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Review

The BCL-2 Family Reunion

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B cell CLL/lymphoma-2 (BCL-2) and its relatives comprise the BCL-2 family of proteins, which were originally characterized with respect to their roles in controlling outer mitochondrial membrane integrity and apoptosis. Current observations expand BCL-2 family function to include numerous cellular pathways. Here we will discuss the mechanisms and functions of the BCL-2 family in the context of these pathways, highlighting the complex integration and regulation of the BCL-2 family in cell fate decisions.

A Death in the Family

Like all living things, cells die. In animals, the predominant mode of cell death during development and tissue homeostasis is apoptosis. During this process, the caspase proteases effectively package and label (e.g., by inducing cellular blebbing and shrinkage, DNA fragmentation, and plasma membrane changes) dying cells for rapid clearance.

In vertebrates, the BCL-2 family regulates the mitochondrial pathway of apoptosis by complex interactions that dictate the integrity of the outer mitochondrial membrane (OMM) (Green and Evan, 2002). This pathway is initiated by mitochondrial outer membrane permeabilization (MOMP), which allows soluble proteins (e.g., cytochrome *c*) in the mitochondrial intermembrane space (IMS) to diffuse into the cytosol. Cytochrome *c* engages apoptotic protease activating factor-1 (APAF-1) to oligomerize into a caspase activation platform termed the apoptosome. This binds and promotes the activation of initiator caspase-9, which then activates executioner caspases-3 and -7. The caspases cleave cellular substrates to elicit the apoptotic phenotype (Figure 1A) (Riedl and Salvesen, 2007). Temporally, MOMP indeterminately occurs following proapoptotic stress, but studies suggest that this timing is dependent on the concentrations of diverse cellular proteins (Spencer et al., 2009). After MOMP, caspase activation and apoptosis ensue often within minutes.

Here we discuss the BCL-2 family and its regulation of mitochondrial integrity, apoptosis, and other cellular processes including mitochondrial dynamics, endoplasmic reticulum (ER) calcium stores, and autophagy. Although the BCL-2 family is conserved among animals, its role in controlling the integrity of the OMM is only described for the vertebrates, and limited data exist for functions in invertebrates. In *C. elegans*, the BCL-2 protein cell death abnormality-9 (CED-9) does not control MOMP, but instead inhibits apoptosis by sequestering the APAF-1 homolog, CED-4 (reviewed in Lettre and Hengartner, 2006). Similarly, the *Drosophila* BCL-2 homologs do not appear to control cell death, and their functions remain obscure (reviewed in Mollereau, 2009). For these reasons, our discussion focuses on vertebrate BCL-2 family function.

A Family Portrait

BCL-2 and its relatives are functionally classified as either antiapoptotic or proapoptotic BCL-2 proteins, and the regulation of their interactions dictates survival or commitment to apoptosis (Figure 1C).

Antiapoptotic BCL-2 proteins contain four BCL-2 homology domains (BH1–4) and are generally integrated within the OMM, but may also be in the cytosol or ER membrane (Figure 2A). BCL-2-related gene A1 (A1), BCL-2, BCL-2-related gene, long isoform (BCL-xL), BCL-w, and myeloid cell leukemia 1 (MCL-1) are the major members of the antiapoptotic BCL-2 repertoire and preserve OMM integrity by directly inhibiting the proapoptotic BCL-2 proteins.

The proapoptotic BCL-2 members are divided into the effector proteins and the BH3-only proteins. The effector proteins BCL-2 antagonist killer 1 (BAK) and BCL-2-associated x protein (BAX) were originally described to contain only BH1-3; however, structure-based alignment of globular BCL-2 family proteins revealed a conserved BH4 motif (Figure 2A) (Kvansakul et al., 2008). Upon activation, BAK and BAX homo-oligomerize into proteolipid pores within the OMM to promote MOMP (Figure 2B). There is a potential third effector molecule, BCL-2-related ovarian killer (BOK), but no biochemical evidence supports a function akin to BAK or BAX.

