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Review

Structural biology of the Bcl-2 family and its mimicry by viral proteins

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Intrinsic apoptosis in mammals is regulated by protein–protein interactions among the B-cell lymphoma-2 (Bcl-2) family. The sequences, structures and binding specificity between pro-survival Bcl-2 proteins and their pro-apoptotic Bcl-2 homology 3 motif only (BH3-only) protein antagonists are now well understood. In contrast, our understanding of the mode of action of Bax and Bak, the two necessary proteins for apoptosis is incomplete. Bax and Bak are isostructural with pro-survival Bcl-2 proteins and also interact with BH3-only proteins, albeit weakly. Two sites have been identified; the in-groove interaction analogous to the pro-survival BH3-only interaction and a site on the opposite molecular face. Interaction of Bax or Bak with activator BH3-only proteins and mitochondrial membranes triggers a series of ill-defined conformational changes initiating their oligomerization and mitochondrial outer membrane permeabilization. Many actions of the mammalian pro-survival Bcl-2 family are mimicked by viruses. By expressing proteins mimicking mammalian pro-survival Bcl-2 family proteins, viruses neutralize death-inducing members of the Bcl-2 family and evade host cell apoptosis during replication. Remarkably, structural elements are preserved in viral Bcl-2 proteins even though there is in many cases little discernible sequence conservation with their mammalian counterparts. Some viral Bcl-2 proteins are dimeric, but they have distinct structures to those observed for mammalian Bcl-2 proteins. Furthermore, viral Bcl-2 proteins modulate innate immune responses regulated by NF-κB through an interface separate from the canonical BH3-binding groove. Our increasing structural understanding of the viral Bcl-2 proteins is leading to new insights in the cellular Bcl-2 network by exploring potential alternate functional modes in the cellular context. We compare the cellular and viral Bcl-2 proteins and discuss how alterations in their structure, sequence and binding specificity lead to differences in behavior, and together with the intrinsic structural plasticity in the Bcl-2 fold enable exquisite control over critical cellular signaling pathways.
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Facts

- Heterodimerization and conformational change of cellular Bcl-2 proteins underpins the initiation and modulation of the intrinsic apoptosis.
- Viral Bcl-2 proteins can adopt different homodimeric configurations that allow modulation of the Bcl-2-mediated pathway as well as NF-κB signaling.
- In contrast to cellular pro-survival Bcl-2 proteins, their viral counterparts have lower affinities for their BH3-only binding partners.

Open Questions

- The structural basis of full-length Bak/Bax engagement by pro-survival Bcl-2 proteins has yet to be elucidated.
- Does the Bax/Bak oligomeric pore have a defined architecture in the MOM?
- Is there a role for cellular Bcl-2 proteins in NF-κB signaling, analogous to that observed for viral Bcl-2 proteins?
- What are the key cellular pro-apoptotic Bcl-2 proteins requiring neutralization during viral infection?

Cues that initiate apoptosis, the programmed elimination of cells no longer required, infected or dangerous to an organism may arise from stimuli external to the cell (extrinsic or death receptor-induced apoptosis) or intracellular (intrinsic, mitochondrial or Bcl-2-regulated apoptosis).[1,2] The result of activation of either mode of apoptosis is the induction of a proteolytic cascade of cysteine aspartyl proteases (caspases) that dismantles cells before their engulfment by phagocytes and destruction in lysozomes.[3] The final fate of cells broken

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Abbreviations: Bcl-2, B-cell lymphoma-2; BH, Bcl-2 homology; IDP, intrinsically disordered protein; IDR, intrinsically disordered region; MOM, mitochondrial outer membrane; MOMP, MOM permeabilization; vBcl-2, viral Bcl-2
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down and packaged during apoptosis is to be recycled through phagocytic clearance. Bcl-2 proteins may also have a part in autophagic cellular destruction,[4] though the crosstalk between autophagy and apoptosis is not yet well understood[5] and probably does not have a more general role in programmed cell death.[6] Molecular mechanisms of apoptotic cell death are highly conserved, and the extrinsic and intrinsic pathways intersect at the mitochondria by direct regulation of the Bcl-2 family.

Approximately 20 proteins define the mammalian B-cell lymphoma-2 (Bcl-2) family and regulate intrinsic apoptosis.[2,7] This family has been traditionally defined by the presence of a set of short conserved sequences (<20 residues) known as Bcl-2 homology (BH) motifs (also commonly called domains though they are not *bona fide* discreetly folded compact entities, but rather represent conserved sequence motifs within the Bcl-2 protein). Responding to diverse apoptotic stimuli Bax or Bak, the two key Bcl-2 proteins, initiate cell death by mitochondrial outer membrane permeabilization (MOMP).[8] Release of cytochrome *c* and other factors from the mitochondrial intermembrane space, by an as yet ill-defined mechanism of MOMP, initiates the critical caspase cascade.[7] Bcl-2 proteins either inhibit or activate MOMP and interactions between pro-survival and pro-apoptotic proteins of the family adjudicate cell death. Many of these interactions have been elucidated biochemically and there is now a well-understood pedigree of interactions within this family.[9–11]

Viruses have evolved multiple strategies to subvert host cell apoptosis in response to cellular infection.[12] Viral interference with apoptotic signaling can occur at multiple control points, such as inhibition of death receptor activation (extrinsic apoptosis),[13] direct caspase inhibition,[14] or by mimicking pro-survival Bcl-2 family action (intrinsic apoptosis)[15] (Figure 1). Here we discuss recently determined structures in the mammalian Bcl-2 family and compare mammalian and viral Bcl-2 proteins and viral Bcl-2 mimicry. Not all features of mammalian Bcl-2 structure and function need be retained by viral Bcl-2 proteins, as the essential goal is to maintain host cell viability while permitting viral replication to proceed in the absence of apoptosis. Indeed, there are remarkable similarities between some viral Bcl-2 proteins and their mammalian counterparts. There are also perplexing differences between the dimers observed in the viral Bcl-2 family from those of their mammalian equivalents.

