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Biochimica et Biophysica Acta 1644 (2004) 83–94
BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Structural biology of the Bcl-2 family of proteins

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Received 28 April 2003; accepted 18 August 2003

Abstract

The proteins of the Bcl-2 family are important regulators of programmed cell death. Structural studies of Bcl-2 family members have provided many important insights into their molecular mechanism of action and how members of this family interact with one another. To date, structural studies have been performed on six Bcl-2 family members encompassing both anti- (Bcl-x_L, Bcl-2, KSHV-Bcl-2, Bcl-w) and pro-apoptotic (Bax, Bid) members. They all show a remarkably similar fold despite an overall divergence in amino acid sequence and function (pro-apoptotic versus anti-apoptotic). The three-dimensional structures of Bcl-2 family members consist of two central, predominantly hydrophobic α-helices surrounded by six or seven amphipathic α-helices of varying lengths. A long, unstructured loop is present between the first two α-helices. The structures of the Bcl-2 proteins show a striking similarity to the overall fold of the pore-forming domains of bacterial toxins. This finding led to experiments which demonstrated that Bcl-x_L, Bcl-2, and Bax all form pores in artificial membranes. A prominent hydrophobic groove is present on the surface of the anti-apoptotic proteins. This groove is the binding site for peptides that mimic the BH3 region of various pro-apoptotic proteins such as Bak and Bad. Structures of Bcl-x_L in complex with these BH3 peptides showed that they bind as an amphipathic α-helix and make extensive hydrophobic contacts with the protein. These data have not only helped to elucidate the interactions important for hetero-dimerization of Bcl-2 family members but have also been used to guide the discovery of small molecules that block Bcl-x_L and Bcl-2 function. In the recently determined structure of the anti-apoptotic Bcl-w protein, the protein was also found to have a hydrophobic groove on its surface capable of binding BH3-containing proteins and peptides. However, in the native protein an additional carboxy-terminal α-helix interacts with the hydrophobic groove. This is reminiscent of how the carboxy-terminal α-helix of the pro-apoptotic protein Bax binds into its hydrophobic groove. This interaction may play a regulatory role and for Bax may explain why it is found predominately in the cytoplasm prior to activation. The hydrophobic groove of the pro-apoptotic protein, Bid protein, is neither as long nor as deep as that found in Bcl-x_L, Bcl-2, or Bax. In addition, Bid contains an extra α-helix, which is located between α1 and α2 with respect to Bcl-x_L, Bcl-2, and Bax. Although there are still many unanswered questions regarding the exact mechanism by which the Bcl-2 family of proteins modulates apoptosis, structural studies of these proteins have deepened our understanding of apoptosis on the molecular level.

Keywords: Apoptosis; Bcl-x_L; Bcl-2; Bcl-w; Bax; Bak; Bid

1. Introduction

Programmed cell death (apoptosis) plays a vital role in normal development, tissue homeostasis, and the removal of damaged and infected cells. Disruption of the normal apoptotic process is implicated in a variety of human diseases [1,2]. For example, in many forms of cancer, an imbalance between pro- and anti-apoptotic proteins leads to the accumulation of cells and the inability to respond correctly to apoptotic stimuli. As a consequence, these cancers do not respond well to cancer therapies that rely on the induction of apoptosis for their effect [3]. Removal of autoreactive T cells after an immune response also depends on apoptosis. Disruption of the process can lead to the auto-immune lymphoproliferative syndrome (ALPS) [4]. Insufficient apoptosis can also lead to persistent infections due to a failure to eradicate bacteria or virus-infected cells. Equally important are diseases characterized by inappropriate or excessive apoptosis. Several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases are characterized by the premature loss of specific neurons

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doi:10.1016/j.bbamcr.2003.08.012

that can lead to irreversible memory loss, uncontrolled muscular movements, and depression [3]. Excessive apoptosis also contributes to the damage caused by inflammation, spinal muscular atrophy, myocardial infarction, and stroke [1,5,6].

Because of its fundamental importance, programmed cell death is a highly regulated pathway. One important set of proteins involved in the regulation of apoptosis is the Bcl-2 family. To date, over 25 Bcl-2 family members have been identified [2,7]. These can be broadly divided into two classes: those that inhibit apoptosis and those that promote apoptosis. Homeostasis is maintained by controlling the amount of active pro- and anti-apoptotic family members along with tissue-specific patterns of expression. Stimuli, such as DNA damage, lead to increased expression of pro-apoptotic family members. This disrupts the fine balance between pro- and anti-apoptotic proteins and leads to programmed cell death.

The pro-survival class of Bcl-2 family members has been divided into two subclasses based on the presence of one or more "Bcl-2 homology" (BH) regions (Fig. 1) [7]. Four of these regions (BH1–4) of sequence homology have been identified, and each Bcl-2 family member contains at least one of them. Several members of the pro-survival subclass, such as Bcl-2, Bcl-x_L, Bcl-w, and the Ced-9 protein from *C. elegans*, possess all four BH regions. Others, such as Mcl-1, the BHRF1 protein from Epstein–Barr virus, and KSHV-Bcl-2 from Kaposi sarcoma virus, only possess strong sequence homology in the BH1, BH2, and BH3 regions. Mutagenesis studies indicate that the BH1, BH2, and BH3

