Building blocks of the apoptotic pore:

How Bax and Bak are activated and oligomerize during apoptosis

Running title: Regulation of Bax and Bak pore-formation

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Abstract

The central role of the Bcl-2 family in regulating apoptotic cell death was first identified in the 1980s. Since then, significant in-roads have been made in identifying the multiple members of this family, characterizing their form and function and understanding how their interactions determine whether a cell lives or dies. In this review we focus on the recent progress made in characterizing the proapoptotic Bcl-2 family members, Bax and Bak. This progress has resolved longstanding controversies, but has also challenged established theories in the apoptosis field. We will discuss different models of how these two proteins become activated and different "modes" by which they are inhibited by other Bcl-2 family members. We will also discuss novel conformation changes leading to Bak and Bax oligomerization and speculate how these oligomers might permeabilize the mitochondrial outer membrane.

Keywords: apoptosis, Bak, Bax, conformation change, oligomerization

Abbreviations: BH, Bcl-2 homology; EPR, electron paramagnetic resonance; MOM, mitochondrial outer membrane; tBid, truncated Bid; TM, transmembrane domain

FACTS

- Bax and Bak are activated by BH3-only proteins and inhibited by prosurvival Bcl-2 proteins via direct interactions

- Bax and Bak undergo major conformation changes during transition from inactive monomers to activated oligomers

- Bax and Bak oligomers are responsible for permeabilization of the mitochondrial outer membrane

OPEN QUESTIONS

- Are both proposed binding sites on Bax essential for its activation by BH3-only proteins?

- What molecular features present in Bax and Bak but not in the prosurvival proteins allow conformation change, oligomerization and pore formation?

- Does Bax and Bak pore formation involve insertion of their $\alpha5/\alpha6$ helices as a membrane-spanning hairpin?

- What protein:protein interfaces allow Bax and Bak to form high molecular weight oligomeric pores?

The Bcl-2 family: Guardians at the "mitochondrial gate"

Mitochondria have a critical role in cell survival through their generation of ATP via oxidative phosphorylation. However, these organelles also have a dark side, as lurking at their surface are the Bcl-2 family of proteins that second mitochondria into the cell death pathway. Two key players of the Bcl-2 family are the proapoptotic proteins Bax and Bak, which convert from harmless monomers into deadly oligomers that form pores in the mitochondrial outer membrane (MOM). These pores are conduits for proapoptotic factors such as cytochrome *c* to translocate to the cytoplasm. The result is 2-fold: the loss of cytochrome *c* from mitochondria disables energy production; and cytosolic cytochrome *c* instigates a proteolytic cascade that dismantles the cell.[1]

Given their critical role in mediating mitochondrial apoptosis,[2,3] Bax and Bak have to be strictly regulated by other members of the Bcl-2 family (Figure 1a). At least five mammalian "prosurvival" proteins have been identified that can inhibit Bax and Bak via three distinct mechanisms: MODE 0, MODE 1 and MODE 2 (Figure 1b). MODE 1 and MODE 2 were recently assigned to describe the indirect and direct inhibition of Bax and Bak, respectively.[4] Here we assign MODE 0 to the newly described mechanism in healthy cells by which prosurvival proteins regulate Bax mitochondrial localization. On the other hand, eight or more proapoptotic "BH3-only" proteins have been found to initiate apoptosis by triggering Bax and Bak activation (Figure 1c). Historically, BH3-only proteins have been placed into discrete subsets, with those able to activate Bax and Bak directly termed "activators" and those that target prosurvival proteins to indirectly activate Bax and Bak called

"sensitizers".[5] However, recent evidence suggests that this strict categorization may no longer be appropriate.[6-10] We therefore discuss BH3-only proteins only by their ability to either "activate" Bax and Bak (Box 1) or "derepress" prosurvival proteins.

Dys-regulated Bcl-2 proteins are associated with a plethora of diseases, making the Bcl-2 family members and their interactions a focus for novel therapeutics.[11,12] BH3-mimetics such as ABT737, its derivative ABT263, and the newly developed ABT199, which bind and inhibit prosurvival Bcl-2 proteins, provide the stepping stones towards customized, selective drugs that activate the apoptotic program in cancer cells[13-15] and have encouraged the search for anti-cancer drugs that directly activate Bax and Bak.[16]

Conversely, as excess apoptosis contributes to ischemia reperfusion injury and diseases such as amyotrophic lateral sclerosis[17,18] there is interest in developing inhibitors of Bax and Bak.[19] Although treating chronic degenerative conditions may not be tractable, acute inhibition of apoptosis, for example in preventing reperfusion injury following organ transplantation, may be of significant benefit. Thus, defining the regulatory landscape that exquisitely controls Bax and Bak will unlock the clinical potential of these critical effector proteins.

In this review, we focus on recent insights into the structural transitions of Bax and Bak from inactive monomers to active oligomers, and the major checkpoints employed by the cell to regulate this transition. The findings are summarized in a model that incorporates both biochemical and structural data supporting the transitions (Figure 2).

Bax and Bak in healthy cells: wolves in sheep's clothing

Like the prosurvival proteins, Bax and Bak protein sequences contain all four Bcl-2 homology (BH) domains, including the re-defined BH4 domain[20] (Figure 2a). In addition, several structures of inactive Bax and Bak[21-23] reveal that the protein fold of the two proapoptotic proteins is strikingly similar to that of their prosurvival counterparts.[24,25] Thus, based on sequence and structure, what distinguishes Bax and Bak from the prosurvival Bcl-2 proteins is unclear.

