Bid-induced Cytochrome c Release Is Mediated by a Pathway Independent of Mitochondrial Permeability Transition Pore and Bax*

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Bid, a pro-apoptosis “BH3-only” member of the Bcl-2 family, can be cleaved by caspase-8 after Fas/TNF-R1 engagement. The p15 form of truncated Bid (tBid) translocates to mitochondria and induces cytochrome c release, leading to the activation of downstream caspases and apoptosis. In the current study, we investigated the mechanism by which tBid regulated cytochrome c release in terms of its relationship to mitochondrial permeability transition and Bax, another Bcl-2 family protein. We employed an in vitro reconstitution system as well as cell cultures and an animal model to reflect the physiological environment where Bid could be functional. We found that induction of cytochrome c release by tBid was not accompanied by a permeability transition even at high doses. Indeed, inhibition of permeability transition did not suppress the activity of tBid in vitro nor could they block Fas activation-induced, Bid-dependent hepatocyte apoptosis in cultures. Furthermore, Mg²⁺, although inhibiting permeability transition, actually enhanced the ability of tBid to induce cytochrome c release. We also found that tBid did not require Bax to induce cytochrome c release in vitro. In addition, mice deficient in bax were still highly susceptible to anti-Fas-induced hepatocyte apoptosis, in which cytochrome c release was unaffected. Moreover, although Bax-induced cytochrome c release was not dependent on tBid, the two proteins could function synergistically. We conclude that Bid possesses the biochemical activity to induce cytochrome c release through a mechanism independent of mitochondrial permeability transition pore and Bax.

Apoptosis is a cellular process of self-destruction with distinctive morphological features, which is important to embryonic development, maintenance of homeostasis, and pathogenesis of a number of diseases (1–3). The genetic pathway and biochemical machinery of apoptosis are conserved in Caenorhabditis elegans, Drosophila, and mammals, in which caspases are the key effector molecules (4–6). Some death signals, such as TNF-α or FasL, induce activation of caspase-8 upon binding to the death receptors (7), whereas other death signals, such as growth factor deprivation or ionizing radiation, induce mitochondrial damage, including the release of cytochrome c into cytosol, which binds to Apaf-1 and activates caspase-9 in the presence of dATP (8). The activation of the initiator caspases, caspase-8 or caspase-9, leads to the activation of downstream effector caspases, such as caspases-3, -6, and -7, which cleave a number of cellular proteins, facilitating the final destruction of the cell (7, 9).

Bcl-2 family proteins consist of both pro-apoptotic and anti-apoptotic proteins, which regulate caspase activation mainly at the mitochondria level (10, 11). Release of cytochrome c is inhibited by the anti-apoptotic Bcl-2 or Bcl-xL but is induced by the pro-apoptotic Bax, Bak, Bid, or Bik (12–19). Interestingly, Bid, a BH3-only member of the Bcl-2 family of proteins, is a substrate of caspase-8 and is activated by the Fas/TNF-R1 pathway (20–22). Once translocated to mitochondria, the cleaved C-terminal Bid (tBid) potently induces cytochrome c release (20–22). Thus, Bid connects the death receptor pathway and the mitochondrial pathway and is responsible for cytochrome c release and the downstream caspase activities after Fas/TNF-R1 activation. This Bid-mediated mitochondrial pathway seems to be critical in certain types of cells, such as hepatocytes. Thus, bid-deficient mice are resistant to anti-Fas-induced hepatic failure and lethality (23).

Despite the fact that many death stimuli can induce cytochrome c release, the pro-apoptosis Bcl-2 family proteins are the only well defined proteins that possess the biochemical capability to do so. However, the molecular mechanisms are still unclear. One major hypothesis is that the opening of the mitochondrial permeability transition (PT) pore is involved, as shown for Bax by a number of investigators (18, 24, 25). However, others have not found an importance of the PT pore for the cytochrome c-releasing activity of Bax (14, 17, 26). It is not clear how this discrepancy may be resolved, although it may be related to different experimental conditions. Alternatively, Bax may work in either mode depending on the cellular environment.

