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Change in oligomerization specificity of the p53 tetramerization domain by hydrophobic amino acid substitutions

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Abstract

The tumor suppressor function of the wild-type p53 protein is transdominantly inhibited by tumor-derived mutant p53 proteins. Such transdominant inhibition limits the prospects for gene therapy approaches that aim to introduce wild-type p53 into cancer cells. The molecular mechanism for transdominant inhibition involves sequestration of wild-type p53 subunits into inactive wild-type/mutant hetero-tetramers. Thus, p53 proteins, whose oligomerization specificity is altered so they cannot interact with tumor-derived mutant p53, would escape transdominant inhibition. Aided by the known three-dimensional structure of the p53 tetramerization domain and by trial and error we designed a novel domain with seven amino acid substitutions in the hydrophobic core. A full-length p53 protein bearing this novel domain formed homo-tetramers and had tumor suppressor function, but did not hetero-oligomerize with tumor-derived mutant p53 and resisted transdominant inhibition. Thus, hydrophobic core residues influence the oligomerization specificity of the p53 tetramerization domain.

Keywords: hydrophobic effect; oligomerization specificity; p53 tumor suppressor; protein design

Wild-type p53 is a sequence-specific transcription factor that induces cell cycle arrest or programmed cell death in response to DNA damage (Kuerbitz et al., 1992; Clarke et al., 1993; Lowe et al., 1993; Levine, 1997). The N-terminus of p53 contains a transactivation domain (Fields & Jang, 1990), the central region a sequence-specific DNA binding domain (Bargonetti et al., 1993; Halazonetis & Kandil, 1993), and the C-terminus a tetramerization domain (Sakamoto et al., 1994; Wang et al., 1994). The tetramerization domain mediates homo-oligomerization, which is required for high affinity sequence-specific DNA binding activity and tumor suppressor function (Halazonetis & Kandil, 1993; Hainaut et al., 1994; Pietenpol et al., 1994).

In about half of all human tumors, the sequence-specific DNA binding domain of p53 is inactivated by point mutations (Hollstein et al., 1991; Levine, 1997). The tumor-derived p53 mutants fail to suppress tumor growth (Eliyahu et al., 1989; Finlay et al., 1989) and also transdominantly inhibit wild-type p53 (Martinez et al., 1991; Milner & Medcalf, 1991; Bargonetti et al., 1992; Kern et al., 1992). There is significant evidence that transdominant inhibition of wild-type p53 is mediated by sequestration of wild-type p53 into inactive mutant/wild-type hetero-tetramers. First, tumor-derived p53 mutants require an intact tetramerization domain to transdominantly inhibit wild-type p53; second, the isolated p53 tetramerization domain also inhibits wild-type p53 function (Shaulian et al., 1992; Reed et al., 1993; Unger et al., 1993); and third, chimeric p53 proteins that contain heterologous oligomerization domains, instead of the native p53 tetramerization domain, are not transdominantly inhibited by tumor-derived mutant p53 (Waterman et al., 1996; Conseiller et al., 1998).

Induction of wild-type p53 function in tumor cells leads to growth arrest or apoptosis (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Yonish-Rouach et al., 1991; Shaw et al., 1992). Thus, introduction of wild-type p53 into tumor cells could in principle be utilized for therapy (Roth et al., 1996; Favrot et al., 1998; Nielsen & Maneval, 1998). One obstacle to the effectiveness of such therapy, however, is that about half of all human tumors express dominant negative mutant p53 (Hollstein et al., 1991; Martinez et al.,

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1991; Milner & Medcalf, 1991; Bargonetti et al., 1992; Kern et al., 1992; Levine, 1997). We therefore attempted to engineer a p53 protein with wild-type function that would not be susceptible to transdominant inhibition.

