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Functional Domains of Wild-Type and Mutant p53 Proteins Involved in Transcriptional Regulation, Transdominant Inhibition, and Transformation Suppression

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The wild-type (wt) p53 protein has transcriptional activation functions which may be linked to its tumor suppressor activity. Many mutant p53 proteins expressed in cancers have lost the ability to function as transcriptional activators and furthermore may inhibit wt p53 function. To study the mechanisms by which mutant forms of p53 have lost their transactivation function and can act in a dominant negative manner, a structure-function analysis of both mutant and engineered truncated forms of p53 was carried out. We show that different mutant p53 proteins found in cancers vary in the ability to inhibit the transcriptional transactivation and specific DNA binding activities of wt human p53. This transdominant effect was mediated through the carboxy-terminal oligomerization region. The role of the transactivation activity in transformation suppression by wt p53 was also examined by constructing an N-terminal deletion mutant lacking the transactivation domain. This mutant was unable to transactivate but could bind specifically to DNA. Although it was impaired in its ability to suppress transformation of primary rat embryo fibroblasts by adenovirus E1A plus activated *ras*, the N-terminal deletion mutant still had some suppression activity, suggesting that additional functions of p53 may contribute to transformation suppression.

Wild-type (wt) p53 is a tumor suppressor protein which has the ability to block cell cycle progression. Mutation of the p53 gene is the most common specific genetic event detected in human cancer and has been found in many different types of human malignancy, including cancers of the colon, liver, breast, lung, brain, and bladder (19, 27). In most cases, both alleles of the normal gene for p53 are lost or mutated (2). Many of the mutated p53 genes found in cancers can act as oncogenes in that they are able to cooperate with an activated *ras* gene to transform primary rodent cells (18). The mechanisms involving negative regulation of cell growth by wt p53, the loss of this property by mutant forms of p53, and the stimulation of cell growth by some forms have not yet been elucidated. The finding that wt p53 has the properties of a sequence-specific transcription factor (14, 24, 33, 44), however, may indicate that p53 affects cellular proliferation by regulating the expression of genes that are in turn involved in cell growth control. The effect of wt p53 on cell growth has been studied in several cell culture systems. wt p53 can induce differentiation of B lymphocytes (47) and induce programmed cell death (apoptosis) in certain cell types (48, 61). Further evidence for a role of p53 in cell cycle control came from studies showing that it can induce cell cycle arrest in G_1 (6, 28, 31). The levels of wt p53 increase in response to DNA-damaging agents such as ionizing radiation, leading to G_1 arrest. As a result of high levels, p53 may transcriptionally induce the expression of GADD45 (growth arrest and DNA damage protein) by specific binding to a p53-responsive element within the GADD45 gene, resulting in the inhibition of progression from G_1 into S (22).

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wt p53 was initially shown to possess transcriptional transactivation properties as a fusion protein with the Gal4 DNA binding domain (10, 37, 40, 56). Most mutant p53 proteins found in human cancers do not have this activity (39, 56). By using fusion proteins containing the Gal4 DNA binding domain, the transactivation activity was mapped to the N terminus of p53 (56), a region which is rich in acidic amino acids and proline residues (50, 51). Other studies have shown that p53 has sequence-specific DNA binding properties (3, 14, 23), and several sequences to which it can bind have now been identified (7). One sequence was identified in the ribosomal gene cluster (23) and in the promoter of the muscle creatine kinase gene (58). wt p53 can stimulate the expression of the muscle creatine kinase gene through binding to a specific DNA sequence in the enhancer-promoter region of the gene (62). wt p53 can also activate the expression of reporter genes containing its cognate binding sites in yeast (44) and mammalian (14, 24, 33) cells. Interestingly, some mutant p53 proteins from human cancers that fail to transactivate reporter genes with p53 binding sites were transactivation competent as fusion proteins in the Gal4 system (10, 39). This apparent discrepancy can be explained by the finding that all mutant proteins tested so far are defective for specific DNA binding (3). The DNA binding domain of p53 is located in the carboxy-terminal region of the molecule (49). Bacterially expressed wt human p53 protein has weak sequence-specific DNA binding activity that can be activated by a number of factors, including phosphorylation of the C-terminal region by casein kinase II (20).

In addition to DNA binding and transcriptional transactivation, a number of other biochemical properties have been described for p53. Among these are the ability to complex with several viral and cellular proteins, homo-oligomerization, and oligomerization with mutant p53 proteins. Several viral oncoproteins, such as simian virus 40 (SV40) tumor

antigen, the 55-kDa E1B proteins of adenovirus types 2 and 5, and the E6 proteins of the high-risk human papillomaviruses (types 16 and 18), can form complexes with p53 (26, 29, 43, 59). The sequence-specific transactivation function of wt p53 is inhibited by these viral proteins (9, 33, 60), suggesting a possible mechanism by which these oncoproteins may contribute to transformation of virus-infected cells. In addition, p53 can form complexes with cellular proteins such as the *mdm-2* gene product (35). The *mdm-2* gene encodes a protein found to have transforming properties in cultured cells (11) and is frequently amplified in certain sarcomas (36). Like the viral oncoproteins, it can inhibit the transcriptional activity of p53 (35). Thus, the transactivation function of p53 appears to be functionally linked with its property as a negative regulator of cell growth.

p53 exists as an homo-oligomer (25, 34), most likely a tetramer (53), and can form heteromeric complexes with mutant p53 molecules. The oligomerization domain has been mapped to the C terminus (34). Mutant p53 proteins can complex with wt p53 when cotranslated in vitro and drive the wt p53 into a mutant conformation, as determined by immunoreactivity with conformation-specific p53 monoclonal antibodies (32, 34). A truncated p53 protein lacking the carboxy-terminal 47 amino acids (aa) was unable to drive wt p53 in a mutant conformation (34), indicating that oligomerization is necessary for this effect. Transfection studies have shown that some mutants of p53 can transform cells, presumably by inhibiting endogenous wt p53 function in a dominant negative fashion (8, 12, 31). The ability of mutant p53 proteins to disrupt the transcriptional function of wt p53 was tested initially by examining the effect of mutant p53 on the transcriptional activity of a chimeric Gal4-wt p53 fusion protein (56). Studies with p53-responsive reporter genes containing p53 binding sites have provided evidence for a dominant negative effect of some p53 mutant proteins on the transcriptional activity of wt p53 in vivo (24) and in vitro (9) and on the DNA binding activity of wt p53 in vitro (3).

