

A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein

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p53 is a sequence-specific DNA-binding oligomeric protein that can activate transcription from promoters bearing p53-binding sites. Whereas the activation region of p53 has been identified within the amino terminus, the location of the specific DNA-binding domain has not been reported. Thermolysin treatment of p53 protein generates a stable protease-resistant fragment that binds with marked specificity to p53 DNA-binding sites. Amino-terminal sequencing of the fragment located the thermolysin cleavage site to residue 91. Because the fragment does not contain the cdc2 phosphorylation site at Ser-315, we conclude that the site-specific DNA-binding domain of p53 spans the central region of the protein. The vast majority of the mutations in oncogenically derived p53 proteins are located within this central portion of the molecule. Such mutant p53 proteins exhibit defective sequence-specific DNA-binding. Although thermolysin digestion of mutant p53 proteins generates proteolytic patterns that differ from wild-type protein, one mutant tested, His-273, generates a resistant fragment that migrates with a similar electrophoretic mobility to the wild-type protease-resistant fragment. Interestingly, although intact mutant His-273 protein binds to DNA at 20°C, the thermolysin-resistant mutant fragment does not. In addition, the central protease-resistant, site-specific binding region of wild-type p53 does not demonstrate nonspecific DNA-binding. Thus, although sequences outside of the central region of p53 contribute to both nonspecific DNA-binding and oligomerization, they are not required for sequence-specific DNA-binding.

[Key Words: p53; DNA-binding domain; sequence-specific DNA binding]

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The p53 gene encodes a protein that can function to suppress progression through the cell cycle in response to DNA damage (for review, see Hartwell 1992; Lane 1992; Prives and Manfredi 1993). Mutations in p53 can cause cells to become oncogenically transformed (for review, see Lane and Benchemol 1990; Levine et al. 1991). The normal function of p53 is regulated, at least in part, by the ability of the protein to bind site specifically to DNA (for review, see Vogelstein and Kinzler 1992). Mutation of the p53 gene is frequently found in many forms of human cancer (Nigro et al. 1989; for review, see Hollstein et al. 1991), and the tumor-derived mutant p53 proteins that have been tested thus far do not bind specifically to DNA in vitro (Bargonetti et al. 1991; Kern et al. 1991a). Wild-type p53 binds nonspecifically to DNA (Steinmeyer and Deppert 1988; Kern et al. 1991b), as well as specifically to diverse DNA sequences that contain forms of the consensus sequence 5'-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3' (El-Deiry et al. 1992; Funk et al.

1992). Wild-type, but not mutant, p53 can activate transcription from constructs containing strong p53-binding sites both in vitro (Farmer et al. 1992) and in vivo (Funk et al. 1992; Kastan et al. 1992; Kern et al. 1992; Scharer and Iggo 1992; Zambetti et al. 1992). Therefore, there is a strong connection between p53 DNA binding and normal p53 function.

The p53 protein, which is phosphorylated at residues within the amino- and carboxy-terminal regions, is composed of three separate structural regions: an acidic proline-rich amino terminus that mediates transcriptional activation (Fields and Jang 1990; Raycroft et al. 1990; Unger et al. 1992), a hydrophobic internal region, and a region within the carboxyl terminus that contains an oligomerization domain (Shaulian et al. 1992; Sturzbecher et al. 1992; Pavletich et al., this issue). Present in the central portion of the protein are four regions that are evolutionarily conserved within all vertebrate species examined (Soussi et al. 1990). Additionally,

approaches using antibodies have suggested that the central region contains determinants that regulate the conformation of p53 [Hinds et al. 1987; Cook and Milner 1990; Gannon et al. 1990]. To date, the specific DNA-binding domain of p53 has not been identified. The mutant p53 proteins found in human cancer contain amino acid substitutions or deletions that fall within the central portion of p53 and tend to cluster within the four conserved regions (for review, see Hollstein et al. 1991). This, coupled with the fact that oncogenically derived mutant p53 proteins do not bind to DNA, suggests that the central domain of p53 may be important for DNA-binding activity. Additionally, SV40 large T antigen binds to the central region of p53 [Tan et al. 1986; Jenkins et al. 1988; Ruppert and Stillman 1993] and, as such, inhibits the *in vitro* DNA-binding ability of the wild-type protein [Bargonetti et al. 1992].

Because the DNA-binding property of p53 is central to the function of the protein, it is important to determine what region of the protein contains the sequence-specific DNA-binding domain. Because distinct domains of proteins are frequently resistant to broad-range proteases [Heinrikson 1977], we examined the binding of p53 proteolytic fragments to oligonucleotides containing strong p53-binding sites. Using thermolysin digestion as a means to generate p53 fragments we have found that a stable p53 proteolytic fragment, ~27 kD in mass, maintains marked sequence specificity for DNA binding. In addition, we have compared the protease cleavage products generated from mutants with those seen with wild-type p53. In some cases, the fragments generated were very similar to wild type, whereas in others they were different. In addition to determining the site-specific DNA-binding domain of p53, our data address the variability between different oncogenically derived mutant forms of p53.

