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p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain

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ABSTRACT

We have previously reported that wild-type p53 can bind single-stranded (ss) DNA ends and catalyze renaturation of ss complementary DNA molecules. Here we demonstrate that p53 can also bind to internal segments of ss DNA molecules via a binding site (internal DNA site) distinct from the binding site for DNA ends (DNA end site). Using p53 deletion mutants, the internal DNA site was mapped to the central region (residues 99–307), while the DNA end site was mapped to the C-terminal domain (residues 320–393) of the p53 protein. The internal DNA site can be activated by the binding of ss DNA ends to the DNA end site. The C-terminal domain alone was sufficient to catalyze DNA renaturation, although the central domain was also involved in promotion of renaturation by the full-length protein. Our results suggest that the interaction of the C-terminal tail of p53 with ss DNA ends generated by DNA damage *in vivo* may lead to activation of non-specific ss DNA binding by the central domain of p53.

INTRODUCTION

Point mutation or deletion of the p53 gene is the most common genetic change in human cancer (1,2), indicating that loss of wild-type p53 function is a critical step in tumorigenesis. In keeping with this notion, p53-deficient mice are prone to the development of a variety of tumors at high frequency (3). Work in several laboratories has implicated p53 in the cellular response to DNA damage (4–8). p53 is expressed at low levels under normal circumstances. However, p53 protein levels rise in cells that have suffered DNA damage. This causes growth arrest in the G1 phase of the cell cycle, allowing DNA repair prior to cell division, or elimination of cells with extensive DNA damage by apoptosis. Cells with deficient wild-type p53 function fail to carry out either of these functions. As a result, they will continue to replicate their DNA and divide in the presence of damaged DNA, leading to the accumulation of mutations and selection of clones with more malignant properties.

Wild-type p53 binds sequence-specifically to DNA (9,10) and stimulates transcription from a downstream promoter (11). Most mutant forms of p53 lack specific DNA binding activity. Among the several genes known to be transactivated by wild-type p53, the WAF1/CIP1 gene is likely to be a critical downstream effector in a p53-dependent growth control pathway (12–14). The specific DNA binding activity resides in the central core domain of the p53 molecule (15–18). Analysis of the crystal structure of a complex containing this p53 domain (amino acid residues 102–292) and a specific DNA binding motif has demonstrated that the core domain consists of a β sandwhich that functions as a scaffold for three loop structures, and that residues within two of these loops interact directly with DNA. Amino acid residues frequently mutated in human tumors are located in the regions of the core domain that interact with DNA, further supporting the view that specific DNA binding is essential for p53-mediated tumor suppression (19).

We and others have shown that wild-type p53 can promote renaturation of complementary single-stranded (ss) DNA and RNA (20–22), and catalyze DNA and RNA strand transfer (21,20). We also found that the p53 protein binds preferentially to ss DNA ends (21). On the basis of these findings, we proposed that p53 could play a direct role in DNA repair by binding ss DNA ends that appear after DNA damage and promote their joining.

Here we have further analyzed the interaction of p53 with ss DNA. We demonstrate that p53 can bind to internal portions of ss DNA in a sequence-independent fashion via a binding site distinct from the binding site for ss DNA ends. Using p53 deletion mutants, we have mapped the internal DNA binding site to the central domain and the DNA end site to the C-terminal domain of p53. Electron microscopy allowed direct visualization of the joining of an ss DNA end and an internal region of ss DNA by the p53 protein.

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MATERIALS AND METHODS

Plasmids and proteins

pCAT-Promoter was from Promega, WN. pCAT1 carries a 0.2 kb human genomic DNA fragment cloned in the *Bgl*II site of pCAT-Promoter. The plasmids encoding human wild-type p53 and GST–wild-type and mutant p53 fusion proteins have been described elsewhere (21,23). Plasmids encoding GST–p53 deletion mutant proteins containing the N-terminal domain (residues 1–100) or the middle domain (residues 99–307) of p53 were constructed by PCR amplification of human wild-type p53 cDNA using appropriate primers. The GST–C2 plasmid, containing the C-terminal domain of p53 (residues 320–393), was provided by Dr Thomas Shenk, Princeton University. Proteins were produced in *E.coli* and purified as outlined (23).

Western blot analysis

Twenty μg of bacterially produced GST–wild-type p53 protein or GST–p53 deletion mutants containing the N-terminal, middle or C-terminal domains as indicated above were run on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Biorad, CA). The membranes were probed with monoclonal antibodies that recognize the N-terminal region of p53 (DO7; residues 19–25), the middle domain (PAb240; residues 212–217), and the C-terminal domain (PAb421; residues 373–381). Antibodies in the form of ascites or purified immunoglobulin were diluted 1:500 in blocking buffer (23). Proteins were visualized using the ECL method according to the instructions of the manufacturer (Amersham, UK).

Oligonucleotides and DNA

Oligonucleotides were synthesized on a Gene Assembler Plus synthesizer (Pharmacia) and purified by polyacrylamide gel electrophoresis. Their sequences have been published elsewhere (oligonucleotides N1, N2, N3 and N4) (21) or are shown below. N5 is a mutant fragment of the human immunodeficiency virus enhancer (5′-GTGATCCTGGAAAGTGAATAGCGGAA AGTGAATGATC-3′, [–] strand, 37 nt) (24); N8 is an oligonucleotide with random sequence, 5′-CAGGTCAGTTCAGCG-GATCCTGTCG(n26)GAGGCGAATTCAGTGCAACTGCAG C-3′ (76 nt). HD3 is a 462 bp fragment from the 5′ end of the dopamine D3 receptor gene (21). The 1150 bp DN fragment, derived from nt 676–1826 of the human prodynorphin gene (25), was obtained by PCR amplification of genomic DNA using appropriate primers. *Escherichia coli* ss and ds DNA (4–15 kb) were obtained from Sigma, MO. None of the DNA oligonucleotides and DNA fragments used contained a p53 consensus binding motif.

