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Tight DNA binding and oligomerization are dispensable for the ability of p53 to transactivate target genes and suppress transformation

Eitan Shaulian[1], Arie Zauberman[1], Jo Milner[2], Elizabeth A.Davies[2] and Moshe Oren[1,3]

[1]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel and [2]Department of Biology, University of York, York, YO1 5DD, UK
[3]Corresponding author

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The p53 tumor suppressor protein can bind tightly to specific sequence elements in the DNA and induce the transactivation of genes harboring such p53 binding sites. Various lines of evidence suggest that p53 binds to its target site as an oligomer. To test whether oligomerization is essential for the biological and biochemical activities of p53, we deleted a major part of the dimerization domain of mouse wild-type p53. The resultant protein, termed p53wtΔSS, was shown to be incapable of forming detectable homo-oligomers *in vitro* and is, therefore, likely to be predominantly if not exclusively monomeric. In agreement with the accepted model, p53wtΔSS indeed failed to exhibit measurable DNA binding *in vitro*. Surprisingly, though, it was still capable of suppressing oncogene-mediated transformation and of transactivating *in vivo* a target gene containing p53 binding sites. These findings indicate that dimerization-defective p53 is biologically active and may engage in productive sequence-specific DNA interactions *in vivo*. Furthermore, p53 dimerization probably leads to cooperative binding to specific DNA sequences.

Key words: dimerization/p53/protein-DNA interaction/ tumor suppressor

Introduction

The p53 protein is the product of a tumor suppressor gene, whose inactivation is likely to play a major role in the development of many types of cancer (reviewed in Hollstein *et al.*, 1991; Levine *et al.*, 1991; Oren, 1992; Vogelstein and Kinzler, 1992). The continued expression of the wild-type (wt) form of the protein (wt p53) therefore appears to counteract the tumorigenic process. This realization has spurred extensive investigation into the biochemical nature of the p53 protein.

p53 is capable of sequence-specific DNA binding (Bargonetti *et al.*, 1991; Kern *et al.*, 1991, 1992; El-Deiry *et al.*, 1992; Funk *et al.*, 1992; Zambetti *et al.*, 1992; Zauberman *et al.*, 1993). Furthermore, the presence of a p53 binding site can confer upon a gene the ability to be positively regulated by wt but not mutant p53 (Farmer *et al.*, 1992; Funk *et al.*, 1992; Kastan *et al.*, 1992; Kern *et al.*, 1992; Zambetti *et al.*, 1992; Zauberman *et al.*, 1993). Although not formally proven, it is conceivable that at least part of the biological effects of p53 stem from its ability to modulate the expression of appropriate target genes (Fields and Jang, 1990; Raycroft *et al.*, 1990; Lane, 1992; Oren, 1992; Vogelstein and Kinzler, 1992). While many of the putative target genes of p53 still remain to be identified, a number of p53-responsive genes are already known. At least some of these genes encode proteins whose function is most likely to be related to the biological activities of wt p53. These include the *mdm2* gene (Barak *et al.*, 1993), whose products appear to antagonize the activity of wt p53 (Momand *et al.*, 1992; Oliner *et al.*, 1992; Finlay, 1993) and whose activation by p53 may thus serve to terminate p53-mediated signaling (Barak *et al.*, 1993), and also the *gadd45* gene (Kastan *et al.*, 1992), whose pattern of expression is consistent with the proposed role of p53 in mediating a G1 growth arrest upon DNA damage (Kuerbitz *et al.*, 1992; Lane, 1992). There also exist other genes which bind p53 and can be transactivated by it, but whose relevance to the proposed functions of p53 is less obvious. Examples of this class are the muscle creatine kinase gene (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992) and the GLN family of retrovirus-like elements (Zauberman *et al.*, 1993).

The elucidation of factors regulating the ability of p53 to bind to and transactivate potential target genes appears to be of central importance in understanding the mode of action of this tumor suppressor. Recent work has suggested that this ability can potentially be modulated through a variety of mechanisms, including phosphorylation, binding of antibodies (and presumably other more relevant proteins), interaction with heat shock proteins and proteolytic cleavage (Hupp *et al.*, 1992).

The structure of the consensus p53 binding site is consistent with the binding of a p53 tetramer to each full site (El-Deiry *et al.*, 1992). However, recent work has suggested that while p53 dimerization may be important for binding, tetramerization may actually inhibit binding to at least certain DNA targets (Hupp *et al.*, 1992). Both dimerization and tetramerization depend on the integrity of the C-terminal domain of p53 (Milner *et al.*, 1991; Stürzbecher *et al.*, 1992). More specifically, the dimerization domain of p53 appears to comprise residues 334-356 of the human protein, whereas further assembly into trimers, tetramers and larger complexes requires residues located further towards the C-terminus of the protein, between positions 363 and 386 of human p53 (Stürzbecher *et al.*, 1992).

To determine whether homo-oligomerization is obligatory for the proper activity of wt p53, we introduced a small deletion into the dimerization domain of mouse p53. The resultant protein appeared to be monomeric and could not form detectable interactions with target DNA sequences *in vitro*. Nevertheless, it could suppress oncogene-mediated transformation and transactivate a target promoter carrying p53 binding sites. These findings suggest that dimerization-defective p53 can still interact with p53-responsive DNA elements, at least *in vivo*, and that dimerization results in cooperative DNA binding and thus in a much tighter

interaction with the consensus target DNA sequence. Importantly, the data argue that stable dimerization is not a prerequisite for the biological and biochemical functions of p53, but rather serves as a mechanism for making the protein more efficient in carrying out these functions.