Bax and Bak contain nine α-helices, with a hydrophobic α5 at the protein core, surrounded by amphipathic helices (Figure 2 and 3a). The C-terminal α9 helix contains a transmembrane domain (TM) that anchors the proteins in the MOM. The hydrophobic BH3 domain, located in α2, is nicely tucked away in inactive Bax and Bak and becomes exposed during activation to facilitate hetero- and homo-oligomerization.[9,26,27] In addition, a hydrophobic groove is located on the surface of Bax and Bak involving residues from the C-terminus of α2 to the N-terminus of α5 and residues in α8. Notably, a similar surface groove in the prosurvival proteins has been characterized as the receptor site for the BH3 domain of proapoptotic Bcl-2 members.[28-34] Given the importance of this structural motif in regulating protein-protein interactions of the prosurvival proteins, one may predict that Bax/Bak regulation could also occur via binding of proapoptotic BH3 domains to the groove of Bax and Bak. However, in inactive Bax, the groove is normally occupied by its own TM.[22] Interestingly, on the opposite side of Bax, and masked by the α1/α2 loop, is a shallower groove or "rear pocket" involving α1/α6 helices (Figure 2a and 3a). As

this rear pocket has a similar distribution of hydrophobicity and charge as the canonical surface groove, it may be a trigger site for Bax activation.[35,36]

Sequestration of the TM of Bax into its own groove renders the protein mainly cytosolic in healthy cells. However, a small portion of Bax is loosely attached to mitochondria,[37-39] as it can be extracted using sodium carbonate.[40] Until very recently, it was thought that after an apoptotic stimulus, Bax actively translocates to mitochondria.[41,42] However, elegant studies using FLIP (fluorescence loss in photobleaching) and FRAP (fluorescence recovery after photobleaching) indicate that Bax localizes to mitochondria in healthy cells, but is actively retro-translocated to the cytosol (discussed below).[37,40,43] In such a model, inhibition of retro-translocation following apoptotic insults causes Bax to accumulate at mitochondria, thereby sensitizing cells to apoptosis.

In contrast to Bax, Bak is constitutively inserted into the MOM in healthy cells,[44,45] presumably via α9. The Bak α9 is more hydrophobic than that of Bax, and appears to prefer the hydrophobic membrane environment rather than the amphipathic environment of the Bak groove. Consistent with this, when the Bak tail was mutated to make it more hydrophilic (Bax-like), the mutant Bak protein became more cytosolic with binding of the tail to the groove.[44,46]

Bax and Bak activation: the Jekyll to Hyde metamorphosis

When Bax and Bak become activated, they undergo major conformation changes involving the C-terminus (of Bax only), N-terminus, the BH3 domain and the α5 and α6 helices, resulting in proteins that have been literally turned inside out (Figure

2b).[26,35,45,47-49] How these changes are triggered, the sequence of events and which changes are actually required for oligomer formation and apoptotic function is still under investigation (reviewed in [50]).

a) Direct activation of Bax and Bak by BH3-only proteins

Bax and Bak can be activated by direct binding of certain BH3-only proteins (Figure 1c). While BH3-only family members bind readily to prosurvival Bcl-2 proteins, an interaction of the former with Bax and Bak has been difficult to capture. In the "hit and run" model it was proposed that binding of BH3-only proteins to Bax and Bak is only transient,[51] presumably because the induced changes in Bax and Bak conformation lead to the disengagement of the activating proteins from Bax and Bak. Some of the first evidence for direct activation derived from a Bid mutant that was unable to bind to prosurvival proteins, but could still bind and activate Bax.[52] Since then, several studies have captured or implicated direct binding and activation of Bax and Bak by BH3-only proteins using biochemical, structural, as well as genetic approaches.[4,7,35,36,46,53-56] However, Bax and Bak activation may also be caused by other factors such as changes in intracellular environment, post-translational modification and interaction with mitochondrial membrane components and non-Bcl-2 proteins (Box 2). Thus, whether direct activation of Bax and Bak by BH3-only proteins is needed in all circumstances remains to be elucidated.[57,58]

The MOM may play a significant role in direct activation, as two landmark papers using full-length recombinant Bcl-2 proteins in liposomes in combination with FRET (Förster resonance energy transfer) showed that Bax and tBid only

interact when a membrane is present.[59,60] After membrane insertion, tBid could then drive the membrane insertion of Bax.[59-61] This led to the "embedded together" model, which proposed an important role for the mitochondrial outer membrane in all interactions between the Bcl-2 family members.[62]

b) Bax and Bak conformation changes triggered by BH3-only proteins

An early conformation change in Bax is eversion of α9 from the Bax groove, to allow the peripherally attached protein to integrate into the MOM. From the structure of inactive Bax it was inferred that BH3-only proteins may not be capable of directly displacing α9 from the groove.[22] However, several studies indicate that BH3-only proteins or peptides can bind to the α1/α6 side (rear pocket) of Bax,[6,9,36,46,63] and thus might provoke Bax α9 exposure indirectly. Indeed, an NMR study showed that binding of a stapled BimBH3 peptide to the rear pocket of Bax induced chemical shifts in residues belonging to the α9 helix (Figure 3b).[35] As Bak is constitutively localized at mitochondria, presumably with its α9 helix already inserted into the membrane, initial activation at the rear pocket would not be required. In support of this, BH3 peptides from activating BH3-only proteins do not bind to the α1/α6 region of Bak.[46]

As well as Bax α9 exposure, several other conformation changes were associated with binding of a stapled BimBH3 peptide to the Bax rear pocket (Figure 3b). Displacement of the loop between α1 and α2 coincided with exposure of the 6A7 epitope in α1 and the BH3 domain, both hallmarks of Bax activation.[35] While these hallmark conformation changes may have been a direct consequence of Bim

binding to the rear pocket they may also have been caused by subsequent binding of Bim to the canonical groove once it is vacated by α9. This sequential binding was first highlighted by Kim et al,[9] and is supported by linkage of BH3 peptides to Bax residues in the rear pocket as well as the canonical groove.[46] A two-step activation for Bax would also explain why the Bax S184V variant that constitutively inserts α9 in the MOM, is inactive until stimulated by proapoptotic factors.[43,49]

