Biochimica et Biophysica Acta 1813 (2011) 521–531

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Molecular biology of Bax and Bak activation and action ☆

Dana Westphal, Grant Dewson, Peter E. Czabotar, Ruth M. Kluck *

Walter & Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia

ARTICLE INFO

Article history:
Received 10 August 2010
Received in revised form 17 December 2010
Accepted 19 December 2010
Available online 30 December 2010

Keywords:
Apoptosis
Bak
Bax
Conformation change
Mitochondrion
Mitochondrial permeability
Oligomerization

ABSTRACT

Bax and Bak are two nuclear-encoded proteins present in higher eukaryotes that are able to pierce the mitochondrial outer membrane to mediate cell death by apoptosis. Thus, organelles recruited by nucleated cells to supply energy can be recruited by Bax and Bak to kill cells. The two proteins lie in wait in healthy cells where they adopt a globular α-helical structure, seemingly as monomers. Following a variety of stress signals, they convert into pore-forming proteins by changing conformation and assembling into oligomeric complexes in the mitochondrial outer membrane. Proteins from the mitochondrial intermembrane space then empty into the cytosol to activate proteases that dismantle the cell. The arrangement of Bax and Bak in membrane-bound complexes, and how the complexes porate the membrane, is far from being understood. However, recent data indicate that they first form symmetric BH3:groove dimers which can be linked via an interface between the α6-helices to form high order oligomers. Here, we review how Bax and Bak change conformation and oligomerize, as well as how oligomers might form a pore. This article is part of a Special Issue entitled Mitochondria: the deadly organelle.

1. Introduction

A major means by which mitochondria can be "deadly" is by releasing pro-apoptotic proteins from the intermembrane space as an early step in apoptotic cell death (Fig. 1, mitochondrial pathway). The principal killing factor is cytochrome *c*, which once in the cytosol initiates the activation of caspases (aspartate-specific proteases) and cleavage of multiple cellular proteins. A second protein released from the intermembrane space, Smac/DIABLO, enhances caspase activation. While cytochrome *c* and Smac induce apoptotic cell death and loss of cell membrane integrity within 4–24 hours, if their action is blocked, the damage to mitochondria still results in a slower, necrotic death. In apoptosis initiated by death receptor signaling (Fig. 1, death receptor pathway), mitochondrial damage is not required in most cells (type I cells) as caspase-8 can directly activate downstream caspases. However, in certain cells (type II cells), recruitment of the mitochondrial pathway by caspase-8 cleaving and activating Bid to its truncated form (tBid) is required for cell death [1,2].

The mitochondrial pathway of apoptosis is regulated principally by the Bcl-2 protein family, whose members fall into three subclasses: the pro-apoptotic BH3-only proteins; the prosurvival Bcl-2-like proteins; and the pore-forming Bax and Bak proteins (Figs. 1 and 2) [3]. Four Bcl-2 homology domains (BH1–BH4) characterize this family of proteins (Fig. 2). Note that the BH4 domain as defined in this review refers to a recently described structural motif ($\phi_1\phi_2XX\phi_3\phi_4$, where X is any amino acid, ϕ is a hydrophobic residue, and ϕ_3 is an aromatic residue) present in a wide range of Bcl-2 proteins [4], rather than to the BH4 domain originally reported only in Bcl-2, Bcl-x_L, and Bcl-w [5]. The prosurvival proteins (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1) each contain four BH domains [4] and are "prosurvival" due to their ability to bind and sequester their pro-apoptotic relatives. The BH3-only proteins (Bid, Bim, Puma, Noxa, Bad, Bmf, Hrk, and Bik) are pro-apoptotic and act as sensors of specific types of cellular stress [6,7]. Bax, Bak, and perhaps Bok, which also contain four BH domains [4], are the critical effectors of apoptosis acting downstream of both the prosurvival and BH3-only members to permeabilize the mitochondrial outer membrane (OM) [8,9]. Additional Bcl-2 family members have been described recently with potential pro-apoptotic (Bcl-G, Bcl-Rambo) or prosurvival (Bcl-B) capacity. Defining their precise roles in regulating apoptosis is the subject of significant interest [73].

Death or survival is determined by the levels of pro-apoptotic and prosurvival proteins in each cell, as well as by binding between the triad of Bcl-2 subfamilies. Binding in most cases involves the BH3 domain being sequestered in the hydrophobic surface groove on another family member (Fig. 3C) [10–12]. In addition, sequence differences confer specific binding [13,14]. For example, the BH3-only proteins Bid, Bim, and Puma bind strongly to all prosurvival proteins, while others exhibit specificity for particular prosurvival homologues. Binding of activated Bax and Bak by prosurvival proteins also involves a BH3:groove interaction [10]. Thus, a BH3:groove interaction is involved when prosurvival proteins block apoptosis upstream by sequestering BH3-only proteins or when they block apoptosis downstream by sequestering activated Bax and Bak.

Abbreviations: BH, Bcl-2 homology; OM, outer membrane; tBid, truncated Bid; TM, transmembrane

☆ This article is part of a Special Issue entitled Mitochondria: the deadly organelle.

* Corresponding author. Tel.: +61 3 9345 2487; fax: +61 3 9347 0852.
E-mail address: kluck@wehi.edu.au (R.M. Kluck).

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doi:10.1016/j.bbamcr.2010.12.019

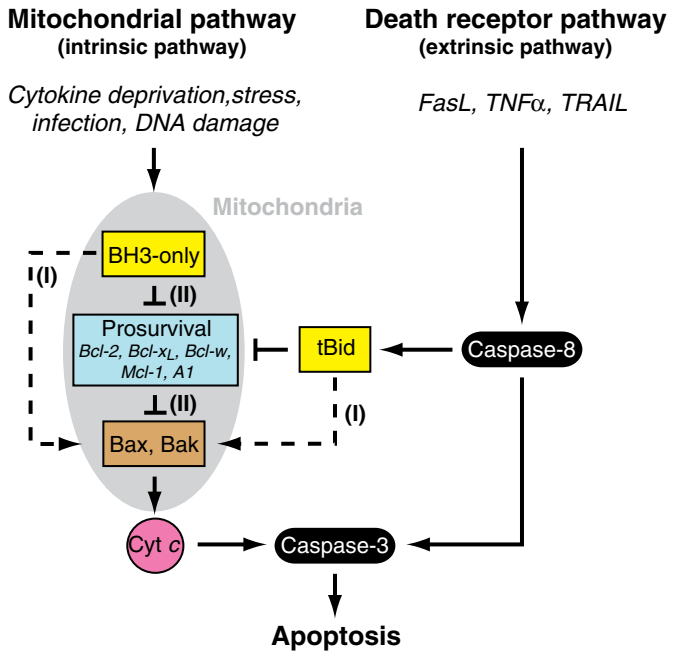

Fig. 1. Multiple pathways to apoptosis. The *mitochondrial* (or intrinsic) pathway is induced as a response to cellular stress and results in the activation of the pro-apoptotic BH3-only proteins. BH3-only proteins may *directly* bind and activate Bax and Bak (I, dashed lines), and may also bind to the prosurvival Bcl-2-like proteins to *indirectly* activate Bax and Bak (II). Once activated, Bax and Bak oligomerize to form pores in the mitochondrial outer membrane that release cytochrome *c*. Cytosolic cytochrome *c* leads to caspase activation and subsequent cell death. The *death receptor* (extrinsic) pathway is initiated by death ligands such as FasL, tumor-necrosis factor α (TNFα), or TNF-related apoptosis inducing ligand (TRAIL) binding to cell surface receptors, resulting in the activation of caspase-8. Active caspase-8 can either activate downstream caspases directly (in type I cells) or engage the intrinsic pathway via a cleaved form of the BH3-only protein Bid (tBid) (in type II cells).

