High Thermostability and Lack of Cooperative DNA Binding
Distinguish the p63 Core Domain from the Homologous Tumor Suppressor p53*

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The p53 protein is the major tumor suppressor in mammals. The discovery of the p53 homologs p63 and p73 defined a family of p53 members with distinct roles in tumor suppression, differentiation, and development. Here, we describe the biochemical characterization of the core DNA-binding domain of a human isoform of p63, p63-δ, and particularly novel features in comparison with p53. In contrast to p53, the free p63 core domain did not show specific binding to p53 DNA consensus sites. However, glutathione S-transferase-fused and thus dimerized p83 and p53 core domains had similar affinity and specificity for the p53 consensus sites p21, gadd45, cyclin G, and bax. Furthermore, the fold of p63 core was remarkably stable compared with p53 as judged by differential scanning calorimetry (Tm = 61 °C versus 44 °C for p53) and equilibrium unfolding (l(urea)100% = 5.2 M versus 3.1 M for p53). A homology model of p63 core highlights differences at a segment near the H1 helix hypothetically involved in the formation of the dimerization interface in p53, which might reduce cooperativity of p63 core DNA binding compared with p53. The model also shows differences in the electrostatic and hydrophobic potentials of the domains relevant to folding stability.

The tumor suppressor gene p53 is the most frequent site of genetic alterations found in human tumors (1). The p53 protein functions primarily as a transcription factor regulating the expression of genes involved in cell cycle arrest, cellular senescence, anti-angiogenesis, and apoptosis (reviewed in Refs. 2 and 3).

Recently, two homologous genes, p63 (also called KET, p51, p40, AIS, and p73L) (4–9) and p73 (10, 11), were identified (reviewed in Refs. 12–16). Several studies showed that some isoforms of p63 and p73 can bind to p53-responsive elements, transactivate p53-responsive genes, and induce apoptosis upon overexpression (7, 10, 11, 17). This structural and functional homology defined a new superfamily of transcription factors, in which p63 and p73 seem to be ancestral genes of the phylogenetically younger p53. The genes targeted by the family members differ (17, 18), and p63 and p73 do not appear to be responsive to the same type of genotoxic damage as p53 (11). Instead, the two p53 homologs play a more fundamental role associated with development and differentiation, as shown in cellular systems (4, 7, 19, 20) and with the respective knockout mice (20–24).

Unlike p53, p63 is essential for embryonic development; mice lacking the p63 gene exhibit severe defects in ectodermal differentiation (20). Based on this phenotype, a role for p63 in stem cell regeneration to sustain epithelial development was suggested (22). Mutations in the p63 DNA-binding domain are the cause of the autosomal dominant EEC1 syndrome and the EEC-like limb mammary syndrome (23).

All members of the p53 family possess a modular architecture with an N-terminal transactivation domain, an ~60% homologous core DNA-binding domain (DBD) that is followed by a tetramerization domain, and a regulatory C-terminus (25, 26). Several isotypes of p63 and p73 have a conserved C-terminal extension of ~100 residues that is not present in human p53 and that might be a protein-protein interaction module with a regulatory function. The structure of this region in p73 has been determined recently (27, 28) and revealed structural homology to the sterile α-motif domain. Alternative splicing of the C-terminal region of p63 and p73 leads to the expression of a variety of splice variants (29). In addition, N-terminally deleted isoforms of p63 and p73 were identified that are not capable of transcriptional transactivation and that have an anti-apoptotic role as antagonists of their full-length counterparts (7, 30).

The p53 DBD contains several hot spot regions for mutation. In addition to the loss of DNA-binding function, a gain of function for mutant p53 DBDs is described (31, 32). The crystal structure of the p53 DBD in complex with DNA (33) shows that almost all mutations described target residues that either directly contact DNA or stabilize the tertiary structure. Recently, the crystal structure of the free mouse p53 DBD was solved (34). Wild-type and mutant p53 DBDs have been studied biophysically in detail (35–40). Several studies tried to rescue the mutant or to stabilize the wild-type p53 DBD conformation based on structural information (41) or by generating second-site suppressor mutations (42, 43). These attempts and others using semirational design (44) or directed evolution (45, 46) yielded protein of increased thermodynamic stability. Very recently, pharmacologically active low molecular mass com-

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1 The abbreviations used are: EEC, ectodermal dysplasia, ectrodactyly and cleft palate; DBD, DNA-binding domain; GST, glutathione S-transferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; pBS, pBluescript II SK+; FCS, fluorescence correlation spectroscopy; DSC, differential scanning calorimetry.
pounds were reported to stabilize the wild-type conformation of human p53 (47).

So far, little is known about the biochemical properties of the homologous DBDs, which share ~60% identity with the p53 DBD and are highly conserved in residues involved in Zn2+ coordination and stabilization of the DNA-binding conformation (4, 7, 33, 48) (see Fig. 1). In contrast to p53, mutational inactivation of p73 and p63 in human tumors is rare and does not seem to be important for carcinogenesis (49). In this study, the p63 DBD was biochemically characterized in comparison with the p53 DBD. In contrast to p53, the free p63 DBD is not capable of binding to specific p53 DNA consensus sites; however, in a dimerized state, GST-p63 DBD as well as GST-p53 DBD show comparable affinity and specificity for p53 consensus sites, supporting the notion that the DBDs might differ with respect to dimerization and consequently cooperative DNA binding, but not with respect to DNA specificity. Differential scanning calorimetry and urea-induced equilibrium unfolding showed that the p63 DBD is markedly stabilized in relation to p53. To understand these results on a molecular basis, a homology model of the p63 DBD was created. Some properties of the p63 DBD may rationalize the lack of cooperativity and enhanced thermostability.