DNA renaturation and band shift assays

Proteins were assayed for DNA binding and ability to catalyze renaturation as previously described (21).

Electron microscopy

pUC18 or pCAT1 plasmid DNA (60 ng), digested with *Pvu*II or *Bgl*II, was heat-denatured and incubated with 20 ng of bacterially produced p53 or GST–p53 fusion proteins in 30 μl of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT for 30 min at 37°C. Unfixed samples or samples fixed in 0.1% glutaraldehyde were spread using benzylalkyldimethylammonium chloride (BAC) (26) or the Kleinschmidt method (27) as described (21). For the BAC procedure, 4 μl samples were diluted with 20 vol of 0.2% BAC in 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT. A 20 μl sample was pipetted onto the surface of a 50 μl droplet of double distilled water placed on Parafilm. After 2–5 min, a carbon-coated grid was allowed to briefly touch the surface of the droplet. The grids were then washed in water for a few minutes, dehydrated in ethanol, and air-dried. Rotary shadowing was performed with platinum–palladium at an angle of 7°. Micrographs were taken in a JEOL TEM-SCAN 100-CX electron microscope at 60 keV. In order to calculate the proportion of molecules that had bound p53, at least 500 molecules were analyzed for each sample.

RESULTS

Band shift analysis of p53 binding to long DNA fragments

We previously showed that p53 binds to different short ss DNA oligonucleotides in a sequence-independent manner (21). Short ss DNA oligonucleotides, but not longer ss DNA fragments, competed efficiently with a short labeled probe for p53 binding, indicating that short ss DNA fragments and/or DNA ends were the binding targets for p53 in these experiments. Here we examined the ability of the p53 protein to interact with longer DNA molecules using the 462 nt HD3, the 1150 nt DN, and the 23 kb λ *Hin*dIII DNA fragments as labeled probes. Incubation of the labeled ds or ss HD3, DN and λ DNA fragments with the wild-type p53 protein resulted in the formation of large complexes or aggregates that did not enter polyacrylamide gels (labeled HD3, Fig. 1A, lanes 2 and 5; labeled DN, Fig. 1C, lane 12) or agarose gels (labeled 23 kb λ *Hin*dIII DNA fragment, data not shown). Both the ds and ss DNA–p53 protein complexes were dissociated by addition of stop solution (21) containing SDS and EDTA (Fig. 1A, lanes 3 and 6). Incubation of GST–wild-type p53 with labeled ss HD3 DNA also gave rise to large DNA–protein complexes. Complex formation was dependent on protein concentration (Fig. 1A, lanes 8, 12 and 17). In the presence of 1 ng of GST–wild-type p53, the protein:DNA ratio corresponds to ~1.6 molecules of protein per molecule of ss HD3 DNA, or 1 molecule of protein per 290 nucleotides. The GST protein alone (lane 13) did not give rise to any complexes. The GST–mutant p53 proteins carrying amino acid substitutions Glu213 (lanes 9 and 14), Ile237 (lanes 10 and 15), and Tyr238 (lanes 11 and 16) were ~10-fold less potent than the GST–wild-type p53 protein in forming retarded DNA–protein complexes that did not enter the gel.

Non-labeled ss *E.coli* DNA efficiently competed for binding of the labeled ss HD3 DNA to the wild-type p53 and GST–wild-type p53 fusion proteins (Fig. 1B, lanes 4–6 and 13–15), as shown by the fact that the labeled DNA now entered the gel (lanes 6 and 15). *Escherichia coli* DNA in ds form (lanes 2–3) competed for p53 binding 5- to 20-fold less efficiently than ss *E.coli* DNA. The ss HD3 DNA fragment itself (lanes 9 and 10) also competed for p53 binding. In contrast, the ds HD3 DNA fragment (lanes 7 and 8) demonstrated weak or no competition with the labeled ss HD3 DNA fragment for binding. The short oligonucleotides N4 (37 nt; lanes 16 and 17) and N8 (76 nt; lanes 18–20) were 1000- and

