Specific Interaction of p53 with Target Binding Sites Is Determined by DNA Conformation and Is Regulated by the C-terminal Domain*

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Transcriptional activation of p53-regulated genes is initiated by sequence-specific DNA binding of p53 to target binding sites. Regulation of sequence-specific DNA binding is complex and occurs at various levels. We demonstrate that DNA topology is an important parameter for regulating the selective and highly specific interaction of p53 with its target binding sites. Specific binding of wild-type p53 is greatly enhanced when cognate binding sites are present in a non-linear stem-loop conformation. The C-terminal domain plays a key role in regulating the specific interactions of p53 with target binding sites in a DNA conformation-dependent manner. The C-terminal domain is required for binding to target sites in a non-linear DNA conformation in contrast to the strong inhibitory effects of the C terminus on p53 interaction with linear DNA. We propose that selective binding of p53 to various promoters may be determined by the DNA conformation within p53 cognate sites.

A major function of the tumor suppressor p53 is that of a pleiotropic transcription factor controlling the expression of target genes by either activating or inhibiting the activity of p53-responsive promoters. p53 has several DNA binding activities of which sequence-specific DNA binding (SSDB) is the most important because it is indispensable for the transcriptional activity of the protein. The importance of SSDB is highlighted by the fact that the most frequent p53 mutations found in human cancers are located within the DNA binding domain encoding region (2, 3) and abrogate the SSDB of p53. The specific interaction of p53 with target DNA is complex and tightly regulated. Promoter recognition by p53 is determined by the presence of p53-response elements (PREs), which share homology with the consensus sequence 5'-PuPuPuC(A/T)(T/T/AGPyPyPy)-3' (1). C-terminally unmodified p53 seems to be inactive in SSDB; however, modifications at the C terminus, like binding of the C-terminal-specific antibody PAb421, phosphorylation, or deletion of the 30 C-terminal amino acids, strongly enhance SSDB under certain in vitro conditions (4). The data indicate that the C terminus regulates binding of p53 to target DNA, although the underlying mechanisms are not entirely understood. Several models have been proposed to explain the influence of the C-terminal domain on SSDB by p53. The conformation model postulates that the p53 protein exists in two conformationally distinct forms termed latent (for DNA-binding inactive p53) and activated (for DNA-binding active p53). According to this model, the C-terminal domain in latent p53 interacts directly with the core domain and thereby inhibits SSDB of p53 (5). A conformational switch was proposed as the mechanism that relieves the allosteric inhibition by the C terminus and converts p53 from latent into an activated form. However, such a hypothesis is not supported by the results of structural studies that recently demonstrated that the conformation of DNA binding-inactive and DNA binding-active p53 forms is largely identical (6). These findings indicate that the C terminus may regulate SSDB by other mechanisms. The competition model explains the inhibitory effect of the C-terminal domain by its ability to bind DNA unspecifically (7). According to this model, unspecific DNA binding of the C terminus interferes with the sequence-specific interaction with DNA mediated by the core domain. Modifications of the C terminus, such as phosphorylation or binding of PAb421, may displace unspecific bound DNA and thereby facilitate SSDB (7).

Both models suggest that SSDB is inhibited by the C terminus directly (conformation model) or indirectly (competition model), thereby necessitating the activation of p53 for SSDB. However, an alternative view is that SSDB of p53 is not inhibited by the C terminus and therefore does not need activation under more physiological conditions. Espinosa and Emerson (8) observed that the p53 C terminus inhibited SSDB to the p21 promoter only in short naked DNA, but not when the p21 promoter was assembled into chromatin in vitro (8). Moreover, deletion of the C terminus decreased SSDB and, consequently, transactivation of the chromatin-assembled p21 promoter (8). The findings indicate that SSDB of p53 requires a certain level of structural DNA organization, which may be missing in short linear DNA.

