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Fluorescence Study Gives a Hint to Understand How the p53 DNA Binding Domain Recognizes Its Specific Binding Site on DNA Fragments

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The p53 protein, which is known as the tumor suppressor protein, displays various cellular functions such as regulating the expression of diverse downstream genes, apoptosis, cell cycle arrest, DNA repair, cell differentiation, and other related functions through binding sequence specifically to the target DNA.[1-3] Mutation of the p53 protein has been reported to be strongly implicated in cancers.[4-6] Therefore studies on p53 look important to understand the mechanism of inducing cancer. The cellular levels of p53 are strictly controlled, and the activity of p53 is regulated post-translational modification *in vivo*.[6]

The p53 protein having 393 amino acid residues consists of the N-terminal domain containing the transactivation domain (TAD) and the proline-rich region (PR), the sequence-specific DNA binding domain (DBD), and the C-terminal domain containing the tetramerization domain and the regulatory domain.[7-10] Uniquely, mutations on p53 occurs mostly in the DNA binding domain.[11]

p53 is reported to bind DNA in the tetrameric binding mode *in vivo*, but p53DBD binds DNA in the monomeric mode *in vitro*.[9,10,12] The cellular levels of p53 are strictly controlled, and the activity of p53 is regulated post-translational modification *in vivo*.[6] The p53 binds DNA in two different ways, base-sequence specifically and nonspecifically.[6,9,13] The base sequence-specific binding occurs through various noncovalent weak interactions between DNA and p53 residues. In contrast, non-specific binding occurs through ion-ion interactions between negatively charged phosphates on the phosphodiester chain of DNA and positively charged residues of p53.[13,14] According to previous reports,[6,15] for specific binding between p53DBD and DNA, both specific and nonspecific bindings should occur altogether, and weirdly, specific binding affinity of p53 to DNA is not so different compared to nonspecific binding affinity. For that reason, we tried to observe the difference in the binding properties between wild type p53DBD and the p53DBD variant containing three-point substitutions (G245S, R248Q and R249S) among six most probable mutations, upon binding to each of

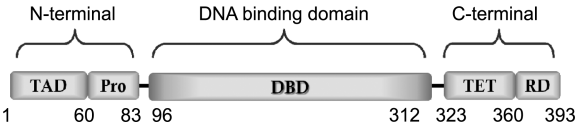

Figure 1. The human p53 protein contains various functional domains.

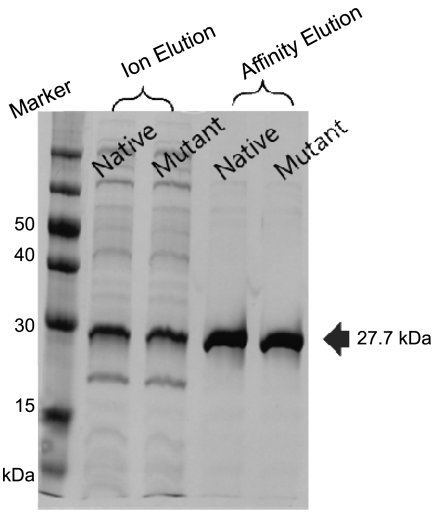

Figure 2. Bands representing pure wt p53DBD and p53DBD variant observed after purification with Ni-NTA column.

two different DNA fragments, DNA1 and DNA2. We prepared very pure wild-type p53DBD and p53DBD variant successfully by the method described in the experimental section (Figure 2).

DNA1 is 5'-GAGCATGCTCGAGCATGCTC-3', and it contains two specific target sequences of 5'-PuPuPuCATG PyPyPy-3'. DNA2 does not contain any consensus sequence and its sequence is 5'-GAGCGCGCTCGAGCGCGCTC-3'.