The ability of Bid to induce cytochrome c release has been suggested to be mediated by Bax, for Bid can facilitate the insertion of Bax into the mitochondrial membrane to form functional oligomers (27, 28). If this is true, then the potential involvement of the PT pore in Bid-mediated cytochrome c re-

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¶The abbreviations used are: TNF, tumor necrosis factor; tBid, truncated Bid; PT, permeability transition; CsA, cyclosporin A; PAGE, polyacrylamide gel electrophoresis; DiOC₆(3), 3,3’-dihexyloxacarbocyanine iodide.
Bid-induced Cytochrome c Release

lelease will also depend on how Bax will work under various conditions, although the available in vitro evidence suggest that the PT pore is not involved (19, 26). How Bid induces cytochrome c release, particularly under the physiological conditions, thus remains to be determined.

In the present study, we decided to investigate Bid-dependent mechanisms under conditions that likely reflected the physiological environment where Bid was functional. Thus an in vitro reconstitution system was employed in which truncated Bid was incubated with mitochondria isolated from murine liver, components that are known to be physiologically relevant (23). Both cytochrome c release and mitochondrial permeability transition were measured under the same experimental conditions to examine mechanistic relationships. Results obtained from these in vitro studies were further verified in both primary hepatocyte cultures and animal experiments after Bid-dependent death stimulation (23).

We found that mitochondrial permeability transition was not induced by tBid and that classical PT pore inhibitors could not block cytochrome c release induced by tBid. In addition, Bid-dependent hepatoocyte apoptosis induced by anti-Fas antibodies could be inhibited by caspase inhibitors but not by PT pore inhibitors. Furthermore, we found that Mg$^{2+}$ could be inhibited by caspase inhibitors but not by PT pore dependent hepatocyte apoptosis induced by anti-Fas antibodies.

The bacterial pellets were resuspended and sonicated in the buffer for 10 min at 4 °C and then treated with various reagents as indicated in the figure legends at 30 °C. Mitochondria were recovered after centrifugation at designated time points and resuspended in ice-cold water. The fluorescence intensity of the totally released calcein was measured with a fluorescence spectrophotometer (PerkinElmer Life Sciences LS-50B) at 485-nm excitation and 520-nm emission (18). Data were standardized to the untreated control group at the time 0 point.

To determine whether Bid has any effects on mitochondrial transmembrane potential, isolated murine liver mitochondria were first incubated in Buffer B with 5 μM Calcein-AM for 15 min at 30 °C. Mitochondria were then washed with Buffer B three times to remove unloaded Calcein-AM and then treated with various reagents as indicated in the figure legends at 30 °C. Mitochondria were recovered after centrifugation at designated time points and resuspended in ice-cold water. The fluorescence intensity of the totally released calcein was measured with a fluorescence spectrophotometer (PerkinElmer Life Sciences LS-50B) at 485-nm excitation and 520-nm emission (18). Data were standardized to the untreated control group at the time 0 point.

Establishment of Primary Hepatocyte Culture and Analysis of Apoptosis Induced by Anti-Fas Antibodies—Murine hepatocytes were isolated from 8-to-10-wk-old male C57BL/6 mice by a perfusion method as described previously (31), with minor modification (22). Briefly, livers of wild-type or bid-deficient mice were first perfused with a Ca$^{2+}$-, Mg$^{2+}$-free Hanks’s balanced salt solution containing 50 mM HEPES, pH 7.3, 0.5 mM EGTA for 10 min at a flow rate of 10 ml/min. Livers were then perfused with 100 ml of Lebowitz L15 solution containing 5 mM CaCl$_2$ and 50 mg of collagenase H for 10 min at the rate of 10 ml/min. Livers were then harvested and dispersed in digestion buffer (1 ml of Buffer F) for flow cytometry analysis. Mitochondrial depolarization was induced by 50 μM protonophore carbonyl cyanide m-chlorophenylhydrazone, (Sigma) in control samples.

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homogenized in Buffer A, and the cytosolic fractions were isolated by centrifugation at 10,000 × g for 20 min. For Western blot analysis, 50 μg of cytosol were subject to 15% SDS-PAGE followed by transfer to polyvinylidene difluoride membrane and immunoblotting with antibodies against Bid (34) or cytochrome c (PharMingen). Caspase-3 activities in the cytosol (20 μg of protein) were evaluated by measuring the proteolytic cleavage of a synthetic fluorescent substrate benzoyl-DEVD aminofluoromethylcoumarin (50 μM) at 37 °C for 30 min and subjected to Western blotting with an anti-cytochrome c antibody. C, release of sulfite oxidase was detected in supernatant by an anti-sulfite oxidase antibody after mitochondrial isolation. Mitochondria were incubated with 3 ng of tBid in 30 μl of Buffer B for 1 h at 30 °C.

