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P Friedlander, Y Haupt, C Prives and M Oren
A Mutant p53 That Discriminates between p53-Responsive Genes Cannot Induce Apoptosis

P. FRIEDLANDER,1 Y. HAUPT,2 C. PRIVES,1* AND M. OREN2

Department of Biological Sciences, Columbia University, New York, New York 10027;1 and Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel2

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Human wild-type (wt) p53 can induce apoptosis in transiently transfected H1299 cells maintained at 37°C, whereas tumor-derived mutant forms of p53 (with the mutation Ala-143, His-175, or Trp-248) fail to do so. At 37°C, p53 with a mutation to Ala at amino acid 143 (p53Ala143) was transcriptionally inactive. However, at 32°C, p53Ala143 strongly activated transcription from several physiologically relevant p53-responsive promoters, to extents similar or greater than that of wt p53. Unexpectedly, p53Ala143 was defective in inducing apoptosis in H1299 cells at 32°C. Concomitantly with the loss of apoptotic activity, p53Ala143 was found to be inefficient in its ability to activate transcription from the p53-responsive portions of the Bax and insulin-like growth factor-binding protein 3 gene promoters. It is proposed that there may exist distinct classes of p53-responsive promoters, whose ability to be activated by p53 can be regulated differentially. Such differential regulation may have functional consequences for the effects of p53 on cell fate.

The number of cells in a given tissue depends upon a delicate balance between cellular proliferation, growth arrest, and apoptosis. Deregelation of this balance by activation of oncoproteins, inactivation of tumor suppressor proteins, or amplification of proteins which inhibit apoptosis can lead to the formation and progression of tumors. The p53 protein is an important player in this process (reviewed in reference 17), as about half of all human tumors produce aberrant p53 protein (41).

The levels and activity of p53 have been shown to increase in response to irradiation and other DNA-damaging agents (31, 46, 47, 57, 58). In the absence of functional wild-type (wt) p53, cells fail to arrest in G1 in response to DNA damage (53). Activation of the p53 protein can affect cell fate through the induction of either growth arrest at G1/S or G2/M cell cycle checkpoints or apoptotic cell death (reviewed in references 28 and 30).

Cell cycle arrest and apoptosis by p53 appear to be differentially regulated functions. Murine hematopoietic cell lines undergo G1 arrest in response to irradiation in the presence of interleukin 3, but when this survival factor is absent, p53-dependent apoptosis ensues (10, 26, 27). The uncoupling between growth arrest and apoptosis has also been demonstrated through the use of several mutant forms of p53 (42, 51, 65, 66). Furthermore, the apoptosis-blocking protein, bcl2, has been shown to prevent p53-mediated apoptosis without impairing p53-mediated G1/S arrest (11, 14).

Studies with p53-null mice show that p53 is necessary for thymocytes to undergo apoptosis in response to DNA damage (12, 54, 56). Induction of apoptosis in fibroblasts by overexpression of c-myc requires wt p53 protein (38, 72), p53-dependent apoptosis can modulate the cytotoxicity of chemotherapeutic agents (55) and can inhibit tumor growth and progression (70). Recently, hypoxia has been shown to induce p53-dependent apoptosis, and hypoxic conditions are believed to provide a selective pressure for p53 mutations (29).

One well-studied characteristic of p53 is its ability to function as a DNA-binding dependent transcriptional activator (reviewed in reference 71). Transactivation by p53 is dependent on specific recognition of DNA sequences containing two copies of the motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy3′. In addition, p53 is capable of repressing transcription in a manner that is independent of specific DNA recognition (24, 43, 59, 67, 69). Of central importance for growth arrest by p53 is transactivation of the waf1/p21 gene (7, 16). The product of this gene binds to and inactivates cyclin-dependent kinase complexes, exhibiting its most potent inhibitory effect on G1 cyclin-dependent kinases (18, 20, 33, 78). A number of other p53-responsive genes, including GADD45, mdr2, the cyclin G gene, and the insulin-like growth factor-binding protein 3 gene (IGF-BP3), have been identified (reviewed in reference 50a).

