Indian Journal of Biochemistry & Biophysics
Vol. 60, November 2023, pp. 828-835
DOI: 10.56042/ijbb.v60i11.5393

FUSE binding protein1 interacts with Tumor Suppressor p53 and p53-Isoforms through their DNA Binding domain: Mapping the FBP1 binding site

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Received 06 September 2023; *revised 04 November 2023*

We have earlier demonstrated that a cellular factor, FUSE binding protein1 (FBP1), physically interacts and effectively suppresses the function of tumor suppressor p53 and promotes persistent HCV replication (Dixit *et al*. JVI 89:7905, 2015). In the present study, we demonstrate that FBP1 interacts with various naturally occurring p53-isoforms isolated from different cancers that carry large deletions at the N- and C-terminal regions but still have an intact DNA binding domain (DBD). We discovered that FBP1 specifically interacts with the DNA binding domain (DBD) of p53 and its isoforms. We further mapped the FBP1-interaction site and identified a 21-residue-long motif spanning amino acid residues 163-183 in the p53-DBD. We further confirmed that Arg175/Cys176, within this motif, is necessary for FBP1 interaction. Arg175/Cys176, located at the junction of the β4 and H1 helix of the L2 Loop, is required for the DNA binding function of p53. Occupying this site containing Arg175/Cys176 by FBP1 may block the DNA binding function of p53.

Keywords: DNA binding domain, Fuse Binding Protein 1 (FBP1), p53

The FUSE-binding protein1 (FBP1) had been initially characterized as the transactivator of c-mycproto-oncogene[1]. FBP1 is a transcription factor that binds to an upstream element (FUSE) of the c-myc gene and transactivates its transcription. FBP1 gene is highly conserved, exhibiting more than 90% sequence homology among mammalian species. The 75 kDa FBP1 protein consists of three distinct functional domains with activation and repression domains at the C- and N- terminals. The central region contains sequence specific DNA binding domain[2-4]. Several reports, including ours, have shown that FBP1 is also an RNA binding protein that influences the stabilization of specific viral and cellular mRNAs[5-7]. FBP1 is autoregulated in cells; the N-terminal repression domain regulates the C-terminal activation domain[8]. FBP1 is specifically expressed in undifferentiated cells during embryonic development and influences cell differentiation, proliferation, and apoptosis[4,9].

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Abbreviations: FBP1, FUSE binding protein1; FUSE, far upstream element; NI-NTA column, nickel-nitrilotriacetic acid affinity column; p53-DBD, p53 DNA binding domain; WAF1 DNA, p53-binding site on the 2.4 kb upstream promoter of p21/WAF1/Cip1 gene

FBP1 is now considered a new proto-oncogene. Its expression is linked with tumor development[8]. It plays a significant role in tumorigenesis in the liver and renal carcinomas[10,11]. FBP1 is also overexpressed in hepatocellular carcinoma and is required for tumor growth[12]. Recently, FBP1 is identified as a long tail cancer driver and widespread regulator of tumor suppressor function[13]. We have earlier demonstrated that FBP1 strongly suppresses p21 expression by inhibiting the DNA binding function of the tumor suppressor, p53[14]. FBP1 strongly binds to p53 and inhibits its binding to the target DNA. Recently Frost *et al.* have also shown that depletion of FBP1 expression enhances the p53 promoter occupancy and upregulates the p53 targeted genes[15]. In this study, we have mapped the interaction site on the DNA binding domain (DBD) of p53 and identified a specific motif site of FBP1 interaction.

Materials and Methods

Plasmids

The mammalian expression clone of wild-type p53 (pcDNA3-p53^{WT}) constructed by Loughery *et al*[16] was obtained from Addgene. The naturally occurring isoforms of p53 (Fig. 1) carrying deletions at N- and C-terminals were a kind gift from Dr. Jean-Christophe Bourdon[17,18].

