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The flexible loop of Bcl-2 is required for molecular interaction with immunosuppressant FK-506 binding protein 38 (FKBP38)

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Abstract

Bcl-2 contains an unusually long loop between the first and the second helices. This loop has been shown to be highly flexible based on NMR and X-ray crystallographic analyses of this region. Bcl-2 is regulated at the posttranslational level through phosphorylation of specific residues within the flexible loop. The biological role and posttranslational modifications of the loop of Bcl-2 is currently unclear. FK-506 binding protein 38 (FKBP38) has been reported to interact with Bcl-2, suggesting that FKBP38 could act as a docking molecule to localize Bcl-2 at the mitochondrial membrane [Shirane, M. and Nakayama, K.I. (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat. Cell Biol.* 5, 28–37]. Here, we investigated the molecular interaction between FKBP38 and Bcl-2, and demonstrated that Bcl-2 interacts with FKBP38 through the unstructured loop, and the interaction appears to regulate phosphorylation in the loop of Bcl-2.

Keywords: Apoptosis; Bcl-2; FK-506 binding protein 38; Chaperone; Peptidylprolyl *cis-trans* isomerase; Phosphorylation

1. Introduction

Apoptosis is an essential and well-orchestrated cellular regulatory mechanism in which pro- and antiapoptotic proteins are involved at various stages. The proteins of Bcl-2 family are central to the regulation of apoptosis [1–4]. Three-dimensional structures of Bcl-Xl and Bcl-2 reveal that such proteins contain an elongated hydrophobic cleft through which the antiapoptotic proteins and their ligands interact to form heterodimers [5,6]. Another intriguing feature from the three-dimensional structures is that Bcl-2 and Bcl-Xl contain a disordered long loop with about 60 amino acid residues between the first and the second helices. This flexible loop has been shown to be unstructured based on NMR or X-ray crystallographic analyses on this region [5].

Recently, this flexible loop has been shown to be regulated at the posttranslational levels, such as phosphorylation and ubiquitin-dependent degradation, in response to diverse external stimuli

[7–10]. Proteins containing regions of denatured or random coil structure do not normally exhibit long half-lives due to cleavage by cellular protease [11,12]. Therefore, it has been suspected that the long disordered loop of Bcl-2 and Bcl-Xl is shielded or otherwise protected from rapid degradation by other cellular proteases [7]. Currently, the association of putative chaperones or regulatory molecule including kinases with the unstructured loop is not well understood. Recently, it has been shown that immunosuppressant FK-506 binding protein 38 (FKBP38) is co-localized with Bcl-2 and Bcl-Xl at the mitochondria, suggesting that FKBP38 is a potential docking molecule for the antiapoptotic proteins [14]. However, details and characteristics of the molecular interaction between FKBP38 and the antiapoptotic proteins remain to be explored. In this study, we have performed the characterization of recombinant human FKBP38 and investigated the nature of molecular interaction between FKBP38 and Bcl-2 to understand a possible, alternative mechanism of apoptotic regulation Bcl-2.

2. Materials and methods

2.1. Materials

Antibodies against Bcl-2 and Bcl-Xl were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibody against human FKBP38 was a kind gift from Keiichi I. Nakayama. Ni²⁺-NTA resin and RNeasy Mini Kit were from Qiagen (Hilden, Germany). Immune-Star Chemiluminescent protein detection system and protein molecular weight marker were from Bio-Rad Laboratories (Hercules, CA, USA). c-Jun-N-terminal kinase 1 α 1 (JNK) was from Upstate (Lake Placid, NY, USA). [γ -³³P]ATP (3000 Ci/mmol), HiPrep 16/60 Sephacryl S200, Superdex 75 gel filtration column, and GST Purification Module were from Amersham Biosciences (Buckinghamshire, UK). Phenylmethylsulfonyl fluoride, reverse transcription-polymerase chain reaction (RT-PCR) kit and restriction enzymes were from Roche (Indianapolis, IN, USA). Ethylene glycol-bis-(β -aminoethyl ether)- *N,N,N',N'*-tetraacetic acid (EGTA) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21 (DE3) and carbenicillin were from Invitrogen (Carlsbad, CA, USA). pET29b was from Novagen (Madison, WI, USA). Synthetic Dropout (SD) medium was from Clontech (Palo Alto, CA, USA). Isopropyl-thio- β -D-galactopyranoside (IPTG) was from Promega (Madison, WI, USA).

2.2. cDNA constructs

The cDNAs coding for human FKBP38 and Bcl-2 lacking the trans-membrane domains (FKBP38 Δ TM and Bcl-2 Δ TM) were amplified using mRNA from MCF-7 breast cancer cell by RT-PCR. The cDNA coding for human Bcl-Xl has been previously described [5]. The amplified PCR products were digested with restriction enzymes *Nde*I and *Xho*I and engineered into pACYC184 and pET29b, respectively. The cDNA of FKBP38 Δ TM was also cloned into pGEX-4T-1-m1 to generate GST fusion protein. The following primers were used for amplifying the

human Bcl-2 and FKBP38: 5'-CACTCAGCATATGGCGCACGCTGGGAGAACGGGGTAC-3' (forward primer for Bcl-2); 5'-GCGAAGCTCTCGAGCTATCACCGCATGCTGGGGCCGTA-CAGTTC-3' (reverse primer for Bcl-2); 5'-CACTCAGCATATGGG-ACAACCTCCGGCGGAGGAGGCT-3' (forward primer for FKBP38); 5'-GCGAAGCTCGAGGTTGCCAGCATTTTCCGGTACAA-3' (reverse primer for FKBP38). For the construction of deletion mutants of Bcl-2 in the flexible loop region, PCR was performed according to the published protocol [6] using pET29/Bcl-2 as a template and 5' phosphorylated forward and reverse primers as shown in Table 1. The amplified DNA fragments were transferred into pET16b to generate His-tag at the N-terminus of proteins. Mutations were confirmed by dideoxy DNA sequencing.

