

Caspase-1 activator Ipaf is a p53-inducible gene involved in apoptosis

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The tumor suppressor protein p53 regulates transcription of many genes that mediate cell cycle arrest, apoptosis, DNA repair and other cellular responses. Here we show that Ipaf, a human CED-4 homologue and an activator of caspase-1, is induced by p53. Overexpression of p53 by transfection in U2OS and A549 cells increased Ipaf mRNA levels. Treatment of p53-positive cell lines U2OS and MCF-7 with the DNA damaging drug, doxorubicin, which increases p53 protein level, induced expression of Ipaf mRNA but similar treatment of MCF-7-mp53 (a clone of MCF-7 cells expressing mutant p53) and p53-negative K562 cells showed much less induction of Ipaf gene expression. Expression analysis for Ipaf mRNA in doxorubicin-treated human tumor cell lines suggests that p53-dependent as well as p53-independent mechanisms are involved in the regulation of Ipaf gene expression in a cell-type-specific manner. The Ipaf promoter was activated by normal p53 but not by His²⁷³ mutant of p53. A functional p53-binding site was identified in the Ipaf promoter. A dominant-negative mutant of Ipaf inhibited p53-induced and doxorubicin-induced apoptosis by about 50%. Ipaf-directed small hairpin RNA downregulated p53-induced Ipaf gene expression and also reduced p53-induced apoptosis. Doxorubicin-induced apoptosis was also inhibited by Ipaf-directed small hairpin RNA. Our results show that p53 can directly induce Ipaf gene transcription, which contributes to p53-dependent apoptosis in at least some human cells.

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Introduction

The tumor suppressor protein p53 is a regulator of transcription, which is activated under conditions of cellular stress such as that induced by genotoxic agents and hyperproliferative signals (Vogelstein *et al.*, 2000;

Hickman *et al.*, 2002; Vousden and Lu, 2002; Sax and El-Deiry, 2003). Upon activation, p53 induces either cell cycle arrest or apoptosis. The apoptosis-inducing activity of p53 is very important for tumor suppressor activity of this protein (Vousden and Lu, 2002). Induction of apoptosis by activated p53 involves transcriptional activation of many target genes such as Bax, p53AIP, Noxa, Puma, PIGs, Apaf-1, PIDD, Fas, Killer/DR5, caspase-1, caspase-6, caspase-8, etc. (Vogelstein *et al.*, 2000; Gupta *et al.*, 2001; Hickman *et al.*, 2002; MacLachlan and El-Deiry, 2002; Matsuda *et al.*, 2002; Vousden and Lu, 2002; Liedtke *et al.*, 2003; Sax and El-Deiry, 2003). It is likely that other as yet unidentified p53-inducible genes contribute to apoptosis and other biological functions that are regulated by this protein.

Caspases are cysteine proteases, that are involved in apoptosis, development, cytokine production, etc. (Cryns and Yuan, 1998; Los *et al.*, 1999; Zheng *et al.*, 1999; Degterev *et al.*, 2003). Caspase-1, also known as interleukin-1 β converting enzyme, is primarily involved in the production of cytokines (interleukin-1 β and interleukin-18) and also in some forms of apoptosis including p53-dependent apoptosis (Cryns and Yuan, 1998; Los *et al.*, 1999; Zheng *et al.*, 1999; Gupta *et al.*, 2001, 2002; King *et al.*, 2003; Zhang *et al.*, 2003). There are several regulators of caspase-1 activity, which have activating or inhibitory function (Humke *et al.*, 2000; Damiano *et al.*, 2001; Druilhe *et al.*, 2001; Geddes *et al.*, 2001; Poyet *et al.*, 2001; Martinon *et al.*, 2002; Srinivasula *et al.*, 2002; Burns *et al.*, 2003; Masumoto *et al.*, 2003). Ipaf (also known as CLAN, Card 12) is a recently identified positive regulator of caspase-1 (Damiano *et al.*, 2001; Geddes *et al.*, 2001; Poyet *et al.*, 2001). It is a member of the CED4 family of proteins, which includes Apaf-1, Nod1 and Nod2. The N-terminal caspase activation and recruitment domain (CARD) of Ipaf is involved in interaction with itself and caspase-1 but not with any other caspase (Geddes *et al.*, 2001; Poyet *et al.*, 2001). The central domain consists of the nucleotide-binding domain (NBD), which appears to be essential for activation of caspase-1 by Ipaf (Poyet *et al.*, 2001). The C-terminal leucine-rich repeat (LRR) domain of Ipaf is believed to be a protein-interaction domain, which has a negative regulatory role because deletion of this domain results in a truncated protein that constitutively activates caspase-1 processing and

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also strongly induces caspase-1-dependent apoptosis (Poyet *et al.*, 2001). Ipaf mRNA is highly expressed in bone marrow and at a lower level in lymph nodes, placenta, spleen, lung, brain and peripheral blood lymphocytes (Damiano *et al.*, 2001; Geddes *et al.*, 2001; Poyet *et al.*, 2001). Ipaf gene expression is induced upon differentiation of CD34⁺ progenitor cells to granulocytes and monocytes (Gutierrez *et al.*, 2004). Treatment of HL-60 cells with ultraviolet radiation or tumor necrosis factor- α induces activation of Ipaf gene transcription (Gutierrez *et al.*, 2004). Expression of Ipaf in immune tissues and cells and its interaction with caspase-1 suggests that it has a role in immune response. Macrophages from Ipaf-deficient mice are defective in caspase-1 activation and apoptosis induction in response to infection by *Salmonella typhimurium* (Mariathasan *et al.*, 2004). However, very little is known about the mechanisms that regulate Ipaf gene expression.

Previously, we have shown that caspase-1 is a direct transcriptional target of p53 and it is one of the mediators of p53-dependent apoptosis in human cells because blocking caspase-1 function either by a chemical inhibitor or by mutant caspase-1 inhibits p53-induced as well as doxorubicin-induced apoptosis (Gupta *et al.*, 2001, 2002). However, overexpression of caspase-1 by itself induces much less apoptosis than p53 (Gupta *et al.*, 2002). Therefore, it is likely that an activator of caspase-1 may be induced by p53. Here, we have analysed the regulation of Ipaf gene expression by endogenous, as well as exogenous, p53. By blocking Ipaf function using a dominant-negative mutant and also by using short hairpin RNA (shRNA), we show that Ipaf plays a role in p53-dependent apoptosis.

