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Completing the family portrait of the anti-apoptotic Bcl-2 proteins: Crystal structure of human Bfl-1 in complex with Bim

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Abstract **Evasion of apoptosis is recognized as a characteristic of malignant growth. Anti-apoptotic B-cell lymphoma-2 (Bcl-2) family members have therefore emerged as potential therapeutic targets due to their critical role in proliferating cancer cells. Here, we present the crystal structure of Bfl-1, the last anti-apoptotic Bcl-2 family member to be structurally characterized, in complex with a peptide corresponding to the BH3 region of the pro-apoptotic protein Bim. The structure reveals distinct features at the peptide-binding site, likely to define the binding specificity for pro-apoptotic proteins. Superposition of the Bfl-1:Bim complex with that of Mcl-1:Bim reveals a significant local plasticity of hydrophobic interactions contributed by the Bim peptide, likely to be the basis for the multi specificity of Bim for anti-apoptotic proteins.**

Keywords: Apoptosis; B-cell lymphoma-2; Cancer; Bfl-1; A1; Crystal structure

1. Introduction

The B-cell lymphoma-2 (Bcl-2) protein family contains both pro- and anti-apoptotic members that play critical roles in the mitochondrial apoptotic pathways [1]. Cancer cells frequently over-express anti-apoptotic Bcl-2 family members that suppress apoptotic signals. Members of the Bcl-2 family interact through their Bcl-2 homology (BH) motifs [2,3]. Bax, Bak and Bok, share three BH motifs (BH1, BH2 and BH3) and are proposed to induce permeabilization of the mitochondrial outer membrane, resulting in cytochrome *c* release and the subsequent activation of caspases [4]. The anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and Bfl-1, contain four BH motifs and a membrane-anchoring sequence at their C-terminus [3]. The pro-apoptotic BH3-only proteins contain a single BH motif.

While it is known that apoptosis is regulated by interactions between the different Bcl-2 family members, the exact hierarchy of these interactions in different cell contexts remains controversial [5,6]. Some of the BH3-only proteins (Bad, Bik, Hrk, Bmf and Noxa) most likely execute their pro-apoptotic effects by directly binding and inactivating anti-apoptotic Bcl-2 proteins [7]. The BH3-only proteins Bim, Bid and Puma could work in a similar fashion but have been shown to also work as direct activators of Bak, Bax and Bok [3]. The main function of the anti-apoptotic Bcl-2 members would then be to sequester Bim, Bid and Puma, thereby blocking their activation of Bax, Bak and Bok. This scenario is in contrast to earlier proposal where the anti-apoptotic members have been suggested to act and interact directly on Bax, Bak and Bok [8]. In either case, it is certain that BH3-only proteins function is to regulate apoptosis by binding though the BH3 motif to its different partners.

Structural studies on several of the multi-BH motif Bcl-2 proteins have revealed a common fold constituted by two central hydrophobic helices surrounded by six or seven amphipathic helices [2,3]. The binding site for the BH3 regions of pro-apoptotic BH3-only proteins is located at a hydrophobic groove formed by the BH1, BH2 and BH3 motifs [9,10]. Structural studies of complexes between anti-apoptotic proteins and BH3 peptides of pro-apoptotic BH3-only proteins such as mouse Bcl-xl:Bim [11], human Bcl-xl:Bak [10], human Bcl-w:Bid [12], mouse Mcl-1:NoxaB and of human Mcl-1:Bim [13], have revealed insights into the specificity determinants for BH3 interactions.

This paper presents the crystal structure of Bfl-1, the only mammalian anti-apoptotic Bcl-2 family member lacking structural information, in complex with a BH3 peptide from Bim. Bfl-1 confers protection against various apoptotic stimuli such as activation of the TNF receptor, oxidative stress, overexpression of Bax and Bid and chemotherapeutic treatments, [14]. It has been demonstrated that Bfl-1 interacts with the BH3-only proteins Bim, Bid, Puma and Noxa in vivo [5] and in vitro [7,15]. The structure of Bfl-1 in complex with Bim provides new information of the structural basis for Bim recognition and can serve as a basis for defining epitopes for the design of anti-apoptotic inhibitors.

