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Bid, a Bcl2 Interacting Protein, Mediates Cytochrome c Release from Mitochondria in Response to Activation of Cell Surface Death Receptors

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Summary

We report here the purification of a cytosolic protein that induces cytochrome c release from mitochondria in response to caspase-8, the apical caspase activated by cell surface death receptors such as Fas and TNF. Peptide mass fingerprinting identified this protein as Bid, a BH3 domain–containing protein known to interact with both Bcl2 and Bax. Caspase-8 cleaves Bid, and the COOH-terminal part translocates to mitochondria where it triggers cytochrome c release. Immunodepletion of Bid from cell extracts eliminated the cytochrome c releasing activity. The cytochrome c releasing activity of Bid was antagonized by Bcl2. A mutation at the BH3 domain diminished its cytochrome c releasing activity. Bid, therefore, relays an apoptotic signal from the cell surface to mitochondria.

Introduction

Apoptosis, a form of cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, is essential for development, maintenance of tissue homeostasis, and elimination of harmful cells in metazoan organisms (reviewed by Jacobson et al., 1997; Nagata, 1997). Malfunctions of apoptosis have been implicated in many forms of human disease such as cancer, neurodegenerative disease, AIDS, and ischemic stroke (reviewed by Thompson, 1995).

Caspases, a group of cysteine proteases that cleave protein substrates after aspartic acids, play a central role in the regulation and execution of apoptosis (reviewed by Cryns and Yuan, 1998). Mutations in the *C. elegans* caspase CED-3 abolished apoptosis in this animal (Yuan et al., 1993). Homologous deletion of caspase-3 from the mouse genome resulted in an accumulation of excess neurons in the mouse brain due to a lack of apoptosis (Kuida et al., 1996). Several important morphological features of apoptosis are caused by caspase activity. For example, cleavage of DFF45/ICAD by caspase-3 results in characteristic chromatin condensation and fragmentation during apoptosis (Liu et al., 1997; Enari et al., 1998; Liu et al., 1998); cleavage of fodrin, gelsolin, and PAK2 by caspases causes morphological changes in the cell membrane and cytoskeleton (Martin et al., 1995; Kothakota et al., 1997; Rudel and Bokoch, 1997); and cleavage of nuclear lamins by caspase-3 and caspase-6 may contribute to nuclear membrane breakage (Lazebnik et al., 1995).

Caspases that function in the apoptotic pathway exist as inactive zymogens in the cytosol of living cells and become activated through proteolysis when cells receive apoptotic signals. These signals include activation of cell surface death receptors such as Fas and TNF, growth factor deprivation, DNA damage, and treatment with a variety of chemotherapeutic drugs (reviewed by Salvesen and Dixit, 1997; Cryns and Yuan, 1998). Caspase activation during apoptosis is presumed to occur through a caspase cascade that is most likely initiated by caspases containing long NH_2-terminal domains that mediate interaction with other caspase-activating factors. For example, during Fas activation, caspase-8 is recruited to the *d*eath *i*nduced *s*ignaling *c*omplex (DISC) on the cell surface through interaction with FADD/MORT-1 (Boldin et al., 1996; Muzio et al., 1996). Caspase-9, on the other hand, forms a complex with Apaf1 and cytochrome c in the presence of dATP/ATP (Li et al., 1997). Caspase-8 and caspase-9 presumably become activated through autocatalysis within these multiprotein complexes (Cryns and Yuan, 1998). The activated caspase-8 or caspase-9 in turn cleaves and activates other caspases such as caspase-3, caspase-6, and caspase-7, which lack the long NH_2-terminal domain but constitute the main caspase activity in apoptotic cells (Srinivasula et al., 1996; Faleiro et al., 1997; Li et al., 1997; Muzio et al., 1997).