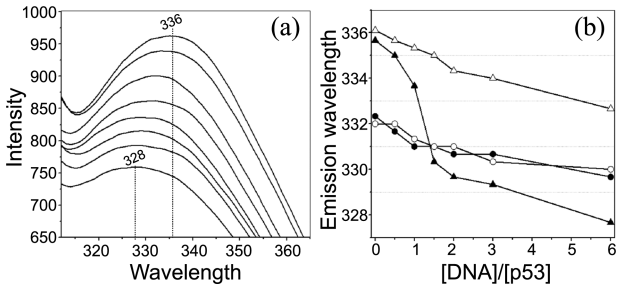

Figure 3. (a) Blue shift shown in emission fluorescence upon binding of wt p53DBD to DNA1. [DNA]/[wt p53DBD] were as following: From the top, [DNA]/[wt p53DBD] = 0, 0.5, 1.0, 1.5, 2.5, 3.0, 6.0. (b) Changes in emission wavelength for various binding interactions were presented: (▲) wt p53DBD-DNA1, (△) wt p53DBD-DNA2, (●) p53DBD variant-DNA1, and (○) p53DBD variant-DNA2.

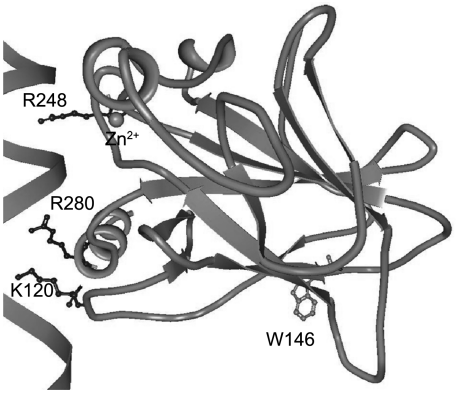

Figure 4. The position of the tryptophan residue (146) which was used for fluorescence measurement, and the specific DNA binding site of p53DBD. This figure was prepared by modifying the 1TSR structure from PDB.

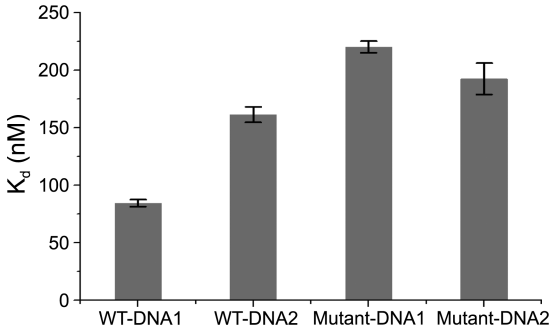

Figure 5. K_d values for various binding interactions. WT-DNA1, WT-DNA2, Mutant-DNA1, and Mutant-DNA2 represented binding of wild-type p53DBD to DNA1 (specific binding), binding of wild-type p53DBD to DNA2, binding of p53DBD variant to DNA1, and binding of p53DBD variant to DNA2, respectively. Each K_d was the mean value obtained from six experimental data.

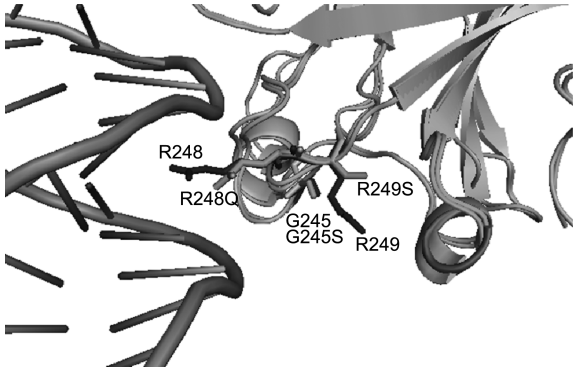

Figure 6. Comparison of the regional structure containing three mutation points, G245, R248, and R249 of wild-type p53DBD (dark) to that of p53 DBD variant (gray). The structure of p53DBD variant was created using the homology modeling program MODELLER and PyMOL, and the structure of 1TSR as a template.[20]

Wild-type p53DBD Showed a Blue-Shift Effect Upon Binding to DNA Containing the Consensus Sequence *in vitro*. The blue-shift effect appeared upon binding of the wild-type p53DBD to DNA1 containing the consensus sequence, 5'-pupupuCATGpypypy-3'. The extent of the blue-shift was about 8 nm (Figure 3(a)). It was not so large, but was observed clearly. In contrast, very little blue-shift could be observed upon binding the wild-type p53DBD to DNA2 and binding of p53DBD variant to either DNA1 or DNA2.