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Abbreviations: Bcl-2, B-cell lymphoma-2; BH, Bcl-2 homology motif

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2. Materials and methods

2.1. Protein production

Human Bfl-1 protein (residues 1–149), lacking the C-terminal 26 residues, was expressed in *Escherichia coli* strain BL21 (DE3) using the pET-based vector pNIC-Bsa4a (Novagen). The recombinant protein contained an N-terminal 6 × His-tag followed by a TEV-protease site. Cell cultivation and protein expression were performed as described previously [16]. Human Bim-BH3 peptide (DMRPEIWIAQELRRIGDEFNAYYAR), corresponing to residues 141–165, was synthesized by GenScript Corporation, Scotch Plains USA. Cell extract preparation, purification protocol and buffer compositions were as previously described [16]. Final gel filtration was performed in 20 mM HEPES, 300 mM NaCl, 10% glycerol, 2 mM TCEP, pH 7.5. The His-tag was removed by TEV-protease treatment. The purity (>98%) of the protein was estimated by SDS–PAGE (data not shown). Molecular weight (19.7 kDa) and protein identity was verified by mass-spectrometry. The protein was concentrated to 8 mg/ml, frozen in liquid nitrogen and stored at −80 °C until further handling. The same methods were used for purifying selenomethionine-labeled protein used for MAD phasing. Mass-spectrometry confirmed the incorporation of three Se-Met.

2.2. Crystallization and data collection

Bfl-1 protein was mixed with an equimolar amount of Bim-BH3 peptide. The protein complex was crystallized in hanging drops containing 1 μl of protein solution (8 mg/ml) and 1 μl well solution (0.1 M BisTris pH 5.8 and 1.8–2.0 M $(NH_4)_2SO_4$), at 20 °C. Crystals were harvested into a cryo-protecting solution composed of the reservoir solution supplemented with 25% glycerol and frozen in liquid nitrogen. Native and MAD data sets were collected at European Synchrotron Ring Facility (ESRF) at beam lines BM14.1 (2.2 Å resolution) and ID29 (2.5 Å resolution), respectively.

2.3. Structure solution and refinement

Data sets were processed with XDS and scaled with XSCALE [17]. The structure of Bfl-1 was solved by MAD phasing using peak and inflection point data sets (Table S1). SOLVE [18] located 2 of the 3 selenium sites in the asymmetric unit. RESOLVE [18] was used to carry out solvent flattening and subsequent initial model building. Molecular replacement was done using MOLREP [19]. The model was improved using ARP/wARP [20], and refined in REFMAC5 [21]. Manual model building was done using Coot. Crystal data and refinement statistics are shown in Table S1. Structure analysis was aided by Coot. Coordinates and structure factors for Bfl-1 were deposited to the PDB with the accession code 2VM6.

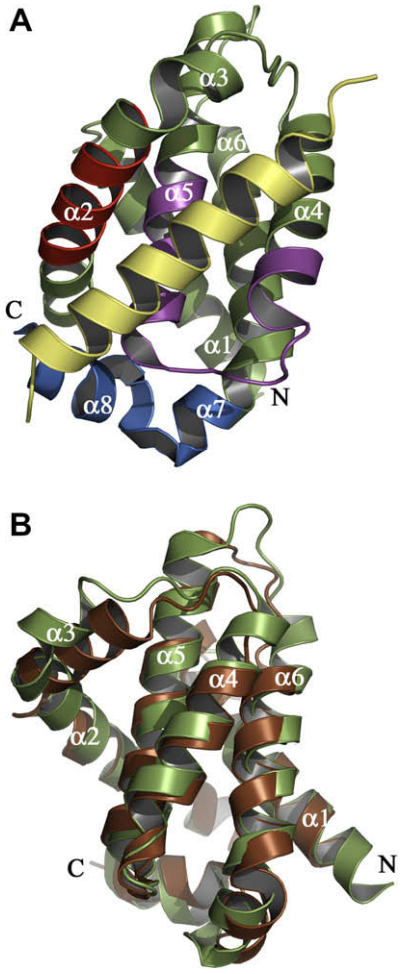

Fig. 1. Overall structure. (A) The structure of Bfl-1 with Bim-BH3 peptide, where Bim peptide is colored in yellow and Bfl-1 in green with motif BH1, BH2 and BH3 colored in magenta, blue and red, respectively. (B) Superposition of the Bfl-1 structure (green) with that of Mcl-1 (2PQK) (brown). The Bim peptide has been omitted from both structures, for clarity.

