A Conserved Mechanism for Binding of p53 DNA-Binding Domain and Anti-Apoptotic Bcl-2 Family Proteins

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The molecular interaction between tumor suppressor p53 and the anti-apoptotic Bcl-2 family proteins plays an essential role in the transcription-independent apoptotic pathway of p53. In this study, we investigated the binding of p53 DNA-binding domain (p53DBD) with the anti-apoptotic Bcl-2 family proteins, Bcl-w, Mcl-1, and Bcl-2, using GST pull-down assay and NMR spectroscopy. The GST pull-down assays and NMR experiments demonstrated the direct binding of the p53DBD with Bcl-w, Mcl-1, and Bcl-2. Further, NMR chemical shift perturbation data showed that Bcl-w and Mcl-1 bind to the positively charged DNA-binding surface of p53DBD. Noticeably, the refined structural models of the complexes between p53DBD and Bcl-w, Mcl-1, and Bcl-2 showed that the binding mode of p53DBD is highly conserved among the anti-apoptotic Bcl-2 family proteins. Furthermore, the chemical shift perturbations on Bcl-w, Mcl-1, and Bcl-2 induced by p53DBD binding occurred not only at the p53DBD-binding acidic region but also at the BH3 peptide-binding pocket, which suggests an allosteric conformational change similar to that observed in Bcl-X$_L$. Taken altogether, our results revealed a structural basis for a conserved binding mechanism between p53DBD and the anti-apoptotic Bcl-2 family proteins, which shed light on to the molecular understanding of the transcription-independent apoptosis pathway of p53.

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INTRODUCTION

The tumor suppressor p53 is a transcription factor that mediates cell cycle arrest, DNA repair, apoptosis, and senescence in response to diverse cellular stresses such as DNA damage, ultraviolet light, and oncogene activation (Harris and Levine, 2005; Vogelstein et al., 2000; Vousden and Lu, 2002). In more than 50% of human cancers, p53 function is inactivated through p53 gene mutation or through abrogation of p53 pathway signaling (Brown et al., 2009; Hollstein et al., 1991). Thus, reactivating p53 pathway is an attractive strategy for cancer therapy (Brown et al., 2009; Fesik, 2005). p53 can mediate apoptosis in the transcription-dependent as well as transcription-independent mechanisms. Within the nucleus, p53 induces transcription-dependent apoptosis through transcriptional activation of various pro-apoptotic target genes, such as Bax and Puma. While most studies have concentrated on the transcription-dependent apoptotic pathway of p53, there are also some recent evidences supporting a transcription-independent apoptotic pathway of p53 (Chi, 2014; Chipuk et al., 2004; 2005; Green and Kroemer, 2009; Jiang et al., 2006; Leu et al., 2004; Matissek et al., 2013; Mihara et al., 2003). In this transcription-independent pathway, p53 plays a direct apoptogenic function at the mitochondria in response to apoptotic stress (Mihara et al., 2003). This mechanism is mediated through the interaction of mitochondrial p53 with anti-apoptotic as well as pro-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins (Moll et al., 2005; Vaseva and Moll, 2009).

The Bcl-2 family proteins play a key role in mitochondrial apoptosis as they regulate the permeability of the outer mitochondrial membrane and the release of cytochrome c (Adams and Cory, 1998; Danial, 2007; Lessene et al., 2008; Youle and Strasser, 2008). The Bcl-2 family protein members can be classified into pro- and anti-apoptotic members, based on the modular structure of Bcl-2 homology (BH) domains. Pro-apoptotic Bcl-2 family proteins contain three conserved BH domains named BH1, BH2, and BH3 (e.g. Bak, Bax, and Bok) or a single BH3 domain (called BH3-only protein). Anti-apoptotic Bcl-2 family proteins such as Bcl-X$_L$, Bcl-2, Bcl-w, and Mcl-1, have four BH domains named BH1-BH4. Interactions between anti-apoptotic and pro-apoptotic Bcl-2 family members are important in maintaining the balance between cell death and survival. In its transcription-independent apoptotic pathway, p53 has been

