p53 domains: identification and characterization of two autonomous DNA-binding regions

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We have investigated the DNA-binding, oligomerization, and *trans*-activation functions of isolated segments of murine p53. We find that p53 has two autonomous DNA-binding regions. One domain, from amino acid 280 to 390, forms stable tetramers and binds DNA nonspecifically. The biological significance, if any, of this DNA-binding activity is not known. A second domain, from amino acid 80 to 290, does not form stable tetramers under stringent conditions but binds DNA both specifically and nonspecifically. The specific DNA-binding function of p53, therefore, resides in the highly conserved central region of the protein and does not require stable tetramerization. Amino acids 1–290, which include both the specific DNA-binding domain and the amino-terminal acidic region, activate a p53-specific promoter *in vivo*. This finding strongly argues that the DNA-binding activity of p53 segment 80–290 is physiologically significant. The role of tetramerization in p53 function remains to be determined.

[Key Words: p53; domains; tetramers; DNA binding; *trans*-activation]

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The p53 gene suppresses cell proliferation (Eliyahu et al. 1989; Finlay et al. 1989; Hinds et al. 1989), and the loss of p53 function is associated with a broad spectrum of human cancers (Hollstein et al. 1991; Levine et al. 1991). Although the mechanism of action of p53 is not well understood, many studies have implicated p53 in the regulation of transcription. The protein has an acidic amino-terminal region and binds to specific DNA sequences (Bargonetti et al. 1991; Kern et al. 1991; El-Deiry et al. 1992; Funk et al. 1992). When a DNA-binding site for p53 is placed upstream from a basal promoter, p53 strongly activates transcription of a variety of reporter genes *in vivo* and *in vitro* (Farmer et al. 1992; Funk et al. 1992; Kern et al. 1992). p53 also activates transcription of several natural promoters (Weintraub et al. 1991; Kastan et al. 1992; Zambetti et al. 1992; Barak et al. 1993; Wu et al. 1993). Paradoxically, p53 represses the transcription *in vivo* of a number of genes that have no apparent p53 binding sites (Ginsberg et al. 1991; Mercer et al. 1991; Santhanam et al. 1991; Shiio et al. 1992). A number of investigators have shown that p53 interacts with TBP (Seto et al. 1992; Liu et al. 1993; Mack et al. 1993; Ragimov et al. 1993; Truant et al. 1993), the TATA-binding protein that initiates the formation of transcriptional complexes at many promoters. The binding of p53 to TBP may activate or repress tran-

scription depending on the context of the promoter (Seto et al. 1992). p53 has also been shown to form tetramers (Milner et al. 1991; Stenger et al. 1992; Sturzbecher et al. 1992; Friedman et al. 1993), and several investigators have suggested that oligomerization may contribute to p53–DNA interactions (El-Deiry et al. 1992; Funk et al. 1992).

A number of mutant forms of p53 interfere with the suppressor function of endogenous wild-type p53 and cause tumors. These mutations cluster in hot spots in the center of the polypeptide chain (Hollstein et al. 1991; Levine et al. 1991) and overlap segments of the protein that are highly conserved among a wide variety of species (Soussi et al. 1990). The position of the oncogenic mutations probably reflects a selective pressure in tumors to inactivate functions crucial to the negative regulation of cell proliferation. This idea is consistent with the finding that most transforming mutations severely reduce site-specific DNA binding (Bargonetti et al. 1991; Kern et al. 1991; El-Deiry et al. 1992) and *trans*-activation (Raycroft et al. 1990; Raycroft et al. 1991; Farmer et al. 1992; Kern et al. 1992; Unger et al. 1992; Zambetti et al. 1992). Aside from the identification of transforming mutations, mutational analysis of p53 has been limited. The first 73 amino acids are highly acidic and contribute to the activation of transcription (Fields and Jang 1990; Raycroft et al. 1990). The carboxy-terminal region is moderately basic, and mutations in this region reduce nonspecific DNA binding and oligomerization (Foord et

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al. 1991; Milner et al. 1991; Sturzbecher et al. 1992). Furthermore, Sturzbecher et al. (1992) have shown that carboxy-terminal segments of p53 can oligomerize.

Recently, we investigated the suppression, transformation, and *trans*-activation functions of isolated segments of wild-type murine p53 (Reed et al. 1993). Intact p53 suppressed cellular transformation by the activated Ras and adenovirus E1A proteins, but deletion of the amino-terminal *trans*-activation domain blocked suppression. Nevertheless, the *trans*-activating domain of herpesvirus protein VP16 was able to substitute for the amino-terminal *trans*-activating domain of p53 in cellular suppression. Thus, unless the interchanged p53 and VP16 acidic segments share additional functions, *trans*-activation is required for suppression by p53. Consistent with the studies of Shaulian et al. (1992), we also found that small carboxy-terminal p53 segments containing amino acids 320–360 enhanced transformation by Ras and E1A, whereas no p53 segments lacking amino acids 320–360 transformed cells. An overlapping region has been associated with the oligomerization of p53 (Milner et al. 1991; Sturzbecher et al. 1992). Intact p53 and chimeric VP16–p53 *trans*-activated the CAT gene under control of a p53-specific promoter, whereas transforming segments of p53 interfered with *trans*-activation by wild-type p53. These findings argue strongly that *trans*-activation by p53 is required for cellular suppression and that any non-*trans*-activating p53 that retains the capacity to oligomerize with wild-type p53 would have transformation potential. In the present study, we have investigated the oligomerization, DNA-binding, and *trans*-activation functions of p53 segments. We find that p53 has two autonomous DNA-binding regions, one that is both specific and nonspecific, and the other that is apparently nonspecific. The specific DNA-binding domain does not form stable tetramers but supports the *trans*-activation function of p53 *in vivo*.