**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. Cloning, Expression, and Purification—The 705-base pair pair cDNA encoding the DNA-binding domain (amino acids 114–349) of the human p63 protein was amplified from a human placenta cDNA library (CLONTECH) by standard polymerase chain reaction with gene-specific primers 5′-GCA GCC CCA TAT GGC ATC CAC CTG CGA TGG CCA TGG CAG and 5′-GCG GCT CCA GTC GTC ATG TCT CAT GCA TAT GGG ATC CTC CAC CTT CGA-3′. The C-terminal part of the amplification product was ligated into a pGEX-4T-1 vector (Amersham Pharmacia Biotech). The resulting recombinant expression vector p404/p63 DBD) codes for the human p63 DBD without tags, including an additional N-terminal glycine; the corresponding vector pGEX-4T(GST-p63 DBD) codes for a GST-p63 DBD fusion protein including a Gln-Arg-Gly linker that remains on the N terminus after digestion of the fusion protein with thrombin to produce a GST-p63 DBD fusion protein. All vector constructions were verified by sequencing. Proteins were expressed in Escherichia coli strains HB101 and uet600, which were grown at 37 °C in LB medium supplemented with ampicillin (100 μg/ml) and/or kanamycin (50 μg/ml) to an absorbance of 0.5–0.8 before overnight induction at 37 °C with 1 mM (for p53 DBD) or 0.1 mM (for GST-p63 DBD) isopropyl-β-D-thiogalactopyranoside. After induction, cells were harvested by centrifugation, resuspended in 50 mM Tris (pH 8.0), 5 mM DTT, 1 mM benzamidine, and Complete protease inhibitor mixture (EDTA-free; Roche Molecular Biochemicals); and disrupted by high-pressure dispersion using an APV-Gaulin Lab 40 Homogenisator. For purification of the p63 DBD, soluble lysate was loaded onto an SP-Sepharose Fast Flow cation-exchange column (Amersham Pharmacia Biotech) and eluted with a linear KCl gradient (0–0.5 M). Final purification was achieved by preparative size-exclusion chromatography on a Superdex 75 HiLoad 26/60 column (Amersham Pharmacia Biotech) in 50 mM Tris (pH 7.0), 150 mM KCl, 5 mM DTT, and 5% glycerol. Final purification was achieved by size-exclusion chromatography on a Superdex 200 HiLoad 26/60 column (Amersham Pharmacia Biotech) in 50 mM Tris (pH 7.0), 150 mM KCl, 5 mM DTT, and 5% glycerol.

Cloning, expression, and purification of the p53 DBD and GST-p53 DBD for comparison studies were performed with minor modifications of the procedure given for the p63 constructs and published elsewhere (e.g., using an additional heparin HiTrap column (Amersham Pharmacia Biotech) for the purification of the p53 DBD) (39). All proteins were concentrated using 5K Ultrafree 4 centrifugal filter devices (Millipore Corp.), flash-frozen in liquid nitrogen, and stored at −80 °C.

**Analytical Methods**—Electrospray mass spectrometry confirmed the identity of all proteins used in this study. The N-terminal methionine was cleaved off after translation. Protein concentration was measured spectrophotometrically according to the method of Bradford (51) or using extinction coefficients of ε280 nm = 14,650 M−1 cm−1 for the p63 DBD and ε280 nm = 15,930 M−1 cm−1 for the p53 DBD, calculated according to the method of Edelhoch (52). SDS-polyacrylamide gel electrophoresis was performed with 12% gels. Size-exclusion chromatography was performed to determine the oligomeric state of proteins using a TSK Gel G 3000SW analytical gel filtration column (TosoHaas) on an analytical Gynkotek high-pressure liquid chromatography instrument (Dionex Corp.) equipped with Chromleon software (Dionex Corp.) at a flow rate of 0.5 mL/min in 50 mM sodium phosphate (pH 7.0), 150 mM KC1, 5 mM DTT. Dynamic light scattering to measure hydrodynamic parameters was performed with a DynaPro molecular sizing instrument (Protein Solutions Inc.) equipped with a temperature controller. All samples (protein concentration of 1–2 mg/ml) were diluted in 40 mM sodium phosphate (pH 7.0) and 5 mM DTT and filtered through a 0.02-μm membrane before analysis at 20 °C.

Electrophoretic Mobility Shift Assay (EMSA)—The DNA-binding activity of the protein constructs was analyzed as described (53). Briefly, specific complementary oligonucleotides containing the 20-mer p53 consensus DNA-binding site PG (golypgrig) (54) were end-labeled and annealed. The DNA binding buffer contained 40 mM HEPES (pH 8.0), 50 mM KC1, 20% (v/v) glycerol, 5 mM DTT, 0.1% Triton X-100, 10 mM MgCl2, and 1.0 mg/ml bovine serum albumin. In general, 50 ng of purified proteins in 10 μl of DNA binding buffer containing 10 ng of double-stranded end-labeled specific p53 consensus site oligonucleotide and 5 μM supercoiled unlabeled nonspecific pBluescript II SK′ (pBS) competitor DNA (Stratagene) and incubated for an additional 15 min on ice. The full preincubation details are given in the figure legends. The reaction mixture was loaded onto a 4% native polyacrylamide gel and separated at 200 V for 2 h at 4 °C. The running buffer consisted of 30 mM Tris-HCl (pH 7.5), 30 mM boric acid, and 1 mM EDTA with 0.01% Triton X-100. The gel was dried, and the labeled DNA was detected by autoradiography.