shown to interact with anti-apoptotic Bcl-2 family proteins at the mitochondria, leading to lipid pore formation by pro-apoptotic effectors, such as Bak and Bax, at the outer mitochondrial membrane and the release of cytochrome c into the cytoplasm (Moll et al., 2005; Vaseva and Moll, 2009). However, the details of the molecular interactions between p53 and the anti-apoptotic Bcl-2 family proteins are unclear.

While the p53 DNA binding domain (p53DBD) was initially suggested to the binding site for anti-apoptotic Bcl-2 family proteins (Sot et al., 2007; Tomita et al., 2006), recent biochemical and structural studies have shown that the MDM2-binding motif in the p53 transactivation domain (p53TAD) binds to the anti-apoptotic Bcl-2 family proteins in a similar mode of binding to MDM2 (Ha et al., 2009; 2011; 2013; Shin et al., 2013; Xu et al., 2009; Yao et al., 2013). More recently, it was also shown that p53DBD induces a structural change in the pro-apoptotic BH3 peptide-binding region of Bcl-X_L to prompt further interaction with pro-apoptotic BH3-only proteins (Hagn et al., 2010). However, it is still unknown whether this binding mechanism is specific to Bcl-X_L or if it is common to the other Bcl-2 protein family members.

As the anti-apoptotic Bcl-2 family proteins, including Bcl-X_L, have similar three-dimensional structures (Petros et al., 2004), we hypothesized that the other anti-apoptotic Bcl-2 family members might also interact with p53DBD in a manner analogous to that found for Bcl-X_L. To test this hypothesis, we performed p53DBD binding experiments for several anti-apoptotic Bcl-2 family members using GST pull-down assays and NMR spectroscopy. The refined structural models reported here for the p53DBD in complex with Bcl-w, Mcl-1, and Bcl-2 indicated that these anti-apoptotic Bcl-2 family members bind to the DNA-binding surface of p53DBD in a manner similar to that found for Bcl-X_L. These results have revealed a structural basis for a conserved binding mechanism of p53DBD among the anti-apoptotic Bcl-2 family proteins, which might provide further insights into the transcription-independent apoptotic pathway of p53.

MATERIALS AND METHODS

Cell culture and transfection
BOSC 23 cells (human embryonic kidney cells) were cultured in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For transfection, each well of a six-well plate was seeded with 7×10^5 BOSC 23 cells. The cells were transfected 20 h later with the plasmids described below, by using Lipofectamine LTX (Invitrogen, USA) according to the protocol provided by the manufacturer.

Cloning
The p53DBD (residues 94-312), fused with an N-terminal glutathione S-transferase (GST) tag, was expressed in BOSC 23 cells using a pEBG vector. As described previously (Ha et al., 2011), the full-length Bcl-2, Bcl-w, and Mcl-1 were expressed using an N-FLAG CMV2 vector (Invitrogen) that expresses proteins with an N-terminal FLAG tag.

GST pull-down assay
After transfection, BOSC 23 cells were cultured for 24 h and then washed with PBS followed by cell lysis with a buffer containing 10 mM HEPES (pH 7.4), 150 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA and 1% NP-40 supplemented with complete EDTA-free® protease inhibitor cocktail tablets (Roche). As previously described (Ding et al., 2012; Wang et al., 2013; Yoon et al.,

2011), cell extracts were then incubated with 20 μl of Glutathione Sepharose TM 4B beads (Amersham Biosciences, USA) at 4°C overnight. The beads were washed three times for 5 min each with 1 ml of NP-40 buffer containing protease inhibitors and then resuspended in 35 μl of SDS sample buffer and boiled for 5 min. The precipitated complexes were analyzed by western blot with an anti-FLAG antibody (Sigma).

