On the Interaction Between p53 and MDM2: Transfer NOE Study of a p53-Derived Peptide Ligated to MDM2

M. J. J. Blommers,*,† G. Fendrich,‡
C. García-Echeverría,§ and P. Chêne‡

Physics, Core Drug Discovery Technologies, and Oncology Research, Ciba-Geigy AG, P.O. Box, CH-4002 Basel, Switzerland

Received September 23, 1996

The tumor suppressor gene p53 is the most frequently mutated gene found in conjunction with human cancer.[1,2] The p53 protein can also be inactivated by binding to viral or cellular proteins[3] such as the human homologue of the mouse double-minute 2 gene product (MDM2) that upon binding inactivates the transcription activity of p53. Thus, in certain human cancers (carcinomas,[7] sarcomas,[8] and glioblastomas[9]), the overexpression of the oncogene MDM2 inhibits p53 tumor suppressor activity. Chemotherapeutic agents that could prevent the binding of p53 to MDM2 in such tumors would release p53 active for tumor suppression. We report herein an NMR study by transfer NOE experiments of a p53-derived peptide in complex with MDM2. The data reported in this paper may be useful in the rational drug design of specific highly active inhibitors of the p53−MDM2 interaction.

The interaction between p53 and MDM2 has been previously characterized by mutagenesis. These experiments show that residues 14, 19, 22, and 23 of p53 (Scheme 1) are crucial for the interaction.[10] In addition, it was shown that a short synthetic peptide corresponding to amino acids 18−23 of p53 can bind MDM2, revealing that this region of the protein is most important for the interaction.[11] Finally, the p53 binding domain has been localized in the N-terminal domain (about 100 amino acids) of MDM2.[12−14] This formed the starting point of our investigation which led to the presented structural information about the interaction between the two proteins.

The N-terminal domain of MDM2, residues 1−188, was expressed as a glutathione *S*-transferase fusion protein (GST-MDM2) in *Escherichia coli.* To ensure that the MDM2 moiety of the fusion protein is functional, ELISA experiments were performed. The protein p53 indeed interacts only with the immobilized GST-MDM2 protein but not with immobilized GST alone (Figure 1A). The specificity of the interaction was analyzed in competition experiments where p53 was incubated in the presence of a peptide derived from p53 comprising the amino acids 15−25 of the p53 sequence. Binding of p53 to the GST-MDM2 protein was inhibited by this peptide (IC_{50} 260 μM) (cf., Figure 1B) strongly suggesting that the fusion protein interacts with p53 in a similar way and with the same specificity as the full-length MDM2 protein. Subsequently, several peptides based on the sequence shown were analyzed in this assay. It was found that the peptide p53(17−24; K24 → P) binds stronger (IC_{50} 100 μM), but its size is reduced to an octapeptide. It has been described that the incorporation of proline residues on either or both sides of the interaction sites of peptides increases its biological activity.[15] The tumbling time of this peptide meets the requirement for transfer NOE experiments favorably since no significant NOEs were detected for the free peptide in solution. Furthermore, chemical shifts and coupling constants were found close to random coil values, suggesting that the peptide lacks a predominant conformation in its unligated state.

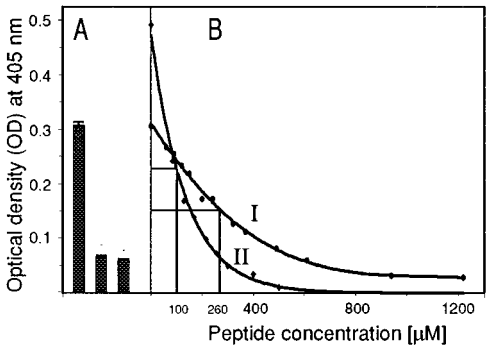

Figure 1. Specific interaction between human MDM2(1−188) and human p53 in ELISA.[26] (A) ELISA plates were coated with either GST-MDM2 (left and right bar) or with GST (middle bar). The plates were subsequently incubated with p53 (left and middle bar) or buffer alone (right bar). (B) A constant amount of GST-MDM2 was immobilized on ELISA plates and a constant quantity of p53 was incubated in the presence of several concentrations of the peptides p53(15−25) (I) and p53(17−24; K24 → P) (II).