Holocytochrome c, which normally resides exclusively in the intermembrane space of mitochondria, is released into the cytosol during apoptosis (Liu et al., 1996b). Release of cytochrome c from mitochondria inactivates the electron transfer chain and triggers caspases activation through Apaf1 (Krippner et al., 1996; Li et al., 1997). Cytochrome c release from mitochondria has been observed in cells undergoing apoptosis induced by signals including the activation of Fas and TNF, growth factor deprivation, excessive DNA damage, and treatment of chemotherapeutical drugs (reviewed by Reed, 1997). Overexpression of Bcl2 or Bcl-x_L blocks the release of cytochrome c and aborts the apoptotic response (Kluck et al., 1997; Yang et al., 1997). Overexpression of Bax, a proapoptotic member of the Bcl2 family of proteins, or its Bcl2 homologous domain 3 (BH3 domain), promotes cytochrome c release (Cosulich et al., 1997; Jurgensmeier et al., 1998; Rosse et al., 1998; Pastorino et al., 1998).

The mechanism of cytochrome c release in response to apoptotic stimuli and its regulation by the Bcl2 family of proteins is unclear. Inasmuch as the structure of Bcl-x_L is reminiscent of pore-forming proteins of bacterial toxins such as diphtheria toxin and colicins, it has been hypothesized that Bcl-x_L may function as an ion channel that regulates the permeability of mitochondria (Muchmore et al., 1996; Minn et al., 1997; Vander Heiden et al., 1997). Such an ion channel could minimize osmotic stress and in doing so prevent cytochrome c release due to mitochondrial matrix swelling and outer membrane

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disruption (Vander Heiden et al., 1997). Indeed, both swelling of the mitochondrial matrix and bursting of the outer membrane were observed in cells treated with agonistic antibody against Fas (Vander Heiden et al., 1997). However, whether such a phenomenon is the cause of cytochrome c release or an effect of the apoptotic program is unclear.

Activation of cell surface receptor Fas leads to rapid inactivation of the electron transfer activity of cytochrome c and subsequent release of cytochrome c from mitochondria (Krippner et al., 1996; Vander Heiden et al., 1997; Scaffidi et al., 1998; Srinivasan et al., 1998). The inactivation and release of cytochrome c induced by Fas activation is sensitive to z-VAD-fmk, a broad range caspase inhibitor (Krippner et al., 1996; Vander Heiden et al., 1997; Scaffidi et al., 1998). Since activation of cell surface death receptor leads to rapid activation of caspase-8, the apical caspase in the Fas-induced apoptotic pathway, the loss of cytochrome c from mitochondria is likely a result of caspase-8 activation. Indeed, addition of active caspase-8 to a *Xenopus* cell-free system induces rapid cytochrome c release from mitochondria (Kuwana et al., 1998).

The activation of caspase-8, therefore, initiates two pathways leading to the activation of downstream caspases. Caspase-8 can activate downstream caspases like caspase-3, caspase-6, and caspase-7 by directly cleaving them (Srinivasula et al., 1996; Muzio et al., 1997). In addition, caspase-8 activates these downstream caspases indirectly by causing cytochrome c release from mitochondria that triggers caspase activation through Apaf1. The latter pathway is regulated by Bcl2 or Bcl-x_L while a caspase-8 inhibitor like CrmA blocks both pathways (Strasser et al., 1995; Srinivasula et al., 1996; Boise and Thompson, 1997; Vander Heiden et al., 1997; Medema et al., 1998; Srinivasan et al., 1998). The contributions of these two pathways to Fas-induced cell death vary between different cell types, presumably due to different levels of activated caspase-8 and its downstream substrates in a particular cell type (Scaffidi et al., 1998).

In the current report, we have isolated a protein factor from normal HeLa cell cytosol that mediates cytochrome c release from isolated mitochondria in response to caspase-8. Mass fingerprinting analysis revealed this protein to be Bid, a protein known to interact with both Bcl2 and Bax through its BH3 domain (Wang et al., 1996). Bid usually exists in an inactive form in the cytosolic fraction of living cells and becomes cleaved and activated by caspase-8. The COOH-terminal part of Bid then translocates onto mitochondria and is sufficient to trigger cytochrome c release. Elimination of Bid from cell extracts by immunodepletion abolished the cytochrome c releasing activity. This study establishes Bid as a direct molecular link between the activated caspase-8 and mitochondrial death machinery.