One approach to prevent the interaction of wild-type p53 with tumor-derived mutants would be to substitute its native tetramerization domain with a heterologous oligomerization domain. We recently reported the design and analysis of a p53 chimeric protein in which the native p53 oligomerization domain is substituted with a modified leucine zipper that assembles as a tetramer (Waterman et al., 1996). The engineered p53 protein is not transdominantly inhibited by tumor-derived p53 mutants, but is not as active as wild-type p53. To overcome this limitation, we decided to adopt a different strategy: introduce amino acid substitutions within the p53 tetramerization domain that change oligomerization specificity. The p53 tetramerization domain modified in this way would still form tetramers, yet not hetero-oligomerize with the unmodified (native) domain.

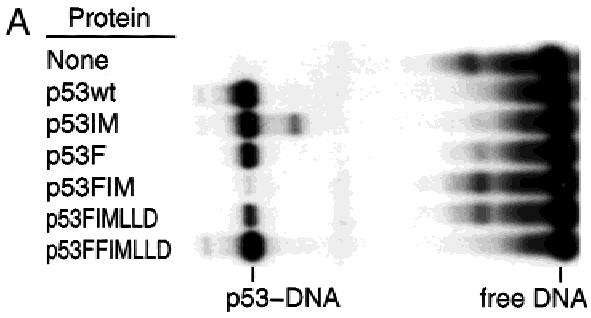
Natural candidates for mutagenesis aiming to change oligomerization specificity of the p53 tetramerization domain are the residues at the interface between subunits. Most of these residues form the core of the p53 tetramerization domain and are hydrophobic in character (Clore et al., 1994, 1995; Lee et al., 1994; Jeffrey et al., 1995). We describe here our efforts to generate p53 domains with altered oligomerization specificity targeting these residues.

Results

Repacking the hydrophobic core of the p53 tetramerization domain

The three-dimensional structure of the p53 tetramerization domain has been determined by X-ray crystallography and NMR spectroscopy (Clore et al., 1994, 1995; Lee et al., 1994; Jeffrey et al., 1995). The domain has a well-defined globular hydrophobic core and contains both β-sheet and α-helical secondary structure elements. The four identical subunits are arranged as a dimer of dimers; in the primary dimer the β-strands pack antiparallel, as do the α-helices (we refer to the two subunits whose β-strands form an antiparallel β-sheet as the primary dimer, because of the large subunit surface area that is buried in this intersubunit interface). Across primary dimers, the α-helices pack parallel at an 81° angle (Fig. 1B).

To construct a domain with altered oligomerization specificity, we substituted residues that map at the interface of the subunits. Most of these residues also form the hydrophobic core of the domain. The substitutions were therefore designed to change the size and/or geometry of the amino acid side chains, while preserving their hydrophobic character. In some cases, the design was guided by the homologous sequences of nonhuman p53 oligomerization domains (Soussi & May, 1996). The amino acid substitu-