Scheme 1. Part of the N-Terminal Amino Acid Sequence of p53 Which Is Important for the Interaction with MDM2

14 19 22 23

Pro-Pro-Leu-Ser-Gln-Glu-Thr-Phe-Ser-Asp-Leu-Trp-Lys-Leu-Leu-Pro

Transfer NOE experiments[16,17] were measured on a 10:1 mixture of this peptide and GST-MDM2. Resonance numbering of the peptide is according to p53: H-Glu[17]-Thr[18]-Phe[19]-Ser[20]-Asp[21]-Leu[22]-Trp[23]-Pro-NH_2. The spectrum was measured using a T_1 ρ filter[18] of 20 ms before t_1 to relax signals from the protein prior to detection. An essential part of the spectrum recorded with a mixing time of 150 ms is presented in Figure 2. No transfer NOEs were detected in control experiments for the peptide and the GST protein alone. Note that various nonsequential crosspeaks are observed, indicating the presence of defined structure (e.g., the aromatic proton Hδ resonance of Phe[19] experiences NOEs with proton resonances of Pro, Asp[21], and Leu[22] as indicated in this part of the spectrum). In total, 18 nontrivial experimental NOEs were used to calculate structures by means of distance geometry calculations. The calculations converge to one family of structures, in which the residues Phe[19], Leu[22], Trp[23], and Pro come together as shown in Figure 3.

† Physics.
‡ Core Drug Discovery Technologies.
§ Oncology Research.

(1) Hollstein, M.; Sidransky, D.; Vogelstein, B.; Harris, C. C. *Science* **1991**, *253*, 49−53.
(2) Hollstein, M.; Shomer, B.; Greenblatt, M.; Soussi, T.; Hovig, E.; Montesano, R.; Harris, C. C. *Nucleic Acid Res.* **1996**, *21*, 141−146.
(3) Ko, L. J.; Prives, C. *Genes Dev.* **1996**, *10*, 1054−1072.
(4) Momand, J.; Zambetti, G. P.; Olson, D. C.; George, D.; Levine, A. *Cell* **1992**, *69*, 1237−1245.
(5) Chen, J.; Lin, J.; Levine, A. *Mol. Med.* **1995**, *1*, 142−152.
(6) Haupt, Y.; Barak, Y.; Oren, M. *EMBO J.* **1996**, *15*, 1596−1606.
(7) Jennings, T.; Bratslavsky, G.; Troshina, K.; Bronstein, M.; Dedov, I.; Alexandrova, G.; Figge, J. *Exp. Mol. Pathol.* **1995**, *62*, 199−206.
(8) Florenes, V. A.; Maelandsmo, G. M.; Forus, A.; Andreassen, A.; Myklebost, O.; Fodstad, O. *J. Natl. Cancer Inst.* **1994**, *86*, 1297−1302.
(9) He, J.; Reinfenberg, G.; Liu, L.; Collins, V. P.; James, C. D. *Genes, Chromosomes Cancer* **1994**, *11*, 91−96.
(10) Lin, J.; Chen, J.; Elenbaas, B.; Levine, A. *Genes Dev.* **1994**, *8*, 1235−1246.
(11) Picksley, S. M.; Lane, D. P. *BioEssays* **1993**, *15*, 689−690.
(12) Chen, J.; Marechal, V.; Levine, A. *Mol. Cell. Biol.* **1993**, *13*, 4107−4114.
(13) Leng, P.; Brown, D. R.; Shivakumar, C. V.; Deb, S.; Deb, S. P. *Oncogene* **1995**, *10*, 1275−1282.
(14) Brown, D. R.; Deb, S.; Munoz, R. M.; Subler, M. A.; Deb, S. W. *Mol. Cell. Biol.* **1993**, *13*, 6849−6857.
(15) Kini, R. M.; Evans, H. J. *FEBS Lett.* **1995**, *375*, 15−17.
(16) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402−417.
(17) Lian, L. Y.; Barsukov, I. L.; Sutcliffe, M. J.; Sze, K. H.; Roberts, G. C. K. In *Methods in Enzymology*; James, T. L., Oppenheimer, N. J., Eds.; Academic Press, Inc.: San Diego, CA, 1994; Vol. 239, pp 657−700.
(18) Scherf, T.; Anglister, J. *Biophys J.* **1993**, *64*, 754−761.

