NMR Spectroscopy Reveals the Solution Dimerization Interface of p53 Core Domains Bound to Their Consensus DNA*

The p53 protein is a transcription factor that acts as the major tumor suppressor in mammals. The core DNA-binding domain is mutated in about 50% of all human tumors. The crystal structure of the core domain in complex with DNA illustrated how a single core domain specifically interacts with its DNA consensus site and how it is inactivated by mutation. However, no structural information for the tetrameric full-length p53-DNA complex is available. Here, we present novel experimental insight into the dimerization of two p53 core domains upon cooperative binding to consensus DNA in solution obtained by NMR. The NMR data show that the p53 core domain itself does not appear to undergo major conformational changes upon addition of DNA and elucidate the dimerization interface between two DNA-bound core domains, which includes the short H1 helix. A NMR-based model for the dimeric p53 core-DNA complex incorporates these data and allows the conclusion that the dimerization interface also forms the actual interface in the tetrameric p53-DNA complex. The significance of this interface is further corroborated by the finding that hot spot mutations map to the H1 helix, and by the binding of the putative p53 inhibitor 53BP2 to this region via one of its ankyrin repeats. Based on symmetry considerations it is proposed that tetrameric p53 might link non-contiguous DNA consensus sites in a sandwich-like manner generating DNA loops as observed for transcriptionally active p53 complexes.

The tumor suppressor gene p53 is the most frequent site of genetic alterations found in human tumors (1) and acts as the major tumor suppressor in mammals. In addition to non-transcriptional functions, p53 acts primarily as a transcriptional activator, that regulates the expression of several genes involved in cell cycle arrest, cellular senescence, anti-angiogenesis, and apoptosis (reviewed in Refs. 2–4). Recently, two homologues of p53, p63 and p73, were discovered, coding for a variety of different isoforms. These three p53 family members play distinct roles in differentiation, development, and tumor suppression (reviewed in Ref. 5). p53 possesses a modular architecture with an N-terminal transactivation domain (TAD), a strongly conserved core DNA-binding domain (DBD), a tetramerization domain (TD), and a regulatory C terminus (6, 7). Tetrameric p53 binds specifically to a DNA consensus sequence consisting of two consecutive palindromic 10-bp half-sites, where each half-site is formed by two head-to-head quarter-sites (8–12). The isolated TD forms a symmetric dimer of dimers (13–15), and contrasting models have been proposed that describe how the DBDs of each dimer are attached to DNA, namely with either consecutive or alternating arrangements (16).

The p53 DBD comprises several hot spot regions for mutation that inactivate p53 in more than half of all human tumors (1). Therefore, wild-type and mutant p53 DBDs have been the focus of various studies (17–21). The crystal structure of the p53 DBD in complex with DNA (10) showed that almost all known mutations affect residues that are in direct contact with DNA or maintain the tertiary structure. However, only one out of three p53 DBDs is bound to DNA sequence specifically in this crystal structure. Recently, the crystal structure of free mouse p53 DBD has been solved (22), and NMR studies provided further insight into the folding of wild-type and mutant p53 DBDs (23).

Owing to its prominent role in tumorigenesis, the restoration of wild-type p53 activity for tumor therapy has gained widespread attraction (24). Several studies have used structural information in attempts to rescue mutated or to stabilize the wild-type p53 DBD conformation (25–30). Based on the allosteric model of p53 regulation (31–33), peptides derived from the wild-type p53 DBD conformation (25–30). Based on the allosteric model of p53 regulation (31–33), peptides derived from the wild-type p53 DBD conformation have been devised (34–36) to restore the wild-type activity of mutant p53. Recently, low molecular weight compounds have been reported to stabilize the wild-type conformation of human p53 and show an anti-tumor activity in vivo (37). Several studies have disclosed that four p53 DBDs bind cooperatively to the DNA consensus sequence (11, 17–19, 21, 38). The crystal structure of the p53 DBD-DNA complex is compatible with a model where four p53 DBDs bind to the consensus DNA without steric clashes (Fig. 7B in Ref. 10) and bend the DNA (19, 39–42). Yet, no structural information is available for the intact tetrameric p53 bound to DNA owing mainly due to the difficulties in obtaining suitable protein samples.

Here, we present the results of NMR experiments that allow for the first time the experimental identification of the actual dimerization interface between two p53 DBDs cooperatively

The abbreviations used are: TAD, transactivation domain; DBD, DNA-binding domain; TD, tetramerization domain; DTT, dithiothreitol; FCS, fluorescence correlation spectroscopy; TOESY, transverse relaxation-opimized spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; 53BP2, p53-binding protein 2; TAMRA, 6-carboxytetramethylrhodamine.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Tel.: 49-8856-60-3745; Fax: 49-8856-60-2659; E-mail: silke.hansen@roche.com.