2.3. Co-expression and purification of FKBP38 and Bcl-2 complexes

The recombinants Bcl-2 and FKBP38 were co-transformed into *E. coli* BL21 (DE3) cells. The proteins were induced by adding IPTG to 1 mM when Abs₆₀₀ was 0.8–1.0. After 2-h induction at 30 °C, the cells were harvested by centrifugation at 8000 × *g* for 10 min. The cells were suspended in the suspension buffer (20 mM phosphate buffer, pH 7.8, 0.5 M NaCl, and 5 mM 2-mercaptoethanol) and sonicated for 20 min. The cell lysate was centrifuged at 20000 × *g* for 25 min. The supernatant was loaded to Ni²⁺-NTA column and the column was washed with 30 column volumes of the washing buffer (20 mM phosphate, pH 7.2, 1 M NaCl, 20 mM imidazole, and 5 mM 2-mercaptoethanol) and eluted with the elution buffer (20 mM phosphate, pH 6.0, 0.5 M NaCl, 5 mM 2-mercaptoethanol, and 0.5 M imidazole). The proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The amount of proteins were determined by Bradford dye assay kit from Bio-Rad Laboratories.

2.4. Western blot analysis

Proteins were separated on 12.5% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was first blocked with 1% milk in TBS buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl) and then incubated with primary antibody in TBS buffer containing 0.2% milk for 2 h at 37 °C. The immunoreactivity was detected using Immune-Star Chemiluminescent protein detection system (Bio-Rad Laboratories).

2.5. Phosphorylation of Bcl-2 and Bcl-Xl

0.5 µg of the purified Bcl-2, its mutants, corresponding FKBP38/Bcl2 complex Bcl-Xl, and corresponding FKBP38/Bcl-Xl complexes were incubated with 50 ng of JNK for 4 h at 30 °C in a buffer containing 10 mM Tris–HCl, pH 7.5, 25 mM MgCl₂, 1 mM EGTA, 1 mM ATP, and 1 µCi [γ -³³P]ATP (3000 Ci/mmol). The phosphorylated samples were analyzed on 12.5% SDS–PAGE at room temperature, dried, and followed by autoradiography for visualization.

2.6. Calcineurin assay

The plasmids pBB131/MEKK-C and NpT7-5/ α -SAPK+MEK4 for expression of active JNK in *E. coli* were kindly provided by Prof. Melanie H. Cobb (University of Texas Southwestern Medical Center, USA). The purification of a large amount of active JNK was performed according to their protocol [17]. Phosphorylation of Bcl-2 using same protocol as described above except using 2 mM cold ATP. The phosphorylated Bcl-2 (P-Bcl-2) was further purified by Superdex 75 gel filtration column chromatography. The plasmid pETCN α encoding calcineurin was kindly provided by Prof. Jun O. Liu (Johns Hopkins University, USA). The yeast myristoyl-CoA: protein *N*-myristoyltransferase (NMT) was PCR amplified using yeast genomic as template and resulting PCR product was inserted into pACDUET (Novagen) with *Nco*I and *Hind*III to generate pACDUET/NMT. The pETCN α was co-transformed with pACDUET/NMT. The purification of the calcineurin and the dephosphorylation reaction were carried out as described previously [18].

2.7. Yeast two-hybrid assay

FKBP38 was cloned into pAS2-1 using *Bam*HI and *Xho*I restriction enzymes to generate GAL4 activation domain fusion protein. Bcl-2 and Bcl-2 loop deletion mutants were cloned into pACT2 using *Nde*I and *Sal*I restriction enzymes to generate GAL4 DNA binding domain fusion proteins. *Saccharomyces cerevisiae* strain PJ69-4A (*MATa his 3 leu 2 ura 3 trp 1 gal4 Δ gal 8 Δ met 2:: GAL7-lacZ GAL2-Ade 2 LYS2::GAL1-his 3*) was grown in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose. Cells were grown on the minimal synthetic dropout (SD) medium lacking Leu, Trp or SD medium lacking Leu, Trp, His to check protein–protein interaction.

2.8. GST pull down assay

GST-FKBP38 Δ TM was purified by the glutathione–Sepharose 4B resin, and further purified by Superdex 75 gel filtration column chromatography. 10 μ g of GST-FKBP38 Δ TM was incubated with 3 μ g of Bcl-2 or P-Bcl-2 on ice for 4 h, respectively. Then protein samples were mixed with 40 μ l of glutathione–Sepharose 4B resin on ice for 2 h, and washed with PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). The protein bound resin was eluted with elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl, pH 8.0). The eluted samples were analyzed in 12.5% SDS–PAGE and followed by Western blotting analysis.