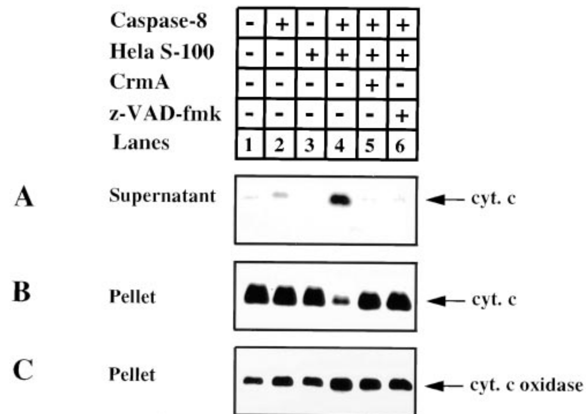

Figure 1. Caspase-8-Induced Cytochrome c Release from Mitochondria Requires a Cytosolic Factor

Mouse liver mitochondria, HeLa S-100, and recombinant caspase-8 were prepared as described in Experimental Procedures. Recombinant CrmA was prepared as in Liu et al., 1996a. Aliquots of 3 μl of mouse liver mitochondria ($\sim$3 μg) were incubated either alone (lane 1), or in the presence of aliquots of 2 μl caspase-8 (20 ng) (lanes 2, and 4–6), or 10 μl (50 μg) of HeLa S-100 extracts (lanes 3–6) in a final volume of 25 μl buffer B. In lanes 5 and 6, an aliquot of 0.3 μg of CrmA (lane 5) or z-VAD-fmk at a final concentration of 20 μM was preincubated with caspase-8 for 15 min prior to the addition of HeLa S-100 and mitochondria. After incubation at 30°C for 1 hr, the samples were centrifuged at 12,000 $\times$ g for 5 min at 4°C to pellet the mitochondria.

(A) The resulting supernatants were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody as described in Experimental Procedures. The filter was then exposed to X-ray film for 3 s.

(B) The resulting pellets were resuspended in 25 μl SDS sample buffer, subjected to 15% SDS-PAGE, and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody and then exposed to X-ray film for 3 s.

(C) The same filter as in (B) was erased and reprobed with a monoclonal anti-cytochrome c-oxidase antibody (Molecular Probes) followed by ECL detection and exposure to X-ray film for 20 s.

Results

Purification of a Cytochrome c Releasing Factor

Upon activation of cell surface death receptor Fas, caspase-8 is recruited into the DISC and becomes activated (Boldin et al., 1996; Muzio et al., 1996). Activation of Fas also results in release of cytochrome c from mitochondria, which can be prevented by inhibitors that block caspase-8 activity (Vander Heiden et al., 1997). Based on these observations, we set up an in vitro assay for cytochrome c release using mitochondria isolated from mouse liver and cytosol from normal HeLa cells. As shown in Figure 1, upon addition of recombinant active caspase-8, HeLa cytosol was capable of inducing the majority of cytochrome c release from the coincubated mitochondria (lane 4). In contrast, no redistribution of cytochrome c oxidase was detected (Figure 1C and data not shown). HeLa cytosol or caspase-8 alone had no detectable cytochrome c releasing activity (lanes 2 and 3). Cytochrome c release was inhibited by the caspase-8 inhibitors z-VAD-fmk and cowpox virus protein CrmA (lanes 5 and 6). Both reagents block Fas and TNF signaling in vivo (Strasser et al., 1995; Tewari and Dixit, 1995; Krippner et al., 1996; Vander Heiden et al., 1997).

Using such an assay, we fractionated HeLa cell cytosolic extracts to purify the *c*aspase-8-dependent *c*ytochrome c *r*eleasing *f*actor (CCRF). The complete purification of CCRF was achieved through an eight-step