Protein expression and purification
Unlabeled and uniformly ^{15}N-labeled p53DBD protein was expressed using the pET21a vector in *Escherichia coli* BL21 (DE3) RIL cells. One hour prior to induction, ZnSO$_4$ was added to give a final concentration of 0.1 mM. After induction with 0.1 mM of isopropyl β-D-thiogalactoside (IPTG), the cells were grown for 20 h at 10°C. The p53DBD protein was then purified using a SP-HiTrap ion exchange column, Heparin HiTrap column, and a Superdex 75 FPLC column. Unlabeled and uniformly ^{15}N-labeled Bcl-2 truncated chimera, Bcl-w (1-157), and hMcl-1^{BLR} chimera were expressed and purified for NMR experiments as previously reported (Czabotar et al., 2007; Denisov et al., 2003; Lee et al., 2011; Petros et al., 2001).

NMR Spectroscopy
The NMR data were acquired using a Bruker Avance II 800 spectrometer equipped with a cryogenic probe at the Korea Basic Science Institute. The 2D ^{15}N-^{1}H HSQC spectra of the ^{15}N-labeled p53DBD were obtained at 20°C in the absence or presence of the anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-w, and Mcl-1). The NMR samples, comprised of 90% H$_2$O/10% D$_2$O, were prepared in 20 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM DTT, and 10 μM ZnSO$_4$ for p53DBD, 20 mM TrisHCl (pH 7.8), and 5 mM DTT for Bcl-2, 50 mM NaCl, 0.5 mM EDTA and 3 mM DTT for Bcl-w; and 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.5 mM benzamidine and 0.1 mM PMSF for Mcl-1. For the chemical shift perturbation experiments with Bcl-2, Bcl-w, and Mcl-1, aliquots of concentrated p53DBD stock solution were added to the ^{15}N-labeled Bcl-2, Bcl-w, and Mcl-1 during titration and the 2D ^{15}N-^{1}H HSQC spectra were collected at 25°C (for Bcl-2 and Mcl-1) or 30°C (for Bcl-w). The chemical shift assignments for p53DBD and the anti-apoptotic Bcl-2 family proteins were performed as previously described (Ha et al., 2011; 2013; Shin et al., 2012; Wong et al., 1999). All the NMR data were processed and analyzed using an NMRPipe/NMRDraw (Delaglio et al., 1995) and SPARKY software.

Structure calculation
The structures of the p53DBD/Bcl-w, p53DBD/Mcl-1, and p53DBD/Bcl-2 complexes were calculated using the programs ZDOCK and RDOCK of the Discovery Studio 3.1 software package (Chen et al., 2003). The binding site between p53DBD and the anti-apoptotic Bcl-2 family proteins was defined as those residues showing a significant chemical shift perturbation value with relatively large per-residues solvent accessibility. Starting from the unbound structures of Bcl-w (PDB code: 1MK3), Mcl-1 (PDB code: 2NLA), Bcl-2 (PDB code: 1GJH), and p53DBD (PDB code: 2FEJ), 3,600 possible binding poses of the complexes were calculated and evaluated by ZDOCK. The top 100 high-scoring and well-clustered complexes resulting from ZDOCK analysis were selected for RDOCK refinement using CHARMM polar H energy. The resulting docking solutions were clustered and the interaction energies of them were calculated and compared. Figures of the complex model were drawn using the PyMOL software package (DeLano, 2002).
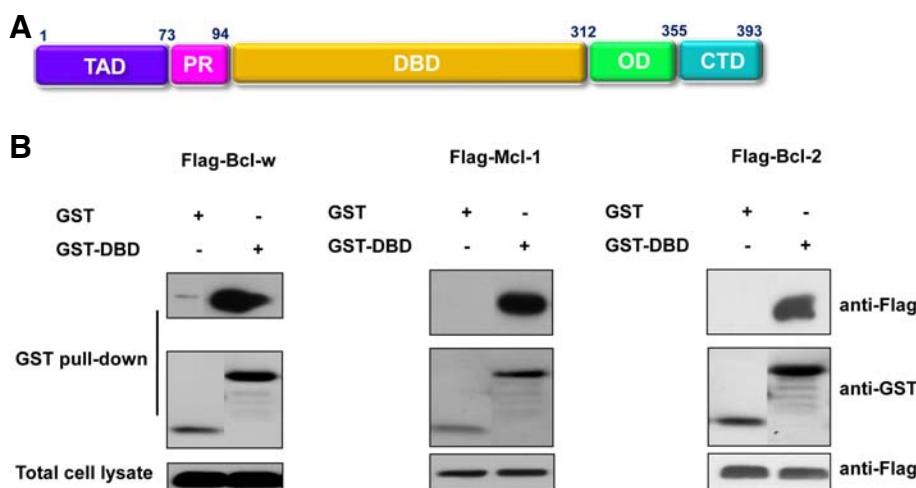
A