3. Results

3.1. Expression and purification of recombinant human FKBP38

The cDNA coding for human FKBP38 was obtained from breast cancer MCF-7 cells, and showed that the sequence is identical with the published sequence. Results from multiple sequence alignments of the FKBP domain and other FKBP family proteins demonstrate the lack of conservation of residues involved in FK-506 binding and peptidyl prolyl *cis*–*trans* isomerase

(PPIase) activity [13] (Fig. 1). Human FKBP38 lacking the transmembrane domain (FKBP38 Δ TM) was expressed in *E. coli* cells, and purified by Ni²⁺-NTA affinity resin and subsequently further purified by Sephacryl S-200 gel filtration column chromatography. As shown in Fig. 2A, we were able to purify the FKBP38 to near homogeneity. The purified protein was stable over a period of one month at 4 °C.

3.2. Complex formation between FKBP38 and Bcl-2

To probe specific molecular interaction between FKBP38 and Bcl-2, the plasmids for FKBP38 with His-tag, and Bcl-2 without His-tag were constructed for the confirmation of complex formation as described in Section 2. The recombinants Bcl-2 and FKBP38 were co-expressed in *E. coli* BL21 (DE3) cells, purified by Ni²⁺-NTA affinity resin (Fig. 2B), and confirmed by Western blot using anti-Bcl-2 and anti-FKBP38 antibodies (Fig. 2C). FKBP38 contains His-tag at the C-terminus, while Bcl-2 has no tag. The only way to purify Bcl-2 on the Ni²⁺-NTA affinity is through the formation of the complex between Bcl-2 and FKBP38. The Western blot results confirmed that the two eluted bands from the affinity column were FKBP38 and Bcl-2, respectively. Taken together, these data suggest that recombinant human FKBP38 bound to Bcl-2, when they were co-expressed in bacterial cells.

3.3. Identification of specific interaction of FKBP38 with Bcl-2

To further define and identify a region on Bcl-2 responsible for the molecular interaction with FKBP38, several mutant forms of Bcl-2 were constructed, based on known three-dimensional structural information (Fig. 3A). In this study, we have focused on the flexible loop of Bcl-2. The wild type Bcl-2 and mutant forms of Bcl-2 with various deletions in the unstructured loop were expressed and tested their abilities to interact with FKBP38. The results showed that all mutants constructed in this study could be purified in soluble forms as shown in Fig. 3B and were stable during the experiment. From the column binding study, the wild type Bcl-2 showed clear binding to FKBP38, whereas the loop deletion mutants, Bcl2 Δ (V35-V89):6A, in which the residues from

V35 to V89 were removed and six alanine residues were replaced instead, and Bcl-2 Δ (H55-V89), in which the residues from H55 to V89 were removed, failed to bind to FKBP38 (Fig. 3C).

Bcl-2 Δ (V35- V89):6A and Bcl-2 Δ (H55-V89) lack three known phosphorylation sites, T56, S70, S87, in the flexible loop of Bcl-2 [10]. The deletion mutants Bcl-2 Δ (P65-V89), Bcl-2 Δ (V35-D64), and Bcl2 Δ (V35-G79) showed binding to FKBP38 to a considerably reduced degree.

To further confirm the interaction of FKBP38 with Bcl-2, yeast two-hybrid experiments were performed (Fig. 3D and E). The wild type Bcl-2-containing yeast cells showed growth in the selection media whereas Bcl-2 Δ loop:6A and Bcl-2 Δ (H55-V89) failed to grow in the selection

media. The results regarding Bcl-2 Δ (V35-V89):6A and Bcl-2 Δ (H55- V89) mutants were consistent with the column binding assays shown in Fig. 3C. Other loop deletion mutants, Bcl-2 Δ (P65- V89), Bcl-2 Δ (V35-D64), Bcl-2 Δ (V35-G79), unlike the column binding experiments,

showed growth under the selection media used in the yeast two-hybrid study, like the same manner as seen in the wild type Bcl-2. The discrepancy between two approaches regarding Bcl-2 Δ (P65-V89), Bcl-2 Δ (V35-D64), Bcl2 Δ (V35-G79) could be attributed to the sensitivity

difference between two procedures. Taken together, our results suggest that the molecular interaction of FKBP38 with Bcl-2 would require at least one phosphorylation site or surrounding sequences of the known three phosphorylation sites (T56, S70, S87) in the unstructured loop of Bcl-2.

3.4. FKBP38 affects phosphorylation of the unstructured loop of Bcl-2

The phosphorylation of Bcl-2 was found to be associated with its flexible loop and JNK has been reported to be one of kinases responsible for the phosphorylation of T56, S70 and S87 within the unstructured loop of Bcl-2 [10]. In this study, to investigate the role of FKBP38 in connection with the posttranslational regulation of the Bcl-2 loop, Bcl-2 phosphorylation experiment was performed using JNK and the purified Bcl-2, its truncation mutants in the flexible loop, and the corresponding FKBP38–Bcl-2 complex as substrates. The purified Bcl-2 mutants were soluble and stable (Fig. 4A). The phosphorylation results showed that JNK was able to phosphorylate the purified Bcl-2 in more than one positions, evidenced by multiple Bcl-2 bands detected (Fig. 4B, lane 1). However, the Bcl-2 in FKBP38–Bcl-2 complex showed a considerable decrease in the phosphorylation by JNK (Fig. 4B, lane 2). To further analyze the effect of FKBP38 on the Bcl-2 phosphorylation, we next examined the phosphorylation of Bcl-2Δ(V35-D64) and Bcl-2Δ(P65-V89). Our data demonstrated that Bcl-2Δ(V35-D64) and Bcl-2Δ(P65-V89) showed a difference in the phosphorylation reaction; Bcl-2Δ(V35-D64) showed a marked reduction in phosphorylation compared to Bcl-2Δ(P65-V89) (Fig. 4B, lanes 4 and 6). When FKBP38 interacts with Bcl-2Δ(V35-D64), the phosphorylation of Bcl-2Δ(V35-D64) by JNK showed a considerable reduction compared to that of Bcl-2Δ(P65-V89). Bcl-X1, a close homolog of Bcl-2, also has been shown to be phosphorylated in response to external stimuli [19,20]. To further study the effect of FKBP38 on other Bcl-2 family protein, we performed JNK-mediated phosphorylation on Bcl-X1, demonstrating that FKBP38 made little effect on the phosphorylation of full length human Bcl-X1 (Fig. 4D, lanes 1 and 2). A mutant form of Bcl-X1, BclX1Δ (M45-A84), showed no phosphorylation by JNK (Fig. 4D, lanes 3 and 4). Taken together, these results suggest that FKBP38 favorably interacts with Bcl-2 and modulates the phosphorylation reactions, in unknown and yet to identified manner, within the unstructured loop, predominantly the region containing S70 and S87, by JNK.

