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REVIEW

Bid: a Bax-like BH3 protein

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Bid, a pro-apoptotic member of the Bcl-2 family, was initially discovered through binding to both pro-apoptotic Bax and anti-apoptotic Bcl-2. During apoptosis, Bid can be cleaved not only by caspase-8 during death receptor apoptotic signaling, but also by other caspases, granzyme B, calpains and cathepsins. Protease-cleaved Bid migrates to mitochondria where it induces permeabilization of the outer mitochondrial membrane that is dependent on the pro-apoptotic proteins Bax and/or Bak, and thus Bid acts as a sentinel for protease-mediated death signals. Although sequence analysis suggests that Bid belongs to the BH3-only subgroup of the Bcl-2 family, structural and phylogenetic analysis suggests that Bid may be more related to multi-BH region proteins such as pro-apoptotic Bax. Analysis of membrane binding by protease-cleaved Bid reveals mechanistic similarities with the membrane binding of Bax. For both proteins, membrane binding is characterized by relief of N-terminal inhibition of sequences promoting migration to membranes, insertion into the bilayer of the central hydrophobic hairpin helices and exposure of the BH3 region. These findings implicate Bid as a BH3-only protein that is both structurally and functionally related to multi-BH region Bcl-2 family proteins such as Bax.
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Introduction

Bid, an acronym for BH3-interacting domain death agonist, is a member of the Bcl-2 family of proteins that regulate the permeabilization of the outer mitochondrial membrane (OMM), a critical event during apoptosis. Members of the Bcl-2 family can be subdivided as either pro- or anti-apoptotic on the basis of their influence over apoptosis, as well as by the number of regions of sequence homology with the anti-apoptotic protein Bcl-2. Most anti-apoptotic proteins, such as Bcl-XL and Bcl-w, share four regions of homology with Bcl-2 (BH regions 1–4). Pro-apoptotic Bcl-2 family proteins can be divided based on the number of BH regions. The multiregion pro-apoptotic proteins, Bax and Bak, contain the BH regions 1–3. Most other pro-apoptotic Bcl-2 family proteins, including Bid, Bim and Bad, contain sequence homology only in the BH3 region and hence are referred to as BH3-only proteins. Some BH3-only proteins (Bid, Bim and Puma) are termed activator BH3-only proteins, as they directly induce Bax/Bak-dependent OMM permeabilization (Kim *et al*., 2006). These BH3-only proteins, or the Bax/Bak molecules they have activated, are sequestered by anti-apoptotic proteins such as Bcl-XL to inhibit OMM permeabilization (Billen *et al*., 2008). Other BH3-only proteins (Bad, Noxa, Bmf, Hrk) are termed as sensitizers, as they promote apoptosis by binding to anti-apoptotic proteins to induce the release of either activator BH3-only proteins (Letai *et al*., 2002; Certo *et al*., 2006) or activated Bax or Bak (Willis *et al*., 2005; Uren *et al*., 2007).

Although Bid has been implicated in participation in a mitosis checkpoint and maintenance of genomic stability (Kamer *et al*., 2005; Zinkel *et al*., 2005), the main function of this protein appears to be to link the death receptor pathway and OMM permeabilization (Li *et al*., 1998; Luo *et al*., 1998). Although full-length Bid contains some pro-apoptotic function, the full activity of Bid is not realized until proteolytic cleavage. During death receptor apoptosis, Bid is cleaved by caspase-8. The C-terminal fragment of this cleavage event, tBid, migrates to and inserts into the OMM (Gross *et al*., 1999), where it subsequently drives the translocation and insertion of Bax into the OMM (Eskes *et al*., 2000), eventually leading to OMM permeabilization that is dependent on either Bax or Bak (Wei *et al*., 2000).

Although Bid, tBid or Bid BH3 peptide are unable to permeabilize the OMM in the absence of both Bax and Bak (Letai *et al*., 2002; Billen *et al*., 2008), it has recently been suggested that Bid may be more related to multidomain Bcl-2 family proteins, such as Bax, than to BH3-only proteins (Youle and Strasser, 2008). Although almost all BH3-only proteins are unstructured (Hinds *et al*., 2007), the structure of Bid (Chou *et al*., 1999; McDonnell *et al*., 1999) is similar to that of pro-apoptotic Bax (Suzuki *et al*., 2000) and Bak (Moldoveanu *et al*., 2006), as well as anti-apoptotic Bcl-2 (Petros *et al*., 2001), Bcl-XL (Muchmore *et al*., 1996), Bcl-w (Denisov *et al*., 2003; Hinds *et al*., 2003) and Mcl-1 (Day *et al*., 2005). Furthermore, phylogenomic analysis suggests an evolutionary link between Bid and the multiregion Bcl-2 proteins, such as Bax and

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Bcl-XL, rather than the BH3-only proteins (Aouacheria *et al*., 2005). In this report we suggest further similarities between Bid and Bax by examining the molecular details surrounding migration to and insertion into the OMM. Bid and Bax share several mechanistic features that regulate migration of the proteins to membranes and that are different from BH3-only proteins such as Bim and Bad. We propose that Bid is specifically designed for rapid migration to and insertion into the OMM after proteolytic cleavage, and does so in a Bax-like manner.

Bid and Bax are structurally similar

Comparison of the NMR structures for human Bid (Chou *et al*., 1999) and Bax (Suzuki *et al*., 2000) proteins reveals remarkable structural similarities (Figure 1). Bid contains eight α-helices, with two central hydrophobic helices (helices 6 and 7) forming a hairpin structure that is surrounded by the remaining six amphipathic helices. This structural arrangement is reminiscent of the bacterial toxins such as the colicins and diphtheria toxin (Parker and Pattus, 1993). The BH3 region of Bid (amino acids 90–98), which comprises a region of sequence homology to other Bcl-2 proteins and is required for interaction with both pro-apoptotic Bax and anti-apoptotic proteins such as Bcl-XL (Wang *et al*., 1996), is located in helix 3. Bid also contains a large unstructured loop (amino acids 42–79) that separates helices 2 and 3. This loop contains a variety of sites that are subjected to posttranslation modifications that regulate Bid localization and apoptotic function (Figure 2, described in detail below). Finally, structural comparison of Bid with multiregion anti-apoptotic proteins, including several viral proteins, suggests that Bid (as well as Bax and Bak) contains a redefined BH4 region at the N terminus, a feature previously believed to be shared only by anti-apoptotic proteins (Kvansakul *et al*., 2008).