B

Fig. 1. Interaction of the p53DBD with anti-apoptotic Bcl-2 family proteins. (A) Structural organization of p53 showing the transactivation domain (TAD), proline-rich domain (PR), DNA-binding domain (DBD), oligomerization domain (OD), and C-terminal domain (CTD) (B) GST pull-down assays for the binding of GST-tagged p53DBD to Flag-tagged Bcl-2 family members (Bcl-w, Mcl-1, and Bcl-2).

RESULTS AND DISCUSSION

p53DBD binds to diverse members of the anti-apoptotic Bcl-2 family proteins

To test whether p53DBD binding is common to multiple anti-apoptotic Bcl-2 family members, we performed GST pull-down assays. BOSC 23 cells were co-transfected with GST-tagged p53DBD and FLAG-tagged Bcl-w, Mcl-1, and Bcl-2 expression plasmids. After transfection, GST-tagged p53DBD and the bound proteins were pulled down by incubation with glutathione agarose beads, and the proteins were immunoblotted with anti-GST and anti-FLAG antibodies. As shown in Fig. 1, GST-tagged p53DBD directly bound FLAG-tagged Bcl-w, Mcl-1, and Bcl-2, indicating universal interactions between p53DBD and diverse members of anti-apoptotic Bcl-2 family proteins.

Mapping of the binding surface of Bcl-w and Mcl-1 with the p53DBD

To define the binding surface of Bcl-w and Mcl-1 in complex with p53DBD, we monitored the binding of the ^{15}N-labeled p53DBD to these anti-apoptotic Bcl-2 family proteins using NMR spectroscopy. In the overlaid 2D ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled p53DBD in the absence or presence of Bcl-w or Mcl-1, we observed significant chemical shift changes and the disappearance of many ^{15}N-^{1}H crosspeaks upon addition of Bcl-w or Mcl-1 (Figs. 2A and 2B). The disappearance of these p53DBD crosspeaks in the spectra seems to be caused by severe line broadening due to intermediate exchange on the NMR chemical shift time scale. The chemical shift assignments for the ^{15}N-^{1}H crosspeaks of p53DBD revealed that, upon the addition of Bcl-w and Mcl-1, significant NMR chemical shift perturbations were localized to the L1, L2/α1, L3, and α2 regions in p53DBD (Fig. 2C), indicating that the binding sites for Bcl-w and Mcl-1 overlap with the positively charged DNA-binding surface of p53DBD. These data are in corroboration with that observed during the binding of p53DBD to Bcl-X$_L$ (Hagn et al., 2010). Although the chemical shift perturbations induced by Mcl-1 were stronger than those induced by Bcl-w, the perturbed regions in the p53DBD induced by both the proteins were similar (Figs. 2B and 2C), indicating that multiple members of the anti-apoptotic Bcl-2 family share a similar binding surface of p53DBD. It is noteworthy that p53DBD utilizes the same DNA-binding surface to mediate transcription-independent interactions with the anti-apoptotic Bcl-2 family proteins at the mitochondria as it does during transcription-dependent interaction with DNA within the nucleus. Thus, p53DBD serves a critical role in both transcription-dependent and transcription-independent apoptosis.

