The p53 tumor suppressor protein binds to DNA as a dimer of dimers to regulate transcription of genes that mediate responses to cellular stress. We have prepared a cross-linked trapped p53 core domain dimer bound to decamer DNA and have determined its structure by x-ray crystallography to 2.3 Å resolution. The p53 core domain subunits bind nearly symmetrically to opposite faces of the DNA in a head-to-head fashion with a loop-helix motif making sequence-specific DNA contacts and bending the DNA by about 20° at the site of protein dimerization. Protein subunit interactions occur over the central DNA minor groove and involve residues from a zinc-binding region. Analysis of tumor derived p53 mutations reveals that the dimerization interface represents a third hot spot for mutation that also includes residues associated with DNA contact and protein stability. Residues associated with p53 dimer formation on DNA are poorly conserved in the p63 and p73 paralogs, possibly contributing to their functional differences. We have used the dimeric protein-DNA complex to model a dimer of p53 dimers bound to icosamer DNA that is consistent with solution bnding data and suggests that p53 core domain dimer-dimer contacts are less frequently mutated in human cancer than intra-dimer contacts.

The p53 tumor suppressor responds to cellular stresses such as DNA damage, in part, by binding to DNA and regulating the transcription of genes involved in cell cycle arrest, apoptosis, or senescence (1). The p53 protein forms a homotetramer, or dimer of dimers (2), with each subunit containing an N-terminal transactivation domain, a DNA-binding core domain (p53DBD) that is the site of the vast majority of tumor-derived substitution mutations, a tetramerization domain structured as (p53DBD) that is the site of the vast majority of tumor-derived substitution mutations, a tetramerization domain structured as a dimer of dimers (2–4), and a C-terminal regulatory domain (5, 6). p53 response elements contain two decamer sequences with the consensus PuPuPuC(A/T)(A/T)PyPyPy (where Pu indicates a purine and Py indicates a pyrimidine) (7) with anywhere from 0 (icosamer) to 20 base pairs between them, although an icosamer is more commonly found in vivo (8). While individual p53DBD subunits can interact with a pentamer sequence, lower order complexes in the presence of a icosamer sequence are not observed, demonstrating that cooperative p53DBD interactions occur on DNA (9, 10). Other studies also reveal that p53DBD exists in both a latent and active DNA-binding form that can be detected in vivo (11–13). This is consistent with structural studies showing that nascent p53DBD forms dimers that are incompatible with simultaneous DNA binding (14), suggesting that the p53DBD, in the context of full-length p53, changes quaternary structure for DNA binding as a dimer of dimers. The functional importance of the p53 dimer is further supported by the observation that replacement of the p53 tetramerization domain with the dimerization domain of GCN4 affords near wild type p53 tumor suppression activity in vivo (15).

The only molecular insights into the mode of DNA recognition by p53 were provided over a decade ago with the structure determination of the p53 core domain monomer bound to DNA (16). While this structure provided important insights into DNA recognition by p53, many questions still remain, leading to several studies aimed at assembling a p53 multimer on DNA but only resulting in several models of this complex (17–22). In this study, we use a cross-linking strategy (23, 24) to trap a p53DBD dimer bound to DNA for structure determination by x-ray crystallography (Fig. 1, A and B). The structure reveals the molecular details of cooperative dimeric p53 binding to DNA that involves a zinc-binding domain. A hot spot for tumor-derived mutations also maps to the dimerization domain of GCN4 affords near wild type p53 tumor suppression activity in vivo (15).