Supporting the importance of the groove as an activation site, a crystal structure of Bax lacking the α9 helix, and thereby representing Bax with an unoccupied groove analogous to Bax S184V, displayed BH3 peptides of Bid and Bax bound to its groove (Figure 3c).[53] The peptides bound to the Bax groove in a similar manner to which they bind to the groove of prosurvival proteins. However, binding to Bax results in increased movement of the α2/α3 side of the Bax groove away from the bound peptide, with a partial displacement of the α2 helix. This opening of the groove might weaken the contact between the BH3 peptide and Bax, providing a structural rationale for transient binding of BH3-only proteins to Bax. The Bax crystal structures also provided evidence for a novel conformation change, termed the "core/latch dissociation" in which α5 and α6 unhinge allowing the α6-α8 helices (termed "latch") to dissociate from the α1-α5 helices (termed "core") (Figure 3c). This conformation change was required for Bax function in mitochondrial assays, as its proapoptotic function was inhibited upon cysteine-tethering of α5 and α6.[53] Interestingly, the structural re-organization that occurs during core/latch dissociation exposes the N-terminus of α1, providing a structural mechanism for the

exposure of the N-terminal epitopes during Bax (and possibly also Bak) activation.[45,49]

The Bax α5 and α6 helices have previously been implicated in its pore-forming function, by everting from their inactive localization and inserting as a hairpin into the MOM, analogous to the pore-forming domains of bacterial toxins such as colicin A and Diphtheria toxin.[24,64] Consistent with this hypothesis, peptides based on Bax α5 and/or α6 helices of Bax have pore-forming activity.[65-67] Most compelling were cell studies in which the Bax α5/α6 helices became buried in the MOM prior to oligomerization.[47] Our recent structural studies indicate that α5 and α6 of Bax dissociate during activation[53] suggesting that membrane insertion of these helices may not occur as a hairpin (see below).

Several biochemical and structural approaches show that, in contrast to Bax, Bak activation involves BH3-only protein or BH3 peptide binding only to the canonical groove.[7,46,55] Firstly, by testing different mutants of Bak and BH3-only proteins (including reciprocal size-swap variants) in binding and Bak oligomerization assays, the activation site of BH3-only proteins was mapped to the Bak groove.[7] Secondly, BH3 peptides can link to the groove of Bak, but not to its α1/α6 region.[46] Thirdly, in a very recent NMR structure, a BidBH3 peptide bound to the groove of Bak[55] in a similar manner to its engagement with the Bax groove.[53] Binding of BH3 peptides to the Bak groove coincided with the exposure of the Bak BH3 domain and the N-terminus,[55] both of which are similar conformation changes to those seen in Bax.[53] Intriguingly, these Bak conformation changes were blocked by tethering the peptide to the groove, suggesting that Bak can only change

conformation when the activating peptide leaves the groove,[55] consistent with the hit and run model. Whether Bak undergoes core/latch dissociation like Bax remains to be determined.

c) Restraint of Bax and Bak by prosurvival proteins

The prosurvival Bcl-2 family members have long been recognized as critical guardians of Bax/Bak activity. It has now become clear that they can act via different "MODES" to prevent Bax and Bak activation and oligomerization.[4]

MODE 0: The level of Bax at mitochondria is tightly regulated in healthy cells as Bax is trafficked away from the MOM to the cytosol,[37,40,43] As Bcl-x_L was found to ferry peripherally associated Bax to the cytosol,[37,40] this represents a new way of keeping Bax in check and is defined here as "MODE 0" inhibition (Figure 1b). The molecular mechanism underpinning this newly described "retro-translocation" remains obscure. Although binding of Bax and Bcl-x_L during retro-translocation has not been shown, mutagenesis of the BH3 domain of Bax and the groove of Bcl-x_L inhibited retro-translocation implicating a direct interaction.[37] This BH3:groove interaction indicates that the peripheral Bax "cargo" has undergone at least certain activation steps including exposure of its BH3 domain, but has not yet reached the stage of membrane integration or oligomerization. Such conformation changes that are normally associated with activation during apoptosis may have been induced by the association of Bax with membranes.[68]

Once retro-translocated, the complex of the cargo (Bax) and ferry (Bcl-x_L) must dissociate as Bax in the cytosol is monomeric and does not require interactions with other proteins to maintain its inactive conformation.[69,70] Further, as cytosolic Bax does not expose its N-terminus or its BH3 domain, Bax must revert to its inactive conformer consistent with the reversible conformation change in Bax induced by interaction with membranes.[68]

Bax retro-translocation independent of prosurvival Bcl-2 proteins has recently been reported.[43] However, retro-translocation in this case involved Bax that was tail-anchored in the MOM rather than peripherally associated, suggesting that different populations of Bax may exist in a healthy cell with distinct mechanisms governing their subcellular localization.

Although the molecular mechanism governing Bax retro-translocation and whether prosurvival Bcl-2 proteins are necessary requires further investigation, controlling Bax subcellular localization clearly represents an important mechanism to regulate apoptotic function. Considering that only 100 molecules of Bax per mitochondria are necessary for pore formation,[71] retro-translocation might be critical in fine-tuning a cell's response to apoptotic stimuli.

MODE 1: In response to apoptotic stimuli prosurvival proteins can sequester BH3-only proteins to prevent them from activating Bax and Bak. This "MODE 1" inhibition[4] (Figure 1b) is likely facilitated by sequestering the BH3 domain of the BH3-only proteins into the prosurvival groove.[28,29,34] However, recent evidence suggests that regions other than the BH3 domain might also be involved, as

mutation in the BH3 domain of Bim (Bim2A) that was sufficient to abrogate binding of a BimBH3 peptide to Bcl-x_L *in vitro*[72] did not abolish interaction of full-length Bim with Bcl-x_L in live cells.[73]

MODE 2: Irrespective of the activating mechanism (see Box 2), once Bax and Bak are activated, prosurvival proteins directly bind to the activated proteins to prevent their homo-oligomerization resulting in MODE 2 inhibition (Figure 1b).[4] Again, MODE 2 occurs via sequestration of the exposed BH3 domain of Bax and Bak into the groove of the prosurvival proteins,[27,31,32] putting the BH3:groove interaction central to the myriad of interactions that govern cell fate.

