Lipidic pore formation by the concerted action of pro-apoptotic BAX and tBID

Oihana Terrones\textsuperscript{a}, Bruno Antonsson\textsuperscript{b}, Hirohito Yamaguchi\textsuperscript{c}, Hong-Gang Wang\textsuperscript{c}, Jihua Liu\textsuperscript{d}, Ray M. Lee\textsuperscript{d}, Andreas Herrmann\textsuperscript{c}, and Gorka Basañez\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a}Unidad de Biofísica (Centro Mixto Consejo Superior de Investigaciones Científicas–Universidad del País Vasco/Euskal Herriko Unibertsitatea), Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), P.O. Box 644, 48080 Bilbao, Spain.

\textsuperscript{b}Serono Pharmaceutical Research Institute, Serono International S.A., 14, chemin des Aulx, CH-1228 Plan-les-Ouates, Geneva, Switzerland.

\textsuperscript{c}Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida 33612

\textsuperscript{d}Huntsman Cancer Institute and Departments of Internal Medicine and Oncological Sciences, University of Utah, Salt Lake City, UT, 84112

\textsuperscript{e}Institut für Biologie, Molekulare Biophysik, Humboldt-Universität Berlin, Invalidenstr. 42, D-10115, Berlin, Germany

*Corresponding author. Phone: 34-946013355, Fax:34-94 6013360

E-mail:gbzbaasg@lg.ehu.es

Running Title: Lipidic pore formation by BAX and tBID
Summary

BH3-only proteins of the BCL-2 family such as tBID and BIM_{EL} assist BAX-type proteins to breach the permeability barrier of the outer mitochondrial membrane, thereby allowing cytoplasmic release of cytochrome $c$ and other active inducers of cell death normally confined to the mitochondrial intermembrane space. However, the exact mechanism by which tBID and BIM_{EL} aid BAX in this mitochondrial protein release remains enigmatic. Here, we provide evidence supporting the notion that tBID collaborates with BAX in pure lipid vesicles to (1) form large membrane openings through both BH3-dependent and BH3-independent mechanisms, (2) cause lipid transbilayer movement concomitant with membrane permeabilization, and (3) disrupt the lipid bilayer structure of the membrane by promoting positive monolayer curvature. None of these effects were observed with BAX when BIM_{EL} was substituted for tBID. Based on these data, we propose a novel model in which tBID assists BAX not only via protein-protein but also via protein-lipid interactions to form lipidic-pore-type non-bilayer structures in the outer mitochondrial membrane through which intermembrane pro-death molecules exit mitochondria during apoptosis.
Abbreviations

LUV, large unilamellar vesicles; cBID, caspase-8-cleaved BID; tBID, the carboxyl-terminal fragment of caspase-8-cleaved BID; OG, octylglucoside; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOG, dioleoylglycerol; O-LPC, oleoylphosphatidycholine; O-LPE, oleoyl-phosphatidylethanolamine; TOPG, tetraoleoyl-phosphatidylglycerol (cardiolipin); CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; C_{12}E_{18}, dodecyl octaethyleneglycol mono ether; D_2O, deuterium oxide; FD-10, fluorescein isothiocyanate-labeled dextrans of 10 kDa average molecular mass; FD-70, fluorescein isothiocyanate-labeled dextrans of 70 kDa average molecular mass; ANTS, 8-aminonaphtalene-1,3,6 trisulfonate (ANTS); DPX, p-xylene-bis-pyridinium bromide; QELS, Quasi—Elastic Light Scattering; and pyPC, 1-lauroyl-2-(1’ pyrenebutyroyl)-sn-glycerol-3-phosphocholine.
Introduction

Mitochondria play a crucial role in the cellular commitment to apoptosis through the release of a number of apoptogenic proteins into the cytosol, leading to caspase-dependent and/or -independent cell death cascades (1-3). This process is under the control of BCL-2 family proteins, which exert their function primarily, although not exclusively, at the level of the outer mitochondrial membrane (1-3). These proteins share up to four conserved regions called BCL-2 homology (BH) domains, and can be divided into three subgroups based on structural and functional properties (4). Members of the first subgroup, exemplified by BCL-2, contain four BH domains and act predominantly as death inhibitors. Members of the second subgroup, exemplified by BAX, contain BH1-BH3 domains and promote apoptosis in most cellular contexts. Finally, members of the third subgroup share only the BH3 domain (BH3-only proteins) and appear to function invariably as death agonists. Two of the most highly studied and important BH3-only proteins are BID and BIM.

BID and BIM require the presence of BAX-type proteins to exert their pro-apoptotic function (5-7), but exactly how BID and BIM assist BAX and its close homologues in the release of mitochondrial apoptogenic factors is still unresolved. One popular model holds that BID and BIM share a common mode of action through direct binding to BAX-type proteins through their BH3 domains (8). This interaction would trigger oligomerization and membrane insertion of BAX-type proteins, thereby unleashing their intrinsic pore-forming activity. Other, not necessarily mutually exclusive mechanisms of action proposed for BID and BIM include (i) binding to and neutralization or reversal of pro-survival BCL-2-type family member function (4,
6, 7, 9, 10), (ii) modulation of resident mitochondrial channels, such as VDAC and ANT (11-13), and (iii) autonomous pore-forming activity (14-20).

Accumulating evidence indicates that lipids can play important roles at different stages of the molecular pathway culminating with outer mitochondrial membrane permeabilization. First, an early, reversible conformational change of BAX associated with apoptosis induction that occurs prior to BAX oligomerization can be reproduced in vitro upon interaction of monomeric BAX with large unilamellar vesicles (LUV), without requiring any particular lipids (22). Second, specific lipids have been shown to favor membrane association of activated forms of BAX and BID. For example, apoptogenic fatty acids augment binding to LUV of an oligomeric form of BAX capable of inducing mitochondrial cytochrome \( c \) release (23). Upon engagement of cell surface death receptors, BID is proteolytically cleaved by activated caspase 8, generating caspase-8-cleaved BID (cBID) which subsequently dissociates into a truncated C-terminal fragment (tBID) harboring the molecule’s full apoptogenicity and an N-terminal inert fragment (24-26). Cardiolipin (CL) increases binding of both cBID and tBID to purely lipidic as well as mitochondrial membranes (27, 28), and myristoylation of tBID further enhances its membrane avidity (29). Third, BID and cBID possess lipid-transfer activity, raising the possibility that BID/cBID may transport specific lipids to the outer mitochondrial membrane during apoptosis to facilitate breaching of this permeability barrier (17, 21, 30, 31). Finally, lipids have been implicated in forming a lesion in the outer mitochondrial membrane large enough to allow passage of intermembrane pro-death molecules. BAX-type proteins destabilize planar lipid bilayers through reduction of membrane line tension and permeabilize LUV to cytochrome \( c \) through a mechanism sensitive to changes in membrane monolayer curvature (32-34). Based on these findings, we proposed that BAX-type proteins form large pores in the outer mitochondrial
membrane containing lipid molecules arranged in a non-bilayer structure (lipidic pores) (32-34). Along the same lines, using vesicular systems of decreasing complexity, Kuwana et al. showed CL-dependent supramolecular pore formation by cBID-activated BAX, and hypothesized that BAX-type proteins cooperate with CL to form non-bilayer lipid structures such as lipidic pores or inverted micelles (35). Interestingly, in the course of apoptosis BAX-type proteins co-localize with components of the mitochondrial fusion and fission machineries at outer mitochondrial membrane scission sites (36), a likely location for occurrence of non-bilayer lipid structures.

In the present study, we investigated the mechanism(s) through which biologically active forms of BID and BIM assist BAX to create a membrane passageway large enough to permit transit of mitochondrial intermembrane pro-death proteins. Our investigations suggest that tBID aids BAX in this task through protein-protein as well as through protein-lipid interactions. Several lines of evidence support the view that the functional collaboration between tBID/cBID and BAX culminates with formation of large lipidic pores possessing net positive curvature. We also show that phosphatidylglycerol, but not phosphatidylinositol or phosphatidylserine can substitute for cardiolipin in this process. None of these effects were observed with BIM EL, but BIM EL reversed the inhibitory effect of BCL-2 on the LUV permeabilization induced by the concerted action of BAX and cBID.