With regard to apoptosis, the need for transactivation by p53 appears to depend on cell type. Certain cell types undergo p53-mediated apoptosis even in the presence of transcriptional inhibitors (9). Furthermore, a truncated p53 protein (residues 1 to 214), no longer capable of binding DNA, can induce apoptosis in HeLa cells (37) and Saos-2 cells (35) but not in H1299 cells (34). However, other studies have demonstrated that p53 transactivation function is indispensable for inducing apoptosis (66, 80).

One gene that is transactivated by p53 and which might be important for p53-mediated apoptosis is the Bax gene (60). The Bax promoter contains a p53-binding element which is sufficient for transactivation by wt p53 (60). Studies with transgenic Bax-null mice show that loss of Bax can cause hypoplasia in some tissues and hyperplasia in others (50). This variable phenotype may result from Bax being a member of a family of homologous proteins, the relative roles of each member of which may fluctuate by tissue and cell type. Importantly, however, thymocytes of Bax-null mice retain a p53-dependent response to DNA damage (50). Bax is a 21-kDa protein with 43% homology to bcl2 (61). Communoprecipitation experiments show that Bax interacts with the bcl2 protein (61). Bcl2 is an integral membrane protein localized to the inner mitochondrial, endoplasmic reticulum, and nuclear membranes (15, 39, 44, 52). Overexpression of bcl2 inhibits p53-mediated apoptosis (11, 21, 66, 74) and prevents the apoptosis seen with
deregulated c-myc, which is also believed to be p53 dependent (6, 22, 72). Overexpression of Bax accelerates the rate of cell death, probably through altering the ratio of Bax to bcl2 (60, 61).

In tumors, p53 mutation is frequently manifested as deletion of one p53 allele and a missense mutation in the other allele. The point mutations almost exclusively map within the DNA binding domain of p53, and the mutant p53 proteins are defective for transactivation and consequently growth supression. One p53 mutant that has been detected in tumors contains a substitution of alanine for valine at amino acid 143. This mutant, p53Ala143, displays a temperature-sensitive phenotype, with functional sequence-specific DNA binding and transcriptional activation seen at the permissive temperature (32°C) but not at the restrictive temperature (37°C) (22a, 51, 84).

We report here that while wt p53 can induce apoptosis in transiently transfected H1299 cells at both 32 and 37°C, p53Ala143 fails to do so even at the permissive temperature. p53Ala143 does activate transcription from numerous physiologically relevant p53-responsive promoters at 32°C as efficiently as wild-type p53. Unexpectedly, however, p53Ala143 is very defective in its ability to activate transcription from a reporter construct containing the wt p53-responsive portion of the Bax promoter. This defect in Bax promoter transactivation may result, in part, from the very weak binding of p53Ala143 to the p53-binding element in this promoter. It is proposed that there may exist distinct classes of p53-responsive promoters. Qualitative and quantitative differences in the ability of p53 to regulate each class of promoters may have functional consequences for the decision of a cell to undergo apoptosis (or not) in response to p53 activation.

MATERIALS AND METHODS

Cells and transfections. H1299 cells were maintained at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Prior to transfection, cells were seeded at either 1.2 × 10^6 cells per 10-cm-diameter dish (for fluorescence-activated cell sorting [FACS] analysis) or 0.6 × 10^6 cells per 6-cm-diameter dish (for protein expression and luciferase assays). The medium was changed to Dulbecco modified Eagle medium supplemented with 10% FCS, and cells were transfected by the calcium phosphate method, with the precipitate left on cells for 6 h. Next, cells were glycine shocked for 1 min and plated in RPMI 1640 supplemented with 10% FCS. After an additional hour at 37°C, cells were either left untreated or transfected with DNA for transfection or for the isolation of cytomelagolavis (CMV)-driven plasmid DNA were used for the transfection of 10- and 6-cm-diameter dishes, respectively. In luciferase assays, 1 μg of reporter plasmid was also included. When appropriate, DNA of the parental vector pCMVneoBam was used to keep the total amount of transfected DNA constant in each sample.