The final interaction between the triad of Bcl-2-type proteins, between BH3-only proteins and Bax or Bak, appears to be transient and thus more difficult to validate. However, its importance may be paramount as this may be a major mechanism for triggering Bax and Bak conversion to their activated and oligomerized forms. The binding site on Bax and Bak may be the canonical hydrophobic surface groove [15–17], arguing for a BH3:groove interaction being involved in all interactions between Bcl-2 family members [18]. However, other groups have proposed the N-terminus of Bax as a binding site [19,20]. In particular, a transient binding site for a stapled Bim BH3 peptide has been mapped to an α1/α6 "rear" pocket of Bax, from which a model for the Bax:BimBH3 complex has been proposed [20]. The transient nature of these interactions may be due to a decrease in affinity once Bax and Bak convert to the activated conformations.

Based on the binding between different Bcl-2 members, two models of Bax and Bak activation, the *direct* and *indirect* models, have been proposed [73]. Briefly, in the *direct* model, BH3-only proteins such as Bid, Bim, and Puma (called "activators") bind directly to Bax and Bak (Fig. 1, step I). In the *indirect* model, Bax and Bak become activated after being displaced from prosurvival proteins by BH3-only proteins (Fig. 1, step II). Alternatively, aspects of both the direct and indirect models may be important [18,21,22].

As alluded to, the point of no return in mitochondrial apoptosis occurs when Bax or Bak generate the "apoptotic pore" in the mitochondrial OM. While it is known that pore formation involves major conformation changes in Bax and Bak, followed by homo-oligomerization, there are as yet no structures of Bax or Bak in their activated or oligomerized forms. Thus, a major goal in the apoptosis field is to identify the step-wise activation of Bax and Bak to form a pore in the mitochondrial OM—a pore that converts mitochondria into a "deadly organelle." Here, we review recent progress towards this goal, including our recent findings and those of others, that Bax and Bak oligomerization involves two distinct interfaces, a BH3:groove interface to form symmetric dimers and an α6:α6 interface that can link dimers to higher order oligomers. Eventual atomic resolution of the oligomeric interfaces may assist the current design of therapeutics that remove cancer cells, or conversely, rescue normal cells [23,24].

2. Bax and Bak in healthy cells and Bax translocation to mitochondria

In healthy cells, Bak is inserted in the mitochondrial OM, whereas Bax is predominantly cytosolic with a minor population loosely attached to the OM [25–27]. A small portion of Bax and Bak can also locate to the endoplasmic reticulum [28,29], although the physiological role of this population is unclear [30]. During apoptosis, Bax translocates from the cytosol to insert into the OM, and both Bax and Bak convert from the non-activated to the activated conformation (see section 3).

2.1. Molecular structures of non-activated Bax and Bak

The structures of non-activated Bax and Bak [17,31,32] exhibit the same fold as Mcl-1 and other prosurvival proteins (Fig. 3A–C). Essentially, both Bax and Bak are globular proteins comprising 9 helices. The hydrophobic α5 helix is protected within a bundle of 7 amphipathic helices. The remaining α9 at the C-terminus acts as a transmembrane domain that can anchor Bax and Bak in the mitochondrial OM. In the Bax structure, α9 is sequestered in its own hydrophobic groove, explaining why Bax is predominantly cytosolic (Fig. 3B, D). In the Bak structures [31,32], α9 is absent and the groove is narrow and occluded by side chains that potentially restrict docking of α9 (Fig. 3A). However, as other members of the Bcl-2 family demonstrate dynamic structural plasticity within the groove region [33], this may also apply to Bak.

In the structures, two other functionally important hydrophobic regions are also buried prior to activation. In α1, the newly described BH4 sequence motif contains hydrophobic and aromatic residues that make several contacts with α2, α5, and α6, apparently stabilizing the tertiary structure (Figs. 2 and 3E). In α2, four hydrophobic residues in the BH3 domain that are important for binding between Bcl-2 family members face the hydrophobic core (not shown).

2.2. Triggering mitochondrial Bax translocation and activation

Clearly, Bax translocation from the cytosol to the mitochondrial outer membrane is an important feature of apoptosis biology [34]. However, a consensus on the mechanism(s) involved has not been reached for perhaps three reasons. First, the different experimental approaches used may generate different outcomes. Ideally, Bax function should be analyzed in cells stably expressing full-length Bax at normal levels, with translocation initiated by a physiological apoptotic stimulus. However, due to obvious experimental limitations of this approach, many studies have utilized cell-free approaches and truncated or overexpressed Bax. In these circumstances, at least a portion of Bax may adopt conformations capable of non-Bax-like function or partial Bax function. For example, if the Bax BH3 domain was exposed but the hydrophobic groove destroyed, this Bax may act like a BH3-only protein and heterodimerize with prosurvival proteins or directly activate other Bax and Bak molecules [35]. Finally, the function of Bax and Bak variants should be tested in cells lacking endogenous Bax and Bak to exclude interference by those proteins.

Second, at least two populations of Bax co-localize with mitochondria, with peripherally attached and membrane-inserted forms

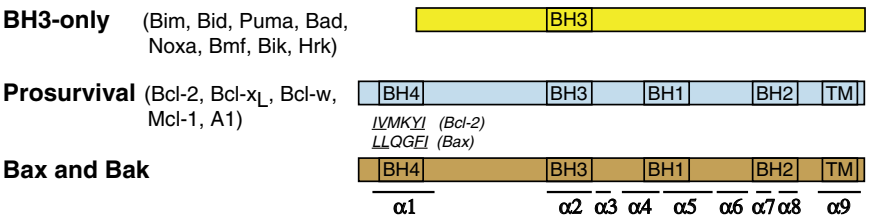

Fig. 2. The Bcl-2 protein family. Mammalian Bcl-2 proteins can be divided into three subfamilies, the prosurvival proteins, the pro-apoptotic BH3-only proteins, and the pro-apoptotic Bax and Bak proteins. The presence of four Bcl-2 homology (BH) domains is as indicated. Note that the BH4 domain refers to a recently described structural motif ($\phi_1\phi_2XX\phi_3\phi_4$, where X is any amino acid, ϕ is a hydrophobic residue, and ϕ_3 is an aromatic residue) that is present in each prosurvival protein and in Bax and Bak [4]. As examples, the BH4 sequences in human Bcl-2 and human Bax are shown. Many members also contain a C-terminal transmembrane (TM) domain. While most BH3-only proteins are unstructured, the prosurvival proteins as well as Bax and Bak each adopt an α-helical structure (Bax α-helices are indicated).

characterized by carbonate extraction [27]. Distinct membrane-inserted forms may also exist (see below). Unless carbonate extraction experiments are performed, it is difficult to discern events that *translocate/address/target/localize* Bax to the membrane from events that *insert* Bax into the membrane.