**Fluorescence Correlation Spectroscopy (FCS)**—FCS allows the determination of free diffusion coefficients in solution. If the difference in diffusion coefficients of the free and bound molecules is sufficiently large, the free and bound molecules can be resolved into two distinct populations. Quantitative analysis of the DNA-binding properties of the protein constructs was performed with a ConfoCor fluorescence correlation spectrometer (Carl Zeiss Jena and Evotec OAI). For affinity determinations, 5′-TAMRA-labeled DNA oligonucleotides (PG, 5′-AGC TTA GCC AGC ATG TCT AGC CAT GTG TCA-3′; 5′-GAC AGC CCT TGCC GAC AGA CCT CGG-3′; cyclic G, 5′-TAC GCA CAA CCG CAG CAT ATG TCA TGA GC-3′; and gadd45, 5′-TAC GCA CAA CCG CAG CAT ATG TCA TGA GC-3′) were end-labeled and complexed with complementary unlabeled oligonucleotides. The concentration of the annealed double-stranded DNA oligonucleotides was determined using FCS and adjusted to 1 nM for all measurements. The concentrations of DBD constructs for determinations of apparent binding constants were in the range of 0.1–1000 nM. To suppress nonspecific DNA binding, measurements were performed in the presence of 10 nM psbs plasmid competitor DNA. All measurements were taken at 20 °C in 40 mM HEPES (pH 7.4), 5 mM DTT, and 0.1% Triton X-100 with typical sample volumes of 40 μl in a 384-well chamber and sampling times of 15 s. Experimental autocorrelation curves were fitted using the FCS-Plus 1.0 software package (Evotec OAI), yielding the diffusion times of free and bound fluorescent DNA oligonucleotides from the time-dependent fluctuations of the fluorescent signal recorded in the confocal volume element. For the determination of apparent binding constants, the values were fitted using the program Prism 3.0 to the following equation: \[ f(\frac{[DBD]}{[DNA]} + K_d) \] valid under conditions when \[ [DBD] \ll [DNA] \ll K_d \].
Comparison of the p53 and p63 DNA-binding Domains

Fig. 1. Primary structure alignments of the p63 DBDs and the homologous domains of p53 and p73. The sequence of the human p53 DBD is aligned with the DBDs of human p63-δ identified in this study, human p63-αβγ, murine p63-αβ, murine p63-γ, and human p73. Residues identical in all DBDs are colored yellow; residues identical in two DBDs are colored cyan. Secondary structure is assigned according to ISTR, chain B (33), as follows: H, helix; B, residue in isolated β-bridge; E, extended β-strand; G, 310 helix; T, hydrogen-bonded turn; S, bend (92). The H1 helix is rendered green. Residues not resolved in the crystal structure of the p53 DBD are labeled (*). The six hot spot point mutations in p53 are highlighted in red. Residues coordinating Zn²⁺ are marked (&), and residues making DNA contacts are labeled (#). The multiple sequence alignment was prepared with ClustalW 1.7 (93).
The p63 DBD was purified using a combination of cation-exchange and size-exclusion chromatography. This purification scheme yielded ~30 mg of >98% homogeneous and monomeric p63 DBD/liter of culture as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2A, lanes 1–5), analytical size-exclusion chromatography (Fig. 2B), and mass spectrometry. Analysis of the hydrodynamic parameters by dynamic light scattering showed that the p63 DBD exhibits a hydrodynamic radius of 2.9 nm versus 2.74 nm for the p53 DBD; however, both DBDs exhibit a monomodal distribution with low polydispersity indices (data not shown). Purification of GST-p63 DBD fusion protein was performed using a combination of affinity chromatography and a final size-exclusion step. Yields were ~20 mg of >95% homogeneous protein (Fig. 2A, lane 9) in a dimeric state (Fig. 2B) per liter of culture. An overview of the purified proteins used throughout this study is given in Fig. 2 (A, lanes 6–9; and B).

DNA-binding Activity and Specificity of the p63 DBD

DNA-binding Activity of p63—Several studies showed that tetrameric p63 isotypes interact with p53 consensus sequences and that p63 can act as a transcription factor that activates promoters of several p53-responsive genes in cotransfection experiments (including p21, bax, and the artificial consensus site PG) (7, 61). Therefore, the DNA-binding activity of the isolated p63 DBD for the p53-responsive consensus site PG (53, 54) was analyzed in comparison with the p53 DBD. The p53 DBD bound to a single consensus quarter-site only with very low affinity so that DNA binding could not be detected by EMSA (data not shown). Upon addition of the PG p53 consensus site, the p53 DBD tetramerized and cooperatively bound with high affinity (Fig. 3A, lane 1) (35, 36, 40, 62–64). In contrast, the free p63 DBD was not able to bind to the PG p53 consensus site under identical conditions (Fig. 3A, lane 2). Co-incubation of the p63 DBD with the p53 DBD did not interfere with DNA binding of the p53 DBD (data not shown). Consequently, the ability of GST-p63 DBD to bind to the PG p53 consensus site was analyzed since the GST part of the fusion protein acts as an artificial dimerization domain (Fig. 3B) and thereby should enhance the affinity for DNA. Dimerized GST-p63 DBD fusion protein was capable of binding to the PG p53 consensus sequence with an affinity similar to that of GST-p53 DBD (Fig. 3A, lanes 3 and 4), indicating that the affinity and specificity of the p63 and p53 DBDs are similar. To confirm that the p63 DBD is not differently folded in the context of the GST fusion protein, the fusion proteins were digested with thrombin, and the free DBDs were released. As expected, thrombin-cleaved GST-p53 DBD retained the DNA-binding activity of the released p53 DBD, whereas thrombin-cleaved GST-p63 DBD completely lost DNA-binding activity (Fig. 3A, lanes 5–8). The lack of measurable DNA-binding activity of the p63 DBD might therefore be explained by its failure to bind to DNA cooperatively. Fig. 9 depicts a schematic model for the interpretation of the experimental results (see “Discussion” for details).