Commonly, two identical repeats of the 5'-PuPuPuC(A/T)(T/T/AGPyPyPy)-3' sequence are used in DNA binding assays as a p53 target binding site. However, the comparison of such “optimized” binding sites with functional PREs identified in various p53-regulated promoters reveals that “natural” p53 binding sites differ significantly from the optimized version. Notably, known PREs are never composed of identical decamers.
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Decamers that perfectly conform to the p53 consensus are shown in italic capital letters and are underlined. Bases violating the consensus are shown in small letters. Boldface and underlined sequences correspond to decamers that perfectly conform to the p53 consensus.

| p53 target | PREs |
|------------|------|
| p21 (52)   | GAACATGTCG cAACATGTT |
| pcna (53)  | GAACAGTTCC GGCAATcGgT |
| cyclin G (54) | AGACCTGCCC GAGCTAGGCC |
| msh2 (55) | ATGCTGGCCC GAGCTAGGCC |
| gml (56)  | GGCAGAGTTG GAAGCAAGGCTC |
| mdm2 (57) | AGAACTAGGAGGGCA |
| egf (58)  | AGAAAGAGc GAGCTAGGCC |
| p2xm (59) | GGCAGAGCTT ccCAAAGTGC |
| po26 (60) | GGGCAGGTG AcCAATGCTC |
| bax1 (62) | tGCAGAGTTA AGAACAGTCC |
| puna-BS2 (63) | cTGGAGGGCC |
| mcm10-PRE1 (64) | GGGCTGACGGC |
| mcm10-PRE2 (64) | cAACACTAGG |
| fusa (65) | cACAAGTGTg |
| h-HA-18 (66) | gag GGCAATGcG |
| mck (67)  | GCGAGGTCGt |
| pig5 (68) | cAAACAAGCCac |
| tgf-a (69) | cAACATGCTT |
| ipo (70)  | GTCAGACGGC |
| p292 (71) | cTACAGTg |

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ers (Table I). Furthermore, because of the high frequency of bases deviating from the consensus, PREs exhibit significant sequence diversity (Table I). Typically, only one decamer (half-site) stringently adheres to the consensus, whereas the other decamer(s) deviate from the consensus to a varying extent. In PREs of some p53-regulated genes, all decamers contain bases that violate the consensus (e.g. c-Ha-ras, pig5, fusa1, tgf-a). Contente et al. (11) have recently reported that the p53-dependent translocation of the pig5 gene is critically dependent on the presence of a (TGYCC)₅ pentanucleotide repeat and not on the p53-consensus sequence identified previously (9). The (TGYCC)₅ pentanucleotide repeat is part of a microsatellite sequence and bears only very limited sequence homology to the p53 consensus. Nevertheless, this sequence mediates sequence-specific transactivation of pig5 by p53, whereas the consensus-like binding site from the pig5 promoter appears to be dispensable (9). The sequence variance among PREs is puzzling because it remains unclear how the sequence heterogeneity of PREs is compatible with the required high specificity for their interaction with p53. As an explanation, we and others have proposed that the sequence variance of PREs may be important to provide DNA with structural flexibility, which appears to be an important parameter determining p53 interactions with DNA (8, 10, 11). p53 binding to unusual DNA structures has been demonstrated in studies investigating the involvement of p53 in repair and recombination pathways (12–14). So far, the two modes of p53 interaction with DNA, sequence-specific and DNA structure-dependent, have been studied independently from each other. With regard to SSDB, only the presence of the specific cognate motif has been considered. However, it is likely that the presence of the specific sequence motif is not the only parameter determining specific binding of p53 to its cognate sites, which may also be influenced by structural features of DNA (8, 10, 15).

In this study we analyzed the impact of DNA structure on the sequence-specific interaction of p53 with PREs derived from known p53 regulated promoters and with target binding sites containing variations of the consensus motif. We demonstrate that the presentation of specific binding sites in a non-linear stem-loop conformation determines strong binding of p53 which does not require C-terminal modification by PAb421. Our data demonstrate that the C-terminal domain exerts composite effects on the interaction of p53 with target sites depending on the conformation of the DNA; while inhibiting the specific interaction of p53 with linear DNA, the C terminus is required for the specific binding to stem-loop DNA. Our data may therefore provide a base for understanding the complex regulation of p53 interactions with DNA. With regard to transcriptional activation, selective promoter recognition might be determined by both binding of other proteins at the C terminus of p53 and by structural organization of the DNA within the p53 target binding sites.