Results

Purification of p53 segments

We wanted to identify autonomous DNA-binding domains of p53 to develop a better understanding of p53 suppression of cellular proliferation. We used an existing collection of p53 segments that was previously used to investigate the suppression and transformation functions of p53 (Fig. 1A). The p53 segments are identified by their amino acid components, which are numbered according to Pennica et al. (1984). In previous studies, where the relatively weak Moloney sarcoma virus (MSV) promoter was used for p53 expression in animal cells (Reed et al. 1993), segments 1–320 and 80–320 neither suppressed nor enhanced transformation significantly while segments 80–390, 280–390, 315–390, and 315–360 enhanced transformation. We moved these p53 segments and an additional segment, 1–360, into baculovirus expression vectors to overproduce the p53 segments with small amino-terminal tags containing 6 histidine residues for purification by metal-affinity chromatography. Figure 1B shows the purified protein fragments after SDS-gel electrophoresis and Coomassie blue staining. Although the proline- and histidine-rich amino-terminal tag slows the migration of all segments, the p53 segments have the expected relative gel migrations. The smaller segments are resolved into two species; only the upper species is phosphorylated (data not shown).

Phosphorylation of p53 made in insect cells

We determined that wild-type, untagged p53 made in insect cells is phosphorylated in a pattern similar to that described previously for p53 made in animal cells. Figure 2 shows a two-dimensional tryptic peptide map of purified wild-type p53 radiolabeled in baculovirus-infected cells. The labeled peptides closely resemble peptides of

Figure 1. Segments of p53. (A) Map of segments relative to conserved regions of p53. The solid lines represent p53 segments expressed by different plasmids. (B) p53 segments purified from insect cells infected with recombinant baculoviruses as described in Materials and methods. (Lane M) The positions of molecular mass standards.

Figure 2. Peptide map of wild-type murine p53 made in insect cells. Purified protein labeled with ^{32}P was digested with trypsin and analyzed in two dimensions as described in Materials and methods. Phosphopeptides are identified.

murine p53 made in animal cells; the phosphopeptides have been identified on the basis of the predicted behavior of the appropriate peptides during electrophoresis and chromatography (Meek and Eckhart 1990). Because the amino-terminal peptides have multiple phosphorylation sites and a complex pattern of phosphorylation, we have not identified individual amino-terminal phosphopeptides. We conclude, however, that p53 made in insect cells is phosphorylated in a pattern similar to that seen in animal cells.

Oligomerization of purified p53 segments

Previously, we used chemical cross-linking coupled with SDS-gradient gel electrophoresis to show that purified wild-type p53 forms tetrameric structures (Stenger et al. 1992). Figure 3 shows that the p53 segment from amino acids 80–320 forms only monomers under these experimental conditions regardless of the concentration of glutaraldehyde used to cross-link the preparations. The migration of segment 80–320 during SDS-gel electrophoresis increases with increasing glutaraldehyde concentrations. This finding confirms that the cross-linking reaction was successful and resulted in a more compact intramolecular structure. Furthermore, the absence of oligomers of segment 80–320 shows that the cross-linking conditions did not lead to artifactual intermolecular cross-linking under these conditions. In the absence of glutaraldehyde, the 280–390 segment electrophoresed as a monomer in the SDS-gel. At concentrations of 0.001–0.01% glutaraldehyde, p53 tetramers were only partially cross-linked and, therefore, partially dissociated during SDS-gel electrophoresis. Monomers, dimers, trimers, and tetramers were evident under these conditions and had the expected molecular masses (molecular mass

markers not shown). At higher glutaraldehyde concentrations, preformed tetramers of segment 280–390 were fully cross-linked and were not dissociated by the SDS-gel buffer at all. Under these conditions, p53 segment 280–390 formed tetramers and only tetramers. The complete absence of other oligomeric forms confirms that the glutaraldehyde did not cause artifactual intermolecular cross-linking. We conclude that p53 segment 80–320 does not form stable tetramers under the same conditions that favor the efficient tetramerization of segment 280–390. Our findings do not exclude the possibility that p53 segment 80–320 can form oligomers under less stringent conditions or in the presence of DNA.

DNA binding by wild-type p53

We examined DNA binding by wild-type p53 using a mobility retardation assay. We used a similar specific DNA substrate and the same binding conditions as those described by Hupp et al. (1992). However, we used phosphorylated murine p53 made in insect cells, whereas Hupp et al. (1992) used unphosphorylated human p53 made in bacterial cells. In addition, our proteins were purified without denaturation. We also used a small double-stranded DNA that contains no known p53 recognition sequences as a probe and as a nonspecific competitor. When used as a nonspecific competitor, this DNA had characteristics similar to those of poly[d(A-T)] and poly[d(I-C)] (data not shown). Because Hupp et al. (1992) showed that pAb421 enhances the DNA binding of wild-type p53, we added the monoclonal antibody to selected samples.

