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# Pore formation by dimeric Bak and Bax: an unusual pore?

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Apoptotic cell death via the mitochondrial pathway occurs in all vertebrate cells and requires the formation of pores in the mitochondrial outer membrane. Two Bcl-2 protein family members, Bak and Bax, form these pores during apoptosis, and how they do so has been investigated for the last two decades. Many of the conformation changes that occur during their transition to pore-forming proteins have now been delineated. Notably, biochemical, biophysical and structural studies indicate that symmetric homodimers are the basic unit of pore formation. Each dimer contains an extended hydrophobic surface that lies on the outer membrane, and is anchored at either end by a transmembrane domain. Membrane-remodelling events such as positive membrane curvature have been reported to accompany apoptotic pore formation, suggesting Bak and Bax form lipidic pores rather than proteinaceous pores. However, it remains unclear how symmetric dimers assemble to porate the membrane. Here, we review how clusters of dimers and their lipid-mediated interactions provide a molecular explanation for the heterogeneous assemblies of Bak and Bax observed during apoptosis.

This article is part of the themed issue 'Membrane pores: from structure and assembly, to medicine and technology'.

## 1. Introduction

Apoptosis is essential for normal development and tissue homeostasis, and its perturbed regulation contributes to numerous pathological conditions, including cancer and autoimmune and degenerative diseases [1]. Apoptosis is regulated principally by interactions within the Bcl-2 family of proteins, whose members fall into three subclasses (figure 1*a*). The eight or more pro-apoptotic BH3-only proteins (e.g. Bid and Bim) act as sensors of specific types of cellular stress, and signal by engaging other family members. The pro-survival proteins (e.g. Bcl-2 and Mcl-1) act by sequestering the pro-apoptotic members. Finally, pro-apoptotic Bak and Bax act as critical effectors of apoptosis, as they are required for mitochondrial permeabilization in cells and in mice [2,3]. As illustrated in figure 1*b*, upon receiving an apoptotic stimulus, upregulated BH3-only proteins bind to Bak and Bax to induce major conformation changes, resulting in Bak and Bax oligomerization and subsequent outer membrane permeabilization. This leads to the release of mitochondrial proteins including cytochrome *c*, which in turn triggers caspase-driven cell demolition (reviewed in [4]).

The three-dimensional structures of non-activated Bak and Bax resemble those of the pro-survival proteins, comprising nine α-helices that form a tight globular bundle (figure 2). Two important features are a surface hydrophobic groove (α2–α5) and a buried BH3 domain in α2 that mediate contact with other family members. A major distinction between Bak and Bax is that Bak is mostly inserted into the mitochondrial outer membrane in healthy cells, whereas Bax is mostly cytosolic and translocates to mitochondria following apoptotic stimuli (figure 1*b*). Bax translocation is triggered by binding of

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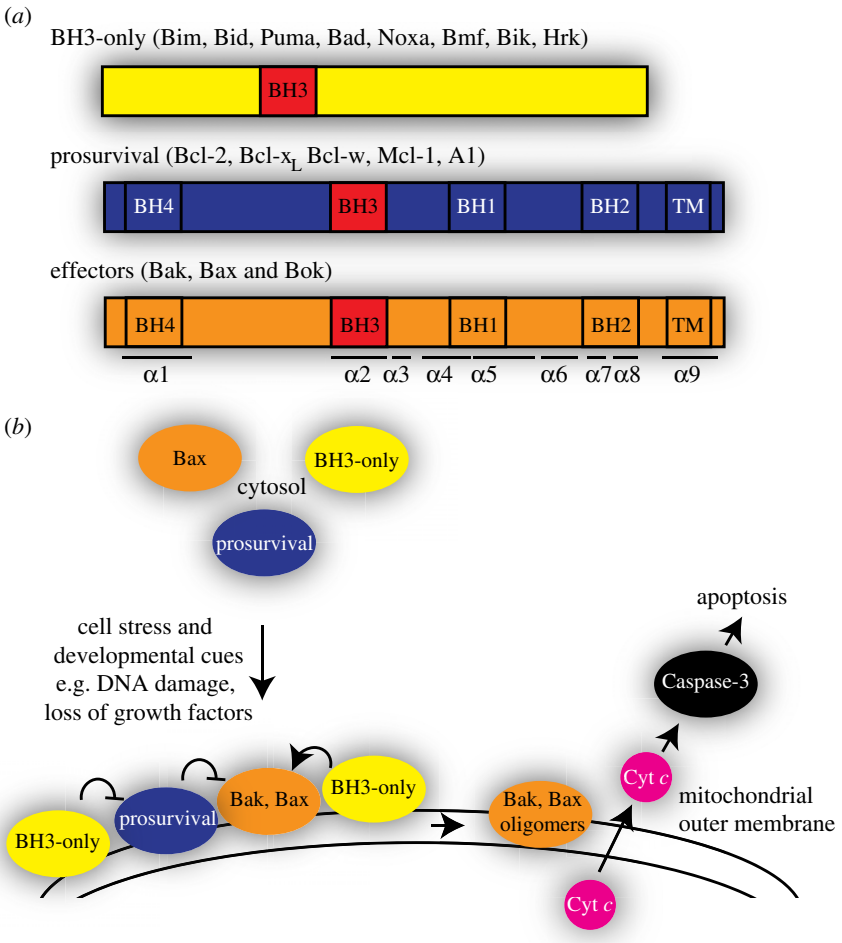