The BH3-only proteins function in distinct cellular stress scenarios and are subdivided based on their ability to interact with the antiapoptotic BCL-2 repertoire or both the antiapoptotic proteins and the effectors (Figures 1C, 3A–3C). BH3-only proteins that only bind to the antiapoptotic repertoire are referred to as "sensitizer" and/or "derepressor" BH3-only proteins; e.g., BAD (BCL-2 antagonist of cell death) and Noxa. BID (BCL-2-interacting domain death agonist) and BIM (BCL-2-interacting mediator of cell death) interact with the antiapoptotic repertoire as well as the effectors, and can directly induce BAK and BAX oligomerization and MOMP. These BH3-only proteins are referred to as "direct activators" (Figure 3B). The interactions between the antiapoptotic repertoire, direct activator/sensitizer/derepressor BH3-only proteins, and effectors determine MOMP and apoptosis (Figures 1C, 3A–3C).

BAK and BAX Activation

Central to the initiation of apoptosis is BAK/BAX activation at the OMM. While there are competing models explaining the control

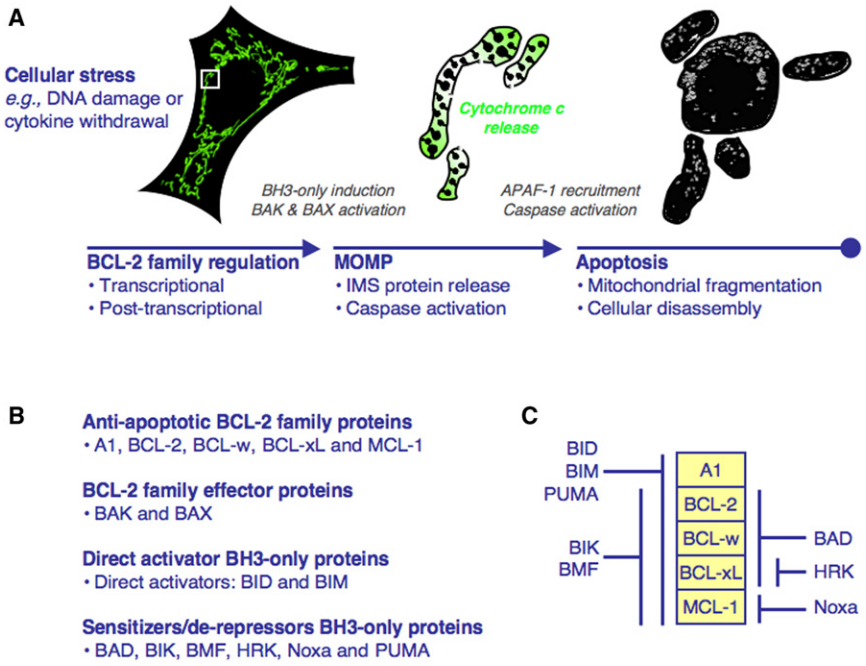

Figure 1. The Mitochondrial Pathway of Apoptosis and the BCL-2 Family

(A) Cellular stress causes transcriptional and post-transcriptional regulation of the BCL-2 family to promote MOMP. MOMP is induced by interactions between the BH3-only and effector proteins and leads to cytochrome *c* release, APAF-1 recruitment, and caspase activation. At the time of MOMP (middle), the intact mitochondrial network (green, left) undergoes fragmentation (gray, right), and soon after the cell is disassembled. The mitochondria in the middle are enlarged from the white box.

(B) The BCL-2 family is divided into antiapoptotic, effector, and direct activator/sensitizer/derepressor BH3-only proteins.

(C) The antiapoptotic BCL-2 protein binding profiles for the BH3-only proteins.