MODE 1 and MODE 2 may require a conformation change in the prosurvival proteins induced by binding of BH3-only proteins.[59] This "activation" of prosurvival Bcl-2 proteins may simply anchor α9 in the MOM,[74] or involve a more drastic insertion of the α5 and α6 helices,[75,76] similar to the proposed conformation change in Bax.[47] That proapoptotic proteins "activate" prosurvival proteins seems at first glance to be counter-intuitive. However, BH3-only proteins may bind to and induce conformation change in any of the structurally similar multi-domain Bcl-2 proteins, regardless of whether they are prosurvival or proapoptotic. The prosurvival proteins might thus act as a dominant negative form of Bax, competing with Bax for binding of BH3-only proteins and later on for activated Bax to prevent Bax homo-oligomerization (reviewed in [77]).

d) Derepression of MODE 1 and MODE 2: Indirect activation of Bax and Bak by BH3-only proteins

MODE 1 and MODE 2 inhibition by prosurvival proteins can be overcome by up-regulating BH3-only proteins, which bind and inhibit the prosurvival Bcl-2 family members and thus indirectly promote Bax and Bak activation (Figure 1c). When these up-regulated BH3-only proteins interact with prosurvival proteins to compete off activating BH3-only proteins,[5,8,60,78,79] the process is termed "MODE 1 derepression".[4] In addition, derepression also takes place when the BH3-only proteins compete off activated Bax and Bak from the prosurvival proteins, termed "MODE 2 derepression",[4] akin to the "indirect activation" model.[58,80,81] Notably, according to Llambi et al,[4] derepression of MODE 2 complexes is more difficult than MODE 1 complexes.

Taken together, the prosurvival proteins, the BH3-only proteins, and Bax/Bak partake in a dynamic triad of competitive interactions (Figure 1a). The relative affinities of these interactions and the cellular concentration of each player determine whether Bax and Bak (i) remain inactive, (ii) become activated but bind prosurvival proteins (in which case the cell is reprieved) or (iii) become activated and self-associate to form a pore (in which case the cell is generally doomed).

Bax and Bak oligomerization: making doughnuts and daisy chains

Once activated, Bax and Bak have exposed hydrophobic regions that need to be buried in a membrane or a protein interface, leading to the formation of membrane spanning high molecular weight oligomers.

Two main oligomer models have been proposed. An asymmetric, single interface oligomer model, or "daisy-chain" model, was originally suggested for Bax[82] and is supported by studies that define a BH3:α1/α6 interface.[35] A recent model of a Bak octameric pore was also based on a single-interface mechanism.[83]

An alternative symmetric or two-interface model was proposed for Bak, based on a cysteine-linkage approach in mammalian cells[26] and was later supported for Bax in different linkage studies.[84,85] In this model, the exposed Bak BH3 domain engages the canonical hydrophobic surface groove of a partner Bak molecule, in a similar manner to its interaction with the prosurvival groove. However, the BH3:groove interaction in a Bax or Bak homodimer is symmetric, with the BH3 domain of the second Bax/Bak molecule binding into the groove of the first in a reciprocal fashion (Figure 2b). The symmetric BH3:groove dimer model was supported by electron paramagnetic resonance (EPR)-spin labeling of recombinant Bax and Bak in liposomes,[86,87] and more recently by evidence that the basic oligomeric unit of activated Bak is a homodimer under native conditions.[88]

The symmetric model is also supported by a recent crystal structure of the Bax α2-α5 region.[53] The α2-α5 of Bax, which contains the BH3 domain and the hydrophobic surface groove, was shown to be sufficient for oligomerization.[89] Intriguingly, when a similar α2-α5 Bax construct was expressed as a GFP fusion protein and crystallized, it spontaneously formed a symmetric BH3:groove dimer (Figure 3d).[53] In this dimer structure, the BaxBH3 domain bound to the Bax groove in a similar fashion as activating BH3 peptides (Figure 3e), indicating that similar BH3 domain residues might be crucial for Bax activation as well as Bax dimerization.

This also indicates that for a Bax homodimer to form, the activating BH3 domain must first leave the Bax groove, consistent with the hit and run model.

Interestingly, one half of the BH3:groove homodimer closely resembles the "core" of the Bax:BidBH3 complex (Figure 3e), suggesting that no major rearrangements of the Bax core, other than the BaxBH3 exposure, are necessary to form the BH3:groove dimer. Thus, the α5 helix remains adjacent to α3 and α4, and, together with α4, lines the base of the BH3:groove dimer, projecting a high concentration of hydrophobic aromatic residues into the milieu (Figure 3d). One may therefore speculate that core/latch dissociation exposes lipophilic residues of α4, α5 and α6 and that BH3:groove homodimers nucleate the oligomerization of this activated form. The locally concentrated lipophilic residues could then penetrate the membrane bilayer to displace the phospholipid headgroups to provoke positive membrane curvature and eventually membrane rupture. Such a mechanism is potentially analogous to the "carpet model" of pore formation described for certain bacterial toxins such as melittin, whereby antimicrobial peptides aggregate in the plane of the membrane leading to membrane rupture.[90,91]

This proposed mechanism for permeabilization of the MOM by Bax and Bak by an in-plane interaction with the membrane (Figure 2b) is consistent with the reduced labelling of the Bax α5/α6 helices with a hydrophilic label during activation.[47,87] However, it suggests that Bax and Bak α5/α6 helices may not traverse the MOM as a membrane-spanning hairpin and therefore may not function analogously to colicin A and Diphtheria toxin. That the Bax or Bak α5/α6 helices may not insert as a hairpin is supported by several lines of evidence. Firstly, the α5

is not everted in the structure of dimerized Bax.[53] Secondly, a disulphide tether between α4 and α5 helices of Bax did not inhibit apoptotic function, providing further support that the α5 remains associated with the "core" domain.[53] Thirdly, α5 and α6 must separate for Bax to mediate cell death.[53] And finally, EPR spin labelling indicates that α5 remains associated with the core in activated Bak and that α6 exhibits only shallow insertion into liposomal membranes.[87]

That the basic oligomeric unit of Bax and Bak is a symmetrical homo-dimer,[26,53,85-88] is not consistent with the daisy-chain model of oligomerization. Rather in a symmetric model a second interface is necessary for dimers to multimerize in order to form the higher order oligomeric pore.[84] Regions outside of the core α2-5 domain such as the α6 helices have been implicated in this requisite second interface in Bax and Bak.[85,87,88,92] Therefore, core-latch dissociation may not only serve to expose a lipophilic surface to engage the MOM, it may also reposition the latch domain, including α6, to facilitate higher order oligomerization. However, how this second interface enables dimers to multimerize, and consequently the structure of the putative apoptotic pore, is unknown. Whether the apoptotic pore involves dimers assembled in a closed conformation (a "doughnut"), a linear assembly, or a disordered aggregate, as well as whether lipids are critical constituents of the pore remains to be determined.