Results

Proteolysis of human p53 yields a sequence-specific DNA-binding fragment

To define the region of p53 that binds to DNA, we tested the ability of a broad range protease, thermolysin, to generate protein fragments capable of binding a strong p53 DNA-binding sequence using the electrophoretic mobility shift assay (EMSA). Immunopurified wild-type human p53 was treated with increasing quantities of the enzyme under DNA-binding conditions; subsequently, oligonucleotides containing the strong p53-binding site from the murine muscle creatine kinase promoter (MCK), identified by Zambetti et al. [1992], were added to each reaction mixture. These samples contained a sufficient amount of protein to be analyzed by SDS-PAGE to identify the p53 proteolytic fragments (Fig. 1A); aliquots were then examined by EMSA to determine whether any of the proteolytic fragments could bind to DNA (Fig. 1B). The proteolytic fragments detected after digestion demonstrated that the higher levels of thermolysin generated a single resistant p53 fragment mi-

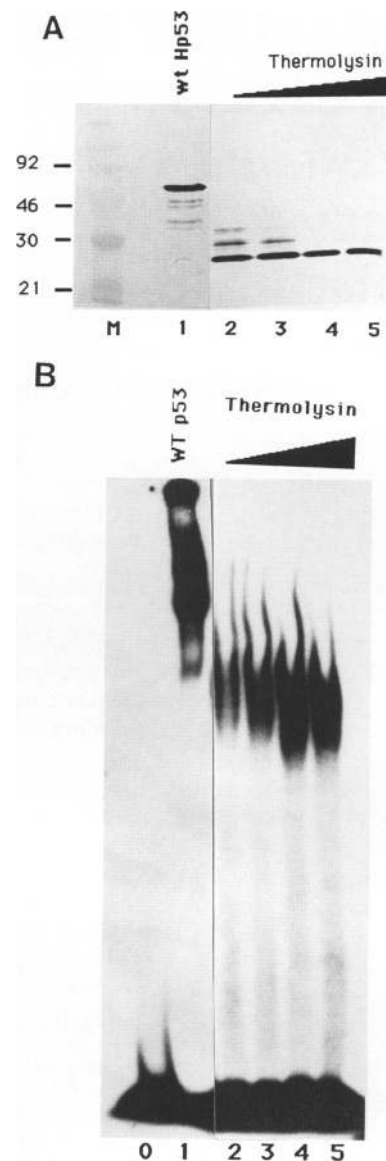


Figure 1. Proteolysis of human p53 yields a fragment that binds DNA. Human p53 (7 μ g) was digested with increasing amounts of thermolysin at 37°C for 15 min such that the ratios (wt/wt) of protease/p53 were 1:7, 1:3.5, 1:2.3, and 1:1.75 (lanes 2–5, respectively). 32 P-Labeled MCK oligonucleotide (1.5 pmole) was added to each reaction, and incubation was continued at 20°C for 30 min. Aliquots of the reaction mixtures were then analyzed for p53 proteolytic digestion patterns (A) and DNA-protein complexes (B). (A) Proteolyzed products were resolved on a 10% polyacrylamide gel followed by electrotransfer to nitrocellulose and probing with pAb 240. [Lane 1] Untreated p53; [lane M] prestained standards (molecular mass in kD at left). (B) EMSA of the proteolysis product reaction mixtures on a 4% native gel. [Lanes 1–5] The same protease reaction mixtures show in A; [lane 0] no p53 protein.

grating at ~27 kD by comparison with standard prestained polypeptides (Fig. 1A, lanes 4,5). Although the detection of these fragments was by Western blot anal-

ysis with the p53-specific antibody pAb 240, silver staining of gels containing such digests showed similar products in this size range (e.g., see Figs. 4B and 6A). The EMSA of aliquots of these proteolysis reaction mixtures showed that the thermolysin-digested protein bound well to the oligonucleotide, demonstrating an affinity that was in the range of full length p53 (Fig. 1B, lanes 2–5). It is interesting to note that the binding ability of the thermolysin treated protein increased with the higher levels of protease used. This was commensurate with the disappearance of larger detectable p53 fragments (cf. Fig. 1, A and B, lanes 2–5), and the appearance of smaller fragments (data not shown), suggesting the possibility that such fragments either mask or inhibit the DNA-binding ability of the major protease-resistant product.

To further analyze the DNA-binding properties of the thermolysin-resistant fragment of p53 we examined its ability to bind to another strong DNA site for p53 from the human ribosomal gene cluster (RGC) identified by Kern et al. (1991). Previously, we have shown that p53 binds with similar affinity to MCK and RGC (Bargonetti et al. 1992). In this experiment wild-type (RGC-W) and mutant (RGC-M) oligonucleotides were incubated with either full-length or thermolysin-treated p53 in the presence of increasing amounts of competitor DNA poly[d(I-C)]; Fig. 2). EMSA analysis demonstrated that under the conditions of the binding assay, although full-length p53 binding to RGC-W was clearly stronger, the protein was also capable of binding to RGC-M (Fig. 2, cf. lanes 9–11 with lanes 2–4). In contrast, the thermolysin-digested p53, which demonstrated strong binding to RGC-W, displayed virtually no detectable binding to RGC-M (Fig. 2, cf. lanes 12–14 with lanes 5–7). PhosphorImaging of the shifted DNA species showed that in the absence of competitor DNA the thermolysin-resistant fragment bound to RGC-W 50% as efficiently as full-length protein (Fig. 2, cf. lanes 9 and 12). At the highest level of competitor DNA, full-length p53 bound to RGC-M 27% as efficiently as RGC-W (Fig. 2, cf. lanes 4 and 11), whereas the p53 fragment bound RGC-M only 1% as efficiently as RGC-W (Fig. 2, cf. lanes 7 and 14). The binding displayed by full-length p53 to RGC-M may be controlled by sequences outside the central-core, thermolysin-resistant fragment. These findings indicate that the thermolysin-resistant p53 fragment displays strong sequence-specific DNA-binding activity and displays little nonspecific DNA-binding activity as compared with the full-length, wild-type protein. This suggests that when excised from the intact protein, the central region of p53 has lost some of the determinants that stabilize DNA-binding.

