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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.

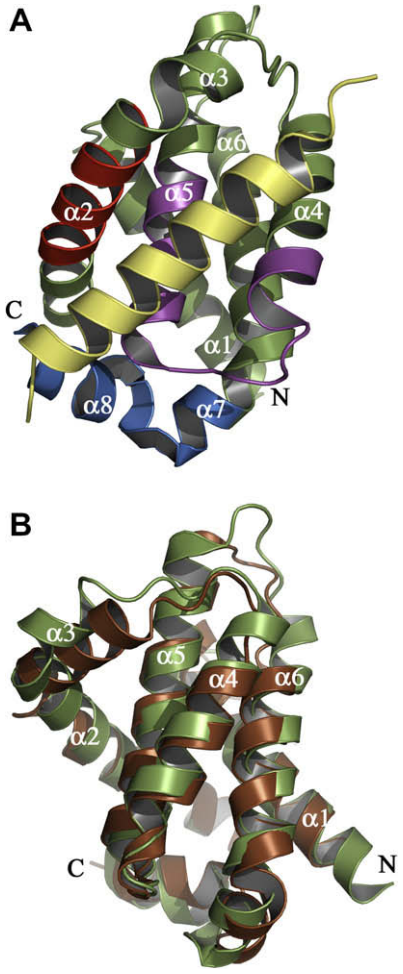

Fig. 1. Overall structure. (A) The structure of Bfl-1 with Bim-BH3 peptide, where Bim peptide is colored in yellow and Bfl-1 in green with motif BH1, BH2 and BH3 colored in magenta, blue and red, respectively. (B) Superposition of the Bfl-1 structure (green) with that of Mcl-1 (2PQK) (brown). The Bim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