Figure 4 demonstrates the specificity of our assay. In Figure 4A we added a 25M excess of p53 to the p53 recognition sequence 5'-AGACATGCCTAGACATGCCT-3' within a labeled, 28-bp synthetic DNA. In the absence of pAb421 and competitor DNA, wild-type p53 retarded

Figure 3. Analysis of the quaternary structures of p53 segments 80–320 and 280–390. Purified proteins were examined by SDS-gradient gel electrophoresis without cross-linking and after partial or complete cross-linking at the glutaraldehyde concentrations shown. The positions of monomers, dimers, trimers, and tetramers were determined by molecular mass standards (not shown).

Figure 4. DNA binding by purified wild-type p53 made in insect cells. Protein–DNA complexes were identified by the mobility retardation assay described in Materials and methods. (A) The labeled probe is specific DNA. (B) The labeled probe is nonspecific DNA. p53 (100 ng) was incubated with labeled probe (2 ng) in the presence or absence of nonspecific and specific competing DNA. The amount of competing DNA is indicated. pAb421 was added to selected samples as indicated.

the migration of the labeled specific probe to multiple positions in the gel. These complexes were only partially competed by 10- to 90-fold excesses of nonspecific double-stranded DNA but were efficiently competed by specific DNA. In the presence of pAb421, p53 retarded the labeled probe more efficiently and caused a supershift to higher positions in the gel. DNA binding was not significantly competed by nonspecific DNA but was inhibited almost completely by specific DNA. p53 binding, therefore, is specific in the absence of pAb421, but the specificity is enhanced in the presence of pAb421. In Figure 4B we used a small, double-stranded DNA containing no p53 recognition sequence as the labeled probe for DNA binding. Wild-type p53 retarded migration of the nonspecific DNA to multiple positions in the gel, and pAb421 caused a supershift in the gel and enhanced binding as it did in the case of specific DNA. Binding to the nonspecific probe, however, was inhibited significantly by nonspecific DNA in either the absence or presence of pAb421. As expected, the specific DNA was somewhat more effective as a competitor.

We conclude that intact wild-type p53 binds DNA in multiple species, that binding is both specific and nonspecific, and that pAb421 enhances binding. These conclusions are consistent with and extend the findings of Hupp et al. (1992), who used nonspecific labeled DNA rather than serial competition studies as a control for specificity.

DNA binding by p53 segments

We used the mobility retardation assay to determine the specific and nonspecific DNA-binding characteristics of p53 segments (Fig. 5). For the sake of consistency, we

initially added pAb421 to all binding reactions even though most of the p53 segments have no corresponding epitope. The specific and nonspecific DNAs were the same synthetic DNAs used in the experiments described above (Fig. 1). Wild-type p53 bound DNA specifically, as shown in Figure 4. Segment 80–390, containing the pAb421 epitope, bound DNA with the same characteristics as wild-type p53. Specific, but not nonspecific competitor, inhibited binding to the specific labeled probe. Segment 280–390, which has the pAb421 epitope, failed to bind to the specific DNA fragment in the presence of pAb421. Segments consisting of amino acids 315–390 and 315–360 also failed to bind DNA (data not shown). Segment 1–360, which has no pAb421 epitope, bound the specific probe in multiple species, and the binding was inhibited much more efficiently by specific than by nonspecific competitor. Surprisingly, segments 1–320 and 80–320, which lack both a pAb421 epitope and a carboxy-terminal tetramerization domain, bound DNA specifically to the same extent as wild-type p53 did at this protein concentration. Additional assays will be required to compare the binding affinities of the proteins. We conclude that the large conserved central region of p53 is sufficient for specific DNA binding in the absence of stable tetramerization.

There were qualitative differences in the patterns of gel retardation by wild-type p53 and various p53 segments. In contrast to the wild-type protein (Figs. 4 and 5) and segment 1–360 (Fig. 5), the 1–320 and 80–320 segments failed to bind DNA as multiple species. Surprisingly, segment 80–320 retarded DNA to a position similar to that of the lowest species of DNA bound to segment 1–360 even though the p53 segments are significantly different in mass. These findings could

Figure 5. DNA binding by purified wild-type p53 and segments of p53. Protein–DNA complexes were identified by the mobility retardation assay described in Materials and methods. The labeled probe is specific DNA. p53 (100 ng) was incubated with labeled probe (2 ng) in the presence or absence of nonspecific and specific competing DNA. The amount of competing specific or nonspecific DNA is indicated. pAb421 was added to all samples.

mean that four subunits of segment 80–320 bind the p53 recognition sequence. Alternatively, charge and shape differences in segment 80–320 could slow the electrophoretic migration of single subunits of segment 80–320 bound to DNA. Clearly, the absence of the acidic and proline-rich amino-terminal region would cause significant differences in the charge and possibly the shape of segment 80–320 relative to segment 1–360.