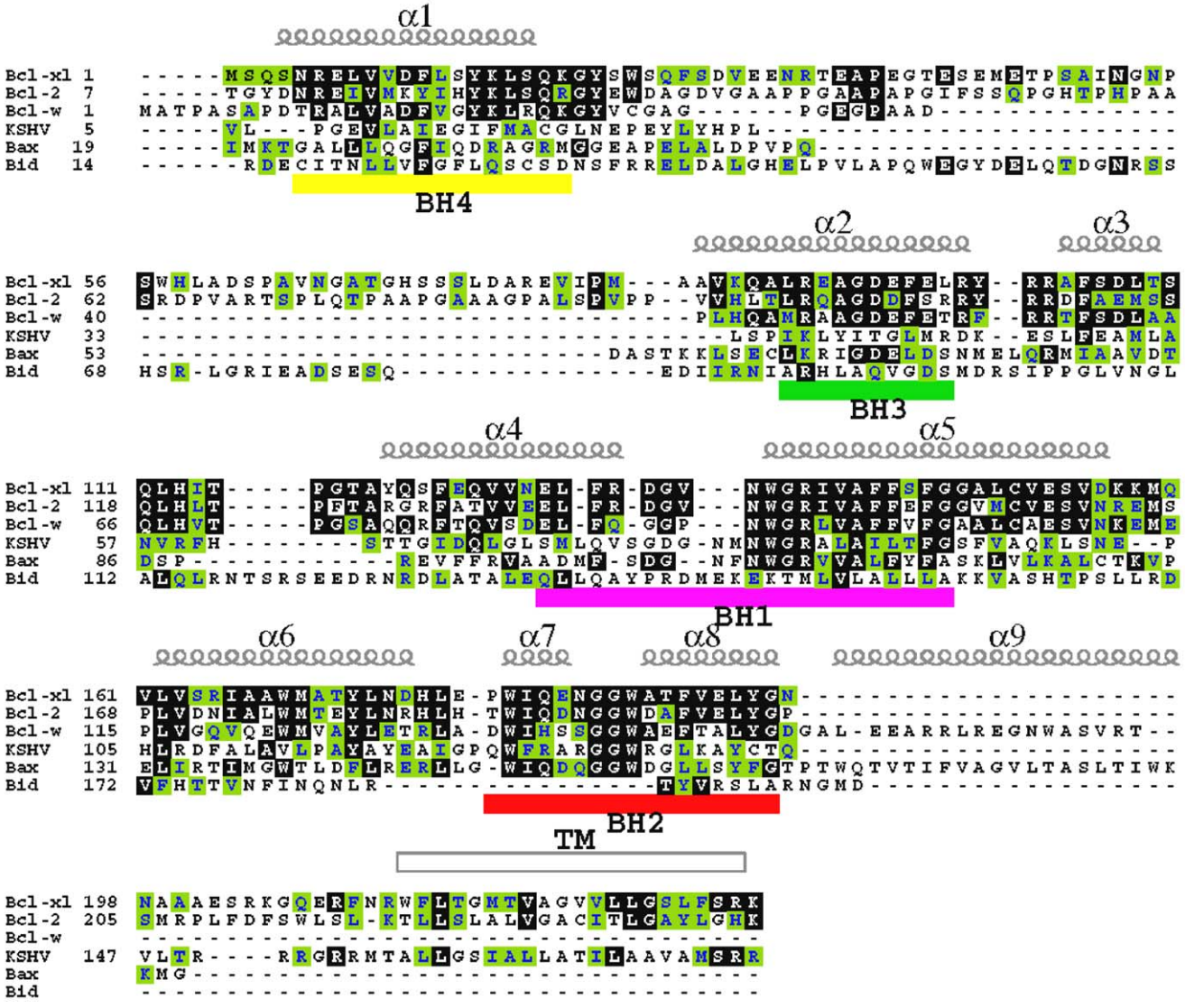

Fig. 1. Sequence alignment of Bcl-2 family proteins for which structural information is known. Bcl-2 homolgy "BH" regions present in Bcl-x_L are indicated with rectangular boxes. Helices 1–8 for Bcl-x_L are shown above the sequence along with helix 9 of Bax and helix 9 of Bcl-w. In addition, the putative transmembrane helix of Bcl-x_L, Bcl-2, and KSHV-Bcl-2 is shown above the sequences. For the aligned sequences, black boxes highlight strictly conserved residues, while green boxes highlight conservative substitutions.

domains strongly influence homo- and hetero-dimerization of these proteins [7]. For the pro-apoptotic proteins, members of the Bax subclass possess sequence homology for the BH1, BH2, and BH3 regions while members of the BH3 subclass have strong homology only in the BH3 region. Pro-apoptotic family members that contain BH1 and BH2 regions such as Bax can promote apoptosis through their interactions with mitochondrial membranes. This activity is independent from their ability to interact with anti-apoptotic proteins. The BH3 region is responsible for mediating the interactions with anti-apoptotic proteins and the ability of the proteins to promote programmed cell death [8]. Peptides derived from the BH3 regions of pro-apoptotic Bcl-2 family members can bind to anti-apoptotic family members such as Bcl-x_L and modulate Bcl-2 regulated apoptotic pathways in living cells [9].

Besides the BH regions, many of the Bcl-2 family members possess a carboxy-terminal hydrophobic domain, which is predicted to be responsible for membrane localization [7,10]. Patterns of membrane localization differ between the anti-apoptotic and pro-apoptotic proteins. For example, Bcl-2 has been shown to reside on the cytoplasmic face of the mitochondrial outer membrane. This membrane anchoring may play a key role in the ability of Bcl-2 to modulate mitochondrial membrane integrity. Mitochondrial dysfunction, in which cytochrome *c* is released from the intermembrane space, is a critical step in the initiation of apoptosis [10] and is inhibited by Bcl-2 and Bcl-x_L. In contrast to Bcl-2 and Bcl-x_L, pro-apoptotic family members such as Bax and Bid are found mainly in the cytosol and are only localized to the mitochondrial membrane upon activation [11,12].

Structural studies of Bcl-2 family members have provided many insights into the molecular mechanism of apoptosis and how Bcl-2 family members interact with one another. Here we review the three-dimensional structures of the Bcl-2 proteins and how this structural information has improved our understanding of apoptosis at the molecular level. We describe the structures of the anti-apoptotic proteins, Bcl-x_L, Bcl-2, a Bcl-2 homolog from the Kaposi Sarcoma virus, and Bcl-w, as well as the structure of Bcl-x_L in complex with BH3-derived peptides from Bak and Bad. In addition, we review the structural data on the pro-apoptotic proteins Bax and Bid.

2. Bcl-x_L

The first published structure of a Bcl-2 family member was that of human Bcl-x_L determined by X-ray crystallography and NMR spectroscopy [13]. The study was conducted on a biologically active form of the protein that lacked the carboxy-terminal transmembrane region. The overall structure of the protein consists of eight α-helices connected by loops of varying length (Fig. 2A). Two central helices (α5 and α6) form the core of the protein. These two helices are predominately hydrophobic and are flanked on one side by α3 and α4, and on the other side by α1 and α2. The signature "NWGR" sequence, which is highly conserved among Bcl-2 family members, directly precedes α5. In Bcl-x_L, this region appears to play both an important structural and functional role. Structurally, the tryptophan residue makes extensive hydrophobic contacts with residues in α7 and α8. The arginine residue also plays a key functional role in the binding of Bcl-x_L to pro-apoptotic proteins and peptides such as Bak and Bad (see below).

An unusual feature of the Bcl-x_L protein is the presence of a long loop between α1 and α2. This loop is largely unstructured as evidenced by the lack of electron density for residues 28 to 80 and the lack of medium- and long-range NOEs for these residues. In addition, the amino acid sequence of this region is highly variable among Bcl-2 family members.

Based on these observations, a mutant protein was constructed in which residues 26–83 of Bcl-x_L were deleted and replaced by four alanines. The mutant protein was able to inhibit programmed cell death better than the wild-type protein upon interleukin-3 withdrawal in FL5.12 cells [13]. These results suggested that the loop is not involved in the anti-apoptotic activity of Bcl-x_L, and may even play a role in the negative regulation of Bcl-x_L function. Indeed, it has been shown that phosphorylation of Ser 70 of Bcl-2, which possesses an analogously long loop, abrogates its anti-apoptotic function [14]. Furthermore, proteolytic cleavage of the Bcl-2 loop at Asp 34 by caspase-3 converts it from an anti-apoptotic protein to a pro-apoptotic protein [15].

The three-dimensional structure of Bcl-x_L also provided insight into the significance of the BH homology regions (Fig. 2A). The BH1, BH2, and BH3 regions are proximal to one another and define the top of an elongated hydrophobic groove on the surface of the protein (Fig. 2B). The bottom of the groove is formed by α3 and α4. This hydrophobic groove was postulated to be the interaction site for the pro-apoptotic members of the Bcl-2 family such as Bak and Bad, which was later confirmed by the NMR structure of the Bcl-x_L/Bak peptide complex [16].