What's next?

Although recent novel approaches have provided significant insight into how Bax and Bak are activated to kill cells, further research is clearly needed. A structure of a

high molecular weight oligomer of Bax and/or Bak would be a major step forward and would determine whether Bax and Bak form proteinaceous pores (reviewed in [50,93]). The alternative is that they form lipidic pores.[67,94] If so intercalated lipids will significantly hinder characterization of the pore by conventional structural approaches and so elegant biophysical approaches in the presence of a membrane may be needed to study the oligomeric pore.

Exciting times lie ahead. We anticipate that future advances in our understanding of how Bax and Bak are activated and how they function will expose these critical apoptotic proteins as targets for novel therapeutics.

Box 1: Molecular features of activating BH3-only proteins

In early studies, only Bid and Bim were categorized as BH3-only proteins that can activate Bax and Bak.[5] This has been challenged with evidence that Puma can activate Bax,[6,95] that Noxa can bind to and induce Bak activation[7] and that peptides from all BH3-only members can trigger Bax- and Bak-mediated permeabilization of liposomes if used at high concentrations.[10]

Recent structural and mutational studies allow a more detailed definition of which residues in the BH3 domain promote binding and activation of Bax[53] and Bak.[55] In general, BH3-only proteins bind in a similar manner to the grooves of Bax/Bak and the prosurvival proteins. Specifically, as seen in the BidBH3 peptide binding to Bax, four hydrophobic residues (h1 to h4) in the BH3 make contact with four hydrophobic pockets in the groove, and a salt bridge forms between a conserved aspartate (BidD95) and a conserved arginine (BaxR109) (Figure 4). Notably, a new h0 position in Bid and Bim as well as Noxa and Bad contributes to their activating function.[53,55]

Mutagenesis has indicated that both the hydrophobic interactions and the salt bridge are essential for the binding of activating BH3 peptides to the Bax groove.[53,55] A single substitution of the h1 residue in the Noxa BH3 domain (NoxaC25I) was sufficient to turn Noxa into an activating BH3 peptide,[53] whereas Bad required at least 3 substitutions to be able to activate Bax and Bak.[53,55] At the other end of the BH3 domain (beyond h4), there is little contact with Bax, and mutation did not affect binding to Bax.[8,53] However substitution at the h5 of Bad and Noxa seemed to contribute to their gain-of-function activity for Bak.[55] In addition,

the glycine in the GDE sequence was important, as Bid G94A lost binding to Bax[52] and the Bad S118G change contributed to its gain-of-function for Bax and Bak.[53,55] Finally, BidA91W lost its ability to activate Bak.[55] Together the data suggest that not one single residue, but rather a combination of residues determines whether a BH3-only protein can activate Bax and Bak.

Binding of BH3 peptides to the rear pocket of Bax requires at least some of the above described residues,[6,35,36,96] consistent with the rear pocket and hydrophobic groove displaying a similar distribution of charge and hydrophobicity. Further analysis may identify mutations that distinguish between the two potential activation sites in Bax to better understand their role in triggering Bax anchorage into the membrane versus Bax conformation change and oligomerization.

Box 2: Non-Bcl-2 factors regulating Bax and Bak

Several factors have been implicated in regulating Bax and Bak independently of, or in concert with, other Bcl-2 family members.

Stimuli that initiate Bax and Bak activation include mild heat, hydrogen peroxide, and low or high pH,[97-100] as well as non-Bcl-2 proteins such as p53 and Bif-1.[101-103] In addition, post-translational modification such as dephosphorylation of Bax and Bak has been implicated in their activation and oligomerization.[104-106] However, an absolute requirement for this dephosphorylation seems unlikely, given that recombinant Bax and Bak can permeabilize liposomes[79,107,108] and that Bak proapoptotic function does not rely on dephosphorylation.[109] In addition, membrane components such as cardiolipin (reviewed in [110]) and sphingolipids[111] may co-operate with BH3-only proteins to promote Bax and Bak activation.

On the other hand, certain interactions can regulate Bax and Bak negatively. For example, in healthy cells, the voltage-dependent-anion-channel2 (VDAC2)[112,113] is proposed to enhance recruitment of Bak to the mitochondria[114] and to keep Bak in an inactive state.[112,113,115] Likewise, Pin1 and the E3 ligase IBRDC2 may function as negative regulators of Bax, presumably by affecting its conformation or targeting it for degradation.[116,117]

Finally, the mitochondrial fission and fusion machinery may remodel the membrane environment to regulate Bax and Bak activation and oligomerization[118,119] (reviewed in [110]).

Whether all of the above-mentioned non-Bcl-2 factors are necessary for regulating Bax and Bak requires further investigation. However, it is conceivable

that these factors may tweak an apoptotic response in certain cell types under certain conditions.

Titles and legends to Figures

Figure 1 Schematic representation of the Bcl-2 family interaction network. (a) The Bcl-2 family can be divided into 3 classes: the proapoptotic Bax/Bak proteins, the proapoptotic BH3-only proteins and the prosurvival proteins. The prosurvival proteins inhibit the activity of the proapoptotic Bcl-2 family members. The BH3-only proteins activate Bax and Bak either directly (activation) or indirectly (derepression). Despite Bok's amino acid sequence similarity to Bax and Bak, its apoptotic role is currently unclear.[120] **(b)** Inhibition by prosurvival proteins may occur via three "MODES". During MODE 0 inhibition (M0), prosurvival proteins such as Bcl-x_L bind to peripheral Bax at mitochondria and retro-translocate it to the cytosol. During MODE 1 inhibition (M1), prosurvival proteins sequester BH3-only proteins to stop them activating Bax and Bak. During MODE 2 inhibition (M2), prosurvival proteins bind to activated Bax and Bak to prevent their homo-oligomerization. **(c)** The BH3-only proteins can cause Bax and Bak activation in two ways. While certain BH3-only proteins can directly bind to and activate Bax and Bak, other BH3-only proteins interact with prosurvival proteins to compete off the activating BH3-only proteins (Derepression of MODE 1, D1) or activated Bax/Bak (Derepression of MODE 2, D2).

