Cell Death and Differentiation (2012) 19, 661–670

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Bax dimerizes via a symmetric BH3:groove interface during apoptosis

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During apoptotic cell death, Bax and Bak change conformation and homo-oligomerize to permeabilize mitochondria. We recently reported that Bak homodimerizes via an interaction between the BH3 domain and hydrophobic surface groove, that this BH3:groove interaction is symmetric, and that symmetric dimers can be linked via the α6-helices to form the high order oligomers thought responsible for pore formation. We now show that Bax also dimerizes via a BH3:groove interaction after apoptotic signaling in cells and in mitochondrial fractions. BH3:groove dimers of Bax were symmetric as dimers but not higher order oligomers could be linked by cysteine residues placed in both the BH3 and groove. The BH3:groove interaction was evident in the majority of mitochondrial Bax after apoptotic signaling, and correlated strongly with cytochrome *c* release, supporting its central role in Bax function. A second interface between the Bax α6-helices was implicated by cysteine linkage studies, and could link dimers to higher order oligomers. We also found that a population of Bax:Bak heterodimers generated during apoptosis formed via a BH3:groove interaction, further demonstrating that Bax and Bak oligomerize via similar mechanisms. These findings highlight the importance of BH3:groove interactions in apoptosis regulation by the Bcl-2 protein family.
Cell Death and Differentiation (2012) **19,** 661–670; doi:10.1038/cdd.2011.138; published online 21 October 2011

Bax and Bak are pivotal effectors of the intrinsic or mitochondrial apoptosis pathway, as either Bax or Bak is needed to permeabilize the mitochondrial outer membrane (MOM).[1,2] Accordingly, the two proteins exhibit significant sequence (Figure 1a) and structural homology.[3,4] Aspects of Bax and Bak regulation are distinct, however, as Bak is integrated into the MOM in healthy cells, whereas Bax is predominantly cytosolic and translocates to mitochondria after an apoptotic stimulus.[5,6] Furthermore, Bax and Bak are countered by different pro-survival Bcl-2 proteins.[7,8]

To form the apoptotic pore in the MOM, both Bax and Bak undergo conformation change, including exposure of N-terminal epitopes, that allows them to homo-oligomerize.[6,9–11] Bax conformation change also involves insertion of α5 and α6 helices into the MOM.[12] We recently reported that Bak activation requires exposure of the BH3 domain, which then binds to the hydrophobic surface groove of another activated Bak molecule to form homodimers.[13] Reciprocity of the BH3:groove interaction generates symmetric dimers that can be linked via a second interface between α6–helices to form the higher order oligomers thought necessary to permeabilize the MOM.[14]

In Bax, a role for the BH3 domain in oligomerization and pro-apoptotic function was implicated by mutagenesis studies.[15,16] More recently, in liposomes Bax formed symmetric dimers via the BH3 domains, as detected by electron paramagnetic resonance spectroscopy.[17] In addition, in Bax oligomers induced by the detergent Triton X-100, BH3 domain residues could cross-link to another Bax molecule, although the receptor surface was not identified.[18] Thus, it remains unclear how Bax oligomerizes within the MOM during apoptosis, including whether the BH3 domain binds to the hydrophobic groove.

Here we use cysteine linkage and blue native PAGE (BN-PAGE) to examine how Bax oligomerizes at the MOM following apoptotic signaling. We show that symmetric BH3:groove dimers of Bax form within the MOM following etoposide treatment of cells, and following truncated Bid (tBid) -treatment of mitochondria. The symmetric dimers can be linked via a second interface at α6 to form higher order complexes. Heterodimers of Bax and Bak also form via a BH3:groove interaction.

Results

Bax conformation change during apoptosis reflects that of Bak. To monitor Bax conformation change, we first considered whether linkage of endogenous cysteines could identify different Bax conformations, as we and others have reported for Bak.[13,14,19] For example, in non-activated Bak, C14 and C166 form an intramolecular disulfide bond when oxidant is added (M_x; Figure 1b), whereas after etoposide C14 and C166 reposition to allow linkage of oligomers

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Keywords: apoptosis; Bax; BH3 domain; heterodimer; oligomer; mitochondria
Abbreviations: BH3, Bcl-2 homology 3; BN-PAGE, blue native PAGE; CuPhe, copper(II)(1,10-phenanthroline)$_3$; DTT, dithiothreitol; GFP, green fluorescent protein; HA, haemagglutinin; hBak, human Bak; hBax, human Bax; IRES, internal ribosome entry site; LOF, loss-of-function; MEFs, mouse embryonic fibroblasts; MOM, mitochondrial outer membrane; tBid, truncated Bid; TNF-α, tumour necrosis factor alpha; wt, wild-type
Received 11.3.11; revised 08.8.11; accepted 20.8.11; Edited by R Youle; published online 21.10.11