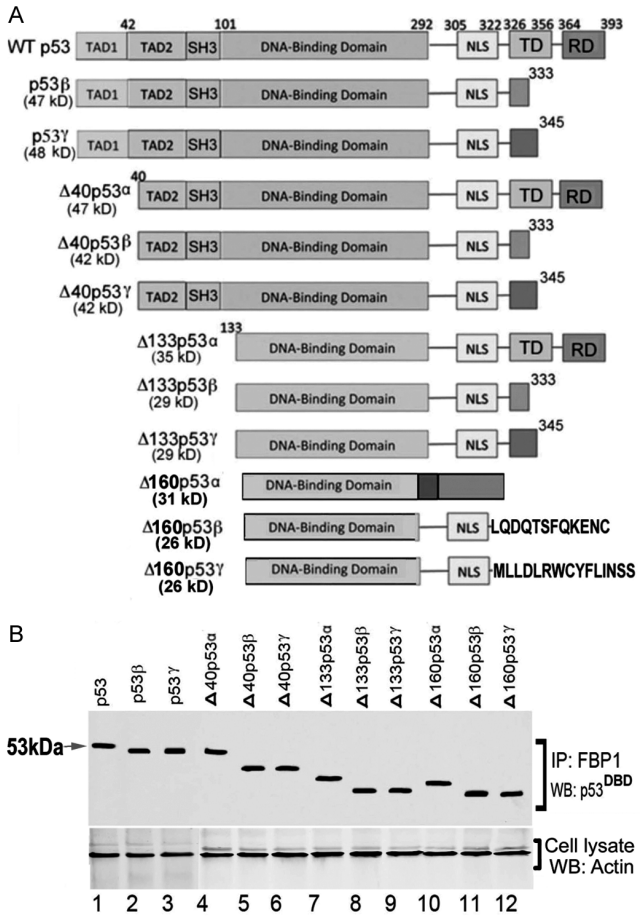

Fig. 1 — Interaction of FBP1 with the wild type p53 and its naturally occurring isoforms. (A) Schematic representation of p53 protein structure and its multiple splice variants isoforms encoded by human p53 gene; and (B) A molecular clone of p53, and its isoforms were separately transfected into p53 null HCT116 cells (p53-/-). Seventy-two hours post-transfection, FBP1-IP was performed on cell lysate and Western blotted for p53 using p53 antibody against DNA binding domain mapping epitope between amino acids 213-217 (PAb 240, Abcam, USA)

Cell culture

p53 null colon cancer cell line HCT116 (p53-/-) were maintained in Dulbecco's modified Eagle medium (DMEM) from Sigma (Saint Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL of nonessential amino acids (Sigma), and 100 µg/mL of penicillin and streptomycin sulfate (Sigma). Cells were grown at 37°C under 5% CO2.

Transient expression of the wild-type p53 and its isoforms in p53 null HCT116 (p53-/-) cells

Two micrograms of each plasmid were separately transfected in HCT116 (p53-/-) cells using Lipofectamine 2000 as previously described[14,19]. Cells were grown and harvested after 72h. Lysates were then prepared. An aliquot equivalent to 15 µg protein was used for SDS-PAGE, followed by WB for p53 and its isoforms.

Preparation of cell extract and Western blotting

HCT116 p53-/- cells were harvested, washed with phosphate-buffered saline (PBS), and then lysed at room temperature in the lysis buffer containing 1% SDS, 10% glycerol, 1 mM DTT, and 10 mM Tris-HCl, pH 6.8. The cell lysates were heated at 100°C for 5 min, cooled at room temperature, and then centrifuged at 13000 rpm for 10 min at 4°C. The clear supernatant was saved, and protein concentration was determined. An aliquot of the supernatant equivalent to 15 µg protein was resolved on 8% SDS-PAGE and then transferred to a nitrocellulose membrane (Schleicher and Schuell Bioscience) and Western blotted for the target protein as previously described[14,19].