Figure 2 Bax and Bak transitions are regulated by other Bcl-2 family members. **(a)** Schematic representation of the Bax/Bak protein sequence indicating the location of the nine α-helices. Helices of interest are colored in red (α2), green (α5),

black (α9) or blue (latch helices α6, α7, α8). In addition, the four Bcl-2 homology (BH) domains and regions that constitute the groove and rear pocket are marked. **(b)** Schematic representation of a model for Bax and Bak activation and oligomerization. Bax and Bak proteins are represented as cylindrical bundles using the same color scheme as in (a). **Bax in healthy cells:** peripheral Bax shuttles between the mitochondrial outer membrane (MOM) and cytosol mediated by prosurvival Bcl-2 proteins (blue box) (MODE 0 inhibition, M0). Note that in healthy cells a tail-anchored population of Bax has also been observed to retro-translocate independent of prosurvival Bcl-2 proteins. Bak is constitutively inserted in the MOM in healthy cells. **Bax/Bak activation:** BH3-only proteins (yellow box) activate Bax and Bak and cause α9 exposure and membrane insertion (Bax only), α2/BH3 domain exposure, N-terminal exposure, α1/α2 loop displacement and core/latch dissociation. To prevent Bax and Bak activation, prosurvival proteins sequester the activating BH3-only proteins (MODE 1 inhibition, M1). In turn, certain BH3-only proteins promote apoptosis by binding the prosurvival proteins and thereby compete off the activating BH3-only proteins (Derepression of MODE 1, D1). **Bax/Bak dimer/oligomerization:** Activated Bax and Bak dimerize by interaction of the exposed BH3 domain of one molecule with the groove of a second molecule and *vice versa*. These symmetric dimers further oligomerize to form a complex of unknown size (n) that permeabilizes the membrane. By directly binding and sequestering activated Bax and Bak, prosurvival Bcl-2 proteins prevent dimerization (MODE 2 inhibition, M2). In turn, BH3-only proteins interfere with the prosurvival

inhibition by binding the prosurvival proteins and thereby competing off activated Bax and Bak (Derepression of MODE 2, D2).

Figure 3 Binding of BH3 peptides to the groove and rear pocket of Bax promotes conformation change and oligomerization. Cartoon and surface overlays of Bax structures and models that support the transitions of Bax and Bak described in Figure 2. Color scheme of the helices as in Figure 2. **(a)** NMR structure of the full-length Bax monomer (1F16). Note, the locations of the hydrophobic surface groove, rear pocket, BH3 domain and transmembrane domain (TM) are indicated. **(b)** Model of a BimBH3 peptide (magenta tube) bound to the rear pocket of full-length Bax calculated from NMR data. Note that binding of the BimBH3 peptide displaced the α1/α2 loop (red arrow). Bim BH3 binding coincided with the exposure of the N-terminal 6A7 epitope (marked in yellow), α9 helix and the BH3 domain (black arrows). **(c)** Crystal structure of a BidBH3 peptide (magenta tube) bound to the groove of BaxΔC21 (4BD2). Note that BidBH3 peptide binding induced partial α2 displacement (short red arrow) and dissociation of the C-terminal α6-α8 helices (latch) from the N-terminal α1-α5 helices (core) (long red arrow). **(d)** Crystal structure of the α2-α5 Bax "core" forming a BH3:groove dimer (4BDU). Note that aromatic residues (dark grey) on helices α4 and α5 form a lipophilic surface. Although the lipophilic surface is concave in the homodimer structure, whether this curvature is retained when Bax is associated with the MOM and whether the curvature is important for membrane permeabilization is unknown. **(e)**. Overlay of the Bax:BidBH3 complex (colored:magenta, from c) with one half of the Bax

BH3:groove dimer (in grey, from d). Note, to form the BH3:groove dimer, no major rearrangements of the "core" helices occur other than the exposure of the BH3 domain (α2, red arrow).

Figure 4 BH3 domain residues involved in binding to the groove of Bax or Bak. **(a)** Surface representation of a Bax structure that has a BidBH3 peptide bound to the Bax groove (4BD2). Note that the hydrophobic residues (grey side chains) and the aspartate residue (red side chain) in the BidBH3 peptide make contact with the hydrophobic pockets and the arginine (indicated in blue) in the groove of Bax, respectively. **(b)** Sequence alignment of the indicated BH3 domains, highlighting five conserved hydrophobic residues (grey bars) and the invariant aspartate (red bar). Residues in orange are discussed in the text.

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Conflict of interest statement:

The authors declare no conflict of interest.