BH3-only proteins in this process is undisputed (Chipuk and Green, 2008). At least two of the BH3-only proteins, BID and BIM, are capable of directly inducing effector function. The active form of BID (discussed below) promotes BAK and BAX oligomerization, MOMP, and cytochrome *c* release (Kuwana et al., 2002; Wei et al., 2000). Similar evidence exists for BIM-mediated BAK/BAX activation; and while PUMA was also suggested to promote BAK/BAX activation, this effect may not be direct (Chipuk et al., 2008; Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002). The BH3 domains of BH3-only proteins can be synthesized and represent the minimal unit of BH3-only protein function (referred to as BH3 peptides). BID and BIM BH3 peptides induce BAK and BAX oligomerization and pore-forming activity with isolated mitochondria or large unilamellar vesicles (LUVs, lipid vesicles that mimic the OMM) (Kuwana et al., 2002, 2005; Letai et al., 2002).

The series of events leading to BID-mediated activation of BAX has been elucidated in vitro (Lovell et al., 2008). BAX is soluble and undergoes activation in the presence of a direct activator and suitable membrane (e.g., the OMM or LUV); this results in oligomerized BAX and membrane permeabilization. The first step for BID-induced BAX activation is the association of BID with a membrane, followed by BAX recruitment, insertion, and oligomerization (Figure 2B) (Leber et al., 2007). Binding between BID and BAX has been difficult to study, but FRET analysis revealed the interaction in vitro (Lovell et al., 2008).

The interaction between BIM and BAX was demonstrated by NMR (Gavathiotis et al., 2008). Several other proteins are described to directly activate BAX, and it will be interesting to determine if they utilize a similar mechanism. BAK and BAX activation can also be triggered by nonprotein factors: e.g., mild heat, detergents, and high pH (Hsu and Youle, 1997; Khaled et al., 2001; Pagliari et al., 2005). Whether or not these mechanisms physiologically occur remains to be proven. However, observations that BAK/BAX-dependent apoptosis proceeds in the absence of BID and BIM argues that either direct activation

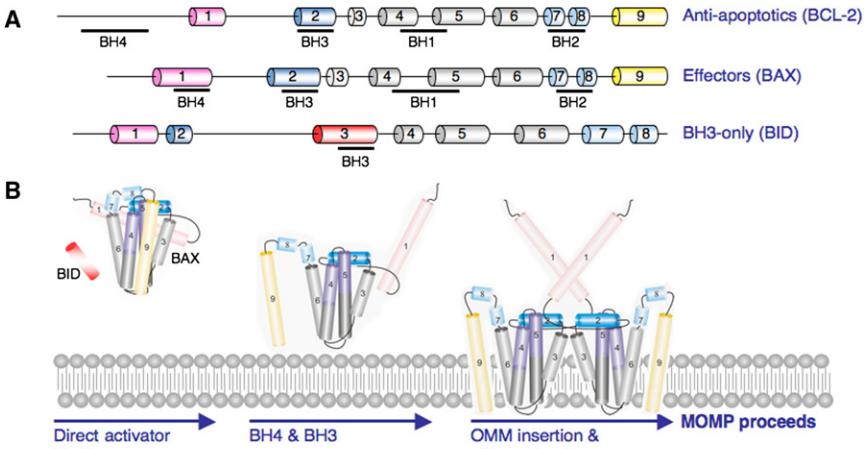

Figure 2. BCL-2 Family Composition and Membrane Permeabilization

(A) The BCL-2 proteins are comprised of BCL-2 homology (BH) domains. A representation of an antiapoptotic (BCL-2), effector (BAX), and BH3-only (BID) protein is shown with the BH1-4 designated underneath the corresponding α helices.

(B) Proposed model of BAX activation. Soluble BAX interacts with a direct activator and the OMM to promote stable N-terminal exposure, and BAX α5, α6, and α9 insert within the OMM.