For luciferase assays, cells were rinsed with cold phosphate-buffered saline (PBS), resuspended in cell lysis buffer (Promega), and incubated for 15 min at room temperature. Samples were centrifuged, and the luciferase activity was determined in the presence of luciferin (Promega) and ATP. Each transfection experiment was performed in triplicate.

For Western blot (immunoblot) analysis, H1299 cells were transfected with 1 μg of reporter construct plus 2 μg of pCMVp53wt, pCMVp53Ala143, pCMVp53HinII175, pCMVp53Tpr248, pCMVp53Ser249, or pCMVp53HinII273. The total amount of DNA in each transfection mixture was kept constant by transfection of pCMVneoBam. Twenty-four hours after incubation at either 32 or 37°C, cells were collected and lysed. Total cellular protein was quantitated by the Bio-Rad assay, and 50 μg of protein of each sample was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Transfected p53 was probed with a mixture of pAb1801 and DO-1 and visualized with the aid of the Amersham ECL system. The probes were 32P-labeled by using the Klenow fragment of E. coli DNA polymerase. The reaction mixtures contained 4 μl of 5× EMSA buffer (100 m MHEPES [pH 7.9], 125 m M KCl, 0.5 m M EDTA, 50% glycerol, 10 m M MgCl2, 1 m M of 40 m M spermidine, 1 m M of 10 m M dithiothreitol, 1 m M of 0.5% NP-40, 1 μl of double-stranded poly(dI-dC) (60 m M), 1 m M bovine serum albumin (2 m g/ml), and 3 m g of 32P-labeled probe DNA, protein samples in dialysis buffer, and H2O to 20 μl. The amounts of protein are noted in the figure legends, and reaction volumes were adjusted with dithiothreitol buffer. Mixtures were incubated for 30 min at 25°C, then loaded on a nondenaturing 4% polyacrylamide gel containing 0.5% Tris-borate-EDTA buffer, 1 m M EDTA, and 0.05% Nonidet P-40, and electrophoresed in 0.5% Tris-borate-EDTA at 4°C at 180 to 200 V (not to exceed 40-mA current) for ~2 h.

RESULTS

Transient overexpression of p53Ala143 does not induce apoptosis in H1299 cells even at the permissive temperature. As previously reported (37), certain cell types do not require the sequence-specific transactivation (SST) activity of p53 in order to undergo p53-mediated apoptosis. However, p53 SST does appear to be required for apoptosis in the p53-null cell line H1299 (34). Since p53Ala143 has been shown to bind and transactivate p53 consensus sites at 32 but not 37°C, we elected to see if it could induce apoptosis at 32°C in H1299 cells. To study the effects of increased p53Ala143 levels in these cells, expression plasmids for human wt p53 and p53Ala143 were introduced by transient transfection using the calcium phos-
at 32°C, the percentage of sub-G₁ cells approached that seen at 37°C, but by 65 h posttransfection, the rate of increase was less than that seen at 37°C. The upper left frames in Fig. 1A and B and 2A and B show the FITC intensities of individual cells as a function of cell size (forward scatter) in the total cell population of transiently transfected exponentially growing H1299 cells. By using a gate to define a region of high FITC intensity, cells expressing high levels of p53 were recorded separately (lower left frames). These cells represent the subpopulation of successfully transfected cells. The gating was set up so as to exclude unusually large cells believed to be cell doublets and multicellular clumps. In addition, very small cellular debris was also excluded through the use of an appropriate threshold parameter. Equal numbers of cells from the total culture and from the analyzed. The cell cycle distribution of the total cell population was determined by propidium iodide staining, as a measure of cell cycle distribution.

Figures 1 and 2 show the effects of transient overexpression of wt p53 and p53Ala143 at 37 and 32°C, respectively. The upper left frames in Fig. 1A and B and 2A and B show the FITC intensities of individual cells as a function of cell size (forward scatter) in the total cell population of transiently transfected exponentially growing H1299 cells. By using a gate to define a region of high FITC intensity, cells expressing high levels of p53 were recorded separately (lower left frames). These cells represent the subpopulation of successfully transfected cells. The gating was set up so as to exclude unusually large cells believed to be cell doublets and multicellular clumps. In addition, very small cellular debris was also excluded through the use of an appropriate threshold parameter. Equal numbers of cells from the total culture and from the high-level p53-expressing subpopulation were acquired and analyzed. The cell cycle distributions of the total cell population (upper right frames of Fig. 1A and B and 2A and B) and of the high-level p53-expressing cells (bottom right frames) are shown.