1 The abbreviations used are: TAD, transactivation domain; DBD, DNA-binding domain; TD, tetramerization domain; DTT, dithiothreitol; FCS, fluorescence correlation spectroscopy; TOESY, transverse relaxation-opimized spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; 53BP2, p53-binding protein 2; TAMRA, 6-carboxytetramethylrhodamine.
bound to their consensus DNA in aqueous solution. This dimerization interface resides in the short H1 helix previously suggested to be involved in protein-protein interaction (10). Based on NMR data, we have created a consistent model for the dimeric p53 DBD-DNA complex. The findings that inactivating hot spot p53 mutations map in the dimerization interface (43) and that the putative p53 inhibitor 53BP2 binds to this region via one of its ankyrin repeats (44) further support this model. We therefore conclude that the experimentally identified region forms the actual p53 interface in the tetrameric p53-DNA complex. Based on the symmetry of the dimeric p53 DBD-DNA complex a sandwich-like model (12) is discussed for the intact tetrameric p53-DNA complex. This model is characterized by tetrameric p53 binding as a dimer of dimers to two separate juxtaposed DNA consensus sites, implying an inherent ability of p53 to link DNA strands, e.g. in transcriptionally active complexes (45). It brings into accord the symmetry of the p53 TD with the structural requirements of p53 DBD binding to the palindromic DNA consensus sequence without assuming a conformational switch upon DNA binding.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of analytical grade and obtained from major commercial suppliers. TAMRA-labeled DNA oligonucleotides were purchased from MWG-BIOTECH and TIB Molbiol. 15NH4Cl and [13C6]glucose was obtained from Martek Biosciences. C-terminal residues 361–382 (GSHRSSHLKKSGQSTSRHKK-NH2) and N-terminal residues 79–94 (APAAPPTAAAPAPAPSPLWS-NH2) p53 peptides were synthesized using standard Fmoc peptide chemistry and purified by reversed phase high performance liquid chromatography.

Cloning, Expression, and Purification of the p53 DBD—Residues 94–312 of human p53 coding for the wild-type p53 DBD (10, 21, 23) were amplified from plasmid pT7.7Hup53 (46) by polymerase chain reaction and cloned into a modified pQE40 vector (Qiagen). p53 DBDs were amplified from plasmid pT7.7Hup53 (46) by polymerase chain reaction and cloned into a modified pQE40 vector (Qiagen). p53 DBD was refolded according to standard procedures (48) (representing molecules as sets of bead-like spherical friction centers) and purified as published (21, 23). Due to the high purity (＞98%), 5% (v/v) 2H2O was added to the NMR sample. Generally, a 1.2–1.5 M excess of consensus DNA was refolded according to standard procedures (48, 55) and purified as published (21, 23). In the presence of 15N, the nuclear magnetic resonance and nitrogen and hydrogen isotope enrichment up to an optical density of 0.8, followed by overnight induction at 37 °C with 1 mM isopropylthio-galactoside. After induction, cells were harvested by centrifugation, resuspended, and ruptured by high-pressure dispersion. Inclusion bodies were isolated, washed, and solubilized in 100 mM Tris, pH 7.5, 6 M guanidine HCl, and 10 mM DTT as described previously (48). In the following, p53 DBD was refolded according to standard procedures (48, 55) and purified as published (21, 23). Due to the high purity (＞98%) of the inclusion body preparation, no additional Heparin Hi-Trap column was necessary during the purification. Refolded and concentrated p53 DBD was dialyzed into 50 mM potassium phosphate, pH 6.8, 50 mM KC1, and 5 mM DTT, loaded onto a SP-Sepharose Fast Flow cation exchange column (Amersham Bioscience, Inc.) and eluted with a linear KCl gradient (Amersham Bioscience, Inc.) in 50 mM potassium phosphate, pH 6.8, 150 mM KC1, and 5 mM DTT. The purity of p53 DBD was ＞98%. 5% (v/v) 1H2O was added to the p53 DBD and samples were concentrated using 5 K Ultrafree 4 Centrifugal Filter Devices (Millipore) to 200–500 μl, dialyzed into 50 mM potassium phosphate, pH 6.8, and 5 mM DTT containing 40–100 mM KC1, flash-frozen in liquid nitrogen, and stored at －80 °C. Final yields were some 60 mg of uniformly 15N-labeled and some 45 mg of uniformly 13C,15N-labeled p53 DBD per 1-liter culture.

Analytical Procedures—Electrospray mass-spectrometry confirmed the identity and complete isotopic labeling of p53 DBD, as well as cleavage of the N-terminal methionine after translation. The protein concentration was measured spectrophotometrically according to Bradford (49) or using an extinction coefficient of ε280 nm = 15,830 M－1 cm－1 (50). SDS-polyacrylamide gel electrophoresis was performed with 12.5% gels. The monomeric state of all p53 DBD preparations was confirmed by analytical size exclusion chromatography with a TSK gel G 2000SW (TosoHaas) analytical gel filtration at a flow-rate of 0.5 ml/min in 50 mM potassium phosphate, pH 7.0, 150 mM KC1, and 5 mM DTT. Specific DNA binding activity of the p53 DBDs was confirmed by electrophoretic mobility shift assays (51). The 15N,13C,H-HSQC spectrum of the refolded U-15N-labeled p53 DBD was identical to the one published recently (23).