The third reason for the lack of consensus on Bax translocation is that it often occurs as part of activation and a complex set of conformation changes. Indeed, distinct translocation mechanisms may exist as a range of biochemical stimuli can trigger activation of Bax (and Bak). Aside from direct activation by BH3-only proteins such as Bim and Bid (or the related BH3 peptides) [14,19,36,37], other triggers include chemical stimuli such as H_2O_2, as well as low and high pH, mild heat, proteolytic cleavage, and post-translational modification such as phosphorylation [38–43]. In the next section, we attempt to address the most significant findings on Bax and Bak conformation changes during activation.

3. Conformation changes of Bax and Bak during activation

A number of biochemical studies have demonstrated that several regions of Bax and Bak undergo conformation rearrangements during apoptosis. These include the N-terminus, BH3 domain, and hydrophobic groove. In Bax, α-helices 5, 6, and 9 also change conformation. We are just starting to understand whether each conformation change is necessary for pro-apoptotic function and in which order these changes might occur. In this section, we discuss each change and then consider whether Bax and Bak activation might be initiated from the N-terminus, the C-terminus, or both.

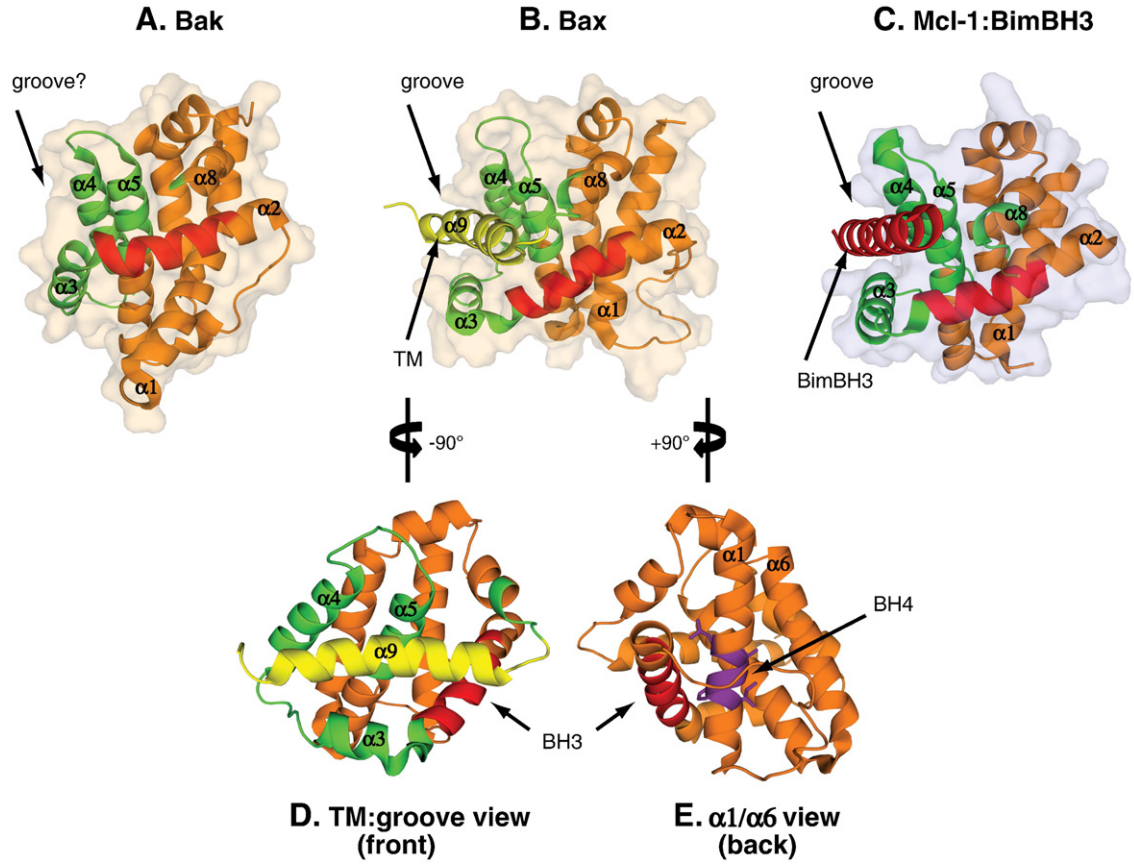

Fig. 3. Structures of Bax and Bak. (A, B, C) Cartoon and surface overlays of Bak (2IMT), Bax (1F16; the flexible N-terminal 12 residues have been omitted for clarity), and Mcl-1 complexed with a Bim peptide (2NL9). α-Helices 1–8 are in orange except for those regions contributing to the hydrophobic groove (green) and BH3 domain (red). The Bak groove appears closed. The Bax groove harbors α9 (yellow) which acts as a transmembrane (TM) domain, while the Mcl-1 groove harbors the Bim BH3 peptide (red). (D, E) Rotation of the Bax structure to view the TM:groove interaction and the α1/α6-helices on opposite sides of the molecule. Color coding is as in (B), with the newly defined BH4 motif and side chains (LLQGFI) in α1 also indicated (purple).

3.1. The C-terminus: function and conformation change

As for most Bcl-2 members, Bax and Bak contain a C-terminal hydrophobic region (α9) that can act as a transmembrane domain within the OM (reviewed in references 16 and 34). This α9 helix is sequestered in the hydrophobic groove in cytosolic Bax (Fig. 3B, D) [17] and becomes exposed in order to facilitate translocation and insertion during apoptosis. Mutations at the C-terminus support the importance of this region for Bax translocation. For example, replacement of serine at position 184 in α9 with valine targets Bax to mitochondria [44]. In addition, mutation of proline at position 168 in the linker region prior to α9 decreases Bax translocation and activity, arguing that this linker region is important for α9 exposure and membrane insertion [45,46].