**Figure 1.** Bcl-2 proteins regulate the mitochondrial pathway of apoptotic cell death. (*a*) Three subfamilies of Bcl-2 proteins. (*b*) Bak and Bax activation by the BH3-only proteins is followed by their oligomerization in the mitochondrial outer membrane to release cytochrome *c* and induce apoptosis.

BH3-only proteins, which releases α9 from the hydrophobic groove [5]. Once the released α9 inserts as a transmembrane domain into the mitochondrial outer membrane, Bax has the same topology as non-activated Bak [6].

## 2. Conversion of Bak and Bax into symmetric homodimers with flexible extremities

Bak and Bax undergo major conformation changes as they convert into pore-forming proteins (figure 2; reviewed in [4]). The changes are triggered by the binding of BH3-only proteins to a hydrophobic surface groove, which generates a cavity underneath both the N- and C-termini [7–9]. Destabilization allows the protein to unfold as three segments: the α1-helix dissociates [10], and the core (α2–α5) separates from the latch (α6–α9) [7,8]. Several newly exposed hydrophobic regions then associate with the mitochondrial outer membrane to lie in-plane (figure 2, activated Bak monomer) [11,12]. The core remains largely folded, but within it the newly exposed hydrophobic BH3 domain (in α2) then binds to the hydrophobic groove of another activated Bak or Bax molecule in a reciprocal manner to form symmetric homodimers (figure 2, BH3:groove dimer). Evidence for symmetric homodimers originated from biochemical studies in mitochondria [13–15] and is supported by X-ray structures of the α2–α5 dimers [7,8] and biophysical studies[12,16–20].

Together these studies support the in-plane model of a Bak dimer (figure 3*a*) [11,22]. The region in contact with the membrane (α2–α9) resembles an extended flexible amphipathic polypeptide anchored at either end with a transmembrane domain—an unusual structure for a pore-forming protein. Several helices may embed into the outer leaflet of the membrane, encouraged by aromatic residues on one surface of the α2–α5 core dimer (figure 3*b*) and on one face of the α6–α8 helices (figure 3*c*). At the N-terminus, the first 70 residues become exposed and do not re-engage with either membrane or protein [10,22,23]. Bax dimers also display aromatic residues on one surface (figure 3*b*,*c*) and a similar membrane topology [6,11,16,19], although complete solvent exposure of the N-terminus has not yet been shown. Thus, Bak and Bax homodimers show several features of antimicrobial peptides such as human LL-37 and magainin 2 (figure 3*d*) that are proposed to form toroidal pores, rather than of the α-helical ClyA and actinoporin proteins that form more structured proteinaceous pores [24,25].

There are few examples of a homodimer as the building block of a pore that might provide insight into pore formation by Bak and Bax. One such example is the plant defensin NaD1, whose structure comprises seven antiparallel dimers [26]. However, unlike Bak and Bax, the NaD1 complexes are not promoted by major conformation change but by binding of the $PIP_2$ phospholipid, and the oligomers lack transmembrane domains and flexible membrane-associated regions. Members of the colicin family of pore-forming proteins may also assemble as multiples of dimers to form small ring-shaped oligomers (approx. 8 nm in diameter) [25,27,28]. Curiously, the first structure of a Bcl-2 protein