BH Motifs—a Distinguishing Feature of the Bcl-2 Family?

Sequence analysis of the Bcl-2 family identified conserved regions,[16] later named as BH motifs. BH1 and BH2 motifs were initially identified in Bcl-2[17] and the BH3 motif in Bak,[18] with a fourth poorly conserved[19] motif, BH4, proposed in the N-terminal region of Bcl-2 and the closely related Bcl-x_L and Bcl-w.[16,20] Searches for BH motifs in numerous genomes led to the identification of many Bcl-2 family members[21,22] and potential, though experimentally unproven, members.[23] The mammalian Bcl-2 family can be classified into the multi-motif Bcl-2 proteins that bear multiple BH motifs with pro-survival (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, Bcl-B) and pro-apoptotic (Bax, Bak) activity. The pro-apoptotic BH3-only proteins (Bim, Bad, Bmf, tBid, Noxa, Bik, Puma, Hrk) bear only a single motif, a BH3.

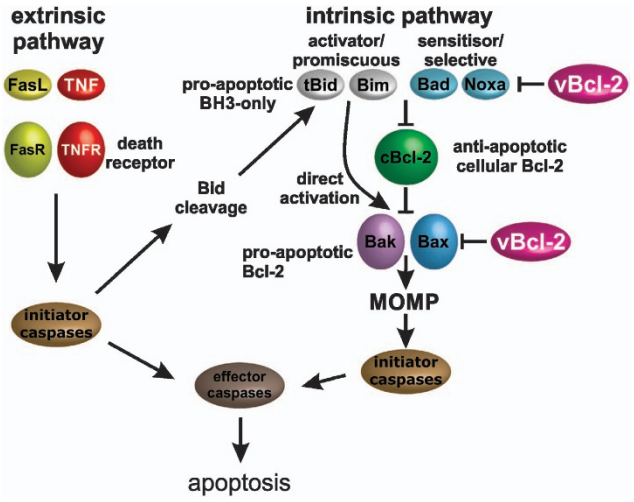

Figure 1 Points of intervention of viral Bcl-2 proteins in mammalian Bcl-2-regulated apoptosis. Shown is a simplified schematic view of apoptosis induction in mammalian cells and its inhibition by pro-survival viral Bcl-2 (vBcl-2) proteins. Apoptosis may be initiated by activation of cell surface receptors (extrinsic apoptosis or death receptor-initiated apoptosis[7]) or by intracellular mechanisms ('intrinsic' or mitochondrial pathway). In either case, a proteolytic cascade of caspases is initiated that destroys the cell. A network of interactions between Bcl-2 proteins controls mitochondrial membrane integrity. BH3-only proteins may either inhibit pro-survival proteins or activate the two key Bcl-2 family members, Bax and Bak, to disrupt the mitochondrial outer membrane and release caspase-activating factors. Viral Bcl-2 proteins bind Bax or Bak or BH3-only proteins to prevent apoptosis induction. cBcl-2, cellular Bcl-2

The presence of a BH3 motif defines all pro-apoptotic Bcl-2 proteins,[24] although it may or may not be present in pro-survival proteins. Analysis of BH3-sequence proteins delineates a motif that bears four hydrophobic residues spanning ~2 heptads. One of these residues is an almost invariant leucine, separated by three residues from a Xaa-Asp dyad, where Xaa is Gly, Ala or Ser, and is a feature that distinguishes BH3 motifs.[9,25] The BH4 motif appears to be less defining, early classifications suggested that this motif was only present in certain pro-survival proteins but not in the pro-apoptotic members.[26] However, more recently, the BH4 was re-defined as a conserved structure-sequence motif, ϕ1-ϕ2-X-X-ϕ3-ϕ4, found in all folded Bcl-2 proteins, where X is any amino acid, ϕ1, ϕ2 and ϕ4 are aliphatic residues and ϕ3 an aromatic residue.[27] Being present on both pro-survival, including viral Bcl-2 homologs, and pro-apoptotic Bcl-2 proteins, it cannot distinguish them and as an isolated parameter does not identify Bcl-2 proteins.[28] Not all BH motifs are present within a given Bcl-2 protein and several viral Bcl-2 fold proteins lack obvious BH motifs (e.g., M11L, N1L, F1L)[27,29–32] altogether indicating BH motifs are not an essential feature for Bcl-2-fold proteins. As structures of more distantly related Bcl-2 family proteins emerge, our understanding of the sequence and structural diversity permitted in the Bcl-2 family will be refined.

An α-Helical Fold Provides a Binding Site and Defines Multi-Motif Bcl-2 Proteins

Structural studies have defined the Bcl-2 family interactions at atomic resolution.[33–36] The structures of the multi-motif

proteins are highly homologous α-helical bundles. Figure 2 shows the basic fold, as exemplified by Bcl-x_L, shared by both the pro-apoptotic and anti-apoptotic multi-motif proteins. Structures of BH3 complexes of the pro-survival proteins show that the BH3 ligand binds as an amphipathic helix in a hydrophobic groove formed from residues in the BH1, BH2 and BH3 motifs (or their structural equivalents where the BH sequence motif is absent) of the pro-survival protein.[35,37] The majority of intermolecular contact residues reside in a 13-residue segment of the BH3 motif, including the 4 hydrophobic residues,[25] and both molecules contribute almost equally to the binding surface (~1000 Å^2 buried surface each). Although the topology of the pro-survival proteins is nearly identical, each protein has a different specificity for BH3 targets (summarized in Table 1).