Since there are differences in the biochemical and biological properties exerted by the various mutants harboring missense alterations, we have examined the effects of specific mutations on the transactivation and DNA binding properties of p53. Mutant p53 proteins were found to act in a transdominant fashion through the carboxy-terminal oligomerization region. The correlation of the transactivation and transformation suppression activities of wt p53 was examined by using an N-terminal deletion mutant lacking the transactivation domain. This mutant was somewhat but not fully impaired in its ability to suppress the transforming activity of activated *ras* and E1A in primary rat embryo fibroblasts (REF). These results support the hypothesis that the transactivation property of wt p53 is important to its suppressor activity but also suggest that the transactivation activity is not the only function involved in p53-mediated tumor suppression.

MATERIALS AND METHODS

Plasmids and plasmid constructions. All p53 cDNAs were expressed from the cytomegalovirus (CMV) immediate-early promoter. The wt p53 expression plasmid, C1N, and the codon 273 mutant (hereafter referred to as the 273 mutant) construct contain the β-globin intron as well as the first four introns of p53, while the p53 codon 248 mutant (hereafter referred to as the 248 mutant) construct contains only the human β-globin intron upstream of the p53 cDNA sequences (18). The wt and codon 179 mutant (hereafter referred to as 179 mutant) constructs, CMV-$p53_{wt}$ and CMV-$p53_{179}$, have been previously described (56). The plasmid encoding the truncated C-terminal mutant p53, CMV-$p53t_{179}$, was generated by digestion of CMV-$p53_{179}$ with *Apa*I in the polylinker, subjected to a fill-in reaction, and recut with *Stu*I enzyme to remove the C terminus of the p53 coding sequence. The resulting truncated plasmid, verified by sequence analysis, contains a stop codon 8 bases after codon 345 of p53, resulting in addition of 3 aa (Ala, Ile, and Leu). The N-terminal deletion mutant, CMV-$p53_{\Delta 43}$, was constructed by polymerase chain reaction (PCR) amplification, using wt human p53 cDNA as the template and primers with *Eco*RI sites at their 5′ ends. The sense primer (5′-ATGCGAAT TCTTGATGCTGTCCCCGGACGATATT-3′) started at aa 43 of the human p53 cDNA, and the antisense primer (5′-ATGCGAATTCGGGAACAAGAAGTGGAGAATGT CA-3′) contained the stop codon of p53 and several nucleotides of 3′ untranslated region. The PCR products were digested with *Eco*RI and ligated into the *Eco*RI site of pGEM-7 (Promega). This intermediate construct was then cleaved with *Xba*I and *Hin*dIII and recloned in these sites in the expression vector pRC/CMV (Invitrogen). The resulting gene was sequenced to ensure that no misincorporations occurred during PCR amplification. The wt human expression plasmid, C1N, and the two mutant human p53 expression plasmids, CMV-$p53_{248}$ and CMV-$p53_{273}$, were generously provided by Arnold Levine (Princeton University).

Cells and transfections. Transient transfection assays were performed in H358 cells, a human lung cancer cell line containing a homozygous deletion of p53 genomic sequences which therefore lacks p53 transcripts and protein (55). Transfections were carried out as described previously (56) except that cell extracts or nuclear extracts were prepared 36 h posttransfection. Fifty micrograms of Lipofectin (GIBCO BRL) was used in each sample when the amount of transfected DNA was 10 μg or less, and 60 μg of Lipofectin was used for more than 10 μg of transfected DNA. A β-galactosidase expression plasmid, pCMVβ (2 μg; Clontech Laboratories, Palo Alto, Calif.), was included in each DNA mixture to monitor transfection efficiency and to standardize the amounts of extract used. One-fifth of the transfected cells were pelleted and analyzed for β-galactosidase activity (41). Chloramphenicol acetyltransferase (CAT) activity (16) was measured by the conversion of ^{14}C-labeled chloramphenicol to its acetylated forms. The percent conversion was analyzed by counting the radioactive spots from the thin-layer chromatography. The exact amounts of plasmids used in each experiment are indicated in the figure legends. For each experiment, the total amount of transfected DNA was the same, adjusted by the addition of salmon sperm DNA. Transfections were repeated independently at least twice.

Primary REF were prepared from 14- to 15-day-old Fisher 344 rat embryos. Secondary or tertiary REF were plated at 5×10^5 cells per 10-cm-diameter dish. The cells were transfected by the calcium phosphate procedure (17) with 5 μg of pEJRAS (38), expressing the activated *ras* oncogene, 5 μg of the different p53 plasmids, and 10 μg of salmon sperm DNA as carrier. In the transfections in which E1A was included, 5 μg of activated *ras*, 5 μg of adenovirus type 2 pE1A (21), and 10 μg of the different p53 plasmids were used. Forty-eight hours after transfection, cells were expanded 1:3. Transformed foci were scored 10 days after transfection.

