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Bax Crystal Structures Reveal How BH3 Domains Activate Bax and Nucleate Its Oligomerization to Induce Apoptosis

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SUMMARY

In stressed cells, apoptosis ensues when Bcl-2 family members Bax or Bak oligomerize and permeabilize the mitochondrial outer membrane. Certain BH3-only relatives can directly activate them to mediate this pivotal, poorly understood step. To clarify the conformational changes that induce Bax oligomerization, we determined crystal structures of BaxΔC21 treated with detergents and BH3 peptides. The peptides bound the Bax canonical surface groove but, unlike their complexes with prosurvival relatives, dissociated Bax into two domains. The structures define the sequence signature of activator BH3 domains and reveal how they can activate Bax via its groove by favoring release of its BH3 domain. Furthermore, Bax helices α2–α5 alone adopted a symmetric homodimer structure, supporting the proposal that two Bax molecules insert their BH3 domain into each other's surface groove to nucleate oligomerization. A planar lipophilic surface on this homodimer may engage the membrane. Our results thus define critical Bax transitions toward apoptosis.

INTRODUCTION

In response to most kinds of stress, the tripartite Bcl-2 family of proteins governs commitment of cells to apoptosis (Youle and Strasser, 2008). The death warrant is conveyed by distant cousins of Bcl-2, termed BH3-only proteins because they share with the family only the third of the four *B*cl-2 *H*omology (BH) domains. They use the BH3 domain, an amphipathic α helix, to engage and neutralize their prosurvival relatives and probably also to activate the critical effectors Bax and Bak, which seal the cell's fate by oligomerizing on the mitochondrial outer membrane (MOM) and provoking its permeabilization (Wei et al., 2001). The resulting release of cytochrome *c* and other pro-apoptotic proteins initiates a proteolytic cascade that ensures the cell's demise.

In healthy cells, Bax is largely cytosolic, but it translocates to the MOM when cells receive an apoptotic stimulus (Edlich et al., 2011; Wolter et al., 1997). Like the prosurvival Bcl-2 proteins (Muchmore et al., 1996), cytosolic Bax comprises a globular bundle of nine α helices (Suzuki et al., 2000). The last helix (α9) may regulate Bax activity, as it either anchors Bax in the MOM or resides in a hydrophobic groove on the surface of cytosolic Bax (Suzuki et al., 2000). The homologous groove on the prosurvival proteins, comprising mainly α2 through α5, is the canonical binding site for BH3 domains from both BH3-only proteins and Bax or Bak (Czabotar et al., 2011; Sattler et al., 1997).

Defining how Bax metamorphoses from an inert cytosolic monomer into the cytotoxic MOM-perforating oligomer has been deemed the "holy grail" of apoptosis research (Youle and Strasser, 2008). This pivotal step remains poorly understood, largely because no structure of any activated form of Bax (or Bak) has been available. Biochemical studies, however, have provided insights. First, certain nonionic detergents induce many of its conformational changes, including exposure of an N-terminal epitope, translocation to the MOM, and oligomerization (Hsu et al., 1997; Hsu and Youle, 1997). Also, studies with BH3 peptides, or full-length BH3-only proteins, in mitochondrial or liposomal systems suggest that certain BH3-only proteins termed "activators," notably Bid and Bim, can bind transiently to Bax and induce its activation, whereas the others, termed "sensitizers" (e.g., Bad), act instead by freeing the activators or Bax from prosurvival relatives (Certo et al., 2006; Kim et al., 2006; Kuwana et al., 2002, 2005; Letai, 2009; Llambi et al., 2011). The sequence characteristics distinguishing activators from sensitizers are unknown. Some experiments suggest that an activator BH3 domain binds transiently to a "rear pocket" on Bax, distal to its canonical surface groove (Gavathiotis et al., 2008, 2010; Kim et al., 2009), but how this interaction would displace α9 from the canonical site and lead to Bax oligomerization remains unclear. Recent biochemical studies suggest that certain BH3 domains instead activate the constitutively

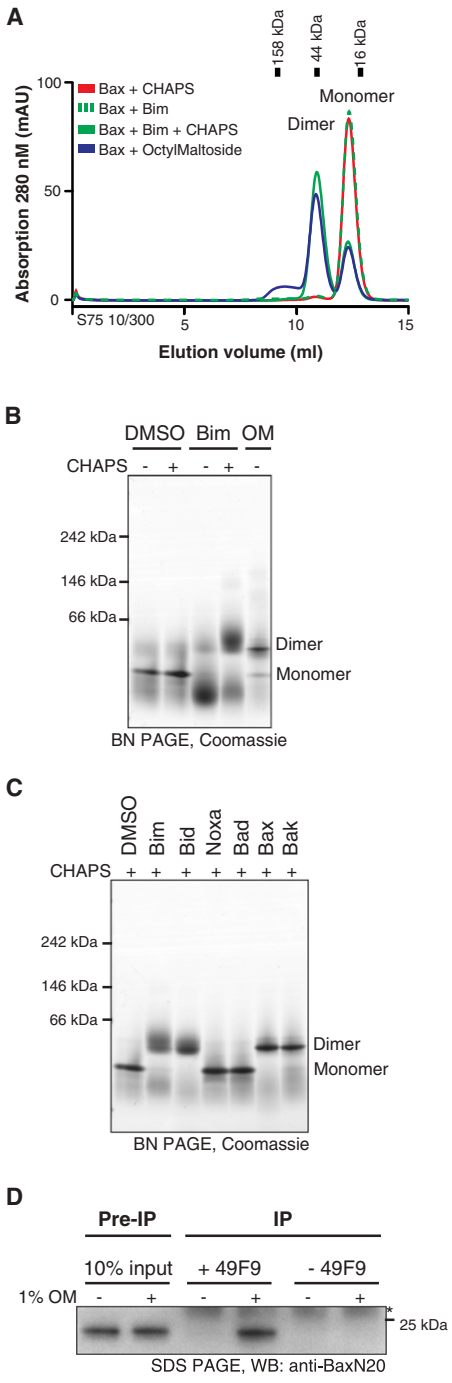


Figure 1. Dimerization of BaxΔC21 Promoted by Octylmaltoside or BH3 Peptides

(A) Gel filtration profile of BaxΔC21 treated with CHAPS, BimBH3, octylmaltoside (OM), or CHAPS and BimBH3.

(B) Blue native PAGE of BaxΔC21 showing dimerization with OM or with CHAPS plus BimBH3 peptide. The Bim-induced dimer probably is more diffuse than the OM one because the peptide partially disassociates during the run. The faster migrating monomer produced by BimBH3 alone may be indicative of an altered monomer conformation.

(C) Blue native PAGE of BaxΔC21, showing dimers in the presence of CHAPS and certain BH3 peptides, but not Noxa or Bad.

(D) SDS PAGE showing exposure of the N-terminal epitope 49F9 by immunoprecipitation of BaxΔC21 during treatment with octylmaltoside. As noted later, the epitope is exposed only transiently during the transition from monomer to dimer (Figure 5). Asterisk indicates immunoglobulin light chain. Data are representative of three experiments.

See also Figure S1.

MOM-associated Bak by binding to its canonical surface groove (Dai et al., 2011).

Homo-oligomerization of Bax requires its BH3 domain (the α2 helix) (Wang et al., 1998). Deletion of Bax segments suggests that its α2–α5 helices alone can oligomerize and that this core, together with α9, suffices for MOM permeabilization (George et al., 2007). Recent crosslinking studies suggest that the homo-oligomerization of both Bax and Bak starts when the BH3 domain of one monomer is exposed and engages the canonical binding groove (mainly α3–α5) of another activated monomer, forming "BH3-in-groove" dimers that multimerize by a separate interface (Bleicken et al., 2010; Dewson et al., 2008, 2009, 2012; Oh et al., 2010; Zhang et al., 2010). Other models of the oligomerization have also been suggested (Bogner et al., 2010; Pang et al., 2012; Westphal et al., 2011).