At 37°C, wt p53 induced a significant increase in the sub-G₁ fraction (Fig. 1A and C), which represents apoptotic cells. At 38 h posttransfection, 15% of the high-level p53-expressing cells displayed sub-G₁ DNA content; at 50 h posttransfection, this number rose to 43% (Fig. 1C).

While wt p53 induced apoptosis at 37°C, expression of p53Ala143 did not increase the fraction of sub-G₁ cells more than expression of the hot spot mutants, p53His175 and p53Trp248 (Fig. 1C). Hence, p53Ala143 exhibited no significant apoptotic activity in H1299 cells maintained at 37°C. That p53Ala143 does not induce apoptosis at the restrictive temperature (37°C) was expected, since under that condition, the mutant protein is defective for DNA binding and transactivation (84) (data not shown). It was of interest, however, to determine the apoptotic effects of p53Ala143 at the permissive temperature of 32°C. It was found that p53Ala143 expression increased the sub-G₁ population only minimally. This effect was practically indistinguishable from that seen with the typical hot spot mutants p53His175 and p53Trp248 (Fig. 2B and C). By contrast, expression of wt p53 significantly increased the percentage of cells with sub-G₁ DNA content (Fig. 2A and C). The rate of increase was less than that seen at 37°C, but by 65 h at 32°C, the percentage of sub-G₁ cells approached that seen with cultures incubated for 50 h at 37°C (compare Fig. 1C and 2C). Hence, even at the permissive temperature of 32°C, p53Ala143 cannot induce apoptosis and behaves like other mutant p53 proteins.

p53Ala143 can transactivate numerous p53 response elements but is specifically defective in transactivating Bax and IGF-BP3 promoter elements. Since overexpression of p53Ala143 did not induce apoptosis at 32°C, we looked at the ability of this mutant to transactivate p53 response elements in H1299 cells. Zhang et al. (84) reported that p53Ala143 transactivates plasmids containing either an idealized p53 consensus binding site, p53CON, or the p53 consensus site located in the ribosomal gene cluster. Neither of these sites is derived from regulatory regions of identified p53 target genes. Thus, it remained possible that p53Ala143 is defective with respect to the activation of physiologically relevant p53-responsive genes. We therefore tested the abilities of different mutant forms of p53 to activate transcription, using a reporter construct containing the luciferase gene driven by the murine mdm2 intronic promoter (3, 45). At 32°C, both wt p53 and p53Ala143, but not several hot spot mutant p53 proteins, transactivated this reporter (Fig. 3). In fact, the extent of transactivation by p53Ala143 was consistently greater than that of wt p53 (Fig. 3A and 4). In this experiment, the ratio of transfected plasmid DNA to cells was the same as that used in the apoptosis assays discussed above. In all transactivation experiments, cells were harvested 22 h after a temperature shift to 32°C in order to minimize the effect of cell loss through p53-mediated apoptosis. Western blot analysis of cells transfected with the p53-expressing plasmids revealed that the lack of transactivation by the p53 mutants was not due to decreased protein expression (data not shown; see Fig. 3B for a representative example). Indeed, the mutants were expressed at higher levels than wt p53.

To assess the generality of these observations, we compared the relative abilities of wt p53 and p53Ala143 to activate several additional authentic p53-responsive promoters in transfected H1299 cells. At 32°C, p53Ala143 activated, to the same extent as or greater extent than wt p53, the transcriptional regulatory regions of the human mdm2 gene, the human waf1/p21 gene, and the rat cyclin G gene (Fig. 4). In these experiments, the ratio of transfected plasmid DNA to cells was the same as that used in the apoptosis assays discussed above. Experiments in which a range of p53 expression plasmid DNA amounts were transfected revealed that the values shown here are plateau levels of transactivation (data not shown). Additionally, p53Ala143 and wt p53 activated to similar extents transcription of a reporter construct containing the luciferase gene driven by a minimal promoter linked to the GADD45 p53-binding element (Fig. 4).

