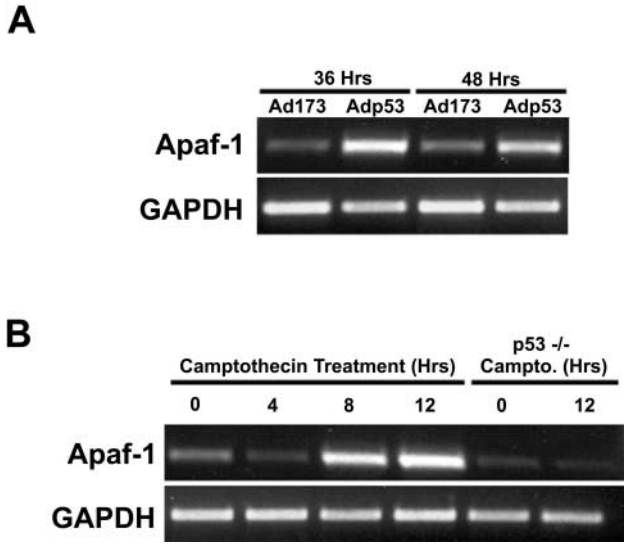

Figure 1. **p53-mediated induction of Apaf1 mRNA in neurons.** (A) RNA was extracted from neurons 36 or 48 h after infection with Ad-p53 or Ad-p53-173L and analyzed for Apaf1 or GAPDH expression using semiquantitative RT-PCR. (B) RNA was extracted from wild-type or p53-deficient neurons at the indicated times after treatment with 10 μM camptothecin and analyzed for Apaf1 or GAPDH expression using semiquantitative RT-PCR.

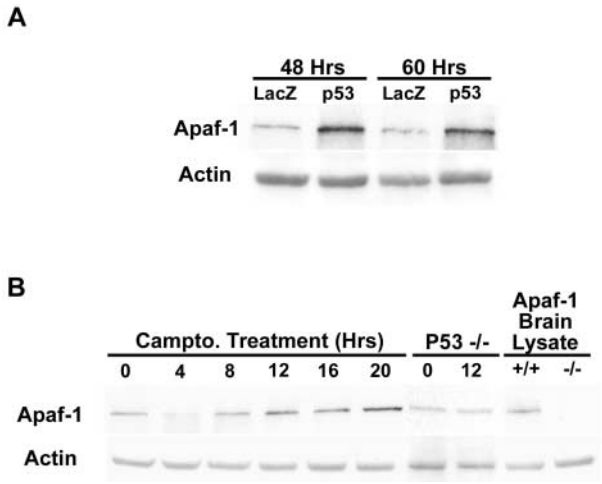

Figure 2. **p53-mediated induction of Apaf1 protein in neurons.** (A) Protein was extracted from neurons 48 or 60 h after infection with Ad-p53 or Ad-p53-173L and assayed for Apaf1 levels by Western blot analysis. (B) Protein was extracted from wild-type or p53-deficient neurons at the indicated times after treatment with 10 μM camptothecin and assayed for Apaf1 levels by Western blot analysis. Specificity of the Apaf1 antibody is demonstrated by lack of immunoreactivity in extracts derived from Apaf1 knockout brain and loading is standardized with actin.

was due to p53, the levels of Apaf1 mRNA were examined in p53-deficient neurons treated with camptothecin. Unlike samples derived from wild-type littermates, Apaf1 mRNA levels did not increase in p53-deficient neurons, thereby confirming the requirement of p53 for Apaf1 induction.

To determine whether the increase in Apaf1 mRNA levels was accompanied by an increase in protein expression, we conducted Western analysis on neurons undergoing p53-induced apoptosis. Cell extracts were obtained from neurons infected at 20 MOI with Ad-p53 or the control vector Ad-LacZ and Apaf1 protein expression was examined. A significant increase in Apaf1 protein levels was evident at 48 and 60 h after infection with Ad-p53 relative to control (Fig. 2 A). We next examined Apaf1 protein levels in camptothecin-induced apoptosis that activates endogenous p53 using wild-type and p53-deficient cortical neurons. A time-dependent increase in Apaf1 protein levels was found in wild-type neurons treated with camptothecin, whereas Apaf1 protein levels did not increase in p53-deficient neurons treated under identical conditions (Fig. 2 B).

To determine whether Apaf1 induction occurs in neuronal injury, models in which the involvement of p53 has been demonstrated previously, we examined mice subjected to ischemia via middle cerebral artery occlusion (McGahan et al., 1998; Watanabe et al., 1999). Mice were subjected to 2 h of focal ischemia followed by reperfusion for 24 h. This procedure generates an infarct in the striatum and cortex on the side of the brain ipsilateral to the occluded middle cerebral artery. Immunohistochemical staining of ischemic mice brain demonstrated increased Apaf1 immunoreactivity in the infarct region relative to the respective contralateral hemisphere (Fig. 3 A). To corroborate immunohistochemical results, Western analysis was performed to measure Apaf1 protein levels from brain tissue after ischemia. The cortex and striatum were removed at 24 h posttreatment, protein was extracted, and Apaf1 protein levels were examined. In support of our immunohistochemical results, a significant increase in the level of Apaf1 expression was evident in the ipsilateral cortex and striatum (Fig. 3 B). Consistent with our in vitro results, p53-mediated neuronal injury in vivo also results in the induction of Apaf1 protein.

