Biochem. J. (2015) **467**, 495–505 (Printed in Great Britain) doi:10.1042/BJ20141291

Distinct lipid effects on tBid and Bim activation of membrane permeabilization by pro-apoptotic Bax

Aisha Shamas-Din*, Scott Bindner*, Xiaoke Chi†, Brian Leber*‡, David W. Andrews*§[1] and Cécile Fradin*‖[1]

*Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8S 4K1, Canada
†Department of Chemical Biology, McMaster University, Hamilton, Ontario L8S 4M1, Canada
‡Department of Medicine, McMaster University, Hamilton, Ontario L8S 4K1, Canada
§Sunnybrook Research Institute and Department of Biochemistry, University of Toronto, Toronto, Ontario M4N 3M5, Canada
‖Department of Physics and Astronomy, McMaster University, Hamilton, Ontario L8S 4M1, Canada

After exposure to stressful stimuli, apoptotic signals can be relayed to mitochondria by pro-apoptotic activator proteins, tBid (truncated Bid/p15) and Bim (Bcl-2 interacting mediator), which activate Bax (Bcl-2 associated X protein) and or Bak (Bcl-2 antagonist/killer) to induce mitochondrial outer membrane (MOM) permeabilization (MOMP). These protein–protein and protein–membrane interactions are critical for apoptosis regulation, since MOMP irreversibly leads to cell death. Whereas the distinct roles of tBid and Bim as sensors of different types of stress are well recognized, it is not known whether the molecular mechanisms whereby they initiate MOMP are the same. In the present study, we compare membrane permeabilization by Bax activated by either cBid [cleaved Bid (p7 and p15)] or Bim and we examine the role of membrane lipids in the recruitment and activation of these three Bcl-2 (B-cell lymphoma 2) pro-apoptotic proteins. We employ fluorescently-labelled proteins and liposomes to quantify the effects of specific lipids on each of the well-characterized steps in Bax-mediated membrane permeabilization. We show that high levels of cholesterol in the membrane inhibit permeabilization by categorically identifying the recruitment of Bax by the activators and Bax insertion in the membrane as the steps being hindered by cholesterol. Furthermore, we show that binding of both cBid and Bim to membranes is facilitated by electrostatic interactions with anionic phospholipids. However, whereas Bim does not require any particular anionic lipids, the conformational change in tBid depends on cardiolipin (CL). This suggests that CL can activate tBid in a similar manner to Mtch2 (mitochondrial carrier homologue 2). Thus, lipids modify multiple aspects of Bax-mediated membrane permeabilization.

Key words: apoptosis, B-cell lymphoma 2 (Bcl-2) proteins, Bax, cardiolipin, cholesterol, fluorescence.

INTRODUCTION

Bcl-2 (B-cell lymphoma 2) proteins play a pivotal role in apoptosis by regulating mitochondrial outer membrane (MOM) permeabilization (MOMP), the first event that commits a eukaryotic cell to undergo apoptosis [1–3]. Bcl-2 proteins are sub-divided as anti- and pro-apoptotic based on the role they play in opposing or promoting cell death. Upon receiving an apoptotic stimulus, pro-apoptotic Bcl-2 activator proteins are post-translationally modified. For example, full-length Bid (BH3 interacting domain death agonist) is cleaved to produce cleaved Bid (cBid), consisting of the two fragments (p7 and p15) that remain bound [4]. The fragments separate when cBid peripherally binds to the MOM and the larger piece, called tBid (p15/truncated Bid), changes conformation to insert into the membrane [5]. After activators translocate to the MOM [6–9], they recruit the pore-forming pro-apoptotic proteins Bax and Bak, which oligomerize to permeabilize the membrane releasing intermembrane space proteins including cytochrome *c*, Smac (second mitochondria-derived activator of caspases) and AIF (apoptosis-inducing factor) into the cytoplasm [10–14]. This process leads to the activation of caspases that cleave multiple substrates resulting in cell death.

The interplay between the different Bcl-2 proteins regulating MOMP involves dynamic protein–protein and protein–membrane interactions [15–17]. Many Bcl-2 proteins undergo conformational changes upon activation and binding to the MOM that alter their primary function [18,19]. As mentioned above, the two fragments of cBid separate upon binding to the membrane and tBid unfolds to insert the hydrophobic α6 and α7 helices in the MOM [5,20–22]. Only after binding to membranes and changing conformation does tBid bind to and activate Bax. Another example is the conformational change in Bax that unmasks the N-terminal domain and inserts the membrane-binding helices (α5 and α6) in the MOM after activation [23–25]. This conformational change in Bax triggers oligomerization and permeabilization of the membrane. Several reports suggest that specific lipids can regulate the function of individual Bcl-2 proteins and therefore MOMP [26,27]. However, the issue has not been addressed systematically for both proteins and lipids.

Mitochondrial phospholipid content is dominated by phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which contribute 45 %–50 % and 27 %–30 % of the total lipid content respectively, with lesser amounts of phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (5 %–10 % each). The anionic phospholipid exclusive to mitochondria,

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; cBid, cleaved Bid (p7 and p15); CL, cardiolipin; DAC, *N*-(7-dimethylamine-4-methylcoumarin-3-yl) maleimide; DPX, *p*-xylene-bis-pyridinium bromide; K_D, dissociation constant; MLCL, monolysocardiolipin; MOM, mitochondrial outer membrane; MOMP, mitochondrial outer membrane permeabilization; NBD, *N,N′*-dimethyl-*N*-(Iodoacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; *P*, partition coefficient; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; tBid, truncated Bid/p15; WT, wild-type.

[1] Correspondence may be addressed to either author (email fradin@physics.mcmaster.ca or david.andrews@sri.utoronto.ca).

Table 1 Physical properties of lipids used in assays

Lipids	Source	Predominant chain composition	Overall charge	Curvature
PC	Egg	16:0/18:1	0	≈0
PE	Egg	16:0/18:1	0	Negative
PI	Bovine liver	18:0/20:4	−1	≈0
PS	Synthetic	18:1/18:1	−1	Positive
CL	Synthetic	18:1/18:1/18:1/18:1	−2	Very negative
MLCL	Bovine heart	18:2/18:2/18:2	−2	Negative
PG	Egg	16:0/18:1	−1	≈0
Cholesterol	Plant derived	Not applicable	0	Very negative

cardiolipin (CL), amounts to 15 % of the total mitochondrial lipid content [28]. Whereas most of the CL is present in the inner membrane, a small amount is present in the outer leaflet of the MOM (3 % of the total MOM lipid content) [29,30]. Furthermore, at the contact sites between the mitochondrial inner and outer membranes, CL content can reach 20 %–25 % [31]. Other lipids are distributed uniformly between the MOM and the inner membrane. To address a potential role for the physical properties of mitochondrial lipids in Bax-mediated permeabilization, we varied the amounts of different lipids based on the physical properties summarized in Table 1, with a focus on CL and other negatively charged lipids and cholesterol.

CL has been implicated in the recruitment and activation of both tBid and Bax at the MOM [32–39]. However, several reports have challenged an obligatory role of CL at the MOM for the induction of MOMP, as over 95 % of CL and its metabolites are present in the mitochondrial inner membrane [29,40]. Other investigators suggest that the overall negative charge of the lipids, rather than the individual lipids, promotes tBid targeting to the membrane [41] and the interaction of Bax C-terminus with the membrane [42]. Additionally, tBid translocates to the MOM during apoptosis despite the progressive loss of CL, which has been attributed to an enhanced binding to monolysocardiolipin (MLCL), a metabolite of CL [43]. Presently, it remains unclear if the CL present at the MOM (even considering the high CL content at the contact sites) is necessary and sufficient to efficiently recruit tBid and Bax. Since targeting of tBid to the MOM must precede the assembly of Bax pores in the membrane [5,10], it is essential to determine the importance of specific interaction(s) of tBid and CL that drive the onset of apoptosis.

Although only ∼5 % of the total cellular cholesterol resides in the mitochondria, it plays a major role in regulating the integrity of the membranes during MOMP [28,44]. At the mitochondria, cholesterol contributes 4 %–8 % of total lipid mass and most of it is present at the MOM [28,31]. The increased mitochondrial cholesterol noted in cancer cells promotes resistance to both chemotherapy and Bax-mediated MOMP. Lowering cellular cholesterol content of cells induces p53 dependent activation of Bax and sensitizes cancer cells to apoptosis [45,46]. Cholesterol is well known to control the fluidity of lipid membranes and it is likely that this effect contributes to the inhibition of Bax-mediated MOMP. However it is not clear if cholesterol achieves MOMP inhibition solely by directly hindering the insertion of Bax into the membrane as suggested in previous studies [47,48] or by influencing a step upstream of Bax insertion.

In contrast with tBid and Bax, not much is known about the interactions of the pro-apoptotic BH3 protein Bim at the MOM. Although both tBid and Bim induce Bax-/Bak-mediated apoptosis, they are not functionally identical: Bim shows a preference to bind to Bax, whereas tBid shows a preference to bind to Bak [49]. In addition, whereas tBid shares its 3D-fold with Bax and Bcl-XL, Bim is intrinsically unstructured and evolutionarily distant from tBid [50–52]. Therefore, it is quite possible that the interactions of tBid and Bim with membranes necessary for Bax activation differ for membranes with different compositions of lipids.