EXPERIMENTAL PROCEDURES

Preparation of p53 Binding Sites Presented in Linear or Non-linear DNA Conformation—All DNA substrates were prepared from synthetic oligonucleotides (ODNs) listed in Table II. To obtain the PRE-p21 in a linear form, the PRE-p21(lin/as) DNA was end labeled with T4 polynucleotide kinase and α-²⁵P]ATP and annealed with unlabelled complementary PRE-p21(lin/as) DNA at a molar ratio of 1:1. Non-linear DNA substrates containing short stem-loop structures on one strand were obtained by annealing of two oligonucleotides from which only the shorter, T₃-oligonucleotide was radiolabeled at the 5'-end. The longer oligonucleotides contained 13 bases at the 5'-end which were complementary to 13 bases at the 3'-end of T₃-1 oligonucleotide. The central parts of the longer oligonucleotides were comprised of self-complementary sequences either containing or lacking p53-specific cognate motifs (Table II). Annealing of the T₃-1 ODN with a stem-loop-forming oligonucleotide allowed the formation of a three-way junction structure that contained a stem-loop on one strand. Such DNA structures can be easily distinguished from single-stranded or linear double-stranded DNA molecules in 8% native polyacrylamide gels (shown in Fig. 2A) allowing their purification. Eluted DNA was recovered by ethanol precipitation, resuspended in buffer containing 10 mM Tris (pH 7.8) and 60 mM NaCl, and used in electromobility shift assay (EMSA) or DNase I protection experiments.

Protein Purification—Recombinant p53 proteins expressed in insect cells were isolated as described by Bessard et al. (16) and purified by ion exchange chromatography (FPLC, Amersham Biosciences). EMSA—DNA binding experiments were performed using 50 ng of recombinant p53 proteins in a reaction mixture containing 5 ng of poly(dI-dC) (Amersham Biosciences) and 2 μg of bovine serum albumin in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 50 mM NaCl. After a 20-min preincubation at room temperature, 20,000 cpm of the labeled DNA probe was added, and the incubation was continued for another 25 min. Samples were loaded onto a 4% native polyacrylamide gel and separated by electrophoresis in 10 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 1.25 mM NaOAc, and 8 mM acetic acid.
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| ODNs for linear dsDNA* |
|------------------------|
| **PRE-p21(lin/s)** | 5’- gctctggc GAACATGTCC cAACATGTTg cggctctg-3’ |
| **PRE-p21(lin/as)** | 5’- cagacgcg cAACATGTTg GAACATGTCC gcggagag-3’ |

* ds, double-stranded. Single-stranded ODNs PRE-p21(lin/s) and PRE-p21(lin/as) represent sense and antisense strands of the PRE-p21 in linear conformation.

**RESULTS**

**Wild-type p53 Binds Strongly and Specifically to Target Sites in Nonlinear DNA Conformation**

We selected PREs from the p53-regulated p21, eif-r, and tgf-a promoters to analyze the binding of recombinant human wt p53 protein. The PREs share little homology in sequence and in the number of decamers composing the p53 binding site (Table 1). However, they all exhibit a 2-fold symmetry that allows intrastrand base pairing of self-complementary regions either between different decamers (structures (b) in Fig. 1) or within the same decamer (structures (c)). For example, PRE-p21 has the potential to form two different stem-loop structures. Structure (b) would be formed by both decamers aligned symmetrically against each other; in structure (c) only one decamer would be fully located within stem-loop, and the other

acid at 200 V for 2.5 h at room temperature. After electrophoresis gels were dried and subjected to autoradiography.

**DNAse I Protection Assay**—DNA binding was performed in the same binding buffer as in EMSA. After the binding step, the samples were supplemented with equal volumes of 10 mM CaCl2 and 20 mM MgCl2 solution and treated with 10 units of DNase I (Promega) for 10 min at 37°C. Digestion was stopped by adding an inactivating buffer containing 0.15 M NaOAc, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% SDS, 5 mM EDTA. The 0.1 mg/ml proteinase K, and 0.05 mg/ml yeast DNA. After treatment with phenol-chloroform, labeled DNA was recovered by ethanol precipitation and dried. After resuspension, samples were analyzed by electrophoresis in 20% denaturing gels containing 8M urea. After electrophoresis, the gels were dried and subjected to autoradiography.

**Cross-linking and Immuno blotting of p53 Oligomers**—The oligomeric status of p53 bound to DNA was analyzed by glutaraldehyde cross-linking as described by Wang et al. (17). Briefly, p53 was incubated with radioactively labeled DNA to allow complex formation. The p53-DNA complex was resolved by electrophoresis in a 4% native polyacrylamide gel (TBE buffer, 20°C at room temperature) along with the mixture of p53 samples with 100% p53.

