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Activation of p53 Sequence-Specific DNA Binding by Short Single Strands of DNA Requires the p53 C-Terminus

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Summary

Upon cellular DNA damage, the p53 tumor suppressor protein transmits a signal to genes that control the cell cycle and apoptosis. One function of p53 that is important for its role in this pathway is its ability to function as a sequence-specific transcriptional activator. We demonstrate here that short single DNA strands can markedly stimulate the ability of human and murine p53 proteins to bind specifically to a p53 response element in supercoiled DNA. We also show that single-stranded DNA does not stimulate binding by a truncated p53 that lacks the C-terminal domain. Finally, we establish that a peptide spanning the p53 C-terminus has the ability in *trans* to stimulate sequence-specific DNA binding by p53 dramatically. These data taken together suggest a model in which the p53 C-terminus can recognize DNA structures resulting from damage-induced lesions, and this interaction can be propagated to regulate positively p53 sequence-specific DNA binding.

Introduction

Signals emanating from damaged DNA cause the p53 response pathway to be activated (reviewed by Donehower and Bradley, 1993). Disruption of this pathway, which is clearly of central importance for maintaining the integrity of the genome of higher eukaryotes, correlates with oncogenesis. It is well established that p53 recognizes sequences conforming to the p53 consensus-binding site (reviewed by Vogelstein and Kinzler, 1992). The importance of the DNA binding property of p53 is underscored by the fact that the vast majority of mutations in p53 genes cloned from tumors are located within the centrally located sequence-specific p53 DNA-binding domain (see Hollstein et al., 1994). It is therefore important to understand how DNA binding by p53 is regulated.

The overall arrangement of p53 functional domains has been defined (reviewed by Prives et al., 1994). Like many other transcriptional activators, p53 contains an activation region, a specific DNA-binding domain, and an oligomerization domain. The structures of the DNA-binding (Cho et al., 1994) and oligomerization domains (Clore et al., 1994; Lee et al., 1994; Jeffrey et al., 1995) have been solved. The DNA-binding domain alone can bind well and highly specifically to DNA (Pavletich et al., 1993; Bargonetti et al., 1993; Wang et al., 1993). Yet, as part of the full-length protein, it is subject to regulation by sequences outside of it (Hupp et al., 1992; Halazonetis et al., 1993; Pietenpol et al., 1994; Wang and Prives, submitted). Cells subjected to DNA-damaging agents accumulate p53, and, depending on factors such as cell environment or genotype, either undergo arrest primarily in G1 or apoptosis (reviewed by White, 1994). Indeed, a number of genes have been identified that are clearly activated in cells by p53 after DNA damage ensues. These include *GADD45* (Kastan et al., 1992), *MDM2* (Perry et al., 1993; Chen et al., 1994), *WAF1*/p21/*CIP1* (El-Deiry et al., 1993; Dulić et al., 1994), and cyclin G (Okamoto and Beach, 1994). Each of these genes can be rationalized to be functional in a DNA damage-initiated pathway, and all have been shown to contain p53-binding sites.

Less well understood in the p53 pathway is how the signal is relayed from damaged DNA to p53. One feature of damaged DNA that appears essential for accumulation of p53 is the appearance of strand breaks (Lu and Lane, 1993; Nelson and Kastan, 1994). But it is not unlikely that more than one determinant can transmit the signal. We have considered the possibility that one recipient of a signal from damaged DNA is p53 itself. Our rationale is based on several studies showing that the C-terminus of p53 can recognize features such as short single strands and DNA ends that are likely to be present in irradiated cells. In particular, p53 has a potent DNA strand-reannealing activity (Oberosler et al., 1993; Bakalkin et al., 1994), and this activity is a function exclusively of the C-terminus since this region alone is sufficient to reanneal complementary single strands (Brain and Jenkins, 1994; Prives et al., 1994; Wu et al., 1995; Bakalkin et al., 1995). The C-terminus of p53 (spanning residues 311–393) contains at least two structural determinants, the tetramerization region (amino acids 319–360) and an adjacent region (amino acids 363–393) that is rich is basic residues. This region is also necessary for the ability of p53 to bind and reanneal complementary DNA single strands (Prives et al., 1994). We have considered the possibility that the C-terminus may interact with single-stranded DNA and that this interaction may activate the p53 DNA-binding domain and therefore the transcriptional activity of p53.

Results

Short Single Strands of DNA Stimulate p53 Binding to Its Cognate Site

We reasoned that, if found, a stimulatory effect of short DNA would be most evident (and perhaps more physiologically relevant) if p53 were bound to a site in closed circular DNA. To test this, we examined the effects of long (2.6 kb) native or heat-denatured (mostly single-stranded) or short (40 nt) single-stranded or double-stranded oligonucleotides on purified human p53 binding to a supercoiled plasmid into which the *GADD45* p53-binding site was cloned (Figure 1). p53 binding alone was adjusted (after preliminary binding curves were established) such that protection of the *GADD45* site was relatively weak (Figure 1A, lane 2). We observed that long denatured DNA not only failed to stimulate binding but actually completely inhibited

