

# Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis

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## Summary

We report here that BID, a BH3 domain-containing proapoptotic Bcl2 family member, is a specific proximal substrate of Casp8 in the Fas apoptotic signaling pathway. While full-length BID is localized in cytosol, truncated BID (tBID) translocates to mitochondria and thus transduces apoptotic signals from cytoplasmic membrane to mitochondria. tBID induces first the clustering of mitochondria around the nuclei and release of cytochrome c independent of caspase activity, and then the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion. Coexpression of Bcl<sub>X</sub>L inhibits all the apoptotic changes induced by tBID. Our results indicate that BID is a mediator of mitochondrial damage induced by Casp8.

## Introduction

Caspase 8 (Casp8), a member of a mammalian caspase family, has been demonstrated to play a key role in mediating Fas-induced apoptosis (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Cryns and Yuan, 1998). Cross-linking of the Fas receptor by engagement of the Fas ligand or agonistic antibodies results in the formation of so-called death-inducing signal complex (DISC), which includes adaptor protein FADD/MORT-1 and Casp8 (Kischkel et al., 1995). The formation of the DISC leads to the activation of Casp8, an initiator of the downstream apoptotic process that includes the activation of Casp3, -6, and -7 and mitochondrial damage (Salvesen and Dixit, 1997). Recently, Scaffidi et al. (1998) have shown that there may be two alternative Fas signaling pathways. In so-called Fas type I cells, a relatively large amount of Casp8 is recruited to DISC upon receptor cross-linking. The activated Casp8 propagates the apoptotic signal by activating downstream caspases through proteolytic cleavage, as well as by triggering mitochondrial damages that in turn activate a proteolytic cascade. In so-called Fas type II cells, a small amount of Casp8 is recruited to the DISC upon receptor cross-linking, and activated Casp8 mediates downstream apoptotic events mainly through inducing mitochondrial damage. These studies, however, did not reveal how active Casp8 induces mitochondrial damage.

A critical role of mitochondria in mediating apoptotic signal transduction pathway has been demonstrated recently (Vander Heiden et al., 1997). Biochemical and structural changes of mitochondria in apoptosis include mitochondrial swelling, disruption of mitochondrial outer

membrane, mitochondrial depolarization, and the release of cytochrome c (Liu et al., 1996; Vander Heiden et al., 1997). The release of cytochrome c may trigger the interaction of Apaf1, a mammalian CED-4 homolog, and Casp9, which in turn results in the activation of Casp9 (Li et al., 1997c; Zou et al., 1997). Activated Casp9 then cleaves and activates pro-Casp3, an event that leads to the cleavage of other death substrates, cellular and nuclear morphological changes, and ultimately, cell death. An active site mutant of Casp9 (C287A) is able to block activation of Casp3 by Casp9 (Li et al., 1997c). Overexpression of Bcl2/Bcl<sub>X</sub>L has been shown to block all apoptosis-induced mitochondrial changes (Kluck et al., 1997; Vander Heiden et al., 1997; Yang et al., 1997). Since mitochondrial damage is an obligatory step in mediating the Fas signaling in type II but not type I cells, Bcl2/Bcl<sub>X</sub>L can inhibit Fas-induced apoptosis in type II but not type I cells (Scaffidi et al., 1998).

Bcl2 and Bcl<sub>X</sub>L, two members of the Bcl2 family, prevent apoptosis induced by a variety of death stimuli (Merry and Korsmeyer, 1997). They are localized mainly to the outer mitochondrial, nuclear membranes and endoplasmic reticular membrane through their carboxy-terminal membrane anchorage domains. Both of them contain the Bcl2 homology domains designated BH1, BH2, BH3, and BH4, all of which are essential for the antiapoptotic activity of Bcl2/Bcl<sub>X</sub>L (Merry and Korsmeyer, 1997; Huang et al., 1998). The Bcl2 family also includes a class of BH3 domain-containing death agonists, which may promote apoptosis by inhibiting the death antagonist members of the Bcl2 family (Merry and Korsmeyer, 1997). Mutational analyses indicate that the BH3 domain of death agonists is required for their proapoptotic activity and their interactions with Bcl2/Bcl<sub>X</sub>L (Chittenden et al., 1995; Wang et al., 1996; Kelekar et al., 1997). It was recently reported that in a cell-free *Xenopus* oocyte system, the BH3 domain alone can induce cytochrome c release and the activation of caspases that can be inhibited by Bcl2 (Cosulich et al., 1997). Thus, these BH3-containing death agonists may play an important regulatory role in mediating apoptotic mitochondrial damage. It is not clear, however, how these BH3 domain-containing proteins fit into the signal transduction pathway of apoptosis and how they act to induce mitochondrial damage.

In this study, we demonstrate that BID, a death agonist member of the Bcl2/Bcl<sub>X</sub>L family (Wang et al., 1996), is a specific proximal substrate of Casp8 in the Fas signaling pathway. Cleavage of BID by Casp8 releases its potent proapoptotic activity, which in turn induces mitochondrial damage and, ultimately, cell shrinkage and nuclear condensation. Expression of Bcl<sub>X</sub>L inhibits all the apoptotic phenotypes induced by truncated BID (tBID), whereas caspase inhibitors inhibit the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation, but not mitochondrial clustering and cytochrome c release. Our study identified the first specific proximal substrate of Casp8 in the Fas pathway and a critical missing link between the activation of Casp8 and mitochondrial damage.

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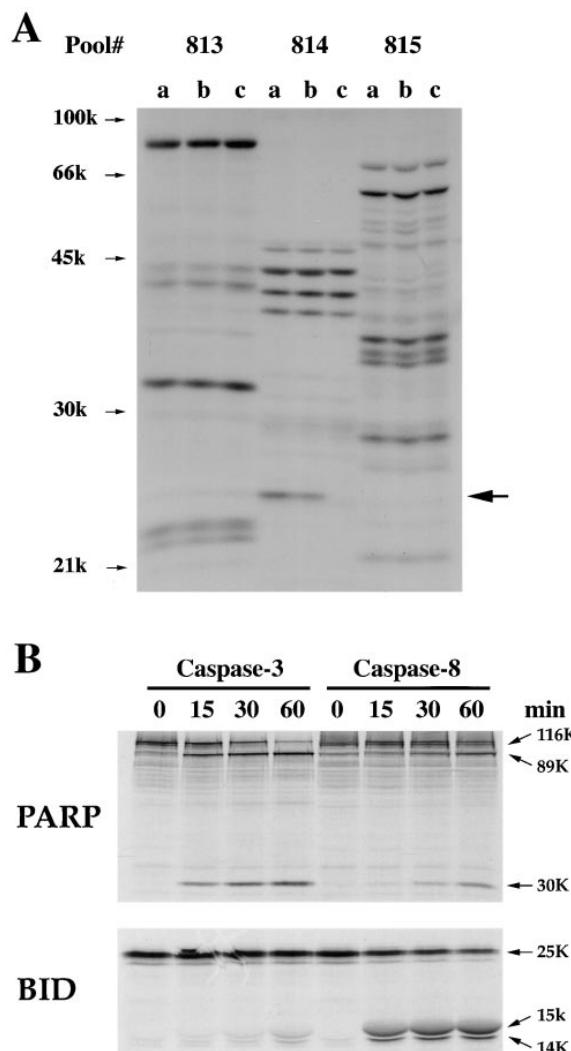
## Results