Results

p53-dependent and independent expression of Ipaf mRNA

Overexpression of wild-type p53 by transfection in the human osteosarcoma cell line, U2OS, increased Ipaf mRNA levels, as determined by reverse-transcriptase polymerase chain reaction (RT-PCR), using primers specific for Ipaf (Figure 1a, b). Transfection with a plasmid encoding p53DD (a dominant inhibitor of wild-type p53) showed no induction of Ipaf. U2OS cells treated with the apoptosis-inducing agent, staurosporine, which induces apoptosis in a p53-independent manner (Villunger *et al.*, 2003), did not show any increase in the mRNA levels of Ipaf (Figure 1c). Therefore, induction of Ipaf gene expression by wild-type p53 is not an outcome of the apoptotic process. Overexpression of p53 in human lung carcinoma A549 cell line also increased the level of Ipaf mRNA (Figure 1d). The identity of Ipaf PCR product was confirmed by carrying out Southern hybridization of the PCR product with Ipaf cDNA labeled with ³²P (Figure 1d). Caspase-1, a known transcriptional target of p53 (Gupta *et al.*, 2001) was also induced by p53 in A549 cells (Figure 1d).

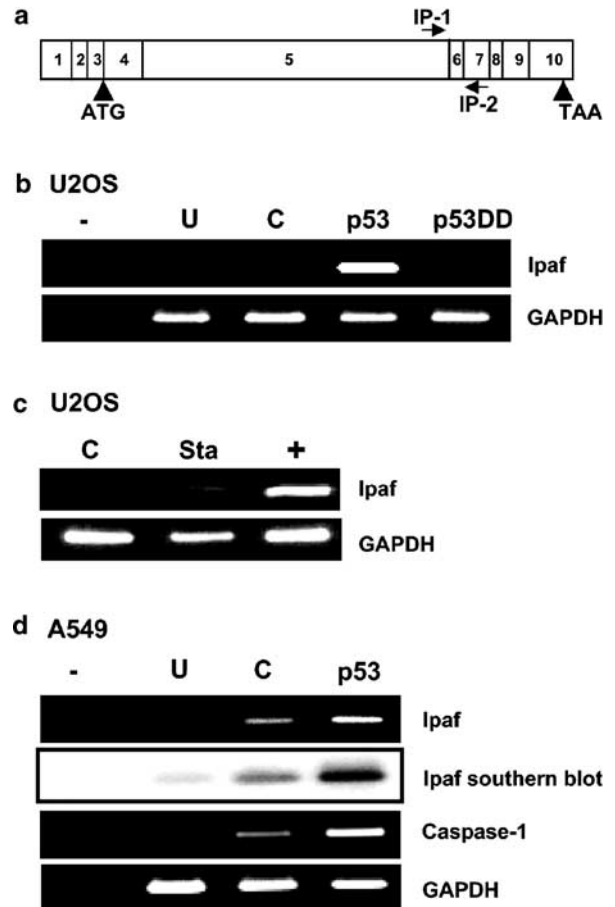


Figure 1 p53-dependent expression of Ipaf mRNA. (a) Schematic of the Ipaf cDNA with the numbers inside representing the exons. IP-1 and IP-2 represent the primers used in the study for detecting Ipaf levels. (b) RT-PCR analysis of total RNA isolated from U2OS cells transfected with a control plasmid (C), wild-type human p53 (p53), p53 dominant-negative (p53DD) and untransfected (U), using primers for Ipaf and GAPDH. (c) Effect of the protein kinase inhibitor and apoptosis-inducing agent, staurosporine on Ipaf mRNA levels in U2OS. The cells were treated with 0.1 μ M staurosporine (Sta) for 8 h or were untreated (C); + is a positive control (U2OS cells treated with doxorubicin for 24 h). (d) Effect of overexpression of p53 on Ipaf and caspase-1 mRNA levels in A549 cells as determined by RT-PCR. Cells transfected with control plasmid (C) and untransfected (U) were used as controls. A Southern blot of Ipaf PCR products is also shown, which was carried out using ³²P-labeled Ipaf cDNA as probe

MCF-7 cells treated with the DNA damaging drug, doxorubicin, which causes increase in p53 protein levels, showed an induction of Ipaf mRNA (Figure 2a). The induction of Ipaf mRNA by doxorubicin was compromised in a clone made from MCF-7 cells expressing His²⁷³ mutant of p53. This mutant of p53 is known to function as a dominant inhibitor of wild-type p53 function (Aurelio *et al.*, 2000). Treatment of other p53-positive cell lines U2OS, HCT-116 and A549 with doxorubicin also induced expression of Ipaf mRNA (Figures 2b and 3a). There was no induction of Ipaf by doxorubicin in the p53-negative cell line, K562. These results clearly implicate wild-type p53 as a regulator of

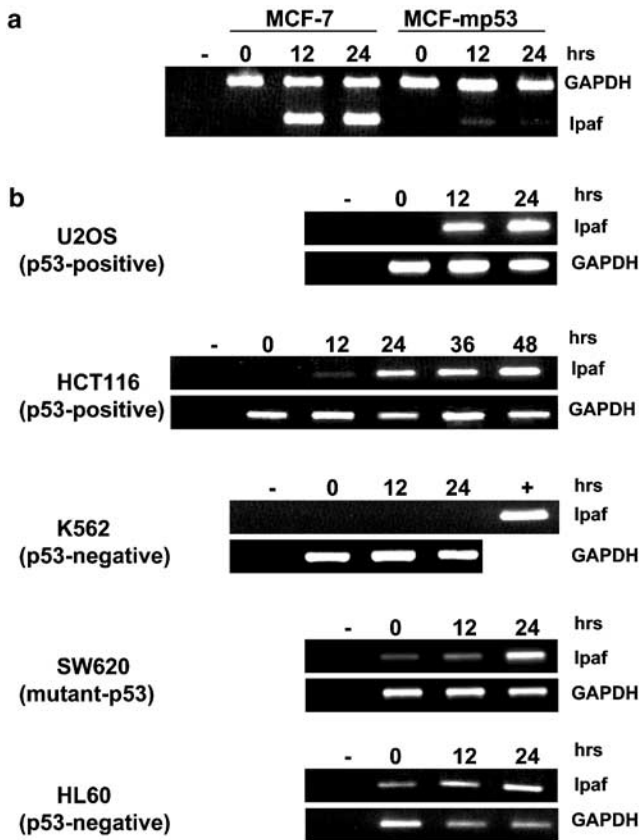


Figure 2 Induction of Ipaf gene expression by doxorubicin. (a) MCF-7 and MCF-mp53 cells were treated with 500 ng/ml of doxorubicin for 12 and 24 h. Ipaf mRNA levels were then analysed by RT-PCR. MCF-7 cells are p53 positive, and MCF-mp53 cell line was derived from MCF-7 cells by transfecting with His²⁷³ mutant of p53 followed by selection in G418. MCF-mp53 cells are functionally p53-negative. (b) The indicated cell lines were treated with 500 ng/ml of doxorubicin, RNA was isolated at the mentioned time points and RT-PCR was performed to analyse the levels of Ipaf mRNA. U2OS and HCT116 cell lines are p53 positive whereas K562, SW620 and HL-60 are p53 negative

Ipaf gene expression. Ipaf mRNA levels were also increased following doxorubicin treatment of the p53-negative cell lines, HL-60 and SW-620 (Figure 2b). In HL-60 cells, the p53 gene has a deletion whereas SW-620 cells carry a point mutation (His²⁷³) in p53. This indicates the existence of a p53-independent pathway, also induced by genotoxic stress that controls Ipaf gene expression in certain cellular contexts.