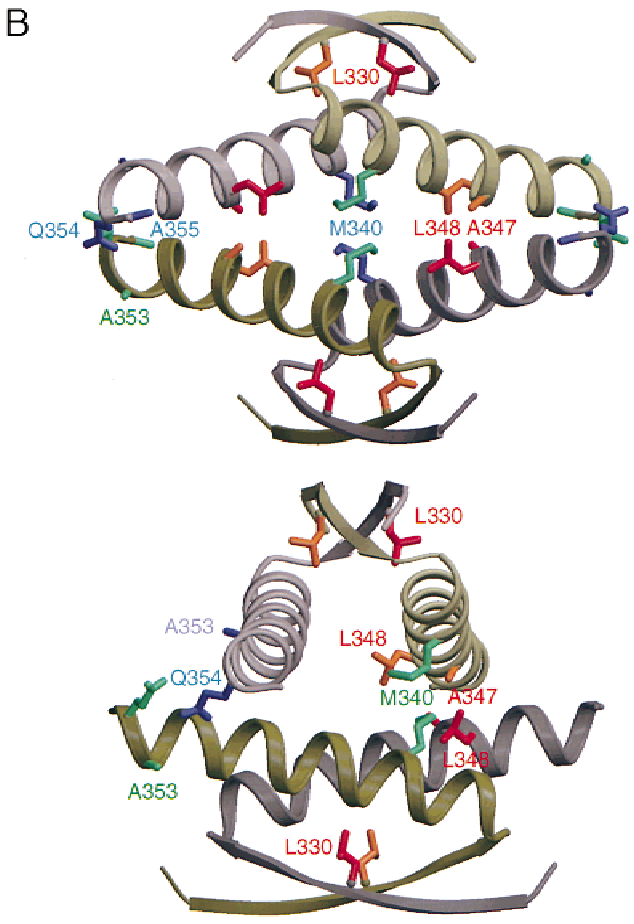


Fig. 1. Effect of hydrophobic amino acid substitutions within the p53 tetramerization domain on the sequence-specific DNA binding activity of full-length p53 protein. **A:** Binding of in vitro translated p53 proteins to a ^{32}P-labeled oligonucleotide containing a p53-binding site assayed by native gel electrophoresis. The names of the p53 mutants indicate the amino acid substitutions, which are abbreviated as follows: p53F, Leu330 to Phe; p53IM, Ala347 to Ile and Leu348 to Met; p53FIM has the substitutions present in p53F and p53IM; p53FIMLLD, has the substitutions present in p53FIM and Ala353 to Leu, Gln354 to Leu, and Ala355 to Asp; p53FFIMLLD, has the substitutions present in p53FIMLLD and Met340 to Phe. **B:** Three-dimensional structure of the p53 tetramerization domain (Protein Data Bank code: 1SAK) showing the hydrophobic residues that affect sequence-specific DNA binding. A triple substitution targeting Leu330, Ala347, and Leu348 abolishes DNA binding and the side chains of these residues are colored red or orange. Substitutions targeting Met340, Ala353, Gln354, and Ala355 restore DNA binding and the side chains of these residues are colored green. The primary dimers are shaded blue and gray and the amino acids are labeled using the single letter code followed by the codon number. The two views are related by a 45° rotation along the *y* axis.

tions were performed in the context of full-length p53, and the mutant p53 proteins were expressed by in vitro translation. As a rapid screen for the effect of the amino acid substitutions on oligomerization, we analyzed the sequence-specific DNA binding activities of the mutant p53 proteins; sequence-specific DNA binding requires oligomerization and many p53 mutants can be analyzed in a single experiment (Halazonetis & Kandil, 1993; Hainaut et al., 1994; Waterman et al., 1995). A large number of amino acid substitutions targeting hydrophobic residues were introduced within the p53 oligomerization domain. For clarity and brevity, only those substitutions that led to the design of a domain with altered oligomerization specificity will be presented. Leu330, whose α-carbon maps to the center of the β-strands and which is involved in intersubunit interactions within the primary dimers (Fig. 1B), was substituted with Phe. This substitution had no apparent effect on the sequence-specific DNA binding activity of p53 (Fig. 1A). Residues Ala347 and Leu348, which map toward the C-termini of the α-helices and whose side chains are involved primarily in hydrophobic interactions across primary dimers (Fig. 1B), were subsequently substituted with Ile and Met, respectively. This double substitution also did not affect DNA binding. However, when combined with the substitution of Leu330 with Phe, p53 failed to bind DNA, suggesting loss of oligomerization (Fig. 1A).