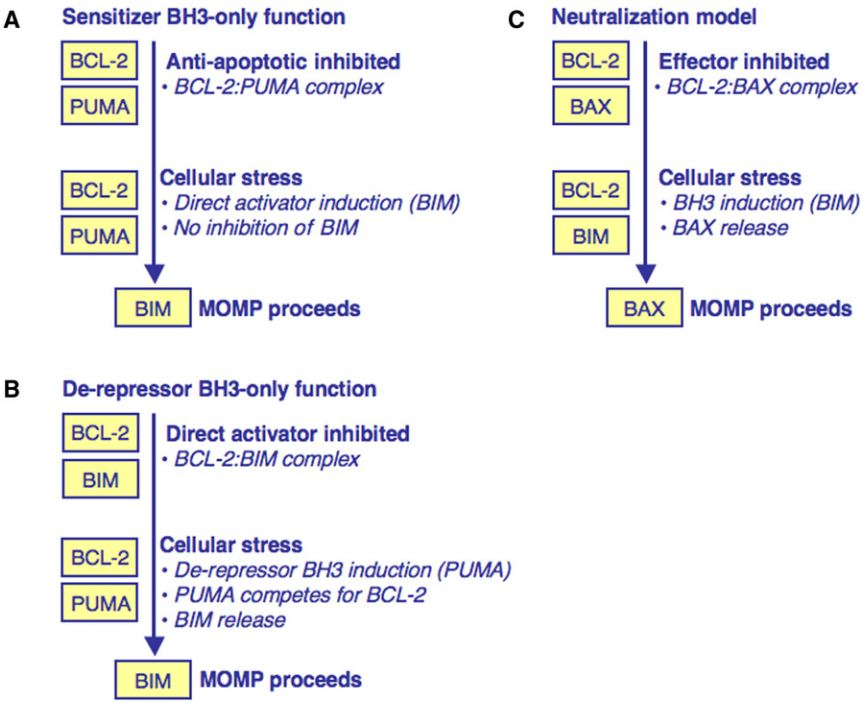

Figure 3. BH3-Only Protein Function

(A) Sensitizer BH3-only protein function. A sensitizer BH3-only protein inhibits the antiapoptotic BCL-2 repertoire. Following minimal cellular stress, a direct activator is induced but cannot be inhibited and MOMP proceeds.

(B) Derepressor BH3-only protein function. A direct activator is sequestered by an antiapoptotic BCL-2 protein. Following cellular stress, a derepressor BH3-only protein is induced and competes with the direct activator for binding to the antiapoptotic repertoire. When the direct activator is released, MOMP proceeds.

(C) The neutralization model of BCL-2 family function. In this model, BAK/BAX are always competent to promote MOMP but are actively inhibited by the antiapoptotic BCL-2 repertoire to promote survival. Following cellular stress, BH3-only proteins are induced, bind the antiapoptotic proteins, and displace effectors to promote MOMP.

by proteins is unnecessary, or alternative mechanisms for activation exist (Willis et al., 2007).

Sensitization and Derepression

Other BH3-only proteins, such as BAD, BCL-2-interacting killer (BIK), Harakiri (HRK), Noxa, and p53-upregulated modulator of apoptosis (PUMA) function predominantly by binding to the antiapoptotic repertoire and not by directly activating BAK or BAX (Chen et al., 2005; Chipuk et al., 2008; Kuwana et al., 2005; Letai et al., 2002). The terms ''sensitizer'' and ''derepressor'' are used to indicate the consequences of binding between a BH3-only protein and an antiapoptotic BCL-2 protein. Each sensitizer/derepressor BH3-only protein has a unique binding profile for the antiapoptotic repertoire, which was primarily determined by using BH3 peptides (Figure 1C). These BH3-only proteins establish two distinct mechanisms that promote BAK/BAX activation: sensitization and derepression (Figures 3A and 3B) (Chipuk et al., 2008; Kuwana et al., 2005; Letai et al., 2002).