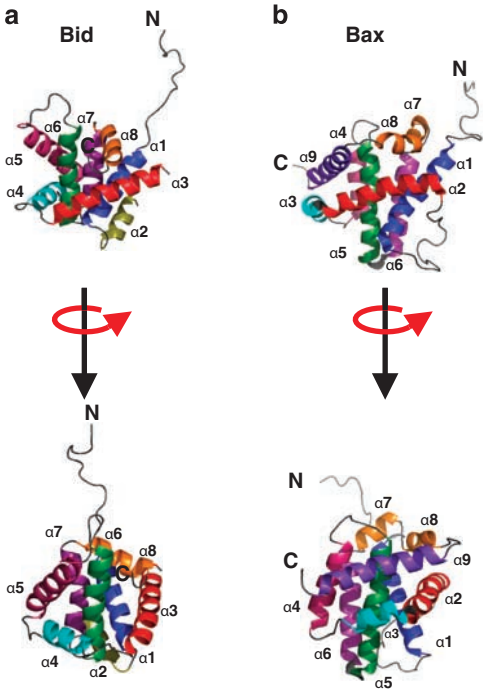

Figure 1 Molecular structures of Bid and Bax. The structures of human Bid (**a**) and human Bax (**b**) are represented. The large, unstructured loop region of Bid (amino acids 43–77) is omitted. The α-helices are color coded to show directly corresponding helices between Bid and Bax. The helices α1, α3, α4, α5, α6, α7 and α8 of Bid directly correspond to helices α1, α2, α3, α4, α5, α6 and α7/α8 of Bax. There are two α-helices that do not correspond between Bid and Bax: α2 of Bid (olive color) is not found in the Bax structure whereas α9 of Bax (purple color) is not found in the Bid structure.

Bax contains nine α-helices that adopt a similar structural arrangement to that seen for Bid, with helices 5 and 6 forming the central hydrophobic hairpin. Unlike Bid, Bax contains three regions of sequence homology to other Bcl-2 family proteins (BH1–3, see Figure 2). The BH regions 1–3 have been shown to form a hydrophobic cleft that can presumably bind the BH3 region of a variety of other Bcl-2 family proteins, although this observation has been made only for the anti-apoptotic proteins (Sattler *et al*., 1997; Petros *et al*., 2000; Liu *et al*., 2003; Smits *et al*., 2008) and not for Bax. Although Bid does not contain sequence similarity to the Bax BH regions 1 and 2, a similar hydrophobic cleft appears in the Bid structure. Surprisingly, in the solution structure of Bid this cleft is solvent exposed, whereas Bax contains an extra helix (helix 9) that fits in the hydrophobic cleft and shields the hydrophobic residues from solvent (Figure 1). With the exception of helix 9 of Bax, the only other major structural difference between Bid and Bax is the presence of an extra helix near the N terminus of Bid. This helix, helix 2, does not appear in the structure of Bax. The function of helix 9 of Bax and helix 2 of Bid in the functions of the respective proteins will be discussed below.

Although structure of the BH3-only protein Bid is remarkably similar to the multiregion pro-apoptotic protein Bax as well as anti-apoptotic proteins such as Bcl-XL, most other BH3-only proteins can be classified as intrinsically unstructured proteins (Hinds *et al*., 2007). In fact, sequence analysis of the BH3-only proteins Bid, Bim, Bad, Bik, Noxa, Puma, Hrk and Bmf suggested that other than Bid, only Bik might be a structured protein. Using the BimL isoform, the authors showed that upon binding to an anti-apoptotic protein, localized conformational changes occur to increase the helical nature of the BH3 region, consistent with the structure of Bim BH3 in complex with Bcl-XL (Liu *et al*., 2003). These results suggest that increased helical content of the BH3 region in most BH3-only proteins, shown to be important for stable interactions between BH3 peptides and both Bax and Bcl-XL (Walensky *et al*., 2006), occurs during binding to an appropriate target. This is in stark contrast to Bid, which contains a constitutively helical BH3 region (Chou *et al*., 1999; McDonnell *et al*., 1999).