**EXPERIMENTAL PROCEDURES**

Mouse p53 Core Domain Purification—The mouse p53 core domain was purified essentially as described previously with minor modification (14). Briefly, the pRSET A (Invitrogen) bacterial expression vector encoding the p53DBD (residues 92–292) was transformed into Escherichia coli BL21 (DE-3). Cells were grown in LB medium at 37 °C, and when cultures reached an absorbance at 595 nm of ~0.4–0.6, cells were induced by the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside supplemented with 100 μM Zn(OAc)2, and grown overnight at 15 °C. Cells were isolated by centrifugation and resuspended in low salt buffer A (20 mM sodium citrate, pH 6.1, 100 mM NaCl, 10 μM ZnOAc2, and 1 mM tris(2-carboxyethyl-
yl)phosphine hydrochloride). The cells were sonicated, and cellular debris was removed by centrifugation. The supernatant containing soluble mouse p53DBD was loaded onto a SP-Sepharose (Amersham Biosciences) ion exchange column that was washed with low salt buffer A and eluted with a 0.1–1.0 M NaCl gradient. Peak fractions containing p53DBD were pooled, concentrated, and further purified by gel filtration using a Superdex-75 preparative column (Amersham Biosciences). Peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis analysis and concentrated to ~10 mg/ml for storage at ~80 °C until use.

Preparation of DNA with a Cross-linkable Tether—The convertible nucleoside strategy (25–27) was used to attach a sulfur-protecting group on the unmodified cytosines and a 5′-isobutyryl group on N4 of cytosine was obtained from Trilink Biotechnologies (San Diego, CA) containing the phenoxyacetyl protecting groups on the adenosine and guanosine bases, the isobutyryl-protecting group on the unmodified cytosines and a 5′-dimethoxytrityl group. The DNA, supplied on resin, was treated with 0.5 M cystamine HCl (Sigma) at pH 13 for approximately 2 h to cleave from resin. The supernatant was separated from the resin by centrifugation and then incubated at 55 °C for 18 h to remove the base protecting groups. The DNA was dialyzed in deionized water overnight using Spectrapor 2000 MWCO dialysis tubing (Spectrum Laboratories) to remove unreacted reagents, lyophilized to dryness, and redissolved in 500 μl of deionized water. The DNA was further purified by reverse-phase HPLC using a Dynamax 300 Å Pure DNA column as described by the vendor (Varian). HPLC fractions containing DNA were dialyzed overnight in deionized water to remove residual trifluoroacetic acid and acetonitrile. The resulting solid was dissolved in ~40 μl of deionized water and quantitated by UV absorbance, and the products were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry yielding an observed molecular weight of 5015.30 daltons consistent with the predicted molecular mass of 5018.11 daltons. The DNA duplex was annealed by heating to 80 °C and slowly cooling to 4 °C for storage until use.

Preparation of the p53DBD Dimer-DNA Complex—Duplex DNA and protein were mixed in a 1:2 molar ratio in cross-linking buffer (20 mM sodium citrate, pH 6.1, 100 mM NaCl, and 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride) to a volume of 2 ml and incubated at 21 °C for 3 h. The cross-linked protein-DNA complex was purified by fast protein liquid chromatography gel filtration using a Superdex-75 preparative column. Fractions corresponding to the protein-DNA complex were copped with 40 mM methane methylthiol sulfonate and analyzed using a denaturing, non-reducing SDS-PAGE gel to confirm the homogeneity of a cross-linked p53DBD-DNA complex. Fractions containing pure complex were pooled and concentrated using a Vivaspin concentrator with a 30,000 MWCO membrane (Viva Science) to a concentration of about 7.5 μM as determined by UV absorbance and stored at ~80 °C until use for crystallization. To determine the extent of nonspecific cross-linking, a C274S p53DBD mutant was prepared and incubated with duplex DNA in cross-linking buffer under identical conditions and the resulting mixture was run on a denaturing, non-reducing SDS-polyacrylamide gel to confirm the absence of DNA cross-linking to the p53DBD C274S mutant.