Sensitization lowers the threshold for BAK and BAX activation and MOMP but does not cause apoptosis itself (Figure 3A). In this scenario, an antiapoptotic protein is in complex with a sensitizer BH3-only protein, which prevents the inhibition of subsequent direct activators. For example, if BCL-2 is associated with PUMA, any future induction of BIM is not inhibited and MOMP proceeds. In the absence of PUMA, BIM would be sequestered and the cell may survive.

For derepression, a direct activator is bound by an antiapoptotic BCL-2 protein, and a subsequent BH3-only protein releases the direct activator to promote MOMP (Figure 3B). For example, reparable cellular stress can induce BIM function, but this activity is inhibited by the antiapoptotic repertoire and the cell survives. If a derepressor BH3-only protein is induced while allowing for MOMP. Studies using FRET demonstrated that derepression and consequential direct activation occur via protein·protein interactions that are not readily detected in the absence of membranes (Lovell et al., 2008). For example, activated BID was bound by BCL-xL, and this interaction was disrupted by BAD. BID then interacted directly with BAX, followed by BAX oligomerization and LUV permeabilization. These studies highlight the rapidity of the interactions once the conditions for derepression and MOMP are satisfied. Derepression also represents a means of pharmacological regulation in certain tumors, such as chronic lymphocytic leukemia (CLL), which undergo MOMP when treated with a derepressor BH3 mimetic (Certo et al., 2006; Del Gaizo Moore et al., 2007).

An alternative hypothesis to the direct activator requirement is the neutralization model (Figure 3C). In this case, MOMP can proceed following inhibition of the antiapoptotic BCL-2 repertoire independently of direct activator·effector interactions (Uren et al., 2007; Willis et al., 2007). This model assumes that cells harbor activated forms of BAK and BAX that are sequestered by the antiapoptotic BCL-2 repertoire. BH3-only proteins then compete for the antiapoptotic proteins and apoptosis ensues. We recognize that inhibition of the antiapoptotic repertoire contributes to MOMP, but contend it most efficiently occurs following the combined efforts of direct activator and sensitizer/derepressor BH3-only proteins, as recently suggested by elegant experiments in vivo (Merino et al., 2009).

Communicating with the BCL-2 Family

The BH3-only proteins are the major sentinels for cellular stress, and diverse signaling pathways converge upon these proteins. BH3-only proteins share little homology, and this may explain the variety of mechanisms capable of regulating their function. The multidomain BCL-2 proteins can also be regulated during apoptotic signaling by changes in stability or cooperation with other family members. Here we highlight a few of the pathways that regulate the BCL-2 family (see Table S1, available online,

The proapoptotic function of the BID BH3 domain is revealed by a unique mechanism involving the proteolytic cleavage of the large unstructured loop joining the inhibitory N terminus and the BH3-containing C terminus. Cleavage can be achieved by a variety of proteases; e.g., caspase-8 (via death receptors), granzyme B (cytotoxic lymphocytes), and caspase-2 (via heat shock). The proapoptotic function of BID is also enhanced by N-myristoylation, which promotes OMM targeting and BAK activation.

BIM is regulated both at the transcriptional and posttranslational levels. BIM is expressed as three alternatively spliced isoforms (BIM-S, BIM-L, and BIM-EL), although the latter two are most often observed. Levels of *bim* mRNA are positively regulated by the forkhead transcription factor FOXO3A upon cytokine deprivation, and by C/EBPα and CHOP following ER stress. Translation of *bim* mRNA is negatively regulated by the miRNA cluster miRNA-17-92, and overexpression of miRNA-17-92 induces a *bim*-deficient phenotype (Xiao et al., 2008). BIM function is also controlled by a series of posttranslational modifications via ERK1/2 and βTrCP that differentially regulate the major BIM isoforms.