Two striking exceptions were identified when we used constructs containing the proximal region of the p53-responsive Bax promoter, including the p53 binding sequence (60) or p53 response element (box A and box B) in the IGF-BP3 promoter (8). In this case, p53Ala143 consistently transactivated these reporters to a much lesser degree than did wt p53 (Fig. 4). Furthermore, this quantitative difference was also observed clearly when decreasing amounts of p53 expression plasmids were transfected into H1299 cells (data not shown).

One possible explanation for the discrepancy between wt p53 and p53Ala143 was that activation of Bax or IGF-BP3 requires more p53 protein, and p53Ala143 accumulates to lower levels than wt p53 and is thus present in insufficient amounts. However, Western blot analysis of cells transfected with p53 expression plasmids and a reporter construct and incubated at either 32 or 37°C revealed that the p53Ala143 protein was expressed at 32°C to the same level as wt p53 (Fig. 3B). Consequently, the relatively weak activation by p53Ala143 of the constructs containing the Bax and IGF-BP3 promoter elements was not due to limited levels of protein expression.

The p53Ser249 mutant exhibited very high levels of expression at both 37 and 32°C, while the level of wt p53 expression was also constant but significantly lower (Fig. 3B). However, significantly more p53Ala143 was expressed at 37°C than at 32°C, with the level approaching that of p53Ser249 at 37°C and...
FIG. 1. Overexpression of wt but not mutant p53 proteins induces cell death in transiently transfected H1299 cells at 37°C. Shown is flow cytometric analysis of H1299 cells transiently transfected with a CMV-driven plasmid expressing wt p53, p53Ala143, p53Trp248, or p53His175. All cells were transfected and incubated for either 38 h (C) or 50 h (A to C) at 37°C. Cells were stained for p53 and analyzed for FITC intensity (fluorescence) as a function of cell size, and patterns are shown for the total unsorted cell population (top left frames in panels A and B) and the successfully transfected, high-level p53-expressing cells (bottom left frames). A total of 2,000 events from each population were acquired. Note that p53 fluorescence is plotted on a logarithmic scale. DNA content as determined by propidium iodide staining was used to ascertain the cell cycle distribution for the total cell population (upper right frames in panels A and B) and the transfected high-level p53-expressing cells (lower right frames). Results are shown for samples incubated for 50 h at 37°C following transfection with a CMV-driven wt p53 expression plasmid (A) or p53Ala143 expression plasmid (B). (C) The percentage of cells with a sub-G1 DNA content is shown graphically for cells incubated for either 38 or 50 h at 37°C following transfection with a CMV-driven plasmid expressing wt p53, p53Ala143, p53His175, or p53Trp248.
that of wt p53 at 32°C (Fig. 3B). This result is consistent with mutant p53 proteins being more stable than wt p53 and with p53Ala143, but not p53Ser249, exhibiting a temperature-sensitive phenotype (25).

These results demonstrate that p53Ala143 is capable of transactivating numerous physiologically relevant p53-responsive promoters as efficiently as wt p53. However, p53Ala143 is specifically defective in activating other p53-responsive promoters derived from the Bax and IGF-BP3 genes. Hence, this mutant form of p53 can discriminate between different p53 target genes.