**ODNs for short stem-loop DNAAs**

| T3-1 | 5’- ccggcgtttata gctcttggccaggg gcgcggagag-3’ |
| p21-dec1 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| p21-dec2 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| p21-dec1 + 2 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| EGF-R | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |

**ODNs for a long stem-loop DNA used in electron microscopy**

| Stem-1 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| Stem-2 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| Loop | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |

* a, antisense. b, sense. ds, double-stranded. Single-stranded ODNs PRE-p21(lin/s) and PRE-p21(lin/as) represent sense and antisense strands of the PRE-p21 in linear conformation.

| DNA contexts used for the preparation of DNA substrates in linear or in stem-loop DNA conformation | p53 cognate motives are shown in bold characters. Underlined sequences are complementary to a 12 bp at the 5’-end and 13 bp at the 3’-end of the T3-1 oligonucleotide. |
|-----------------------------------------------|--------------------------------------------------------------------------------|
| **ODNs for linear dsDNA** | **ODNs for short stem-loop DNAAs** |
| **T3-1** | 5’- ccggcgtttata gctcttggccaggg gcgcggagag-3’ |
| **p21-dec1** | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| **p21-dec2** | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| **p21-dec1 + 2** | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| **EGF-R** | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |

**ODNs for a long stem-loop DNA used in electron microscopy**

| Stem-1 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| Stem-2 | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |
| Loop | 5’- ggcgccttaggta cctggctgctct ggtgcccaggg gcggagag-3’ |

* a, antisense. b, sense. ds, double-stranded. Single-stranded ODNs PRE-p21(lin/s) and PRE-p21(lin/as) represent sense and antisense strands of the PRE-p21 in linear conformation.

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FIG. 1. Putative stem-loop structure formation by symmetric PREs of the p53-regulated p21, TGF-α, and EGF-R genes. Individual decamers (designated as dec) composing the full PRE in linear form of DNA (a) are separated by a slash. Bases that violate the consensus rule are shown in lowercase letters. Symmetric PREs have the potential to form various secondary structures (DNA isoforms (b) and (c)).
decamer would mostly be located at the junction of the structure. We reconstituted both types of stem-loops that may be formed by either two or by one decamer from PRE-p21 by using an approach commonly used for the preparation of cruciform DNA and stem-loop structures (21, 22). Briefly, two ODNs of different length are annealed, with only the longer ODN containing the PRE-specific sequence. A shorter oligonucleotide is complementary only to the PRE-flanking sequences in the longer oligonucleotide but is lacking the PRE-complementary sequence. Annealing of the longer and the shorter oligonucleo-
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We analyzed binding of p53 to linear or to stem-loop DNA by EMSA using recombinant human wt p53 protein purified from insect cells (Fig. 2B). The results showed that p53 did not bind to PRE-p21 when it was present in linear DNA conformation unless antibody PAb421, directed against a C-terminal epitope of p53, was added (Fig. 2C, compare lanes 5 and 6). In contrast, when two decamers of PRE-p21 formed a stem-loop, p53 bound strongly in the absence of PAb421 (Fig. 2C, lane 2). Moreover, in contrast to the enhancement of p53 binding to linear PRE-p21, PAb421 inhibited binding to stem-loop DNA (compare lanes 2 and 3). Complex formation with PRE-p21 in stem-loop conformation was strictly dependent on the p53 concentration. As shown in Fig. 2D, nearly all DNA probe representing stem-loop PRE-p21 was bound in the presence of 100 ng of p53 in the absence of PAb421, whereas with linear PRE-p21, no binding could be detected under the same conditions (compare lanes 10 and 20). Surprisingly, stem-loop DNA formed by only one decamer of the PRE-p21 was also bound strongly by p53. The binding did not require PAb421 (Fig. 2E, lane 2); in contrast, addition of PAb421 led to a significant inhibition of p53 binding (lane 3). The same decamer in linear DNA form was not bound by p53 at all, regardless of the presence or absence of PAb421 (Fig. 2E, lanes 4–6). The results demonstrate that the presence of only one specific decamer may already be sufficient for strong binding of p53 provided that it is presented in a stem-loop and not in a linear conformation of DNA. A similar binding pattern was observed with stem-loop structures containing p53 cognate motifs derived from PRE-TGF-α and PRE-EGF-R (Fig. 2F), indicating that the interaction of p53 with target binding sites in non-linear DNA conformation is a general phenomenon, and not a peculiar feature of PRE-p21.