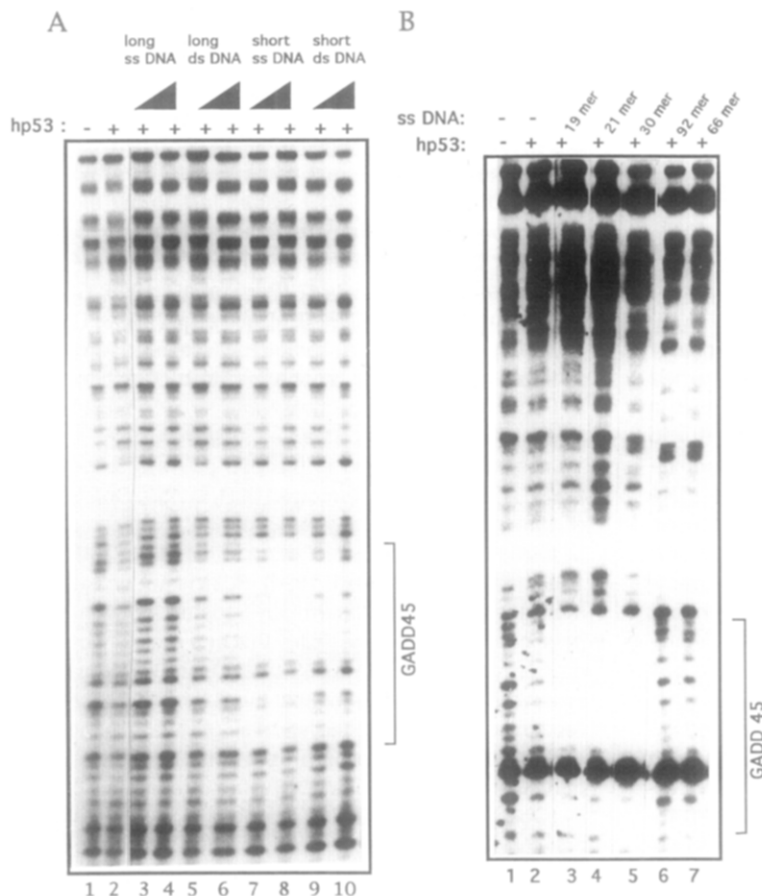

Figure 1. Short Single-Stranded DNA Fragments Stimulate Sequence-Specific Binding by Wild-Type Human p53

(A) Human p53 protein (hp53) (1 μg) was bound to a wild-type *GADD45* site-containing plasmid (100 ng), and primer extension footprinting was carried out as described in Experimental Procedures. Binding reaction mixtures contained either no protein (lane 1) or 1 μg of p53 (lanes 2–10) and either no DNA fragments (lanes 1 and 2) or 400 ng and 800 ng, respectively, of long single-stranded DNA (lanes 3 and 4), long double-stranded DNA (lanes 5 and 6), short single-stranded DNA (lanes 7 and 8) or short double-stranded DNA (lanes 9 and 10).

(B) Human p53 protein (hp53) (1 μg) was bound to a *GADD45* site-containing plasmid (100 ng), and primer extension footprinting was performed as in (A). Binding reactions contained either no protein (lane 1) or 1 μg of p53 (lanes 2–7) and either no DNA fragments (lanes 1 and 2) or 1000 ng of a 19-mer (lane 3), a 21-mer (lane 4), or a 30-mer (lane 5) or 2200 ng of a 66-mer (lane 6) or a 92-mer (lane 7). The very dark band in the lower region of the *GADD45*-protected site is a primer artifact.

any protection of this region, while long or short duplex DNA stands were without significant effect. By contrast, in the presence of a short single-stranded oligonucleotide i.e., a 40-mer, p53 binding to the *GADD45* site was markedly and consistently increased such that the two 10 nt half-sites that make up the p53-binding site were each very well protected. The quantity of short single-stranded DNA that was required to produce the stimulatory effect was in the stoichiometric rather than the catalytic range (1–4 mol of single-stranded DNA per mole of p53). To obtain further information on the size of single-stranded DNA molecules that could stimulate p53 binding a range of oligonucleotides (containing 19, 21, 30, 66, or 92 nucleotides) were then tested (Figure 1B). The results were striking: taken together with the data shown in Figure 1A, oligomers up to 40 bases in length were stimulatory. However, those containing 66 or 92 nucleotides were actually inhibitory to binding. This inhibition was observed when these longer oligomers were tested at either mass or molar equivalence to the 30-mer. Thus, there is a sharp transition between the length of a single-stranded DNA molecule that will activate p53 DNA binding and one, merely 25 bases longer, that not only fails to stimulate but actually inhibits. The sequences of the single-stranded oligonucleotides were most likely unimportant because there was no obvious relationship between the various oligonucleotides that stimulated or inhibited.

We tested whether purified murine p53 binding to supercoiled DNA could also be stimulated by short oligonucleotides (Figure 2). Additionally, since the C-terminus of p53 contains the region that binds to and anneals single-stranded DNA, we asked whether (murine) p53 that lacks the C-terminus but retains the ability to bind to a cognate site can be stimulated by single-stranded DNA. Baculoviruses encoding either murine full-length p53 or p53 containing amino acids 1–320 (Wang et al., 1993) were used as a source of immunopurified p53 proteins. Note that the murine p53 produced a substantially different footprint from human p53: murine p53 protected sequences within (solid line in Figure 2) and also adjacent to (dotted line in Figure 2) the *GADD45* site. While the short oligonucleotide stimulated full-length murine p53 protection of the *GADD45* site in supercoiled DNA, there was no increase in protection of this site when p53(1–320) was used. Thus, the p53 response to short single strands is conserved between human and murine species. Perhaps of greater significance, these data suggest that the p53 C-terminus is necessary for stimulation by short single strands of DNA.