RESULTS

Overall Structure of Dimeric p53-DNA Complex

Crystallization, Data Collection, and Structure Determination—Crystals were prepared using hanging drop vapor diffusion against a reservoir containing 0.2 M NH4Cl, 0.01 M CaCl2, 0.05 M Tris-HCl, pH 8.5, 28% polyethylene glycol 4000 and appeared after 2 days, growing to a maximum size of 0.4 × 0.05 × 0.025 mm after 4–5 days. Crystals were flash-frozen in 0.2 M NH4Cl, 0.01 M CaCl2, 0.05 M Tris-HCl, pH 8.5, 28% polyethylene glycol 4000, and 15% glycerol. Data collection was initially carried out on a Rigaku Raxis IV detector with a rotating copper anode. Phases were initially obtained at 4 Å resolution with molecular replacement using the program Molrep (28) and a human p53 core domain model bound to its cognate pentamer sequence (1TSR) as a search model, yielding a solution with a correlation coefficient of 48.1% and an Rfactor of 44.8%. Further inspection of the solution revealed that the two p53DBD-pentamer complexes were aligned to form a continuous decamer helix. In addition, Fo − Fc electron density corresponding to where the phosphates should be to connect the two pentamers was observed, although none was input into the starting model. Crystals were diffraction to a maximum useful resolution of 2.3 Å at Cornell High Energy Synchrotron Source beamline F-1 and were indexed and scaled using HKL2000 (29), respectively. The molecular replacement model was refined to 2.3 Å in CNS (30) and employed simulated annealing or minimization with NCS (relating the two halves of the homodimer complex) and DNA restraints. The NCS restraints were decreased over time and eventually dropped. Between refinement cycles, the model underwent manual rebuilds with the program Coot (31) or O (32). Toward the end of refinement, individual B-factors, two Tris molecules, and solvent molecules were added. The final model was checked for errors using a composite omit map and Procheck (33). Analysis of the DNA was done using the program 3DNA (34). Figures were generated using Pymol (35). Data collection and refinement statistics are summarized in Table 1.

The abbreviation used is: HPLC, high performance liquid chromatography.
FIGURE 1. Structure of the p53DBD dimer-DNA complex. A, overall structure of mouse p53DBD (blue and cyan) dimer bound to DNA in ribbons representation. The DNA consensus pentamer sequence is colored in gray, and bases not involved in p53DBD interactions are colored in red. Zn$^{2+}$ ions are represented as yellow spheres. B, structure looking down the DNA helix (rotated 90° toward the viewer from A). C, sequence alignment of mouse and human p53DBD. The alignment was generated using ClustalW and then rendered using ESPript. Identical residues are shaded in black, and similar residues are shown in bold. Relative frequencies of tumor-derived mutations are represented by the bar graph above the sequence, with secondary structural elements depicted as black bars (α-helix), arrows (β-sheet), and lines (loop). Residues that are involved in DNA binding interactions (green dot), dimer contacts (red dot), zinc ligation (orange dot), or protein stability (blue dot) are indicated. Potential residues involved with protein stability were assessed by observation of interactions with other residues in the protein; nonconserved residues were not included in this analysis. Residues of p53DBD that are implicated in dimer-dimer interactions within the tetramer are indicated with a yellow dot. D, DNA sequence used in crystallization. The consensus pentamer sequences are boxed, and cross-linked bases are colored in red. Bases that are not observed in the electron density are colored in gray.
Structure of Dimeric p53-DNA Complex

Minor groove via a zinc-binding domain. The DNA adopts B-form geometry with a bend of about 20° at the site of protein dimerization. Except for a disordered L1 loop region (residues 114–118) in each subunit of the dimer (Fig. 1C), the overall fold of the p53DBD core is similar to previously published structures of the nascent human (36, 37) and mouse core domains (14), and the human core domain bound as a monomer to DNA (16). Briefly, the core domain contains a sandwich of anti-parallel β-sheets with the H1 and H2 helices on one end of the molecule. The longer H2 helix, along with the preceding loop, interacts with the DNA major groove of the pentamer sequence, while the shorter H1 helix, along with the preceding L2 loop and the L3 loop, ligate a zinc ion that together forms the p53DBD dimerization interface over the DNA minor groove. Thirteen or 15 of the 16 bases on each strand of the DNA are visible in the electron density map forming a 13-base pair duplex in which two of the 5'-nucleotides on one strand are flipped out at one end of the DNA helix. The ends of the DNA make protein lattice contacts and do not arrange themselves in an extended helix within the crystal lattice as is often observed in DNA-protein coocrystals.

