An Engineered Four-Stranded Coiled Coil Substitutes for the Tetramerization Domain of Wild-Type p53 and Alleviates Transdominant Inhibition by Tumor-derived p53 Mutants

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ABSTRACT

The tetramerization domain of p53 is required for efficient tumor suppressor activity. This domain, however, also allows wild-type p53 to hetero-oligomerize with dominant negative tumor-derived p53 mutants. We explored the feasibility of substituting the native tetramerization domain of wild-type p53 with an engineered leucine zipper that assembles as a four-stranded coiled coil. The engineered zipper drove p53 tetramerization in vitro and p53 function in vivo. Furthermore, it alleviated transdominant inhibition by tumor-derived p53 mutants, implying that dominant negative mutants act by hetero-oligomerizing with wild-type p53. The ability of the engineered zipper to drive tetramerization was critical for p53 function, since p53 dimers, formed by substituting the p53 tetramerization domain with a native leucine zipper, were weak tumor suppressors.

INTRODUCTION

Wild-type p53 is a sequence-specific transcription factor that induces cell cycle arrest or programmed cell death in response to DNA damage (1–6). The NH2 terminus of p53 contains a transactivation domain (7, 8), the COOH terminus contains a tetramerization domain (9–12), and the central region contains a sequence-specific DNA-binding domain (13–16). The latter domain is inactivated by point mutations in about one-half of all human tumors (17–20).

The tumor-derived p53 mutants fail to suppress tumor growth (21–25) but in addition act as dominant negatives by inhibiting the function of wild-type p53 (26–29). This latter property may account for their oncogenic activity in primary cells (30–32). Mechanistically transdominant inhibition of wild-type p53 could be mediated by sequestration of wild-type p53 into inactive mutant/wild-type heterotetramers or by titration of targets required for wild-type p53 function. In favor of the first mechanism, the oncogenic activity of tumor-derived p53 mutants requires an intact tetramerization domain, and the isolated tetramerization domain is as transforming as full-length p53 mutants (33–35).

Induction of wild-type p53 function in tumor cells leads to growth arrest or apoptosis (21–22, 24, 36, 37). Thus, introduction of wild-type p53 into tumor cells could in principle be used for therapeutic purposes (38–40). One obstacle to the effectiveness of such therapy, however, is that about one-half of all human tumors express dominant-negative p53 mutants (17–20). Therefore, we wondered whether it would be possible to engineer a p53 protein with wild-type function that would not be susceptible to transdominant inhibition.

One approach to prevent wild-type p53 from interacting with tumor-derived mutants would be to substitute its native tetramerization domain with a heterologous oligomerization domain. Recently, an analysis of mutant GCN4 LZs3 has identified domains with altered subunit stoichiometries (41). One such engineered zipper, which will hereafter be referred to as the TZ, assembles as a parallel tetramer. This domain has not yet been shown to drive tetramerization of any protein. Furthermore, its ability to form tetramers in vivo has not been characterized.

In an effort to generate a functional p53 protein that is not transdominantly inhibited by tumor-derived p53 mutants, we constructed and characterized a number of p53 proteins, the oligomerization of which is driven by the tetrameric or by the native GCN4 LZ. Our results indicate that the engineered TZ, but not the LZ, can drive p53 function in tumor cells and furthermore can alleviate transdominant inhibition by tumor-derived p53 mutants. We have thus demonstrated the feasibility of designing a p53 protein with dominant wild-type function.

MATERIALS AND METHODS

Recombinant Plasmids. Standard cloning procedures were used, and all mutants were generated by PCR-directed mutagenesis (42). Plasmids pGEMhp53wtB, pGEMhp53A344, and pGEMhp53LZ335Q encode human wild-type p53, p53Ala344, and p53LZ335Q, respectively, and have been described (43). Plasmid pGEMhp53LZ343RMKQ encodes a hybrid protein consisting of residues 1–343 of human p53 and residues 249–281 of GCN4 (44). Because the LZ segment in p53LZ335Q refers to residues 253–281 of GCN4, for uniformity we consider p53LZ343RMKQ as having a LZ corresponding to residues 253–281 of GCN4 and a linker consisting of residues 249–252 of GCN4, which are Arg-Met-Lys-Glu (RMKQ). Plasmid pGEMhp53TZ334NR was derived from pGEMhp53wtB by replacing the Sall-SalI fragment with synthetic oligonucleotides encoding the TZ (41) corresponding to residues 250–281 of GCN4 (44) via an Asn-Arg (NR) sequence. Plasmid pGEMhp53TZ334NR/J1352 was derived by inserting sequences encoding an Ile (I), followed by residues 352–393 of human p53 after the last codon in pGEMhp53TZ334NR.