Immunoprecipitation of FBP1 and Western blotting of p53

The Cell lysate treated with RNase-A or Benzonase was immunoprecipitated with anti-FBP1 antibody (FBP1 C-20; Santa Cruz Biotechnology) and Western blotted for p53 as described before[14,20] using p53 antibody against either N-terminal epitope mapping between amino acids 32-79 (sc-98; Santa Cruz, USA) or against DNA binding domain mapping epitope between amino acids 213-217 (PAb 240, Abcam, USA).

Cloning, expression, and purification of recombinant 25kDa DNA-binding-domain (DBD) of p53

We constructed a clone of p53-DBD by cloning a PCR amplified DNA fragment encoding 25 kDa $p53^{DBD}$ (aa 101-292) and ligating it into a pET-28a vector (pET28a-$p53^{DBD}$) at HindIII and BamH1 restriction site. *E. coli* Rosetta (DE3) cells were transformed with recombinant pET28a-$p53^{DBD}$ and grown at 37°C in LB medium containing 30 µg/mL kanamycin until an OD_{595} of 0.4 was achieved. The culture medium was cooled to 26°C and then supplemented with IPTG to a final concentration of 1.0 mM. Cells were further incubated at 26°C for 16 h with vigorous shaking, then chilled and harvested by centrifugation. The cells were washed, lysed, and His-tagged recombinant protein was isolated by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) column as described[14].

Construction of p53 mutants carrying deletion or point mutation in the DNA binding domain

The mammalian expression clone of WT human p53 was used as the template to generate deletion and

point mutations in the DBD using Agilent site-directed mutagenesis kit. Deletions were carried out by introducing stop codon at positions267, 207, 184, 163, and 136in the DBD spanning residue 101-292. Point mutations were carried out at positions 175 (Arg→His) and 176 (Cys→Ser) in the p53 DBD.

Expression and purification of FBP1

The recombinant clone of FBP1 (pET28a-FBP1) was expressed in *E. coli* Rosetta (DE3) cells and affinity purified using Ni-NTA and Hi-Trap Heparin columns as previously described[14].

P53-DNA binding assay

The p53-DNA binding assay was done by the electrophoretic mobility shift assay using Cy3 labeled (top strand) 30-mer duplex WAF side DNA **(**top strand- 5'CGA GGA ACA TGT CCC AAC ATG TTG CTG GAG-3'; bottom strand 3'-GCT CCT TGT ACA GGG TTG TAC AAC GAG CTC-5')as previously described[14].

Results

FBP1 interacts with all naturally occurring isoforms of p53

Recently, several naturally occurring isoforms of the human p53 have been identified in cancer cells[18]. These isoforms are formed due to alternative usage of splicing, promoter, and translation initiation sites. These isoforms carry deletions at either the N-terminus, C-terminus, or both but contain nearly intact DNA binding domain (Fig. 1A). Since we have shown that FBP1 physically interacts with the wild type p53 and inhibits its DNA binding function[14], we examined whether FBP1 interacts with these naturally occurring isoforms of p53. We individually transfected mammalian expression clones of each p53-isoforms into p53 null HCT116 cells (HCT116 p53-/-) in which FBP1 is abundantly expressed.

The wild type p53 clone was also transfected as a positive control. The FBP1 immunoprecipitation (IP) was carried out on cell lysates, and Western blotted for p53 using an antibody sensitive against the p53 DNA binding domain's epitope. The results shown in (Fig. 1B**)** indicate that like the wild type p53, all the p53 isoforms were co-immunoprecipitated with FBP1 during the IP. This implies their interaction with FBP1. Since two of the p53-isoforms, Δ160p53β and Δ160p53γ (Fig. 1A), carry a large deletion at both N- and C-termini but have nearly intact DBD, the possible site of FBP1 interaction must reside within this region.

