Sequence-specific Interaction of a Conformational Domain of p53 with DNA

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Abstract

Mutations within a conserved “conformational” domain of the p53 protein have frequently been observed in a wide variety of human cancers. A hybrid protein containing the wild-type conformational domain of p53 fused to protein A bound to calf thymus DNA and a specific p53 DNA-binding motif. Hybrid proteins containing mutations in p53 bound to DNA less efficiently than wild-type hybrid protein. In addition, competition experiments showed that mutated p53 DNA-binding motif failed to interact with p53 hybrid proteins. The DNA-binding activity of wild-type p53 hybrid protein was inhibited by the metal chelator 1,10-phenanthroline. These results demonstrate that DNA-binding activity resides in the conformational domain of p53, providing a structural model for disruption of DNA binding by mutation. Furthermore, metal ions may regulate binding of p53 to DNA by modulating its conformation.

Introduction

Mutation or deletion of the p53 gene is the most common genetic denominator among human cancers (1-3). A region of p53 between amino acids 100 and 300 containing several highly conserved domains is a hot spot for missense mutations (1-3). This region can be referred to as a “conformational” domain because of the observed effects of mutations on the conformation of the protein (4-12). The mutation-induced changes in tertiary structure interfere with the ability of p53 to bind DNA (13-16) and to trans-activate reporter genes (15, 17, 18). The conformational domain also has two sites (between amino acids 123 and 215 and between amino acids 236 and 289) that bind to the SV40 large T-antigen (19, 20); this binding results in stabilization and overexpression of p53 in SV40-transformed cells (21, 22). The functional consequences of binding T-antigen, like those of missense mutations, are the loss of the DNA-binding and trans-activation activities of p53 (23).

Although the 80 COOH-terminal amino acid residues of p53 were mapped for DNA binding, other sequences further upstream must be involved in perfect DNA interaction, since a wild-type and a mutant p53 sequence was in the correct translational reading frame with protein A cDNA in the pRIT2T protein A gene fusion vector (Pharmacia LKB Biotechnologie). Automated DNA sequencing confirmed that both wild-type and mutant p53 sequences were in the correct translational reading frame with protein A cDNA sequences.

Materials and Methods

Construction of Protein A-p53 Hybrid Proteins. The cDNA domain encoding p53 amino acids 115-295 was amplified by polymerase chain reaction by using flanking primers containing artificial terminal BamHI restriction sites. Amplified p53 (115-295) polymerase chain reaction DNA was subcloned in frame downstream to protein A cDNA in the pRIT2T protein A gene fusion vector (Pharmacia LKB Biotechnologie). Automated DNA sequencing confirmed that both wild-type and mutant p53 sequences were in the correct translational reading frame with protein A cDNA sequences.

Expression and Harvesting of Protein A-p53 Hybrid Proteins. Hybrid p53 proteins were induced by heat shock and incubation of transformed Escherichia coli at 42°C for 3 h. Bacteria were pelleted and disrupted in bacterial lysis buffer (50 mM Tris-HCl, pH 8.0-1 mM EDTA-0.1% Triton X-100-1 mM phenylmethylsulfonyl fluoride) containing 1 mg/ml lysozyme. Bacteria were allowed to lyse on ice for 1 h followed by two 1-min bursts of sonication to complete lysis. Lysates were clarified at 10,000 X g for 10 min. Human IgG-Sepharose was added and the protein A-containing molecules were allowed to bind for 1 h by gentle shaking at 4°C. IgG-Sepharose and bound protein A-containing molecules were washed 4 times with wash buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 0.2% Triton X-100. Hybrid p53 proteins were eluted from the IgG-Sepharose into 0.5 M ammonium acetate buffer (pH 3.4), neutralized with Tris base buffer, and washed with wash buffer in a Centricon-10 (Amicon).