Complete elimination of essential lipids in cells is not compatible with survival, whereas experiments based on partial lipid elimination have failed to definitively establish a role for specific lipids in MOMP [53]. Therefore, to rigorously examine these issues, we used a well-established system of purified full-length proteins and liposomes that authentically reproduces the core features of MOMP. To dissect the mechanism of permeabilization of membranes containing different lipids, we used a range of fluorescence techniques to discriminate the individual steps [5,10]. The composition of the liposomes we used is shown in Table 2. We found that anionic phospholipids in the membrane recruit both cBid and Bim. However, whereas CL is not required for the initial binding of cBid to the membrane, it is necessary to promote an activating conformational change in tBid, a step that in cells is also facilitated by Mtch2. In contrast, increases in membrane cholesterol impede both the recruitment of Bax by the activators and Bax insertion into the membrane, thus inhibiting membrane permeabilization.

EXPERIMENTAL

Protein purification and labelling

Recombinant full-length human wild-type (WT) Bax was purified and the single-cysteine mutant, Bax 126C was purified and labelled with the environment sensitive dye *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (NBD; Life Technologies) as described [10,54]. Recombinant full-length N-terminal 6×-histidine-tagged murine WT Bid and the single-cysteine mutants, Bid 126C and 163C were purified, labelled and cleaved with caspase-8 as described [5]. Bid 126C was labelled with *N*-(7-dimethylamine-4-methylcoumarin-3-yl) maleimide (DAC) and Bid 163C was labelled with NBD (Life Technologies) as described [5]. Recombinant full-length N-terminal 6×-histidine-tagged human WT BimL (Bim large) and the single-cysteine mutant BimL 104C were purified as described [49]. BimL 104C was labelled with DAC by following the same labelling protocol as used for labelling Bid [5]. Briefly, the protein was incubated with a 10–15 molar excess of dye in the dialysis buffer for 2–3 h at room temperature with rotation in the presence of 0.5 % CHAPS. The dye was dissolved in DMSO and the final percentage DMSO content of the reaction was kept below 10 %. Then BimL was subjected to nickel-affinity chromatography and washed with buffer to remove the excess unreacted dye. The labelled BimL was then eluted, dialysed and stored at −80 °C until use. For simplicity, BimL is referred to as Bim.

Mitochondrial permeabilization assay

Mitochondria were isolated from the livers of $bak^{-/-}$ mice and stored at −80 °C, as described [5]. MOMP was assessed by the release of cytochrome *c* from mitochondria after incubation with indicated concentration of Bax with cBid or Bim for 30 min at 37 °C. Detection of cytochrome *c* and quantification of the immunoblots were performed as described [5].

Table 2 Nomenclature and lipid composition of liposomes used in assays

Name	Average negative charge/Lipid	mol % composition							
		PC	PE	PI	PS	CL	MLCL	PG	Cholesterol
Mitochondria-like	0.28	48	28	10	10	4	–	–	–
8 % Chol	0.28	40	28	10	10	4	–	–	8
20 % Chol	0.28	28	28	10	10	4	–	–	20
Low charge	0.10	62	28	10	–	–	–	–	–
No charge	0	72	28	–	–	–	–	–	–
14 % CL	0.38	48	28	10	–	14	–	–	–
24 % PS	0.24	48	28	–	24	–	–	–	–
14 % PS	0.24	48	28	10	14	–	–	–	–
10 % PS	0.20	52	28	10	10	–	–	–	–
0 % PS, 4 % CL	0.18	58	28	10	–	4	–	–	–
4 % MLCL	0.28	48	28	10	10	–	4	–	–
14 % PG	0.24	48	28	10	–	–	–	14	–
24 % PI	0.24	48	28	24	–	–	–	–	–

Liposome preparation

Liposomes with mitochondria-like lipid composition were prepared as described previously, using the following lipids (all from Avanti Polar Lipids) in the mol% ratio outlined in Table 2: PC, PE, PI, PS, CL [5,54]. This mixture reflects the MOM phospholipid composition [28–31] and has been shown numerous times to support Bid and Bax-mediated pore formation [5,10,11,17,54–57]. Other lipids were added to the membrane as indicated: MLCL, phosphatidylglycerol (PG) and cholesterol. The surface charge of the membrane was determined for each different lipid composition based on the pK_a values of the functional groups at pH 7 [58]. Liposome concentration was estimated using ∼84000 lipids per 100 nm diameter liposome [54]. See Table 1 for a summary of the physical properties of the lipids used in the present study.

Liposome permeabilization assay

Liposomes encapsulating the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and the collisional quencher *p*-xylene-bis-pyridinium bromide (DPX) were prepared as described [54,56]. ANTS–DPX liposomes (0.5 nM) were incubated with the indicated concentration and combination of proteins for 3 h at 37 °C without stirring. Control experiments for liposomes alone and for liposomes with one of cBid, Bim or Bax yielded 5 %–15 % release.

Membrane-binding assay

Membrane binding was assessed using a protein-membrane FRET assay described previously [10,17]. The position of the label is indicated for each of the proteins as follows: cBid 126C DAC indicates that the DAC fluorophore is covalently attached to the side chain of a cysteine at position 126 in cBid. All the labelled proteins used in the present study are single-cysteine mutants. Assays containing 20 nM cBid 126C DAC or Bim 104C DAC (donor) were incubated with different concentrations of unlabelled liposomes (no acceptor control) or with liposomes containing 1 mol% of PE labelled with the acceptor NBD at the tail (Avanti Polar Lipids) for 20 min at 37 °C without stirring. The donor was excited at 380 nm (5 nm bandwidth) and its fluorescence emission was monitored at 460 nm (20 nm bandwidth). The fluorescence intensity recorded in the sample containing both the donor and the acceptor was denoted F_{+A} (t) and that recorded in the sample containing unlabelled liposomes was denoted F_{-A} (t). The FRET efficiency is given by: E (t) = $\{1 - [F_{+A}$ (t)/F_{-A} (t)]$\}$ × 100. The final FRET efficiency, E (t = 20 min), is proportional to the fraction of proteins bound to the liposomes at equilibrium. For each experiment, the data from several independent repeats were averaged to yield a titration curve showing the average FRET efficiency ± S.E.M as a function of liposome concentration. These data were then fit with the software GraphPad Prism using the free ligand approximation, as described in [5]. This fit returned a value and error for the dissociation constant (K_D) associated with the binding of the protein to the liposomes. The binding equilibrium of the protein to the lipid phase was also described by a partition coefficient (P), $P = 1/(V_L K_D)$, where V_L is the molar volume of the liposomes (volume of the lipid phase for 1 mole of liposomes), V_L = 7.6×10^4 l/mol [57].

Protein–protein binding assay

Protein–protein binding was assessed using a FRET assay carried out as described previously [10]. cBid 126 DAC or Bim 104 DAC (donor) was incubated with Bax 126 NBD (acceptor) or unlabelled Bax (no acceptor control) at the indicated concentrations in the presence of liposomes for 2 h at 37 °C without stirring. The donor was excited at 380 nm (5 nm bandwidth) and its emission was monitored at 460 nm (20 nm bandwidth). The binding of the two proteins was assessed using the percentage of FRET efficiency, given as before by: $E = \{1 - [F_{+A}$ (t)/F_{-A} (t)]$\}$ × 100.

Conformational change assay

The assay was carried out as described using the environment-sensitive fluorescence of NBD [5,10]. Liposomes were added to the indicated concentration of cBid 163 NBD or Bax 126 NBD and incubated for 20 min or 2 h respectively at 37 °C without stirring. Measurements with Bax 126 NBD were obtained simultaneously with the protein–protein FRET assay described above. The change in the fluorescence intensity of NBD was monitored using excitation at 475 nm (5 nm bandwidth) and emission at 530 nm (20 nm bandwidth). Normalized change in NBD fluorescence was calculated as F/F_0, where F is the fluorescence of NBD at the reaction end-point and F_0 is the fluorescence at time $t = 0$.

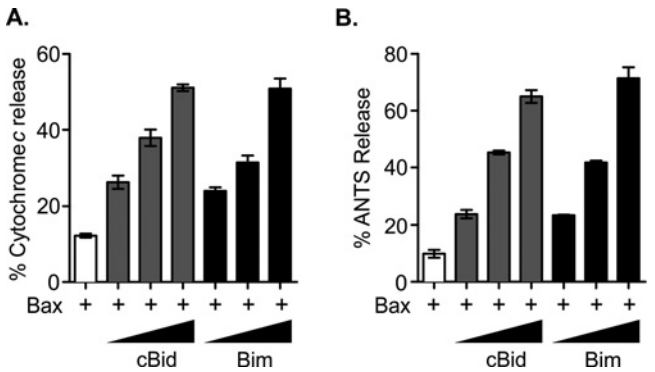

Figure 1 cBid and Bim directly activate Bax in a dose-dependent manner

(**A**) Membrane permeabilization of mitochondria isolated from *bak*$^{-/-}$ mice measured by the release of cytochrome *c*. Mitochondria (1 mg/ml) were incubated with either 50 nM Bax alone or with increasing concentration of cBid (20, 60 and 400 pM, grey) or of Bim (100, 300 and 2000 pM, black). Cytochrome *c* release was measured by comparing the amount of protein in the pellet and the supernatant as quantified by immunoblotting (mean ± S.E.M., $n = 3$). (**B**) Membrane permeabilization of liposomes encapsulating ANTS and DPX. Mitochondria-like liposomes were incubated with either 100 nM Bax alone or with increasing concentration of cBid (1, 3 and 20 nM, grey) or of Bim (1, 3 and 20 nM, black), (mean ± S.E.M., $n = 3$).

RESULTS

cBid and Bim induce Bax dependent membrane permeabilization in mitochondria and liposomes

To compare the activity of cBid and Bim in catalysing Bax-mediated membrane permeabilization, we tested the two activators using isolated mitochondria from *bak*$^{-/-}$ mice that do not contain endogenous membrane bound Bax. As expected, for both proteins there was a dose-dependent effect of sub-nanomolar concentrations of cBid or Bim on cytochrome *c* released in the presence of Bax (Figure 1A). Using a model system to study membrane permeabilization in more detail with liposomes encapsulating both a fluorophore (ANTS) and a quencher (DPX), we examined the effects of increasing cBid or Bim on Bax activation [55,56]. Similar to results obtained with isolated mitochondria, both cBid and Bim directly activated Bax to permeabilize the membrane to a comparable extent and in a dose-dependent manner (Figure 1B).