Additional testing of whether the Bax C-terminus acts as an addressing and/or insertion motif has involved two approaches, 1) fusing the Bax C-terminus to a GFP molecule and testing whether it targets to mitochondria and 2) truncating this region and testing whether Bax no longer targets to mitochondria. However, both approaches gave contrasting findings presumably due to different experimental conditions as discussed above. For example, the C-terminal 21 or 24 residues of Bax failed to target GFP to mitochondria in some studies [44,47], but 23, 24, or 27 residues were able to do so in others [45], including in *bax*$^{-/-}$ *bak*$^{-/-}$ cells [46,48]. In the other set of experiments, after C-terminal truncation, Bax still localized to mitochondria in some studies[47,49,50] but remained cytosolic in others, even after apoptotic signaling [27,44,45]. A more recent detailed study found that Bax mutants that were unable to target mitochondria in *bax*$^{-/-}$ *bak*$^{-/-}$ cells could do so in the presence of endogenous Bax [46], suggesting that endogenous Bax and Bak may have become activated and affected the localization of the truncated proteins. Another recent study found that the Bax C-terminus was essential for mediating Bax OM insertion but was not the only addressing signal [48].

In Bak, the C-terminus is membrane-inserted prior to apoptosis, perhaps because this hydrophobic region inserts more readily into the OM than into the Bak groove. The Bak C-terminus can target GFP to mitochondria [51] and is essential for Bak insertion into the OM as its truncation abrogated Bak mitochondrial localization and function after stable expression in *bax*$^{-/-}$ *bak*$^{-/-}$ cells [52].

3.2. The N-terminus: function and conformation change

The N-termini in Bax and Bak contain two regions, the N-segment and the α1 helix. The N-segment in Bax (residues 1–15) is flexible and exposed to solvent [17], while that in Bak (residues 1–23) is partially structured according to antibody and crosslinking studies [52]. The N-segment in Bak seems to play no role in stability or function as its truncation did not affect these characteristics [52]. However, Bak stability and function was greatly decreased by removing most of α1 [52]. The stability of Bax after N-terminal truncation has not been examined; however, truncation of the first 20 residues (the ART domain) increased mitochondrial-targeting of *in vitro* translated Bax [53], and a natural Bax variant (Bax ψ) lacking the first 19 residues localized mainly to mitochondria [54]. Indeed, Vallette et al. [47] found that removing the ART domain exposes a mitochondrial-targeting sequence within residues 20–37 as this region fused to GFP targets to mitochondria. However, a longer Bax sequence (residues 1–50) failed to target YFP to mitochondria [46], and at least in some systems, Bax can translocate without detectable N-terminal epitope exposure [44,55]. A possible explanation for these findings is that removing the BH4 domain in Bax α1 destabilizes the protein (as shown for Bak) to expose regions that target mitochondria.

During apoptosis, the N-terminus in both Bax and Bak undergoes conformation change, although the extent of this change is not clear. For example, α1 may reposition entirely, rotate slightly, or become less helical. Perhaps, the clearest data are based on the 6A7 antibody whose epitope has been carefully mapped to Bax residues 13–19 at the start of α1 [56]. This and other antibodies to α1 are conformation-specific as they only recognize Bax after an apoptotic stimulus [57]. In Bak, antibodies to the N-segment are also conformation-specific [25,52,58].

3.3. The BH3 domain and hydrophobic groove: function and conformation change

A key step in Bax and Bak activation is exposure of the BH3 domain as this domain is critical for oligomerization and pro-apoptotic function [59,60] (see section 4). As discussed above, prior to apoptosis, the hydrophobic BH3 residues face the core of Bax and of Bak [17,31]. Following apoptotic signaling, the Bak BH3 domain is exposed transiently before becoming re-buried in Bak homo-oligomers [60]. For example, antibody to the Bak BH3 domain did not recognize Bak before or after mitochondria were treated with tBid but did so if added during tBid treatment [60]. Very recent studies provide evidence that the Bax BH3 domain is also exposed during activation [61,62]. Whether the Bax BH3 domain then binds to the hydrophobic groove in another activated Bax molecule has not been demonstrated.

3.4. The Bax α-helices 5 and 6

Structural similarity of Bcl-2 proteins to pore-forming proteins such as diphtheria toxin suggested that Bax and Bak form pores by inserting α-helices 5 and 6 into the OM [63]. In support of this hypothesis, these two helices in Bax become membrane-inserted following apoptotic signaling in *myc*$^{-/-}$ cells [64]. Notably, insertion was an early event as the 6A7 epitope was not exposed, and oligomerization had not occurred. Thus, a conformation change that essentially turns the Bax and Bak proteins inside-out may be involved in their activation and be required for self-association and pore formation.

3.5. Possible sequence of conformation changes

As outlined, a number of conformation changes are associated with Bax and Bak activation, although it remains unclear in which order these changes occur. Based on the extensive molecular contacts made between α-helices 1, 2, 5, 6, and 9, changes in conformation of any of these regions could theoretically trigger changes in other regions. For example, as α2 containing the BH3 domain is juxtaposed with both terminal helices (α1 and α9), movement of either terminus may trigger BH3 exposure. Indeed, different sites may be targeted by different triggers of Bax and Bak conformation change (e.g. Bim, heat, oxidants, phosphorylation) and consequently involve distinct sequences of conformation change. As refolding, once triggered, will be driven towards the lowest free energy state, it is likely that the final conformation in each case is equivalent.

In terms of activation by BH3-only proteins, two binding sites on Bax have been proposed, the hydrophobic groove and the α1/α6 rear pocket on the other side of the molecule (Fig. 3D, E). The Bax hydrophobic groove is a potential binding site provided the C-terminus can be displaced [15–18]. Thus, a hypothetical sequence of changes in Bax starting at the C-terminus (Fig. 4, dashed arrows) would be Bim or Bid displacing the C-terminus (α9) and binding to the groove, followed by exposure of the Bax BH3 domain (in α2). Once the BH3 domain is exposed, conformation change of the groove seems likely as α2 is adjacent to, or even forms part of, the groove in the non-activated structures (Fig. 3). Modification of the groove may dislodge Bim or Bid, accounting for the transient nature