Apaf1 is a direct transcriptional target of p53 in neurons

To determine whether Apaf1 is a direct transcriptional target for p53 in neuronal apoptosis, we examined the Apaf1 promoter recently characterized in the Helin laboratory (Moroni et al., 2001). Analysis of the Apaf1 promoter sequence revealed the existence of two putative p53 consensus binding sites located at −572 to −604 (p53 BS1) and −739 to −765 (p53 BS2) relative to the transcription initiation site (Fig. 4 A). To determine whether p53 can interact with these consensus elements, oligonucleotides derived from these proposed binding sites were synthesized and used in electrophoretic mobility shift assays (EMSAs). Protein extracts were examined from neurons undergoing p53-induced apoptosis, including: (a) treatment with camptothecin and (b) direct adenovirus-mediated p53 gene delivery. 48 h after infection with an adenovirus vector carrying wild-type p53 or the DNA-binding mutant p53-173L, protein was extracted and examined by EMSA. EMSA demonstrates that neuronal extracts exhibit p53–DNA binding activity at both putative binding sites, although greater activity was found on BS1 relative to the BS2 (Fig. 4 B). Furthermore, specific DNA binding activity to BS1 was observed with cell extracts prepared from camptothecin-treated neurons, suggesting that endogenous p53 can also interact with these binding sites (Fig. 4 C). The specificity of p53 binding from neu-

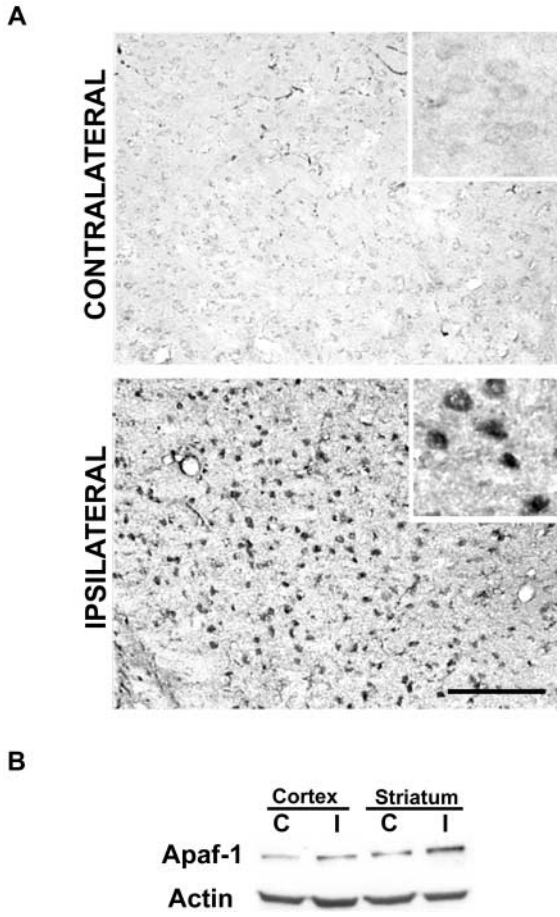

Figure 3. **Induction of Apaf1 in neurons after focal ischemia in mice.** Mice were subjected to 2 h of middle cerebral artery occlusion followed by 24 h of reperfusion. (A) Sections from ipsilateral and contralateral forebrain were prepared and immunostained for Apaf1. (B) Western blot analysis of Apaf1 expression in contralateral (C) versus ipsilateral (I) cortex and striatum after focal ischemia.