### p53 Binds Stem-Loop DNA as a Tetrramer

Transcriptionally competent p53 must be present as a tetramer (23, 24). Therefore, the question of whether or not p53 binds to stem-loop DNA in its transcriptionally active (tetrameric) form is of physiological relevance. We analyzed the oligomerization status of p53 bound to stem-loop DNA by using an approach optimized for the analysis of the oligomeric status of p53 in complex with DNA (17). The results show that in complex with stem-loop DNA (Fig. 3, lanes 3 and 3′) p53 was present predominantly as a tetramer (lane 3 in the protein blot panel). We conclude that the interaction of tetrameric p53 with linear and stem-loop DNA is determined by different modes of DNA binding: p53 tetramer binds readily to stem-loop DNA, but its binding to linear DNA requires modification at the C terminus such as interactions with other proteins, which mimic binding of PAb421.

### Sequence-specific and Structure-specific DNA Recognition Determines the Strong Interaction of p53 with Target Sites in Stem-Loop Conformation

An important question arising from the high affinity interaction of p53 with stem-loop DNA is whether stem-loop binding is sequence-specific. To address this question we prepared a set of stem-loops containing either a perfect consensus decamer derived from the RGC-sequence (CON-loop) or its mutated versions in which either one or both highly conserved bases, C₄ or G₄, were mutated. Specific decamer in CON-loop DNA is derived from the RGC sequence (25), which is capable of mediating p53-dependent transactivation and often used as a model p53 binding site in DNA binding studies. The results show that mutation of the conserved bases reduced binding of p53 (Fig. 4A), indicating that the strong interaction of p53 with stem-loop DNA requires the presence of the p53 cognate motif. Specifically, maintenance of both conserved bases appears crucial to determine strong binding of p53 to stem-loop DNA.

All stem-loop structures tested in our experiments contained single-stranded DNA within the loop. Therefore, the possibility remained that the strong interaction of p53 with stem-loop DNA might have been determined by its ability to bind single-stranded DNA in a sequence-independent fashion (26). Accordingly, enhanced binding of p53 to stem-loop DNA containing the consensus sequence would result from the presence of single-stranded DNA within the loop rather than from specific sequence recognition. If this would be the case, the presence of single-stranded DNA should be the determining parameter for the interaction of p53 with stem-loop DNA, whereas the presence of the specific sequence motif would rather have a modulating effect. We analyzed binding of p53 to various stem-loop structures (depicted in Fig. 4B) by DNase I protection assay, which allows a precise mapping of the protein-bound regions in the target DNA. In the absence of p53, DNase I treatment resulted in a characteristic cleavage pattern at hypersensitive bases, which was consistent with the presence of a stem-loop structure (Fig. 4B, lanes 2 and 5). In the presence of p53, the loop and part of the stem were protected in CON-loop DNA, in which the loop is formed by a perfect consensus decamer (Fig. 4B, lane 3). Mutation of the conserved bases of the consensus abrogated binding to the loop in MUT-loop DNA (Fig. 4B, lane 6), indicating that the presence of a specific sequence, but not the presence of single-stranded DNA as such, determined the
Fig. 4. Strong interaction of p53 with stem-loop structures is determined by sequence- and structure-specific recognition of DNA. A, binding of p53 to stem-loops containing either a perfect (lanes 1–3) or mutated (lanes 4–6, 7–9, and 10–12) consensus decamer. Loop regions differing in four stem-loops are depicted underneath the gel image. The consensus motif is typed in capital letters; mutated conserved bases are circled. Arrows indicate p53-specific complexes formed with CON-loop DNA. Entire CON-loop and MUT-loop stem-loops are depicted in B. B and C, mapping of p53-bound regions within stem-loop DNAs by DNase I protection. Stem-loop structures are illustrated below the corresponding gel.

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binding of p53 to the loop region. However, binding to the stem region of stem-loop structures was maintained in CON-loop and MUT-loop DNA (regions designated stem), regardless of the presence (CON-loop) or absence (MUT-loop) of the consensus motif. In agreement with our EMSA experiments, the results showed that p53 binds to the stem MUT-loop DNA, albeit much weaker compared with CON-loop DNA (Fig. 4A). In addition, the data demonstrate that p53 binds at two independent sites on CON-loop DNA: the consensus motif and at the stem.

