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ORIGINAL PAPERS

A positive feedback mechanism in the transcriptional activation of Apaf-1 by p53 and the coactivator Zac-1

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p53 exerts its tumor suppressor effects by activating genes involved in cell growth arrest and programmed cell death. The p53 target genes inducing growth arrest are well defined whereas those inducing apoptosis are not fully characterized. Proapoptotic activity of p53 was shown to involve several genes like Bax, Noxa and Puma, which may function in the release of cytochrome *c* from the mitochondria. Cytochrome *c* associates with Apaf-1 and caspase 9 to form the apoptosome. Genetic and cellular data indicate that Apaf-1 deficiency abrogates the apoptotic effect of p53 and substitutes for p53 loss in promoting tumor formation. Here we show that Apaf-1, the mammalian homologue of *C. elegans* CED4, is a direct target of p53 as demonstrated by gel shift analysis of the target site sequence in the presence of p53 and by Apaf-1 promoter-luciferase assays. We also show that the p53 activation of the Apaf-1 luciferase construct can be enhanced by the putative tumor suppressor gene product, Zac-1, a transcription factor that has previously been shown to inhibit cell proliferation. Furthermore, we demonstrate that Zac-1 is a possible direct target of p53 since the sequence upstream to the first coding exon of Zac-1 contains a p53 recognition site and the luciferase construct containing this region is activated by p53. These results suggests the existence of a tightly controlled self amplifying mechanism of transcriptional activation leading to apoptosis by p53.
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Introduction

Cell transformation and cancer result from failure of potent tumor suppression mechanisms, that prevent growth deregulation due to mutations and epigenetic events. In most cancer cases, inactivation of p53 function is an important step in the process that results in selecting mutant cells for tumor development. The tumor suppressor effect of p53 stems mainly from its transcriptional activation of genes regulating cell growth and programmed cell death (PCD) (Levine, 1997; Gottlieb and Oren, 1998; Bates and Vousden, 1999; Jimenez *et al*., 2000). While the p53 downstream genes that cause growth arrest, like $p21^{WAF}$ are well defined, the p53 effector genes involved in apoptosis are not fully identified. It is also likely that the irreversible process of apoptosis may require a more complex regulatory mechanism than that of growth arrest which is potentially reversible.

Recent experiments indicate that the proapoptotic activity of p53 mainly involves the mitochondrial pathway and is greatly dependent on the activity of Apaf-1 and caspase 9 (casp9). In response to death stimuli, cytochrome *c* is released from the mitochondria and associates with Apaf-1 and pro-casp9 to form the apoptosome which is central for the PCD pathway. This results in activation of casp9 and the subsequent proteolytic activation of casp3 followed by the triggering of apoptosis (Cecconi *et al*., 1998; Yoshida *et al*., 1998; Zou *et al*., 1999; Jiang and Wang, 2000).

Disruption of Apaf-1 overcomes the proapoptotic effect of p53 as was shown by colony growth of Apaf-1 negative cells in agar and tumor formation in mice, by Apaf-$1^{-/-}$ cells in spite of p53 expression (Soengas *et al*., 1999). The inactivation of Apaf-1 may substitute for the loss of p53 in enhancing tumor formation. It has been shown that in a majority of melanoma cases Apaf-1 is inactivated by methylation of its promoter whereas p53 is present in the wild-type conformation. These Apaf-1 negative melanomas are defective in their response to chemotherapeutic drugs. However treatment with 5aza2dc can restore Apaf-1 expression and chemosensitivity (Soengas *et al*., 2001). Similarly, leukemia cell lines K562 and CEM and the bladder cancer cell line J82 are UV resistant, due to low expression of Apaf-1 and transfection of Apaf-1 conferred sensitivity to UV driven apoptosis (Jia *et al*., 2001). We found that a high proportion of cells from CML patients at blast crisis showed marked reduction in Apaf-1 expression as compared to chronic phase CML patients (Rozenfeld-Granot unpublished data). The correlation between a low frequency of p53

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mutation and a high frequency of Apaf-1 inactivation in melanoma and other cancers suggests that Apaf-1 may be a downstream effector of p53 driven apoptosis and that this apoptosis can be obliterated in the absence of Apaf-1 expression, allowing for tumor development.

We recently used the DNA microarray technique to compare the p53-activated genes in two cell lines expressing the ts p53val135; H1299 that does not undergo apoptosis and exhibits G1 arrest and LTR6 that shows significant apoptosis at 32°C when p53Val135 is activated (Kannan *et al*., 2001a,b). In this analysis we found that in H1299 several known proapoptotic genes like Fas were upregulated. Notably the gene encoding the 'bcl2 binding component' (Accession No. U82997), later characterized as the most effective p53 target for apoptosis and renamed PUMA (Yu *et al*., 2001; Nakano and Vousden, 2001) was significantly activated as a primary target in H1299 (Kannan *et al*., 2001a). Nevertheless H1299 did not undergo apoptosis upon temperature shift to 32°C. On the other hand, the mouse cells LTR6 expressing the ts p53Val135 did undergo apoptosis and showed elevation of Apaf-1 expression on the microarray (Kannan *et al*., 2001b). This raised the possibility that Apaf-1 is a downstream target of p53 dependent apoptosis (Kannan *et al*., 2001b). While this work was in preparation Moroni *et al*. (2001) demonstrated that Apaf-1 promoter is indeed a target for p53 activation.