We rationalized that the triple substitution abrogated oligomerization by disrupting the hydrophobic interactions that stabilize the three-dimensional structure of the native domain. Accordingly, introducing novel intersubunit interactions might restore oligomerization and possibly lead to a domain with altered oligomerization specificity due to repacking of the hydrophobic interactions. Toward this goal, the three C-terminal residues of the α-helix, Ala353, Gln354, and Ala355 (Fig. 1B), were substituted with Leu, Leu, and Asp, respectively. Remarkably, these three substitutions restored sequence-specific DNA binding activity to the Phe330–Ile347–Met348 triple mutant, although not to wild-type p53 levels (Fig. 1A). The additional substitution of Met340 at the interface of all four subunits (Fig. 1B) with Phe further restored sequence-specific DNA binding to essentially wild-type levels (Fig. 1A). The engineered p53 tetramerization domain with the seven amino acid substitutions, is hereafter referred to as IND (independent tetramerization domain), because as demonstrated below it does not interact with wild-type p53 or tumor-derived p53 mutants.

Oligomerization stoichiometry

The migration of p53-DNA complexes on native gels is affected by the molecular size of the complex (Hedrick & Smith, 1968; Waterman et al., 1995). Thus, the DNA binding assay of Figure 2 suggests that p53IND assembles as a tetramer, because its complex with DNA comigrates with the wild-type p53-DNA complex. To directly address what is the oligomerization stoichiometry of p53IND, we expressed and purified to homogeneity a recombinant polypeptide that encompasses the IND tetramerization domain. Oligomerization stoichiometry was examined by gel filtration chromatography, which has been shown to be sensitive to changes in oligomerization stoichiometry of the p53 tetramerization domain (McCoy et al., 1997). The elution time of the IND domain was the same as that of the corresponding wild-type domain (Fig. 2). Since the wild-type domain is known to assemble as a tetramer (Halazonetis & Kandil, 1993; Clore et al., 1994, 1995; Lee et al., 1994; Sakamoto et al., 1994; Wang et al., 1994; Jeffrey et al., 1995), we conclude that the IND domain is also a tetramer.

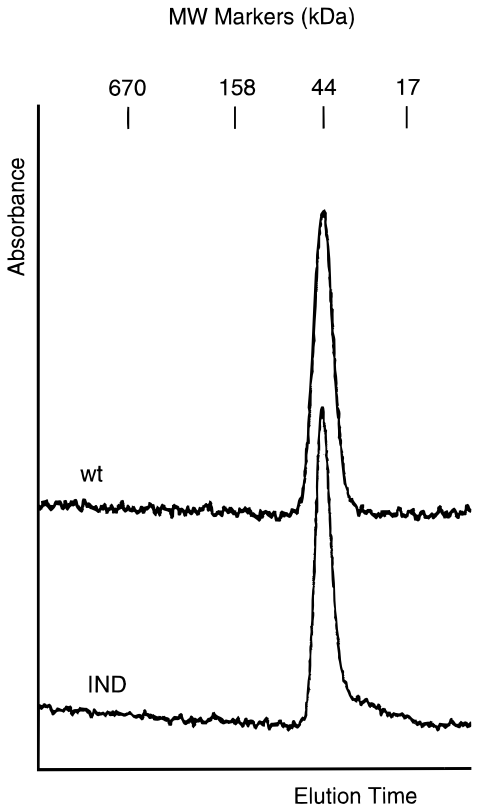


Fig. 2. Stoichiometry of the p53 IND oligomerization domain. Gel filtration chromatography elution profiles of wild-type (wt) and IND oligomerization domains corresponding to residues 304–363 of human p53. The elution profiles of molecular weight (MW) markers are also indicated.

Transcriptional and tumor suppressor activities

The transcriptional and tumor suppressor activities of wild-type p53 require homo-oligomerization (Pietenpol et al., 1994). Since p53IND has amino acid substitutions in the tetramerization domain, we examined whether it retains transcriptional and tumor suppressor activities. Transcriptional activity was assayed by cotransfecting Saos-2 cells, which lack endogenous p53 (Diller et al., 1990), with a plasmid directing p53-expression and a reporter plasmid containing the p53-binding site of the *p21/cip1/waf1* gene (Waterman et al., 1996). p53IND activated transcription with almost the same efficiency as wild-type p53 (Fig. 3A). Tumor suppressor activity was assayed by cotransfecting Saos-2 cells with a plasmid directing expression of p53 and a plasmid conferring G418 resistance (Waterman et al., 1996). p53IND suppressed colony formation almost as efficiently as wild-type p53 (Fig. 3B).

