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The single-stranded DNA end binding site of p53 coincides with the C-terminal regulatory region

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ABSTRACT

p53 is a transcription factor that binds double-stranded (ds) DNA in a sequence-specific manner. In addition, p53 can bind the ends of single-stranded (ss) DNA. We previously demonstrated that ssDNA oligonucleotides interact with the C-terminal domain of p53 and stimulate binding to internal segments of long ssDNA by the p53 core domain. Here we show that the p53 C-terminal domain can recognize staggered ss ends of dsDNA. We have mapped the binding site for ssDNA ends to residues 361–382 in human p53 using a p53 deletion mutant (p53-Δ30) lacking the 30 C-terminal amino acid residues and a series of 22mer peptides. The binding site for DNA ends coincides with a region previously implicated in regulation of sequence-specific DNA binding by the core domain. The interaction of the C-terminal regulatory domain with the ends of ssDNA or with the protruding ends of dsDNA stimulates both sequence-specific and non-specific DNA binding via the core domain. Electron microscopy demonstrated the simultaneous binding of p53 to dsDNA and a ssDNA end. These results suggest a model in which interaction of the p53 C-terminal tail with DNA ends generated after DNA damage causes activation of sequence-specific p53 DNA binding *in vivo* and may thus provide a molecular link between DNA damage and p53-mediated growth arrest and apoptosis.

INTRODUCTION

The p53 protein plays a crucial role in the cellular response to DNA damage (for a review, see 1). p53 is normally expressed at low levels but accumulates by post-transcriptional mechanisms in cells exposed to DNA damaging agents (2–6). The increase in p53 protein levels causes G1 arrest, allowing DNA repair prior to S phase entry and DNA replication. Alternatively, p53 may trigger cell death by apoptosis (7–9). In both cases, p53 acts to prevent the propagation of cells with genomic injury that may lead to tumour development.

p53 is a transcription factor that has sequence-specific DNA binding activity (10,11). Genes containing p53 consensus binding sites in their promoter regions include WAF1/p21 (12), MDM2 (13,14), GADD45 (15), cyclin G (16) and bax (17). The specific DNA binding activity resides in the core domain of p53, approximately corresponding to amino acid residues 102–290 (18–21). A vast majority of p53 mutations found in human tumours are clustered within this domain (22). The transactivating activity is located in the N-terminal region of p53 (23,24), whereas the C-terminal domain harbours a region required for oligomerization (20,21,25,26).

Wild-type p53 is structurally flexible and appears to reversibly adopt distinct conformations *in vivo* during cell division (27). Under normal conditions, p53 is present in cells in a latent form which is unable to bind DNA specifically (28). Cryptic specific DNA binding of p53 can be activated *in vitro* by several modifications of the very C-terminal region, including binding of the monoclonal antibody PAb421, deletion of the 30 C-terminal residues, interaction with dnaK and phosphorylation by casein kinase II and protein kinase C (29,30). The activation of cryptic specific DNA binding was suggested to involve an allosteric mechanism (28). The recent finding that a small peptide, corresponding to the C-terminal residues 369–383 in p53, can activate latent p53 for specific DNA binding (31,32) lends further support to this notion. Thus, the C-terminal region of p53 appears to play an important role as regulator of specific DNA binding.

The C-terminal domain of p53 can bind DNA (20,21,33) and re-anneal complementary DNA strands (34,35). We found that human p53 can bind single-stranded (ss) DNA ends through the C-terminal residues 320–393 (35,36) and showed that short ssDNA oligonucleotides can stimulate binding of the p53 core domain to internal segments of ssDNA (35). Studies by Jayaraman and Prives (37) demonstrated that sequence-specific binding of p53 to supercoiled double-stranded (ds) DNA can be activated by short ssDNA oligonucleotides, as shown by DNase I footprinting. Moreover, the C-terminal domain of p53 can bind insertion/deletion (IDL) mismatches in DNA (38) and DNA damaged enzymatically or by ionizing radiation (39). Taken together, these findings suggest that the recognition of damaged

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DNA by the C-terminus can lead to activation of specific DNA binding of p53 *in vivo*.

In order to further characterize the biological role of the interaction between the C-terminus of p53 and ssDNA ends, we have precisely mapped the region within the C-terminal domain of p53 that interacts with ssDNA ends and found that it coincides with the negative regulatory domain of p53. In addition, we show that ssDNA end binding stimulates both sequence-specific and non-specific dsDNA binding through the p53 core domain. This supports the hypothesis that the direct interaction with the ends of damaged DNA may trigger an allosteric shift in p53 after DNA damage *in vivo*.

MATERIALS AND METHODS

Plasmids

The plasmids encoding the GST–human wild-type p53 fusion protein and the GST–p53-Δ30 deletion mutant fusion protein lacking amino acid residues 364–393 were constructed by PCR amplification of human wild-type p53 cDNA using appropriate primers and subsequent cloning in-frame in the *Bam*HI and *Eco*RI sites of the vector pGEX-2T (Pharmacia, Sweden). DNA sequencing confirmed that no mutations had been introduced in the p53 coding sequence during PCR. The PG-CAT plasmid containing 13 repeats of a p53 consensus site and the MG-CAT plasmid containing mutated p53 binding sites were provided by Dr Bert Vogelstein and have been described elsewhere (40).

Production of GST–p53 fusion proteins in bacteria

GST–p53 proteins were produced in *Escherichia coli* and purified as described by Smith and Johnson (41), except that bacteria were grown at room temperature for 2 h after induction with 0.2 mM IPTG and lysed by sonication in 1% Triton X-100 in PBS.