Cholesterol inhibits Bax membrane permeabilization by impeding the interaction of activators with Bax and its subsequent insertion in the membrane

Cholesterol has been shown to affect the binding of cBid and Bax to liposomes and isolated mitochondria [45,47,48]. To further characterize the mechanism by which cholesterol regulates membrane permeabilization by Bcl-2 proteins, we prepared liposomes with two different amounts of cholesterol: 8 % and 20 % (see Table 2 for details on membrane composition) [44]. In agreement with the above studies, high cholesterol levels (20 % Chol) caused a significant reduction in the final membrane permeabilization (ANTS–DPX release) of liposomes by cBid and Bax compared with liposomes with mitochondria-like composition. Moreover, our results demonstrate that Bim-mediated Bax activation is similarly inhibited (Figure 2A).

To determine which step of Bax pore formation was inhibited by cholesterol, we employed dedicated fluorescence based assays to probe each step separately [5,10]. First, we investigated the effect of cholesterol on cBid or Bim binding to the membrane using a protein to membrane FRET assay. DAC-labelled cBid or Bim was used as a donor, whereas liposomes were labelled with the acceptor fluorophore NBD. For mitochondria-like lipid composition, we obtained a protein to liposome K_D of 0.36 nM for Bim and 0.76 nM for cBid (a value that is very close to our previously reported K_D of 0.9 nM using a different technique) [5]. From these K_D values, the P associated with the binding of cBid and Bim to liposomes with different lipid compositions were also calculated (Table 3). Binding to the membrane was slightly lowered by cholesterol, for both cBid and Bim, as indicated by minor changes in the value of the K_D (Table 3). This result is in contrast with a previous report that cholesterol enhances cBid binding to membranes, as measured by a targeting assay to liposomes with supra-physiological CL content [48]. To investigate subsequent steps in the pathway independently of this slight change in K_D, a liposome concentration higher than the K_D was used, such that the total amount tBid–Bim bound to membrane at equilibrium remained the same within error for different cholesterol concentrations. We thus examined the conformational change of tBid after binding the membrane, the rate-limiting step of tBid activation [5]. Using cBid labelled with the environment-sensitive dye, NBD, we observed a higher level of conformational change for tBid at 8 %, but not in 20 % cholesterol liposomes. Thus a moderately increased level of cholesterol may be more conducive to the activating tBid conformational change (Figure 2B).

Table 3 K_D and P associated with the binding of cBid and Bim to liposomes with different lipid compositions. The K_D is reported in liposome concentration (nM)

	cBid		Bim	
Liposomes	K_D (nM)	10^3 P	K_D (nM)	10^3 P
14 % CL	0.58 ± 0.10	22.7 ± 3.9	0.32 ± 0.04	41.1 ± 5.1
Mitochondria-like	0.76 ± 0.17	17.3 ± 3.9	0.36 ± 0.10	36.5 ± 10
14 % PS	1.06 ± 0.22	12.4 ± 2.6	0.68 ± 0.08	19.3 ± 2.3
0 % PS, 4 % CL	1.83 ± 0.48	7.2 ± 1.9	0.84 ± 0.22	15.7 ± 4.1
Low charge	2.66 ± 0.5	4.9 ± 0.9	1.48 ± 0.31	8.9 ± 1.9
No charge	>40	<0.3	8.24 ± 3.92	1.6 ± 0.8
8 % Chol	1.35 ± 0.27	9.7 ± 2.0	0.80 ± 0.25	16.4 ± 5.2
20 % Chol	1.20 ± 0.15	11.0 ± 1.4	0.75 ± 0.15	17.5 ± 3.5
4 % MLCL	1.91 ± 0.30	6.9 ± 1.1	0.29 ± 0.06	45.4 ± 9.4
14 % PG	3.50 ± 0.81	3.8 ± 0.9	0.42 ± 0.10	31.3 ± 7.5
24 % PI	7.31 ± 2.0	1.8 ± 0.5	0.98 ± 0.13	13.4 ± 1.8

The effect of cholesterol on tBid–Bax and Bim–Bax interaction is unknown. Therefore, we used a FRET-based protein–protein binding assay with DAC-labelled cBid or Bim as a donor and NBD-labelled Bax as acceptor. The extent of tBid–Bax and Bim–Bax interaction was notably lower in liposomes containing high amounts of cholesterol, although the kinetics of these interactions remained largely unaffected (Figure 2C). We then tested the effect of cholesterol on the next step in the process, Bax insertion into membranes, using NBD-labelled Bax. As shown in Figure 2D, both the extent and the kinetics of Bax insertion were significantly reduced by increasing cholesterol in the membrane, maybe as a direct consequence of the decrease in Bax interaction with activator proteins. In either case, these effects led to lower Bax oligomerization and membrane permeabilization.

Anionic lipids are required for binding of activator proteins to membranes

As mentioned above, the role of negatively charged lipids in MOMP is unresolved. Therefore, we tested the hypothesis that

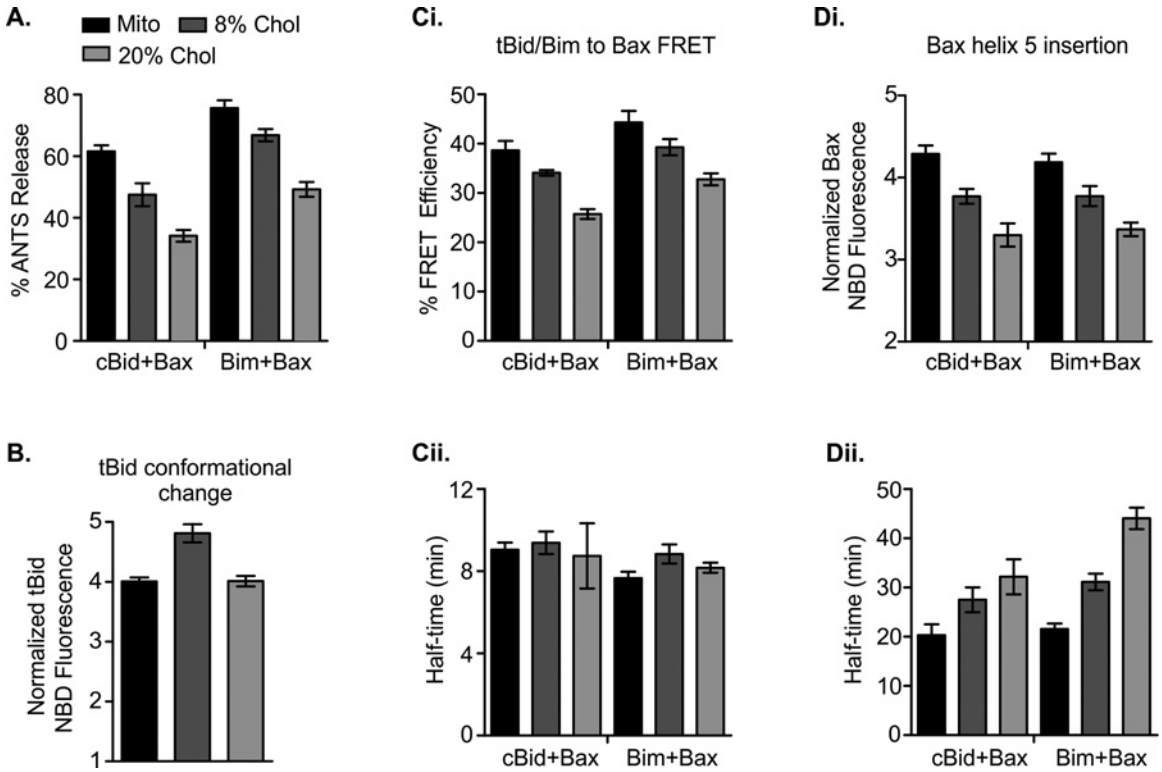

Figure 2 Cholesterol impedes membrane permeabilization by hindering Bax activation and insertion into the membrane

(**A**) Liposomes encapsulating ANTS and DPX with mitochondria-like (Mito), 8% and 20% cholesterol (8% and 20% Chol respectively) lipid composition were used to assay membrane permeabilization by 80 nM Bax and 20 nM of either cBid or Bim (mean ± S.E.M., $n = 3$). (**B**) The effect of lipids on the tBid conformational change as assayed by an increase in the NBD fluorescence, resulting from residue 163 moving to a more hydrophobic environment (membrane). Twenty nanomoles of cBid 163 NBD was added to 1.5 nM liposomes of the indicated lipid composition, (mean ± S.E.M., $n = 5$). (**C**) Interaction of Bax with membrane-bound tBid or Bim measured using FRET. Twenty nanomoles of cBid 126 DAC or Bim 104 DAC (donor) with 80 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with the indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid–Bax or Bim–Bax (**Ci**) and the half-time obtained from the fit of the data with a single-exponential function is shown in (**Cii**) (mean ± S.E.M., $n = 5$). (**D**) Insertion of Bax into the membrane after activation. The change in the environment of the NBD-label of Bax 126 NBD (80 nM) activated with cBid or Bim from the same reaction as (**C**). A decrease in NBD intensity signifies less insertion of Bax in the membrane. The end-points of the data are shown in (**Di**), whereas the half-time obtained from the fit of the data with a single-exponential function is shown in (**Dii**) (mean ± S.E.M., $n = 5$).

electrostatic interactions play a crucial role in the recruitment of Bax activators and the activation of Bax. We used liposomes with a lipid composition resulting in either a low average negative surface charge of 0.10 per lipid (low charge) or no negative charge (no charge; Table 2). In agreement with the hypothesis, membrane permeabilization with 'low charge' and 'no charge' liposomes was drastically reduced compared with liposomes with a mitochondrial lipid composition (average negative surface charge of 0.28 per lipid). This reduction was more noticeable when cBid was used as an activator (Figure 3A).