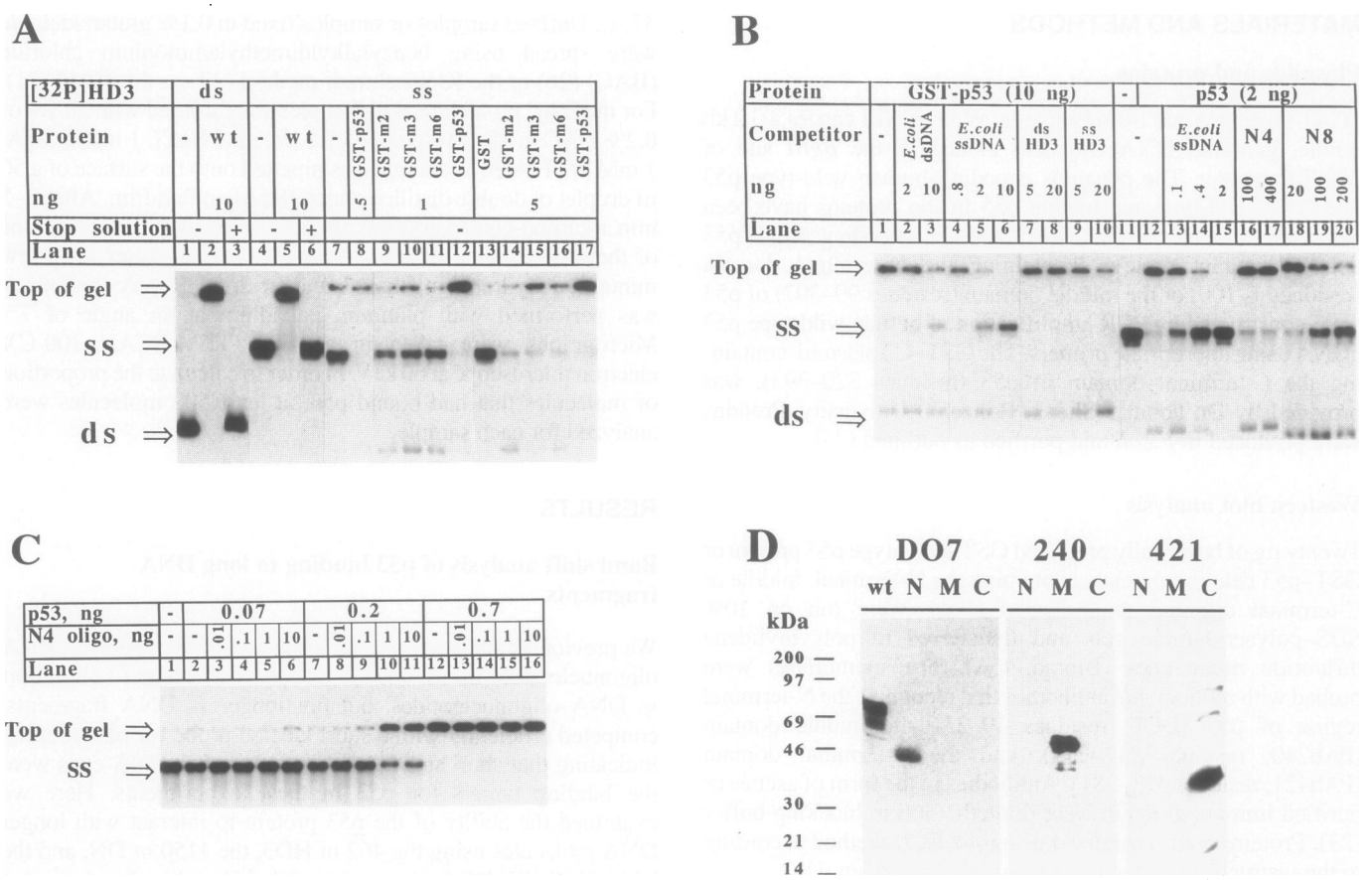

Figure 1. Interaction of p53 with long DNA fragments. (**A**) Band shift analysis of the binding of wild-type p53 (lanes 2, 3, 5 and 6), GST–wild-type p53 (lanes 8, 12 and 17), GST–point mutant p53 fusion proteins carrying amino acid substitutions Arg213 to Gln (m2), Met237 to Ile (m3) or Cys238 to Tyr (m6) (lanes 9–11 and 14–16), and GST alone (lane 13) to the ^{32}P-labeled ss HD3 fragment. In lanes 3 and 6, the reaction mixture was treated with stop solution. (**B**) Retardation of labeled DNA by the GST–wild-type p53 fusion protein (lanes 1–10) or wild-type p53 protein (lanes 12–20) in the presence of ds and ss *E.coli* or HD3 DNA or the N4 or N8 oligonucleotides. (**C**) Retardation of the labeled 1150 nt DN DNA fragment by 0.07, 0.2 or 0.7 ng wild-type p53 in the presence of different concentrations of the N4 oligonucleotide. The ds (A, lanes 1–3) or denaturated (A, lanes 4–17; B and C) ^{32}P-labeled DNA fragments (1.2 ng HD3 in A and B, 0.8 ng DN fragment in C) were incubated with protein and analyzed on non-denaturing 5% polyacrylamide gels. Arrows indicate the positions of ss and ds DNA and the retarded complexes at the top of the gel. The long ds DNA fragments migrated faster than the long ss DNA fragments.

500-fold less efficient, respectively, than ss *E.coli* DNA as competitors. Similar results were obtained with the N5 oligonucleotide (37 nt; data not shown). Thus, the p53 protein can form high molecular weight complexes with long DNA molecules. This binding is competed for by long ss DNA at low concentrations, but not by short oligonucleotides, even at high concentrations. These results suggest that the p53 molecule has a distinct binding site for internal ss DNA segments in addition to the binding site for ss DNA ends.

Analysis of wild-type p53–DNA complexes by electron microscopy

The interaction between full-length wild-type p53 and ss DNA was also studied by electron microscopy (Fig. 2). Approximately 50% of all DNA molecules scored were found in DNA–protein complexes consisting of a few (≤4) DNA molecules (A–I). Most of the remaining DNA molecules were found in larger DNA–protein aggregates (K and L), although rare ss DNA fragments that did not bind p53 were also seen (not shown). In agreement with our previous experiments (21), p53 did not interact efficiently with ds DNA (data not shown).