Results

Deletion of residues 330–344 of mouse wt p53 abolishes the ability to form homo-oligomers in vitro

A miniprotein comprising the 89 C-terminal residues of mouse p53 can associate efficiently with full-length p53. However, an internal deletion which substitutes residues 330–344 by a single proline (see Figure 1) causes the miniprotein to lose its homo-oligomerization potential (Shaulian *et al.*, 1992). A similar deletion was subsequently introduced into the full-size mouse p53, giving rise to mutant p53wtΔSS (Figure 1). To assess the potential of p53wtΔSS to form homo-oligomers, it was translated *in vitro* together with full-length human wt p53. The translation products were then precipitated with either of the following monoclonal antibodies: PAb421, reactive with both human and mouse p53; PAb1801, reactive with human p53 only; or the SV40 large T antigen-specific PAb419, serving as a negative control (Figure 1C). As expected, PAb1801 reacted selectively with human p53 (lane 8) and did not precipitate directly intact mouse wt p53 (lane 5) or mouse p53wtΔSS (lane 2). Upon co-translation with human p53, a fraction of intact mouse p53 became precipitable by PAb1801 (lane 14, p53m). This implies the formation of a tight association between the two p53 species. However, no such co-precipitation was detectable when p53wtΔSS was co-translated with human p53 (lane 11). Thus, as in the C-terminal miniprotein (Shaulian *et al.*, 1992), deletion of residues 330–344 abolishes the ability of mouse wt p53 to form stable oligomers with other full-length p53 molecules, at least *in vitro*.

These results strongly suggest that p53wtΔSS can no longer form stable homo-oligomers. Yet, one could still formally argue that the deletion eliminates only the ability of p53wtΔSS to associate with heterologous p53 molecules, while retaining the capacity to self-associate. To address this issue more directly, different variants of mouse wt p53 were translated *in vitro* at 30°C and subjected to size fractionation by gel filtration using FPLC (Milner *et al.*, 1991). Under those conditions, full-length wt mouse p53 (Figure 2A) could be clearly resolved into monomers (peak 1), dimers (peak 2) and higher-order forms. In contrast, p53wtΔSS was practically exclusively monomeric (Figure 2B). In fact, the profile exhibited by p53wtΔSS was virtually indistinguishable from that of p53Ala^{stu} (Figure 2C), which lacks the last 47 C-terminal residues and has previously been shown to be incapable of self-association (Milner *et al.*, 1991). The FPLC analysis thus further confirmed that deletion of residues 330–344 rendered p53wtΔSS unable to undergo efficient dimerization, resulting in the generation of an essentially monomeric mutant.

The properties of p53wtΔSS are consistent with the work of Stürzbecher *et al.* (1992), who have recently demonstrated that a α helical domain comprising residues 334–356 of human p53 (and by analogy residues 331–353 of mouse p53) is the major determinant for p53–p53 dimerization, and that mutation of key residues within this region converts the protein into a dimerization-defective form.

Deletion of residues 330–344 of mouse wt p53 abolishes detectable sequence-specific DNA binding in vitro

The structural features of DNA elements which can be recognized specifically by p53 suggest that p53 binds to DNA

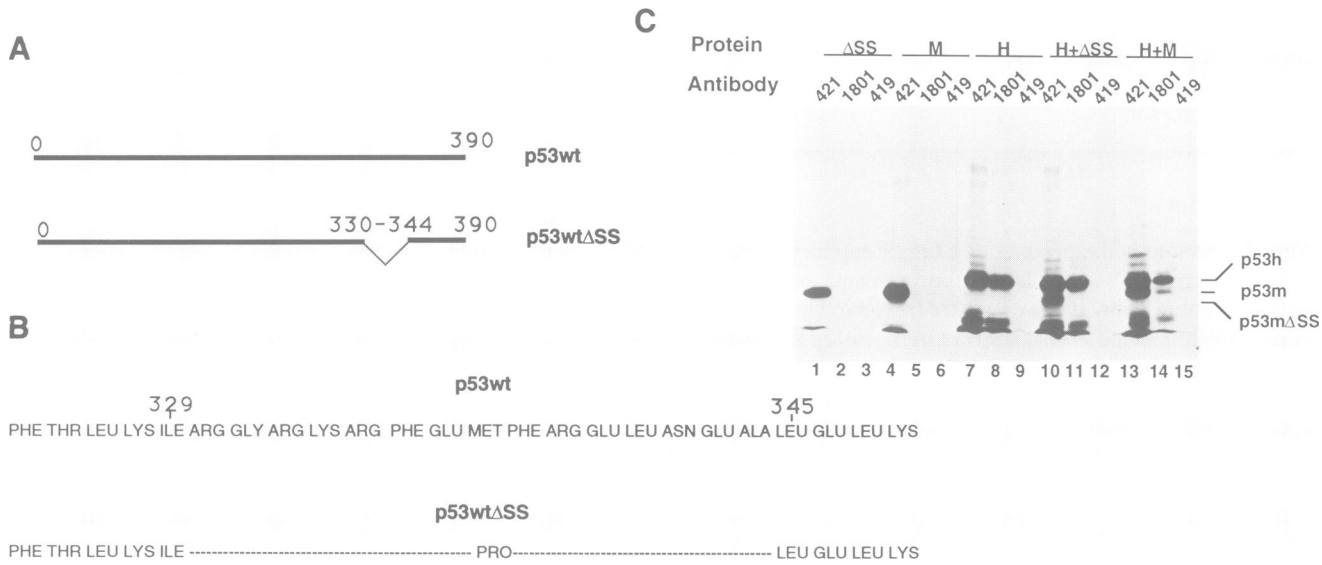

Fig. 1. **(A)** Schematic illustration of p53wtΔSS, indicating the position of the internal deletion. **(B)** Amino acid sequence of residues 325–348 in intact wt p53 and of the corresponding region in p53wtΔSS; the internal deletion substitutes residues 330–344 by a single proline. **(C)** p53wtΔSS fails to associate with co-translated human wt p53. RNA molecules encoding either murine p53wtΔSS (ΔSS), mouse wt p53 (M) or human wt p53 (H) were generated by *in vitro* transcription and translated in a rabbit reticulocyte lysate either separately (lanes 1–9) or in pairs (lanes 10–15), as indicated above each set of lanes. Translation reactions included [^{35}S]methionine. Each translation reaction was then divided into three equal portions, which were subjected to immunoprecipitation with the following monoclonal antibodies: PAb421, which recognizes directly both human and mouse p53; PAb1801, which binds only to human p53; and the SV40 large T antigen-specific monoclonal antibody PAb419, which served as a specificity control. The positions of the following polypeptides are indicated on the right: p53h, human wt p53; p53m, mouse wt p53; p53mΔSS, mouse p53wtΔSS.