The ~27-kD thermolysin-resistant DNA-binding fragment spans the central region of p53

The proteolytic p53 fragments shown in Figure 1 were detected by immunostaining with pAb 240. The epitope of pAb 240 has been mapped to residues 212–217 (Stephan and Lane 1992), suggesting that this region of p53 is contained within the DNA-binding portion. This

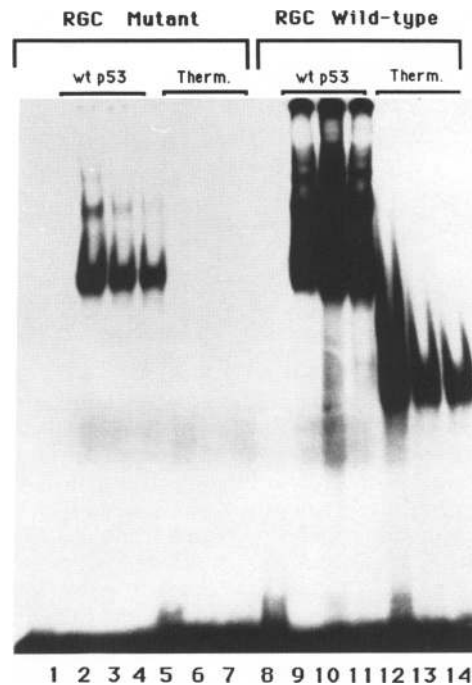


Figure 2. The thermolysin-resistant p53 fragment binds sequence specifically to DNA. Untreated p53 (3.5 μ g; lanes 2–4 and 9–11) or p53 treated at 37°C for 30 min with thermolysin [1:1.75 wt/wt; lanes 5–7 and 12–14] were incubated with 25 ng of either RGC-M (lanes 2–7) or RGC-W (lanes 9–14) 32 P labeled oligonucleotides in the presence of no competitor (lanes 2,5,9,12), or 150 ng (lanes 3,6,10, 13) or 300 ng (lanes 4,7,11, 14) of poly [d(I-C)]. Lanes 1 and 8 contain no p53 protein.

is consistent with additional immunochemical data from Western blot analysis, which showed that the ~27-kD thermolysin-resistant p53 product lacks both the pAb 1801 epitope between 32 and 79 (Banks et al. 1986) at the amino terminus, and the pAb 421 epitope between residues 371 and 381 at the carboxyl terminus (Wade-Evans and Jenkins 1985; data not shown). Before obtaining further information about the sequence of the stable proteolytic p53 fragment, it was necessary to determine whether it was actually bound to DNA. This was accomplished by isolating DNA–protein complexes containing either full-length or proteolyzed p53 from acrylamide gels, eluting the protein from such complexes, and analyzing the eluted protein by SDS-PAGE (Fig. 3A). Because the only polypeptide detectable in the thermolysin-treated p53–DNA complex migrated as a fragment within the size range of 27–29 kD, the likelihood that this fragment contained the site-specific DNA-binding domain of p53 was high (for discussion of the size of the p53 fragment, see below). Accordingly, a larger preparation of the the thermolysin-resistant product was generated, electrophoresed on an SDS–polyacrylamide gel, electroblotted onto a polyvinylidene difluoride (PVDF) membrane, stained with Coomassie blue, and then amino-terminally sequenced by gas-phase analysis. The

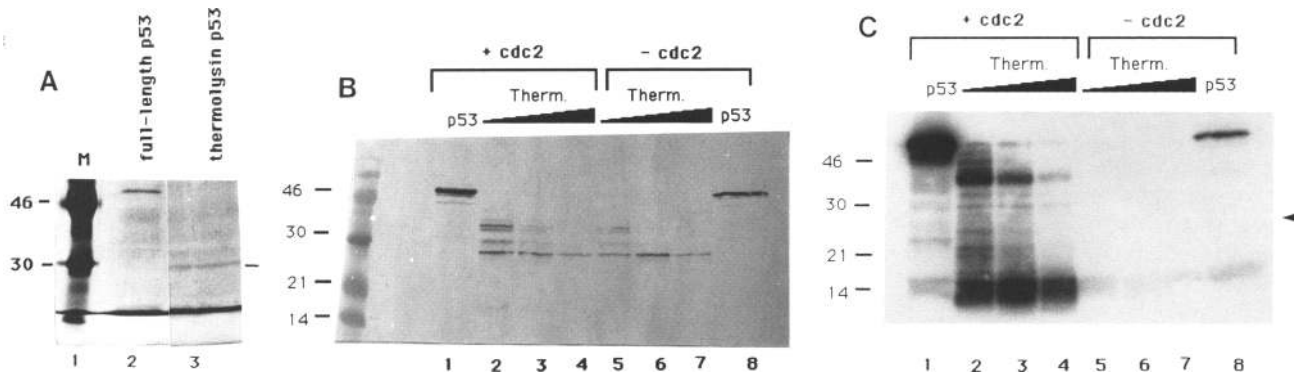


Figure 3. The ~27-kD thermolysin-resistant fragment spans the central region of p53. (A) Intact or thermolysin-treated p53 proteins were bound to ^{32}P -labeled MCK oligonucleotide and DNA-protein complexes were resolved on a 4% native polyacrylamide gel. ^{32}P -labeled bands corresponding to the shifted species (identified by wet autoradiography) were excised from the gel, and the protein was eluted. Shown here is a silver-stained 10% SDS-polyacrylamide gel with the eluted polypeptides from the untreated- (lane 2) and the thermolysin-digested- (lane 3) p53. Lane 1 contains markers for size. (B) p53 protein was incubated for 30 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and other reagents as described in Material and methods with (lanes 1–4) or without (lanes 5–8) cdc2 kinase before incubation with thermolysin. Subsequent thermolysin treatment of p53 was at a ratio of 1:1.75 for 30 min (lanes 2,5), 45 min (lanes 3,6), or 60 min (lanes 4,7) at 37°C. Samples were analyzed by 15% SDS-PAGE, followed by electrotransfer to nitrocellulose and were probed with pAb 240. (C) autoradiograph of the blot described in B. The arrowhead marks the position of the ~27-kD thermolysin-resistant fragment detected in lanes 2–7 of B.

sequence of the polypeptide LSSVPSQKT identified the thermolysin cleavage site to be at amino acid 91.

