Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome c release

1,2Tae-Hyoung Kim, 1Yongge Zhao, 1Wen-Xing Ding, 2Jin Na Shin, 1Xi He, 3Young-Woo Seo, 4Jun Chen, 1Hannah Rabinowich, 1Andrew A. Amoscato and 1Xiao-Ming Yin

1Department of Pathology and 4Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261
2Dept. of Biochemistry, Chosun University School of Medicine, Gwangju, 501-759, Korea
3Korea Basic Science Institute Gwangju branch, Chonnam National University 300 Yongbong-Dong, Book-Gu, Gwangju, 500-757, Korea

T-H Kim, Y. Zhao and W-X Ding contributed equally to this manuscript.

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Correspondence should be addressed to:

Dr. Xiao-Ming Yin
Department of Pathology
University of Pittsburgh School of Medicine
3550 Terrace Street, Pittsburgh, PA 15261
Tel: 412-648-8436
Fax: 412-648-9564
e-mail: xmyin@pitt.edu
Abstract

Release of cytochrome $c$ from the mitochondrial intermembrane space is critical to apoptosis induced by a variety of death stimuli. Bid is a BH3-only pro-death Bcl-2 family protein that can potently activate this efflux. In the current study, we investigated the mitochondrial localization of Bid and its interactions with mitochondrial phospholipids, focusing on their relationships with Bid-induced cytochrome $c$ release. We found that Bid binding to the mitochondria required only three of its eight helical structures ($\alpha_4$-$\alpha_6$), but not the BH3 domain, and the binding could not be inhibited by the anti-death molecule Bcl-x$_L$. Membrane fractionations indicated that tBid bound to mitochondrial outer membranes at both contact and non-contact sites. Bid could interact with specific cardiolipin species on intact mitochondria as identified by mass spectrometry. Like the binding to the mitochondria, this interaction could not be blocked by the mutation in the BH3 domain or by Bcl-x$_L$. However, a cardiolipin-specific dye, 10-N-nonyl acridine orange, could preferentially suppress Bid binding to the mitochondrial contact site and inhibit Bid-induced mitochondrial cristae reorganization and cytochrome $c$ release. These findings thus suggest that interactions of Bid with mitochondrial cardiolipin at the contact site can contribute significantly to its functions.
Introduction

The Bcl-2 family proteins regulate apoptosis at the level of mitochondria and consist of both anti-death and pro-death members (Gross et al., 1999). Bid belongs to the BH3-only subgroup of the pro-death molecules (Wang et al., 1996), which serve as sentinels to diverse apoptotic signals (Kelekar and Thompson, 1998; Huang and Strasser, 2000). Bid can be activated by multiple proteases, including caspases (Li et al., 1998; Luo et al., 1998), granzyme B (Barry et al., 2000; Wang et al., 2001), lysosomal enzymes (Stoka et al., 2001; Reiners et al., 2002) and calpains (Chen et al., 2001; Mandic et al., 2002), in response to various death stimuli. The C-terminal part of the truncated Bid is able to translocate to the mitochondria and to induce release of apoptogenic factors including cytochrome c and Smac/DIABLO (reviewed in (van Gurp et al., 2003)), which are critical to the initiation and promotion of cell death (Green and Reed, 1998; Budiardjo et al., 1999). The Bid-mediated mitochondrial events are also important to the successful completion of the death program in the so-called Type II cells, such as hepatocytes, following death receptor engagement (Yin et al., 1999; Zhao et al., 2001; Li et al., 2002).

Release of the apoptogenic factors is one of the key events of mitochondria activation during apoptosis, for which the mechanisms are still largely elusive. Because of its potent effects in activating mitochondria, Bid has been studied as a model molecule to understand how mitochondria could be activated by the BH3-only pro-death Bcl-2 family proteins. Bid
can induce oligomerization of Bak or Bax, leading to mitochondrial outer membrane permeabilization (Eskes et al., 2000; Wei et al., 2000). The BH3 domain of Bid is required for its interaction with Bak or Bax (Eskes et al., 2000; Wei et al., 2000), and is also the target of the anti-death Bcl-2 or Bcl-xL (Wang et al., 1996; Cheng et al., 2001). However, other mechanisms can be involved. Bid can cause mitochondrial cristae reorganization, which mobilizes a major portion of cytochrome c for a maximal release (Scorrano et al., 2002). In addition, acidic phospholipids, particularly cardiolipin, may also participate in the function of Bid based on in vitro liposome permeabilization study (Schendel et al., 1999; Kudla et al., 2000; Lutter et al., 2000; Zha et al., 2000; Esposti et al., 2001; Zhai et al., 2001; Epand et al., 2002). Only cardiolipin has been implicated in the in vivo function of Bid in one study, in which binding of Bid to the mitochondria derived from cells with diminished cardiolipin synthesis was significantly reduced, accompanied with a reduced cytochrome c release (Lutter et al., 2000). These studies, however, have not demonstrated that Bid could actually interact directly with cardiolipin on intact mitochondria and have not shown how cardiolipin may contribute to Bid-induced mitochondria permeabilization.

Localization of Bid in the mitochondria has been shown to be restricted in the mitochondria contact site by immunoelectron tomography (Lutter et al., 2001). However it is not known how Bid could induce mitochondrial outer membrane permeabilization at this site. For example, if Bid induced Bak or Bax oligomerization at the contact site, the process might
lead to permeabilization of both outer and inner membranes, as the two membranes are closely juxtaposed at this unique site. However, permeabilization of inner membranes has not been observed (Kluck et al., 1999; Van Loo et al., 2002).