Four types of ss DNA–protein structures were discernable among the complexes containing a few DNA molecules (Fig. 2). The first type of complex, constituting 45–50% of all complexes, contained p53 bound to the ends of shorter (0.32 kb) or longer (2.4 kb) ss DNA molecules (Fig. 2C, F and I). This type of complex has previously been described by us (21). The second type of complex (30% of all complexes) contained predominantly long DNA fragments that formed small (Fig. 2A–C) or large (Fig. 2D) loops with p53 molecules situated at the forks of the loops. These loop structures are most likely due to the binding of p53 to both the ss DNA end and an internal segment of a DNA molecule. The presence of DNA loops of different sizes may indicate that the p53–DNA end complex was able to translocate along the DNA strand. The second DNA end was often bound to another p53 molecule (Fig. 2C). The third type of nucleoprotein complex (10% of all complexes) contained two to three long or short ss DNA fragments joined end-to-end with p53 molecules located at the junctions (Fig. 2E and F). Such complexes are probably formed due to p53 binding and joining of two ss DNA ends. The protein complexes located at the junctions were larger than the complexes bound to DNA ends, suggesting that more than one

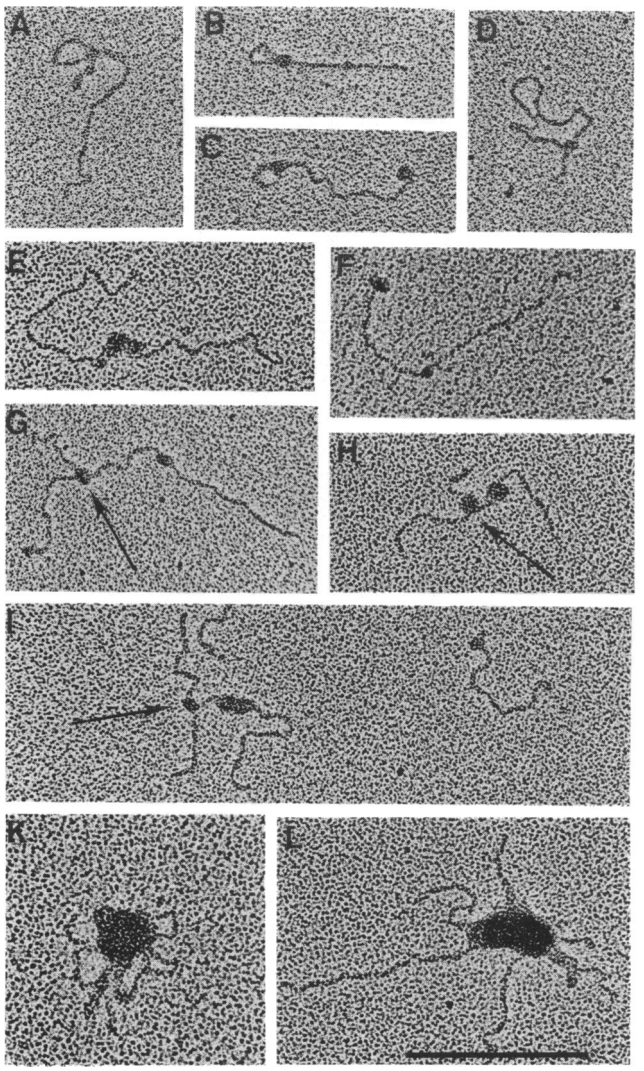

Figure 2. Electron micrographs of p53–ss DNA complexes. Bacterially produced p53 was incubated with denaturated pUC18 DNA fragments (2378 and 322 bp) generated by digestion with *Pvu*II. (**A–D**) Long ss DNA fragments with small (A–C) or large (D) loops. The p53 molecules are located at the junction of the ss DNA end and an internal segment of the same DNA molecule. The second DNA end often bound to p53 as well (C). (**E** and **F**) Complexes with intermolecular ss DNA end-to-end joints formed in the presence of p53. The protein knobs are located at the DNA junctions and are sometimes larger in size than the p53 molecules bound to DNA ends. (**G–I**) Complexes with intermolecular ss DNA end–internal ss DNA joints formed in the presence of p53. Three free DNA ends are clearly seen in each complex. Protein knobs are located at ss DNA forks as well as in internal regions of DNA molecules. In the latter case, the length of DNA molecules suggests that the p53 molecules are most likely located at the junction of two different DNA molecules. (**K** and **L**) Large DNA–protein complexes probably containing multiple p53 and DNA molecules. Arrows indicate the DNA end–internal DNA joints with p53 molecules. Bar represents 250 nm.

p53 molecule is involved in formation of the DNA end-to-end junctions. The fourth type of structure (10% of all complexes) included the intermolecular complexes with a DNA end of one molecule joined to the internal segment of another DNA molecule (indicated by arrows in Fig. 2G, H and I). p53 molecules were found at these junctions, suggesting that a p53 molecule or oligomer can bind to both a ss DNA end of one DNA fragment and an internal region of another DNA fragment. The same results were obtained with both bacterially produced wild-type p53 and GST–wild-type p53 fusion proteins.

Mapping the p53 domains that bind ss DNA ends and internal DNA segments

In order to identify which regions of the p53 protein are important for binding ends or internal segments of ss DNA molecules, the N-terminal (residues 1–100), middle (residues 99–307) and C-terminal (residues 320–393) p53 domains were expressed as GST fusion proteins and analyzed in the band shift assay (Fig. 3). Incubation of GST–full-length wild-type p53 or the GST fusion protein containing the C-terminal p53 domain with a short labeled ss oligonucleotide (N1; 37 nt) produced a retarded complex (Fig. 3A, lanes 3, 4 and 6) in our band shift assay, whereas the GST fusion protein containing the N-terminal p53 domain (lane 1) or GST alone (data not shown) did not bind DNA. The GST fusion protein containing the middle p53 domain had weak affinity for the short oligonucleotide (lane 2).

As can be seen in Figure 3A, lanes 8 and 9, the unlabeled N1 oligonucleotide (37 nt) competed efficiently with labeled N1 for binding to the C-terminal domain. The ability of long ss *E.coli* DNA to compete for p53 binding was 5- to 10-fold lower (compare lanes 8 and 9 with 10 and 11). This result indicates that the binding site for ss DNA ends resides in the C-terminal domain of p53 (residues 320–393).

