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Supporting Information

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Cooperative Binding of p53 to DNA is regulated by protein-protein interaction forming a double salt bridge

*Alexander Dehner, Christian Klein, Silke Hansen, Lin Müller, Johannes Buchner, Manfred Schwaiger and Horst Kessler**

Cloning, Expression and Purification

Residues 94-312 of human p53 coding for the wild-type p53DBD[1-3] were amplified from plasmid pT7.7Hup53[4] by polymerase chain reaction and cloned into a modified pQE40 vector (Quiagen, Hilden, Germany). Single and double mutations were introduced by Quick-Change site-directed mutagenesis (Stratagene, La Jolla, CA, USA) according to the specifications. Gene-specific oligonucleotide primers were synthesized by MWG Biotech AG (Ebersberg, Germany). Mutant p53DBD cDNA was PCR-amplified, sequenced using the Big Dye Terminator Sequencing Kit (Perkin Elmer) and introduced into the NdeI/XhoI site of a modified pQE40 expression vector. The mutant p53DBD expression vectors were identical to the wild-type vectors except for specific single and double site nucleotide substitutions. p53DBD wild-type and p53DBD mutants were expressed in *Escherichia coli* co-transfected with pUBS520[5] at 37 °C in Luria broth medium as inclusion bodies, refolded *in vitro* and purified as described elsewhere.[6] For the preparation of uniformly ^{15}N-labeled p53DBDs, bacteria were grown at 37 °C in M9 minimal medium containing antibiotics, minerals and vitamins supplemented with 2 g/L $^{15}NH_4Cl$ as sole nitrogen source. For the GST-p53DBD mutants the polymerase chain reaction product was subcloned into a pGEX-4T-1 vector (Amersham Pharmacia Biotech, Frankfurt, Germany). The resulting recombinant expression vector pGEX-4T(GST-p53DBD) codes for the corresponding GST-p53DBD (mutant) fusion protein including a Gly-Ser-Gly linker that remains at the N-terminus after digestion of the fusion protein with thrombin. All vector constructs were confirmed by sequencing. Fusion proteins were expressed in *Escherichia coli* strains HB101 and UT5600, which were grown at 20 °C in Luria broth medium, and soluble protein was purified as described elsewhere.[7] All proteins were concentrated to 100-200 µM and dialyzed against 50 mM potassium phosphate, pH 6.8, 50 mM KCl and 5 mM DTT, flash-frozen and stored at –80 °C.

Human p63DBD was expressed and purified as described elsewhere.[7]

Electrophoretic Mobility Shift Assay (EMSA)

DNA binding was examined via electrophoretic mobility shift assay (EMSA) as described.[8] For each lane 200 ng of p53DBD wild-type, p63DBD wild-type or mutant p53DBD as indicated were

incubated on ice for 15 min in 10 µL DNA binding buffer (40 mM HEPES (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mg/mL bovine serum albumin, 5 mM DTT, 15% glycerol) with a 1.1 µM solution of fluorescence labeled double-stranded CON4x5 oligonucleotide (5'-IRDye700-AGCTTAGACATGCCTAGACATGCCTA-3' and 3'–ATCTGTACGGATCTGTACGGATTCG-A-5') in the presence of 200 ng nonspecific unlabeled pBluescript II SK^+ competitor DNA (Stratagene La Jolla, CA, USA) in 10 µL DNA binding buffer. DNA complexes were separated from unbound oligonucleotides on native 4% polyacrylamide gels; 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 and quantified with a Odyssey Imager (LI-COR).

Fluorescence Anisotropy Measurements

Fluorescence anisotropy measurements were recorded on a Jobin Yvon FluoroMax-3 spectrometer (Edison, FL, USA). The excitation and emission wavelengths used were 495 nm and 515 nm, respectively. Each experiment was performed in a total time of 102 s, using a time increment of 5.1 s and an integration time of 1 s each. The initial concentration of double-stranded fluorescein-labeled CON4x5 oligonucleotide was 25 nM. The concentrations of the p53 / mutant titrant were 10 nM – 25 µM. Experiments were performed at 10 °C in 50 mM potassium phosphate, pH 6.8, 50 mM KCl and 5 mM DTT. Data were analyzed using a simple cooperative model,

$$2A + B \rightarrow A_2B; \qquad K_d = \frac{[A]^2 \cdot [B]}{[A_2B]} \qquad (\mathbf{1})$$

yielding the following expression for a nonlinear least-squares regression:

$$a_{obs} = \frac{a_B \cdot K_d + a_{A_2B} \cdot [A]^2}{K_d + [A]^2}, \qquad (\mathbf{2})$$

where a_B and a_{A2B} are the maximum anisotropy values for free fluorescein-labeled DNA and A_2B-complex-bound DNA, respectively. A is the concentration of p53 / mutant and K_d is the dissociation constant.

NMR Spectroscopy

NMR investigations were carried out on a Bruker DMX700 spectrometer equipped with a triple channel (^1H, ^{13}C, ^{15}N) inverse probe. Standard sample conditions were 200 – 800 µM ^{15}N-labeled protein in 50 mM potassium phosphate, pH 6.8, 50 mM KCl and 5 mM DTT. Standard temperature was 298 K. DNA oligonucleotides containing one 10-mer p53 consensus half site, 16-meric CON2x5 (5´-CCTAGACATGCCTAAT-3´) were annealed with complementary oligonucleotides, diluted into the NMR buffer, and titrated to the NMR sample. A 1.2 molar excess of consensus

quarter sites relative to the p53DBD was used to achieve a stoichiometric 1:2 = dsCON2x5:p53DBD binding.

Spin-echo Diffusion Measurements

Spin-echo diffusion measurements were performed on *apo* p53DBD wild-type and mutants and on their DNA complexes using a standard stimulated echo pulse train with sine-shaped bipolar diffusion-encoding gradients, one *z*-spoil gradient and a WATERGATE water suppression scheme.[9] 64 ^{1}H spectra were recorded using a gradient ramp ranging from 1.07 to 50.80 G/cm and stored as a pseudo-2D experiment. The dephasing and refocusing gradient length *LD* was set to 4 ms, and the diffusion delay *BD* was 120 ms. Convergent non-exchangeable signal decays between 4.0 ppm and 0 ppm were averaged and a nonlinear least-squares regression onto the signal decay yielded the translational diffusion coefficient.

Hydrodynamic Calculations - Hydrodynamic calculations were performed with HYDRONMR Version 5a,[10] using the published crystal structure of the p53DBD (pdb-ID: 1TSR, chain B) with and without DNA and a dimeric p53DBD-DNA complex as proposed by Klein et al.[11] to build up the bead model.

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