Here, we describe crystal structures that clarify the structural transitions of Bax. Because the hydrophobic α9 helix can compromise such studies by causing aggregation, we have primarily studied the C-terminally truncated BaxΔC21, which we consider a surrogate for Bax tail-anchored in the MOM. With this construct, we have obtained atomic structures of Bax in complex with BH3 peptides. They provide insights regarding Bax activation and the nature of activator BH3 domains. Extending those insights, we also describe a crystal structure of the Bax domain containing only helices α2 through α5. Significantly, the structure reveals a symmetric homodimer closely akin to that proposed to nucleate Bax and Bak oligomerization (Dewson et al., 2008). The findings not only define important transitions in Bax on the path to apoptosis but also provide a structural basis for eventually manipulating Bax activity pharmacologically.

RESULTS

Dimerization of BaxΔC21

Treatment of BaxΔC21 with the nonionic detergent octylmaltoside produced primarily a dimer, as assessed by gel filtration and blue native PAGE (Figures 1A and 1B). Alone, the detergent CHAPS, which does not itself affect the Bax structure (Hsu and Youle, 1998), did not induce dimers, but CHAPS plus certain BH3 peptides, namely those from Bim, Bid, Bax, and Bak, but not those from Bad or Noxa, readily dimerized BaxΔC21 (Figure 1C). The responses of BaxΔC21 to detergents resemble those of full-length Bax in cell lysates (Hsu and Youle, 1997, 1998), and the observed specificity of the BH3 peptides is consistent with peptide-induced activation of the full-length protein (Letai et al., 2002), suggesting that this construct is relevant to the behavior of the native protein and that the assay reflects Bax activation. Furthermore, the N-terminal epitope for antibody 49F9 (Figure 1D and Figure S1 available online), which only recognizes Bax in etoposide-treated cells (Figure S1B), was exposed in BaxΔC21 during treatment with octylmaltoside and

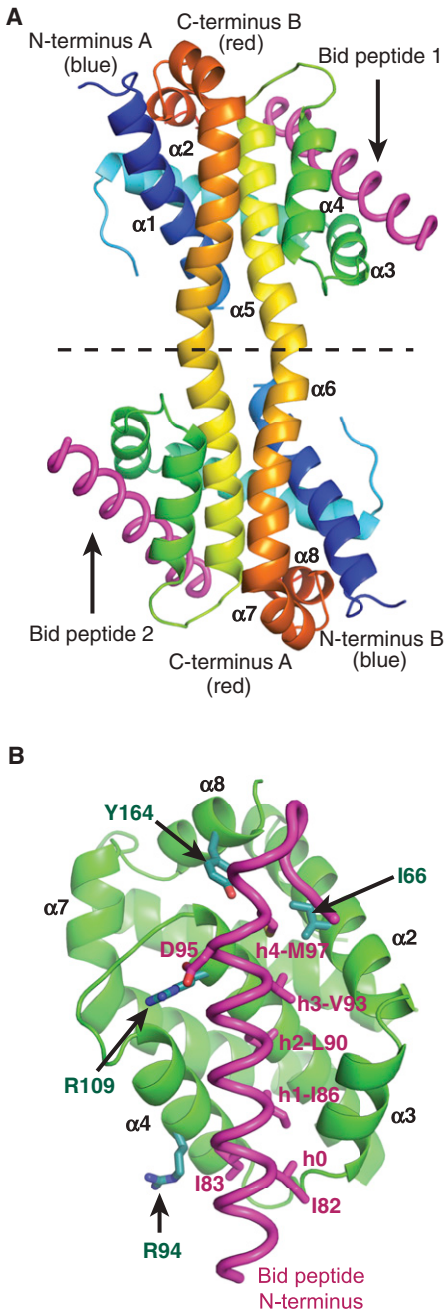


Figure 2. Structure of BidBH3:BaxΔC21 Complex

(A) Dimer of BaxΔC21, colored rainbow (blue N terminus to red C terminus) in complex with BidBH3 (magenta), illustrating domain swapping of helices 6–8 (colored orange and red) across the dotted line.

(B) BidBH3 peptide (magenta) projects four canonical hydrophobic residues (h1 through h4) into the canonical binding groove of Bax (green). The h0 residues I82 and I83 make additional interactions with the Bax groove. Bid D95 forms a salt link to Bax R109 (cyan). Other Bax side-chains shown (cyan) are R94 (close to Bid I83) and I66 and Y164 (both close to Bid M97).

See also Figure S2.

modestly by BimBH3 plus CHAPS (Figure S1C), just as other nonionic detergents expose the related 6A7 epitope in full-length Bax (Hsu and Youle, 1997).

BH3 Peptides Bind BaxΔC21 in Its Groove

Purified Bax dimer induced by the BidBH3 peptide and CHAPS (as in Figure 1A) was reconstituted with a 2-fold molar excess of the peptide and crystallized. The structure (Table S1 and Figure 2A) reveals a domain-swapped dimer topologically identical to that reported for Bcl-x_LΔC exposed to high pH (O'Neill et al., 2006). As is evident with that Bcl-x_LΔC dimer, we conclude that the globular Bax structure, comprising helices α1–α5 from one polypeptide and helices α6–α8 from the other (region above dotted line in Figure 2A), represents the structure of the BaxΔC21 monomer, except for the altered conformation at the α5–α6 corner. We address the significance of this domain-swapped dimer later.

Notably, the BidBH3 peptide occupies the canonical surface groove (Figure 2B) rather than the proposed BH3 peptide-binding site on Bax (Gavathiotis et al., 2008) (see Discussion). The association resembles that of BH3 peptides engaging prosurvival relatives. The peptide is α-helical (Figures S2A and S2B) from its N terminus (S76) to M97 but is loosely ordered thereafter. It interacts with Bax by inserting the four signature BH3 hydrophobic residues, h1 through h4 (Bid I86, L90, V93, and M97), into the hydrophobic groove. These contacts, and the salt bridge between Bid D95 and Bax R109 in α5, mirror those of BH3 peptides with prosurvival proteins (Sattler et al., 1997) (see also Figure S2B). Notably, we observed additional hydrophobic interactions between residues near the N terminus of the Bid peptide (I82/I83), designated "h0," with the Bax α3 and α4 helices (Figure 2B). No significant interactions appeared C-terminal to Bid M97 (h4).

We similarly crystallized BaxΔC21 in complex with the BaxBH3 peptide and determined its structure (Table S1 and Figures S2B–S2E), which closely resembles that of the BidBH3 complex. Again, the peptide is loosely ordered beyond the h4 residue (L70). This complex is relevant to Bax oligomerization because three independent lines of evidence suggest that it mimics the Bax structure formed during apoptosis. First, in the Bax oligomer formed by an apoptotic stimulus, Bax BH3 residue T56 comes close enough to Bax R94 in the groove to allow efficient disulfide linkage of T56C to R94C (Dewson et al., 2012), in accord with the short distance between their Cβ atoms (4.9 Å) in the BaxBH3:BaxΔC21 complex (Figure S2D). Second, electron paramagnetic resonance (EPR) studies on activated Bax in liposomes (Bleicken et al., 2010) place the two C62 residues within a dimer 24 Å apart, similar to the 21.5 Å in the BaxBH3:Bax structure (Figure S2E). Finally, site-specific photocrosslinking also places the Bax BH3 region in the interface of a symmetric Bax homodimer (Zhang et al., 2010). These results are in full accord with the BH3-in-groove homodimeric structure described below.