Zn$^{2+}$ Coordination Is Required for p63 DNA Binding—The
presence of Zn$^{2+}$ is required for the DNA-binding activity of the p53 protein (36). The crystal structure of the p53 DBD-DNA complex shows that the structure of the p53 DBD is stabilized by Zn$^{2+}$ that is tetrahedrally coordinated by three cysteines (residues 176, 238, and 242) and one histidine (residue 179) (33). Treatment of wild-type p53 with metal chelators causes the removal of Zn$^{2+}$ and oxidation of essential cysteine residues. This results in the disruption of the tertiary structure, with loss of DNA-binding activity, exposure of amino acids cryptic in the fully folded protein, and adoption of the monoclonal antibody PAb 240-positive phenotype identical to that of mutant forms of p53 (65, 66). Primary structure data suggest that this metal-dependent structure is conserved in the p53 homolog p63 (4, 7, 33, 48), as all residues involved in Zn$^{2+}$ coordination are conserved (Fig. 1). Consequently, the effect of metal-chelating agents on DNA binding was examined. Fig. 3 shows that incubation with the metal-chelating agent 1,10-phenanthroline reduced the DNA-binding activities of GST-p53 DBD and GST-p63 DBD (lanes 1–10). It was surprising that the Zn$^{2+}$ seemed to be bound with higher affinity by GST-p53 DBD, as concentrations of 1,10-phenanthroline $>20$ mM were needed to abolish DNA binding, whereas GST-p63 DBD lost DNA-binding activity at lower concentrations. As the effect of Zn$^{2+}$ chelation and loss of DNA-binding activity varied with incubation time and temperature, the effect of 1,10-phenanthroline on the DNA-binding activity was not quantitatively evaluated.

Quantitative Analysis of the DNA-binding Activity and Specificity of p63—FCS was applied to measure the apparent binding constants of fluorescently labeled DNA consensus oligonucleotides for p63 and p53 DBDs. In the first experiments, the effect of supercoiled unlabeled nonspecific pBS competitor DNA on the DNA-binding activities of p63 and p53 DBDs for a specific TAMRA-labeled PG oligonucleotide was determined (Fig. 4, A and B). Whereas binding of the p53 DBD and GST-p53 DBD (Fig. 4A) as well as GST-p63 DBD (Fig. 4B) to PG was specific and almost unaffected over a wide concentration range, DNA binding of the p63 DBD was nonspecific and could be competed at 5 nM nonspecific pBS DNA (Fig. 4B). This concentration of nonspecific pBS DNA was comparable to that used in the EMSA (Fig. 3A), which accounts for the lack of DNA binding of the p63 DBD. In the following experiments, the specific binding affinity of the GST-DBDs for 1 nM TAMRA-labeled p53 consensus oligonucleotides was therefore determined in the presence of 10 nM pBS. For comparative reasons, the question of DNA specificity was addressed using different p53 consensus oligonucleotides. An exemplary binding curve for binding of GST-p53 DBD and GST-p63 DBD to the TAMRA-labeled p21 oligonucleotide is shown in Fig. 4C. The apparent $K_d$ values for binding to the different p53 consensus oligonucleotides are given in Table I. Dimerized GST-p63 DBD and GST-p53 DBD showed affinities in a comparable range and an almost identical specificity pattern. With the exception of the bax consensus oligonucleotide, GST-p63 DBD displayed an affinity for the p53 consensus oligonucleotides $\sim$3–5-fold higher compared with GST-p53 DBD, so the relative affinities with regard to the selected consensus sites are comparable. Both GST-DBDs showed the highest affinity for the artificial PG site, followed by (lanes 7 and 8) were analyzed for their DNA-binding activity before (−) and after (+) cleavage with 1 μg of thrombin. BG probably indicates background binding of a small portion of monomeric GST-p53 that is in equilibrium with dimeric GST-p53 and not detectable by size-exclusion chromatography; FP indicates free probe. B, influence of 1,10-phenanthroline on the DNA-binding activity. 50 ng of GST-p53 DBD (lanes 1–5) and GST-p63 DBD (lanes 6–10) were incubated for 10 min with the indicated concentrations of 1,10-phenanthroline at 4 °C and subsequently subjected to an EMSA at 4 °C.
and labeled as follows: for 1 nM specific TAMRA-labeled GST-p63 DBD and p63 DBD (see below). The far-UV spectrum of the p63 DBD indicates a high content of β-sheets as secondary structure, which was lost upon denaturation with 6 M urea. The p53 DBD shows a less typical spectrum. The results of secondary structure estimation with the program CDNN (55) (α-helical content: 8.1% for p53 and 9.3% for p63; β-sheet content: 46.4% for p53 and 45.7% for p63) are comparable for both DBDs. Within the limits of secondary structure prediction from CD spectra, these values indicate a similar overall fold of the DBDs and are in accordance with the secondary structure content as deduced by the p53 DBD crystal (33) and the p63 model structure (see below). As the three-dimensional structure and dynamics of the p53 DBD are unknown, the differences in the far-UV CD spectra of the p53 and p63 DBDs cannot be unambiguously explained. Differences in the unique amino acid sequence and composition of the two domains might contribute to the observed differences. However, as the α-helical content of the p53 and p63 DBDs is small, even slight differences in the ratio and stability of β-sheet and coil segments as well as slight changes in the helical composition of the domains can well account for the observed differences. On the other hand, the increased signal intensity of the p63 DBD spectrum may reflect the significantly higher thermostability of the p63 DBD in comparison with p53 (see Fig. 7). An increased conformational stability of p63 DBD secondary structure elements over time may be the major reason for the observed higher signal intensities of the far-UV CD spectra. On the other hand, the higher signal intensity observed may also be indicative of the existence of slight differences in the structures of the two DBDs regardless of the assumed similar overall fold of the two DBDs (see Fig. 8). The near-UV CD spectra of the DBDs are shown in Fig. 5B. They are indicative of stable tertiary structures of both DBDs. In distinction to p53, the p63 DBD showed a band in the positive range.