Another key observation was that the three-dimensional structure of Bcl-x_L resembles the membrane insertion domains of diphtheria toxin and of the colicins [13]. Like Bcl-x_L, these domains contain two hydrophobic α-helices which are long enough to span a bilayer. Based on this structural similarity, it was postulated that the Bcl-2 family of proteins may also form pores in membranes [13]. Subsequent studies have shown that Bcl-x_L, Bcl-2, and Bax can all form ion-conducting channels when incorporated into synthetic lipid bilayers [17–19]. However, it is unclear how the pore-forming ability of the Bcl-2 family of proteins regulates the release of cytochrome *c* and controls apoptosis at the molecular level. In an attempt to provide information on the conformation of Bcl-x_L in a lipid environment, the structure of Bcl-x_L in lipid micelles was characterized [20]. Upon interaction with dodecylphospho-

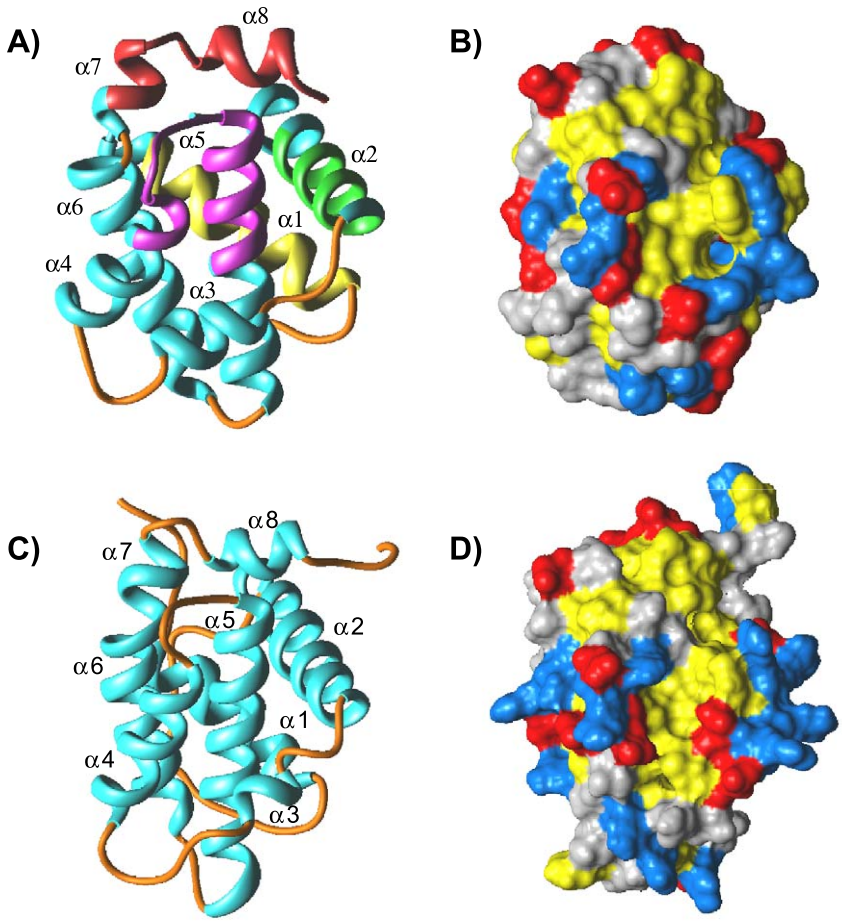

Fig. 2. Ribbon representations of (A) Bcl-x_L [13] and (C) Bcl-2 [21]. For Bcl-x_L, the BH1 region is colored magenta, the BH2 region is colored red, the BH3 region is colored green, and the BH4 region is colored yellow. The large unstructured loop between α1 and α2 is omitted for clarity. Helices are numbered with respect to Fig. 1. Connolly surfaces for (B) Bcl-x_L and (D) Bcl-2 were calculated using a probe radius of 1.4 Å and are colored as follows: leucine, valine, isoleucine, phenylalanine, tyrosine, tryptophan, methionine, and alanine are colored yellow; arginine, lysine, and histidine are colored blue; aspartic acid and glutamic acid are colored red, while all other residues are colored gray. The hydrophobic groove can be clearly seen (yellow) in the surface representations of both Bcl-x_L and Bcl-2.

choline (DPC) micelles, Bcl-x_L undergoes a conformational change. Based upon an analysis of protein-detergent NOEs and limited proteolysis, α1, α6, and the initial part of the long loop after α1 were shown to be embedded in the hydrophobic core of the micelle. In addition, Bcl-x_L is a monomer when incorporated in the micelle. These results contradict the findings that Bcl-x_L and other family members form multimers when incorporated in lipids and question the biological relevance of the structural conclusions obtained from Bcl-x_L in micelles.

3. Bcl-2

The three-dimensional structure of Bcl-2 has also been determined [21]. Like Bcl-x_L, the NMR structure of Bcl-2 is composed of eight α-helices with a hydrophobic groove on the surface (Fig. 2C and D). The overall backbone RMSD between Bcl-2 and Bcl-x_L is ~ 1.85 Å, excluding the loop between α1 and α2. The largest difference between the proteins is in the region of α3, which forms part of the hydrophobic groove. If this region is excluded, the backbone RMSD drops to ~ 1.66 Å. In Bcl-2 this region consists of a 3^{10}-helix while in Bcl-x_L it is a regular α-helix. In addition, the portion of the hydrophobic groove defined by α3 on one side is somewhat wider in Bcl-2 than in Bcl-x_L. This difference is likely due to hydrophobic contacts in Bcl-x_L between the side chains of Tyr 120 in α4 and Tyr 173 in α6, which pull the amino-terminal portion of α4 and hence the carboxy-terminal portion of α3 toward α6. In Bcl-2 the residue corresponding to Tyr 120 is an arginine, which forms a different set of interactions.

There are other differences in the primary sequence of the proteins, which change the character of the binding groove. Most notable are differences in sequence at position 104 (Ala in Bcl-x_L, Asp in Bcl-2), 108 (Leu in Bcl-x_L, Met in Bcl-2), and 122 (Ser in Bcl-x_L, Arg in Bcl-2) (Fig. 3). The alanine-to-aspartic acid and serine-to-arginine substitutions would be expected to have the most profound consequences, since they change the electrostatic character of the groove. This can be seen by comparing the color-coded surfaces of Bcl-x_L and Bcl-2 in Fig. 2B and D, respectively. In the surface representation, the wider more elongated groove of Bcl-2 is also evident. These differences in the topology and electrostatic character of the binding groove affect the binding of BH3 peptides from the pro-apoptotic proteins Bak and Bad. Both peptides bind about 10-fold more weakly to Bcl-2 than to Bcl-x_L [21], suggesting that these proteins possess a different specificity for binding to pro-apoptotic family members. It may also have important implications in the design of small-molecule modulators of apoptosis. Based on the structures, it may be possible to design Bcl-x_L selective, Bcl-2-selective, or inhibitors that bind to both of these proteins.