FBP1 physically interacts with the DNA binding domain of p53

To further confirm that FBP1 interacts explicitly with the DBD of p53, we constructed a recombinant clone of the 25-kDa-p53-DBD and expressed it in *E. coli* (Fig. 2A). The two proteins co-immunoprecipitated each other in a reciprocal IP on a mixture of recombinant FBP1 and 25 kDa p53-DBD. This confirms the interaction site of FBP1 is within the DBD (Fig. 2B).We then examined whether FBP1 inhibits the DNA binding activity of the recombinant $p53^{DBD}$. Results shown in Figure 2C (lanes 2-8) indicate that the recombinant p53-DBD efficiently binds to Cy3-labeled WAF-side target DNA in a concentration-dependent manner. We further confirmed that DNA binding of p53-DBD is strongly inhibited in the presence of increasing concentrations of FBP1 (Fig. 2C, lanes 10-14).

Mapping of theFBP1 interaction site on p53-DBD

We then generated several deletion mutants of the wild-type p53 by step wise C-terminal deletion in the

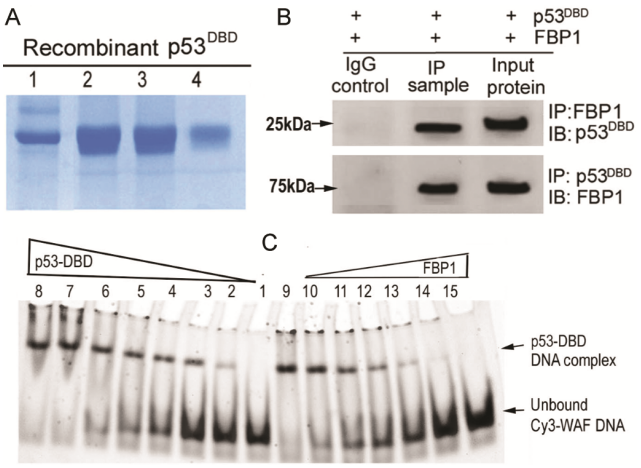

Fig. 2 — FBP1 binds to the central core DNA binding domain of p53 and disrupts its DNA binding function. (A) Thirty microliters of Ni-NTA column fractions of recombinant His-tagged 25-kDa p53-DBD (aa 101-292) expressed in E. coli was used to examine the purity of recombinant protein; (B) Reciprocal FBP1-IP (upper panel) and p53-IP (lower panel) on a mixture of purified protein. We used 2μg each of purified recombinant FBP1 and p53 and carried out reciprocal IP and Western blotted for either FBP1 or p53; and (C) EMSA of recombinant p53-DBD bound to WAF side target DNA. A fixed concentration of Cy3-labelled WAF side duplex DNA (50 pmol) was incubated with an increasing concentration of recombinant p53-DBD (100-300 ng) in the absence of FBP1 (lanes 2-8) and a fixed concentration of both p53-DBD (300 ng) and Cy3-WAF side DNA (50 pmol) was incubated with increasing concentration of FBP1 (lanes 10-14). Lanes 1 and 9 represent control without and with p53-DBD, respectively

DNA binding domain. We used a mammalian expression clone of the wild-type human p53 as the template to introduce a stop codon at positions 267, 207, 184, 163, and 136 in the DNA binding domain. The choice of insertion of stop codons was such that secondary structure of the region remained unperturbed. For example, insertion of stop codons at position 267, 206 were at the end of β9 and β5, respectively. This generated Δp31, Δp24, Δp21, Δp19, and Δp16 deletion mutants of p53, respectively, (Fig. 3A). The stop codons were introduced at the end of indicated secondary structure so that the folding of the deletion derivatives remained unaffected after the deletion (Fig. 3A). These p53 deletion derivatives were then individually transfected in HCT116 (p53-/-) cells. Seventy-two hours post-transfection, FBP1-IP was performed on the cell lysates, followed by Western blotting for p53 using p53-antibody recognizing the N-terminus epitope. The results shown in Figure 3B indicate that FBP1 efficiently binds to Δp31, Δp24, and Δp21 deletion mutants but is unable to bind to Δp19 and Δp16 mutants carrying stop codons at the 163 and 136 positions, respectively. This indicates that the motif spanning from 162-182 in the p53-DBD is the site for FBP1 interaction. This region of p53-DBD contains mutational hot spots, R175 and C176, that affect the DNA binding function of p53[21],which are essential for the DNA binding function of p53[22].