RESULTS

Induction of Cytochrome c Release by tBid in Vitro—To explore the mechanism by which tBid induces cytochrome c release from mitochondria, we generated recombinant full-length murine Bid (amino acids 1–195), tBid (amino acids 60–195), and mutant tBid (G94E) tagged with 6 histidines at the N terminus. Proteins were purified to homogeneity by His-Bind resin affinity chromatography. Purified proteins were examined by 15% SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining or by Western blotting (WB) with an anti-Bid antibody. The first lane of the Coomassie Brilliant Blue-stained gel represents molecular mass markers. B, recombinant wild-type (wt) full-length Bid, wild-type (wt) tBid, and mutant (mt) tBid (G94E) at indicated amounts were incubated with 15 μg of liver mitochondria in Buffer B in a total volume of 30 μl for 1 h at 30 °C. Supernatants were collected after centrifugation at 10,000 × g for 10 min and subjected to Western blotting with an anti-cytochrome c antibody. C, release of sulfite oxidase was detected in supernatant by an anti-sulfite oxidase antibody after mitochondrial isolation. Mitochondria were incubated with 3 ng of tBid in 30 μl of Buffer B for 1 h at 30 °C.

protein with a molecular mass of 104 kDa (a dimer of 52-kDa subunits) was also released, suggesting that the effect of tBid was not specific to cytochrome c (Fig. 1C). A number of pro-apoptotic Bcl-2 family proteins other than Bid possess the ability to induce cytochrome c release (14, 15, 17, 19, 26), among which Bax has been extensively studied. Although some investigators have proposed the importance of Ca2+ (16, 19) or Mg2+ (14, 28) in Bax-mediated cytochrome c release, others found that Bax retained its capacity in buffers that contain EGTA (14) or lack Mg2+ (15, 19) or in buffers under both conditions (17). We thus sought to determine whether these ions are important to the cytochrome c-releasing activity of tBid.

In all the experiments performed, we used Buffer B as the basic reaction buffer for both cytochrome c-releasing and mitochondrial permeability assays, which does not contain exogenous calcium but does contain 25 μM EGTA to prevent spontaneous mitochondrial swelling caused by trace amounts of Ca2+ in the solution. Under this condition and when there was no exogenous Mg2+ added, tBid was still able to induce cytochrome c release in a dose-dependent manner (Fig. 2A), suggesting that neither Ca2+ nor Mg2+ is absolutely required for the activity of tBid. However, Mg2+ (4 mM) significantly enhanced the ability of tBid to induce cytochrome c release (Fig. 2A), so that even a low dose of tBid (1 ng/30 μl) achieved the maximal effect of releasing more than 95% of cytochrome c (Fig.
Bid-induced Cytochrome c Release

Fig. 3. tBid does not induce mitochondrial swelling or permeability transition. A, isolated mitochondria (1 mg/ml) were treated with buffer (○), 50 μM CaCl₂ (□), 50 μM CaCl₂ plus 50 μM CsA (△), or different amounts of tBid (0.1 μg/ml (●), 1 μg/ml (■), or 5 μg/ml (▲)). Absorbance at 540 nm was measured at designated time points after incubation at 30 °C. The data shown are representative of four independent experiments. B, absorbance was measured as described above but in the presence or absence of Mg²⁺ (buffer alone (○), 4 mM MgCl₂ alone (●), 50 μM CaCl₂ alone (△) or with 4 mM MgCl₂ (▲) and 5 μg/ml tBid alone (□) or with 4 mM MgCl₂ (■)). The data shown are representative of four independent experiments. C, isolated mitochondria (1.5 mg/ml) were loaded with 5 μM calcein-AM by incubation at 30 °C for 15 min and then washed three times with Buffer B by centrifugation at 10,000 × g for 5 min. Calcein-AM-loaded mitochondria were resuspended in Buffer B and treated with buffer (○), 50 μM CaCl₂ (□), 50 μM CaCl₂ plus 50 μM CsA (△), or tBid at different concentrations (0.1 μg/ml (●), 1 μg/ml (■), or 5 μg/ml (▲)) for the indicated times at 30 °C. The data shown are representative of three independent experiments. D, isolated mitochondria were treated with tBid (0.1 or 0.5 μg/ml) in the absence or presence of MgCl₂ for 60 min at 30 °C before 40 mM DIOC₃(3) was added for another 10 min. The depolarization control was treated with 50 μM protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) before the addition of DIOCl₃(3). Samples were analyzed by flow cytometry, and the mean fluorescence intensity of incorporated DIOCl₃(3) was determined. The data represent one of three experiments with similar results. OD, optical density.