4. Bcl-x_L/Bak peptide complex

Mutational studies of Bcl-x_L suggested that an extensive surface, including the BH1 and BH2 regions, is necessary for hetero-dimerization with pro-apoptotic family members such as Bak. However, corresponding studies of pro-apoptotic members indicated that only a small portion of the protein involving the BH3 region is required for binding to Bcl-x_L [8,22,23]. Indeed, a short 16-residue peptide from the BH3 region of Bak was found to bind to Bcl-x_L with an affinity (K_d) of 340 nM [16]. Furthermore, peptides comprising the BH3 domain of Bak were shown to induce apoptosis in HeLa cells [24].

To determine the molecular basis for hetero-dimerization between members of the Bcl-2 family, a three-dimensional structure was determined by NMR of a complex between Bcl-x_L and a peptide from the BH3 region of the pro-apoptotic protein Bak [16]. The structure was solved using a truncated version of Bcl-x_L in which both the membrane-spanning helix and the large, unstructured loop between α1 and α2 were deleted. The three-dimensional structure of the truncated Bcl-x_L protein in the complex was found to be very similar to that of the free protein with the wild-type loop intact that was described in Section 2. The Bak peptide binds to the hydrophobic groove formed on the surface of the protein by the BH1-3 regions of Bcl-x_L and adopts an amphipathic α-helix (Fig. 4A). Several hydrophobic residues of the peptide (Val 74, Leu 78, Ile 81, and Ile 85) point into this groove and make contact with hydrophobic residues (Phe 97, Tyr 101, Phe 105, Leu 108, Val 126, Leu 130, Val 141Val) of Bcl-x_L (Fig. 4B).

The importance of specific interactions between Bcl-x_L and the Bak peptide was investigated by preparing alanine mutants of the Bak peptide and testing them for binding to Bcl-x_L [16]. The largest effect on binding was observed by substituting Leu 78 with alanine which caused a decrease in the affinity of the peptide nearly 800-fold. Substitution of Ile 85, which is located on the same side of the amphipathic α-helix, also significantly decreased peptide affinity. These results clearly demonstrate the importance of hydrophobic interactions in complex formation. In addition to hydrophobic interactions, charged side chains of the peptide (Arg 76,

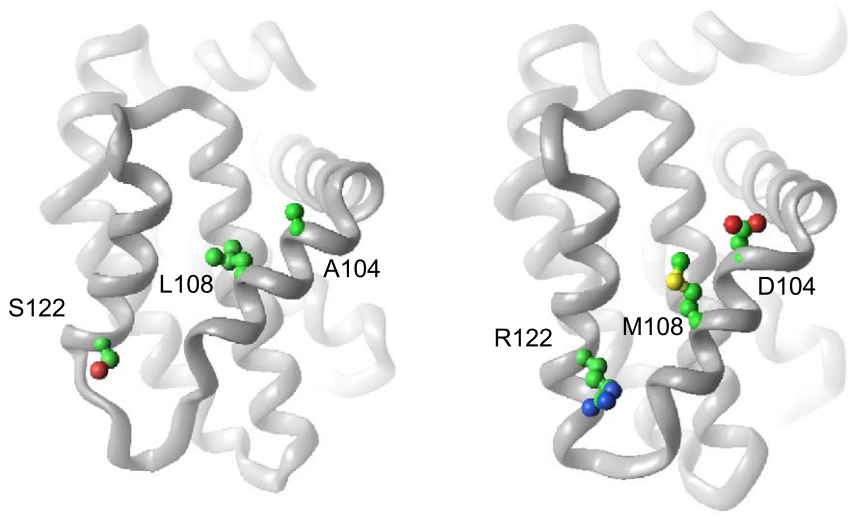

Fig. 3. Hydrophobic groove of Bcl-x_L [13] (left) and Bcl-2 [21] (right) highlighting residues which differ between the two proteins. Differences are located at positions 104 (Ala in Bcl-x_L, Asp in Bcl-2), 108 (Leu in Bcl-x_L, Met in Bcl-2), and 122 (Ser in Bcl-x_L, Arg in Bcl-2). Of these differences, the serine-to-arginine change would be expected to have the largest effect on the character of the groove.

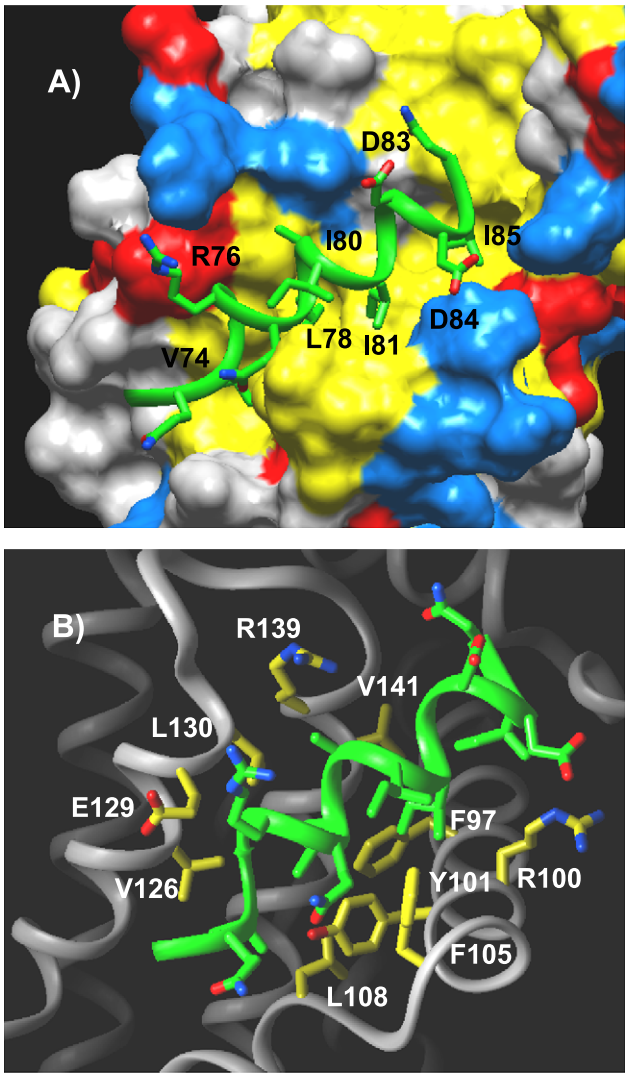

Fig. 4. (A) Connolly surface and (B) ribbon representation for Bcl-x_L complexed to a 16-residue peptide from the Bak protein [16]. The sequence of the Bak peptide is: GQVGRQLAIIGDDINR. The protein surface is color-coded as in Fig. 2. The Bak peptide binds to Bcl-x_L as an amphipathic α-helix which is stabilized by a number of hydrophobic contacts with the protein and via an electrostatic interaction between Asp 83 of the peptide and Arg 139 of Bcl-x_L. In (A) the key hydrophobic residues of the peptide are numbered with respect to the Bak protein while in (B) the key residues of the Bcl-x_L hydrophobic groove are labelled.

Asp 83, and Asp 84) also appeared to make significant contributions to binding based on the structure. In the structure of the complex, Asp 83 of the peptide is close to Arg 139 of Bcl-x_L, which is consistent with the 120-fold decrease in binding affinity observed when Asp 83 was mutated to an alanine. Analogously, mutation of Arg 139, which is completely conserved within the BH3 domains of Bcl-2 family members, to glutamine reduces both the anti-apoptotic activity of Bcl-x_L and its affinity for the pro-apoptotic Bax protein [23]. On the other hand, mutation of Asp 84 of the Bak peptide to alanine has no effect on the affinity of the peptide for Bcl-x_L despite the fact that it is proximal to Arg 100 of the protein.