The p53 C-Terminus Stimulates Sequence-Specific DNA Binding by the p53 DNA-Binding Domain

The experiments described above suggested that the p53 C-terminus, through its interactions with short single strands, may be capable of increasing the affinity of the

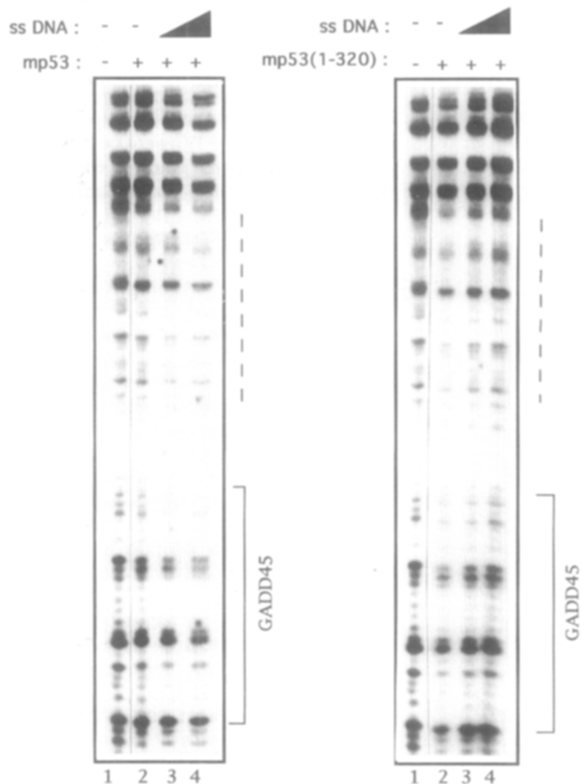

Figure 2. Binding by Full-Length Murine p53 but Not C-Terminal Truncated (1–320) Murine p53 Is Stimulated by Short Single Strands

Binding and primer extension footprinting reactions were carried out as in Figure 1.

(Left) Binding reaction mixtures contained 300 ng of murine full-length p53 (mp53) (lanes 2–4) and either no DNA fragments (lanes 1 and 2) or 500 ng (lane 3) and 1000 ng (lane 4) of short single-stranded DNA (ssDNA). Lane 1 contains no protein.

(Right) Binding reactions contained 400 ng of murine p53(1–320) (lanes 2–4) and either no added DNA fragments (lanes 1 and 2) or 500 ng (lane 3) or 1000 ng (lane 4) of short single-stranded DNA (ssDNA). Lane 1 contains no protein.

central sequence-specific DNA-binding domain with a cognate site. Since the interactions of the C-terminus with single strands are the result of an independent functional domain, it is possible that this region might have an effect on the rest of the p53 protein in *trans*, i.e., as a separate entity. We examined whether adding a peptide comprising the p53 C-terminus (amino acids 311–393) to full-length p53 affects the ability of p53 to bind to DNA. Additionally, since the C-terminal 30 amino acids of this region are required for the p53 DNA reannealing function, we also tested the effects of a C-terminal p53(311–367) peptide that lacks this basic region at the end. The purified proteins that we used are shown in Figure 3.

The purified C-terminal p53(311–393) peptide was added to full-length p53, and DNA binding to an oligonucleotide containing the *GADD45* p53 site was examined by the electrophoretic mobility shift assay (EMSA). The C-terminus dramatically stimulated the ability of p53 to bind to the *GADD45* oligonucleotide (Figure 4A). The magnitude of the stimulation, which varied among different experi-

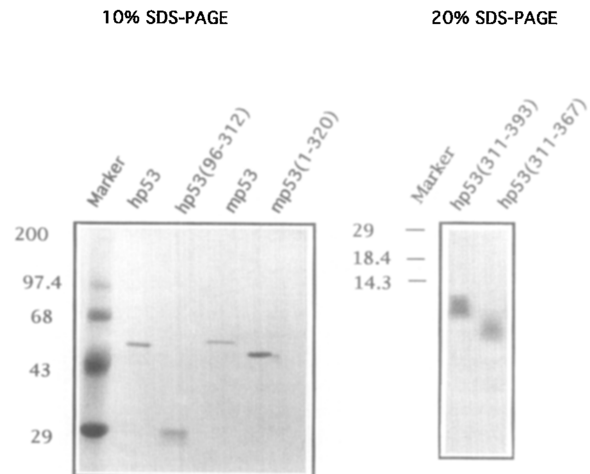

Figure 3. Full-Length and Truncated p53 Proteins

Proteins were analyzed by 10% or 20% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by Coomassie blue staining. Abbreviations: h, human; m, murine.

ments and different preparations of proteins, ranged between approximately 10- and 80-fold. The effect was dose dependent, reaching a maximum at a ratio of approximately 1000 ng of C-terminus per 100 ng of full-length p53 (Figure 4B). The C-terminus peptide also stimulated binding of p53 to the *GADD45* site in supercoiled DNA (unpublished data). Importantly, not only was full-length p53 binding increased in the presence of the 311–393 peptide, but the binding by the central core domain, i.e., p53 residues 96–312, was also consistently increased, although to a somewhat lesser extent, i.e., approximately 5- to 13-fold (Figure 4C). The murine C-terminus (amino acids 315–390) was also capable of stimulating human p53 to the same extent as the human p53 C-terminus (compare lane 1 with lanes 10 and 11 in Figure 4D), and DNA binding by full-length murine p53 was also stimulated by both the human p53 and murine C-terminus peptides (data not shown). This leads us to conclude that the effect of the C-terminus on p53 DNA binding is a conserved property of p53. A p53 C-terminal peptide that lacks the 30 terminal acids (311–367) did not stimulate binding detectably (compare lane 1 to lanes 6 and 7 in Figure 4D). Thus, the region at the distal end of the C-terminus that contributes to stimulation of DNA binding is the same as that required for DNA reannealing. This suggests that this region of the protein is required both for recognition of short single strands and regulation of DNA binding by the central sequence-specific DNA-binding domain.