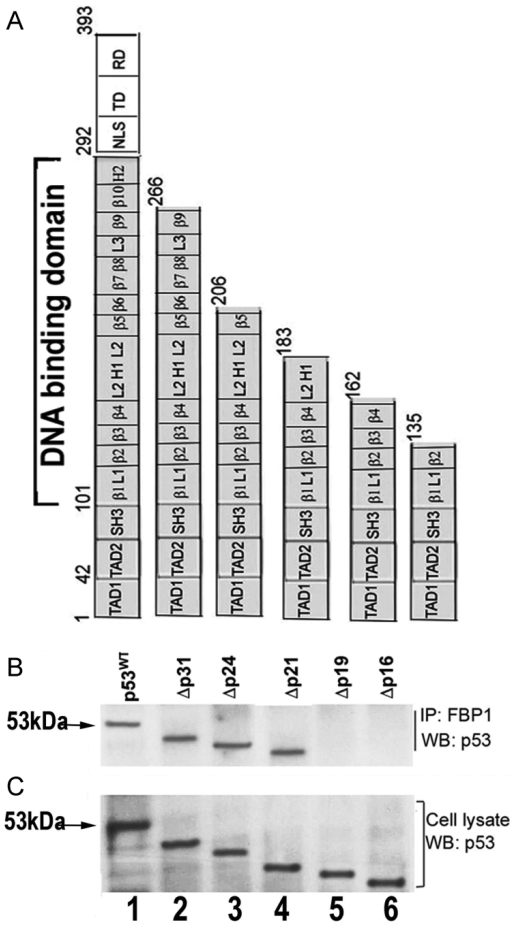

Fig. 3 — Mapping of FBP1-interacting motif of the p53-DBD (A) Using wild type p53 clone, specific deletions mutants were constructed by introducing stop codons after β9, β5, L2 Loop, helix H1, β4, and β2 at positions 267,207,184,163 and 136, respectively. (B) The wild type p53 and its deletion derivatives were transfected in p53 null HCT116 cells. Seventy-two hours post-transfection, cell lysates were prepared, and FBP1-IP was performed followed by Western blotting for p53 using p53 antibody against N-terminal epitope mapping between amino acids 32-79 (sc-98; Santa Cruz, USA). (C) An aliquot of the cell lysate was Western blotted to examine the expression of the p53 and its deletion mutants.

R175H/C176S mutations abolish FBP1 interaction with p53 in p53-DBD

After establishing that the FBP1 interacts explicitly with a motif spanning residue 162-182 in the p53-DBD, we examined whether mutational changes in this motif can affect the interaction of p53 with FBP1. This region in the p53-DBD is the site for a mutational hot spot affecting the DNA binding function of p53. R175 and C176 are the two critical residues on the L2 Loop spanning residues 164-176 in p53-DBD[21]. Mutational changes at these positions (R175H and C176Y, C176S or C176H) affect the L2 loop conformation[23] and DNA binding function of p53[22]. We, therefore, carried out R175H/C176S mutations in the wild type p53 as well as in two of its deletion derivatives, Δp31 (1-266aa) and Δp24 (1-206aa). We transfected these wild-type and mutant clones into HCT116 (p53-/-) cells. After 72 h post-transfection, we irradiated the cells with 3-Gy gamma rays to activate the p53. We prepared the benzonase-treated cell lysate six hours later and performed FBP-IP and immunoblotted for p53. The results, shown in Figure 4, indicate that the interaction of FBP1 with $p53^{WT}$ and its deletion derivatives Δp31 and Δp24, carrying wild type R175 and C176, remained unaffected (Fig 4, lanes 1-3). In contrast, FBP1 binding with each of these was drastically reduced by substituting Arg→His and Cys→Ser at positions 175 and 176, respectively, (Fig. 4, lanes 4-6). This result indicated that Arg175/Cys176, within this motif, is necessary but traces of FBP1 binding suggest that Arg175/Cys176 not sufficient for FBP1 interaction. We confirmed that mutant p53 carrying R175H/C176S mutations lost its ability to bind the