3.5. Phosphorylation of Bcl-2 influences molecular interaction with FKBP38

To address how JNK-mediated phosphorylation in the flexible loop of Bcl-2 affects the interaction between Bcl-2 and FKBP38, the phosphorylated Bcl-2 was prepared by JNK-mediated phosphorylation reaction in vitro (Fig. 5A), and followed by GST pull down assay (Fig. 5B). The phosphorylated Bcl-2 was a good substrate for calcineurin after phosphorylation by JNK, evident from the band shift from the higher migrating species to lower migrating species, which is same as the unphosphorylated form of Bcl-2 (Fig. 5A, lanes 2 and 3). Our pull down results demonstrated that the phosphorylated Bcl-2 showed a dramatic decrease in molecular interaction with FKBP38 (Fig. 5B, lanes 3 and 4), compared to that of the

unphosphorylated form of Bcl2 (Fig. 5B, lanes 1 and 2). These data suggest that JNK-mediated phosphorylation in the highly flexible, disordered loop of the anti-apoptotic protein Bcl-2 might influence its interaction with FKBP38.

4. Discussion

FKBP38 is a unique protein. It is different from typical FKBP family proteins; it contains a FKBP domain – a domain which is conserved among FKBP family proteins, a calmodulin-binding domain and three tetratricopeptide repeats, but it appears to lack conserved residues for FK-506 binding and PPIase activity conserved in other FKBP family proteins [13– 15]. Recently, its co-localization with antiapoptotic proteins Bcl-2 and Bcl-Xl and the inhibition of calcineurin activity in the absence of FK-506 have been reported [14]. Bcl-2 family proteins are frequently regulated by posttranslational modification that can control their activity and conformation. Bcl-2 has been shown to be phosphorylated on some specific residues within its unstructured loop under diverse stimuli [7–10]. It was reported that phosphorylation of Bcl-2 is associated with its inactivation and mutation of the phosphorylation sites was reported to enhance the antiapoptotic activity of Bcl-2 [16]. The phosphorylated Bcl-2 was found to be localized at ER and inhibit its binding to proapoptotic proteins [10]. Currently, biochemical function and specific molecular regulatory mechanism of FKBP38 with the antiapoptotic proteins are not well understood.

In this study, we were able to express and purify recombinant FKBP38 from bacteria for biochemical and structural studies in connection with Bcl-2 family proteins. From our co-expression, column binding, yeast two-hybrid, and phosphorylation studies, we demonstrated that the unstructured loop region of Bcl-2 was important for the binding between FKBP38 and Bcl-2. We showed that the stress kinase JNK was efficiently able to phosphorylate the purified Bcl-2 *in vitro* and the phosphorylation of Bcl-2 was extensively inhibited in the presence of FKBP38. On the other hand, to ask how JNK-mediated phosphorylation on Bcl-2 would affect its interaction with FKBP38, we performed GST pull down experiments. Clearly, our data revealed that the phosphorylated Bcl-2 showed a considerable reduction in its binding with FKBP38. Taken together, these results suggest that FKBP38 might modulate the phosphorylation in the unstructured loop of Bcl-2 and could prevent Bcl-2 from being phosphorylated. On the other hand, a kinase or kinases, including JNK, might regulate molecular interaction between Bcl-2 and FKBP38 through the phosphorylation.

To address the specificity of interaction, we carried out JNK-mediated phosphorylation on Bcl-Xl, which is similar in function with Bcl-2. Our data showed that Bcl-Xl did not appear to be affected in the presence of FKBP38; rather our results showed an indication of a slight increase in the phosphorylation. This suggests that FKBP38 might show a difference in molecular interaction with the phosphorylation sites in the flexible loops of Bcl-2 family proteins. From our deletion mutant analyses, our data suggest that the sites containing or surrounding S70 and S87 in the flexible of Bcl-2 appear to be important for the physical interaction with FKBP38. Addi-

tional studies need to be done to further characterize the regulation of FKBP38 on the phosphorylation of Bcl-2 with respect to other sites.

In summary, we demonstrated that FKBP38 protein can interact with Bcl-2, which is consistent with the earlier observation made by Shirane and Nakayama [14]. The unstructured loop of Bcl-2 is important for the binding between FKBP38 and Bcl-2 and the binding between Bcl-2 and FKBP38 appears to affect the phosphorylation of Bcl-2 by JNK. Also, the JNK-mediated phosphorylation appears to influence molecular interaction between Bcl-2 and FKBP38. These new results might provide an important clue for the additional regulatory mechanism of Bcl-2 in apoptosis.