Fluorescence emission came from excitation of the Trp residue at 146 position of p53DBD (Figure 4).

This meant that binding of wild-type p53DBD to DNA1 induced a structural change shifting Trp at 146 position toward more nonpolar β–sheet region. We did not determine the detailed structural change of p53DBD induced by binding to DNA yet. Our fluorescence data showed that binding of p53DBD to DNA1 containing the consensus sequence caused clearly the structural change in the region containing Trp, although it was not close to the binding site. The data also indicated that the sequence-specific binding might be able to cause the structural change in the Trp region, but nonspecific bindings between wild-type p53DBD and DNA2, and between p53DBD variant and DNA1 or DNA2 might be not, as shown in Figure 3(b).

p53DBD Variant Bound DNA Weakly Compared to Wild-Type p53DBD. As stated elsewhere previously,[15,16] the difference in binding affinities of p53 for specific and nonspecific DNA binding is considered to be not so large. We also obtained consistent binding affinities represented by dissociation constants (K_d) based on fluorescence data. The K_d for binding of wild-type p53DBD to DNA1, which is a specific binding, was about 80 nM, but were about 200 nM for non-specific bindings (Figure 5).

As shown here, the difference in binding affinity between specific and nonspecific binding is not so large. For this reason, it is still highly controversial how p53 recognizes its specific target site on DNA.[6,15,17-19] According to the previously reported data, G245, R248, and R249 residues on wild-type p53DBD might be able to form well-defined hydrogen bonds, and then R248 could interact with DNA at the minor groove properly.[6] If so, 3-point mutations at G245S, R248Q, and R249S might make it unable to form hydrogen bonds among them, therefore there might be a minor structural change at the region near G245S, R248Q, and R249S. In addition, Q248 has a shorter side chain than the arginine residue. Consequently, Q248 residue might be not able to interact with DNA at the minor groove properly (Figure 6).

Experimental

All primers for performing PCR experiments were obtained from Bioneer Inc. (Daejeon, Korea). The PCR product was cloned into a pET28a vector and then excised with restriction enzymes *EcoR1* and *Xho1*, followed by treatment with ligase. The DNA fragment was inserted into the *EcoR1-Xho1* site of the pET28a vector. The DNA product was then sequenced to identify the correct sequence at Bioneer Inc. (Daejeon, Korea). *Escherichia coli* strain BL21 (DE3) transformed with the pET28a vector was grown at 37 ºC in Luria-Bertani (LB) medium containing kanamycin (25 μg/

mL) to an A_{600} of 0.6 followed by overnight induction at 25 ºC with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, all procedures for p53DBD preparation were carried out as described elsewhere.[21] And then p53DBD was purified using a SP-Sepharose column followed by a Ni-NTA column. The p53DBD variant was prepared as the same method described above (Figure 6). All p53DBD and its variant were dialyzed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.001% sarkosyl and 500 mM NaCl for fluorescence measurement.[22] DNA1 and DNA2 were purchased from Bioneer Inc.(Daejeon, Korea) and desalted by dialysis and dissolved in 50 mM phosphate buffer (pH 7) for fluorescence experiment. Collection of fluorescence data were performed on a JASCO FP-750 spectrofluorometer using an excitation wavelength of 274 nm at room temperature. Emission spectra were recorded from 295 nm to 500 nm, the bandwidths for excitation and emission were set on 10 nm and 5 nm, respectively. We derived K_d values from fluorescence data using Sigma Plot (SPSS Inc., USA) and Origin (Origin Lab., USA). The structure of p53 DBD variant was created using the homology modeling program MODELLER and PyMOL, and the structure of 1TSR as a template.

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