A time course of the induction of Ipaf gene expression following doxorubicin treatment of MCF-7 and A549 cells revealed Ipaf to be an early induced gene (Figure 3a, b). The induction of Ipaf paralleled the induction of the p21 gene, a previously well-characterized transcriptional target of p53 (El-Deiry *et al.*, 1993). However, the induction of Ipaf gene expression preceded the induction of PUMA, a recently described transcriptional target of p53, which is one of the mediators of p53-dependent apoptosis (Han *et al.*, 2001; Yu *et al.*, 2001; Nakano and Vousden, 2001). Apaf-1, another proapoptotic gene regulated by p53 (Moroni *et al.*, 2001), was induced early like Ipaf and p21 (Figure 3a, b).

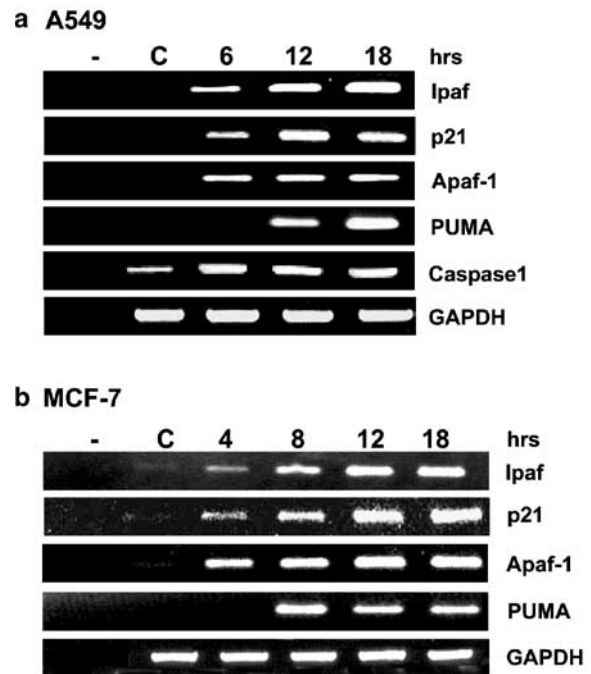


Figure 3 Time course of induction of Ipaf and other p53 target genes in doxorubicin-treated cells. A549 and MCF-7 cells were treated with 500 ng/ml of doxorubicin and RNA was isolated at the mentioned time points. The levels of Ipaf, p21, Apaf-1, PUMA and GAPDH (control) were analysed by RT-PCR. C, untreated control cells

Analysis of Ipaf promoter

Two transcription start sites were mapped by primer extension analysis using RNA from doxorubicin-treated MCF-7 cells. Transcription from these sites was induced by doxorubicin treatment of cells (data not shown). The major transcription start site is indicated in Figures 4 and 5. The putative promoter region of Ipaf (from -2.37 to +0.227 kb relative to the major transcription start site) was amplified by PCR using human genomic DNA as template. It was cloned in a promoter-less vector (pCAT-BASIC) upstream of the CAT reporter gene (Figure 4a). This promoter-reporter vector was co-transfected with or without wild-type p53 in HeLa cells. Reporter assays showed that this promoter was active in HeLa cells and wild-type p53 activated this promoter by over 50-fold (Figure 4b). A DNA-binding deficient mutant of p53 (His²⁷³) was not able to activate this promoter significantly (Figure 4b) showing the specificity of activation by wild-type p53. In order to identify the p53-responsive site in the Ipaf promoter several deletion mutants were made, some of which are shown in Figure 4a. The nucleotide sequence of the promoter region (from -507 to +227 bp) is shown in Figure 5. The deletion mutants pCAT-P2 and pCAT-P3 were tested for their ability to be activated by p53. Wild-type p53 but not mutant p53 was able to activate pCAT-P2 promoter (Figure 4b). The promoter construct pCAT-P3 (-507 to -17 bp relative to the transcription start site) in which the 3' end of the promoter was deleted did

not show any activation by wild-type or mutant p53 (Figure 4b). These results showed that the major p53-responsive site in Ipaf promoter is present in the pCAT-P2 promoter downstream of -507bp relative to the transcription start site. The nucleotide sequence of Ipaf promoter showed a putative p53-responsive site very close to the transcription start site (Figure 5). This site showed 17 nucleotides (out of 20) matching with the p53-binding consensus sequence. Mutation of this site, by replacing C and G nucleotides with A and T, respectively, in the 5'-half-site resulted in a promoter that showed drastic reduction in activation by wild-type p53 (Figure 4c). Wild-type promoter (pCAT-P2) was

activated 30–37-fold by p53 whereas the mutant promoter (pCAT-mutP2) showed about sixfold activation. These results showed that the p53-responsive site present very close to the transcription start site (bold and underlined in Figure 5) is the major p53-responsive site in the Ipaf promoter.

Direct binding of the p53-responsive site identified by reporter assays, to p53 *in vitro* was assessed by gel shift assays using synthetic oligonucleotides. Recombinant FLAG-p53 purified from *Escherichia coli* bound the p53-responsive site in Ipaf as evidenced by the shift produced (Figure 6a, b). In addition to a shifted band that matched with the consensus-shifted band, a lower band was also seen (Figure 6b). Both the shifted bands were entirely competed out by a 100-fold excess of self or consensus oligonucleotides indicating a p53-specific binding. The lower band is likely to represent the shift produced by the binding of p53 dimers. p53 is a dimer of dimers and binds DNA with each dimer of the tetramer contacting its own half-site (McLure and Lee, 1998). In the identified p53-responsive site, one half-site is a perfect match with the consensus while the other has three mismatches, with one of them being in the core of the half-site. The higher affinity of p53 for one of the half-sites is thus probably reflected in the *in vitro* binding to p53 dimers. The *in vitro* binding of p53 to sequences in the Ipaf promoter and its responsiveness to p53 in reporter assays suggest a direct mechanism of p53-dependent upregulation of Ipaf gene expression.