Oligonucleotides

Synthetic oligonucleotides were purchased from Eurogenthec (Belgium). The following ss oligonucleotides were used: 5′-CTGATCCATG-3′ (10mer); 5′-AAGAGAGGTCCGAGGA-GGGG-3′ (20mer); 5′-GGACGAATGCGCCGCATGCGAATA-TAGCGTTTGT-3′ (37mer); 5′-TCAACGTCCATTACAGTGC-ATCAAAGTCCATTACAGTGCGCACATTAACGCGCATTA-ATGTGCATCTTCAACACT-3′ (75mer). The BC blunt end dsDNA oligonucleotide 5′-CCGGGCATGTCCGGGCACGTC-CGGGCACGT-3′ contains a p53 consensus binding site (42). The ds oligonucleotide 5′-CCTTAATGGACTTTAATGG-3′ (40) was used as control. The sequence of the dsDNA oligonucleotide MN with protruding ss ends of 2 nt was 5′-GAAGTGGGCGTGG-TTTAAAGTATATAAGCA-3′ (+ strand) and 5′-GTTGCTTATA-TACTTTAAACCACGCCCACT-3′ (– strand).

Cell culture and preparation of cell extracts

MCF-7 human breast carcinoma cells and HL60 human pro-myelocytic leukaemia cells were grown in IMDM medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL). Subconfluent cell cultures were used for preparation of nuclear extracts as described (43).

DNA binding assays

Band shift assays were performed as described (31) except that 5 mM $MgCl_2$ was added to the binding reaction. Circular pUC19 plasmid DNA (20–100 ng/reaction) was used as non-specific competitor. Typically, 0.5 ng ^{32}P-end-labeled probe, 10 ng purified GST–p53 protein and 1 µg nuclear extract proteins were mixed in a 20 µl binding reaction. The reactions were incubated for 30 min at room temperature when purified proteins were used or on ice when nuclear extracts were added. The samples were analysed on 4% native polyacrylamide gels containing 0.1% Triton X-100 and run at 200 V (constant voltage) for 1 h 45 min at 4°C. The gels were fixed in 10% acetic acid and 10% methanol (v/v), dried and autoradiographed.

GST pull-down assays were performed with PG-CAT plasmid DNA linearized with *Bam*HI and labelled by filling in 5′ overhangs with Klenow polymerase in the presence of radio-labelled dATP. Binding reactions (400 µl total volume) containing 40 ng GST protein or GST–wild-type p53 fusion protein immobilized on glutathione–Sepharose beads and 100 ng labelled PG-CAT plasmid DNA were performed in the buffer for band shift assays. After 30 min incubation at room temperature, beads were collected by centrifugation and washed twice at 0°C. DNA was eluted from beads by addition of 0.2% SDS and 25 mM EDTA followed by 10 min incubation at 50°C. The radiolabelled DNA was subjected to agarose gel electrophoresis and visualized by autoradiography.

For filter binding assay, overlapping peptides (10 mg) covering the p53 C-terminus were immobilized on a PVDF filter (BioRad) blocked with 10 mg/ml denatured salmon sperm DNA. The filters were incubated for 30 min at room temperature with the labelled ss 37mer (10 ng/ml) in the binding buffer used for band shift assays containing 2% BSA. Filters were then washed five times and autoradiographed.

Antibodies

The p53-specific mouse monoclonal antibodies PAb421 and PAb1801 were from Oncogene Science Inc. (New York, NY).

Electron microscopy

Analysis of p53/DNA complexes by electron microscopy was performed essentially as previously described (35,36). The PG-CAT plasmid was digested either with *Pvu*II to generate blunt ends or with *Bam*HI to generate 5′ protruding ends.

Synthetic peptides

22mer peptides covering the C-terminal domain of p53 (residues 337–393) were synthesized using the Merrifield solid phase method (44). Each peptide overlapped the previous peptide by 14 residues. Peptides were dissolved in dimethylsulfoxide at a concentration of 1 mg/ml and stored at –20°C. Purification by HPLC was performed on a Super Pac pep-S column.

RESULTS

The C-terminal 30 amino acid residues of p53 are required for recognition of ssDNA ends or protruding ends of dsDNA

Studies of complex formation of wild-type GST–p53 protein with different DNA substrates in a band shift assay revealed two major types of p53/DNA complexes. A slowly migrating complex was