To gain information about the carboxyl terminus of the protease-resistant fragment, we took advantage of the single cdc2 kinase site on human p53 at Ser-315 (Bischoff et al. 1990). The full-length p53 was treated with purified cdc2 kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and the labeled protein was digested with thermolysin to generate the resistant fragment. After SDS-PAGE, electrotransfer to nitrocellulose, and Western blotting with pAb 240 (Fig. 3B), the blot was exposed to X-ray film to ascertain the location of the radioactive phosphate (Fig. 3C). The p53 preparation, when incubated alone with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, displayed a small amount of endogenous-associated protein kinase activity (Fig. 3C, lane 8). However, upon treatment with cdc2 kinase, the protein incorporated significantly more label (Fig. 3C, lane 1). The highest level of thermolysin digestion, where the only species detected by Western blot analysis was the ~27-kD fragment, also revealed a ~14-kD species by autoradiography that was strongly labeled with $\gamma\text{-}^{32}\text{P}$ and several additional minor labeled species (Fig. 3C, lanes 2–4). PhosphorImaging showed that the minor labeled species were relatively insignificant when compared with the full-length protein. Whereas the autoradiograph showed that the labeled species in the vicinity of the position of the ~27-kD polypeptide contained far less radioactivity than had been incorporated into full-length p53 (Fig. 3, cf. B and C), the Western blot demonstrated that an abundant quantity of the ~27-kD thermolysin-resistant fragment had been generated (Fig. 3B, lanes 2–7). We therefore conclude that the p53 thermolysin-resistant fragment does not contain the Ser-315 site that was phosphorylated by cdc2 kinase. Additionally, because human p53 contains a potential thermolysin cleavage

site between Asn-306 and Leu-307, it is likely that Asn-306 is the carboxy-terminal amino acid of the DNA-binding fragment. The endogenously labeled p53 also yielded the ~14-kD $\gamma\text{-}^{32}\text{P}$ labeled material (Fig. 3C, lanes 5–7) suggesting that the endogenous kinase phosphorylates the protein at the carboxyl terminus. This is consistent with the fact that Herrman et al. (1991) have identified a casein kinase II activity associated with p53, and that casein kinase II was shown by Meek et al. (1990) to phosphorylate a single site at Ser-389.

Taken together, our data with antibodies, amino-terminal sequencing, cdc2 kinase treatment, and relative SDS-PAGE migration show that the DNA-binding domain of p53 is located within a protease-resistant fragment of p53 that extends from amino acid 91 to approximately amino acid 306. Thus, the specific DNA-binding domain resides within the central conserved portion of the protein. We first determined that the thermolysin-resistant p53 fragment has a molecular mass close to that of 27 kD by comparison with prestained markers, particularly with the 29-kD carbonic anhydrase marker polypeptide. However, in cases where the same digests were analyzed on gels containing unstained markers (such as that shown in Fig. 3A), the apparent migration of the single stable thermolysin-generated p53 fragment was somewhat closer to that of the 30-kD marker polypeptide, indicating a somewhat greater molecular mass of ~28 kD. Examination of the two kinds of standards run in adjacent wells on polyacrylamide gels showed that prestained markers migrate more slowly than their unstained counterparts, possibly because of the added mass of the dyes, thus explaining possible discrepancies between different experiments. Nevertheless, because the thermolysin-resistant fragment extends from residue 91 at the amino terminus to, at the uppermost, residue

306 at the carboxyl terminus, thus containing a maximum of 205 amino acids, this would correspond to a polypeptide with an actual molecular mass in the vicinity of 23–24 kD, rather than the estimated molecular mass of 27–28 kD that was derived from electrophoretic mobility. This discrepancy may be related to the fact that intact p53 itself, whose migration on gels would suggest that it has a molecular mass of ~53 kD, has an actual molecular mass of ~44 kD.

Proteolysis of mutant p53 proteins demonstrates variability in cleavage patterns

We have constructed recombinant baculoviruses that express tumor-derived mutant p53 proteins Ala-143, Trp-248, His-273, and His-175, with amino acid substitutions that are each located within a different conserved region. When assayed by DNase I footprinting, these mutant proteins did not exhibit site-specific DNA-binding ability (Bargonetti et al. 1992). It was therefore of interest to examine whether differences in the thermolysin cleavage patterns of the mutant proteins could be discerned. Partial digestion of the immunopurified p53 proteins revealed that both the His-273 and Trp-248 mutant proteins have proteolytic patterns similar (but not identical) to wild-type protein (Fig. 4A, lanes 1–9). These two mutant proteins show additional similarity to wild-type p53 in that more extensive proteolysis by thermolysin generates a stable fragment that also migrates at ~27 kD in SDS-PAGE (Fig. 4B, lanes 7–12). Partial proteolysis of the His-175 mutant p53 (Fig. 4A, lanes 10–12) demonstrated a proteolytic pattern substantially different from that of the wild-type protein; and after more extensive treatment virtually no His-175 p53 proteolytic fragments remained (Fig. 4B, lanes 13–15). The Ala-143 mutant protein also appeared to be consistently more sen-

sitive to proteolysis; therefore, it was not possible to determine whether this mutant generated a partial pattern similar to or different from that of wild type (data not shown). However, it was clear that as with His-175 p53, Ala-143 p53 did not yield a stable thermolysin-resistant fragment (Fig. 4B, lanes 4–6). These data, obtained with thermolysin treatment of mutant proteins, suggest that His-273 and Trp-248 mutant p53 proteins maintain a structure similar to wild-type while Ala-143 and His-175 mutant p53 proteins do not.