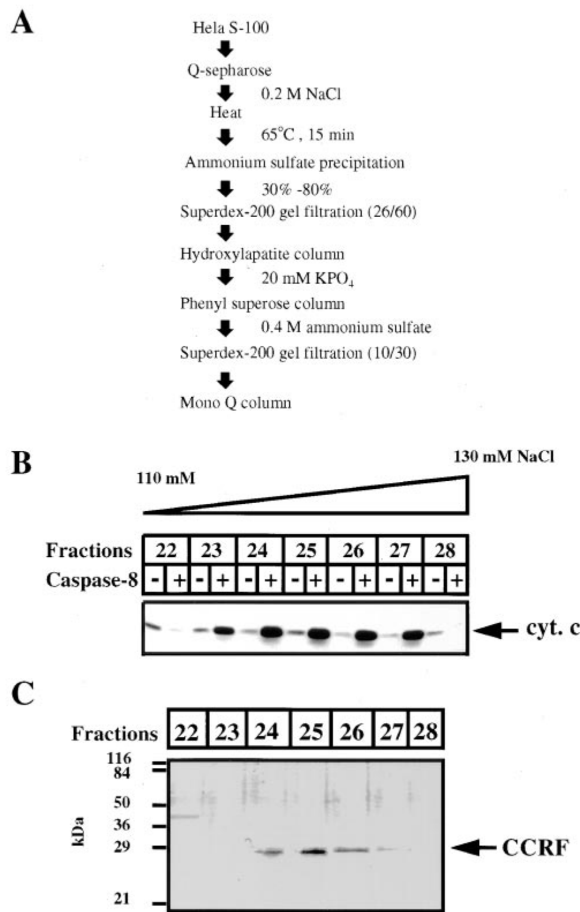

Figure 2. Purification of a Caspase-8-Dependent Cytochrome c Releasing Factor (CCRF)

(A) Diagram of the purification scheme for CCRF.

(B) Aliquots of 10 μl fractions from the Mono Q column were incubated with aliquots of 3 μl of mouse liver mitochondria (3 μg) in the absence or presence of aliquots of 2 μl caspase-8 (20 ng) in a final volume of 25 μl buffer B. After incubation for 30 min at 30°C, the samples were centrifuged at 12,000 × g for 5 min at 4°C. The resulting supernatants were subjected to 15% SDS-PAGE. After transferring to a nitrocellulose filter, the filter was probed with a monoclonal anti-cytochrome c antibody as described in Experimental Procedures. The filter was exposed to X-ray film for 3 s.

(C) Aliquots of 30 μl of the indicated fractions from the Mono Q column were subjected to 15% SDS-PAGE followed by silver staining using a Silver Stain Plus kit from Bio-Rad.

procedure (Figure 2A). The last step of purification, a Mono Q column chromatographic step, is shown in Figure 2B. CCRF was eluted from the Mono Q column at ~120 mM NaCl (fractions 24–27). The same protein fractions were subjected to SDS-PAGE followed by silver staining. A protein band of 26 kDa correlated with the CCRF activity.

CCRF Is Identical to Bcl2 Interacting Domain Death Agonist, Bid

To reveal the identity of CCRF, the 26 kDa protein was subjected to tryptic digestion, and the resulting peptides were subjected to mass spectral analysis. As shown in Table 1, eight peptides from the tryptic digestion of CCRF matched with a known human protein, BH3-containing death agonist, Bid (Wang et al., 1996).

To verify that CCRF is indeed Bid, we generated recombinant Bid by bacterial expression and purified the recombinant Bid to homogeneity. As shown in Figure 3A, Bid displayed caspase-8-dependent cytochrome c releasing activity in a concentration similar to purified CCRF. At higher concentrations (20–60 ng/25 μl), Bid induced cytochrome c release from the coincubated mitochondria independent of caspase-8 (upper panel). A point mutation at the conserved BH3 domain of Bid (G94E), which has previously been shown to abolish its interaction with both Bax and Bcl2, drastically decreased its cytochrome c releasing activity (bottom panel; Wang et al., 1996).

Since the cytochrome c releasing activity of Bid is activated by caspase-8, it is likely that Bid is a substrate of caspase-8. To test that directly, we incubated the recombinant Bid with caspase-8. Shown in Figure 3B, caspase-8 cleaved wild-type and G94E Bid into two fragments of 15 kDa and 11 kDa as measured by SDS-PAGE (lanes 2 and 6). Protein sequencing analysis of the 15 kDa cleaved product revealed that the cleavage is at the aspartic acid 59 after LQTD. Consistently, a mutation that changed Asp-59 to Ala abolished the cleavage of Bid by caspase-8 (lane 4). Such a mutant Bid lost the caspase-8-dependent cytochrome c releasing activity but maintained caspase-8-independent activity when higher concentrations of this protein were used (Figure 3A, middle panel).