In fact, under this condition, as little as 0.22 nm (0.1 ng/30 μl) tBid was able to induce detectable levels of cytochrome c release from mitochondria (see Fig. 8). A lower dose of 1 mM MgCl₂ was also effective in our experiments (data not shown). To determine whether other cations may have the similar effect, we added KCl at various concentrations to the reaction mixture. We found that K⁺ did not enhance the release of cytochrome c induced by tBid at a concentration of 4 or 10 mM but could do so at 40 mM or higher (Fig. 2C). Similar observations were made when NaCl was used (data not shown). It is possible that these cations may exert their effects in a similar fashion.

Bel-2 and Bcl-xL may inhibit apoptosis by modulating several mitochondrial functions. In particular, by blocking cytochrome c release from mitochondria, these anti-apoptotic proteins can effectively inhibit the caspase activation through this pathway (12, 13). To test whether Bel-xL could block tBid-induced cytochrome c release by directly antagonizing the effect of tBid or the enhancing effect of Mg²⁺, we incubated the mitochondria with recombinant human Bel-xL and then tBid. We found that Bel-xL completely blocked tBid-induced cytochrome c release, either in the presence or absence of Mg²⁺ (Fig. 2D). This indicated that Bel-xL inhibited the activity of tBid per se, although it might also be able to block the secondary enhancement effect of Mg²⁺.

tBid Does Not Induce Mitochondrial Permeability Transition in Vitro—It has been suggested that certain death stimuli cause an increase in mitochondrial permeability by activating the PT pore, which then leads to mitochondrial swelling, rupture of the outer membrane, and release of cytochrome c as well as other proteins normally localized in the inter-membrane space (16, 18, 19, 35–37). To investigate whether tBid-induced cytochrome c release was mediated by the mitochondrial PT pore, we examined whether tBid could induce permeability transition in isolated mitochondria in vitro.

We first tested whether tBid could induce the large amplitude swelling of mitochondria by measuring light absorbance at 540 nm. A decrease in the absorbance represents an increase in mitochondrial volume (30). To eliminate the interference from the small amount of Ca²⁺ released spontaneously from the mitochondria, we routinely included 25 μM EGTA in Buffer B, which was titrated to be sufficient to suppress the nonspecific swelling effect of residual Ca²⁺. Under this condition, however, mitochondria remained sensitive to as low as 50 μM Ca²⁺ added exogenously (Fig. 3A), which could be completely suppressed by cyclosporin A (CsA), a PT inhibitor, as reported previously (30, 38–40). Truncated Bid, however, did not induce mitochondrial swelling even at a concentration of roughly 335 nM, 50 times higher than the effective concentration to induce cytochrome c release (Fig. 3A). Furthermore, although 4 mM MgCl₂ strongly enhanced the release of cytochrome c induced by tBid (Fig. 2A), it did not potentiate tBid to induce mitochondrial swelling (Fig. 3B). In contrast, Mg²⁺ suppressed the mitochondrial swelling effect of residual Ca²⁺, which was titrated to be sufficient to suppress the nonspecific swelling effect of residual Ca²⁺.
Bid-induced Cytochrome c Release

It has been suggested that permeability may not change in a synchronous manner across the mitochondrial population upon the application of low doses of inducers and that the PT pore may return to the closed state after opening for a period of time (39, 41). These events may result in failure to detect mitochondrial swelling by light absorbance at 540 nm. We therefore decided to employ a more sensitive fluorescence-based assay (18, 41, 42). Isolated mitochondria were pre-loaded with 5 μM Calcein-AM at 30 °C for 15 min, washed, and re-suspended in Buffer B before treatment. The opening of the PT pore, even transiently, would result in the loss of the fluorescent dye, Calcein, and therefore decreased fluorescence intensity associated with the mitochondria. However, our results again indicated that tBid, even at very high concentrations, did not induce PT, whereas Ca^{2+} did (Fig. 3C).