The proteins representing different p53 domains were also separately incubated with the labeled 462 nt HD3 ss DNA fragment and analyzed in the band shift assay. The middle domain produced retarded complexes or aggregates with the labeled DNA fragment that did not enter the gel (Fig. 3B, lane 2), whereas both the N- and C-terminal domains failed to retard labeled DNA on the top of the gel (lanes 1 and 3). This shows that the middle p53 domain is responsible for the interaction with internal segments of ss DNA.

We also examined the ability of the p53 domains to interact with ss DNA ends and internal segments of ss DNA using electron microscopy. As shown in Figure 4, both the central and C-terminal domains of p53 bound to ss DNA, while the N-terminal domain of p53 did not bind at all. The central p53 domain bound preferentially to internal segments of ss DNA (C–H). Between 35 and 40% of DNA molecules were found in complex with the protein representing the central p53 domain. Moreover, several protein molecules were frequently observed along one ss DNA strand. The C-terminal p53 domain, in contrast, bound to ss DNA ends, but did not show any significant binding to the internal regions of the ss DNA (Fig. 4I and K). Around 20% of DNA molecules were found in complex with the protein representing the C-terminal p53 domain. These findings confirm the results obtained by the band shifts experiments described above, and clearly demonstrate that p53 has two distinct binding sites for ss DNA ends and internal DNA segments.

Activation of the binding site for internal DNA segments by short oligonucleotides

In an experiment using long ss DN DNA (1150 nt) as labeled probe (Fig. 1C), retarded DNA–protein complexes were not observed at low p53 concentrations (0.07 and 0.2 ng/10 µl; lanes

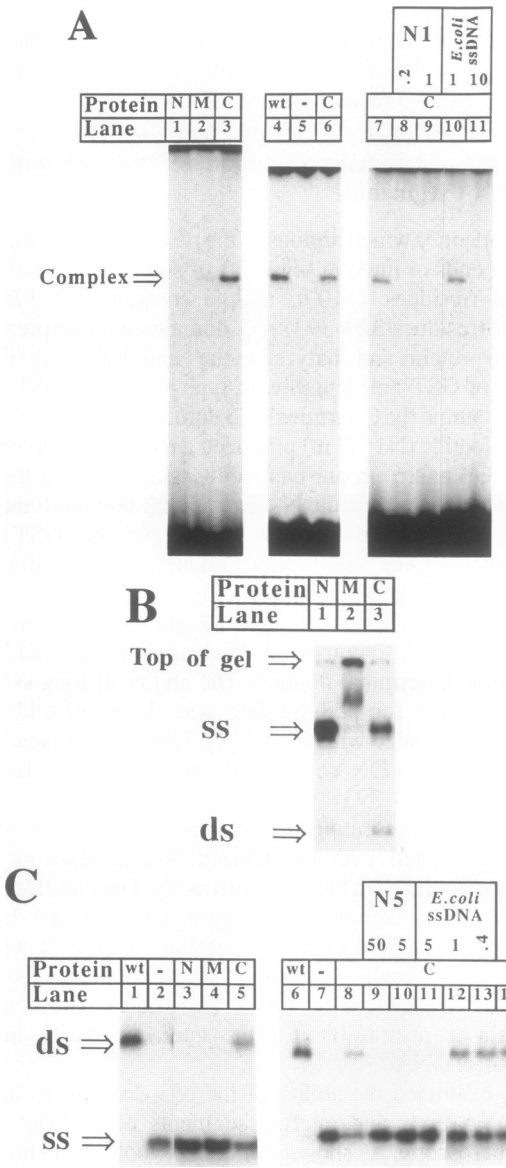

Figure 3. DNA binding and renaturation activity of the separate p53 domains. (**A**) Binding of GST–wild-type p53 (wt) and GST–p53 deletion mutant proteins representing the N-terminal (N), middle (M), and C-terminal (C) domains to the short labeled ss N1 oligonucleotide (0.32 ng; lanes 1–6). N1 itself competed more efficiently than ss *E.coli* DNA with labeled N1 for binding to the C-terminal p53 domain (lanes 7–11). The arrow indicates the position of retarded complexes. (**B**) Retardation of labeled long ss HD3 DNA fragment (0.8 ng) by the GST–p53 deletion mutant proteins representing the three domains. The positions of ds and ss HD3 DNA fragments and complexes on top of the gel are indicated by the arrows. (**C**) DNA renaturation activity of the same proteins (lanes 3–5). The labeled oligonucleotide N2 (0.02 ng) and the non-labeled oligonucleotide N3 (0.6 ng) were incubated in the absence or presence of GST fusion proteins and treated with stop solution. The non-complementary oligonucleotide N5 (lanes 9 and 10), but not ss *E.coli* DNA (lanes 12 and 13), inhibited DNA renaturation catalyzed by the C-terminal p53 domain. Arrows indicate the positions of the ss oligonucleotide N2 and the N2/N3 duplex. Five ng of p53 protein were used for binding and renaturation assays. Reaction mixtures were analyzed in non-denaturing 5% polyacrylamide gels. The long ds HD3 DNA fragment migrated faster than ss HD3 DNA (B), whereas the short ds oligonucleotide N2/N3 migrated slower than the short ss oligonucleotide N2 (C). Amounts of competitors are shown in ng. (**D**) Western blot analysis of the GST–p53 deletion mutants. Bacterially produced GST–wild-type p53 (wt) and the GST–p53 deletion mutants containing amino acid residues 1–100 (N), 99–307 (M), and 320–393 (C), were loaded on 10% SDS–polyacrylamide gels and separately probed with monoclonal antibodies specific for the N-terminal (DO7), middle (PAb240), and C-terminal (PAb421) domains of p53.