The distinction between the BH3-only and the multi-motif proteins is not only in their biological activity and sequences but also in their structures. The BH3-only proteins are intrinsically disordered proteins (IDPs)[38,39] or become disordered after processing.[40] In contrast, the multi-motif proteins are folded entities, but bear intrinsically disordered regions (IDRs).[39] The sequence and structural differences between the multi-motif and BH3-only proteins lead to the conclusion that they are separate phylogenetic classes.[41]

Structures of full-length Bax[42] and Bid[43,44] have been determined, but generally structural investigations have focused on 'transmembrane' (TM)-region truncated proteins due to their better expression and solubility properties. Bid, the only BH3-only protein with a well-defined structure, has a hydrophobic core structure similar to the Bcl-2 fold.[43,44] Activation of Bid by proteolytic cleavage in the α1-α2 loop by multiple proteases, including caspase-8, Granzyme B, calpains and cathepsins,[45] to form truncated Bid (tBid) containing the BH3 region leaves it stably folded.[44] Like Bak and Bax, the Bid BH3 motif is released for binding in the presence of a membrane environment.[46] In Bax, the C-terminal α-helix occupies a similar position to the BH3-only binding site on pro-survival proteins and blocks the groove. Bcl-w[47,48] has a similar structure to Bax and biochemical and kinetic evidence is consistent with the C-terminal residues of other Bcl-2 proteins, such as Bcl-x_L and Mcl-1, also occupying the groove.[47,49] These findings indicate that the binding groove is hindered or occupied in the multi-motif proteins.

Two distinct roles are performed by BH3-motif residues: one as a ligand, the other as part of the receptor site groove, in each case different atomic interactions are observed for the BH3. This BH3-in-groove interaction, where the groove is formed by helices α2-α5 (Figure 2) and occupied by the BH3 motif is a key mechanistic aspect of Bcl-2-regulated apoptosis and one that viral Bcl-2 proteins mimic to prevent cell death initiation.

Bcl-2 Proteins BH3-Ligand Specificity

Investigation of the affinity and specificity of interactions between Bcl-2 proteins[9–11] (Table 1) led to proposals for the mechanisms of apoptosis initiation. Two main mechanisms have been hypothesized. The 'indirect activation'

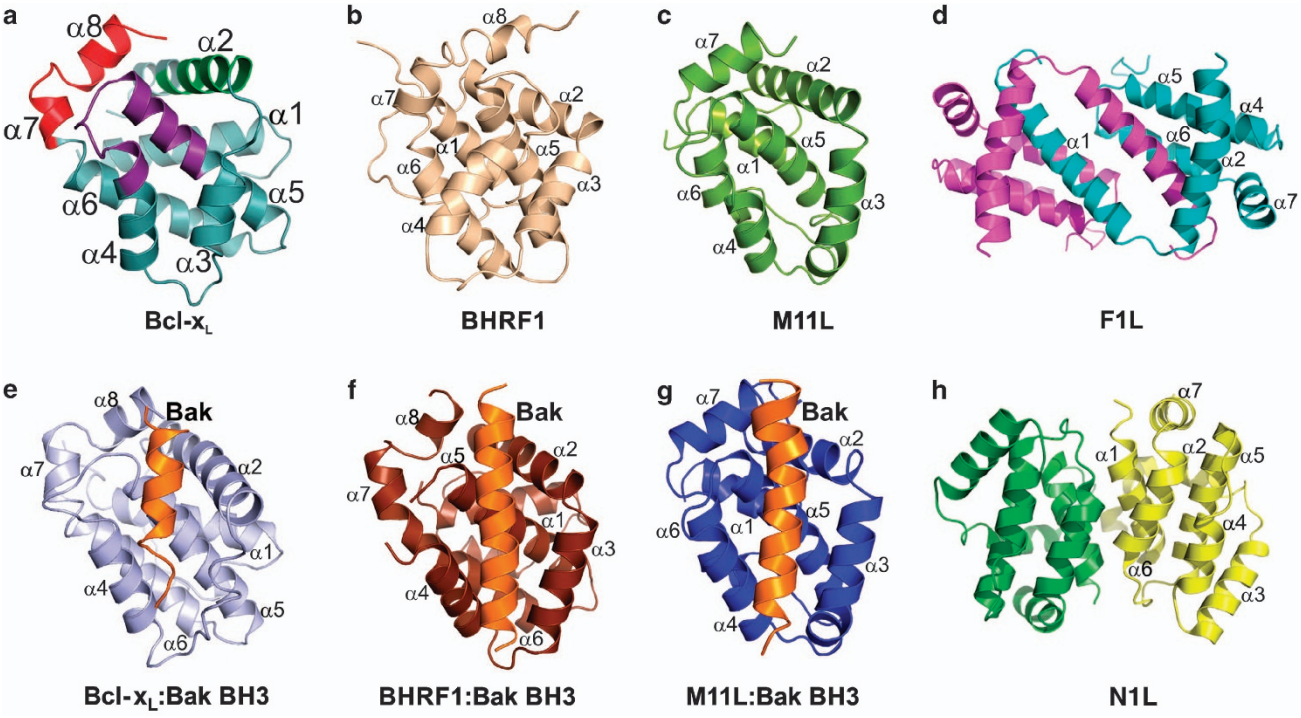

Figure 2 Structural comparison of human and viral Bcl-2 proteins and their complexes with Bak BH3 peptides. (**a**) Human Bcl-x_L (pdb accession code: 1MAZ) with the BH-motifs colored BH1 purple; BH2, red; BH3, green. (**b**) Epstein-Barr virus BHRF1 (1Q59), (**c**) Myxoma virus M11L (2BJX), (**d**) Vaccinia virus F1L (2VTY), (**e**) Bcl-x_L:Bak (1BXL), (**f**) BHRF1:Bak (2XPX), (**g**) M11L:Bak (2JBY), (**h**) Vaccinia virus N1L (2UXE). The Bak BH3 peptide is shown in orange. Structures were aligned on Bcl-x_L in **a**. Protomers of the F1L and N1L dimers are depicted in different colors