3. Results

3.1. Overall structure

The crystal structure of Bfl-1 was solved at 2.2 Å resolution with one Bfl-1:Bim complex in the asymmetric unit. Bfl-1 shows a compact fold composed of eight α-helices, which constitute the canonic Bcl-2 fold (Fig. 1A). All amino acids from residues 1 to 149 was modeled into the electron density, except for residues 25–30. This disordered stretch connects helices α1 and α2 – a region which differs greatly in length between Bcl-2 family members, and which is usually disordered [2,3]. The binding groove for the Bim peptide is composed of a cleft formed between the α2, α3, α4, α5, α7 and α8 helices, that also contains the BH1, BH2 and BH3 motifs. Some of the most conserved residues in these motifs play structural roles while others contribute directly to the peptide-binding pocket. Although the overall sequence identity between Bfl-1 and other anti-apoptotic members of the Bcl-2 family is relatively low, ranging from 19% (Bcl-w) to 35% (Mcl-1) (Table S2), their three-dimensional architectures are very similar. Root mean square deviations are in the range of 3.2 Å (Bcl-w, 115 residues) to 1.8 Å (Bcl-xl, 144 residues). The superposition of human Bfl-1 with human Mcl-1 (2PQK) is shown in Fig. 1B. The largest structural differences are seen in helices α2 and α3 and the connecting region between helices α5 and α6. The structural changes in these two regions are partially concerted – helix α3, which line the Bim peptide-binding pocket, are partially packing on top of the region where helices α5 and α6 connect.

3.2. The Bfl-1:Bim interaction

The Bfl-1 Bim-BH3 peptide-binding pocket has similar overall properties as the corresponding groove in other Bcl-2 proteins. Several hydrophobic patches line along the pocket at positions conserved in the Bcl-2 proteins, and have been labeled h1–h4 (Fig. 2A). These hydrophobic patches interact with highly conserved residues on the amphipathic helix of BH3-only proteins (Fig. 2B). In the Bim peptide these residues are represented by Ile148, Leu152, Ile155 and Phe159. The Bim peptide makes two additional hydrophobic interactions; Trp147 of Bim stacks onto a surface patch formed by Leu52 and Cys55 of Bfl-1, and Tyr163 of Bim makes hydrophobic