Since the C-terminus itself can bind nonspecifically to DNA (Wang et al., 1993), it was important to ascertain whether the increase in DNA binding was specific. To examine the specificity of the stimulated DNA binding, we employed both a competition EMSA and DNase I footprinting (Figure 5). In each case, it was clear that the increased binding by p53 was markedly sequence specific. By EMSA, all detectable shifted labeled p53 consensus site (PCS) DNA was completely competed away by excess unlabeled wild-type *GADD45* oligonucleotides, while there

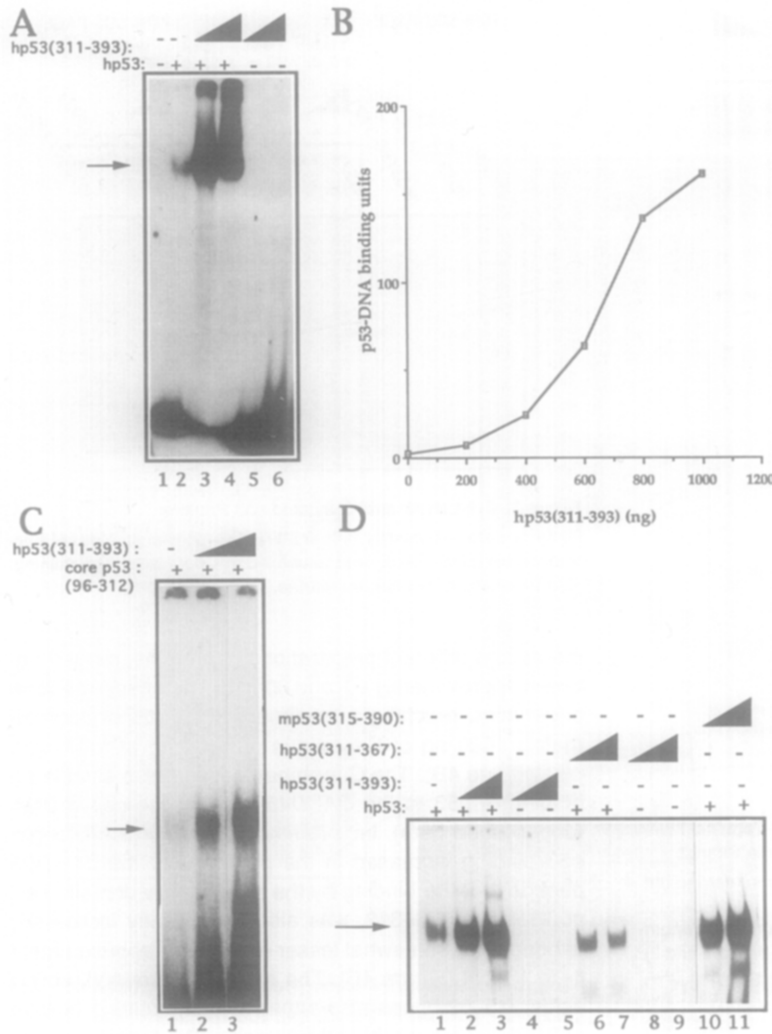

Figure 4. A Peptide That Spans the C-Terminus p53(311–393) Stimulates DNA Binding by Full-Length p53

(A) Human p53 (hp53) (50 ng) (lanes 2–4) and 300 ng (lanes 3 and 5) or 500 ng (lanes 4 and 6) of human p53(311–393) were incubated with a ^{32}P-labeled wild-type *GADD45* oligonucleotide (3 ng) and reaction mixtures analyzed by EMSA. Lane 1 contains DNA probe without protein. The arrow indicates the p53–DNA complex.

(B) Increasing quantities of p53(311–393) were added to p53 (100 ng) in EMSA reaction mixtures containing ^{32}P-labeled wild-type *GADD45* oligonucleotides (3 ng), and after gel electrophoreses the resulting protein–DNA complexes were quantitated by phosphorimaging using Image-Quant software.

(C) Human core p53(96–312) (60 ng) (lanes 1–3) and 300 ng (lane 2) or 500 ng (lane 3) of human p53(311–393) were incubated with a ^{32}P-labeled wild-type *GADD45* oligonucleotide (3 ng) and reaction mixtures analyzed by EMSA. The arrow indicates the p53–DNA complex.

(D) Human p53 (100 ng) (lanes 1–3, 6, 7, 10, and 11) was bound to a ^{32}P-labeled wild-type *GADD45* oligonucleotide (3 ng) in the presence of 200 ng (lanes 2 and 4) or 400 ng (lanes 3 and 5) of human p53(311–393); 200 ng (lanes 6 and 8) or 400 ng (lanes 7 and 9) of human p53(311–367); or 160 ng (lane 10) or 240 ng (lane 11) of murine C-terminus p53(315–390) peptide as indicated above each lane. The arrow indicates the p53–DNA complex.

was virtually no competition by similar quantities of mutant *GADD45* oligonucleotides (Figure 5A). Similarly, in a DNase I footprint assay, when an amount of p53 was used that only weakly protected the *GADD45* site in an end-labeled DNA fragment (Figure 5B, lane 1), the protection of this region was complete in the presence of the C-terminal peptide (lanes 6 and 7). Interestingly, the DNase I hypercutting induced by p53 (indicated by the arrow in Figure 5B) was reduced in the presence of p53(311–393). As expected, the C-terminal peptide itself showed absolutely no protection of this region (Figure 5B, lane 5).

To confirm further the specificity of the p53 C-terminus for stimulation of p53-specific DNA binding, we asked whether unrelated sequence-specific DNA-binding proteins might be affected in a similar manner by the p53 C-terminus (Figure 6). We used purified GAL4-VP16 and serum response factor (SRF) proteins and tested their binding to oligonucleotides containing their cognate sites by EMSA. Under all conditions examined, the interaction of neither protein with DNA was significantly stimulated by the p53 C-terminus peptide.