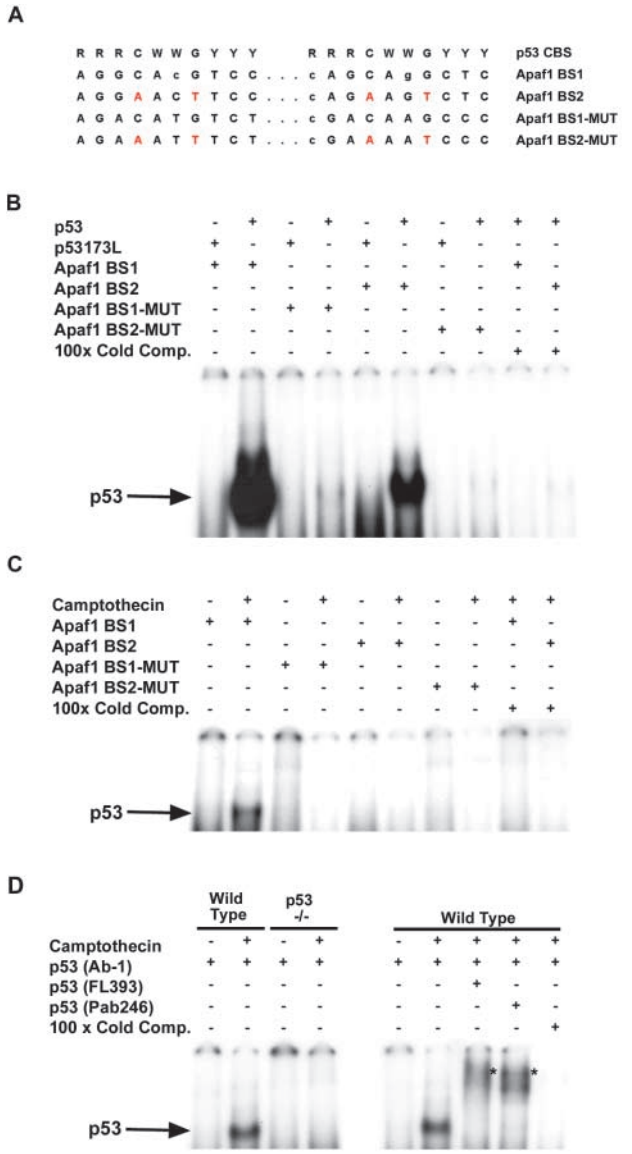

Figure 4. **Specific binding of p53 to Apaf1 promoter elements in neuronal extracts.** (A) Comparison of p53 consensus binding sequence (p53 CBS; el-Deiry et al., 1992), with two putative p53 recognition sequences located in the Apaf1 promoter (Apaf1 BS1 and BS2). The sequence of the corresponding mutated versions of these oligonucleotides (Apaf1 BS1-mut and BS2-mut) used in the electrophoretic mobility shift assays are also indicated. (B) Protein was extracted from neurons 48 h after infection with Ad-p53 or Ad-p53-173L and p53 binding activity to the Apaf1 promoter elements was assayed by electrophoretic mobility shift assay. Binding reactions were carried out with neuronal extracts (10 μg protein) and the indicated oligonucleotides in the presence of p53 antibody (pAb1). (C) Cell extracts (20 μg protein) obtained from untreated neurons or neurons exposed to camptothecin (10 μM) for 12 h were tested for p53 binding activity to the Apaf1 promoter elements as described above. (D) Specificity of p53 binding activity to the Apaf1 promoter was examined in p53+/+ and p53−/− neurons treated with camptothecin. Supershifts with two antibodies directed against p53 were carried out on p53+/+ neurons to further confirm the presence of p53 binding to the Apaf1 promoter.

ronal extracts is supported by several control experiments: (a) the p53 DNA binding mutant (p53-173L) did not exhibit binding to either recognition site; (b) mutation of four key residues within each proposed p53 binding sites on the Apaf1 promoter (Fig. 4 A) abolished p53 binding activity; (c) DNA binding activity could be competed out by incubation with excess unlabeled probes; (d) the bands were supershifted by the addition of two different p53-specific antibodies (Fig. 4 D); and (e) no DNA binding activity was seen in extracts obtained from p53-deficient neurons treated under identical conditions (Fig. 4 D).

The ability of p53 to activate the Apaf1 promoter in neurons was further examined by using a luciferase reporter assay regulated by the Apaf1 promoter. Three different Apaf1-luc reporter constructs were tested, including the full length Apaf1 promoter (−871 to +208), a truncated promoter missing one p53 recognition sequence (−715 to +208), and a truncated Apaf1 promoter deleted for both proposed p53 recognition sequences (−396 to +208; Fig. 5 A). Cultured neuronal cell lines cotransfected with the intact Apaf1 promoter construct and wild-type p53 exhibited a fourfold increase in luciferase activity (Fig. 5 B). In contrast, no induction of luciferase activity occurred upon cotransfection with the DNA binding mutant p53-173L. Deletion of BS2 from the Apaf1 promoter resulted in an ∼25% decrease in p53-induced luciferase activity, and deletion of both p53 recognition sites essentially abolished all p53-induced pro-

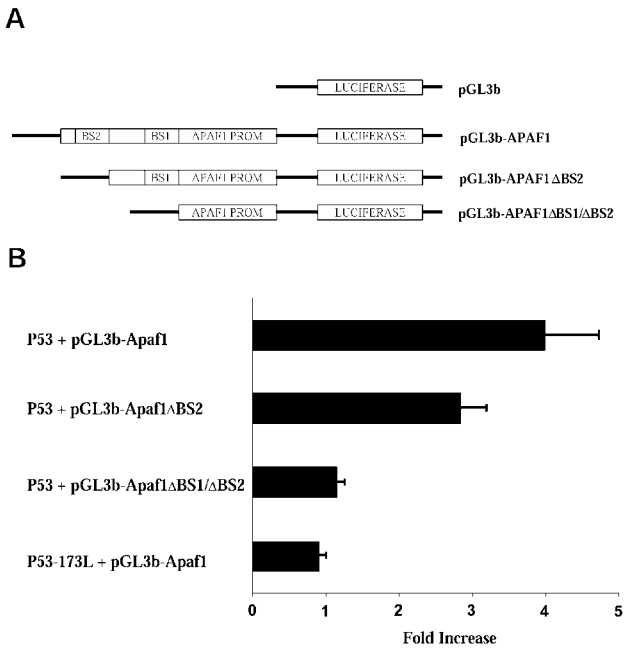

Figure 5. **Activation of the Apaf1 promoter by p53 in a neuronal cell line.** (A) p53 responsiveness of the Apaf1 promoter was tested using luciferase reporter constructs (pGL3b; Promega) consisting of the luciferase gene fused to an Apaf1 promoter fragment containing both p53 binding sites (BS1 and BS2), or truncated promoter fragments deleted for one or both p53 recognition sequences. (B) SN48 cells were cotransfected with the indicated luciferase reporter construct, a CMV–β-gal reporter construct, and an expression plasmid for either wild-type p53, DNA binding–defective p53-173L, or empty vector as control. Luciferase activity was measured in cell lysates obtained 48 h after transfection and normalized to β-galactosidase activity. Fold increase indicates the ratio of normalized luciferase activity of each Apaf1 promoter construct in the presence of p53 expression vector versus empty vector control. Data represent the mean and standard error of triplicate samples from three independent experiments.