To determine how electrostatics may influence Bax-mediated membrane permeabilization, we investigated each step separately. With 'low charge' liposomes, the K_D corresponding to the dissociation from cBid and Bim to membrane increased 3–4-fold. With 'no charge' liposomes it increased over 20-fold for Bim and over 50-fold for cBid (Figure 3B; Table 3). Thus, the dependence of membrane binding on the anionic lipids is stronger for cBid than for Bim. Since the binding of the activator proteins to membranes with low negative charge is significantly compromised, we observed low levels of tBid–Bax and Bim–Bax FRET and Bax helix five insertion into membranes (Figures 3C and 3D). The FRET between Bim and Bax in solution is ∼20% (result not shown); therefore, we observe higher Bim–Bax FRET than tBid–Bax FRET in low-charge and no-charge liposomes.

To further study the effect of anionic lipids on the interaction of Bax activators with the membrane, we adjusted the lipid composition to generate liposomes with a range of average negative surface charge. Liposomes were prepared by replacing one or several of the three different anionic lipids PI, PS and CL with the neutral lipid PC (see Table 2 for detailed lipid compositions). We observed a robust positive correlation between the densities of anionic lipids with membrane permeabilization using Bax activated by cBid or Bim, regardless of the anionic lipids that were used (Figure 4A). The strong linear correlation between the amount of anionic phospholipids and the binding affinities of both cBid and Bim for membranes suggests that binding of tBid and Bim to membranes limits membrane permeabilization in our assays (Figure 4B). These results also suggest that binding of both cBid and Bim to membranes is regulated by electrostatic interactions with anionic phospholipids.

Cardiolipin accelerates the conformational change in tBid to mediate membrane permeabilization

To determine the specific effect of CL on Bax activation by cBid or Bim, we replaced CL with PS in the membrane composition. As noted above, we observed membrane permeabilization in liposomes without CL for both cBid and Bim, as long as the average negative charge of the membrane was preserved (Figure 5A). Decreasing the average negative charge of the membrane resulted

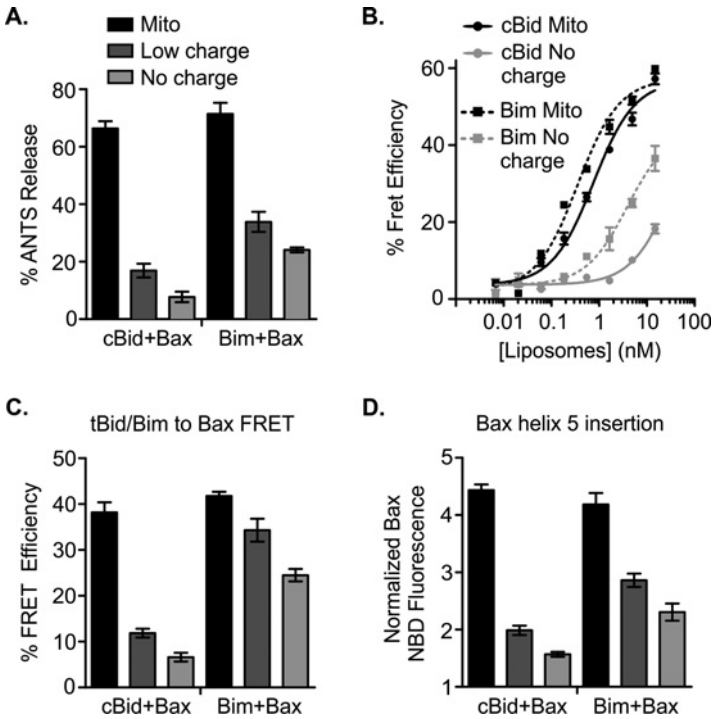

Figure 3 Membrane permeabilization requires anionic lipids at the membrane

(**A**) Mitochondria-like (Mito), Low charge (with average negative charge/lipid of 0.10) and No charge (no negative charge/lipid) liposomes encapsulating ANTS and DPX were used to assay membrane permeabilization by 100 nM Bax and 20 nM of either cBid or Bim (mean ± S.E.M., $n = 3$). See Table 2 for details on lipid compositions. (**B**) Binding of cBid and Bim to Mito (black) and No charge (grey) liposomes as measured by FRET. Twenty nanomoles of cBid 126 DAC (solid line) or Bim 104 DAC (dashed line) labelled with the donor dye were incubated with different concentrations of liposomes containing the acceptor, NBD–PE. An increase in the percentage FRET efficiency indicates more binding of protein to liposomes. The data (dots/squares) are fit with an equation described in [5] (line; mean ± S.E.M., $n = 3$) See Table 3 for the K_D values. (**C** and **D**) Interaction of Bax with membrane-bound tBid or Bim measured using FRET (**C**) and insertion of Bax in the membrane after activation (**D**). Twenty nanomoles of cBid 126 DAC or Bim 104 DAC (donor) with 100 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes of the indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid–Bax or Bim–Bax (**C**) and increasing NBD intensity signifies more insertion of Bax in the membrane (**D**) (mean ± S.E.M., $n = 3$).

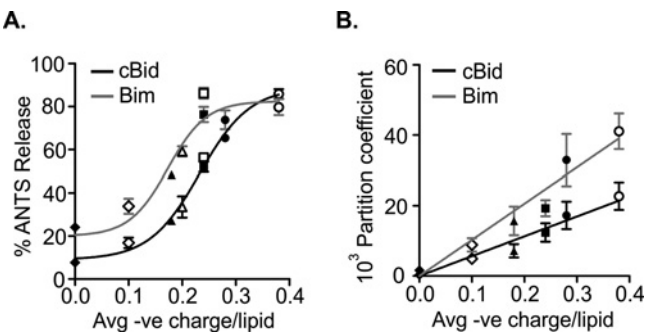

Figure 4 Electrostatic interactions mediate cBid and Bim binding to membranes

(**A**) Extent of liposome permeabilization as a function of average negative charge/lipid for cBid–Bax and Bim–Bax. These data are also presented in Figures 3A and 5A. (**B**) *P* of cBid and Bim binding to membranes as a function of average negative charge/lipid. These data are also presented in Table 3. The data points shown are as follows (name, average negative charge): ◆ No charge, 0.0; ◇ Low charge, 0.10; ▲ 0 % PS, 4 % CL, 0.18; △ 10 % PS, 0.20; ■ 14 % PS, 0.24; □ 24 % PS, 0.24; ● mitochondria-like, 0.28; and ○ 14 % CL, 0.38. See Table 2 for complete lipid compositions.

in lower amounts of membrane permeabilization; and this effect was stronger when using cBid as the activator.

However, a specific role for CL emerged when the interaction of cBid and Bim with the membrane was examined in more detail. The binding affinity of cBid and Bim for membranes with mitochondria-like composition was very similar to their binding affinity for membranes with similar surface charge but containing no CL (14 % PS, no CL), but higher than their affinity for membranes with slightly lower surface charge but containing 4 % CL (0 % PS, 4 % CL; Table 3). This demonstrates that CL does not affect binding to membranes of activators beyond its effect on the membrane surface-charge and maybe curvature. By contrast, tBid conformational change upon binding to membranes was fully impaired in liposomes lacking CL, but reduced only by half in liposomes with a surface charge less negative than the mitochondria-like liposomes but still containing CL (Figure 5B). Thus CL clearly facilitates this particular step of tBid activation.

We also examined the effect of CL on tBid–Bax and Bim–Bax interactions. Lowering the average negative charge of the liposomes impaired the interaction between tBid and Bax markedly and had some effect on the interaction between Bim and Bax, as seen by a decrease in final efficiency of FRET and an increase in the half-time of the reaction recorded for both tBid–Bax and Bim–Bax (Figure 5C). This effect can be mostly attributed to the inefficient binding of cBid and Bim to the membranes with lower average negative charge, as membrane binding by activator proteins precedes or coincides with their interaction with Bax. The only exception was 4 % CL membranes, where the final extent of activators–Bax FRET is lower due to less cBid/Bim binding to the membrane with a lower negative charge (Figure 5Ci); strikingly, CL increased the kinetics of this interaction for tBid–Bax and Bim–Bax (Figure 5Cii), in perfect agreement with its proposed role as an accelerator of membrane permeabilization.