Immunoblotting. H358 cells were transfected with 6 μg of various CMV-p53 construct DNAs and 2 μg of pCMVβ, using 50 μg of Lipofectin. At 36 h posttransfection, the cells were washed twice with phosphate-buffered saline, a sample

was taken for β-galactosidase determination, and the remainder was used to prepare nuclear extracts as described elsewhere (45). About half of the nuclear extract of each sample (standardized to the level of β-galactosidase activity) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to nitrocellulose. Mouse monoclonal antibody PAb1801 (Oncogene Sciences, Mineola, N.Y.) was used to detect p53, followed by ^{125}I-labeled sheep anti-mouse antibody (Amersham).

Electrophoretic mobility shift assay (EMSA). H358 cells were transfected with Lipofectin as described above. Each transfection included 14 μg of total DNA, 2 μg of control plasmid pCMVβ, and either 6 μg of the various CMV-p53 constructs plus 6 μg of salmon sperm DNA or 6 μg of CMV-p53$_{wt}$ plus 6 μg of the different CMV-p53 mutants. Nuclear extract was prepared (45) and subjected to gel shift assay (4). About half of the nuclear extract of each sample (as judged from β-galactosidase activity) was incubated in 25 μl of binding buffer at room temperature for 30 min with 15,000 cpm of end-labeled double-stranded oligonucleotide (0.2 to 1 ng) in the presence of 1 μg of poly(dI-dC) (Boehringer Mannheim). Twenty nanograms of unlabeled oligonucleotide was used as a competitor when indicated in the figures. Protein-DNA complexes were resolved on 4% acrylamide gels (1× Tris-borate-EDTA).

RESULTS

Mutant p53 genes from human cancers vary in their dominant negative effects on the transcriptional activity of wt p53. Several mutant p53 proteins were analyzed for the ability to affect the wt p53 transactivation of a p53-responsive CAT reporter. Three mutant proteins were tested: a p53 mutant with a His-to-Gln substitution at codon 179 isolated from a human lung tumor (55); a mutant at codon 248 changing Arg to Trp found in some Li-Fraumeni syndrome patients (30); and a codon 273 mutation changing Arg to His isolated from a colon cancer (2). The transactivation properties of the three mutant proteins have been previously analyzed when the proteins were fused to the Gal4 DNA binding domain; it was found that the 179 mutant had low transactivation levels, and the 248 and 273 mutants resembled wt p53 (10, 39, 56). Each of the p53 genes analyzed in the present study was expressed from the CMV promoter (Fig. 1A). The p6FSVCAT reporter (33) contains six copies of the p53-binding element TGCCT, which is located in the human ribosomal gene cluster and in the promoter of the muscle creatine kinase gene (23, 62). H358 cells, a human non-small cell lung cancer cell line lacking an endogenous p53 gene (55), were used throughout this study. Cells were transfected with the wt p53 expression plasmid alone, with the individual mutant p53 constructs, or with a combination of the wt and mutant p53 constructs in a wt/mutant p53 DNA molar ratio of 1:3. As previously shown, expression of wt p53 transactivated the CAT reporter plasmid (33), whereas expression of each of the mutant p53 proteins did not (Fig. 1B). Cotransfection of wt p53 along with the 179 mutant resulted in nearly complete inhibition of transactivation by wt p53. Cotransfection of wt p53 plus the 248 mutant resulted in moderate inhibition, and only a slight reduction in the transactivation activity of wt p53 was detected with the 273 mutant (Fig. 1B). The diminished ability of the 248 and 273 mutants to act in a dominant negative manner is consistent with previous observations that the biochemical and biological properties of these two mutants differ from some of the other more oncogenic p53 mutants (18). It has been shown

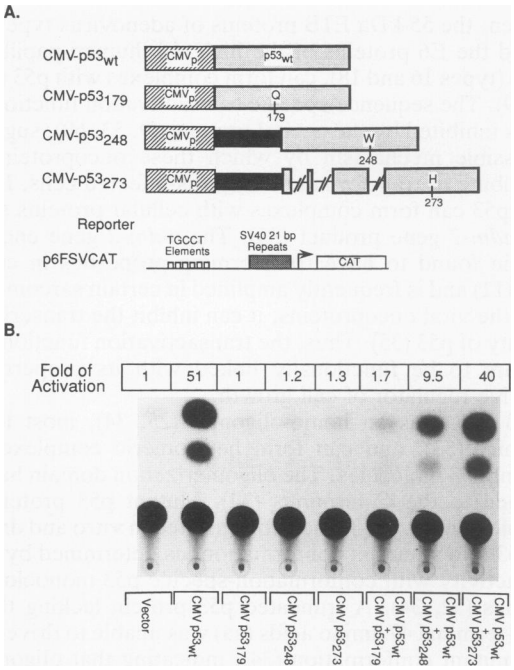

FIG. 1. Mutants of p53 differ in the ability to inhibit transcriptional transactivation by wt p53. (A) Schematic representation of p53 expression plasmids. wt and mutant p53 cDNAs (gray boxes) were expressed from the CMV promoter. The position of the amino acid substitution is indicated for each mutant. wt p53 and p53 mutants with mutations at codons 179 and 248 contain cDNA sequences; the 273 mutant contained some p53 genomic sequences which are indicated by the interrupted lines. In addition, two constructs (CMV-p53$_{248}$ and CMV-p53$_{273}$) contain the human β-globin intron sequences indicated by black boxes. The reporter plasmid (p6FSVCAT) contains six copies of the TGCCT element serving as a p53 binding site (small open boxes) upstream of the SV40 minimal promoter (hatched box) directing expression of the CAT gene (large open box). (B) Transactivation of reporter construct p6FSVCAT by wt p53 in the presence of three different mutant p53 proteins. H358 lung cells were transfected with the reporter plasmid p6FSVCAT and a wt or mutant p53 expression plasmid or with a combination of the wt p53 expression plasmid (0.5 μg) and individual p53 mutant DNAs (1.5 μg). CAT activity was assayed 48 h posttransfection. As a control, the vector pRC/CMV was transfected alone with the reporter plasmid. Activities relative to that of the vector alone are indicated above the lanes and are expressed as fold activation. A β-galactosidase expression plasmid was included in each transfection to monitor transfection efficiency.