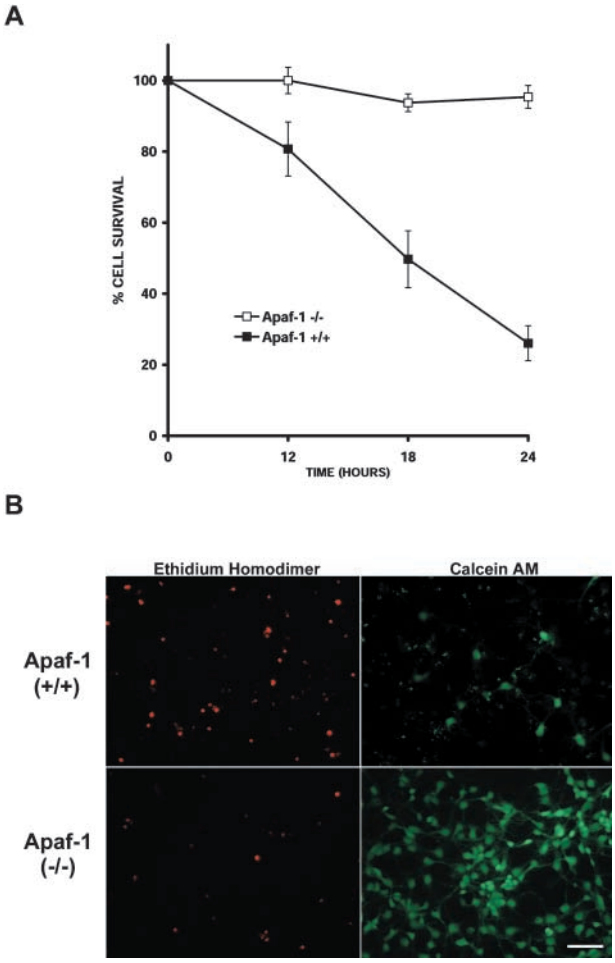

Figure 6. **Apaf1-deficient neurons exhibit increased resistance to p53-mediated cell death.** (A) Cortical neurons obtained from Apaf1-deficient mice or wild-type littermates were treated with 10 μM camptothecin and cell survival was determined by LIVE/DEAD assay (Molecular Probes) at the indicated times. Survival is reported as a percentage of corresponding untreated control cultures. Data represents the mean values obtained from independent cultures involving three separate Apaf1 knockout mice and matching wild-type littermates, and error bars indicate standard deviation of the mean. (B) Cortical neurons from Apaf1+/+ and Apaf1−/− littermates were treated with camptothecin and after 24 h neurons were stained in a LIVE/DEAD assay. Live cells exhibit positive staining for calcein AM activity (green fluorescence), whereas dead cells stain positive for ethidium homodimer (red fluorescence). Bar, 100 μm.

moter activity. Together, our EMSA results demonstrating DNA binding activity at p53 consensus sites in extracts derived from neurons undergoing p53-induced apoptosis, as well as the direct activation of the Apaf1 promoter by p53 show that Apaf1 is a direct target for p53 in the regulation of neuronal cell death.

Apaf1 plays an important role in the regulation of p53-mediated neuronal cell death

To determine whether Apaf1 plays an important role in the regulation of neuronal cell death, we treated wild-type and Apaf1-deficient neurons with camptothecin, a DNA-damaging agent known to induce neuronal cell death through a p53-dependent mechanism (Xiang et al., 1998). Primary cortical neurons were cultured from E14.5 Apaf1-deficient embryos in parallel to their corresponding wild-type littermates. After 24 h in vitro, neurons were treated with camptothecin and cell survival was examined after 0, 12, 18, and 24 h. In Apaf1+/+ neurons, loss of cell viability became apparent at ~12 h after camptothecin treatment and by 24 h only ~25% of neurons survived (Fig. 6). In contrast, Apaf1−/− neurons treated with camptothecin remained viable throughout this time frame, such that by 24 h there was little difference in cell survival relative to untreated controls.

To determine whether this enhanced survival of Apaf1-deficient neurons was associated with a decrease in apoptotic cell death, Apaf1+/+ and Apaf1−/− neurons were treated with camptothecin and the frequency of TUNEL-positive cells was determined after 24 h. In wild-type neurons, camptothecin treatment resulted in a significant increase in the number of TUNEL-positive cells (~70%) relative to untreated controls (Fig. 7 A). Immunofluorescence revealed that TUNEL-positive cells exhibited the typical pyknotic nuclear morphology (Fig. 7 B). In contrast, camptothecin treatment of Apaf1 null neurons did not reveal a significant increase in TUNEL-positive cells. This suggests that within the 24 h time frame examined, in which the majority of