Comparison with the Monomeric Human p53DBD in Complex with DNA—Not surprisingly, a superposition of a p53DBD subunit bound to a p53 pentamer with the previously published p53 core domain monomer bound to DNA (1TSR) shows a high degree of structural similarity with root mean square deviations of 0.732 or 0.748 Å, for Cα atoms of the protein (chains A and B, respectively) and 1.213 or 1.171 Å for all non-hydrogen atoms of the decamer sequence of DNA, (chains C and D, respectively) (Fig. 2A). In addition, nearly identical protein-DNA interactions are observed (Fig. 2, B and C). This occurs despite the fact that the sulfur atom of Cys274 is cross-linked to the N4 atom of cytosine 8 and 8' through a sulfur-bearing three-carbon linker that has a maximal length of 7.5 Å (23). Indeed, the two cross-linked atoms are about 3.8 Å apart, only about 0.6 Å further than the corresponding atomic distances in 1TSR, and the linker is not visible in the electron density map (Fig. 2B). These observations suggest that the tether remains flexible and accommodates native protein-DNA interactions.

In particular, like in 1TSR, within the loop H2 region, Arg277 makes base specific contacts with guanosine 7 in the DNA major groove and residues Ala276, Arg277, and Arg280 (residues 280, 281, and 283 in human) make analogous DNA backbone contacts (Fig. 2C). In addition, residues Ser238 and Arg245 (residues 241 and 248 in human) from the L3 loop make interactions to the DNA minor groove.

The one region that shows significant deviation in protein-DNA contacts between the p53DBD dimer-DNA complex and 1TSR is the L1 loop (Fig. 2D). In 1TSR, Lys120 from the human protein makes base-specific contacts to a purine base in the major groove (the equivalent to adenosine 2 in the p53DBD dimer-DNA structure). In the p53DBD dimer-DNA complex, residues 114–118 of the L1 loop harboring the corresponding lysine (Lys117) are disordered in both protein subunits of the complex. Interestingly, the L1 loop shows considerable flexibility in the mouse and human p53DBD domains in the absence of DNA; and in the worm p53 homologue, CEP-1, the L1 loop has recessed conformation that is not in position to make DNA contacts (38). Moreover, although mutations of other residues that contact DNA are the most frequently observed to be mutated in human cancers, the frequency of Lys120 mutations is rather low. Taken together, these data suggest that the L1 loop does not make a significant contribution to DNA binding by p53. Otherwise, this structural comparison reveals that p53 dimer formation on DNA does not significantly alter the protein-DNA contacts made by each p53 subunit.

A comparison of the DNA conformation within the p53DBD dimer-DNA complex and 1TSR reveals that protein dimer binding on DNA induces a bend of about 20°, and this is in contrast to the essentially straight decamer DNA observed in 1TSR (Fig. 2E). This bend is centered around the dimerization region of the protein illustrating that dimer formation induces DNA bending. Based on this observation, we extrapolate that the p53 tetramer bound to DNA may induce a DNA bend of about 2 × 20°, or 40°, which is consistent with solution studies (9, 18).