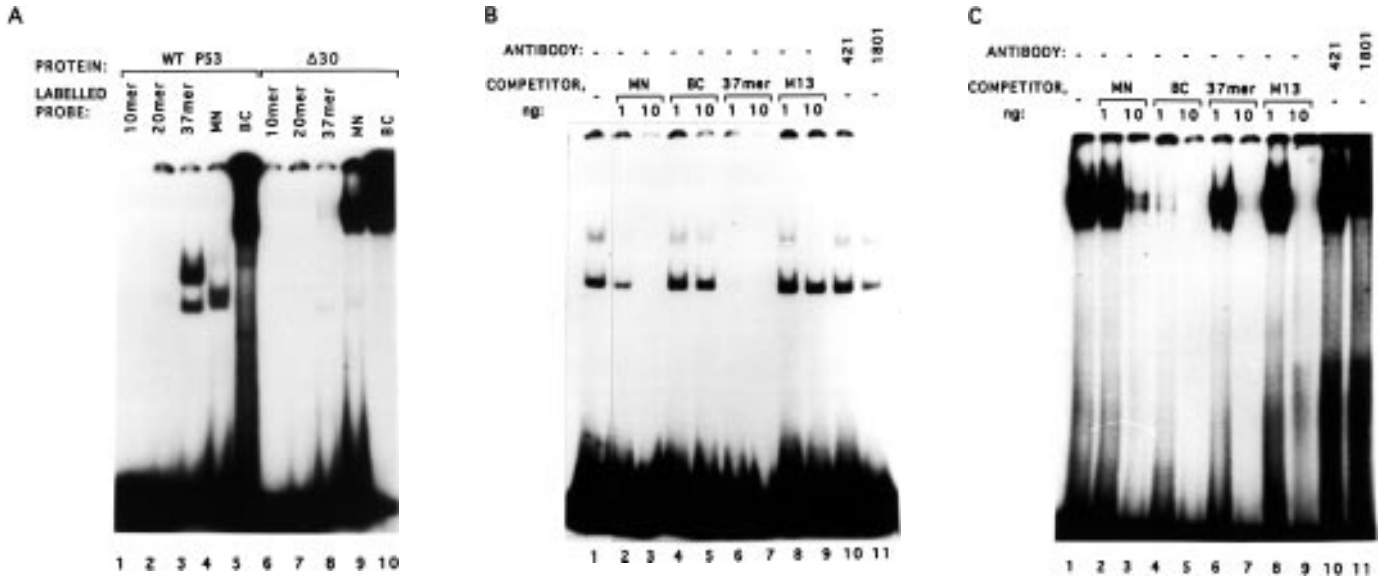

Figure 1. Wild-type p53 protein interacts with ss ends of DNA whereas the p53-Δ30 deletion mutant lacks ssDNA end binding activity. (**A**) GST–wild-type p53 fusion protein (lanes 1–5) or GST–p53-Δ30 protein (lanes 6–10) was incubated with different labelled DNA oligonucleotides and analysed in a band shift assay. The following labelled DNA oligonucleotides were used: ss 10mer (lanes 1 and 6); ss 20mer (lanes 2 and 7); ss 37mer (lanes 3 and 8); ds 30mer MN with 2 nt ss overhangs (lanes 4 and 9); blunt end ds 30mer BC containing a p53 consensus binding site (lanes 5 and 10). (**B**) Competition of p53/MN complex formation by different unlabelled DNA. The experiment was performed as described for (A), using MN as labelled probe. Competitor DNA was added to the reaction mixture before addition of labelled probe. Lane 1, no competitor DNA; lanes 2 and 3, 1 or 10 ng MN; lanes 4 and 5, 1 or 10 ng BC; lanes 6 and 7, 1 or 10 ng ss 37mer; lanes 8 and 9, 1 or 10 ng M13 circular ss DNA; lane 10, 50 ng PAb421 added; lane 11, 100 ng PAb1801 added. (**C**) Competition of p53-Δ30/MN complexes by different unlabelled DNA (for experimental conditions, see above). Competitor DNA and antibodies were added in the same order as in (B).

formed upon incubation of p53 protein with the labelled ds oligonucleotide BC containing a p53 consensus binding site (Fig. 1A, lane 5). This complexing, which is due to specific interaction of the core domain with DNA, was enhanced by addition of PAb421 antibody and could be competed by the BC oligonucleotide (data not shown). In contrast, interaction of p53 with the labelled 37mer ss oligonucleotide gave rise to faster migrating complexes (Fig. 1A, lane 3). p53 interacts with the ends of the ss 37mer oligonucleotide through the C-terminal domain, since these complexes were competed by short ssDNA (35; data not shown). p53 did not form any detectable complexes with the 10mer and 20mer ssDNA oligonucleotides (lanes 1 and 2), although complexes between p53 and the 20mer could be seen after longer exposure (data not shown). Thus, the minimal length of ssDNA oligonucleotide that can be stably bound by the ssDNA end binding site in the C-terminal domain of p53 is at least 20 nt.

As shown in Figure 1A, lane 4, p53 also formed a retarded complex with the labelled 30mer dsDNA oligonucleotide MN with ss overhangs of 2 nt at both ends. The mobility of the p53/MN complex is similar to that of the p53/ss 37mer complex. To ascertain that MN binds to the ssDNA end site of p53, competition experiments using various ssDNA and dsDNA oligonucleotides were performed. Figure 1B shows that only competitors containing ssDNA ends (MN and the ss 37mer; lanes 2, 3, 6 and 7) but not dsDNA with blunt ends (BC; lanes 4 and 5) or circular ssDNA (M13; lanes 8 and 9) were able to challenge p53/MN complex formation. These data indicate that p53 can bind ssDNA ends as short as 2 nt through its C-terminal DNA binding site.

In order to further map the ssDNA end site, we tested whether a deletion mutant of p53, p53-Δ30, lacking the C-terminal 30 amino acid residues, is able to bind ssDNA ends. This deletion mutant is constitutively activated for specific DNA binding through the central core domain (29). Figure 1A shows that p53-Δ30 failed to bind short ssDNA oligonucleotides (lanes 6–8), but formed complexes with both MN and BC, which had the same mobility (lanes 9 and 10). According to PhosphorImager quantitation, specific binding of p53-Δ30 to BC was 7- to 10-fold stronger than binding of p53-Δ30 to MN.

To confirm that p53-Δ30 binds to the MN oligonucleotide through the core domain, we performed additional competition experiments (Fig. 1C). The p53-Δ30/MN complex was completely competed away by a 2-fold excess of unlabelled BC oligonucleotide (lanes 4 and 5), but not by the same amount of non-specific competitors, including the ds MN oligonucleotide (lanes 2 and 3), the ss 37mer oligonucleotide (lanes 6 and 7) and M13 ssDNA (lanes 8 and 9). These results demonstrate that deletion of the 30 C-terminal amino acid residues abolishes the recognition of ssDNA ends by p53. It is also clear from these experiments that C-terminal truncation not only causes activation of specific DNA binding, as shown by Hupp *et al.* (29), but also stimulates non-specific dsDNA binding by the core domain.

Interestingly, recognition of p53 in complex with DNA by the p53-specific antibodies PAb421 and PAb1801, whose epitopes reside in the C- and N-terminal domains respectively, varied depending on whether the core or C-terminal domain of p53 was involved in interaction with DNA. For example, PAb1801 supershifted the p53-Δ30/MN complex (Fig. 1C, lane 11), but not the wild-type p53/MN complex formed through the C-terminal domain (Fig. 1B, lane 11). PAb421 did not recognize the p53/MN complex, but supershifted the p53/BC complex formed through the core domain (Fig. 1B, lane 10; data not shown). Taken together with the observation that p53/DNA complexes formed through the C-terminal or core domains differ significantly in

their mobility, these results suggest that p53 may adopt distinct conformations characterized by different orientation of the N- and/or C-terminal domains in p53 tetramers upon interaction with DNA.