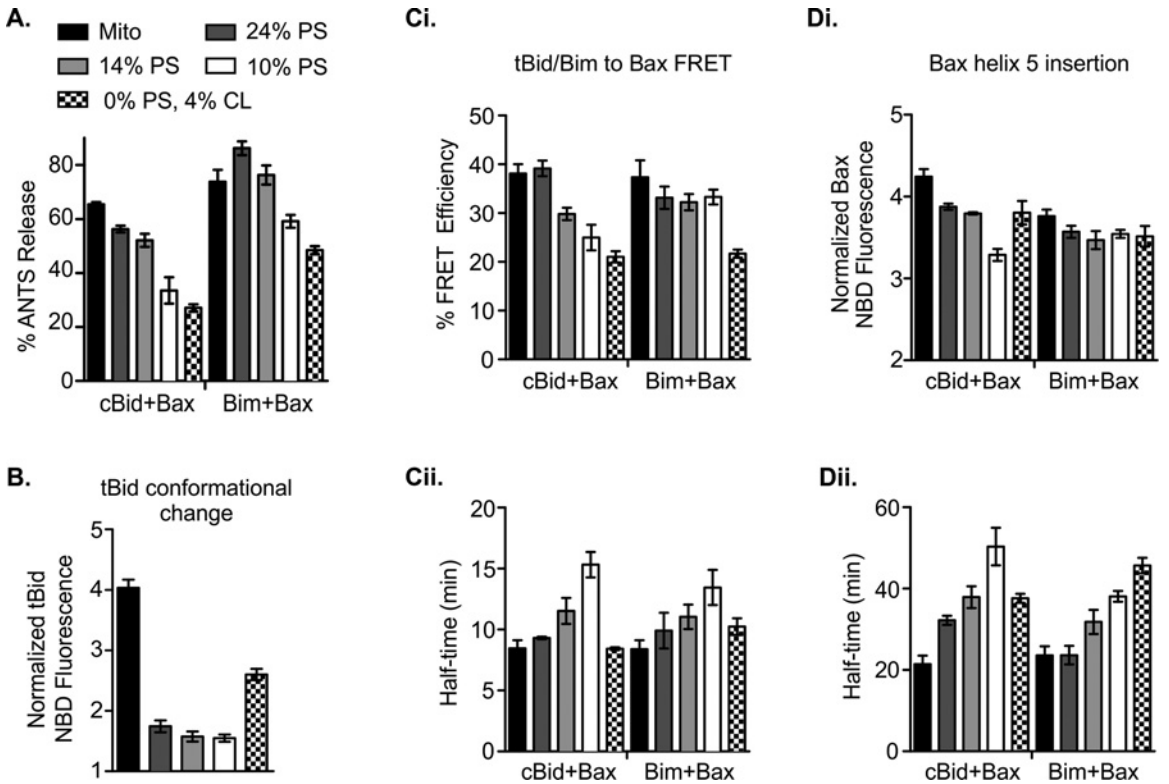

Figure 5 CL accelerates the conformational change in tBid to mediate membrane permeabilization

(**A**) Liposomes encapsulating ANTS and DPX in mitochondria-like (Mito), 24% PS, 14% PS, 10% PS and 0% PS, 4% CL lipid compositions were used to assay membrane permeabilization by 100 nM Bax and 20 nM of either cBid or Bim (mean ± S.E.M., $n = 3$). See Table 2 for details on lipid compositions. (**B**) tBid conformational change measured as an increase in the NBD fluorescence resulting from the residue moving to a more hydrophobic environment (membrane). Twenty nanomoles of cBid 163 NBD was added to 1.5 nM liposomes of indicated lipid composition (mean ± S.E.M., $n = 3$). (**C**) Interaction of Bax with membrane-bound tBid or Bim measured using FRET. Twenty nanomoles of cBid 126 DAC or Bim 104 DAC (donor) were added together with 100 nM Bax 126 NBD (acceptor) to 1.5 nM liposomes with the indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid–Bax or Bim–Bax (**Ci**) and the half-time obtained from the fit of the data with a single-exponential function is shown in (**Cii**), (mean ± S.E.M., $n = 3$). (**D**) Insertion of Bax in the membrane after activation. The change in the environment of the NBD-labelled residue of 100 nM Bax 126 NBD that was activated with cBid or Bim from the same reaction as (**C**). A higher fold change in NBD intensity signifies more insertion of Bax in the membrane. The end-points of the data are shown in (**Di**), whereas the half-time obtained from the fit of the data with a single-exponential function is shown in (**Dii**) (mean ± S.E.M., $n = 3$).

For the next downstream step, Bax helix 5 insertion, it was the rate rather than the eventual extent of insertion into the membrane that depended on the anionic lipids (Figure 5D). There was less effect on the final Bax helix 5 insertion probably due to the 'amplification' that occurs with Bax auto-activation (not to be confused with Bax baseline activity) (Figure 5Di). However, the specific dependence of tBid activation on CL was still discernable at this stage by differences in the reaction rate, since as little as 4% CL could induce as much Bax insertion and as quickly as with membranes with much more negative surface charge but no CL (compare the results for the '24% PS, no CL' and '14% PS, no CL' liposomes with those for the 'no PS, 4% CL').

Activation of Bax by Bim is independent of the identity of anionic phospholipids

To further investigate the role of specific lipid composition compared with overall electrostatic interactions, we prepared liposomes in which CL was replaced with MLCL, PG or PI (Table 2). As a predominant catabolic product of CL in mitochondrial membranes in both healthy and apoptotic cells [43,59], MLCL could be expected to fully substitute for CL. When using cBid to activate Bax, we found that permeabilization of membranes containing MLCL, PG or PI instead of CL was significantly compromised compared with mitochondria-like membranes. On the other hand, when Bax was activated by Bim, membrane permeabilization was not affected by replacing CL by MLCL and only slightly impaired when replacing it with PG or PI (Figure 6A). This result again points to unexpected differences between the lipid requirements for Bim and Bax to activate Bax.

To determine which step was most sensitive to the lipid type, we first measured protein-membrane affinity. We found that whereas the K_D of Bim binding to these membranes was not affected, the K_D of cBid binding to membranes increased ~4 and ~10-fold with 14% PG and 24% PI respectively, in spite of these membranes having a similar net negative charge to mitochondria-like membranes (Table 3). Consequently, very little tBid conformational change was observed with these liposomes (Figure 6B). In contrast, MLCL affected membrane binding of cBid only minimally, yet it severely hindered the subsequent conformational change of tBid (Table 3; Figure 6B). This underlines the exquisite structural specificity associated with CL enhancement of tBid conformational change.

The conformational equilibrium of tBid on membranes affects primarily the kinetics of Bax activation rather than the final extent of membrane permeabilization [5]. Therefore, we examined the effect of MLCL, PG and PI on activator–Bax interactions by FRET. Membranes containing MLCL showed no significant difference in the kinetics or the end-points of tBid/Bim to Bax FRET (Figure 6C). This result demonstrates that tBid binds to Bax on membranes lacking CL, but that the absence of the tBid

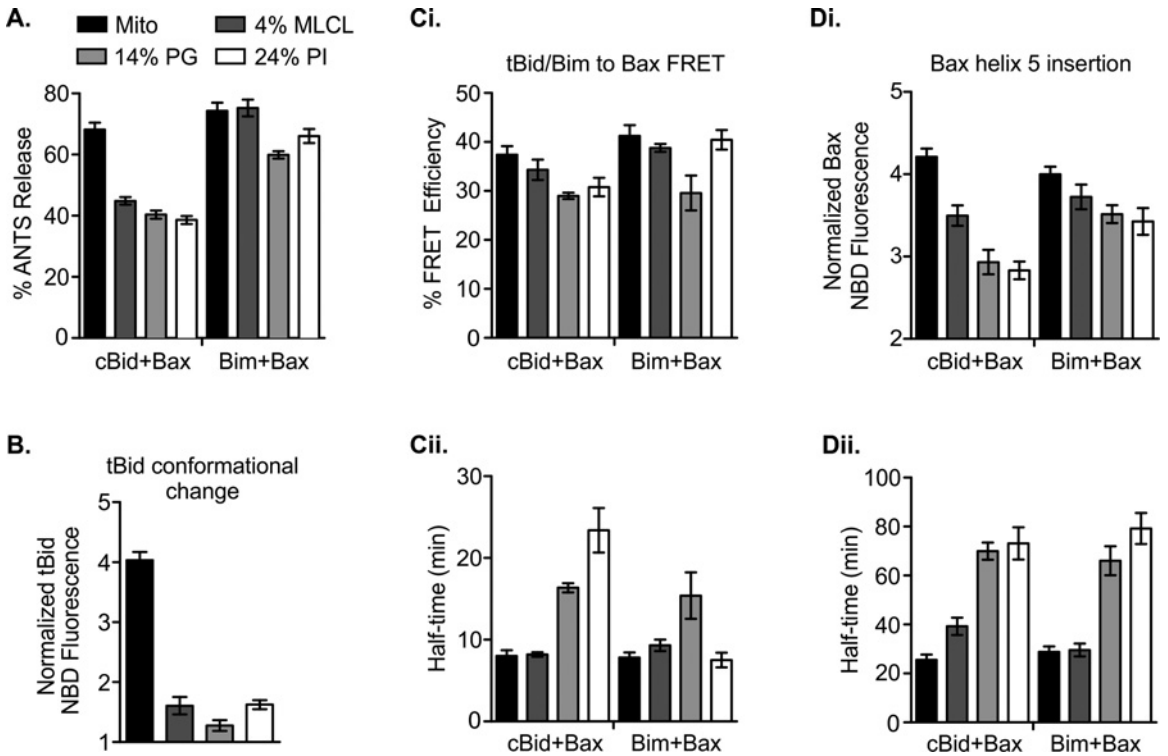

Figure 6 Specific anionic lipids are required to replace CL for efficient cBid-mediated Bax membrane permeabilization

(**A**) Liposomes encapsulating ANTS and DPX in mitochondria-like (Mito), 4 % MLCL, 14 % PG and 24 % PI lipid composition were used to assay membrane permeabilization using 100 nM Bax and 20 nM of either cBid or Bim (mean ± S.E.M., $n = 3$). See Table 2 for details on lipid compositions. (**B**) tBid conformational change as assayed by an increase in the NBD fluorescence reflecting the residue moving to a more hydrophobic environment (membrane). Twenty nanomoles of cBid 163 NBD was added to 1.5 nM liposomes with the indicated lipid composition (mean ± S.E.M., $n = 3$). (**C**) Interaction of Bax with membrane-bound tBid or Bim measured using FRET. Twenty nanomoles of cBid 126 DAC or Bim 104 DAC (donor) and 100 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with the indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid–Bax or Bim–Bax (**Ci**) and the half-time obtained from the fit of the data with a single-exponential function is shown in (**Cii**) (mean ± S.E.M., $n = 3$). (**D**) Insertion of Bax in the membrane after activation. The change in the environment of the NBD-labelled residue of Bax 126 NBD (100 nM) activated with cBid or Bim from the same reaction as (**C**). A higher fold change in NBD intensity signifies more insertion of Bax in the membrane. The end-points of the data are shown in (**Di**), whereas the half-time obtained from the fit of the data with a single-exponential function is shown in (**Dii**) (mean ± S.E.M., $n = 3$).

conformational change impaired Bax activation. Thus in this case, the decrease in membrane permeabilization seems predominantly due to a decrease in Bax helix 5 insertion (Figure 6D).