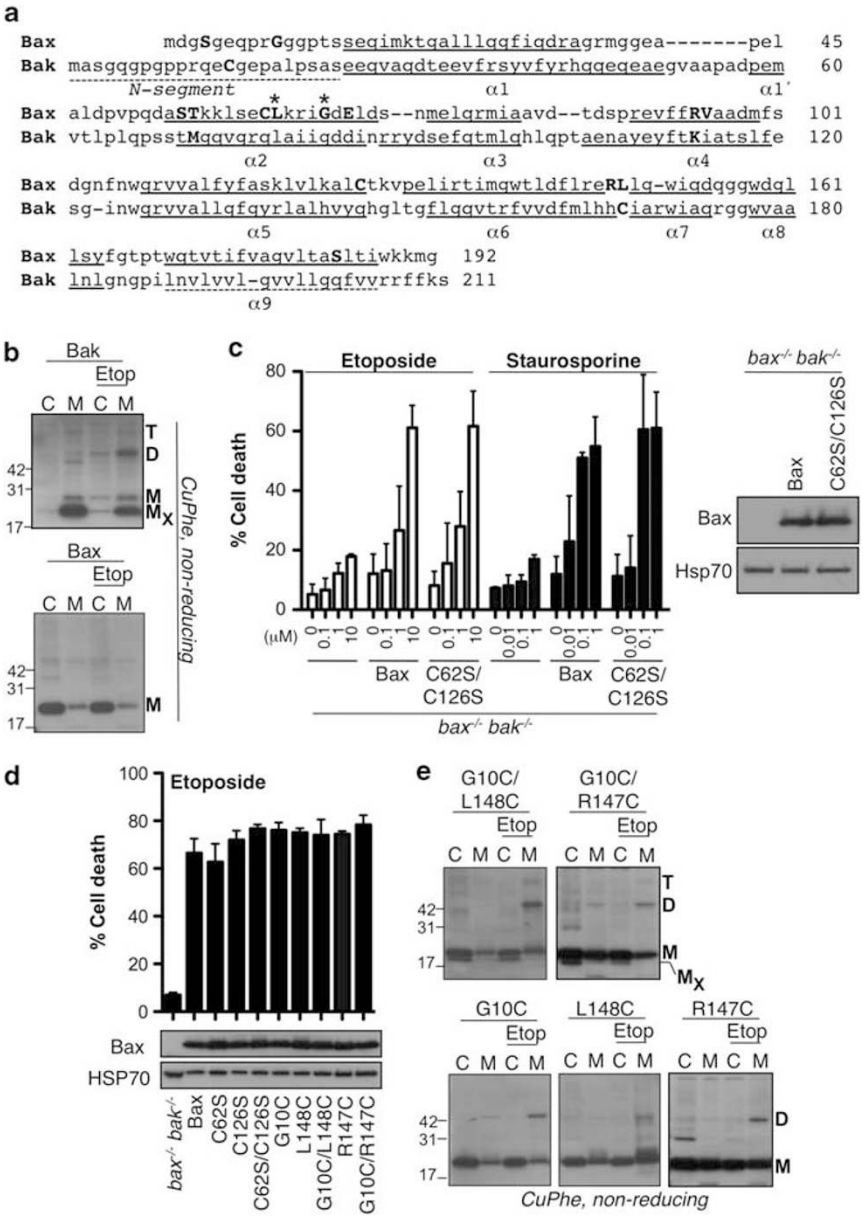

Figure 1 Bax conformation change reflects that of Bak. (**a**) Sequence alignment of the human Bax and Bak sequences based on their structures.[3,4] Residues related to the current study are indicated (*bold upper case loss-of-function mutations indicated with **). α-helices 1-9 are as indicated (*underline*), including the predicted Bak α9 helix (*broken underline*). (**b**) Unlike Bak activation, Bax activation during apoptosis cannot be monitored by disulfide-linkage of endogenous cysteines. Cells expressing Bak or Bax were left untreated, or treated with etoposide (10 μM). The oxidant, CuPhe, was used to induce disulfide bonding in cytosol (C) and membrane (M) fractions, and samples analyzed by non-reducing SDS-PAGE and western blot for Bak (*top*) or Bax (*bottom*) to reveal monomers (M), intramolecular disulfide-linked monomers (M_x), dimers (D) and trimers (T). Data are representative of three independent experiments. (**c**) Endogenous cysteines in Bax are not required for apoptotic function. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs stably expressing Bax or BaxC62S/C126S were treated with the indicated concentrations of etoposide or staurosporine. Cell death is expressed as mean ± S.D. of three independent experiments. (**d**) Bax variants with cysteine substituted at Bak-equivalent positions retain apoptotic function. *bak*$^{-/-}$*bax*$^{-/-}$ MEFs expressing the indicated Bax variants were treated with 10 μM etoposide and assessed as in (c). Cell death is expressed as mean ± S.D. of three independent experiments. (**e**) Bax activation can be monitored by disulfide-linkage of cysteines substituted at Bak-equivalent positions. Cells expressing double- or single-cysteine Bax variants were treated and analyzed as in (b). Data are representative of three independent experiments

(dimers, D; trimers, T; Figure 1b). In contrast, the two endogenous cysteines in Bax (C62 and C126) could not be linked either before or after apoptosis (Figure 1b).

To more directly compare Bax conformation change with that of Bak, cysteine was introduced in Bax at sites equivalent to those in Bak, after first substituting the two endogenous cysteines with serine. Cys-null Bax (C62S/C126S) retained normal pro-apoptotic function when stably expressed in *bax*$^{-/-}$ *bak*$^{-/-}$ MEFs (Figure 1c), as reported by others.[20,21] Curiously, in those studies Cys-null Bax failed to respond to oxidative stress, suggesting that the cysteines act as redox sensors.[20,21] This redox sensing is unlikely to involve disulfide bonds, as cysteine linkage in wild-type Bax was not evident following oxidant (Figure 1b). Cysteines were then

re-introduced in Cys-null Bax at S4 or G10 and L148 or R147. Each variant was expressed at levels similar to that of wild-type Bax, and retained pro-apoptotic function (Figure 1d). Before apoptosis, the two introduced cysteines were nearby in the same Bax molecule as oxidant induced intramolecular linkage (M_x; Figure 1e and Supplementary Figure 1). Efficiency of intramolecular linkage was lower in Bax than in Bak (M_x; Figure 1b), perhaps owing to sub-optimal cysteine positions, in particular within the Bax N-segment, as this region in Bax and Bak differs significantly in sequence (Figure 1a), and its absence in the Bak structure precludes structural alignment.[3] Inefficient intramolecular linkage in Bax may also be explained by flexibility of the Bax N-segment[4] compared with the Bak N-segment.[14] Nevertheless, the observed intramolecular linkage indicates that the N-segment and α6 regions are close in the non-activated forms of Bax and Bak.

After apoptosis, the Bax N-segment and α6 regions had repositioned, as after oxidant dimers and trimers were evident in the double-cysteine variants (Figure 1e and Supplementary Figure 1). Dimers and trimers were only detected in mitochondrial fractions suggesting that Bax converts from a monomer in cytosol to an oligomer once at mitochondria, as reported previously.[22,23] Single-cysteine variants could also link to dimers after apoptosis (Figure 1e), indicating that the N-segments and α6 regions can each self-associate in oligomerized Bax, as observed for oligomerized Bak.[14]

In summary, by introducing two cysteines in Bax at the cysteine positions in Bak and monitoring their ability to form disulfide bonds, we show that Bax and Bak adopt similar conformations before apoptosis, and again after apoptosis.

Bax forms BH3:groove homodimers during apoptosis. We next tested whether Bax oligomerization involves a BH3:groove interaction. Cysteine was introduced in Cys-null Bax at positions predicted to be in close proximity in a BH3:groove interaction (Figure 2a). Cysteine substitutions were also generated in a mitochondria-targeted form of Bax, S184L.[24] Each single-cysteine BH3 and groove variant (bearing FLAG or HA epitope tags at the N terminus) retained pro-apoptotic function (Figure 2b). To test for a BH3:groove interaction, single-cysteine variants were co-expressed, apoptosis was initiated, and disulfide bonding was induced. FLAG variants were then immunoprecipitated from membrane fractions and blotted for the associated HA-variant (Figure 2c, *top panel*). When run under non-reducing conditions the co-precipitated HA-variant was evident either as a monomer, or as a dimer linked to the FLAG-variant. Notably, linkable dimers were generated if BH3 and groove cysteine variants were co-expressed, but not if two BH3 variants were co-expressed (Figure 2c), indicating that a BH3:groove interaction is present in oligomerized Bax. Dimers were again absent in cytosolic fractions (data not shown).