Insight into the Mechanism by Which the p53 C-Terminus Stimulates Specific DNA Binding by the Central Core Domain

The specificity of the effect of the p53 C-terminus on p53 sequence-specific DNA binding suggested that there might be protein–protein interactions between the C-terminus and the central core of p53. However, we have been unable to demonstrate an interaction between the C-terminal peptide and either full-length p53 or the central "core" of p53 (data not shown). Furthermore, there is no change in the mobility of p53 protein–DNA complexes in the presence of p53(311–393). The C-terminus may thus increase specific p53 DNA binding by competing for and thereby removing the nonspecific carrier DNA from reaction mixtures. However, we have three lines of evidence against this possibility. First, the fact that the highly sequence-specific central core domain of p53 is strongly stimulated by the C-terminus (see Figure 4C) argues against competition for nonspecific DNA. Second, even when carrier DNA was absent from the DNA-binding reaction mixtures, p53(311–393) was highly effective in stimu-

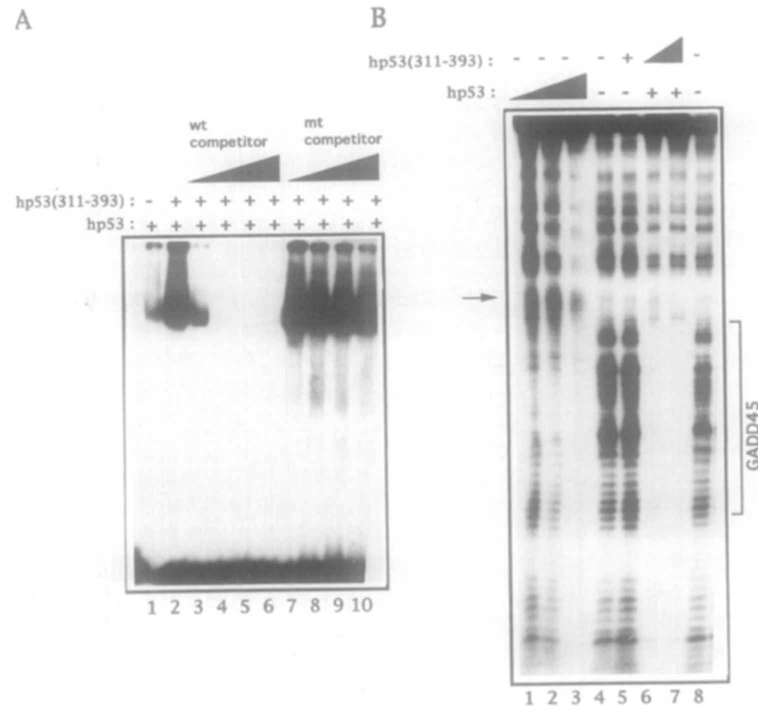

Figure 5. The C-Terminus p53(311–393) Stimulates Sequence-Specific Binding by Full-Length p53

(A) EMSA competition analysis was performed using ^{32}P-labeled p53 consensus site (PCS) oligonucleotide (3 ng). Reaction mixtures contained 50 ng of p53 (lanes 1–10) and 300 ng of p53(311–393) (lanes 2–10) and either no competitor (lanes 1 and 2) or a 13.3-, 50-, 75-, and 100-fold excess of unlabeled wild-type (wt) *GADD45* oligonucleotide (lanes 3–6, respectively) or a 13.3-, 50-, 75-, and 100-fold excess of mutant (mt) *GADD45* oligonucleotide competitor (lanes 7–10, respectively).

(B) DNase I footprinting was carried out using a ^{32}P-labeled wild-type *GADD45* DNA fragment (0.3 ng) as described in Experimental Procedures. Reaction mixtures contained no protein (lanes 4 and 8), or 250 ng (lanes 1, 6, and 7), 500 ng (lane 2), or 1000 ng (lane 3) of full-length p53 and 1 μg (lanes 5 and 6) and 2 μg (lane 7) of p53(311–393). DNase I hypercutting induced by p53 is indicated by arrow.

lating sequence-specific DNA binding by p53 (Figure 7). Third, results with the p53-specific monoclonal antibody PAb421, shown previously to stimulate the DNA binding properties of p53 (Hupp et al., 1992; Halazonetis et al., 1993), argued against the notion that the C-terminus peptide is simply competing for nonspecific DNA (Figure 7). In this experiment we determined the effects of PAb421 on DNA binding by full-length p53 and by p53(311–393). Since no carrier DNA was present, it was possible to detect C-terminus-bound DNA (Figure 7A, lane 1, thick arrow).

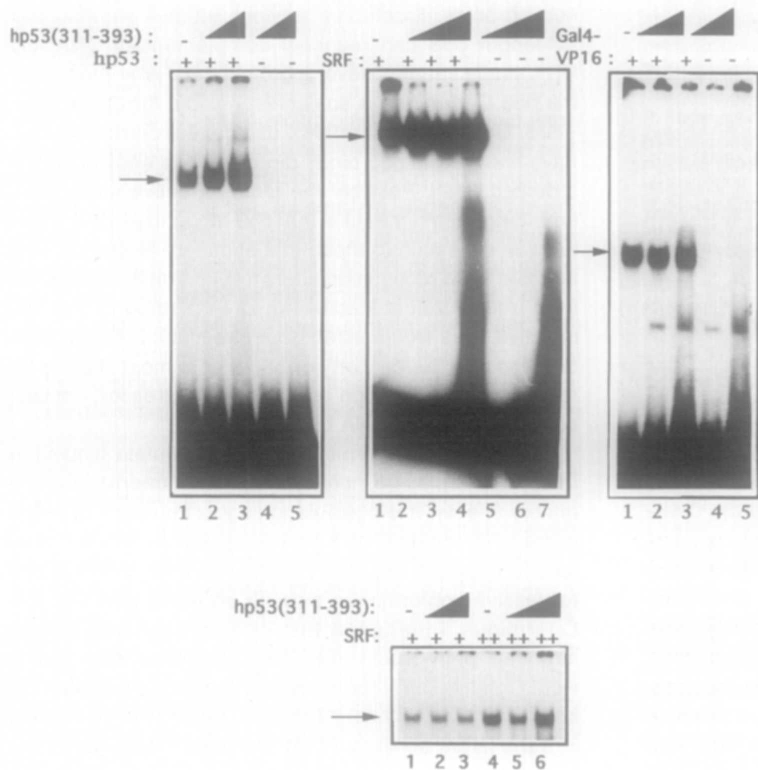

Figure 6. C-Terminus p53(311–393) Does Not Significantly Stimulate DNA Binding by SRF or GAL4–VP16 Proteins

EMSAs were performed with ^{32}P-labeled oligonucleotides (3 ng) containing either wild-type GADD45-, SRF-, or GAL4-binding sites. Arrows indicate positions of specific protein–DNA complexes.