To address these issues, we carried out an in-depth analysis of the binding of Bid to the mitochondria, the distribution of Bid in the mitochondrial subcompartments, the interactions of Bid with cardiolipin on intact mitochondria and the effects of such interactions on Bid localization, Bid-induced Bak oligomerization and cristae reorganization, and Bid-induced cytochrome c release. Our study indicates that Bid-cardiolipin interaction at mitochondrial contact site could contribute significantly to Bid-induced mitochondrial permeabilization.
Materials and Methods

Expression and purification of recombinant proteins. Recombinant Bid proteins were prepared as described previously (Kim et al., 2000). Briefly, murine full length Bid (α1-8, amino acid 1-195), wild-type tBid (α3-8, amino acid 60-195), mutant tBid (amino acid 60-195, G94E), Bid α3-6 (amino acid 60-166), Bid α3-5 (amino acid 60-145) were amplified by PCR and cloned into pET23dw, an expression vector modified from pET23d(+) with the polyhistidine tag. Human Bcl-xL ΔTM (amino acid 1-209) was also cloned in pET23dw as described previously (Kim et al., 2000). All the constructs were expressed in E. Coli BL21(DE3) and purified using His-Bind nickel agarose affinity column chromatography. However, truncated Bid (α4-6, amino acid 105-166) was fused to EGFP in the vector pEGFP-c1 (BD ClonTech). The fusion protein was analyzed in vivo with confocal microscopy.

Preparation of mitochondria. Murine liver mitochondria were isolated as previously described (Kim et al., 2000) with Buffer A (250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES-NaOH, pH 7.2) and resuspended in Buffer B (250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH$_2$PO$_4$, 5 mM sodium succinate, 25 µM EGTA, 0.1 mM phenylmethylsulfonyl fluoride). This preparation was used in all studies except for the mass spectrometric analysis. For the latter, isolated mitochondria were resuspended in Buffer A and further purified by a sucrose step gradient consisting of 2 ml each of 1.2M and 1.6M sucrose through centrifugation at 40,000 x g for 1 hour at 4°C. The mitochondria, which
appear as a brownish band at the interface of 1.2 M and 1.6 M sucrose, were recovered, washed once with Buffer A, and resuspended in Buffer B.

**Mitochondrial membrane fractionation with digitonin.** This was performed as previously reported (Greenawalt, 1974) and modified (Ohlendieck *et al.*, 1986; Hovius *et al.*, 1990). Briefly, ten mg of mitochondria (0.5mg/ml) were treated as indicated in the figure legend. The mitochondria were then pelleted by centrifugation at 10,000 x g for 15 minutes, and resuspended in Buffer A. Freshly-prepared 2% digitonin in Buffer A was added to the mitochondrial suspension to a final ratio of 0.2%, 0.3% or 0.4% (w/w, digitonin/mitochondrial protein). The mixtures were then gently rotated (about 35 rpm) at 4°C for 15 minutes to solubilized the outer membranes. Following the centrifugation at 10,000 x g for 15 minutes, the outer membrane fraction was collected from the supernatant, and the inner membrane fraction was collected from the pellets. These fractions were analyzed by SDS-PAGE followed by immunoblot with antibodies against VDAC (mAb4, Calbiochem), COX IV (clone 20E8-C12, Molecular Probe), Bid (Wang *et al.*, 1996), Bak (Upstate Biotechnology) or Bcl-x<sub>L</sub> (Upstate Biotechnology).

**Mitochondrial membrane fractionation by linear sucrose gradient centrifugation.** This was mainly based on the swell-shrink-sonicate procedure previously described (Ohlendieck *et al.*, 1986; Adams *et al.*, 1989; Pon *et al.*, 1989; Ardail *et al.*, 1990) with modifications. Briefly, mitochondria suspended in Buffer B (0.5 mg/ml) were treated as
indicated in the figure legends before washed and resuspended in 0.5 ml of hypotonic Buffer C (10 mM KH$_2$PO$_4$, pH 7.4, 1 mM EDTA) at a concentration of 10 mg/ml. The mitochondria were allowed to swell at 4°C with gentle rotation for 15 minutes. The suspension was then admixed with 121 µl of 2.3 M sucrose per 0.5 ml of volume so that the final concentration of the sucrose was 0.45 M. After 15 minutes of incubation at 4°C, the shrunken mitochondria were sonicated (Sonic Vibracell, 3 x 10 seconds at 40% amplitude or 5 watts output). The mitochondria suspension was centrifuged at 10,000 x g for 10 minutes to remove debris and intact mitochondria. The supernatants were then loaded onto a sucrose linear gradient (1.8 M-1.4M, 4.0 ml) prepared in Buffer C, and centrifuged at 100,000 x g in a SW60Ti rotor (Beckman) for 20 hours at 4°C. Fractions were then collected from the bottom of the gradient. Thus, the heavier inner membranes were eluted first, followed by the lighter outer membranes. Fractions were sequentially labeled and each contained about 100 µl of sample. The sucrose concentration of each fraction was derived from linear regression analysis with the first fraction set at 1.8M and the last fraction (#40) set at 1.4M. Protein concentrations were determined. A 20 µl of sample containing similar amounts of proteins for each fraction was analyzed by SDS-PAGE followed by immunoblot with antibodies against VDAC (mAb4, Calbiochem), COX IV (clone 20E8-C12, Molecular Probe), Bid (Wang et al., 1996), or Bak (Upstate Biotechnology).

**Analysis of mitochondrial targeting, cytochrome c release and Bak oligomerization.**
Isolated mitochondria (0.5mg/ml) in Buffer B were incubated with various recombinant proteins as indicated in the figure legends for 1 hour at 30°C. The supernatants were separated by centrifugation at 10,000 x g for 15 minutes at 4°C and analyzed for cytochrome c release by immunoblot with an anti-cytochrome c antibody (BD PharMingen). For analysis of protein insertion into the membrane, the mitochondrial pellets were resuspended in Buffer B containing 0.1 M Na2CO3, pH 11.5 and incubated on ice for 30 minutes. The mitochondria were re-pelleted by centrifugation at 100,000 x g for 30 minutes, and analyzed by immunoblot for Bid (Wang et al., 1996) or Bcl-xL (Upstate Biotechnology). To analyze cytochrome c release induced by tBid, mitochondria were suspended in Buffer B with 4 mM MgCl2 and treated with tBid for 60 minutes at 30°C. The supernatant were then separated and analyzed by immunoblot for cytochrome c. To determine Bak oligomerization, the mitochondria were treated with tBid as above. After centrifugation, the mitochondria were resuspended in Buffer B and incubated with the homobifunctional cross-linker, bismaleimidohexane (BMH) (10 mM) or the solvent DMSO for 60 minutes at room temperature followed by SDS-PAGE and immunoblot for Bak (Upstate Biotechnology)(Wei et al., 2000). To determine the effect of 10-N-nonyl-acridine orange (NAO) on cytochrome c release and Bak oligomerization, mitochondria were pre-treated with NAO (5-15 µM) for 10 minutes at 30°C before being treated with various recombinant proteins as described above.