NMR Chemical shift perturbations in the anti-apoptotic Bcl-2 family proteins induced by p53DBD binding

For mapping of the p53DBD binding site on the anti-apoptotic Bcl-2 family proteins, we examined the binding of the ^{15}N-labeled Bcl-w, Mcl-1, and Bcl-2 to p53DBD by using NMR spectroscopy. Upon the addition of p53DBD, we found severe line broadening and significant chemical shift changes on ^{15}N-^{1}H crosspeaks of Bcl-w, Mcl-1, and Bcl-2 (Fig. 3A). NMR chemical shift perturbations induced by p53DBD binding were mapped onto the structures of Bcl-w, Mcl-1, and Bcl-2 to determine the binding site at the three-dimensional level (Fig. 3B). Significant chemical shift perturbations on Bcl-w, Mcl-1, and Bcl-2 induced by p53DBD binding occurred in the acidic regions of these proteins, composed of α1/2, α3/4, and α5/6 loops, at the bottom side of the molecules. For example, the strongly perturbed residues of Bcl-2 included Arg26, Gly27, Trp30, Asp31, Ala32, and Gly33 between helix 1 and 2 (α1/2), Thr122 between helix 3 and 4 (α3/4), and Arg164, Glu165, Leu169, and Val170 between 5 and 6 (α5/6). Interestingly, noticeable chemical shift perturbations were also observed at the BH3 peptide-binding pocket surrounded by the BH1, BH2 and BH3 domains. In accordance with the previously observed chemical shift perturbation profile of Bcl-X$_L$ (Hagn et al., 2010), these results suggest that p53DBD binding leads to similar structural changes at the BH3 peptide-binding pocket in diverse members of the anti-apoptotic Bcl-2 protein family.

Structural basis of the interaction between p53DBD and the anti-apoptotic Bcl-2 family proteins