(Left) Reaction mixtures contained p53 (100 ng; lanes 1–3) and either 300 ng (lanes 2 and 4) or 500 ng (lanes 3 and 5) of C-terminus p53(311–393) peptide.

(Middle) Reaction mixtures contained SRF (30 ng; lanes 1–4) and either 100 ng (lanes 2 and 5), 300 ng (lanes 3 and 6), or 500 ng (lanes 4 and 7) of p53(311–393).

(Bottom) Reaction mixtures contained 200 ng (lanes 2 and 4) or 400 ng (lanes 3 and 6) of p53 C-terminus peptide and either 4 ng (lanes 1–3) or 8 ng (lanes 4–6) of SRF.

(Right) Reaction mixtures contained 20 ng of GAL4–VP16 (lanes 1–3) and 300 ng (lanes 2 and 4) or 500 ng (lanes 3 and 5) of C-terminus p53(311–393) peptide.

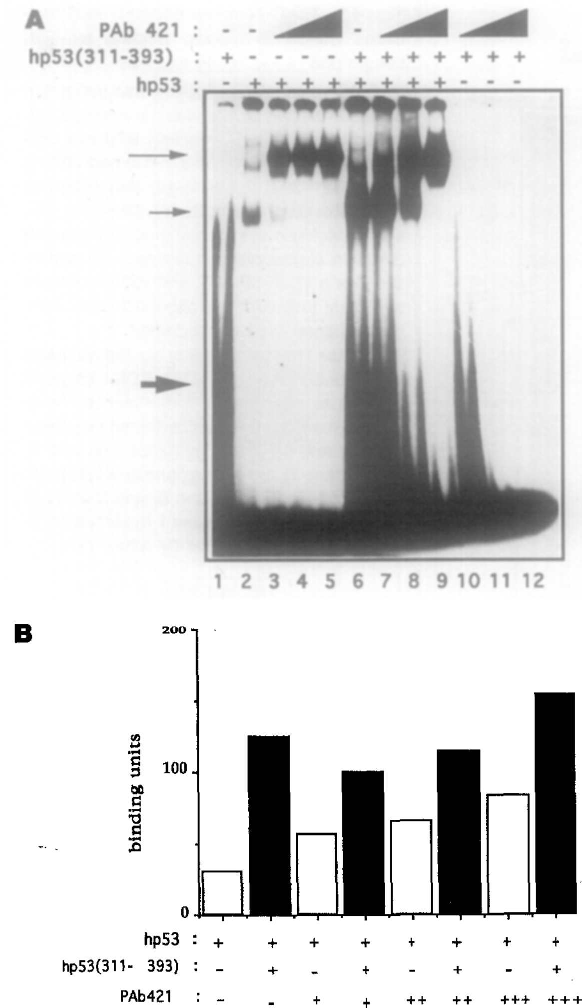

Figure 7. C-Terminus p53(311–393) Stimulates DNA Binding by p53 in the Presence of Monoclonal Antibody PAb421

(A) EMSA was performed after p53 (50 ng) was bound to a ^{32}P-labeled p53 consensus site oligonucleotide (3 ng) as described in Experimental Procedures. However, reaction mixtures contained no poly(dI–dC). Where indicated above lanes, 300 ng of C-terminus p53(311–393) peptide was added; 2 μl (lanes 3, 7, and 10), 3 μl (lanes 4, 8, and 11), or 4 μl (lanes 5, 9, and 12) of p53-specific monoclonal antibody PAb421 were added as indicated.

(B) Graphic representation of protein–DNA complexes (all forms) shown in (A) quantitated by phosphorimaging. Thick arrow indicates C-terminus-bound DNA; long arrow indicates PAb421-shifted p53–DNA complex; short arrow indicates the p53 complex that is stimulated by the C-terminal peptide (311–393).

Additionally, we measured binding by p53 in the presence of both PAb421 (long arrow in Figure 7A) and the C-terminal peptide (short arrow in Figure 7A). As shown previously, PAb421 stimulated and shifted the p53–DNA complex (Figure 7A, lanes 3–5). However, under conditions that allowed detection of the binding of the oligonucleotide by p53(311–393) (see Figure 7A, lane 1), PAb421 inhibited such binding (compare lanes 1 and 10–12). In the presence of both full-length p53 and p53(311–393), increasing levels of PAb421 led to increased binding over what was seen with PAb421 alone (compare lanes 5 and 9 in Figure 7A). Therefore, PAb421, while inhibiting DNA binding by p53(311–393), did not block its ability to stimulate full-length p53 DNA binding. By contrast, at lower concentrations of PAb421, the C-terminus apparently reduced the extent to which PAb421 shifted the p53–DNA complex in the EMSA. Taken together, our data suggest that p53(311–393) transiently (but undetectably) interacts with full-length p53, leading to augmentation of its ability to bind specifically to its cognate sites.