### BID Is a Specific Substrate of Caspase 8 In Vitro

To identify the substrates of Casp8, we transcribed and translated in vitro a small pool mouse spleen cDNA library in the presence of  $^{35}$ S-methionine, using the methods of Lustig et al. (1997). The radioactively labeled protein pools were incubated with either recombinant Casp8 in bacterial lysates or control lysates and analyzed by SDS-PAGE. cDNA small pools (1700) were screened, and the individual cDNA clones containing candidate Casp8 substrates were isolated from positive pools. In pool #814, a 24 kDa protein disappeared after incubation with Casp8 (Figure 1A). This pool was subdivided, and the cDNA encoding this 24 kDa protein was isolated and sequenced. The 24 kDa protein was identified to be murine BID, a proapoptotic Bcl2 family member whose homology to other members of the family is limited to a BH3 domain (Wang et al., 1996). N-terminal T7-tagged BID was cleaved by Casp8 into 15 kDa and 14 kDa fragments in vitro (due to the different contents of methionine in these two fragments, the two  $^{35}$ S-Met-labeled fragments did not appear to be stoichiometric) (Figure 1B). As the majority of the caspase substrates identified so far are the substrates of Casp3, we determined the specificity of BID cleavage by Casp3 and -8, using PARP as a control.  $^{35}$ S-labeled proteins were incubated with limited amounts of Casp3 and -8 for different lengths of time, and the cleavage efficiencies were evaluated by SDS-PAGE. As shown in Figure 1B, Casp8 cleaved most of BID but very little of PARP in 15 min, while the contrary was true for Casp3. This in vitro cleavage result indicates that BID is a much better substrate of Casp8 than of Casp3.

### BID Is Cleaved In Vivo during Fas- and TNF $\alpha$ -Induced Apoptosis

To examine the cleavage of BID in vivo, we generated a polyclonal antibody against human BID protein. Using this antibody for Western blots, we found that when Jurkat cells were induced to undergo apoptosis by anti-Fas antibody, BID was initially cleaved to a 15 kDa fragment and further to a 13 kDa fragment. The cleavage event occurred in the early stage of apoptosis and was comparable to the time courses of Casp7, -8 activation and PARP cleavage in Jurkat cells (Figure 2A). To rule out the possibility that the 15 kDa and 13 kDa BID fragments are newly synthesized novel protein products recognized by the BID antibody, we induced Jurkat cells to undergo apoptosis in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, and anti-Fas antibody. The cleavage patterns of BID, Casp7, -8, and PARP were examined by immunoblotting. We found that the appearance of the BID fragments was not altered by the presence of CHX (data not shown). Thus, the 15 kDa and 13 kDa BID fragments are likely to be the cleavage products of full-length BID induced by apoptosis. The amount of the 15 kDa peptide was constant during the time course of apoptosis, possibly due to its rapid conversion to the 13 kDa peptide and subsequent degradation. The cleavage of BID in Fas-induced apoptosis was inhibitable by 100  $\mu$ M of zVAD-fmk, a peptide



**Figure 1. BID Is a Preferred Substrate of Caspase 8 In Vitro**  
**(A)** Primary screening of caspase substrates by small pool in vitro expression cloning. Different pools of  $^{35}$ S-labeled proteins were incubated with bacterial lysates containing either no caspase (a), Casp2 (b), or Casp8 (c) for 2 hr at 37°C. The reactions were terminated and analyzed by 12% SDS-PAGE. An arrow points to a band, later identified as murine BID, that disappeared in the presence of Casp8.  
**(B)** BID is a better substrate of Casp8 than of Casp3.  $^{35}$ S-labeled N-terminal T7-tagged BID was incubated with either Casp3 or Casp8 for indicated time periods, and the cleavage products were analyzed by 15% SDS-PAGE.

inhibitor of caspases (Figure 2B). To confirm the cleavage of BID in vivo and to determine the approximate site of cleavage, we transiently transfected C-terminal Flag-tagged murine BID into HeLa cells, which were then treated by TNF $\alpha$  and CHX to induce the activation of Casp8 and apoptosis. A Western blot of the cell lysate was blotted by anti-FLAG antibody (Figure 2C). A 16 kDa Flag-tagged peptide was present in the apoptotic lysate and absent in the control lysate, confirming our in vivo cleavage data and suggesting that the cleavage site of BID is in the N-terminal portion of the protein.

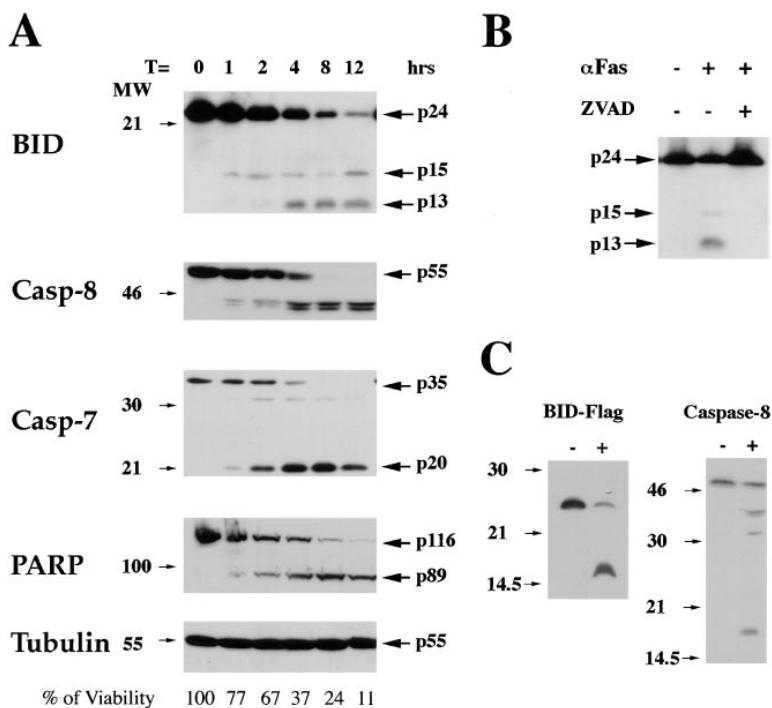


Figure 2. BID Is Cleaved In Vivo during Fas- and TNF $\alpha$ -Induced Apoptosis

(A) Time course of BID cleavage in Fas-induced apoptosis of Jurkat cells. The cell viability was determined by MTT assay and shown at the bottom of the figure.

(B) BID cleavage was inhibited with zVAD-fmk. Jurkat cells were preincubated with 100  $\mu$ M of zVAD-fmk for 30 min and then incubated with anti-Fas antibody for 5 hr. Total cell lysates were prepared and blotted with anti-BID antibody. Arrows point to the full-length BID (p24) and two cleavage products, p15 and p13.

(C) C-terminal Flag-tagged BID was cleaved in TNF $\alpha$ -induced apoptosis of HeLa cells. Twenty-four hours after transient transfection of BID-Flag, HeLa cells were treated with either 1  $\mu$ g/ml CHX alone (−) or 10 ng/ml TNF $\alpha$  and 1  $\mu$ g/ml CHX (+) for 4 hr. Total cell lysates were subjected to Western blotting using either anti-Flag or anti-Casp8 antibodies. The molecular weight standard is shown on the left. A 16 kDa fragment is present specifically in the apoptotic HeLa cells transfected with BID-flag.