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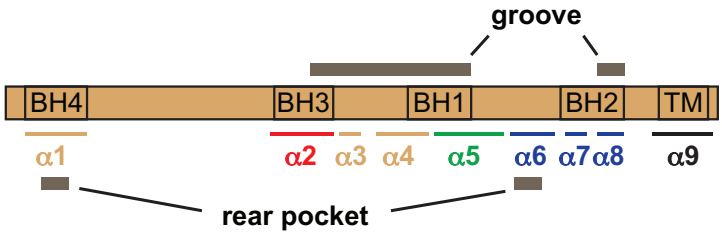
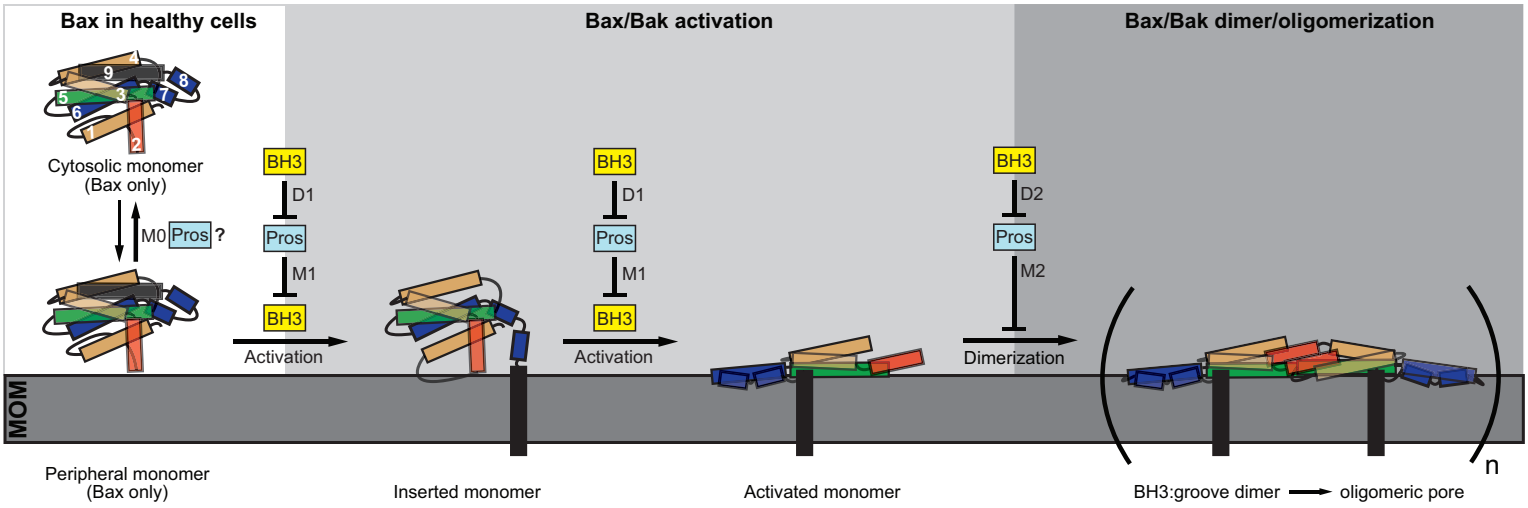
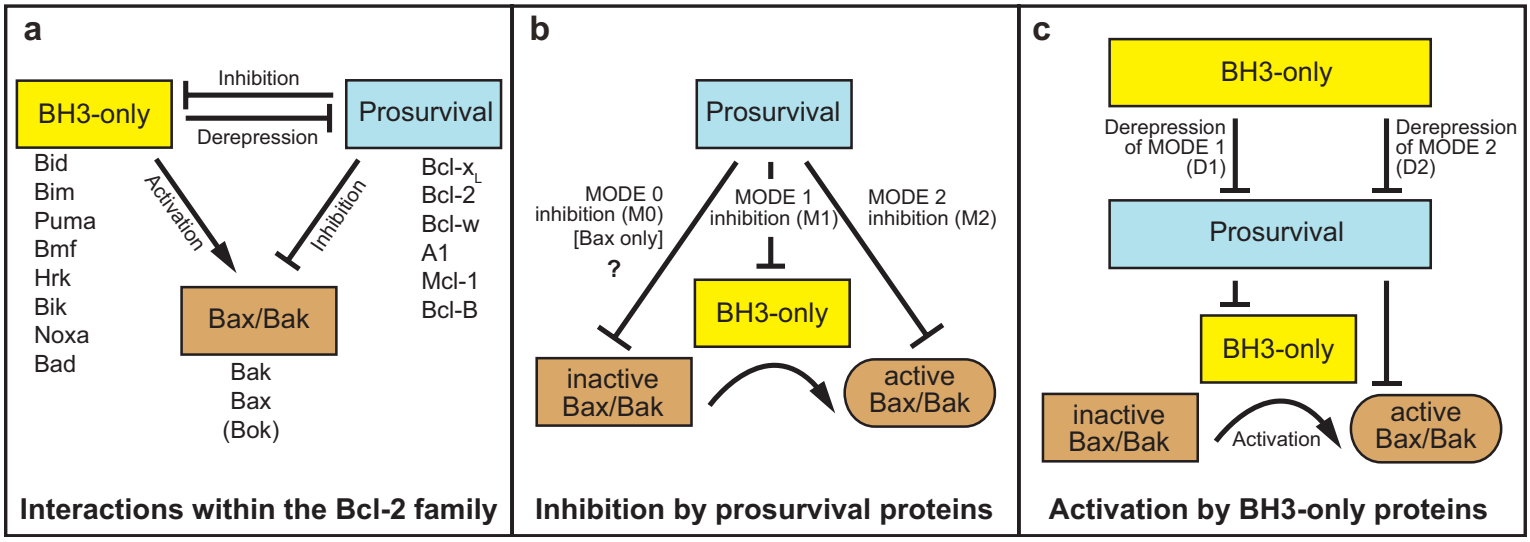
a

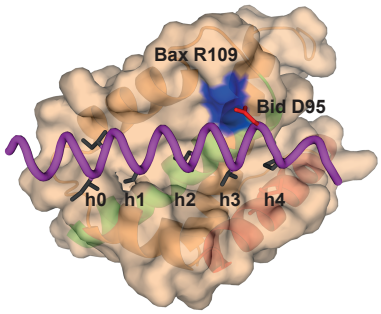

groove
BH4
BH3
BH1
BH2
TM
α1
α2
α3
α4
α5
α6
α7
α8
α9
rear pocket

b


Bax in healthy cells
Bax/Bak activation
Bax/Bak dimer/oligomerization
Cytosolic monomer
(Bax only)
M0
Pros
?
BH3
D1
Pros
M1
BH3
Activation
BH3
D1
Pros
M1
BH3
Activation
BH3
D2
Pros
M2
Dimerization
MOM
Peripheral monomer
(Bax only)
Inserted monomer
Activated monomer
BH3:groove dimer
oligomeric pore
n