Electron microscopy analysis of the interaction of full-length p53 and p53-Δ30 with ssDNA

Since band shift experiments showed that wild-type p53 and p53-Δ30 interacted with DNA in a different manner, we compared their binding to ssDNA using electron microscopy. Our previous electron microscopy analyses demonstrated that both full-length wild-type p53 and the C-terminal domain alone (residues 320–393) can bind to the ends of ssDNA, whereas the core domain (residues 90–307) binds to internal ssDNA segments, producing a beads-on-a-string-like pattern (35,36). Both the full-length wild-type p53 and p53-Δ30 proteins were incubated with *Pvu*II-digested and heat-denatured MG-CAT plasmid and analysed by electron microscopy. As can be seen in Figure 2G and H, wild-type p53 bound to the ends of ssDNA. The p53-Δ30 mutant protein, in contrast, associated with internal ssDNA in a manner indistinguishable from that of the core domain and did not show any binding to ssDNA ends (Fig. 2B–F). Up to 10 p53-Δ30 molecules were bound along one DNA molecule (Fig. 2B). This confirms the data obtained by the band shift assays, demonstrating that deletion of the very C-terminal 30 residues causes loss of ssDNA end binding. In addition, C-terminal truncation leads to activation of non-specific binding to internal segments of ssDNA by the core domain.

Mapping of the DNA end binding site using synthetic peptides

In order to map the ssDNA end binding site in p53 in closer detail, a series of overlapping 22mer peptides spanning most of the C-terminal domain (residues 337–393) were examined for their ability to bind ssDNA oligonucleotides in band shift assays. The ss 37mer and the ss 75mer oligonucleotide were used as labelled probes. As can be seen in Figure 3A, lanes 4 and 10, peptide 46, containing amino acid residues 361–382 of p53, binds to both the ss 37mer and the ss 75mer.

Since the complex between the ss 37mer oligonucleotide and peptide 46 did not appear as a distinct band in the band shift gel, perhaps due to dissociation of the complex during gel electrophoresis, we also tested binding of the labelled ss 37mer to peptides immobilized on a PVDF filter. Only peptide 46, but not overlapping peptides, was able to retain the labelled ss 37mer oligonucleotide on the filter (data not shown).

To determine whether peptide 46 interacts with the ends of ssDNA, we performed a phosphatase protection assay. The ^{32}P-end-labelled ss 75mer DNA oligonucleotide or blunt end ds BC oligonucleotide was incubated with peptide 45 or peptide 46 in the presence of increasing amounts of calf intestine alkaline phosphatase (CIAP). Peptide/DNA complexes were then analysed in a band shift assay. Binding of the peptide to the ssDNA ends of the 75mer should prevent removal of the labelled phosphate by CIAP. As shown in Figure 3B, peptide 45 did not protect the ss 75mer or BC from dephosphorylation, since the amount of labelled oligonucleotide decreased at increasing concentrations of CIAP (compare lane 1 with lanes 2–4 and lane 8 with lanes 9–11). Incubation with peptide 46, on the other hand, protected

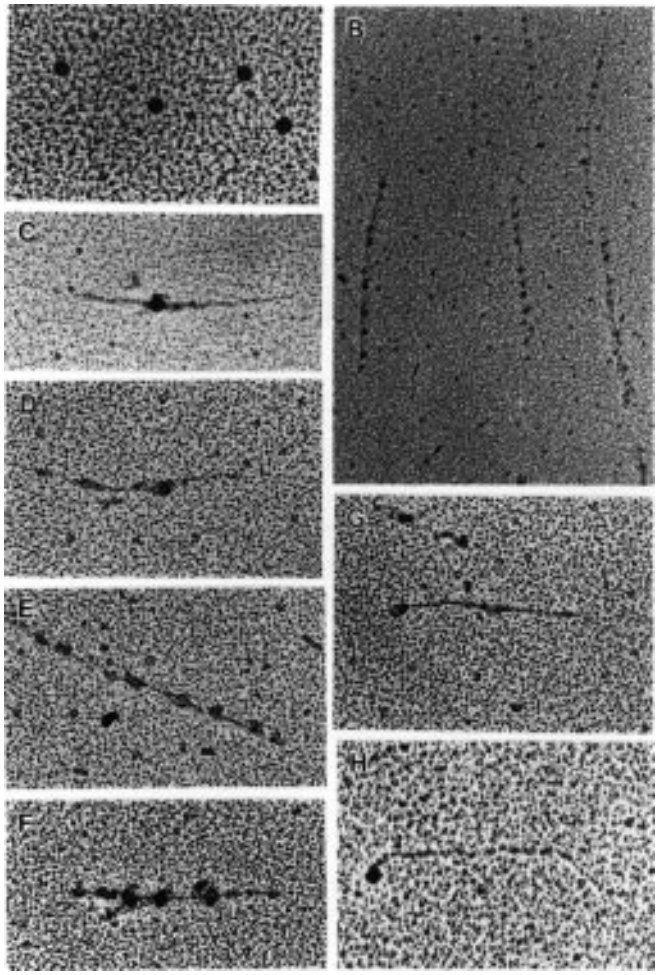

Figure 2. Electron micrographs of GST–wild-type p53/ss DNA and GST–p53-Δ30/ss DNA complexes. (**A**) GST–p53-Δ30 protein without DNA. (**B**–**F**) GST–p53-Δ30 protein incubated with MG-CAT plasmid DNA linearized with *Pvu*II and heat denatured before use. p53-Δ30 binds to the internal portions of ss DNA; several p53-Δ30 molecules were bound along one DNA strand. (**G** and **H**) The wild-type p53 protein binds to the ends of ssDNA. Magnification: 30 000× (B); 90 000× (A and C–F).

the ss 75mer from dephosphorylation in the presence of increasing concentrations of CIAP (lanes 5–7). Peptide 46 did not prevent dephosphorylation of the BC oligonucleotide, however (lanes 12–15). The full-length p53 protein prevented dephosphorylation of the ss 75mer as efficiently as peptide 46 (data not shown). These results suggest that peptide 46 binds to the ss ends of DNA.