Table 1 Summary of BH3 motif affinities for Bcl-2 proteins

Pro-death	Pro-survival										
	SPPV14	M11L	F1L	BHRF1[a]	KsBcl-2[b]	N1L[b*]	Bcl-2	Bcl-w	Bcl-x_L	Mcl-1	A1[a]
Bad	>2000	>1000	NB	>2000	>1000	>1000	16	30	5.3	>100 000	15 000
Bid	341 ± 16	100	NB	109	112	152	6800	40	82	2100	1
Bik	>2000	>1000	NB	>2000	>1000	n/a	850	12	43	1700	58
Bim	26 ± 4	5	250	18	29	72	2.6	4.3	4.6	2.4	1
Bmf	67 ± 6	100	NB	>2000	>1000	n/a	3	9.8	9.7	1100	180
Hrk	63 ± 6	>1000	NB	>1000	>1000	n/a	320	49	3.7	370	46
Noxa	>2000	>1000	NB	>2000	>1000	n/a	>100 000	>100 000	>100 000	24	20
Puma	65 ± 1	>1000	NB	70	69	n/a	3.3	5.1	6.3	5	1
Bak	46 ± 3	50	4300	150	<50	71	>1000	500	50	10	3
Bax	32 ± 5	75	1850	1400	980	n/a	100	58	130	12	n/a

[a]Indicates affinities determined by isothermal calorimetry
[b]Indicates affinities determined by florescence polarization
Interactions were determined using SPR methods except where stated. Sources: SPPV14,[115] M11L,[31] F1L,[27] BHRF1,[107] KsBcl-2,[132] N1L,[30] Bcl-2,[9,133,134] Bcl-w,[9,133,134] Bcl-x_L,[9,133,134] Mcl-1,[9] A1[135]

or 'displacement' model poses that the BH3-only proteins release Bax and Bak from regulation by the pro-survival proteins to initiate MOMP. Whereas the 'direct activation' mechanism proposes two roles for BH3-only proteins: they may act either as 'sensitizers' (Bad, Bmf, Noxa, Hrk, Bik) that interact only with pro-survival proteins or as 'activators' (Bim, Puma, Bid) that interact with pro-survival Bcl-2 proteins and with Bax and Bak[50] (Figure 1). The sensitizer BH3-only proteins sequester the pro-survival proteins preventing interaction with Bax or Bak, leaving them to interact with the activator BH3-only proteins to induce the conformational changes necessary for MOMP. Composite mechanisms have been proposed and the evidence recently reviewed.[51] Bim is the universal pro-survival Bcl-2 family antagonist,[37] whereas other BH3-only family members are more selective and the combination of Mcl-1 and Bcl-x_L potentially inhibits all BH3-only initiated intrinsic apoptotic pathways.[37]

Things Change at the Membrane

The least well-understood aspect of Bcl-2 family protein-mediated apoptosis is their interactions at intracellular (mitochondrial, endoplasmic reticulum, golgi and nuclear envelope) membrane surfaces. Many Bcl-2 proteins bear a hydrophobic C-terminal TM sequence, frequently assumed to be helical, for membrane anchorage. Some are constitutively membrane bound (Bak, Bok), whereas others (Bax, Bcl-x_L, Bcl-w, Mcl-1, A1) are partitioned between the membrane and the cytosol and become integrated subsequent to apoptotic stimulus.[51,52] Like Bim, Noxa, Puma and Bmf, tBid is also found to be membrane associated, though Bad is not.[53] Most Bcl-2 proteins are targeted to the MOM but Bok[54] and Bik[53] locate to the endoplasmic reticulum.

Pro-survival proteins appear to require their TM regions for full pro-survival activity[47] and recent evidence shows the TM region of Bcl-x_L has a role in transferring Bax between the MOM and cytosol.[55] Before apoptotic stimuli, Bax exists in equilibrium between the cytosol and the MOM and is shuttled between them by Bcl-x_L in a process dependent on the BH3 motif of Bax, a competent BH3-binding groove of Bcl-x_L and the TM region of Bcl-x_L,[55,56] but structural details are not available. Other evidence suggests certain BH3-only proteins directly bind Bax and Bak[57–61] and in the case of Bax results in membrane integration. Some viral Bcl-2 homologs also bear a hydrophobic TM region, suggesting that they too act at the MOM.[62,63] How pro-apoptotic Bax and Bak affect MOMP remains uncertain. A 'hit and run mechanism'[59,64,65] has been proposed based on a weak interaction with a BH3-only protein inducing a poorly defined and structurally uncharacterized membrane insertion transition. Although biochemical evidence shows the BH3 motif of Bax (or Bak) is necessary for interaction with pro-survival proteins, currently there is no high-resolution structural data available on any complexes of full-length Bax/Bak: pro-survival Bcl-2 proteins. Further structures will be required to answer this question.