Oligomerization specificity

The oligomerization specificity of p53IND was first examined in vitro by monitoring hetero-oligomerization between cotranslated p53 proteins with wild-type and IND tetramerization domains. Formation of hetero-oligomers was evaluated by immunoprecipi-

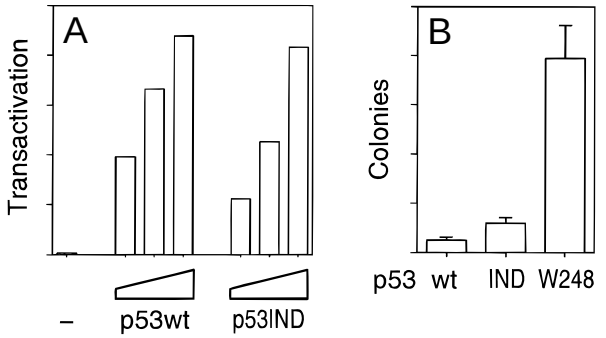


Fig. 3. Functional activities of p53IND in Saos-2 cells. **A:** Transactivation of p53-reporter plasmids cotransfected either with a control plasmid (−) or increasing amounts (0.5–2 μg) of plasmids expressing wild-type p53 or p53IND. **B:** Tumor suppressor activities of wild-type p53, p53IND and the tumor-derived mutant p53W248 using the colony-forming assay. Activities are presented as means +1 SE of tumor cell colonies per plate.

tation with antibody DO1, which recognizes full-length, but not N-terminally truncated p53. DO1 precipitated N-terminally truncated wild-type p53 when it was cotranslated with full-length wild-type p53, but not when it was cotranslated with full-length p53IND, indicating that p53 proteins with wild-type and IND tetramerization domains do not hetero-oligomerize (Fig. 4A).

The oligomerization specificity of p53IND was subsequently evaluated in cells. HA-tagged wild-type p53 or p53IND were expressed in U2-OS human osteosarcoma cells and their propensity to hetero-oligomerize with the endogenous wild-type p53 protein was monitored by co-immunoprecipitation. Hetero-oligomers were readily detected between endogenous p53 and HA-tagged wild-type p53, but were rare between endogenous p53 and HA-tagged p53IND (Fig. 4B).

Since the mechanism by which tumor-derived p53 mutants inhibit wild-type p53 function involves formation of inactive wild-type/mutant heterotetramers (Shaulian et al., 1992; Reed et al., 1993; Unger et al., 1993; Waterman et al., 1996; Conseiller et al., 1998), we subsequently examined whether p53IND, which has altered oligomerization specificity, was susceptible to transdominant inhibition by mutant p53. The experiments monitored the transcriptional activity of p53IND and were performed in both Saos-2 and U2-OS cells. Saos-2 cells lack endogenous p53; expression of wild-type p53 or p53IND in these cells led to transactivation of a reporter gene that contains a p53-binding element. Co-expression of a tumor-derived p53 mutant inhibited transactivation of wild-type p53, but not p53IND (Fig. 5A). p53IND also escaped transdominant inhibition by mutant p53 in U2-OS cells. These cells express endogenous wild-type p53, which activates transcription of a p53-reporter gene. Transactivation mediated by the endogenous p53 protein was suppressed by cotransfection of a plasmid that expresses tumor-derived mutant p53 (Fig. 5B). However, p53IND transactivated the reporter plasmid even when the tumor-derived p53 mutant was co-expressed (Fig. 5B).