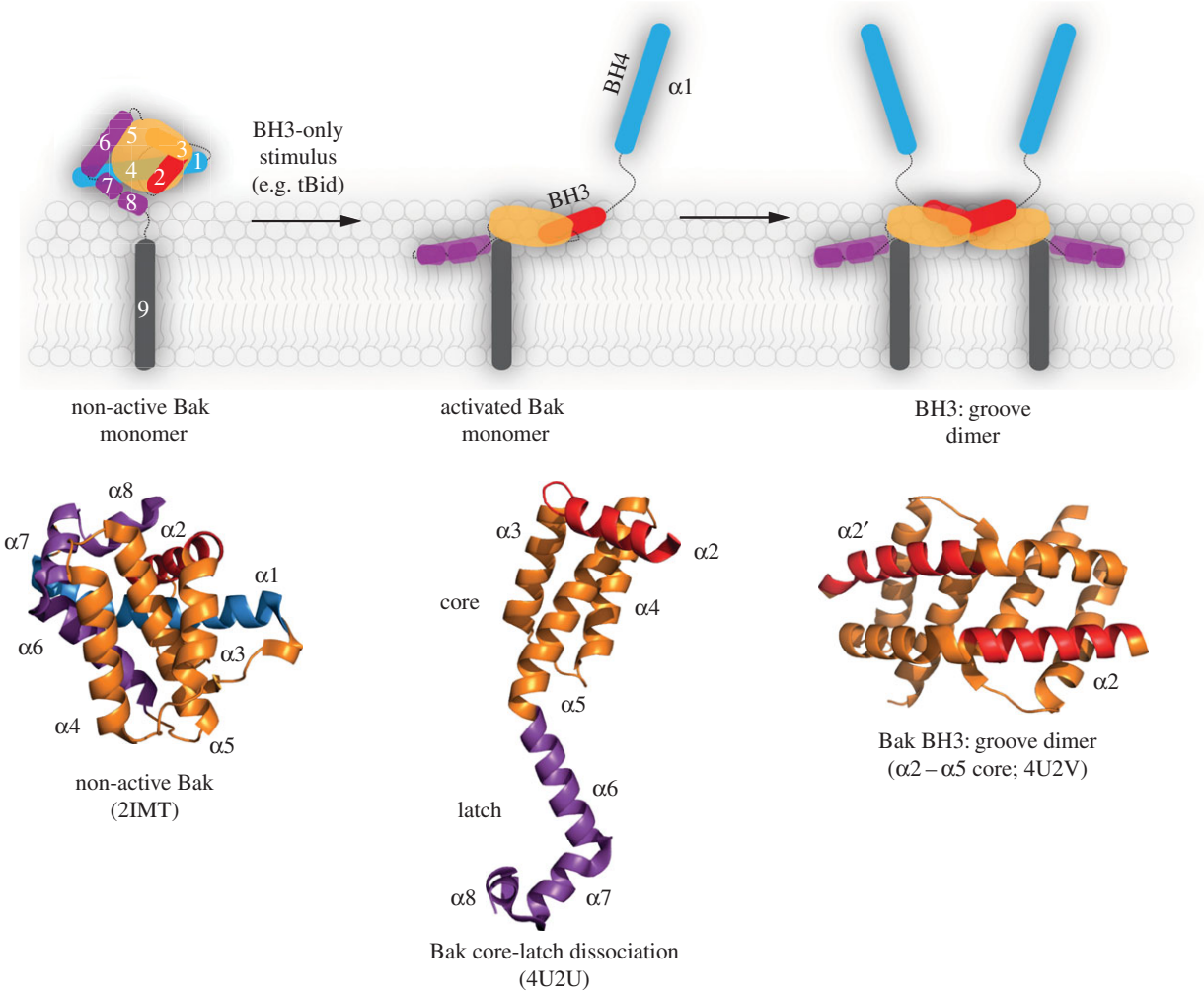


**Figure 2.** Bak activation and conformation change results in symmetric homodimers. A schematic showing that Bak unfolds by the N-terminus (α1, blue) and the C-terminal latch (α6–α8, magenta) separating from the α2–α5 core (orange, red). Hydrophobic regions of the core and latch then collapse onto the membrane, while the exposed BH3 domain (in α2) binds to the hydrophobic groove in another activated Bak molecule. Reciprocal BH3:groove binding results in symmetric homodimers. The indicated crystal structures demonstrate the major conformation changes involved. Equivalent changes are observed for Bax.

and its similarity to the colicins and diphtheria toxin prompted the idea that, as proposed for those proteins, Bak and Bax might form channels or pores by inserting a helical hairpin (α5/α6) through the membrane [29–31]. Notably, hairpin insertion is not consistent with the in-plane model in which α5 remains with the α2–α5 dimer and α6 lies in-plane in the outer membrane (figures 2, 3 and 4) [11,12]. A recent study proposed that Bax α2–α5 dimers can progress to α2–α3–α4 dimers after separation of α5 to allow α5/α6 insertion [16]. However, it was not clear whether those dimers were functional [16], and in other studies separation of Bax α5 from α4 was not required for cytochrome *c* release [7]. Even so, further comparison of pore formation by the Bcl-2 and colicin families may prove informative.