Stimulation of specific DNA binding *in vitro* by ss ends of DNA oligonucleotides

The localization of the ssDNA end binding site to the C-terminal region of p53, previously implicated in modulation of specific DNA binding by the core domain (29,31), raised the possibility that the interaction of ssDNA ends with the C-terminal region positively regulates sequence-specific DNA binding by inducing an allosteric shift in the p53 molecule. Jayaraman and Prives (37) have shown that short ssDNA oligonucleotides can indeed stimulate the specific binding of p53 to supercoiled plasmid DNA using DNase I footprinting. To explore this idea further, we

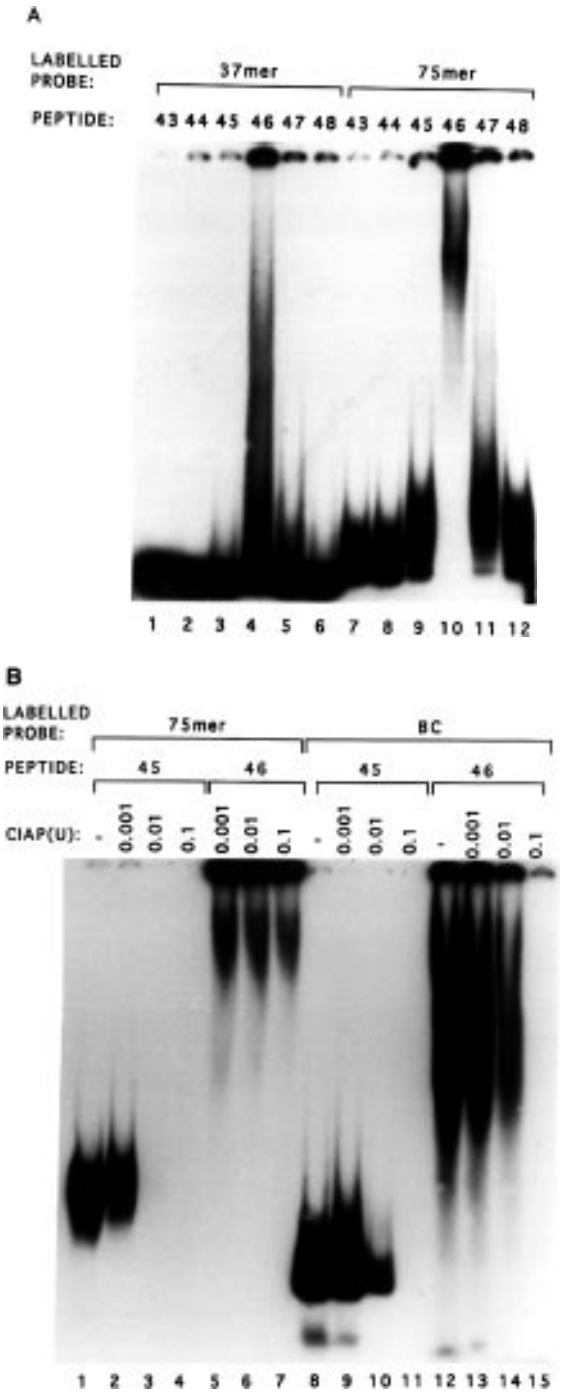

Figure 3. Mapping of the ssDNA end binding site in the p53 protein using synthetic peptides. Overlapping peptides spanning the C-terminal region of p53 were analysed for DNA binding in band shift assays. Peptides (100 ng) were incubated with labelled ssDNA as described for the p53 protein (see Materials and Methods and legend to Fig. 1A) and analysed by electrophoresis on a native polyacrylamide gel. (**A**) The ss 37mer (lanes 1–6) or ss 75mer (lanes 7–12) oligonucleotides were used as labelled probes. Lanes 1 and 7, peptide 43; lanes 2 and 8, peptide 44; lanes 3 and 9, peptide 45; lanes 4 and 10, peptide 46; lane 5 and 11, peptide 47; lanes 6 and 12, peptide 48. (**B**) Phosphatase protection assay using synthetic peptides: 100 ng of peptides 45 (lanes 1–4 and 8–11) or 46 (lanes 5–7 and 12–15) were incubated with labelled ssDNA (ss 75mer, lanes 1–7) or blunt end dsDNA (BC, lanes 8–15) in the presence of increasing amounts of CIAP and analysed in a band shift assay. Lanes 1, 8 and 12, no CIAP; lanes 2, 5, 9 and 13, 0.001 U CIAP; lanes 3, 6, 10 and 14, 0.01 U CIAP; lanes 4, 7, 11 and 15, 0.1 U CIAP.

analysed the specific DNA binding of bacterially produced GST–wild-type p53 in band shift experiments in the presence of short ssDNA (the 37mer). Since the binding of p53 to a consensus binding motif can be stabilized by nuclear extracts (45), suggesting that some unknown cellular factor or factors are needed for specific binding, HL60 nuclear extracts were added to the reaction mixture. In our initial experiments we observed only weak stimulation of specific DNA binding by the ss 37mer (data not shown). However, addition of the ssDNA oligonucleotide together with the activating antibody PAb421 had a synergistic effect on p53 DNA binding. The amount of complexes between p53 and the consensus BC oligonucleotide increased in the presence of ss 37mer DNA in a dose-dependent manner (Fig. 4A, lanes 3–5). According to Western blot analysis, the GST–p53 protein levels did not change during incubation with the HL60 nuclear extract in the presence or absence of ssDNA (data not shown).

Similar experiments were performed using nuclear extracts from MCF-7 cells, a breast carcinoma cell line that carries wild-type p53 (46). Figure 4B shows that complex formation between endogenous p53 and the specific BC oligonucleotide was stimulated by the addition of increasing amounts of 37mer ssDNA (lanes 1–3) in the presence of PAb421. The same amount of unlabelled BC (lane 4) competed efficiently with labelled BC for complexing with p53, whereas a ds blunt end control oligonucleotide had no effect, confirming that the increased p53 DNA binding was sequence specific (lane 5).