BAD is involved in apoptotic and nonapoptotic processes, and these dual activities are regulated by posttranscriptional modifications. Growth factors inhibit the proapoptotic function of BAD through phosphorylation by several kinases, such as Akt. Phosphorylation results in cytoplasmic sequestration and inactivation of BAD by 14-3-3 proteins, which prevents interaction with antiapoptotic BCL-2 proteins. The BAD BH3 domain phosphorylation status also regulates a nonapoptotic function of BAD through the direct regulation of glucokinase and glucose-driven mitochondrial respiration. Accordingly, mice deficient in *bad* or expressing a mutant with mutated phosphorylation residues display abnormal glucose homeostasis and deficient insulin responses, an effect that is reversed by BAD BH3 peptide treatment.

BAK and BAX expression levels are generally sufficient to promote MOMP. Hence, posttranslational modification of BAK and BAX likely regulates interactions within the BCL-2 family. In line with this hypothesis, survival signaling through ERK1/2 causes BAX phosphorylation and inhibition of its proapoptotic activity. Interestingly, the ubiquitously expressed β isoform of human BAX is capable of inducing MOMP and apoptosis, apparently in a BH3-only protein-independent manner. To thwart unwarranted MOMP, BAXβ is constitutively degraded in the absence of proapoptotic stress, suggesting strict posttranslational control (Fu et al., 2009).

Among the antiapoptotic proteins, the stability and function of MCL-1 have been extensively studied. After genotoxic stress, MCL-1 is ubiquitinylated by MULE, a HECT domain-containing E3 ligase, and rapidly degraded. MULE contains a BH3 domain that binds to MCL-1 similarly to the Noxa BH3, which can also promote MCL-1 degradation. MCL-1 stability is regulated by glycogen synthase kinase-3 (GSK-3), which phosphorylates MCL-1 to promote its degradation. The E3 ligase responsible for MCL-1 ubiquitination following GSK-3 phosphorylation is βTrCP, which can regulate BIM-EL stability. Also, removing ubiquitin groups conjugated to MCL-1 thwarts degradation and enhances cellular survival; this can be achieved by the deubiqui-

Family Form: Structural Considerations of the BCL-2 Family

The BCL-2 Core

The BCL-2 family is structurally categorized into folded globular and intrinsically unstructured proteins (IUPs) (see Table S2 for a listing of structures and references). Among the globular proteins, all of the multidomain antiapoptotic and effector BCL-2 proteins share a conserved "BCL-2 core." This is also preserved in BID, even though it has the least structural homology to the folded members. The remaining BH3-only proteins are IUPs and likely fold upon binding to a globular BCL-2 protein. MCL-1 is structurally distinct, as it contains an N-terminal unstructured domain followed by the BCL-2 core.

The BCL-2 core was revealed by the X-ray and NMR structures of BCL-xL and represents a ~20 kDa globular domain comprised of seven or eight amphipathic alpha (α) helices, arranged around a central buried helix, α5 (Figure 4A). The BH1 (portions of α4–α5) and BH2 (α7–α8) regions on one side, and the BH3 region (α2) and α3 on the other side, coalesce to delimit a hydrophobic groove (referred to as the BCL-2 family BH3 and C-terminus-binding groove, BC groove) at the "front" of the BCL-2 core. In BCL-w, and probably BCL-xL (as well as BAX, see below) the C-terminal α helix binds to the BC groove. The BH4 region, which is structurally defined by the conserved α1 positioned alongside α6, stabilizes the BH1-BH3 regions. All together, the BC groove and α1/α6 structural components comprise the BCL-2 core. The major differences between antiapoptotic and effector proteins are likely distinguished via the structural features of the individual BCL-2 cores, which reveals how the front pocket geometry, amino acid composition, and degree of obstruction by the C-terminal transmembrane (TM) tail modulate interactions with the BH3-only proteins.

Conformational Changes during BAK and BAX Activation

In unstimulated cells, BAX is cytosolic and the BC groove accommodates the C-terminal TM region as an amphipathic α helix (Figure 2B). To initiate MOMP, BAX undergoes a cytosolic-to-mitochondrial redistribution, initially implicating the exposure of TM region with OMM targeting. In contrast, BAK is constitutively targeted to the OMM, likely explained by an occluded BC groove, which impedes binding of the TM tail.