#### Determination of Cleavage Sites of BID

Two potential caspase cleavage sites reside in the N-terminal portion of murine BID:  $^{56}$ Leu-Gln-Thr-Asp-Gly $^{60}$  (LQTDG) and  $^{72}$ Ile-Glu-Pro-Asp-Ser $^{76}$  (IEPDS) (the corresponding sequences for human BID are  $^{57}$ LQTDG $^{61}$  and  $^{72}$ IEADS $^{76}$ ). They match perfectly with the preferred cleavage sites for Casp8 and granzyme B, respectively, as determined by combinatorial peptide library screening (Thornberry et al., 1997). To confirm that they are indeed the cleavage sites for Casp8 and granzyme B, we mutated the two Asp residues to Glu either individually or both (namely mutant D59E, D75E, and DM). As shown in Figure 3A, N-terminal T7-tagged wild-type BID was completely cleaved by Casp1, -8, and granzyme B, and partially cleaved by Casp2 and –3. D59E BID mutant was only cleaved by granzyme B, not by caspases, while D75E BID mutant was cleaved efficiently by caspases, but not by granzyme B, suggesting that D75 is the cleavage site for granzyme B while D59 is the caspase cleavage site. As expected, the double mutant BID (DM BID) was cleaved by neither the caspases nor granzyme B. Two fragments of BID, a 15 kDa and a 14 kDa, were generated by caspase cleavage. The 14 kDa peptide was shifted to 13 kDa when a wild-type BID without N-terminal tag was cleaved by Casp8 (data not shown), suggesting that the 15 kDa peptide is the C-terminal part of BID and the 14 kDa peptide is the N-terminal portion with an anomalous mobility in SDS-PAGE. As an additional proof, the cleavage of BID by granzyme B generated a smaller C-terminal fragment and a larger N-terminal fragment that overlapped each other on SDS-PAGE (Figure 3A).

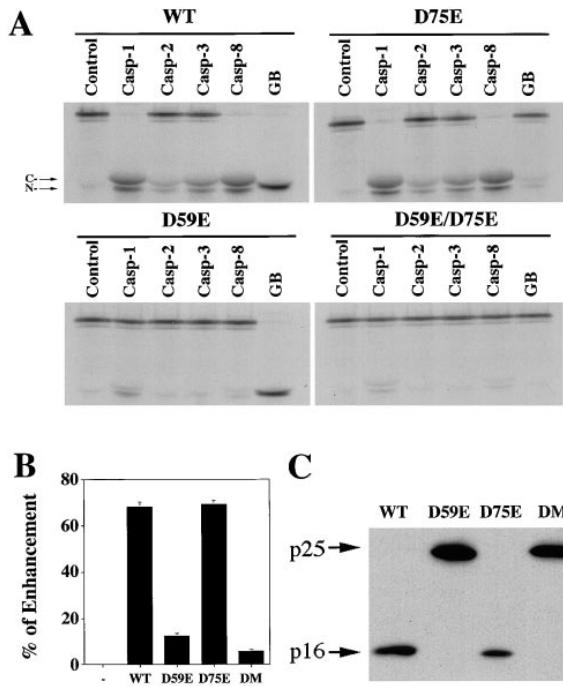
#### The Proapoptotic Activity of BID Strongly Depends upon Its Cleavage

BID was previously identified as a death agonist (Wang et al., 1996). Thus, it is interesting to examine whether

its proapoptotic activity is enhanced by its cleavage. We cotransfected a LacZ construct with either wild-type BID or its cleavage-defective mutants into HeLa cells and then treated the cells with TNF $\alpha$  and CHX for 4 hr. The percentages of cell death expressing BID was determined by X-Gal staining. While 4 hr treatment of TNF $\alpha$  and CHX induced 54% of LacZ-transfected cells to die (LacZ-transfected control cells without treatment showed 3.8% of cell death, and WT BID-transfected cells without treatment showed 9.3% of cell death), the same TNF $\alpha$  and CHX treatment induced 91.5% of WT and 92.2% of D75E BID-transfected cells to die, respectively. Thus, the expression of wild-type and D75E BID increased TNF $\alpha$ -induced cell death by 69.4% and 69.5%, respectively, as shown in Figure 3B. In contrast, transfection of D59E and DM mutant BID had insignificant effects on TNF $\alpha$ -induced HeLa cell death (12.5% and 5.9%, respectively). The expression of BID and its mutants in transfected HeLa cells after TNF $\alpha$  treatment was also examined. Wild-type and D75E BID were cleaved into a 16 kDa fragment, but D59E and DM BID were not (Figure 3C). Thus, the proapoptotic activity of BID is strongly dependent upon its cleavage by Casp8 at D59.

#### The Cleaved BID Is a Potent Apoptosis-Inducing Agent

Most of the death agonists in the Bcl2 family contain a BH3 domain that is essential for their Bcl2 binding and proapoptotic activity (Chittenden et al., 1995; Wang et al., 1996; Kelekar et al., 1997) and capable of inducing caspase activation in a cell-free *Xenopus* oocyte system (Cosulich et al., 1997). It is possible that once BID is cleaved, the truncated portion containing the BH3 domain becomes lethal to cells. To investigate this possibility, we transiently transfected the C-terminal portion of BID (residue 60–195) (tBID) into Rat-1 fibroblast cells.



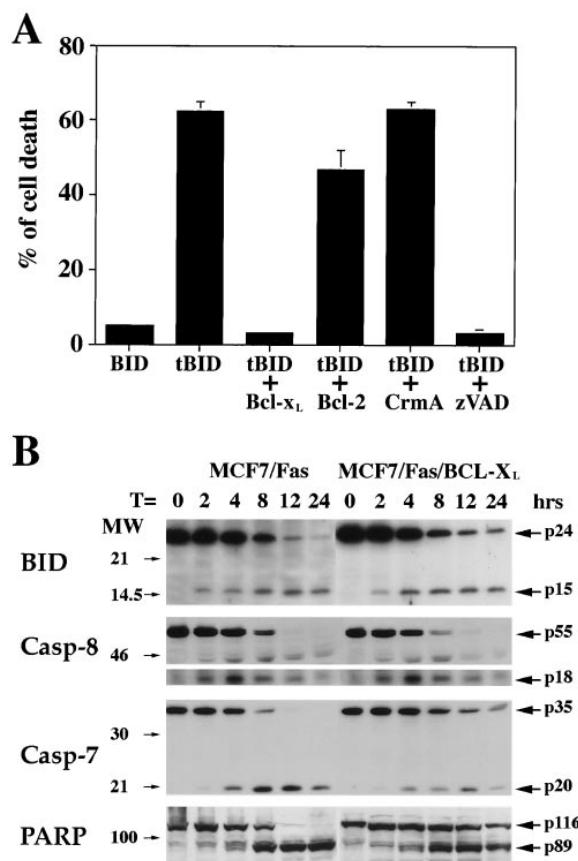
**Figure 3.** Proapoptotic Activity of BID Strongly Depends upon Its Cleavage

(A) Determination of cleavage sites of BID.  $^{35}$ S-labeled wild-type BID or its mutants were incubated with either caspases or granzyme B and analyzed by SDS-PAGE. BID was cleaved by caspases into two fragments: the larger one (15 kDa) was the C-terminal fragment, and the smaller one (14 kDa) was the N-terminal fragment. The cleavage products of granzyme B overlapped each other.