Fluorescence Correlation Spectroscopy (FCS)—Quantitative analysis of the DNA binding properties of p53 DBD was performed with a ConfoCor fluorescence correlation spectrometer (Carl Zeiss Jena and Evotec OAI). 5' TAMRA-labeled CON2x5 and CON4x5 DNA oligonucleotides containing one or two 10-mer p53 consensus half-sites, 18-mer CON2x5 (5'-CCTACATGCTATA-3') and 26-mer CON4x5 (5'-CCTACATGCTATA-3') (18) were annealed with complementary oligonucleotides. The concentrations of the annealed double-stranded DNA oligonucleotides were determined using FCS and adjusted to an equimolar ratio in relation to quarter-sites (3 nt for CON2x5, 1.5 nt for CON4x5). Measurements were performed at 20 °C in 50 mM potassium phosphate, pH 7.0, 50 mM KC1, 5 mM DTT, and 0.1% Triton X-100 in the presence of 1 nM supercoiled, nonspecific pbLuescript (pBS) DNA (Stratagene) to suppress nonspecific DNA binding. Experimental autocorrelation curves were fitted using the FCS plus 1.0 software package (Evotec OAI). For the determination of apparent binding constants by the program Prism 3.0, the values were fitted to the equation: f = [DBD][[DBD] + Kd], for [DNA] ≪ [DBD] and [DNA] ≪ Kd, where f is the fraction of complexed TAMRA-labeled oligonucleotide and Kd is the apparent equilibrium binding constant.

NMR Spectroscopy—NMR investigations on p53 DBD were carried out on Bruker DMX750 and DMX600 spectrometers equipped with a triple channel (1H, 13C, 15N) and quadruple channel (1H, 13C, 15N, 2H) inverse probe head, respectively. Water suppression was achieved by means of a water suppression scheme (56) for a 1.2-1.5 3t excess of consensus DNA relative to the p53 DBD was necessary to achieve stoichiometric binding. The pH of the solution was verified during measurements by monitoring the 31P NMR chemical shift of the phosphate buffer.

Hydrodynamic Calculations—Hydrodynamic calculations were performed with the DIFPCG module of the DASHA software package (version 3.4.8b) (56), using the published crystal structure of the p53 DBD (PDB-ID: 1ITSR, chain B) and the modeled structure of the dimeric p53 DBD-DNA complex described in this article (see below). The bead model (representing molecules as sets of bead-like spherical friction centers) was used with beads centered in all CA, CG, and C2 (side chains of Arg, Tyr, Phe, and Trp) atoms of the protein as well as in all C2 (nucleobases), C3' (ribose), and phosphor atoms of the DNA. Beads were scaled equally and a hydration shell of 0.5 atoms thickness was added.

Molecular Modeling—Molecular modeling was performed with the program X-PLOR (57) using the paralldlg force field. The crystal structure of the p53 DBD-DNA complex (PDB-ID: 1ITSR) was the basis of the calculations (10). From chain B, which is the only subunit bound specifically to the DNA in the crystal structure, a starting structure of the dimeric p53 DBD was generated by applying C2 symmetry and using the homomomer. First, the structure was optimized with fixed internal atom coordinates until no clashes between the subunits could be detected. Second, the internal atom coordinates were tethered according to chemical shift changes observed by the NMR experiment, i.e. atoms, which show only little shift changes were fixed, and no restraint was put on atoms, which show the strongest shift changes. The system was minimized using 300 steps of conjugate gradient, relaxed using 5000 steps with a time step of 3 fs, and at a temperature of 300 K and finally another 300-step minimization was performed. Figures were created with InsightII (MSI Inc.).


**RESULTS**

**Probing the Allosteric Model of p53 Regulation by NMR Spectroscopy**—The allosteric model of p53 regulation proposes that the C-terminal regulatory domain of p53 interacts with the p53 DBD and keeps it in a latent state incapable of specific interaction with DNA. Upon cellular stress the C terminus is subjected to phosphorylation and acetylation, releasing the inhibitory interaction such that p53 can bind to its specific consensus sites and activate the corresponding target genes (31–33). In addition, several publications have suggested that an N-terminal region of p53 participates in its regulation (36, 58, 59). The published NMR assignment for p53 DBD (23) allowed us to probe for the postulated interaction between the C terminus and the p53 DBD (60) by NMR spectroscopy and to map possible shift differences upon the protein structure. The experiments were carried out by titrating U-15N-labeled p53 DBD (residues 94–312) with the C-terminal peptide (residues 361–382, phosphorylated and unphosphorylated) under varying conditions (i.e., varied temperatures, pH, and ionic strengths). These NMR experiments included protein-detected (15N,1H-HSQC) and peptide-detected methods (1H spectra, diffusion-ordered spectroscopy, inversion recovery, and saturation transfer difference experiments with selective saturation of p53 DBD methyl groups). None of the performed experiments, however, produced any indication of molecular interactions between the C-terminal peptide and p53 DBD. In addition, no direct interactions were found between a peptide covering the N-terminal polyproline-rich part of p53 (residues 76–94) (59) and the U-15N-labeled p53 DBD. Likewise, no contacts were found between the C-terminal peptide (residues 361–382) and the polyproline-rich region of a U-15N-labeled N-terminal extended p53 DBD construct (residues 40–312) (36), which proved to be highly flexible in the NMR spectra. We then verified the hypothesis that consensus DNA might be involved in the interaction between the C terminus and the p53 DBD. While no direct interaction of the C-terminal peptide with p53 DBD in complex with DNA consensus oligonucleotide could be detected, we observed clear evidence of weak unspecific interactions between the peptide and the oligonucleotide as titratable NMR shift changes of the isolated DNA imino protons (data not shown).