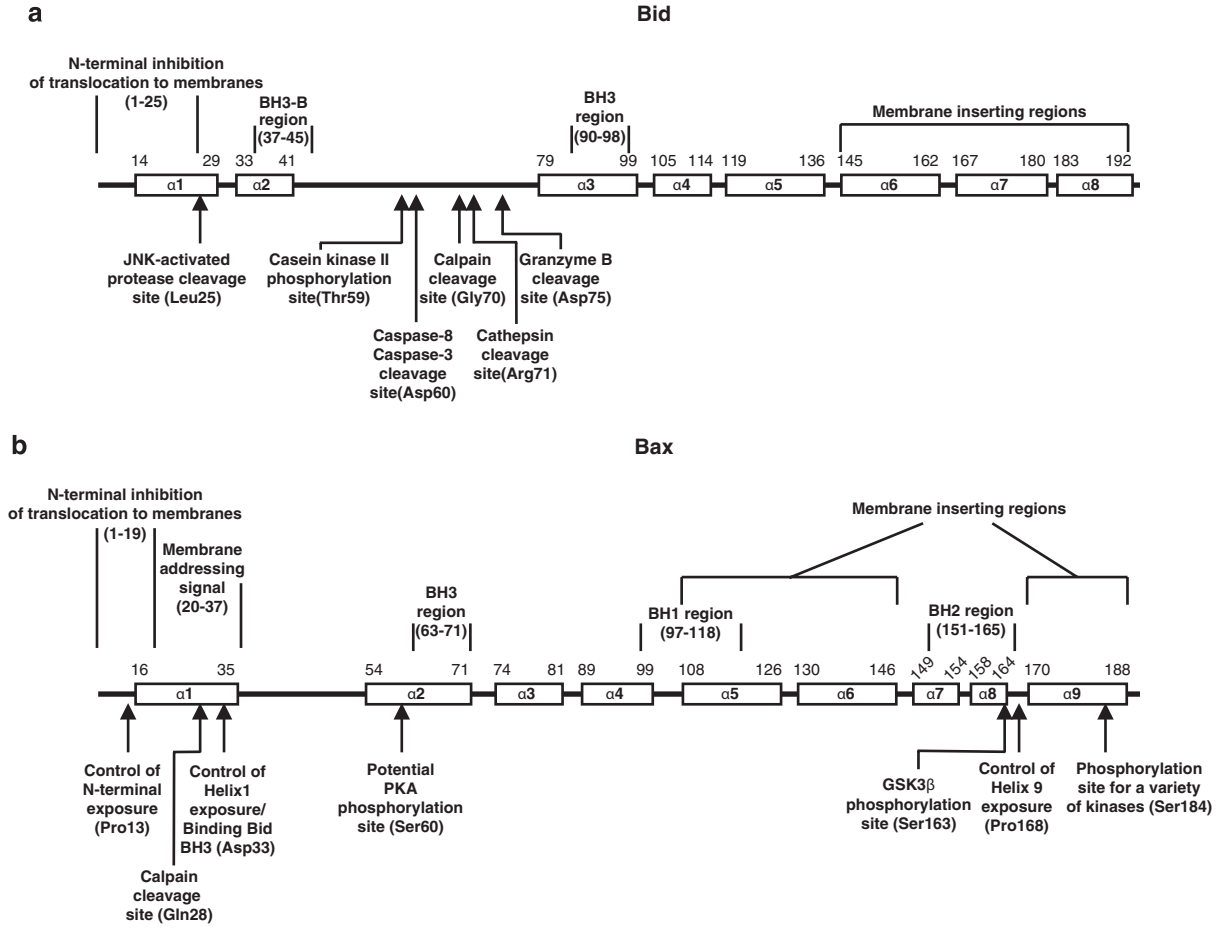

Figure 2 Regions and residues of Bid and Bax that regulate binding to membranes and apoptotic function. The human Bid protein (**a**) and the human Bax protein (**b**) are depicted. Bid contains 195 amino acids and 8 α-helices whereas Bax contains 192 amino acids and 9 α-helices. The numbers above each α-helix indicate the starting and ending amino acids for that helix. The regions of Bid and Bax that control recruitment of the proteins to membranes and function, including the BH regions and domains believed to insert into membranes, are indicated above the proteins. Where appropriate, parentheses indicate the amino acids that correspond to a particular region. Individual amino acids that regulate binding to membranes or are subjected to posttranslational modifications that regulate binding to membranes are indicated below the proteins, with parentheses indicating the identity and number of each amino acid.

Migration to and insertion into membranes of the BH3-only proteins Bim and Bad

Like many Bcl-2 family proteins, but unlike Bid, the BH3-only protein Bim has been suggested to contain a C-terminal transmembrane (TM) region (O'Connor *et al*., 1998). Although this proposed sequence contains several charged residues, it has been shown to function as a mitochondrial-specific tail-anchor sequence (George Hacker, personal communication). Removal of the proposed TM region of BimEL abrogated mitochondrial localization when expressed in HeLa cells (Yamaguchi and Wang, 2002), however only a minimal loss of function was observed for this mutant. These results indicate that insertion of Bim into the membrane is not absolutely required for the function of Bim, consistent with the unstructured nature of these proteins allowing their BH3 regions to be accessible in solution. Similarly, the largely unstructured nature of the proteins suggests that even in the presence of an appropriate binding partner, insertion of Bim and Bad into the OMM is unlikely to be controlled through conformational changes required to expose the membrane binding sequence. Bad does not even contain an amino-acid sequence that resembles a membrane anchor and thus it has not been suggested to have a C-terminal TM region. Nevertheless, in some circumstances this protein still migrates to and inserts into the OMM (Jeong *et al*., 2004). Deletion studies have indicated that many sections of the Bad protein can be removed with little effect on its function as long as an intact BH3 region is present (Zha *et al*., 1997).

A mutation in the BH3 region of full-length BimEL, which severely diminished binding to Bcl-XL, also caused only minimal loss in the activity of BimEL (Yamaguchi and Wang, 2002). However, the combination of a mutation in the BH3 region and removal of the TM region completely abolished BimEL activity. The unstructured nature of this protein and its BH3 region suggests that in the absence of binding to membranes,

binding to anti-apoptotic proteins is possible; however, local concentrations at the OMM are low. In the presence of a mutation in the BH3 region of the full-length protein, local concentrations at the OMM are high; however, the binding affinity for the target is low. Although data on the binding to membranes of unstructured BH3-only proteins are lacking, these results raise the possibility that BH3-only proteins, such as Bim and Bad, insert into the OMM to raise the local concentration of their unstructured BH3 regions to promote binding to relevant targets.

Bid and Bax interact with membranes by similar mechanisms

As described above, the solution structure of Bid shares many features with multiregion anti- and pro-apoptotic proteins, including Bax. Further analyses of the activities of Bid and Bax (presented below) suggest that Bid is conceptually more similar to Bax than it is to BH3-only proteins, such as Bim and Bad. Both Bid and Bax contain sequences of amino acids at the extreme N terminus (20–25 amino acids) that negatively regulate binding to membranes, as loss of these amino acids leads to constitutive localization at membranes (Goping *et al*., 1998; Deng *et al*., 2003). Bid does not contain a C-terminal TM region, and membrane insertion after cleavage by caspase-8 or other proteases is dependent on the central helices, primarily helix 6 (Lutter *et al*., 2000; Kim *et al*., 2004). Although Bax was initially suggested to contain a C-terminal TM region (Suzuki *et al*., 2000), recent evidence indicates membrane binding probably involves coordinated insertion of helices 5, 6 and 9 (Garcia-Saez *et al*., 2004; Annis *et al*., 2005). Moreover, it appears that although helix 9 may insert into membranes, it is the insertion of helices 5 and 6 during apoptosis that is essential for apoptotic function (Nouraini *et al*., 2000; Heimlich *et al*., 2004; Cartron *et al*., 2005). Furthermore, the highly helical BH3 regions of Bid and Bax are not fully exposed until after the protein inserts into membranes (Cartron *et al*., 2005; Oh *et al*., 2005). An overview of the critical regions/residues of both Bid and Bax that are involved in recruiting the proteins to membranes is shown in Figure 2. The sections below provide a more detailed discussion of the regulation of the recruitment of Bid and Bax to membranes, as well as of the function of phosphorylation in regulating the recruitment of Bid, Bax and the BH3-only proteins Bim and Bad to membranes. Although there are differences in both the methods and extent to which these processes are regulated, there are enough similarities between Bid and Bax to suggest that Bid behaves like a pseudo-Bax molecule to initiate the cascade of Bax recruitment to membranes required for OMM permeabilization.