A Bax BH3:groove interaction was also tested using mitochondria isolated from cells expressing the mitochondria-targeted BaxS184L.[24] Incubation with tBid triggered Bax activation and MOM permeabilization (see below), and provided a model corresponding to that used for Bak.[13] tBid treatment induced linkable dimers if BH3 and groove cysteine variants were co-expressed, but not if two BH3 variants were co-expressed (Figure 2c). A nearby BH3 cysteine, S55C, showed a similar linkage pattern (Supplementary Figure 2), while cysteine introduced at a more distal position in the BH3, E69C, was unable to link to either groove cysteine (Supplementary Figure 3), as predicted (Figure 2a). Notably, near 100% linkage occurred when the T56C or S55C and R94C variants were co-expressed, indicating that all mitochondrial Bax had adopted a BH3:groove interaction. As the equivalent pairing in Bak (M71C:K113C; Figure 1a) also yielded near 100% dimers in response to tBid,[13] the BH3:groove interactions in both proteins appear equivalent at the molecular level. The BH3:groove interaction occurred only after Bax had become activated, as disulfide-linkable dimers were absent before tBid treatment (Figure 2e).

The BH3:groove interaction in Bax dimers is symmetric. To test whether the Bax BH3:groove homodimer is symmetric, as we have shown for the Bak homodimer,[14] we generated BH3:groove double-cysteine variants (S55C/R94C and T56C/R94C) of Bax and of BaxS184L. A symmetric BH3:groove interaction (Figure 3a) predicts that cysteine linkage would generate dimers but not trimers or higher order oligomers.

The double-cysteine variants in Bax and in BaxS184L retained normal function (Figure 3b). They also oligomerized like wild-type Bax when analyzed by BN-PAGE (Figure 3c), a technique that utilizes digitonin to solubilize membrane proteins without denaturing proteins or disrupting supramolecular complexes.[25,26] For example, before apoptosis, Bax variants in the cytosolic fraction migrated at low molecular weight, consistent with cytosolic Bax being monomeric,[5] while Bax variants in the membrane fraction were either monomeric or within a high molecular weight complex, as observed in HeLa cells.[26] After apoptosis, each Bax variant formed a ladder in the membrane fraction, similar to those observed in TNF-α-treated HeLa cells.[26] Thus, the double-cysteine substitutions did not alter Bax pro-apoptotic function or oligomerization.

When assessed by cysteine linkage, the double-cysteine Bax variants formed linkable dimers after etoposide treatment (Figure 3d). As for the single-cysteine variants in Figure 2, dimers were only in the mitochondrial fractions, with essentially all mitochondrial Bax forming a BH3:groove dimer. Notably, trimers and higher order oligomers were not observed despite the presence of two cysteines in each protein. For comparison, the two cysteines in Bax G10C/R147C could link to complexes larger than dimers (Figure 3d). As BH3:groove linkage of single-cysteine variants was near complete (Figure 2d), yet linkage of double-cysteine variants generated only dimers (Figure 3d), these data argue that the Bax BH3:groove interaction is reciprocal, as predicted by symmetric dimers (Figure 3a).

When tested following tBid treatment of mitochondrial fractions, the double-cysteine BaxS184L variants were also able to link only as dimers (Figure 3e). In addition, dimer formation correlated with cytochrome *c* release, strongly supporting a role for Bax BH3:groove dimers in pore formation.

To further assess the physiological relevance of the BH3:groove interaction we tested whether loss-of-function

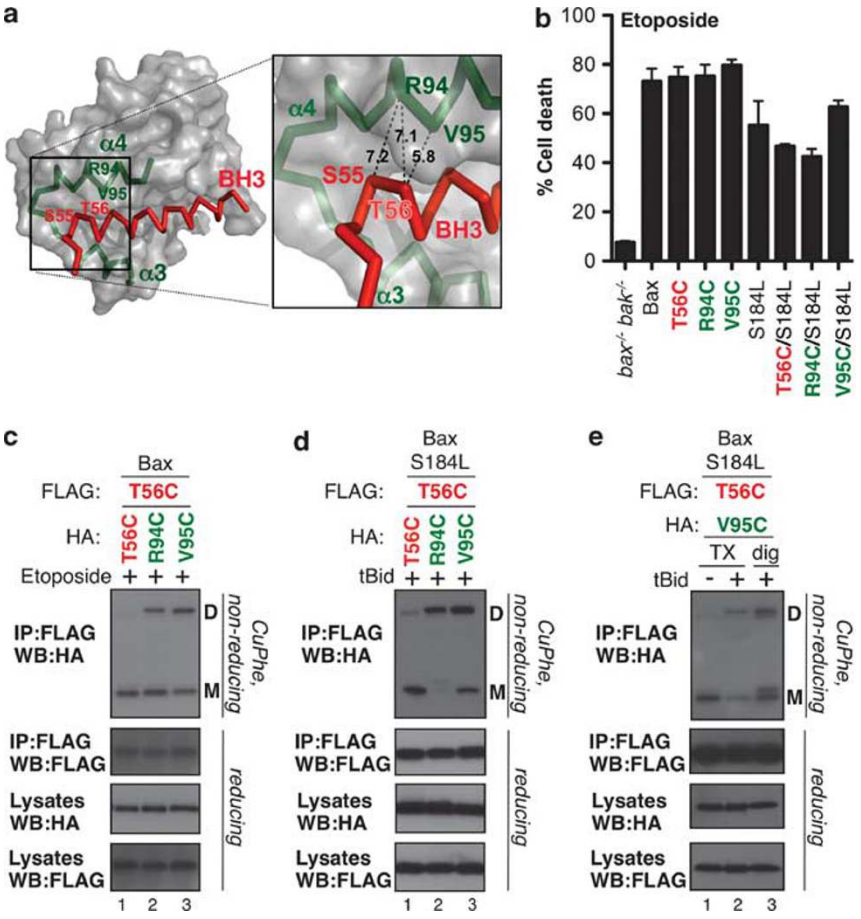

Figure 2 Bax homodimerizes via a BH3:groove interaction during apoptosis. (**a**) Model of BH3:groove interaction in Bax dimers. A model of a BH3 domain (based on Bim; *red*) bound to the Bax hydrophobic groove (α3 and α4; aa74-100; *green*) is based on an alignment of the Bax and Bcl-x_L:Bim structures.[4,40] Residues mutated to cysteine and the predicted molecular distances (Å) between them are indicated. (**b**) Bax BH3 and groove cysteine variants retain apoptotic function. $bax^{-/-}bak^{-/-}$ MEFs expressing the indicated Bax variants were treated with etoposide (10 μM). Cell death is expressed as mean ± S.D. of three independent experiments. (**c**) Bax BH3 residues are juxtaposed with α4 residues after etoposide treatment. Cells expressing pairs of FLAG- and HA-tagged single-cysteine Bax variants were treated with etoposide (10 μM). Mitochondrial fractions were incubated with CuPhe, solubilized in 1% Triton X-100 and immunoprecipitated for FLAG before non-reducing or reducing SDS-PAGE. Immunoprecipitates (IP) and cell lysates were western blotted (WB) for HA or FLAG, as indicated. Data are representative of two independent experiments. (**d**) Bax BH3 residues are juxtaposed with α4 residues after tBid treatment. Membrane fractions from MEFs expressing BaxS184L BH3 and groove cysteine variants were treated with tBid and examined as in (**c**). Data are representative of two independent experiments. (**e**) BH3:groove interaction is specific for activated Bax. Membrane fractions from MEFs expressing BaxS184L BH3 and groove cysteine variants were treated with or without tBid before induction of disulfide-linkage, solubilization in either 1% Triton X-100 (TX) or 1% digitonin and immunoprecipitation as in (c). Data are representative of two independent experiments