Additional evidence to indicate that PT was not induced by tBid was that tBid did not cause significant changes in mitochondrial transmembrane potential in vitro (Fig. 3D). Thus, the potential-dependent incorporation of DiOC<sub>6</sub>(3) into mitochondria as measured by mean fluorescence intensity was only slightly reduced after treatment with tBid, either in the presence or absence of Mg<sup>2+</sup> (Fig. 3D). This low level of depolarization may be due to a decrease in the efficiency of respiration after a portion of cytochrome c is released.

PT Inhibitors Suppress Ca<sup>2+</sup>- and Atractyloside-induced but Not tBid-induced Cytochrome c Release—Based on our results, it seems likely that tBid may induce cytochrome c release independent of mitochondrial permeability transition. To test this hypothesis, we determined whether cytochrome c release induced by tBid could be subject to the same regulation as PT. Ca<sup>2+</sup>, a mitochondrial PT inducer, released cytochrome c (Fig. 4A), which could be completely blocked by CsA or Mg<sup>2+</sup> (Fig. 4, A and B), suggesting that its activity was mediated by opening of the PT pore. However, tBid-induced cytochrome c release was not inhibited by CsA and was, in fact, enhanced by Mg<sup>2+</sup> (Figs. 2 and 4). Furthermore, other PT pore inhibitors, such as ADP (data not shown) and bongkrekic acid, had little effect on tBid-induced cytochrome c release either in the absence (Fig. 4C) or presence of MgCl<sub>2</sub> (data not shown). Bongkrekic acid binds to adenine nucleotide translocator, a key component of the PT pore, and stabilizes it in the m conformation, which favors pore closure (39). This chemical inhibits cytochrome c release induced by atractyloside (Fig. 4C), which also binds to adenine nucleotide translocator, but stabilizes it in the c conformation, leading to pore opening (39).

Taken together, these results strongly indicated that tBid did not induce opening of the PT pore and thus caused the release of cytochrome c through an alternative pathway. On the other hand, Ca<sup>2+</sup> and atractyloside induced cytochrome c release through the PT pore. When both Ca<sup>2+</sup> and tBid (Fig. 3A) or atractyloside and tBid (data not shown) were present in the absence of other regulators, we observed enhanced cytochrome c release, suggesting that the two pathways could work simultaneously.

Caspase Inhibitors, but Not PT Inhibitors, Suppress Anti-Fas-induced, Bid-dependent Hepatocyte Apoptosis—Previously reported that mice deficient in bid were resistant to anti-Fas-mediated hepatocyte apoptosis and lethality (23). In vivo culture of primary hepatocytes also demonstrated that this Fas-induced killing was dependent on Bid (23). If tBid-induced cytochrome c release is mediated by permeability transition pore, then PT inhibitors may be able to block the effect of tBid and rescue cells from death, as reported in other cases (43). To test this hypothesis, we treated hepatocytes isolated from wild-type and bid-deficient mice with an anti-Fas antibody, a general caspase inhibitor, or DEVD-aldehyde, a caspase inhibitor more specific to the effector caspases-3 and -7 (44). In contrast, PT inhibitors, bongkrekic acid, or CsA did not inhibit this type of cell death under the same conditions (Fig. 5). Combination of CsA and aristolochic acid, a phospholipase A2 inhibitor, also failed to inhibit cell death (data not shown).
Thus, the cell culture data are consistent with the in vitro findings. Together they suggest that the mitochondrial PT may not be the major mechanism by which tBid induces cytochrome c release and apoptosis.

**tBid-induced Cytochrome c Release Is Independent of Bax**—Bid can facilitate the insertion of Bax into the mitochondrial membrane, followed by conformational changes in Bax and cytochrome c release (28). It has thus been suggested that the activity of Bid is dependent on Bax (27, 28). To determine whether Bid could induce cytochrome c release independently of Bax, we prepared mitochondria from wild-type or bax-deficient mouse livers (29) (Fig. 6B). tBid induced a similar level of cytochrome c release from mitochondria of either genotype (Fig. 6B). These results suggest that induction of Bax oligomerization in the mitochondrial membrane is not a prerequisite for tBid to induce cytochrome c release.