P53Ala143 binds very weakly to the Bax-binding element. Since p53Ala143 activates transcription from the Bax promoter to a significantly lesser degree than wt p53, we examined the ability of this p53 mutant to bind to the Bax p53 response element. The p53 proteins used in these assays were immunopurified from Sf21 insect cells infected with appropriate recombinant baculoviruses. (The insect cells were maintained and infected at 26°C, and extraction and purification were conducted at 4°C. Thus, both the mutant and wt proteins are not likely to be thermally inactivated at any stage of their preparation.) EMSAs were performed to determine the relative abilities of wt p53 and p53Ala143 to bind to short oligonucleotides containing the p53 binding sites found in the waf1/p21, GADD45, cyclin G gene, and Bax promoters. We observed that p53Ala143 bound more weakly to all of the oligonucleotides tested, displaying 25 to 45% of the extent of binding seen with wt p53 (Fig. 5A and B). Similar results were obtained when an oligonucleotide containing the ribosomal gene cluster (RGC) binding site was used (data not shown). This finding suggests that under the conditions of the EMSA, p53Ala143 was still partially defective for DNA binding. Strikingly, however, the wt p53 protein itself displayed a significantly lower affinity for the Bax site compared with the other oligonucleotides tested (Fig. 5A and data not shown). As seen with the other sites, the binding of p53Ala143 to the Bax site oligonucleotide was also less than that of wt p53, and thus its overall binding to this site was extremely weak. Note, however, that these data do not show that p53Ala143 is specifically defective for binding to the Bax element. Competition experiments with unlabeled wt and mutant GADD45 oligonucleotides demonstrated that the binding to the Bax fragment by p53Ala143, although very weak, was nevertheless sequence specific (data not shown).

Next we examined p53 binding to larger fragments encompassing the p53 binding sites. DNase I footprinting analysis revealed that wt p53 and p53Ala143 made identical contacts with the 132-bp fragment containing the GADD45 p53 binding site, since they displayed similar patterns of protection and hypercutting (Fig. 5C). Consistent with the less efficient binding by p53Ala143 seen by EMSA, approximately threefold more p53Ala143 than wt p53 was required for protection of the GADD45 fragment from DNase I. Also in agreement with the data obtained with short synthetic oligonucleotides, the larger GADD45 fragment was bound more effectively by wt p53 than was a 93-bp fragment containing the Bax promoter region including the p53 binding site. The slower mobility of the p53-Bax fragment complexes may be related to the possibility of unusual structural features caused by an unusually long stretch of A’s in the Bax promoter fragment (60) (see Discussion). Significantly, the binding of p53Ala143 to the Bax fragment was extremely poor (Fig. 5D and E).

**DISCUSSION**

In the study described here, transient-transfection assays were used to evaluate the relationship between p53-induced apoptosis and the ability of p53 to activate the transcription of various target promoters. Transient transfection of p53 expression plasmids can induce apoptosis in a variety of cell types (34, 36, 37, 80). This assay typically entails the delivery of the transfected plasmid DNA into the cells by the calcium phosphate method. This procedure probably imparts a strong DNA damage signal (64, 79) which may be required for allowing the transfected p53 to serve as an efficient effector of apoptosis. In support of this view, a particular p53 mutant (p53Gln22,Ser23) causes apoptosis in transiently transfected cells (37) but not in cells which express it from a stably integrated transgene (66).

As reported previously, the SST function of p53 is dispensable for the apoptotic activity of excess p53 in certain cell types (9, 72), including HeLa (37) and Saos-2 (35) cells. However, in other cell types, retention of SST appears to be tightly correlated with p53-mediated apoptosis and may therefore be obligatory for this response. This latter category includes the human lung carcinoma-derived cell line H1299 (34). It could therefore be expected that SST-competent forms of p53 will be capable of eliciting efficient apoptosis in H1299 cells.

Surprisingly, p53Ala143, which can activate transcription in a temperature-sensitive manner in transiently transfected H1299 cells, was unable to induce apoptosis in the same cells at the permissive temperature of 32°C. A similar observation was recently described with respect to K562 cells; in that case, p53Ala143 failed to induce apoptosis despite activating the waf1 gene (51). However, in our study, more extensive examination of the SST ability of p53Ala143 revealed that while it activates strongly several p53-responsive promoters at 32°C, this mutant is defective in transactivating the Bax and IGF-BP3 (box A and box B) promoter elements. This observation might also explain several earlier reports documenting the surprising failure of SST-competent p53 mutants to exhibit transformation-inhibitory activity (13, 42, 62). It now appears that at least in some common assays of p53’s tumor suppressor function, it is the apoptotic rather than growth-arresting activity of p53 which is crucial (37, 65).