Interactions at the p53DBD Dimer Interface—Previously reported structural studies on the p53 core domain had noted a zinc ion that is ligated by three cysteine residues and one histidine residue from the H1 helix and L2 and L3 loops (Fig. 1, A and C). The resulting zinc-binding domain was proposed to play a role in maintaining p53 core domain stability, consistent with solution studies showing that disruption of this domain destabilizes the p53 protein (39). The p53DBD dimer-DNA structure reveals that this zinc-binding domain also plays a pivotal role in p53DBD dimer formation on DNA. The zinc domains of the dimer interact over the minor groove between the two DNA pentamer sites, forming a clamp-like structure over the DNA. Dimer interactions involve van der Waals,
hydrogen bonding, and electrostatic interactions. van der Waals interactions are made between Pro174 and His175 from the H1 helices of opposing subunits of the dimer (Fig. 3A). An intermolecular salt bridge is mediated by the NH1 atom of Arg178 and the OD1 atom of Glu177 from the H1 helices of the dimer (Fig. 3B). Additionally, the NH2 atoms of both Arg178 residues are observed at close enough distances to the NE atoms (3.31 and 3.46 Å) to participate in dipole-dipole interactions. The Arg178 salt bridge interaction with Glu177 may serve to localize the positive charge of Arg178 toward the NH1 and NH2 atoms allowing an electron pair to localize at the NE atoms, thereby creating a dipole within Arg178. Additionally, there are water-mediated (W75 and W141) hydrogen bonding interactions in the inter-dimer interface via the backbone carbonyl of Met180 from both monomers. Correlating with our finding that the H1 helix plays a particularly important role in p53DBD dimer formation on DNA, previously reported NMR studies of a human p53DBD dimer bound to DNA also observed involvement of residues in the H1 helix in dimer formation (17, 21).

The functional importance of the observed p53DBD dimer interactions on DNA is supported by recently reported in vitro studies. In these studies, mutation of Arg181 and His178 in human p53 (corresponding to mouse residues 175 and 178) to alanine decreases the cooperative DNA binding of p53DBD and an Arg181 mutation to Glu completely abolished DNA binding, even in the presence of a glutathione S-transferase dimerization domain fusion (22). In addition, residues that form the p53DBD dimer interface on DNA, Pro174, His175, Glu177, and Arg178 (residue numbers 177, 178, 180, and 181 in human) are found to be mutated in human cancers (Figs. 1C and 3C). These observations reinforce
the importance of the observed dimer interactions for p53 function in vivo. Taken together with previous structural studies, it appears that tumor-derived p53 core domain mutations fall into three classes, those that disrupt protein stability, protein-DNA interaction, or dimer formation on DNA. The greater mutational sensitivity of the residues that make protein-DNA contacts suggests that mutation of DNA contact residues are less easily compensated for by other p53 residues.

**DISCUSSION**

**Comparison with p63 and p73 Paralogs**—The DNA-binding domain of p63, a paralog and evolutionary ancestor of p53, shares ~60% identity with the p53DBD, which includes the residues that coordinate the zinc ion and that contact DNA (40–42). Despite this similarity, the isolated p63DBD domain alone cannot bind p53 response elements, while full-length p63 can (42). In contrast, introduction of a glutathione S-transferase dimerization fusion allows p63DBD to bind cooperatively to p53 response elements, showing that p63 dimerization is a key component of its DNA binding properties. Interestingly, key residues for p53DBD dimerization, His175 and Arg178, are not present in p63. Instead, p63 contains an asparagine and leucine at these positions (Fig. 3D). p73, another p53 paralog, has identical sequence with p63 in the H1 helix. Based on these observations, we hypothesize that p63 and p73 do not make the same dimer interactions as the p53DBD on DNA and propose that this may represent an important functional distinction of these p53 paralogs. It is interesting to speculate that p53DBD dimer interactions could have evolved later to enhance DNA binding ability by “fixing” the positions of the p53DBD domain relative to each other when bound to DNA. It may have been evolutionarily economical for p53 to utilize an already existing structural feature (Zn$^{2+}$-binding helix) to fine tune DNA binding.