**Experimental procedures**

*Materials*—Recombinant full-length monomeric BAX with an N-terminal His$_6$ tag (BAX) (37), His$_6$-tagged monomeric BAX with the 20-aminoacid carboxyl-terminal domain truncated (BAXΔC) (38), caspase-8-cleaved BID with an N-terminal His$_6$ tag (cBID) (15), cBID mutant
mIII-2 (G93DE96→AAAA) (15), cBID mutant mIII-3 (G94→A) (15), the carboxyl-terminal fragment of caspase-8-cleaved BID (aa 60-195) (tBID) (29), the cardiolipin-binding domain of BID (aa 103-162) (CBD) (28), BIMEL with an N-terminal His6 tag and devoid of the carboxyl-terminal hydrophobic domain (BIMEL) (20), and BCL-2 devoid of the carboxyl-terminal hydrophobic domain (BCL-2) (38), were obtained as described previously. All proteins were >90% pure electrophoretically. Octylglucoside (OG) activated BAX (OG-BAX) was obtained by incubating BAX in 100 mM KCl, 10 mM Hepes, 0.1 mM EDTA, pH 7.0 buffer (KHE buffer) containing OG (2% w/v), for 1 hour at 4°C. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylglycerol (DOG), oleoyl-phosphatidycholine (O-LPC), oleoyl-phosphatidylethanolamine (O-LPE), tetraoleoyl-phosphatidylglycerol (cardiolipin) (TOPG), heart cardiolipin (CL), egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), egg phosphatidylglycerol (PG), brain phosphatidylserine (PS), and liver phosphatidylinositol (PI) were purchased from Avanti Polar Lipids (Alabaster, AL). KCl, HEPES, EDTA, dodecyl octaethyleneglycol mono ether (C12 E18), deuterium oxide (D2O), melittin, S. aureaus α-toxin, and fluorescein isothiocyanate-labeled dextrans (FD) of 10 kDa (FD-10) and 70 kDa (FD-70) were obtained from Sigma (St. Louis, MO). 8-aminonaphtalene-1,3,6 trisulfonate (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were from Molecular Probes (Eugene, OR).

Release of Cytochrome c from Isolated Mitochondria—Mitochondria were isolated from livers of male Harlan Sprague-Dawley rats as described in Basañez et al. (33). Isolated mitochondria (200 µg of protein/ml) were incubated for 20 min with recombinant proteins in 50 µl of 125 mM KCl, 5 mM KH2PO4, 25 µM EGTA, 5 mM succinate, 5 µM rotenone, and 10 mM HEPES-KOH,
pH 7.2. Reaction mixtures were centrifuged at 10,000 × g for 10 min. Supernatant fractions (20 µl) were subjected to 4-20% SDS-PAGE, followed by Western blotting using anti-cytochrome c 7H8.2C-12 antibody (Pharmingen, San Diego, CA), and visualization by the ECL method (Amersham Pharmacia Biotech). Mitochondria were kept on ice and used within 3 hours of preparation.

**Preparation of LUV**—Lipid mixtures at indicated ratios were co-dissolved in chloroform/methanol (2/1). Organic solvents were removed by evaporation under an argon stream, followed by incubation under vacuum for 2 h. Dry lipid films were resuspended in the following buffers. For assays of vesicular contents release: KHE buffer supplemented with either 100 mg/ml FD or 12.5 mM ANTS, 45 mM DPX, 20 mM KCl, 10 mM HEPES, 0.1 mM EDTA, pH 7.0. For assays of vesicular size, BAX oligomerization and transbilayer lipid redistribution: KHE buffer. For assays of membrane-binding and integral membrane insertion of BAX: KHE buffer using D2O instead of H2O. LUV were formed by the method of Mayer et al. (39), using 20 freeze/thaw cycles and two polycarbonate membranes of 0.2-µm pore size for extrusion (Nucleopore, San Diego, CA). Untrapped ANTS/DPX and FD were removed by gel filtration in Sephadex G-25 and Sephacryl S-500 HR columns, respectively, with KHE running as elution buffer. Lipid concentration was determined by the method of Bartlett et al. (40). Unless otherwise stated, liposome composition was DOPC/TOPG (cardiolipin) (80/20 mol/mol).

**Release of LUV-entrapped Markers**—Release of LUV-encapsulated fluorescent markers was monitored in an SLM-2 Aminco-Bowman luminescence spectrometer (Spectronic Instruments, Rochester, NY), in a thermostatted 1-cm path length cuvette with constant stirring, at 37 °C. For
FD, $\lambda_{ex}$ was 490, and $\lambda_{em}$ was 520 nm (slits, 4 nm); for ANTS/DPX, $\lambda_{ex}$ was 350 nm, and $\lambda_{em}$ was 520 nm (slits, 8 nm). A 515 nm cut-off filter was placed between the sample and the emission monochromator to avoid scattering interferences. The extent of marker release was quantified on a percentage basis according to the equation: 

$$\left(\frac{F_t - F_0}{F_{100} - F_0}\right) \times 100,$$

where $F_t$ is the measured fluorescence of protein-treated LUV at time t, $F_0$ is the initial fluorescence of the LUV suspension before protein addition, and $F_{100}$ is the fluorescence value after complete disruption of LUV by addition of C$_{12}$E$_8$ (final concentration, 0.5 mM). Unless otherwise stated, lipid concentration was 20 $\mu$M.

Assays of BAX Binding to and Insertion into the LUV Membrane—To measure the amount of BAX bound to LUV, a method was used based on the fact that lipid-associated protein, but not free protein, floats in D$_2$O-based KHE buffer. Briefly, proteins and LUV were incubated together at 37°C in D$_2$O-based KHE buffer (final volume of reaction mixture, 100 $\mu$l), followed by centrifugation of the mixture for 1 hour at 100 000 $\times$ g, room temperature. Under these conditions, LUV remained in the upper fraction of the buffer, whereas free BAX protein sedimented. The top 20-$\mu$l fraction of the gradient, corresponding to the lipid-associated fraction, was subjected to SDS-PAGE in 15% Tris-glycine gels, followed by BAX visualization by Western blotting using N20 anti-BAX polyclonal antibody (Santa Cruz Biotechnology, Inc.). To discriminate between protein inserted into and only peripherally adsorbed to the membrane surface, the same protocol as above was followed except that a second incubation was performed for 30 minutes at pH 11.5, the alkaline pH being maintained during sample centrifugation. Upon alkaline pH incubation, the fraction of protein integrated into the membrane hydrophobic matrix.
remains associated to LUV, whereas the fraction of protein only peripherally associated with the membrane is detached from vesicles.

**Size-Exclusion Chromatography Analysis of BAX Oligomerization in LUV**—Experiments were performed in a Superdex-200 (15/45) column equilibrated with 100 mM KCl, 10 mM HEPES, 0.2 mM EDTA (pH, 7.0), with or without 2% (w/v) CHAPS (J.T. Baker, Phillipsburg, NJ) at a 1 ml/min flow rate. The column was calibrated using protein gel-filtration standards (Bio-Rad, Hercules, CA). Samples of 300 µl were loaded onto the column, followed by collection of 2-ml elution fractions. Aliquots of individual fractions were subjected to SDS-PAGE in 15% Tris-glycine gels, followed by visualization of BAX using N20 anti-BAX antibody.

**Measurements of LUV Size by Quasi—Elastic Light Scattering (QELS)**—Vesicle size was determined by QELS at a fixed angle of 90° and room temperature, using a Malvern Zetasizer 4 instrument (Malvern, UK). A 64-channel correlator was used capable of estimating particle sizes in the range from 5 nm to 5000 nm. Data were analyzed by the cumulant method using Malvern Application Software. The hydrodynamic radius of the particle was obtained from the first cumulant, whereas the polydispersity of the sample was obtained from the second cumulant.

**Assays of Lipid Transbilayer Movement in LUV**—Assays of lipid transbilayer motion were done using 1-lauroyl-2-(1’pyrenebutyroyl)-sn-glycero-3-phosphocholine (pyPC), as described before (41, 42). Briefly, pyPC dissolved in an ethanolic solution was added externally to the liposome suspension at a final concentration of 5 mol % (pyPC/total lipid), leading to its incorporation only in the external monolayer of the membrane. When pyPC translocates from the external
membrane monolayer to the internal membrane monolayer the probe is diluted, leading to a decrease in the ratio of fluorescence intensities of pyPC excimers (I_E) and pyPC monomers (I_M). The degree of pyPC transbilayer redistribution (q) was estimated from measured I_E/I_M ratios, using a calibration curve obtained with LUV containing different molar amounts of pyPC, as described before (41). Fluorescence was monitored in an SLM-2 Aminco-Bowman luminescence spectrometer (Spectronic Instruments, Rochester, NY), in a thermostatted 1-cm path length cuvette with constant stirring, at 37 °C. λ_ex was set at 345 nm, λ_em_M at 395 nm, and λ_em_E at 465. Unless otherwise stated, lipid concentration was 20 µM.