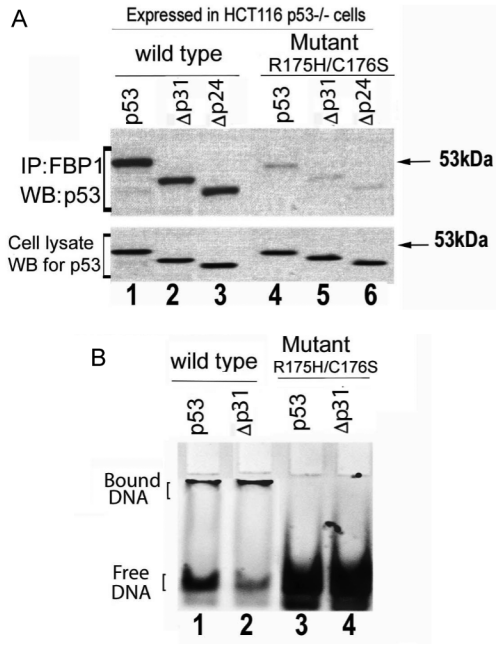

Fig. 4 — FBP1 binding to p53 carrying R175H and C176S mutations in the p53-DBD. (A) Two mutations at positions 175 (Arg→His) and 176 (Cys→Ser) were introduced in the wild type p53 and its Δp31 deletion derivative. The wild-type and mutant derivatives were transfected into p53-null HCT116 cells. Seventy-two hours post-transfection, cells were irradiated with 3-Gy gamma rays, and cell lysate were subjected to FBP-IP followed by Western blotting for p53; and (B) B) DNA binding ability of R175H/C176S mutant of p53was examined by EMSA using Cy3 labeled WAF side DNA

WAF side DNA target (Figure 4B, lanes 3, 4). Both R175 and C176 are located at the junction of β4 and the H1 helix of the L2 Loop in p53-DBD[24]. Mutational changes at these positions in p53-DBD have the potential to destabilize the L2 Loop resulting in loss of binding of both FBP1 and DNA. These results suggest that the interaction of FBP1 may be either specific to the conformation of the L2 Loop or FBP1 directly interacting and sequestering these residues to disrupt the overall stability of the L2 Loop resulting in the inhibition of the DNA binding function of p53.

Discussion

FBP1 is abundantly expressed in various malignant tumors in the liver, prostate, bladder, colon, breast, lung, gastric and glial cells of the brain and spine [10,12,14,25-27]. FBP1 influences several critical cellular functions in the initiation and progression of tumor cells[8]. FBP1 is overexpressed in chronic hepatitis C (CHC) and CHC-cirrhotic livers. It facilitates their progression into human hepatocellular carcinoma by inhibiting the p53 mediated apoptotic stimuli by direct or indirect repression of cell cycle inhibitors [12,14]. FBP1 facilitates HCV replication by inhibiting p53 which promotes E6AP mediated ubiquitination and degradation HCV Core protein[28]. P53 transactivates the transcription of cell cycle inhibitor p21, also known as p21/WAF/cip1or p21CDKN1A. P21 arrests the cell cycle progression at G1/S and G2/M transitions and promotes apoptosis[29]. FBP1 strongly suppresses the transactivation activity of p53 by blocking its binding to the promoter site DNA of WAF/ p21/cip1and thus facilitates HCV replication[14,20]. Recently Frost *et al*., have shown that *FBP*1 mediated suppression of p53 is required for human adenovirus (HAdV) replication. The early 1A (E1A) protein of HAdV down regulates p53 function by in interacting with p53 *via* FBP1[15].