a
BH3-only
Inhibition
Derepression
Prosurvival
Bid
Bim
Puma
Bmf
Hrk
Bik
Noxa
Bad
Activation
Inhibition
Bax/Bak
Bak
Bax
(Bok)
Bcl-x$_L$
Bcl-2
Bcl-w
A1
Mcl-1
Bcl-B
Interactions within the Bcl-2 family
b
Prosurvival
MODE 0
inhibition (M0)
[Bax only]
?
MODE 1
inhibition (M1)
MODE 2
inhibition (M2)
BH3-only
inactive
Bax/Bak
active
Bax/Bak
Inhibition by prosurvival proteins
c
BH3-only
Derepression
of MODE 1
(D1)
Derepression
of MODE 2
(D2)
Prosurvival
BH3-only
inactive
Bax/Bak
Activation
active
Bax/Bak
Activation by BH3-only proteins

a



b

```
                 'h0' h1   h2  h3   h4   h5
Bid       79 QEDIIRNIARHLAQVGDSMDRSIPPG
Bim      141 DMRPEIWIAQELRRIGDEFNAYYARR
Noxa      18 PAELEVECATQLRRFGDKLNFRQKLL
Bad      103 NLWAAQRYGRELRRMSDEFVDSFKKG
Bax       52 QDASTKKLSECLKRIGDELDSNMELQ
Bak       67 PSSTMGQVGRQLAIIGDDINRRYDSE
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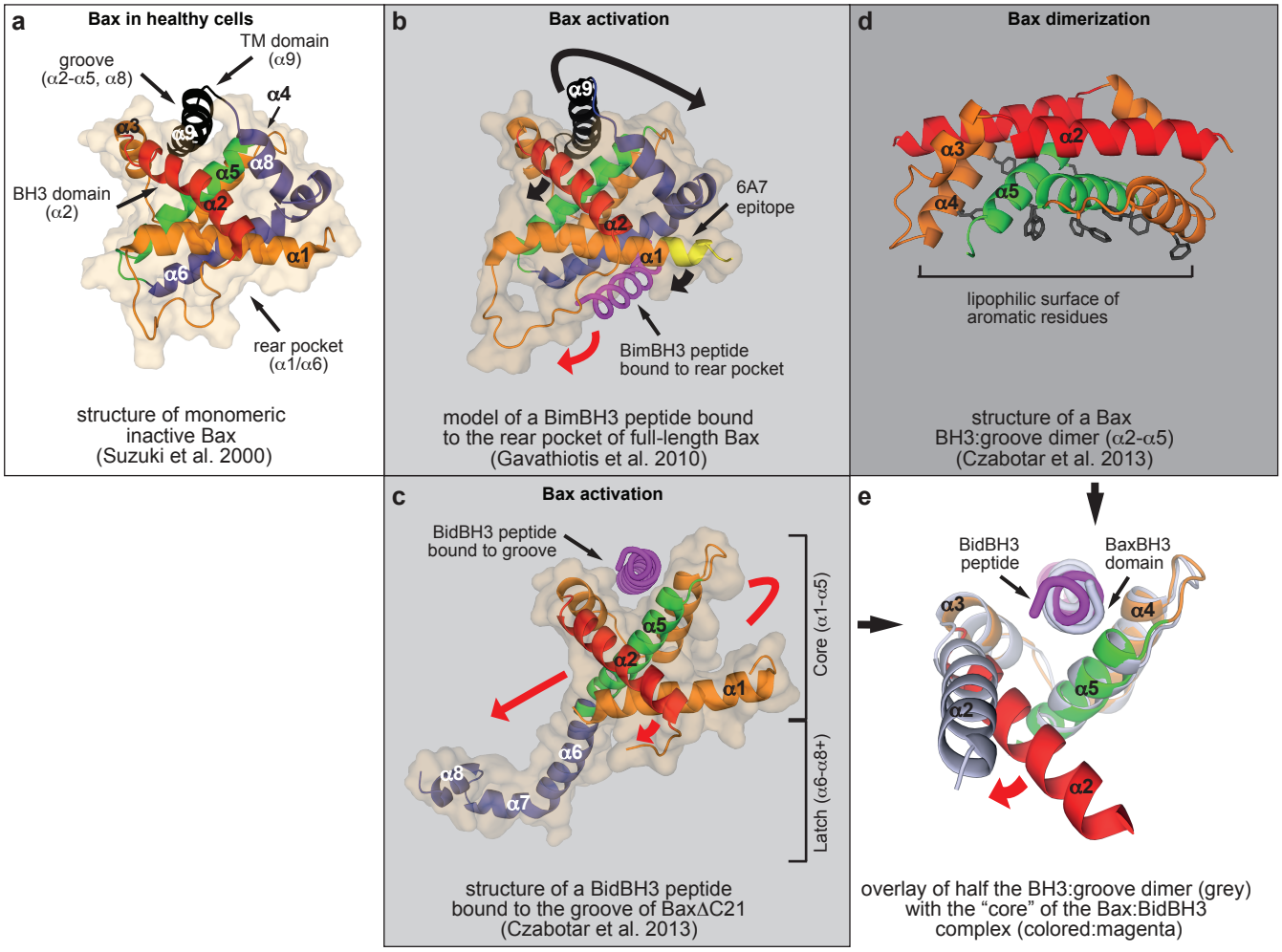
a
Bax in healthy cells
groove
(α2-α5, α8)
TM domain
(α9)
α4
α3
α9
α5
α8
BH3 domain
(α2)
α2
α1
α6
rear pocket
(α1/α6)
structure of monomeric
inactive Bax
(Suzuki et al. 2000)
b
Bax activation
α9
6A7
epitope
α2
α1
BimBH3 peptide
bound to rear pocket
model of a BimBH3 peptide bound
to the rear pocket of full-length Bax
(Gavathiotis et al. 2010)
d
Bax dimerization
α3
α2
α4
α5
lipophilic surface of
aromatic residues
structure of a Bax
BH3:groove dimer (α2-α5)
(Czabotar et al. 2013)
c
Bax activation
BidBH3 peptide
bound to groove
α5
α2
α1
α6
α8
α7
Core (α1-α5)
Latch (α6-α8+)
structure of a BidBH3 peptide
bound to the groove of BaxΔC21
(Czabotar et al. 2013)
e
BidBH3
peptide
BaxBH3
domain
α3
α4
α5
α2
α2
overlay of half the BH3:groove dimer (grey)
with the “core” of the Bax:BidBH3
complex (colored:magenta)