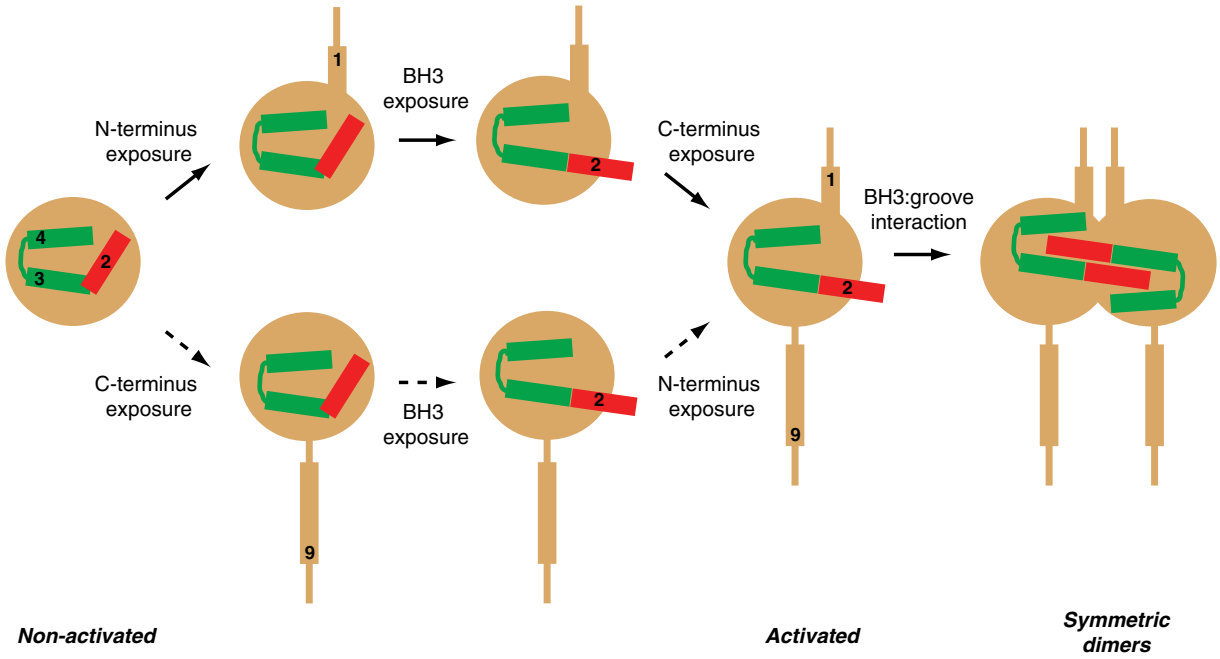

Fig. 4. Two proposed sequences of Bax conformation change during apoptosis. Schematic of the sequence of Bax conformation changes initiated by Bim binding at the N-terminus, as recently reported [61] (solid arrows). A different sequence may occur if Bim binds to the hydrophobic groove after displacement of the C-terminus (dashed arrows). Both sets of conformation change may generate an activated monomer with an exposed BH3 domain that can then form symmetric BH3:groove dimers. The non-activated form of Bax is represented as a globular molecule (orange) containing a hydrophobic surface groove (α3/α4; green) and a buried BH3 domain (α2; red). Two other regions, the N-terminal α1 helix and the C-terminal α9 helix, are also initially buried but become exposed either as early or late events in the two models. See text for a detailed description of the possible binding and conformation changes involved. Note that exposure of the α5/α6 hairpin is not included in these models. Bak is likely to undergo similar conformation changes.

of this binding. In addition, BH3 exposure and groove conformation change may occur roughly simultaneously, allowing the BH3 in one molecule to bind to a modified groove in another molecule [18] (see section 4). Finally, changes to the BH3 and groove may alter the N-terminus.

Binding to the α1/α6 rear pocket of Bax would result in roughly the reverse sequence of conformation changes (Fig. 4, solid arrows). A stapled Bim BH3 peptide first exposes the N-terminal epitope (6A7 epitope in α1) and the BH3 domain (α2), followed by exposure of α9 [20,61]. Lalier et al. [65] proposed a similar but distinct sequence of Bax conformation changes initiated at the N-terminus. Clearly, further studies are required to test these hypotheses, including how exposure of the α5/α6 hairpin and its insertion into the OM may relate to the two models presented.

4. Homo-oligomerization: two distinct interfaces

After Bax and Bak become activated, the newly exposed regions allow new interactions and thus oligomerization. Membrane insertion itself may also drive conformation changes [66]. Anchorage of activated Bax and Bak in the OM may assist oligomerization by concentrating the proteins and by aligning the protein regions that are to form interfaces. The formation of Bax and Bak homooligomers during apoptosis has been observed for some time [67,68] and more recently shown to be necessary for pore formation [59,60].

An early speculative model of Bax/Bak oligomerization involved a single interface, in which the front of one molecule interacts with the back of the next molecule to form a "daisy-chain" [69]. A single interface was also proposed recently in the "asymmetric autoactivation" model where the exposed Bax BH3 domain binds to the α1/α6 rear pocket [22]. However, our recent biochemical studies with Bak support a "two interface" model involving the BH3:groove and α6:α6 interfaces (Fig. 5) [52,60]. Very recent *in vitro* experiments also support the concept of two distinct interfaces in oligomers of Bax as well as Bak [62,70,71].

4.1. A BH3:groove interface forms symmetric Bak dimers

As discussed above, following Bak activation in mitochondria or in cells, the exposed BH3 domain binds to the hydrophobic groove of another activated Bak molecule [52,60]. This interaction was identified by different means, including cysteine linkage between the BH3 domain and groove only after apoptotic signaling. This linkage could be attributed to functional oligomeric Bak rather than to a minor non-functional population of oligomers as one pair of cysteine residues (in the BH3 domain and in the groove) essentially linked all activated Bak as BH3:groove dimers [60].

As the groove in non-activated Bak is occluded (Fig. 3A) [31], it must convert to a more open conformation to allow a BH3:groove interaction. As discussed above, the BH3 domain and groove are adjacent in the molecule, suggesting that BH3 exposure and groove opening occur simultaneously during Bak conformation change, thereby coordinating exposure of the BH3 domain with its binding to the groove of the partner Bak molecule.

Notably, BH3:groove interactions between two activated Bak molecules are reciprocal (Figs. 4 and 5) as Bak containing cysteine in both the BH3 domain and the groove can be linked as dimers but not as higher order oligomers [52]. Reciprocal binding is feasible from a structural point of view as an exposed BH3 domain and the groove would be on the same side of activated Bak (Fig. 3D). Reciprocal BH3:groove interactions would strengthen the interaction by increasing the buried surface area between the two proteins. Symmetric Bak dimers are distinct from the domain swap dimers reported for several Bcl-2 homologs [4,72,73] as the exposed BH3 domain does not return to a similar position in its Bak partner.

4.2. An α6:α6 interface can link symmetric Bak dimers to higher order oligomers

The formation of symmetric (BH3:groove) dimers specifies that a second interface is needed to form higher order oligomers of Bak (and