Results and discussion

tBID, but not BIM_EL, works in conjunction with BAX to form large membrane lesions in LUV—To elucidate the mechanism(s) by which BID and BIM assist BAX in the release of mitochondrial intermembrane apoptogenic factors, we first used an in vitro reconstitution system consisting of mitochondria freshly isolated from rat liver (which contain minimal amounts of BAX and BAK) (43) and various combinations of recombinant purified pro-apoptotic proteins. We used physiologically-relevant concentrations of full-length monomeric BAX (BAX) (44), together with increasing concentrations of tBID or a form of BIM previously shown to induce BAX-dependent mitochondrial cytochrome c release in cultured cells (BIM_EL) (19). Soluble fractions were separated from mitochondria by centrifugation, and assayed for the presence of freed cytochrome c by immunoblotting. As shown in Fig. 1A, the combination of BAX with either tBID or BIM_EL led to release of mitochondrial cytochrome c, whereas none of these
proteins alone released substantial cytochrome \(c\) under conditions tested (Fig. 1A, and data not shown). However, tBID released mitochondrial cytochrome in the presence of BAX with \(~5\)-fold higher potency compared to BIM\(_{EL}\) (Fig. 1A).

Next, we wished to test whether in the absence of additional proteins, tBID and/or BIM\(_{EL}\) can assist BAX to release macromolecules of the size of cytochrome \(c\) (~14 kDa), as well as other larger pro-death proteins released from mitochondria during apoptosis, i.e. Smac/DIABLO (~23 kDa), EndoG (~30 kDa), Omi/HtrA2 (~37 kDa), and AIF (~57 kDa) (3). To this aim, we used large unilamellar vesicles (LUV) loaded with self-quenching concentrations of fluorescein isothiacyanate-labelled dextrans (FD) of average molecular mass 70 kDa (FD-70). Release of LUV-entrapped FD-70 was monitored as an increase in the fluorescence signal due to marker dilution in the external medium. None of the pro-apoptotic proteins alone caused significant FD-70 release from LUV (data not shown). However, when LUV were treated with a BAX+tBID mixture efficient efflux of vesicular FD-70 ensued (Fig. 1B). Kuwana et al have previously shown that the combination of BAX and cBID can elicit the release of 2000-kDa dextrans from pure lipid vesicles (35). We found that the combination of BAX and tBID is much more potent in the release of vesicular FD-70 than the BAX+cBID combination (Fig. 1B). This finding is consistent with evidence showing that BID possesses an auto-inhibitory N-terminal domain which remains attached to the apoptogenic C-terminal part of the molecule after caspase-8 cleavage (15). In sharp contrast to the situation found with tBID/cBID, addition of BIM\(_{EL}\) together with BAX resulted in virtually no vesicular FD-70 release, even at concentrations of BIM\(_{EL}\) 10-fold higher than those used in the tBID/cBID assays (Fig. 1B).

Anti-apoptotic proteins such as BCL-2 antagonize the release of mitochondrial pro-death factors during apoptosis (1-4, 7). To analyze this issue in our in vitro reconstitution systems,
isolated rat liver mitochondria and LUV were incubated with BCL-2 prior to the BAX+tBID treatment. BCL-2 effectively blocked the release of mitochondrial cytochrome c as well as that of LUV-entrapped FD-70 induced by the BAX+tBID mixture (Fig. 1, C, D). Moreover, the finding that BCL-2 inhibition occurred with a similar dose-dependence in both experimental setups adds credit to the physiological significance of the LUV system.

BH3-only proteins are thought to heterodimerize with other BCL-2 family members through insertion of their BH3 domain into a hydrophobic groove localized on the surface of binding partners (2, 4, 9). The three-dimensional structure of BAX revealed that a carboxyl-terminal helix folds into the groove to which BH3 ligands presumably bind (45). To test whether this could explain the lack of effect of the BAX+BIM\textsubscript{EL} mixture in LUV, we used a deletion mutant of BAX lacking its carboxyl-terminal hydrophobic region (BAX\textsubscript{ΔC}). Additionally, we decided to examine the size of the membrane lesion caused by various combinations of apoptotic proteins in this system. To achieve this, LUV were prepared that encapsulated markers of different molecular weights, i.e., ANTS/DPX (~0.4 kDa), FD-10, and FD-70. When BIM\textsubscript{EL} was added together with BAX\textsubscript{ΔC} to the liposome suspension, significant amounts of vesicular ANTS, but not FD-10 or FD-70 was released (Figure 1E, left panel). However, considering that the outer mitochondrial membrane is normally permeable to molecules <5kDa (3), the physiological significance of this vesicular ANTS release induced by BAX+BIM\textsubscript{EL}, if any, remains to be determined. On the other hand, when either BAX or BAX\textsubscript{ΔC} were added together with tBID or cBID to LUV not only ANTS, but also FD-10 and FD-70 were released (Fig. 1E, central and right panels). Surprisingly, despite the availability of the BH3-binding pocket in the BAX\textsubscript{ΔC} molecule, tBID/cBID induced vesicular marker release with lower efficiency in the presence of BAX\textsubscript{ΔC} relative to its full-length counterpart.
In summary, these results indicate that cBID and, more efficiently, tBID cooperate with BAX to form membrane openings in pure lipid vesicles large enough as to allow transit of mitochondrial intermembrane apoptogenic proteins. In sharp contrast, BIM\textsubscript{EL} lacks this capacity.

tBID can work in concert with BAX to induce membrane damage in a BH3-independent manner—It is well established that the BH3 domain of tBID is crucial for its bioactivity. To address this, we used two BH3 mutants of BID with reduced affinity for BAX: cBid mIII-2 (\textsuperscript{93}IGDE\textsuperscript{96}→AAAA) and cBid mIII-3 (G\textsuperscript{94}→A) (46, 47). We compared the effects of increasing concentrations of cBID, cBID mIII-2 and cBID mIII-3 on the release of FD-70 from LUV treated with fixed amounts of BAX. cBID mIII-2 showed ~3-4-fold reduced efficiency in vesicular FD-70 release relative to cBID, whereas cBID mIII-3 showed similar efficiency in FD-70 release relative to cBID (Fig. 2). Previous studies showed that BID mIII-3 and BID mIII-2 possess at least 10-fold lower affinity for BAX (47). This observation, together with the paradoxical finding that exposure of the BH3-binding cleft of BAX did not increase but actually decreased tBID/cBID-assisted vesicular contents release, prompted us to investigate the possibility that tBID utilizes BH3-independent mechanisms to aid BAX in membrane damage induction.

In healthy cells BAX exists in a monomeric inactive state (2). BAX activation during apoptosis has been related to a change in conformation of the protein leading to its mitochondrial membrane insertion and oligomerization, and tBID has been suggested to trigger both events through binding to BAX via its BH3 domain (47-49). Specific detergents such as octylglucoside (OG) can induce BAX membrane integration and oligomerization (22, 34, 35, 38), and several groups have shown that such OG-activated BAX (OG-BAX) permeabilizes both outer mitochondrial (35, 38, 50, 51) as well as pure lipid membranes (13, 22, 34, 35, 38). Moreover,
OG-activated BAX and tBID-activated BAX display similar degrees of membrane integration and oligomerization in LUV (22, and data not shown). Thus, we decided to use OG-BAX as a tool to test whether tBID can collaborate with BAX through mechanisms other than BH3-triggered BAX membrane integration and oligomerization. OG-BAX alone released LUV-entrapped FD-70 efficiently (data not shown), consistent with previous findings (35). Remarkably, when LUV were treated with suboptimal doses of OG-BAX, subsequent addition of tBID, but not BIMEL or tBID buffer alone, led to additional vesicular FD-70 release (Fig. 3A). Since OG-BAX inserted in the LUV membrane in an alkali-resistant manner to the same degree with or without tBID treatment (Fig. 3D), and OG-BAX formed multimers of similar sizes in CHAPS-solubilized LUV with or without tBID treatment (Fig. 3E), we concluded that tBID did not alter the membrane-insertion capacity or oligomeric status of OG-BAX under conditions in which it increased vesicular FD-70 release.