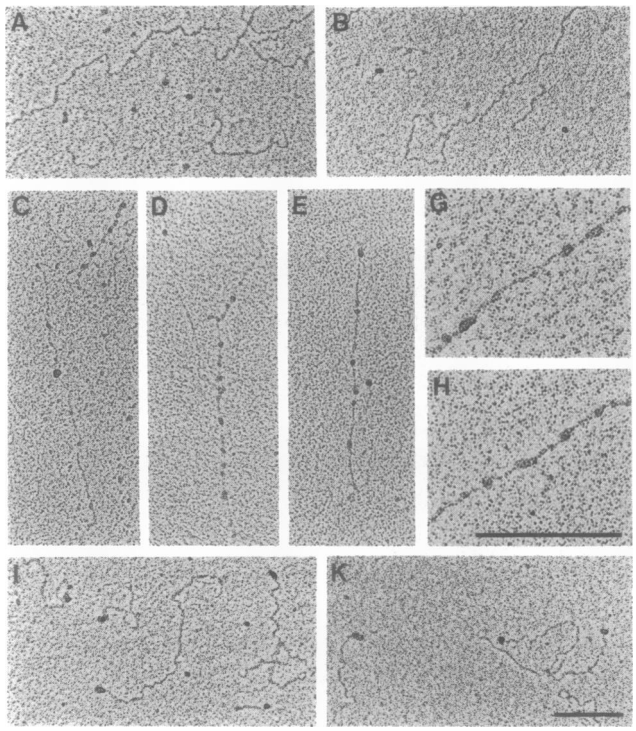

Figure 4. Electron microscopy analysis demonstrating the interaction of GST–p53 deletion mutant proteins with ss DNA. Sixty ng of denatured 4.5 and 0.2 kb pCAT1 DNA fragments generated by *Bgl*II digestion were incubated with GST–p53 deletion mutant proteins representing the three p53 domains. (**A** and **B**) Incubation of the N-terminal p53 domain (residues 1–100) with ss DNA. Less than 1% of the DNA molecules were found in complex with the protein. (**C–H**) Interaction between the central p53 domain (residues 99–307) and ss DNA. This domain bound efficiently to ss DNA, and was localized primarily to internal segments of ss DNA. (**I** and **K**) Interaction between the C-terminal p53 domain (residues 320–393) and ss DNA. The C-terminal domain bound preferentiallly to ss DNA ends. Since the p53 domains were produced as GST fusion proteins, their sizes do not differ significantly on the electron micrographs. The magnification in G and H is twice that in the other electron micrographs. Bars represent 250 nm.

2 and 7). However, the short ss oligonucleotide N4 (37 nt) stimulated the formation of large DNA–protein aggregates that did not enter the gel (Fig. 1C; compare lane 6 with lane 2, and lanes 10 and 11 with lane 7). The labeled DNA–protein complex gradually appeared at the top of the gel as increasing concentrations of non-labeled N4 (0.01–10 ng or 0.08–80 nM; lanes 3–6 and 7–11) were used. No retarded complexes were produced when the same labeled probe was incubated with the N4 oligonucleotide (0.01–10 ng) in the absence of p53, or with ss *E.coli* DNA (0.001–10 ng) in the presence of p53 (0.07 or 0.2 ng) (not shown). Thus, a short ss oligonucleotide can stimulate DNA–protein complex formation.

Renaturation of complementary DNA fragments in the presence of excess heterologous DNA

The interaction of p53 with ss DNA promotes renaturation of two complementary DNA strands (20–22). In order to assess the relative role of the two distinct ss DNA binding sites in this process, we performed renaturation experiments in the presence of short or long non-complementary DNA fragments. These

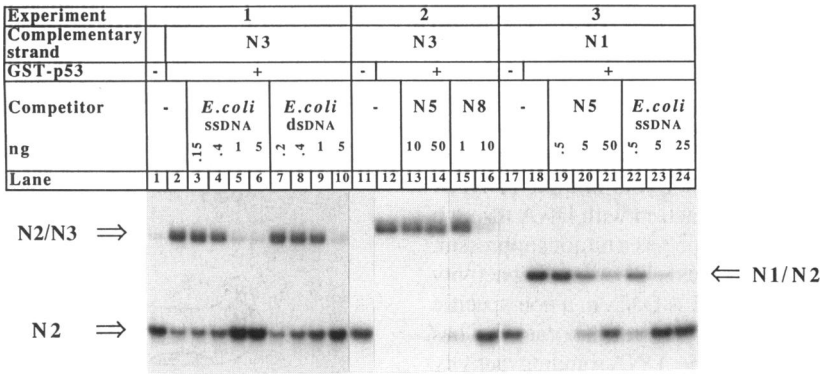

Figure 5. Renaturation of complementary oligonucleotides in the presence of non-complementary oligonucleotides and DNA fragments. ^{32}P-labeled oligonucleotide N2 (0.05 ng) and non-labeled oligonucleotides N3 (1.2 ng; experiments 1 and 2) or N1 (0.06 ng; experiment 3) were incubated in the presence or absence of 10 ng GST–p53, treated with stop solution containing SDS and EDTA (21), and analyzed on a non-denaturing 10% polyacrylamide gel. Arrows indicate the positions of ss oligonucleotide N2 and the resulting duplexes N1/N2 and N2/N3. The ds oligonucleotides migrated slower than the ss oligonucleotides. Duplex formation was gradually reduced in the presence of increasing concentrations of ss *E.coli* DNA (lanes 3–6 and 22–24). ds *E.coli* DNA inhibited duplex formation less efficiently (lanes 7–10). Oligonucleotide N8 (76 nt; lanes 15 and 16), but not the shorter N5 oligonucleotide (37 nt; lanes 13 and 14), also inhibited formation of the N2/N3 duplex. The N5 oligonucleotide inhibited formation of the N1/N2 duplex (lanes 19–21). Identical results were obtained with wild-type p53 and GST–wild-type p53 protein, and with the N4 oligonucleotide (37 nt) instead of N5 (data not shown).

fragments should selectively inhibit the binding of complementary ss oligonucleotides to the DNA end site and internal DNA site of p53.