Discussion

It is likely that there are at least two ways in which p53 is up-regulated after DNA damage. That p53 levels are increased and stabilized after treatment of cells with agents that induce DNA damage has been well documented. Additionally, it is reasonable to assume that p53 in cells is intrinsically more active for DNA binding and transcriptional activity after DNA damage. This latter possibility is supported by the following p53 features. First, p53 can exist in at least two conformations that differ in their ability to interact with DNA (Hupp and Lane, 1994; Halazonetis and Kandil, 1993). Furthermore, the C-terminus of p53 contains a determinant that is responsible for these alternate conformations. Indeed, the region of the C-terminus that is most likely to be regulatory, i.e., the last 30 amino acids, is also the region that we have shown is necessary for the stimulation of p53 by the C-terminal peptide. The likely possibility that p53 exists in latent and active forms implies that there are effectors in cells that can bring about a shift between these two forms. Second, the C-terminus of p53 displays properties that suggest that it can interact in a unique way with short single-stranded nucleic acids. Extending previous studies showing that full-length p53 can reanneal complementary DNA and RNA single strands, several groups have now shown that the p53 C-terminus is solely responsible for this activity. Third, Bakalkin et al. (1994), using electron microscopy, have shown that p53 binds directly to the ends of single-stranded DNA. Possibly the most attractive speculation emerging from our data is that p53 recognizes DNA structures in cells akin to those that stimulate binding in vitro and that such recognition alters it such that it is converted from a form that is inactive for DNA binding to one that is active. This would establish a direct link between DNA damage and the property of p53 that is most likely to be required for its function as a tumor suppressor, namely, sequence-specific DNA binding.

How does the C-terminal peptide stimulate binding in *trans*? There may be more than one component to its stimulatory effects. It is possible to suggest a model in which some other region of p53, perhaps at the N-terminus, contains a negative regulatory sequence that is held in this repressive conformation through an interaction with the C-terminus. Interaction with effectors such as single-stranded DNA would alter the C-terminus such that it would release the N-terminus and thus make available the central region for binding with DNA. Adding the C-terminus in *trans* might similarly titrate the N-terminus away from its negative regulatory conformation. However, since the

C-terminus of p53 is capable of stimulating, albeit to a lesser extent, the ability of the central site-specific p53 core to bind to DNA, there is clearly a direct effect of the C-terminus on the DNA-binding domain. The most reasonable view of all this, notwithstanding, is that full understanding of the roles of the C-terminus is not yet available and that, however it acts, the C-terminal 30 amino acids are clearly an important regulatory part of the protein.

In our initial experiments, we used EMSA to test the effects of long or short single- or double-stranded DNA on p53 sequence-specific binding to a labeled *GADD45* site duplex oligomer. Our results showed that while short single strands had no effect, all other forms of DNA reduced sequence-specific binding (unpublished data). One explanation for our inability to detect a stimulatory effect by EMSA is that the short labeled oligomers characteristically used in this assay consist almost entirely of the p53 site, while with a supercoiled plasmid, p53 would have to "seek and find" its site over a considerable distance of DNA. It is therefore possible that short single-stranded oligonucleotides stimulate binding by increasing the relative affinity of p53 for a specific site in nonspecific contiguous DNA or, more speculatively, by facilitation of movement along DNA to find a binding site.

It is remarkable that only short oligonucleotides were effective in stimulating p53 sequence-specific binding. Although we do not know the precise length of the shortest stimulatory oligonucleotide, we have found that one containing only 16 nucleotides is also capable of activating DNA binding (data not shown). However, at the upper limit, an oligonucleotide with as few as 66 nucleotides was inhibitory to p53 sequence-specific binding. This is consistent with results of Bakalkin et al. (1995), who have evidence suggesting that two discrete domains of p53 interact with single-stranded DNA: the C-terminus, which recognizes short oligonucleotides, and the central domain, which binds to long single strands. Since we found that the 66-mer is inhibitory to site-specific binding, this would imply that this oligonucleotide is of sufficient length to bind preferentially to the central region of p53 and to prevent its binding to a cognate site. By contrast, oligomers ranging between 16 and 40 nucleotides bind the C-terminus of p53, and this facilitates the interaction of the central domain with p53 response elements. Whether or not the short single-stranded oligomers remain associated with the p53 C-terminus when the protein is bound to its site in double-stranded DNA is currently under investigation.

One consideration in the interpretation of our data is the improbability that within cells subjected to DNA-damaging agents DNA lesions would occur within the vicinity of a p53-responsive gene. Clearly, therefore, a brief transient effect of single-stranded DNA on p53 would not be relevant to the cellular p53 DNA damage response. However, if the effect of single strands on existing p53 molecules in cells were to be of significant duration or even irreversible, then it is possible to envision a more long-range propagation of the effect of DNA damage on p53. The fact that purified p53 associates and dissociates from DNA rapidly (Bargonetti et al., 1992) suggests that mechanisms may exist in cells for stabilizing its binding.

How would p53 find short single strands resulting from DNA damage in cells? Indeed, there are aspects of the DNA repair process that might well intersect with p53. p53 itself has the ability to recognize and bind to some forms of DNA lesions, such as certain mismatches (Lee et al., 1995 [this issue of *Cell*]) or single strand ends. Different components of the excision repair complex have been reported to interact with p53. The single-stranded binding protein RPA has been shown to associate with the activation domains of p53 and VP16 and possibly other activators as well (Dutta et al., 1993; He et al., 1993; Li and Botchan, 1993). Additionally, the basal transcription factor TFIIH (Xiao et al., 1994) and the *ERCC3* polypeptide component of TFIIH (Wang et al., 1994) have been reported to bind p53. Human excision repair is carried out by a large complex of polypeptides that includes both RPA and TFIIH (reviewed by Sancar, 1994). The human excinuclease complex first recognizes ultraviolet-damaged DNA and then introduces single strand incisions at positions 3′ and 5′ to the mismatch (Huang et al., 1992). A product of the subsequent repair of the lesion is the release of a single-stranded 29-mer. This short oligonucleotide is then an excellent candidate for a physiologically relevant activator of p53 DNA binding. It is tempting to speculate that the interaction of p53 either with proteins such as RPA or TFIIH or with DNA lesions directly may result in its recruitment to the site of damaged DNA so that it can be activated by the oligonucleotide product of the excision repair reaction.