One possibility to explain the above observations is that a membrane-inserted portion of tBID assists OG-BAX in membrane damage induction. A domain was identified in BID, distinct from BH3, which possesses potential for membrane insertion and targets the molecule to CL-containing membranes, the so-called cardiolipin-binding-domain (CBD) of BID (27, 28). When FD-10-loaded vesicles were pre-treated with sub-optimal OG-BAX doses, subsequent addition of recombinant CBD induced marker release without increasing the degree of BAX membrane-insertion or multimerization (Fig. 3B-E). CBD did not induce substantial FD-10 release in OG-BAX-treated LUV in which cardiolipin had been substituted for by equimolar amounts of phosphatidylserine (data not shown), consistent with the selective preference of CBD for CL (27, 28). Of note, however, CBD was less efficient than tBID in eliciting FD-10 and FD-70 release from OG-BAX-treated LUV (Fig. 3C). Also of interest, CBD did not cause vesicular contents
release from LUV pre-treated with monomeric BAX (Fig. 3B), in agreement with the notion that the BH3 domain of BID (absent in CBD) is required for activation of BAX membrane insertion and oligomerization.

Collectively, these results suggest that tBID works in concert with BAX to form large membrane openings in LUV through two distinct mechanisms: (1) activation of BAX integral membrane insertion and oligomerization via physical interaction of the BH3 domain of tBID with BAX, and (2) a heterodimerization-independent mechanism via non-BH3 regions of tBID which include, but are not limited to, the CBD.

Lipidic pore formation by the concerted action of BAX and tBID— The finding that the membrane lesion caused by BAX in conjunction with tBID/cBID did not discriminate permeants according to size (Fig. 1E) raised the possibility that pro-apoptotic proteins may act by solubilizing LUV in a “detergent-like” manner. For example, BAX together with tBID/cBID may induce micellization of the membrane of LUV, breaking it up into small fragments or disk-like structures as seen with a number of membrane-disrupting antimicrobial peptides, including melittin (52). Evidence for such a detergent-like action can be obtained by analysing changes in the size distribution of LUV using QELS. This approach was utilized here to evaluate whether pro-apoptotic proteins caused FD-10 release through vesicle fragmentation. Results were compared to those obtained with melittin, used as a positive reference. As shown in Table I, addition of melittin caused, as expected, a decrease in the size of vesicles together with near complete release of vesicular FD-10. However, at concentrations either of BAX+tBID or BAX+cBID causing comparable FD-10 release, the size distribution of LUV did not change significantly with respect to untreated samples.
An alternative mechanism to explain the large size and non-selectivity of the membrane lesion caused by BAX together with tBID/cBID is destabilization of the bilayer structure through non-lamellar lipid structures. In this scenario, pro-apoptotic proteins would act either globally or in a localized manner on the membrane to change its material elastic properties, forcing membrane lipids to adopt a curved, non-bilayer disposition. Two main types of non-bilayer structures have been related to BAX-induced membrane permeabilization: (i) inverted-type lipid structures, such as inverted intrabilayer micelles (35, 53), and (ii) lipidic pores (1, 2, 32-35, 53). One distinguishing feature between these two types of non-bilayer structures is their net curvature. By definition, inverted-type lipid structures possess net negative curvature (54). In opposition, lipidic pores possess net positive curvature (55). Hence, increasing membrane monolayer curvature toward more negative values should lower the energy required to form inverted-type lipid structures, whereas increasing membrane monolayer curvature toward more positive values is predicted to favour lipidic pore formation.

To assess whether membrane monolayer curvature is an important contributor of the vesicular FD-70 release elicited by BAX and tBID/cBID and to try distinguishing between the inverted-micelle model and the lipidic pore model, non-bilayer lipids of known intrinsic curvature were employed. Positive curvature-inducing lipids such as O-LPC and O-LPE potentiated vesicular FD-70 release, whereas negative curvature-inducing lipids such as DOG and DOPE inhibited vesicular FD-70 release (fig. 4A, Table II, and data not shown). O-LPC possesses an intrinsic curvature more positive than O-LPE (56), and O-LPC potentiated vesicular FD-70 release to a higher extent than O-LPE (Table II). DOG possesses an intrinsic curvature more negative than DOPE (57), and DOG inhibited vesicular FD-70 release to a higher extent than DOPE (Table II). Since the intrinsic curvature of O-LPC is opposite to that of DOG, next
we examined whether O-LPC and DOG cancel each others effect on LUV permeabilization. The levels of vesicular FD-70 release obtained in the presence of equimolar amounts of O-LPC and DOG were intermediate between those obtained with either DOG or O-LPC alone (Table II). Changes in neither the degree of BAX membrane insertion nor its oligomeric status could provide a complete explanation for the effects of non-bilayer lipids on vesicular FD-70 release (data not shown). Based on analogous results, we previously proposed that OG-activated BAX induces lipidic pore formation by promoting positive membrane monolayer curvature (34). We now extend this proposal to the more physiologically relevant form of BAX activated by tBID/cBID. Importantly, O-LPC and DOG also potentiated and decreased, respectively, tBID-induced FD-70 release from OG-BAX-treated LUV suggesting tBID also promotes positive membrane monolayer curvature (Fig. 4B, and Table II). Thus, tBID may facilitate release of vesicular FD-70 downstream of BAX membrane insertion and oligomerization by decreasing the energy required to nucleate and/or expand a positively-curved lipidic pore. In apparent contradiction with our proposal, however, is the finding that cBID can decrease the bilayer-to-non-bilayer lipid phase transition temperature implying cBID promotes negative, not positive membrane monolayer curvature (19). However, such experiments were done in the absence of BAX, and using DOPC/DOPE/TOPG (1/1/1) lipid mixtures with millimolar calcium concentrations in the medium. Biophysical studies are in progress in our laboratory to determine the concerted effect of BAX and tBID/cBID on bilayer-to-non-bilayer lipid phase transitions under more physiologically-relevant conditions. Finally, the effects of non-bilayer lipids on vesicular contents release were tested for *S. aureaus* α-toxin, a bacterial toxin that forms protein channels rather than lipidic pores (58). Unlike the situation found with pro-apoptotic proteins,
O-LPC did not affect and DOG promoted the release of vesicular contents induced by *S. aureus* α-toxin (Table II).

If, as suggested by the results described above, the concerted action of BAX and tBID/cBID creates lipidic pores in the membrane, its constituent monolayers would become continuous via the pore-lining lipids. Thus, one implication of the lipidic pore concept is that such a structure should allow the movement of lipid molecules from one monolayer of the bilayer to the other. In order to test for this possibility, we applied an assay developed by Muller et al. (41) recently used in our laboratory (42). In this assay, LUV are labeled with the fluorescent phosphatidylcholine analogue pyPC exclusively in the external monolayer of the membrane. Membrane-incorporated pyPC displays two distinct peaks in the fluorescence spectrum, one arising from excited monomeric pyPC molecules and the other arising from excited dimeric (excimer) pyPC molecules. Any redistribution of pyPC to the internal monolayer is accompanied by changes of analogue concentration and, by that, of the excimer to monomer ratio in each monolayer. Thus, changes in the ratio of the excimer to monomer fluorescence intensity signals of pyPC (I_E/I_M) can be used to monitor the outward-inward transbilayer movement of the PC analogue.