The deficient transactivation of the Bax promoter by p53Ala143 may be due, at least in part, to the very low affinity of this mutant p53 for the Bax p53-binding element. Under the conditions of the EMSA, p53Ala143 exhibited a reduced interaction with all p53 target sites tested. This finding might imply that under these in vitro conditions, the conformation of p53Ala143 was still not fully identical with that of authentic wt p53. However, whereas other p53 binding sites were still bound by p53Ala143 with a fairly high affinity, the binding of this mutant to the Bax site was remarkably poor. This very weak
FIG. 2. The mutant p53 protein p53Ala143 does not induce apoptosis in transiently transfected H1299 cells at 32°C. Shown is flow cytometric analysis of H1299 cells transiently transfected with a CMV-driven plasmid expressing wt p53, p53Ala143, p53Trp248, or p53His175. All cells were transfected at 37°C and incubated for either 25 h (C), 50 h (C), or 65 h (A to C) at 32°C. Cells were stained and analyzed as for Fig. 1. Results are shown for samples incubated for 65 h at 32°C following transfection with a CMV-driven wt p53 expression plasmid (A) or p53Ala143 expression plasmid (B). (C) The percentage of cells with a sub-G1 DNA content is shown graphically for cells incubated for 25, 50, or 65 h at 32°C following transfection with a CMV-driven wt p53, p53Ala143, p53His175, or p53Trp248 expression plasmid.
binding correlates well with the very minimal, albeit detectable, activation of the Bax reporter by p53Ala143.

It is of interest that wt p53 itself revealed lower affinity for the Bax site than for sites from other p53-responsive promot-
strongly by the same amounts of p53 even without irradiation (27). Furthermore, maximal induction of endogenous Bax RNA, but not GADD45 RNA, was seen in irradiated cells under conditions in which they presumably expressed only submaximal levels of p53 (83) despite the apparently lower affinity of wtp53 to the Bax site (this report). Thus, activation of the Bax promoter by p53 may be favored in cells exposed to DNA damage, overriding the inherent low affinity of p53 for this promoter. While the mechanism of this preferential activation remains unclear, it is conceivable that p53Ala143 is defective also in some aspect of this mechanism.

FIG. 5. wtp53 and p53Ala143 interact with the p53-binding element in the Bax promoter with weaker affinity than other p53 binding sites. (A and D) Gel mobility shift assay showing abilities of wt p53 (lanes 3 to 6 and 11 to 14) and p53Ala143 (lanes 7 to 10 and 15 to 18) to bind 32P-labeled oligonucleotides (A) or fragments (D) containing the p53-binding element found in either the GADD45 promoter (lanes 1 and 3 to 10) or the Bax promoter (lanes 2 and 11 to 18). The amounts of p53 added were 0 ng (lanes 1 and 2), 40 ng (lanes 3, 7, 11, and 15), 80 ng (lanes 4, 8, 12, and 16), 160 ng (lanes 5, 9, 13, and 17), and 320 ng (lanes 6, 10, 14, and 18). (B) Gel mobility shift assay showing abilities of wt p53 (lanes 2 to 4, 8 to 10, and 14 to 16) and p53Ala143 (lanes 5 to 7, 11 to 13, and 17 to 19) to bind 32P-labeled oligonucleotides found in the GADD45 promoter (lanes 2 to 7), the cyclin G gene promoter (lanes 8 to 13), or the p21 promoter (14 to 19). Lane 1 represents a reaction with no p53 protein and using the GADD45 oligonucleotide. (C) DNase I footprinting analysis of wt p53, p53Ala143, and p53His273 proteins. A 32P-labeled fragment containing the GADD45 p53 binding site was incubated with 250 ng (lane 2), 500 ng (lane 3), or 800 ng (lane 4) of wt p53 protein and 1,500 ng (lanes 6 and 9), 2,000 ng (lanes 7 and 10), or 2,500 ng (lanes 8 and 11) of either p53His273 or p53Ala143 protein. NP, reactions to which no p53 protein was added (lanes 1, 5, and 12). (E) Graphical representation of DNA binding by wt p53 and p53Ala143 to the GADD45 and Bax fragments. DNA binding units represent the values (10⁻³) obtained by quantification of shifted bands by using ImageQuant phosphor imaging. Equivalent specific activities of the 32P-labeled GADD45 and Bax fragments, as determined by acid precipitation, were used.
The correlation between transactivation of the Bax promoter and induction of apoptosis suggests that the Bax-encoded protein may play a role in p53-mediated apoptosis. This suggestion is consistent with earlier reports (59, 60, 68, 83) and with the documented apoptotic effect of Bax overexpression (61). However, the exact relationship between physiological p53-dependent apoptosis and Bax overexpression remains unresolved. In particular, it is of note that the apoptotic death of irradiated thymocytes, which is strongly p53 dependent (12, 56), takes place with apparently normal kinetics in Bax-null (knockout) mice (50). It is therefore possible that the relative role of Bax, as well as of the other members of the Bcl2 family, will vary with cell type and specific environmental cues (reviewed in reference 76). This possibility also agrees with the variable phenotype of both hyperplasia and hypoplasia in mice (50). It is therefore possible that the relative role of Bax in the regulation of apoptosis varies with cell type and specific environmental cues. Nevertheless, the apoptotic death of irradiated thymocytes, which is strongly p53 dependent (12, 56), takes place with apparently normal kinetics in Bax-null mice (50).