Migration of Bid to membranes

The BH3-only protein Bid was first cloned in 1996 based on its binding to both Bcl-2 and Bax (Wang *et al*., 1996) and was identified again in 1998 as a caspase-8 substrate (Li *et al*., 1998; Luo *et al*., 1998). These studies, among others (Gross *et al*., 1999), identified that the C-terminal p15 fragment (tBid) resultant from caspase-8 cleavage of Bid rapidly accumulated at mitochondria and initiated cytochrome *c* release. The site of caspase-8 cleavage in human Bid is Asp60 (Li *et al*., 1998). Caspase-8 cleavage of Bid drives membrane binding of tBid by relieving the inhibition mediated by the uncleaved N-terminal p7 fragment. Removal of this fragment from Bid has been suggested to increase the number of exposed hydrophobic residues thereby facilitating binding of the protein to membranes (McDonnell *et al*., 1999). Consistent with hydrophobic interactions holding the two fragments of Bid together, incubation with octyl glucoside is required to separate the fragments when purified Bid is cleaved with recombinant caspase-8 (Zha *et al*., 2000). However, our recent data reveal that in the presence of membranes, the p7 and tBid fragments spontaneously dissociate allowing tBid to insert into the membrane (Lovell *et al*., 2008). Therefore, we presume that in the presence of a membrane target, caspase-8-cleaved Bid undergoes a conformational change that displaces the p7 fragment and exposes sufficient additional hydrophobicity to drive insertion of the tBid fragment into the membrane.

Caspase-3, activated downstream of mitochondrial permeabilization, can also cleave Bid at residue 60 (Slee *et al*., 2000). Bid cleavage has also been shown to be mediated by several non-caspase proteases. A granzyme B cleavage site was detected at Asp75 of human Bid (Li *et al*., 1998; Sutton *et al*., 2000). Furthermore, granzyme B treatment of cells resulted in cleavage of Bid, accumulation of tBid at mitochondria and release of cytochrome *c* that was not inhibited by caspase inhibitors (Heibein *et al*., 2000; Sutton *et al*., 2003). Similar effects were observed when Bid was cleaved at Gly70 by the protease calpain (Chen *et al*., 2001; Mandic *et al*., 2002). Unlike tissue extracts from normal mice, similar extracts from *Bid*−/− mice failed to release cytochrome *c* from the component mitochondria upon treatment with lysosomal extracts, indicating that Bid may also be cleaved by cathepsins (Stoka *et al*., 2001; Reiners *et al*., 2002). Consistent with these results, treatment of neutrophils with type 1-fimbriated *Escherichia coli* induced lysosomal permeabilization, the release of cathepsins, cleavage of Bid and apoptosis (Blomgran *et al*., 2007). In this system, cleavage of Bid and mitochondrial permeabilization could be inhibited by cathepsin inhibitors, but not by caspase inhibitors. Similar to cleavage by caspases, granzyme B and calpain, cathepsins cleaved Bid in the loop region between helices 1 and 2, predominately at Arg71 for human Bid (Cirman *et al*., 2004). Finally, a small fraction of Bid becomes cleaved to generate a large C-terminal fragment by an unknown protease that is dependent on the kinase JNK (Deng *et al*., 2003). In this system, cleavage of Bid has been suggested to occur at Leu25 and the C-terminal fragment, termed jBid, accumulated at mitochondria. Expression of jBid in HeLa cells did not induce apoptosis, but did induce

preferential release of SMAC/DIABLO, and not cytochrome *c*, from the mitochondrial intermembrane space.

In all of these studies, when Bid was cleaved by any protease the C-terminal cleavage product accumulated at mitochondria. As the smallest N-terminal fragment removed from Bid occurs upon JNK-induced cleavage to produce jBid, removal of as little as the first 25 amino acids from Bid is sufficient to result in recruitment of the C-terminal fragment to mitochondria. This implicates the extreme N terminus of Bid as a negative regulatory sequence that ensures the cytoplasmic localization of Bid.

Although binding of jBid to mitochondria released SMAC/DIABLO from the mitochondrial intermembrane space, cytochrome *c* was not released even though it is a smaller protein than SMAC/DIABLO. Furthermore, apoptosis did not occur in this experimental setting (Deng *et al*., 2003). Cleavage of Bid with any of the other proteases described above led to full-blown mitochondrial permeabilization and apoptosis, indicating that the sequence located between amino acids 25 and 59 may contain a region that inhibits the pro-apoptotic function of Bid either directly or indirectly. Alternatively, cleavage at this site might lead to alternate folding of the C-terminal fragment. Sequence analysis of this region indicated the presence of a BH3-like region (termed BH3-B) that encompasses amino acids 35–43 and roughly overlaps with helix 2 of Bid (Tan *et al*., 1999). Separate expression of the N- and C-terminal fragments of caspase-8-cleaved Bid indicated that these fragments bind each other through interactions of the BH3-B region of the N-terminal fragment and the true BH3 region of the C-terminal fragment. Accordingly, mutations of the Bid BH3-B region that inhibit the binding interaction between this region and the true BH3 region created Bid variants that show apoptotic activity similar to that of tBid when expressed in cells. Taken together, these results suggest that the N-terminal fragment of Bid, which is lost upon caspase-8 cleavage, contains regions that negatively regulate the binding of Bid to membranes and prevent exposure of the Bid BH3 region.

Examination of the regions of Bid required for it to insert into membranes suggested that the central hairpin helices of Bid (helices 6 and 7) may be involved. Glycosylation mapping suggested that helix 6, or the combination of helices 6 and 7, could insert into membranes (Garcia-Saez *et al*., 2004). Furthermore, expression of various Bid fragments, either alone or as fusion proteins, strongly suggested the involvement of helix 6 in binding to membranes (Lutter *et al*., 2000; Hu *et al*., 2003; Kim *et al*., 2004; Garcia-Saez *et al*., 2005). Determination of the structure of membrane-bound tBid by electron paramagnetic resonance (EPR), NMR and CD spectroscopy indicated that although helices 6, 7 and the short C-terminal helix 8 all insert into the membrane, none is membrane-spanning and only a portion of each helix inserts into the bilayer (Gong *et al*., 2004; Oh *et al*., 2005). Furthermore, EPR studies indicate that insertion of helices 6–8 of Bid into the membrane accompanied by full exposure of the BH3 region is vital to the apoptotic function of tBid.

As will be described below, the features of Bid recruitment to and insertion into membranes are reflected in the mechanism by which Bax inserts into membranes. However unlike Bid, for Bax neither binding to nor insertion into membranes requires prior proteolytic cleavage. Instead the insertion of Bax into membranes is mediated by interactions with BH3-only proteins.

Insertion of Bax into membranes

Unlike Bid, the pro-apoptotic multi-BH region protein Bax does not require proteolytic cleavage to insert into membranes or for activation of apoptotic function. Rather, the binding of Bax to membrane-bound tBid can drive the insertion of Bax into the membrane bilayer. However, similar to Bid, the migration of Bax to membranes and the activation of Bax appear to require conformational changes at the N terminus of the protein.