Plasmid pSV2 hp53wtB was prepared by cloning into the SalI-BglII sites of pSV2 humjun (45) a blunted EcoRI-HindIII p53 insert from pGEMhp53wtB (43). Plasmids expressing p53 fusion proteins were similarly derived from the corresponding pGEM plasmids. A pSV2 plasmid without insert was prepared by ligating pSV2 humjun (45) linearized with SalI and BglII. The tumor-derived p53 mutation Trp248 was introduced in the context of pGEMhp53wtB by ligating pSV2 humjun (45) linearized with SalI and BglII. The tumor-derived p53 mutation Trp248 was introduced in the context of pGEMhp53wtB and pSV2 hp53wtB by site-directed mutagenesis.

Plasmids p6p21/TKseap and pEmdm2/TKseap have one copy of oligonucleotides Ep21 and Emdm2, respectively, cloned in the EcoRV site of pTKseap (46) and express secreted alkaline phosphatase in a p53-responsive manner. The sequences of oligonucleotides Ep21 and Emdm2 are CCC-GAACA-TGTCG-CAGAG-GG and GCCGGTGGCAGAG-GAGCT-AAGTCC-TGACA-TG, respectively. The repeats recognized by p53 are underlined.

In Vitro Translation, Subunit Stoichiometry, and DNA Binding. Plasmids of the pGEM series were used to generate in vitro-translated p53 proteins (16, 43, 47). For analysis of subunit stoichiometry, the p53 proteins were translated in the presence of [35S]methionine and subjected to electrophoresis on native Tris-glycine gels (16). The logarithms of electrophoretic migration were plotted as a function of the percentage of polyacrylamide by linear regression analysis (48).

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3 The abbreviations used are: LZ, leucine zipper; TZ, tetrameric zipper.

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For analysis of DNA-binding activity, the p53 proteins were incubated with 32P-labeled oligonucleotides and subjected to electrophoresis (16, 43, 47). Oligonucleotides BC.V4A.S10 (43), Ep21, and Em2 (see above) each contain a p53-binding site, whereas oligonucleotide TF3 is a nonspecific DNA (47).

Transcription and Tumor Suppression Assays. Transcriptional activity was determined by transfecting in quadruplicate Saos-2 cells with 5 μg of p53 expression and 25 μg of reporter plasmids. Alkaline phosphatase activity was determined 48 h later (46). To assay inhibition of transcriptional activities of functional p53 proteins by p53Trp248, Saos-2 cells were cotransfected in triplicate with 1 μg of p53 expression plasmid, 9 μg of plasmid expressing the p53Trp248 mutant, or 9 μg of pSV2 plasmid without insert and 20 μg of the Ep21/TK-seap reporter plasmid.

Tumor suppressor activity was assayed by cotransfecting Saos-2 cells in quadruplicate with 5 μg of p53 expression plasmid, 1 μg of pSV7 neo, a plasmid that confers neomycin resistance (45), and 24 μg of pBC12/PlsHseap (46), a carrier plasmid. The transfected cells were selected for G418 resistance, and 2 weeks later, the resistant colonies were stained with crystal violet and counted. To assay inhibition of tumor suppressor activities of functional p53 proteins by p53Trp248, Saos-2 cells were cotransfected in triplicate with 2.5 μg of p53 expression plasmid, 10 μg of plasmid expressing p53Trp248 or 10 μg of pSV2 plasmid without insert, 1 μg of pSV7 neo, and 16.5 μg of pBC12/PlsHseap carrier plasmid.