The thermolysin product of His-273 mutant p53 does not bind to DNA

When we showed previously that the immunopurified mutant p53 proteins described above do not display sequence-specific DNA-binding activity, the conditions used for those experiments included incubation of proteins and DNA at 37°C. However, more recently we have found that some mutant p53 proteins bind to DNA at lower temperatures (Friedlander et al., unpubl.). The DNA-binding ability of one of these mutants, His-273, was compared with wild-type p53 by EMSA in both the absence and presence of competitor DNA at 20°C (Fig. 5). The wild-type and mutant p53 proteins displayed similar over-all binding when no competitor DNA was present (Fig. 5, cf. lanes 2 and 7). It should be noted that differences in the wild-type and mutant p53 oligomeric forms associated with DNA were observed frequently. However, when unlabeled competitor DNA was added there was a marked difference between the binding ability of the two proteins. As expected, wild-type p53 binding was strongly competed by the specific oligonucleotide RGC-W and very poorly competed by the mutant RGC-M (Fig. 5, lanes 8–11). In contrast, in the case of His-273, the unlabeled mutant oligonucleotide RGC-M

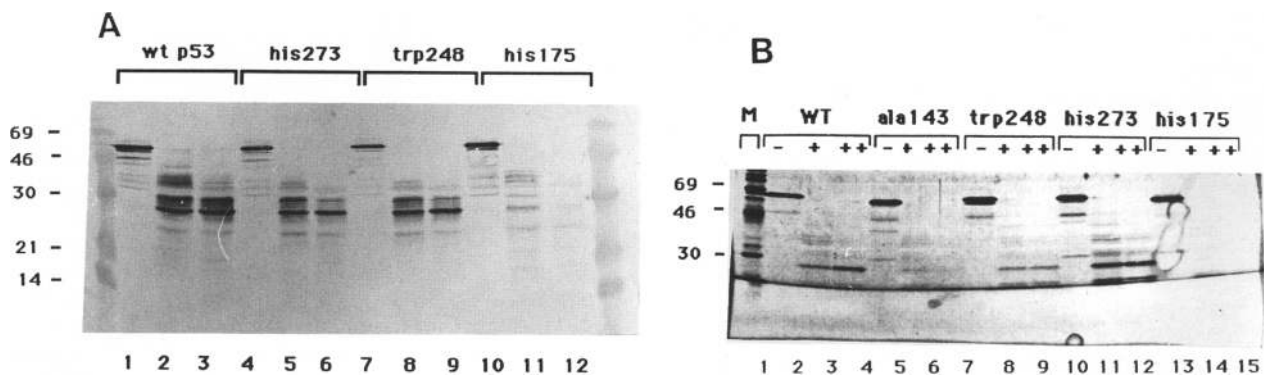


Figure 4. Proteolysis of mutant p53 proteins demonstrates variability in the cleavage patterns. (A) Limited digestion of wild-type and mutant p53 proteins. Wild-type human p53 (lane 1), His-273 (lane 4), Trp-248 (lane 7), and His-175 (lane 10) proteins (3 μ g) were digested with thermolysin at a protease to p53 ratio (wt/wt) of 1:0.4 (lanes 2,5,8,11) or 1:0.2 (lanes 3,6,9,12), respectively, for 30 min at 20°C. Samples were analyzed by 15% SDS-PAGE, followed by electrotransfer and immunoblotting with pAb240. (B) Extensive proteolysis of mutant p53 protein. Approximately 1 μ g of wild-type human p53 (lane 1), Ala-143 (lane 4), Trp-248 (lane 7), His-273 (lane 10), and His-175 (lane 13) was digested with thermolysin at an enzyme/p53 ratio of 1:0.5 (lanes 2,5,8,11,14) or 1:0.25 (lanes 3,6,9,12,15) for 15 min at 37°C. Reaction mixtures were electrophoresed on a 15% SDS-PAGE, and the gel was silver-stained. The arrowhead indicates the 35-kD thermolysin protein, which silver-stains negatively because it is a metalloprotease.



Figure 5. The tumor-derived mutant p53 (His-273) binds DNA at 20°C. EMSA, comparing the DNA binding of wild-type and mutant His-273 p53 proteins at 20°C. Reaction mixtures contained 1.5 pmole of labeled MCK oligonucleotide and 1 μ g of either His-273 (lanes 2–6) or wild-type (lanes 7–11) p53 with no competitor DNA (lanes 2,7), or 20 (lanes 3,8) or 40 (lanes 4,9) pmoles of unlabeled RGC-W competitor, and 20 (lanes 5,10) or 40 (lanes 6,11) pmoles of unlabeled RGC-M competitor. Lane 1 contains 1.5 pmoles of labeled MCK oligonucleotide with no p53 protein.

was fairly effective at competing for binding to the labeled probe (Fig. 5, cf. lanes 5 and 6 with lanes 10 and 11). This result indicates that, although His-273 exhibits binding activity at 20°C as monitored by EMSA, the binding displayed is less specific than that demonstrated by wild-type p53.

We then determined whether the thermolysin-resistant fragment of His-273 mutant p53 was capable of binding to the 32 P-labeled MCK oligonucleotide (Fig. 6). The mutant protein yielded a predominant ~27-kD migrating fragment that was somewhat more resistant to proteolysis at 20°C than at 37°C (Fig. 6A). However, virtually no DNA binding by the mutant proteolytic digest was observed at either temperature (Fig. 6B, lanes 2–9). This was in marked contrast to the highly specific binding displayed consistently by the wild-type p53 thermolysin fragment (e.g., see Fig. 2). Thus, although similar protease-resistant fragments are generated from both His-273 mutant and wild-type p53, only the wild-type fragment binds to DNA. The binding displayed by the intact His-273 mutant protein at 20°C appears to be of a significantly different nature from wild-type p53 and may be controlled, to some extent, by sequences outside the central-core thermolysin-resistant fragment.