Binding of p53 Hybrid Proteins to Calf Thymus DNA. Binding of p53 hybrid proteins to calf thymus DNA was investigated in both crude bacterial lysates or in purified form. For analysis in lysates, hybrid p53 proteins were induced and bacteria were lysed as described above. Calf thymus DNA cross-linked to cellulose (25 µg) was added and the mixture was shaken at 4°C for 3 h. Calf thymus DNA-cellulose and bound proteins were washed with DNA-protein-binding buffer (100 mM NaCl; 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5; 1.5 mM MgCl2; 10 mM dithiothreitol; 0.1% Triton X-100; 20% glycerol; 1 mM phenylmethylsulfonyl fluoride; 1% apotinin; and 10 µg/ml leupeptin). DNA-binding proteins were eluted into 2% SDS sample gel loading buffer (0.125 M Tris-HCl, pH 6.8; 2% SDS; 5% 2-mercaptoethanol; and 10% glycerol) and immunoblotted by using p53 monoclonal antibody PAb240 (Oncogene Science, Inc.). To analyze purified p53 hybrid proteins for binding calf thymus DNA, hybrid proteins were harvested from bacterial lysates by using human IgG-Sepharose (Pharmacia LKB Biotechnologie, Inc.) and eluted into 0.5 M ammonium acetate buffer (pH 3.4). Eluates were diluted and neutralized with 2 ml DNA-protein-binding buffer and concentrated to 200 µl. The concentrate was then diluted to 1 ml with DNA-protein-binding buffer. Calf thymus DNA-cellulose was added and bound p53

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3 The abbreviations used are: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; OP, 1,10-phenanthroline.

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hybrid protein was processed as described above. Quantitation of the amount of p53 hybrid protein bound to DNA was performed by using a Molecular Dynamics personal densitometer. A calibration curve consisting of different amounts of p53 hybrid protein was determined to ensure that data were obtained within the linear curve of the densitometric analysis. Data are expressed as the ratio of the volume of peak area of p53 hybrid protein bound to DNA to the total amount of p53 hybrid present in the assay.

Binding of p53 Hybrid Proteins to the p53CON Motif (GGACATGC-CGGGCATGTCC). Hybrid p53 proteins were induced and harvested from bacterial lysates by using IgG-Sepharose as described earlier. IgG-Sepharose beads with attached hybrid p53 proteins were incubated with 1 ng of 32P-end-labeled p53CON DNA and 0.5 mg of polydeoxyinosinate-polydeoxyoctylate as nonspecific competitor in DNA-protein-binding buffer. The reaction was allowed to proceed at room temperature for 20 min. The IgG-Sepharose beads with attached hybrid p53 protein/DNA complexes were washed 3 times in DNA-protein-binding buffer and the complexes were eluted in 0.5 M ammonium acetate. The eluates were neutralized, digested with proteinase K, and electrophoresed on a 4% agarose gel. Data were quantitated by both densitometric and phosphorimager analyses as described above.

Results

To determine if the conformational or SV40 large T-antigen-binding domain of p53 has the ability to interact with DNA, a hybrid protein containing p53 amino acids 115 to 295 fused to protein A was constructed and investigated for binding to calf thymus DNA and a p53 DNA-binding motif (p53CON) (13). Hybrid proteins containing wild-type p53 sequence or sequences with a mutation at amino acid 175 (His175) or 273 (His273) of p53 were expressed in E. coli, purified from bacterial lysates by using IgG-Sepharose, and immunoblotted with p53 monoclonal antibody directed against an epitope within the conformational domain (PAb240) (12, 28). Selection of the mutants was based upon two criteria: they have been frequently observed in human cancers and are localized within the putative zinc loops. The hybrid proteins migrated at the expected size of 50K M, (Fig. 1A, Lanes 2–4), whereas protein A migrated at 30K M, (Fig. 1A, Lane 1). Protein A bound to the p53 monoclonal antibody in the immunoblot by virtue of its affinity for IgG.

The protein A and p53 hybrid proteins were investigated for binding to calf thymus DNA cellulose. DNA cellulose was added to bacteria cell lysates and bound proteins were eluted with SDS electrophoresis buffer. DNA-binding proteins were then immunoblotted with p53 monoclonal antibody PAb240. All of the p53 hybrid proteins bound to DNA (Fig. 1B, Lanes 2–4). The DNA interaction of the hybrid proteins appeared to be through the p53 sequence, since an overexposure of the immunoblot shown in Fig. 1B did not detect any protein A bound to calf thymus DNA (Fig. 1C, Lane 1). Protein A would have been detected in this analysis since it binds to IgG in the immunoblot (Fig. 1A, Lane 1). The specificity for the p53 sequence binding to DNA is further demonstrated in that the hybrid p53 proteins containing mutations were less efficient in binding DNA than the wild-type protein. By determining the ratio of intensity of hybrid p53 bound to DNA (Fig. 1B) to the amount of total hybrid p53 protein in the assay (Fig. 1A) by densitometric analysis, the p53 protein containing a mutation at amino acid 175 bound to DNA 45% less than the wild-type protein, and that with a mutation at position 273 was reduced by 35% (Fig. 1D). Although the amount of the His273 protein binding DNA looks somewhat comparable to wild-type p53 hybrid protein in the enhanced chemiluminescence immunoblot exposure (Fig. 1B; compare Lane 4 with Lane 2), densitometry of Fig. 1A revealed that 20% more His273 protein was present in the assay than wild-type p53 hybrid protein. These data are consistent with previous work showing that mutant p53 has a reduced affinity for DNA (14). Therefore, the conformation and DNA-binding properties of the hybrid p53 protein are similar to those of full-length protein.