We also tested the ability of the ss 37mer oligonucleotide to stimulate specific DNA binding of p53 in a GST pull-down assay, using linearized PG-CAT plasmid DNA carrying 13 tandem copies of a p53 consensus target site (40) as a labelled probe. Addition of the ss 37mer oligonucleotide caused a significant increase in the amount of PG-CAT DNA retained on glutathione–Sepharose beads with immobilized GST–p53 (Fig. 4C, lanes 4 and 5) in the absence of PAb421 antibody. Next, we asked whether the binding to staggered ends of dsDNA can stimulate specific p53 DNA binding. As shown in Figure 4C, lane 6, the ds oligonucleotide MN with 2 nt ss overhangs caused even stronger stimulation of p53 DNA binding than the ss 37mer oligonucleotide. Thus, ssDNA ends rather than just ssDNA of a certain length are important for the activation of p53.

Electron microscopy analysis of p53 binding to dsDNA containing tandem consensus p53 binding sites in the presence of ssDNA ends

To analyse the effect of ssDNA ends on the sequence-specific DNA binding of p53 in closer detail, we studied the binding of bacterially produced GST–full-length wild-type p53 protein to linearized plasmid dsDNA in the presence of the ss 75mer oligonucleotide by electron microscopy. This technique allows direct visualization of individual protein/DNA complexes at a given moment. As can be seen in Figure 5B, p53 bound very poorly to dsDNA. In agreement with our previous results (35), only ~2% of the dsDNA molecules were found in complex with p53. In contrast, p53 bound to the ends of the ss 75mer oligonucleotide (Fig. 5C–E), which is consistent with the results from our band shift experiments (data not shown). Sometimes p53 could be observed joining two ss 75mer DNA molecules together (Fig. 5F). Around 30% of the p53 molecules were bound to the ss 75mer oligonucleotide.

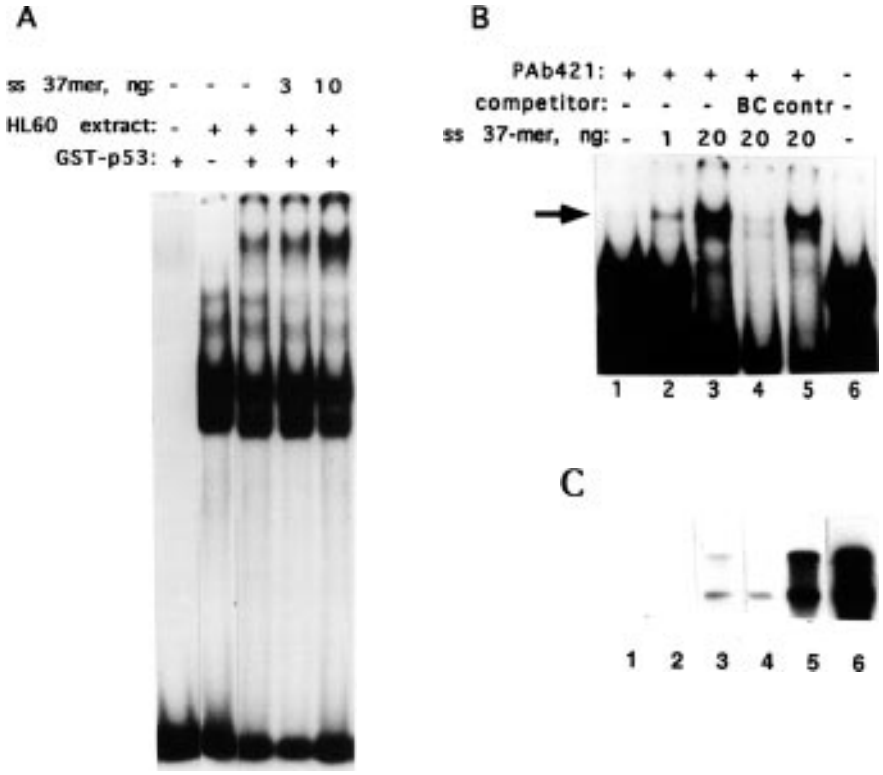

Figure 4. Activation of specific DNA binding of p53 by ssDNA ends. (**A**) GST–wild-type p53 protein was incubated with labelled specific oligonucleotide (BC, 1 ng) in the presence (lanes 3–5) or absence (lane 1) of HL60 nuclear extract (1 µg total protein). DNA/protein complexes were analysed in a band shift assay. Increasing amounts of short ssDNA (37mer) were added to the reaction mixtures: lane 4, 3 ng; lane 5, 10 ng. Fifty nanograms of PAb421 antibody was added to all reactions. Lane 2, HL60 extract without GST–wild-type p53. (**B**) MCF-7 nuclear extract (1 µg total protein), containing endogenous wild-type p53, was incubated with the labelled specific oligonucleotide BC (1 ng) in the absence (lanes 1 and 6) and presence of ss 37mer oligonucleotide (lane 2, 1 ng; lanes 3–5, 20 ng). DNA/protein complexes were analysed in a band shift assay. Twenty nanograms of unlabelled BC (lane 4) or control oligonucleotide (lane 5) were used as specific and non-specific competitor respectively. Fifty nanograms of PAb421 were added to the reactions in lanes 1–5. (**C**) Forty nanograms of GST (lanes 1 and 2) or GST–p53 protein (lanes 4–6) immobilized on glutathione–Sepharose beads were incubated with labelled PG-CAT plasmid DNA containing 13 copies of a p53 binding site in the presence (lanes 2, 5 and 6) or absence (lane 4) of 1 ng ssDNA end-containing oligonucleotides: lanes 2 and 5, ss 37mer; lane 6, ds MN with ss overhangs. After 30 min incubation at room temperature, DNA/protein complexes were precipitated and bound DNA was eluted from the Sepharose beads and analysed by agarose gel electrophoresis. Lane 3, 5% of input DNA.