Addition of BAX together with tBID to LUV containing pyPC localized only in the external monolayer led to a rapid decrease of I_E/I_M, indicating transfer of the analogue to the internal monolayer (Fig. 5A). Addition of BAX together with cBID also triggered pyPC transfer to the internal monolayer, albeit to a lower extent. In sharp contrast, the BAX+BIM_{el} mixture caused negligible transbilayer movement of pyPC. Of note, the time courses of pyPC transbilayer redistribution induced by BAX together with either tBID or cBID were comparable to those obtained in analogous experiments of vesicular FD-70 release (compare Fig. 1B and Fig.
Next, the degree of pyPC transbilayer redistribution between outer and inner monolayer (q) was determined. The results obtained with different combinations of apoptotic proteins correlated with those obtained in analogous experiments of vesicular FD-70 release (Fig. 5B). Last, the influence of membrane monolayer curvature on pyPC transbilayer movement was analyzed. As in the case of vesicular contents release, positive and negative membrane monolayer curvature increased and decreased, respectively, the degree of pyPC transbilayer motion induced not only by the BAX+tBID mixture, but also by the OG-BAX+tBID mixture (Table II). Importantly, S. aureus α-toxin induced little pyPC transbilayer redistribution under conditions in which it caused extensive vesicular contents release, indicating opening of a purely proteinaceous channel in the membrane of LUV does not cause substantial lipid transbilayer redistribution (Table II).

Taken together, these observations strongly suggest that the release of vesicular contents induced by BAX and tBID in LUV is mechanistically related to lipid transbilayer movement, and add further support to the notion that BAX and tBID cooperate at different stages of a molecular pathway culminating with lipidic pore formation.

Studies with biomembrane mimetic LUV of outer mitochondrial membrane contact site composition—BAX (59, 60), tBID (61) and BCL-2 (62, 63) have been shown to localize at zones of close proximity between the outer and the inner mitochondrial membranes, the so-called mitochondrial membrane contact sites. Thus, we decided to examine the effect of these proteins in LUV bearing the phospholipid composition determined for the outer mitochondrial membrane at such contact sites (64). Neither BAX nor tBID alone had any effect on such LUV (data not shown). However, addition of the two proteins together caused vesicular FD-70 release and
pyPC transbilayer redistribution with similar time courses and comparable susceptibilities to BCL-2 inhibition (Fig. 6A and Table III). cBID functioned together with BAX in a similar manner causing both FD-70 release and lipid transbilayer movement, although with reduced potency compared to tBID (Fig. 6B and Table III). Once again, however, BIMEL was unable to cooperate with BAX to induce any of these effects in LUV of outer mitochondrial membrane contact site composition (Table III).

Next, we sought to determine whether CL is required for the membrane actions of BAX and tBID. To this aim, LUV were prepared of outer mitochondrial membrane contact site composition but excluding all acidic lipids (-), and with CL being substituted by PS, phosphatidylinositol (PI), or phosphatidylglycerol (PG) (at same mole ratios of acidic lipids) (Fig. 6A). The BAX+tBID mixture did not permeabilize LUV of outer mitochondrial membrane contact site composition in which all acidic lipids had been excluded. Substitution of CL by PI or PS caused much reduction in the release of vesicular FD-70 elicited by BAX together with tBID. Interestingly, when CL was substituted by PG, the BAX+tBID mixture caused vesicular FD-70 release with only slightly lower efficiency as compared to CL-containing LUV (Fig. 6A). Moreover, pyPC transbilayer redistribution accompanied the release of vesicular FD-70 in PG-containing LUV, and both processes were inhibited by BCL-2 (Figure 6A, and Table III). Thus, we concluded that PG can replace CL in formation of large lipidic pores by BAX and tBID. This finding may be of physiological relevance considering that (i) PG and CL are structurally- and biosynthetically-related lipids primarily localized to mitochondrial membranes (65), (ii) mitochondrial PG levels increase early during Fas and radiation-induced apoptosis (21, 66), and (iii) BAX can induce cytochrome c release in mitochondria from mutant yeast lacking CL, but containing increased PG levels (67).
What remains to be addressed is the underlying mechanism of CL and PG selectivity in the concerted action of BAX and tBID. Because CL, PG, PS and PI, all possess near-zero intrinsic curvature under conditions tested here (68), membrane curvature changes implicated in lipidic pore formation are unlikely to be the source of the CL/PG preference over PS/PI. Interestingly, recent studies showed that during Fas-induced apoptosis the amount of mitochondrial CL decreases whereas those of monolysocardiolipin, lysophosphatidylglycerol and lysophosphatidylcholine increase coincidentally with cytochrome c release (21, 31). Considering that lysolipids, in general, possess positive intrinsic curvature (54), it is tempting to speculate that during apoptosis such lysoderivatives rather than their parental species may be sequentially and/or cooperatively implicated in lipidic pore formation by BAX and tBID/cBID.

Finally, we performed additional studies to gain more insight on the mechanism of action of BIMEL. Since no evidence was found that BIMEL can assist BAX in the release of large molecular weight dextrans from LUV, yet BIMEL was able to assist BAX in cytochrome c release from isolated rat liver mitochondria, we reasoned that additional endogenous mitochondrial factors may be required to reconstitute the BIMEL-BAX cooperation in LUV. It has been proposed that BH3-only proteins can be subdivided into two classes: those that can bind to and activate BAX-type proteins directly, and those that can render cells more susceptible to apoptotic stimuli through binding to and inactivation of BCL-2-type proteins (8, 10). Although it has been difficult to capture BIMEL-BAX complexes in vivo (8, 20, 69-72), evidence indicates that BIMEL can bind to BCL-2 in several cellular contexts (69-72). Thus, we assessed whether, by virtue of its putative capacity to bind to BCL-2, BIMEL could reverse BCL-2 inhibition of LUV permeabilization by the concerted action of BAX and cBID. BCL-2 exerted a dose-dependent inhibition on the release of vesicular FD-70 elicited by the BAX+cBID mixture
in LUV of outer mitochondrial membrane contact site composition (Fig. 6B). Addition of equimolar concentrations of BIM\textsubscript{EL} compared to cBID did not affect vesicular FD-70 release. However, augmentation of BIM\textsubscript{EL} concentration led to a progressive increase of vesicular FD-70 release, and at a 6-fold higher concentration of BIM\textsubscript{EL} relative to cBID a complete reversal of the BCL-2 inhibition was achieved (Fig. 6B). BIM\textsubscript{EL} did not enhance the release of vesicular FD-70 mediated by the BAX+cBID combination in the absence of BCL-2, arguing that BIM\textsubscript{EL} acts through BCL-2 rather than through BAX and/or cBID.

Concluding remarks— In summary, we showed that tBID/cBID and BIM\textsubscript{EL} use different mechanisms to aid BAX in making lipid vesicles permeable to macromolecules as large as pro-death proteins released from mitochondria during apoptosis. Several findings support the idea that tBID/cBID assists BAX directly in LUV permeabilization through formation of large, lipid-containing pores: (i) the observation that BAX together with tBID/cBID induce the release of all molecules entrapped within LUV irrespective of their size; (ii) the strong coupling between this vesicular contents release and the redistribution of lipids from one monolayer of the membrane to the other, (iii) the dependence of both vesicular contents release and lipid transbilayer redistribution on membrane monolayer curvature, and (iv) the lack of effect of pro-apoptotic proteins on LUV size, which argues against a detergent-like mechanism of action. An additional important highlight of our study is that tBID utilizes BH3-dependent as well as BH3-independent mechanisms to assist BAX in LUV permeabilization. Based on these observations, we propose a new model in which tBID aids BAX to elicit outer mitochondrial membrane permeabilization not only by triggering BAX oligomerization and membrane insertion, but also by increasing
outer membrane curvature in such a way as to allow formation/expansion of a positively-curved lipidic pore.

In contrast to tBID/cBID, our results with LUV suggest that BIM\textsubscript{EL} aids BAX in outer membrane permeabilization indirectly, through binding to and neutralization of BCL-2-type proteins. However, we cannot rule out the possibility that other isoforms of BIM or a post-translationally modified form of BIM\textsubscript{EL} may assist BAX in a more direct manner. Additionally, it is possible that mitochondrial components unrelated to BCL-2 family proteins (e.g. VDAC) play an important role in BIM\textsubscript{EL} bioactivity (12). Further studies are required to test these possibilities, as well as to examine the applicability of the lipidic pore model and its postulates for the increasing number of molecules proposed to collaborate with BAX-type proteins in permeabilization of the outer mitochondrial membrane during apoptosis (36, 73-75).

Acknowledgments

The authors thank Dr. B. Polster for critically reading the manuscript and Dr. M. D. Esposti for helpful comments. This work was supported in part by funds from the Ministerio de Ciencia y Tecnologia, Spain, Grant No. BMC 2002-00784. O. T. was a pre-doctoral student supported by the Basque Government.