as an oligomer (El-Deiry *et al.*, 1992). It was therefore of interest to find out whether the apparently monomeric p53wtΔSS can engage in sequence-specific DNA binding. To that end, the experiment shown in Figure 3 was carried out. Full-length wt p53 and p53wtΔSS were generated by *in vitro* translation in a reticulocyte lysate in the presence of [^{35}S]methionine. Part of each reaction was subjected to SDS−PAGE (panel B), revealing the presence of comparable amounts of each protein in the corresponding translation reaction. In parallel, an equal aliquot was assayed for tight binding to a ^{32}P-labeled p53 target site, derived from mouse genomic DNA (Zauberman *et al.*, 1993). This site conforms in 20 out of 20 positions with the consensus site as defined by El-Deiry *et al.* (1992). The results of the binding assay, in which p53−DNA complexes were immunoprecipitated with PAb421 and the bound DNA was then released and analyzed by gel electrophoresis, are shown in panel A. It is clear that while wt p53 bound the radiolabeled probe with high efficiency, p53wtΔSS failed to exhibit detectable sequence-specific binding to this target site. Thus, a deletion which inhibits p53 dimerization also results in loss of the ability to engage in tight specific DNA binding *in vitro*.

It has previously been shown that some p53 target sites are incapable of reacting with *in vitro* translated p53, unless the latter is mixed with a cellular extract (Funk *et al.*, 1992). This may reflect a need for a covalent modification of p53 or for an interaction with another protein present in the extract (Funk *et al.*, 1992), and is consistent with the demonstration that the DNA binding capacity of wt p53 can be activated by phosphorylation, proteolysis or exposure to the bacterial heat shock protein dnaK (Hupp *et al.*, 1992). It was therefore conceivable that the DNA binding activity of p53wtΔSS might also be revealed in the presence of other cellular proteins. Therefore, Saos-2 cells, which do not express endogenous p53 (Masuda *et al.*, 1987), were transiently transfected with expression vectors encoding either p53wtΔSS or full-length mouse wt p53. Cell extracts were prepared 48 h later and assayed for binding to the same radiolabeled DNA target as in Figure 3. The results of the binding assay are depicted in Figure 4B. In parallel, the steady-state levels of p53 in each extract were monitored by Western blotting (Figure 4A). It is obvious that the extract from cells transfected with p53wtΔSS contained at least as much p53 as that of cells transfected with wt p53. Nevertheless, the p53wtΔSS-containing extract did not

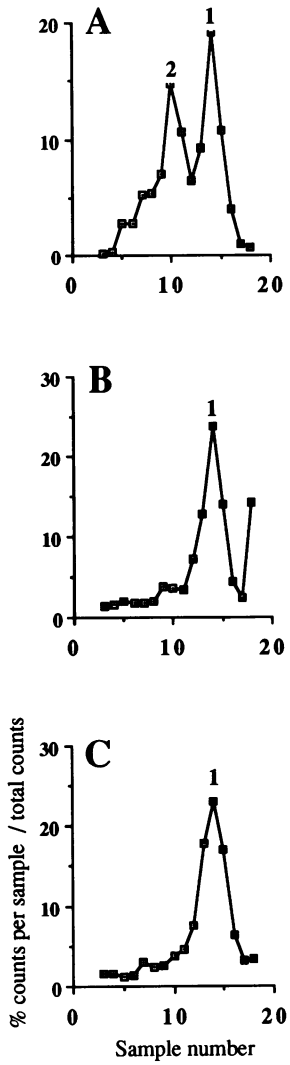

Fig. 2. Analysis of *in vitro* translated p53-derived polypeptides by FPLC. RNA molecules encoding either murine wt p53 **(A)**, p53wtΔSS **(B)** or the C-terminally deleted p53Ala^{stu}, comprising only the first 343 residues of mouse wt p53 (**C**; see Milner *et al.*, 1991) were generated by *in vitro* transcription and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Milner *et al.*, 1991). Translation products were resolved by FPLC. Peaks 1 and 2 denote monomeric and dimeric p53, respectively; larger oligomeric forms can be seen as shoulders to the left of peak 2 in panel A (Milner and Medcalf, 1991).

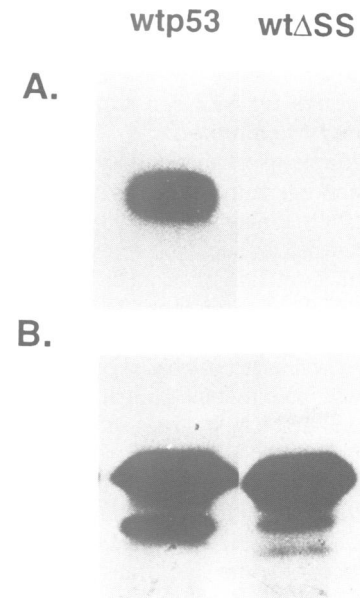

Fig. 3. Analysis of sequence-specific binding by *in vitro* translated p53 polypeptides. RNA encoding either wt p53 or p53wtΔSS was generated by *in vitro* transcription and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, in a total reaction volume of 25 μl. Aliquots containing 10 μl of each reaction were either taken for a DNA binding assay **(A)** or applied directly to SDS−PAGE, followed by autoradiography **(B)**. The DNA binding assay was performed as described under Materials and methods, with a high-affinity p53 binding site derived from mouse genomic DNA serving as a radiolabeled probe. Only the regions of the corresponding autoradiograms which contain the precipitated DNA probe (A) and the p53 polypeptides (B) are shown in the figure.