Figure 5G–J shows protein/DNA complexes observed after incubation of GST–p53 with the ss 75mer oligonucleotide and *Pvu*II-digested PG-CAT plasmid dsDNA. The presence of the ss 75mer oligonucleotide resulted in a dramatic increase in the fraction of p53 bound to dsDNA. Around 80% of the dsDNA molecules were coated with p53 protein molecules. At least some of the p53 protein molecules bound to the PG-CAT dsDNA had short 'tails', consisting of the ss 75mer bound to p53, as indicated by the arrows in Figure 5H and J. p53 molecules were distributed along most of the PG-CAT plasmid DNA, rather than being confined to a region corresponding to the tandem p53 consensus sites. Similar results were obtained when incubating p53 with PG-CAT DNA in the presence of the ss 37mer oligonucleotide, although this oligonucleotide is too short to be readily visible in complex with p53 (not shown).

Since p53 can recognize a ss protruding end of 2 nt (Fig. 1A), we predicted that p53 would be able to bind to the 4 nt ss protruding ends generated by *Bam*HI cleavage. To determine whether binding to the ends of *Bam*HI-digested PG-CAT plasmid DNA could stimulate specific DNA binding of p53, we incubated GST–p53 with *Bam*HI-digested PG-CAT DNA and examined complexes by electron microscopy (Fig. 5K–N). At least 40–50% of the DNA molecules formed loops of different sizes, with p53 located at the junctions. Again, these observations are consistent with the idea that binding of ssDNA ends by the C-terminus causes activation of DNA binding through the core domain.

DISCUSSION

We have previously shown that p53 has affinity for ssDNA ends (36). We suggested that the interaction of p53 with ssDNA ends that appear in cells exposed to DNA damaging agents may trigger a conformational change in the p53 molecule, leading to p53 activation and subsequent cell cycle arrest and/or apoptosis (1). Here we have characterized the interaction of p53 with ssDNA ends in more detail. Our observation that p53 not only binds to the ends of ssDNA, but also efficiently interacts with dsDNA containing ss protruding ends supports the idea that p53 can directly recognize DNA damage in the form of staggered DNA strand breaks *in vivo*.

Deletion of the C-terminal 30 amino acid residues of p53 abolishes recognition of ssDNA ends. The efficient competition of p53-Δ30/MN complexing by the BC oligonucleotide indicates that p53-Δ30 interacts with DNA through the core domain.

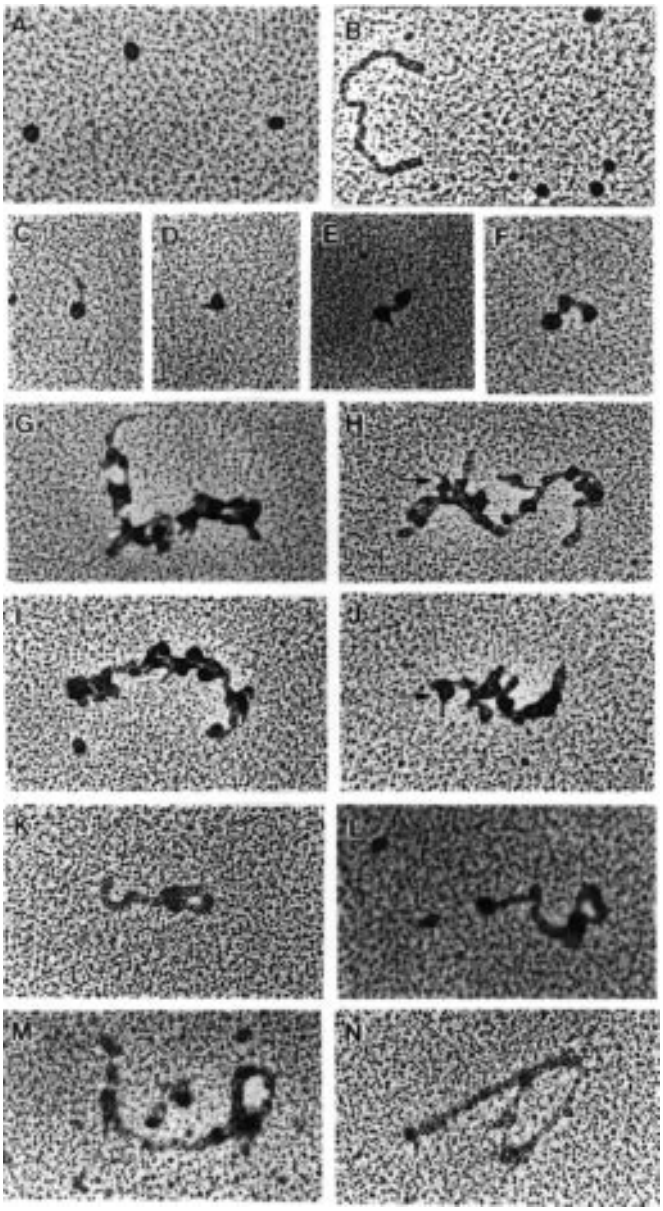

Figure 5. Electron microscopy analysis of p53/dsDNA complexes in the presence of ssDNA ends. (**A**) GST–wild-type p53 protein without DNA. (**B**) GST–wild-type p53 protein incubated at room temperature for 30 min with *Pvu*II-digested plasmid DNA (blunt ends). No significant binding was observed. (**C**–**F**) GST–wild-type p53 incubated with 75mer ssDNA (1 µg). The p53 protein is located preferentially at the DNA ends. (**G**–**J**) GST–wild-type p53 was incubated with *Pvu*II-digested PG-CAT plasmid DNA in the presence of the ss 75mer oligonucleotide. Arrows indicate p53 molecules bound simultaneously to the ss 75mer and dsDNA. (**K**–**N**) GST–wild-type p53 complexes with *Bam*HI-digested PG-CAT plasmid DNA. p53 protein molecules bound simultaneously to the ssDNA end and ds segments of the PG-CAT DNA. Magnification: 90 000×.