Next, we administrated anti-Fas antibodies to wild-type, bid−/− and bax−/− mice and compared the apoptotic responses in the livers. As expected, severe hepatocyte apoptosis was observed in wild-type but not in bid−/− mice. Mice deficient in bax exhibited liver damage essentially identical to that of wild-type mice (Fig. 7A). Not surprisingly, Bid was cleaved in bax−/− livers as well as in wild-type livers after Fas activation, and similar levels of cytochrome c were released from the liver mitochondria (Fig. 7B). This could explain the high caspase-3 activities seen in both types of mice but not in the bid−/− mice (Fig. 7C). Thus these in vivo results again demonstrated that Bid possessed a Bax-independent activity capable of inducing cytochrome c release and apoptosis in the proper physiological context.

**tBid Is Not Required by Bax to Induce Cytochrome c but the Two Can Work Synergistically**—We next tested whether Bax-induced cytochrome c release was dependent on Bid. Mitochondria isolated from bid−/− deficient mouse livers were incubated with Bax. We found that Bax could induce cytochrome c release from bid−/− mitochondria, indicating that the activity of Bax did not absolutely require the participation of Bid (Fig. 8A).

However, the possibility is not excluded that Bid and Bax can work in a collaborative way. In fact, when Bax-ΔTM (50 nM) and tBid (0.7 nM) were added together at sub-optimal doses, significantly more cytochrome c was released than when either protein was added alone (Fig. 8B). This collaborative effect suggests that if a death stimulus is too weak to activate sufficient amounts of Bid or Bax, it may still be able to elicit an effective apoptotic response if both proteins are present in the cell.

**DISCUSSION**

The Bcl-2 family proteins regulate apoptosis mainly at the level of mitochondria (8, 10, 11). The mitochondria apoptosis pathway is signified by the release of cytochrome c, changes in the transmembrane potential and mitochondrial permeability (11, 43). Bid is a BHS-only Bcl-2 family protein that promotes cell death (34). It can be activated by caspase-8 through proteolytic cleavage in response to Fas/TNF-R1 signaling. Truncated Bid translocates to mitochondria and induces cytochrome c release and downstream caspase activation (20–23). How Bid induces cytochrome c release is not clear. The current study...
Bid-induced Cytochrome c Release

FIG. 8. The induction of cytochrome c release by Bax is not dependent on but can be enhanced by tBid. A, mitochondria isolated from wild-type (WT) or bid-deficient mouse livers were incubated with Bax-ATM (3 μM) in Buffer B. Cytochrome c release was determined by Western blotting using the anti-cytochrome c antibody. B, liver mitochondria isolated from wild-type mice were incubated with Bax-ATM and/or tBid at the designated amounts in Buffer B with 4 mM MgCl2. Released cytochrome c was detected in supernatants by Western blot. The densitometric measurement was performed, and the density relative to the blank control was indicated at the bottom of the gel.

was aimed at addressing the role of permeability transition and Bax in this process.

We employed assay systems where a clear pathophysiological significance of Bid could be related (23). Bid was incubated with murine hepatocyte mitochondria to release cytochrome c, which was dependent on its Bcl-2 domain and could be inhibited by Bcl-xL. The truncated Bid was much more potent than the full-length molecule, perhaps due to an easier insertion into mitochondrial membranes. This could be due to the exposure of a large previously buried hydrophobic surface and a reduction of negative charges in the truncated molecule (45). The enhancement effect of certain cations, particularly Mg2+, on Bid-induced cytochrome c release, could be also related to the promotion of membrane integration due to the alteration of mitochondrial surface charges (46–48). Alternatively, altered surface charges by the cations may facilitate the translocation of cytochrome c without affecting the permeabilization by Bid.

To determine whether tBid could induce mitochondrial PT, we measured the light absorbance of mitochondria at A540 to detect large amplitude swelling (30) and the ability of mitochondria to retain an incorporated fluorescent dye, Calcein, after various treatments. The latter is a more sensitive method to address the concern that the non-synchronous nature of permeability transition across the mitochondrial population may not allow its detection by the swelling-based assay (18, 41, 42). We found that at a dose from around 6.7 to 335 nM, tBid did not induce permeability transition or mitochondrial swelling, whereas 6.7 nM tBid was sufficient to induce significant release of cytochrome c. In contrast, Ca2+ added exogenously induced significant PT and mitochondrial swelling, which could be blocked by CsA or Mg2+ (49). The observation that tBid can induce cytochrome c release at a low dose but not PT even at a much higher dose supports the notion that tBid does not release cytochrome c by inducing mitochondrial PT. This conclusion is further strengthened by the following observations. 1) tBid-induced cytochrome c release is enhanced by Mg2+, which is a PT inhibitor that blocks PT and cytochrome c release induced by Ca2+ (Figs. 3 and 4); 2) neither CsA, bongkrekic acid (Fig. 4), or ADP (data not shown) can inhibit the cytochrome c-releasing activity of tBid, whereas they effectively inhibit the activity of Ca2+ or atractyloside; 3) tBid, unlike Bax, does not bind to voltage-dependent anion channel and does not modulate its activity (19); 4) tBid does not induce significant depolarization of mitochondria in vitro (Fig. 3).