Sequence Signature of Activator BH3 Domains

To identify specific BH3 residues that convey activator function, we tested structure-guided mutant peptides (Figure 3A) for capacity to bind to BaxΔC21 and dimerize it, as well as to induce full-length Bax to permeabilize liposomes and mitochondria

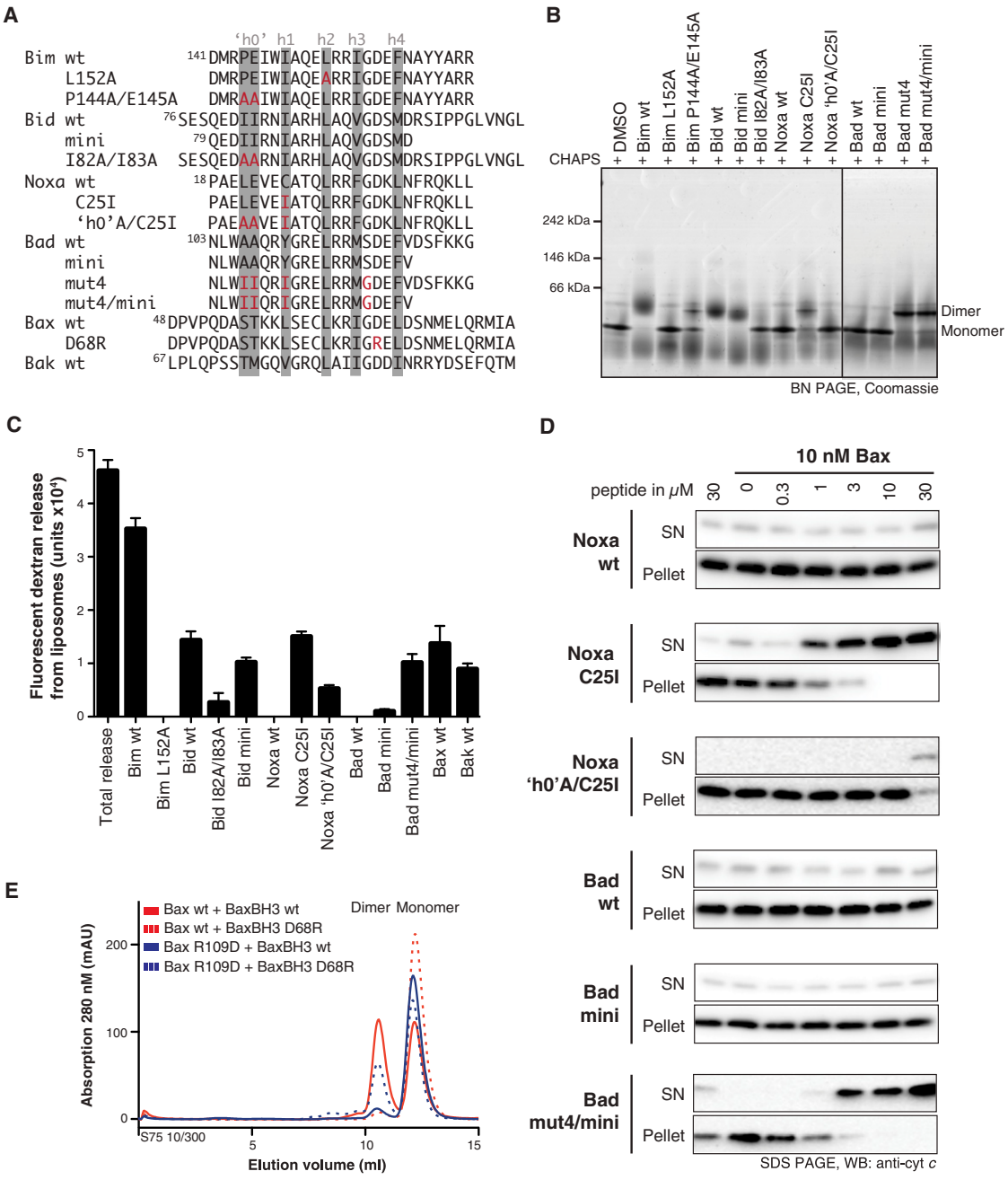


Figure 3. Characteristics of Activator BH3 Domains

(A) BH3 peptide sequences.

(B) Blue native PAGE assays of CHAPS/peptide-induced dimerization of BaxΔC21.

(C) Release of fluorescent dextran from liposomes exposed to full-length Bax and BH3 peptides. Note especially loss of function in Bid I82A/I83A and gain of function in Noxa C25I and Bad mut4/mini. Error bars represent SEM of at least three independent experiments.

(D) Noxa mutant C25I and Bad mut4/mini, but not wild-type peptides nor NoxaC25I with Ala at h0, release cytochrome *c* from mouse liver mitochondria derived from *Bak*$^{-/-}$ mice and reconstituted with recombinant wild-type, full-length Bax. SN, supernatant. See also Table S2. (Bad mut4 was not included in liposomes or MLM experiments as it permeabilized these in the absence of Bax.)

(E) The Bax groove mutant R109D is not dimerized by CHAPS with wild-type BaxBH3 peptide but, rather, is with the Bax peptide D68R, which cannot dimerize wild-type Bax. Data are representative of three experiments.

See also Figure S3.

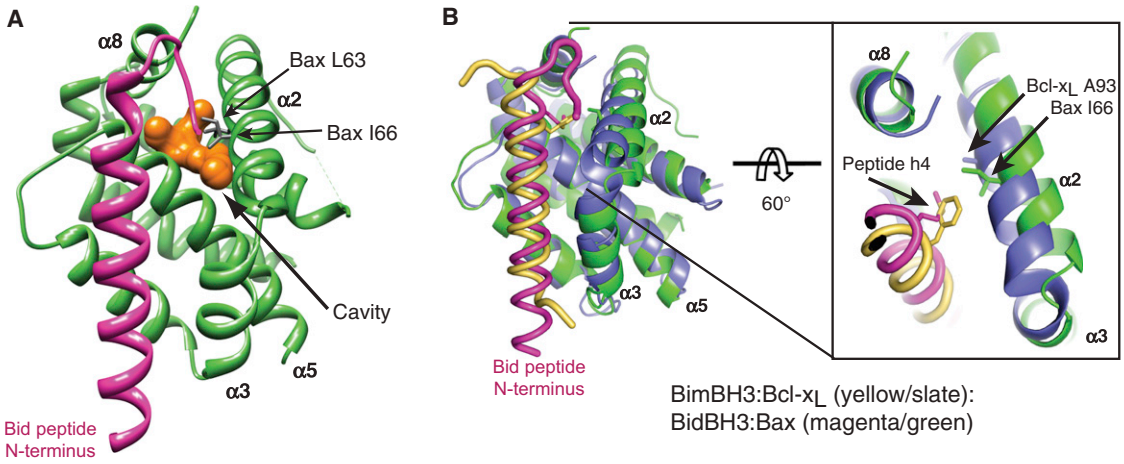


Figure 4. Comparison with Structures of Bcl-x_L:BH3 Complexes

(A) Cavity (orange, 140 Å^3) in the hydrophobic core of BidBH3:Bax complex between α2, α5, and α8. The cavity is adjacent to Bax α2 residues L63 and I66 (side chains shown in gray). The largest comparable cavity in BimBH3:Bcl-x_L is 20 Å^3.

(B) Overlay of BidBH3:Bax with BimBH3:Bcl-x_L and zoom showing relative displacement of the Bax α2 and the contacts between hydrophobic residues on the BH3 domains of protein (h3 residues Bcl-x_L A93/Bax I66) and peptide (h4 residues Bid M97/Bim F159).

See also Figure S4.