Moreover, electron microscopy revealed that p53-Δ30 interacts with ssDNA in a manner similar to that of the protein representing the p53 core domain, i.e. p53-Δ30 binds internal segments of ssDNA but not ssDNA ends (Fig. 2; 35). In contrast, the full-length p53 protein binds to the ends of ssDNA (Fig. 2; 36).

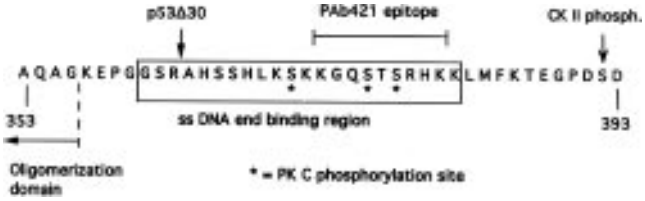

Figure 6. Schematic representation of the C-terminal region of human p53. Numbers indicate amino acid residues. Peptide 46 that binds ssDNA ends is indicated by the box. The C-terminal end of the p53-Δ30 truncation mutant is indicated, as well as the PAb421 epitope and phosphorylation sites for casein kinase II (CKII) and protein kinase C (PKC).

Mapping of the ssDNA end site using overlapping peptides allowed a more precise localization of this site to residues 361–382 (peptide 46) in the C-terminus of p53. The basic region of the p53 C-terminal domain was previously shown to bind DNA (20,21,33). Our data suggest that the biological function of this C-terminal DNA binding site in p53 is to recognize DNA damage *in vivo*.

The C-terminal region of p53 has several features of interest (Fig. 6). Hupp *et al*. (29) showed that various modifications of the C-terminus can activate cryptic specific DNA binding by the p53 core domain. The allosteric model for negative regulation of specific DNA binding by the C-terminal p53 domain proposed by Hupp and Lane (28) has been further substantiated by the finding that a small peptide corresponding to amino acid residues 369–383 of the p53 C-terminus can activate specific DNA binding of p53 *in vitro*, presumably by disrupting contacts between the regulatory domain and another region in p53 (31). Thus, the ssDNA binding site, localized to residues 361–382 in p53, coincides with a region implicated in regulation of specific DNA binding. Peptide 46, representing the ssDNA end binding site, can also activate latent p53 for specific DNA binding (G. Selivanova, unpublished results). Taken together, these observations suggest that ssDNA end binding to the C-terminal site may unleash the sequence-specific DNA binding activity of the p53 core domain from the negative control imposed by the C-terminal domain.

We tested this idea in band shift experiments, GST pull-down assays and by electron microscopy. Although ssDNA oligonucleotides were not able to activate p53 potently in band shift assays, a synergistic activation of both bacterially produced p53 and endogenous p53 from nuclear extracts by the ss 37mer oligonucleotide and the PAb421 antibody was observed. This implies that additional cellular factors besides ssDNA are needed for activation of sequence-specific DNA binding and/or stabilization of p53/DNA complexes *in vivo*. Our data are in agreement with results reported by Jayaraman and Prives (37) obtained in DNase I footprinting experiments. However, the fact that the MN oligonucleotide, which has 2 nt ss overhangs, can stimulate specific DNA binding of p53 implies that ssDNA ends, rather than ssDNA of a certain length, are crucial for activation of p53.

Electron microscopy analyses showed a significantly increased binding of p53 to dsDNA in the presence of the ss 75mer oligonucleotide, with up to 10 p53 molecules bound to one DNA molecule. Simultaneous binding of several p53 molecules to one dsDNA molecule was not detected in the absence of ssDNA oligonucleotides in our previous experiments (35), nor was it was observed by Stenger *et al*. (49). Since the on/off rate of p53 DNA binding is of the order of seconds (47), the probability that at least

one p53 molecule is bound to DNA at a given moment is significantly higher when a long target DNA containing several p53 binding sites is used. This may explain why we observed a much more potent stimulation of p53 binding to PG-CAT plasmid DNA containing multiple tandem p53 binding sites in GST pull-down assays and electron microscopy analysis than to the short BC oligonucleotide in band shift assays.

Notably, the p53 protein did not show any obvious preference for the tandem p53 consensus binding sites in the PG-CAT plasmid (Fig. 5). This is consistent with the notion that the interaction with ssDNA ends activates both specific and non-specific DNA binding by the core domain. C-terminal truncation, which activates specific binding (29), can also activate non-specific DNA binding by the p53 core domain. The crystal structure of the core domain in complex with a DNA oligonucleotide containing a consensus p53 binding site revealed that while two of the p53 molecules contact DNA specifically, the third p53 molecule makes non-specific contacts with DNA (48). This suggests that non-specific DNA binding may also be important for specific protein/DNA complex formation by the core domain. It is conceivable that p53 makes non-specific DNA contacts and 'slides' along the DNA before finding a specific target site.

A prediction from the allosteric model for regulation of p53 activity (31) is that the switch between a latent and an active conformation results in re-positioning of the C-terminal domain in the p53 tetramer. Our data are consistent with and, in fact, provide further evidence for this notion. PAb421 and PAb1801 do not recognize p53 complexed with DNA through its C-terminus but supershift p53/DNA complexes formed through the core domain, indicating that the epitopes detected by these antibodies are masked when p53 is complexed with DNA through the C-terminus.

Our results suggest that recognition of ssDNA ends generated in cells that have suffered DNA damage may cause activation of specific DNA binding of p53 and subsequent transactivation of p53-responsive genes. To prove this model, it would be necessary to identify point mutations or short deletions in the ssDNA end binding region that knock out ssDNA end binding without interfering with any other activity of p53. Introduction of such mutants in p53-negative cells and analyses of their ability to restore a normal DNA damage response may ultimately provide an answer as to the biological role of the ssDNA end binding activity of p53.

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