 Arrest of cell cycle progression was determined by transfecting Saos-2 cells in duplicate with 30 μg of p53 expression plasmid. Thirty-six h later, the cells were trypsinized, washed with PBS, and fixed with 0.4% paraformaldehyde at 37°C for 10 min and then with methanol at 4°C for 10 min (49). The cells were then washed with PBS/0.1% Tween 20 (PBS/T) and incubated with antibody DO-1 (Oncogene Sciences, Uniondale, NY) in PBS/T with 50% fetal bovine serum at 30°C for 3 h. Excess antibody was washed with PBS/T, and the cells were incubated with fluorescein-conjugated anti-mouse F(ab')2 (Boehringer Mannheim, Indianapolis, IN) diluted 1:100 in PBS/T with 50% fetal bovine serum at 37°C for 1 h. Excess secondary antibody was washed with PBS/T, and the cells were incubated with RNase A at 37°C for 30 min and stained with propidium iodide. The cells were analyzed on a Becton Dickinson FACScan.

RESULTS

An Engineered Coiled Coil Drives p53 Tetramerization in Vitro. The tetramerization domain of human p53 maps to residues 322–355 at the p53 COOH terminus (11, 12). In an effort to generate p53 proteins that do not rely on the native p53 tetramerization domain for oligomerization, we substituted the p53 COOH-terminal sequences with heterologous oligomerization domains derived from the transcription factor GCN4 (Fig. 1).

Oligomerization of GCN4 is mediated by a LZ, a domain characterized by heptad repeats of the general sequence α.b.c.d.e.f.g, with hydrophobic residues at positions α and d and polar residues at all other positions. The native GCN4 LZ has mostly valines at positions a and leucines at positions d and assembles as a parallel dimer (44, 50–53). Interestingly, a mutant GCN4 zipper (tetrameric zipper) with leucines at positions a and assembles as a parallel dimer (43), (10–12, 16); p53A344 has a single amino acid substitution (Leu 344 to Ala) in the tetramerization domain and assembles as a dimer (43), whereas p53A333–393 corresponds to p53Trz334NR without the TZ and is a monomer, because it is missing most of the p53 tetramerization domain (9, 58). On native gels, p53Trz334NR resolved as two species (17, 334 and 352) and the p53 sites of the p21 and mdm2 genes present within oligonucleotide TF3. The number of the p53 zipper oligomer proteins refers to amino acid positions of wild-type p53.

p53LZ343RMKQ, which is also named p53-CC 1–343 (58), and p53LZ335Q (16, 43) assemble as dimers and can serve as controls for the TZ hybrids (Fig. 1).

To examine if the engineered GCN4 zipper could drive tetramerization of p53, we determined the subunit stoichiometry of p53Trz334NR by plotting the logarithm of its electrophoretic migration on native gels relative to gel polyacrylamide content. The slope of this curve is proportional to molecular size (59, 60). As standards we used wild-type p53, p53A344, and p53A333–393. Wild-type p53 assembles as a tetramer (10–12, 16); p53A344 has a single amino acid substitution (Leu 344 to Ala) in the tetramerization domain and assembles as a dimer (43), whereas p53A333–393 corresponds to p53Trz334NR without the TZ and is a monomer, because it is missing most of the p53 tetramerization domain (9, 58). On native gels, p53Trz334NR resolved as two species (Fig. 2A). Comparison of the migration of these two species relative to the migration of the p53 standards revealed that the major species of p53Trz334NR (slow form) was a tetramer, while the minor species (fast form) was a dimer (Fig. 2B).

Functional Analysis of p53-TZ Hybrids. The ability of p53Trz334NR to bind DNA was examined in an electrophoretic mobility shift assay (Fig. 3A). Oligonucleotide Ep21, which contains the p53 site of the p21 gene (61), was recognized efficiently by p53Trz334NR. DNA binding was sequence specific, since it was not inhibited by excess of nonspecific competitor DNA (oligonucleotide TF3). The monomeric p53A333–393, which is identical to p53Trz334NR except that it lacks the TZ, failed to bind DNA (Fig. 3A), highlighting the importance of the TZ and confirming previous observations that oligomerization facilitates p53 DNA binding (16, 62). Like p53Trz334NR, p53Trz334NR/I352 also recognized DNA with high affinity and in a sequence-specific manner. However, efficient DNA-binding activity was dependent on the presence of antibody PAb421 (Fig. 3A). p53Trz334NR/I352 contains the COOH terminus of wild-type p53 (residues 352–393), which suppresses p53 DNA binding in vitro, unless masked by antibody PAb421 (43, 47, 55–57). This COOH-terminal region is absent in p53Trz334NR (Fig. 1).