We repeated selected assays in the presence and absence of pAb421 (Fig. 6). The binding of wild-type p53 was specific in the absence of pAb421 and was enhanced by pAb421. These results reproduce those shown in Figures 4 and 5. As expected, pAb421 had no effect on the binding of p53 segments 1–360 or 80–320, which lack the appropriate epitope. Competition analysis indicated that binding by these two fragments was highly specific. In the absence of pAb421, segments 280–390 and 315–390 bound DNA. In contrast to binding by wild-type p53, pAb421 almost completely blocked the DNA binding of segments 280–390 and 315–390. The fact that both non-specific and specific competitors inhibited binding to similar extents in the absence of pAb421 indicates that binding by the carboxy-terminal fragments was nonspe-

cific. Segment 315–360 failed to bind DNA in either the absence or the presence of pAb421. We conclude that amino acids 315–390 have an autonomous, nonspecific, DNA-binding domain that can be blocked by the monoclonal antibody pAb421.

Fine mapping of the specific DNA-binding domain

We identified the amino- and carboxy-terminal boundaries of the DNA-binding domain by progressively shortening the p53 segment from either end (Fig. 7). We overexpressed the segments in bacteria and purified them using metal-affinity chromatography. After confirming that segment 80–320 made in bacterial and insect cells has the same DNA-binding characteristics (data not shown), we determined the DNA-binding efficiency of the truncated polypeptides with the mobility retardation assay. Figure 7 shows our results, where selected truncation mutants smaller than segment 80–320 were used. The p53 segment from amino acid 80 to 320 served as a positive control for p53-specific DNA binding in the absence of pAb421. It bound the specific DNA probe and retarded its migration to a single position in the gel.

Figure 6. The effects of pAb421 on the DNA-binding properties of p53 and segments of p53. Protein–DNA complexes were identified by the mobility retardation assay described in Materials and methods. pAb421 was added to selected samples as indicated. The labeled probe is specific DNA. p53 (100 ng) was incubated with labeled probe (2 ng) in the presence or absence of nonspecific and specific competing DNA. The amount of competing specific or nonspecific DNA is indicated.

Binding was not significantly inhibited by a 30-fold excess of nonspecific competitor but was completely inhibited by the same concentration of specific competitor. p53 segment 80–290 behaved in an identical way except that it retarded migration of the specific probe to a lower position in the gel. In contrast, p53 segments 80–260 and 110–320 failed to bind DNA under these conditions. Smaller segments (140–320, 160–320, 80–230, 110–290, and 140–260) also failed to bind DNA at these protein concentrations (data not shown). We conclude that the minimal, specific DNA-binding domain of p53 is included within segment 80–290 but not in the smaller segments that we tested. This autonomous DNA-binding region contains the entire highly conserved central region of p53.

Trans-activation by p53 domains

We investigated *trans*-activation by wild-type p53 and segments of p53 to determine how the *trans*-activation function correlates with the DNA-binding functions (Fig. 8A). We used the chloramphenicol acetyl transferase (CAT) gene under the control of a p53-specific promoter as a reporter for *trans*-activation. Kern et al. (1992) have shown that 13 DNA recognition sequences for p53 upstream from a TATA sequence act as a strong promoter. We used a reporter plasmid with two copies of this segment upstream from a TATA sequence and the CAT gene. We cotransfected plasmids that express wild-type p53 or segments of p53 and the reporter plasmid into the H358 cell line that is null for p53 function (Funk et al. 1992). Transfection of the control plasmid pCMH6K did not induce the *trans*-activation of the reporter CAT plasmid (<500 cpm). Wild-type p53 stimulated CAT activity ~190-fold above background. In the

absence of the p53 recognition sequences in the test promoter, wild-type p53 failed to induce any transcription of the CAT gene (data not shown). The 1–320 and 1–290 segments stimulated CAT expression by the complete promoter ~90- and 40-fold, respectively. In contrast, segments 1–260, 80–320, and 280–390 failed to stimulate the reporter promoter. We conclude that the autonomous, specific DNA-binding segment is sufficient for strong *trans*-activation in vivo if it is joined to the amino-terminal acidic region. This experiment provides the best possible evidence that segments 1–320 and 1–290 are expressed in animal cells and bind specific DNA sequences in vivo under physiological conditions.

We used a combination of metal-affinity selection and immunoblotting techniques to establish p53 expression levels in cells transiently transfected by plasmids expressing p53 under the control of the cytomegalovirus (CMV) promoter (Fig. 8B). The immunoblot demonstrates that with the exception of segment 1–260, the p53 segments are expressed at significant levels and that the segments have appropriate sizes. Therefore, with the possible exception of segment 1–260, levels of *trans*-activation reflect intrinsic protein function rather than protein accumulation. Because segment 1–260 is either insoluble or does not accumulate intracellularly, however, the precise carboxy-terminal limit of the DNA-binding region required for *trans*-activation cannot be determined with certainty. More importantly, it is clear that segment 1–290 is sufficient for both DNA binding and *trans*-activation.