**Analysis of tBid-lipid interactions by mass spectrometry.** Purified mitochondria were
treated with wild-type tBid or mutant tBid (G94E) (0.1 µg/ml) in the absence or presence of Bcl-xL∆TM (13 µg/ml) for 1 hour at 30°C. Lipids were extracted from untreated or treated mitochondria and analyzed by mass spectrometry as previously described (Matsko et al., 2001). Briefly, mitochondria were extracted in chloroform:methanol (2:1, v/v) overnight at 4°C under a N2 atmosphere in the presence of butylated hydroxytoluene. Sodium chloride (0.15M) was added and the sample was vortexed and centrifuged. The chloroform layer was removed and the lipids were dried under a stream of N2 and stored under a N2 atmosphere at –20°C until analysis. Lipids were analyzed by direct infusion into a Quattro II triple quadrupole mass spectrometer (Micromass, Inc.) equipped with an electrospray ionization source. The electrospray probe was operated at a voltage differential of −3.5 keV in the negative ion mode. Mass spectra were obtained by scanning in two mass/charge ranges, 400-950 Da/e and 1200-1800 Da/e every 1.6s and summing individual spectra. Source temperature was maintained at 70°C. Scanning in the range of 400-950 Da/e in the negative ion mode affords identification of the major phospholipid species including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylglycerol (PG), running as singly-charged (M-H)− ions. Some species may also run as chloride adducts. In addition, the 400-950 Da/e range also detects cardiolipin (CL) that runs as the doubly-charged (M-2H)2− species due to its additional phosphate. Due to is higher mass, cardiolipin may also be
detected in the singly-charged (M-H)$^-$ state in the range of 1200-1800 Da/e. Daughter ion spectra were obtained by selecting the ion of interest and performing daughter ion scanning in Q3 at 400 Da/s. Collision gas (argon) and collision energy were adjusted accordingly. The mass spectrometer was operated at unit resolution in the ms mode and slightly below unit resolution in the tandem ms mode.

**Electron microscopy.** This was performed as previously described (Scorrano et al., 2002) with modification. Briefly, About 150 µl of mitochondria (0.5 mg/ ml) in Buffer B with 4 mM MgCl$_2$ were treated with NAO (10 µM) or buffer for 5-10 minutes, washed and then incubated with wild-type or G94E mutant tBid (0.3 µg/ml) for 10 minutes at 30°C. Alternatively, mitochondria were pre-incubated with Bcl-xLΔTM (20 µg/ml) for 10 minutes before tBid was added for another 10 minutes. Mitochondria were then separated by centrifugation at 10,000 × g for 10 min and washed three times with isotonic potassium phosphate buffer to remove sucrose, which otherwise would interfere with the microscopy. The pellets were then fixed with 1.25% glutaraldehyde at room temperature for 1 hr, rinsed in cacodylate buffer, post-fixed with 1% osmium tetroxide, dehydrated, embedded and thin-sectioned for electron microscopic analysis at the Electron Microscopy Facility of the Department of Pathology, University of Pittsburgh School of Medicine.
Results

Binding of tBid to the mitochondria does not require the BH3 domain, but requires the α4, α5 and α6 helices.

To study the interaction of Bid with the mitochondria and the subsequent events, we first defined the region of Bid responsible for the binding to the mitochondria. Bid is composed of eight alpha helices, namely α1 to α8 (Chou et al., 1999; McDonnell et al., 1999). We constructed several deletional Bid mutants, encompassing different alpha helices (Fig. 1A). Purified recombinant Bid proteins were then incubated with isolated mouse liver mitochondria. The membrane-inserted Bid on alkali-treated mitochondria and the cytochrome c released into the supernatant were determined by immunoblot analysis (Fig. 1B, 1C).

Full length Bid (α1-α8) was able to bind to and insert into the mitochondria but its ability to induce cytochrome c release was minimal. Bid (α3-α8), the truncated form of Bid after caspase-8 cleavage (tBid) (Li et al., 1998; Luo et al., 1998), bound to the mitochondria equally well, and caused a much stronger cytochrome c release, indicating that the N-terminal α1 and α2 did not participate in membrane binding and was in fact inhibitory to the activity of Bid (Tan et al., 1999). The central hydrophobic helix 6 was required for Bid to interact with the mitochondria, thus α7 and α8, but not α6, could be dispensed. On the other hand, BH3 domain-containing α3 helix seemed to be required for the step subsequent to membrane
insertion, \textit{i.e.}, cytochrome \textit{c} release, but not for the binding itself, as indicated by the behavior of the BH3 domain mutant tBid (G94A) (Fig. 1B and C). None of these Bid recombinant proteins could be detected in the absence of mitochondria in the same fraction (data not shown). To further confirm that only helices 4-6 of Bid was required for its binding to the mitochondria, we constructed this mutant Bid (amino acid 105-166) for expression in \textit{E. coli}. However, the protein was not stable and we were not able to conduct the \textit{in vitro} experiment. Thus, we constructed Bid (\(\alpha_4-\alpha_6\)) in fusion with the green fluorescence protein (GFP) gene and transiently expressed the fusion molecule in \textit{HeLa} cells. Confocal microscopy showed that GFP-Bid (\(\alpha_4-\alpha_6\)) was located on the mitochondria (Fig. 1D), indicating that these three helices were structurally sufficient for Bid to localize to the mitochondria. However, because of the lack of the BH3 domain, GFP-Bid (\(\alpha_4-\alpha_6\)) would not be able to induce cytochrome \textit{c} release (Lutter \textit{et al.}, 2000).