(B) Wild-type BID and D75E mutant promoted TNF $\alpha$ -induced apoptosis of HeLa cells, while caspase cleavage-defective mutants (D59E and DM) did not. HeLa cells were transfected with either the expression constructs of Bid or its mutants with LacZ construct overnight and treated with 10 ng/ml TNF $\alpha$  and 1  $\mu$ g/ml CHX for 4 hr. After fixation, X-Gal staining was performed, and the percentage of cell death was scored by cell morphology of blue cells (flat, well-attached versus round blue cells) as before (Miura et al., 1993).

(C) Cleavage of wild-type BID and its mutants in vivo. HeLa cells were transfected with the expression constructs of Bid or its mutants overnight and then treated with TNF $\alpha$  and CHX for 4 hr. The cell lysates were blotted with anti-Flag antibody. Arrows point to the full-length Flag-tagged BID (p25) and its cleavage product, p16.

As shown in Figure 4A, full-length BID could not induce apoptosis, while tBID induced apoptosis very rapidly and efficiently. tBID-induced cell death was inhibited completely by Bcl $x_L$  and zVAD-fmk, partially by Bcl2, but not at all by CrmA. This result suggests that the N terminus of BID has an inhibitory effect to its proapoptotic activity, and its removal by caspase cleavage yields tBID that is an effective inducer of downstream apoptotic events. The apoptosis-inducing activity of tBID is completely inhibited by Bcl $x_L$ , suggesting that BID may function upstream of Bcl $x_L$ /Bcl2. As CrmA can inhibit Casp1 and Casp8, but not other caspases (Zhou et al., 1997), the failure of CrmA to inhibit tBID-induced apoptosis is consistent with the hypothesis that BID acts downstream of Casp8.



**Figure 4.** BID Acts Downstream of Casp8 and Upstream of Bcl $x_L$  in Fas-Induced Apoptosis

(A) tBID-induced apoptosis of Rat-1 cells. Rat-1 cells seeded in 6-well plate were transfected with either 1  $\mu$ g pcDNA-Bid or 0.25  $\mu$ g pcDNA-tBid along with 1  $\mu$ g LacZ construct. For different cell death inhibitors, 0.25  $\mu$ g tBID was cotransfected with either 1  $\mu$ g Bcl $x_L$ , 1  $\mu$ g Bcl2, or 1  $\mu$ g CrmA-expressing constructs, along with 1  $\mu$ g LacZ construct. zVAD-fmk (100  $\mu$ M) was added half an hour before transfection of tBID was performed. After 8 hr transfection, cells were fixed and X-Gal stained, and cell death percentage was scored by cell morphology of blue cells.

(B) Time course of BID cleavage in vivo. MCF7/Fas and MCF7/Fas/Bcl $x_L$  cells were incubated with anti-Fas monoclonal antibody 7C11 (1:500) in the presence of 1  $\mu$ g/ml CHX for different periods of time as indicated. Total cell lysates were then subjected to Western blotting analysis using antibodies against BID, Casp8, Casp7, and PARP. Arrows point to p24 (full-length BID), p15 (cleaved BID), p55 (full-length Casp8), p18 (cleaved Casp8), p35 (full-length Casp7), p20 (cleaved Casp7), p116 (full-length PARP), and p89 (cleaved PARP).

#### BID Acts Downstream of Caspase 8 and Upstream of Bcl $x_L$ in Fas-Induced Apoptosis

To examine whether BID is a specific proximal substrate of Casp8 and whether it operates upstream of Bcl $x_L$ /Bcl2, we used MCF7/Fas and its Bcl $x_L$  stable cell lines. It has been shown that in MCF7 cells, Bcl $x_L$  can prevent Fas- and TNF $\alpha$ -induced cell death and inhibit downstream caspase activation even though Casp8 is activated (Medema et al., 1998; Srinivasan et al., 1998). As shown in Figure 4B, the cleavage profile of BID correlated perfectly with the activation of Casp8, both of

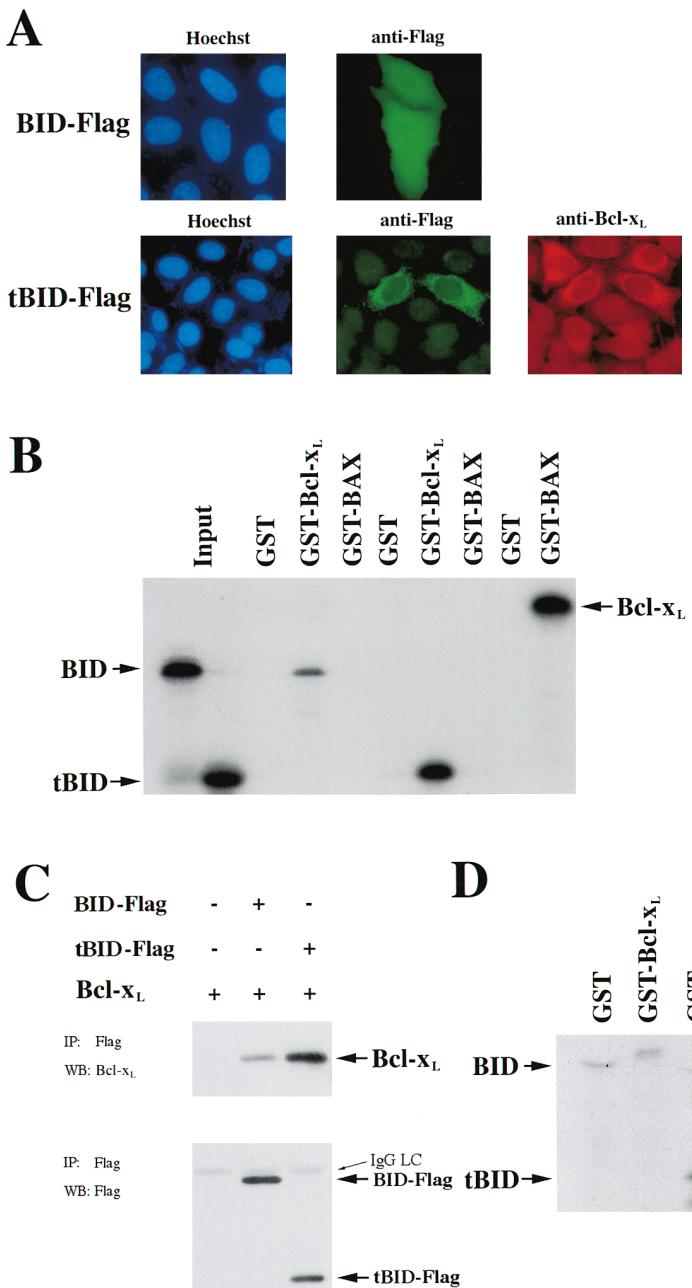


Figure 5. Truncated BID Has a Stronger Affinity for Bcl-x<sub>L</sub> than Its Precursor

(A) tBID was colocalized with Bcl-x<sub>L</sub>. Constructs expressing BID-Flag or tBID-Flag fusion proteins were transiently transfected into HeLa/Bcl-x<sub>L</sub> stable cells. Immunohistochemistry was performed using anti-Flag antibody M2 and anti-Bcl-x<sub>L</sub> antibody.