A dominant-negative mutant of Ipaf compromises p53- and doxorubicin-induced apoptosis

To examine the role of Ipaf in p53- and doxorubicin-induced apoptosis, we sought to construct a mutant of Ipaf that could function as a dominant inhibitor of the wild-type Ipaf protein. Ipaf oligomerizes with itself (and caspase-1) through CARD–CARD interaction. It has been reported that while Ipaf deletion mutants lacking the C-terminal LRR region cause pro-caspase-1 processing and apoptosis, the Ipaf (1-256) deletion mutant, which lacks most of the central nucleotide-binding

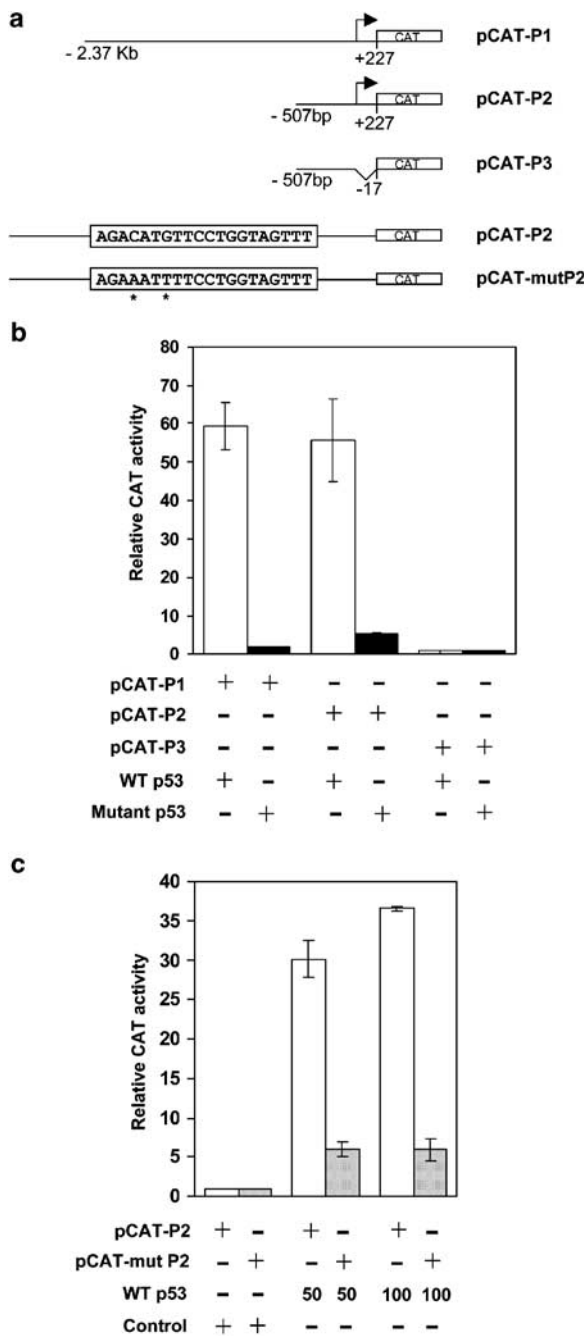


Figure 4 Transactivation of the Ipaf promoter by p53 and identification of a p53-responsive site. (a) Schematic showing different promoter constructs made in the pCAT-BASIC promoterless vector. The arrow represents the major transcription start site identified by primer extension analysis. The two asterisks shown in the schematic of pCAT-mutant P2 (*pCAT-mutP2*) construct represent the two nucleotides that have been changed in comparison to the pCAT-P2 construct ('C' to 'A' and 'G' to 'T') in the putative p53-responsive site. (b) Ipaf promoter is transactivated by wild-type (WT) p53. The different promoter constructs were cotransfected along with WT p53, mutant p53 (His²⁷³) or control plasmid (150 ng each) in HeLa cells. CAT activities relative to the control are shown (*n* = 3). The ratio of WT p53 and mutant p53 to the promoter plasmid was 1:1. pCMV.SPORT.β-gal was included in all these transfections to normalize for the transfection efficiency. (c) Identification of a p53-responsive site in the Ipaf promoter. pCAT-P2 and pCAT-mutP2 constructs (200 ng each) were cotransfected along with 50 or 100 ng of WT p53 or control plasmid and pCMV.SPORT.β-gal. CAT activities relative to the control are shown

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-507   GGGAGGC AGAGGTTGCA GTGAGCCAAG ATTGAGCCAC TGCACTCCAC
-460  TCCAGCCTGG GCAACAGAAC GAGACTCTGT CTCAAATAAA TAAATAAATA
-410  AATAAATAAA CAAATAAAAT AAAAAATAAA TAAGTTGATA AATAAAATTA
-360  GAATATACAG ATAAGCCAAA GACAGAAATT TAAAATTGCT TATAATTGTA
-310  TTCCCCAGCA ATAAGCATCA TTAATAATTTT GGTGGCTGTC TTTGCAGTTT
-260  TTTCTCTTGA ACATATGTTT ATATATAGGT TTCATTACAA TTTAAAAATC
-210  TAGCTGATAG TGTAGCCCTC AATCTCACTT CAGAGAAACC CACCTCACAA
-160  GTTGGGAAAC ACTGACACTG ATGCCCCCTT AGAAAACCCA GGATGTAGAC
-110  ATTTATTTGC CGGGAAACTT TGGTATCCCT CTATGGCAGA TAGGCTACTG
-60   TCAGAATGCT CTTTTTGAAA TAAGAAGTTA TGTAACACAA AGACATGTT
      ────────────┬───────────
-10   CTGGTAGTTT ATGCAAAGAC AGAGGAAAGA GTATGCAAGA ATGTCATCCT
+41   CAAGGGAAGT GCAGAGAGAT TTCTTCAGTC CTCAGCTGAG TATAAGCTGG
+91   CCTCCTGGAG TCTGTGAACA CAAACGTCCA ATGTGAGTGT GCCTGTGCAA
+141  GCCCCTGGCT GTTTATACTC CGGAGGGTGT CCCCGTGCGT CATCGGTGGA
+191  GTGGACCAAA ACTGGTGATC TGTTTGCCCT GTGTGAC