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List of Tables

Table 1 Primers used for constructing the mutants of Bcl-2 in the flexible loop region

Bcl-2 wt	Forward	5' - CACTCAGCATATGGCGCACGCTGGGAGAACGGGGTAC
	Reverse	5' - GCGAAGCTCTCGAGCTATCACCGCATGCTGGGGCCGTACAGTTC
Bcl-2Δ(V35-V89);6A	Forward	5' - AGCAGCAGCAGCAGCAGCATCTCCCGCATCCCACTCGTAGCC
	Reverse	5' - TCGCCGCTGCAGACCCCGGCTGCC
Bcl-2Δ(P65-V89)	Forward	5' - CCTGTGGTCCACCTGGCCCTGCGC
	Reverse	5' - CCCGGGCTGGGAGGAGAAGAT
Bcl-2Δ(H55-V89)	Forward	5' - CCTGTGGTCCACCTGGCCCTGCGC
	Reverse	5' - GTCCCGGATGCGGCTGG
Bcl-2Δ(V35-D64)	Forward	5' - CCGGTCGCCAGGACCTCGCCGCTG
	Reverse	5' - ATCTCCCGCATCCCACTCGTAGCC
Bcl-2Δ(V35-G79)	Forward	5' - TCGCCGCTGCAGACCCCGGCTGCC
	Reverse	5' - ATCTCCCGCATCCCACTCGTAGCC

All forward and reverse primers were phosphorylated at the 5'-terminus.

Table.1

List of Figures

- Fig. 1 Multiple sequence alignment of FKBP family proteins: FKBP51(JC5422), FKBP52(A46437), FKBP12(A35780), FKBP25(JQ1522), and FKBP38(AAB00102). The multiple protein alignment was generated by Vector NTI Align X (Informax). Based on three-dimensional structure of FKBP/FK506 [15] and sequence alignment among FKBP family proteins, the residues in the active pocket for binding FK506 are indicated by reverse triangles (▼), and those which are involved in interaction with FK-506 through hydrogen bonding are indicated by filled circles (●). NCBI protein data base accession number is indicated in parenthesis corresponding to the FKBP family proteins.
- Fig. 2 Molecular interaction between FKBP38 and Bcl-2. (A) Human FKBP38 lacking the transmembrane domain was expressed in *E. coli* BL21 (DE3) cells, purified by Ni²⁺-NTA affinity resin, and subsequently further purified by Sephacryl S-200 (Pharmacia) gel filtration column chromatography. (A) Protein samples at various stages were analyzed by 12.5% SDS-PAGE: 1, MW markers; lanes 2, 3, before and after IPTG induction; lanes 4, 5, loading sample before Ni²⁺-NTA and eluted fraction after Ni²⁺-NTA affinity purification, respectively; lane 6, FKBP38 after S-200. (B) The FKBP38 with His-tag and Bcl-2 with no-tag were co-expressed in *E. coli* BL21(DE3) cells, purified by Ni²⁺-NTA affinity resin, and analyzed in 12.5% SDS-PAGE: 1, MW markers; lanes 2, 3, before and after IPTG induction; lanes 4, 5, loading sample before Ni²⁺-NTA and eluted fraction after Ni²⁺-NTA affinity. (C) Complex formation of FKBP38 with Bcl-2. The loading sample and eluted fraction from Ni²⁺-NTA were analyzed by Western blot with antisera to FKBP38 and Bcl-2.
- Fig. 3 FKBP38 interacts with the unstructured loop of Bcl-2. (A) Loop deletion mutants used in this study are graphically displayed. (B) Co-expressed complexes of FKBP with a His-tag and Bcl-2 and the Bcl-2 loop deletion mutants with no-tag were purified by Ni²⁺-NTA. (C) Loading (L lanes) and purified samples from Ni²⁺-NTA resin (P lanes) were analyzed on 12.5% SDS-PAGE, and subjected to Western blot with anti-Bcl-2 and FKBP38 antisera. (D) and (E) Yeast two-hybrid experiments were carried out to analyze molecular interaction between FKBP38 and Bcl-2 using *Saccharomyces cerevisiae* PJ69-4A with two different selection media: SD/(-)Leu/(-)Trp (D); SD/(-)Leu/(-)Trp/(-)His (E). For (D) and (E), the wild type and the deletion mutants of Bcl-2 in the flexible loop were tested: 1, wild type Bcl-2; 2, Bcl-2Δloop:6A; 3, Bcl-2Δloop(H55-V89); 4, Bcl-2Δloop(P65-V89); 5, Bcl-2Δloop(V35-D64); 6, Bcl-2Δloop(V35-G79).

Fig. 4 FKBP38 affects the phosphorylation of Bcl-2 by JNK. (A) The N-terminal His-tagged wild type Bcl-2 protein (N-His-Bcl-2-wt), the loop deletion mutant forms of Bcl-2 (N-His-Bcl-2Δ(p65-V89), N-His-Bcl-2Δ(V35-D64)), and corresponding Bcl-2-FKBP38 complexes were expressed *E. coli* BL21 (DE3) cells, and purified by the Ni²⁺-NTA resin, subjected to Western blot using anti-Bcl-2 antisera. (B) The purified Bcl-2-wt and mutants were subjected to phosphorylation by JNK with [γ -³³P]ATP, and analyzed on 15% SDS-PAGE followed by autoradiography for visualization. (C) His-Bcl-X1 and Bcl-X1Δ(M45-A84) were purified, subjected to Western blot using anti-Bcl-X1 antisera, and (D) JNK-mediated kinase reaction, under the same condition described in (A) and (B), respectively.