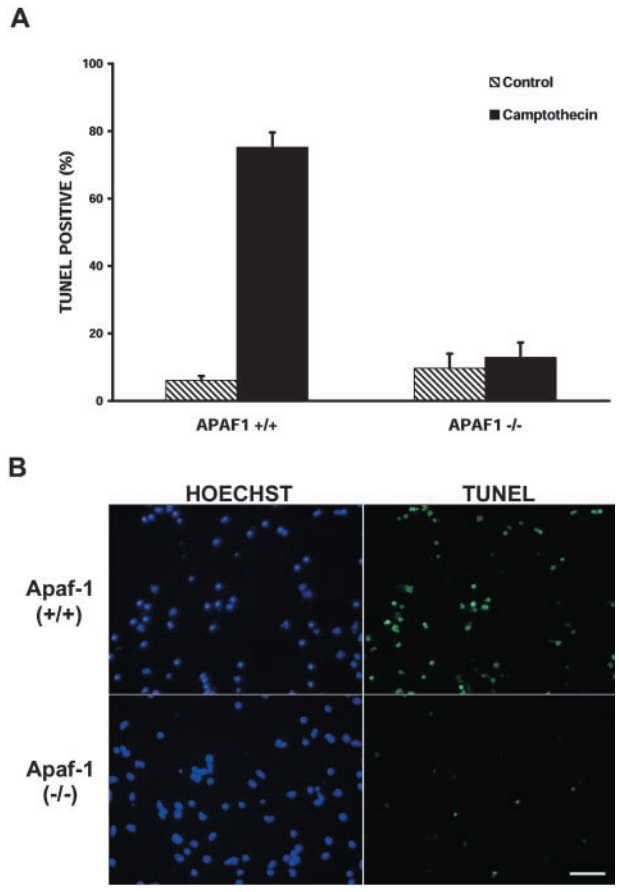

Figure 7. **Apaf1 deficiency decreases p53-mediated apoptosis in neurons.** (A) Cortical neurons obtained from Apaf1-deficient mice or wild-type littermates were treated with 10 μM camptothecin and the extent of apoptotic cell death was determined by Tunel assay. Data represents the mean and standard deviation of three independent experiments. (B) Cortical neurons from Apaf1+/+ and Apaf1−/− littermates were treated with camptothecin and after 24 h cells were fixed, stained for Tunel, and counterstained with Hoechst. Bar, 100 μm.

wild-type cells have undergone apoptosis, Apaf1-deficient neurons were resistant to DNA damage–induced cell death.

In summary, the results of our studies demonstrate that Apaf1 is a direct transcriptional target regulated by p53 and that it plays a prominent role in the execution of p53-mediated neuronal cell death.

Discussion

In postmitotic neurons, the mechanisms by which p53 induces apoptosis are not well understood and appear to be distinct from those in proliferating cells. We and others have shown that p53-mediated cell death in mature neurons works through a Bax-dependent mechanism (Johnson et al., 1998; Xiang et al., 1998; Cregan et al., 1999). It has been shown previously that p53-mediated cell death can function through transcriptional induction of the Bax gene (Miyashita and Reed, 1995); however, we and others (Johnson et al., 1998; Xiang et al., 1998; Cregan et al., 1999) have shown that neurons undergoing p53-induced apoptosis require Bax without significant upregulation of Bax protein levels. The question as to the transcriptional targets for p53 in neuronal cell death remains unknown. Therefore, we set out to conduct a DNA microarray analysis in search of p53-responsive genes important in the regulation of neuronal cell death and have identified Apaf1 as a potential target. In the present study, we examined the role of Apaf1 in the regulation of p53-induced neuronal cell death. Apaf1 is a molecule thought to link the mitochondrial death–signaling events to the initiation of the caspase cascade by forming, along with cytochrome c and dATP, an apoptosomal complex that recruits and activates caspase 9 (Li et al., 1997; Hu et al., 1999). Caspase 9, in turn, goes on to activate other effector caspases such as caspase 3. Accordingly, in the present study we have shown: (a) that Apaf1 is a direct regulatory target for p53 in neuronal cell death and (2) that the upregulation of Apaf1 by p53 plays an important role in the apoptosis-signaling cascade after neuronal injury.

Several lines of evidence suggest that p53 is a key player in the regulation of neuronal cell death in acute neurological disease (for review see Hughes et al., 1997). Studies have demonstrated that p53 protein levels are upregulated after excitotoxicity, hypoxia, and ischemia (Xiang et al., 1996; Banasiak and Haddad, 1998; McGahan et al., 1998). Mice carrying a p53 null mutation exhibited almost complete protection against glutamate or kainic acid–induced excitotoxic injury (Morrison et al., 1996; Johnson et al., 1998). Similarly, ischemic brain damage was reduced in the absence of functional p53 (Crumrine et al., 1994). Furthermore, we and others (Slack et al., 1996; Xiang et al., 1998; Cregan et al., 1999) have shown that enforced expression of p53 alone is sufficient to trigger the apoptotic cascade in postmitotic neurons. Taken together, the observations that: (a) p53 is upregulated after acute neuronal injury, (b) p53 overexpression is sufficient to induce apoptosis in neurons, and (c) the loss of functional p53 reduces neuronal cell death after ischemia and excitotoxicity, strongly implicate this molecule as a key regulator of the death cascade in injured neurons.

The mechanism by which p53 regulates transcription is complex and varies depending on the cell type and can lead to either growth arrest or apoptosis (for review see Prives and Hall, 1999). Although many of the transcriptional targets involved in p53-mediated cell cycle arrest have been identified (for review see el-Deiry, 1998) those involved in the regulation of apoptosis have not been well defined. Several p53 target genes capable of inducing cell death have been identified, including BAX (Miyashita and Reed, 1995), NOXA (Oda et al., 2000a), AIP-1α (Oda et al., 2000b), and PUMA (Nakano and Vousden, 2001; Yu et al., 2001); however, their role in p53-mediated cell death appears to be cell type–dependent. For example, although Bax appears to be important for p53-induced cell death in neurons (Xiang et al., 1998; Cregan et al., 1999) and certain tumor cell lines (Miyashita and Reed, 1995), it does not appear to be required in p53-mediated thymocyte cell death (Brady et al., 1996). Likewise, although the induction of AIP-1 appears to play a role in p53-mediated cell death of Saos-2 osteosarcoma cells (Oda et al., 2000b) it does not seem to function in p53-mediated cell death of several colorectal cancer cell lines (Yu et al., 2001). The proteins encoded by these genes have all been reported to target the mitochondria and initiate caspase

activation, suggesting that they may all function through Apaf-1, which has been identified as a critical cofactor in the mitochondrial caspase activation pathway (Li et al., 1997; Cecconi et al., 1998; Hu et al., 1999). Indeed, we have shown here that Apaf-1 plays an important functional role in p53-mediated cell death in neurons.