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Figure 5 The nucleotide sequence of Ipaf promoter. The nucleotide sequence of the Ipaf promoter as represented in the construct pCAT-P2 (Figure 4a) is shown. The nucleotides underlined and in bold represent the p53-responsive site identified in the promoter. The arrow represents the approximate transcription start site as determined by primer extension analysis. The nucleotides in *italics* comprise the 5' end of the longest reported cDNA of Ipaf or CARD12 (GenBank Accession number-AF 376061 version 1). The Ipaf promoter sequence depicted here corresponds to nucleotides 89363-90096 (734 bases) of the human BAC sequence at 2p21-2p22 (GenBank Accession number-AL 121653 version 2)

domain and the LRR domain, has a much reduced ability to cause pro-caspase-1 processing. We therefore chose to delete most of the NBD (253–659 amino acids), which would compromise the function of Ipaf, but retained the N-terminal CARD domain needed for oligomerization and the C-terminal LRR domain (Figure 7a). This mutant (Mt-Ipaf) is expected to oligomerize with wild-type Ipaf (and caspase-1) through CARD–CARD interaction but the resulting complex is expected to be functionally inactive due to the absence of the NBD. Expression of mutant Ipaf in Cos-1 cells by transient transfection produced a polypeptide of expected size (Figure 7b).

Apoptosis induced by expression of p53 in U2OS cells was inhibited by over 50% upon coexpression of mutant Ipaf but not by wild-type Ipaf (Figure 7c). Expression of Ipaf or mutant Ipaf alone induced only low-level apoptosis (Figure 7c). Overexpression of Ipaf did not significantly enhance apoptosis induced by p53 or doxorubicin (Figure 7b, c). Apoptosis induced by doxorubicin treatment of U2OS cells was also inhibited

by mutant Ipaf (by about 50%) but not by wild-type Ipaf (Figure 7d).

A shRNA inhibits p53-induced Ipaf gene expression and apoptosis

Mutant Ipaf is likely to inhibit caspase-1 in addition to inhibiting Ipaf. To provide more clear evidence for the requirement of Ipaf gene expression in p53-induced apoptosis, shRNA was used to downregulate Ipaf gene expression. Coexpression of this shRNA by a mouse U6 promoter-based vector resulted in reduced Ipaf protein level (Figure 8a). As a control, C3G expression plasmid was cotransfected but shRNA had no inhibitory effect on C3G protein level (Figure 8a). Induction of Ipaf gene expression by p53 was reduced by coexpression of shRNA in U2OS cells (Figure 8b). Induction of p21 mRNA by p53 was not inhibited by shRNA directed to inhibit Ipaf (Figure 8b). A mutant of shRNA for Ipaf was made by substituting two bases so as to serve as an additional control. This mutant did not inhibit

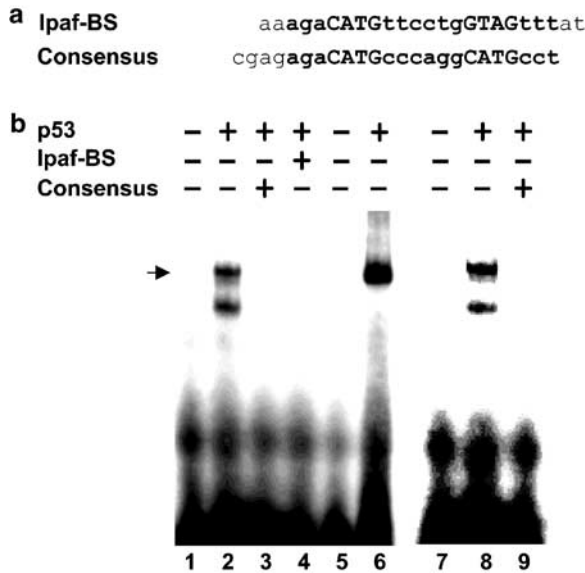


Figure 6 Electrophoretic mobility shift assay. (a) Sequence of the oligonucleotide corresponding to the p53-responsive site (*Ipaf-BS*) in the *Ipaf* promoter region. Sequence of the p53-binding consensus oligonucleotide (*Consensus*) is also shown. (b) Electrophoretic mobility shift assays were performed using radiolabeled *Ipaf-BS* oligonucleotide with recombinant purified p53 protein. Radiolabeled *Ipaf-BS* (lanes 1–4 and 7–9) or consensus oligonucleotide (lanes 5, 6) were used for binding with 200 ng (lanes 2–4, 6) or 400 ng (lanes 8, 9) of purified p53. Lanes 1, 5 and 7 are binding reactions without p53. The arrow shows the p53-specific shifted band that was competed out by 100-fold excess of unlabeled *Ipaf-BS* oligonucleotide (lane 4) and consensus oligonucleotides (lanes 3, 9)

p53-induced *Ipaf* gene expression (Figure 8c), showing sequence specificity of the inhibitory effect of shRNA for *Ipaf*. The effect of *Ipaf*-directed shRNA was determined on p53-induced apoptosis in U2OS cells. Expression of shRNA for *Ipaf* reduced p53-induced apoptosis from $25 \pm 2\%$ to $9.2 \pm 2.5\%$ (63% inhibition), whereas expression of mutant shRNA had no effect on p53-induced apoptosis (Figure 8d). p53-induced apoptosis in A549 cells was also inhibited partially by shRNA for *Ipaf* but not by the mutant shRNA (Figure 8e). The inhibition of p53-induced apoptosis by shRNA was not due to its effect on p53 protein level as determined by Western blotting (Figure 8f).

The effect of *Ipaf*-directed shRNA on DNA damage-induced apoptosis was examined. A549 cells, which are p53 positive, were transfected with shRNA or its mutant or control plasmid, and after 24 h treated with doxorubicin for 24 h. It was found that shRNA reduced doxorubicin-induced apoptosis from 34 to 19% whereas mutant shRNA had no significant effect (Figure 8g). The expression of shRNA or its mutant was monitored by coexpression with GFP.

Discussion

The results presented here show that exogenous p53 can induce *Ipaf* gene expression in U2OS and A549 cells.

Ipaf gene expression was induced by doxorubicin treatment of MCF-7 cells in a p53-dependent manner. These results suggest that *Ipaf* gene expression is induced by exogenous as well as endogenous p53. However, doxorubicin could also induce *Ipaf* gene expression in some but not all p53-negative cell lines, which suggests that p53-dependent as well as p53-independent mechanisms are involved in the induction of *Ipaf* gene expression by doxorubicin in a cell-type-specific manner. Activation of *Ipaf* promoter by normal p53 but not by His²⁷³ mutant of p53 suggests that *Ipaf* promoter activation by p53 requires sequence-specific DNA binding. We have identified a p53-responsive site in the *Ipaf* promoter, which is present very close to the transcription start site. The time course of the induction of *Ipaf* mRNA upon doxorubicin treatment of cells showed that *Ipaf* mRNA is induced early, at about the same time as p21 and Apaf-1, which are also regulated directly by p53.