Currently, no structural data are available for full-length Bak. However, a model of the Bak protein was constructed based on its homology with Bcl-x_L [16]. In this model, the key hydrophobic side chains of the BH3 region of Bak (Val 74, Leu 78, Ile 81, and Ile 85) point toward the interior of the protein and would thus be unavailable to interact with Bcl-x_L. This model for full-length Bak would require a conformational change in Bak to occur in order for these residues to be exposed.

Due to their importance in keeping cancer cells alive, Bcl-x_L and Bcl-2 are considered relevant targets for cancer chemotherapy. In fact, Bcl-2 anti-sense nucleotides are currently being tested in clinical trials for the treatment of cancer [25]. In addition, by utilizing the structure of the Bcl-x_L/Bak peptide complex, small molecule inhibitors of Bcl-x_L and Bcl-2 have been designed.

Wang et al. [26] were the first to report a small-molecule inhibitor of Bcl-2. These workers built a model of Bcl-2 based on the structure of the Bcl-x_L/Bak peptide complex and subsequently employed a computer docking strategy to screen 193,833 compounds from the Available Chemicals Directory. They identified the molecule shown in Fig. 5A and using a fluorescence polarization-based assay determined its IC_{50} for Bcl-2 to be ~ 9 μM. In addition, they showed that this compound does in fact induce apoptosis in HL-60 cells. In another study using computer-based screening, Enyedy et al. [27] searched the National Cancer Institute 3D database of 206,876 organic compounds to identify Bcl-2 binders. A model of Bcl-2 was created based

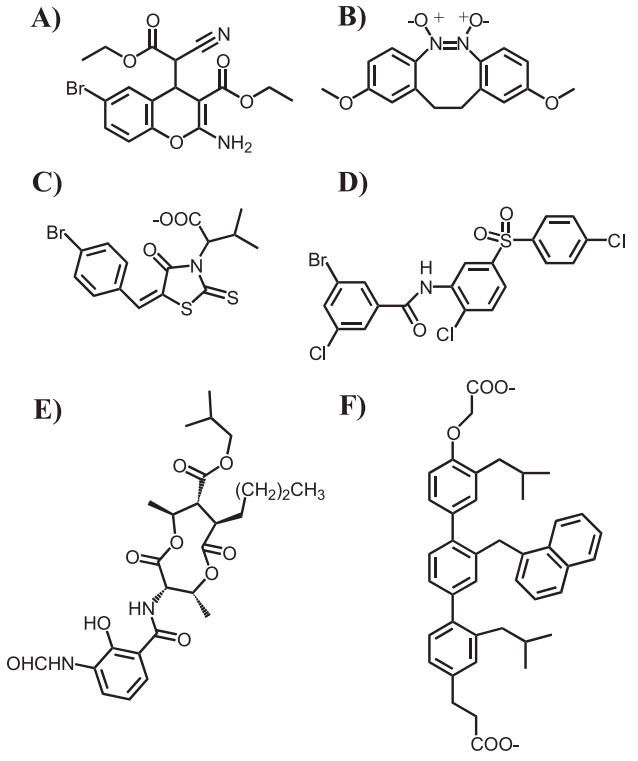

Fig. 5. (A–F) Small-molecule inhibitors of either Bcl-x_L or Bcl-2 [26–32].

on the NMR-derived structure of the Bcl-x_L/Bak peptide complex. The docking exercise yielded 35 potential binders, seven of which were shown to bind to Bcl-2 with IC_{50}s between 1.6 and 14.0 μM. The compound shown in Fig. 5B was the most effective in an anti-proliferation assay using HL-60 cells with an IC_{50} of 4 μM.

The structure of the Bcl-x_L/Bak peptide complex has also been used to complement the results of high-throughput screening. In 2001, Degterev et al. [28] carried out a fluorescence polarization-based screen of compound binding to Bcl-x_L. Out of 16,320 compounds tested, two series emerged with single-digit micro molar potency (Fig. 5C and D). The binding of these compounds to Bcl-x_L was confirmed by NMR, and, models were constructed of the complex using the chemical shift perturbation method [29]. These compounds were shown to induce apoptosis in Jurkat cells which overexpressed Bcl-x_L.

Based on the observation that members of the Bcl-2 family can affect mitochondrial integrity, Tzung et al. [30] hypothesized that small molecules which are known to effect mitochondrial respiration might directly trigger an apoptotic response. To test this hypothesis, they screened various small molecule inhibitors of respiration in isogenic hepatocyte cell lines with graded expression of Bcl-x_L. For antimycin A_3 (Fig. 5E), an inhibitor of electron transfer at complex III, they found that rather than protect cells from apoptosis, Bcl-x_L actually enhanced antimycin A_3-induced apoptosis. They went on to show that this effect is mediated through the binding of antimycin A_3 to the hydrophobic groove of Bcl-x_L and using the structure of the Bcl-x_L/Bak peptide complex, they constructed a model for the binding of antimycin A_3 to the hydrophobic groove of Bcl-x_L.

Another approach for identifying small molecules that bind to Bcl-x_L involved the design of peptidomimetics [31]. Based on the structure of the Bcl-x_L/Bak peptide complex, Kutzki et al. [32] synthesized a number of terphenyl derivatives designed to mimic the amphipathic α-helix of Bak that was shown to bind into the hydrophobic groove of Bcl-x_L. The best of these is shown in Fig. 5F. It binds to Bcl-x_L with a K_d of 114 nM. Docking studies involving this compound suggest that this molecule fits into the hydrophobic groove of the protein in a similar orientation as the Bak peptide. Further evidence to support this hypothesis involved NMR experiments to identify key residues in the groove that experienced chemical shift changes upon binding of the compound.

5. Bcl-x_L/Bad peptide complex

Like Bak, the pro-apoptotic protein Bad binds to Bcl-x_L. However, unlike the tight binding observed with the Bak 16-mer, an analogous 16-residue peptide from the BH3 region of Bad displayed only weak affinity for Bcl-x_L (K_d>50 μM). It was found that tight binding of the Bad peptide (K_d ~ 6 nM) requires five additional residues at the amino terminus and four residues at the carboxy terminus [33,34]. The rationale for the importance of the residues for complex formation was examined in structural studies of the complex and binding studies of mutant Bad peptides [35].