that the 273 mutant does not adopt a mutant conformation, as determined by immunoreactivity to specific p53 monoclonal antibodies, whereas the 248 mutant has an altered conformational structure but is unable to drive wt p53 into a mutant conformation (34). Thus, not all of the naturally occurring mutants are able to fully abrogate the transactivation activity of wt p53.

p53 protein levels in transfected cells. Since abrogation of transactivation by p53 mutants is dose dependent (reference 24 and data not shown), the steady-state protein levels of the different p53 mutants were examined to determine whether the effects observed could be due to p53 protein levels. Levels of p53 were determined by Western blot (immunoblot) analysis using the monoclonal antibody 1801 (Fig. 2). Transfected wt p53 had lower levels of protein expression than did the different transfected mutants. p53 mutants at codons 179, 247, and 248 showed comparable levels about

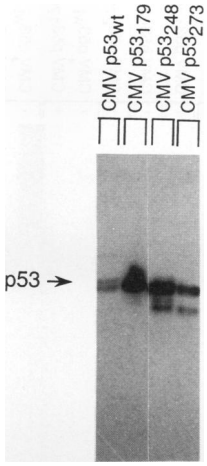

FIG. 2. Levels of wt and mutant p53 proteins. H358 cells were transfected with the indicated p53 expression constructs (6 μg of each). Nuclear extracts of cells were prepared 36 h posttransfection and subjected to immunoblot analysis using the p53 monoclonal antibody PAb1801. The band corresponding to p53 is indicated.

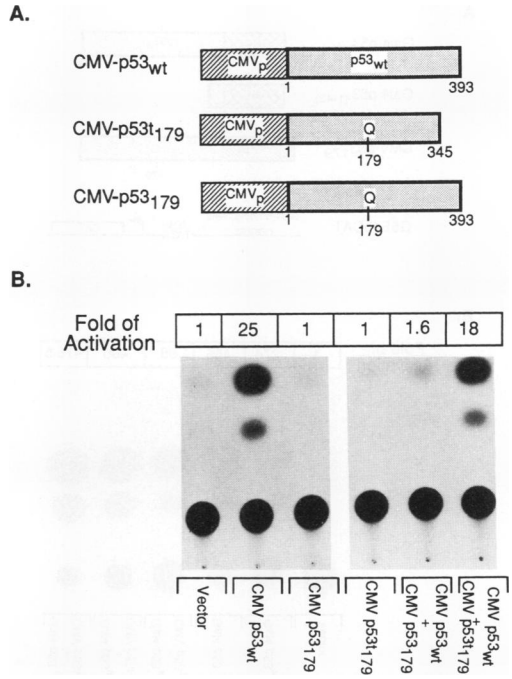

FIG. 3. Inhibition of transactivation activity by mutant p53 requires the C-terminal domain. (A) The full-length (aa 1 to 393) and truncated (aa 1 to 345) constructs used. All symbols are the same as in Fig. 1A. (B) Transactivation activity of wt p53 in the presence of the 179 or truncated 179 mutant construct. The p53 constructs used for cotransfections (0.5 μg of CMV-$p53_{wt}$, 0.5 μg of CMV-$p53_{179}$, and 5 μg of CMV$p53t_{179}$) are indicated below the lanes, and CAT activities are shown above the lanes. Activity was determined by normalizing for transfection efficiency, using a β-galactosidase expression plasmid as described for Fig. 1B.

10-fold higher than that of wt p53, whereas the 273 mutant level was closer to the wt level (2.5 times higher expression for the 273 mutant than for wt p53). However, when five times more DNA of the 273 mutant construct than of the other mutant plasmids was used, similar levels of protein expression were observed. Under these conditions, the same results were obtained with the 273 mutant in cotransfection experiments. Therefore, the differences observed above for the inhibition effect of the transactivation activity of wt p53 by the different mutants were not due to differences in protein levels but rather were due to intrinsic functional differences among the mutant proteins.

The C terminus of p53 is necessary for the transdominant inhibition of wt p53. One proposed mechanism for the abrogation of wt transactivation by some p53 mutants is that wt and mutant p53 can form a complex through the oligomerization domain, and that this heteromeric complex is functionally inactive. To test this hypothesis, we constructed a mutant p53 containing a mutation at codon 179 and lacking the carboxy-terminal 48 aa (Fig. 3A). The oligomerization domain has been mapped in vitro to this carboxy-terminal region (34). The truncated protein, $p53t_{179}$, like the full-length 179 mutant, was unable to transactivate a p53-responsive promoter. However, although transactivation by wt p53 was fully inhibited by the full-length 179 mutant, the truncated 179 mutant had no effect (Fig. 3B). This result is consistent with the model that the transdominant effect of mutant p53 proteins on wt p53 is through oligomer formation. A difference in protein levels was observed between the intact and the truncated 179 mutants, with the truncated mutant form expressing 10 times less protein than the intact mutant (data not shown). Therefore, different amounts of the intact (0.5 μg) and the truncated (5 μg) mutant expression plasmids were used for transfection to achieve equal levels of protein expression. However, even with the increased amount of transfected DNA, the C-terminal truncated 179 mutant did not inhibit wt p53 transactivation, confirming that the inhibition in the transactivation activity of wt p53 requires an intact carboxy-terminal domain, presumably by requiring heteromer formation. A possible explanation of the inhibitory effect of the 179 mutant is that heteromerization with wt p53 eliminates the sequence-specific DNA binding capacity of wt p53 (see below).