(LOF) mutations in the Bax BH3 domain precluded disulfide-linkage of the BH3:groove interface. Bax L63A and G67K failed to function when expressed in $bax^{-/-}bak^{-/-}$ MEFs (Figure 4a), and failed to oligomerize after etoposide as assessed by BN-PAGE and by BH3:groove linkage (Figure 4b). However, as these variants also failed to translocate to mitochondria after etoposide (Figure 4b), the mutations appear to block Bax activation, consistent with a previous study.[23] To bypass the step needed for etoposide-induced Bax activation we used Triton X-100 to induce Bax oligomerization. Notably, cell lysis in Triton X-100 (but not in digitonin) allowed BH3:groove linkage of double-cysteine Bax, but not if the BH3 was mutated (Figure 4c). In another test of physiological relevance, Bcl-2 expression blocked both Bax BH3:groove linkage and cytochrome *c* release (Figure 4d). These findings each support the importance of the BH3:groove interaction in Bax apoptotic function.

Bax BH3:groove dimers are symmetric and can be linked via an α6:α6 interface. Oligomerized Bax exhibited linkage between the N termini (e.g., G10C:G10C) and between the α6-helices (e.g., R147C:R147C) in Figure 1e suggesting that these interfaces may be important for Bax oligomerization and function. However, truncating 5 or 10 residues did not affect apoptotic function or oligomerization, indicating that an N-terminal interface is not important for Bax function (Supplementary Figure 4). The observed N-terminal linkage may be due to the flexibility of the N-segment as indicated by the structure of non-activated Bax[4] and by exposure of the N-terminal 6A7 epitope in activated Bax and Bax G10C (Supplementary Figure 4c).[6]

To test whether an interface between the α6 helices is involved in Bax oligomerization, we introduced single cysteines in α6, either along the surface or at I136 that orientates toward the core in non-activated Bax (Figure 5a),[4]

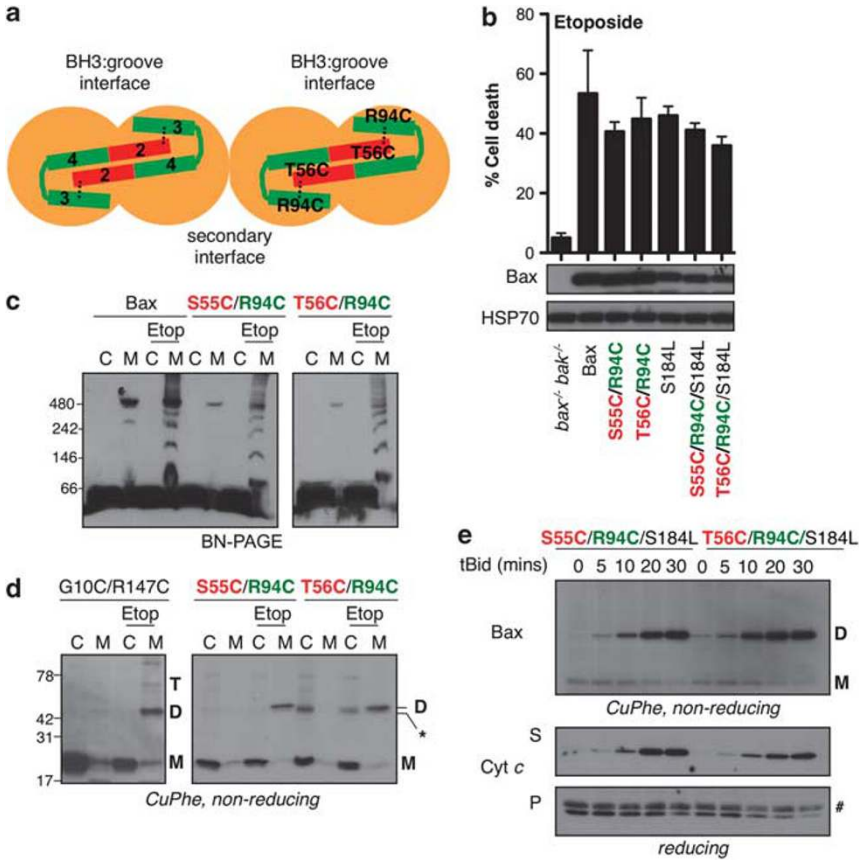

Figure 3 Bax BH3:groove dimers are symmetric and their formation correlates with cytochrome *c* release. (**a**) Schematic of Bax oligomerization. Symmetric dimer formed by reciprocal BH3:groove interactions with α-helices 2-4 indicated (*left*). Bax with cysteine in the BH3 and in the groove (T56C/R94C) would form disulfide bonds within a dimer, but not between dimers (*right*). Symmetric dimers may link via a secondary interface (e.g. between the α6-helices; Figure 1e) to form higher order oligomers. (**b**) BH3/groove double-cysteine variants retain apoptotic function. $bax^{-/-}bak^{-/-}$ MEFs expressing the indicated Bax variants were treated with etoposide (10 μM). Cell death is expressed as mean ± S.D. of three independent experiments. (**c**) BH3/groove double-cysteine variants oligomerize like wild-type Bax on BN-PAGE. MEFs expressing Bax or the indicated cysteine variants were treated with or without etoposide, and cytosol (C) and membrane (M) fractions generated. Fractions were solubilized in 1% digitonin, analyzed by BN-PAGE and immunoblotted for Bax. Data are representative of three independent experiments. (**d**) BH3/groove double-cysteine variants form disulfide-linkable dimers but not higher order oligomers. Cells expressing the indicated Bax variants were left untreated or treated with etoposide (10 μM). Cytosol (C) and membrane (M) fractions were treated with CuPhe and analyzed as in Figure 1b. Note that a band slightly smaller than dimer size (*) in the cytosolic T56C/R94C fractions does not relate to apoptosis as it occurs before and after etoposide treatment. Data are representative of two independent experiments. (**e**) Formation of Bax BH3:groove dimers correlates with cytochrome *c* release. Membrane fractions from cells expressing BaxS184L variants were treated with tBid for the indicated times prior to CuPhe addition and analysis as in (d) (*top panel*). Alternatively, samples were separated into supernatant (S) and membrane (P) fractions and assessed for cytochrome *c* (Cyt *c*; *bottom panels*). #Non-specific band. Data are representative of two independent experiments

and tested whether each cysteine could disulfide-link to the same residue in another Bax molecule after apoptosis. Each α6 cysteine mutant retained apoptotic function (Figure 5b). After etoposide, disulfide-linkable dimers were particularly evident in W139C and E146C in the membrane fractions (Figure 5c, *top panels*). Linkage of these α6 residues, and of R147C in Figure 1e, indicates that the α6-helices are in close proximity in a Bax oligomer.