To understand the structural basis for the interaction between p53DBD and the anti-apoptotic Bcl-2 family proteins, we calculated refined structural models for p53DBD in complex with Bcl-w, Mcl-1, and Bcl-2 using the programs ZDOCK and RDOCK. The structural comparison of these complex models revealed that the overall binding mode of p53DBD is highly conserved among the anti-apoptotic Bcl-2 family proteins (Fig. 4). The refined structural models of the p53DBD complexes are also
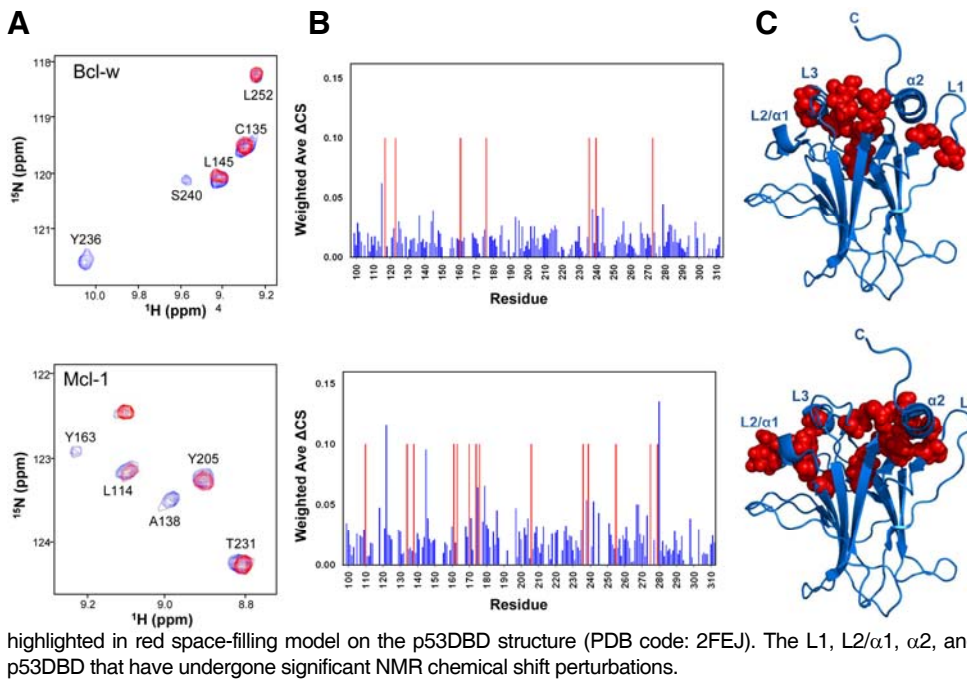
Fig. 2. Mapping of the binding surface of p53DBD for Bcl-w and Mcl-1. (A) The overlaid 2D ^{1}H-^{15}N HSQC spectra for ^{15}N-labeled p53DBD in the absence (blue) or presence of Bcl-w and Mcl-1 (red). (B) NMR chemical shift perturbations on p53DBD induced by binding to Bcl-w and Mcl-1. Resonances of p53DBD that disappeared upon binding of Bcl-w and Mcl-1 are shown as red bars. The weighted ΔCS values were calculated as previously described (Stoll et al., 2001) and plotted against the residue number of the p53DBD. (C) Binding site mapping for Bcl-w and Mcl-1 on the structure of p53DBD. The p53DBD residues that were shown to disappear are highlighted in red space-filling model on the p53DBD structure (PDB code: 2FEJ). The L1, L2/α1, α2, and L3 labels indicate regions of the p53DBD that have undergone significant NMR chemical shift perturbations.
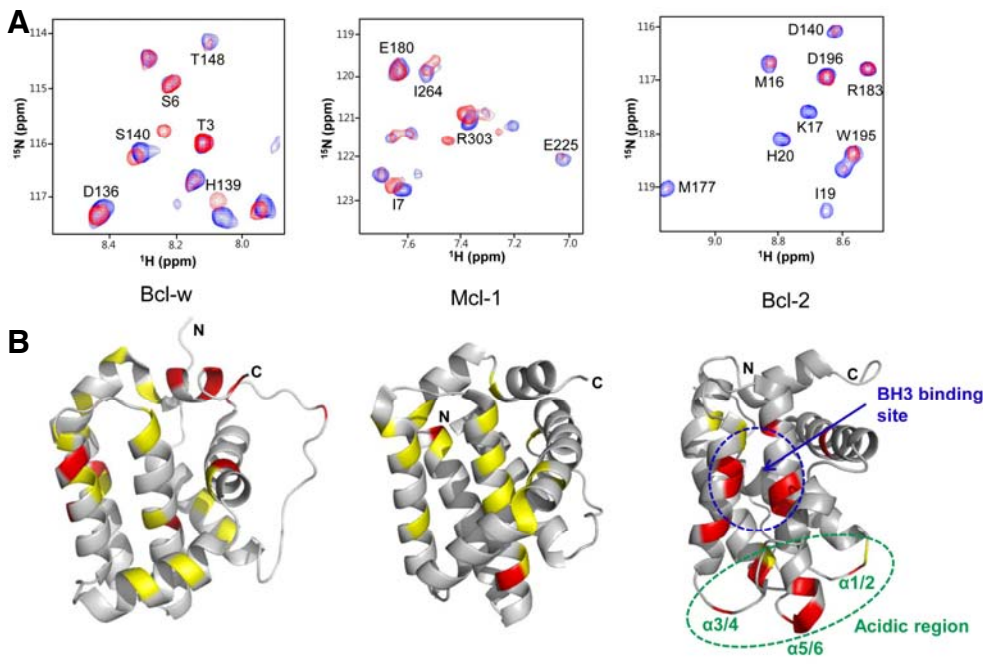