We have shown that one of the direct targets of FBP1 is the tumor suppressor p53, with which FBP1 physically interacts and blocks the ability of p53 to bind its target DNA sequences[14]. P53 is a transcription factor that regulates a wide variety of cell functions involving DNA repair, apoptosis, and senescence in response to stress[30,31]. The central DNA binding core domain of p53 is the site for most mutational changes detrimental to its DNA binding function[32]. The core domain is highly unstable, melting at ≈42–44°C[33]. Many oncogenic mutations in the core domain are responsible for destabilizing or distorting its structure, thus inactivating the DNA binding function of p53[34,35].

This study demonstrates that FBP1 interacts with all the naturally occurring p53-isoforms carrying large deletions at the N- and C-terminal regions with an intact DBD (Fig 1 A and B). This suggests that the core DBD could be the site of FBP1 interaction. To ascertain this, we used a recombinant 25 kDa core DBD and confirmed its interaction with FBP1 (Fig. 2AB). We also demonstrated that the DNA binding function of the recombinant p53-DBD is intact, and its binding to target DNA is strongly inhibited in the presence of FBP1 (Fig. 2C). Mapping of the FBP1-interaction site on the DBD identified a 21-residue long motif spanning amino acid residues 163-183 (Fig. 3AB). In the structure of core p53-DBD complexed with DNA (PDB:4hje), this region encompasses β4 and H1 helix of the extended L2 Loop spanning residues 164-194,which influences the DNA binding function of p53 (Fig. 5A). We further confirmed that R175 and C176, located at the junction of β4 and H1 helix in the L2 Loop, are essential for