Bcl-2 Proteins Dimerize

Heterodimerization of Bak and Bax[66] with pro-survival proteins requires a conformational change in one or both partners,[67] as the key residues in the interacting BH3 motifs are buried in monomeric Bax and Bak.[42,68] Early in the exploration of the Bcl-2 family, it was noted that Bax:Bcl-2 dimers form in the presence of non-ionic surfactants (detergents)[69] and the affinity was recently measured in the presence of the surfactant Brij-35 ($K_d \sim 36$ nM).[70] Given that the core of the Bcl-2 fold largely comprises of hydrophobic interactions, it is not surprising that surfactants have an effect on the structure. Bcl-x_L in dodecylphosphocholine micelles forms a molten globule retaining secondary structure but not a tertiary fold.[71] Conformational change of the Bcl-2 proteins in the membrane is a major feature of their action,[72] and changes have been observed for Bax and Bak through the use of antibodies specific for epitopes that are not exposed before membrane interaction.[73,74]

In addition to heterodimerization, both pro-survival and pro-apoptotic proteins can homodimerize. Under the influence of heat, organic solvents, pH or surfactants, Bcl-x_L dimerizes[75–78] to form an extended structure capable of binding BH3 peptides[75,78] (Figure 3a). An α1 helix swapped dimer of Bcl-x_L retaining an ability to bind BH3 ligands has also been observed (Figure 3b).[79] Higher order oligomers of Bcl-x_L can

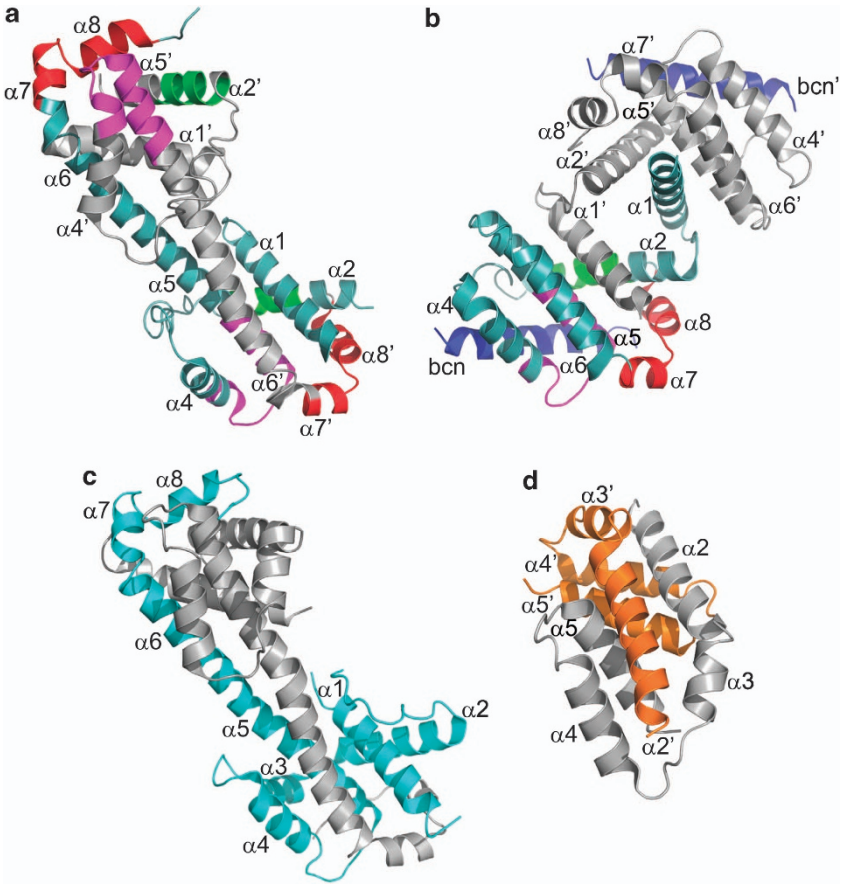

Figure 3 Structures of Bcl-2 dimers. Dimers may be induced by treatment with heat, pH, surfactant or BH3-ligand. (**a** and **b**) Bcl-x_L dimers; (**c** and **d**) Bax dimers. (**a**) Bcl-x_L dimer (2B48). The BH motifs are indicated in color as Figure 1 and the helices indicated with the alternate protomer helices denoted with a prime (′) (**b**) Beclin-1: Bcl-x_L heterotetramer (2P1L). (**c**) Octylmaltoside-induced Bax dimer (4BD7) (**c**) CHAPS-induced alternate dimeric Bax core (4BDU) that contains only the central helices (α2–α5) of Bax and lacks both N-terminal residues and the C-terminal TM region. For clarity, the GFP-fusion tag on each chain is not shown. BH motifs have been colored as in Figure 1. The structures depicted in **a** and **c** have been structurally aligned with Bcl-x_L in the same orientation as Figure 1 and the Bcl-x_L dimer in **b** was aligned over helices α5–α8 on the structure in **a**

be formed and there appear to be differences between dimers formed using surfactant and pH, the acid-formed dimers bind BH3 peptides, whereas surfactant-induced dimer did not, though structural details are lacking.[77] A TM-truncated Bcl-w dimerizes on crystallization, forming a structurally distinct dimer to that of surfactant-induced Bcl-x_L with reduced ability to bind BH3 peptides.[80] Surfactant treatment of Bax followed by dilution or removal of surfactants gives a surfactant-free dimer with a similar structure to surfactant-induced Bcl-x_L dimer, where the central α5 and α6 helices form a single contiguous helix[61] (Figure 3c). A truncated GFP-tagged Bax retaining only the central α2-α5 region forms a dimer with a different structure in the absence of surfactant (Figure 3d). Bak has also been reported to dimerize before MOMP.[81] The functional role, if any, of Bcl-2 protein homodimerization remains unclear, and kinetically pore formation appears to be dependent on monomeric Bax.[82]

Although the multi-motif Bcl-2 monomers are topologically near-identical, the dimers are not, having different architecture depending on the protein and how dimerization was induced (Figure 3). The interaction of proteins with crystallization co-solvents[83] or surfactants[84] is complex and surfactant-generated forms may be artifacts of their preparation.[85] Co-solvents such as methylpentanediol or isopropanol employed in Bax[61] and Bcl-x_L[78] dimer crystallization, respectively, may contribute to dimer formation. In contrast to the mammalian counterparts, some viral Bcl-2 proteins appear to be constitutive dimers and have architectures structurally unique to those observed for mammalian proteins (Figures 2 and 3).