The above studies suggested that the binding domain of Bid (\(\alpha_4-\alpha_6\)) and its BH3 domain (\(\alpha_3\)) could function separately. This notion was further supported by the observation that the anti-death molecule Bcl-x\textsubscript{L} was able to suppress tBid-induced cytochrome \textit{c} release (Fig. 1E), but not the binding of tBid to the mitochondria (Fig. 1F). Cytochrome \textit{c} release by tBid is dependent on its BH3 domain, which is the binding target of Bcl-x\textsubscript{L} (Cheng \textit{et al.}, 2001).

\textbf{Localization of tBid on both contact and non-contact sites of the mitochondria outer membranes.}
Targeted tBid (α3-α8) remained mainly in the mitochondrial outer membranes, as indicated by limited digitonin solubilization. The digitonin-solubilized fraction was enriched with the outer membranes and the insoluble fraction was enriched with the inner membranes, as verified by the immunoblot analysis with antibodies against the outer membrane marker, voltage dependent anion channel (VDAC), and the inner membrane marker, cytochrome c oxidase subunit IV (COX IV) (Fig. 2A). tBid exhibited a digitonin solubilization pattern similar to that of VDAC, but not to that of COX IV, indicating its main presence in the outer membranes (Fig. 2A). Similarly, exogenously targeted Bcl-xL and endogenous Bak were also found in the digitonin-soluble fraction containing the outer membranes (Fig. 2A).

A dichotomy in Bid location and Bid function was implicated in an immunoelectron tomography study finding Bid at the mitochondrial contact site (Lutter et al., 2001), as the formation of Bak or Bax oligomers at this site might lead to the leakage of matrix proteins, but not proteins in the inter-membrane space, due to the lack of inter-membrane space. To address this issue, we separated membranes from tBid-treated mitochondria in a linear sucrose gradient, which segregated the inner membranes, the contact site and the outer membranes to fractions of heavier, intermediate and lighter density, respectively. These fractions had been previously characterized extensively using classical outer membrane markers, such as monoamine oxidase and VDAC, and inner membrane markers, such as succinate dehydrogenase and COX IV (Ohlendieck et al., 1986; Adams et al., 1989; Pon et
Correspondingly, immunoblot analysis of the fractions showed distinguished distribution of COX IV and VDAC, corresponding to the inner and outer membranes, respectively (Fig. 2B). tBid could be found in the lighter density fractions like VDAC, but it could be also found in the intermediate density fractions where the levels of both VDAC and Cox IV were low (Fig. 2B). These fractions were previously characterized to be enriched with mitochondrial contact site (Ohlendieck et al., 1986; Adams et al., 1989; Pon et al., 1989; Simbeni et al., 1991). On the other hand, Bak was mainly found in the lighter density fractions of normal mitochondria (Fig. 2C).

These results suggest that tBid may be present in the two subcompartments of the mitochondria, the contact site and the non-contact site. Since Bak was mainly found in the lighter density fractions corresponding to the non-contact sites, we also speculate that its interaction with Bid as documented in previous studies (Wei et al., 2000; Scorrano et al., 2002) may occur mainly at this subcompartment. On the other hand, those tBid localized at the intermediate fractions (contact sites) might be involved in a different type of function. To address this possibility, we performed the following experiments investigating the interaction of Bid with lipids on the mitochondria.

**tBid binds to mitochondrial cardiolipin at mitochondrial contact sites.**

Previous studies had shown that Bid was able to bind to anionic phospholipids, particularly cardiolipin, in artificial systems, such as in liposomes or on solid surface
Bid targeting to the mitochondria isolated from cardiolipin-deficient cells was reduced significantly (Lutter et al., 2000), direct evidence of Bid interaction with cardiolipin in intact mitochondria was still missing. Cardiolipin is enriched in the inner membranes and the outer membranes at the contact site (Ardail et al., 1990; Simbeni et al., 1991). We thus decided to investigate whether tBid could be associated with cardiolipin on intact mitochondria.

Mitochondria were first incubated with the recombinant proteins under the same conditions that led to their integration into the mitochondrial membranes (Fig. 1B). Total mitochondrial lipids were then extracted and analyzed by electrospray ionization mass spectrometry. If a particular type of lipid was associated with the recombinant proteins, the protein would compromise the lipid’s ability to ionize properly and the signals for the particular lipid species would be reduced. On the other hand, the Bid-binding lipids may partition into the hydrophilic phase with Bid and its signals would be also reduced in the hydrophobic phase, which was used for lipid extraction and analysis. Lipid scans were conducted in two mass/charge (Da/e) ranges, 400-950 and 1200-1800. The 400-950 Da/e range affords identification of the major phospholipid species, which run as singly-charged entities. This range also detects any doubly-charged species of cardiolipin, that run as (M-2H)^- ions. Scanning in this range showed that the only mass ion that had significant changes upon Bid-treatment was the 723.6 Da/e species. Daughter ion analysis indicated that this was...
a doubly charged cardiolipin species. There were no significant signal changes in other major phospholipids including PG, PE, PC, PS and PI (Fig. 3A-B).

Scanning in the 1200-1800 Da/e range would detect any cardiolipin species that carries only a single negative charge (M-H)- in the negative ion mode. Cardiolipin contains two phosphates, each capable of losing one hydrogen upon negative ionization. Electrospray ionization itself promotes multiple charging, hence both minus (-1) and (-2) charge states can exist. We found that signals of several species with mass/charge ratios around 1447-1452 were dramatically reduced in mitochondria treated with wild-type tBid (Fig. 3C-D). These species were identified as cardiolipin by daughter ion analysis. One of the species with a Da/e ratio of 1447 represented the singly charged version of the 723.6 mass ion detected in the 400-950 Da/e range. The decrease in signal intensity of these cardiolipin species were consistently observed, although the extent of the change varied among experiments. The average reduction of signals was more than 50% for the two species (Fig. 3H).