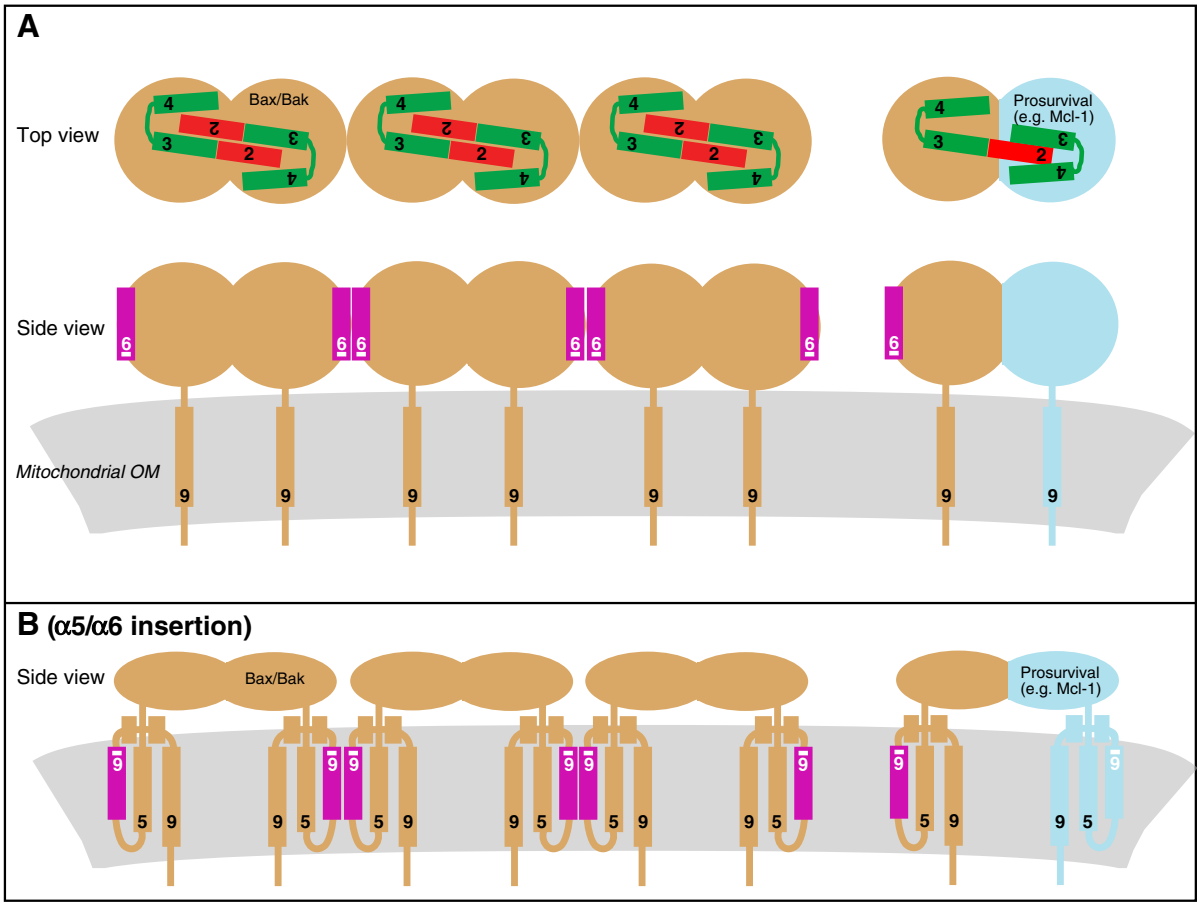

Fig. 5. Models of Bax and Bak oligomerization. (A) During apoptosis, Bax and Bak (orange) become activated and form BH3:groove dimers, possibly with the BH3 domain (α2; red) and groove (α3/α4; green) positioned as indicated looking down on the mitochondrial surface (top view). Reciprocal BH3:groove interactions generate symmetric dimers (left), which can then be linked via an α6:α6 interface (magenta; side view) to form the higher order oligomers thought to form the apoptotic pore. Prosurvival proteins (e.g. Mcl-1) may sequester activated Bax and Bak via a BH3:groove interaction (right) to prevent homo-oligomerization and pore formation. (B) Insertion of the α5/α6 hairpin, as shown for Bax [64], may occur prior to formation of a BH3:groove interface outside the membrane and formation of an α6:α6 interface within the membrane.

of Bax). Accordingly, we found that Bak dimers could be linked via an α6:α6 interface to form higher order oligomers (Fig. 5) [52]. The α6:α6 interface forms after the BH3:groove interface as antibody to the Bak BH3 domain blocked α6:α6 interaction. As α6 is on the opposite side of the molecule from the BH3 domain and groove (Fig. 3D, E), both the BH3:groove and α6:α6 interfaces might form on the mitochondrial surface without major rearrangement of the α3-α6 region (Fig. 5A). However, if the Bak α5/α6 hairpin becomes membrane-inserted, as shown for Bax [64], an α6:α6 interface may form within the OM (Fig. 5B).

Bak oligomers (>18 mer) were observed in our linkage studies where disulphide bonds linked both the BH3:groove and the α6:α6 interfaces [52]. Oligomers of similar size are evident in apoptotic cell lysates examined by both size exclusion chromatography and Blue Native PAGE (see section 5). Our evidence that an α6:α6 interface in Bak forms only after apoptosis is contrary to evidence of a Zn-mediated α6:α6 interface in non-activated Bak that blocks activation [31], possibly because the latter studies were based on Bak lacking the N- and C-termini.

Compared to the BH3:groove interface, the α6:α6 interface is less well defined and not yet shown to be needed for Bax and Bak function. For instance, while mutations in the BH3 domain or groove blocked cytochrome *c* release, mutations in α6 of Bax or Bak have not blocked cytochrome *c* release [52,59]. Moreover, there are no significant hydrophobic surfaces in the α6 region, and three charged residues in α6 would generate significant electrostatic repulsion, especially if the helices were strictly parallel, as discussed [22]. One possibility is that the α6-helices adopt a criss-cross orientation, which may still allow cysteines placed along 3 turns of α6 to link to themselves [52], especially if dimers could rotate slightly. These possibilities require further examination, including whether the α6:α6 interaction is driven by forces outside this region, perhaps by the membrane itself. Alternatively, if the α6:α6 interface occurs within the OM (Fig. 5B), hydrophilic residues along the α6 surface may drive the interface due to the hydrophobic environment of the lipid bilayer.

Very recent analysis of Bax and Bak oligomers formed in non-mitochondrial assays support the “two interface” model of oligomerization [62,70,71]. Two reports utilized electron paramagnetic resonance (EPR) spectroscopy of spin-labeled Bax and Bak to measure residue positions in oligomers that form in liposomes [70,71]. The distances measured between specific BH3 residues were consistent with symmetric BH3:groove dimers as modeled in Fig. 5A. A third report based on photocross-linkage of specific residues found that detergent-induced (Triton X-100) Bax oligomers contained one interface involving the BH3 domain and a second interface involving the other side of the molecule [62]. Together, these studies indicate that Bax and Bak homo-oligomerize via equivalent mechanisms.

4.3. Implications of an exposed BH3 domain and a functioning groove

With their BH3 domain exposed, activated Bax and Bak resemble BH3-only proteins in that the BH3 domain can bind to the groove of prosurvival proteins [74,75]. Indeed, binding of activated Bax and Bak by prosurvival proteins is proposed to be a major mechanism used by

prosurvival proteins to inhibit apoptosis [74,75]. Although there are no structures of full-length Bax and Bak bound to prosurvival proteins, the similar size and structure of Bax and Bak and prosurvival proteins suggests that heterodimers may partly resemble Bax and Bak homodimers (Fig. 5A). Moreover, according to the "embedded together" model, when Bax binds Bcl-2, both proteins have the α5/α6 hairpin inserted in the OM (Fig. 5B) [22,76]. Heterodimers must be unable to multimerize further, as prosurvival proteins block homo-oligomerization of Bax and Bak [62,77]. (As noted in section 1, prosurvival proteins can also block Bax homo-oligomerization indirectly, i.e. by blocking its translocation to mitochondria [78], presumably by sequestering activator BH3-only proteins.) In heterodimers, the BH3:groove interaction may not be symmetric (Fig. 5A) as the structures of Bcl-x_L and A1 bound to Bak BH3 peptides do not exhibit exposed BH3 domains [10,79].