In this report we extend our finding that Apaf-1 is a p53 target and characterize the response of Apaf-1 promoter to p53. Using the gel shift analysis, we show that p53 binds specifically to its target site in the Apaf-1 promoter. We also show that p53 enhances luciferase activity of an Apaf-1 promoter-luciferase construct. This enhancement can be further increased 4–5-fold by the transcription factor Zac-1, which was shown previously to drive cells to growth arrest and apoptosis (Spengler *et al*., 1997; Varrault *et al*., 1998; Huang and Stallcup, 2000). In addition, the sequence upstream to the first coding exon of Zac-1 also contains a p53 target site and the luciferase construct containing this region showed increased luciferase activity due to p53 transactivation. This demonstrates a complex and delicate regulation of apoptosis by p53 through Apaf-1 transactivation.

Results

p53 activates Apaf-1 expression and binds to its target site on the Apaf-1 promoter

Previously we used DNA microarrays to analyse changes in gene expression due to p53 activation in apoptotic cells, using the murine M1 and LTR6 cells (Kannan *et al*., 2001b). Since Apaf-1 expression was elevated seven-fold in LTR6 cells after 12 h at 32°C we verified its expression by Northern blot of RNA from the two cell lines. Figure 1 shows that Apaf-1 mRNA level is elevated only in LTR6 cells that express the ts-p53, beginning 6 h after the temperature shift. Work by others have shown that Apaf-1 protein level is elevated in a p53 dependent manner 12 h after p53 induction (Robles *et al*., 2001; Moroni *et al*., 2001).

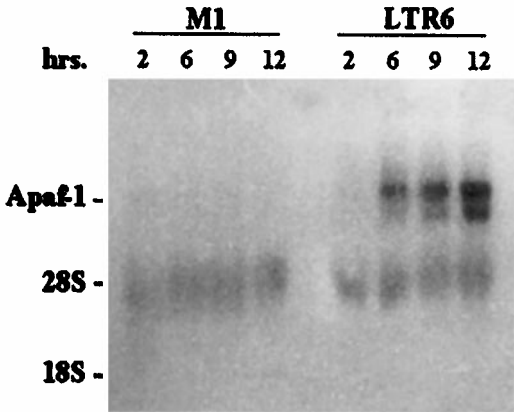

Figure 1 p53 induces Apaf-1 mRNA expression in LTR6 cells. RNA was extracted from M1 cells and LTR6 cells at different time points after temperature shift to 32°C. After separation by electrophoresis in agarose gel/formaldehyde and Northern blotting, the filter was subjected to hybridization with murine Apaf-1 cDNA (Cecconi *et al*., 1998). The increase of Apaf-1 mRNA expression over time is shown in LTR6 cells which express the ts-p53

We extended this finding by searching for a p53 target site in the Apaf-1 promoter. The promoter sequence was obtained by BLASTing Apaf-1 mRNA against the human genome sequence and searching for the sequence upstream to the mRNA start site (Accession No. AC013283). A putative p53 binding site was identified at positions −604 to −572 of the Apaf-1 promoter (Figure 2). This 33 base pair sequence AGACATGTCTGGAGACCCTAGGACGACAAGCCC consists of two conserved decamers that are homologous to the known p53 recognition site, RRRC(A/T)(A/T)GYYY (el-Deiry *et al*., 1992), separated by a 13 bp spacer. The second decamers harbors one mismatch. To demonstrate binding of wild-type p53 to this putative p53 target site, gel shift analysis was performed on double strand oligonucleotides derived from positions −607 to −569 of the Apaf-1 promoter using baculovirus recombinant murine p53. Figure 3 demonstrates that a protein-DNA complex was formed with wild-type p53 (lane 2), but not when mutant p53 was used (lane 6). In addition, an anti p53 specific antibody (pAb421) was able to supershift this protein-DNA complex (lane 3). The addition of a 50-fold excess of unlabeled p53 target site oligonucleotide reduced p53 binding to the labeled probe (lane 5), whereas addition of a 50-fold excess of unlabeled unrelated oligonucleotide had no effect on p53 binding to the labeled probe (lane 4). Deletions of the spacer (lane 7) or of half of the spacer (lane 8) did not obliterate the binding of p53 to the oligonucleotide although some reduction in binding was observed. These data demonstrate that p53 binds to the putative p53 target site of the Apaf-1 promoter and that this binding is specific. Another possible p53 target site in