Fig. 3. NMR chemical shift perturbations of the anti-apoptotic Bcl-2 family proteins, Bcl-w, Mcl-1, and Bcl-2 induced by p53DBD binding. (A) The overlaid 2D ^{1}H-^{15}N HSQC spectra for ^{15}N-labeled Bcl-w, Mcl-1, and Bcl-2 in the absence (blue) or presence (red) of p53DBD. (B) The chemical shift perturbations were mapped onto the molecular surface of Bcl-w (left panel), Mcl-1 (middle panel), and Bcl-2 (right panel). The residues that were observed to disappear and the residues showing significant chemical shift changes are colored in red and yellow, respectively. The α3/4, α5/6, and α1/2 labels indicate regions of the Bcl-2 that have undergone significant NMR chemical shift perturbations.

similar to that of Bcl-X$_L$/p53DBD complex (Hagn et al., 2010). Consistent with the chemical shift perturbation data, the structural models of the p53DBD complexes showed that the L1, L2/α1, L3, and α2 loops of p53DBD come in contact with the α1/2 and α5/6 loops of Bcl-w, Mcl-1, and Bcl-2 (Fig. 4B), indicating that the binding interface is formed by the positively charged DNA-binding surface of p53DBD and the negatively charged bottom region of the anti-apoptotic Bcl-2 family proteins. In particular, the electrostatic interactions between Arg248

in p53DBD and Glu29 in Bcl-2 and between Arg280 in p53DBD and Asp31 and Asp34 in Bcl-2 make important contributions to complex formation (Fig. 4D).

Conserved binding mechanism of p53DBD among the anti-apoptotic Bcl-2 family proteins

In the present study, the GST pull-down assay and NMR data have shown that p53DBD is universally involved in the binding of diverse members of the anti-apoptotic Bcl-2 family proteins.
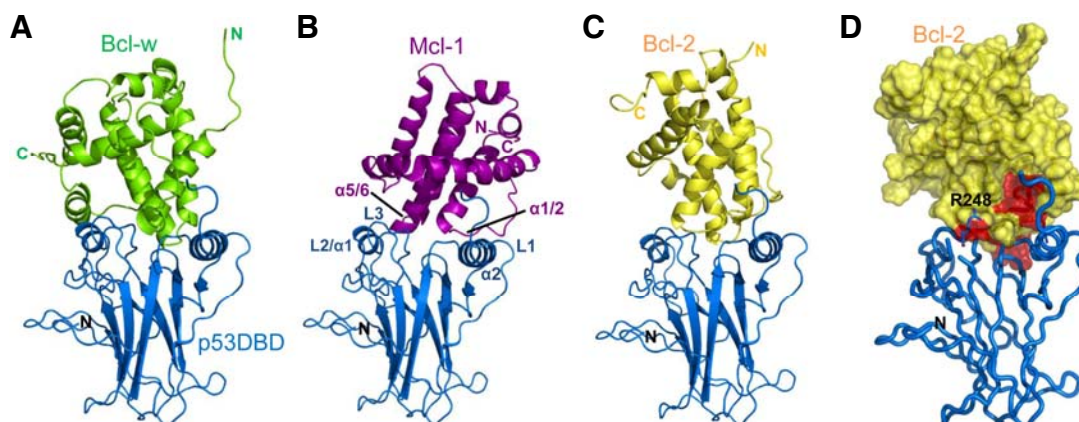
Fig. 4. Refined structural models of the complexes formed between p53DBD and the anti-apoptotic Bcl-2 family proteins. The structural models of the p53DBD/Bcl-w (A), p53DBD/Mcl-1 (B), and p53DBD/ Bcl-2 (C) complexes are shown. (D) Molecular surface representation of the p53DBD/Bcl-2 complex structure. Chemical shift perturbations of Bcl-2 induced by p53DBD binding are mapped onto the surface of Bcl-2 in red. The key binding residue (R248) in Bcl-2 is labeled. The L2/α1, L3, L1, and α2 loops of the p53DBD appear to come in contact with the α5/6 and α1/2 loops present in the anti-apoptotic Bcl-2 family proteins, as highlighted in (B) for Mcl-1.