(Figures 3B–3D and S3A–S3D). Bid mini (truncated at D98) retained activity, suggesting that residues C-terminal to h4 are not essential. Notably, Bid I82A/I83A lost activity in all assays, establishing the importance of these h0 residues (Figure 2B) for Bid's activating function. The h1 position is also important because a single-residue replacement to Noxa at h1 (C25I) rendered it both able to activate Bax in biochemical assays and to trigger Bax-mediated cytochrome *c* release from mouse liver mitochondria (Figures 3B–3D). This result was not due to increased binding of Noxa C25I to prosurvival proteins (Table S2). Similarly, four replacements in Bad at the h0, h1, and to the unusual Ser after h3 made Bad mut4 (A106I/A107I/Y110I/S118G) an activator (Figure 3). The apparent anomaly that some activator sequences (Bim and Noxa C25I) have glutamic acid in h0 rather than isoleucine was resolved when we noted that the BidBH3:BaxΔC21 structure would allow a glutamic acid in h0 to form a salt bridge with Bax Arg94 (Figure 2B). Indeed, Bim and Noxa mutants (Bim P144A/E145A, Noxa h0 A/C25I) confirmed that either glutamate or isoleucine in h0 promotes activator function (Figure 3B).

To confirm that binding of the peptides into the Bax groove was critical, we mutated the conserved arginine (R109) in the Bax groove to aspartate to preclude its association with the invariant aspartate (D68) in the Bax BH3 (Figures 3A and S2B). As expected, Bax R109D was not dimerized by the Bax wild-type BH3 peptide but was dimerized by the charge-swap mutant BaxBH3 peptide D68R (Figure 3E). Hence, the phenomena monitored here require peptide binding in the groove.

Structural Consequences of Activator Peptide Binding to Bax

Although the BidBH3:BaxΔC21 complex resembles prosurvival proteins with bound BH3 peptides, notable differences explain how activator BH3-only proteins trigger structural transitions in Bax. A striking anomaly of the BidBH3:BaxΔC21 structure is the presence of a cavity (Dundas et al., 2006) between Bax helices α2, α5, and α8 (Figure 4A), also seen in the BaxBH3:BaxΔC21 complex (Figure S4A). By contrast, the structure of BaxΔC21 without a bound BH3 peptide (see below) and those of prosurvival family members bound to BH3 peptides contain no cavity. Cavities destabilize globular proteins (Baase et al., 2010), in this case promoting extrusion of Bax α2, its BH3 domain. Release of this domain is a critical step in Bax (and Bak) dimerization (see Discussion).

Structural features associated with the cavity are revealed in an overlay of the structures of BidBH3:BaxΔC21 with BimBH3:Bcl-x_L (Lee et al., 2009; Liu et al., 2003). The C-terminal half of Bax α2 is more removed from the Bid peptide than their counterparts in BimBH3:Bcl-x_L (Figure 4B). Furthermore, the Bid peptide is slightly displaced toward its C-terminal end compared to Bim bound to Bcl-x_L. Similar features are seen in the BaxBH3:BaxΔC21 structure (Figure S4B). Sequence differences between Bcl-x_L and Bax help to explain these discrepancies (Figure S4C). First, Bax has two fewer residues than Bcl-x_L in the α2/α3 corner, and this affects the conformation of adjacent residues on α2 and α3. Second, the third signature hydrophobic residue (h3) in the BH3 motif of Bax (I66) and Bak (I81) is invariably a large hydrophobic residue, usually isoleucine, whereas four of the six human prosurvival proteins have a small residue, alanine, in this position. Thus, in the BidBH3:BaxΔC21 structure, Bid h4 residue M97 contacts Bax I66 in α2, whereas in the BimBH3:Bcl-x_L structure Bim F159 (h4) abuts A93 (h3) in Bcl-x_L α2 (Figure 4B). Accordingly, Bim F159A and Bid M97A peptides have reduced capacity to dimerize BaxΔC21 and have even less capacity with BaxΔC21 I66A (Figure S4D).

The other marked effect on BaxΔC21 of the peptide binding was its transformation from monomer to dimer, which is addressed next.

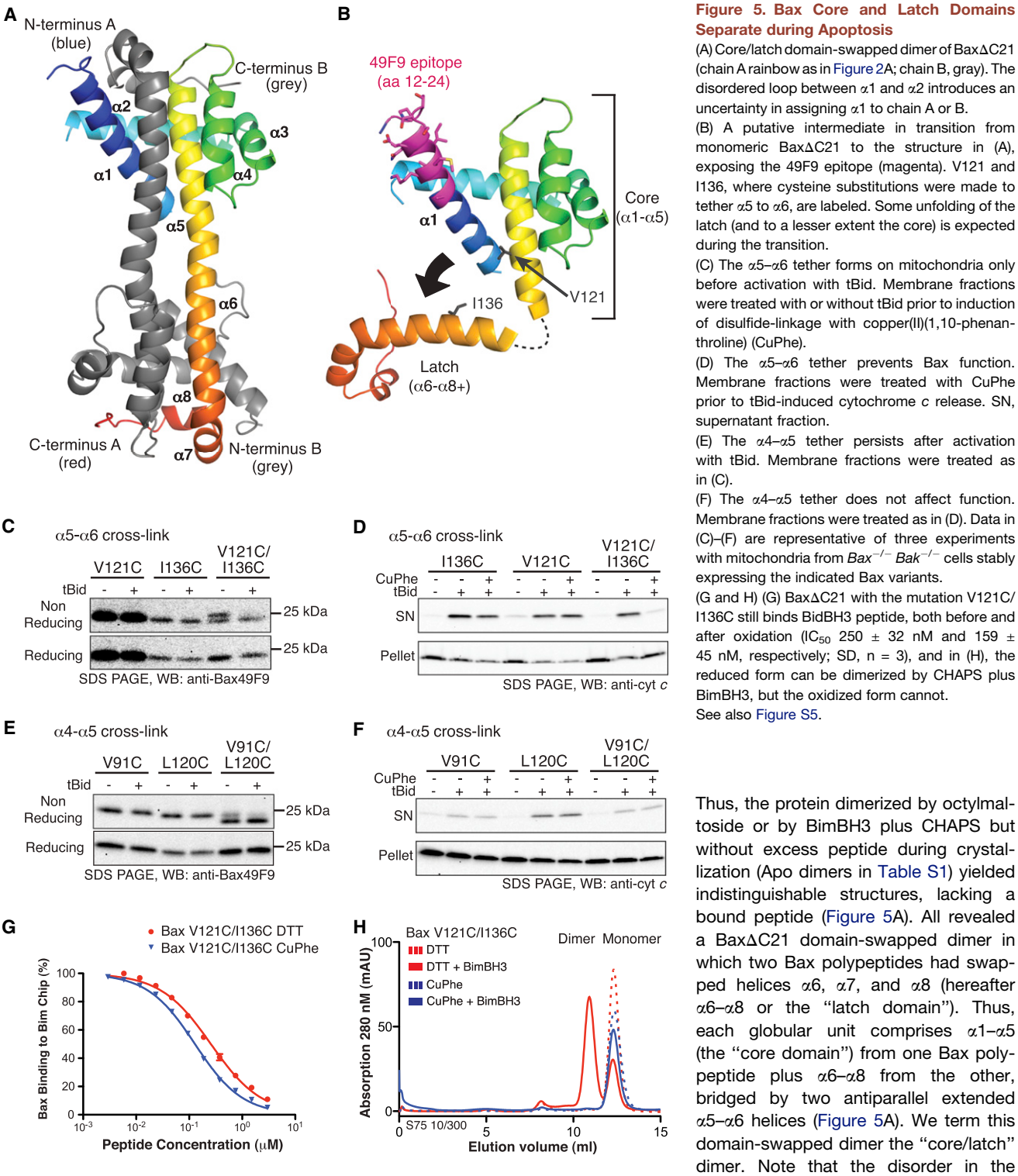


Figure 5. Bax Core and Latch Domains Separate during Apoptosis

(A) Core/latch domain-swapped dimer of BaxΔC21 (chain A rainbow as in Figure 2A; chain B, gray). The disordered loop between α1 and α2 introduces an uncertainty in assigning α1 to chain A or B.