The transdominant inhibition of wt p53 transactivation function by mutant forms of p53 can involve mechanisms other than the inhibition of wt p53 DNA binding to its recognition sites, since the 179 mutant form of p53 can inhibit transactivation by a chimeric Gal4-$p53_{wt}$ protein on a Gal4-responsive promoter (56) (Fig. 4B). This inhibition also requires oligomerization between wt and mutant p53 molecules, since the C-terminal truncated form of $p53_{179}$ does not inhibit transactivation by the chimeric protein of the Gal4 reporter plasmid (Fig. 4). We further tested this by carrying out a reciprocal experiment, using a construct containing only the N-terminal transactivation domain (aa 1 to 42) of p53, $p53_{1-42}$, fused to the Gal4 DNA binding domain, and analyzed the effect of the full-length $p53_{179}$ mutant on the Gal4-responsive plasmid. As shown in Fig. 4, $p53_{179}$ had no effect on the transactivation function of Gal4-$p53_{1-42}$, confirming the observation that transdominant inhibition of the p53 transactivation function is mediated through the C-terminal oligomerization domain.

DNA binding activity of wt and mutant p53 proteins. Next we analyzed the effects of different mutant p53 proteins on the DNA binding properties of transfected wt p53. We performed an EMSA using nuclear extracts from transfected H358 cells and a ^{32}P-labeled oligonucleotide DNA probe containing a palindromic sequence of p53 binding sites (Fig. 5) (14). A specific retarded band that was competed for by a

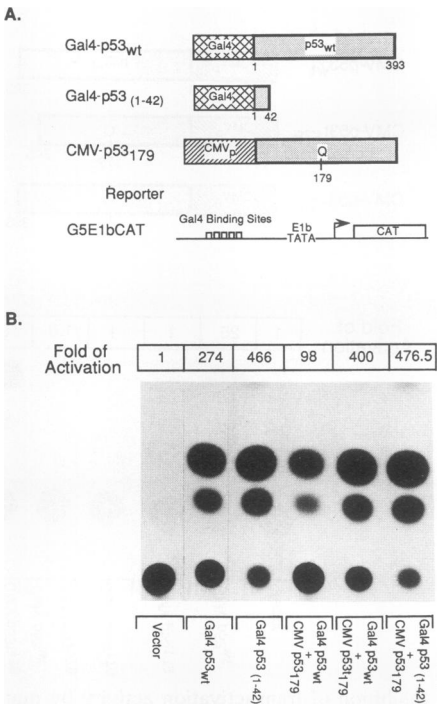

FIG. 4. Mutant p53 does not inhibit transactivation activity of a truncated p53 containing only the transactivation domain. (A) Effector and reporter constructs used. The chimeric Gal4-p53 constructs and the reporter plasmid, G5E1bCAT, have been described in detail elsewhere (56). The Gal4 DNA binding domain (aa 1 to 147; hatched boxes) was fused to the entire coding region or to the activating domain (aa 1 to 42) of wt p53. The reporter plasmid contains five Gal4 DNA binding sites (open boxes) upstream of the E1b promoter sequences and the CAT gene. (B) Effects of mutant $p53_{179}$ on the transcriptional activity of chimeric Gal4-p53 constructs. H358 cells were transfected with reporter plasmid G5E1bCAT along with the different chimeric Gal4-p53 constructs alone or in combination with mutant $p53_{179}$ or $p53t_{179}$ in a 1:3 molar ratio (0.5 and 1.5 μg, respectively), as indicated below each lane. The relative CAT activity is indicated above each lane.

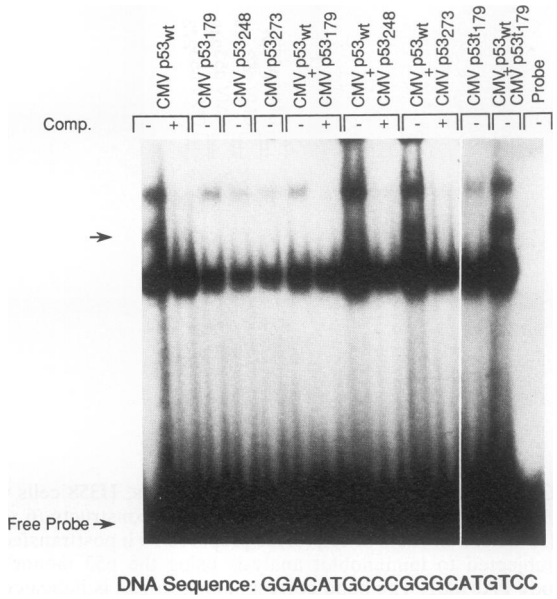

FIG. 5. DNA binding activity of transfected wt p53 in the presence of mutant p53 proteins. H358 cells were transfected with 6 μg of each of the indicated p53 expression plasmids. Nuclear extracts were subjected to gel shift analysis using the labeled oligonucleotide probe shown at the bottom. Unlabeled double-stranded oligonucleotides were used as competitors (Comp.) in 20-fold molar excess in the indicated reactions (+). The upper arrow indicates the position of the p53-specific band; the lower arrow indicates the position of unbound probe. The last lane contains the labeled probe without nuclear extract. The two other bands are not p53 dependent, since they appear in all transfected extracts. The upper of these two bands is competed for by unlabeled probe but is also detected in nuclear extracts from cells transfected with the vector alone (see Fig. 6B).