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-1156  GCAGATGTGTCCCGGAATGGGGCACAGCGGACAGGAAGTAACGGTTTATA
-1106  ACAACGTGAACGCTTCTGACGTGTAGCGGGAGCTTCAGAAAGCGTGGCCG
-1056  AATCTGCAGCTCTTAACAAATGGCTCGTTCCCAAACCTTAGCCTCAACTC
-1006  CTTCTGAGCGATAGGGGCATGCTACCAGCACGGGGGGAAATGAGATACAA
 -956  GAAACGCCCGAAAGGGAAAAACAAGGCTGGGCTGTTTCCTTCCTTTGGGG
 -906  TCAAGACAGAGGCACAGAAGGCCAGGTCTGGGAGGTTGGATCACCTCCGG
 -856  CTGGATGTTGAGCCGGGTGGGAGCCCAAACAGCAGGGGGCCCACAGGAGG
 -806  CCGGCCCAGGCAGCCTCGCGTCCACTTACCAGGCCAGGCCCAGGCACGTC
 -756  CCCAGCGACAGCAGGCTCAGGCACGTTCGGGGGTCTGCCCAGCCCCCGCC
 -706  TCCGCTGCTCCGGGCCACGGGGGTCTTCCCGCCCTCGCTCCGCTTCCCGG
 -656  GCTCCGCAGCAGGGGCTCCCTTGGGCCCCGACTTCTTCCGGCTCTTCACC
 -606  TCAGACATGTCTGGAGACCCTAGGACGACAAGCCCAGGGCAGCTTCTTCA
 -556  CCAGGGGGAGCAGGACGTGGCCGCCTTGGCGTTCGTGGGAACCCTGGGCG
 -506  GTGACCGCGCCCCCTCACAGACTTGGCACCGCCCAGAGCCCAGCCCCTTC
 -456  CCTCTCCCCGGCATCCTCGTTGCTTCACTGAGTCTTTCAGCTGCCAGCTC
 -406  CATAGTTCCCCTAGGAGAGGTGGGCGGCGACCTCAACCCACAGCGCCTTC
 -356  CACTGCGATATTGCTCCAAATCCGAGGAAATTCAAACTCCCGGGCGCGCG
 -306  CAGGCCGACGGGACCCGAGGAGGAGGGGCAGGACGAAGGGGTCGCGCGCG
 -256  CCACGTCGGGCGCGCCGCCGCTGCCCGAGTCCGGCATTGGTGGGAACGCG
 -206  GCGCGTCCCTGAGGCTTAGCCACGCCCCGTCCGCGGGGTAGGCGGGCACT
 -156  TCTACGCGCGCGGGCATGAGCCGTGGCAGGAGTGCGCGGCGGCAGCGGTG
 -106  GCCGCCCCTTGGGGCTTGGGGTGTGTTTATTTGCATAAGCGGGCGCGCGC
  -56  CGTCCGGGCTGGGTGGATCCGGCGGGATTTGACTGCTCCGCTGTCCAGAG
   -6  GCGGAGAAGAAGAGGTAGCGAGTGGACGTGACTGCTCTATCC
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Figure 2 Sequence of the human Apaf-1 promoter. The nucleotides are numbered relative to the transcription start site as +1 (marked with an arrow). The consensus p53 binding site is shown in a box, the two decamers are in bold. Putative Zac-1 binding sites are underlined. Sp1 binding sites are in italics

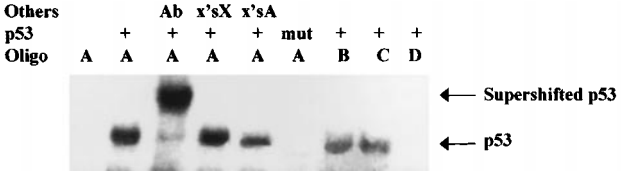

Figure 3 Wild-type p53 binds to the p53 target site of the Apaf-1 promoter. Wild-type (lanes 2–5, 7–9) and mutated (lane 6) p53 proteins were incubated with ^{32}P end labeled double strand oligonucleotides derived from the p53 binding site of the Apaf-1 promoter. (**A**) wild-type oligonucleotide, CTCAGACATGTCTGGAGACCCTAGGACGACAAGCCCAGG (lanes 1–6), (**B**) same as **A** without the spacer, CTCAGACATGTCTCGACAAGCCCAGG (lane 7), (**C**) same as **A** with half the spacer CTCAGACATGTCTGGAGACCGACAAGCCCAGG (lane 8), (**D**) same as **A** with two mutations CTCAGAaATGTCTGGAGACCCTAGGACGAaAAGCCCAGG (lane 9). The p53 target site is underlined, the mutated nucleotides are in lower case. For supershift analysis the mix was incubated with anti-p53 antibody (lane 3). For the competition analysis the mix was incubated with a 50-fold excess of unlabeled non relevant 25 bp double strand oligonucleotide (x'sX, lane 4) or unlabeled wild-type p53 site oligonucleotide A (x'sA, lane 5)

the Apaf-1 promoter is present 101 bp upstream to this target site. This site contains three mismatches, two of them are each in the central tetranucleotide (CWWG) of the decamer which is considered essential for p53 binding. Therefore we did not include this sequence in our gel shift analysis. Experiments with luciferase constructs demonstrated that this sequence is not essential for p53 activation (Figure 4d,e).

p53 and Zac-1 coactivate the Apaf-1 promoter

To evaluate the role of p53 as a regulator of Apaf-1 gene expression, U2OS or H1299 cells were cotransfected with an Apaf-1 promoter-luciferase construct and p53. Wild-type p53, but not the mutant p53, was shown to activate the Apaf-1 promoter (Figure 4a). The luciferase activation however was lower than experienced with other p53 target sites (e.g. Mdm2) and suggested that perhaps additional factors may be involved.