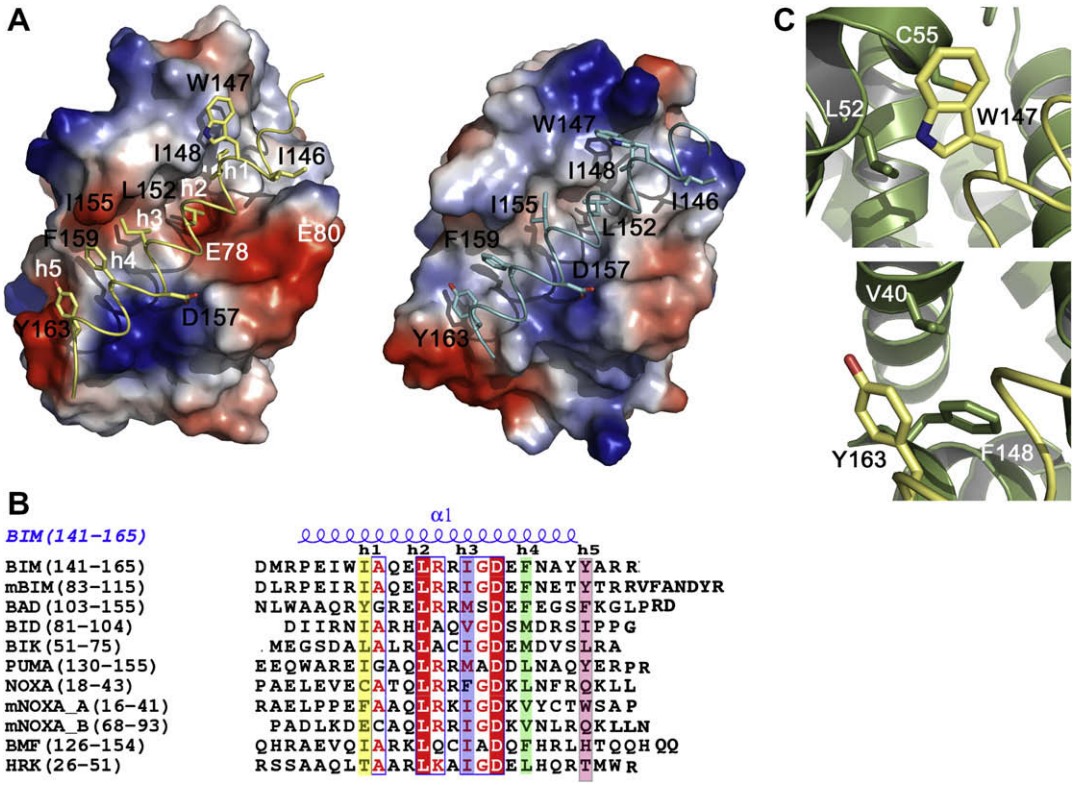

Fig. 2. Bfl-1:Bim interaction. (A) Comparison of the Bfl-1:Bim complex (left, Bim peptide is colored yellow) with the Mcl-1:Bim complex (right, Bim peptide is colored blue). Black labels refers to Bim residues, white labels refers to Bfl-1. Bfl-1 and Mcl-1 are rendered as electrostatic potential surface models. (B) Sequence alignment of BH3 motifs from different BH3-only proteins. The conserved hydrophobic residues h1, h2, h3, h4 and h5 are highlighted in yellow, red, light blue, green and pink, respectively. Figure prepared using the ESPript server [31]. (C) Close views on the interactions of Trp147 and Tyr163 of Bim with the peptide-binding pocket of Bfl-1.

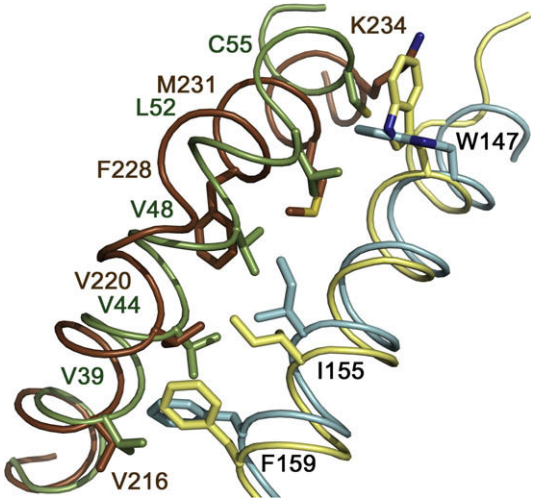

Fig. 3. Bim binding to human Bfl-1 and Mcl-1 induces different side-chain conformations in Bim. Shown is a ribbon representation of the α2 and α3 helices and the Bim peptide from Bfl-1:Bim complex (green:yellow) and the Mcl-1:Bim complex (2PQK) (brown:blue). See text for description.

interactions with Phe148 and Val40 of Bfl-1 (Fig. 2C). Since these latter residues are highly conserved in other anti-apoptotic Bcl-2 proteins, we suggest that this region is labeled the h5 patch. The part of the pocket lined by helix α4 and α5 has several polar interactions similar to those in other Bcl-2 proteins. The most notable is the conserved Arg88 of the WGR motif in BH1, which interacts with the strictly conserved Asp157 of Bim. A characteristic feature of the Bfl-1 BH3-binding groove is the higher negative charge in its central region, contributed by Glu78 and Glu80 at the end of helix α4 (Fig. 2A).

3.3. Comparison of Bim binding in human Bfl-1 and Mcl-1

Comparison of the human Bfl-1:Bim and human Mcl-1:Bim (2PQK) [13] complexes does, for the first time, allow the analysis of the binding of the same pro-apoptotic peptide into two different human anti-apoptotic proteins. The comparison reveals very similar overall peptide backbone conformation and shows that most of the polar side chains in the two Bim peptide structures have similar conformations. However, large differences are seen in the conformations of the hydrophobic side chains of the Bim peptide, particularly notable for the residues Trp147, Ile155 and Phe159 of Bim (Fig. 3). The induction of different conformations in the Bim peptide by the two proteins is likely caused by the specific topology of the hydrophobic patches in Bfl-1 and Mcl-1.