Small differences in the end-points and slower binding kinetics of tBid/Bim to Bax were observed with membranes containing PG (Figure 6C). This may be due to a negative effect of PG on Bax. Consistent with this hypothesis, insertion of Bax helix 5 also proceeded to a lower extent and with slower kinetics for liposomes with PG, for both tBid and Bim (Figure 6D). Similarly, PI decreased the rate of tBid binding to Bax, without significantly changing its final amount (Figure 6C). Insertion of Bax helix 5 also proceeded to a lower extent and with slower kinetics for liposomes with PI for both tBid and Bim. This suggests that the low membrane permeabilization observed with cBid and Bax in PG and PI liposomes is due to the additive effect of the low affinity of cBid for the membrane, the inefficiency of tBid conformational change, the slow kinetics of tBid–Bax binding and the slow and inefficient insertion of Bax helix 5 in the membrane. In contrast, these steps are only slightly-impaired with Bim and Bax.

DISCUSSION

The main locus of the action of Bcl-2 proteins is the MOM, where protein–protein and protein–membrane interactions decide the fate of the cell. During MOMP, the membrane is an active participant that not only contains and recruits Bcl-2 proteins but also promotes conformational changes in Bcl-2 proteins that promote their apoptotic regulatory functions [3,15]. In the present study, we demonstrate that CL induces a conformational change in membrane bound tBid that enhances its activation of Bax. Moreover, we show that high levels of cholesterol inhibit the interaction of both tBid and Bim with Bax as well as interfering with insertion of Bax into the lipid bilayer.

Our results highlight both the similarities and the differences in the dependence of cBid and Bim on specific MOM lipids to efficiently activate Bax. Whereas cBid and Bim binding to membranes is enhanced by anionic phospholipids, a negative surface charge is a stronger requirement for cBid. Nevertheless, there is a very clear positive correlation between the density of negative charges at the membrane and the K_D of cBid and Bim for binding to the membrane (Figure 4B). At one extreme, no stable binding of cBid to neutral liposomes was detected, whereas Bim bound the same liposomes with a K_D of ~8 nM (Table 3). We speculate that the translocation of cBid and Bim to the MOM is mediated by interactions between the anionic lipids at the membrane and the cluster of positively charged residues found between the membrane-binding helices of tBid and adjacent to the C-terminal region of Bim. In agreement with our findings, electrostatic interactions have been reported previously to contribute to binding both cBid and pro-apoptotic sensitizer Bcl-2 protein Bad, to membranes [41,60,61]. In fact, the importance of electrostatic interactions for proteins binding to membranes has been documented for several membrane proteins,

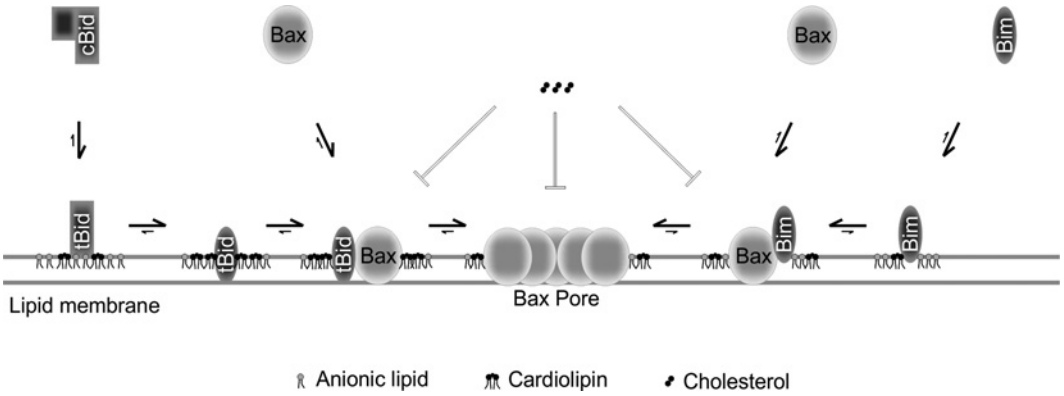

Figure 7 Schematic overview of the control by lipids of membrane permeabilization by Bax when activated by cBid or Bim

Bax activated by cBid or Bim inserts and oligomerizes that result in membranes permeabilization. The initial binding of both cBid and Bim to the membrane is governed by electrostatic interactions between the positively charged residues on the proteins and the anionic lipids at the membrane. Whereas specific lipids do not influence the activity of Bim, tBid requires CL in the membrane to undergo a conformational change required to activate Bax. Binding of tBid to the membrane potentially rearranges and clusters CL, causing a local increase in spontaneous membrane curvature that may promote pore formation by Bax. Cholesterol inhibits membrane permeabilization by impeding the interaction between Bax and the activator proteins and Bax insertion in the membrane. Therefore, the lipid content of the membrane can modulate membrane permeabilization at multiple steps.

such as cytochrome *c* [62], diphtheria toxin [63] and colicin A [64]. Our studies systematically varying the lipid composition of the liposomes suggest that different MOM lipids function additively in recruiting cBid and Bim by contributing to the negative charge density at the membrane surface.

Beyond their common reliance on negative membrane surface-charge, the responses of the two pro-apoptotic activator proteins cBid and Bim to different lipid compositions suggest that their membrane-binding mechanisms are different. Although it does prefer negatively charged liposomes, Bim binds a variety of compositions of membranes, whereas cBid displays a higher sensitivity towards exact lipid makeup. For example, binding of cBid is reduced when PI or PG is the only source of the negative charge of the membrane (Table 3). In agreement with our results, Lutter et al. [32], also observed poor binding of tBid with liposomes containing amounts of PG to match the negative charge of CL. One possible explanation for lower cBid binding to PI or PG containing membranes is inefficient electrostatic interactions with these lipids because of steric hindrance caused by the bulky inositol and glycerol lipid head groups. Although not examined in the present study, it is also possible that a higher degree of saturation of the lipid tails plays a negative role in cBid binding with membranes. Indeed, cBid shows favourable binding to membranes containing CL, MLCL and PS that have two unsaturated chains, but lower binding to membranes containing PG and PI that have only one unsaturated chain. Lipid chain unsaturation has also been shown to influence the insertion of Bax in the membrane [48].

Although negative charges are important, tBid specifically depends on CL to undergo efficient change in its conformation after binding to the membrane (Figure 7). Surprisingly, other negatively charged lipids including MLCL were unable to substitute for CL for the conformational change in tBid (Figure 6). In type II cells, CL has also been reported to be required for recruiting and activating caspase-8 at the MOM, which subsequently leads to the cleavage of full-length Bid to cBid [65]. A CL-rich membrane therefore provides the ideal environment for the full sequence of events pertaining to Bid activation, from cleavage by caspase-8, to binding to the membrane through electrostatic interactions and mediating the conformational change required for tBid-binding Bax. This observation is consistent with reports that relocalization of CL to the MOM provides or at the very least amplifies the signal for MOMP [40]. It is also possible that the favourable interaction of tBid with CL results in the formation of CL clusters, which may further promote subsequent interactions with Bax and Bak and membrane permeabilization (maybe through an effect of CL on membrane curvature) [66]. In support of the formation of CL clusters, the disruption of mitochondrial bioenergetics homoeostasis that follows binding of tBid to the MOM was shown to be mediated by the reorganization of CL within the mitochondrial membranes, whereas CL deficient mitochondria failed to exhibit any respiratory inhibition [34,35]. However, cBid and Bax efficiently permeabilized liposomes that contained PS in place of CL (Figure 5). These data show that the requirement for CL is primarily kinetic. Permeabilization of liposomes without CL probably results from amplification of tBid activation of a small amount of Bax by Bax auto-activation.

In cells, the MOM protein Mtch2 facilitates the conformational change in tBid that is required for the activation of Bax [5,67]. The fact that both Mtch2 and CL mediate the conformational change in tBid strongly suggests that similar to Mtch2, CL is also a receptor for cBid at the MOM and that the function of CL may overlap or synergize with that of Mtch2. Multiple binding partners of Bid, cBid and tBid at the MOM have been identified; however, the picture is often simplified. It now appears that a more complete picture, at the very least, should include cleavage of full-length Bid by caspase-8 to produce cBid at the MOM, cBid fragment separation immediately followed by binding to the MOM and finally membrane insertion of tBid through a conformational change accelerated by Mtch2. In addition, as we previously speculated [14], owing to its considerable structural homology to ANT1 and the presence of a mitochondrial-carrier domain, Mtch2 may bind CL molecules in the MOM and therefore facilitate the conformational change in tBid by providing a CL-rich platform in the MOM. However, this hypothesis remains speculative.