A mutant of *Ipaf* lacking the essential nucleotide-binding domain was made, which is likely to inhibit the function of wild-type *Ipaf* protein (and caspase-1) by oligomerization through CARD domain. This mutant *Ipaf* inhibited p53-induced and doxorubicin-induced apoptosis. Inhibition of *Ipaf* gene expression by small inhibitory RNA also resulted in reduced apoptosis by p53 and doxorubicin. The specificity of *Ipaf*-directed shRNA is shown by the following observations: (a) shRNA but not its mutant downregulates p53-induced *Ipaf* gene expression and apoptosis, (b) shRNA had no effect on general protein synthesis because it did not inhibit the synthesis of C3G, an unrelated protein, or of p53 and (c) shRNA did not affect p53-induced transcription of p21. These observations suggest that the induction of *Ipaf* gene expression contributes in part to p53-dependent apoptosis.

Overexpression of *Ipaf* was not able to induce apoptosis in U2OS cells. This observation is consistent with the results of Poyet *et al.* (2001), who also reported that full-length *Ipaf* does not induce apoptosis in 293T cells. An activated form of *Ipaf* generated by deletion of C-terminal LRR domain has been shown to induce caspase-1 processing and enhance caspase-1-induced apoptosis (Poyet *et al.*, 2001). These observations suggest that *Ipaf* needs to be activated before it can induce caspase-1 activation and caspase-1-dependent apoptosis. We have also observed that the activated form of *Ipaf* lacking LRR domain causes caspase-1 processing and enhances caspase-1-induced apoptosis (data not shown). These observations raise the possibility that the mechanism that activates *Ipaf* (e.g. an activator of *Ipaf* or a post-translational modification) may be induced by p53 and also by doxorubicin.

How does *Ipaf* mediate p53-dependent or doxorubicin-induced apoptosis? It has been reported that *Ipaf* interacts with caspase-1 through CARD–CARD interaction and activates it (Poyet *et al.*, 2001). We have shown earlier that caspase-1 is a direct transcriptional target of p53 in human cells and blocking of caspase-1 function either by a chemical inhibitor or mutant caspase-1 results in reduced apoptosis induced by p53

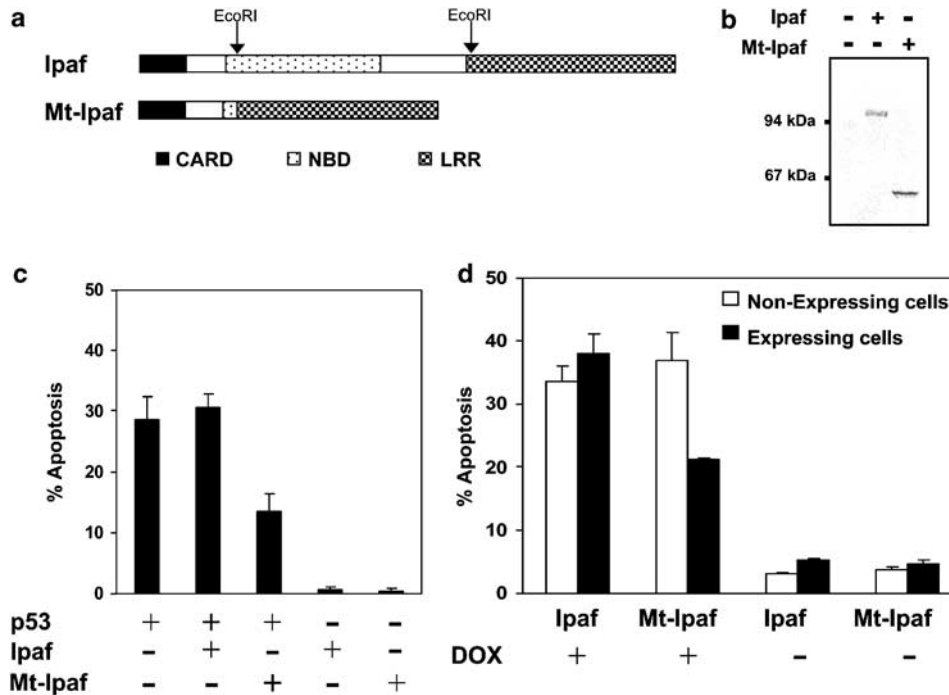


Figure 7 A dominant-negative mutant of Ipaf compromises p53 and doxorubicin-induced apoptosis. (a) Schematic showing the domains present in wild-type Ipaf and the mutant construct of Ipaf (*Mt-Ipaf*). (b) Western blot showing the expression of Ipaf and *Mt-Ipaf* in COS-1 cells. COS-1 cells were transfected with pCDNA3-T7-Ipaf and pCDNA3-T7-mutant Ipaf constructs and cell lysates were made 30 h after transfection. The expression of these proteins was detected by using T7 antibody. (c) Effect of mutant and wild-type Ipaf on p53-induced apoptosis in U2OS cells. U2OS cells grown on coverslips were transfected with indicated plasmids. After 30 h of transfection, the cells were fixed and stained for p53 for determining apoptosis in p53-expressing and nonexpressing cells. The data represent mean \pm s.d. of at least three experiments in duplicate after subtracting the background apoptosis observed in nonexpressing cells. (d) Effect of mutant and wild-type Ipaf on doxorubicin-induced apoptosis. U2OS cells were transfected with Ipaf or mutant Ipaf individually on coverslips. After 24 h of transfection, doxorubicin was added at a concentration of 500 ng/ml. The cells were fixed after 30 h of doxorubicin treatment and stained for Ipaf using the T7 antibody for determining the extent of apoptosis

or doxorubicin in MCF-7 cells and also in A549 cells (Gupta *et al.*, 2001, 2002). Endogenous levels of Ipaf are very low in most of the human tumor cell lines. Therefore, p53-mediated apoptosis appears to depend on induction of caspase-1 as well as its activator, Ipaf. Recently, it has been reported that coexpression of Ipaf and pyrin-card protein ASC (which interacts with Ipaf) induces apoptosis, which is dependent on caspase-8 but not on caspase-1 (Masumoto *et al.*, 2003). We observed that doxorubicin-induced apoptosis was not inhibited by mutant caspase-8 but was inhibited by mutant caspase-1 in U2OS cells (data not shown). Therefore, it seems likely that p53-dependent apoptosis is mediated in part by Ipaf-caspase-1 pathway in U2OS and A549 cells. It has been suggested that in stress-induced apoptosis several caspases such as caspase-1 and caspase-2 may function as the initiators for caspase activation cascade (Marsden *et al.*, 2002; Lassus *et al.*, 2002). Our results, showing that an activator of caspase-1, Ipaf contributes in part to p53-induced apoptosis, are consistent with such a scenario. However, it is also possible that p53-induced or DNA damage-induced Ipaf may have a caspase-1-independent function in apoptosis.