Experimental Procedures

Purification of p53 Proteins

Sf-21 cells were infected with human p53 recombinant virus, harvested 48 hr postinfection, and extracted, and full-length p53 protein was immunopurified essentially as described by Friedman et al. (1990). Baculoviruses expressing His-tagged murine full-length p53 or truncated murine p53(1–320) and p53(315–320) proteins were provided by P. Tegtmeyer. Human central core p53(96–312) and human p53(311–393) and p53(311–367) C-terminus peptides were a gift from N. Pavletich and were prepared as described by Pavletich et al. (1993). SRF (Manak et al., 1990) and GAL4–VP16 (Zhu et al., 1994) proteins were provided by members of the R. Prywes laboratory.

EMSAs

EMSA was carried out as described previously (Chen et al., 1993). Sequences of the oligonucleotide probes containing either wild-type or mutant p53 consensus sequence are as follows: *GADD45* wild type, AATTCTCGAGCAGAACATGTCTAAGCATGCTGGGCTCGAG; *GADD45* mutant, AATTCTCGAGCAGAAAATTTCTAAGAATTCTGG-GCTCGAG; p53 consensus site (PCS) wild type, TCGAGAGACA-TGCCCAGGCATGCCTC. The oligonucleotides containing the SRF-binding site (CAATCCCTCCCCCCTTATGAAAGATGCCCATATATG-GGCATCTTCTGCAGCA) (Manak et al., 1990) and the GAL4–VP16-binding site (TCGAGCGGAGGAC$^{T}/_{A}$GTCCTC) (Johansen and Prywes, 1993) were gifts from R. Prywes and coworkers. Probes were labeled by the Klenow fragment of Escherichia coli DNA polymerase. Reaction mixtures contained 8 μl of 5× EMSA buffer (100 mM HEPES [pH 7.9], 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl), 2 μl of 40 mM spermidine, 2 μl of 10 mM DTT, 2 μl of 0.5% NP-40, 2 μl of 60 μg/ml double-stranded poly(dI–dC), 4 μl of bovine serum albumin (BSA) (1 mg/ml), ^{32}P-labeled probe DNA (3 ng), proteins as indicated, and water in a total volume of 40 μl. Reaction mixtures were incubated at room temperature for 40 min; 20 μl of each reaction mixture was then loaded onto a native 4% polyacrylamide gel containing 0.5× Tris–borate–EDTA (TBE) buffer, 1 mM EDTA, and

0.05% NP-40 and electrophoresed in 0.5× TBE at 4°C at 200–250 V for 2 hr.

DNase I Footprinting Assays

Analysis of p53 bound to supercoiled DNA was carried out as described previously (Gralla, 1985). Reaction mixtures contained 10 µl of 5× footprint buffer (31 mM MgCl, 163 mM HEPES [pH 7.9], 2.5 mM DTT, 250 mM KCl, 500 µg/ml BSA, 0.25 mM EDTA, 25% glycerol, 0.125% NP-40, and 2.5 mM spermidine), 20 µl of water, 1 µl of plasmid DNA (100 ng/µl), buffer BC100 (20 mM Tris [pH 8.0], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA), p53 proteins, and DNA as indicated in a final volume of 50 µl. The plasmid DNA used was pBluescript containing the *GADD45* site (see above) cloned into the EcoRI site. The bottom strand of the *GADD45* mutant oligonucleotide (described earlier) was used for short single-stranded DNA, and the hybridized double-stranded mutant *GADD45* oligonucleotide was used for short double-stranded DNA in Figure 1A. pUC19 plasmid DNA cut at the unique EcoRI site was used for long double-stranded DNA. Long single-stranded DNA was prepared by heating the EcoRI-cut pUC19 plasmid DNA at 92°C for 5 min and then adding it directly to the binding mixture on ice. Sequences of oligonucleotides used in the Figure 1B were as follows: 19-mer, CGCCCACGGATCTGAAGGG; 21-mer, CATGAGCGCTGCTCAGATAGC; 30-mer, CCAGCTGCAGCTCCACTGGATGGAGAATAT; 66-mer, GATCGAATTCACCATGGGCTACCCATACGATGTTCCAGATTACGCTGAGGAGCCGCAGTCAGATCC; 92-mer, AGTAGTGTAGGAATTC-N60-CTCGAGAGGTCACAGT (N is any nucleotide). Reaction mixtures were incubated on ice for 30 min followed by DNase I digestion. The amount of DNase I used was pretested empirically to produce an even pattern of partial cleavage products. DNase I reactions were performed on ice for 2 min and stopped by adding 20 µl of DNase I stop buffer (1% SDS, 20 mM EDTA, 200 mM KCl, and 250 µg/ml yeast tRNA). The DNAs were extracted with phenol and heated at 80°C for 2 min, and the aqueous phase was loaded on a prespun G-50 Sephadex column and centrifuged at 2300 rpm for 5 min. The eluate was collected and the volume made up to 70 µl with water.

Primer extension of the DNase I–digested fragments was done using the 20-mer SK primer (Stratagene). The primer was ^{32}P labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. To half the DNase I–treated samples, 1 µl of primer and 4 µl of 50 mM NaOH were added, and then the mixture was heated at 80°C for 3 min to denature the DNA. The samples were fast-cooled on ice, and 10 µl of primer extension buffer (500 mM Tris [pH 7.2], 100 mM Mg_2SO_4, and 2 mM DTT) was added and incubated at 45°C for 3 min for annealing. A dNTP mixture (2.5 mM of each NTP; 40 µl total) was added to each of the samples and the volume made up to 100 µl with water. Extension reactions were then performed with the Klenow fragment of E. coli DNA polymerase at 52°C for 10 min. Reactions were stopped with 25 µl of stop mix (4 M sodium acetate and 20 mM EDTA), and the DNA was ethanol precipitated and resuspended in deionized formamide. The DNA fragments were electrophoresed on a gel of 8% polyacrylamide and 7 M urea.

DNase I footprinting of p53 bound to a linear ^{32}P-labeled 120 bp DNA fragment from the pBluescript plasmid containing the wild-type *GADD45* site was performed as described previously (Chen et al., 1993).

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