We next wanted to identify the parameters that determine stem recognition by p53. A common feature of all stem-loop structures bound by p53 was the presence of unpaired mismatched bases in the stem. The C-terminal and the DNA binding domains of p53 bind mismatched bases in a sequence-independent fashion (27, 28). Therefore, we assumed that mismatched bases might be important structural marks determining the recognition of the stem in stem-loop DNA. To test this possibility, we compared the binding of p53 to stem-loops formed by scrambled sequences and termed SCR-loop and SCR-paired. Both DNAs contain an identical loop that shares no homology to the consensus, but they differ in their stem region: in SCR-paired DNA the stem is fully paired, whereas SCR-loop DNA contains mismatched bases in the stem.

DNase I protection experiments showed that p53 slightly protected the stem in SCR-loop DNA (Fig. 4C, lanes 1–3), whereas no protection was observed in SCR-paired DNA, in which the stem is fully paired (Fig. 4C, lanes 4–6). The data suggest that the presence of mismatched bases determines weak and sequence-unspecific binding of p53 at the stem of SCR-loop DNA. EMSA experiments confirmed this conclusion because they also showed weak but reproducible binding of p53 to SCR-loop but not to SCR-paired DNA (Fig. 4D).

Therefore, we conclude that mismatched bases might be important structural marks determining the recognition of the stem in stem-loop DNA. To test this assumption, we compared the binding of p53 to CON-loop and CON-paired DNA, which both contain a consensus decamer within the loop but differ in the presence of mismatched bases in the stem. EMSA showed that p53 bound strongly to CON-loop but not to CON-paired DNA lacking mismatched bases in the stem (Fig. 4E). We conclude that structural recognition of mismatched bases in the stem strongly enhances the overall binding of p53 to stem-loop DNA mediated by sequence-specific recognition of the consensus motif.

The C-terminal Domain Positively Regulates Specific Interaction of p53 with Binding Sites in Stem-Loop DNA

Analysis of p53 Deletion Mutant Binding by EMSA—Our EMSA experiments demonstrated that the C terminus plays an important role in the regulation of specific binding to stem-loop DNA. Although strongly enhancing binding of p53 to linear DNA, the C-terminal antibody PAb421 inhibited p53 binding to stem-loops. The inhibition was specific for PAb421 because N-terminal specific antibody DO-1 only retarded the migration of the complex but did not inhibit binding (Fig. 5A).

To characterize further the role of the C-terminal domain in stem-loop DNA binding, we analyzed the binding of p53 deletion mutants to stem-loop DNA. Confirming previously reported observations, binding of the C-terminally truncated p53-Δ30 protein to linear PRE-p21 DNA was much stronger compared with full-length p53 (Fig. 5B, compare lanes 2 and 4 in the lower panel). In striking contrast, binding of p53-Δ30 was abolished when PRE-p21 was presented in a stem-loop conformation (compare lanes 2 and 4 in the upper panel).

Similar patterns were observed with PRE-TGF-α and PRE-EGF-R stem-loop DNAs (data not shown). Oligomerization-deficient p53-Δ70 did not bind to linear or stem-loop forms of PRE-p21 (lanes 5 and 5′). We next analyzed stem-loop DNA binding of the p53-ΔDBD protein, which contains the N- and the C-terminal domains but lacks the DNA binding domain (DBD, amino acid residues 110–280). The results showed that p53-ΔDBD did not bind to PRE-p21 stem-loop DNA at all (lanes 6), indicating that stem-loop binding requires the DBD. In addition, the data show that binding of the C terminus to mismatched bases as such is not sufficient to determine binding of p53 to stem-loop DNA in the absence of the DBD.

Analysis of p53 Deletion Mutant Binding by Electron Microscopy—Several groups, including ours, reported that the use of different assays might greatly influence the outcome of experiments analyzing the interactions of p53 with DNA. For example, apparently discrepant results were obtained when DNA binding of p53 was analyzed using different methods such as EMSA or DNase protection assay (8, 11, 29). In addition, the DNA size strongly influences the specific interaction with target binding sites because different effects of C-terminal deletion on DNA binding by p53 were observed with short or large DNA templates (8).