In conclusion our results show that Ipaf gene expression is regulated by tumor suppressor p53.

Blocking of Ipaf function by a dominant-negative mutant or by small inhibitory RNA resulted in reduced apoptosis by p53 and doxorubicin. On the basis of these observations, we suggest that Ipaf is one of the mediators of p53-dependent apoptosis at least in some human cells.

Materials and methods

Cell culture and transfections

The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin, kanamycin) at 37°C in a CO₂ incubator. The transfections with Qiagen purified plasmids were carried out using Lipofectamine PLUSTM reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Treatment of cells with 500 ng/ml doxorubicin (Sigma) was carried out in T-25 flasks for RNA isolation. MCF-7-derived cell line MCF-7-mp53 has been described previously (Gupta *et al.*, 2001).

RT-PCR

Total RNA was isolated from treated and control cells using Trizol reagent (Life Technologies, Inc.). Semiquantitative RT-PCR was carried out essentially as described previously

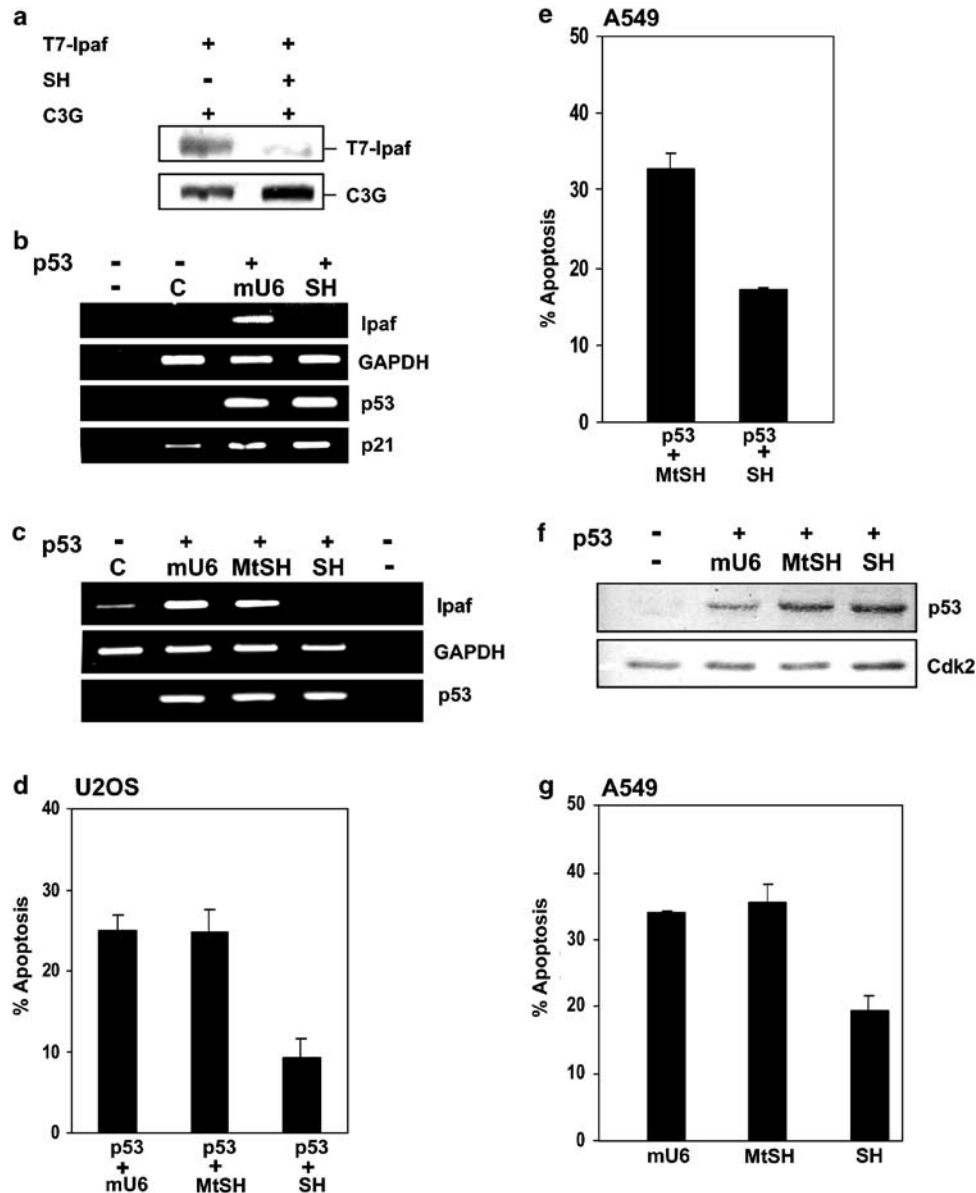


Figure 8 shRNA directed against Ipaf compromises p53-induced apoptosis. (a) shRNA for Ipaf knocks down the level of Ipaf. U2OS cells were transfected with pcDNA-T7-Ipaf along with shRNA expressing vector (SH) or control mU6-pro vector in a 1:3 ratio. pcDNA3-C3G was also cotransfected with the above-mentioned plasmids as a control for the transfection efficiency and specificity. The Ipaf was detected by Western blotting using T-7 antibody and C3G was detected using C3G antibody (from Santa Cruz). (b) shRNA reduces the expression of endogenous Ipaf induced by p53 overexpression. RT-PCR analysis of Ipaf mRNA levels in U2OS cells transfected with p53 and shRNA expression vector (SH) or control mU6-pro (mU6) in a ratio of 1:3. GAPDH, p21 and p53 were also amplified. C is control RNA from untransfected cells. (c) A mutant of shRNA (MtSH) does not inhibit p53-induced Ipaf gene expression. U2OS cells were transfected with p53 along with indicated plasmids in 1:1 ratio and RT-PCR analysis was carried out for Ipaf, GAPDH and p53. (d, e) shRNA but not its mutant compromises p53-induced apoptosis in U2OS and A549 cells. Cells were transfected with p53 along with indicated plasmids in a ratio of 1:3. The cells were fixed 30 h after transfection and stained for p53 and the extent of apoptosis was determined in p53-expressing and nonexpressing cells. (f) U2OS cells were transfected with the indicated plasmids as in (d) and after 30 h of transfection, p53 protein level was determined by Western blotting. (g) Effect of Ipaf-directed shRNA (SH) on doxorubicin-induced apoptosis. A549 cells grown on coverslips were transfected with SH, MtSH or control plasmid (mU6) along with 5 ng of GFP expression plasmid. After 24 h, the cells were treated with 500 ng/ml of doxorubicin and after another 24 h, the cells were fixed and stained with DAPI. Apoptosis was determined in GFP-expressing and nonexpressing cells. The data represent mean \pm s.d. of three experiments