Fig. 5 Phosphorylation of Bcl-2 influences molecular interaction between FKBP38 and Bcl-2. (A) Purified Bcl-2 (lane 1) was phosphorylated by JNK (lane 2) and the phosphorylated Bcl-2 was purified by Superdex 75 gel filtration column. The phosphorylation of Bcl-2 was confirmed by the treatment calcineurin (lane 3). The samples were analyzed on 12.5% SDS-PAGE, and subjected to Western blot with anti-Bcl-2. (B) The purified Bcl-2 and P-Bcl-2 were mixed with 10 μg of GST-FKBP38ΔTM, and the bindings between Bcl-2 or P-Bcl-2 and FKBP38 were analyzed by in vitro pull down assay as described, with 3 μg of Bcl-2 or P-Bcl-2 and 10 μg of GSTFKBP38ΔTM: lane 1, Bcl-2 before GST pull down; lane 2, Bcl-2 after GST pull down; lane 3, P-Bcl-2 before GST pull down; 4, P-Bcl-2 after GST pull down.

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      1                               ▼                               •                               50
FKBP51  --GVLKIVKRVGN-GEETPMIGDKVYVHYK GKLSNGKKFDSSHDRNE---
FKBP52  -EGVLKVIKREGT-GTEMPMIGDRVVFVHYTGWLLDGTKFDSSLDRKD---
FKBP12  -MGVQVETISPGD-GRTFPKRGQTCVVHYTGMLDGGKKFDSSSRDRNK---
FKBP25  -PKYTKSVLKKGD-KTNFPKKGDVVHCWYTGT LQDGT VFDTNIQTSAKKK
FKBP38  NGLLRKKTLPVPGPPGSSRPVKGQVVTVHLQTSLENGTRVQEEP-----
      51   ▼           •••           ▼                               •                               100
FKBP51  ----PFVFSLGKQVIKAWDIGVATMKKGEICHLLCKPEYAYGSAGS-LP
FKBP52  ----KFSFDLGKGEVIKAWDIAIATMKVGEVCHITCKPEYAYGSAGS-PP
FKBP12  ----PFKFMLGKQEVIRGWEEGVAQMSVQORAKLTISPDIYAYGATGH-PG
FKBP25  KNAKPLSFKVGVGKVIKAWDEALLTMSKGEKARLEIEPEWAYGKKGQPD
FKBP38  ----ELVFTLGDCDVIQALDLSVPLMDVGETAMVTADSKYCYGPQGSRSP
      101           ▼                               122
FKBP51  KIPSNATLFFEIELLDFKGE--
FKBP52  KIPP NATLVFEVELFEFKGE--
FKBP12  IIPPHATLVFDVELLKLE----
FKBP25  KIPPNAKLTFEVELVDID----
FKBP38  YIPPHAALCLEVTLKTAVDGPD