4. Discussion

The affinities of anti-apoptotic Bcl-2 family members for BH3-only protein derived peptides has been measured by

several groups [5–7,15]. Although these studies yield somewhat different quantitative results due to the use of different samples and methodologies, they indicate that the qualitative binding profiles of Bcl-2, Bcl-w and Bcl-x_L for BH3-only proteins or BH3 peptides are very similar, whereas Mcl-1 and Bfl-1 exhibits a distinct pattern, creating a subclass in the anti-apoptotic family. The most notable difference in affinity is seen for the binding of Bmf and Bad peptides to Bfl-1 and Mcl-1, which are 3–4 orders of magnitudes weaker than the binding of these peptides to Bcl-2, Bcl-x_L and Bcl-w [5]. The modeling of these peptides into the Bfl-1 pocket (not shown) does not reveal obvious steric hindrances or charge repulsions that could explain these differences in affinities. It is therefore unlikely that the lower affinity of the Bad and Bmf peptide can be explained by single amino acid substitution and is probably due to cooperative effects resulting from several sequence differences. It is noteworthy that Bfl-1 shows a lower degree of conservation of residues in the BH3 motif, encompassing helices α2 and α3, as compared to other Bcl-2 proteins (Fig. S1). This might contribute to the structural differences seen in helices α2 and α3 and is likely to be an important determinant for the Bfl-1 peptide-binding selectivity. It has previously been shown that different BH3 peptides have different helical propensities [3,9]. The binding energies are therefore likely to also be dependent on how well the Bcl-2 proteins are able to enhance these propensities, which is not easily derived from the available structural data.

The binding affinities of Bim-BH3 to Bfl-1 and Mcl-1 are, however, very similar, in spite of the differences in the detailed binding of the Bim peptide to the two proteins. The polar interactions are likely to confer selectivity and help in orienting the peptide, where the conserved Asp157:Bim-Arg88:Bfl-1 salt bridge plays a key role. The hydrophobic interactions contributed by h1–h5 probably play a major role in determining the affinity of the Bim peptides. The similarity in binding affinities can be explained by a similar extent of burial of the hydrophobic side chains in the two complexes, since the major determinant for the binding energy is likely to be the hydrophobic effect [22].

Bfl-1 activity is regulated by post-translational modification such as phosphorylation and ubiquitinylation at the C-terminal region, or proteolytic removal of the N-terminal BH4 motif by μ-calpain [23]. These modifications allow Bfl-1 to act as an anti- or pro-apoptotic protein depending on the apoptotic stimuli. The C-terminal region of Bfl-1 is proposed to form an amphipathic helix that interacts with mitochondrial membranes [24,25], although this is still a matter of debate [26]. All other multidomain Bcl-2 family members have hydrophobic C-terminal segments that are suggested to form transmembrane helices [3]. The C-terminal helix of Bax binds in a pocket corresponding to the BH3-intreacting groove [27]. Similarly, half of the hydrophobic C-terminus of Bcl-w is found in the peptide-binding pocket, and was shown to interfere with BH3-only protein interaction [28]. In the Bfl-1 structure, the C-terminus ends close to the Bim binding pocket and it is possible that the C-terminal helix could insert into the pocket in a similar manner as in Bcl-w. Post-translational modifications of the C-terminal region could be a means to modify its affinity for the pocket, making it available for BH3-only proteins.

Extensive efforts are being put into developing Bcl-2 inhibitors to be used in cancer therapies [4]. ABT-737 is such an inhibitor, modeled on the Bad BH3 peptide, which have been extensively studied and which binds to Bcl-2, Bcl-w and Bcl-xL, but not Mcl-1 and Bfl-1 [29,30]. Superposition of the Bcl-xL:ABT-737 complex onto the Bfl-1 structure (Fig. S2) reveals sterical hindrance for the chloro-biphenyl and sulphoamide groups of ABT-737, which could explain the low affinity for Bfl-1. Future design will be focused on both member-specific and family-wide inhibitors that allow several anti-apoptotic signals to be blocked simultaneously. The completion of the structural gallery of anti-apoptotic Bcl-2 family proteins now allows for the evaluation of the different regions of the binding pockets to aid the design of such family-wide inhibitors. To achieve this broader specificity, a possible strategy is to mimic the action of BH3-only peptides hitting on all anti-apoptotic Bcl-2 proteins, such as Bim and Bid. This might involve introduction of some of the flexibility seen in the Bim peptide, allowing the compounds to adjust for local differences in the BH3-binding groove.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.028.

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