High levels of cholesterol in the membrane have been reported to inhibit Bax insertion and therefore membrane permeabilization [47,48]. By using fluorescence assays to monitor each step of the mechanism of MOMP, we were able to identify that cholesterol inhibits both the binding of Bax to the activator proteins tBid and Bim. This decreased binding may be sufficient to impair subsequent insertion of the hairpin helices of Bax into the

membrane (Figure 2). Therefore, inefficient initial steps in the Bax activation cascade negatively affect Bax oligomerization and membrane permeabilization (Figure 7). In agreement with previous reports [47,48], we propose that cholesterol inhibits MOMP due to changes in the physical properties of the membrane, thereby requiring more activation energy for proteins to insert into the membrane or to assemble as complexes in the membrane.

Additional lipids found at the MOM, such as ceramide, have been reported to induce membrane permeabilization [68]. However, we found that the addition of ceramide, sphingosine and sphingosine-1-phosphate in liposome membranes had no effect on cBid and Bax-mediated membrane permeabilization (result not shown). In addition, increasing PE to increase the negative curvature of the membranes had no effect of cBid and Bax-mediated membrane permeabilization (result not shown).

Taken together, our data suggest that the lipid content of the membrane can modulate permeabilization by Bcl-2 pro-apoptotic proteins by various means: recruitment of cBid and Bim to the membrane, the conformational change in tBid, binding of Bax to the activator proteins and insertion of Bax into the membrane (Figure 7). We have previously shown that the conformational changes in tBid and Bax at the membrane are the rate-limiting steps for MOMP [5,10]. It is therefore remarkable that different membrane lipids effectively modulate both of these important steps.

Using a system of liposomes and purified proteins to understand the core mechanism of Bcl-2 proteins has allowed us characterize the individual steps in the MOMP cascade. By using this system, we have uncovered mechanisms that can explain observations in cultured cells and in mice [5,10,11,69]. Taken together, the results presented in the present study show that tBid and Bim employ different mechanisms of interacting with membranes and activating Bax. This suggests that they may function very differently depending on the destination membrane. In particular, Bim might be a much better activator than tBid of Bax at the endoplasmic reticulum, since this organelle does not contain CL. This is an important observation because the endoplasmic reticulum is a known site of Bax activity [70]. Our results therefore open avenues to modulate activator specific apoptosis. In addition, the dependence of tBid on CL and Mtch2 to undergo a conformational change at the membrane leads us to postulate that CL and Mtch2 may have a redundant role in the recruitment of tBid at the MOM. It will be interesting to compare tBid-mediated MOMP in cells lacking both CL and Mtch2.

AUTHOR CONTRIBUTION

Aisha Shamas-Din designed and performed research, analysed data and wrote the paper. Scott Bindner and Xiaoke Chi performed experiments and analysed data. Brian Leber, David Andrews and Cécile Fradin conceived and designed the study and wrote the paper. All authors approved the final manuscript.

FUNDING

This work was supported by the Canadian Institute of Health Research [grant numbers FRN86657 (to C.F.) and FRN12517 (to D.W.A. and B.L.)]; and the Natural Sciences and Engineering Council [grant number RGPIN/250205-2010 (to C.F.)].

REFERENCES

1 Adams, J.M. (2003) Ways of dying: multiple pathways to apoptosis. Genes Dev. **17**, 2481–2495 CrossRef PubMed
2 Youle, R.J. and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. **9**, 47–59 CrossRef PubMed
3 Shamas-Din, A., Kale, J., Leber, B. and Andrews, D.W. (2013) Mechanisms of action of Bcl-2 family proteins. Cold Spring Harb. Perspect. Biol. **5**, a008714–a008735 CrossRef PubMed
4 Chou, J.J., Li, H., Salvesen, G.S., Yuan, J. and Wagner, G. (1999) Solution structure of BID, an intracellular amplifier of apoptotic signaling. Cell **96**, 615–624 CrossRef PubMed
5 Shamas-Din, A., Bindner, S., Zhu, W., Zaltsman, Y., Campbell, C., Gross, A., Leber, B., Andrews, D.W. and Fradin, C. (2013) tBid undergoes multiple conformational changes at the membrane required for bax activation. J. Biol. Chem. **288**, 22111–22127 CrossRef PubMed
6 Schinzel, A., Kaufmann, T. and Borner, C. (2004) Bcl-2 family members: integrators of survival and death signals in physiology and pathology [corrected]. Biochim. Biophys. Acta **1644**, 95–105 CrossRef PubMed
7 Puthalakath, H. and Strasser, A. (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. **9**, 505–512 CrossRef PubMed
8 Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the fas pathway of apoptosis. Cell **94**, 491–501 CrossRef PubMed
9 Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell **94**, 481–490 CrossRef PubMed
10 Lovell, J.F., Billen, L.P., Bindner, S., Shamas-Din, A., Fradin, C., Leber, B. and Andrews, D.W. (2008) Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell **135**, 1074–1084 CrossRef PubMed
11 Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. and Newmeyer, D.D. (2002) Bid, bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell **111**, 331–342 CrossRef PubMed
12 Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science **292**, 727–730 CrossRef PubMed
13 Kim, H., Tu, H.C., Ren, D., Takeuchi, O., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J. and Cheng, E.H. (2009) Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. Mol. Cell. **36**, 487–499 CrossRef PubMed
14 Shamas-Din, A., Brahmbhatt, H., Leber, B. and Andrews, D.W. (2011) BH3-only proteins: orchestrators of apoptosis. Biochim. Biophys. Acta **1813**, 508–520 CrossRef PubMed
15 Leber, B., Lin, J. and Andrews, D.W. (2007) Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes. Apoptosis **12**, 897–911 CrossRef PubMed
16 Bogner, C., Leber, B. and Andrews, D.W. (2010) Apoptosis: embedded in membranes. Curr. Opin. Cell Biol. **22**, 845–851 CrossRef PubMed
17 Shamas-Din, A., Satsoura, D., Khan, O., Zhu, W., Leber, B., Fradin, C. and Andrews, D.W. (2014) Involvement of cardiolipin in tBID-induced activation of BAX during apoptosis. Cell Death Dis. **5**, e1277–e1289 CrossRef PubMed
18 Dlugosz, P.J., Billen, L.P., Annis, M.G., Zhu, W., Zhang, Z., Lin, J., Leber, B. and Andrews, D.W. (2006) Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J. **25**, 2287–2296 CrossRef PubMed
19 Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J. and Korsmeyer, S.J. (2003) VDAC2 Inhibits BAK activation and mitochondrial apoptosis. Science **301**, 513–517 CrossRef PubMed
20 Oh, K.J., Barbuto, S., Meyer, N., Kim, R.S., Collier, R.J. and Korsmeyer, S.J. (2005) Conformational changes in BID, a Pro-apoptotic BCL-2 family member, upon membrane binding. J. Biol. Chem. **280**, 753–767 CrossRef PubMed
21 Wang, Y. and Tjandra, N. (2013) Structural Insights of tBid, the caspase-8-activated Bid, and its BH3 domain. J. Biol. Chem. **288**, 35840–35851 CrossRef PubMed
22 Hu, X., Han, Z., Wyche, J.H. and Hendrickson, E.A. (2003) Helix 6 of tBid is necessary but not sufficient for mitochondrial binding activity. Apoptosis **8**, 277–289 CrossRef PubMed
23 Annis, M.G., Soucie, E.L., Dlugosz, P.J., Cruz-Aguado, J.A., Penn, L.Z., Leber, B. and Andrews, D.W. (2005) Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. EMBO J. **24**, 2096–2103 CrossRef PubMed
24 Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J.C. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol. **144**, 891–901 CrossRef PubMed
25 Bleicken, S., Landeta, O., Landajuela, A., Basanez, G. and Garcia-Saez, A.J. (2013) Proapoptotic Bax and Bak proteins form stable protein-permeable pores of tunable size. J. Biol. Chem. **288**, 33241–33252 CrossRef PubMed
26 Zhang, T. and Saghatelian, A. (2013) Emerging roles of lipids in BCL-2 family-regulated apoptosis. Biochim. Biophys. Acta **1831**, 1542–1554 CrossRef PubMed
27 Lindsay, J., Esposti, M.D. and Gilmore, A.P. (2011) Bcl-2 proteins and mitochondria–specificity in membrane targeting for death. Biochim. Biophys. Acta **1813**, 532–539 CrossRef PubMed
28 Horvath, S.E. and Daum, G. (2013) Lipids of mitochondria. Prog. Lipid Res. **52**, 590–614 CrossRef PubMed

29 de Kroon, A.I., Dolis, D., Mayer, A., Lill, R. and de Kruijff, B. (1997) Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the mitochondrial outer membrane? Biochim. Biophys. Acta **1325**, 108–116 CrossRef PubMed

30 Hovius, R., Thijssen, J., van der Linden, P., Nicolay, K. and de Kruijff, B. (1993) Phospholipid asymmetry of the outer membrane of rat liver mitochondria. Evidence for the presence of cardiolipin on the outside of the outer membrane. FEBS Lett. **330**, 71–76 CrossRef PubMed

31 Ardail, D., Privat, J.P., Egret-Charlier, M., Levrat, C., Lerme, F. and Louisot, P. (1990) Mitochondrial contact sites. Lipid composition and dynamics. J. Biol. Chem. **265**, 18797–18802 PubMed

32 Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. and Wang, X. (2000) Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat. Cell Biol. **2**, 754–761 CrossRef PubMed

33 Kim, T.H., Zhao, Y., Ding, W.X., Shin, J.N., He, X., Seo, Y.W., Chen, J., Rabinowich, H., Amoscato, A.A. and Yin, X.M. (2004) Bid–cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. Mol. Biol. Cell. **15**, 3061–3072 CrossRef PubMed

34 Gonzalvez, F., Pariselli, F., Dupaigne, P., Budihardjo, I., Lutter, M., Antonsson, B., Diolez, P., Manon, S., Martinou, J.C., Goubern, M. et al. (2005) tBid interaction with cardiolipin primarily orchestrates mitochondrial dysfunctions and subsequently activates Bax and Bak. Cell Death Differ. **12**, 614–626 CrossRef PubMed