(Gupta *et al.*, 2001). The primers for amplification of Ipaf (CLAN-A) were designed so that shorter isoforms (known as CLAN-B, -C and -D; Damiano *et al.*, 2001) would not be amplified. The primers used for amplifying Ipaf were 5'-CTC TCA TGG TGG AAG CCA GTCC-3' and 5'-GAC AGA

GAC TTG ACT ATG TAA TCC-3'. Primers for caspase-1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) have been described previously (Kamatkar *et al.*, 1996; Gupta *et al.*, 2001). Appropriate gene-specific primers were used for amplification of PUMA, Apaf-1, p53 and p21.

Reporter plasmids and reporter assays

The putative promoter region of Ipaf gene from -4.49 to +0.227 kb relative to the transcription start site was amplified by PCR using human genomic DNA isolated from U2OS cells as template. The primers used were forward, 5'-GGT GTA CAA GTA CAT CCA ATG CTTGC-3' and reverse, 5'-GTC ACA CAG GGC AAA CAG ATC ACC-3'. During cloning, the region upstream of -2.37kb was lost repeatedly. The reporter-construct carrying -2.37 to +0.227 kb (pCAT-P1) was made by restriction digestion and by cloning the required fragment in pCAT-BASIC vector. Other smaller promoter-reporter constructs were made by amplifying the desired region by PCR and cloning in pCAT-BASIC vector. The putative p53-responsive site was mutated by PCR-based site-directed mutagenesis. The nucleotide sequence of all the constructs was determined by using an automated DNA sequencer.

For CAT reporter assays, HeLa cells were grown in 24-well plates and transfected with 150 ng of required reporter plasmid, 150 ng (or indicated amounts) of wild-type p53 or mutant p53 (His²⁷³) and 100 ng of pCMV.SPORT.β-gal (Life Technologies, Inc.). Lysates were prepared after 24 h of transfection using reporter lysis buffer (Promega). Chloramphenicol acetyl transferase (CAT) assays and β-galactosidase assays were carried out as described previously (Gupta *et al.*, 2001). Relative CAT activities were calculated after normalizing with β-galactosidase enzyme activities.

Expression vectors

Full-length Ipaf cDNA cloned in pcDNA3 with T7-tag at the N-terminus was a kind gift from Professor ES Alnemri, Thomas Jefferson University, Philadelphia, USA (Poyet *et al.*, 2001). Mutant Ipaf was made by *EcoRI* digestion of the plasmid expressing full-length Ipaf followed by religation. This resulted in an in-frame deletion of amino acids 253 to 659. The mutant Ipaf gave a polypeptide of expected size as determined by Western blotting with T-7 tag antibody (Novagen).

Construction of vectors expressing shRNA

The shRNA expression vector was constructed using the U6 promoter-based vector essentially as described (Yu *et al.*, 2002). The desired synthetic oligonucleotides were annealed and cloned into the *BbsI*-*XbaI* digested U6 promoter plasmid, mU6-pro. The parent plasmid contains green fluorescence protein coding sequence, which is removed by *BbsI*-*XbaI* digestion. The Ipaf sequence targeted by shRNA was from nucleotide 1294-1312 (accession AY035391). The sequences of the oligonucleotides used for cloning were 5'-tttGGCTCCGAATGAAGTCACTacaAGTGACTTGATTCGGAGCCttttt-3' and 5'-ctagaaaaGGCTCCGAATCAAGT

CACTgtAGTGACTTCATTCGGAGC-3'. This vector expressed antisense, hairpin and sense sequence. For mutant shRNA construct, the oligonucleotides used for cloning were 5'-tttGGCTCCGAAACAAGTCACTacaAGTGACTTCTTTGGAGCCttttt-3' and 5'-ctagaaaaGGCTCCGAAAGAAGTCACTgtAGTGACTTGTTCGGAGC-3'. The sequence of the constructs was confirmed by automated DNA sequencing.

Apoptosis assays

Quantitative analysis of apoptotic cells was carried out essentially as described previously (Radha *et al.*, 1999; Shivakrupa *et al.*, 2003). Cells grown on coverslips were transfected with the required plasmids and fixed after 30 h (or later as indicated) and stained with required antibodies as described earlier (Kamatkar *et al.*, 1996). p53 antibody was from Roche Molecular Biochemicals. Cells were mounted in 90% glycerol containing 1 mg/ml paraphenylenediamine (antifade) and 0.5 μg/ml DAPI (4',6 diamidino-2-phenylindole dihydrochloride) to stain the DNA. Cells showing immunofluorescence staining were counted and those cells that showed loss of cell volume, loss of refractility and condensed chromatin or the presence of apoptotic bodies were scored as apoptotic. At least 200 expressing cells were counted in each coverslip. The data represent mean ± s.d. of the percentage of apoptotic cells among the total number of expressing cells from at least three independent experiments carried out in duplicate. Cells not expressing the transfected protein were also counted in each coverslip for determining the background level of apoptosis.

Electrophoretic mobility shift assay

Double-stranded oligonucleotide corresponding to the putative p53-responsive site of Ipaf (Ipaf BS; see Figure 6a) was end-labelled with polynucleotide kinase using [γ -³²P]ATP. Recombinant p53 expressed in *E. coli* was purified using FLAG resin. Binding reactions with labeled oligonucleotide and purified p53 were performed essentially as described previously (Gupta *et al.*, 2001) in 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4% glycerol, 1 mM EDTA, 2 mM MgCl₂, 40 mM NaCl, 50 ng of poly (dI-dC). Purified p53 (200 ng) was then added followed by the addition of 6 ng of labeled probe (10⁵ cpm). The reaction mix was incubated at 30°C for 30 min.

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