These results seemed to be most compatible with the interpretation of Bid being tightly associated with these cardiolipin species, which compromised the signal detection by mass spectrometry. In support of this argument, the Bid-induced signal reduction of the selected cardiolipin species was not affected by the G94E BH3 mutation (Fig. 3E), in agreement with the mitochondria binding results (Fig. 1). Bcl-xL alone did not seem to have the same effect (Fig. 3G), indicating some specificity of the Bid-cardiolipin interaction. Intriguingly,
treatment of mitochondria with both tBid and Bcl-xL could further reduce the cardiolipin signals (Fig. 3F). This may suggest either that Bcl-xL could further attenuate the cardiolipin signal only in the presence of tBid, or that the tBid-Bcl-xL complex had a higher affinity to cardiolipin than tBid alone.

The interaction between tBid and cardiolipin on intact mitochondria could be further confirmed by the use of a cardiolipin-specific dye, 10-N-nonyl-acridine orange (NAO) (Petit et al., 1992). Pre-treatment of mitochondria with NAO in low concentrations noticeably inhibited the membrane targeting of tBid, but not Bcl-xL, to the mitochondria (Fig. 4A), perhaps by blocking the access of tBid to cardiolipin. These results were consistent with the mass spectrometry finding.

Among the several cardiolipin species that displayed signal reduction, we determined the fatty acyl chain content of two major species (1447 and 1449 Da/e) by collision-induced dissociation tandem mass spectrometry of the selected mass ions. Daughter ion analysis determined that 1447 Da/e contained the following fatty acyl chain composition: C18:2, C18:2, C18:2, C18:2; and 1449 Da/e: C18:2, C18:2, C18:2, C18:1. These compositions are consistent with those reported for mouse liver cardiolipins, which express relatively high linoleoyl content (Hoch, 1992).

Cardiolipins containing 18:2 acyl compositions are more concentrated in the mitochondrial contact site (Ardail et al., 1990). To determine whether tBid association with
the contact site (Fig. 2B) was mediated by the interaction with cardiolipin, mitochondria sequentially treated with NAO and tBid were sub-fractionated. Immunoblot analysis of the various fractions indicated that NAO preferentially blocked tBid targeting to membranes of intermediate density, or the contact sites (Fig. 4B-C), as confirmed by densitometry analysis based on equal amount of protein loading (Fig. 4D). That the G94A mutant of tBid could bind to cardiolipin (Fig. 3C) was also consistent with the finding that this mutant could target to the contact site as shown by immunoelectron tomography (Lutter et al., 2001). Targeting of tBid to the non-contact site might be also mediated by Bid-cardiolipin interactions, as a small amount of cardiolipin can also be found in this part of the outer membrane (Ardail et al., 1990; Simbeni et al., 1991). In addition, although mass spectrometry analysis did not detect signal alterations in other lipid species in tBid-treated mitochondria, the participation of other non-cardiolipin lipid species in Bid-mitochondria interaction, particularly at the non-contact site, remains possible (Esposti, 2002).

Interaction of Bid with cardiolipin contributes to mitochondrial cristae re-organization.

NAO pre-treatment also led to a significant suppression of tBid-induced cytochrome c release (Fig. 5A), indicating that there was a functional consequence of tBid-cardiolipin interaction. This seemed to be less likely caused by a reduced tBid binding to the mitochondria, as a large amount of tBid was still present in NAO-treated mitochondria,
particularly at the non-contact site (Fig. 4C). Thus cardiolipin may not just serve as a docking site for Bid, but also a mediator of functional alterations.

A possible outcome is an enhanced membrane permeabilization. *In vitro* analysis indicates that cardiolipin, as well as other anionic phospholipids, is important for tBid to permeabilize liposomes (Schendel *et al.*, 1999; Kudla *et al.*, 2000; Epand *et al.*, 2002). However, liposomes reconstituted with xenopous mitochondria lipids cannot be permeabilized by caspase-8 activated Bid alone, even if the right amount of cardiolipin was present (Kuwana *et al.*, 2002). Thus permeabilization of membranes may not be the primary consequence of Bid-cardiolipin interactions, particularly on intact mitochondria.

Alternatively, tBid can cause mitochondria cristae reorganization, which is accompanied with the mobilization of a major portion of cytochrome *c* (Scorrano *et al.*, 2002). In line with this notion, we found that while cristae remodeling induced by tBid could not be blocked by the G94A BH3 mutation or by Bcl-xL, it could be significantly suppressed by NAO at a low concentration (10 µm), indicating the participation of cardiolipin in this process (Fig. 5B, C). In contrast, tBid-induced Bak oligomerization was only slightly affected by NAO at high concentrations, although it was completely suppressed by Bcl-xL (Fig. 5A). Cytochrome *c* release and cristae reorganization were thus more susceptible to NAO treatment than Bak oligomerization, indicating the role of cristae reorganization in cytochrome *c* release, as previously indicated (Scorrano *et al.*, 2002). Taken together, our results suggest that Bid at
different mitochondrial sub-compartments could exhibit distinct activities, culminating in a coordinated maximal release of cytochrome c.
Discussion

Bid contains eight alpha helices, including two central hydrophobic helices, $\alpha_6$ and $\alpha_7$, but no transmembrane domain (Chou et al., 1999; McDonnell et al., 1999). Previous study showed that only a region from $\alpha_4$ to $\alpha_6$ is required for Bid binding, excluding the requirement of one central hydrophobic helix, $\alpha_7$ (Lutter et al., 2000). We confirmed this observation in the current study. In addition, we found that the BH3 domain-containing $\alpha_3$ helix was not required for membrane binding, but was needed for the cytochrome c releasing activity. Thus, the minimal functional unit of Bid would include the $\alpha_3$-$\alpha_6$ helices. The present study also indicates that the binding domain of Bid ($\alpha_4$-$\alpha_6$) and its BH3 domain ($\alpha_3$) can function separately. The binding of Bid to the mitochondria through the binding domains can lead to distinctive functional consequences independently of the BH3 domain (see below).