Addendum

While this manuscript was in preparation Epand \textit{et al.} reported that cBID-activated BAX promotes transbilayer lipid diffusion (75).
References

1. Kuwana, T., and Newmeyer, D. D. (2003) *Curr. Opin. Cell Biol.* **15**, 691-699
2. Sharpe, J. C., Arnoult, D., Youle, R. J. (2004) *Biochim. Biophys. Acta* **1644**, 107-113
3. Festjens, N., van Gurp, M., van Loo, G., Saelens, X., and Vandenabeele, P. (2004) *Acta Haematol.* **111**, 7-27
4. Cory, S., Huang, D. C., S., and Adams, J. M. (2003) *Oncogene* **22**, 8590-8607
5. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panautsakopoulou, V., Ross, A. J., Roth, K. A., Mac Gregor, G. R., Thompson, C. B., and Korsmayer, S. J. (2001) *Science* **292**, 727-730
6. Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. (2001) *Genes Dev.* **15**, 1481-1486
7. Cheng, E. H., Wei, M. C., Weiler, .S, Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) *Mol. Cell* **8**, 705-711
8. Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. (2002) *Cancer Cell* **2**, 183-92
9. Puthalakath, H., and Strasser A. (2002) *Cell Death Differ.* **9**, 505-512
10. Terradillos, O., Montessuit, S., Huang, D. C., and Martinou, J. C. (2002) *FEBS Lett.* **522**, 29-34.
11. Zamzami, N., El Hamel, C., Maises, C., Brenner, C., Muñoz-Pinedo, C., Belzacq. A. S., Costantini, P., Vieira, H., Loeffler, M., Molle, G., and Kroemer, G. (2000) *Oncogene* **19**, 6342-6350
12. Sugiyama, T., Shimizu, S., Matsuoka, Y., Moneda, Y., and Tsujimoto, Y. (2002) *Oncogene* **21**, 4944-9456
13. Rostovtseva, T. K., Antonsson, B., Suzuki, M., Youle, R. J., Colombini, M., and Bezrukov, S. (2004) *J Biol Chem.* 279, 13575-13583

14. Schendel, S. L., Azimov, R., Pawlowski, K., Godzik, A., Kagan, B. L., and Reed, J. C. (1999) *J. Biol. Chem.* **274**, 21932-21936

15. Kudla, G., Montessuit, S., Eskes, R., Berrier, C., Martinou, J. C., Ghazi, A., and Antonsson, B. (2000) *J. Biol. Chem.* **275**, 22713–22718

16. Zhai, D., Miao, Q., Xin, X., and Yang, F. (2001) *Eur. J. Biochem.* **268**, 48-55

17. Degli Esposti, M., Erler, J. T., Hickman, J. A., and Dive C. (2001) *Mol. Cell Biol.* 21, 7268-7276

18. Grinberg, M., Sharig, R. Zaltsman, Y., Frumkin, D., Grammatikakis, N., Reuveny, E., and Gross, A. (2002) *J. Biol. Chem.* **277**, 12237-12245

19. Epand, R. F., Martinou, J. C., Fornallaz-Mu lhauser, M., Hughes, D. W., and Epand, R. M. (2002) *J. Biol. Chem.* **277**, 32632-32639

20. Yamaguchi, H., and Wang, H.G. (2002) *J. Biol. Chem.* **277**, 41604-41612

21. Cristea, I. M., and Degli Esposti, M. (2004) *Chem. Phys. Lipids* **129**, 133-160

22. Yethon, J. A., Epand, R. F., Leber, B., Epand, R. M., and Andrews, D. W. (2003) *J. Biol. Chem. Chem.* **278**, 48935-48941

23. Epand, R. F., Martinou, J-C., Montessuit, S., and Epand, R. M. (2004) *Biochem. J.* **377**, 509-516

24. Luo, X., Budlhardjo I., Zou, H., Slaughter, C., and Wang, X. (1998) *Cell* **94**, 481-490

25. Li, H., Zhu, H., Xu, C.-J., and Yuan, J. (1998) *Cell* **94**, 491-501

26. Gross, A., Yin, X.-M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P. and Korsmeyer, S. J. (1999) *J. Biol. Chem.* **274**, 1156-1163
27. Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., and Wang, X. (2000) Nat. Cell Biol. 2, 754-761

28. Liu, J., Weiss, A., Durrant, D., and Lee, R.M. Submitted

29. Zha, J., Weiler, S., Oh, K. J., Wei, M. C., and Korsmeyer, S. J. (2000) Science 290, 1761-1765

30. Degli Esposti, M. (2002) Biochim. Biophys. Acta 1553, 331-340

31. Degli Esposti, M., Cristea, I. M., Gaskell, S. J., Nakao, Y., and Dive C. (2003) Cell Death and Differ. 10, 1300-1309

32. Basañez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K. A., Hsu, Y., Zimmerberg, J., and Youle, R. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5492–5497

33. Basañez, G., Zhang, J., Chau, B. N., Maksaev, G. I., Frolov, V. A., Brandt, T. A., Burch, J., Hardwick, J. M., and Zimmerberg, J. (2001) J. Biol. Chem. 276, 31083–31091

34. Basañez, G., Sharpe, J. C., Galanis, J., Brandt, T. B., Hardwick, J. M., and Zimmerberg, J. (2002) J. Biol. Chem. 277, 49360-49365

35. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R., and Newmeyer, D. D. (2002) Cell 111, 331-342

36. Karbowski, M., Lee, Y.-J., Gaume, B., Jeong, S.-Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C.L., and Youle, R. J. (2002) J. Cell Biol. 159, 931-938

37. Montessuit, S., Mazzei, G., Magnenat, E., and Antonsson, B. (1999) Protein Expr. Purif. 15, 202-206

38. Antonsson, B., Montessuit, S., Lauper, S., Eskes, R., and Martinou, J.-C. (2000) Biochem. J. 345, 271–278
39. Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986) *Biochim. Biophys. Acta* **858**, 161–168

40. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-468

41. Muller, P., Schiller, S., Wieprecht, T., Dathe, M. and Herrmann, A. (2000) *Chem. Phys. Lipids* **106**, 89-99

42. Basañez, G., Shinnar, A. E., and Zimmerberg, J. (2002) *FEBS Lett.* **532**, 115-120

43. Polster B. M., Kinnally, K. W., and Fiskum, G. (2001) *J. Biol. Chem.* **276**, 37887-37894

44. Polster, B. M., Basañez, G., Young, M., Suzuki, M., and Fiskum, G. (2003) *J. Neurosci.* **23**, 2735–2743

45. Suzuki, M., Youle, R. J. and Tjandra, N. (2000) *Cell* **103**, 645-654

46. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L. and Korsmeyer, S. J. (1999) *Genes Dev.* **15**, 2859-2869

47. Desagheger, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J. C. (1999) *J. Cell Biol.* **144**, 891-901

48. Eskes, R., Desagher, S., Antonsson, B., and Martinou, J.-C. (2000) *Mol. Cell Biol.* **20**, 929-935

49. Roucou, X., Montessuit, S., Antonsson, B., and Martinou, J.-C. (2002) *Biochem. J.* **368**, 915-921

50. Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky B., and Orrenius S. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 1259-1263

51. Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J. C., Cecconi, F., and Youle, R. J. (2003) *EMBO J.* **22**, 4385-4399

52. Dufourcq, J., Faucon, J.-F., Fourche, G., Dasseaux, J.-L., Le Maire, M., and Gulik-Krzywicki, T. (1986) *Biochim. Biophys. Acta* **859**, 33-48
53. Hardwick, J. M., and Polster, B. M. (2002) *Mol. Cell* **10**, 963-965

54. Basañez G. (2002) *Cell. Mol. Life Sci.* **59**, 1478-1490

55. Chernomordik, L. V., Melikyan, G. B., and Chizmadzhev, Y. A. (1987) *Biochim. Biophys. Acta.* **906**, 309-352

56. Fuller, N., and Rand, R. P. (2001) *Biophys. J.* **81**, 243-54

57. Szule, J. A., Fuller, N. L., and Rand, R. P. (2002) *Biophys. J.* **83**, 977-984

58. Menestrina, G., Serra, M. D., and Prevost, G. (2001) *Toxicon* **39**, 1661-1672

59. Zamzami, N., Brenner, C., Marzo, I., Susin, S. A., Kroemer, G. (1998) *Oncogene* **30**, 2265-2282

60. Capano, M., and Crompton, M. (2002) *Biochem. J.* **367**, 169-178

61. Lutter, M., Perkins, G. A., and Wang, X. (2001) *BMC Cell Biol.* **2**, 22

62. Krajeski, S., Tanaka, S., Takayama, S., Schiber, M. J., Fenton, W., and Reed J. C. (1993) *Cancer Res.* **53**, 4701-4714