This is in contrast with the observation that the anti-apoptotic Bcl-2 family proteins have specific binding affinity for the pro-apoptotic Bcl-2 family members, despite their having similar overall structures (Petros et al., 2004). For example, previous studies indicated that Bcl-2, Bcl-X$_L$ and Bcl-w bind to Bad whereas Mcl-1 and A1 bind to Noxa (Adams and Cory, 1998; 2007; Youle and Strasser, 2008). Indiscriminate binders such as Bim, Puma and tBid are known to be much more potent inducers of apoptosis. In this study, we have shown that p53DBD contains a universal binding site for diverse members of the anti-apoptotic Bcl-2 family proteins. Thus, we suggest that p53DBD can inhibit multiple anti-apoptotic Bcl-2 family members simultaneously, which may be more effective in neutralizing the anti-apoptotic functions of this protein subfamily during transcription-independent apoptosis of p53.

The refined structural models of the complexes formed by the p53DBD and the anti-apoptotic Bcl-2 family proteins reveals a molecular basis for how the p53DBD universally binds to diverse members of this protein family. A structural comparison of the p53DBD/Bcl-2 family protein complexes with the Bcl-X$_L$/p53DBD complex (Hagn et al., 2010) showed that the binding mechanism of the p53DBD is highly conserved among the anti-apoptotic Bcl-2 family proteins. First, the binding sites of Bcl-w, Mcl-1, Bcl-2, and Bcl-X$_L$ are commonly located at the positively charged DNA-binding surface of p53DBD. Second, similar to Bcl-X$_L$, p53DBD binding induced the chemical shift perturbations at the p53DBD-binding acidic region as well as at the BH3 peptide-binding pocket in Bcl-w, Mcl-1, and Bcl-2. This suggests a similar p53DBD-binding mechanism to that of Bcl-X$_L$, whereby p53DBD binding induces a structural change at the BH3 peptide-binding pocket in Bcl-X$_L$, facilitating the interaction with pro-apoptotic BH3-only proteins in an allosteric mode of action (Hagn et al., 2010).

Previously, we also showed that p53TAD binds to diverse members of the anti-apoptotic Bcl-2 protein family to mediate transcription-independent apoptosis (Ha et al., 2009; 2011; 2013; Xu et al., 2009). Taken with our current results, the biological significance for having two anti-apoptotic Bcl-2 family protein binding domains in p53, p53TAD and p53DBD, is not clear. We speculate that having two tandemly linked binding domains,

capable of binding to distinct sites of a single binding partner, may work in a cooperative manner to increase the overall binding affinity. It is possible that both p53DBD and p53TAD simultaneously bind to the two distinct sites (p53DBD binds to the acidic region, while p53TAD the BH3 peptide-binding site) in the anti-apoptotic Bcl-2 family proteins. This mechanism is reminiscent of the double-hitting models of p53 binding to MDM2 (Yu et al., 2006) and BRCA2 (Rajagopalan et al., 2010). Such a binding mechanism mediated by the two domains in p53 may be more effective for binding site competition with the BH3 motif of the pro-apoptotic Bcl-2 family proteins, which bind to the same site on the anti-apoptotic Bcl-2 family proteins.

Taken together, the data presented here indicate a role for the p53DBD in the binding of multiple anti-apoptotic Bcl-2 family members. The mechanism of this binding provides important insight into the sequestration of anti-apoptotic Bcl-2 family proteins leading to the transcription-independent mitochondrial apoptosis of p53.

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