We examined binding of p53 to the consensus binding site present in linear and in stem-loop DNA by electron microscopy. The approach allows the analysis of protein-DNA interactions over a wide range of DNA length. A 474-bp fragment, containing a 20-bp consensus binding site located ~40% from one end of the DNA, was used as a linear binding substrate (19). Consensus-containing stem-loop DNA with an elongated 64-bp stem was prepared using the strategy depicted in Fig. 6A. Binding of full-length and various deletion mutant p53 proteins was analyzed as described under “Experimental Procedures.”

In accordance with previously published results (19), we observed that the isolated DBD (p53-DBD, amino acid residues 94–312) formed a globular complex at the consensus binding site in linear DNA (Fig. 6B, upper panel, a–c). Similarly, the C-terminally truncated p53-Δ30 bound to the consensus binding site in linear DNA (upper panel, d–f) in agreement with EMSA experiments. With stem-loop DNA, both p53-DBD and full-length wt p53 formed a complex located asymmetrically at one side of the stem-loop (lower panel, b–f and g–i, respectively). The asymmetric location of the p53-DBD complexes most likely reflects binding of p53 at the consensus decamer located within the loop of the stem-loop DNA. The conclusion is supported further by the fact that neither p53-DBD nor full-length wt p53 formed a complex at DNA ends (see upper panel). p53-DBD protein that lacks the C and the N terminus formed...
Role of the C-terminal domain in regulation of p53 interaction with structurally distinct target binding sites analyzed by EMSA. A, CON-loop DNA was incubated with 50 ng of p53 in the absence (lane 1) or in the presence of the C-terminal PAb421 (lane 2) or the N-terminal DO-1 (lane 3) antibodies. B, binding of p53 deletion mutants to PRE-p21 in stem-loop (upper panel) or in linear (lower panel) conformation. Amino acid residues 360–393 (regulatory C-terminal domain) or 110–280 (DNA binding domain) are deleted in p53-Δ30 and p53-ΔDBD mutants, respectively. The p53-Δ70 mutant is lacking both the C-terminal and the oligomerization domains (amino acid residues 320–393). In all lanes except lane 1, DNA was incubated with 50 ng of the indicated p53 protein. Control lanes 1 and 1’ show DNA in the absence of p53 or PAb421. In lanes 3 and 3’, binding mixtures contained PAb421.
FIG. 6. Analysis of interactions of p53 with linear and stem-loop DNA by electron microscopy. A, preparation of stem-loop DNA for electron microscopy (for a detailed description, see “Experimental Procedures”). 5'-End phosphorylated oligonucleotides are marked with P. DNA III resulting after step 5 was used for electron microscopy. B, the upper panel represents images of the complexes formed with a linear 474-bp DNA fragment bearing the p53 recognition sequence (19). Complexes were formed by the DNA binding domain p53-DBD (a–c) or by p53-Δ30 mutant (d–f) at molar protein:DNA ratio of ~4 for p53-DBD and ~3.5 for p53-Δ30 (calculated for p53 tetramer). The arrows indicate p53-DBD and p53-Δ30 bound at the specific cognate motif. The lower panel represents images of the complexes formed with stem-loop DNA III. Complexes were formed by the DNA binding domain p53-DBD (b–f) or by wt p53 (g–i) at molar protein:DNA ratio ~0.5–1.0 and ~2.5 (calculated for p53 tetramer), respectively. a, DNA III alone.
decamer and that the cooperative interaction between DNA-bound dimers may further stabilize the p53-DNA complex (31). However, the higher stability of the complex was only observed in the presence of PAb421, whereas in the absence of PAb421 p53 did not bind, regardless of the number of decamers. Our finding that a single decamer in stem-loop DNA conformation is sufficient to mediate high affinity binding of p53 in the absence of PAb421 raises the question of why PREs should contain more than one decamer to be recognized by p53. As shown here, p53 bound to a single decamer only when this decamer was present in the loop of a stem-loop structure. Within PREs, the potential to form a secondary structure that is compatible with high affinity binding of p53 depends on the presence of self-complementary regions. The structural plasticity of a single-decameric PRE would be much lower than that of a multidecameric PRE in which the potential to undergo structural changes is provided by complementary regions shared by different decamers (see Fig. 1). Therefore, the multidecameric composition of PREs provides a structurally flexible context that allows PREs to adopt different conformations. Thereby, individual decamers could be converted from poor binding sites into structurally favorable sites strongly bound by p53. Such a mechanism could also explain why in most of PREs only one decamer strictly conforms to the consensus, whereas the others deviate to a variable degree.