**NMR Studies of the p53 DBD-DNA Interaction**—Earlier studies have demonstrated that p53 DBD cooperatively binds to DNA consensus sequences covering one or two consensus half-sites, whereas binding to a single quarter-site cannot be detected by electrophoretic mobility shift assays (11, 16, 18, 19, 21). Electrophoretic mobility shift assays, FCS, and NMR confirmed that p53 DBD does not bind with detectable affinity to pentameric DNA oligonucleotides covering only one quarter-site (data not shown), whereas it does bind cooperatively to decameric CON2x5 (i.e., one half-site, apparent \( K_d = 519 \pm 65 \) nM) and with higher cooperativity and affinity (apparent \( K_d = 124 \pm 26 \) nM) to dodecameric CON4x5 DNA oligonucleotides (i.e., two half-sites, see Fig. 1). Consequently, the latter was used first for NMR spectroscopic examinations of the p53-DNA complex. Upon addition of a 0.25 M equivalent of the CON2x5 oligonucleotide to U-15N-labeled p53 DBD, dramatic intensity losses and line broadenings were observed in the 15N,1H correlation spectrum of p53 DBD. These were initially attributed to the large size of the ensuing macromolecular complex of some 120 kDa (four p53 DBDs per CON4x5 oligonucleotide), entailing very slow molecular rotation. To improve the poor spectral quality, further NMR experiments were performed in the following with CON2x5 oligonucleotide covering only one p53 consensus half-site. The expected ternary p53-DNA complex consisting of two p53 DBDs cooperatively bound to one CON2x5 oligonucleotide, subsequently denoted as dimeric p53 DBD-DNA complex, was confirmed by NMR diffusion experiments (see below).

Addition of a 0.5 m equivalent of the CON2x5 oligonucleotide to the U-15N-labeled p53 DBD binding led to shift changes both in the protein 15N,1H correlation spectrum and on the well separated imino protons (12.2–13.8 ppm) of the DNA, while the 31P signals of the DNA were too broad and overlapped to disclose any shift changes. Most prominently, however, 15N,1H-HSQC signal intensities again dropped dramatically, albeit to a somewhat lesser extent than with CON4x5. Treatment of the dimeric p53 DBD-DNA complex with DNase II restored the original spectrum of the free p53 DBD, excluding irreversible effects such as protein aggregation or degradation as reasons for the poor spectral quality. Therefore, the signal reductions were initially also attributed to relaxation losses due to the increased size of some 60 kDa and decreased global mobility of the dimeric p53 DBD-DNA complex. Such size-dependent relaxation effects can efficiently be suppressed by recording a subspectrum of the 15N,1H-HSQC, known as 15N,1H-TROSY (61), in which only the one 15N,1H-coupled spin state is selected that experiences a negative interference of two major relaxation pathways. The resulting cancellation of relaxation losses improves with increasing magnetic field strength. However, the application of 15N,1H-TROSY to the dimeric p53 DBD-DNA complex failed to produce the desired signal enhancement even at 750 MHz. Instead, signal intensities increased 3-fold on the average in a fast standard 15N,1H-HSQC with minimized duration, i.e., without time consuming coherence-selective gradients and with a \( J_{\text{HN}} \) coupling evolution delay set to only half its theoretical value (2.8 versus 5.5 ms; a WATERGATE water suppression scheme was implemented during the final ReINEPT step without additional lengthening of the pulse sequence). While shortening of a pulse sequence will generally minimize relaxation losses, the failure of TROSY excluded that size-dependent relaxation mechanisms are important for the dimeric p53 DBD-DNA complex. This leaves only slow chemical or conformational exchange processes as the primary source for the observed drastic line broadenings of partly more than 100 Hz (1H-line broadening at 600 MHz), indicating lifetimes for the (associated) complex and/or for a specific conformation in the range of micro- to milliseconds.

Attempts to shift the system out of the coalescence region into either the slow or the fast exchange regime failed. For instance, neither cooling (from 293 to 283 K) nor heating the system to 303 K had any decisive effect upon the 15N,1H-HSQC spectra, which was also verified on corresponding TROSY spectra at 750 MHz. While an increase in the concentration of potassium chloride from 100 to 250 mM again yielded the 15N,1H correlation spectrum of free p53 DBD, indicating com-
plex dissociation, a decrease in the salt concentration to 40 mM did not lead to the desired stabilization of the ternary complex and concomitant spectral improvement either. Minor improvements of $^{15}$N,$^1$H-HSQC and $^{15}$N,$^1$H-TROSY spectra were, however, observed upon changing from 600 to 750 MHz, indicating a slight magnetic field-dependent shift toward the slow exchange region.