The truncated Bid is generally considered to be localized in the outer membranes of the mitochondria as it is able to permeabilize the outer membranes without affecting the permeability of the inner membranes (Kluck et al., 1999; Van Loo et al., 2002). Both limited digitonin solubilization and sucrose linear gradient fractionation studies now provide the actual evidence to support this notion. Furthermore, the fractionation study indicates that Bid can be present in both mitochondrial contact site and non-contact site. In addition, it is possible that some tBid proteins may diffuse into the inner membranes as they could be found in fractions with an even higher density, which are more enriched with COX IV (Figs. 2 and
4). However, the significance of this observation has yet to be determined. The finding of tBid in the contact site of the outer membranes by the gradient fractionation method is consistent with the study using immunoelectron tomography (Lutter et al., 2001). However, in the latter study, localization of tBid in the non-contact site was apparently not readily observable, perhaps reflecting the lower sensitivity of this method.

Notably, Bak was found mainly in the non-contact site (Figs. 2, and 4). Since Bak can be activated by tBid (Wei et al., 2000), we speculate that tBid-Bak interaction may occur mainly at the non-contact site. On the other hand, our data indicate that tBid at the contact site are involved in the interaction with cardiolipin. Bid-cardiolipin interactions had been reported previously only in artificial conditions (Lutter et al., 2000; Zha et al., 2000; Epand et al., 2002). Through the use of electrospray ionization mass spectrometry and a cardiolipin-specific dye, NAO (Petit et al., 1992), we now found that Bid could indeed interact with cardiolipin on functional mitochondria and furthermore we were able to define the location where such interactions occurred by mitochondria fractionation analysis.

The mass spectrometry had been successfully used to analyze lipid-protein interactions (Elviri et al., 2001; de Brouwer et al., 2002; Demmers et al., 2003) and it allowed us to identify at least two cardiolipin species, whose signals were affected significantly by tBid. We consider that this signal reduction is due to Bid-cardiolipin interactions, which cause an inadequate ionization of the affected cardiolipin species. This interpretation is most
compatible with other findings reported here and in the literature (Lutter et al., 2000; Kuwana et al., 2002). Although we can not completely exclude other possibilities, the signal reduction does not seem to be due to a general degradation, as only selected cardiolipin species were affected (Fig. 3).

Cardiolipin is predominantly present in the inner membranes, but can also be found in the outer membranes in smaller amounts (Daum, 1985; Ardail et al., 1990; Hovius et al., 1990; Simbeni et al., 1991; Hoch, 1992; Schlame et al., 2000). It is much more concentrated in the outer membranes at the contact site (Ardail et al., 1990; Simbeni et al., 1991) and has a higher 18:2 acyl content (Ardail et al., 1990). Indeed, two of the cardiolipin species identified in this study (1447 and 1449 Da/e) were found to be enriched in linoleic acid (C18:2). Thus, the ability of tBid to bind to cardiolipin may allow tBid to localize to the contact site. In support of this notion, pre-treatment of the mitochondria with the cardiolipin-specific dye NAO preferentially inhibited the binding of tBid to the mitochondria at the contact site (Fig. 4). Furthermore, that the G94A mutant of tBid could still bind to cardiolipin (Fig. 3C) is also consistent with the finding that it retained its ability to bind to mitochondria (Fig. 1B) and with the finding that this mutant could be found at the contact site (Lutter et al., 2001). It has to be pointed out that although mass spectrometry analysis did not detect signal alterations in other lipid species in tBid-treated mitochondria, we cannot completely rule out the participation of other non-cardiolipin lipids in tBid-mitochondria interaction (Esposti et
However, interaction of tBid with cardiolipin is unique in that it contributes to tBid-induced cytochrome c release via a defined mechanism.

NAO has a high affinity to cardiolipin ($K_a = 2 \times 10^6 \text{M}^{-1}$) and each cardiolipin molecule can bind to two NAO molecules (Petit et al., 1992). NAO can also bind to two other mono-acidic phospholipids, PI and PS, at a much lower affinity ($K_a = 7 \times 10^4 \text{M}^{-1}$) in an equal molar ratio (Petit et al., 1992). Comparison of the binding kinetics between cardiolipin-containing liposomes and mitochondria indicates that the main target of NAO in mitochondria was cardiolipin (Petit et al., 1992). NAO is thus quite specific to cardiolipin. When mitochondria were pre-treated with NAO, it reduced tBid binding to the mitochondria at the contact site and also reduced cytochrome c release. This effect is likely resulted from the blockage of tBid-cardiolipin interaction by NAO, as suggested by the available data (Fig. 2-4) (Petit et al., 1992). Furthermore, NAO pre-treatment only affected Bid-induced mitochondrial cristae reorganization, but not Bak oligomerization (Fig. 5), the two major mechanisms responsible for tBid-induced cytochrome c release (Wei et al., 2000; Scorrano et al., 2002). Previous studies have suggested a model in which Bid-induced Bak oligomerization is essential for outer membrane permeabilization and Bid-induced cristae reorganization is required to maximally mobilize cytochrome c for release (Scorrano et al., 2002). However, it is not known how Bid induces cristae reorganization, which is apparently independent of Bak and the BH3 domain of Bid ((Scorrano et al., 2002) and Fig. 5). Our finding now suggests that
cristae reorganization may be resulted from tBid-cardiolipin interaction at the mitochondrial contact site. At the present time, it is not clear how such an interaction could lead to the alteration of cristae structures. We speculate that this may be related to the non-lamellar tendency of cardiolipin due to its intrinsic negative curvature (Epand et al., 2002), which can render cardiolipin in the hexagonal phase. This conversion from lamellar to non-lamellar structure may be facilitated by the interaction with tBid and may lead to alterations of the cristae membrane structure.