**Fluorescence Spectroscopy**—The characteristic fluorescence spectra of the native and denatured p63 DBD in comparison with the spectra of the p53 DBD are shown in Fig. 5C. Upon excitation at 280 nm, the p53 DBD spectrum is dominated by the contribution of several tryptophan residues at 304 nm, whereas the single tryptophan residue is quenched and shows low fluorescence in the native state; upon urea denaturation, tryptophan fluorescence at 354 nm is strongly enhanced (37). On the other hand, the p63 DBD displays a fluorescence spectrum that is dominated by the single nonconserved tryptophan residue present in the p63 DBD. Upon denaturation, the maximum of emission is red-shifted from 342 to 354 nm.

**Enhanced Thermodynamic Stability of the p63 DBD**

**Thermostability of the p63 DNA-binding Activity**—The effect of temperature on the DNA-binding activity of GST-p63 DBD and GST-p53 DBD was examined after 5 min of incubation at 30, 34, 37, 40, 45, 50, 55, 60, and 70 °C. Proteins were preincubated at the indicated temperatures and subsequently kept on ice until analysis of the DNA-binding activity by EMSA. Fig. 6C shows that GST-p53 DBD activity was reduced following preincubation at 37 °C and abolished after preincubation at 40 °C. In contrast, GST-p63 DBD lost DNA-binding activity after preincubation at 52 °C for 5 min.

**p63 DBD Is Stabilized against Thermal Denaturation**—DSC experiments were performed with the p63 and p53 DBDs. As

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**TABLE I**

Apparent binding constants for the binding of GST-p63 DBD and GST-p3 DBD to TAMRA-labeled p53 DNA consensus oligonucleotides

| p53 DNA consensus oligonucleotide | $K_d$ GST-p63 DBD | $K_d$ GST-p3 DBD |
|----------------------------------|------------------|------------------|
| PG                              | 4.4 ± 0.5        | 0.7 ± 0.04       |
| p21                             | 10.6 ± 1.6       | 1.9 ± 0.3        |
| gadd45                          | 15.2 ± 2.3       | 3.6 ± 0.5        |
| cyclin G                        | 72.2 ± 11.4      | 13.8 ± 2.0       |
| bax                             | 99.7 ± 15.0      | 68.7 ± 11.9      |
Comparison of the p53 and p63 DNA-binding Domains

for the p53 DBD (37), thermal denaturation of the p63 DBD
was irreversible and occurred during a single DSC run. However,
the melting point $T_m$ can be used as a semiquantitative
indicator of thermostability. For both proteins, thermal unfold-
ing could be measured, and the melting points were highly
reproducible. The p63 DBD showed significantly enhanced
thermostability with an apparent $T_m$ of 61.0 ± 0.1 °C versus
44.0 ± 0.1 °C for the p53 DBD (Fig. 7). Thus, the DNA-binding
activities of GST-p63 DBD and GST-p53 DBD are lost before
the DBDs show obvious signs of thermal denaturation. Due to
the irreversibility of the denaturation, no quantitative deter-
mination of $\Delta H_{\text{cal}}$ and $\Delta G(T)$ was performed. It should be noted
that the isolated GST fusion protein unfolded at an apparent
$T_m$ of 57.3 °C and completely lost enzymatic activity after in-
cubation for 5 min at 58 °C (data not shown), whereas GST-p63
DBD lost DNA-binding activity after incubation at 52 °C.

**p63 DBD Is Stabilized against Urea-induced Equilibrium Unfolding**—The observation of enhanced thermodynamic sta-
bility of the p63 DBD was confirmed using urea-induced equi-
librium unfolding. The unfolding of the p63 DBD was measured
at 15 °C based on the decrease in the CD signal at 222 nm upon
unfolding (Fig. 5A). Unfolding transitions are found to be re-
versible under these conditions, so the data can be fitted ac-
cording to a two-state transition model (57). As the fluorescence
spectra of the p53 DBD are better suited for a quantitative
analysis than the CD spectra, equilibrium unfolding of the p53
DBD was measured for comparative reasons based on the in-
crease in the normalized fluorescence signal at 356 nm (Fig.
5C) upon unfolding. Unfolding transitions were performed un-
der the stated conditions at 15 °C and were found to be unaf-
efected by the protein concentrations used. Despite the inferior
quality of CD unfolding data, however, unfolding transitions
yielded comparable results to those monitored by fluorescence
spectroscopy. Table II summarizes the thermodynamic param-
eters of the equilibrium unfolding of the p63 and p53 DBDs.

The p63 DBD is characterized by a $[\text{urea}]_{50\%}$ of 5.15 ± 0.01 m,
whereas the less stable p53 DBD has a $[\text{urea}]_{50\%}$ of 3.06 ± 0.02 m.
In relation to p53, the p63 DBD is therefore markedly
stabilized by a calculated $\Delta G([\text{urea}]_{50\%})$ of $-4.57 ± 0.21$ kcal
mol$^{-1}$. The $[\text{urea}]_{50\%}$ and the slope of the transition from native
to denatured state ($m$ value) for the equilibrium unfolding of
the p53 DBD at 15 °C are in the range of the values determined
for the p53 DBD under comparable, but not identical, condi-
tions ($[\text{urea}]_{50\%} = 3.33–2.66$ m at 10–25 °C; $m$ value of 2.40–
3.25 kcal mol$^{-1}$ m$^{-1}$ at 10 °C) (37, 39, 43, 44).