Our studies using DNA microarray analysis have identified Apaf1 as a target for p53 in the regulation of neuronal cell death. The Apaf1 promoter contains two p53 consensus sites, and p53 DNA binding activity is dramatically enhanced when neurons are induced to undergo apoptosis. p53-mediated Apaf1 promoter activation is robust in neuronal cell lines and Apaf1 transcripts are induced in wild-type, but not p53 null, neurons undergoing DNA damage–induced apoptosis. Indeed, Apaf1 protein is enhanced after cerebral ischemia, an injury model previously shown to involve p53 (Li et al., 1994; McGahan et al., 1998; Watanabe et al., 1999). Furthermore, the present studies demonstrate that, not only is Apaf1 a direct transcriptional target for p53 in neurons, but that Apaf1 upregulation is an important event leading to the demise of injured neurons.

Recently, transcriptional regulation of the APAF1 gene was reported by Moroni et al. (2001). APAF1 was identified as an E2F1 target gene using DNA microarray analysis and characterization of the APAF1 promoter revealed the existence of both E2F1 and p53 response elements. Both E2F1 and p53 are known to cooperate in certain neuronal death paradigms; for example, pRB-deficient mouse embryos exhibit widespread apoptosis within the central nervous system and this cell death is suppressed in the absence of p53 (Macleod et al., 1996) or E2F1 (Tsai et al., 1998). Although E2F1 is known to induce p53 stabilization (Bates et al., 1998) the Helin laboratory has demonstrated that E2F1 can also activate the apoptotic machinery directly. Furthermore, we have shown previously that E2F1 can induce cell death in postmitotic neurons in the absence of p53 (O'Hare et al., 2000). Thus, it appears that E2F1 and p53 can also induce caspase activation and cell death independent of one another, with one possible mechanism being through APAF1 induction.

The studies of Moroni et al. (2001) also identified potential p53 response elements within the APAF1 promoter and demonstrated APAF1 promoter activation in tumor cell lines overexpressing p53. Our studies extend this work by demonstrating that endogenous p53 is capable of activating the APAF1 promoter in models of neuronal injury. Our results reveal that primary neurons induced to die by DNA damage or after ischemia in vivo exhibit a robust upregulation of APAF1. Furthermore, we demonstrate using EMSAs from neuronal extracts that endogenous p53 is capable of binding wild-type but not mutated p53 response elements on the APAF1 promoter. Parallel studies with p53-deficient neurons confirmed that this binding activity is due to p53 and not other p53 family genes such as p73. Finally, we have demonstrated that APAF1 plays an important functional role in p53-mediated neuronal cell death. Thus, the results of our study establish a novel mechanism by which p53 induces neuronal cell death.

The importance of the caspase-signaling cascade has been demonstrated in many models of neuronal injury as well as neurodegeneration. For example, caspase 3 activation has been demonstrated in traumatic brain injury and inhibition of caspase activity was shown to reduce posttraumatic apoptosis and improve neurological function (Yakovlev et al., 1997). Induction of caspase 3 levels and activity has been demonstrated in the hippocampus after transient global forebrain ischemia (Gillardon et al., 1997; Ni et al., 1998), and, caspase inhibitory peptides have been reported to block neuronal cell death in several models of ischemia (Hara et al., 1997; Cheng et al., 1998; Endres et al., 1998). Indeed the IAPs (the inhibitor of apoptosis protein family) that have been shown previously to be potent inhibitors of caspase activity, exert neuroprotective effects when expressed in neurons induced to die by ischemia (for review see Robertson et al., 2000). Thus, the caspase family of cysteine proteases appears to play an important role in the execution of neuronal cell death, and identification of upstream targets of this cascade is critical for the development of therapeutic strategies for the treatment of acute neuronal injury. In this regard, future studies will be conducted to investigate the role of APAF1 as an upstream regulator of the caspase cascade in in vivo models of neuronal injury using APAF1 conditional knockout mice.

In summary, the results of these studies identify a key mechanism of p53 action in the regulation of neuronal cell death. First, we show that Apaf1 mRNA is upregulated in response to exogenous and endogenous p53 after neuronal injury. Second, we show that Apaf1 protein is upregulated in neurons undergoing p53-induced apoptosis. Third, we demonstrate through EMSA and luciferase reporter assays that p53 directly transactivates the Apaf1 promoter in neuronal cells. Finally, our results show that Apaf1 is an important target for p53 that plays a pivotal role in the regulation of neuronal apoptosis.

Materials and methods

Transgenic mice

Apaf1-deficient transgenic mice have been described previously (Cecconi et al., 1998) and were maintained on a C57BL6 background to maintain genetic uniformity. Apaf1 null mice were genotyped by PCR as described previously. The primers for the wild-type and knockout Apaf1 alleles were 5′-AGATAGCCTAGGGGGTGCAT-3′ (sense) and 5′-ATCAGTTTCCA-ATCGCTGCT-3′ (antisense). Conditions were set as follows: 94°C, 5 min (1 cycle); 94°C, 1.5 min, 66°C, 1 min, 72°C, 1.5 min (30 cycles); 72°C, 10 min (1 cycle). p53-deficient transgenic mice were obtained from The Jackson Laboratory and were maintained on a C57BL6 background to maintain genetic uniformity. p53-deficient mice were genotyped by PCR. The primers for the wild-type were 5′-GTATCTGGAAGACAGGCAGAC-3′ (sense) and 5′-TGTACTTGTAGTGGATGGTGG-3′ (antisense) and for the knockout p53 alleles were 5′-TTCCTCGTGCTTTACGGTATC-3′ (sense) and 5′-TATACTCAGAGCCGGCCT-3′ (antisense). Conditions were set as follows: 94°C, 5 min (1 cycle); 94°C, 1 min, 55°C, 1 min (30 cycles); 72°C, 1 min, 72°C, 10 min (1 cycle).