Constitutive targeting of BAK or induced redistribution of BAX to the OMM does not imply that effectors are active once the TM regions are embedded in the OMM. Instead, direct activation of BAK and BAX induces numerous conformational changes: (1) α1 exposure, (2) transient BH3 exposure, (3) protection of the membrane-embedded BCL-2 core, and (4) increased proximity of embedded monomers. Furthermore, analysis of BCL-xL/BAX chimeras identified the regions of BAX required for membrane insertion and MOMP (George et al., 2007). Together, these activation-induced conformational changes correlate with MOMP. These studies also revealed that the α5 helix was both necessary and sufficient for BAX oligomerization and MOMP.

Supporting the sequence of events postulated for direct activation of the effectors, subtle conformational changes in BAX instigated by the BIM BH3 peptide were modeled using NMR (Figure 4H) (Gavathiotis et al., 2008). To improve the affinity of

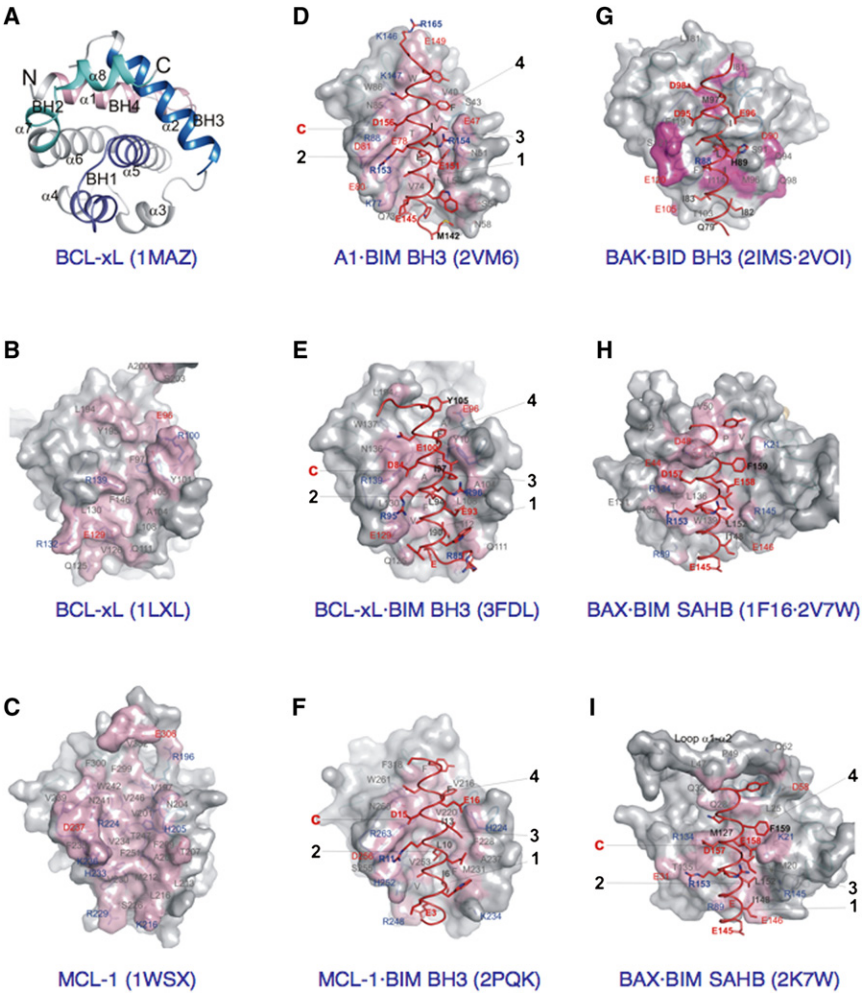

Figure 4. Structural Highlights for the BCL-2 Family

(A) The "front" view of BCL-xL identifies the BCL-2 core and the respective locations of the four BH regions. The PDB identifier is in parentheses.