While the failure to perceptibly stabilize the complex by reducing the salt concentration was a first indicator that internal dynamics, rather than complex dissociation, were responsible for the observed strong line broadening, this hypothesis was subsequently verified by convection-compensated NMR diffusion measurements (54). These yielded a reduction in the diffusion coefficient of the dimeric p53 DBD-DNA complex by roughly one-third compared with free p53 DBD, i.e. from $7.3 \times 10^{-11}$ m$^2$/s to $5.0 \times 10^{-11}$ m$^2$/s (at 293 K; errors are in the range of 5–10%); the reference diffusion coefficients determined for H$_2$O congruently were $1.9 \times 10^{-9}$ m$^2$/s, some 5% below the tabulated value for pure water). The experimentally determined reduction in the diffusion coefficient ($-31.5\%$) upon DNA binding and dimerization of p53 DBD agrees excellently with the theoretical reduction of $-30\%$ derived by hydrodynamic calculations (see “Experimental Procedures”) (62), but in principle might still reflect an averaging of higher and lower oligomeric states. The CON2x5 oligonucleotide, however, makes higher-order oligomerization of p53 DBD most improbable due to steric hindrance and lack of further binding sites. Moreover, we could not establish that any saturation exchanged between DNA and p53 DBD in the complex got carried into the free state (see “Experimental Procedures”). The failure of this saturation transfer difference experiment (55) indicates that the interchange between bound and free species is rather low, i.e. that the complex does not readily dissociate, implying a $K_D$ smaller than some $\mu$M. As affinity and lifetime ranges of both experimental methods are comparable, a postulated averaging of measured diffusion coefficients should entail an observable saturation transfer between bound and free states. In summary, all of the experimental results strongly indicate that p53 DBD dimerizes upon CON2x5 DNA binding, the ensuing complex remaining stably associated with an estimated minimal lifetime of some $10^{-2} \cdot 10^{-1}$ s. In contrast, the complex displays substantial internal dynamics on the micro- to millisecond timescales, as indicated by massive line broadening in the NMR spectra. The nature of the underlying motions or conformational changes remains speculative.

To study the interaction of p53 DBD with its consensus DNA and the interface of two p53 DBDs upon DNA-mediated cooperative dimerization by chemical shift perturbation mapping, we acquired $^{15}$N,$^1$H correlation spectra of U-13C,$^{15}$N-labeled p53 DBD before and after addition of a 0.6 M equivalent of the CON2x5 oligonucleotide. Fig. 2 shows a superposition of these spectra, demonstrating that selective chemical shift changes are clearly discernible despite the weak intensities, low resolution, and broad line shapes of the complex. Fig. 3 shows the effects of titration on $^{15}$N and $^1$H chemical shifts plotted versus residue number. Chemical shift changes map into four distinct regions that coincide almost perfectly with the four conserved regions II (residues 117–142), III (residues 171–181), IV (residues 234–258), and V (residues 270–286) of the p53 DBD. These conserved residues form the DNA binding interface (L2 loop (residues 163–195), L3 loop (residues 236–251), and the loop-sheet-helix motif formed by L1 loop (residues 112–124), S2-S2’ hairpin (residues 124–141), S10 β strand (residues 271–274), and H2 helix (residues 278–286)) and a putative dimerization interface made up by the H1 helix (residues 177–182) in the L2 loop (10). The chemical shift changes upon addition of DNA were then mapped onto the crystal structure of p53 DBD (10) (Fig. 4A, see below). Further attempts to characterize the DNA binding and dimerization interface via direct or indirect methods failed owing to the low spectral quality of the ternary complex. It was thus impossible to observe by NOESY experiments neither p53 DBD-DNA contacts (using U-13C,$^{15}$N-labeled p53 DBD) nor intermolecular p53 DBD contacts across the dimerization interface (using a 1:1 mixture of unlabeled

**Fig. 2.** Superposition of $^{15}$N,$^1$H correlation spectra at 750 MHz of the free (blue) and DNA-bound (red) U-15N-labeled p53 DBD after addition of an 0.6 M equivalent of CON2x5 DNA consensus oligonucleotide. The peak assignment according to Wong et al. (23) is shown in green.
Residues His178, Arg181, and Cys182 are labeled ns, ms, and ws, shifts stronger than half line width; ss, shifts stronger than the line width; ms, shifts stronger than half line width; ws, shifts lower than half line width; ns, no shifts observable; na, residue not assigned. The conserved residues His178, Arg181, and Cys182 are labeled +, residues involved in intermolecular hydrogen bonding with DNA are marked by asterisks.

and U-13C,15N-labeled p53 DBD. It was likewise impossible to indirectly map the interface region with sufficient reliability by probing the solvent accessible protein surface via H2O exchange rates or paramagnetic relaxation quenching following the addition of 20 mM 4-hydroxy-TEMPO.