(B) In vitro interactions between BID and Bcl-x<sub>L</sub> or Bax. <sup>35</sup>S-labeled BID or its cleaved form was incubated with either GST-Bcl-x<sub>L</sub> or GST-Bax bound to glutathione-agarose beads. After 3 hr incubation and three washes, the bound BID or tBID was analyzed by SDS-PAGE and autoradiography.

(C) In vivo interactions between BID and Bcl-x<sub>L</sub>. Constructs expressing BID-Flag or tBID-Flag were cotransfected with Bcl-x<sub>L</sub> construct into HEK 293T cells. BID-Flag or tBID-Flag was immunoprecipitated by anti-Flag M2 affinity gel in the presence of 0.25% nonionic detergent IGEPAL CA-630 and Western blotted using anti-Bcl-x<sub>L</sub> antibody and M2 anti-Flag antibody, respectively.

(D) In vitro interaction between BID and Bcl-x<sub>L</sub> in the absence of nonionic detergent. <sup>35</sup>S-labeled BID or tBID was incubated with GST-Bcl-x<sub>L</sub> in a binding buffer without any nonionic detergent, and the bound BID or tBID was subjected to SDS-PAGE and autoradiography.

which were not altered by expression of Bcl-x<sub>L</sub>. In contrast, the activation of Casp7 and the cleavage of PARP were delayed and partially inhibited by overexpression of Bcl-x<sub>L</sub>. This result strongly supports our hypothesis that BID is a specific proximal substrate of Casp8 during Fas-induced apoptosis that functions upstream of Bcl-x<sub>L</sub>.

#### Truncated BID Has a Stronger Affinity for Bcl-x<sub>L</sub> than the Full-Length BID

How does tBID induce apoptosis? It was proposed that BID, which localizes in cytoplasmic as well as in membrane fractions, acts as a ligand to Bcl2/Bcl-x<sub>L</sub> and Bax (Wang et al., 1996). We examined whether tBID behaves

the same as its precursor. When tBID was overexpressed in HeLa/Bcl-x<sub>L</sub> stable cells, tBID colocalized with Bcl-x<sub>L</sub> (Figure 5A), suggesting that BID is translocated from cytoplasm to the membrane compartments when cleaved by Casp8. In contrast, the full-length BID was evenly distributed in every cellular compartment (Figure 5A). As BID does not have a membrane anchorage domain, one possible mechanism of translocation is that tBID has a stronger affinity to Bcl-x<sub>L</sub> than its precursor. To test this hypothesis, we examined the interaction between tBID and Bcl-x<sub>L</sub> in vitro and in vivo. We incubated <sup>35</sup>S-labeled BID or tBID with GST-Bcl-x<sub>L</sub> in vitro in the presence of 0.25% nonionic detergent IGEPAL CA-630,

and the amount of bound BID or tBID was analyzed by SDS-PAGE. As estimated by intensities of the corresponding bands, tBID has approximately 10-fold higher affinity toward GST-Bcl $\alpha$ <sub>L</sub> fusion protein compared to its full-length precursor (Figure 5B), while the interaction between tBID and GST-Bax was undetectable. To examine the interaction of BID with Bcl $\alpha$ <sub>L</sub> in vivo, we cotransfected the C-terminal Flag-tagged BID or its truncated form with Bcl $\alpha$ <sub>L</sub> into HEK 293T cells and immunoprecipitated with anti-Flag M2 affinity gel in the presence of 0.25% nonionic detergent IGEPAL CA-630. The expression level of tBID was much lower than that of its precursor (data not shown). As shown in Figure 5C, much more Bcl $\alpha$ <sub>L</sub> was coimmunoprecipitated with tBID than with full-length BID, and the difference was estimated around 20-fold by phosphoimager. It was reported that the presence of detergent in coimmunoprecipitation experiments affects the conformation of Bax and its interaction with Bcl2/Bcl $\alpha$ <sub>L</sub> (Hsu and Youle, 1997), so we examined the interaction between BID and Bcl $\alpha$ <sub>L</sub> in the absence of detergent. The interaction between the full-length BID and GST-Bcl $\alpha$ <sub>L</sub> in the absence of detergent was not significantly different from the background, while the tBID C-terminal fragment, but not the N-terminal fragment, still bound effectively to Bcl $\alpha$ <sub>L</sub> (Figure 5D). Thus, our in vivo and in vitro data indicate that the N-terminal domain of BID may have an inhibitory function, and the removal of the N-terminal domain exposes BID's BH3 domain to allow efficient interactions with other proteins.

#### Truncated BID Induces Mitochondrial Damage, Cell Shrinkage, and Nuclear Condensation

Mitochondrial damage and release of cytochrome c have been identified as key events in mediating activation of downstream caspases in apoptosis (Liu et al., 1996; Shimizu et al., 1996; Susin et al., 1996). Occurrence of these events is prevented by overexpression of Bcl2/Bcl $\alpha$ <sub>L</sub> (Kluck et al., 1997; Vander Heiden et al., 1997; Yang et al., 1997). We postulated that tBID may be able to mediate mitochondrial damage that is inhibitable by Bcl $\alpha$ <sub>L</sub>. To illustrate the downstream events resulting from the cleavage of BID, we used an ecdysone-inducible system in which the tBID-GFP fusion gene was placed under the control of an ecdysone-inducible promoter. After transient transfection of tBID-GFP and transactivator pVgRXR overnight, the expression of tBID-GFP was induced by addition of 1  $\mu$ M of muristerone A, an ecdysone analog, and examined by appearance of green fluorescence. Green fluorescence of tBID-GFP could be detected as early as 2 hr after induction (data not shown). Mitochondrial integrity was examined by fluorescent dye mitotracker, whose uptake depends on mitochondrial membrane potential, immunostaining of cytochrome c, which resides between inner and outer mitochondrial membranes, and cytochrome c oxidase subunit V<sub>c</sub> (COX V<sub>c</sub>), which resides within the inner mitochondrial membrane. The change of nuclear morphology was monitored by Hoechst dye staining. In the early stage of tBID-induced apoptosis, when nuclear morphology was largely normal, the signals of mitotracker, immunostaining of cytochrome c, and COX V<sub>c</sub>

began to fade in the periphery of the cells and became clustered around nuclei forming a ring (data not shown). As the apoptotic process proceeded, cells shrank and nuclei began to condense and fragment, and both signals of mitotracker and cytochrome c immunostaining became diffused, while immunostaining of COX V<sub>c</sub> became intensified around shrunk nuclei (Figure 6A). This result suggests that tBID induces the clustering of mitochondria, release of cytochrome c, loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation. Green fluorescence of tBID-GFP exactly overlapped with COX V<sub>c</sub> immunostaining in the early and late stages, further suggesting that tBID is targeted to the mitochondria.