Primary cortical neuron cultures

Cortical neurons were cultured from dissociated cortices of E14.5 mice as described previously (Xiang et al., 1996) with certain modifications. Cortices from individual embryos were dissected and incubated for 25 min at 37°C in 1× Hank's balanced salt solution (GIBCO BRL) containing 0.50 mg/ml trypsin. Trypsinization was stopped by incubating with 0.2 mg/ml trypsin inhibitor (Boehringer) and 0.2 mg/ml DNase I (Boehringer) for 2 min at 25°C. Cells were pelleted and triturated in Neurobasal medium (GIBCO BRL) containing 0.2 mg/ml trypsin inhibitor and 0.25 mg/ml DNase1. The cell suspension was centrifuged and the pellet was resuspended in Neurobasal medium containing B-27 supplement, N-2 supple-

ment, 0.5 mM glutamine, and 0.05 U/ml 0.05 mg/ml penicillin-streptomycin (GIBCO BRL). Cells were plated in either Nunc 4-wells (3×10^5 cells/well) or 35 × 10 mm (1.5×10^6 cells) dishes (GIBCO BRL) coated with poly-D-lysine (Sigma-Aldrich). Cortices from each embryo were cultured individually and remaining tissue was used for genotyping, after which the appropriate cultures were selected for experimentation.

Murine SN48 cells, which were derived by fusing septal cells from postnatal day 21 mice to N18TG2 neuroblastoma cells (Lee et al., 1990), were maintained in DME supplemented with 10% fetal calf serum at 37°C in 5% CO_2. Cells were grown to 50–60% confluence, and the media was replaced 12 h before transfection by calcium phosphate coprecipitation as described previously (Storring et al., 1999).

Semiquantitative RT-PCR analysis

Total RNA was isolated from cells using Tripure isolation reagent according to the manufacturer's instructions (Boehringer). Pilot experiments were done to determine the linear range of amplification with respect to amount of starting template and PCR cycles. 2 ng of total RNA was used for cDNA synthesis and targeted gene amplification using the SuperScript One-Step RT-PCR kit (GIBCO BRL). cDNA synthesis was carried out at 48°C for 45 min followed by a 2 min initial denaturation step at 94°C. This was followed by 35 cycles (Apaf1) or 25 cycles (GAPDH) at 94°C for 30 s, 56–58°C for 30 s, and 72°C for 1 min. Primers were designed to amplify nucleotides 582–1352 of the Apaf1 transcript and 139–740 of the GAPDH transcript.

Recombinant adenovirus infection

Recombinant adenoviral vectors carrying the human p53, DNA binding mutant p53-173L, or LacZ expression cassettes were constructed, purified, and titered as described previously (Cregan et al., 2000). All experiments were performed at a MOI of 20 pfu/cell. Recombinant adeniviral vectors were added to cell suspensions immediately before plating.

Surgical procedures

All animal procedures conformed to guidelines endorsed by the Medical Research Council of Canada and were approved by the Animal Care Committee of the University of Ottawa. Male C57BL/6 mice weighing 20–22 g were subjected to 2 h of middle cerebral artery occlusion as described (Nagasawa and Kogure, 1989). Mice were anesthetized with a mixture of 30% oxygen, 70% nitrous oxide containing 1.5% halothane. The left common carotid artery was exposed through a ventral midline incision in the neck and permanent ligature placed around the external carotid artery. A temporary ligature was placed around the left common carotid artery and a microaneurysm clip was placed across the internal carotid artery. A silicon-coated -8-0 nylon suture was inserted into the external carotid artery through an incision in the arterial wall, the microaneurysm clip was removed, and the suture was advanced ~9 mm to the origin of the middle cerebral artery. The suture was left in place for 2 h, then withdrawn and the ligature around the common carotid artery was removed. The wound was then sutured with topical application of bupivicaine HCL (0.5 mg/ml).

Cell viability assays

Cell survival was measured by two methods, LIVE/DEAD staining and TUNEL assay. At the times indicated, neuronal viability was determined using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) following manufacturer's instructions. Representative samples were photographed using a ZEISS Axiovert 100 with a Northern Eclipse Sony power HAD 3CCD color video camera. TUNEL labeling was used to visualize cells with fragmented DNA. Cells were harvested 24 h after camptothecin treatment and fixed in 4% paraformaldehyde for 20 min, washed in three changes of PBS, and then incubated for 1 h at 37°C with 75 μl of a cocktail (Boehringer) consisting of 0.5 μl terminal transferase, 0.95 μl biotin-16-dUTP, 6.0 μl $CoCl_2$, 15.0 μl 5× TdT buffer, and 52.55 μl distilled water. The reaction was stopped by incubation in 4× SSC buffer followed by three washes in PBS. Cells were then labeled with a streptavidin Cy2 secondary antibody (Jackson ImmunoResearch Laboratories) for 45 min at room temperature and counterstained with Hoechst 33258 (1 μg/μl) for 5 min. The fraction of TUNEL-positive cells as a percentage of total cell number was determined. A minimum of 500 cells was scored for each treatment and the data represents the mean and SD from three independent experiments.