The overall structure of the Bcl-x_L/Bad peptide complex was found to be very similar to that of the Bcl-x_L/Bak peptide complex (Fig. 6). Like the Bak peptide, the Bad 25-mer binds as an amphipathic α-helix, and similar interactions stabilize complex formation. The additional asparagine and tryptophan residues at the amino terminus of the Bad peptide are completely solvent exposed; whereas, Leu 141, Ala 144, and Ala 145 contact the protein. At the carboxy terminus of the Bad peptide, only the added Phe makes contact with the protein. Surprisingly, mutation of the five additional residues at the amino terminus or the four additional residues at the carboxy terminus to either alanine (or glycine, in the case of Ala 144 and Ala 145) had essentially no effect on the ability of the Bad 25-mer to bind to Bcl-x_L. Furthermore, mutation of individual residues of the Bad peptide that contact the protein in the Bcl-x_L/Bad structure to the corresponding residue in Bak did not improve the affinity of the Bad 16-mer for Bcl-x_L with the exception of the carboxy-terminal aspartic acid. When this residue was mutated to an arginine, the corresponding residue in the Bak peptide, the affinity of the Bad 16-mer for Bcl-x_L improved about 15-fold. Examination of the structure of the Bad 25-mer bound to Bcl-x_L revealed that this aspartic acid residue (Asp 160) is predicted to be one helical turn away from another aspartic acid (Asp 156) (Fig. 6). A repulsive interaction between these negatively charged residues would be expected to disfavor helix formation. This

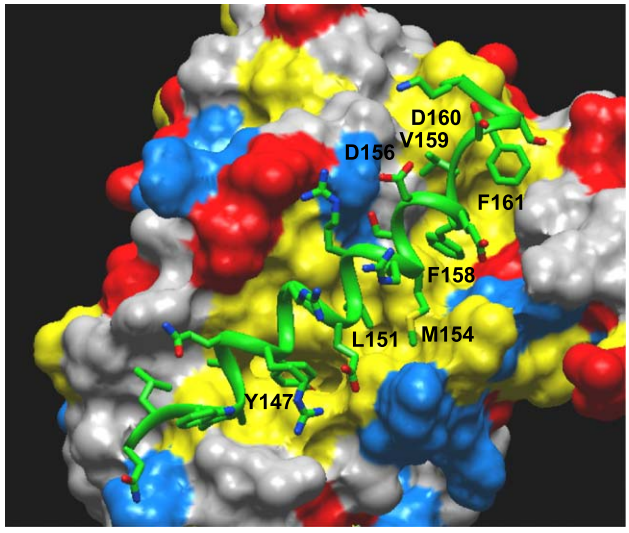

Fig. 6. Connolly surface for the complex of Bcl-x_L with a 25-residue peptide derived from the Bad protein [35]. The sequence of the Bad peptide is: NLWAAQRYGRELRRMS DEFVDSFKK. As with Bak peptide binding to Bcl-x_L, the Bad peptide binds into the groove as an amphipathic α-helix. Protein surface is color-coded as in Fig. 2. The key hydrophobic residues of the peptide are labelled along with the critical aspartic acid residue (Asp 156).

observation suggests that helix propensity plays an important role in the binding of the Bad peptide to Bcl-x_L. To test this hypothesis, two mutant 16-residue Bad peptides were designed which would be predicted to maintain the protein contacts of the longer Bad peptide but have an increased propensity to form an α-helix compared to the wild-type 16-mer. These peptides showed a 100-fold increase in affinity for Bcl-x_L compared to the original 16-residue Bad peptide and thus confirmed the importance of helix propensity for the binding of BH3 peptides to Bcl-x_L.

6. Bax

The overall fold of the pro-apoptotic protein Bax resembles that of Bcl-x_L and Bcl-2 [36]. Bax has seven amphipathic helices clustered around two central, mostly hydrophobic α-helices (Fig. 7). Like its anti-apoptotic counterparts, a long unstructured loop connects α1 with α2. Helices 2, 3, and 4 are in the same relative orientation as in Bcl-x_L and form a hydrophobic groove. The orientation and lengths of α5, α6, α7, and α8 are also very similar to those in Bcl-x_L. Helix 9 of Bax, which corresponds to the transmembrane α-helix of Bcl-x_L and Bcl-2, binds into a hydrophobic groove of the protein. The binding site corresponds to the same site on Bcl-x_L which binds to the Bak and Bad peptides. However, the α-helix from Bax binds in the groove in the opposite direction compared to how the Bak and Bad peptides bind to Bcl-x_L. In binding into the groove, α9 not only covers the hydrophobic residues of the groove, but also buries its own hydrophobic residues. This may be important for increasing the solubility of the protein and contributes to the fact that Bax exists predominantly in the cytosol before apoptosis induction.

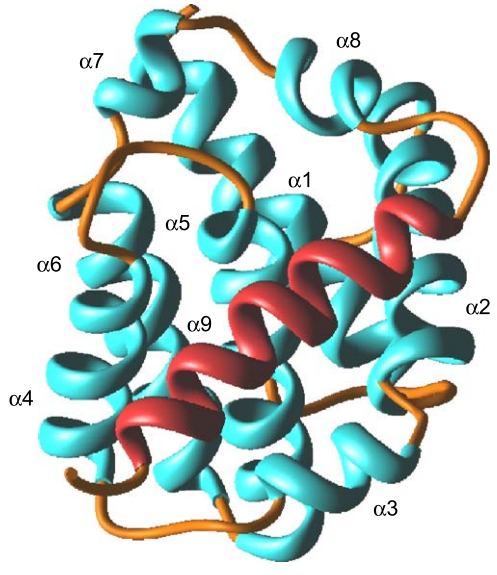

Fig. 7. Ribbon representation of Bax [36]. Helices are numbered with respect to Bcl-x_L. Helix 9 (colored red), which corresponds to the putative transmembrane helix of Bcl-x_L and Bcl-2, sits in the groove formed by α2, α3, α4, and α5. It sits in the hydrophobic groove in a similar manner to that observed for the Bak and Bad peptides bound to Bcl-x_L with the exception that its direction is reversed.

One mechanism proposed for how Bcl-x_L and Bcl-2 maintain the apoptotic balance in the cell and prevent apoptosis is through their ability to hetero-dimerize with pro-apoptotic Bcl-2 family members. On a molecular level this involves binding of BH3-containing pro-apoptotic proteins such as Bak and Bax into a hydrophobic groove on the surface of Bcl-x_L or Bcl-2. In the case of Bax, the hydrophobic side chains of the BH3 helix, which would presumably be involved in binding to the anti-apoptotic family members, point inward toward the hydrophobic core of the protein and are covered by α9. To expose these residues, a conformational change of Bax would be required in which α9 disengages from the binding groove and α2 rotates about its axis to expose these key residues. This conformational change is analogous to the change proposed for Bak based on homology modeling studies [16]. It is clear from the Bax structure that such a rearrangement would disrupt the hydrophobic core of the protein and is thus unlikely to occur without a stimulus [36].

It has been suggested that a rapid increase in intracellular pH upon cytokine withdrawal serves as a trigger for apoptosis and causes the translocation of Bax into the mitochondria [37]. In solution, no structural change in Bax was observed over the pH range of 6 to 8 [36]. Detergents are also known to induce complex formation of Bax [11]. Titration of Bax with β-octyl glucoside triggers oligomer formation, which subsequently induces a major conformational change in the protein. However, the exact nature of this conformational change has not yet been elucidated.

7. Bcl-w

The structure of the anti-apoptotic protein Bcl-w has recently been determined by two groups using NMR spectroscopy [38,39]. Its overall fold is similar to that found for Bcl-x_L, Bcl-2, and Bax (Fig. 8). Like the other familiy members, Bcl-w is composed of two, mostly hydrophobic α-helices surrounded by amphipathic α-helices. Also, analogous to Bcl-x_L, Bcl-2, and Bax, Bcl-w contains a hydrophobic groove on its surface.