63. de Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B., and Kluin, P.M. (1994) *Cancer Res.* **54**, 256-260

64. Ardail, D., Privat, J. P., Egret-Charlier, M., Levrat, C., Lerme, F., and Louisot, P. (1990) *J. Biol. Chem.* **265**, 18797-18802

65. Malisan, F., and Testi, R. (2003) *Curr. Med. Chem.* **10**, 1573-1580

66. Matsko, C. M., Hunter, O. C., Rabinowich, H., Lotze, M. T., and Amoscato, A. A. (2001) *Biochem. Biophys. Res. Commun.* **287**, 1112-1120

67. Iverson, S. L., Enoksson, M., Gogvadze, V., Ott, M., and Orrenius, S. (2004) *J. Biol. Chem.* **279**, 1100-1107
68. Cullis, P. R., Tilcock, C. P., and Hope M. J. (1999) In *Membrane Fusion*, pags. 35-64 (Eds. J. Wilschut, D. Hoekstra, Marcel Dekker Inc., New York)

69. O’Connor, L.A., Strasser, A., O’Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., and Huang, D. (1998) *EMBO J.* 17, 384-395

70. Sheau, Y. H., Lin, P., and Hsueh, J. W. (1998) *Mol. Endocrin.* 12, 1432-1440

71. Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N. R. (2002) *Mol. Cell Biol.* 11, 3577-3589

72. Mouhamad, S., Besnault, L., Auffredou, M. T., Leprince, C., Bourgeade, M. F., Leca, G. and Vazquez, A. (2004) *J. Immunol.* 172, 2084-2091

73. Konishi, A., Shimizu, S., Hirota, J., Takao, T., Fan, Y., Matsuoka, Y., Zhang, L., Yoneda, Y., Fujii, Y., Skoultchi, A.I., and Tsujimoto, Y. (2003) *Cell* 114, 673–688

74. Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) *Science* 313, 1010-1014

75. Lin, Z., Kolluri, S. K., Lin, F., Liu, W., Hang, Y.-H., Cao, X., Dawson, M. I., Reed, J. C., and Zhang, X. (2004) *Cell* 116, 527-541

76. Epand, R. F., Martinou, J.-C., Montessuit, S., and Epand, R. M. (2003) *Biochemistry* 42, 14576-14582
Table I: Effects of pro-apoptotic proteins and melittin on LUV size and on vesicular FD-10 release

| Additive       | % FD-10 Release | LUV size (nm) | polydispersity |
|----------------|-----------------|---------------|----------------|
| None           | 0               | 153±34        | 0.09           |
| Melittin\(^a\) | 89±4            | 49±31         | 0.64           |
| BAX+tBID\(^b\) | 94±6            | 167±27        | 0.21           |
| BAX+cBID\(^c\) | 81±8            | 145±45        | 0.14           |

\(^a\)Melittin concentration was 10\(\mu\)M, and LUV composition was PC. \(^b\)Concentrations of BAX and tBID were 60 nM each. \(^c\)Concentrations of BAX and cBID were 150 nM each. In all cases, lipid concentration was 60 \(\mu\)M.
Table II: Effect of non-bilayer lipids on the release of vesicular contents and on the transbilayer lipid redistribution induced by apoptotic proteins and by *S. aureaus* α-toxin.

| LUV<sup>d</sup> | BAX+tBID<sup>a</sup> | OG-BAX+tBID<sup>b</sup> | *S. aureaus* α-toxin<sup>c</sup> |
|----------------|----------------------|-----------------------|----------------------------------|
|                | % Release<sup>e</sup> | % Release<sup>f</sup> | % Release<sup>h</sup>          |
| Control        | 59±4                 | 34±3                  | 57±4                            |
| O-LPE          | 70±5                 | ND                    | ND                              |
| O-LPC          | 93±6                 | 50±5                  | 52±6                            |
| DOPE           | 40±6                 | ND                    | ND                              |
| DOG            | 10±2                 | 7±2                   | 77±5                            |
| O-LPC+DOG      | 34±5                 | ND                    | ND                              |

<sup>a</sup>Concentrations of BAX and tBID were 20 nM and 10 nM, respectively. <sup>b</sup>Concentrations of OG-BAX+tBID were 20 nM and 50 nM, respectively. <sup>c</sup>*S. aureaus* α-toxin concentration was 80 nM. <sup>d</sup>For (OG-)BAX+tBID, LUV compositions were DOPC/TOPG (8/2) (Control), DOPC/TOPG/O-LPE (7/2/1) (O-LPE), DOPC/TOPG/O-LPC (7/2/1) (O-LPC), DOPC/TOPG/DOPE (5/2/3) (DOPE), DOPC/TOPG/DOG (7/2/1) (DOG), and DOPC/TOPG/O-LPC/DOG (6/2/1/1) (O-LPC+DOG). For *S. aureaus* α-toxin, LUV compositions were DOPC/CHOL (5/5) (Control), DOPC/CHOL/O-LPC (4/5/1) (O-LPC), and DOPC/CHOL/DOG (4/5/1) (DOG). <sup>e</sup>Total extents of vesicular FD-70 release. <sup>f</sup>Degree of transbilayer redistribution of pyPC (q). <sup>g</sup>Extents of FD-70 release induced by tBID in LUV pre-treated with OG-BAX; <sup>h</sup>Total extents of vesicular ANTS release. ND, not determined. Data correspond to means±S.E.M. of 2-5 independent measurements.
Table III: A comparison of the vesicular FD-70 release and the lipid transbilayer redistribution induced by different combinations of BCL-2 family proteins in CL- and PG-containing LUV emulating the composition of outer mitochondrial membrane contact sites.

| Protein mixture       | CL-containing LUV<sup>a</sup> | PG-containing LUV<sup>b</sup> |
|-----------------------|-------------------------------|-----------------------------|
|                       | % Release | q       | % Release | q       |
| BAX+BIM<sub>EL</sub>  | 0.4±0.1  | 0.02±0.01 | 0.2±0.1   | ND      |
| BAX+tBID              | 78±5     | 0.38±0.04 | 72±7      | 0.40±0.09 |
| BAX+tBID+BCL-2        | 11±1     | 0.06±0.02 | 8±1       | 0.12±0.04 |
| BAX+cBID              | 13±2     | 0.13±0.02 | 15±3      | ND      |

<sup>a</sup>PC/PE/PI/CL (26/21/8/20, weight/weight), and <sup>b</sup>PC/PE/PI/PG (26/21/8/10, weight/weight). BAX, tBID and cBID concentrations were 20 nM, BIM<sub>EL</sub> concentration was 200 nM, and BCL-2 concentration was 300 nM. Data correspond to mean values±S.E.M. of 2-3 independent experiments.
Figure legends

Fig. 1. Influence of recombinant BCL-2 family proteins on the release of both cytochrome c from isolated mitochondria and fluorescent markers from LUV. 

A. Freshly isolated rat liver mitochondria were incubated with indicated amounts of recombinant apoptotic proteins for 20 minutes at 30 °C. The 10000g supernatants (Sup.) were collected and analyzed by western blotting for cytochrome c (Cyt. c) release. Total cytochrome c release was determined by 0.5% Triton X-100 solubilization of mitochondria. 

B. Representative time courses of FD-70 release from LUV elicited by BAX (20 nM) combined with either tBID (20 nM), cBID (20 nM), or BIMEL (200 nM). The arrow denotes the time of addition of apoptotic proteins to the liposome suspension.

C. Freshly isolated mitochondria from rat liver were incubated for 5 minutes with antiapoptotic BCL-2 prior to treatment with BAX plus tBID, followed by analysis of freed cytochrome c as explained in Panel A. 

D. Dose-dependent inhibition by BCL-2 of the vesicular FD-70 release induced by BAX (20 nM) plus tBID (20 nM). Maximum extents of marker release were obtained when the fluorescence signal reached a plateau. Mean values ± S.E.M. shown for three independent experiments.