Several early studies of the recruitment of Bax to membranes during apoptosis suggested that it might be regulated solely by a proposed C-terminal TM region (Wolter *et al*., 1997; Nechushtan *et al*., 1999). In these studies, removal of this TM region from GFP-Bax attenuated both the function and binding of Bax to membranes during apoptosis. Mutations in the putative TM region were also discovered that either attenuated or enhanced Bax binding to membranes (Nechushtan *et al*., 1999). In particular, removal of Ser184 of Bax or substitution of the residue with valine caused GFP-Bax to become constitutively localized at mitochondria and enhanced Bax cytotoxicity. On the basis of the structure of cytosolic Bax in which the putative TM sequence occludes the BH3 binding pocket (Suzuki *et al*., 2000), these results would suggest that activating mutations at position 184 perturb this binding. Releasing the putative TM sequence not only frees this sequence, potentially allowing it to insert into membranes, but also uncovers the hydrophobic binding pocket in which it normally resides.

When it was first discovered, exposure of an N-terminal Bax epitope (amino acids 14–23, recognized by the antibody 6A7) was thought to coincide with (or result from) Bax binding to mitochondria during apoptosis (Hsu and Youle, 1997, 1998). Further studies into Bax translocation to mitochondria indicated that recruitment to membranes could occur long before the cytochrome *c* release (Valentijn *et al*., 2003) and was reversible upon removal of the apoptotic stimulus (Gilmore *et al*., 2000). Using recombinant Bax (Yethon *et al*., 2003) suggested that Bax undergoes a reversible N-terminal conformational change that exposes the 6A7 epitope upon interaction with the surface of a membrane. Cocrystallization of a Bax peptide containing this epitope complexed with the 6A7 antibody revealed that the N-terminal conformational changes in Bax that expose the epitope involve unwinding of at least part of Bax helix 1 (Peyerl *et al*., 2007). These studies suggest that similar to Bid, N-terminal conformational changes in Bax may normally be prerequisite to, rather than a product of, Bax inserting into membranes.

In another approach to understanding the recruitment of Bax to membranes, an N-terminally truncated Bax, which arose from an internal translation initiation, was observed to insert into mitochondria spontaneously (Goping *et al*., 1998). This truncated Bax, termed BaxΔART for apoptosis-regulating targeting sequence, is equivalent to a Bax variant, Bax ψ, that is constitutively localized to mitochondria in human gliomas (Cartron *et al*., 2002b). Removal of the first 19 or 20 amino acids of Bax have consistently been shown to result in spontaneous insertion of the protein into mitochondria and in enhanced cytotoxicity in yeast (Priault *et al*., 2003; Arokium *et al*., 2004), in mammalian cells and using IVTR-produced proteins and rat or mouse liver mitochondria (Cartron *et al*., 2003, 2005). Similarly, calpain cleavage of Bax at Gln28 has been shown to cause spontaneous insertion of Bax into the OMM (Toyota *et al*., 2003). The Pro13 residue has been shown to be vital for the first 20 amino acids of Bax to inhibit Bax binding to membranes (Cartron *et al*., 2002a), as a P13V mutant of Bax displayed a similar phenotype to Bax ψ. Taken together, these data suggest that the first 20 amino acids of Bax, similar to the first ~25 amino acids of Bid, contribute to cytoplasmic localization in the absence of an apoptotic stimulus.

Although the first 20 amino acids of Bax have been shown to negatively regulate localization of Bax at mitochondria, Bax amino acids 20–37, which comprise the first helix of Bax, contribute to localization of the protein at mitochondria. Removal of helix 1 from Bax ψ completely ablates the constitutive mitochondrial localization of this protein (Cartron *et al*., 2003). Furthermore, when fused to the N terminus of RFP, this helix was shown to be sufficient to target the protein to (but not insert it into) mitochondria.

Studies of the first helix of Bax have also implicated it in binding to the BH3 region of Bid (Cartron *et al*., 2004). A mutation in helix 1 of Bax, D33A, completely abolished this interaction, as did several mutations in the Bid BH3 sequence, including an R84G mutant. The opposing charges of these two residues suggested that they may interact, and charge-swapping mutations (D33R of Bax and R84D of Bid) restored the interaction. In this study, the first helix of Bax was also shown to interact with the BH3-only protein Puma, another protein that may in some circumstances directly activate Bax (Kim *et al*., 2006). These studies indicate that the N terminus of Bax contains regions that both positively and negatively regulate targeting of the protein to membranes, and in binding to proteins such as tBid. However, it is Bax binding to tBid that drives the subsequent insertion of Bax into the OMM. Unlike Bid, regulation of Bax appears to require coordinated conformational changes at both the N terminus and the C terminus of the protein.

The function of the C-terminal helix of Bax, helix 9, in directing Bax to and inserting it into the OMM has been highly controversial. C-terminal fusion of the last 21 amino acids of Bax to GFP did not result in colocalization with mitochondria (Nechushtan *et al*., 1999). Although some studies have suggested that longer C-terminal fragments of Bax (23–24 amino acids) result in the insertion of soluble proteins into the OMM (Goping *et al*., 1998; Schinzel *et al*., 2004), other studies have indicated that even these fragments are insufficient (Cartron *et al*., 2003). When overexpressed in HeLa cells, comparisons of various Bax or GFP-Bax constructs showed that removal of the C-terminal 24 amino acids attenuated both the localization at mitochondria and function, even for a highly toxic Bax mutant that is missing the first 20 amino acids (Schinzel *et al*., 2004). This study also suggested that the regulated exposure of helix 9 of Bax required Pro168, as deletion of this residue or substitution with alanine had an effect similar to the removal of the C-terminal 24 amino acids. However, unlike this study and others described above (Wolter *et al*., 1997; Nechushtan *et al*., 1999), several studies using either mammalian cells or *in vitro* systems containing recombinant or IVT proteins synthesized in reticulocyte lysate and isolated mitochondria, C-terminal Bax deletions or mutation of Pro168 did not inhibit the translocation and toxicity of Bax upon apoptotic stimulus (Desagher *et al*., 1999; Cartron *et al*., 2002a, 2005).