The DNA-binding activities of p53Trz334NR and wild-type p53 were subsequently compared using a panel of DNA sites: an artificial suboptimal p53 site present within oligonucleotide BC.V4A.S10 (43); and the p53 sites of the p21 and mdm2 genes present within oligonu-
cleotides Ep21 and Emdm2, respectively (61, 63). Both p53TZ334NR and wild-type p53 recognized these sites with equal efficiency (Fig. 3B). Binding of wild-type p53 to oligonucleotides BC.V4A.S10 or Ep21 required the presence of monoclonal antibody PAb421, consistent with previous reports on regulation of p53 DNA binding in vitro by its COOH terminus (43, 47, 55—57).

We subsequently examined the DNA-binding activities of the p53-LZ hybrids p53LZ335Q and p53LZ343RMKQ. Both recognized oligonucleotides Ep21 and Emdm2 less efficiently than wild-type p53 (Fig. 3C). Since p53LZ335Q and p53TZ334NR contain essentially the same p53 segment fused either to a LZ or to a TZ, we attribute the lower affinities of the LZ hybrids for DNA to their dimeric subunit stoichiometry.

The transcriptional activities of p53TZ334NR and p53TZ334NR/I352 were examined by transient transfection in Saos-2 osteosarcoma cells, which lack endogenous p53 (21). Both proteins activated transcription from reporter plasmids containing the p21 or mdm2 p53 sites (Fig. 4A). The dimeric p53-LZ hybrids were less potent transcriptional activators, especially with the reporter plasmid conferring G418 resistance. The number of G418-resistant colonies is inversely related to tumor suppressor activity. Both p53TZ334NR/I352 and p53TZ334NR suppressed tumor growth almost as efficiently as wild-type p53 (Fig. 4B). A single amino acid substitution, Trp248 (W248), which maps to the p53 DNA-binding domain and is associated with human cancer, was sufficient to abrogate the tumor suppressor activity of p53TZ334NR (Fig. 4B). The same substitution also abrogated the tumor suppressor activity of wild-type p53, as expected (21—25).

Interestingly, the dimeric proteins p53LZ343RMKQ and p53LZ335Q were weak tumor suppressors (Fig. 4B).

The differences in transcriptional and tumor suppressor activities between the examined p53 proteins could not be attributed to differences in expression. They were all expressed at equivalent levels as determined by flow cytometry (data not shown, but see Fig. 4C below).

To analyze cell cycle arrest, Saos-2 cells were transfected with p53 expression plasmids and examined by flow cytometry 36 h later. Cells expressing p53 were identified with DO-1, a monoclonal antibody that recognizes the NH2 terminus of human p53 (56). DNA content was determined by propidium iodide staining and plotted relative to
p53 content. Cells expressing wild-type p53, p53TZ334NR, or p53TZ334NR/I352 were arrested in G1 (Fig. 4C). In contrast, cells expressing the tumor-derived mutant p53Trp248 (p53W248) or p53TZ334NR with the Trp248 substitution were distributed in all phases of the cell cycle, as were cells that were not transfected or did not express significant levels of p53 (Fig. 4C).

To determine if the T2 alleviates transdominant inhibition by tumor-derived mutants, we examined the transcriptional and tumor suppressor activities of p53-T2 hybrids in the presence of p53Trp248 (p53W248). Transcriptional activity was assayed with a reporter plasmid containing the p53 site of p21; tumor suppressor activity was assayed by the colony-forming assay. In contrast to wild-type p53, neither p53TZ334NR nor p53TZ334NR/I352 were transdominantly inhibited by p53Trp248 (Fig. 5). Other dominant-negative, tumor-derived p53 mutants also failed to inhibit p53TZ334NR and p53TZ334NR/I352 (data not shown).