Alternate Functions for Bcl-2 Proteins?

Although the main protagonists of the Bcl-2 family now have well-understood roles in apoptosis signaling, even if the exact details of MOMP are not understood, others are less well characterized. This includes Bcl-2 proteins such as A1,[86] Bcl-B[37] and Bok.[54] Structurally Boo, the mouse homolog of human Bcl-B (Bcl-2L10), has a Bcl-2 fold[87] but only binds the known BH3-only proteins with very weak affinity,[87,88] suggesting that the human and mouse homologs have diverged functionally. The main difference between Bcl-B and other multi-motif proteins is the presence of an unstructured loop between helices α5 and α6[37] shown to be important for regulating its protein levels in cells through ubiquitylation and proteasomal degradation.[89]

Bcl-2 proteins may modulate biochemical pathways other than mitochondria-regulated apoptosis.[90] Bcl-x_L, Bcl-2 and Bax interact weakly with the tumor repressor and transcription factor p53.[78,91–93] NMR studies have shown weak specific interaction between p53 and Bcl-x_L[91] with a K_d of 370 nM[78] that is released on Puma binding Bcl-x_L in the groove,[78] providing a potential allosteric mechanism for p53 activation of apoptosis. Bcl-2 family proteins may be involved in regulating mitochondrial remodeling through fusion and fission.[94,95] It has been proposed that Bcl-x_L regulates autophagy through interaction with Beclin-1 in a BH3-like interaction ($K_d \sim 2\,\mu$M).[79] Other potential pathways regulated include energy metabolism[41] and cell cycle progression,[96] but details are yet to emerge on interactions and structures. At present, the molecular details of these processes are not firmly established, with many likely to be indirect effects.

Viral Bcl-2 Proteins: Keeping Cells Alive when Hosting a Pathogen

Molecular mimicry through expression of sequence, structural and functional orthologs of Bcl-2 proteins is an important survival strategy of viruses.[97] The significance of successful subversion of host cell apoptosis has been demonstrated by construction of deletion mutants for several viruses. Adenovirus,[98] murine cytomegalovirus,[99–101] Epstein-Barr virus (EBV),[102] γ-herpesvirus,[103] myxomavirus[104] and vaccinia virus[63] trigger apoptosis during infection in the absence of functional viral Bcl-2 proteins. By retaining key structural, but not necessarily sequence features, viral Bcl-2 homologs can inhibit both BH3-only and Bax-like death signals. Readily identifiable viral Bcl-2-like proteins include those found in adenovirus (vBcl-2),[105] Kaposi sarcoma-associated herpesvirus (KS-Bcl-2),[106] Epstein-Barr virus (BHRF1),[107,108] murine γ-herpesvirus 68 (γHV68-vBcl-2),[109] herpesvirus saimiri ORF16[110] and the recently identified FPV039 from Fowlpox virus,[111] CNPV058 in canarypox virus[112] and vNR13, a Bcl-B homolog from avian α-herpesvirus.[113] However, in general, the sequence divergence of vBcl-2 proteins from recognized Bcl-2 proteins has made them difficult to identify by sequence comparison alone. Structures of these vBcl-2 molecules show they are highly similar to mammalian family members. KS-Bcl-2[106] and BHRF1[107,108] adopt Bcl-2 folds comprising 8 alpha helices and have a TM region indicating that membrane interaction and perhaps interactions in the membrane are necessary for their activity.

More recently, a number of viral Bcl-2 members were identified in pox viruses that, while displaying virtually no sequence identity with mammalian Bcl-2 members adopt a Bcl-2 fold. These include myxoma virus M11L[31,32] and vaccinia virus F1L[27] and N1L.[29,30] These three proteins comprise only seven alpha helices, with helix 7 (numbering based on the structure of Bcl-x_L; Figure 2) being absent. Solving the structures of N1L and F1L demonstrated the considerable structural diversity to the Bcl-2 fold. N1L and F1L adopt dimeric configurations; with F1L forming a domain-swapped dimer mediated by an exchange of α1 helices between two F1L protomers. Intriguingly, the topology of the F1L dimer is reminiscent of the Bcl-x_L:Beclin-1 BH3 domain-swapped dimer observed by Oberstein *et al.*,[79] where truncating the α1-α2 loop results in helix α1 no longer being accommodated within the Bcl-2 fold but located in a neighboring protomer. In contrast, N1L forms a dimer by association of two independently folded N1L chains.[29,30] Lacking a TM region, N1L has a cytosolic location[114] and no mammalian Bcl-2 protein has been shown to associate in a similar configuration (Figures 2 and 3).