Although conflicting, the results presented above suggest that the C-terminal TM region of Bax inserts into the OMM during apoptosis, as has been shown by chemical labeling of single-cysteine mutants (Annis *et al*., 2005) and by glycosylation mapping (Garcia-Saez *et al*., 2004). However, helix 9 of Bax does not appear to be absolutely necessary for the insertion of Bax into the OMM and the subsequent permeabilization of the membrane. Rather, this helix may primarily function in concealing the pore-forming helices 5 and 6 before an apoptotic stimulus. It appears that removal of helix 9 by itself is insufficient to drive Bax to membranes in the absence of an apoptotic stimulus or to inhibit localization of Bax at the membrane in the presence of an apoptotic stimulus. Forced displacement of this helix from its hydrophobic binding pocket through mutations overrides the need for N-terminal conformational changes to drive Bax to membranes. Finally, helix 9 does very little to inhibit the spontaneous binding of Bax to membranes and toxicity caused by N-terminal deletions/mutations. It is therefore likely that for Bax both recruitment to and insertion into the membrane are regulated by changes at the N and C terminus. The N-terminal conformational changes that have been indicated in the addressing of mitochondria and exposure of the Bax BH3 (Gilmore *et al*., 2000; Valentijn *et al*., 2003; Cartron *et al*., 2005) likely also result in the displacement of the C-terminal helix, allowing the exposure of the pore-forming helices 5 and 6 and the insertion of Bax into the membrane.

Subcellular localization of Bax, Bid and the BH3-only proteins Bim and Bad can be regulated by phosphorylation

Posttranslational modifications of Bcl-2 family proteins, in particular phosphorylation, can affect the function of

these proteins through a variety of mechanisms, including modulation of binding partners, alterations in subcellular localization and increased/decreased degradation rates (reviewed in Kutuk and Letai (2008)). Comparing the effects of phosphorylation on localization of Bcl-2 family proteins suggests that Bid is functionally more similar to multidomain pro-apoptotic proteins, such as Bax, than it is to the prototypical BH3-only proteins Bad and Bim.

Although phosphorylation regulates the conformational changes that ultimately lead both Bid and Bax to insert into membranes, temporal differences between the rate of insertion of tBid and Bax into membranes (Lovell *et al*., 2008) have resulted in different phosphoregulation strategies. Although phosphoregulation of Bax alters individual conformational changes that are involved in both the addressing and insertion of Bax into a membrane bilayer, phosphoregulation of the localization of Bid is achieved solely through modulation of the caspase-8 cleavage event. By allowing Bid to change conformation upon interaction with membranes, cleavage by caspase-8 triggers rapid accumulation of tBid at membranes. Similarly, phosphorylation of the BH3-only proteins Bad and Bim regulates their accumulation at membranes, although by a completely different mechanism. Phosphorylation of the BH3-only proteins Bad and Bim alters their interactions with other proteins that sequester them and thereby prevents their interaction with membranes.

Phosphorylation inhibits Bid cleavage by caspase-8

In human Bid there are two potentially phosphorylated residues (Thr59 and Ser65) in the vicinity of the caspase-8 cleavage site (Bid is cleaved after Asp60). Phosphorylation of Thr59 severely inhibited cleavage of Bid by caspase-8, whereas phosphorylation of Ser65 had no effect (Degli Esposti *et al*., 2003). Phosphorylation of Thr59 was achieved by casein kinase II, and presumably other cellular kinases. Similarly, phosphorylation of murine Bid (at Ser61 and Ser64) by casein kinase I and II also attenuated cleavage by caspase-8 cleavage (Desagher *et al*., 2001). Because cleavage of Bid is an essential prerequisite to its binding to membranes, phosphorylation effectively prevents Bid from binding to membranes.

Phosphorylation alters the conformational changes at both the N terminus and the C terminus of Bax that regulate its binding to membranes

There are several residues in Bax for which phosphorylation can either enhance or inhibit Bax binding to membranes. The most potent response to phosphorylation occurs at Ser184 in helix 9 of Bax. Mutations of this residue have been shown to alter the localization of Bax at membranes, either positively or negatively depending on the mutation (Nechushtan *et al*., 1999). Phosphorylation of Bax Ser184 by one of a variety of kinases, including Akt (Gardai *et al*., 2004; Xin and Deng, 2005) and PKCζ (Xin *et al*., 2007), leads to a decrease in the amount of Bax found at OMM during apoptosis and a corresponding reduction of apoptotic function. As reported above, similar results were obtained with a phospho-mimicking S184D mutation (Nechushtan *et al*., 1999). The proximity of Ser184 to Lys94 in the Bax structure (5.3 Å) (Suzuki *et al*., 2000) has been suggested to account for the effects of phosphorylation (Arokium *et al*., 2007), as an ionic interaction between phospho-Ser184 and Lys94 would inhibit the release of helix 9 required for Bax to insert into membranes.

In cerebellar granule neurons undergoing apoptosis, phosphorylation of Ser163, located in the loop between helix 8 and 9, increases the migration of Bax to mitochondria (Linseman *et al*., 2004). As expected, an S163A mutant Bax showed deficiencies in targeting to mitochondria. The location of this residue suggests that phosphorylation could be involved in either or both N- and C-terminal conformational changes. Ser163 is proximal to both Pro168 (a residue involved in the control of helix 9 exposure (Cartron *et al*., 2005)) and the weak ionic interaction between Arg9 and Asp154 that has been implicated in controlling the ART region (Arokium *et al*., 2007). It is possible that phosphorylated Ser163 might compete with Asp154 for interaction with Arg9 thereby destabilizing the structure of this region of Bax.

Finally, a PKA phosphorylation site has been predicted at Ser60 of Bax (Arokium *et al*., 2007). Ser60 is close to the ionic interaction between Asp33 and Lys64 that has been suggested to stabilize helix 1 of cytoplasmic Bax (Cartron *et al*., 2004). When expressed in yeast, Bax containing a phospho-mimicking S60D mutation spontaneously accumulated at mitochondria (Arokium *et al*., 2007). However, unlike other Bax mutations that localize Bax to mitochondria in yeast (that is, P168A), the S60D mutation did not cause release of cytochrome *c*, consistent with mutation of Ser60 inducing only N-terminal conformational changes in Bax.

Phosphorylation regulates the localization of the BH3-only proteins Bad and Bim

As described in previous sections, there are several key differences between the BH3-only proteins Bad and Bim and the Bax-like BH3-only protein Bid. The lack of defined structures for Bad and Bim suggests that it is unlikely that localization is regulated by conformational changes in the proteins. Hence, phosphoregulation of the subcellular localization of Bim and Bad probably occurs through another mechanism.