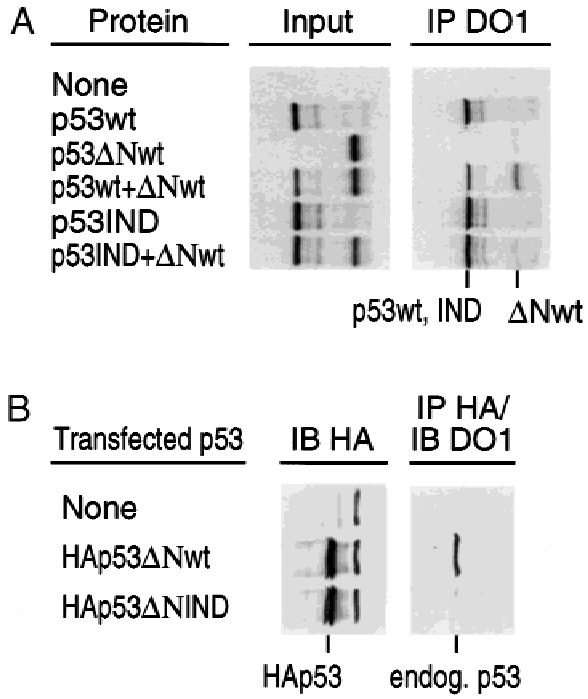


Fig. 4. Oligomerization specificity of p53IND. **A:** Co-immunoprecipitation assay of individually translated or cotranslated ^{35}S-labeled wild-type (wt) p53 and p53IND proteins. After synthesis the proteins were detected by autoradiography (Input) or immunoprecipitated (IP) with antibody DO1 and detected by autoradiography. **B:** Co-immunoprecipitation assay of endogenous wild-type p53 in U2-OS cells with transfected HA-tagged p53 proteins bearing an N-terminal deletion of residues 1–39 and wt or IND tetramerization domains. Cell extracts were immunoblotted (IB) with anti-HA antibodies or immunoprecipitated (IP) with anti-HA antibodies followed by immunoblotting with antibody DO1 to detect coprecipitated endogenous p53.

Discussion

The goal of this study was to develop p53 proteins that escape transdominant inhibition by tumor-derived mutant p53. Engineered p53 proteins whose function are not suppressed by mutant p53 were previously constructed by substitution of the entire p53 tetramerization domain with modified leucine zippers (Waterman et al., 1996; Conseiller et al., 1998). However, these chimeric p53-leucine zipper proteins were not very potent tumor suppressors. Here we used a different strategy to change the oligomeriza-

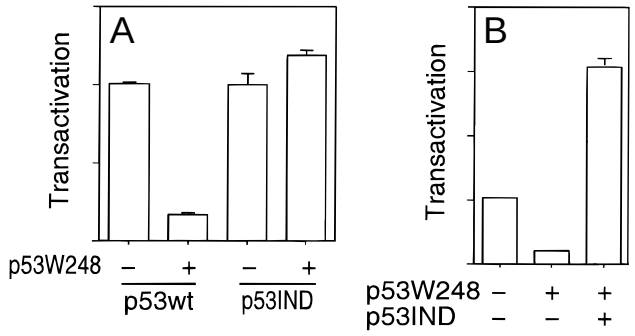


Fig. 5. Resistance of p53IND transactivation to transdominant inhibition by the tumor-derived mutant p53W248. **A:** Saos-2 cells. Transactivation of wt p53 and p53IND in the presence or absence of excess tumor-derived p53 mutant p53W248. **B:** U2-OS cells. Inhibition of the transcriptional activity of endogenous wt p53 by the tumor-derived mutant p53W248 and high activity of p53IND in the presence of p53W248. Activities are presented as means +1 SE.