S0002-7863(96)03317-3 CCC: $14.00

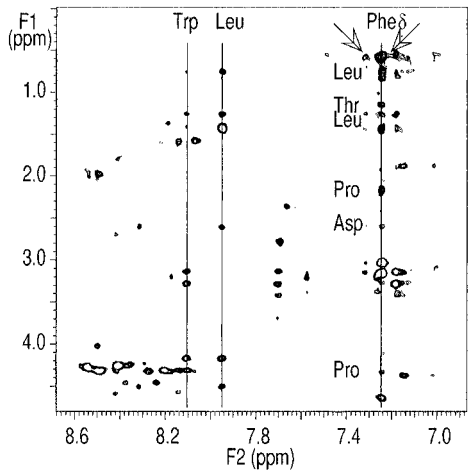

Figure 2. Part of the transfer NOE spectrum recorded for the peptide H-Glu[17]-Thr[18]-Phe[19]-Ser[20]-Asp[21]-Leu[22]-Trp[23]-Pro-NH_2, derived from p53, complexed to the MDM2 fusion protein GST-MDM2(1−188) (46.8 kDa) in aqueous solution (95% H_2O, 5% D_2O), 50 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA-d_{12}, and 10 mM 2-mercaptoethanol-d_6, pH 7.6. The concentrations of ligand and receptor were 1.9 mM and 190 μM, respectively. The mixing time was 150 ms. The spectrum was measured on a Varian Unity-plus 600 spectrometer. Phase sensitive free induction decays (FID) were aquired using 512 and 2048 data points in t_1 and t_2, respectively. Thirty-two transients per FID were recorded. Data were processed using the VNMR software. Arrows highlight intermolecular NOEs.

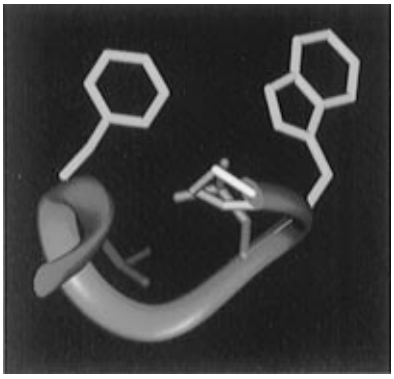

Figure 3. View of the structure of the peptide, derived from p53(17−24; K24 → P), when complexed to MDM2. The structure is characterized by a close proximity of Phe[19] and Trp[23] (both yellow), which are exposed. This typical orientation of these aromatic residues is stabilized by hydrophobic interactions with Leu[22] (blue), Thr[19] (red), and Pro (white). The structure is a representative member of a family of 100 structures which is calculated by means of 18 nontrivial NOE distance restraints collected from two transfer NOE experiments recorded with a mixing time of 80 and 150 ms. Upper bounds of 3.3 and 5.0 Å were set for medium and small NOEs, respectively. Structures were generated using the distance geometry program DGII,[24] using sequential tetrangle smoothing, embedding in 4 dimensions, and 20 000 steps simulated annealing. Finally, structures were energy minimized using DISCOVER. Five-hundred steps conjugate gradient minimization and the cvff91 force field[25] were applied. All structure calculations were done on a Silicon Graphics using the software package InsightII (Molecular Simulations, Inc.).

In this binding loop, the interactions between the amino acids are mostly hydrophobic (i.e., Phe[19] and Trp[23] come together in a manner similar to the hydrophobic core in many proteins). We observe packing of the side chains of Leu[22], Pro, and the methyl group of Thr[18] together with the previously mentioned Phe[19] and Trp[23]. Interestingly, the two aromatic residues are still exposed to a certain extent. This leads to the suggestion that this part of the structure is actually involved in binding the MDM2 domain. Therefore, the transfer NOE experiment was repeated without suppression of the protein signals, in the hope to see dipolar interaction between the ligand and the protein. Indeed, intermolecular NOEs were observed involving resonances of Phe[19] and Trp[23] and at least two upfield resonances of the MDM2 fusion protein, presumably methyl resonances (cf., Figure 2).