Discussion

We have investigated the oligomerization, DNA-binding, and *trans*-activating properties of isolated segments

Figure 7. Fine mapping of the specific DNA-binding domain of p53 using truncations of the p53 segment 80–320. The p53 truncated segments and their positions relative to the most highly conserved regions of p53 are shown at the top. Protein–DNA complexes were identified by the mobility retardation assay described in Materials and methods. The labeled probe is specific DNA. p53 (100 ng) was incubated with labeled probe (2 ng) in the presence or absence of nonspecific and specific competing DNA. The amount of competing specific or nonspecific DNA is indicated.

of wild-type murine p53. We find that the protein has two autonomous DNA-binding domains. These domains map within amino acids 315–390 and 80–290. Shortening of the domains from either end by 30–35 amino acids causes loss of their DNA-binding functions.

The 315–390 domain appears to recognize DNA non-specifically. These findings are consistent with reports that mutations in the carboxyl terminus reduce nonspecific DNA binding (Foord et al. 1991). Because this binding is blocked by pAb421, it is distinct from the specific and nonspecific DNA-binding functions of the intact protein that are stimulated by pAb421. We do not know the function, if any, of this autonomous domain. It could be an artifact of our assay and of no biological significance. Its close association with the tetramerization domain of p53, however, leads us to suspect that it may be important to p53 function. Nonspecific DNA-binding by p53 tetramers could explain the ability of p53 to promote the annealing of complementary single-stranded nucleic acids. Oberosler et al. (1993) have suggested that this function may play a role in DNA repair or replication.

Alternatively, this nonspecific DNA-binding activity may even be associated with a specific DNA-binding activity distinct from that already identified by many groups. Weissker et al. (1992) have presented evidence that p53 may have a second specific DNA recognition site. Lambda phage integrase, which has autonomous DNA-binding domains that recognize two different sequence families, provides a precedent for this possibility (Moitoso de Vargas et al. 1988).

The second autonomous domain from amino acids 80–290 lacks the carboxy-terminal tetramerization domain but binds DNA both specifically and nonspecifically. Therefore, a major DNA-binding function of p53 resides in the highly conserved central region of p53. These results are consistent with reports that p53s encoded by mutant DNAs isolated from human tumors are defective for specific and nonspecific DNA binding (Bargonetti et al. 1991; Kern et al. 1991; El-Deiry et al. 1992). These mutant p53s have missense mutations in the conserved central region of p53. p53 segment 80–290 contains no obvious conventional DNA-binding motif, but the conformation of this region is highly sensitive to amino acid

A Transactivation

B Protein Expression

Figure 8. (A) *Trans*-activation by wild-type p53 and segments of p53. H358 cells were transfected with plasmids expressing the CAT gene under the control of a p53-specific promoter and wild-type p53 or segments of p53. The histogram shows levels of CAT activity induced by the various p53s. (B) Expression of tagged p53. H358 cells were transfected with plasmids expressing wild-type p53 or segments of p53. The tagged p53s were concentrated by metal-affinity selection and analyzed by immunoblotting with monoclonal antibodies against the hemagglutinin tag as described in Materials and methods.

substitutions and to the binding of antibody to distant sites (Gannon et al. 1990; Hupp et al. 1992). A similar region of p53 is necessary for the binding of SV40 large T antigen (Ruppert and Stillman 1993). This overlap in p53 functions would explain how large T antigen blocks the binding of p53 to DNA (Bargonetti et al. 1992). Because the optimal DNA recognition sequence for p53 consists of four imperfect repetitions, it has been proposed that the sequences would accommodate binding by a p53 tetramer (El-Deiry et al. 1992; Funk et al. 1992). It is therefore somewhat surprising that the central domain maintains significant DNA-binding function in the apparent absence of p53 tetramerization. Perhaps the conformation of this domain, rather than tetramerization, contributes to the affinity of the protein–DNA interaction. We find that pAb246, specific for wild-type p53 (Gannon et al. 1990), reacts with p53 segments that bind DNA specifically but not with segments that fail to bind DNA (data not shown). Other investigators have suggested that the conformation in the conserved region of p53 may depend on zinc binding (Hainaut and Milner 1993) or a reactive sulfhydryl group (Hupp et al. 1993).

In agreement with others (Funk et al. 1992; Hupp et al. 1992), we have shown that pAb421 increases the specificity of DNA binding by p53. Hupp et al. (1992) interpreted this finding to mean that the binding of pAb421 to the carboxyl terminus of p53 leads to conformational changes that enhance DNA binding. Our data are consistent with that interpretation and offer an additional explanation for enhancement by pAb421. By interfering with nonspecific binding by the carboxyl terminus, pAb421 may make more p53 available for specific binding by the central domain of p53. Possibly, a similar competition between p53 DNA-binding domains *in vivo* may play a role in regulation of p53 function. Investigation of this possibility will require a better understanding of the carboxy-terminal DNA-binding function.

Wild-type p53 and some p53 segments that tetramerize form multiple species with both specific and nonspecific DNA in the mobility retardation assay. At the same protein concentrations, segments 80–290 and 80–320, which do not form stable tetramers, bind DNA as a single species. In theory, the multiple protein–DNA species could represent binding of multiple proteins to a single DNA, structural changes in p53–DNA complexes, binding of a single protein to multiple DNAs, or a combination of these interactions. Because wild-type p53 seems to form predominantly tetrameric forms, we think it unlikely that the multiple species represent binding by monomers, dimers, and tetramers. It also seems improbable that multiple tetramers could bind the small 28-bp DNA probes. Oberosler et al. (1993) have shown that p53 promotes annealing of complementary single-stranded DNAs. Thus, the possibility that p53 may induce structural changes in DNA cannot be excluded. All the data presented here, however, are consistent with the possibility that tetramers can bind multiple DNAs while monomers with a single DNA interaction site can bind only single DNA molecules. The number of DNAs in the

complexes would clearly influence gel mobilities of the complexes.