## 3. Dimer–dimer interactions are not via a single protein–protein interface

It is thought that homodimers of Bak or Bax must then associate to higher order oligomers to porate the mitochondrial outer membrane. Such oligomers of Bak and of Bax are generated in mitochondria during apoptosis, as evident by gel filtration, blue native PAGE and linkage studies [32–34]. In addition, recombinant Bak and Bax form high order oligomers in liposome experiments (table 1). High order oligomers observed biochemically correlate with the clusters observed in early microscopy studies (table 1). And these clusters are sometimes apparent at points of mitochondrial fission/fusion [59]. Higher-resolution microscopy shows Bak and Bax complexes of various shapes and sizes in liposomes and mitochondria, including clusters, rings and arcs (table 1). Thus, there is strong correlation between high order oligomers and pore formation. Nevertheless, it is yet to be shown that specifically inhibiting dimer–dimer interaction blocks pore formation.

While either Bak or Bax is sufficient to form pores (table 1), the two proteins locate to the same complexes in apoptotic cells [32,34], suggesting that mixtures of the two proteins may be able to generate pores. The mixtures may include heterodimers of Bak and Bax, although heterodimers form only a minor population compared with homodimers [13,60]. The low frequency of heterodimers may be explained by a degree of incompatibility due to the limited sequence similarity of the BH3 domains and grooves of the two proteins. Mixtures may also include homodimers of Bak and of Bax, as Bax is able to intermingle with pre-formed Bak dimers [22]. If mixtures of Bak and Bax homodimers can