In the presence of survival signals (growth factors and cytokines), Bad is phosphorylated at three serine residues (Ser112, Ser136 and Ser155). Phosphorylation of these residues, primarily by Akt kinase, causes the cytoplasmic sequestration of Bad by the 14-3-3 proteins, preventing mitochondrial localization and interaction with membrane-bound anti-apoptotic proteins (Zha *et al*., 1996; Datta *et al*., 2000; Tan *et al*., 2000; Zhou *et al*., 2000). The initiation of apoptosis by a variety of mechanisms, including Ca^{2+} mobilizing agents or IL-3 withdrawal, leads to the activation of phosphatases

including PP2A (Chiang *et al*., 2003), PP2C (Klumpp *et al*., 2003) and the Ca^{2+}-dependent phosphatase PP2B/calcineurin (Wang *et al*., 1999). Dephosphorylation of Bad by these phosphatases releases the protein from 14-3-3 proteins and thereby leads to the accumulation of Bad at mitochondria. Alternately, phosphorylation of Bad by JNK or Cdc2 at an alternate serine residue, Ser128, has been reported to inhibit binding of Bad by 14-3-3 proteins and to increase its mitochondrial localization (Donovan *et al*., 2002; Konishi *et al*., 2002).

Similar to the results observed with Bad, Akt-mediated phosphorylation of BimEL at Ser87 causes sequestration by 14-3-3 proteins and inhibits localization at mitochondria (Qi *et al*., 2006). In addition, activation of JNK during apoptosis results in phosphorylation of human BimL at three residues (Ser44, Thr56 and Ser58) and induces relocalization of the protein to mitochondria (Lei and Davis, 2003). However unlike Bad, the Bim isoforms BimL and BimEL both contain a region that promotes binding to the dynein light chain of microtubules (Puthalakath *et al*., 1999) and sequesters these proteins in the cytoplasm. Thus, phosphorylation by JNK promotes mitochondrial localization of Bim primarily by releasing BimL and BimEL from dynein light chain.

Bid: the Bax-like BH3-only protein

The evidence outlined above suggests that Bid and Bax share several structural features and similar modes of recruitment to and insertion into membranes. Current evidence suggests that once initiated, Bax/Bak membrane permeabilization proceeds by an auto-activation pathway (Ruffolo and Shore, 2003; Tan *et al*., 2006). It appears that Bax auto-activation results from the interaction of cytoplasmic Bax with membrane-bound, activated Bax. In this model, the exposed BH3 of membrane-bound Bax acts as a ligand for another cytoplasmic Bax protein, triggering its insertion into membranes. An analogous model was invoked above to explain recruitment of Bax to membranes through an interaction with membrane-bound tBid, which can trigger the Bax/Bak auto-activation process. Thus in this model, membrane-bound tBid represents a pseudo-Bax molecule that recruits cytoplasmic Bax to the membrane.

A hypothetical representation of Bid and Bax migration to and insertion into membranes is presented in Figure 3. Although Bax translocation to membranes is tightly regulated by both the N-terminal and the C-terminal fragments of the protein, several features of Bid suggest that proteolytic cleavage primes this protein to bind membranes and trigger Bax/Bak activation. The N-terminal fragment of Bid generated by cleavage by caspase-8 contains helices 1 and 2, and both have been suggested to negatively regulate the apoptotic function of Bid. In the solution structure of human Bid, helix 1 makes contacts with the BH3 region located in helix 3 (Chou *et al*., 1999). Helix 2, although physically separated from the BH3 region by helix 1, has been suggested to contain a BH3-B region that physically interacts with and inhibits the true BH3 region of Bid (Tan *et al*., 1999).

In solution, the conserved hydrophobic BH3 residues of Bax, Leu59, Leu63, Ile66 and Leu70, are all facing the hydrophobic interior of the protein (Suzuki *et al*., 2000). In Bid these residues, Ile86, Leu90, Val93 and Met97, are more exposed, with the first two being involved in hydrophobic contacts with helix 8 (Chou *et al*., 1999) that are relieved during tBid insertion into membranes (Oh *et al*., 2005). Furthermore, although the hydrophobic binding groove of Bax is shielded in solution by helix 9, the absence of this helix in Bid leaves the surface of this protein rather hydrophobic. Taken together, these observations indicate that the primary barrier to the apoptotic function of Bid is N-terminal inhibition of binding to membranes, which is relieved by cleavage of Bid to produce tBid.

Although the molecular details of the conformational changes that underlie tBid binding to membranes after Bid cleavage are still vague, we propose that they may occur similarly to Bax (Figure 3a). Given that the N-terminus of Bax is highly flexible and undergoes reversible conformational changes upon exposure to a membrane surface (Yethon *et al*., 2003), it is not unlikely that similar events occur in Bid. Conformational changes that cause the further exposure of helix 1 of Bid at the surface of a membrane would lead to further exposure of the BH3 region, although this may then be masked by the BH3-B region located in helix 2. This hypothesis is supported by the fact that mutations in the BH3-B region can increase to toxicity of uncleaved Bid to levels similar to tBid (Tan *et al*., 1999). For full-length Bid, like Bax, these conformational changes are reversible; however, the transition from intramolecular to intermolecular regulation after caspase-8 cleavage likely drives further conformational changes that result in displacement of the N-terminal p7 fragment, the insertion into membranes of the C-terminal p15 (tBid) fragment and irreversible exposure of the BH3 region.

Although Bax interactions with membranes (Figure 3b) may be mechanistically similar to that of Bid/tBid, there are several features of Bax that suggest that the barrier to Bax insertion into membranes may be greater. First, the presence of helix 9 in the hydrophobic binding region blocks the exposure of the central hydrophobic helices 5 and 6 (Suzuki *et al*., 2000). Second, although the Bid helices 6,7 and 8 embed into the membrane bilayer (Oh *et al*., 2005), Bax helices 5, 6 and 9 are thought to span the bilayer (Annis *et al*., 2005), which would suggest a much larger conformational change. Finally, relief of N-terminal inhibition of binding to membranes occurs without proteolytic cleavage. The increased barrier to Bax insertion into membranes is likely overcome through its interaction with membrane-inserted tBid or another activating protein(s).