Recent work reported that the transcription factor Zac-1, which displays anti-proliferative properties (Spengler *et al*., 1997) may function as a coactivator of p53 (Huang *et al*., 2001). Three putative Zac-1 binding sites were identified upstream of the p53 binding site in the Apaf-1 promoter (Figure 2). To determine whether Zac-1 activates the Apaf-1 promoter and whether it enhances p53 activation of this promoter, U2OS cells or H1299 were cotransfected with an Apaf-1 promoter-luciferase construct and Zac-1 alone or in combination with p53. Zac-1 alone does not appear to have any effect on Apaf-1 promoter activity as compared to the empty vector, whereas it does significantly enhance the activation of the Apaf-1 promoter by p53 (Figure 4b,c). This enhancement by Zac-1 seems to depend on the amount of Zac-1 construct used in the transfection in relation to p53 so that a low ratio of p53 to Zac-1 is inhibitory for the coactivation. It has previously been shown that there is a narrow range of optimum p53 concentration that supports Zac-1 coactivation and that p53 overexpression inhibits the coactivation effect of Zac-1 (Huang *et al*., 2001). It is of interest that activation of the Bax promoter by p53 requires Sp1 as a coactivator (Thornborrow and Manfredi, 1999). We analysed the effect of Sp1 on the Apaf-1 promoter-luciferase construct with and without p53 and found that it had no effect on luciferase expression (data not shown).

Zac-1 has been shown to exert its transcriptional activation effects either through direct enhancement by binding to a putative Zac-1 recognition site: GGGGCCCC (Varrault *et al*., 1998) or through p53, serving as a p53 coactivator (Huang *et al*., 2001). In order to test whether the enhancement effect of Zac-1 on the Apaf-1 promoter required the putative Zac-1 recognition sites of this promoter or whether it required only p53 binding, U2OS cells were cotransfected with various Apaf-1 promoter-luciferase constructs that were deleted for the Zac-1 consensus sites or mutated for the p53 binding site (Figure 4d) together with Zac-1 alone or in combination with

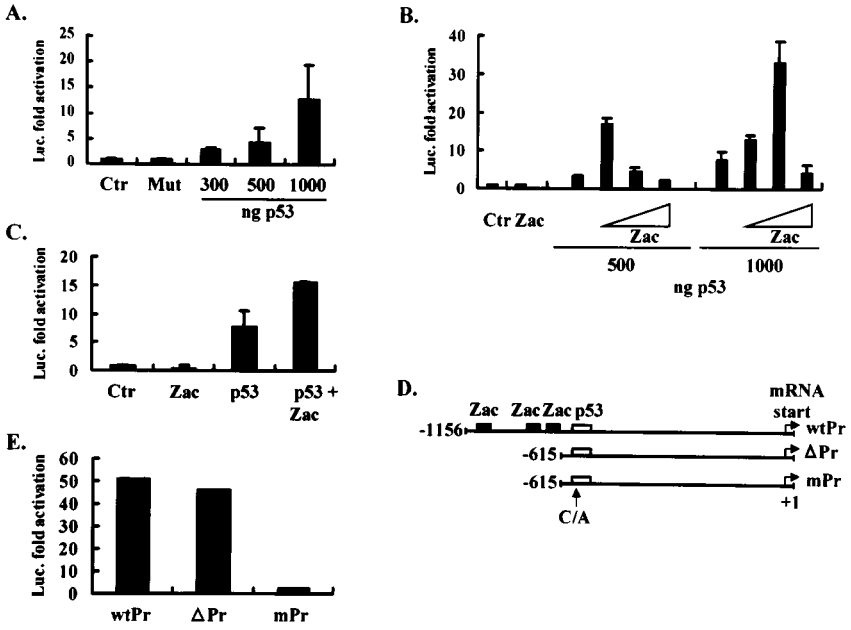

Figure 4 p53 and Zac-1 co-activate the Apaf-1 promoter. (**A**) p53 activates the Apaf-1 promoter. U2OS cells were cotransfected with 2 μg of Apaf-1 luciferase construct and either with an empty vector (ctr), mutant p53 (mut) or wild-type p53. One μg of ctr and mut vectors was used. (**B**) Zac-1 enhances the activation of the Apaf-1 promoter by p53 depending on the amount of Zac-1 construct used in the transfection in relation to p53. U2OS cells were cotransfected with 2 μg of Apaf-1 luciferase construct and either with an empty vector (ctr), or Zac-1 either alone or in combination with p53. The triangles represent transfection of 100, 300 and 500 ng of Zac-1. One μg of ctr and Zac-1 vectors was used. (**C**) H1299 cells were cotransfected with 500 ng of Apaf-1 luciferase construct alone (ctr), or either with Zac-1 (300 ng) or p53 (300 ng) or a combination of p53 (300 ng) and Zac-1 (300 ng). (**D**) a schematic presentation of the different Apaf-1 promoter constructs used for the luciferase assays. The putative p53 and Zac-1 binding sites are indicated. The mRNA start site is indicated by an arrow. The C to A mutation in the conserved CATG region of the p53 binding site in the Apaf-1 promoter is also indicated. WtPr, wild-type promoter; ΔPr, deleted promoter lacking the putative Zac-1 sites; mPr, promoter lacking the putative Zac-1 sites and harboring a mutation in the p53 binding site. (**e**) the putative Zac-1 binding sites are not required for Zac-1 mediated enhancement of Apaf-1 promoter activation by p53. U2OS cells were cotransfected with 2 μg of the indicated Apaf-1 luciferase construct, 1 μg of p53 and 300 ng of Zac-1. A–B, E, transfections were performed with the calcium phosphate technique, promoter activity was measured 48 h after transfection. C, transfection was performed with lipofectamine, promoter activity was measured 24 h after transfection. Luciferase values were normalized to the β-galactosidase values. A–C, the results are mean$\pm$s.d. of three independent transfection assays