20-fold excess of unlabeled probe (Fig. 5) and was supershifted by the p53 monoclonal antibody PAb421 (data not shown) was observed with extracts from cells transfected with wt p53, whereas the 179, 248, and 273 mutants did not bind specifically to the DNA probe (Fig. 5). Transfection of the 179 mutant together with wt p53 resulted in a loss of specific DNA binding by wt p53. In contrast, weak binding by wt p53 was detected in the presence of the 248 mutant and strong binding was observed with p53 in the presence of the 273 mutant. Since the protein levels of the 273 mutant were lower than those achieved with the other mutants (Fig. 2), we cannot rule out the possibility that high levels of this mutant decrease the binding of wt p53 to DNA, although in several repeated EMSA, the binding of wt p53 was actually enhanced by the presence of low levels of this 273 mutant (unpublished results). In addition, transfection of the C-terminal truncated 179 mutant did not affect the binding of wt p53 to the DNA.

An N-terminal deletion mutant lacking the first 42 aa is not able to transactivate but is able to bind DNA specifically. The N-terminal region of p53 comprising the first 42 aa is sufficient for transactivation using the Gal4 system (56) (Fig. 4). To test whether this region is also necessary for the transactivation function of p53 on promoters containing p53 binding sequences or whether other regions of p53 could independently confer transactivation, a mutant p53 deleted of the first 43 aa was constructed ($p53_{\Delta 43}$). This mutant protein failed to transactivate with use of the reporter plasmid p6FSVCAT (Fig. 6A), confirming that the N-terminal 42 aa are necessary for transactivation by p53.

Since this N-terminal deletion mutant failed to transactivate, we tested whether it could still bind specifically to DNA. Nuclear extracts from H358 cells transfected with $p53_{\Delta 43}$ showed a stronger binding to the DNA probe by EMSA than did extracts from cells transfected with wt p53 (Fig. 6B). The effect of $p53_{\Delta 43}$ on wt p53 transactivation was then analyzed. Cotransfection of $p53_{\Delta 43}$ with wt p53 resulted in inhibition of transactivation by wt p53 (Fig. 6A). This inhibition could result from competition by the deleted p53 protein for the p53 binding sites, since it can bind DNA but not transactivate.

The protein levels of $p53_{\Delta 43}$ were determined by Western blot analysis using the p53 monoclonal antibody 1801. This truncated mutant was expressed at 40 times the level of wt p53 and at 5 times the level of the 179 mutant (Fig. 6C). This higher level of expression achieved with $p53_{\Delta 43}$ resulted in the stronger binding by $p53_{\Delta 43}$ than by wt p53 seen in the EMSA analysis (Fig. 6B). When the extracts were adjusted for p53 protein concentration, no difference in binding affinity could be observed between $p53_{\Delta 43}$ and wt p53 (data not shown). A likely explanation for the inhibition of wt p53 transactivation by $p53_{\Delta 43}$ is therefore the efficient competi-

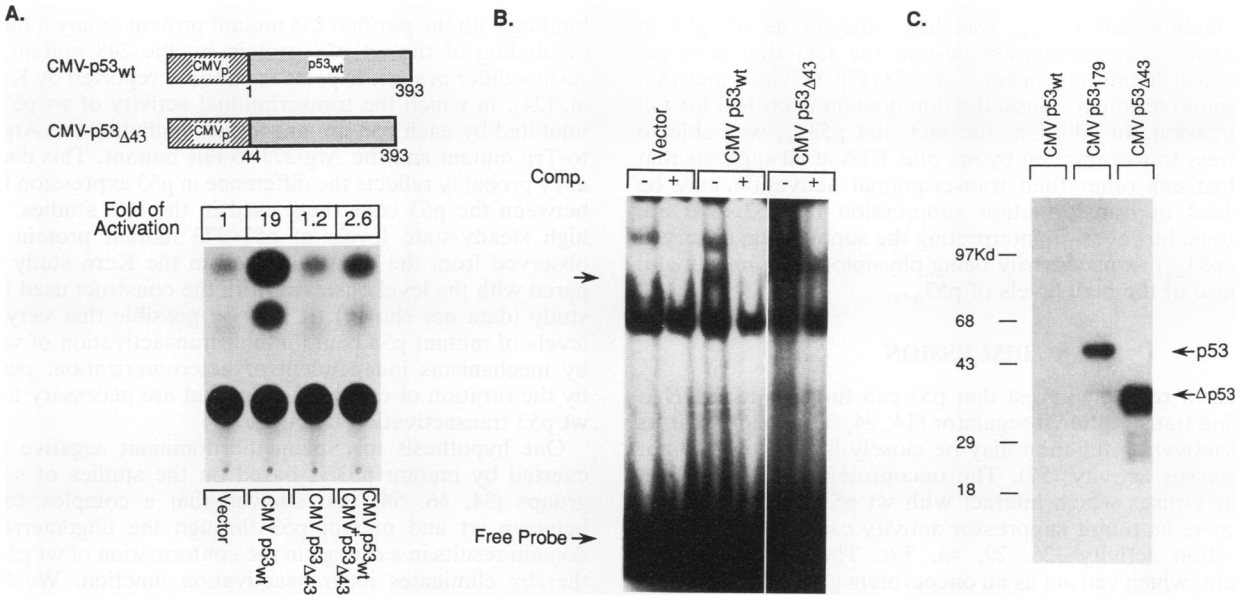

FIG. 6. A deleted p53 protein lacking the N-terminal 43 aa is transactivation defective but can bind specifically to DNA. (A) $p53_{\Delta 43}$ is unable to transactivate reporter plasmid p6FSVCAT. H358 cells were transfected with $p53_{\Delta 43}$ alone or in combination with wt p53, using an excess of deletion mutant over wt p53 (3:1). The p53 constructs shown schematically at the top are indicated below the lanes. The relative CAT activity is indicated above each lane. (B) DNA binding activity of the N-terminally deleted mutant, $p53_{\Delta 43}$. Shown is gel shift analysis of nuclear extract from H358 cells transfected with $p53_{\Delta 43}$ or wt p53 and assayed for binding to an oligonucleotide probe as described in the legend to Fig. 5. Transfection of the vector pRC/CMV alone was used as a control for nonspecific bands with the probe. Comp., competitor; upper arrow, position of the p53 shifted band; lower arrow, position of the unbound probe. Nonradioactive DNA probe was added to the indicated reactions (+). (C) Protein levels of transfected $p53_{\Delta 43}$. Immunoblot analysis of nuclear extracts from transfected H358 cells with p53 constructs (6 μg of each), using p53 monoclonal antibody PAb1801. Protein markers are shown at the left, and the p53-specific bands are indicated at the right.