Our data for the IGF-BP3 promoter elements demonstrate that the transactivation defect of p53Ala143 is clearly not limited to Bax. While Bax overexpression is linked to the apoptotic response, the role of IGF-BP3 in cell death is less well established. However, IGF-BP3 antagonizes the activity of insulin-like growth factor 1 (IGF-1), and lowered activity of IGF-1 or reduced levels of IGF-1 receptor have been shown to result in apoptosis in some cases (reviewed in reference 4a).

Indeed Bax and IGF-BP3 may be members of a subset of p53 target genes, all of which are impaired with regard to transactivation by this mutant at 32°C. Failure to activate any of these putative genes might underlie the inability of p53Ala143 to elicit apoptosis in H1299 cells. If this is the case, it is conceivable that reconstitution of normal Bax expression may not, by itself, suffice to restore full apoptotic competence to p53Ala143. It will be of great interest to identify additional p53-responsive genes which exhibit a selective deficiency in transactivation by p53Ala143; such genes may play an important role in mediating p53-dependent apoptosis, at least in cells in which the SST function is clearly required for this biological activity.

The properties of p53Ala143 are very reminiscent of those ascribed to another p53 mutant, p53Pro175. Like p53Ala143, p53Pro175 is a potent transactivator of many target promoters, yet it does not inhibit oncogene-mediated transformation (13). A recent study has revealed that p53Pro175, too, is incapable of triggering apoptosis in transiently transfected H1299 cells (65). This contrasts with its ability to act as an effective blocker of cell cycle progression, which is consistent with the efficient activation of the waf1 promoter and possibly also the promoters of other growth-related genes (65). Importantly, p53Pro175 was also recently found to be severely impaired with regard to activation of the Bax and IGF-BP3 promoters in transiently transfected cells (57a). This observation defines a class of transcriptionally competent p53 mutants which share the inability to trigger apoptosis and to induce transcription from the Bax promoter and strengthens the conviction that these two properties are related.

Finally, our data suggest the existence of discrete classes of p53-responsive genes. Although the experiments reported here and in parallel by Ludwig et al. (57a) were carried out with mutant forms of p53, it is tempting to speculate that a similar promoter discriminatory capacity is inherent to wt p53. This model would propose that wt p53 possesses a potential for the differential recognition of distinct subclasses of target genes. Dependent upon the specific environmental cues and intracellular context, p53 might interact with and activate either its entire panel of target genes or only a subset thereof. Interestingly, a temperature-sensitive p53 protein, p53Val135, induced equal amounts of waf1/p21 mRNA but very different amounts of mdm2 mRNA when introduced into different cell lines (49).

Indeed, it has been demonstrated that phosphorylation of p53 by S-phase and G1/M-phase cyclin-dependent kinases stimulates binding to only a subset of p53 response elements (75). This observation suggests the possibility that p53 structure can be regulated such that binding site selectivity is altered. Possibly mutant forms of p53 are incapable of such regulation. This differential recognition and activation may explain how stimulation of wt p53 can lead to apoptosis in certain situations and to viable growth arrest in others.

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