The 15 kDa COOH-terminal cleaved product contains the BH3 domain, suggesting that it is the functional part of Bid for cytochrome c release. To test this directly, we generated a recombinant Bid protein truncated at the caspase-8 cleavage site Asp-59 and purified it to homogeneity (Figure 3D). This protein is sufficent to cause cytochrome c release from purified mitochondria, and such activity was no longer sensitive to caspase-8 and its inhibitors z-VAD-fmk and CrmA (Figure 3C, lanes 1–5).

To further confirm that Bid is the caspase-8-dependent cytochrome c releasing factor, an antibody generated against recombinant Bid was used to immunodeplete Bid from HeLa cell S-100 extracts. This antibody recognizes a single 26 kDa protein in the HeLa cell S-100 extracts by Western blot analysis (data not shown). As shown in Figure 4, S-100 extracts depleted of Bid lost the cytochrome c releasing activity (lanes 5 and 6). Preimmune serum from the same animal did not deplete any cytochrome c releasing activity (lanes 3 and 4).

COOH Terminus of Bid Translocates onto Mitochondria after Caspase-8 Cleavage

To study the mechanism of Bid-induced cytochrome c release, we translated Bid in vitro in the presence of ^{35}S-methionine and incubated the translated product with mitochondria in the presence of caspase-8. After incubation, the mitochondria were pelleted through centrifugation, and the resulting supernatants and pellets were analyzed by SDS-PAGE followed by autoradiography.

Table 1. Mass Fingerprinting of CCRF and Its Identification as Human Bid

Peptide #	Data Submitted	MH^+ Matched	Peptide Sequences	Amino Acid Position in Bid
1	1080.54[a]	1080.62[a]	VASHTPSLLR	159–168
2	1185.67[a]	1185.76[a]	TMLVLALLLAKK	147–157
3	1209.34[b]	1209.4[b]	KVASHTPSLLR	158–168
4	1504.63[a]	1504.71[a]	IEADSESQEDIIR	72–84
5	1549.15[b]	1548.9[b]	SIPPGLVNGLALQLR	100–114
6	1702.85[b]	1703.0[b]	DLATALEQLLQAYPR	126–140
7	1818.74[b]	1819.0[b]	DVFHTTVNFINQNLR	169–183
8	3167.57[b]	3167.4[b]	ELDALGHELPVLAPQWEGYDELQTDGNR	36–63

Peptides were analyzed by mass fingerprinting in a MALDI-TOF mass spectrometer. The values for m/z of the protonated molecular ions corresponding to eight tryptic peptides were matched to the sequence of human BH3 interacting domain death agonist (BID) with mass errors of 0.005–0.016%. These peptides covered 55% of the BID sequence.
[a] Monoisotopic mass values.
[b] Average mass values.

As shown in Figure 5, when the cleaved Bid was incubated with mitochondria, the COOH-terminal part became associated with the mitochondria (Figures 5A and 6C, lane 6). In contrast, minimal full-length Bid and no NH_2-terminal part of Bid were found to be associated with mitochondria (Figures 5A–5C, lanes 4–6). Interestingly, the BH3 mutant of Bid (G94E) that showed markedly reduced cytochrome c releasing activity associated