Mitochondrial depolarization occurs during apoptosis induced by a variety of death stimuli and has often been considered an in vivo sign of permeability transition (8, 43). The current study indicated that tBid only induced a minor degree of mitochondrial depolarization in vitro, which seemed to be the outcome rather than the cause of cytochrome c release. A recent study using cells stably expressing green fluorescent protein-conjugated cytochrome c found that mitochondrial transmembrane potentials did not dissipate immediately after cytochrome c was released, possibly due to the presence of an inner membrane-bound fraction of cytochrome c (49, 50). Thus it is possible that tBid may cause significant cytochrome c release, but only minor mitochondrial depolarization, as shown in this and another (19) study. The contribution of Bid to mitochondrial depolarization in vivo may be amplified by additional factors, as seen in the case of Fas-induced thymocyte death or TNF-α-induced embryonic fibroblast death (23).

At this moment, we cannot rule out the possibility that tBid may induce PT and mitochondrial swelling in vivo, e.g. by working with other cytosolic factor(s). Such a factor has been recently reported to be present in the Xenopus egg cytosol (26). What seems certain is that PT is not a prerequisite for tBid to induce cytochrome c release at least in vitro. Although our study using hepatocyte culture suggests that PT may not be the major factor in determining the cell fate in Fas activation-induced apoptosis, other studies have shown that it could contribute to the process at least partially, so that PT inhibitors could delay the death process (51). The contribution of PT may be more significant in vivo when the anti-Fas antibody is injected into mice, where mitochondrial swelling could be identified and blocked by CsA (52). Thus it is likely that factors other than Bid may be involved in determining mitochondrial swelling and/or hepatocyte death in vivo, particularly if the death signal is very potent. In view of this possibility, we did observe Bid-independent death in cultured hepatocytes treated with anti-Fas antibody plus cycloheximide, which provided a strong death signal that might enhance the Bid-independent caspase pathway of Fas activation. Cycloheximide, however, is necessary in this in vitro culture system to induce Fas-dependent apoptosis (53). Nevertheless, the death of bid−/− hepatocytes in this scenario, like wild-type hepatocytes, was only marginally affected by PT inhibitors (data not shown). Further studies are needed to reveal all the molecular components involved in the in vivo process.

If Bid does not activate PT, what may be the possible mechanism for its cytochrome c-releasing activity? Recently, it has been suggested that Bid may work through Bax (27, 28), since it can bind to Bax and promote the insertion of Bax into the mitochondrial outer membrane, followed by conformation changes in Bax and cytochrome c release (28). However, our experiments using mitochondria isolated from bax-deficient livers indicated that Bid possessed the ability to induce cytochrome c release independent of Bax. In addition, bax−/− mice were as susceptible as wild-type mice to Fas-activated, Bid-mediated hepatocyte apoptosis. Cytochrome c release was not affected in the absence of Bax, indicating that Bid was fully functional in the bax−/− environment. Interestingly, Bax-induced cytochrome c release was not dependent on tBid either, suggesting that a Bid/Bax functional complex might not be required for the activity of either molecule. Bax could thus work through a different mechanism from that employed by tBid, such as the one involving PT (18, 24, 25). However, it is possible that tBid and Bax work synergistically, so that an
Bid-induced Cytochrome c Release

ever, this type of pore is not likely specific to cytochrome proteins may be involved to form a unique type of pore. How-
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How tBid induces cytochrome c independently of both PT pore and Bax remains to be determined. It is possible that other mitochondrial components or other pro-apoptosis Bcl-2 family proteins may be involved to form a unique type of pore. However, this type of pore is not likely specific to cytochrome c, since other inter-membrane proteins with larger molecular masses, such as sulfite oxidase (Fig. 1C) (26) and adenylate kinase (26), can also be released by tBid. Although the biochemical components of this pathway have yet to be determined, our work provides a framework for the future characterization of this mechanism.

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