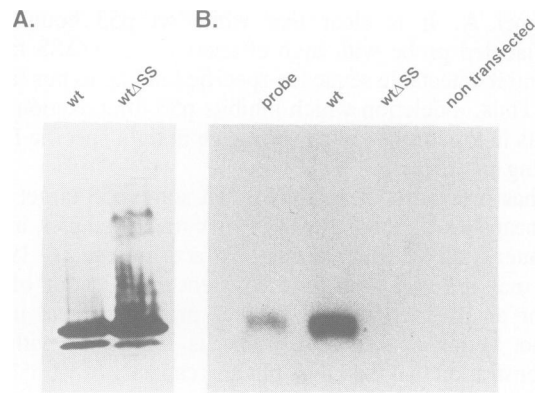

Fig. 4. Analysis of sequence-specific DNA binding by transfected p53. Saos-2 cells were seeded at a density of 2×10^6 cells/10 cm dish and transfected 24 h later with 15 μg of either pCMVp53wt (wt) or pCMVp53ΔSS (wtΔSS). After an additional 48 h, the transfected cells as well as a parallel dish of non-transfected Saos-2 were harvested and extracted. Portions of each extract containing equal amounts of total protein were either subjected to Western blotting followed by probing with PAb421 **(A)**, or taken for a DNA binding assay **(B)**. The first lane in panel B ('probe') contains unreacted probe (2% of the amount employed for each DNA binding assay). The probe was the same as in Figure 3.

display any measurable DNA binding, whereas the wt p53-containing extract bound the radiolabeled target very efficiently. Hence, dimerization-defective p53 is not activated to form tight protein−DNA complexes even when expressed within transfected cells. It is noteworthy that all DNA binding assays reported here (Figures 3 and 4) also included PAb421, which can greatly potentiate DNA binding by wt p53 (Funk *et al.*, 1992; Hupp *et al.*, 1992; Zauberman *et al.*, 1993).

Dimerization-defective wt p53 can suppress oncogene-mediated transformation

One of the manifestations of the activity of wt p53 is its ability to suppress oncogene-mediated transformation *in vitro* (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). If tight DNA binding is a prerequisite for such anti-oncogenic properties, one could expect that deletion of residues 330−344 should result in the loss of these properties. This issue was next addressed experimentally.

The first set of experiments was based on the temperature-sensitive (ts) mouse p53 mutant p53val135 (Michalovitz *et al.*, 1990). At elevated temperatures, p53val135 behaves, both biochemically and biologically, like other p53 mutants (Michalovitz *et al.*, 1990; Milner and Medcalf, 1990). At permissive temperatures, on the other hand, it assumes the features of wt p53. Thus, like authentic wt p53, it can strongly suppress oncogene-mediated transformation at 32°C (Michalovitz *et al.*, 1990). We therefore reconstructed the C-terminal internal deletion into a p53val135 expression vector. The resultant plasmid, pLTRcGval135ΔSS, was then tested at 32°C for its behavior in transformation assays (Table I). Like plasmids specifying full-length p53val135, pLTRcGval135ΔSS also did not cooperate with mutant *ras* in the induction of transformed foci when both were transfected together into primary rat embryo fibroblasts (REF). Under the same conditions, the non-ts mutant p53phe132 cooperated with *ras* very efficiently (Table I). Thus, not surprisingly, the dimerization-defective p53val135ΔSS did not exhibit transforming activity at 32°C.

Table I. Effects of various p53-derived plasmids on oncogene-mediated transformation

Transfected plasmids	Foci per dish	Average ± SD
ras + pLTRp53dl	0,0,0	0
ras + pLTRcGval135	0,0,0,0	0
ras + pLTRcGval135ΔSS	0,0,0,0,0,0,0,0	0
ras + pLTRcGphe132	3,10,24,27	16.0 ± 5.7
ras + *myc* + pLTRp53dl	27,28,14,14	20.7 ± 7.8
ras + *myc* + pLTRcGval135	0,0,0,3	0.7 ± 1.5
ras + *myc* + pLTRcGval135ΔSS	0,0,0,0,0,1	0.2 ± 0.4
ras + *myc* + pLTRcGphe132	41,56,23,20	35.0 ± 16.8

REF were transfected with 1.5 μg of pEJ6.6, encoding activated *ras*, with or without an equal amount of plasmid pLTRmyc, in combination with the indicated p53 expression plasmids (5 μg/dish). Cells were maintained at 32°C throughout the experiment and foci were scored 10 (with *myc*) or 14 (without *myc*) days post-transfection. See Materials and methods for details of the plasmids and of the transformation assay. The values were compiled from several separate experiments. SD = standard deviation.

A more surprising result was obtained when pLTRcGval135ΔSS was assayed for its ability to suppress oncogene-mediated focus formation at 32°C. At this permissive temperature, full-length p53val135 suppressed transformation of REF by *myc* plus mutant *ras* very efficiently, whereas the non-ts mutant p53phe132 actually enhanced transformation (Table I). These observations are in line with earlier studies (Halevy *et al.*, 1990; Michalovitz *et al.*, 1990). Most notably, however, p53val135ΔSS also practically completely suppressed *myc* plus *ras*-mediated transformation at 32°C, thereby behaving identically to its non-deleted ts parent (Table I).

To rule out the possibility that this result reflected a peculiarity of the ts p53, a similar approach was employed for authentic wt p53 harboring the ΔSS deletion. The ability of p53wtΔSS to suppress oncogene-mediated transformation was compared to that of full-length wt p53 over a range of transfected DNA concentrations. The assay was performed in parallel with *myc* plus *ras* and with mutant p53 plus *ras* as the transforming oncogene combinations, and the results are displayed in Figure 5. It can be seen clearly that the internally deleted wt p53 suppressed oncogene-mediated focus formation in a concentration-dependent manner. The inhibitory effect was consistently, though not dramatically, less pronounced than with authentic full-length wt p53, and was exerted in both transformation systems. It is noteworthy that transformation by *myc* plus *ras* was somewhat more sensitive to inhibition by wt p53 (both intact and deleted) than transformation by mutant p53 plus *ras*. In conclusion, deletion of the dimerization domain does not abrogate the anti-oncogenic effects of wt p53 in this experimental system.