We then tested whether the α6:α6 interface could link symmetric dimers to form high order multimers of Bax, as we had found for Bak.[14] Triple-cysteine mutants were generated to allow linkage at both the BH3:groove (S55C:R94C) and α6:α6 (e.g., E146C:E146C) interfaces, with each variant retaining apoptotic function (Figure 5b). Consistent with our hypothesis, disulfide linkage trapped higher order complexes of Bax in the membrane of etoposide-treated cells, again particularly with the W139C and E146C variants (Figure 5c, *bottom panels*). The disulfide-linked oligomers converted to monomers when run under reducing conditions (Supplementary Figure 5a), confirming that the complexes were disulfide-linked Bax. Two features of the linkage pattern indicate that BH3:groove dimers are linked by an α6:α6 interface. First, the dimer in the triple-cysteines is a BH3:groove dimer, running slightly slower than an α6:α6 dimer (Supplementary Figure 5b). Second, the absence of trimers indicates that the BH3:groove dimers are symmetric, with cysteines in an alternative interface necessary to link multimers.

Heterodimers of Bax and Bak also contain a BH3:groove interaction, but form a minor population in apoptotic cells. Given the conserved BH3:groove mechanism of homodimer formation in Bax and Bak, we tested whether Bak and Bax also heterodimerize via a BH3:groove

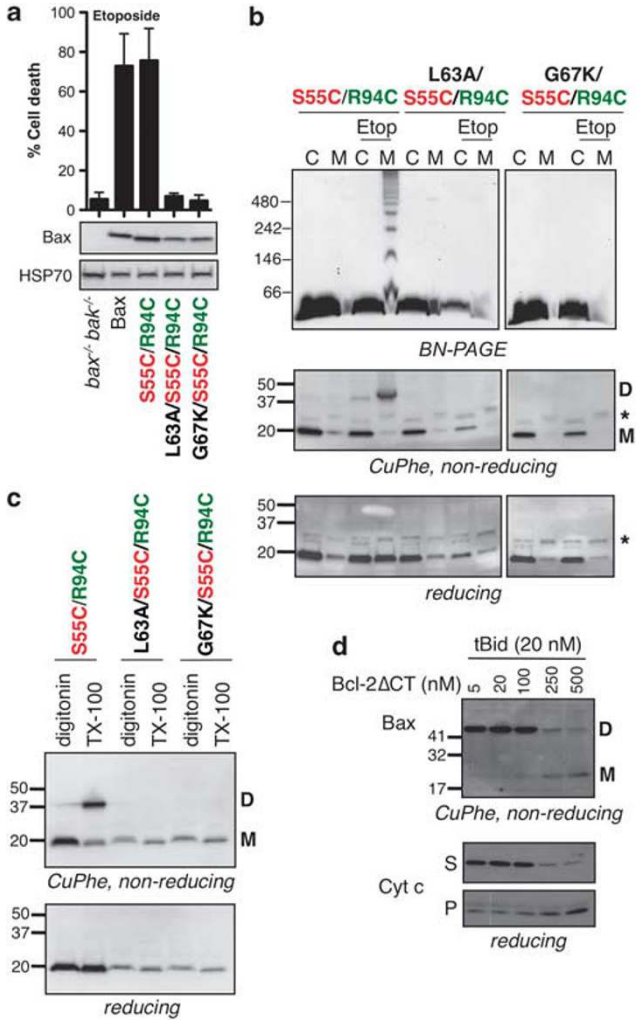

Figure 4 Bax BH3:groove interaction correlates with apoptotic function. **(a)** Mutation of key residues in the Bax BH3 domain disrupts function. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs stably expressing the indicated Bax variants were assessed for protein expression (*lower panels*), and for cell death following treatment with etoposide (10 μM). Cell death is expressed as mean ± S.D. of three independent experiments. **(b)** Loss-of-function Bax BH3 mutants fail to oligomerize. MEFs expressing the indicated Bax variants were left untreated or treated with etoposide (10 μM). Cytosol (C) and membrane (M) fractions were separated and either solubilized in 1% digitonin for BN-PAGE, or treated with CuPhe and analyzed by SDS-PAGE under non-reducing or reducing conditions. Data are representative of two independent experiments. Note that the BH3:groove disulfide-linked dimer is abolished under reducing conditions, while the indicated bands (*) were not disulfide-linked Bax complexes as they were resistant to reduction. **(c)** BH3:groove interaction induced by detergent is blocked by mutation of the BH3 domain. Cells expressing the indicated Bax variants were lysed in 1% Triton X-100 or 1% digitonin before induction of disulfide linkage and analysis by non-reducing SDS-PAGE. **(d)** Bax BH3:groove interaction is inhibited by Bcl-2. Membrane fractions from MEFs expressing Bax S55C/R94C were incubated with tBid and with increasing concentrations of recombinant Bcl-2ΔCT before disulfide linkage and analysis by non-reducing SDS-PAGE. Samples were also separated to supernatant and pellet fractions for analysis of cytochrome *c* release (*lower panels*). Data are representative of two independent experiments