NMR-based Modeling and Description of the Dimerization Interface—1H,15N chemical shift perturbation mapping onto the crystal structure of p53 DBD (10) (Fig. 4A) allowed the construction of a NMR-based model of the p53 DBDs bound to their consensus DNA (see Fig. 4B). The chemical shift changes occurring upon addition of DNA, which either indicate intermolecular contacts or induced conformational changes, are restricted to one side of the protein. Interestingly, chemical shift changes are particularly pronounced for the H1 helix near the zinc coordination site. Yet, according to the crystal structure (10), this region does not participate in DNA binding. Nonetheless, as confirmed by our NMR investigations (see above), DNA binding occurs in a cooperative manner (11, 17–19, 38, 64), necessitating an intermolecular dimerization interface between both p53 DBD molecules. Only one model for the dimeric p53 DBD-DNA complex fulfills all the experimental results and steric requirements: a C2-symmetric arrangement for both p53 DBDs on the DNA, reflecting the C2-symmetry of the two pentameric DNA consensus sites (Fig. 4B). The strongest 1H,15N shift differences map onto the protein-DNA and putative dimerization interfaces. The latter includes the structurally important L2 loop (Arg174 to Gly187) with the short H1 helix (Pro177–Cys182) and the nearby zinc-coordinating residues Cys176 and His179. Without substantial conformational changes, this contact region would only comprise one side of the short H1 helix and thus be at the surface limit proposed for protein-protein interfaces (42, 65). The strong NMR shift changes in the H1 helix could, however, indicate its disintegration and conformational rearrangement, allowing an increase in the interface surface. It is noteworthy that the solvent-exposed residues His178, Arg181, and Cys182, which point outward in the free p53 DBD monomer, nevertheless display a high degree of conservation (43). Therefore, this conservation can best be rationalized by involvement of these residues in intermolecular contacts, such as the proposed p53 DBD dimerization.

**DISCUSSION**

*Probing the Allosteric Model of p53 Regulation*—The solution structure of the C-terminal peptide bound to the regulatory protein S100B(ββ) was recently solved (66). The allosteric model of p53 regulation proposes that the C-terminal regulatory domain of p53 interacts with the p53 DBD and keeps it in a latent state until the C terminus is subjected to phosphorylation or acetylation and the inhibitory interaction is released (31–33, 67). No direct experimental proof has been published for the allosteric model so far. In contrast, a just recently published NMR study showed on the basis of chemical shifts that designed latent and active p53 dimers are identical in conformation and that the C terminus does not interact with other p53 domains (68, 69). Initially, our NMR studies were intended to gain further insight into the mode of p53 regulation. These, however, failed to produce any indication of binding either between isolated C-terminal peptide and free or DNA-bound p53 DBD (60); between the N-terminal p53 peptide and p53 DBD (69); or between the C-terminal peptide and the polyproline-rich region of the N-terminal extended p53 DBD (36). We could, however, detect unspecific binding of the C-terminal peptide to DNA oligonucleotides (70) in complex with p53 DBD. In the light of our results, the presence of unspecific DNA contamination resulting from protein preparation might thus have interfered in experiments reporting on the interaction between p53 DBD and C-terminal peptide (60). Our results support the results of the recent NMR study (69) and are
Solution Dimerization Interface of p53 Core Bound to DNA

49025

contradictory to the allosteric model of p53 regulation (71). On the contrary, these data seem to favor alternative models of p53 regulation based on long nonspecific competitor DNA that do not assume an inhibitory interaction between the C terminus and the DBD (72–75) or propose an as yet unidentified inhibitory factor (76).

Solution Dimerization Interface of p53 DBDs Bound to Their Consensus DNA—Our NMR studies on the p53 DBD-DNA complex provide the first experimental definition of the dimerization interface of p53 DBD. The studies complement current knowledge of the p53-DNA interaction derived from the p53-DNA crystal structure (10) and corroborate published models of p53 organization upon DNA binding (39, 41, 42). NMR diffusion measurements show that the p53 DBD dimerizes upon addition of decameric CON2x5 DNA consensus oligonucleotide. The quality of the 15N,1H correlation spectra of the dimeric p53 DBD-DNA complex is strongly impaired by coalescence phenomena due to chemical or conformational exchange, leading to strong line broadening. The results of the diffusion measurements and the lack of any observable intermolecular saturation transfer between p53 DBD and DNA imply a complex stability in the submicromolar range which was also confirmed by FCS. Therefore, complex dissociation (i.e. chemical exchange) can be ruled out as the likely reason for the observed line broadening which thus has to be attributed to internal mobility of the system. A possible way to minimize the detrimental coalescence effects and improve the quality of the NMR spectra would be to try shifting the system into the slow exchange regime by, e.g. switching to even higher magnetic field strengths than employed (750 MHz). Attempts to achieve line narrowing by changes in the temperature or salt concentration did not produce the desired results either.