35 Gonzalvez, F., Bessoule, J.J., Rocchiccioli, F., Manon, S. and Petit, P.X. (2005) Role of cardiolipin on tBid and tBid/Bax synergistic effects on yeast mitochondria. Cell Death Differ. **12**, 659–667 CrossRef PubMed

36 Lucken-Ardjomande, S., Montessuit, S. and Martinou, J.C. (2008) Contributions to Bax insertion and oligomerization of lipids of the mitochondrial outer membrane. Cell Death Differ. **15**, 929–937 CrossRef PubMed

37 Petit, P.X., Dupaigne, P., Pariselli, F., Gonzalvez, F., Etienne, F., Rameau, C. and Bernard, S. (2009) Interaction of the alpha-helical H6 peptide from the pro-apoptotic protein tBid with cardiolipin. FEBS J. **276**, 6338–6354 CrossRef PubMed

38 Sani, M.A., Dufourc, E.J. and Grobner, G. (2009) How does the Bax-alpha1 targeting sequence interact with mitochondrial membranes? The role of cardiolipin. Biochim. Biophys. Acta **1788**, 623–631 CrossRef PubMed

39 Raemy, E. and Martinou, J.C. (2013) Involvement of cardiolipin in tBID-induced activation of BAX during apoptosis. Chem. Phys. Lipids. **179**, 70–74 CrossRef PubMed

40 Gonzalvez, F. and Gottlieb, E. (2007) Cardiolipin: setting the beat of apoptosis. Apoptosis **12**, 877–885 CrossRef PubMed

41 Zha, J., Weiler, S., Oh, K.J., Wei, M.C. and Korsmeyer, S.J. (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science **290**, 1761–1765 CrossRef PubMed

42 Ausili, A., Torrecillas, A., Martinez-Senac, M.M., Corbalan-Garcia, S. and Gomez-Fernandez, J.C. (2009) The interaction of the Bax C-terminal domain with negatively charged lipids modifies the secondary structure and changes its way of insertion into membranes. J. Struct. Biol. **164**, 146–152 CrossRef

43 Esposti, M.D., Cristea, I.M., Gaskell, S.J., Nakao, Y. and Dive, C. (2003) Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. Cell Death Differ. **10**, 1300–1309 CrossRef PubMed

44 Montero, J., Mari, M., Colell, A., Morales, A., Basanez, G., Garcia-Ruiz, C. and Fernandez-Checa, J.C. (2010) Cholesterol and peroxidized cardiolipin in mitochondrial membrane properties, permeabilization and cell death. Biochim. Biophys. Acta **1797**, 1217–1224 CrossRef PubMed

45 Montero, J., Morales, A., Llacuna, L., Lluis, J.M., Terrones, O., Basanez, G., Antonsson, B., Prieto, J., Garcia-Ruiz, C., Colell, A. and Fernandez-Checa, J.C. (2008) Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. Cancer Res. **68**, 5246–5256 CrossRef PubMed

46 Lee, S.K., Kim, Y.C., Song, S.B. and Kim, Y.S. (2010) Stabilization and translocation of p53 to mitochondria is linked to Bax translocation to mitochondria in simvastatin-induced apoptosis. Biochem. Biophys. Res. Commun. **391**, 1592–1597 CrossRef PubMed

47 Christenson, E., Merlin, S., Saito, M. and Schlesinger, P. (2008) Cholesterol effects on BAX pore activation. J. Mol. Biol. **381**, 1168–1183 CrossRef PubMed

48 Lucken-Ardjomande, S., Montessuit, S. and Martinou, J.C. (2008) Bax activation and stress-induced apoptosis delayed by the accumulation of cholesterol in mitochondrial membranes. Cell Death Differ. **15**, 484–493 CrossRef PubMed

49 Sarosiek, K.A., Chi, X., Bachman, J.A., Sims, J.J., Montero, J., Patel, L., Flanagan, A., Andrews, D.W., Sorger, P. and Letai, A. (2013) BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. Mol. Cell. **51**, 751–765 CrossRef PubMed

50 Hinds, M.G., Smits, C., Fredericks-Short, R., Risk, J.M., Bailey, M., Huang, D.C. and Day, C.L. (2007) Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. Cell Death Differ. **14**, 128–136 CrossRef PubMed

51 Billen, L.P., Shamas-Din, A. and Andrews, D.W. (2009) Bid: a Bax-like BH3 protein. Oncogene. **27** (Suppl 1), S93–S104 CrossRef

52 Aouacheria, A., Brunet, F. and Gouy, M. (2005) Phylogenomics of life-or-death switches in multicellular animals: Bcl-2, BH3-only, and BNip families of apoptotic regulators. Mol. Biol. Evol. **22**, 2395–2416 CrossRef PubMed

53 Choi, S.Y., Gonzalvez, F., Jenkins, G.M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P.X. and Frohman, M.A. (2007) Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Differ. **14**, 597–606 CrossRef PubMed

54 Satsoura, D., Kucerka, N., Shivakumar, S., Pencer, J., Griffiths, C., Leber, B., Andrews, D.W., Katsaras, J. and Fradin, C. (2012) Interaction of the full-length Bax protein with biomimetic mitochondrial liposomes: a small-angle neutron scattering and fluorescence study. Biochim. Biophys. Acta **1818**, 384–401 CrossRef PubMed

55 Yethon, J.A., Epand, R.F., Leber, B., Epand, R.M. and Andrews, D.W. (2003) Interaction with a membrane surface triggers a reversible conformational change in bax normally associated with induction of apoptosis. J. Biol. Chem. **278**, 48935–48941 CrossRef PubMed

56 Billen, L.P., Kokoski, C.L., Lovell, J.F., Leber, B. and Andrews, D.W. (2008) Bcl-XL inhibits membrane permeabilization by competing with bax. PLoS Biol. **6**, 1268–1280 CrossRef

57 Shivakumar, S., Kurylowicz, M., Hirmiz, N., Manan, Y., Friaa, O., Shamas-Din, A., Masoudian, P., Leber, B., Andrews, D.W. and Fradin, C. (2014) The proapoptotic protein tBid forms both superficially bound and membrane-inserted oligomers. Biophys. J. **106**, 2085–2095 CrossRef PubMed

58 Tsui, F.C., Ojcius, D.M. and Hubbell, W.L. (1986) The intrinsic pKa values for phosphatidylserine and phosphatidylethanolamine in phosphatidylcholine host bilayers. Biophys. J. **49**, 459–468 CrossRef PubMed

59 Schlame, M., Rua, D. and Greenberg, M.L. (2000) The biosynthesis and functional role of cardiolipin. Prog. Lipid Res. **39**, 257–288 CrossRef PubMed

60 Veresov, V.G. and Davidovskii, A.I. (2007) Monte Carlo simulations of tBid association with the mitochondrial outer membrane. Eur. Biophys. J. **37**, 19–33 CrossRef PubMed

61 Hekman, M., Albert, S., Galmiche, A., Rennefahrt, U.E., Fueller, J., Fischer, A., Puehringer, D., Wiese, S. and Rapp, U.R. (2006) Reversible membrane interaction of BAD requires two C-terminal lipid binding domains in conjunction with 14–3-3 protein binding. J. Biol. Chem. **281**, 17321–17336 CrossRef PubMed

62 Muga, A., Mantsch, H.H. and Surewicz, W.K. (1991) Membrane binding induces destabilization of cytochrome c structure. Biochemistry **30**, 7219–7224 CrossRef PubMed

63 London, E. (1992) Diphtheria toxin: membrane interaction and membrane translocation. Biochim. Biophys. Acta **1113**, 25–51 CrossRef PubMed

64 Lakey, J.H., Parker, M.W., Gonzalez-Manas, J.M., Duche, D., Vriend, G., Baty, D. and Pattus, F. (1994) The role of electrostatic charge in the membrane insertion of colicin A. Eur. J. Biochem. **220**, 155–163 CrossRef PubMed

65 Gonzalvez, F., Schug, Z.T., Houtkooper, R.H., MacKenzie, E.D., Brooks, D.G., Wanders, R.J., Petit, P.X., Vaz, F.M. and Gottlieb, E. (2008) Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. J. Cell Biol. **183**, 681–696 CrossRef PubMed

66 Bergstrom, C.L., Beales, P.A., Lv, Y., Vanderlick, T.K. and Groves, J.T. (2013) Cytochrome c causes pore formation in cardiolipin-containing membranes. Proc. Natl. Acad. Sci. U.S.A. **110**, 6269–6274 CrossRef PubMed

67 Zaltsman, Y., Shachnai, L., Yivgi-Ohana, N., Schwarz, M., Maryanovich, M., Houtkooper, R.H., Vaz, F.M., De Leonardis, F., Fiermonte, G., Palmieri, F. et al. (2010) MTCH2/MIMP is a major facilitator of tBID recruitment to mitochondria. Nat. Cell. Biol. **12**, 553–562 CrossRef PubMed

68 Siskind, L.J. and Colombini, M. (2000) The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. J. Biol. Chem. **275**, 38640–38644 CrossRef PubMed

69 Merino, D., Giam, M., Hughes, P.D., Siggs, O.M., Heger, K., O'Reilly, L.A., Adams, J.M., Strasser, A., Lee, E.F., Fairlie, W.D. and Bouillet, P. (2009) The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. J. Cell. Biol. **186**, 355–362 CrossRef PubMed

70 Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T. and Korsmeyer, S.J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science **300**, 135–139 CrossRef PubMed

Received 15 October 2014/12 February 2015; accepted 25 February 2015
Published as BJ Immediate Publication 25 February 2015, doi:10.1042/BJ20141291