Renaturation of the N2 (36 nt) and N3 (70 nt) complementary oligonucleotides was not inhibited by a 1000-fold excess (50 ng) of the non-complementary oligonucleotide N5 over labeled oligonucleotide (Fig. 5, lanes 12–14). In contrast to N5, the longer oligonucleotide N8 (76 nt) and *E.coli* DNA (4–15 kb) strongly inhibited GST–p53-catalyzed DNA renaturation of N2/N3. The N8 oligonucleotide and ds *E.coli* DNA demonstrated similar inhibitory activity (lanes 10 and 16), and were 5- to 10-fold less potent than ss *E.coli* DNA (lane 5) as inhibitors. The N8 oligonucleotide, which is more than twice as long as the N5 oligonucleotide (76 versus 37 nt), was at least 100-fold more potent an inhibitor molar-wise than N5 (compare lanes 14 and 15).

E.coli ss DNA was equally efficient in inhibiting renaturation of the N2/N3 (36 and 70 nt; 36 complementary nt) and N1/N2 (37 and 36 nt; 35 complementary nt) oligonucleotides (Fig. 5, lanes 3–6 and 22–24). In contrast, 50 ng of the N5 oligonucleotide could inhibit renaturation of N1/N2 (Fig. 5, lane 21), but not renaturation of N2/N3 (Fig. 5, lane 14). This suggests that the mechanisms of renaturation of the N2/N3 and N1/N2 pairs of complementary oligonucleotides are different (see Discussion).

These results demonstrate that the renaturation reaction can proceed in the presence of a 300- to 450-fold molar excess of non-complementary short oligonucleotide/oligonucleotide ends over substrate DNA/DNA ends. The ability of DNA fragments to inhibit the reaction depends on their length; longer oligonucleotides or long DNA fragments are more potent inhibitors than short oligonucleotides.

Mapping the p53 domain responsible for catalyzing DNA renaturation

In the presence of GST–wild-type p53, N2 and N3 were quantitatively converted to the ds form (Fig. 3C, lanes 1 and 6). The ds product was formed neither in the absence of protein (lanes 2 and 7) nor in the presence the N-terminal (lane 3) or central (lane 4) domains of p53. In contrast, the C-terminal p53 domain was able to catalyze DNA renaturation, although less efficiently than the full-length protein (lane 5).

A short unrelated oligonucleotide (N5; 37 nt) in 100-fold excess (5 ng/10 µl) over labeled oligonucleotide (lane 10) significantly inhibited the formation of duplex molecules catalyzed by the C-terminal p53 domain. The recovery of duplex product was also reduced in the presence of 5 ng, but not 0.4–1 ng, of ss *E.coli* DNA. However, renaturation catalyzed by the full-length protein was inhibited by 0.4–1 ng of ss *E.coli* DNA (Fig. 5, lanes 3–6). Hence, renaturation catalyzed by full-length p53 is more sensitive to inhibition by ss *E.coli* DNA than renaturation catalyzed by the C-terminal domain.

DISCUSSION

p53 has two distinct binding sites for ss DNA ends and internal ss DNA segments

We previously found that long DNA fragments do not compete for p53 binding to short ss DNA (21). Here we have shown that the formation of high molecular weight ss or ds long DNA–p53 protein complexes is not inhibited by an excess of short oligonucleotides. This suggests that p53 possesses two distinct ss DNA binding sites: one site for binding short ss DNA fragments or DNA ends (DNA end site), and another site for binding to internal segments of long ss DNA molecules (internal DNA site). This notion was corroborated by the mapping of the DNA binding domains using p53 deletion mutants in our band shift assay and in the electron microscopy analysis. These experiments localized the internal ss DNA binding site to the central domain (residues 99–307) and the ss DNA end binding site to the C-terminal region (residues 320–393) of the p53 protein.

The fact that a short oligonucleotide stimulated the formation of high molecular weight p53–DNA complexes suggests that the binding of ss DNA ends to the C-terminal domain of p53 activates the internal DNA site located in the central domain. In accordance with this idea, electron microscopy demonstrated the binding of full-length p53 to ss DNA ends or to both ss DNA ends and

internal regions of ss DNA molecules, but failed to demonstrate binding of full-length p53 to only internal segments of ss DNA molecules.

Three mutant p53 proteins derived from human tumors had lower affinity for internal DNA segments. This is consistent with the fact that all three mutants carry amino acid substitutions located in the central domain that harbors the internal ss DNA binding site, and suggests that the interaction with DNA through this site is important for the function of p53 as a tumor suppressor.

The p53 protein has sequence-specific ds DNA binding activity (9,10). However, it can also bind ds and ss DNA in a non-specific manner (28–30). Recent work from several laboratories has demonstrated that the sequence-specific DNA binding activity resides in the central domain of p53 (15–18). In addition, both the central domain (18) and the C-terminal domain (18,29) of p53 can bind DNA non-specifically. Our findings are consistent with these observations. The ss DNA end binding site that we mapped to the C-terminal domain may correspond to the non-specific DNA binding site identified in the same domain by Foord *et al.* (29) and Wang *et al.* (18). The internal ss DNA binding site described here was mapped to the central p53 domain. Since the sequence-specific DNA binding function of p53 also resides in the central domain, we suggest that this domain has only one DNA binding site that can interact specifically with motifs in ds DNA and non-specifically with internal ss DNA segments. Alternatively, the central region of p53 may contain a non-specific DNA binding site distinct from the specific DNA binding site.