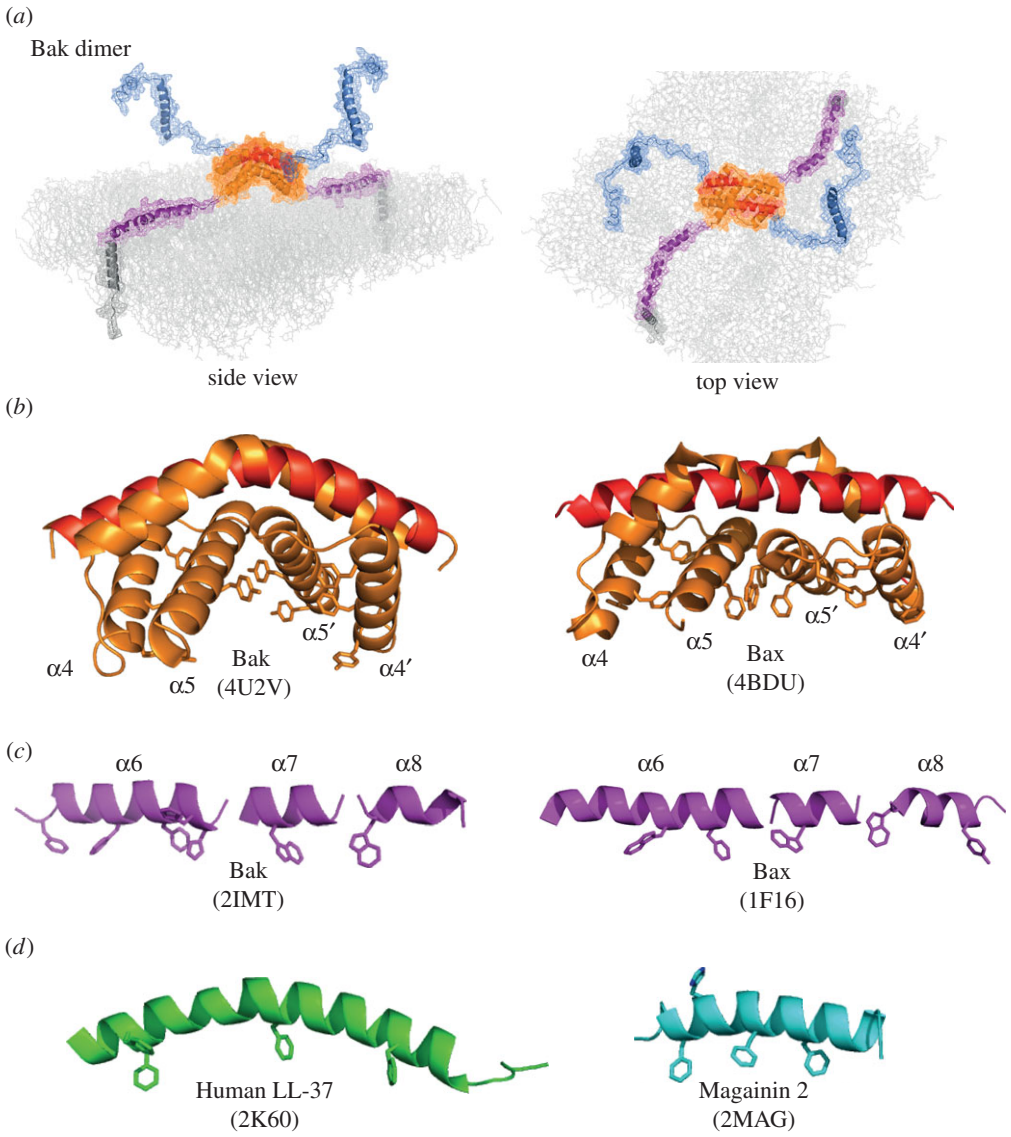


**Figure 3.** Membrane topology of the Bak dimer. (*a*) The in-plane model of the Bak dimer. The N-terminal regions become solvent-exposed while the remainder of the Bak dimer resembles a flexible extended amphipathic peptide that lies in-plane with the membrane, anchored at either end by transmembrane domains. Note that α1 may unfold after it dissociates, decreasing the hydrophobicity of the BH4 structural motif (VFrsYV) therein [10,21]. Images were assembled in PyMol using the structures of Bak (2IMT) and the Bak dimer (4U2V), and represented as cartoon and mesh. (*b*) Aromatic residues are concentrated on the bent surface of the Bak and Bax α2–α5 core dimers. (*c*) Aromatic residues can position on one edge of the flexible α6–α8 latch. (*d*) Examples of antimicrobial peptides thought to form lipidic pores, with aromatic residues indicated. Colour coding as in figure 2.

actually generate pores, this would provide further evidence that protein–protein interactions between dimers are not important for high order oligomers or pore formation.

Molecular structures of Bak or Bax as high order oligomers or pore complexes are currently not available. However, a range of biochemical approaches have been used to examine how activated Bak and Bax interact to generate pores. Most prominent have been linkage studies showing that homodimers can associate via interactions at α-helices 1, 3, 5, 6 and 9 [6,12,13,15,16,19,61–64]. Our initial studies showed that linkage between the α6-helices could link dimers of Bak and Bax [13,15], suggesting that an α6:α6 interface may drive high order oligomers and pore formation. However, there was no evidence that mutations in α6 could block apoptosis [4,65], and several groups reported linkage between additional regions. Thus, some or all of these linkages may be due to collisions rather than to stable complex formation. Flexibility of the N- and C-termini, as depicted in figure 4*a*, may allow linkage between multiple regions, and would also limit interaction between the α2–α5 core dimers [8,22]. Based on the linkage pattern throughout the full-length of Bak, we recently proposed that dimers form disordered clusters during apoptosis (figure 4*a*), and this was supported by mathematical simulation of linkage within the whole population of Bak dimers in the sample [22]. It is yet to be determined if only a small subpopulation of dimers directly participates in a pore complex. If so, within this subpopulation the core dimers may adopt an ordered arrangement, e.g. end-to-end or side-by-side.

## 4. Formation of lipidic (toroidal) pores

Several lines of evidence indicate that Bak and Bax form lipidic rather than proteinaceous pores (table 1). Amphipathic peptides based on the Bak and Bax α5 and α6 helices can