(B) A putative intermediate in transition from monomeric BaxΔC21 to the structure in (A), exposing the 49F9 epitope (magenta). V121 and I136, where cysteine substitutions were made to tether α5 to α6, are labeled. Some unfolding of the latch (and to a lesser extent the core) is expected during the transition.

(C) The α5–α6 tether forms on mitochondria only before activation with tBid. Membrane fractions were treated with or without tBid prior to induction of disulfide-linkage with copper(II)(1,10-phenanthroline) (CuPhe).

(D) The α5–α6 tether prevents Bax function. Membrane fractions were treated with CuPhe prior to tBid-induced cytochrome *c* release. SN, supernatant fraction.

(E) The α4–α5 tether persists after activation with tBid. Membrane fractions were treated as in (C).

(F) The α4–α5 tether does not affect function. Membrane fractions were treated as in (D). Data in (C)–(F) are representative of three experiments with mitochondria from $Bax^{-/-}$ $Bak^{-/-}$ cells stably expressing the indicated Bax variants.

(G and H) (G) BaxΔC21 with the mutation V121C/I136C still binds BidBH3 peptide, both before and after oxidation (IC_{50} 250 ± 32 nM and 159 ± 45 nM, respectively; SD, n = 3), and in (H), the reduced form can be dimerized by CHAPS plus BimBH3, but the oxidized form cannot.

See also Figure S5.

The Domain-Swapped Dimer Suggests that Bax Separates into Two Domains

Untreated BaxΔC21 is a monomer (Figure 1), as NMR confirms (Figure S5A), but BaxΔC21 activated in various ways routinely yielded crystal structures of domain-swapped dimers. Thus, the protein dimerized by octylmaltoside or by BimBH3 plus CHAPS but without excess peptide during crystallization (Apo dimers in Table S1) yielded indistinguishable structures, lacking a bound peptide (Figure 5A). All revealed a BaxΔC21 domain-swapped dimer in which two Bax polypeptides had swapped helices α6, α7, and α8 (hereafter α6–α8 or the "latch domain"). Thus, each globular unit comprises α1–α5 (the "core domain") from one Bax polypeptide plus α6–α8 from the other, bridged by two antiparallel extended α5–α6 helices (Figure 5A). We term this domain-swapped dimer the "core/latch" dimer. Note that the disorder in the loop connecting α1 to α2 leaves open the possibility that α1 is part of the latch rather than the core domain.

We suggest that the unfolding event that dissociates the core and latch domains (Figure 5B) is on the pathway to Bax oligomerization, but the core/latch dimer itself is off pathway because its

structure (Figures 2A and 5A) is inconsistent with all data on interfaces in Bax (or Bak) oligomers formed during apoptosis (Bleicken et al., 2010; Dewson et al., 2008, 2012; Zhang et al., 2010). In our experiments, the absence of orienting membranes and the increased stability of domain-swapped structures over their monomeric counterparts (Bennett and Eisenberg, 2004) most likely promoted formation of the off-pathway core/latch dimer.

We propose that, when an activator such as tBid or Bim engages Bax, for example, when anchored in the MOM via α9, it triggers release of the core from the latch, and this suffices to expose the N-terminal epitope (Figures 1D and 5B). In our biochemical experiments, this exposure is transient due to the formation of the core/latch dimer. At the MOM, the membrane environment, together with the activator BH3 domain (Lovell et al., 2008), helps to "unlatch" the core domain and direct the oligomerization along a path that precludes the core/latch dimer (see Discussion).

Tethering Helix α5 to α6 Inactivates Bax

Because the proposed "unlatching" model suggests that Bax activation disengages α5 from α6 (Figure 5B), tethering the two helices together by a disulfide bond might inactivate Bax. To test this hypothesis, we engineered a cysteine into α5 (V121C) and α6 (I136C) and introduced these single mutations and the double mutation V121C/I136C within full-length Bax (C62S, C126S) also bearing the mutation S184L in α9 to render Bax MOM-associated (Fletcher et al., 2008; Nechushtan et al., 1999). All mutants retained apoptotic function when stably expressed in $Bax^{-/-}Bak^{-/-}$ fibroblasts (Figures S5F and S5G), and the oxidized, recombinant V121C/I136C mutant retains structure (Figure S5H) and capacity to bind BH3 peptides, though not to dimerize (Figures 5G and 5H). Consistent with dissociation of α5 and α6 upon Bax activation, disulfide linkage of the double cysteine mutant, as indicated by aberrant migration on nonreducing SDS-PAGE (Ruiz et al., 2010), was lost after tBid treatment (Figure 5C). A disulfide tether between α5 and α6 blocked tBid-induced cytochrome *c* release, whereas oxidation of the single cysteine variants had no effect (Figure 5D). Bax with cysteines introduced instead into both α4 and α5 remained functional even when disulfide tethered (Figures 5E, 5F, S5F, and S5G). These results argue that α5 and α6 must dissociate during Bax activation and that V121 and I136 are not proximal after activation (Figure 5B).

Structure of a Bax BH3-in-Groove Homodimer

To study the structural properties of the unlatched core domain while precluding formation of the off-pathway core/latch dimer, we made Bax constructs lacking the latch domain. Because the isolated core domain (α1–α5) would expose hydrophobic surfaces that might promote aggregation, we fused it to green fluorescent protein (GFP) to facilitate crystallization (Suzuki et al., 2010). We also made a GFP-Bax(α2–α5) fusion because α2–α5 suffices for oligomerization (George et al., 2007).

The GFP was dimeric when expressed alone, but both GFP-Bax fusions behaved mainly as a tetramer on gel filtration (Figure S6F), suggesting that the Bax core domain, with or without α1, had dimerized and linked GFP dimers. The purified GFP-Bax(α2–α5) tetramer was crystallized, and its structure was determined by molecular replacement, phasing the Bax(α2–α5) from the GFP (Table S1 and Figures S6A and S6B). Although the diffraction data extend to 3 Å resolution, that of the Bax(α2–α5) domain is significantly lower (estimated ~4 Å). In the crystal, two dimers of Bax(α2–α5) have indeed linked two GFP dimers into tetramers (Figures S6C and S6D). Because the two Bax polypeptides within each dimeric unit are fused to different GFP dimers, GFP dimerization did not cause Bax(α2–α5) dimerization.