Dimerization-defective mutant p53 cannot participate in transformation

Mutant forms of p53 can transform REF in concert with activated *ras* (see the preceding section). In general, the oncogenic effects of mutant p53 may be elicited either through a gain of function or through a negative dominant interference with the normal functions of endogenous wt p53 (Michalovitz *et al.*, 1991). In the case of REF transformation, there are strong indications that negative dominance is sufficient to account for the transforming

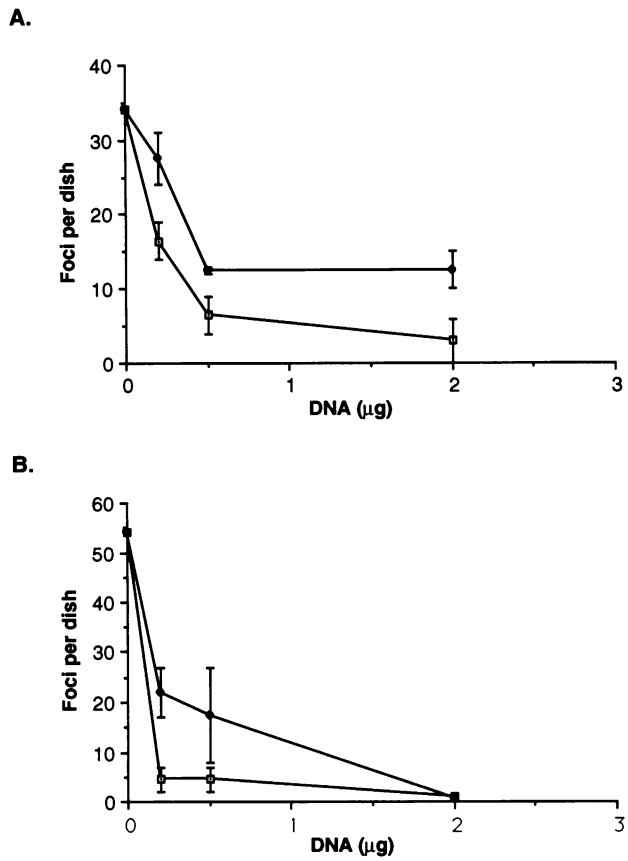

Fig. 5. Suppression of oncogene-mediated transformation by full-length and internally deleted wt p53. Transformation assays were performed as described in Materials and methods. REF were transfected with a combination of either pEJ6.6 (encoding mutant human *ras*) plus pLTRp53cGphe132, encoding an oncogenic p53 mutant (**panel A**), or pEJ6.6 plus pLTRmyc (**panel B**). The amounts of DNA per dish were: pEJ6.6 and pLTRmyc, 1.5 μg each; pLTRp53cGphe132, 5 μg. In addition to these oncogenic plasmids, each dish also contained the indicated amount of either pCMVp53wt (empty squares) or pCMVp53wtΔSS (filled diamonds). Transfections were performed in duplicate and the standard error is indicated for each value.

Table II. Transforming activity of various p53-derived expression plasmids

Transfected plasmids	Foci per dish	Average ± SD
ras + pLTRp53dl	0,0,0,1	0.25 ± 0.5
ras + pLTRcGval135	17,18,47,52	33.5 ± 18.5
ras + pLTRcGval135ΔSS	0,0,0,0,0,0	0
ras + pLTRcGval135t360	13,16	14.5 ± 2.1

REF were transfected with 1.5 μg of pEJ6.6 plus 5 μg of the indicated p53 expression plasmid. Experimental details were as in Table I, except that the experiment was performed at 37.5°C, a temperature at which p53val135 exhibits mutant properties (Michalovitz *et al.*, 1990; Milner and Medcalf, 1990). Foci were scored 14 days post-transfection. The values were compiled from three separate experiments. SD = standard deviation.

potential of mutant p53. Thus, a short C-terminal fragment of p53 can cooperate with *ras* in REF transformation at least as efficiently as full-length tumor-derived mutant p53 (Shaulian *et al.*, 1992). Moreover, this transforming effect of the C-terminal miniprotein appears to rely on its ability to oligomerize with full-length wt p53: deletion of the dimerization motif (the same ΔSS deletion introduced here into full-length p53) renders such miniprotein completely transformation incompetent (Shaulian *et al.*, 1992).

To test whether transformation by full-length mutant p53 also requires the dimerization motif, we assessed the ability of p53val135ΔSS (see the preceding section) to cooperate with *ras* in the transformation of REF at 37.5°C. At this temperature, intact p53val135 behaves like other non-conditional mutants and transforms REF very efficiently (Michalovitz *et al.*, 1990). As seen in Table II, deletion of residues 330–344 completely abolished the ability of mutant p53 to exert oncogenic effects in this assay. On the other hand, a derivative of p53val135 from which the most C-terminal 30 residues had been truncated still retained its transforming potential. This mutant, p53val135t360 (Barak and Oren, 1992), is expected to be capable of forming only dimers, but no higher order complexes (Stürzbecher *et al.*, 1992). Thus, while tetramerization is dispensable for the transforming activity of full-length mutant p53, dimerization appears obligatory. Combined with earlier work (Shaulian *et al.*, 1992), these results further strengthen the notion that the oncogenic potential of mutant p53 in the REF system is achieved through a negative dominant mode of action.

Dimerization-defective wt p53 can function as a sequence-specific transcriptional activator

The capacity of the internally deleted p53 to suppress transformation, despite its failure to exhibit measurable interactions with a specific DNA target, was seemingly inconsistent with the notion that tumor suppression by wt p53 entails the transactivation of relevant target genes. This apparent conflict could, however, be resolved by assuming that dimerization-defective p53 can still interact sequence specifically with cognate DNA elements, albeit with much lower affinity than dimeric wt p53. Such weak interaction may escape detection in standard *in vitro* DNA binding assays, but may nevertheless be sufficient for transactivation within living cells. To address this possibility, we asked whether p53wtΔSS could activate a promoter linked to a p53 binding region. The reporter plasmid was 100/T-CAT, containing the SV40 early promoter and the CAT coding region, preceded by three tandem repeats of a p53 binding element from mouse genomic DNA (Zauberman *et al.*, 1993; a single copy of the same element was also used in the DNA binding assays depicted in Figures 3 and 4). This reporter plasmid was transfected into p53-deficient Saos-2 cells, together with effector plasmids encoding each of the following polypeptides: DΔSS (a short C-terminal fragment of p53 harboring the same internal deletion as p53wtΔSS; Shaulian *et al.*, 1992), mouse wt p53, p53wtΔSS, or a tumor-derived mutant p53 (p53m). As seen in Figure 6, neither the deleted short C-terminal fragment (lane 1) nor the mutant p53 (lane 4) stimulated the expression of the p53-binding reporter gene. However, p53wtΔSS (lane 3) transactivated the reporter gene as efficiently as intact wt p53 (lane 2). As expected, a promoterless CAT gene did not exhibit any activity (lanes 5–8). Similarly, a plasmid conaining only the enhancerless SV40 promoter in front of the CAT gene was not stimulated by either wt p53 or p53wtΔSS (data not shown). These results strongly argue that despite the apparent lack of DNA binding in *in vitro* assays, dimerization-defective p53 can indeed engage in functional interactions with specific DNA targets within the cell.