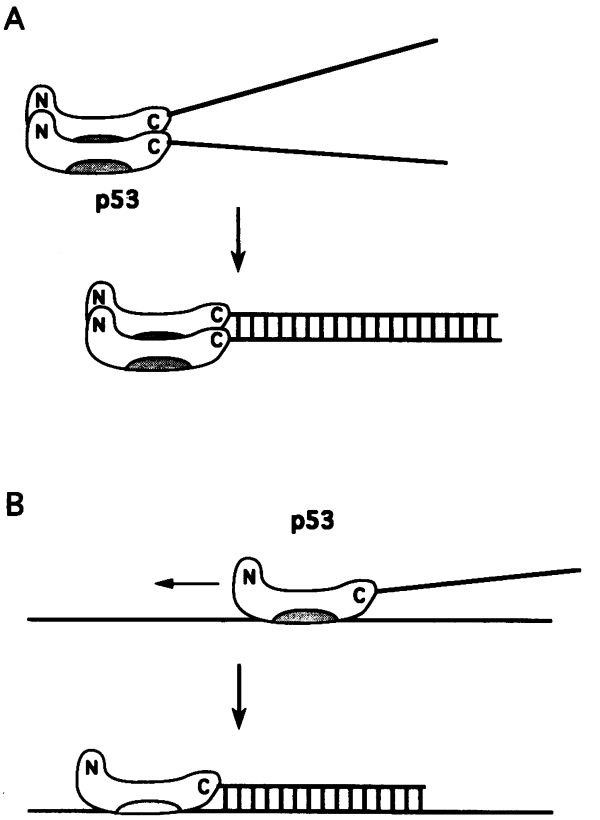

Figure 6. A model for p53-mediated DNA renaturation. (**A**) p53 oligomers bind ss DNA ends and align complementary DNA strands, thereby promoting their renaturation. (**B**) p53 binds the end of one ss DNA molecule and internal segments of another ss DNA molecule via the DNA end binding site and the internal DNA binding site. Renaturation may occur if the p53–ss DNA end complex slides along the ss DNA or occupies different internal positions on this DNA strand due to multiple association–dissociation steps until complementary sequences are aligned.

The DNA end and internal DNA binding sites in p53 are involved in DNA renaturation

Our data strongly suggest that the DNA end site in p53 participates in DNA renaturation. This is supported by the fact that p53 can promote the renaturation of short oligonucleotides that do not bind to the internal DNA site, but should bind to the DNA end site. Moreover, p53 catalyzed renaturation of short oligonucleotides much more efficiently than it catalyzed renaturation of longer DNA fragments, at least under the experimental conditions used (G. Bakalkin, unpublished results). The rate of p53-promoted renaturation of short oligonucleotides was very high, even at a high DNA:protein ratio, indicating that the binding of p53 to short oligonucleotides or DNA ends is not a limiting step in the renaturation reaction. Finally, the C-terminal p53 domain that binds short ss oligonucleotides, but not internal segments of long ss DNA fragments, can catalyze the DNA renaturation reaction.

Although the C-terminal p53 domain containing the DNA end site is sufficient for catalyzing DNA renaturation, several observations suggest that the internal DNA site located in the central domain is also involved in this process. (i) p53 can catalyze the renaturation of longer DNA fragments (G. Bakalkin, unpublished results) that have a high number of potential internal binding sites relative to DNA ends. Similar results were reported by Brain and Jenkins (22). (ii) Unrelated long ss DNA fragments inhibited renaturation of short complementary oligonucleotides catalyzed by full-length p53 much more efficiently than short ss oligonucleotides. (iii) Renaturation of short oligonucleotides catalyzed by the C-terminal p53 domain that lacks the internal DNA site was less sensitive to inhibition by long ss DNA than renaturation catalyzed by the full-length protein.

Binding of the ends of two DNA strands to a p53 oligomer containing two or more DNA end sites may align the complementary strands and promote their renaturation (Fig. 6A). However, since both the DNA end site and the internal DNA site appear to be involved in DNA renaturation, we suggest that p53 can also catalyze renaturation in a different way. The binding of both the end of one ss DNA molecule and an internal segment of another DNA strand to a p53 oligomer via distinct sites may allow p53 to search for complementarity by translocating along the DNA until complementary sequences are aligned, and thereby promote DNA renaturation (Fig. 6B). This notion is supported by our electron microscopy analysis, which demonstrated the joining of a ss DNA end and an internal region of another ss DNA molecule by the p53 protein. The ability of long DNA fragments to inhibit p53-mediated renaturation of short complementary oligonucleotides is probably a result of the binding of DNA end–p53 complexes to internal segments of long competitor DNA.

Biological significance of the interaction of p53 with ss DNA

DNA damage in the form of strand breaks causes nuclear accumulation of p53 (31). We have previously suggested that p53 may function as a sensor of DNA strand breaks that binds ss DNA

ends and triggers a DNA repair pathway (21). This process could conceivably involve stabilization of p53 following interaction with ss DNA ends. Moreover, the high levels of p53 protein may promote the joining of staggered DNA strand breaks by catalyzing their renaturation via the DNA end and internal DNA sites. In general, it is possible that a p53 oligomer can join any ss DNA ends via its DNA end sites in a manner similar to the joining shown in Figure 2E and F. This could allow repair by other mechanisms. Our results indicate that the internal DNA site can in fact be activated due to the binding of a ss DNA end to the C-terminal domain, resulting in stimulation of DNA renaturation. In cells with mutant p53, DNA breaks that arise spontaneously would not be repaired by this mechanism, leading to the accumulation of mutations and tumor progression. Thus, interaction with ss DNA may be an additional p53 function relevant to tumor suppression, apart from the sequence-specific DNA binding and transcriptional activation.

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