Discussion

The p53 tumor suppressor protein can function as a checkpoint factor (Hartwell 1992), causing cells that were exposed to DNA-damaging agents to arrest in G₁ (Kastan et al. 1992; Hall et al. 1993). p53 presumably brings about this arrest, in part, by activating one or more genes that are part of a DNA damage-response

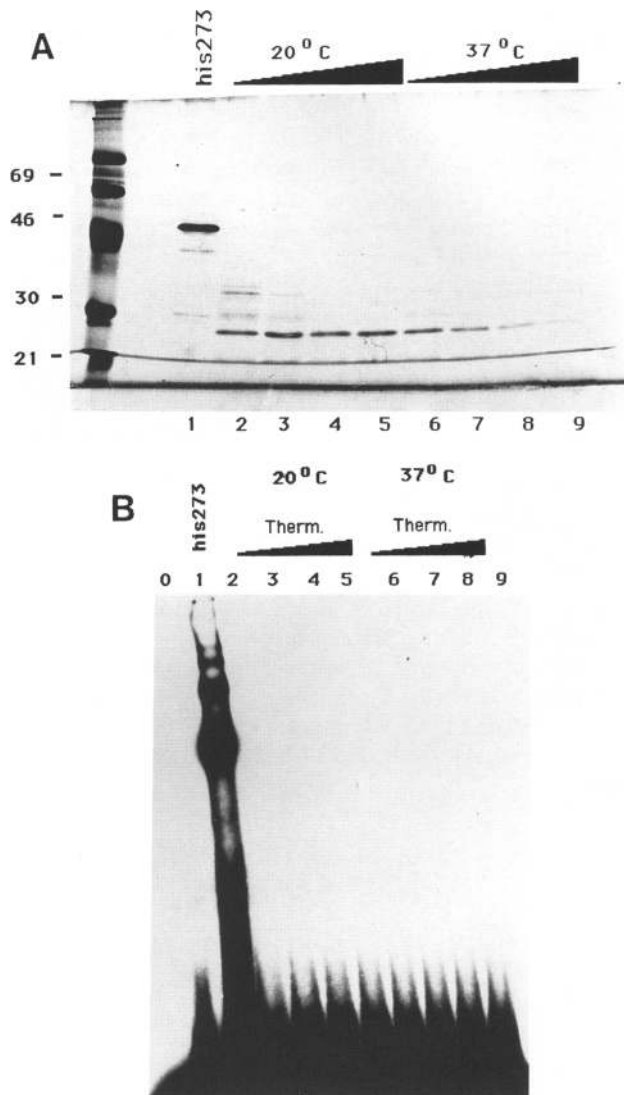


Figure 6. The thermolysin proteolytic fragment of mutant p53 (His-273) does not bind to DNA. Thermolysin digestion of 1 μ g of mutant His-273 p53 was carried out at 20°C and 37°C as indicated with protease/p53 weight ratios of 1:0.5 (lanes 2,6), 1:0.25 (lanes 3,7), 1:0.125 (lanes 4,8), and 1:0.06 (lanes 5,9) for 30 min. 32 P-labeled MCK oligonucleotide (1.5 pmole) was then added to each reaction mixture and incubated for 15 min more. Half of each reaction mixture was either electrophoresed on a 10% SDS-polyacrylamide gel and then silver stained to detect the appearance of the ~27-kD thermolysin-resistant fragment (A) or electrophoresed on a 4% acrylamide gel. The proportion of bound to free DNA was detected by autoradiography (B). (Lane 1) Full-length untreated p53; (lane 0) no His-273 protein.

pathway. The fact that tumor-derived mutant p53 proteins do not activate transcription (Farmer et al. 1992; Kern et al. 1992) nor induce G₁ arrest (Kastan et al. 1992) may contribute to the genetic instability that is a hallmark of the oncogenic state (Livingstone et al. 1992; Yin et al. 1992). A key feature of transcriptional activation by p53 is the binding of the protein to DNA. Therefore, defining the region of p53 that is responsible for sequence-specific DNA binding will further our understanding of the normal function of p53. In this study we have isolated a thermolysin-resistant p53 fragment that exhibits strong sequence-specific DNA-binding activity in mobility shift assays. This fragment is the only polypeptide detected in the DNA-protein complex isolated from acrylamide gels and is not immunoreactive in Western blots with either the amino-terminal p53-specific antibody pAb 1801 or the carboxy-terminal-specific antibody pAb 421. Gas-phase sequencing has determined that the amino-terminal cleavage site is at position 91, whereas treatment of intact p53 with cdc2 kinase and then cutting with thermolysin has demonstrated that the resistant fragment does not contain Ser-315. These data locate the site-specific DNA-binding domain to within the central conserved region of the protein (see Fig. 7). Our findings are consistent with the work of Pavletich et al. (this issue), showing that a bacterially expressed p53 polypeptide able to bind to DNA contains amino acid residues 102–292. A feature that is common to all mutants tested is that they are defective in DNA binding. The fact that the vast majority of oncogenic mutations within p53 are located within the central portion of the protein fits extremely well with the location of the site-specific DNA-binding region that we have identified. Furthermore, our observation that SV40 T antigen, which was shown to bind to the central region of p53, abolishes specific DNA-binding by p53 provides further support for our conclusions.

Foord et al. (1991) proposed that the DNA-binding domain of p53 is contained within the carboxy-terminal 83 amino acids of the protein. This was based on their observation that truncated wild-type p53 lacking the carboxy-terminal 83 amino acids was unable to bind to DNA. However, in that study they neither used a defined p53-binding sequence nor demonstrated directly that the missing 83 amino acids were capable of specifically bind-