tion specificity of p53, namely modify, rather than replace, the p53 tetramerization domain. The designed domain, which we refer to as IND, differs from the native domain by seven amino acid substitutions. The wild-type and IND domains are functionally quite similar; they both assemble as tetramers and both support the sequence-specific DNA binding, transactivation, and tumor suppressor functions of p53. Furthermore, the IND domain is anticipated to have a three-dimensional structure that is very similar to the structure of the wild-type domain, because their amino acid sequences are very similar. The high functional and structural similarity between the IND and wild-type tetramerization domains suggest that p53IND could be used instead of wild-type p53 for cancer gene therapy (Roth et al., 1996; Favrot et al., 1998; Nielsen & Maneval, 1998). Due to its altered oligomerization specificity, p53IND would be clearly superior to wild-type p53 for such therapy, since about half of all human tumors express mutant p53 proteins that can inactivate wild-type p53 (Hollstein et al., 1991; Martinez et al., 1991; Milner & Medcalf, 1991; Bargonetti et al., 1992; Kern et al., 1992; Levine, 1997).

Because the design of p53IND was aided by the sequences of nonhuman p53 tetramerization domains, six of the seven substitutions introduce nonhuman residues within p53IND. Specifically, substitutions Leu330 to Phe, Gln354 to Leu, and Ala355 to Asp introduce residues present in xenopus p53; substitutions Ala347 to Ile and Leu348 to Met introduce residues present in squid p53; and substitution Ala353 to Leu introduces a residue present in trout p53 (Soussi & May, 1996). Our understanding of how the seven amino acid substitutions in p53IND change oligomerization specificity is as yet incomplete, but could involve two steps. In the first step, the substitutions targeting Leu330, Ala347, and Leu348 disrupt oligomerization. In the second step, the substitutions targeting Met340, Ala353, Gln354, and Ala355 restore oligomerization resulting in a domain with altered oligomerization specificity. Of the three substitutions required to disrupt oligomerization, the role of the substitution of Leu330 with Phe is difficult to understand based on the three-dimensional structure of the wild-type p53 tetramerization domain; for the other two substitutions, Ala347 with Ile and Leu348 with Met, we propose that they weaken the hydrophobic interactions that stabilize the packing of the two primary dimers. Regarding the four substitutions that restore oligomerization, we envision that they establish novel intersubunit hydrophobic interactions (substitution of Ala353 and Gln354 with Leu) or enhance existing hydrophobic interactions (substitution of Met340 with Phe). Thus, a redistribution of hydrophobic interactions may underlie the altered oligomerization specificity.

Oligomerization specificity has been studied extensively in the context of leucine zippers. These amphipathic coiled coils are characterized by heptad repeats of the general sequence (abcdefg)n; where a and d are hydrophobic amino acids, and b, c, e, f, and g are polar or charged residues (O'Shea et al., 1989, 1991; Landschulz et al., 1988). Leucine zipper dimerization is mediated by the hydrophobic residues at positions a and d. These hydrophobic interactions are critical for stability of the dimer, but do not contribute to oligomerization specificity. Rather, specificity is determined by the polar and charged residues at positions e and g, which participate in intersubunit electrostatic interactions (O'Shea et al., 1992; Lumb & Kim, 1995; Zeng et al., 1997a, 1997b; Kohn et al., 1998).

In contrast to leucine zippers, the oligomerization specificity of the p53 tetramerization domain can be altered by substitutions of hydrophobic residues. The differences between the structures of leucine zippers and the p53 tetramerization domain may explain the discrepancy. Coiled coils have a single secondary structure element and a nonglobular hydrophobic core, whose geometry provides very limited tolerance for substitutions at the intersubunit interface (O'Shea et al., 1989, 1991; Landschulz et al., 1988). In contrast, the p53 tetramerization domain, which contains β-strand, turn, and α-helical secondary structure elements, has a globular hydrophobic core, whose geometry can accommodate hydrophobic amino acid substitutions (Clore et al., 1994, 1995; Lee et al., 1994; Jeffrey et al., 1995; Waterman et al., 1995). Such substitutions can redistribute the intersubunit hydrophobic interactions resulting in altered oligomerization specificity.