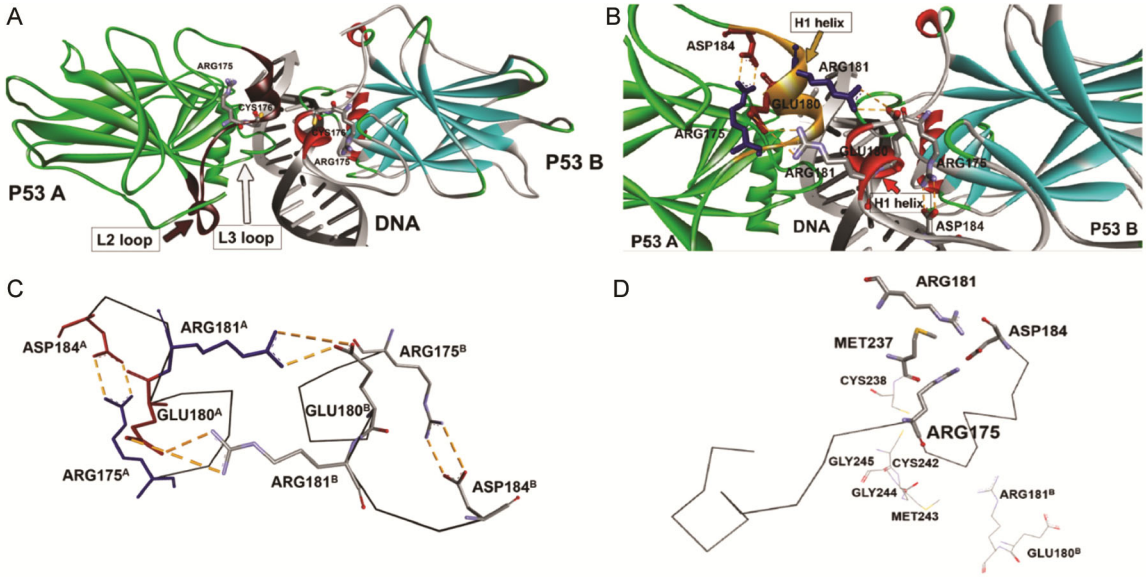

Fig. 5 —Structural location of pertinent features of FBP1-interacting motif of p53-DBD. Representations of the 3-D crystal structure of the p53-DNA complex (PDB# 4hjeA) were performed in Biovia Discovery Studio Visualizer V19.1.0.18287 from Dassault Systems. (A) Location of L2-loop predicted to be essential for FBP1 binding. P53 chain A and Chain B are shown in green with their L2 loops in maroon. Residues R176 and C176 are labeled and shown in heavy-atom stick representation; (B and C) Saltbridge interaction between R175 and D184. The salt-bridge interaction between R175 and D184 within the L2 Loop shown in the dotted line seems to stabilize the extended L2 loop H1 helix, which is essential for P53 dimerization and DNA binding; and (D) Helix H1 interaction outside of L2 loop. Residues interacting with the H1 helix are shown in small font, while residues interacting with R175 are labeled in large font and in heavy-atom stick representation

FBP1 interaction with p53. Both R175 and C176 are mutational hotspots in p53-DBD. The R175H mutation is one of the most common somatic mutations detected in most human cancers[32,36]. The p53-DBD carrying the R175H mutation in the L2 Loop is thermodynamically unstable at physiological temperature and unable to bind to its target DNA. This indicates that native conformation of L2 Loop in the DBD is required for the DNA binding function of p53.

Using the biological assembly of the p53 tetramer structure in complex with DNA (PDB: 4hje), we examined the positioning of R175 and C176 and their interactions with adjacent residues in a ground energy state. The DNA binding surface is constituted by two large loops, L2 and L3 intercalated by a short helix, H1. These loops are stabilized by the tetrahedral coordination of a single zinc ion by C176 and H179 of L2 and C238 and C242 of L3[37]. R175 also mediates the interaction between L2 and L3 loops and confers structural stability to p53-DBD[38]. The most notable interaction of R175 is with D184 within the L2 Loop (Fig. 5B). It forms a well-aligned salt bridge with D184, which appears to pin the L2-loop into a kink. R175 not only forms a salt-bridge with D184, but it also forms a salt-bridge with the backbone C=O of P191, which may be more important than the salt-bridge with D184 since proline residues are known to disrupt loop structures. This presumably stabilizes both L2-loop and H1-helix in a conformation appropriate for the DNA binding function of p53 (Fig. 5B). Why FBP1 does not bind to these mutants of p53? It is possible that native conformation of L2 Loop is required for interaction with FBP1. TheR175 also displays hydrophobic interaction with R196 in the beta-sheet that follows the L2 Loop. The H1 helix interacts with R181 and E180 on the adjacent p53 subunit (Fig. 5D).

In the DNA-bound tetrameric p53 structure, H1 helix of one p53 subunit interacts with H1 helix of the adjacent p53 subunit via the formation of the inter-subunit salt bridge between their E180 and R181 (Figs. 5B and 5C).Substitution of Arg→Pro at position 181 has been shown to affect the stability of the L2 Loop in the p53-DBD[23]. This suggests a critical role of native conformation of L2 Loop in the dimerization of p53.FBP1 binding to this region may destabilize the conformation of the L2 Loop and thus affect the dimerization process of p53.

Conclusion

We discovered that FBP1 inhibits the DNA binding activity of p53 by physically interacting with 21

residues long motif within the L2-loop of p53-DBD. The Arg 175 and Cys 176 within this motif stabilizes the conformation of the L2-loop, which is necessary for DNA binding. Sequestering of these residues by FBP1 destabilizes the L2-loop and blocks the DNA binding function of p53.

Acknowledgment

This work was supported by NIDDK, the National Institute of Health Grant DK083560 (to VNP).

Conflict of interest

All authors declare no conflict of interest.

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