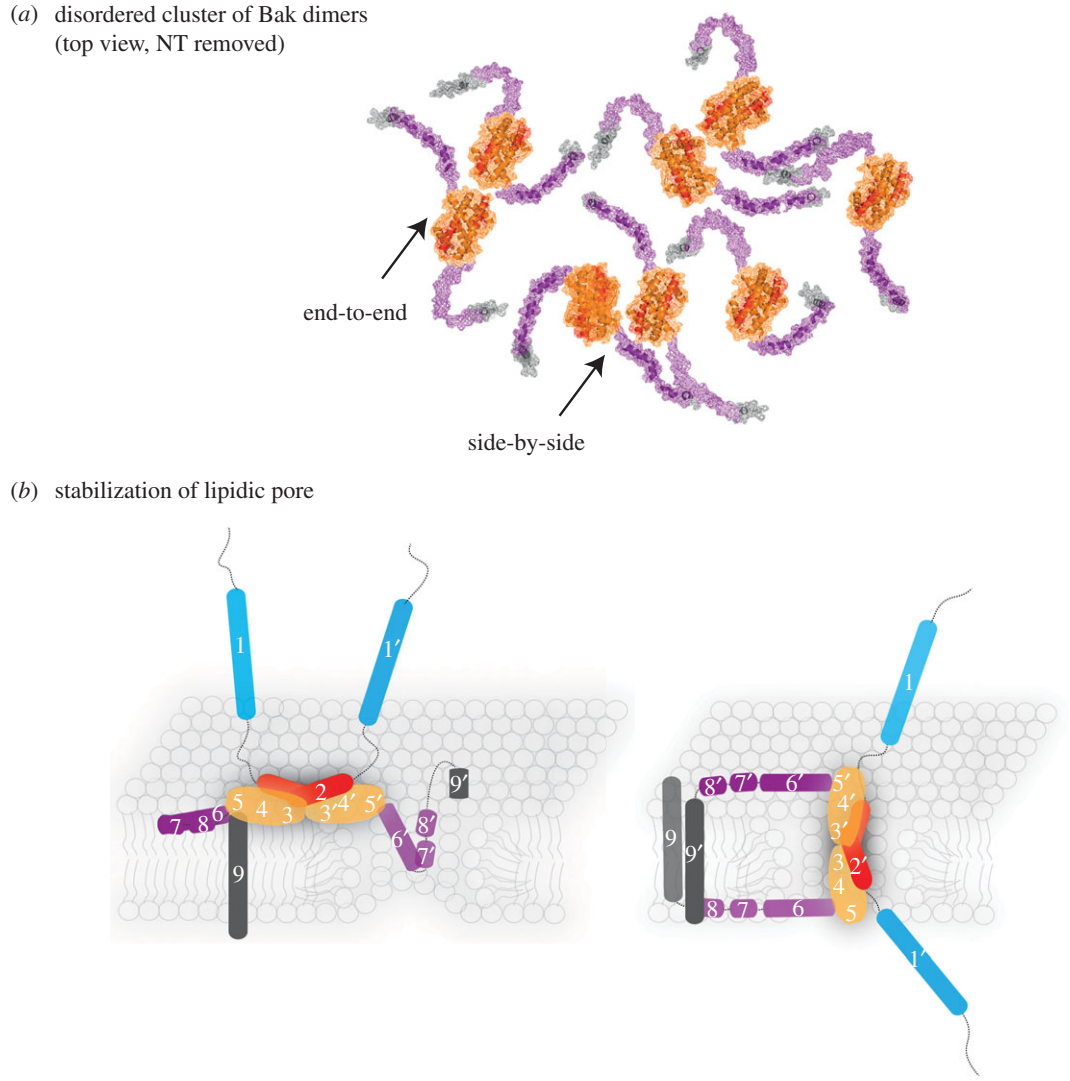


**Figure 4.** Possible mechanisms involved in lipidic pore formation and stabilization by homodimers of Bak or Bax. (*a*) Schematic of Bak dimers forming a disordered cluster on the mitochondrial outer membrane, encouraged by flexibility of the α6–α8 latch. Note that end-to-end or side-by-side contact between the core regions is possible. Images were assembled as in figure 3*a*. (*b*) Parts of the dimer may line a lipidic pore. The flexible amphipathic latch may slide into a nascent pore to partially line and stabilize the pore (left; in-plane model [4]). The amphipathic core dimer (α2–α5) may also line the pore generating antiparallel α9-helices (right; clamp model [19]). Colour coding as in figure 2.

permeabilize membranes (table 1), as do amphipathic antimicrobial peptides that act via forming lipidic pores. As the Bak and Bax dimers resemble a flexible amphipathic polypeptide (figure 3*a*), their shallow insertion into the outer leaflet [11,18,19] may destabilize the lamellar structure of the bilayer to induce lipidic pores. This mechanism of pore formation may be related to the 'carpet' model proposed for antimicrobial peptides. In the Shai–Matsuzaki–Huang version of the carpet model [66–68], peptides can disrupt membranes without disintegrating the membranes in a detergent-like manner [69]. The peptides insert close to the membrane surface to promote a convex curvature of the outer leaflet. As the peptide concentration increases, membrane defects occur, and in some cases may be resolved by peptide (or phospholipid) equilibrating across the bilayer. As Bak and Bax are unlikely to equilibrate across the bilayer due to their size and transmembrane domains, the membrane defects may progress to pore formation. There is evidence that pore formation is associated with lipid transbilayer movement [35,42,46].