In summary, we found that targeting of tBid to the mitochondria through its $\alpha_4$-$\alpha_6$ domain led to its localization to the mitochondrial outer membranes. Our data indicate that tBid could be distributed into different mitochondrial subcompartments with distinctive functional consequences. In particular, tBid-cardiolipin interactions on functional mitochondria seemed to occur mainly in the contact site, which contributes to mitochondrial cristae reorganization and cytochrome $c$ release. These data also imply that Bid-Bak interaction may occur at the non-contact site. Thus our studies support a model in which Bid can initiate distinctive molecular events at specific mitochondrial subcompartments to induce a maximal cytochrome $c$ release.
Acknowledgments

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Figure Legends

Figure 1. Different structures of tBid are required for membrane binding and cytochrome c release. (A). A schematic diagram of various recombinant Bid molecules. Designation of alpha helices in relation with the deletional mutants is shown. The solid box in the alpha 3 helix indicates the wild type BH3 domain, whereas the dotted box in the same helix indicated the BH3 domain with a point mutation (G94E). (B). Binding of Bid to the mitochondria depends on specific alpha helices. Various Bid proteins (1 µg/ml) were added to isolated mitochondria (0.5 mg/ml), and incubated for 1 hour at 30°C. Mitochondria were separated, treated with alkali and analyzed by immunoblot with an anti-Bid antibody (Wang et al., 1996) for Bid targeting (upper panel). This antibody was able to recognize the various truncated recombinant Bid mutants in purified form (lower panel). (C). Supernatants isolated from Bid-treated mitochondria as in (B) were analyzed by immunoblot for released cytochrome c. (D). Bid binding to the mitochondria can be BH3-domain independent. Bid (α4-6)-EGFP was transfected into HeLa cells for 8 hours in the presence of 2 mM of z-VAD-fmk (left panel: green color). Mitochondria were identified by MitoTracker (100 nM, middle panel: red color). Localization of Bid to the mitochondria is indicated by the orange color (right panel) by confocal microscopy. (E). Bcl-xL inhibits tBid-induced cytochrome c release. Mitochondria (0.5 mg/ml) were incubated with tBid (0.1 µg/ml) and Bcl-xL (3.3, 6.7, 13 or 26 µg/ml) for one hour at 30°C. The supernatants were separated and analyzed by immunoblot for cytochrome c release. (F). Bcl-xL does not interfere with tBid binding to the
mitochondria. Mitochondria were pre-treated with Bcl-xL (27 µg/ml) and then tBid (0.1 µg/ml). The treated mitochondria were separated, alkali-treated and analyzed by immunoblot with the anti-Bid antibody.

**Figure 2. Localization of tBid in the mitochondrial outer membranes. (A).**
Localization of tBid, Bak and Bcl-xL in the mitochondrial outer membranes. Mitochondria were incubated with tBid (0.1 µg/ml) or Bcl-xL (13 µg/ml) for one hour at 30°C before being treated with different amount of digitonin (w/w, 0-0.4%). Mitochondria were then separated by centrifugation. The supernatant, containing the outer membranes, and the pellet, containing the inner membranes, were analyzed by SDS-PAGE followed by immunoblot for the proteins indicated. (B). Distribution of tBid on different mitochondrial fractions. Mitochondria were incubated with tBid as in (A), and then disrupted and fractionated on a sucrose linear gradient. Eluted fractions were analyzed by SDS-PAGE and immunoblot. (C). Mitochondria without tBid treatment were fractionated as in (B) and analyzed by immunoblot with antibodies against VDAC and Bak. For B and C, the sucrose concentration of each fraction is indicated.

**Figure 3. Binding of tBid to mitochondrial cardiolipin. (A-G).** Mitochondria were incubated with buffer (A, C), tBid (0.1 µg/ml) (B, D), G94A mutant tBid (0.1 µg/ml) (E), tBid plus Bcl-xL (13 µg/ml) (F), or Bcl-xL (13 µg/ml) (G) for 30 minutes, and then purified for lipid extraction. Extracted lipids were analyzed by electrospray ionization mass
spectrometry. Lipid scans in the 400-950 Da/e range (A, B) and in the 1200-1800 Da/e range (C-G) were conducted. Typical profiles are shown. Note the reduction in the signals of certain cardiolipin species in panels B (723.6 Da/e) and D-F (1447-1452 Da/e).

Abbreviations used are CL for cardiolipin, PC for phosphatidylcholine, PE for phosphatidylethanolamine, PS for phosphatidylserine, PI for phosphatidylinositol and PG for phosphatidylglycerol. (H). The relative signal levels of the two major affected cardiolipin species are shown based on the scan in the range of 1200-1800 Da/e. The cardiolipin signal level of the control sample (Ctr) was invariably set at 100% in each experiment, to which the signal levels of the treated groups were compared. The numbers shown on the top of each bar are Mean (SD). Data are representative of 2-5 experiments performed.

**Figure 4. Interaction of tBid with cardiolipin contributes to its targeting to the mitochondrial contact site.** (A). NAO inhibits binding of tBid, but not Bcl-xL, to the mitochondria. Mitochondria (0.5 mg/ml) were pretreated with different amounts of NAO, washed, incubated with tBid (0.1 µg/ml) or Bcl-xL (27 µg/ml) for 1 hour at 30°C, alkali-treated and analyzed by immunoblot with an anti-Bid or anti-Bcl-xL antibody. (B-C). NAO preferentially blocks tBid targeting to the contact site. Mitochondria were pre-treated with buffer (B) or with NAO (10 µM) (C) for 10 minutes, washed and then incubated with tBid (0.1 µg/ml) for 30 minutes at 30°C before disrupted and fractionated. Eluted fractions were quantified for protein concentrations and aliquots containing similar amount of proteins were
separated by 12% SDS-PAGE and immunoblotted with anti-COX VI, VDAC or Bid antibodies. For B and C, the sucrose concentration of each fraction is indicated. (D).

Densitometry analysis of the Bid blots from panels B (without NAO, filled triangle) and C (with NAO, open circle). The relative intensity of the Bid signal is plotted against the sucrose gradient. Data were normalized to the amount of proteins loaded. Densitometric values of those fractions with immunoblot results compromised by gel artifacts (fractions 17 and 19 from panel B and fractions 28 and 31 from panel C) were not included for a clearer presentation. Note the significant reduction of tBid binding at 1.65-1.67M of sucrose gradient (fractions 14-16 in panel C). Data are representative of four experiments performed.