The DNA-binding properties of the 80–290 domain have biological significance. Expression of amino acids 1–290 and 1–320 results in strong activation of a reporter CAT gene *in vivo*. The reporter gene has p53 recognition sequences upstream from a TATA box, and deletion of these DNA recognition sequences results in loss of CAT expression. Our p53 segments that lack either the amino-terminal acidic region or the central DNA-binding domain of p53 fail to *trans*-activate completely. In contrast, p53 segments 1–290 and 1–320, which lack known tetramerization sequences, bind to DNA and activate p53-responsive promoters. Our findings are consistent with those of Aoyama et al. (1992), who showed that human p53 segment 1–326 (equivalent to amino acids 1–323 in the mouse) is sufficient for *trans*-activation, and those of Shaulian et al. (1993), who showed that mutation of the carboxy-terminal oligomerization domain does not block *trans*-activation. We have presented evidence previously that *trans*-activation is necessary for the suppression of transformation (Reed et al. 1993). Deletion of the p53 acidic region within amino acids 1–80 completely abolished p53 suppression of transformation by E1A and Ras. Substitution of the *trans*-activation domain of herpesvirus VP16 for the p53 acidic region fully restored the suppression function. Aside from a net negative charge, the amino acid sequences of the acidic regions of VP16 (Pellet et al. 1985) and p53 (Soussi et al. 1990) are quite different. Recent evidence, however, indicates that the acidic regions of both p53 and VP16 interact with the replication protein RF-A (Dutta et al. 1993; Li and Botchan 1993). Thus, unless RF-A binding is needed for suppression, our earlier findings implicate *trans*-activation in the suppression of cellular proliferation by p53.

Because p53 segment 1–290 has no carboxy-terminal nuclear localization signals (Shaulsky et al. 1990), it is surprising that it *trans*-activates a transfected promoter *in vivo*. Shaulsky et al. (1991) have presented evidence that mutation of the nuclear localization signals blocks suppression of transformation by wild-type p53 expressed under the control of the Moloney murine leukemia virus promoter. In contrast, Slingerland et al. (1993) report that when expressed by the strong CMV promoter, nuclear translocation-defective p53 proteins retain suppressor functions. Because suppression requires *trans*-activation, this latter study, in support of our present data, indicates that the carboxy-terminal nuclear localization signals of p53 may not be absolutely essential for *trans*-activation. It remains to be determined how p53, apparently lacking a nuclear localization signal, accomplishes *trans*-activation. Perhaps, the overexpression of p53 under the control of the strong CMV promoter allows a small fraction of p53 to enter the nucleus, or p53 may be transported into the nucleus in association with another protein or in association with the reporter plasmid DNA itself. Alternatively, p53 may activate the p53 reporter plasmid by an indirect mechanism unrelated to the DNA-binding function of p53. Because the promoter in

the reporter plasmid contains no transcriptionally active sequences in the absence of the p53-binding site, we think this latter alternative is very unlikely.

Figure 9 summarizes the results of this study and that of Reed et al. (1993) and compares the molecular and biological functions of p53 autonomous domains. The protein is divided into three regions with distinct molecular properties. The amino-terminal segment 1–80 is highly acidic. The large conserved central domain within segment 80–290 binds to DNA both specifically and nonspecifically but lacks the carboxy-terminal tetramerization sequences. The carboxy-terminal p53 segment 280–390 forms tetramers autonomously and binds to DNA nonspecifically. The molecular activities in the carboxy-terminal domain are necessary and sufficient for cellular transformation in conjunction with other cellular oncogenes. The acidic amino-terminal region and the central DNA-binding regions are necessary and sufficient for efficient *trans*-activation. We have found that segment 1–360 is sufficient for suppression of transformation by Ras and E1A (data not shown) and that segment 1–320 is not sufficient for suppression when expressed under the control of the relatively weak MSV promoter (Reed et al. 1993). Because protein levels may influence the suppression function, the precise limits of the suppression domain remain to be determined.

A number of p53 functions not examined in this study may contribute to the suppression of cellular proliferation. Our *trans*-activation assay utilizes a minimal promoter consisting of a p53 recognition element and a TATA sequence. Although natural target promoters of p53 (Weintraub et al. 1991; Kastan et al. 1992; Zambetti et al. 1992; Barak et al. 1993; Wu et al. 1993) have not yet been identified in any detail, they almost certainly contain additional DNA regulatory elements for transcription. It is possible that these elements modify the effects of p53 binding. Similarly, interactions of p53 with cellular proteins, like mdm2 (Momand et al. 1992; Oliner et al. 1993) or TBP (Seto et al. 1992; Liu et al. 1993; Mack et al. 1993; Ragimov et al. 1993; Truant et al. 1993), may modify the *trans*-activation or repression of p53-sensitive promoters. Other investigators have suggested that

p53 may also play roles in DNA replication (Friedman et al. 1990) and in DNA repair (Lane 1992). Finally, Dittmer et al. (1992) have presented evidence that p53 mutations sometimes confer new or additional phenotypes on cells that express no endogenous p53. The mechanisms underlying these gain-of-function mutations remain unknown. This study not only provides tools for dissecting the molecular activities of p53 but also for relating its activities to regulation of cellular growth.