E. Effects of different combinations of proapoptotic proteins on the release of vesicular ANTS/DPX (0.4 kDa) (black bars), FD-10 (light grey bars), and FD-70 (dark grey bars). Mean values ± S.E.M. shown for three to five independent experiments. BAX, BAXΔC, tBID, and cBID concentrations were 20 nM, and BIMEL concentration was 200 nM. Appropriate controls demonstrated that none of the protein buffers induced significant release of mitochondrial cytochrome c or LUV-entrapped markers, at conditions used in these assays.
Fig. 2. Role of the BH3 domain of cBID on vesicular FD-70 release. LUV were treated with BAX (20 nM) together with increasing concentrations of indicated proteins, and extents of vesicular FD-70 release were determined when the fluorescent signal reached a plateau. Data correspond to means (± S.E.M) of at least three independent experiments.

Fig. 3. tBID can cooperate with BAX in a BH3-independent manner to induce LUV permeabilization. A, B, C. tBID and the cardiolipin binding domain of BID (CBD) release FDs from LUV pre-treated with octylglucoside-activated BAX (OG-BAX). A, FD-70-loaded LUV were treated as follows, OG-BAX+tBID: first OG-BAX (large arrow), then tBID (small arrow); OG-BAX: first OG-BAX (large arrow), then tBID buffer (small arrow); OG-BAX+BIMEL: first OG-BAX (large arrow), then BIMEL (short arrow); tBID: first OG-BAX buffer (large arrow), then tBID (short arrow). Concentrations of OG-BAX, tBID, and BIMEL were 20 nM, 150 nM, and 200 nM, respectively. B. FD-10-loaded LUV were treated as follows, OG-BAX+CBD: first OG-BAX (large arrow), then CBD (short arrow); OG-BAX: first OG-BAX (large arrow), then CBD buffer (short arrow); CBD: first OG-BAX buffer (large arrow), then CBD (short arrow); BAX+CBD: first monomeric BAX (large arrow), then CBD (short arrow). OG-BAX, monomeric BAX and CBD concentrations were 20 nM, 20 nM, and 150 nM, respectively. C, Dose dependence of the tBID- and CBD-mediated vesicular FD release in LUV pre-treated with OG-BAX (20 nM), as explained in Panels A and B. tBID/CBD concentrations were 20 nM (black bars), 50 nM (light grey bars), and 150 nM (dark grey bars). Data represent means (± S.E.M) of at least two independent experiments. D, Effect of tBID and CBD on the membrane-binding and membrane-insertion capacities of OG-BAX. LUV (40 µM) were treated first with OG-BAX (40nM) and subsequently with tBID/CBD (300 nM), as described in Panels A and B.
“Total” corresponds to a sample of 40 nM OG-BAX. E, Migration patterns of apoptotic proteins after incubation with LUV as described in Panels A and B, followed by solubilization of membranes with 2% (w/v) CHAPS. Samples containing indicated proteins were subjected to gel filtration in the presence of 2% (w/v) CHAPS, elution fractions of 2 ml were collected, and BAX migration profiles were determined by immunoblotting. Arrows denote elution peaks of standard proteins: thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). OG-BAX, tBID and CBD protein concentrations were 80 nM, 600 nM and 600 nM, respectively. Lipid concentration was 80 µM.

**Fig. 4. tBID cooperates with BAX in vesicular FD-70 release through a mechanism sensitive to intrinsic membrane monolayer curvature.** A, Typical kinetics of vesicular FD-70 release induced by the concerted action of BAX and tBID in DOPC/TOPG (8/2) (Control), DOPC/TOPG/O-LPC (7/2/1) (O-LPC), and DOPC/TOPG/DOG (7/2/1) (DOG) LUV. BAX and tBID concentrations were 20 nM and 10 nM, respectively. B, Representative kinetics of vesicular FD-70 release in LUV treated first with OG-BAX (20 nM) (large arrow), and then with tBID (50 nM) (small arrow).

**Fig. 5. Effects of BCL-2 family proteins on transbilayer lipid redistribution.** A, Representative time courses of pyPC transbilayer redistribution in LUV after addition of BAX combined either with cBID, tBID, or BIM_{EL}. LUV were labeled exclusively in the outer leaflet by external addition of pyPC (5 mol% of total lipid) to the liposome suspension. After a 5-minute pre-incubation of the lipid mixture, proteins were added (corresponding to time=0 s), and the ratio of excimer to monomer fluorescence intensity signals (I_E/I_M) was recorded at any given
time. Values of $I_E/I_M$ are given normalized to those obtained in pure lipid vesicles. BAX, cBID
and tBID concentrations were 20 nM. Concentration of BIM$_{EL}$ was 200 nM. B, Comparison of
the degree of pyPC transbilayer redistribution elicited by various combinations of apoptotic
proteins. The degree of pyPC transbilayer redistribution was estimated from $I_E/I_M$ values as
explained in Experimental Procedures. Proapoptotic protein concentrations were as follows:
BAX+BIM$_{EL}$, 20nM+200 nM; BAX+cBID, 20nM+20nM; OG-BAX, 20 nM; OG-BAX+tBID,
20nM+50nM; and BAX+tBID, 20 nM+20nM. Data represent means (± S.E.M) of 2-4
experiments. Appropriate controls demonstrated that (a) none of the buffers in which the proteins
were suspended caused substantial pyPC transbilayer movement at conditions used in these
assays, and (b) addition of proteins to liposomes symmetrically labeled with pyPC caused much
lower changes in the $I_E/I_M$ ratio as compared to asymmetrically labeled liposomes.

Fig. 6. Effects of BCL-2 family proteins in LUV reflecting the physiological phospholipid
composition of outer mitochondrial membrane contact sites. A, Phosphatidylglycerol (PG)
can replace cardiolipin (CL) in the vesicular FD-70 release and the transbilayer pyPC
redistribution induced by the concerted action of BAX and tBID. LUV were prepared mimicking
the phospholipid composition of outer mitochondrial membrane contact sites including
cardiolipin (26 PC/21 PE/8 PI/20 CL, by weight) (CL) (64), with CL being replaced by equal
molar amounts of phosphatidylglycerol (26 PC/21 PE/8 PI/10 PG, by weight) (PG),
phosphatidylinositol (26 PC/21 PE/20 PI, by weight) (PI), or phosphatidylserine (26 PC/21 PE/
8 PI/11 PS, by weight) (PS), or excluding all acidic lipids (26 PC/21 PE, by weight) (-). Then,
BAX (20 nM) and tBID (20 nM) were added to the vesicle suspensions (arrow), and the time-
dependence of vesicular FD-70 release (continuous lines) in each type of LUV, as well as the
transbilayer redistribution of pyPC in CL-containing LUV (filled circles) and PG-containing LUV (empty circles) were monitored. Experiments were performed three times, each yielding similar results. B, Effects of various BCL-2 protein combinations on the release of FD-70 from LUV of outer mitochondrial membrane contact site composition (26 PC/21 PE/8 PI/20 CL, by weight). Data represents means (± S.E.M) of two independent experiments.
FIGURE 1
FIGURE 1 (cont.)
FIGURE 1 (cont.)
FIGURE 2
FIGURE 3
FIGURE 3 (cont.)
**FIGURE 4**

(A) % FD-70 release over time (s) for different conditions: O-LPC, Control, and DOG.

(B) % FD-70 Release over time (s) for O-LPC, Control, and DOG conditions.
FIGURE 5
FIGURE 6

Degree of pyPC transbilayer redistribution (○)

% FD-70 Release

Time (s)

BAX+
tBID

CL

PG

PI

PS

(-)

Degree of pyPC transbilayer redistribution (○)

% FD-70 Release

BAX (nM)               20   20   20   20   20   20   20   20

cBID (nM)               50   50   50   50   50   50   50   50

BCL-2 (nM)             -   100  300 100 100 100 100   -

BIM\textsubscript{EL} (nM)              -      -      -     50  100 200 300 300

FIGURE 6
Lipidic pore formation by the concerted action of pro-apoptotic BAX and tBID
Oihana Terrones, Bruno Antonsson, Hirohito Yamaguchi, Hong-Gang Wang, Yihua Liu, Ray M. Lee, Andreas Herrmann and Gorka Basanez

J. Biol. Chem. published online May 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313420200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/05/11/jbc.M313420200.citation.full.html#ref-list-1