(B) Surface representation of free BCL-xL, emphasizing amino acids participating in BH3 peptide binding at the "front" face, identifies the BC groove. Representations are partially transparent, permitting identification, beneath the surface, of contact side chains positioned 4 Å from a peptide. With the exception of free BCL-xL and MCL-1 (C), both having surface coloring based on all atoms of peptide amino acids, surface coloring highlights strictly the atoms within 4 Å from a peptide. PDB residue numbering has been maintained. Labels of acidic and basic amino acids are colored red and blue, respectively.

(C) Free MCL-1.

(D) A1 bound to BIM BH3. The four conserved hydrophobic residues/sites and the conserved charged interactions are marked. Peptide amino acid labels are bold.

(E) BCL-xL bound to BIM BH3.

(F) MCL-1 bound to BIM BH3.

(G) A model between BID BH3 (from the A1·BID BH3 complex) and BAK-ΔTM, overlapping sites of interaction (colored) identified by NMR spectroscopy.

(H) BIM SAHB modeling to the "back" face of free BAX.

(I) BIM SAHB binding site on BAX.

of BCL-2 domain'' peptide) was used, in which the BH3 α helix is fixed by a chemical staple. The BAX·BIM SAHB complex was nevertheless unstable, precluding structural determination by conventional NMR. Instead, paramagnetic relaxation enhancement NMR oriented the BIM SAHB on the back face opposite the BC groove of BAX. A shallow hydrophobic groove in BAX devoid of pronounced ridges was defined by α1 and α6. This occurred by displacing the α1-α2 loop and rearranging the peripheral side chains (e.g., BAX Lys21 next to BIM Glu158) to accommodate the BIM SAHB; the newly identified groove appears similar to antiapoptotic BC grooves. Binding of the BIM SAHB also induced subtle conformational changes to the BAX BC groove, where α9 became loosely attached compared to the free BAX structure. In addition, the BID and PUMA BH3 can promote remodeling of the BAX structure (Kim et al., 2009).

Whether a similar direct activator-binding strategy mediates BAK activation is unknown. Modeling of the BIM SAHB on the back face identified fewer clashes in BAK compared to BAX (Figure 4I). However, NMR revealed a low-affinity complex between the BID BH3 peptide and BAK-ΔTM (which mimics the globular domain of membrane-targeted BAK) (Figure 4G). BC groove of BAK, and major conformational changes were not detected. The BAK BC groove is also implicated in homodimerization, as illustrated by cross-linking studies at cysteine residues engineered within the BH3 and the BC groove (Dewson et al., 2008). These studies indicated that the formation of BAK dimers involves reciprocal BH3·BC groove interactions; and most recent studies revealed that high-order BAK oligomer formation requires α6·α6 interactions to promote MOMP (Dewson et al., 2009).

Structures of Antiapoptotic and BH3-Only Protein Complexes

The antiapoptotic BCL-2 family members have been extensively studied at the molecular level by both NMR spectroscopy and X-ray crystallography. A comprehensive review of antiapoptotic BCL-2 family structures described free BCL-2, BCL-xL, BCL-w, and the complexes between BCL-xL and the BAK and BAD BH3 peptides (Petros et al., 2004). The current portrait of antiapoptotic proteins now includes A1 and MCL-1 structures in complex with an extensive panel of BH3 peptides (Figures 4C–4F and 5A). These recent structural insights are significant to cancer drug discovery because MCL-1 and A1 are not targeted by the most promising BH3 mimetics (e.g., ABT-737 and its analogs), which only inhibit BCL-2, BCL-w, and BCL-xL (Oltersdorf et al., 2005).

There are structural similarities and differences between the BCL-2 cores of A1, BCL-xL, and MCL-1 in the free and BH3 peptide-bound states (Figures 4B–4F). Analyses of their BC

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