An interesting feature of the Bcl-w structure is that it has an additional carboxy-terminal α-helix which sits in the hydrophobic groove of the protein. This is similar to the carboxy-terminal α-helix of the pro-apoptotic protein Bax which also lies in its hydrophobic groove. As would be expected, this additional carboxy-terminal helix causes a moderate decrease in the affinity of BH3 proteins and peptides to Bcl-w. For example, the affinity of the pro-apoptotic Bim protein for Bcl-w increases threefold upon removal of this helix [39]. This helix is distinctly more mobile than the other α-helices of Bcl-w and more mobile

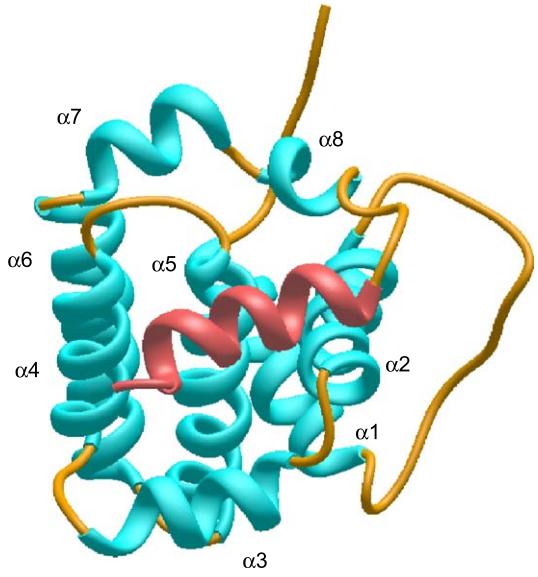

Fig. 8. Ribbon representation of Bcl-w [38]. Helices are numbered with respect to Bcl-x_L. Helix 9 (colored red) binds into the groove formed by α2, α3, α4, and α5.

than α9 of Bax based on an analysis of $^{15}N\{^1H\}$ heteronuclear NOE measurements [39]. The carboxy-terminal α-helix of Bax has more hydrophobic side chains in contact with the groove than α9 of Bcl-w, which likely accounts for its restricted mobility. While the extra α-helix of Bax is believed to play a regulatory role, it is currently unclear whether the extra helix of Bcl-w is also involved in regulation of the protein's activity.

8. Bid

The pro-apoptotic Bid protein is considered to be part of the "BH3 only" subfamily of Bcl-2 proteins [40] since it is only homologous to the BH3 region of Bcl-2. Bid is normally present in an inactive form in the cytosol. In response to stimuli of the extrinsic, death receptor, pathway, Bid is proteolytically cleaved by caspase 8 and thus activated [12]. The activated protein, tBid, translocates to the mitochondria and serves to link signaling through the FAS receptor with the mitochondrial death machinery. In the absence of detergents, only the activated form of the protein, tBid, binds to Bcl-x_L [12].

The solution structure of the Bid protein was determined using NMR spectroscopy by two groups independently. The overall fold is remarkably similar to Bcl-x_L and Bcl-2 [40,41]. Although it is considered to be a "BH3 only" protein based on sequence homology with Bcl-2, it clearly contains all of the structural elements of Bcl-2 and Bcl-x_L. Like these anti-apoptotic proteins, Bid has two central, mostly hydrophobic α-helices surrounded by six amphipathic helices (Fig. 9A). The main difference in overall fold is in the amino-terminal portion of the protein. Bid contains an extra α-helix compared to Bcl-x_L (α1′) that packs in an anti-parallel fashion against α1. This α-helix is followed by a long unstructured loop like that found in Bcl-x_L, which connects it to α2. Thus, this third helix of Bid corresponds to the BH3 region of Bcl-x_L. A hydrophobic patch is located on the surface of Bid (Fig. 9B). This patch is flat and does not form a deep cleft similar to that observed in the structures of Bcl-x_L and Bcl-2. This difference may explain why unprocessed Bid cannot form homo- or hetero-dimers with other Bcl-2 family members. Ile 88, Leu 92, Val 95, and Met 99 in Bid are homologous to the hydrophobic residues of the Bak BH3 peptides that were shown to be important for binding to Bcl-x_L [16]. Both Val 95 and Met 99 are exposed in the Bid structure; however, Ile 88 and Leu 92 are partially buried (Fig. 9B). Thus, a change in conformation would be necessary in order for the Bid BH3 domain

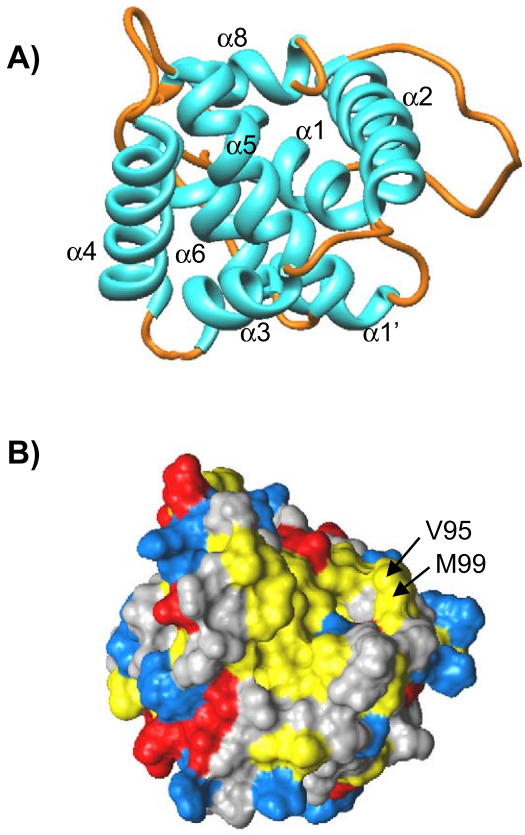

Fig. 9. (A) Ribbon representation of Bid [40]. Helices are numbered with respect to Bcl-x_L. Bid contains an extra α-helix between α1 and α2 of Bcl-x_L. Structurally, the third helix of Bid corresponds to the second helix of Bcl-x_L. This extra helix is denoted α1′ . (B) Connolly surface of Bid with atoms color-coded as in Fig. 2. The hydrophobic groove (yellow) present on the surface of the protein is neither as long nor as deep as that found on the surface of Bcl-x_L and Bcl-2.

to bind in the same manner as the Bak BH3 peptide binds to Bcl-x_L [40]. Another difference between Bid and Bcl-x_L is the absence of an α-helix corresponding to α7. In Bid, α6 is followed by the final C-terminal helix of the protein. The position of this helix is similar to that of α8 in Bcl-x_L (Fig. 2A).

Like Bcl-x_L, the long loop in Bid that precedes the BH3 region is susceptible to caspase cleavage. However, in contrast to Bcl-x_L, for which this cleavage converts the function of the protein from anti-apoptotic to pro-apoptotic, caspase-8 cleavage of Bid converts it from an inactive form to a pro-apoptotic form [12]. The activated fragment, tBid, lacks the first helix, the small additional helix, and part of the unstructured loop. The first α-helix of Bid has hydrophobic interactions with the BH3 region in the native protein. Removal of this helix would expose a large hydrophobic surface on the BH3 helix [40,41]. This activating cleavage causes a change in the localization of the protein and the ability of the protein to bind to Bcl-x_L.