ing to DNA; therefore, their original conclusion must be reinterpreted. It is now clear that the specific DNA-binding domain of p53 is not located within the extreme carboxyl terminus of the protein. Because p53 fragments containing sequences between residue 91 and 306 (this study), or *Escherichia coli*-expressed p53 polypeptide 102–292 (Pavletich et al., this issue), or a baculovirus-expressed p53 polypeptide containing residues 97–305 (Jayaraman et al., unpubl.) all bind specifically to p53 DNA-binding sites, it is evident that the oligomerization domain of p53 that is located within the carboxyl terminus (Sturzbecher et al. 1992; Pavletich et al., this issue) is also not required for p53 sequence-specific DNA-binding activity. However, the question must then be posed as to why some forms of p53 that are truncated at, or deleted within, the carboxyl terminus oligomerization region show defective DNA-binding ability despite the fact that they retain the DNA-binding domain (Foord et al. 1991; Hupp et al. 1992; Shaulian et al. 1993; Jayaraman et al., unpubl.). One explanation for this apparent discrepancy is that in the absence of an intact carboxy-terminal region, the amino terminus may negatively regulate interactions by the central specific DNA-binding region. Preliminary experiments support this possibility (Jayaraman et al., unpubl.). This would be akin to the observation that the p53 carboxy-terminal-specific antibody pAb 421 activates p53 DNA binding (Funk et al. 1992; Hupp et al. 1992; Halazonetis et al. 1993; J. Bargonetti et al., unpubl.) and that there is a negative regulatory region within the extreme carboxy-terminal 30 amino acids with repression of DNA-binding that can be relieved by various treatments (Hupp et al. 1992). Thus, discrete sequences outside of the central region of p53 are capable of regulating the sequence-specific DNA-binding ability of the protein. Halazonetis et al. (1993) have suggested that the conformation of intact p53 changes when the protein binds to DNA. The ability to assume this conformational change may be regulated by sequences both within and outside of the central DNA-binding region.

The ~27-kD thermolysin-resistant DNA-binding p53 fragment contains four evolutionarily conserved regions that sustain a high occurrence of mutations in human cancers. The results from thermolysin digestion of the four mutant p53 proteins examined suggests that there

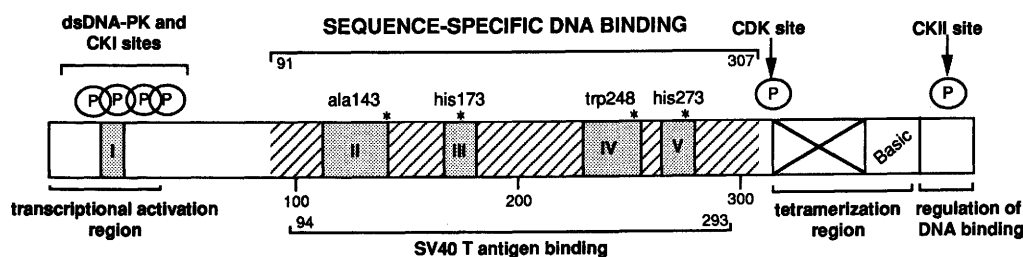


Figure 7. The central region of p53 binds to DNA. The lined area, spanning from 91 to 306, indicates the structural domain of p53 that binds sequence specifically to DNA. A detailed description of the diagram representing the structural domains of p53 is in Prives and Manfredi (1993). The five regions of p53 that are evolutionarily conserved are shown as stippled boxes; the asterisks (*) above them demark the substituted residues present in the oncogenic mutants described in this study.

may be at least two classes of mutations that cause aberrant DNA binding. Two mutants, Trp-248 and His-273, generated thermolysin digestion patterns similar to wild-type p53, suggesting that their structure is similar to wild type. If this is the case, then the inability of this class of mutants to bind to DNA may be attributable to changes in amino acids that make direct contact with the DNA. Interestingly Trp-248 and His-273 are transcriptionally active when fused to the GAL4 DNA-binding domain (Raycroft et al. 1991), which lends support to the above possibility. The two other mutants tested, Ala-143 and His-175, were more highly susceptible to proteolysis, suggesting that they might be less tightly folded. Consistent with this observation is the fact these two mutants are defective in *trans*-activation when fused to the GAL4 DNA-binding domain (Raycroft et al. 1991). Furthermore, Ala-143 and His-175 were reported to bind to the Hsc70 heat shock protein, whereas wild-type p53 and His-273 do not (Hinds et al. 1990).

Other lines of evidence point to the importance of the central region for DNA-binding activity. *Xenopus* p53, which is only homologous to human p53 in the central region, binds specifically to a human p53 consensus sequence (Y. Wang and C. Prives, unpubl.). Additionally, thermolysin treatment of either *Xenopus* or murine p53 protein yields a stable fragment that complexes with a strong DNA-binding site (Y. Wang, J. Bargonetti, and C. Prives, unpubl.). Hainaut and Milner (1993) showed that metal ions bind to the conserved cysteinyl residues in this central region, and this binding stabilizes the tertiary structure of wild-type p53. Pavletich et al. (this issue) have shown that metal binding by p53 is necessary for the central region to exhibit DNA-binding activity.

Although the central portion of p53 exhibits strong sequence-specific DNA-binding activity that is comparable to intact p53, it appears to have far less nonspecific DNA-binding activity than does the full-length protein. While full-length p53 was able to interact with a nonspecific oligonucleotide, the ~27-kD fragment was extremely defective, suggesting that amino acids outside the central domain are important for nonspecific DNA-binding activity. The fact that His-273 mutant p53 was able to bind relatively nonspecifically to DNA when intact, but not when digested to yield the ~27-kD fragment, also suggests that there are two determinants for DNA binding by p53. Shaulian et al. (1992) have reported that the carboxy-terminal portion of p53, extending from residue 302 to 390, is able to bind to DNA cellulose although it is unable to bind site specifically to DNA.

Although oncogenic mutant p53 proteins cannot bind site specifically to DNA, perhaps many maintain the ability to bind nonspecifically to DNA *in vivo*. If so, this property might contribute to the role of mutant p53 proteins in cell transformation (Dittmer et al. 1993). The nonspecific DNA binding of mutant proteins, coupled with their ability to maintain interactions with other regulatory proteins, might incur promiscuous regulation of many promoters, thereby *trans*-activating genes that under normal circumstances would be inactive. If this were the case, inactivating the nonspecific DNA-binding

activity of mutant p53 protein might be a means to inactivate some of the transformation activity demonstrated by these mutants in human tumors.