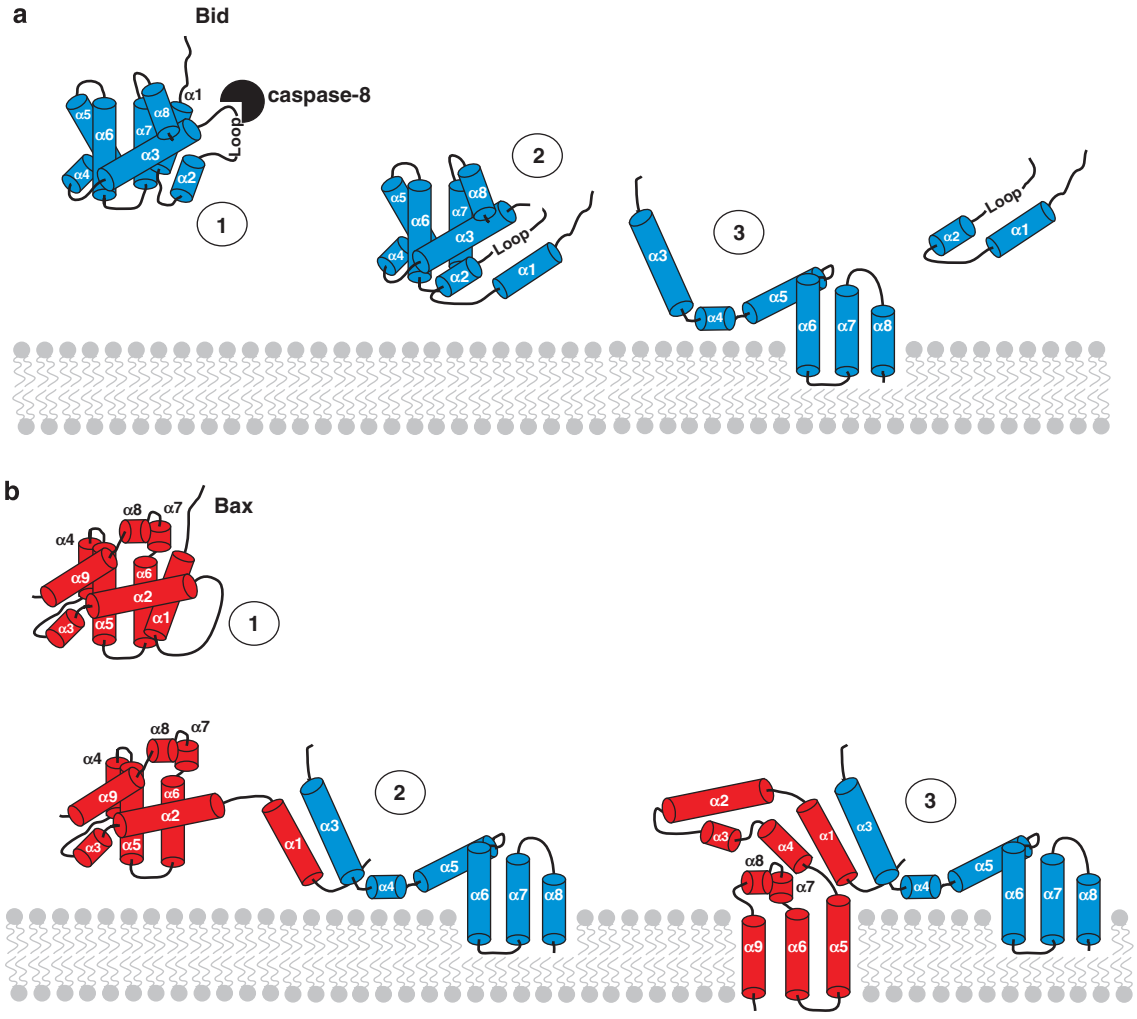

Figure 3 A proposed model for the insertion of Bid (**a**) and Bax (**b**) into membranes. (**a**) After the initiation of apoptosis, Bid is cleaved in the cytoplasm by caspase-8 or other proteases (step 1). As Bid approaches the surface of a membrane, N-terminal conformational changes result in the exposure of helix 1 (step 2). In the absence of proteolytic cleavage, this step is reversible. However, in the presence of proteolytic cleavage, further conformational changes drive the displacement of the p7 fragment, the insertion of helices 6, 7 and 8 as well as the full exposure of the BH3 region (helix 3, step 3). (**b**) Cytoplasmic Bax (step 1) undergoes N-terminal conformational changes that result in the exposure of helix 1 upon contact with the membrane surface (step 2). In the absence of membrane-embedded tBid, another Bax activator or membrane-embedded, active Bax, these conformational changes are reversible. However, membrane-embedded tBid drives the insertion of Bax helices 5, 6 and 9 into the membrane, exposing the Bax BH3 region (step 3). The insertion of Bax into membranes may be driven through interactions between helix 1 of Bax and helix 3 of Bid (the BH3 region) or through binding of the BH3 region of tBid to the hydrophobic cleft of Bax (not depicted).

As a soluble Bax molecule comes in contact with the membrane surface, a series of reversible conformational changes occur at the N terminus (Figure 3b). The presence of membrane-inserted tBid with a fully exposed BH3 region likely drives further conformational changes at the N terminus of Bax. In the solution structure of Bax, Asp33 (helix 1) forms an ionic interaction with Lys64 (helix 2); however, this residue has also been implicated in binding to Arg84 in the Bid BH3 region (Cartron *et al*., 2004). It is currently unclear whether the interaction of helix 1 of Bax and the BH3 region of tBid drives the displacement of helix 9, the membrane insertion of helices 5, 6 and 9 and exposure of the Bax BH3 region, or whether further interaction of tBid with Bax through the tBid BH3 and Bax hydrophobic binding pocket is necessary. Furthermore, although recent data from our lab suggest that tBid and Bax remain bound during membrane permeabilization (Lovell *et al*., 2008), the structure of a membrane-embedded tBid/Bax complex is not known. Currently, it is unknown if Bax auto-activation proceeds through a similar mechanism. However, in spite of all that is still unknown, an examination of the mechanisms of Bid and Bax binding to membranes reveals several similarities. For both Bid and Bax, these events proceed by a mechanism that is initiated by N-terminal conformational changes that direct the proteins to membranes. Insertion into the membrane is critically dependent upon the central hydrophobic helices and both proteins appear to contain a C-terminal helix that also inserts

into the membrane. Finally, insertion into the membrane is required for the full exposure of the BH3 region that propagates the apoptotic signal. The questions provoked by the assignment of Bid to the family of multiregion apoptosis regulators include the following: How have cells capitalized on the similarity in structure between Bid and Bax? What functional differences between Bid and the BH3 proteins depend on the unique structural features of Bid? And finally, do the unique properties of Bid provide previously unappreciated opportunities for pharmacologic intervention?

Conflict of interest

The authors declare no conflict of interest.

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