In the experiment depicted in Figure 6, p53wtΔSS

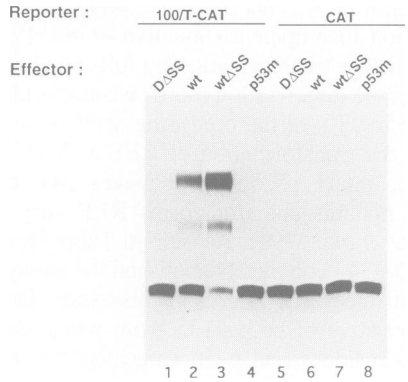

Fig. 6. Transactivation of a p53-responsive promoter by different forms of p53. Saos-2 cells were transfected with a combination of 5 μg of plasmids 100/T-CAT or CAT (see Materials and methods) and 15 μg of a plasmid expressing the indicated p53-derived effector protein. CAT analysis was performed as described in Materials and methods. DΔSS—pCMVDΔSS, encoding a miniprotein extending from positions 302 to 390 of mouse wt p53, but lacking residues 330–344 (Shaulian *et al.*, 1992); wt—pCMVp53wt; wtΔSS—pCMVp53wtΔSS; p53m—pCMVp53m, encoding a double mutated variant of mouse p53. DΔSS lacks the N terminal transactivation domain of p53 and is therefore expected to be inactive in this assay.

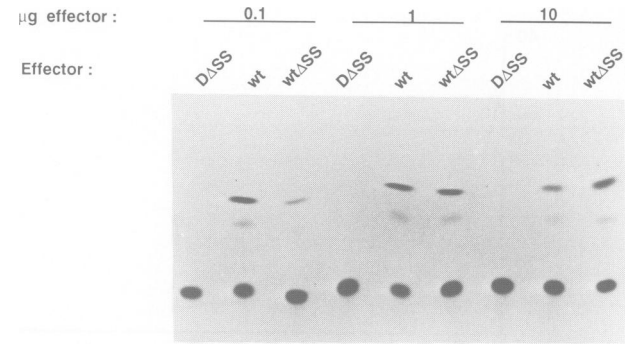

Fig. 7. Dose-dependent transactivation of a p53-responsive promoter by different forms of p53. Saos-2 cells were transfected with 2 μg of reporter plasmid 100/T-CAT plus the indicated amounts of effector plasmids encoding either DΔSS, wt p53 or p53wtΔSS. Experimental details were as in Figure 6.

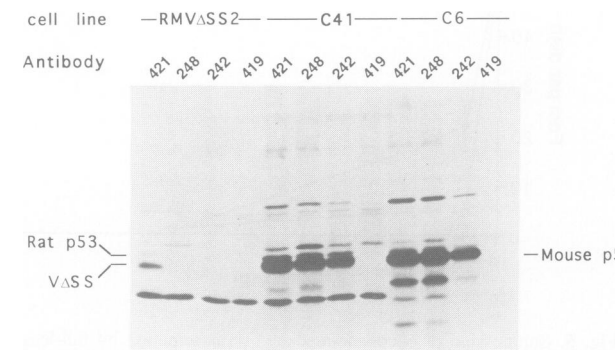

Fig. 8. Immunoprecipitation of p53val135 and p53val135ΔSS with various p53-specific monoclonal antibodies. Cells of line RMVΔSS2, as well as of C41 and C6 (see Materials and methods), were maintained for 24 h at 32°C, and then biosynthetically labeled with [^{35}S]methionine and extracted. Extract portions containing equal amounts of acid-insoluble radioactivity were subjected to immunoprecipitation with the p53-specific monoclonal antibodies PAb421, PAb248 and PAb242, or with the SV40 large T antigen-specific monoclonal antibody PAb419. The positions of endogenous rat p53, intact mouse p53 (carrying the val135 mutation) and the internally deleted p53val135ΔSS (VΔSS) are indicated.

appeared to be as potent as intact wt p53 in transactivating the reporter. This was rather unexpected, given that it fails to bind target DNA tightly and is also less effective in transformation suppression assays (Figure 5). One possible explanation could be that the use of high effector DNA concentrations (15 μg/dish) in the experiment shown in Figure 6 resulted in saturating levels of protein, which masked differences in specific activity between p53wtΔSS and wt p53. Therefore, a similar experiment was repeated with varying concentrations of the effector plasmids. As can be seen in Figure 7, at low effector DNA inputs (0.1 μg/dish) intact wt p53 was indeed more effective than p53wtΔSS. On the other hand, while elevated levels of transfected DNA increased transactivation by the dimerization-defective p53, they actually decreased the effect of wt p53. The latter could reflect adverse cellular consequences of excess p53 or excess DNA, or else could represent a more specific process (see Discussion). The relatively lower specific activity of p53wtΔSS was not due to less efficient expression than wt p53, since both proteins accumulated to similar levels in Saos-2 cells transfected with the corresponding plasmids (Figure 4A). Overall, the data in Figure 7 confirm the notion that while dimerization-defective p53 is not as potent as intact wt p53 in transactivating p53-binding target genes, it can still do so quite efficiently.