During pore formation, parts of the dimer may line and stabilize the pore (figure 4*b*) [11,19,70]. According to the clamp model (figure 4*b*, right) [19] the core dimer positions roughly perpendicular in a circle to line the pore. Several features of this model are attractive. The length of the core dimer (approx. 4 nm) is the approximate width of the MOM, and the bend observed in the structures (Bak and Bax α2–α5 dimers; figure 3*b*) may be accommodated by the curved edge of the pore. In this position, the α6–α8 latch would disturb the outer and inner leaflets equally, and the core dimer could contribute a large surface area to stabilize the pore. In addition, the core dimers could pack tightly side by side around the pore. However, one side-by-side orientation of Bak α2–α5 dimers observed in a crystal structure was not supported by linkage studies in mitochondria [8,22]. Moreover, the clamp model suggests that the α9 transmembrane domains become antiparallel within a dimer, presumably after the charged residues (e.g. RRFFKS in human Bak) at the far C-terminus of one activated molecule flip through the hydrophobic bilayer. While such flipping may be similar to insertion of the transmembrane domains of non-activated Bak (and Bax), direct evidence of antiparallel α9-helices in Bax or Bak oligomers is required to support this model.

**Table 1.** Heterogeneity of Bak and Bax complexes and pores. AFM, Atomic Force Microscopy; CD, Circular Dichroism; Cryo-EM, Cryo-Electron Microscopy; ΔC, C-terminally truncated; FCS, Fluorescence Correlation Spectroscopy; GUV, Giant Unilamellar Vesicles; IVT, In Vitro Translated; LUV, Large Unilamellar Vesicles; OG, Octyl Glucoside; OMV, Outer Membrane Vesicles; PALM, Photo-activated Localization Microscopy; TEM, Transmission Electron Microscopy; TIRF, Total Internal Reflection Fluorescence Microscopy; SMLM, Single Molecule Localization Microscopy; STED, Stimulated Emission Depletion microscopy.

| | Bak/Bax | membrane type | resolution method | characteristics of complexes and pores, including effect on membranes | references |
|---|---|---|---|---|---|
| peptides | Bax α5, α6 | LUV<br>planar bilayer | AFM<br>CD spectroscopy<br>confocal<br>X-ray diffraction | toroidal pores of diameter approximately 5.8 nm<br>decreased membrane line tension at pore rim<br>lipids with positive intrinsic curvature enhance pore formation<br>lipid transbilayer redistribution activity for Bax α5 | Garcia-Saez 2005, 2006, 2007; Qian 2008 [35–38] |
| recombinant proteins | BaxΔC<br>Bax | liposome<br>planar bilayer | patch clamping<br>FI-dextran release | Bax forms pH dependent ion-conduction channels/pores<br>maximum of 4 molecules, pore diameter approximately 2.2 nm | Antonsson 1997, Schlesinger 1997, Saito 2000 [39–41] |
| | BaxΔC<br>IVT-Bax<br>Bax (bovine) | LUV<br>liposome<br>planar bilayer | voltage clamp<br>membrane lifetime measurements | decrease in membrane lifetime and linear tension<br>increase in positive monolayer curvature stress<br>lipid transbilayer redistribution | Basanez 1999, Basanez 2002, Terrones 2004, Landeta 2011 [42–45] |
| | Bax<br>BaxΔC | LUV | AFM | toroidal pores of diameter approximately 100–300 nm<br>small ring-like structures on bilayer surface<br>structures contain clusters of approximately 22 Bax monomers<br>lipid transbilayer redistribution | Epand 2002, 2003 [46,47] |
| | Bax<br>OG-Bax | liposome<br>OMV | TEM<br>dextran release | supramolecular lipid pores<br>allowed release of approximately 2000 kDa dextran | Kuwana 2002 [48] |
| | Bax<br>BakΔC | LUV<br>GUV<br>mitochondria | Cryo-EM<br>FCS | time and protein concentration dependent lipid pores<br>pores of diameter approximately 3–140 nm<br>induction/stabilization of curved membrane structures<br>reduction in vesicle size due to budding, fission, tethering | Bleicken 2013a, 2013b, 2016 [49–51] |
| | Bax | bilayer nanodiscs | Cryo-EM | active Bax monomers form pores of diameter approximately 3.5 nm | Xu 2013 [52] |
| | Bax<br>Nanogold labelled Bax | liposome<br>LUV<br>OMV | TEM<br>Cryo-EM | pore-like openings with diameter approximately 25–100 nm<br>growing pores in the range of approximately 100–300 nm<br>solitary dynamic pores with negative curvature at edges<br>complete rings of Bax exclusively associated with pore rims | Schafer 2009, Gillies 2015, Kuwana 2016 [53–55] |
| | Bax | LUV<br>planar bilayer | AFM<br>TIRF | multiple oligomer species of dimers<br>round heterogeneous pores of diameter approximately 24–176 nm<br>Bax along pore rim | Subburaj 2015, Salvador-Gallego 2016 [17,56] |