We show that the presence of mismatched bases is required for the strong interaction of p53 with target binding sites in stem-loop conformation. Our analysis of DNA binding by various p53 deletion mutants demonstrated that the C-terminally truncated p53 protein bound to linear DNA but was unable to bind to stem-loop DNA. The requirement of the C terminus for stem-loop DNA binding can be explained by the intrinsic affinity of the C terminus to mismatched bases in DNA as demonstrated previously (27, 28). The requirement of the C terminus for p53 to bind to stem-loop DNA was alleviated when the N-terminal domain was also absent, as evidenced by electron microscopy. The observation suggests that the C terminus may only be required for specific interaction of p53 with stem-loop DNA in the presence of the N-terminal domain. It was proposed that the N terminus negatively regulates DNA binding of p53 by increasing the dissociation of the p53-DNA complex (29). Our results are compatible with this hypothesis and suggest that the C- and the N-terminal domains may regulate structure-specific interactions of p53 with target DNA in an interdependent fashion.

An important question relating to the biological relevance of p53 binding to PRE in non-B DNA conformation is whether, and if so, under which physiological conditions, PREs might undergo structural transitions. Thermodynamically, the formation of secondary structures is unfavorable in fully paired double-stranded DNA. However, during actively ongoing transcriptions or replication, DNA strands must be locally separated. Under such conditions, the energetic constraints that impede secondary structure formation in fully paired DNA will be obviated, thereby promoting the formation of secondary DNA structures by intrastrand pairing. Indeed, active replication and transcription are accompanied by an increased formation of unusual DNA structures that have been detected in vivo (32–35). Formation of such structures has been associated with regulation of transcription, replication, and recombination (36, 37; for review, see Refs. 38 and 39).

Several lines of evidence strongly support the idea that the specific interaction of p53 with target binding sites is dependent on DNA structure. First, transcriptionally active p53 binds to target binding sites in highly organized chromatin DNA, whereas it does not bind to linear DNA (8). Second, PREs possess features such as repetitiveness and internal symmetry, which are characteristic for structurally flexible DNA sequences. Third, p53 binds to various target binding sites with different efficiency as observed in vitro (40) and in vivo (30, 41), but these differences do not correlate with the degree of conformation to the consensus. Fourth, the specific interaction of p53 with target binding sites is enhanced by the high mobility group (HMG) family member, HMG1 protein (42), which influences the helical structure of DNA and binds to unusual structures such as cruciform DNA (21, 43, 44). Regulation of protein-DNA interactions by DNA topology is not unique for p53 and resembles the specific interaction with target sites of other transcription factors, e.g. CREB (45, 46). Specific DNA binding of the CREB protein is also enhanced by the formation of stem-loop structures within its binding sites, and the presence of mismatched bases in the stem is required for CREB binding (46).

A plethora of genes with different functions is regulated by p53. Transcriptional regulation of various target genes by p53 must occur in a strictly coordinated manner to avoid the simultaneous triggering of conflicting pathways. The different modes of interaction of p53 with target sites in linear or non-linear DNA conformation represent a plausible mechanism by which p53-regulated promoters could be recognized in a selective fashion. Binding of p53 to various promoters thus would depend on the DNA topology of p53 cognate sites and could be fine tuned by the specific interaction of p53 with DNA topologically modulating proteins that either, like HMG1 (42), stabilize or, like topoisomerases (47, 48) may resolve unusual DNA structures. Structural transitions in DNA mostly occur during the S phase of the cell cycle (32, 49, 50). Our hypothesis that structural transitions in PREs may determine the selective promotor recognition by p53 would predict that p53 binding to some but not all promoters may depend on DNA replication. Indeed, Gottfried et al. (51) recently reported that transcriptional activation by p53 of some, but not all promoters is selectively inhibited when DNA synthesis is blocked. The DNA structure-selective interaction of p53 with target binding sites, as demonstrated here, might provide a new understanding as to how selective promoter recognition by p53 could be achieved.

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