In some aspects, Bax and Bak also resemble prosurvival proteins as the groove in activated Bak is able to bind the BH3 domain of another activated Bak molecule. As noted in section 3.5, it is also possible that the groove in non-activated Bax and Bak is a binding site for activator BH3-only proteins such as Bid and Bim [15–18,30]. It will be interesting to determine exactly how the groove changes upon activation and whether it might convert from a groove with strong affinity for Bid and Bim to one with strong affinity for Bak and Bax BH3 domains.

Further studies are needed to clarify how Bax and Bak interact with themselves, with other members of the Bcl-2 family, and with the OM. These studies may continue to be difficult due to the inherent difficulties of studying membrane proteins and the major conformation changes that occur in Bax and Bak and perhaps also in prosurvival proteins. Defining how and when interfaces allow activated Bax and Bak to oligomerize will bring us closer to understanding the pore complex and how pore formation might be blocked to prevent apoptosis.

5. Porating the mitochondrial outer membrane

How oligomerized Bax and Bak allow passage of large proteins across the OM is an ongoing question. An early hypothesis was that the OM tears as a result of matrix swelling, which was caused by Bcl-2 proteins targeting the "permeability transition" pore, a pore already pre-formed in mitochondria [80]. However, genetic studies have since indicated that while permeability transition may account for cell death induced by signals such as ionized calcium, it is not the mechanism used by the Bcl-2 family to permeabilize the OM [81,82] (see Kinnally et al. in this issue).

A range of biochemical and biophysical studies then addressed whether Bcl-2 family members had direct effects on membranes and were capable of forming channels themselves. These studies often used planar lipid bilayers (to measure ion channels) or liposomes (to measure protein channels). Studies also examined whether Bax and Bak pierce membranes as do bacterial pore-forming toxins (e.g. the colicins and diphtheria toxin) or pore-forming peptides (e.g. alamethicin, melittin, and magainin).

5.1. Do Bax and Bak functionally mimic bacterial pore-forming toxins?

A clue to the mechanism by which Bax and Bak may form pores arose from the first structure of a Bcl-2 protein, that of Bcl-x_L. The α-helical fold of Bcl-x_L resembled that of the colicins and of the pore-forming domain of diphtheria toxin, with core hydrophobic helices cloaked by amphipathic helices [63]. On contacting membranes, the two central helices of the pore-forming domains of colicin and diphtheria toxin are thought to insert as an α-helical hairpin in membranes [83]. This insertion may make the membrane leaky to ions, thereby causing membrane depolarization and death of the target cell, or may facilitate passage of another protein or domain across the membrane [84]. As an example of the latter, pore formation by diphtheria toxin allows passage of the catalytic domain that then blocks protein synthesis in the target cell [85].

Like pore-forming toxins, Bax can undergo significant conformation change on association with the mitochondrial OM (see above), including insertion of the α5/α6 hairpin into membranes [64]. As discussed above, the Bcl-2 α5/α6 hairpin is also proposed to insert into the OM [86]. However, there are important differences between the central helices of the pore-forming proteins and those of the Bcl-2 proteins. Firstly, while the central helices of colicin and of the diphtheria toxin pore-forming domain are hydrophobic, those in Bax, Bak, and Bcl-2 each contain 2 or more charged residues. Secondly, whereas α5 in Bax is potentially long enough to span a lipid bilayer as an α-helix, the central helices of colicin are not. Thus, these structural differences between pore-forming toxins and the Bcl-2 proteins imply distinct mechanisms of pore formation.

5.2. Evidence that Bcl-2 proteins can form ion channels

Bax, Bcl-x_L, and Bcl-2 can each form ion channels in planar lipid bilayers [87–90]. The channels are ion-selective, pH-sensitive, voltage-insensitive, and of variable conductance. Initial studies indicated that channels formed by Bcl-2 and Bcl-x_L were cation-specific whereas those formed by Bax were more anion-specific [87], supporting a model whereby prosurvival and pro-apoptotic Bcl-2 proteins regulate mitochondrial ionic balance. An increase in "Bax-like" channel activity during apoptosis could potentially lead to a disruption of this balance with mitochondrial swelling and rupture of the mitochondrial OM, akin to the opening of the permeability transition pore. However, given the abundance and high conductance of the VDAC channels in the mitochondrial OM, the limited conductance of the Bcl-2 proteins is unlikely to significantly affect ionic balance across that membrane. Nevertheless, the ability of Bax to form ion channels *in vitro* may in some way reflect its pro-apoptotic function in cells, as Bcl-2 blocks both processes [88].

5.3. Evidence that Bax and Bak can form protein channels

It is possible that Bax ion channels in the OM play a minor role in apoptosis and that Bax (and presumably also Bak) forms large protein channels in the OM that act as a direct conduit for cytochrome *c*. A channel comprising oligomers of Bax, termed the mitochondrial apoptosis-induced channel (MAC), was detected by patch clamp analysis of mitochondria isolated from cells undergoing apoptosis [91]. In those studies, MAC was voltage-insensitive and so unlikely to involve VDAC. Its cation selectivity and estimated pore diameter of 4 nm was consistent with passage of the positively charged cytochrome *c*. Indeed, addition of cytochrome *c* reduced the conductance of the MAC, supporting the pore as a direct translocator of cytochrome *c* rather than a mere facilitator. Notably, either Bax or Bak was required for the MAC to form. In addition, MAC-like activity was detected in yeast mitochondria expressing Bax [92], suggesting that Bax was able to form the pore in the absence of other Bcl-2 proteins, as reported for Bax in liposomes [93].

The size of the apoptotic pore has been examined using several model systems. A tetramer was the minimal Bax oligomer to translocate cytochrome *c* across liposomes [94]. The MAC pore formed by Bax has been further characterized to comprise 9 molecules with a pore diameter of 6 nm [95]. Much larger Bax and Bak oligomers (>100 molecules) were reported in dying cells [96,97]. At least 18 molecules were observed in Bak oligomers where disulphide bonds linked both the BH3:groove and the α6:α6 interfaces [52], and in Bax oligomers analyzed by Blue Native PAGE [98]. Such large oligomers are consistent with activated Bax forming supramolecular pores in liposomes [93] and explain the coordinated release of both small and large proteins from mitochondria. Thus, while several approaches

detect large Bax (and Bak) oligomers in apoptotic cells, the size of the functional pore and whether pores can adopt several sizes remain unclear.

A peptide corresponding to the Bax α5/α6 hairpin could form pores in liposomes, although larger pores were formed by full-length Bax [99]. This raises the possibility that Bax permeabilizes the OM in a step-wise fashion, where α5/α6 insertion forms ion channels and subsequent oligomerization forms protein pores. Consistent with this, Andrews et al. [64] found that Bax inserted the α5/α6 hairpin prior to oligomerization and that activated Bax caused limited membrane permeability that increased over time to allow passage of high molecular weight proteins [100].