Materials and methods

Recombinant plasmids

Plasmids used to express in vitro-translated full-length p53 proteins with modified tetramerization domains were derived from pGEMhp53wtB (Waterman et al., 1995) by polymer chain reaction (PCR)-directed mutagenesis. Plasmid pSV2hp53IND was derived from pSV2hp53wt (Waterman et al., 1996) by substituting the Sst I-Sal I fragment that encodes the wild-type p53 tetramerization domain with the corresponding fragments of pGEMhp53IND. Plasmids pSV2HAhp53Δ1-39wt and pSV2HAhp53Δ1-39IND were derived from pSV2hp53wt and pSV2hp53IND, respectively, by PCR-directed mutagenesis to remove the sequences that encode residues 1–39 of p53, followed by inserting, at the Nco I site corresponding to the initiation codon, synthetic oligonucleotides that encode a hemaglutinin (HA) antigen. Plasmid pT5Thp53IND was derived from pT5Thp53wt (McCoy et al., 1997) and was used to express in *Escherichia coli* a polypeptide corresponding to residues 304–363 of p53 that encompasses the IND tetramerization domain. Plasmids pEp21/TKseap and pEmdm2/TKseap are reporter plasmids containing p53 response elements (Wieczorek et al., 1996).

DNA binding assays

Plasmids of the pGEM series were used to generate in vitro translated p53 proteins. For analysis of DNA binding activity, the p53 proteins were incubated with ^{32}P-labeled oligonucleotide BC.V4A and subjected to native gel electrophoresis (Waterman et al., 1995).

Gel filtration chromatography

Polypeptides that contain the wild-type or IND p53 tetramerization domains were expressed in *E. coli* and purified to homogeneity by hydrophobic affinity and ion exchange chromatography (McCoy et al., 1997). The purified proteins were examined by gel filtration chromatography on a Superdex 200 column (Pharmacia, Piscataway, New Jersey).

Transcription and tumor suppression assays

Transcriptional activity was determined by transfecting Saos-2 cells with 0.5–2 μg p53-expression plasmid and 28 μg p53-reporter plasmid. Alkaline phosphatase activity was determined 48 h later (Waterman et al., 1996). To determine whether the transcriptional activities of the p53 proteins were suppressed by a tumor-derived

p53 mutant, Saos-2 or U2-OS cells were transfected in triplicate with 1 μg p53-expression plasmid, 9 (Saos-2) or 5 (U2-OS) μg plasmid encoding the tumor-derived mutant p53W248, 20 μg p53-reporter plasmid, and pBC12/PLseap carrier plasmid—so the total amount of transfected plasmid DNA was 30 μg.

Tumor suppressor activity was assayed by cotransfecting Saos-2 cells in quadruplicate with 5 μg p53-expression plasmid, 1 μg pSV7neo, a plasmid that confers neomycin resistance, and 24 μg pBC12/PLseap carrier plasmid (Waterman et al., 1996). The transfected cells were selected for G418 resistance, and two weeks later colonies were stained with crystal violet and counted.

Interaction of p53IND with wild-type p53

Full-length p53 proteins with wild-type or IND tetramerization domains were translated in vitro with wild-type p53 bearing an N-terminal deletion of residues 3–79 in the presence of ^{35}S-labeled methionine and immunoprecipitated with antibody DO1 (Waterman et al., 1995, 1998). Precipitated proteins were detected by autoradiography. For analysis of interaction in vivo, HA-tagged wild-type p53 or p53IND with an N-terminal deletion of residues 1–39 were expressed in U2-OS cells by transient transfection. Cell extracts were prepared 48 h later, and the HA-tagged p53 proteins were immunoprecipitated with anti-HA antibodies (Waterman et al., 1998). The presence of coprecipitating endogenous wild-type p53 was examined by immunoblotting with antibody DO-1.

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