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Fig. 1

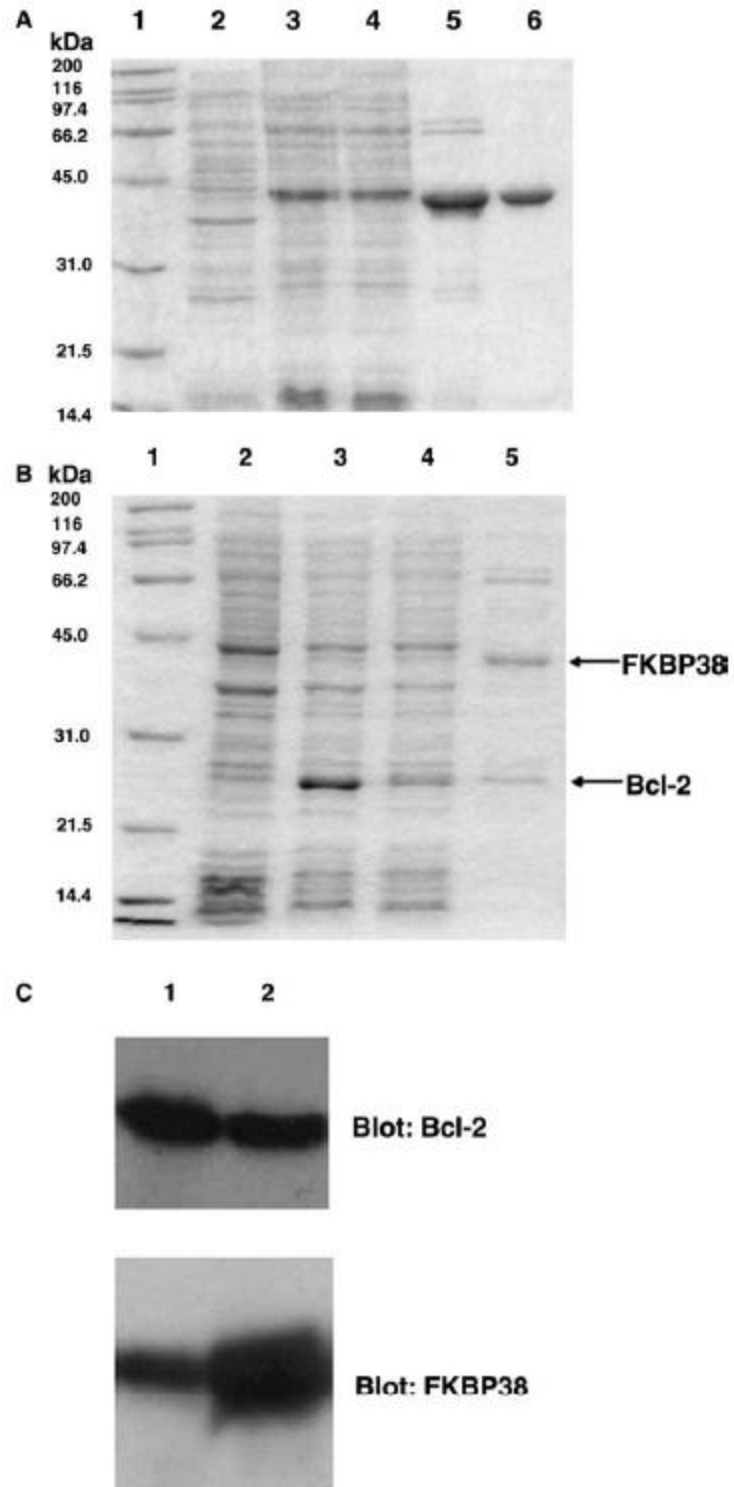


Fig. 2