discussion

Design of a p53 Protein with Dominant Wild-Type Activity. Tumor-derived p53 mutants and wild-type p53 form inactive heterotetramers (26, 29). We, therefore, reasoned that a necessary step in the design of a p53 protein with dominant wild-type activity would be to substitute the tetramerization domain of wild-type p53 with a heterologous oligomerization domain. The design of such a p53 protein was not straightforward. First there is a paucity of independently folding native tetramerization domains that could be grafted to p53. For this reason, we initially focused on LZ hybrid proteins. However, functional analysis of hybrids, such as p53LZ335Q and p53LZ343RMKQ, revealed that their DNA binding and transcriptional activities were impaired relative to wild-type p53. Furthermore, although p53LZ343RMKQ (referred to as p53-CC 1-343 in Ref. 58) suppresses colony formation of H1299 lung adenocarcinoma cells efficiently (58), we only observed weak tumor suppressor activity with Saos-2 osteosarcoma cells (Fig. 4B). The discrepancy may reflect the sensitivities of the two cell lines to wild-type p53 activity or the level of p53 expression. Irrespective of the reason, the dimeric p53 proteins are not as potent as wild-type p53. This conclusion is further supported by comparing the DNA-binding activities of these proteins in vitro (Fig. 3C). The importance of tetrameric subunit stoichiometry can be rationalized since the p53 sites identified in regulatory sequences of genes contain four specific repeats (61, 63—65). If the affinity of a single DNA-binding domain for a repeat is the same in the context of p53 dimers and tetramers, then the avidity for DNA increases in parallel to the number of recruited DNA-binding domains.

Our inability to generate a p53-LZ hybrid protein with the desired properties turned our attention to a mutant GCN4 zipper that contains leucines at positions a of the coiled coil and isoleucines at positions d...
and assembles as a tetramer in vitro (41). Several properties of this mutant zipper make it appealing as a surrogate tetramerization domain for p53: (a) it is an independently folding domain, and therefore, it can be grafted to p53; (b) its tetrameric subunit stoichiometry matches the stoichiometry of the native p53 oligomerization domain; (c) its engineered nature decreases the probability that the hybrid p53 proteins will hetero-oligomerize with cellular proteins; and (d) its topology, a parallel four-stranded coiled coil (41), favors alignment of the p53 DNA-binding domains to DNA (Fig. 6).

Certainly an unknown parameter in selecting the TZ was the affinity with which it would drive p53 tetramerization in vitro and more so in vivo. An engineered heterodimerization domain, the design of which was based on the natural heterodimeric Jun and Fos LZs, forms stable dimers in vitro and in cells (66, 67). However, the TZ has no natural counterpart. Remarkably, it drove efficient tetramerization of p53 (Fig. 2). Although we did not quantitate the association constant, it must compare favorably to the constant of the native p53 tetramerization domain, since both domains drove wild-type p53 function with essentially equal efficiency in vitro and in tumor cells (Figs. 3 and 4).

Insights into p53 Biology and Prospects for Gene Therapy. The functional properties of the p53-zipper hybrid proteins provide insights into the biology of p53: (a) they demonstrate the importance of tetramerization for efficient p53 function, since the dimeric hybrids were significantly less active than the tetrameric ones; and (b) they provide a better understanding of the mechanisms by which tumor-derived p53 mutants transform cells. Tumor-derived p53 mutants lack tumor suppressor activity (17–25) and also transdominantly inhibit wild-type p53 (26–29). Remarkably, they can even enhance the tumorigenic potential of cells that do not express endogenous p53 (68, 69). Substitution of the native tetramerization domain of wild-type p53 by the TZ zipper was sufficient to confer dominant tumor suppressor activity. We can, therefore, conclude that transdominant inhibition of wild-type p53 by tumor-derived p53 mutants is mediated solely through hetero-oligomerization and furthermore that any oncogenic activity that tumor-derived p53 mutants may have independent of transdominant inhibition must be recessive to wild-type p53 function.

The p53-TZ hybrid proteins can be used to study p53 function in the background of dominant-negative, tumor-derived p53 mutants. However, their greatest significance may relate to the possibility of using these proteins in a gene therapy approach for treatment of human cancer (38–40). A p53 protein with dominant wild-type function would be clearly superior to wild-type p53 for such therapy, since about one-half of all human tumors express p53 mutants that can inhibit wild-type p53 (17, 18). Furthermore, it is possible to apply the paradigm established here with p53 and the engineered TZ to other human diseases in which a wild-type protein is sequestered by a dominant-negative mutant.

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