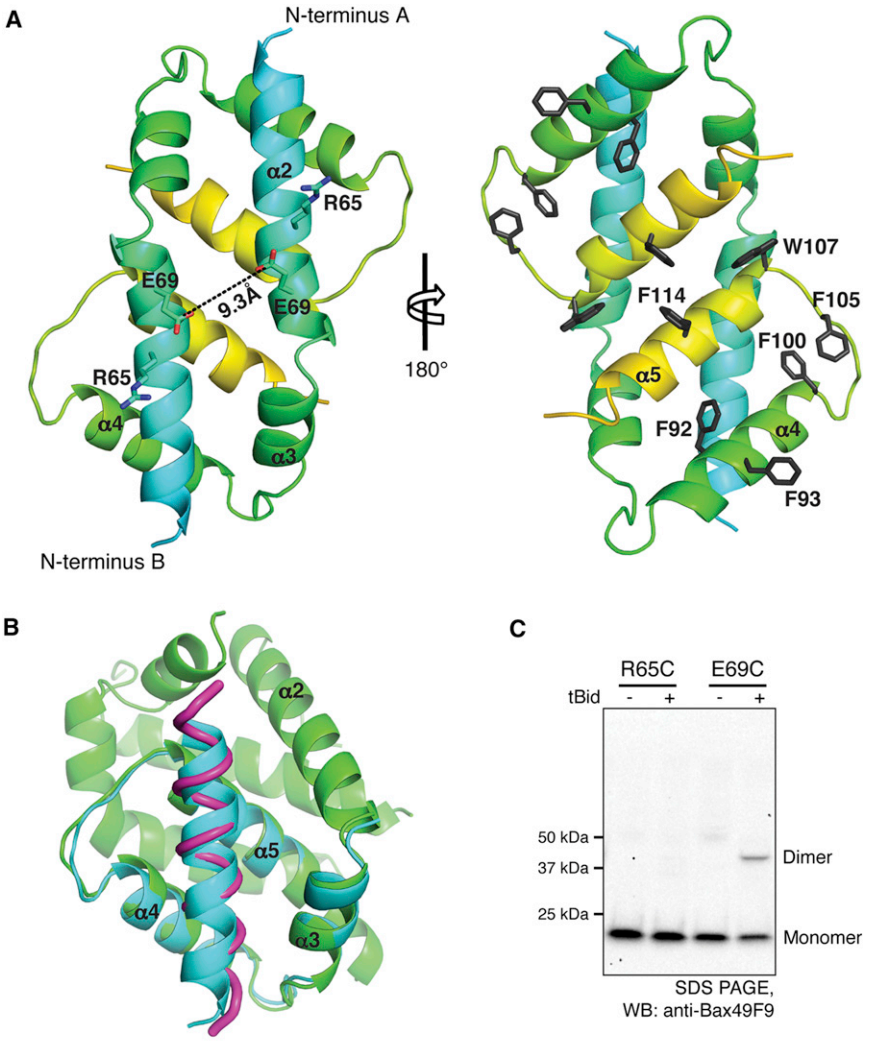
Bax(α2–α5) formed a symmetric dimer in which helix α2 (the BH3 domain) of each monomer engages helices α3–α5 of the other monomer (Figure 6A). One half of the dimer, i.e., α2 of one monomer and α3–α5 of the other, closely resembles the complex between the BaxBH3 peptide and BaxΔC21 (Figure 6B). Thus, this dimer is held together by contacts made in both of its BH3-in-groove interfaces. The two antiparallel α2 helices (BH3 domains) are close together, their E69 residues being only 9.3 Å apart. Accordingly, when stably expressed in $Bak^{-/-}Bax^{-/-}$ MEF, full-length mitochondrial Bax E69C residues exposed to an apoptotic stimulus (tBid) could be crosslinked with the 8 Å linker BMOE, whereas residues farther apart (R65C) could not (Figure 6C).

This homodimer has a layer of two α2 and α3 helices stacked above a layer of two α4 and α5 helices. The angle between helices α2 and α3 within a Bax polypeptide is ~120° versus ~90° in the BaxBH3:Bax complex, juxtaposing the two hydrophobic α5 helices. Whereas in monomeric BaxΔC21 the latch domain buries the hydrophobic surfaces of α4 and α5, here they form a continuous planar surface with their counterparts on the partner molecule (Figure 6A). Six of the eight aromatic amino acids in Bax(α2–α5)—F92, F93, F100, F105, W107, and F114—and their symmetry equivalents in the dimer localize to this lipophilic surface, which may represent a region of membrane contact (see below).

These results strongly support the concept that the BH3-in-groove symmetric dimer is the fundamental unit of the Bax oligomer.

DISCUSSION

Our studies with BaxΔC21 reveal structural transitions that drive inactive, monomeric Bax on the path to an apoptotic oligomer. With this construct, we cannot directly address the earliest Bax transitions, which presumably occur on cytosolic Bax, where α9 occupies the α2–α5 groove (Gavathiotis et al., 2008; Kim et al., 2009). Although the core/latch dimer we describe is unlikely itself to figure in oligomerization, it has revealed an unsuspected Bax transition: its N-terminal core (α1–α5) disengages from the C-terminal latch (α6–α8). This transition (Figure 5B) appears to be critical for Bax function because tethering α5 to α6 ablated cytochrome *c* release by full-length Bax (Figure 5C). That result might seem incompatible with a model in which α5α6 hairpins enter the MOM during its permeabilization (Annis et al., 2005). Our model only requires that these helices separate in an essential early step. We have not addressed whether or not α5 and α6 might reform a hairpin during MOM



half of a BH3-in-groove dimer (both cyan)
BaxBH3:Bax (magenta/green)

Figure 6. The BH3-in-Groove Symmetric Dimer Formed by Bax(α2–α5)

(A) Each polypeptide is colored rainbow as in Figure 5B, cyan at the N terminus of α2 through yellow at the C terminus of α5. The helices of one polypeptide are labeled. E69 and R65 are indicated in the left view, and aromatic residues concentrated on one surface are indicated in the right view, the putative "membrane view."

(B) Overlay of one half of the above dimer (α2 from one polypeptide, α3–5 from the other) with the BaxBH3:BaxΔC21 complex (see Figure S2B).

(C) Bax E69C can be crosslinked on mitochondria. Membrane fractions from *Bax*$^{-/-}$ *Bak*$^{-/-}$ cells stably expressing the indicated Bax S184L variants were treated with or without tBid prior to crosslinking with BMOE. Note that R65C one turn away on helix α2 cannot crosslink.

See also Figure S6.

permeabilization. If they do, the disulfide-linkage data argue that they assume a different orientation.

Activator BH3 Binding Initiates Bax Conformational Changes

We propose that the globular structure of BaxΔC21 complexed with the Bid or Bax BH3 peptide (i.e., the globule above the dotted line in Figure 2A) represents the complex that monomeric Bax forms with a direct activator on the MOM. Notably, cavities appear in the Bax:BH3 peptide structures at the interface between the core and latch domains (Figures 4A and S4A). No such cavities appear in the equivalent Bax structures without the bound peptide, nor do they appear in the complexes of BH3 peptides with prosurvival Bcl-2 relatives. Because such cavities are destabilizing (Baase et al., 2010), these findings strongly suggest that a bound activator BH3 peptide induces a metastable Bax structure that facilitates release of the core and its α2 segment. These structures thus resolve the central mystery of how the Bax BH3 domain is liberated to trigger Bax oligomerization. Whereas prosurvival family members remain intact on binding a BH3 peptide, Bax instead releases its core domain and its own BH3 domain (α2). The freed Bax α2 can then compete with an activator BH3 domain, e.g., that of Bid, for binding another multidomain family member; its sequestration by a prosurvival relative can preserve mitochondrial integrity, whereas its engagement of another Bax molecule sets it on the path to MOM permeabilization.

Exposure of an N-terminal epitope, a hallmark of Bax in dying cells, is thought to require release of α1, but core/latch dissociation offers an alternative mechanism. We suggest that the unlatched form occurs in activated full-length Bax monomers associated with membranes or liposomes. Pertinently, excision of its N-terminal 19 or 20 residues targets Bax to the MOM (Cartron et al., 2002; Goping et al., 1998). Because I19, at the α1 N terminus, packs against L149 and I152 in α7, its absence is likely to facilitate core/latch separation. Thus, N-terminal mutations promoting Bax activation may act by decreasing Bax stability, allowing core/latch disengagement. Certain C-terminal deletions may also promote their dissociation. We find that Bax lacking its C-terminal 25 or 28 residues behaves like BaxΔC21 in the presence of detergents and BH3 peptides but that BaxΔC29, which also removes Y164, does not express as a monomeric protein (data not shown). Pertinently, the constitutively active isoform Baxβ has a distinct sequence after S163 (Fu et al., 2009), and BaxΔC35 aggregates and exposes the N-terminal 6A7 epitope (Liu et al., 2003). These data suggest that Y164 (Figure 2B) makes critical interactions at the core/latch interface to stabilize the inactive form of Bax.

Activator BH3 Sequences and Their Binding Site

We identified sequences distinguishing activator and sensitizer BH3 domains and designed mutants that converted sensitizers into activators (Figure 3). We show that a critical element lies N-terminal to the core BH3 domain in a position we term h0, where either an isoleucine or a glutamate favors activator function on Bax. An isoleucine at h1 is also important, as is the almost invariant glycine between h3 and h4. These findings suggest ways to design BH3 sequences that retain interactions with prosurvival proteins, but not with Bax.