The acquired 15N,1H correlation spectra were nevertheless sufficient to perform a conclusive chemical shift perturbation mapping onto the crystal structure of p53 DBD (10). Based on this perturbation mapping a NMR-based model was created that fully explains the experimental results and is in accordance with a previously suggested model for the tetrameric p53-DNA complex in which the short H1 helix is involved in the formation of a hypothetical protein-protein interface (see Fig. 7B in Ref. 10). The model requires DNA bending to form the putative interface (39, 41, 42). No direct experimental proof had yet been presented for the participation of the o-helical region in the dimerization of the p53 DBDs upon cooperative DNA binding. None of the published crystal structures (10, 22) contain the proposed dimerization interface with C2-symmetry of the p53 DBDs, but show an interface that is incompatible with cooperative DNA binding and cannot reflect the arrangement of p53 DBD in the native p53-DNA complex (22).

Our NMR results point out the essential role of the short H1 helix (Pro177-Cys182) for intermolecular p53 DBD dimerization, which does not occur in the absence of DNA (19). Vice versa, dimerization is a prerequisite for cooperative DNA binding since free p53 DBD exists primarily as a monomer in solution (22) and does not readily bind to DNA with a single quarter-site (16, 42). The model is further supported by the fact that the dimerization interface displays mutation hot spots within the mentioned conserved cluster of codons 173–181, and particularly also for the three solvent-exposed residues Pro177, His178 and Arg181 which were predicted to participate in protein-protein interactions (43). Interestingly, the putative inhibitor 53BP2 binds to p53 (yellow) both at the DNA-binding surface (through its Src homology domain 3, SH3, gray) and at the dimerization interface including the H1 helix (through one of its ankyrin repeats, blue) (Fig. 5) (44). Finally, the model is in accordance with the generally C2-symmetric binding mode of DNA-binding homodimeric proteins (77). These studies on the dimeric p53 DBD-DNA complex provide the first experimental evidence for the mode of cooperative p53 DBD-DNA binding and allow the conclusion that the identified dimerization interface forms the actual interface of the tetrameric p53-DNA complex in solution (64) as well. It is an interesting question whether inhibition or deletion of the dimerization interface of the p53 DBD would prevent DNA binding by full-length p53 with a functional TD. We, furthermore, investigated the p63 DBD which is homologous to p53 DBD but does not display cooperative DNA binding (78). Both DBDs differ in the above mentioned H1 helix region and it was deduced that the conformation of this region might modify the dimerization behavior, thus accounting for the lack of cooperativity in DNA binding.

Sandwich-like Model for the p53-DNA Complex—Several studies have tried to gain insight into the organization of tetrameric full-length p53 bound to DNA (reviewed in Refs. 6, 79, and 80) from structures of the p53 DBD (10) and TD (13, 15, 81). Tetrameric p53 is the predominant form in solution (82). To date, the common model holds that the tetrameric p53 binds to its DNA consensus site with two adjacent DBD dimers of the tetramer binding on one side of the DNA to pairs of half-sites arranged in a regular staggered array having pseudo-dyad symmetry, connected by the TD orientated on the opposite direction of the DNA (10, 11, 16, 39). The DNA is thus enclosed by four p53 DBDs and the TD. This model, however, cannot bring the C2-symmetry of the DNA-bound p53 DBD dimer and the D2-symmetry of the p53 TD into agreement. As a consequence of the mandatory disruption of symmetry, the interfaces between the DBDs and the TD cannot be uniform and/or the four linkers connecting the DBDs and the TD have to be unstructured or of at least two different conformations, e.g. as observed in the crystal structure of the lac repressor core tetramer (83). This break in symmetry has already been noted in earlier studies, which have proposed that free p53 adopts an overall D2 symmetry imposed by the TD, and only switches to the asymmetric conformation upon DNA binding (12). It was also concluded that p53 tetramers with short hinge domain linkers should be unable to undergo the required conformational changes. They should instead form sandwich-like complexes with maintained dihedral symmetry, binding two DNA strands at opposite ends (12, 84). It should be noted that a recent thermodynamic analysis of DNA binding by p53 suggested that only minimal conformational rearrangements of full-length p53 occur upon complex formation (73). As shown by previous studies p53 does not bind to DNA solely as tetramer,
DNA sequence cooperatively with C2-symmetry (see above). (c) Most importantly, electron microscopy showed that a single p53 tetramer can bind to its target sequences (e.g., p21, cyclin G, and MCK) contain proximal and distal copies of the palindromic p53 consensus sequence within their regulatory regions (91–93). DNA looping by linking proximal and distal consensus sites was found to be synergistically involved in transcriptional activation by p53 (45, 90). Sandwich-like complexes associated with transcriptional activation and DNA looping have also been described for the lac repressor (94, 95). (f) Cooperative dimer–dimer interactions stabilize p53 DNA binding independent of the TD (16). Consequently, binding of two p53 tetramers to two juxtaposed consensus sites can be stabilized by stacking interactions between the two tetramers. Electron microscopic pictures show that p53 tetramers stack and bind to DNA in octameric and higher oligomeric states (45, 90), for instance, Fig. 7 shows how p53 tetramers can line up along several consensus sites like a pearl necklace (Fig. 7, B and D) (45).