Western blot analysis

Tissue was extracted in lysis buffer (50 mM Hepes, pH 7.8, 250 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 10% glycerol, 0.1% NP-40, 1.0 mM DTT, 0.5 mM PMSF, 5 μg/mL aprotinin, 2 μg/mL leupeptin, 0.4 mM sodium vanadate) and aliquots containing 40 μg protein were separated on a 10% acrylamide gel and transferred to a nitrocellulose membrane. After blocking for 2 h with 5% skim milk, membranes were incubated for 1 h with either a rat monoclonal antibody directed against Apaf1 (1:500; Chemicon International, Inc.) or a goat polyclonal antibody directed against actin for standardization (Santa Cruz Biotechnology, Inc.). After three washes with TPBS (25 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.9% NaCl, 0.1% Tween-20), membranes were incubated for 1 h at 25°C with the appropriate secondary antibody, washed five times for 5 min each in TPBS, and then developed by an enhanced chemiluminescence system according to the manufacturer's instructions (PerkinElmer).

Immunohistochemistry

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital and transcardially perfused with 5 ml of saline followed by 5 ml of 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed overnight in 4% paraformaldehyde followed by 48 h in 10% sucrose in 0.01 M phosphate-buffered saline. 20-μm thick cryostat sections were cut and processed as free-floating sections as described (Shu et al., 1988). The anti-Apaf1 antibody (MAB3505) was obtained from Chemicon International, Inc.

EMSAs

EMSAs were performed on total protein extracts as described (Macleod et al., 1996), with certain modifications. In brief, cells were harvested, centrifuged, and extracted in lysis buffer (100 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2.5 mM EDTA, 20% glycerol, 0.5 M KCl, 0.5 mM PMSF, 0.1% NP-40, 5 μg/mL aprotinin, 2 μg/mL leupeptin, and 20 μM sodium orthovanadate) and assayed by the method of Bradford (Bio-Rad Laboratories protein assay reagent). 10–20 μg of total cell lysate was incubated with an excess of indicated ^{32}P-labeled double-stranded DNA probes (60,000 cpm/0.2 ng of DNA). Oligonucleotides used included 5′-ATGGAGACATGTCTGGAGACCCTAGGACGACAAGCCC-3′ (BS2) and 5′-ATGGAGGCACGTCCCCAGCGACAGCAGGCTC-3′ (BS1) corresponding to the p53 binding consensus sequences located between −739 to −765 and −572 to −604, respectively, of the Apaf1 promoter. Oligonucleotides 5′-ATGGAGAAATTTCTGGAGACCCTAGGACGAAAATCCC-3′ (BS2-MUT) and 5′-ATGGAGGAACTTCCCCAGCGACAGAAGTCTC-3′ (BS1-MUT) containing mutations within the corresponding p53 consensus sequences were also used as indicated. The binding reaction (25 μl) was carried out 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 μg sonicated herring sperm DNA and 1 uL of p53 Pab421 monoclonal antibody (Ab-1; Oncogene Research Products). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide for BS1 and BS2 was added to the binding reaction and incubated for 20 min before the addition of labeled probe. Furthermore, supershifts were performed with two different p53-specific antibodies, including FL393 and Pab243 (Santa Cruz Biotechnology, Inc.). Complexes were resolved on a 5% polyacrylamide, 1× Tris-Glycine gel, dried, and visualized by autoradiography.

Apaf1 promoter luciferase reporter assays

The Apaf1 luciferase reporter construct (pGL3b-Apaf1) was generated by subcloning the Apaf1 promoter (−871 to +208) into the HindIII site of pGL-3 basic (Promega). Promoter deletion constructs were generated by deleting from the 5′ end with Erase-a-Base nucleotide kit (Promega) (Moroni et al., 2001). The truncated construct pGL3b- Apaf1ΔBS2, missing the most 5′ p53 consensus binding site, contains sequence −715 to +208, and pGL3b-Apaf1ΔBS1/ΔBS2, missing both putative sites, contains sequence −396 to +208. SN48 cells were transfected by calcium phosphate precipitation (Storring et al., 1999) using 15 μg of luciferase construct, 5 μg/plate of either the pCMV p53, the DNA binding mutant pCMV p53-173L, or the empty pCMV vectors, and 5 μg/plate of pCMV LacZ vector as an internal standard. After 14–16 h, cells were passaged into three wells of a 6-well dish/10-cm plate, and incubated for 36 h with fresh medium before assaying for luciferase activity. All plasmids used for transfection were purified using Maxiprep columns (QIAGEN) and quantified by spectrophotometric analysis. Luciferase assays were performed after 36 h, at which time cells were washed once with PBS and lysed in the wells with 200 μL/well of reporter lysis buffer (Promega). Cells were collected by scraping and were subjected to one freeze–thaw cycle followed by centrifugation. Supernatants were collected and assayed for luciferase activity using a BioOrbit 11250 luminometer. A portion of the harvested cell extract (10%) was assayed for β-galactosidase activity based on the conversion of 4-methylumbelliferyl-D-galactoside (MUG) (Sigma-Aldrich) to the highly fluorescent molecule methylumbelliferone. In brief, 30 μl of cell extract was in-

cubated in the dark with 30 μl of 0.3 mM MUG, 15 mM Tris-HCl, pH 8.8, for 30 min after which time a stop solution was added (300 mM glycine, 15 mM EDTA, pH 11.2). After addition of 2 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), fluorescence was quantified using a PerkinElmer LS50 luminescence spectrofluorometer at 350-nm excitation and 450-nm emission settings. The ratio of luciferase to β-galactosidase activity was determined in triplicate samples and normalized to vector-transfected extracts. All data are presented as the mean ± SD of at least three independent experiments.

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