p53. As mentioned before, Zac-1 alone had no effect on the activation of the Apaf-1 promoter-luciferase (Figure 4b,c). Consistent with this, Zac-1 and p53 coactivated the Apaf-1 promoter after deleting the Zac-1 target sites to the same extent as they activated the longer Apaf-1 promoter (Figure 4e). Furthermore, the activity of a promoter harboring a mutation in the p53 recognition site, preventing p53 binding, was also not enhanced by Zac-1 (Figure 4e). Taken together, these data indicate that Zac-1 cannot enhance expression of the Apaf-1 promoter unless p53 is present and that it functions as a coactivator of p53.

p53 and the coactivator Zac-1 form a self amplifying loop

Examination of the sequence upstream to the first coding exon of Zac-1 revealed a 20 nucleotide long p53 binding site, consisting of two conserved decamers homologous to the known p53 recognition site, RRRC(A/T)(A/T)GYYY (el-Deiry *et al*., 1992). The sequence harbors two mismatches within the first decamer: cAACTAGaCTAGACTAGCTT (the mismatches are in lowercase) (Figure 5). To demonstrate binding of wild-type p53 to this putative p53 target site, gel shift analysis was performed on double strand oligonucleotides derived from positions -587 to -562 upstream to the Zac-1 first coding exon with baculovirus recombinant p53 similar to the experiment described in Figure 3 for the p53 target site in Apaf-1 promoter. The results confirm that p53 binds to the target site in the Zac-1 gene and that this binding is specific (Figure 6). New information on the structure of the Zac-1 gene demonstrated that it contains nine exons, the first seven of them are non coding exons that are subjected to extensive alternative splicing. The first Zac-1 exon is $\sim$60 kb upstream to the first coding exon which is exon 8 (Varrault *et al*., 2001). The sequence of intron 7 upstream to exon 8 which is the first coding exon contains this p53 target site. Target sites for p53 in introns were also found in other genes like MDM2 (intron 1), GADD45 (intron 3) and IGFBP3 (intron 1).

To test whether p53 functions as a transcription regulator of Zac-1, we cotransfected U2OS and H1299 cells with p53 and a luciferase construct containing 803 bp derived from the sequence upstream to the first coding exon of Zac-1 (Figure 5). Wild-type p53, but not the mutant p53 increased Zac-1 reporter gene

-869 AGAAAAAGAAGAACCTGGTTTTTGGTGACTCTGGCTGAGTCAGAAGGATG
-819 ATAATGATTTGGGTTTGAGACAAGTTGGGTTTGAGGAGGCAGTAGGCCCA
-769 TCTCCTGGAGGTGGCCTACCAGCAGGTAGCTGGATGGGCCCTCCAGGGTA
-719 GGGCCAGGGAATGAAGAAAGGTCCATAAACAAAAAAGGAAGAGATTAGTA
-669 GAACTTACTAGAGGATGGTGCCTACAAATCTGAACGTGGCCTGGGCTGGT
-619 TCCTGAGCTATCAGCTTTGTGCCACCACATCAGTGCAACTAGACTAGACT
-569 AGCTTCATGCACATTCTGTTTATATCAGCCCTTCTGCAGAGTAATCTTAC
-519 AAACAAATTGACTTAGGTGTATACAAATTATTTCTTATCCTCTGGAAGTT
-469 TAAACAGGTGTGATAAGCAACTAATGTTATGCCAACAATGAGTGATGAGG
-419 AAGAGCCCGTGGTTTGAGTAAGTGTGGGTGATTTTTGTTTATGGACCAGT
-369 AAAAACACTTGAACACACTGAGGGAGGGAGGTTTTTGTTTGTTGTTGTTG
-319 TTGTTGTTGTTGTTGTTTTTCTCTTTGGTCAATGGAGCTTCACACTGACC
-269 TGAAATAGATAATACTGGATCCGTGGAAAGAACTCTCCTTACCAGAGGGA
-219 AAGTGTGCTCCACTGGCCATCAGTGCTTGACTCCTCACCCTCTGAGAAGC
-169 CAAAAGTGTAGACTTGTATGCAGATGGCCCTCTCACATGGAGAACTGTGT
-119 TCCATGAGCACCATTTAAAGGAACGTGTTGATTGTGGGTAGTTGATTCTG
-69 AAGCGGTCAGGGAGGAAAAGCATTGTTTTCAAATGGCTAATGCAAGAAAA
-19 CTGTTCCTCTTCTCTCCACAGCCAATCCGGGGGACCC~308nt~ATG