In addition to nonspecific interaction with DNA, wild-type p53 protein exhibits binding to several distinct DNA motifs (13–16). The interaction of the wild-type and mutant hybrid p53 proteins with the p53CON DNA sequence (13) was therefore investigated. The wild-type p53 hybrid bound to the p53CON DNA in an immunoprecipitation assay (Fig. 2A, Lane 2). Protein A did not appear to bind p53CON (Fig. 2A, Lane 1). Sequence-specific binding to p53CON was shown by a competition experiment using a mutated p53CON sequence (M1; GGAACCTACCGGTCAGTC). Mutations were made within regions of the oligonucleotide required for sequence-specific binding (13). Five- to 20-fold excesses of the M1 oligonucleotide failed to compete with 32P-labeled p53CON for binding to the p53 conformational domain (Fig. 2B, Lanes 1–3), whereas p53CON blocked binding of 32P-p53CON (Fig. 2B, Lanes 4–6). Both mutant p53 hybrid proteins bound less efficiently than wild-type proteins to the p53CON DNA (Fig. 2C; compare Lanes 2 and 3 with Lane 1); the His175 mutant bound the least, indicating that interaction of the hybrid protein with p53CON DNA occurred through the p53 sequence. Densitometric and phosphorimager quantitative analysis showed that the His175 hybrid was approximately 70%, and the His273 mutant 55%, less effective than the wild-type p53 hybrid protein in binding to the p53CON DNA sequence (Fig. 2D). Thus, the conformational domain interacts with a specific p53 DNA-binding motif in a specific manner.

Metal ions, most likely Zn2+, play a role in stabilizing the wild-type conformation of p53, possibly by interacting with conserved cysteinyI residues (27) located within the two domains that bind to large T-antigen (19). We examined the ability of wild-type p53 hybrid proteins...
to bind to calf thymus DNA in the presence of the metal chelator OP to determine if there was any role for metal ions in DNA binding. OP inhibited binding of the wild-type p53 hybrid protein to DNA by greater than 90% (Fig. 3; compare Lane 2 with control Lane 1). The inhibition was not due to a nonspecific effect of OP, since pretreatment of p53 hybrid protein with OP followed by its removal by ultrafiltration still resulted in inhibition of DNA binding (data not shown). Binding of p53CON to wild-type p53 hybrid protein was also reduced by greater than 90% in the presence of OP (data not shown).

**Discussion**

A role for sequences within the large T-antigen-binding domain of p53 (amino acids 115–295) in sequence-specific DNA binding was suggested by other workers (24, 25). The results presented here show that both nonspecific and sequence-specific DNA-binding activity resides between amino acids 115 and 295 of p53. The DNA-binding domain may encompass most of this region, since mutations at either amino acid 175 or amino acid 273 altered DNA interaction. Alternatively, a shorter and more defined DNA-binding domain may be present within this region, which loses its affinity for DNA upon changes in conformation induced by mutation or protein binding.

A dependence on structural Zn\(^{2+}\) for stabilization of the wild-type form of p53 implicates metal ions in regulating the conformation of the protein (27). The ability of the conformational domain to interact with DNA was dependent on the presence of metal ions, since a metal chelator dramatically reduced binding to DNA. Based upon the differences in affinity for DNA between wild-type and mutant p53 hybrid proteins and their dependence on metal ions for binding to DNA, tertiary structure is thus an important factor in the interaction of the conformational domain with DNA.

A structural basis can now be envisioned for the inactivation or interference of the DNA binding of p53 by a broad spectrum of mutations within the conformational domain. Mutations affect the interaction with metal ions or cellular proteins and thus disrupt the tertiary structure of the conformational domain required for sequence-specific DNA binding.

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**References**

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