Certainly, the sandwich-like model cannot explain how p53 binds to a single p53 consensus sequence as a tetramer (11, 16, 45). Actually, the inherent flexibility of p53 DNA binding as evidenced by the diversity of p53-DNA complexes observed by electron microscopy (45) might allow a conformational switch upon DNA binding (12) and result in a quaternary structure having no D$_2$-symmetry (83). Nevertheless the sandwich-like model may represent a p53 DNA binding mode involved in the transcriptional activation of the corresponding target genes. From a structural point of view, the sandwich-model takes advantage of the fact that it does not have to assume different conformations for the p53 subunits and major conformational rearrangements. The sandwich-like model might therefore be dissected into subunits which can be treated separately.

Solution Dimerization Interface of p53 Core Bound to DNA

FIG. 6. A, sandwich-like model for the tetrameric p53-DNA complex. 1, top view. The p53 DBDs and the TD are shown as ribbons. The 4-helix bundle of the tetrameric TD is situated in the center of the complex. To illustrate that the TADs can be integrated into the complex fulfilling the steric and geometric requirements, an undetermined fold was applied to generate a surface representation of the p53 TAD. B, overview of the complex from three different directions. C, schematic representation of the p53-DNA complex illustrating the D$_2$-symmetry of the complex that is made up by three orthogonal C$_2$ axes. The shape of the complex resembles a flat cylinder, so that upon DNA binding several p53 tetramers can line up and assemble cooperatively to higher oligomers that are stabilized by stacking interactions.

but likewise, although with lower affinity and cooperativity, as a dimer (12, 16, 85, 86). Artificial dimers of p53 bind to p53 consensus sequences (16, 87) and transactivate p53 target genes in reporter-gene assays (12, 88, 89). One dimer of the p53 tetramer might thus be able to interact with one half-site of the consensus site whereas the other might remain free to interact with a second consensus site as discussed (45).

To resolve the steric problems resulting from the attempt to combine the different symmetries of the four DBDs and the TD in a complex with a single DNA consensus site we propose that wild-type p53 tetramers might be capable of linking two separated juxtaposed DNA consensus sites via a DNA loop according to the sandwich-like model (see Fig. 6). The sandwich-like model integrates the D$_2$-symmetry of the p53 TD and implies that all p53 subunits have identical conformations. It covers 100 residues without defined tertiary structure (96) and be integrated between the DBD and TD. The N-terminal TD covers 100 residues without defined tertiary structure (96) and folding is probably not induced until complex formation with additional cofactors (97). (f) Cooperative dimer–dimer interactions stabilize p53 DNA binding independent of the TD (16).

Consequently, binding of two p53 tetramers to two juxtaposed consensus sites can be stabilized by stacking interactions between the two tetramers. Electron microscopic pictures show that p53 tetramers stack and bind to DNA in octameric and higher oligomeric states (45, 90), for instance, Fig. 7 shows how p53 tetramers can line up along several consensus sites like a pearl necklace (Fig. 7, B and D) (45).

Certainly, the sandwich-like model cannot explain how p53 binds to a single p53 consensus sequence as a tetramer (11, 16, 45). Actually, the inherent flexibility of p53 DNA binding as evidenced by the diversity of p53-DNA complexes observed by electron microscopy (45) might allow a conformational switch upon DNA binding (12) and result in a quaternary structure having no D$_2$-symmetry (83). Nevertheless the sandwich-like model may represent a p53 DNA binding mode involved in the transcriptional activation of the corresponding target genes. From a structural point of view, the sandwich-model takes advantage of the fact that it does not have to assume different conformations for the p53 subunits and major conformational rearrangements. The sandwich-like model might therefore be dissected into subunits which can be treated separately.

**Acknowledgments**—We are especially grateful to F. Hesse for initial help with protein preparation, for access to analytical instruments, and discussion. We thank H. Rabenseifner and D. Sonnenstuhl for technical assistance, A. Gärtnert and M.-L. Hagmann for performing mass spectrometry, and C. Seidel for peptide synthesis. We are indebted to the members of the p53 team for discussion and D. Ambrosius, J. P. Holck, B. Kalaza, B. Kresse, A. Mertens, and A. Stern (all Roche Diagnostics GmbH) for support.
NMR Spectroscopy Reveals the Solution Dimerization Interface of p53 Core Domains Bound to Their Consensus DNA
Christian Klein, Eckart Planker, Tammo Diercks, Horst Kessler, Klaus-Peter Künkele, Kurt Lang, Silke Hansen and Manfred Schwaiger

J. Biol. Chem. 2001, 276:49020-49027.
doi: 10.1074/jbc.M107516200 originally published online October 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107516200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 96 references, 42 of which can be accessed free at http://www.jbc.org/content/276/52/49020.full.html#ref-list-1