Could peptide binding to an alternate "rear site" (Gavathiotis et al., 2008) have triggered the dimerization we observed, despite Bid and Bax peptides appearing in the groove? That appears untenable because Bax groove mutant R109D was not dimerized by the wild-type BaxBH3 peptide (Figure 3E). Furthermore, the critical h0 residues of Bid and Bim would lie well outside the proposed rear site, and the important h1 residues would lie at its periphery (Figure S3E). Our studies, however, do not address whether some such alternative site contributes to earlier activation steps, such as release of α9 from the groove. Hence, BH3 interactions with two different sites on Bax may play sequential roles in activation, as some biochemical evidence suggests (Kim et al., 2009).

The BH3-in-Groove Dimer as the Central Oligomerization Unit

We suggest that the Bax(α2–α5) dimer (Figure 6A) nucleates Bax oligomerization. This dimer is essentially a head-to-head composite of two BaxBH3:BaxΔC21 peptide complexes, stripped of the α1 helix and the latch domain (Figure 6B). That such a structure forms in dying cells is supported by crosslinking studies on Bax (and Bak) dimers in apoptotic cells (Dewson et al., 2008, 2012), EPR measurements on activated Bax in liposomes (Bleicken et al., 2010), photocrosslinking studies (Zhang et al., 2010), and evidence that Bax M74 mutants (at the h5 position in the Bax BH3), which retain capacity to homo-oligomerize (Czabotar et al., 2011), lie outside the dimerization interface (Figure S6E). These findings argue against the proposal that the central unit of polymerization is a monomer engaging another monomer in a head-to-tail ("daisy-chain") manner (Bogner et al., 2010; Pang et al., 2012). Which of the surfaces exposed on the Bax(α2–α5) dimer promote its association into larger oligomers is unknown, although crosslinking studies suggest that multimerization of Bak or Bax dimers on mitochondria brings their α6 helices into proximity (Dewson et al., 2009; Zhang et al., 2010).

How Bax oligomerization perforates the MOM remains a mystery (Westphal et al., 2011). Known pore-forming proteins that assemble from a soluble monomer create proteinaceous pores with circular n-fold symmetry and pores of a unique or narrowly defined size (Mueller et al., 2009; Tilley and Saibil, 2006). Curiously, the pore-forming domains of bacterial colicins, which resemble the Bcl-2 family fold (Muchmore et al., 1996), seem to function as monomers creating small pores for ions, not macromolecules (Parker and Feil, 2005). No precedent currently exists for assembling a pore-forming oligomer from membrane-anchored dimers. Several reports suggest instead that the Bax pore is lipidic (Epand et al., 2003; Qian et al., 2008; Terrones et al., 2004).

Significantly, the BH3-in-groove homodimer (Figure 6A) has a high concentration of aromatic residues (ten phenylalanines and two tryptophans) on the planar surface comprising the exposed faces of the α4 and α5 helices from each monomer. This finding tempts speculation that this surface lies along the MOM; insertion of these residues between the head groups of the MOM lipids would promote positive curvature and tension in the outer leaflet of that membrane (Fuhrmans and Marrink, 2012), steps thought to promote the opening of a lipidic pore. The latch domain is also amphipathic and might associate similarly with the MOM surface without necessarily actively contributing to pore formation.

Conclusions

Our findings, summarized in Figure 7A, thus support a model (Figure 7B) for activation of MOM-anchored Bax in which an activator BH3-only protein inserts its BH3 domain into the canonical groove of Bax, initiating release of the core domain (α1–α5) from the latch domain (α6–α8) and dislodgement of Bax α2 (its BH3 domain). The freed Bax BH3 domain can then compete with activator BH3-only proteins for the groove of another Bax molecule due to the increased stability of the BH3-in-groove symmetric dimer. These steps further clarify the catalytic role of activator BH3 domains in Bax oligomerization (Billen et al., 2008; Bleicken et al., 2010). The symmetric dimer can then nucleate oligomerization of the Bax core domain (Dewson et al., 2012; George et al., 2007) and hence provoke MOM permeabilization. Similar interactions between BH3 activators and the canonical groove of the MOM-anchored Bak may well initiate its oligomerization (Dai et al., 2011).

The structures of the prosurvival Bcl-2 proteins have guided the development of the promising anticancer "BH3 mimetics" (Lessene et al., 2008). Our results suggest that the groove of Bax and Bak could also be a valid therapeutic target and suggest a rationale for discovering both agonists and antagonists that respectively promote or hinder release of their core domain and/or helix α2 (their BH3 domain). Targeting these critical apoptotic effectors might improve treatment of diseases with attenuated apoptosis (e.g., cancer and autoimmune disease) or those with excessive cell death (degenerative disorders).

EXPERIMENTAL PROCEDURES

Procedures for assigning BaxΔC21 backbone chemical shifts, epitope mapping of antibody 49F9, immunoprecipitation of mitochondrial Bax, cytochrome *c* release from mouse liver mitochondria, release of dextran from liposomes, measuring peptide affinity for Bcl-x_L, and binding of peptides to Bax are in Extended Experimental Procedures.

Peptides and Recombinant Proteins

Peptides were purchased from Mimotopes Australia. Recombinant Bax(ΔC21; C62S, C126S), full-length Bax, and variants thereof were produced by a protocol modified from that described (Suzuki et al., 2000). Bax cloned into pTYB1 was expressed in *E. coli* BL21(DE3) cells. Cells were lysed in TBS (20 mM Tris [pH 8.0], 150 mM NaCl), and proteins were purified by chitin affinity chromatography followed by gel filtration (Superdex75) in TBS.

Dimer Preparation, Crystallization, Data Collection, and Processing

Our first solved structure was the Bax dimer induced by octylmaltoside (OM). To produce it, protein at 7 mg/ml was treated with 1% OM in TBS for 10 min at