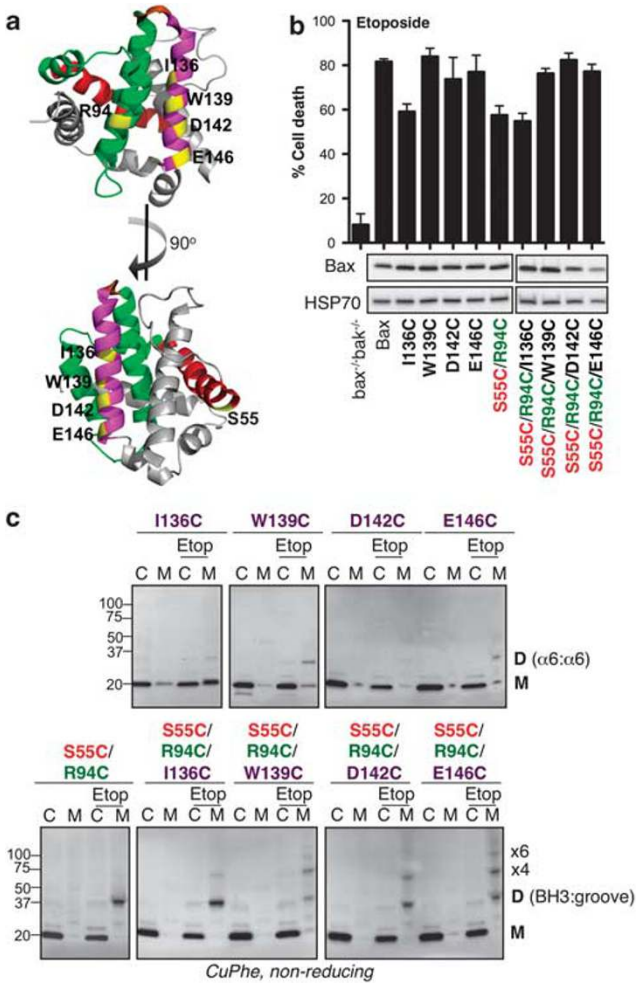

Figure 5 An α6:α6 interface can link symmetric BH3:groove dimers in Bax oligomers. **(a)** Cysteine substitutions in Bax α6. A cartoon representation of Bax showing residues in α6 (*magenta*), BH3 domain (*red*) and groove (*green*) that were mutated to cysteine (*yellow*). **(b)** Bax α6 cysteine variants retain apoptotic function. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing the indicated Bax variants were treated with etoposide (10 μM). Cell death is expressed as mean ± S.D. of three independent experiments. **(c)** BH3:groove dimers can link via an α6:α6 interface to generate higher order complexes. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing the indicated single-, double-, or triple-cysteine Bax variants were left untreated or treated with etoposide (10 μM). Cytosol (C) and membrane (M) fractions were treated with CuPhe and analyzed as in Figure 1b. Samples were also run under reducing conditions (Supplementary Figure 5a). Data are representative of three independent experiments

interaction, and so provide further evidence that the two proteins adopt similar conformations during apoptosis.

To first examine whether heterodimers form in apoptotic MEFs, we used BN-PAGE, reasoning that the different molecular weights of Bax and Bak would distinguish heterodimers from homodimers. Mouse Bax present in mitochondrial extracts from etoposide-treated *bak*$^{-/-}$ MEFs migrated as a series of discrete complexes (Figure 6a), similar to ectopically expressed human Bax (Figure 3c). If Bak was also present (wt MEFs), Bax complexes were largely unaffected except for a minor band just above the Bax:Bax dimer (Figure 6a). On the basis of size this minor band is a Bax:Bak heterodimer, and based on its low intensity it is a minor species in these apoptotic cells.

To confirm that Bak rather than some other protein was associating with Bax in this putative heterodimer band, gel-shift assays using antibodies against Bak were performed.

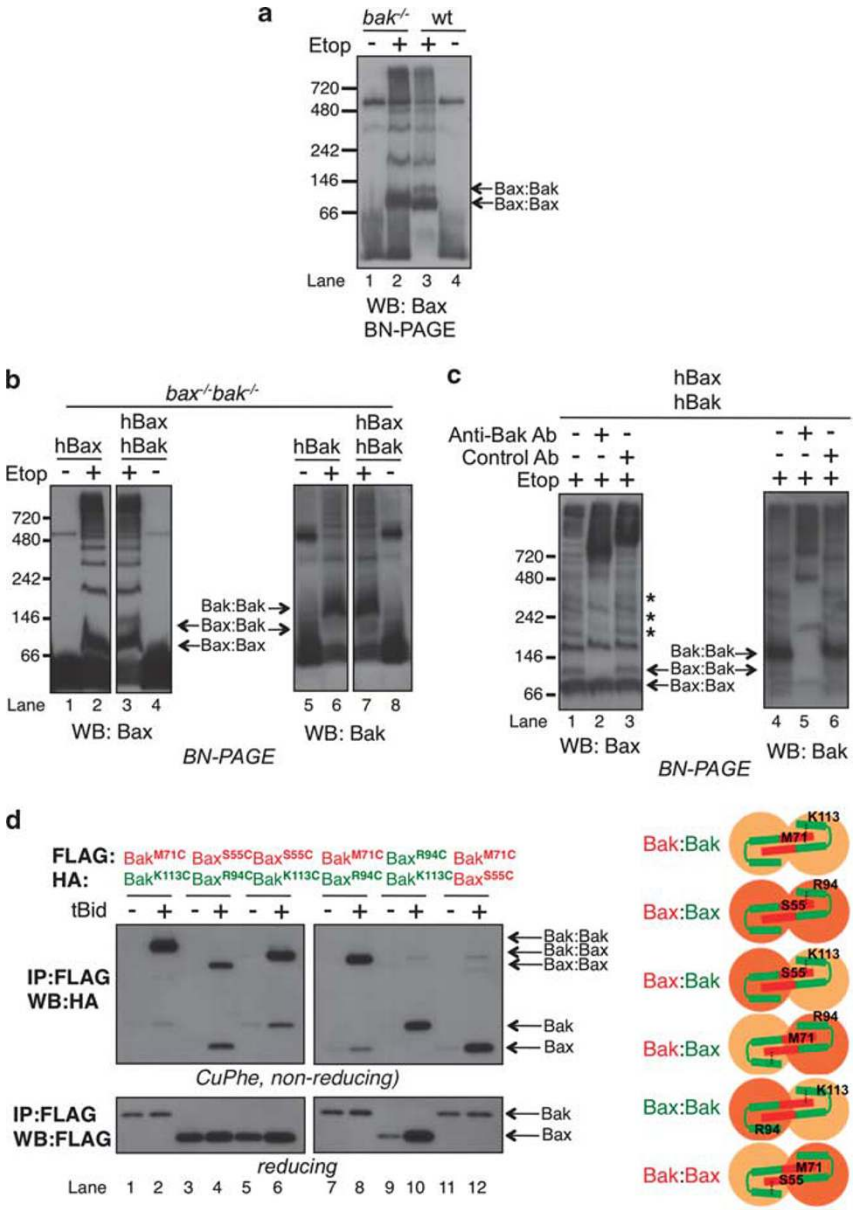

Figure 6 Bax and Bak can heterodimerize following apoptotic signaling and do so via a BH3:groove interaction. **(a)** Mouse Bax predominantly self-associates during apoptosis. Wild-type (wt) and *bak*$^{-/-}$ MEFs were treated or not with etoposide for 24 h. Membrane fractions were then run on BN-PAGE and immunoblotted for Bax. Note the appearance in wt MEFs of a band consistent in molecular weight with a Bax:Bak heterodimer. Data are representative of three independent experiments. **(b)** Human Bax and human Bak predominantly self-associate during apoptosis. *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing hBax and/or hBak were treated with etoposide for 24 h and the membrane fractions run on BN-PAGE and immunoblotted for Bax or Bak. Data are representative of two independent experiments. **(c)** Gel-shift of Bak identifies heterodimers. *bax*$^{-/-}$ *bak*$^{-/-}$ MEFs expressing hBax and hBak were treated with etoposide for 24 h as in (**b**). Membrane fractions were then incubated with anti-Bak antibody (7D10) or a control antibody against Bid (8C3) before BN-PAGE and immunoblotting for Bax or Bak. Data are representative of two independent experiments. *Possible higher order oligomers containing both Bax and Bak. **(d)** Bax and Bak associate via a BH3:groove interaction. Mitochondrial fractions were obtained from cells co-expressing the indicated variants of BaxS184L or Bak. Each variant contains a single cysteine in the BH3 domain (*red*) or groove (*green*), and each has a FLAG or HA tag. Mitochondrial fractions were left untreated or treated with tBid, and CuPhe added to induce disulfide bonds. Samples were then immunoprecipitated in 1% digitonin for FLAG and run on non-reducing (*upper panel*) or reducing (*lower panel*) SDS-PAGE before western blotting for HA (*upper panel*) or for FLAG (*lower panel*). Also shown is a schematic of the disulfide bonding induced in each dimer of Bak (*yellow*) and Bax (*orange).* Data are representative of three independent experiments