tion for the p53 DNA binding sites by the $p53_{\Delta 43}$ which is in large molar excess. Interestingly, the high steady-state level of protein seen with $p53_{\Delta 43}$ may suggest that the N-terminal region contains sequences involved in determining p53 protein stability.

$p53_{\Delta 43}$ is impaired in its transformation suppression activity. Since the transactivation function of p53 is thought to be important for its tumor suppressor activity, we next tested whether the N-terminally deleted p53 protein had the ability to suppress transformation. The activity of the N-terminally deleted protein was compared with those of wt p53 and the 179 mutant protein. We cotransfected primary REF with the p53 expression plasmids along with either the combination of a mutant *ras* gene and adenovirus E1A or *ras* alone. The results of six independent transfection experiments are shown in Table 1. As expected, *ras* alone or *ras* plus wt p53 (C1N) resulted in very few foci. In agreement with previous studies (18), wt p53 reduced the number of foci observed with *ras* plus E1A by about 80%. Typical of some human p53 mutants, the 179 mutant resulted in a small number of foci in the presence of *ras* and could further enhance transformation of *ras* plus E1A. In contrast, $p53_{\Delta 43}$ did not form foci when transfected with *ras* alone and did not enhance transformation of *ras* plus E1A as did some other human p53 mutants. Therefore, elimination of the transactivation function of wt p53 is not sufficient to generate a transforming form of p53. $p53_{\Delta 43}$ was able to suppress transformation by *ras* plus E1A, from 30 to 70% depending on the experiment.

TABLE 1. Effect of N-terminal deletion construct $p53_{\Delta 43}$ on *ras*-plus-E1A transformation of REF

Transfected construct(s)	No. of foci/plate[a]							Ratio[b]
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Avg	
ras	0.3	0.0	0.5	0.0	0.0	0.2	0.2	0.0
ras + wt p53[c]	—[d]	0.0	0.0	0.2	0.2	0.3	0.1	0.0
ras + CMV-$p53_{179}$	4.8	0.0	0.6	1.0	0.2	0.2	1.1	0.1
ras + $p53_{\Delta 43}$	—	0.0	0.6	0.3	0.2	0.0	0.2	0.0
ras + E1A	9.6	8.2	20.8	20.3	19.2	12.8	15.2	1.0
ras + E1A + wt p53	3.0	1.0	2.2	4.2	1.3	3.5	2.5	0.2
ras + E1A + CMV-$p53_{179}$	16.5	9.0	24.0	31.5	14.8	16.3	18.7	1.2
ras + E1A + $p53_{\Delta 43}$	6.8	2.2	9.3	6.2	6.5	3.7	5.8	0.4

[a] Six plates were tested per experiment.
[b] Ratio of average number of foci to number of *ras*-plus-E1A foci.
[c] Construct C1N was used as the wt p53 expression plasmid (see Materials and Methods).
[d] —, contaminated.

The finding that $p53_{\Delta 43}$ was less efficient as wt p53 in suppressing transformation despite the fact that it is expressed at 40 times the level of wt p53 (Fig. 6C) indicates that the amino-terminal transactivation domain is critical for full suppression. In addition, the fact that $p53_{\Delta 43}$ was able to suppress transformation by *ras* plus E1A at all suggests that mechanisms other than transcriptional activation may be involved in transformation suppression by p53. We are cautious, however, in interpreting the suppression observed with $p53_{\Delta 43}$ as necessarily being physiologically meaningful because of the high levels of $p53_{\Delta 43}$.

DISCUSSION

Recent reports suggest that p53 can function as a DNA-binding transcriptional regulator (14, 24, 33, 44) and that its transactivation function may be closely linked to its tumor suppressor activity (57). The oncoproteins of some DNA tumor viruses which interact with wt p53 and presumably eliminate its tumor suppressor activity can inhibit its transactivation activity (26, 29, 43, 59). The cellular mdm-2 protein, which can act as an oncoprotein (11), also binds p53 and inhibits its transactivation function (35). In addition, the mutant forms of p53 isolated from cancers have been shown to act in a dominant negative manner to induce transformation (8, 12, 18) and to inhibit the transactivation activity of wt p53 (9, 24, 56).

Since different p53 mutant proteins vary in their biochemical and biological properties (18), we analyzed the effects of the 179, 248, and 273 mutants on wt p53 activity in transactivation and DNA binding assays. Whereas the 179 mutant abrogated the transcriptional activity of wt p53 by eliminating the binding of wt p53 to its responsive DNA sequence, the 248 and 273 mutants had only moderate and slight inhibitory effects, respectively, on the transcriptional and DNA binding activities of wt p53 (Fig. 1 and 5). The 248 and 273 mutants have properties shared with wt p53, in that they exhibit transcriptional transactivation of Gal4-dependent promoters when fused to a Gal4 DNA binding domain (10, 39), and unlike other mutant p53 proteins, they do not detectably interact with the heat shock protein Hsc70 (13, 18). The 248 mutant is found in Li-Fraumeni syndrome patients harboring an inherited p53 mutation. Patients with this syndrome have one wt p53 allele in addition to the mutant allele and are at significant risk for the development of tumors by the age of 30 (30, 52). In these patients, the 248 mutant does not act in a dominant negative manner since tumors do not form until the wt allele is lost. The 273 mutant with an Arg-to-His mutation is frequently mutated in a variety of tumors; however, its ability to cooperate with *ras* in transformation assays using primary REF is very low (18).