**Modeling of the p63 DBD**

Table III gives a statistical comparison of the p63 and p53
DBDs. Most parameters are comparable; however, the p63
DBD displays a higher hydrophobicity (GRAVY) (67) and a
higher aliphatic index (68) as well as a lower instability index
(69), which can be indicative of elevated thermostability. To
investigate the molecular mechanisms of thermostabilization
of the p63 DBD and to see whether the p63 and p53 DBDs
might differ in their dimerization and thermostability proper-
ties, a model of the p63 DBD was created based on the crystal
structure of the p53 DBD bound to its DNA consensus sequence
(33). Due to the high level of identity and homology between
p63 and its parent molecule, p53 (Fig. 1), the sequence align-
ment is unambiguous, and a similar overall fold for the homol-
ogous DBDs can be assumed. Among the important residues,
*e.g.* residues chelating Zn$^{2+}$, binding to DNA, and frequently
being reported as mutated in p53, all are identical with the
exception of an arginine residue in contact with DNA that is
replaced by a lysine (Fig. 8A). Concerning the residues near
these residues, four non-homologous substitutions are found.
between p63 and p53. First, Gln\textsuperscript{165} (p53) becomes Lys\textsuperscript{194} (p63). In the model, this lysine is exposed to the solvent with a charge
further compensated by a glutamic acid located two amino
residues (LSREFNEGQ) that is located just after the H1 helix
(p63). This arginine residue is part of a region of 10 amino acid
residues is not strictly conserved and shows an insertion of two
amino acids.

A search into the Protein Data Bank for this short sequence
has not revealed a particular homology for this segment. How-
ever, using a data base of C-a three-dimensional coordinate
matrices extracted from the Protein Data Bank, three back-
bone loops are suggested for the nonconserved region. The first
shows an extended hairpin motif on the surface of the protein.
The second one extends the helix containing the above-men-
tioned histidine and adds a subsequent shortened loop. The
third loop proposed has no particular secondary structure. Of
the three models, the one containing the hairpin motif is prob-
ably the most stable (Fig. 8A). Moreover, this strand following
the H1 helix is the one that differs in conformation between
the two known structures of p53 and was suggested to form the
protein-protein interface in the tetrameric p53-DNA complex
(33, 58). Fig. 8B shows the lipophilic surface potentials of the
p53 DBD structure and the p63 model structure in comparison.
In general, a structure-based comparison between the p63 DBD
and p53 parent structure reveals no major differences with
regard to surface area, molecular volume, or packing density.
However, the p63 DBD is apparently less polar than the parent
p53 DBD, particularly on the surface opposite to the DNA-
binding region (lower panels).

### DISCUSSION

Both, the p53 tumor suppressor protein and its homolog p63
contain a tetramerization domain and form homotetramers in
solution. Tetrameric p53 binds specifically to a DNA consensus
sequence consisting of two consecutive 10-base pair half-sites,
which each half-site is formed by two head-to-head quarter-
sites (33, 54, 63, 70, 71). The isolated tetramerization domain
forms a symmetric dimer of dimers (72–74). Several studies
showed that four p53 DBDs bind cooperatively to a DNA con-
sensus sequence (35, 36, 39, 40, 62, 63). The crystal structure
of the p53 DBD-DNA complex is compatible with a model whereby
four p53 DBDs bind to the DNA consensus sequence (33). We
compared the DNA-binding properties of a highly purified,
monomeric p63 DBD with those of the p53 DBD. In contrast to
the p53 DBD, the isolated p63 DBD cannot bind to a specific
DNA consensus oligonucleotide in EMSA. There are two gen-
eral explanations for this result: the p63 DBD either binds

### TABLE II

| Thermodynamic parameters for the urea-induced equilibrium unfolding of the p53 and p63 DBDs |
|------------------------------------------------------------------------------------------------|
| Thermodynamic parameters | p53 DBD | p63 DBD |
| [urea]\textsubscript{50%}, M | 3.06 ± 0.02 | 5.15 ± 0.01 |
| m, kcal mol\textsuperscript{-1} M\textsuperscript{-1} | −2.277 ± 0.110 | −2.098 ± 0.068 |
| ∆G(H\textsubscript{2}O), kcal mol\textsuperscript{-1} | 6.97 ± 0.29 | 10.80 ± 0.33 |
| ∆G(urea\textsubscript{50%}), kcal mol\textsuperscript{-1} | −4.57 ± 0.21 |

### TABLE III

| Statistical comparison of the human p53 and p63 DBDs performed with ProtParam (91) |
|-------------------------------------------------------------------------------------|
| Sequence-based comparison | p53 DBD | p63 DBD |
| Acidic residues (Asp + Glu) | 21 (8 + 13) | 23 (10 + 13) |
| Basic residues (Arg + Lys) | 27 (19 + 8) | 26 (15 + 11) |
| Calculated isoelectric point | 8.83 | 8.42 |
| Nonpolar residues | 90 | 102 |
| Aliphatic index | 62.69 | 76.34 |
| Hydrophobicity (GRAVY) | −0.638 | −0.497 |
| Instability index | 72.2 | 47.8 |

**Note:** The images and diagrams are not included in this text representation.
DNA more weakly than p53 or fails to bind cooperatively. For the first case, the p63 DBD might not be active for DNA binding, e.g. because (a) the cloned domain is not sufficient for DNA binding; (b) it is not properly folded; (c) it is in a conformation that needs activation for DNA binding; or (d) it has a lower affinity or different DNA specificity. For the second case, the p63 DBD might not be active for cooperative DNA binding. In this case, the resultant binding of a single DBD would not be detected by EMSA due to its low affinity. To distinguish between these possibilities and to enhance affinity, p63 DBDs were attached to GST as an artificial dimerization site. Dimerized GST-p63 DBD binds to several p53 consensus sites with comparable affinity and specificity, as does GST-p53 DBD. Upon digestion of GST-p63 DBD with thrombin, it loses DNA-binding activity. These results support the conclusion that the p63 DBD is sufficient and properly folded to bind to DNA; however, under the experimental settings, it is not capable of binding to DNA cooperatively. This discrepancy can be attributed to differences in the putative dimerization interface made up by the H1 helix in p53. Fig. 9 depicts a schematic model illustrating the experimental results. Whereas the p53 DBD contains a dimerization interface, p63 DBD lacks a functional interface and can just bind to DNA upon GST-mediated dimerization (see below).