Materials and methods

Expression plasmids

Previously, we have described the pIT plasmid for recombination of p53 or segments of p53 with baculovirus and expression in insect cells (Reed et al. 1993). For expression of p53 in bacteria, the DNA cassette used for insertion of PCR-generated DNA segments of p53 in pIT was inserted between the unique *NcoI* and *BamHI* sites of pET11d (Dubendorf and Studier 1991) to create the pBT plasmid, which utilizes an inducible T7 promoter for transient expression of p53. Both plasmids produce p53s with a small amino-terminal tag that encodes six histidines for purification by metal-affinity chromatography (see below).

We constructed the pCMH6K plasmid to express tagged proteins in animal cells. The plasmid includes the following sequences inserted between the *Clal* and *BamHI* sites of pBlue-script KS+ (Stratagene): the CMV immediate-early promoter from nucleotide –480 to +1, a cloning and expression cassette flanked by unique *NheI* and *BamHI* sites, SV40 small t-antigen splicing signals (SV40 nucleotides 4710–4100), and a polyadenylation signal (SV40 nucleotides 2770–2533). The cloning and expression cassette has a 5'-GCCATGG-3' translation initiation sequence and encodes amino acids MA-YPYDVPDYA (the hemagglutinin epitope)-R-HHHHHH (for metal-affinity chromatography)-A-RRASV (a site for heart creatine kinase A)-GV. Unique *HpaI* and *BamHI* sites allow directional insertion of PCR-generated DNA segments of the murine p53 gene immediately downstream from the cassette. Plasmids were purified by CsCl centrifugation for transfection studies in animal cells. Expression of the plasmids in animal cells produces p53 with a 26-amino-acid, amino-terminal tag.

Synthesis of p53 domains

Arnold Levine supplied us with wild-type murine p53 cDNA in pMSVcl (Finlay et al. 1988). We copied the entire p53 sequence or segments of it by using PCR with Vent polymerase (New England Biolabs) as described by the supplier. The PCR primers matched the amino and carboxyl termini of chosen segments of p53. Amino-terminal primers were blunt-ended, and carboxy-terminal primers included a termination codon and a *BamHI* site. The PCR fragments were cut with *BamHI* and phosphorylated at their 5' ends. These fragments were directionally ligated into the cloning and expression cassettes of pIT, pBT, and pCMH6K for expression in insect, bacterial, or animal cells, respectively (see above). The entire sequence of wild-type p53 and amino-terminal junctions of all cloned segments was verified by sequencing. We found no PCR-related mutations. To exclude further the possibility of mutations within p53 segments, we isolated and tested two independent clones expressing each p53 segment.

Figure 9. Functional organization of p53.

Overexpression and metal-affinity purification of p53

Sf9 insect cells at 80% confluency in Grace's medium with 10% fetal bovine serum were infected with high-titer recombinant baculoviruses expressing wild-type p53 or segments of p53. Three days after infection, cell monolayers were removed from plastic 175-cm² flasks by vigorous pipetting and pelleted by low-speed centrifugation. After washing four times with phosphate-buffered saline (PBS) at 4°C, the pellets were resuspended and lysed in 0.6 ml of lysis buffer (150 mM Tris-HCl at pH 9.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM PMSF, 50 µg/ml of aprotinin, 50 µg/ml of leupeptin, 10 µg/ml of pepstatin A, 2 mM benzamidine, and 1 mM β-mercaptoethanol) for 30 min on ice. The lysates were cleared by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor at 4°C for 30 min. Wild-type p53 or p53 segments with histidine tags were bound to 0.4 ml of Ni-NTA-agarose (Qiagen Inc.) in Eppendorf tubes by tumbling overnight at 4°C. The Ni-NTA-agarose was transferred to a small column and washed eight times with 1 ml of lysis buffer and six times with 1 ml of lysis buffer adjusted to pH 7.0. The columns were step eluted with lysis buffer at pH 7.0 containing increasing concentrations of imidazole (25, 50, 100, and 250 mM). p53-containing fractions were dialyzed overnight at 4°C against 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 50% glycerol (dialysis buffer). The proteins were quantitated by gel electrophoresis and Coomassie blue staining and were stored in aliquots at -70°C.

Bacterial cells (HMS174 or BL21), transformed by pBT plasmids expressing segments of p53, were grown from a single colony overnight in 10 ml of Luria broth (LB) medium. After transfer to a 500-ml culture for 4 hr at 37°C, the cells were induced with IPTG at a final concentration of 1 mM and incubated for 4 hr at 30°C. The cells were pelleted by centrifugation at 4000g for 20 min. The pellets were suspended in 15 ml of lysis buffer and were broken with a French press at 1000 psi. The lysates were cleared by centrifugation and were passed slowly through 0.8 ml of Ni-NTA-agarose in a small column. The bound p53 was washed, eluted, and dialyzed as described above. Purified p53 segments were the only bands seen in gels stained by Coomassie blue.