Dimerization-defective p53 possesses an altered protein conformation

There are a growing number of indications that conformational changes play a major role in regulating the activities of p53 (reviewed in Milner, 1991; Ullrich *et al.*, 1992). We therefore wished to determine the conformation of the dimerization-defective deletion mutant. To that end, we employed a cell line derived from transformation of REF with *myc* plus activated *ras* plus pLTRcGval135ΔSS (see Table I). This cell line, RMVΔSS2, was generated and maintained at 37.5°C, a temperature at which pLTRcGval135ΔSS does not possess transformation inhibitory properties (data not shown). RMVΔSS2 cells were shifted down to 32°C, a temperature at which the p53val135ΔSS polypeptide becomes biologically activated (Table I), labeled with [^{35}S]methionine and subjected to immunoprecipitation with a variety of monoclonal antibodies that can recognize mouse p53. As seen in Figure 8, p53val135ΔSS failed to react with either PAb248, which recognizes an epitope located within the central portion of the polypeptide, or with PAb242, whose cognate epitope is located more towards the N-terminus (Wade-Evans and Jenkins, 1985). Positive immunoreactivity was obtained only with PAb421, which recognizes an epitope near the C-terminus of p53. Similar results were also obtained with another independent cell line expressing p53val135ΔSS (data not shown). On the other hand, all three antibodies reacted efficiently with extracts from two cell lines harboring full-length p53val135 (C41 and C6). Finally, *in vitro* translated p53wtΔSS also failed to react with PAb248 (E.A.Davies and J.Milner, data not shown). Hence, the internal deletion which eliminates self-oligomerization also abolishes reactivity with at least two monoclonal antibodies (PAb242 and PAb248) whose cognate epitopes are not located within the deleted

segment. This observation indicates that the deletion which impairs the dimerization domain of p53 also causes a major conformational change in the protein. The relationship between these two effects of the deletion is presently unclear; however, it is noteworthy that point mutations within the dimerization domain of human p53, which abrogate self-association, also lead to a concomitant conformational change which affects the accessibility of distant epitopes (Stürzbecher *et al.*, 1992).

Discussion

The work reported here demonstrates that deletion of residues 330–344 of mouse wt p53 generates a form of the protein which has an altered conformation, cannot dimerize efficiently and cannot engage in tight sequence-specific binding to a consensus DNA target *in vitro*. Nevertheless, this internally deleted dimerization-defective p53 is well capable of transactivating *in vivo* a gene containing the same target DNA. Furthermore, it can also suppress oncogene-mediated transformation quite efficiently.

In evaluating these results, one should keep in mind that at least some of the unexpected observations may reflect different sensitivities of *in vitro* and *in vivo* assays. It is likely that relatively weak protein–protein and protein–DNA interactions, while not stable enough to withstand *in vitro* binding assays, may nevertheless suffice for maintaining transcriptional activation and growth suppression *in vivo*. Yet, a number of conclusions may be suggested on the basis of our findings. First, stable dimerization is not essential for transactivation of target genes *in vivo*, and most probably also not for functional sequence-specific interactions with DNA within the cell. Second, dimerization-defective p53 is a rather effective inhibitor of oncogenic processes; this may explain in part why mutations within the dimerization domain of p53 are not selected for in tumors (Caron de Fromentel and Soussi, 1992). Finally, transactivation of target genes and suppression of transformation appear to co-segregate, further strengthening the proposal that the two are functionally coupled (Lane, 1992; Oren, 1992; Vogelstein and Kinzler, 1992).

Our data indicate that p53 does not have to form stable dimers in order to interact with its specific DNA targets and activate transcription. Nevertheless, the dimerization of p53 results in tight DNA binding, and consequently in higher specific transcriptional activity. The simplest interpretation is that the dimerization of p53 results in cooperative binding. For instance, the binding of one member of a dimer to its DNA site could cause it to undergo some conformational change, which will then be transmitted to the other member of the dimer. This, in turn, could switch the second molecule into a form which now binds its target DNA with a much higher affinity, resulting in an overall major increase in the stability of the DNA–protein complex. In fact, it has already been demonstrated that an altered conformation of one p53 molecule can be imposed on a second p53 molecule when the two are co-translated (Milner and Medcalf, 1991). Moreover, oligomers of p53 (believed to be dimers) can be induced to change conformation in a cooperative manner, and it has been suggested that cell growth control may involve allosteric regulation of p53 function (Milner and Watson, 1990; Milner and Medcalf, 1991). Our present findings are consistent with the notion that the formation of p53 homodimers leads to cooperative DNA binding through induction of a new high-affinity state. This notion has precedents in both bacterial and mammalian DNA binding proteins (Dahlman-Wright *et al.*, 1991; Kim and Little, 1992).

It is interesting to note that high concentrations of intact wt p53 actually resulted in less efficient transcriptional activation, whereas such an effect was not observed with the dimerization-defective derivative (Figure 7 and data not shown). One possible explanation is based on the observation that truncation of the extreme C-terminus of p53, which serves as a tetramerization domain (Stürzbecher *et al.*, 1992), leads to constitutive activation of DNA binding (Hupp *et al.*, 1992). This observation has led Hupp *et al.* (1992) to propose that formation of large oligomers by p53 inactivates its function. At high concentrations of wt p53, such oligomers may be favored, resulting in decreased activity. On the other hand, as dimerization through the domain deleted in p53wtΔSS appears to be a prerequisite for further oligomerization via the extreme C-terminus (Stürzbecher *et al.*, 1992), p53wtΔSS is expected to remain dimerization-defective even at high protein concentrations. Consequently, its activity should not be impaired under such conditions.

The nature of the sequence-specific DNA binding domain of p53 still remains unresolved. In many dimeric transcription factors, a basic domain located immediately next to the dimerization domain is responsible for conferring DNA binding specificity (reviewed in Jones, 1990). A highly basic region is indeed present in close proximity to the oligomerization domain of p53, in the extreme C-terminal part of the protein. This domain is probably responsible for the ability of p53 to engage in non-specific DNA–protein interactions, since deletion of the C-terminal 47 amino acids completely abolishes binding to genomic DNA (Shohat-Foord *et al.*, 1991). However, this region is clearly insufficient for, and probably not involved in, sequence-specific DNA binding by p53. This conclusion is supported by several observations. First, deletion of the last 30 residues of p53 does not impair, but rather enhances, sequence-specific DNA binding (Hupp *et al.*, 1992). Second, a miniprotein containing the last 89 residues of wt mouse p53 (DD; Shaulian *et al.*, 1992), namely residues 302–390, lacks demonstrable sequence-specific DNA binding, even though it can dimerize efficiently; on the other hand, it is capable of rather tight, apparently non-specific binding to calf thymus DNA (E.Shaulian, unpublished observations). Finally, the basic C-terminal domain is probably involved in binding to acidic residues within p53, and may function as a tetramerization domain (Stürzbecher *et al.*, 1992). It would thus appear that residues further upstream to position 302 of mouse p53 are required for stable sequence-specific DNA binding. It is of note that several short strings of basic residues are also found immediately upstream to the dimerization domain; one or more of those strings may be involved in the recognition of specific DNA targets. Yet, it is equally conceivable that the determinants for DNA binding specificity are located further towards the N-terminus of p53. Whatever the nature of the DNA binding domain of p53, our data strongly suggest that stable dimerization is not obligatory for specific and functional DNA recognition by p53, even though it makes this process more efficient.