Figure 5 Sequence upstream to the first coding exon of Zac-1. The nucleotides are numbered relative to the start of the first coding exon (exon 8) (marked +1 and with an arrow). The consensus p53 binding site is boxed. DNA sequences that were used to make PCR primers to generate the luciferase construct (Figure 7) are underlined. The number 308 refers to the number of nucleotides to the first Methionine (**ATG**). The sequence was obtained from UCSC golden path and mRNA from Accession No. U72621

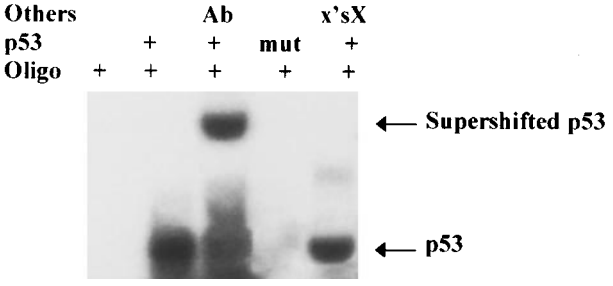

Figure 6 Wild-type p53 binds to the p53 target site in the sequence upstream to the first coding exon of Zac-1. Wild-type (lanes 2, 3, 5) and mutated (lane 4) p53 proteins were incubated with ^{32}P end labeled double strand oligonucleotides derived from the p53 binding site in the sequence upstream to the first coding exon of Zac-1. The oligonucleotide used was GTGCAACTAGACTAGACTAGCTTCAT. The p53 target site is underlined. For supershift analysis the mix was incubated with anti-p53 antibody (lane 3). For the competition analysis the mix was incubated with a 50-fold excess of unlabeled non relevant 25 bp double strand oligonucleotide (x'sX, lane 5)

activity (Figure 7). The elevation of luciferase expression by p53 on the Zac-1-luciferase construct was to a lesser extent than that of p53 on the Apaf-1 promoter construct (compare Figure 4b to Figure 7). The addition of Zac-1 to the transfection mixture had no incremental effect on the activation of the Zac-1-luciferase construct attained by p53 alone. These data suggest that p53 is a transcriptional activator of Zac-1 and that p53 and Zac-1 may operate as an amplifying

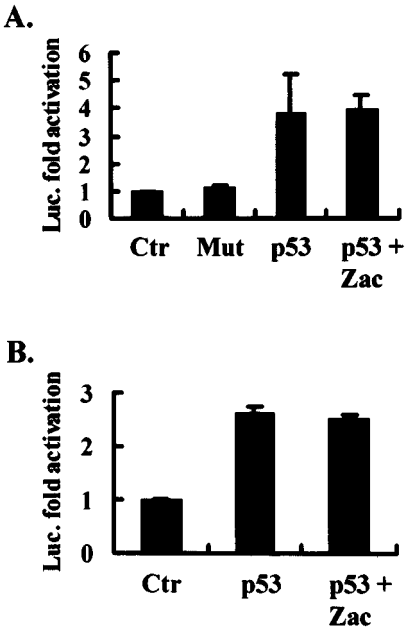

Figure 7 p53 activates the Zac-1 luciferase construct. (**A**) U2OS cells were cotransfected with 2 μg of the Zac-1 luciferase construct containing 803 bp derived from the sequence upstream to the first coding exon of Zac-1 and either with an empty vector (ctr 500 ng), mutant p53 (mut 500 ng), wild-type p53 (500 ng) or wild-type p53 with 100 ng Zac-1. Transfections were performed with the calcium phosphate technique. Luciferase activity was measured 48 h after transfection. Luciferase values were normalized to the β-galactosidase values. (**B**) H1299 cells were cotransfected with 500 ng of the Zac-1 luciferase construct alone (ctr), or either with p53 (300 ng) alone or p53 (300 ng) and Zac-1 (300 ng) together. In this experiment total DNA was balanced with pCMV to 1.6 μg. Transfection was performed with lipofectamine, promoter activity was measured 24 h after transfection. Luciferase values were normalized to the β-galactosidase values. The results are mean$\pm$s.d. of three independent transfection assays

loop where p53 activates the transcription of its own coactivator Zac-1.

Discussion

The discovery that p53 induces apoptosis (Yonish-Rouach *et al*., 1991) directed attention to PCD as a key mechanism by which p53 functions as a tumor suppressor. In the growth arrest function of p53, the major downstream factor is p21^{WAF} and deficiency of p21^{WAF} interferes with p53-driven growth arrest but not with apoptosis (Deng *et al*., 1995). On the other hand, the p53 effectors in apoptosis are not completely defined and most of them are related to the mitochondrial pathway of apoptosis. For example Bax (Miyashita and Reed, 1995; Reed, 1999) Noxa (Oda *et al*., 2000) and Puma (Yu *et al*., 2001; Nakano and Vousden, 2001) belong to the protein family which share the BH3 motif with Bcl2 (Kelekar and Thompson, 1998; Gross *et al*., 1999; Aravind *et al*., 2001) and interact with Bcl2 to inhibit its antiapoptotic effect. Hence, these proapoptotic proteins function to

induce cytochrome *c* release from the mitochondria. The binding of cytochrome *c* to Apaf-1 in the presence of dATP (Zou *et al*., 1999; Jiang and Wang, 2000) and to pro-casp9 leads to the conversion of pro-casp9 to casp9 which activates casp3 and triggers PCD. Since Apaf-1 is a low abundance protein, its transcriptional activation is essential for this process. Indeed, Apaf-1 deficiency interferes with p53-driven apoptosis as well as with sensitivity to chemotherapy, which in many cases activates p53 (Soengas *et al*., 1999, 2001).