#### Bcl $\alpha$ <sub>L</sub> and Caspase Inhibitors Inhibit Distinct Steps in Apoptosis Induced by Truncated BID

To elucidate the mechanism of tBID-induced apoptosis, we examined the effects of various cell death inhibitors, such as CrmA, p35, zVAD-fmk, Casp9 (C287A) dominant negative mutant (DN), and Bcl $\alpha$ <sub>L</sub>, on tBID-induced mitochondrial damage, cell shrinkage, and nuclear condensation. Ninety-eight percent of MCF7/Fas cells underwent cell shrinkage and nuclear condensation and fragmentation at 4 hr after induction of tBID-GFP (Figures 6A and 6B). Among caspase inhibitors, CrmA had no effect, and p35 had a modest effect (32% inhibition), while zVAD-fmk inhibited most of tBID-induced cell shrinkage and nuclear condensation (90% inhibition) (Figures 6B and 6C), indicating that the caspase activity is required for tBID-induced cell shrinkage and nuclear condensation. Casp9 DN mutant also inhibited tBID-induced cell shrinkage and nuclear condensation, but was less effective than zVAD-fmk (71% inhibition) (Figure 6B), suggesting that BID acts upstream of Casp9/cytochrome c/Apaf1 pathway. Thirty hours after induction, most of the cells remained normal in the presence of zVAD-fmk (85%), while only 30% of nuclei were normal in the presence of Casp9 DN mutant (data not shown), indicating that Casp9 DN mutant can only delay, not block, the apoptotic process induced by tBID. Compared to other inhibitors, Bcl $\alpha$ <sub>L</sub>, which completely inhibited tBID-induced cell shrinkage and nuclear condensation, was the most effective antagonist of tBID's function (Figures 6B and 6D).

Although zVAD-fmk, Casp9 DN, and Bcl $\alpha$ <sub>L</sub> have comparable effects on tBID-induced cell shrinkage and nuclear condensation, they had very different effects on tBID-induced mitochondrial damage. As shown in Figure 6D, the distribution of mitotracker, cytochrome c, and COX V<sub>c</sub> in tBID/Bcl $\alpha$ <sub>L</sub>-expressing cells was the same as that in normal cells where green fluorescence of tBID-GFP overlapped with mitotracker signals and immunostaining of cytochrome c and COX V<sub>c</sub>. In contrast, in the presence of zVAD-fmk, the signal of mitotracker disappeared from the peripheral region of cytoplasm and became aggregated around the nuclei, while immunostaining of cytochrome c completely disappeared (Figure 6C), suggesting that zVAD-fmk inhibits the loss of mitochondrial inner membrane potential but not the release of cytochrome c induced by tBID. The Casp9 DN mutant has a similar but less effective inhibitory activity (data not shown). As COX is an enzyme

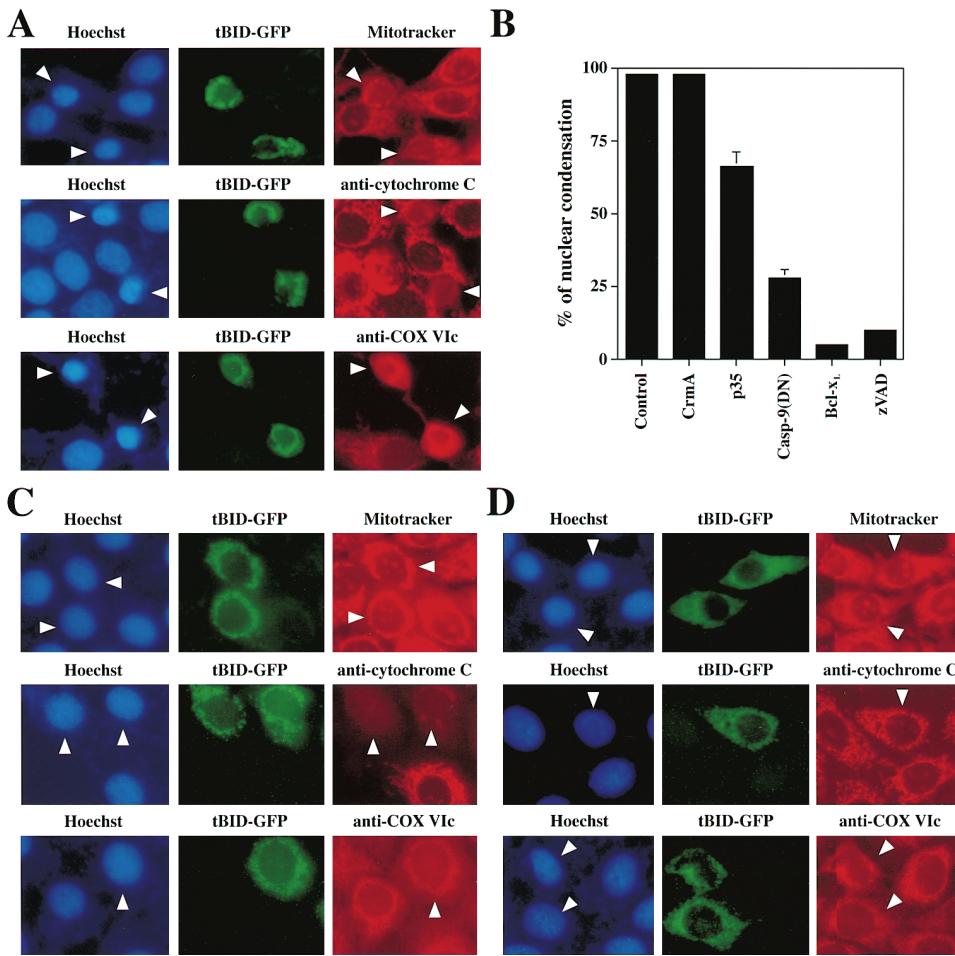


Figure 6. Differential Effects of Bclx<sub>L</sub> and zVAD-fmk on Truncated BID-Induced Apoptosis

(A) tBID-induced mitochondrial depolarization and the release of cytochrome c. MCF7/Fas cells were transfected with pIND-tBID-GFP and pVgRxR overnight and induced with muristerone A for 4 hr. For mitotracker staining, 300 nM Mitotracker CMTMRos was added into medium and then cells were fixed. For immunohistochemistry, cells were then fixed and immunostained with either anti-cytochrome c antibody or anti-COX VIc antibody. Arrows indicate the cells expressing tBID-GFP fusion protein.

(B) Effects of different cell death inhibitors on tBID-induced nuclear condensation. pIND-tBID-GFP and pVgRxR were transfected into MCF7/Fas or in combinations with different cell death inhibitors: CrmA, p35, Casp9 DN mutant, and Bclx<sub>L</sub>. After overnight transfection, 1 μM of muristerone A was added into medium (for zVAD-fmk, cells were incubated with 100 μM of zVAD-fmk for half an hour prior to the addition of muristerone A). After 4 hr induction, cells were fixed and washed with PBS and then incubated with 1 ng/ml of Hoechst dye. Percentage of nuclear condensation was scored by nuclear morphology.

(C) zVAD-fmk-inhibited tBID-induced loss of mitochondrial membrane potential, indicated by the ability of mitochondria to uptake mitotracker, cell shrinkage, and nuclear condensation, but not mitochondrial clustering and cytochrome c release. Cells were incubated with 100 μM of zVAD-fmk for half an hour prior to addition of muristerone A. Similar experiments described in (B) were performed. Arrows indicate the cells expressing tBID-GFP fusion protein.

(D) Bclx<sub>L</sub> completely inhibited all apoptotic morphological changes induced by tBID. Bclx<sub>L</sub> construct was cotransfected into MCF7/Fas cells along with pIND-tBID-GFP and pVgRxR. After overnight transfection, similar experiments described in (B) were performed. Arrows indicate the cells expressing tBID-GFP fusion protein.

localized in the inner membrane of mitochondria, immunostaining of COX VIc is an indication of mitochondrial distribution. In contrast to the disappearance of cytochrome c immunostaining in the presence of zVAD-fmk, the signal of COX VIc immunostaining remained largely clustered around nuclei and overlapped with the tBID-GFP signal (Figure 6C). Taken together, these results suggest that tBID induces the redistribution of mitochondria and the release of cytochrome c in a caspase-independent process but does not cause a complete disruption of mitochondrial inner membrane in the presence of caspase inhibitors.