Biochemical interaction studies revealed significant variations in the ligand-binding profile of vBcl-2 proteins with respect to the range and nature of their ligands as well as their affinities. Vaccinia virus F1L has been shown to engage Bim, Bak and Bax only and with moderate affinities.[27] In contrast, SPPV14 interacts with several BH3-only proteins (Bim, Bid, Bmf, Hrk and Puma) as well as Bak and Bax[115] at considerably higher affinities (Table 1). In the case of vaccinia virus, expression levels of F1L are high and driven by a strong early/late latency promoter. All *vbcl-2* are single genes without splice sites and there is some level of conservation of the location within the genome in the pox viruses, indicating common progenitors. Another contrast with the mammalian Bcl-2 family is that no inhibitors of Mcl-1-specific binders (Noxa) have been found. Immunoprecipitation analysis has indicated N1L associates with Bad and ORFV125 binds Noxa, but no affinities have been determined with purified proteins.[116] Expression of Bcl-x_L and Mcl-1 potentially neutralizes all pro-apoptotic proteins.[37] Viral blocking of the Mcl-1-specific inhibitor Noxa does not appear to be required to maintain host cell viability and Mcl-1 may not be a significant survival signal in the cell types infected.

Recently a structure of the inhibitory peptide from cytomegalovirus Bcl-2 homolog, viral mitochondrial localized inhibitor of apoptosis (vMIA), in complex with Bax demonstrated a unique binding location distinct from the canonical BH3-biding site. Binding was mapped to inter-helical loop regions α1-α2, α3-α4, α5-α6 with residues contributed from α9 and the C-terminus (equivalent to the lowermost loops in Bcl-x_L of Figure 2). Electrostatic interactions have a significant role in the interaction and the affinity was measured at K_d of 22 nM.[117] In contrast to other Bcl-2 members that inhibit recruitment of Bax to mitochondria, vMIA recruits Bax to mitochondria independent of the Bax TM helix and does not rely on the α1 site.[117]

Structural Plasticity of vBcl-2 Proteins Enables Additional Functionality

Although a number of apoptosis-modulating viral Bcl-2 proteins target endogenous Bcl-2 family members, several recently determined structures point to additional biological activities that can be regulated by the Bcl-2 fold. Vaccinia virus N1L is able to subvert Bcl-2-mediated host signaling.[29,30] Further evidence suggests an additional role for N1L as a modulator of NF-κB signaling,[118] an important mediator of the host innate immune response. N1L is thus endowed with dual functionality. To date, no other viral Bcl-2 protein has been shown to harbor both anti-apoptotic and NF-κB modulatory activity.[115,119] However, the ability to modulate NF-κB is not limited to N1L, two additional vaccinia virus factors A52 and B14[120] have also been shown to adopt Bcl-2 folds and act on the NF-κB pathway, but not the apoptotic pathway. Similar to

N1L, both A52 and B14 do not harbor a transmembrane region and appear to be located in the cytosol.[121] For the purpose of NF-κB modulation, the loop-connecting helices 4 and 5 appears to be the site of activity, at least in the case of A52[120] and the hydrophobic BH3-binding groove that mediates Bcl-2 family member interactions is in a closed conformation and is not involved in NF-κB signaling. Mutagenesis data supports the alternative binding mode with the apoptotic and NF-κB functions on independent surfaces in N1L.[122]

Additional activities, other than modulation of NF-κB signaling, have been attributed to viral Bcl-2 proteins. Work on vaccinia virus K7 revealed a Bcl-2-like fold that was utilized to engage RNA helicases.[123] The region on K7 that mediates helicase interactions was mapped using NMR to a shallow groove along the α1 helix, a region that corresponds to the N1L dimer interface. N1L dimerization was shown to be critical for inhibition of NF-κB signaling via a surface patch that covers the dimer interface and comprises the α1 and α6 helices.[122] Non-groove-mediated functions of Bcl-2-like proteins are not limited to viral members of the family and structural evidence has emerged that Bax may also harbor an alternative interaction site for BH3-only proteins.[57] Again, a surface region involving helices α1 and α6 was identified as the alternative site, suggesting that this region of Bcl-2 proteins is available for functional interactions in both viral and mammalian family members. However, the structural data for the α1/α6 interface is still rather weak for Bax.

Comparison of Cellular with Viral Bcl-2 Family Members

Although cellular and viral Bcl-2 proteins adopt an identical fold, a number of differences hint at the different purposes for which these molecules evolved. Mammalian Bcl-2 members typically comprise eight alpha helices, a feature that is also observed for readily identifiable vBcl-2 proteins, such as KsBcl-2[106] and BHRF1.[107,108] However, more distant vBcl-2 proteins, such as M11L,[31] F1L[27] and γHV68-vBcl-2,[109] only contain seven alpha helices leading to a reduction in chain length required to form the Bcl-2 fold. The sequences of mammalian multi-motif Bcl-2 proteins vary in sequence length and are generally long due to the insertion of residues in the inter-helical loops, whereas their viral counterparts are typically shorter adopting a more economical compact fold lacking long inter-helical loops and in the case of the more distantly related vBcl-2 homologs loss of α7. N1L encodes the entire Bcl-2 fold within 115 residues,[29,30] making it the member with the shortest primary sequence of the group, this contrasts with human Bcl-x_L that is 233 residues in length with a 50-residue α1-α2 IDR loop.[36] An exception is provided by vaccinia virus F1L, which is encoded by 226 residues, including a $\sim$60-residue N-terminal extension. The N-terminus of F1L has recently been identified to harbor additional biological activities, notably a caspases-9 inhibitory function[124,125] as well as an unexpected inflammasome regulatory role.[126] This would establish F1L as the first vBcl-2 to modulate inflammasome activity directly by binding NLRP1/NALP1, in a manner reminiscent of Bcl-x_L,[127] pointing toward an additional level of functional mimicry.