Unfortunately, several antibodies failed to gel-shift endogenous mouse Bak (data not shown). As the most anti-Bak antibodies have been generated against the human protein, we stably expressed human Bak (hBak, FLAG-tagged) and human Bax (hBax) in *bax*$^{-/-}$*bak*$^{-/-}$ MEFs. Again, etoposide induced a putative heterodimer band when both proteins were expressed, but not when hBax was expressed alone (Figure 6b), showing that human Bax and Bak stably expressed in MEFs oligomerize in a similar fashion to the endogenous mouse proteins. Gel-shift assays on these MEFs expressing human Bax- and Bak then confirmed that Bak was present in the putative heterodimer complex (Figure 6c).

For example, the minor Bax band was gel-shifted by antibody that recognizes all forms of Bak but not by a control antibody. A duplicate gel blotted for Bak confirmed that the anti-Bak antibody gel-shifted Bak (Figure 6c). Higher order Bax complexes migrating above the predominant Bax:Bax oligomers (*, Figure 6c) were also gel-shifted by the anti-Bak antibodies. Thus, during apoptosis a small portion of Bax and Bak heterodimerize and multimerize to high order complexes.

Cysteine linkage and co-precipitation was then used to test whether heterodimers involve a BH3:groove interface. Bax and Bak cysteine variants were co-expressed with either a FLAG or HA tag, as in Figure 2. Each protein contained cysteine at equivalent positions in the BH3 domain (BaxS55C and BakM71C) or the groove (BaxR94C and BakK113C) (Figures 1a and 6d), with Bax cysteine mutants generated in the mitochondrial S184L variant to allow efficient activation of both Bak and Bax with recombinant tBid. As expected, co-expressed proteins co-precipitated only after tBid, and homodimers of Bak and of Bax efficiently disulfide-linked via a BH3:groove interaction (Figure 6d, lanes 2 and 4). Notably, heterodimers of Bak and Bax identified by their intermediate molecular weight, were also linked via a BH3:groove interaction (Figure 6d, lanes 6 and 8). Indeed, BH3:groove linkage was observed in both the Bax:Bak and Bak:Bax directions, supporting a reciprocal BH3:groove interaction in the heterodimers. As negative controls, disulfide-linkage within heterodimers did not occur in a BH3:BH3 or groove:groove manner, despite co-precipitation (Figure 6d, lanes 10 and 12).

Thus, Bax and Bak can heterodimerize during apoptosis and do so via a reciprocal BH3:groove interaction. However, as heterodimers were consistently a minor species on BN-PAGE, Bax and Bak predominantly homodimerize during apoptosis.

Discussion

These studies are the first to show that when Bax oligomerizes in apoptotic cells the BH3 domain binds to the hydrophobic groove, and that reciprocity of this BH3:groove interaction generates symmetric dimers. The findings are consistent with recent liposome experiments in which a Bax dimerization interface involved two BH3 domains.[17] In addition, in Bax oligomers induced by the detergent Triton X-100, BH3 residues could form a cross-link with another Bax molecule, although the receptor surface was not identified.[18]

Bax is thus similar to Bak which also forms symmetric BH3:groove homodimers in mitochondria of apoptotic cells,[13,14] as well as in liposomes.[27] The BH3 domain and the groove are located in the α2–α5 regions of both proteins, with those regions implicated by mutagenesis studies to be important for homo-oligomerization.[13,15] Even at the molecular level the Bax and Bak BH3:groove interactions are similar, as near complete cysteine linkage could be induced between cysteines substituted at equivalent positions, for example, at T56 and R94C in Bax (Figure 3d) and at M71 and K113 in Bak.[14]

That a BH3:groove interaction in Bax is important for pore formation as well as for oligomerization is supported by several lines of evidence. First, because essentially all mitochondrial Bax adopted a BH3:groove conformation following apoptotic signaling, a BH3:groove interface has to be present in those Bax oligomers responsible for MOM permeabilization rather than in a minor Bax population that might not be responsible for pore formation. Second, BH3:groove dimerization correlated with cytochrome *c* release. Furthermore, when apoptosis was blocked by LOF mutations in the Bax BH3 domain or by addition of Bcl-2, BH3:groove dimerization was also blocked.

The symmetric nature of the Bax BH3:groove dimer implies that a second interface is necessary for Bax to oligomerize into the higher order complexes responsible for MOM permeabilization. In Bak, an α6:α6 interface could link symmetric dimers to higher order complexes.[14] We now show that this also holds for Bax, although linkage at α6 did not trap the very high order complexes of Bax detected by BN-PAGE (Figure 3c). As α6 mutations performed to date did not block Bax (or Bak) function, further analysis is required to determine whether the α6:α6 interface is necessary for Bax (or Bak) function, or whether additional interfaces are involved.[28]

While heterodimerization between Bax and Bak is clearly not required for apoptosis,[2,1] heterodimers are generated during apoptosis as Bax and Bak can co-precipitate, and heterodimers are evident on BN-PAGE (Figure 6).[29,30] Cysteine linkage also showed that heterodimers form via a BH3:groove interaction, and that the interaction was efficient in both the Bak:Bax and Bax:Bak directions, further indicating the similarity of BH3:groove interactions in Bax and Bak. However, homodimers of Bax and Bak predominate, at least as indicated by BN-PAGE, presumably due to higher binding affinities between like molecules. Heterodimers also multimerized to some degree (Figure 6c), although given the minor population of hetero-oligomers it is not possible to conclude whether they contribute to pore formation, or whether they might actually inhibit the process.