Recent studies have examined the ability of the p53 family members to transactivate various p53-responsive promoters in reporter gene assays. p73-α and p73-β were reported to activate some, but not all, previously identified p53 target genes (17, 76). For p63, only minor differences in the transcriptional activation of several cellular p53 target genes mediated by p63

Fig. 8. A, superposition of the backbone representation of the p53 DBD structure (shown in cyan) (33) and the p63 DBD homology model (shown in red). The four residues coordinating the Zn²⁺ are indicated in violet; the six hot spot point mutations in p53 are displayed in green; and the amino acids that are engaged in DNA interactions are highlighted in orange. In gray are the residues located at 2.5 Å of the three first sets. The bound schematic DNA consensus double helix is displayed in yellow. The black oval highlights the region that differs between the two domains. B, lipophilic surface potentials for the p53 DBD (upper panels) and p63 DBD (lower panels) calculated for each atom using Clog P. The coloring is indicated, from blue for negative polar values to red for positive hydrophobic values. For each DBD, the right panel is rotated by 180° regarding the left panel.
Comparison of the p53 and p63 DNA-binding Domains

The p53 DBD (black circles) contains a functional dimerization interface putatively formed by the H1 helix (represented by gray rectangles) and is capable of cooperative binding to its consensus DNA. The p63 DBD (white circles), in contrast, does not bind to DNA cooperatively due to a different or lacking dimerization interface (represented by gray rectangles). Artificial dimerization mediated via fusion with GST (gray circles) restores the DNA-binding activity of GST-p63 DBD. GST-p53 DBD and GST-p63 DBD bind to DNA with similar affinity and specificity. The arrows indicate a further putative intermolecular oligomerization interface.

p53

p63

FIG. 9. Schematic model for the DNA-binding properties of the p63 and p53 DBDs and the respective GST fusion proteins. The p53 DBD (black circles) contains a functional dimerization interface putatively formed by the H1 helix (represented by gray rectangles) and is capable of cooperative binding to its consensus DNA. The p63 DBD (white circles), in contrast, does not bind to DNA cooperatively due to a different or lacking dimerization interface (represented by gray rectangles). Artificial dimerization mediated via fusion with GST (gray circles) restores the DNA-binding activity of GST-p63 DBD. GST-p53 DBD and GST-p63 DBD bind to DNA with similar affinity and specificity. The arrows indicate a further putative intermolecular oligomerization interface.

and p53 could be shown (61). In that study, it was also found that p63 activates the \textit{bax} promoter more efficiently than p53 and at levels comparable to those of p21. We examined the question of DNA specificity and selectivity by comparing the affinity of the GST-DBDs for different natural p53 consensus oligonucleotides. In general, the affinities for the consensus sites differ according to the same pattern, so GST-p53 DBD and GST-p63 DBD show almost identical specificity with regard to the selected representative p53 consensus sites. The affinity of GST-p53 DBD in comparison with GST-p63 DBD is relatively 3–5-fold lower for the PG, p21, \textit{gadd45}, and \textit{cyclin G} consensus sites, with exception of the \textit{bax} consensus site, to which both GST-DBDs bind with similar, but lowest affinity. These results show that GST-p63 DBD binds to known p53 consensus sites with similar specificity and at least the same affinity as GST-p53 DBD. However, as could be seen for \textit{bax}, \textit{in vitro} binding affinities of the DBDs for p53 consensus site oligonucleotides and the degree of reporter gene activation in cellular assays using the natural promoter sequences cannot be directly compared, as the isolated DBDs and the full-length proteins might differ in their specificity and as additional sequence elements are involved in reporter gene activation (77).

The observed differential recognition of p53 promoters might account for the distinctive role of the p53 family members in tumor surveillance and development (14). It was therefore suggested that the differential responses of the target genes might be due to slightly different specificities or conformational states of the DBDs (61). However, our results with the isolated p63 DBD are not in favor of this hypothesis. Rather, the distinctions in target specificity seem to be based upon a differing specificity of the full-length proteins in the cellular context. Part of these differences might be mediated by the C-terminal regions of the proteins (61). Despite the limitations stated above, quantitative \textit{in vitro} studies on the binding to larger sets of DNA target sequences might help to explain the different responses of p53-regulated genes in response to p53 activation (18, 78) and to resolve the question of how target discrimination occurs within the p53 family (25).