#### Truncated BID Induces the Release of Cytochrome c from Purified Mitochondria In Vitro

To examine the mechanism by which BID mediates mitochondrial damage, we evaluated its ability to induce cytochrome c release from partially purified mitochondria in a cell-free system. Partially purified mitochondria were incubated with purified full-length BID or its truncated form, and the release of cytochrome c was examined. As shown in Figure 7, both full-length and tBID were able to induce the release of cytochrome c from mitochondria, but tBID was much more efficient than

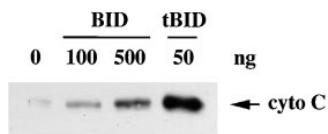


Figure 7. Truncated BID Induced the Release of Cytochrome c from Partially Purified Mitochondria In Vitro

Purified recombinant BID or tBID were incubated with partially purified mitochondria from mouse liver, and the released cytochrome c was examined by immunoblotting.

full-length BID. This result is consistent with our *in vivo* observation that tBID is more potent in its proapoptotic activity than its precursor.

## Discussion

A number of BH3-containing death agonists of the Bcl2 family have been recently identified. Among them, a group of so-called "BH3-only" death agonists, including Bik/Bbk, BID, Hrk/DP5, and newly identified Blk and Bim, seem to be more potent in their apoptosis-inducing activity than other death agonists in the family that contains additional BH1 and BH2 domains such as Bad. Identification of *C. elegans egl-1* as a general component of programmed cell death machinery indicates that BH3-containing death agonists are important regulators in apoptosis (Conradt and Horvitz, 1998). The BH3 domain is the only homologous region shared by these BH3-only death agonists, indicating that they may use a common BH3 domain-mediated mechanism to induce apoptosis, although the regulation of their activities may be different from each other. BID differs from other BH3-only molecules by two characteristics: the absence of a C-terminal hydrophobic membrane anchor, and the presence of two perfect cleavage sites for Casp8 and granzyme B, respectively, which are not found in other BH3-only molecules. Although overexpression of full-length BID can induce apoptosis in certain cells (data not shown; Wang et al., 1996), its truncated form is much more potent, indicating that caspase cleavage is one of the mechanisms to regulate its proapoptotic activity. It was recently reported that death antagonists of the Bcl2 family, such as CED-9, Bcl2, and Bclx<sub>L</sub>, can be cleaved by caspases (Cheng et al., 1997; Xue and Horvitz, 1997; Clem et al., 1998), indicating that caspase cleavage of Bcl2 family members is evolutionarily conserved and diversified.

We presented the evidence of BID translocation from cytosol to mitochondria during propagation of a death signal. Such translocation during apoptosis has been observed for two additional members of the Bcl2 family, Bax and BAD. It was reported that Bax moves from cytosol to mitochondria during apoptosis, although the regulatory mechanism of Bax translocation is unclear (Wolter et al., 1997). BAD exhibits a similar behavior when FL5.12 cells are starved for IL-3 and the translocation is regulated by protein phosphorylation (Zha et al., 1996). Translocation from cytosol to mitochondria may be an important activating mechanism for the proapoptotic members of the Bcl2 family and the propagation of apoptotic signals intracellularly. Caspase cleavage-

induced BID translocation represents a novel mechanism to release the proapoptotic potential of BID, perhaps by removing the inhibitory N-terminal domain, exposing BH3 domain and allowing tBID to interact with its receptor on the mitochondria.

Availability of different cell death inhibitors allowed us to map accurately the position of BID in the Fas signaling pathway and to study the downstream events after BID cleavage. The first detectable morphological change induced by tBID is the clustering of mitochondria around the nuclei at a stage when cytochrome c appears to remain largely within the mitochondria and mitotracker uptake is normal. Such movement of mitochondria has not been detected previously during the Fas-induced apoptosis, which may be because of two reasons. First, Fas-induced apoptosis can activate multiple apoptotic downstream events that occur very fast, and thus it may be difficult to capture such an intermediate step. Second, caspase inhibitors such as CrmA, p35, and zVAD-fmk are all effective inhibitors of Casp8, and thus they inhibit the activation of Casp8 itself and prevent the activation of downstream events altogether. Truncated BID allows us to bypass the requirement of Casp8 to dissect the downstream events of the Fas pathway. Why do mitochondria appear to be clustering around nuclei in the early stage of tBID-induced apoptosis? One possible explanation is that mitochondria are damaged sequentially, with mitochondria closest to the cytoplasmic membrane being damaged first. Alternatively, tBID may be able to induce not only mitochondrial leakage but also their detachment from cytoskeletal structure, resulting in their collapsing around nuclei. More experiments will be done to distinguish these two possibilities.

The second detectable morphological change in tBID-induced apoptosis appears to be the release of cytochrome c while mitotracker uptake is still normal. In the presence of zVAD-fmk, we can see rings of mitochondria that are mitotracker-positive and COX VIc-positive, while cytochrome c immunostaining has disappeared. Since cytochrome c resides between the inner and outer mitochondrial space, mitotracker uptake depends upon mitochondrial membrane potential, and COX VIc resides in the inner mitochondrial membrane, our results suggest that tBID may be able to cause disruption of the outer mitochondrial membrane directly, while the disruption of inner mitochondrial membrane needs the help from caspase. Our results are consistent with the observation of Vander Heiden et al. (1997), who have suggested that in apoptosis the disruption of outer mitochondrial membrane occurs before the loss of mitochondrial membrane potential, which is mainly an indication of the inner mitochondrial membrane integrity. Our results are also consistent with the observation of Bossy-Wetzel et al. (1998), who showed that the loss of mitochondrial membrane potential, but not cytochrome c release of CEM and HeLa cells induced by UVB irradiation or staurosporine treatment, can be inhibited by zVAD-fmk. We suggest that BID or related BH3 domain-containing proteins may be involved in inducing mitochondrial damage in apoptosis induced by other stimuli as well, although the mechanism of activation may be different.

Although caspase inhibitors such as Casp9 DN, p35, and zVAD-fmk are unable to inhibit the early mitochondrial damages induced by tBID, they can inhibit the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation induced by tBID with different effectiveness. While CrmA does not inhibit tBID-induced cell death, and p35 has a modest effect, zVAD-fmk is very effective in inhibiting tBID-induced nuclear condensation and in halting apoptotic process at an early stage when mitochondria cluster around normal-looking nuclei. The inhibitory activity of Casp9 DN mutant is similar to that of zVAD-fmk but less effective. Although this spectrum of inhibitor profile does not allow us to identify positively the critical caspase(s) involved in this process, it does suggest, however, that the release of cytochrome c may not be the only critical event downstream from the mitochondrial damage induced by tBID, since Casp9 DN is only partially effective in inhibition of tBID-induced cell death. This conclusion is also supported by the fact that tBID induces apoptosis of MCF7/Fas cells effectively, while microinjection of cytochrome c to MCF7/Fas cells does not induce apoptosis (Li et al., 1997a). Alternatively, tBID may activate downstream caspases by a cytochrome c-independent pathway in which tBID simply competes Bcl<sub>xL</sub> from Bcl<sub>xL</sub>/Apaf1/caspase complex and activates downstream caspases. The observation that the Apaf1/Casp9/Bcl<sub>xL</sub> complex exists (Hu et al., 1998; Pan et al., 1998) provides additional support for this model. These two mechanisms, however, are not mutually exclusive, and the mechanism by which tBID acts may be cell type-specific.