In this report we extend our previous finding that Apaf-1 is a direct target of p53 transactivation (Kannan *et al*., 2001b) as was also shown by Moroni *et al*. (2001). We focus on the Apaf-1 promoter and show that it contains a p53 target site that binds to p53 in a specific manner. This part of the Apaf-1 promoter ($\sim$0.7 kb upstream to mRNA start) confers transcriptional activation of a luciferase reporter construct by wt p53. Furthermore, we show that p53 transactivation of Apaf-1 promoter can be enhanced by Zac-1, a transcription factor that was previously shown to inhibit cell proliferation through induction of apoptosis and G1 arrest (Spengler *et al*., 1997).

Recent work by Huang *et al*. (2001) demonstrated that Zac-1 is a coactivator of p53 using the pG13 and $p21^{WAF}$ promoter-luciferase constructs as reporters. The coactivation function of Zac-1 was found to be due to direct binding of Zac-1 to p53 (Huang *et al*., 2001). We show here that Zac-1 behaves as a coactivator of p53 in the activation of Apaf-1. Moreover, the sequence upstream to the first coding exon of Zac-1 (exon 8) itself contains a p53 consensus site that binds to p53 specifically and Zac-1 can be activated by p53 as ascertained by a luciferase assay. This may allow for the amplification of p53 function by forming a self amplifying loop of transcription factors.

These results do not imply that Zac-1 is a coactivator of p53 only on promoters of proapoptotic genes since it was also shown to enhance p53 transactivation of $p21^{WAF}$ (Huang *et al*., 2001). The coactivation of p53 as a new mechanism that may direct p53 to distinguish between promoters of growth arrest genes and proapoptotic gene is an important issue and the role of Zac-1 in such a mechanism requires further investigation *in vitro* and *in vivo*. Recent work on ASPP (a protein containing the 53BP2) demonstrated the possibility that p53 binding proteins can shift the affinity of p53 between target genes and enhance specifically the transactivating function of p53 on the promoters of proapoptotic genes, thus directing cells towards apoptosis (Samuels-Lev *et al*., 2001). Zac-1 was shown to bind to p53 and its enhancement of p53 function is due to this direct binding (Huang *et al*., 2001). The question whether Zac-1 binding can alter the target specificity of p53 deserves further investigation.

It is of interest that the activation of Bax by p53 is also enhanced by a coactivator, Sp1 (Thornborrow and Manfredi, 1999). We tested Sp1 as a coactivator of p53 on the Apaf-1 promoter and found that it had no effect, in spite of the putative Sp1 sites in the Apaf-1 promoter. The utilization of diverse coactivators for the regulation of gene expression in the apoptotic pathway implies the need for a tightly controlled regulatory mechanism of gene activation. In contrast to growth arrest, apoptosis is an irreversible process which crosses a point of no return. It therefore requires a more delicate and multistep regulation. The use of different coactivators at various stages of this process (Bax, and Apaf-1) may allow for a more accurate fine-tuning of this regulation by p53.

Zac-1 is a putative tumor suppressor gene, located on chromosome 6q24-25, a region frequently deleted in many solid tumors such as breast and ovarian cancers, astrocytomas, melanomas and renal cell carcinomas (Varrault *et al*., 1998; Bilanges *et al*., 1999). Recently Zac-1 was found to be imprinted and expressed only from the paternal chromosome (Arima *et al*., 2000). p53 is directly inactivated by mutations in approximately half of the human cancers. In many other cases it is indirectly inactivated through the binding of viral proteins or as a result of alterations in genes whose products, like HDM2 or $p14^{ARF}$, modify the p53 protein level or function. Zac-1 appears as a potential tumor suppressor gene that can also affect p53 transcriptional activity in the apoptotic process.

An attractive possibility that emerges from defining Apaf-1 as a p53 target gene, is using the Apaf-1 promoter as a target for reagents that can substitute for p53 and activate this promoter. It is possible that restoring chemosensitivity of tumor cells, by transcriptional activation of Apaf-1 through these reagents, may overcome the deficiency in p53 function characteristic of many human cancers. This may form the basis for p53-replacement therapy of cancer.

Materials and methods

Cell culture and transient transfection

U2OS and H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) and RPMI 1640 medium (Sigma, USA) respectively, supplemented with 10% serum (Gibco BRL Paisley, Scotland) 2 mM glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin (Gibco BRL Paisley, Scotland) at 37°C in a humidified incubator with 5% CO_2.