A possible pitfall in the transfer NOE experiment is the observation of NOEs which origin exclusively through spin diffusion via the protein. These effects can be detected by performing the transfer NOE experiment in the rotating frame because in this type of experiment positive crosspeaks are notorious for these cases.[19] The transfer ROESY experiment recorded for the peptide−MDM2 complex resulted in the disappearance of the crosspeaks in which Trp and Phe resonances participate, whereas for most other resonances negative NOEs were observed. Thus, in our case we do not observe positive NOEs, but cancelling contributions, which indicates that indeed Phe and Trp are close in space and in addition are contacting the MDM2 protein. Because both features results in an opposite sign of the NOE, these signals are cancelled.

For complexes of this size, NOEs may origin from spin diffusion within the ligand or mediated by protons of the receptor. This may result in errors in the upper bounds chosen as input for the structure calculations. When the structure depicted in Figure 3 is subjected to backcalculation of the NOEs using the program CORCEMA,[20] no significant discrepency between experimental and theoretical intensities is found.

In conclusion, the interaction of MDM2 and p53, which inactivates p53 for tumor suppression, can be described as follows: the N-terminal domain of p53 can bind the MDM2 protein through hydrophobic interactions mediated by residues at positions Phe[19], Leu[22], and Trp[23] and hydrophobic side chains at the surface of MDM2. These three amino acids are conserved in 23 species reported for p53 so far,[21] which finds an explanation by the here presented structural model for the recognition of p53 and MDM2.

After this paper was submitted, the crystal structure of a peptide complexed to MDM2 was published.[22] Although we consider the crystal structure to contain more structural details, the conclusions we drew from the transfer NOE experiments of the complex in aqueous solution agree very well with those of Pavletich and co-workers.

JA9633171

(19) Arepalli, S. R.; Glaudemans, P. J.; Daves, G. D.; Kovac, P.; Bax, A. *J. Magn. Reson.* **1995**, *B106*, 195−198.
(20) Moseley, H. N. B.; Curto, E. V.; Krishna, N. R. *J. Magn. Reson.* **1995**, *B108*, 243−261.
(21) Soussi, T.; May, P. *J. Mol. Biol.* **1996**, *260*, 623−637.
(22) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. *Science* **1996**, *274*, 948−953.
(23) Lane, D. P.; Crawford, L. V. *Nature* **1979**, *278*, 261−263.
(24) Havel, T. L. *Prog. Biophys. Mol. Biol.* **1991**, *56*, 43−78.
(25) Dauber-Osguthorpe, P.; Roberts, V. A.; Osguthorpe, D. J.; Wolff, J.; Genest, M.; Hagler, A. T. *Proteins: Struct., Funct., Genet.* **1988**, *4*, 31−47.
(26) The p53 gene was cloned into the NdeI/BamHI sites of a pET-3a vector (Novagen) and was expressed in *E. coli* strain BL21(DE3)pLysS (Novagen). DNA-encoding human MDM2(1−188) was inserted into the EcoRI/BamHI sites of a pGEX-2T vector (Pharmacia). The domain was expressed as a glutathione *S*-transferase fusion protein (GST-MDM2) in *E. coli* strain BL21 (Novagen). The ELISA plates (MaxiSorp-Nunc) were coated with either 250 ng of purified GST-MDM2 protein or 250 ng of GST protein diluted in PBS (0.008 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.14 M NaCl, 0.01 M KCl, pH = 7.4) and incubated overnight at 4 °C. After washing with PBS containing 0.2 M NaCl, the plates were incubated at 37 °C with blocking solution (PBS containing 10 mg of BSA/mL) and washed (PBS containing 1 mg of BSA and 0.05% Tween-20). A solution of 100 ng of p53 protein diluted in 50 mM Tris, 50 mM NaCl, 10% glycerol, 0.1% Triton X-100, 20 mM 2-mercaptoethanol, and 1 mg of BSA/mL, pH = 7.6 was incubated for 30 min at 4 °C. After an additional washing step, the plates were incubated for 1 h at 37 °C with the monoclonal antibody Pab421[23] (Oncogene Science) diluted at 0.2 mg/mL in blocking solution. The plates were again washed and incubated for 1 h at 37 °C with a goat antimouse IgG antibody coupled to alkaline phosphatase (Promega) diluted in blocking solution. The excess of antibody was removed with washing solution, and the coupled antibody was visualized with *p*-nitrophenyl phosphate disodium diluted in diethanolamine. The absorbance was measured at 405 nm.