The p63 DBD can be expressed at 37°C in a soluble form, whereas the p53 DBD is highly temperature-sensitive (39, 79) and is deposited almost exclusively in inclusion bodies at this temperature. This observation supports the notion that the p63 DBD shows enhanced thermostability. In addition, the p63 DBD is highly stable in contrast to the p53 DBD, as it has almost no tendency to aggregate and precipitate in solution even at elevated temperatures. The enhanced thermodynamic stability of the p63 DBD could be confirmed by DSC and urea-induced equilibrium unfolding. The observed gain in stability of the p63 DBD of a calculated $\Delta \Delta G(\text{urea}_{50\%}) = -4.57 \text{ kcal mol}^{-1}$ ($T_m = 61 \text{ °C versus } 44 \text{ °C}$ and $[\text{urea}]_{50\%} = 5.15 \text{ M versus } 3.06 \text{ M}$ for the p53 DBD) is remarkable. It is, however, surprising that Zn$^{2+}$ seems to be more tightly coordinated by the thermodynamically less stable p53 DBD, in particular as all residues directly involved in coordination are highly conserved in the p63 DBD. This slight destabilization might be the consequence of the unique region of 10 amino acid residues that is located just after the H1 helix in p53 with His$^{206}$ coordinating the Zn$^{2+}$. Several attempts to stabilize the p53 DBD have been undertaken so far. Using DNA shuffling, 20 amino acid residues (positions 101–120) could be identified as responsible for a thermostable phenotype (46). A significant stabilization ($\Delta \Delta G(\text{urea}_{50\%}) = -2.65 \text{ kcal mol}^{-1}$, $T_m = 47.2 \text{ °C}$, [urea]$_{50\%} = 4.17 \text{ M}$) (44) was achieved by semirational design of a quadruple mutant (M133L/V203A/N239Y/N268D), two homologous residues (Leu$^{113}$ and Ala$^{203}$) of which can be found in the p63 DBD as well. Matsumura and Ellington (45) created thermostable p53 variants by \textit{in vitro} evolution and found that two of three stabilizing mutations reside in the p53 DBD (N239Y and N268D). Both of these mutations were also found by Nikolova et al. (44), but they are not present in the p63 DBD.

The stability of globular proteins in solution depends on several factors. Generally, the stabilization of proteins is due to only a few molecular interactions (80). A statistical comparison of the p63 and p53 DBDs revealed no major differences. However, the p63 DBD has a higher portion of hydrophobic residues that might stabilize the hydrophobic core and shows a lower instability index (69). The p53 and p63 DBDs probably have a similar overall fold with minor structural differences. By building a model of the p63 DBD, it could be shown that the p63 and p53 DBDs differ mainly in the above-mentioned region of 10 amino acid residues that is located after the H1 helix in p53. We have been able to highlight a particular conformation of an extended hairpin loop on the surface of the p63 DBD. Moreover, the significance of the H1 helix region for the putative dimerization interface in the tetrameric p53-DNA complex has been previously suggested (see Fig. 7B in Ref. 33). Studies on the overall structure of the p53 DBD-DNA complex proposed that DNA bends to avoid steric clashes in this interface when four...
p53 DBDs are bound to DNA (64). The particular constitution and orientation of that hairpin motif in the p63 DBD may reduce cooperativity of DNA binding (see Fig. 9), e.g. by interfering with the H1 helix and/or preventing conformational rearrangements necessary for dimerization. As our model does not integrate the first amino acids and the C-terminal domain of the full-length p63 protein, the thermostable character and the dimerization behavior are perhaps not fully reflected by a partial three-dimensional structure of the DBD. Nevertheless, the hydrophobic character of the protein in conjunction with the packed conformation of the extended hairpin motif may provide a way to explain the enhanced (thermo) stability and the lack of cooperative DNA binding of the p63 DBD. It is tempting to speculate how this distinction in the potential dimerization interface in the DBDs influences the DNA-binding properties of the tetrameric full-length p63. Based on the sequence alignment and the modeled structure, it will be possible to design chimeric p53 DBDs possibly lacking dimerization properties or, vice versa, p63 DBDs displaying dimerization properties of p53 for biochemical and cellular studies.

Knowledge of the molecular basis of thermostabilization of the p63 DBD might allow the identification of essential residues and make it feasible to engineer more thermostable p53 DBDs, e.g. for the application of highly stabilized p53 variants in gene therapy. The p63 protein is possibly the most ancient member of the p53 family (7, 16). The enhanced in vivo thermostability of the p63 DBD might reflect the evolutionary development and the different in vivo functions of p53 and p63 ("regulation of function through protein stability" (13)). Little cellular data on the cellular stability of the p63 isoforms are available (81); however, p63 probably shows a comparable long half-life due to its more constitutive physiological function. In contrast, the tumor suppressor p53 is present only in low concentrations and shows a short half-life under physiological conditions. Following several stress signals, p53 is activated through post-translational mechanisms and rapidly accumulates (82, 83). Consequently, the p53 protein has to be tightly regulated due to its potential "toxicity" for undamaged cells. One mean to achieve this might be the low thermostability of the p53 DBD that leads to a rapid turnover of inactive unfolded p53 protein mediated by ubiquitination and proteolysis. It is in this line of evidence that MDM2 binds to the N-terminal part of p53, thereby inhibiting the transcriptional activity and targeting p53 for degradation by the ubiquitin-proteasome pathway (84–87), whereas p63 levels are independent of MDM2, and neither MDM2 nor MDMX is capable of binding to or targeting p63 for degradation (88).

Very recently, it was demonstrated that certain p63 isoforms form complexes with wild-type p53 and that these interactions are mediated by both DBDs (81). Other experiments support a direct interaction of tumor-derived mutant p53 DBDs with p63 and p73 (89, 90). The availability of highly purified and characterized p63 and p53 DBDs makes it now possible to study the basis of these reported interactions.

In summary, the characterization of a new isoform of the DBD of the p53 homolog p63, p63-δ, revealed several novel features. The free p63 DBD does not bind to DNA cooperatively. However, dimerized GST-p63 DBD shows comparable affinity and specificity for representative p53 consensus sites as GST-p53 DBD. In comparison with p53, the p63 DBD is thermodynamically remarkably stabilized. These results might support biochemical and structural studies to elucidate the molecular basis for the lack of cooperative DNA binding and the high thermostability of the p63 DBD in order to gain further insight into the function of the p53 family members in tumorigenesis and development.
Comparison of the p53 and p63 DNA-binding Domains

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High Thermostability and Lack of Cooperative DNA Binding Distinguish the p63 Core Domain from the Homologous Tumor Suppressor p53
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