Although the dominant negative effect of some mutant p53s over wt p53 can be observed in cotransfection experiments, the role of this phenomenon in carcinogenesis is not yet clear. In most tumors harboring p53 mutations, either the two alleles for p53 are mutated or, as is more often the case, one allele is lost and the other is mutated. If the mutated forms of p53 were truly transdominant, one would expect to find a higher percentage of tumors that contain both a wt and a mutant allele. However, it seems possible that the transdominance could be important at an earlier stage of carcinogenesis and that the loss of the normal allele represents a later step in carcinogenic progression.

The results observed for the 248 mutant are in partial agreement with the in vitro DNA binding activity study by Bargonetti et al. (3). They did not detect DNA-specific binding with the purified 248 mutant protein or any inhibition of binding of the wt p53 protein by the 248 mutant. Our results differ in some aspects from those reported by Kern et al. (24), in which the transcriptional activity of wt p53 was inhibited by each p53 mutant tested, including the Arg-248-to-Trp mutant and the Arg-273-to-His mutant. This discrepancy probably reflects the difference in p53 expression levels between the p53 constructs used in the two studies. Very high steady-state levels of p53 273 mutant protein were observed from the construct used in the Kern study compared with the level observed with the construct used in our study (data not shown). It may be possible that very high levels of mutant p53 could inhibit transactivation of wt p53 by mechanisms independent of heteromerization, perhaps by the titration of cellular factors that are necessary for the wt p53 transactivation function.

One hypothesis to explain the dominant negative effect exerted by mutant p53 is based on the studies of several groups (34, 46, 54) that showed that a complex formed between wt and mutant p53 through the oligomerization domain results in a change in the conformation of wt p53 and thereby eliminates its transactivation function. We tested this hypothesis by using a truncated derivative of mutant $p53_{179}$ ($p53t_{179}$) lacking the carboxy-terminal 48 aa and in a reciprocal experiment by using the N-terminal 42 aa transactivation domain of wt p53 fused to Gal4. In both cases, no inhibition of the transcriptional activity of wt p53 by mutant $p53_{179}$ was detected, indicating that an intact oligomerization domain is required in both the wt p53 protein and the mutant p53 protein for transdominant inhibition of the transactivation function (Fig. 3 and 4). Two recent studies have mapped the oligomerization domain to a precise region in the C-terminal region of p53. A region from aa 302 to 360 of mouse p53 was sufficient for the formation of stable p53 oligomers (46), and in human p53, the minimal domain for oligomerization has been mapped to residues 334 to 356 (54). Thus, mutant p53 proteins deleted of their last 48 aa will not be able to oligomerize with wild-type p53.

We tested the possibility that regions other than the N-terminal 42 aa have transactivation capacity. A deletion mutant lacking the N-terminal 43 aa failed to transactivate a p53-responsive reporter gene (Fig. 6A). This result indicates that the N-terminal region containing the first 42 aa is necessary for the transactivation activity observed by wt p53. Furthermore, this deletion is unlikely to have caused a significant alteration in the functional conformation of the protein, since it can still specifically bind to DNA (Fig. 6B), and it is capable of complex formation with HPV16 E6 and SV40 large T (data not shown).

The fact that $p53_{\Delta 43}$ does not act like the other mutant forms of p53 found in human cancers and can still bind specifically to DNA indicates that deletion of transcriptional activation function is not sufficient to generate a cancer-associated mutant form of p53, even though it can inhibit wt p53 transactivation. Therefore, the mutations in the conserved regions of p53 observed in cancers must result in some additional change in the molecule.

Mutations of p53 isolated from human tumors have been generally found in evolutionarily conserved regions in the central portion of the p53 molecule. Mutations are not generally found either in the N terminus (transactivation domain) or in the C terminus (oligomerization domain) of the molecule. Assuming that the transcriptional and DNA binding activities are both necessary for the tumor-suppressive activity of wt p53, mutations in those regions would be predicted to alter these functions. The absence of mutations

within those regions may suggest that these domains are necessary for additional functions of mutant p53 proteins. The fact that mutations in these regions are not selected for in tumors is consistent with the notion that mutant forms of p53 may have a gain-of-function activity (27) which requires the functional integrity of the N- and C-terminal domains. Alternatively, mutations of the N- and C-terminal domains could result in an increased stability of the p53 protein that may not be tolerated by the cell.

One surprising result from this study was the intermediate level of transformation suppression observed with the $p53_{\Delta 43}$ mutant. On the basis of the notion that the transactivation property of wt p53 is linked to its tumor suppressor function, we did not expect to observe any suppression with this mutant. Why then does $p53_{\Delta 43}$ have any transformation suppression activity? If the observed suppression is not due to nonspecific cell toxicity from the high level of $p53_{\Delta 43}$ expression, then transformation suppression by wt p53 may involve another region of p53 in addition to its transactivation domain. The possible physiological significance of the lower number of transformants observed with $p53_{\Delta 43}$ will, however, require additional study.

Our analysis has focused primarily on the transcriptional transactivation function of wt p53 mediated by its specific binding to DNA. However, wt p53 can also down-regulate the transcription of several promoters lacking a p53-responsive element, presumably by direct interaction with the TATA-binding protein or the CCAAT-binding factor (1, 5, 15, 42). It will be of interest to test whether $p53_{\Delta 43}$, which lacks the ability to transactivate adjacent genes although it can bind specifically to DNA, has the ability to down-regulate the transcription of regulatory genes by interacting with other transcription factors.

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