Viral Bcl-2 Proteins Interact with BH3 Proteins with Relatively Low Affinity

When comparing affinities of cellular Bcl-2 proteins for their ligands with their viral homologs, a striking feature is the higher affinity of most cellular Bcl-2 proteins compared with viral Bcl-2 proteins. Furthermore, cellular pro-survival Bcl-2 proteins tend to engage a much wider range of pro-apoptotic proteins[9] than the viral counterparts. Whereas Bcl-x_L shows high affinity for all pro-apoptotic Bcl-2 members except Noxa,[9] vaccinia virus F1L only binds Bim, Bak and Bax,[27] with affinities that are at least 20-fold lower compared with those of Bcl-x_L. Additionally, two of the most promiscuous BH3-only proteins Bid and Puma, are only engaged by a limited number of viral Bcl-2 proteins (Table 1). It is conceivable that the more selective ligand-binding profiles of a viral Bcl-2 protein reflect the circumstance that only specific pro-apoptotic Bcl-2 proteins require targeting during viral infection. The smaller number of ligands identified for certain vBcl-2 proteins might also be reflected in the changes within the canonical binding groove.

Although the core structural features of ligand engagement by viral Bcl-2 members are conserved with their mammalian counterparts, some intriguing differences exist. The four key hydrophobic interactions with BH3 motifs are retained in all vBcl-2:BH3 motif structures, whereas the conspicuous salt bridge formed by an aspartic acid from the BH3 motif with an arginine residue from the accepting Bcl-2 protein is not observed in the M11L:BakBH3 complex. This suggests that the salt bridge may not be a defining feature for vBcl-2–BH3 interactions as it seems for their cellular counterparts. M11L exhibits only minimal movement in the binding groove when binding a BH3 motif ligand,[27] and displays high-affinity binding to a number of point mutants of Bak BH3 motif. In contrast, Bcl-x_L undergoes substantial changes in the binding groove (in the order of 9 Å) on BH3 motif binding,[35,128] and appears to be unable to engage Bak BH3 point mutants.[31] This suggests that M11L engagement of BH3 ligands is more robust, and not subject to subtle control mechanisms as required for the modulation of apoptosis signaling by endogenous cellular Bcl-2 proteins. The reduced binding groove movement and robustness of ligand binding in the case of M11L could be a consequence of high-level optimization of M11L to engage only a small subset of pro-apoptotic Bcl-2 members.

Concluding Remarks

Bcl-2 proteins couple apoptotic stimuli with mitochondrial membrane permeabilization to initiate the caspase cascade. One of the most striking observations is that of the sequence and structural similarity between the multi-motif pro-apoptotic Bax and Bak and their pro-survival counterparts. This might imply that the mode of action of pro-survival and pro-apoptotic proteins are similar and that specific binding partners and interactions define their activity. Our understanding of this family is evolving, but current research is presenting a more unified mechanism with the canonical BH3-binding groove being the target in both pro-survival and pro-apoptotic proteins with high-affinity BH3 interactions in the former and moderated to low-affinity interaction observed for Bax and Bak.[59–61]

The thermodynamics of these processes remain poorly elucidated and converting the *in vitro* findings to a more physiological setting is required. Many questions also remain as to how Bax and Bak are inhibited by pro-survival proteins from the mammalian and viral Bcl-2 family and the molecular details of their mode of action at intracellular membrane surfaces are currently not available.

The role of viral Bcl-2 proteins in apoptosis is probably more restricted than the mammalian Bcl-2 family, for instance no viral homolog of Mcl-1 has yet been found, as they only have to keep host cell apoptosis in abeyance during replication. Conformational change in viral Bcl-2 proteins is less important for their function with their primary requirement being optimized binding of their main targets, the initiators of apoptosis. What messages can we take from Bcl-2 mimicry? In the most parsimonious case, viral Bcl-2 proteins could simply antagonize the pan-pro-survival antagonist Bim preventing activation of Bax or Bak. The absence of observed specificity for other BH3-only proteins, particularly Bid/tBid and Puma, may suggest that subtle differences exist that govern activation of Bim expression compared with Bid and Puma. However, considering the absence of definitive expression studies that identify differential expression of potent BH3-only proteins during viral infections this remains speculative.

Determination of the structures and binding properties of Bcl-2 family proteins has been a fruitful tool in delineating biological roles and the role of individual Bcl-2 proteins and their viral counterparts. It has allowed predictions of possible functional importance of the members of the Bcl-2 family in regulating cell fate and a detailed molecular understanding is being put to use in designing inhibitors for members of this family to trigger tumor cell death by inactivation of pro-survival proteins.[129,130] Development of antagonists against viral Bcl-2 proteins may prove a viable therapeutic approach to treating diseases etiologically linked to viral infection. For example, EBV expresses the pro-survival Bcl-2 homolog BHRF1, and chronic EBV infection is strongly associated with development of Burkitt's lymphoma. Constitutive BHRF1 expression[131] has a prominent role in B-cell transformation[102] and may contribute significantly to virus-associated lymphomagenesis, in addition to being associated with potent resistance to chemotherapy in mouse models of Burkitt's lymphoma.[107] Viral Bcl-2 proteins only mimic select functions of mammalian proteins, thus presenting the tantalizing prospect of easier therapeutic amenability by limiting the number of potential off-target effects. Given the recent advances in designing BH3 mimetics against mammalian pro-survival Bcl-2 proteins to activate apoptosis for cancer therapies,[129] such structure-based approaches against viral Bcl-2 proteins may well be feasible, exploiting the fact that the Bcl-2 fold is put to work in a similar context but with a rather different purpose.

Conflict of Interest

The authors declare no conflict of interest.

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