**Figure 5.** NAO inhibits Bid-induced cytochrome c release and mitochondrial cristae reorganization, but not Bak oligomerization. (A). NAO inhibits tBid-induced cytochrome c release without significantly affecting Bak oligomerization. Mitochondria were treated with tBid (0.1 µg/ml) alone or with NAO or Bcl-xL (30 µg/ml) as indicated. The supernatants were separated by centrifugation and analyzed by immunoblot for cytochrome c (upper panel). The mitochondria pellets were then washed and incubated with 10 mM BMH (middle panel), or the solvent DMSO (lower panel) for 60 minutes. The pellets were then solubilized in the SDS-PAGE loading buffer and separated by SDS-PAGE. Immunoblot was conducted with the anti-Bak antibody. The BMH cross-linked product that migrated faster than the Bak monomer (indicated as 1x Bak) in lanes 1, 8, 9 and 10 may represent the intra-molecular
cross-linked Bak monomer (indicated by the arrowhead) (Wei et al., 2000). BMH cross-linked Bak dimers are indicated as 2x Bak. Treatment with the solvent control DMSO did not resulted in any cross-linked forms of Bak, although non-specific bands were observed as indicated by the asterisks. The relative intensities (RI) of cytochrome c (upper panel) and Bak dimers (middle panel) as determined by densitometry are indicated. (B). NAO inhibits mitochondrial cristae reorganization. Mitochondria were treated and processed as described in the method section. Representative electron micrographs are shown: (a), Control; (b), tBid only (0.3 µg/ml); (c), G94A mutant tBid only (0.3 µg/ml); (d), tBid plus Bcl-xL (20 µg/ml); (e), NAO (10 µM) and (f), tBid plus NAO. Arrows indicated the mitochondria with typical cristae reorganization. Scale bar, 1 µm. (C). Mitochondria with reorganized cristae were quantified and expressed as the percentage of total mitochondria counted (400-500 total per group). B and C: data are representative of three experiments performed.
A

\[ \begin{array}{cccccccc}
\alpha_1 & \alpha_2 & \alpha_3 & \alpha_4 & \alpha_5 & \alpha_6 & \alpha_7 & \alpha_8 \\
1 & & & & & & & 195 \alpha_1-8 \\
60 & & \boxed{} & & & & & 195 \alpha_3-8 (tBid, WT) \\
60 & & & \boxed{} & & & & 195 \alpha_3-8 (tBid, G94E) \\
60 & & & & \boxed{} & 166 & & \alpha_3-6 \\
60 & & & & & & \boxed{} & \alpha_3-5 \\
\end{array} \]

\[ \text{BH3} \]

B

\begin{align*}
\alpha_1-8 & \\
\alpha_3-8 & \text{ (WT)} \\
\alpha_3-8 & \text{ (G94E)} \\
\alpha_3-6 & \\
\alpha_3-5 & \\
\end{align*}

C

\begin{align*}
\alpha_1-8 & \\
\alpha_3-8 & \text{ (WT)} \\
\alpha_3-6 & \\
\alpha_3-5 & \\
\alpha_3-8 & \text{ (G94E)} \\
\end{align*}

D

\begin{align*}
\text{GFP-Bid (}\alpha_4-6\text{) } & \quad \text{MitoTracker} & \quad \text{Overlay} \\
\end{align*}
A

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B

Sucrose (M): 1.50 1.51 1.52 1.53 1.54 1.55 1.56 1.57 1.58 1.59 1.60 1.61 1.62 1.63 1.64 1.65 1.66 1.67 1.68 1.69 1.70 1.71 1.72 1.73 1.74 1.75 1.76 1.77 1.78 1.79
Fractions: 1 3 5 7 9 11 13 15 17 19 21 23 25 26 27 28 29 30

C

Sucrose (M): 1.53 1.54 1.55 1.56 1.57 1.58 1.59 1.60 1.61 1.62 1.63 1.64 1.65 1.66 1.67 1.68 1.69 1.70 1.71 1.72 1.73 1.74 1.75 1.76 1.77 1.78 1.79 1.80
Fractions: 2 4 6 8 10 12 14 16 18 19 20 21 22 23 24 25 26 27
Graph showing signal level (% of control) for different conditions:

- **1447**
  - Ctr
  - tBid (WT)
  - tBid (G94E)
  - tBid+ (Bcl-xL)

- **1449**
  - Ctr
  - tBid (WT)
  - tBid (G94E)
  - tBid+ (Bcl-xL)

Legend:
- **Signal Level (%)**
- **Cardiolipin (Da/e)**

Values:
- Ctr: 100 (0)
- tBid (WT): 32 (10)
- tBid (G94E): 36 (6)
- tBid+ (Bcl-xL): 20 (0)
- Ctr: 100 (0)
- tBid (WT): 32 (9)
- tBid (G94E): 35.5 (1)
- tBid+ (Bcl-xL): 20 (4)
D

Sucrose Concentration (M)

Relative Intensity of tBid

1.45 1.50 1.55 1.60 1.65 1.70 1.75 1.80 1.85

0

10000 20000 30000 40000 50000

0

10000 20000 30000 40000 50000

1.85 1.80 1.75 1.70 1.65 1.60 1.55 1.50 1.45

Sucrose Concentration (M)
A

| tBid: | - | + | + | + | + | + | - | - | - |
| NAO (µM): | - | - | 5 | 10 | 15 | 20 | - | 10 | 15 | 20 |
| Bcl-xL: | - | - | - | - | - | - | + | - | - | - |

Supernatant

RI: 0.18 1.0 0.87 0.77 0.50 0.15 0.12 0.08 0.09 0.09

Cytochrome c

BMH

RI (Bak dimers): 0.08 1.0 0.98 0.89 0.88 0.77 0.16 0.04 0.03 0.04

1x Bak

2x Bak

DMSO

* * *

1x Bak

Lane: 1 2 3 4 5 6 7 8 9 10

C

Percentage of mitochondria with cristae reorganization (%)

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