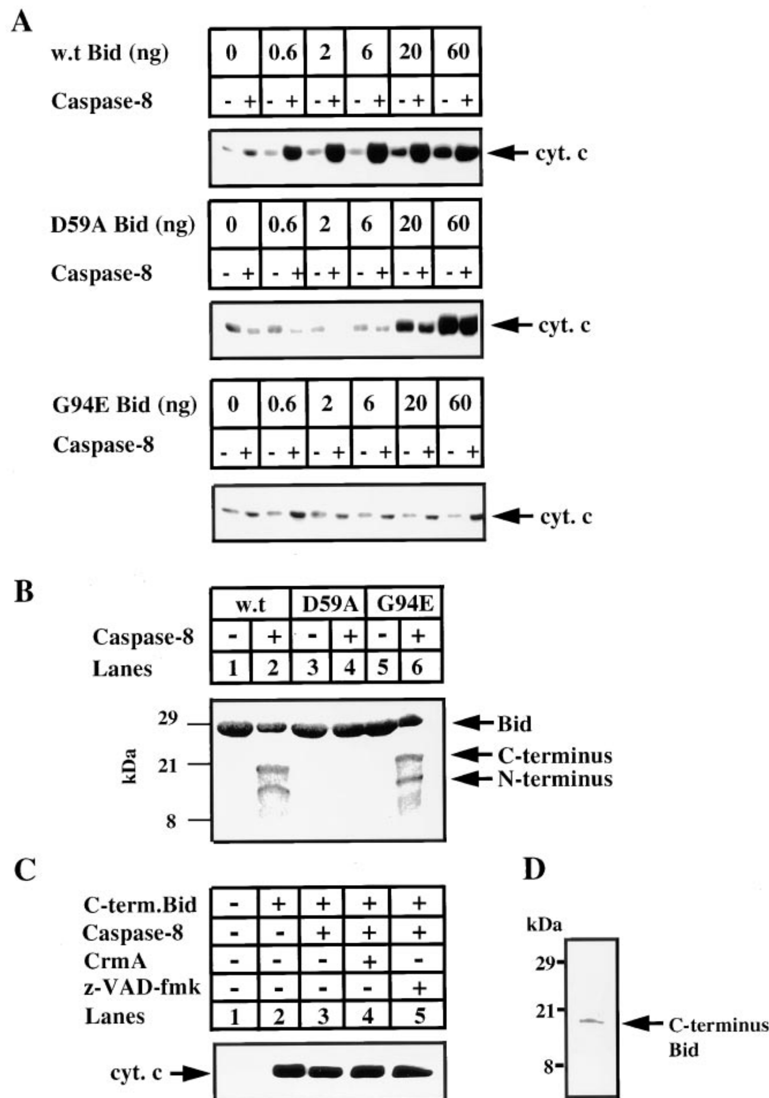

Figure 3. Recombinant Bid Has Caspase-8-Dependent Cytochrome c Releasing Activity

Recombinant wild-type, D59A, G94E, and the truncated Bid (aa 60–195) were generated and purified as described in Experimental Procedures.

(A) Aliquots of 3 μl of mitochondria (3 μg) were incubated with the indicated amounts of recombinant wild-type Bid (top panel), D59A Bid (middle panel), or G94E Bid (bottom panel) in the absence or presence of aliquots of 2 μl caspase-8 (20 ng) as indicated. After 30 min incubation at 30°C, the samples were centrifuged at 12,000 × g for 5 min at 4°C. The resulting supernatants were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody as described in Experimental Procedures. The filters were exposed to X-ray film for 3 s.

(B) Aliquots of 10 μg of recombinant wild-type Bid (lanes 1 and 2), D59A Bid (lanes 3 and 4), or G94E Bid (lanes 5 and 6) were incubated in the presence or absence of aliquots of 2 μl caspase-8 as indicated for 30 min at 30°C. After incubation, the samples were subjected to 15% SDS-PAGE, and the gel was subsequently stained by Coomassie brilliant blue.

(C) Aliquots of 3 μl of mitochondria were incubated with mitochondria resuspension buffer alone (lane 1) or 2 ng of truncated Bid in the absence (lane 2) or presence (lanes 3–5) of aliquots of 2 μl of caspase-8 (20 ng). In lanes 4 and 5, aliquot of 0.3 μg of CrmA (lane 4) or zVAD-fmk at a final concentration of 20 μM was preincubated with caspase-8 for 15 min prior to the addition of mitochondria and Bid. After 60 min incubation at 30°C, the samples were centrifuged at 12,000 × g for 5 min at 4°C, and the resulting supernatants were analyzed by Western blot using an anti-cytochrome c antibody as in (A).

(D) An aliquot of 0.5 μg of truncated Bid (aa 60–195) was subjected to 15% SDS-PAGE, and the gel was subsequently stained by Coomassie brilliant blue.