Peptide mapping of p53

Baculovirus-infected Sf9 cells were labeled with [³²P]orthophosphate (New England Nuclear) in phosphate-free medium 50–54 hr after infection. Wild-type untagged p53 was extracted and purified by immunoaffinity chromatography as described by Stenger et al. (1992). Tryptic digestion and peptide mapping of wild-type p53 were done as described by Meek and Eckhart (1990).

Oligomerization of p53

The quaternary structure of p53 segments was analyzed by protein cross-linking in conjunction with gradient gel electrophoresis as first described by Stenger et al. (1992). Purified p53 segments (3 µg) were incubated for 30 min at 25°C in 30 µl of p53 dialysis buffer containing various concentrations of freshly diluted glutaraldehyde (Sigma). The products of the cross-linking reactions were analyzed by gel electrophoresis. All of the gel components were those described by Laemmli (1970), except that we used an 80:1 ratio of acrylamide to bis-acrylamide at pH 8.8 to prevent protein retardation in the stacking gel. The samples were diluted twofold in sample buffer containing 4% (wt/vol) SDS and 5% (vol/vol) β-mercaptoethanol and heated for 5 min at 100°C immediately before being loaded onto the gel. The

samples were electrophoresed through 8–20% polyacrylamide gels in 0.1% SDS at 200 V for 4 hr at room temperature. Following electrophoresis, the gels were silver stained (Oakley et al. 1980).

Mobility shift assay for DNA binding by p53

Completely double-stranded, radiolabeled DNA probes were made by annealing synthetic oligonucleotides and filling their 4-nucleotide overhangs with DNA polymerase. The 28-bp specific probe had the sequence 5'-CCGGAGACATGCCTAGACATGCCTCCGG-3'; the 40-bp nonspecific probe had the sequence 5'-CATGCCATGAAACGCCCCAGGCCAAGGCCTCCCGGGGATC-3'. The same DNAs were used as specific and nonspecific, unlabeled competitor DNAs. Equal amounts of complementary oligonucleotides were annealed in 20 mM Tris (pH 7.4), 2 mM MgCl₂, and 50 mM NaCl at 80°C for 10 min and cooled slowly to room temperature. The annealed oligonucleotides were diluted to 1 µg/µl in TE buffer. The probes (1 µg) were radiolabeled with [³²P]dCTP and [³²P]dGTP using the Klenow fragment of DNA polymerase I and ethanol precipitated. Annealed radiolabeled oligonucleotides (2 ng), unlabeled competitor DNA (20–180 ng), and wild-type p53 or segments of p53 (100 ng) were mixed in this order at a final concentration of 25 mM HEPES (pH 7.4), 50 mM KCl, 20% glycerol, 0.1% NP-40, 1 mM DTT, and 1 mg/ml of bovine serum albumin. After incubation on ice for 20 min, pAb421 was added for 20 min when appropriate. The samples were loaded onto 4% polyacrylamide gels and subjected to electrophoresis in 0.3× TBE in a cold room for 2 hr. The gels were dried and exposed to X-ray film overnight at -70°C.

CAT assays for p53 trans-activation

NCI-H358 cells that express no endogenous p53 (Funk et al. 1992) were transfected by using liposome-mediated transfection as described previously (Reed et al. 1993). The reporter plasmid contained 26 direct repeats of a p53 recognition sequence 19 bp upstream from an E1B TATA sequence and the CAT reporter gene. It was constructed by inserting two PG₁₃ recognition segments from pPG₁₃CAT (Kern et al. 1992) between the HindIII and PstI sites and a synthetic E1B TATA sequence between the SalI and XbaI sites of pChlorAce-B (U.S. Biochemical). The pCMH6K plasmids were used to express wild-type p53 or segments of p53. H358 cells were cotransfected with 5 µg each of the reporter and p53 expression plasmids. Cells were harvested after 48 hr, and CAT activities were measured by phase extraction using a kit and procedures supplied by U.S. Biochemical. Each assay was repeated four times; there was <25% variation among transfections of the same plasmid using liposome-mediated transfection.

Immunoprecipitation and immunoblotting

H358 cells in two 10-cm² plates were transfected with 20 µg of pCMH6K plasmid DNAs expressing wild-type p53 or segments of p53 using calcium-phosphate precipitation (Chen and Okayama 1987). After 30 hr, the cells were washed with PBS, and p53 was extracted with 0.5 ml of lysis buffer for 30 min at 4°C. The tagged p53s were bound to 100 µl of Ni-NTA-agarose (Qiagen Inc.) overnight at 4°C. After the beads were washed three times with 1 ml of lysis buffer, the tagged p53s were released with 100 µl of SDS loading buffer for gel electrophoresis. The concentrated and partially purified p53s were analyzed by SDS-gel electrophoresis and immunoblotting using a kit for enhanced chemiluminescence (Amersham). We used 600 µg of

monoclonal antibody in ascitic fluid (12CA5 supplied by Babco) in 100 ml against the hemagglutinin epitope within the amino-terminal tag for the immunoblotting procedure.

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