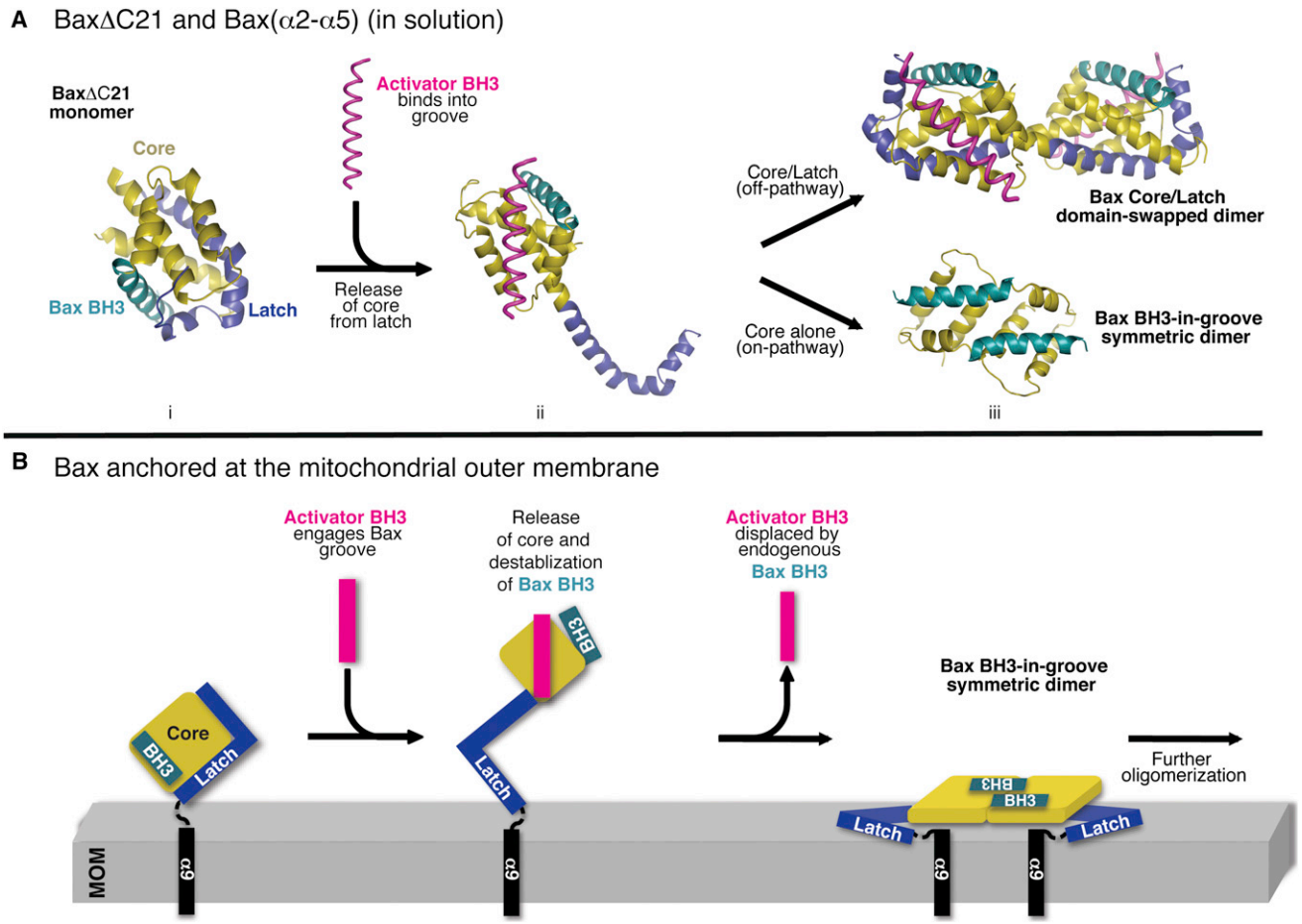


Figure 7. Models Demonstrating Our Conclusions Regarding BaxΔC21 Activation and Bax(α2–α5) Dimerization and Their Implications for Bax Anchored to the MOM

(A) (i) BaxΔC21 is monomeric in the absence of detergents or CHAPS plus BH3 peptides. (ii) Binding of an activator BH3 peptide into the Bax canonical groove unlatches the core domain (α1–α5). (iii) In the absence of an orienting membrane tether, two such unlatched molecules combine ("head to tail") to form the core/latch dimer. Note the change in orientation of the core in these figures once the latch is released (i to ii). The core alone instead forms the on-pathway BH3-in-groove symmetric dimer.

(B) With MOM-anchored native Bax, we propose that activator BH3 domains bind into the groove and unlatch the structure as in (A), releasing the Bax BH3 domain and allowing it to compete for the groove of neighboring Bax molecules on the MOM. The final complex, the BH3-in-groove symmetric Bax dimer, is favored due to the stability afforded by dimerization. In that dimer, the positions of the latch domain and the membrane-anchoring α9 are unknown. The dimer's lipophilic α4–α5 surface may engage the MOM and promote its permeabilization.

room temperature (RT), dialysed for 48 hr against TBS at 4°C, and purified by gel filtration in TBS. Crystals of dimer (5 mg/ml) grew at RT in 1.8 M NH_4SO_4, 0.1 M HEPES (pH 6.5), and 10 mM $PrCl_3$. Crystals were frozen in well solution supplemented with 20% ethylene glycol and X-ray data collected at the Australian Synchrotron beamline MX2 at 100 K. Data were processed with HKL2000 (Otwinowski and Minor, 1997), and the structure was solved by SAD phasing with PHENIX Autosolve (Adams et al., 2010), which found two Pr sites and built 345 residues. Further rounds of building in COOT (Emsley and Cowtan, 2004) and refinement in PHENIX yielded the final model, which was used for molecular replacement in subsequent structure solutions.

BH3-induced Bax dimers were produced by treating protein at 2 mg/ml with 0.5% CHAPS and a 2-fold molar excess of BH3 peptide in TBS (1 hr, RT). The dimer fraction was purified on a Superdex75 column in TBS and crystallized under similar conditions to the OM-induced dimers. Crystals were frozen in cryo-protectant, data were collected on MX2, data were processed with XDS (Kabsch, 2010), and the structure was solved by molecular replacement with PHASER (McCoy et al., 2007). Further rounds of building in COOT and refinement in PHENIX resulted in the final model.

Crystals of Bax:BH3 complexes were obtained by supplementing purified BH3-induced dimers with an excess of the corresponding BH3 peptide. Crystals grew from well solutions containing sodium malonate or sodium citrate in various buffers. The Bax:BidBH3 complex crystal grew in 1.4 M sodium citrate and 0.1 M sodium cacodylate (pH 5.75). Crystals were frozen in well solution supplemented with 20% ethylene glycol, and data were collected on MX2. Data processing, structure solution, building, and refinement were as above. The structures were solved by molecular replacement, the Bax moiety clearly phasing the peptide (Figure S2A). All structures were validated using tools in PHENIX and COOT (Emsley and Cowtan, 2004).

Bax Core Domain Constructs and Crystallography

Sequence encoding α2–α5 of Bax (D53-K128, C62S, C126S) and α1–α5 (P13-K128, C62S, C126S) were cloned into pET28a-GFP (Suzuki et al., 2010). GFP

for the α2–α5 construct encoded an unintentional mutation (A206N). Expression and purification were as above except that affinity purification was via the His Tag, removed by thrombin cleavage before gel filtration. Crystals grown in 10% PEG3350, 20% MPD, 0.5% CHAPS, and 0.1 M Tris (pH 8.0) were frozen in well solution, and data were collected on MX2 and processed with XDS. The structure was solved searching for GFP with PHASER, revealing density for helical regions of Bax (Figure S6A). PHASER was then used to place the relevant regions of the BaxBH3:Bax model followed by rounds of building in COOT and refinement in PHENIX with 4-fold NCS restraints.

Blue Native PAGE

5 μg of Bax dimers induced by BH3 peptides (in presence of 5 mM DTT) or OM as described above were analyzed using Invitrogen blue native (BN) PAGE gels.

Cysteine-Linking and Cytochrome *c* Release from Mouse Embryonic Fibroblasts

Cysteine mutants of Bax S184L were stably expressed in $Bax^{-/-}$ $Bak^{-/-}$ mouse embryonic fibroblasts (MEF), and their function was assessed by analyzing the percentage of propidium iodide-positive cells after etoposide exposure (10 μM, 24 hr). For induction of disulfide linkage, heavy membrane fractions enriched for mitochondria were treated with the oxidant copper(II)(1,10-phenanthroline) (CuPhe 1 mM, 15 min, 4°C). Cytochrome *c* release assays of oxidized Bax S184L in response to tBid (100 nM, 30 min, 30°C) were performed as described for Bak (Dewson et al., 2008). Supernatant and pellet fractions were separated and analyzed for cytochrome *c* by immunoblotting. Cysteine crosslinking with bismaleimidoethane (BMOE, Pierce) was performed as described (Dewson et al., 2008).

Detection of N-Terminal Conformational Changes

1 μg of 49F9, which recognizes Bax only after an apoptotic stimulus (Figure S1), was added to 0.5 μg of BaxΔC21 (2.5 μM) with or without 1% OM (1 hr, RT). Samples were incubated with Protein-G sepharose beads (1 hr, RT) in TBS with 1% CHAPS to minimize nonspecific binding. As a control, Bax was incubated with beads in the absence of 49F9. Samples were washed four times (TBS with 0.1% CHAPS) before resuspension in loading buffer; 100 ng of IP samples or 10 ng of pre-IP samples were run on SDS PAGE and blotted for Bax.

ACCESSION NUMBERS

The PDB accession numbers for the structures reported in this paper are 4BD2, 4BD6, 4BD7, 4BD8, and 4BDU.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.12.031.

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