Finally, while dimerization may be at least partially dispensable for the biological functions of wt p53, it appears to be an absolute requirement for the transforming activity of mutant p53 in the REF system. This observation is in

line with earlier experiments using a series of related p53-derived miniproteins (Shaulian *et al.*, 1992). It further supports the idea that mutant p53 can mediate transformation through a negative dominant mechanism, involving the formation of non-functional hetero-oligomers between transfected mutant p53 and endogenous wt p53 (see Milner, 1991; Oren, 1992). One should keep in mind, though, that certain p53 mutants can also act through a gain of function (Wolf *et al.*, 1984; Chen *et al.*, 1990; discussed in Michalovitz *et al.*, 1991). The role of oligomerization in this type of oncogenic activity remains to be explored.

Materials and methods

Cells and plasmids

Primary REF were prepared and processed as described before (Eliyahu *et al.*, 1984). Saos-2 were a generous gift of Dr R.A.Weinberg (Cambridge, USA). Both cell types were propagated in DMEM containing 10% fetal calf serum in a 6% CO_2 environment. Clone 6 (C6) and Clone 41 (C41) are transformed cell lines derived by transfection of REF with activated *ras* and the temperature-sensitive p53 plasmid pLTRp53cGval135 (Michalovitz *et al.*, 1990, and data not shown). Line RMVΔSS2 was generated from a focus induced by the transfection of REF at 37.5°C with a combination of pEJ6.6, pLTRmyc and pLTRcGval135ΔSS (see below).

Plasmid pCMVp53wt encodes wt mouse p53, pCMVp53dl is a derivative of pCMVp53wt from which the bulk of the p53 coding region has been removed, and pCMVp53m encodes a p53 variant derived from Meth A fibrosarcoma cells, which harbors point mutations at positions corresponding to residues 168 and 234 of the protein; all have been described before (Eliyahu *et al.*, 1989). Plasmid pCMVDΔSS encodes a p53-derived miniprotein, including a very short stretch from the N-terminus of mouse p53, followed by residues 302–390 of the protein, from which residues 330–344 have been deleted (Shaulian *et al.*, 1992). Plasmid pCMVp53ΔSS encodes wt p53 with a deletion of residues 330–344, under the transcriptional control of the CMV immediate early enhancer/promoter. Plasmid pLTRcGval135ΔSS encodes a similarly deleted derivative of the temperature-sensitive p53 mutant p53val135 (Michalovitz *et al.*, 1990), whose expression is under the control of the Harvey sarcoma virus LTR. Plasmids pEJ6.6, encoding mutant human Ha-*ras*, pLTRmyc, encoding deregulated mouse c-*myc*, pLTRp53cGphe132, encoding an oncogenic mouse p53 mutant, and pLTRp53dl, containing a grossly deleted version of mouse p53, have been described before (Michalovitz *et al.*, 1990). Plasmid pLTRp53cGval135t360 encodes a version of p53val135 missing the last 30 residues (Barak and Oren, 1992). For *in vitro* transcription of p53-specific RNA, cDNA specific for either mouse wt p53, p53wtΔSS or wt human p53 (Zakut-Houri *et al.*, 1985) was placed downstream to the SP6 promoter in plasmid pSP65. Reporter plasmid 100/T-CAT contains the SV40 early promoter and the CAT coding region, preceded by three tandem repeats of a p53-binding element from mouse genomic DNA, and plasmid CAT contains a promoterless CAT coding region. Both plasmids have been described (Zauberman *et al.*, 1993).

Cell extracts

Cell extracts employed for DNA binding assays and for immunoprecipitation were prepared by the NP40 method, as described before (Shaulian *et al.*, 1992).

DNA binding assays

Sequence-specific binding to DNA was assayed as described before (Shaulian *et al.*, 1992). Briefly, *in vitro* translation products or cell extracts were incubated with a radiolabeled DNA fragment containing a high-affinity p53 binding site, derived from the LTR of a murine retrovirus-like element (Zauberman *et al.*, 1993). The p53-specific monoclonal antibody PAb421 was then added and, following incubation at 4°C, immune complexes were collected on protein A–Sepharose beads. Bound DNA probe was released by incubation in the presence of 1% SDS, phenol–chloroform extracted and applied to a 5% polyacrylamide gel.

Transfections

Transfection of REF or Saos-2 cells was by the calcium phosphate co-precipitation method, as described before (Michalovitz *et al.*, 1990).

Oncogene-mediated transformation was assayed on primary REF as described before (Shaulian *et al.*, 1992). Briefly, REF were seeded at a concentration of 8×10^5/10 cm dish, and transfected 24 h later with various combinations of oncogenic plasmids, with or without an additional p53 expression vector. Immediately after transfection, the fetal calf serum concentration was lowered to 5% and several days later it was further reduced to 3%. Foci were scored 10–14 days post-transfection.

Transient transactivation assays were performed with Saos-2 cells. Cells were seeded at a density of 1.5×10^6/10 cm dish, and transfected 24 h later with a combination of the appropriate CAT reporter plasmid and various p53 expression vectors. Cells were harvested 48 h post-transfection and subjected to CAT analysis as described before (Ginsberg *et al.*, 1991).

Protein analysis

Immunoprecipitation of radiolabeled proteins with p53-specific monoclonal antibodies was done as described before (Shaulian *et al.*, 1992). Precipitated polypeptides were resolved by SDS–PAGE, employing a 7.5% polyacrylamide gel. Western blotting was done as described before (Shaulian *et al.*, 1992), and detection of p53 was carried out with the ECL (Amersham) chemiluminescence kit.

For size analysis, fresh samples of radiolabeled p53 were fractionated by gel filtration on a Superose 6 column (30×2 cm; Pharmacia) using FPLC (Gilson) as described before (Milner *et al.*, 1991). The presence of radiolabeled p53 in the radiolabeled peaks was confirmed by immunoprecipitation with PAb421, followed by SDS–PAGE and autoradiography.

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