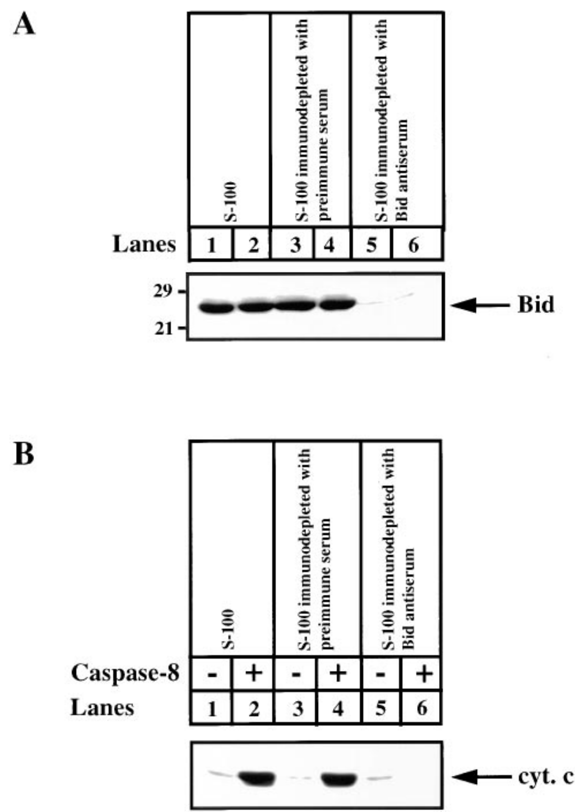

Figure 4. Bid Is Required for Caspase-8-Induced Cytochrome c Release from Mitochondria

An aliquot of 500 μl of Protein A agarose (Santa Cruz) was incubated with an aliquot of 500 μl of preimmune or immune serum against human recombinant Bid at 4°C overnight. The antibody/Protein A agarose beads were collected by centrifugation and washed five times with buffer A. The beads were resuspended in 1 ml buffer A, and aliquots (50 μl) of these beads were then incubated with 1 ml HeLa cell S-100 overnight at 4°C. The beads were subsequently pelleted by centrifugation, and the supernatants were used as extracts immunodepleted of Bid. The cytochrome c present in these extracts was also immunodepleted as in Liu et al., 1996b.

(A) Duplicate samples of HeLa S-100 (50 μg) (lanes 1 and 2), or HeLa S-100 immunodepleted with the preimmune serum (50 μg) (lanes 3 and 4), or HeLa S-100 immunodepleted with an antiserum raised against recombinant Bid (50 μg) (lanes 5 and 6) were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with the anti-Bid antibody as described in Experimental Procedures. The filter was exposed to X-ray film for 3 s.

(B) Aliquots of 50 μg of HeLa S-100 (lanes 1 and 2), or HeLa S-100 immunodepleted with the preimmune serum (lanes 3 and 4), or HeLa S-100 immunodepleted with an antiserum raised against recombinant Bid (lanes 5 and 6) were incubated with 3 μl of mitochondria (3 μg) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 2 μl of caspase-8 (20 ng). After 30 min incubation at 30°C, the samples were centrifuged at 12,000 × g for 5 min at 4°C. The resulting supernatants were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody, and the filter was exposed to X-ray film for 3 s.