As the Bax groove acts as a receptor for the BH3 domain of Bax or Bak, it may also act as a receptor for BH3-only proteins such as tBid, Bim and Puma to cause 'direct' activation of Bax,[23] as suggested,[28,31] and very recently reported for activation of Bak.[32] tBid, Bim and Puma were reported to bind to the Bax groove, but only after first binding to an α1/6 rear pocket.[23] As the Bax groove is normally occupied by the Bax transmembrane domain, that domain would need to be displaced by the activator BH3-only proteins.[4,33] The activator BH3-only protein may itself then be displaced either by the newly exposed Bax BH3 domain or by conformation change in the groove. This complex series of binding and conformation change is one possible explanation for the apparent transient interaction between activator BH3-only proteins and Bax or Bak.[9,34]

Is the symmetric dimer model of Bax oligomerization proposed by these studies consistent with previous studies regarding Bax oligomerization? For example, the Bax α5/6 hairpin is reported to integrate into the MOM during apoptosis,[12] and as α5 forms part of the hydrophobic groove,[4] its eversion and insertion into the MOM may disrupt the groove and preclude Bax BH3:groove dimer formation. However, our data show that the BH3:groove association persists in the fully activated and presumably integrated form of Bax. Thus, loss of α5 from the groove may be offset by the large interacting surface area formed upon BH3:groove

binding being reciprocal.[28] In terms of the sequence of Bax conformation changes, the BH3:groove and α6:α6 interfaces may form after α5/6 inserts into the MOM, as α5/6 insertion occurs before oligomerization.[12]

A recent study reported that the Bax BH3 domain binds to the α1/6 binding pocket in Triton X-100-induced Bax oligomers,[18] suggesting that Bax autoactivates to form an asymmetric 'daisy chain' of monomers.[35]However, our data that the Bax BH3 domain is bound to the canonical hydrophobic groove in fully activated Bax suggest that the BH3:α1/6 interaction may be a transient activating event, analogous to Bax activation at this site by activator BH3-only proteins.[23,36,37]

By defining how Bax and Bak self-associate, the current findings may help identify novel agents that block these interactions. For example, blockade of the BH3:groove interactions in Bax and in Bak may benefit conditions such as ischemic heart disease that are characterized by unwanted cell death.

Materials and Methods

Generation of cell lines and death induction. SV40-immortalized *bax*$^{-/-}$*bak*$^{-/-}$ MEFs were retrovirally infected with human Bax or human Bak variants in internal ribosome entry site (IRES)-GFP or IRES-hygromycin expression constructs, and polyclonal populations of GFP-positive or hygromycin-resistant cells selected as previously described.[13] Apoptosis was induced by incubation with etoposide (10 μM) or staurosporine (1 μM) for 24 h, and the resulting cell death assessed by propidium iodide uptake. The samples were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).[13]

Mitochondrial permeabilization by tBid. For mitochondrial permeabilization assays, membrane fractions prepared by digitonin-permeabilization of untreated cells were incubated for 30 min at 30 °C with 20–100 nM recombinant Bid cleaved with caspase-8 to generate tBid.[13]. Recombinant Bcl-2ΔCT lacked 22 residues at the C-terminus, and was generated as previously described.[38] Supernatant (S) and membrane pellet (P) fractions were separated by centrifugation at 13 000 *g* for 5 min, and immunoblotted for cytochrome *c* (clone no. 7H8.2C12, BD Pharmingen, San Jose, CA, USA).

Detecting Bax oligomerization by disulfide-linkage. Bax oligomerization in cells and in isolated mitochondria was monitored as previously described for Bak.[13] Briefly, after cells were treated with etoposide in the presence of the broad range caspase inhibitor Q-VD.oph (50 μM, Enzyme Systems, San Diego, CA, USA), the cell membrane was permeabilized in permeabilization buffer (20 mM HEPES/KOH pH 7.5, 100 mM sucrose, 2.5 mM MgCl2, 50 mM KCl, 0.025% digitonin and Complete protease inhibitors (Roche, Basel, Switzerland)). Supernatant (S) and membrane pellet (P) fractions were separated by centrifugation at 13 000 *g* for 5 min. To detect cysteine-linked oligomers, fractions were incubated with the oxidant copper(II)(1,10-phenanthroline)$_3$ (CuPhe) on ice for 15–30 min before quenching the oxidation reaction with 10 mM EDTA and 10 mM *N*-ethylmaleimide. Where indicated, samples were incubated in 1% detergent in permeabilization buffer for 30 min on ice before oxidant treatment. Samples were subjected to non-reducing or reducing SDS-PAGE and immunoblotted for Bax (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Bak (aa23-38, B5929, Sigma-Aldrich, St. Louis, MO, USA).

Immunoprecipitation. Cells or cell fractions were solubilized on ice for 30 min in lysis buffer (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol) containing either 1% Triton X-100 or 1% digitonin, and anti-FLAG immunoprecipitation performed as described.[13] Immunoprecipitates and cell lysates were run on SDS-PAGE under non-reducing or reducing conditions, and immunoblotted for HA (16B12, Covance, Princeton, NJ, USA) or FLAG (M2, Sigma-Aldrich). To assess Bax activation by immunoprecipitation cells were solubilized on ice for 30 min in lysis buffer (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol) containing 1% CHAPS, and conformationally active Bax immunoprecipitated with 6A7 antibody.[5]

BN-PAGE and antibody gel-shift assays. BN-PAGE was performed essentially as described.[25]Cytosol and membrane fractions from either untreated or etoposide-treated cells were generated as above, but in the presence of 2 mM DTT. Membrane fractions were then solubilized in 20 mM Bis-tris, pH 7.4, 50 mM NaCl, 10% glycerol, 2 mM DTT and 1% digitonin, before centrifugation at 13 000 *g* to remove debris. 10X BN-PAGE loading dye (5% Coomassie Blue G-250 (Bio-Rad, Hercules, CA, USA) in 500 mM 6-aminohexanoic acid, 100 mM Bis-tris, pH 7.0) was then added. Samples were electrophoresed on 4–13% native gels in anode buffer (50 mM Bis-tris, pH 7.0) and blue cathode buffer (50 mM Tricine, 15 mM Bis-tris unbuffered containing 0.02% Coomassie Blue G-250), with blue cathode buffer replaced with clear buffer (without Coomassie Blue) when the dye front was one third through the resolving gel. Gels were transferred to PVDF and immunoblotted for Bax (21C10, from DCS Huang) or for Bak (aa 23–38, B5929, Sigma-Aldrich). For gel-shift analysis, before addition of BN-PAGE loading buffer, solubilized membrane fractions from etoposide-treated cells were incubated for 30 min on ice with 2 μg of rat monoclonal antibody against Bak (7D10)[13] or Bid (8C3)[39] as a control.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Peter Colman for comments on the manuscript, Michael Ryan and Michael Lazarou for advice on BN-PAGE, Stephanie Fennell and Laura Raiti for technical assistance, and David Huang and W Douglas Fairlie for reagents. The work was supported by grants from the National Health and Medical Research Council of Australia (no. 575559 and no. 637335), and the Association for International Cancer Research (no. 10–230), and operational infrastructure grants through the Australian Government IRISS and the Victorial State Government OIS.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)