Conformational changes that are likely to occur because of this cleavage have not been determined. Initial attempts to process Bid in an NMR tube did not lead to any clear changes in the NMR spectrum. When cleaved, the fragment remained associated with the protein. Chou et al. [40] proposed that although the amino-terminal fragment of Bid remains associated with the core of the protein at NMR concentrations, at low cellular concentrations this fragment might detach to give the active species.

As discussed above, hetero-dimerization of Bcl-x_L or Bcl-2 with Bax through its BH3 helix would require a major conformational change in the protein. In Bid, full exposure of the BH3 domain may be regulated by the posttranslational cleavage event. The conserved hydrophobic residues of the Bid BH3 region are more solvent exposed than those in Bax [36,40]. Model building studies, based on this observation and on the solution structures of the Bcl-x_L/Bak peptide complex and that of Bid, suggest that a hetero-dimer could be formed with only minor stereochemical conflicts between the two molecules, and these could be relieved by a slight movement of the Bid BH3 region [40].

9. Kaposi sarcoma-associated herpes virus (KSHV) Bcl-2

When a host is invaded by a virus, the response is typically the activation of the extrinsic pathway of apoptosis in the infected cells. A few cells are sacrificed in order to spare the organism. To counteract the host response, some viruses have acquired the ability to make anti-apoptotic proteins [42]. For example, many of the large DNA viruses, including the poxviruses or gamma herpesviruses, encode a functional Bcl-2 homolog.

Recently, the NMR structure of a Bcl-2 homolog from KSHV was reported [43]. Despite the low sequence homology (Fig. 1) between the viral protein and Bcl-x_L and Bcl-2, its overall fold is very similar (Fig. 10A). Like Bcl-x_L and Bcl-2, KSHV Bcl-2 is an all α-helical protein. Two central hydrophobic helices (α5 and α6) form the core of the protein. These are sandwiched between the amphipathic helices α3 and α4 on one side and by α1 and α2 on the other side. One difference between the proteins is that the loop connecting α1 to α2 is much shorter in KSHV Bcl-2 than in Bcl-x_L or Bcl-2. In addition, this loop does not contain a caspase cleavage site like that found in Bcl-2 and Bcl-x_L or the inactivating phosphorylation site which may allow KSHV Bcl-2 to escape these extra levels of regulation found in the mammalian proteins [44]. As in other Bcl-2 family members, the signature NWGR sequence at the beginning of α5 is conserved. The tryptophan residue clearly serves the same structural role in KSHV Bcl-2 as it does in Bcl-x_L and Bcl-2 and makes intimate hydrophobic contacts with residues in α7 and α8.

A further similarity of KSHV Bcl-2 to Bcl-x_L and Bcl-2 is the presence of a hydrophobic groove on the surface of

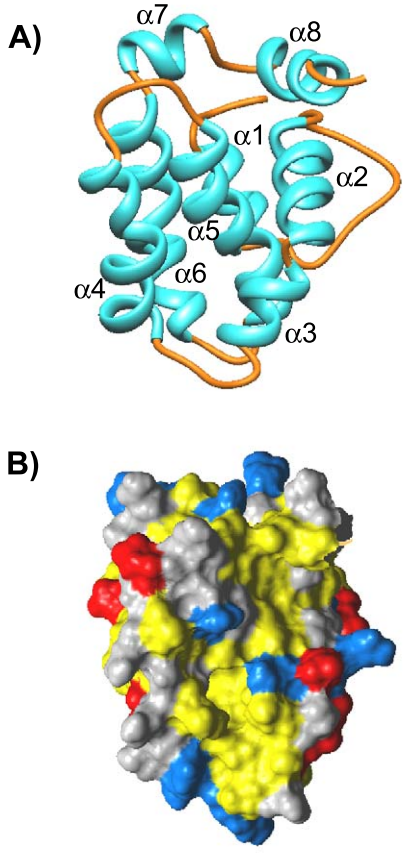

Fig. 10. (A) Ribbon representation of KSHV Bcl-2 [43] in the same orientation as Bcl-x_L and Bcl-2 in Fig. 2. Helices are numbered with respect to Bcl-x_L. (B) Connolly surface of KSHV Bcl-2 with atoms color-coded as in Fig. 2.

the protein composed of side chains from α2, α3, α4, and the loop connecting α4 to α5 (Fig. 10B). The position of α3 in KSHV Bcl-2 is similar to that found in Bcl-2. This causes the hydrophobic pocket to be wider and longer than that found for Bcl-x_L. Hydrophobic residues line the pocket and are located in a similar position to those found in both Bcl-x_L and Bcl-2. In addition, like Bcl-x_L and Bcl-2, KSHV Bcl-2 binds to the BH3 peptides from the pro-apoptotic proteins Bak, Bad, and Bax. The Bak 16-mer binds to KSHV Bcl-2 with a dissociation constant <50 nM while the Bad 25-mer and a 16-residue peptide from Bax bind with affinities of 3900 and 980 nM, respectively. The greater affinity of KSHV Bcl-2 for the Bak and Bax peptides compared to Bad is different from the relative affinities of these peptides for binding to Bcl-x_L and Bcl-2.

10. Conclusions

Structural studies of the Bcl-2 family of proteins have led to a number of key insights on how these proteins function and how they are regulated at the molecular level. Despite the sequence diversity among the family members and the diversity of function, they have a remarkably similar fold. This fold consists of two central, predominantly hydrophobic α-helices surrounded by six or seven amphipathic α-helices. The overall topology of the Bcl-2 proteins is similar to that observed for the membrane translocation domain of the bacterial toxins such as diphtheria toxin and the colicins. This observation led to experiments which showed that Bcl-x_L and Bcl-2 can form pores in artificial membranes. The structure of the Bcl-x_L/Bak peptide complex provided important insights into the mechanism of hetero-dimer formation between pro- and anti-apoptotic family members. In addition, this structure has been used to guide the discovery of small molecule inhibitors of Bcl-x_L and Bcl-2 function. Finally, structural studies carried out on the pro-apoptotic proteins Bax and Bid raised important questions regarding the active form of these proteins in the cell.

From an evolutionary perspective, apoptosis is a very old and highly conserved process. The strong structural and sequence homology between the Bcl-2 family members suggests that they have all descended from a common gene. While structural studies have provided many insights into the molecular mechanism of apoptosis and have been used to guide the design of small molecule that bind to these proteins, there are still many unanswered questions. The exact nature of the conformational change required for Bak or Bax binding to Bcl-x_L and Bcl-2 still needs to be determined as well as any structural changes Bcl-x_L, Bcl-2, and Bax may undergo upon membrane association. In addition, although both Bcl-x_L and Bcl-2 have been shown to form pores in artificial membranes, it is still unclear how this relates to the ability of these proteins to regulate apoptosis. However, despite these and other questions, it is clear that structural studies on the Bcl-2 family of proteins have, and will continue, to deepen our understanding of the apoptotic process and may ultimately lead to the development of novel therapeutic agents.

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