5.4. Proteinaceous or lipidic pore?

Biophysical studies suggest two predominant mechanisms by which α-helical proteins or peptides form pores: proteinaceous (barrel-stave) and lipidic (toroidal) pores (reviewed in reference 101). Here, we briefly describe the two pore types as formed by α-helical pore-forming peptides (e.g. alamethicin, melittin, and magainin) and compare them to pores formed by Bax and Bak.

5.4.1. Proteinaceous (barrel-stave) pores

The prototypical proteinaceous pore is based on the pores generated by the alamethicin 21 residue peptide (produced by the fungus *Trichoderma viride*). The helices align closely in the bilayer so that protein forms the solvent-exposed surface of the pore (Fig. 6A). Proteinaceous pores generally exhibit single conductance and due to their inherent instability are limited to less than 10 peptides with a pore diameter of approximately 2 nm. Alamethicin pores, for example, comprise 5–10 peptides with a pore diameter of 1.8 nm [102], which is too small to traffic proteins released with cytochrome *c* during apoptosis. However, it remains possible that full-length proteins may form stable oligomeric complexes and thus larger pores. It is interesting to note that a dodecameric barrel-stave complex is evident in the crystal structure of the cytolysin A pore, currently the only structure of a membrane-inserted α-helical pore-forming toxin [103].

5.4.2. Lipidic (toroidal) pores

These pores are formed by melittin (from bee venom) and magainin (from *Xenopus laevis* skin) [104]. Prior to pore formation, the amphipathic peptides lie on the membrane surface and once at a certain concentration increase surface tension to invaginate the membrane so that the outer leaflet of the membrane is continuous with the inner leaflet. Thus, the solvent-exposed surface of the pore is lined by lipid headgroups as well as by protein (Fig. 6B). Lipidic pores are stable due to intercalated lipid reducing the impact of charge–charge repulsion between adjacent peptides. As a consequence, lipidic pores can be larger than barrel-stave pores and can increase in diameter [101]. A defining characteristic of lipidic pores is their promotion by positive membrane curvature, such as that induced by non-lamellar lipids [105]. In addition, membrane invagination accommodates shorter helices lining the pore. Accordingly, colicin may form a lipidic rather than a proteinaceous pore as its channel activity is enhanced by positive membrane curvature [106] and its central helices are relatively short.

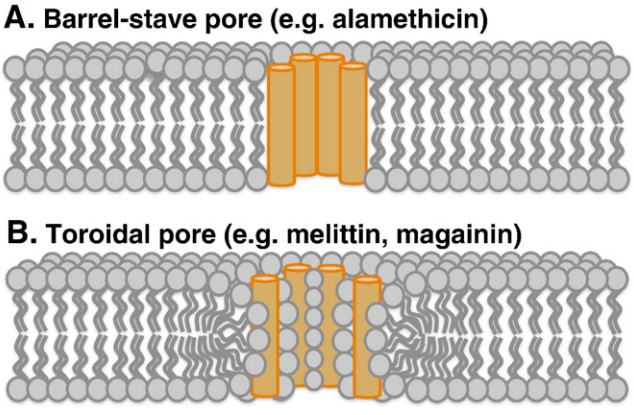

Fig. 6. Two mechanisms by which Bax and Bak oligomers might porate the mitochondrial outer membrane. (A) In the barrel-stave model, α-helical peptide "staves" align to form a barrel-like pore that spans the membrane. Peptides are adjacent to the lipid acyl chains. (B) In the toroidal pore model, α-helical peptides induce membrane curvature such that the outer and inner leaflets are continuous. Peptides are adjacent to the lipid headgroups.

5.4.3. Does Bax form a proteinaceous or lipidic pore?

Based on the above criteria, the membrane-inserted α5/α6 hairpin of Bax [64] could self-associate to form either proteinaceous or lipidic pores. Although a barrel-stave assembly of Bax molecules was proposed for the MAC in mitochondria isolated from apoptotic cells [95], Bax pores formed in planar lipid bilayers and liposomes have characteristics of lipidic pores. Firstly, Bax formed channels with variable conductance states [107]. Secondly, at physiological pH the α5/α6 hairpin of Bax (and of Bak) has a net positive charge, less compatible with barrel-stave pores that are largely restricted to peptides of neutral charge. Thirdly, lipids that induce positive membrane curvature promote Bax pore formation [108,109]. Finally, lipidic pores can expand to actually destabilize the lipid bilayer, which would allow efflux not just of cytochrome *c* but of the larger proteins known to cross the OM during apoptosis [107,109]. As Bcl-x_L does not cause lipid destabilization in planar lipid bilayers [107], it is intriguing to speculate that the propensity of Bax but not Bcl-x_L to self-associate is directly related to membrane destabilization of the mitochondrial OM.

Elegant structural studies are beginning to throw some light on how Bax and Bak associate with, and insert into, model membranes. According to X-ray diffraction in a multiple lipid bilayer system, the Bax α5/α6 hairpin forms lipidic pores up to 5 nm in diameter [110]. This may be a conservative estimate as in liposomes, the Bax α5/α6 peptide formed pores over 11 nm in diameter, and full-length Bax protein formed even larger pores [99,109]. Thus, several liposome studies imply a Bax lipidic pore.

Whether Bax and Bak homo-oligomerization and pore formation is encouraged at specific sites in the OM is unclear. Association of Bax and Bak with components of the mitochondrial fission/fusion machinery suggests their selective recruitment to fission/fusion sites in the OM [111]. The precise role of mitochondrial fission and fusion in apoptosis is controversial and discussed by Martinou et al. in this issue.

6. Perspectives

These two fascinating proteins, Bax and Bak, are just beginning to divulge their secrets. A major challenge is to obtain structures of the activated Bax and Bak as monomers, homodimers, heterodimers, and higher order oligomers, preferably of full-length proteins within membranes. Obtaining structures of membrane-integrated complexes may be technically challenging if the lipidic pore model holds. Nevertheless, even one or two structures of the activated proteins in their non-membrane-integrated forms would allow more informed functional studies to interrogate the apparent multiple steps involved in Bax and Bak activation, including translocation, membrane insertion, and oligomerization. Another challenge is to understand the role of the mitochondrial outer membrane itself, in particular the lipid components, in each step of Bax and Bak function. A combination of biochemical and structural approaches as well as new emerging

technologies will be needed to discern the true nature of Bax and Bak oligomers.

Ultimately, understanding how Bax and Bak regulate mitochondrial permeabilization during apoptosis will allow better design of drugs that target the Bcl-2-regulated pathway. Each step and each interface in Bax and Bak homo- and heterooligomers is a potential new therapeutic target.

Acknowledgments

We thank the Victorian Cancer Council, NHMRC, Australian Research Council, Association for International Cancer Research, and German Research Foundation for funding our work.

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