In contrast to caspase inhibitors, Bcl<sub>xL</sub> can fully block the tBID's activity. Thus, the interaction of tBID with Bcl<sub>xL</sub> must be the key to its ability to induce apoptosis. Kelakar et al. (1997) reported recently that wild-type BAD fails to induce apoptosis in response to growth factor withdrawal when cells are protected by a Bcl<sub>xL</sub> mutant that does not bind to the BAD BH3 domain, suggesting that the interaction between the BH3 domain and Bcl2/Bcl<sub>xL</sub> is critical for BH3 domain's proapoptotic activity. tBID may behave as an intracellular ligand for Bcl<sub>xL</sub>, which acts as the receptor in the Fas pathway. Unlike the conventional ligand-receptor interactions at cytoplasmic membrane, where such interactions usually result in the activation of the receptor, the interaction of tBID with Bcl<sub>xL</sub> results in the inactivation of protective functions of Bcl<sub>xL</sub> (or may we call it the activation of apoptotic-inducing functions of Bcl<sub>xL</sub>?). Alternatively, tBID may be capable of triggering apoptosis on its own, which may be Bcl2/Bcl<sub>xL</sub>-inhibitable, or Bcl2/Bcl<sub>xL</sub> may simply act as a neutralizer of its death-inducing activity. These are interesting questions for future experiments. The dimeric interactions of antiapoptotic members of the Bcl2 family with proapoptotic members of the Bcl2 have been hypothesized to be an important mechanism of regulation (Oltvai and Korsmeyer, 1994). Recently, however, this view has been questioned mainly because of two works. First, Knudson and Korsmeyer (1997) reported that, in a genetic analysis, Bcl2 and Bax are able to act independently. Second, Hsu and Youle (1997) reported that the dimeric interaction of Bcl2 and Bax can only be detected in the presence of detergent, suggesting that detergent may induce specific conformational changes, exposing the interacting domain of Bcl2

and Bax and allowing such dimerization to occur. Interestingly, while we can detect the interaction of full-length BID with Bcl<sub>xL</sub> in the presence of detergent, such interaction appears to be largely absent in the absence of detergent. In contrast, the interaction of tBID with Bcl<sub>xL</sub> is still very strong in the absence of detergent. Our results indicate that the interaction of tBID with Bcl<sub>xL</sub> is not induced by detergent. On the other hand, nonionic detergent appears to enhance the binding of full-length BID with Bcl<sub>xL</sub>, suggesting that detergent may be able to induce the conformational change of BID by removing the inhibition of N-terminal domain, a hypothesis that can be examined directly by further experimentation.

#### Experimental Procedures

##### Construction of Mouse Spleen cDNA Small Pool Library and In Vitro Expression Cloning

A mouse spleen cDNA library of  $2 \times 10^5$  independent clones was constructed by using a Stratagene cDNA synthesis and cloning kit (Stratagene, La Jolla, CA), and cDNAs were inserted into EcoRI and Xhol sites of pCS2. Pools of cDNAs and in vitro translations were done as described by Lustig et al. (1997). The caspase cleavage assay was performed as described previously (Li et al., 1997b). The individual positive cDNA was identified using 96-well plates as described (Lustig et al., 1997), was sequenced, and compared with known sequences by searching the GenBank database.

##### Bacterial and Mammalian Expression Constructs of Murine and Human Bid and Generation of Anti-BID Antibody

The original clone of murine *Bid* obtained from the small pool library contained the full-length open reading frame of *Bid*. The standard molecular cloning methods and PCR were used to construct various mammalian expression vectors of murine *Bid*.

The full-length human *BID* gene was amplified by PCR from EST cDNA clone 52055 (Research Genetics, Inc., Huntsville, AL) and then cloned into the BamHI site of pGEX-2T. GST-BID fusion protein was purified by GST beads, GST was removed by thrombin digestion, and the purified *BID* protein was injected into rats to generate polyclonal antibody.

To establish an inducible expression system of *tBid*, *tBid* was inserted into BamHI site in frame with GFP (green fluorescence protein) in pEGFP-N1 vector (CLONTECH, Palo Alto, CA), generating *tBID-GFP* fusion gene. The cassette of *tBID-GFP* was then cloned into EcoRI and NotI sites of pIND (Invitrogen, San Diego, CA).

##### Site-Directed Mutagenesis of the Cleavage Sites of Bid

Aspartic acid residues at positions 59 and 75 of *Bid* were mutated to glutamic acid individually or in combination, using QuickChange site-directed mutagenesis kit (Stratagene), following the supplier's instruction. Mutated sites were verified by DNA sequencing.

##### Tissue Culture Cells

Jurkat cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and HeLa and HEK 293T cells were cultured in DMEM containing 10% FBS. MCF7/Fas and MCF7/Fas/Bcl<sub>xL</sub> stable cells were cultured in RPMI 1640 containing 10% FBS, 200 µg/ml G418, and 100 µg/ml hygromycin (Srinivasan et al., 1998).

##### Immunohistochemistry

For Mitotracker staining, Mitotracker CMTMRos (Molecular Probes Inc., Eugene, OR) was added to medium in a final concentration of 300 nM, and cells were further cultured for 30 min and fixed in 4% paraformaldehyde for 15 min. Immunohistochemistry of cytochrome c and COX Vlc was done according to Srinivasan et al. (1998), with minor modifications.

##### In Vitro and In Vivo Binding Assays

<sup>35</sup>S-labeled *BID* or its cleaved form was incubated with 10 µg GST-Bcl<sub>xL</sub> in 100 µl of the binding buffer (10 mM HEPES [pH 7.5], 150 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.25% IGEPAL CA-630) at

4°C for 3 hr with agitation. After brief centrifugation, beads were washed three times with 400 µl of binding buffer and resuspended in protein sample buffer. The samples were subjected to SDS-PAGE and autoradiography.

For in vivo binding assay, constructs expressing BID-Flag or tBID-Flag fusion protein were cotransfected with the construct expressing Bcl<sub>xL</sub> into HEK 293T cells by a calcium-phosphate method. After 24 hr, cells were lysed in 1 ml of binding buffer, and total cell lysates were centrifuged at 10,000 × g for 15 min. M2 anti-Flag affinity gel (30 µl) was added into the supernatant, and the resulting mixture was incubated at 4°C for 3 hr with agitation. Following three washes, the beads were resuspended in appropriate amounts of protein sample buffer. The samples were subjected to Western blotting analysis.

#### Cell-Free Assay for the Release of Cytochrome c from Purified Mouse Liver Mitochondria

Mitochondria were purified as described by Shimizu et al. (1998). For in vitro assay, 5 µl of purified mitochondria was incubated with purified BID or tBID at 37°C for 1 hr. The reaction mixture was then centrifuged at 10,000 × g for 10 min, and the supernatant was subjected to SDS-PAGE and immunoblotting using anti-cytochrome c antibody.

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