Materials and methods

Purification of p53 proteins

Recombinant baculoviruses expressing wild-type human p53 or mutant p53 proteins His-273, His-175, Ala-143 and Trp-248 were described previously (Bargonetti et al. 1992, and references therein). Sf21 insect cells (2.5×10^7 /150-mm dish) were infected with recombinant viruses and harvested at 48 hr postinfection. Extracts of infected cells were prepared, and p53 proteins were purified from cell lysates by immunoaffinity procedures as described previously (Wang et al. 1989). Protein A-Sepharose columns cross-linked with p53-specific monoclonal antibody pAb 421 (Harlow et al. 1981) were used to purify p53 proteins. The proteins were eluted with pAb 421 epitope containing p53 peptide (KKGQSTRHKK-OH) (Wade-Evans and Jenkins 1985) and dialyzed into a buffer containing 10 mM HEPES (pH 7.5), 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol.

Proteolytic digests of p53 proteins and DNA-binding assays

Thermolysin digests of purified p53 proteins were carried out at either 37°C or 20°C in 50- μ l reaction volumes containing 40 mM creatine phosphate, 4 mM ATP, 7 mM MgCl₂, and 0.5 mM DTT at enzyme/protein ratios as indicated. Over the course of these experiments the activity of the thermolysin preparations varied and the ratio for digestion was changed accordingly.

After protease treatment of p53, DNA was added as indicated. The synthetic double-stranded oligonucleotides used in this study (RGC-W, RGC-M, and MCK)—RGC-W, 5'-TCGAGTTG-CCTGGACTTGCCTGGCCCTTGCCTTTTC-3'; RGC-M, 5'-TCGAGTTAATGGACTTTAATGGCCCTTAATTTTC-3'; MCK, 5'-TCGAGTGGCAAGCCTATGACATGGCCGGGCCTGCCTCTCTGCTCTGACCCTC-3'—which were described previously (Bargonetti et al. 1992), were labeled using the large fragment of DNA polymerase and [α -³²P]dNTPs. Reaction mixtures (50 μ l) containing 40 mM creatine phosphate, 4 mM ATP, 7 mM MgCl₂, 0.5 mM DTT, and labeled oligonucleotide (3×10^{-7} M), as well as full-length or proteolytically-cleaved p53, were incubated at temperatures and for times as indicated. In some cases, unlabeled competitor poly [d(I-C)], RGC-W, or RGC-M was then added at indicated concentrations and the binding reaction was allowed to continue for the additional time as shown. The protein-DNA complexes were resolved by 4% native acrylamide gel electrophoresis in a buffer of 0.5 \times Tris-borate electrophoresis buffer (TBE). Each reaction mixture (10 μ l) was electrophoresed on a 4% native gel for EMSA, and the remainder of each sample was analyzed by 10% or 15% SDS-PAGE. The gels were either silverstained or electrotransferred to nitrocellulose and probed with p53-specific antibody pAb 240 (Gannon et al. 1990) or with a mixture of p53-specific antibodies [pAb 240, pAb 421, and pAb 1801 (Banks et al. 1986)].

Elution of protein from native gels

Elution of proteins from native acrylamide gels was essentially as described (Hager and Burgess 1980). Full-length purified p53 (7 μ g) or ~25 μ g of thermolysin-digested p53 (at an enzyme to p53 ratio of 1: 1.75) was bound to 3×10^{-7} M labeled MCK for EMSA analysis. The thermolysin-treated protein (a combina-

tion of four reaction mixtures loaded over four lanes) was electrophoresed on a 4% native gel, which was then exposed wet to X-ray film overnight at 4°C. The gel slices corresponding to the shifted complexes were excised, crushed, and soaked in elution buffer containing 0.1 mM EDTA, 5 mM DTT, 0.20 M NaCl, 0.1% SDS, and 0.05 M Tris-HCl (pH 7.9) for 3 hr at 25°C. The crumbled gel was pelleted in a clinical centrifugation for 1 min at maximum speed, and the supernatant was then transferred into a siliconized 15-ml Corex tube and precipitated with four volumes of ice cold acetone for 30 min in a dry ice ethanol bath. After centrifugation at 10,000 rpm for 30 min, protein-electrophoresis sample buffer was used to resuspend the pelleted material and the samples were separated on a 10% SDS-polyacrylamide gel.

Amino-terminal sequence analysis of the p53 DNA-binding fragment

Sequencing of the ~27-kD thermolysin-resistant p53 fragment amino terminus was as described (Matsudaira 1987). Purified wild-type human p53 (60 µg) was digested with thermolysin at a ratio of 1:0.5 at 37°C for 30 min. Unlabeled RGC-W oligonucleotide was added to the reaction mixture (400 µl), and incubation was continued at room temperature for 30 min. The reaction mixture was then concentrated in an Amicon concentrator spun at 5000 rpm for 1 hr. The sample was then electrophoresed on a 10% SDS-polyacrylamide gel and electrotransferred to a ProBlott membrane (Applied Biosystems). The membrane was stained with Coomassie blue to visualize the proteolyzed p53 product before amino-terminal sequence analysis by the Protein Chemistry Core Facility (Columbia University). Gas-phase sequencing was performed using an Applied Biosystems model 470A protein sequencer/120A PTH analyzer for 10 cycles.

Phosphorylation of p53

Phosphorylation of p53 was carried out by incubation of p53 at 30°C for 30 min in buffer containing 50 mM HEPES at pH 7.9, 10 mM MgCl₂, 1 mM DTT, 0.1 mM ATP, and 25 µCi of [γ -³²P]ATP (New England Nuclear, 3000 Ci/mmol), in the absence or presence of 0.8 ng of cdc2 kinase (sp. act. 912 nmoles/min per mg) purified from mitotic HeLa cells and characterized according to Marshak et al. (1991)

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