**Functional Consequences of p53 Dimerization States**—The p53DBD dimer interface that we observe in complex with DNA...
has not been observed in other crystal structures of the p53DBD in the absence of DNA, suggesting that this mode of p53DBD binding is facilitated by DNA binding and is unlikely to occur in the absence of DNA. Interestingly, several other reported crystal structures of the p53DBD in the absence of DNA also show significant dimer interactions in the crystal lattice, but each of these dimers is in a conformation that is incompatible for dimeric binding on DNA (14, 37) (Fig. 4). Specifically, both the DNA-free mouse p53DBD and the human p53DBD monomer bound to DNA show dimer interactions in the crystal lattice that involves the H1 helix and the flanking loops of one subunit with the S6-S7 loop of another subunit (Fig. 4B). These dimer interactions bury a total solvent-excluded surface of 996 and 1432 Å², respectively, while the dimeric p53DBD shows a solvent excluded surface of only 595 Å² between the two protein subunits. This dimeric arrangement of the p53 core domain places the H2 DNA recognition helices of the dimer on the same side of the DNA, as opposed to opposite sides of the DNA as observed in the p53DBD dimer bound to DNA as reported here. This observation suggests that the p53DBD may exist as DNA-binding incompetent dimers in the absence of DNA and DNA competent dimers in the presence of p53 response elements. This proposal is consistent with equilibrium sedimentation data showing that nascent mouse p53DBD forms dimers with a dissociation constant of about 2 mM (14). Although this concentration far exceeds the cellular p53 concentration in the cell, the local p53DBD concentration within the intact p53 tetramer may be high enough to induce dimer formation. The signal that might induce a p53DBD dimer transition from a DNA-binding incompetent to DNA-binding competent form is unknown, although DNA binding alone is likely insufficient since crystallization attempts at trapping a p53 dimer bound to DNA in the absence of cross-linking has not been successful.3 Taken together, the involvement of the zinc-binding region of the p53DBD, which includes the H1 helix and the surrounding loops, in each of the p53 dimerization states observed to date argues for its pivotal role in regulating the DNA binding function of p53. This is consistent with the high degree of mutational sensitivity of this region (Figs. 1C and 3C).

Model for a p53 Dimer of Dimers—All p53 response elements contain two decamer sites that are bound by a dimer of p53 dimers. Although there is variability in the number of bases separating two decamer sites in nature, most sites have no intervening bases (icosamers) (8). Interaction between two p53DBD dimers on DNA would be distinct from the interatomic interactions within the dimer itself, since the former would involve tail to tail interactions instead of the head to head interactions observed in the DNA-bound dimer. To obtain some insights into the nature of these dimer–dimer interactions, we modeled a p53DBD dimer of dimers on an icosamer site by translating the p53DBD dimer along the DNA by 10 base pairs and used the ends of the DNA outside the decamer site for superposition (Fig. 5A). This modeling assumes a duplication of the DNA bend observed for the p53DBD dimer-DNA structure, resulting in an overall bend of about 40°, which is consistent with published reports (43). Interestingly, this bend is not continuous but, instead, is comprised of two bends that occur at the junction of each p53 core domain dimer (Fig. 5B). This modeling reveals that a dimer of p53DBD dimers can be assembled on DNA without molecular clashes (Fig. 5). The modeling also shows that additional DNA bending or protein adjustments would result in tail-to-tail dimer interactions involving the S7-S8 and L2 loops of the p53DBD. The relative paucity of

3 R. Marmorstein, K. Zhao, and W. C. Ho, unpublished data.
tumor-derived mutations in these areas (Figs. 1C, 3C, 5C, and 5D), however, argues against the relative functional importance of p53 dimer-dimer interactions on DNA. This is consistent with the variability of base pair length separating p53 decamer sites that is observed in nature.

Interestingly, a mapping of the frequency of tumor derived mutations onto the surface of the p53 dimer of dimers bound to DNA reveals two spines of mutational sensitivity on the top surface of the p53 dimers that extend across the dimers (Fig. 5, C and D). This highlights a potential functionally important binding surface for either another part of p53 or other p53-associated proteins that might influence the dimerization function of p53. This structure now provides a scaffold for testing this hypothesis.

In summary, this study brings us significantly closer to understanding the molecular details of how a p53 dimer of dimers assembles on DNA to help mediate its tumor suppressor function. This structure also provides new avenues for the structure-based design of small molecule compounds that may shift the equilibrium of mutated p53 to the DNA-binding competent form for tumor suppressor activity.

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Structure of Dimeric p53-DNA Complex

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