(*Continued.*)

**Table 1.** (*Continued.*)

| | Bak/Bax | membrane type | resolution method | characteristics of complexes and pores, including effect on membranes | references |
|---|---|---|---|---|---|
| cells | GFP-Bax | HeLa<br>Cos-7 | TEM<br>confocal | large clusters containing 1000–20 000 Bax molecules | Nechushtan 2001 [34] |
| | CFP-Bax | HeLa | quantitative fluorescence imaging | Bax complexes with approximately 150–1000 molecules per cluster<br>620 nm average cluster size | Zhou 2008 [32] |
| | mEos3-Bak | MEF | PALM | heterogeneous Bak clusters of diameter approximately 70–600 nm<br>each cluster contains approximately 20–2000 Bax molecules<br>no pores evident in clusters (using resolution of 20 nm) | Nasu 2016 [57] |
| | Endog. Bax | HeLa<br>U2OS<br>HT1080<br>SH-SY5Y<br>CV-1 | STED | large compact clusters<br>rings up to 400 nm diameter | Grosse 2016 [58] |
| | GFP-Bax | HeLa<br>HCT-116 | TEM<br>STED<br>SMLM | heterogenous distribution of:<br>clusters<br>rings (35 nm diameter)<br>arcs (100–500 nm diameter)<br>lines | Salvador-Gallego 2016 [56] |

## 5. Heterogeneity of Bak and Bax complexes and pores

Consistent with forming a lipidic pore, significant heterogeneity is observed in the characteristics of the Bak and Bax complexes and the actual pores formed by Bak and Bax (table 1). Differences may be due to the levels of Bak and Bax (and pro-survival Bcl-2 proteins), lipid composition and diameter of the mitochondria or liposome, and even the presence or absence of the mitochondrial inner membrane and matrix. Detectable pores in the membrane were not always evident in the clusters, suggesting that at least some clusters may form upstream or downstream of pore formation. Notably, Bax pore size in liposomes increased with higher protein concentration and over time [39,49,53,71], and pore size in *Xenopus laevis* mitochondria increased in response to a cytosolic factor that was more potent in the presence of caspase inhibitors [72]. Thus, both protein and lipid appear able to enlarge apoptotic pores, a process that would ensure rapid cell death. It will be interesting to determine the role of pores that are not detectable by microscopy. Can a pore stay small, and what is the composition of such a 'minimal' pore? Are there multiple small pores in a mitochondrion, perhaps even in a single cluster? Might a single small pore in each mitochondrion be sufficient for apoptosis? Answers to these questions may help to regulate apoptosis at the step of pore formation, including reversing the process.

## 6. Concluding remarks

Increasing evidence indicates that symmetric homodimers of Bak and of Bax form the structural building block of the apoptotic pore. As there is a strong correlation of higher order oligomers with pore formation, it is important to understand how symmetric dimers can form these oligomers. Within each dimer, the N-terminus is solvent-exposed and flexible, implicating the membrane-associated regions (α2–α9) in driving pore formation. Flexibility of the latch (α6–α9) implies several arrangements of dimers may occur and contribute to the heterogeneity of clusters and pores observed. Notably, insertion of the core and latch into the outer leaflet may remodel the bilayer to form a small lipidic pore, which may then grow considerably. Obtaining structures of small apoptotic pores may yet be possible, and these structures may be the key to understanding how this central step of cell death might be regulated.

Authors' contributions. The authors contributed equally to the planning, writing and illustrating of this article.

Competing interests. We declare we have no competing interests.

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