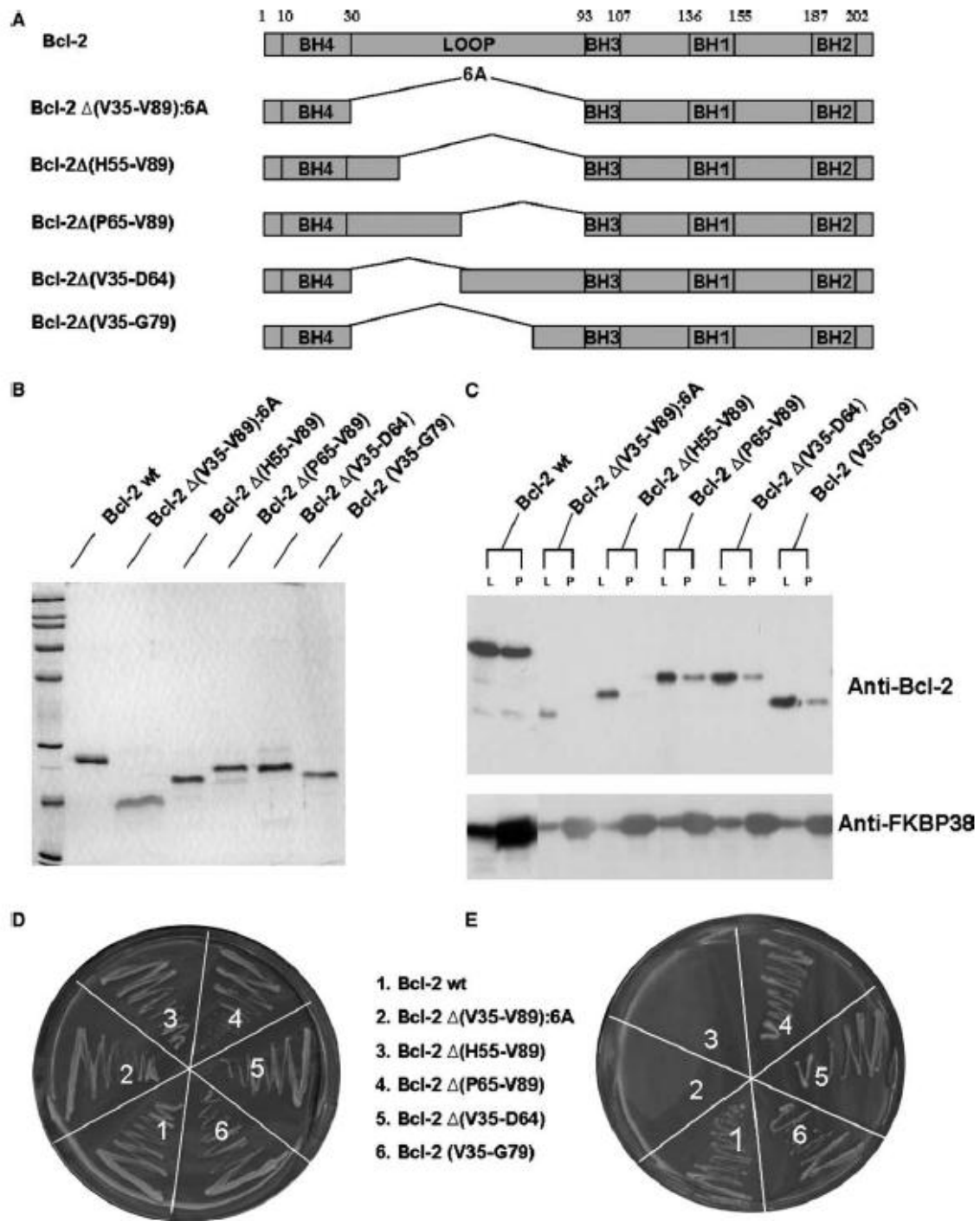


Fig. 3

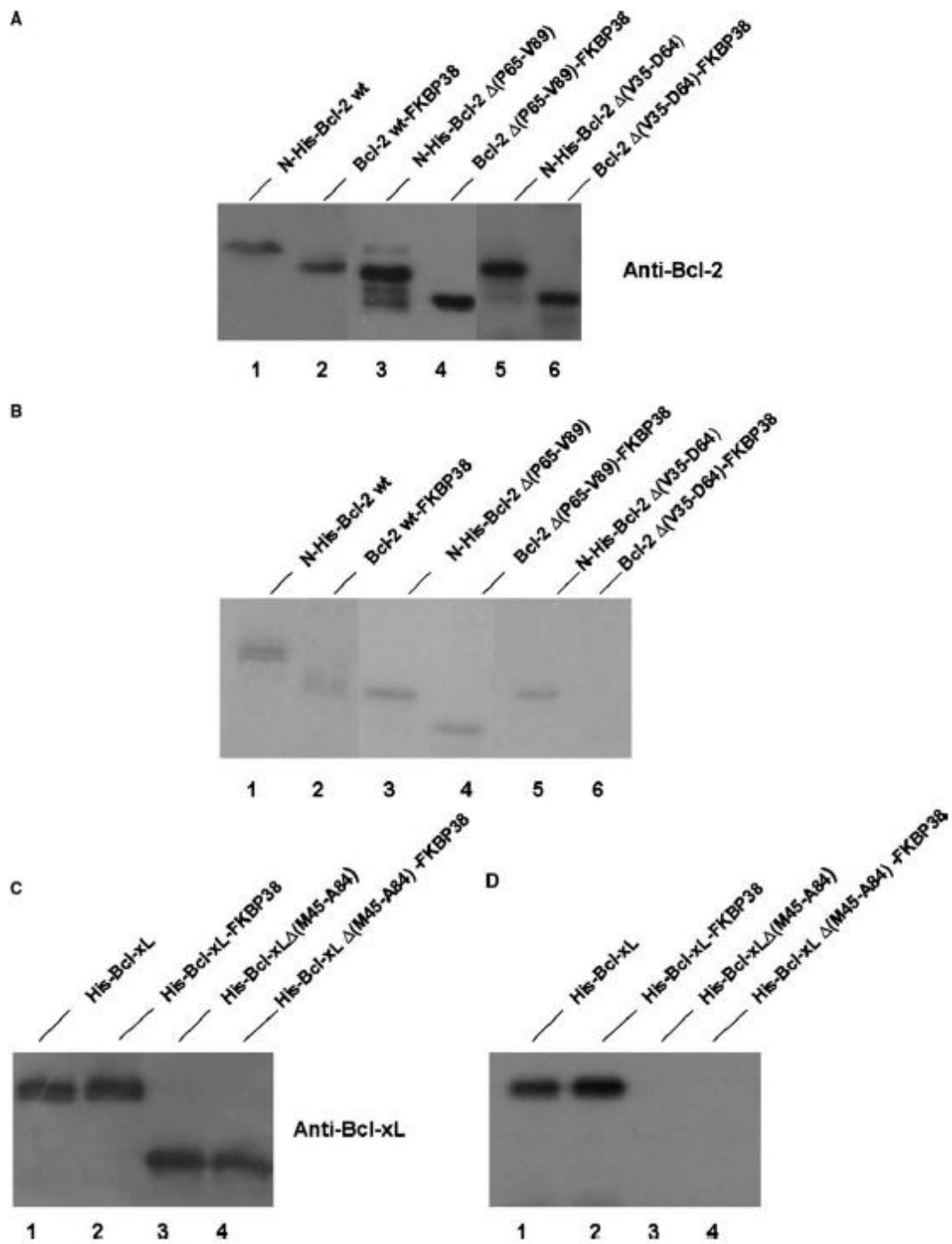


Fig. 4

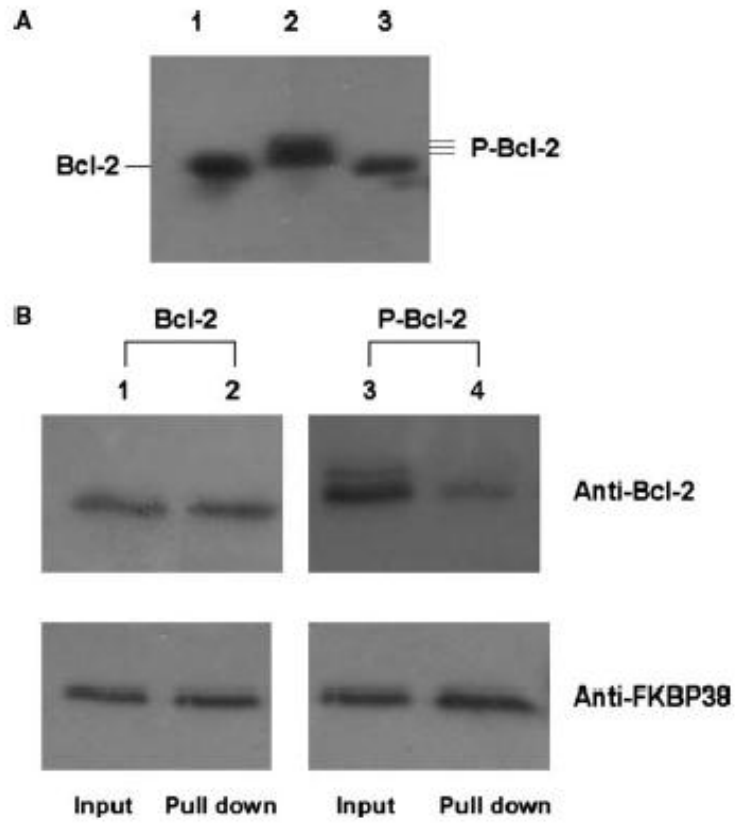


Fig. 5