Transient transfections were performed using the calcium phosphate method or lipofectamine (Gibco BRL). U2OS cells were plated at 1.5×10^5 cells/well in a 6 well plate. Twenty-two hours after plating, the medium was replaced with 2.5 ml of fresh, supplemented DMEM, 2 h later the transfections were performed by adding calcium phosphate precipitates to the cells. Sixteen hours later the cells were glycerol shocked with 10% glycerol in 1 ml of DMEM. Twenty-four hours after the glycerol shock the cells were trypsinized, washed twice with cold PBS and lysed with reporter lysis buffer (Promega, Madison, WI, USA). Luciferase (Promega, Madison, WI, USA) and β-galactosidase expression was assayed immediately. Luciferase values were normalized with the aid of β-galactosidase values to correct variations in transfection efficiencies.

The calcium phosphate precipitates were generated by adding the appropriate amounts of plasmid DNA (total of 3 μg) to 125 μl of 260 mM $CaCl_2$. Next, an equal volume (125 μl) of HBS buffer (50 mM HEPES, 280 mM NaCl and 1.5 mM Na_2HPO4, pH adjusted to 7.05) was slowly added to the mix. The precipitates were allowed to stand at room temperature for 30 s before adding them to the cells. For H1299, 2×10^5 cells were plated in 6 well plate and transfected 24 h after plating using Lipofectamine (Life Technologies) according to manufacturer's instructions. The total amount of DNA for transfection was balanced with pCMV to 2 μg unless stated otherwise.

The cells were transfected with 0.5 μg of CMV-LacZ construct, 2 μg of the various luciferase reporter construct (pGL2-Basic vectors, Promega), 300–1000 ng of wild-type or mutated p53 pcDNA constructs, 100–500 ng of Zac-1 pcDNA construct, and 100–200 ng of Sp1 pcDNA construct.

Plasmid constructs

To create promoter luciferase constructs for Apaf-1 and Zac-1, a 1.18 kb and a 0.65 kb PCR fragments spanning part of the Apaf-1 promoter (nucleotides −1156 to 36, and −615 to 36 respectively, Figure 2) and a 0.8 kb PCR fragment spanning part of the Zac-1 promoter (Figure 5) were isolated from human placenta DNA and cloned into the *Kpn*I/*Hind*III site of the pGL2-Basic vector (Promega, USA). Site directed mutagenesis of the consensus p53 target site was generated by using a mutated primer spanning part of the p53 recognition site for the PCR reaction. This 0.65 kb PCR fragment was also cloned into the *Kpn*I/*Hind*III site of the pGL2-Basic vector. *Kpn*I/*Hind*III sites were added to all primers, at the 5′ end and 3′ respectively.

Primers for cloning

The forward primers for PCR of the Apaf-1 promoter were: (1) Primer for the 1.18 kb fragment: 5′-CGGGGTACCGCAGATGTGTCCCGGAATG; (2) Primer for the 0.65 kb fragment: 5′-CGGGGTACCCTCTTCACCTCAGACATGTC; (3) Primer for the mutant promoter: 5′-CGGGGTACCCTCTTCACCTCAGAaATGTC (the mutated site is shown in lower case). The Reverse primer common for all these reactions was: 5′-CCCCAAGCTTGGATAGAGCAGTCACGTCC. The primers for PCR of the sequence upstream to the first coding exon of Zac-1 were: (1) Forward primer: 5′-CGGGGTACCGTGACTCTGGCTGAGTCAG; (2) Reverse primer: 5′-CCCCAAGCTTCAATGCTTTTCCTCCCTGAC.

Gel mobility shift assay

Double-strand fragments were prepared from sense and antisense oligonucleotides spanning the p53 target site derived from positions −607 to −569 of the Apaf-1 promoter (Figure 2) as follows: (A) 5′-CTCAGACATGTCTGGAGACCCTAGGACGACAAGCCCAGG; (B) Like A without the spacer, 5′-CTCAGACATGTCTCGACAAGCCCAGG; (C) Like A with half spacer, 5′-CTCAGACATGTCTGGAGACCGACAAGCCCAGG; (D) Like A with two mutations, 5′-CTCAGAaATGTCTGGAGACCCTAGGACGAaAAGCCCAGG.

Double-strand fragments spanning the p53 target site in the Zac-1 gene were derived from positions −587 to −562 of the Zac-1 intron (Figure 5) as follows: 5′-GTGCAACTAGACTAGACTAGCTTCAT. The p53 target site is underlined, the mutated nucleotides are in lowercase. ^{32}P end labeled fragments (40 000 c.p.m.) were incubated with 100 ng of baculovirus recombinant murine wild-type p53 or mutant p53 (R270C) prepared in insect cells (kindly provided by Prof Moshe Oren, Weizmann Institute of Science) for 15 min on ice followed by 15 min at room temperature in a binding buffer containing 25 mM Tris-HCl (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 10% glycerol and 1.5 μg poly dIdC (Amersham Life Sciences, Cleveland, Ohio, USA). Supershift assays were performed by adding 1 μl anti-p53 antibody (pAb421) (kindly provided by Prof Moshe Oren, Weizmann Institute of Science) to the reaction. Competition assays were performed by adding a 50-fold excess of unlabeled non relevant double strand oligonucleotide or oligo A to the reaction. The samples were separated on a 5% polyacrylamide gel in 0.4× TBE buffer and visualized by autoradiography after drying.

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