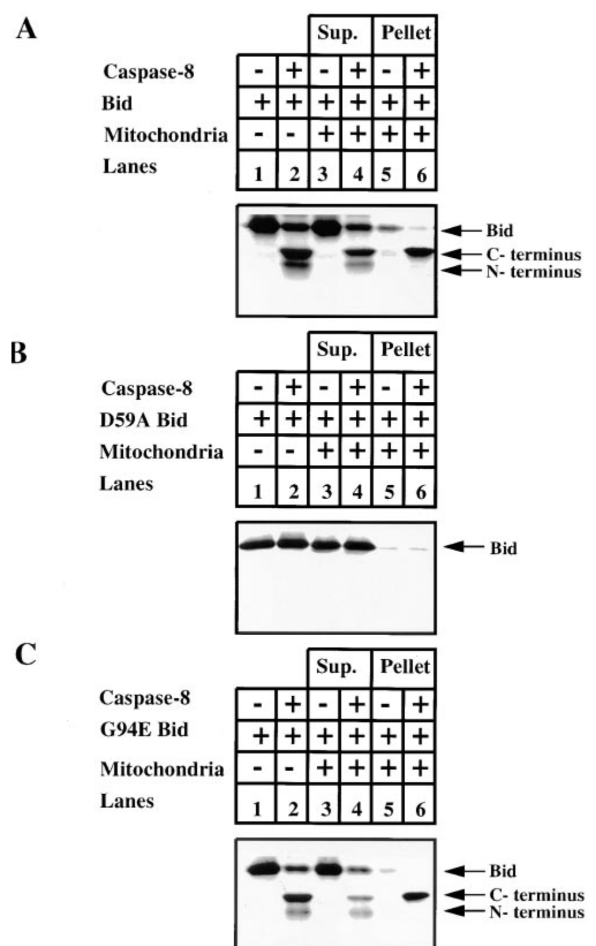

Figure 5. The COOH-Terminal Part of Bid Translocates onto the Mitochondria after Cleavage by Caspase-8

Recombinant wild-type, D59A, and G94E Bid were in vitro–translated in the presence of ^{35}S-methionine and affinity-purified as described in Experimental Procedures.

Aliquots of 2 μl of in vitro–translated, ^{35}S-methionine-labeled, and affinity-purified wild-type Bid (A), D59A Bid (B), or G95E Bid (C) were incubated with (lanes 2, 4, and 6) or without aliquots of 2 μl caspase-8 (lanes 1, 3, and 5) in the presence of aliquots of 6 μl mitochondria. In lanes 1 and 2, mitochondria were omitted from the mixtures. After 30 min incubation at 30°C, the samples were centrifuged at 12,000 × g for 5 min at 4°C, and the resulting supernatants (lanes 3 and 4) and pellets (lanes 5 and 6) were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter that was subsequently exposed to X-ray film for 16 hr at room temperature.

with the mitochondria to the same extent as the wild type (Figure 5C, lane 6).

Mitochondria from Bcl2 Overexpressed Cells Show Decreased Response to Bid

Overexpression of Bcl2 blocks cytochrome c release from mitochondria in response to a variety of apoptotic signals (Kluck et al., 1997; Yang et al., 1997). To test the role of Bcl2 during cytochrome c release induced by Bid, we isolated mitochondria from human leukemia cell line HL60 cells stably transfected with human *Bcl2* cDNA or the neo vector control. The overexpression of Bcl2 protein on the mitochondria isolated from Bcl2 cells was verified by Western blot analysis using an antibody against Bcl2 (Figure 6A). The same amount of mitochondria from these two cell lines was incubated with increasing amounts of Bid in the presence of caspase-8. As shown in Figure 6B, cytochrome c was completely released from mitochondria of neo cells in the presence of 0.2 ng of Bid (lane 2). In contrast, the same concentration of Bid did not induce any cytochrome c release

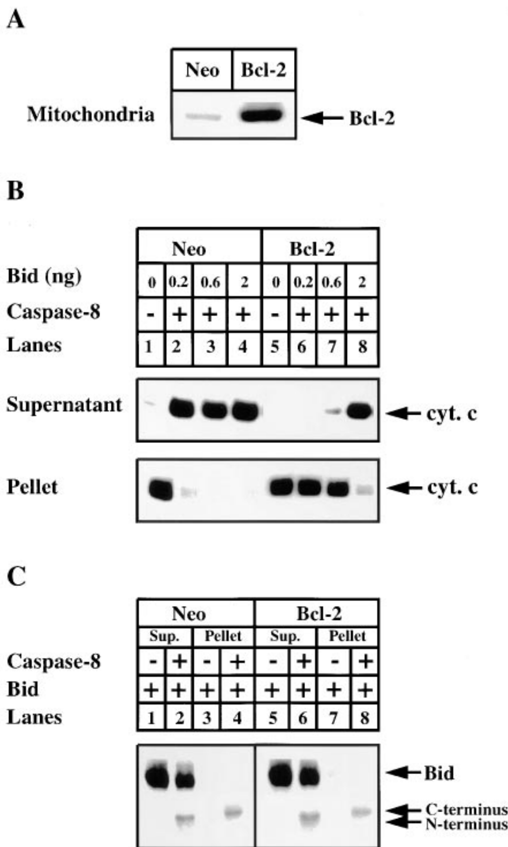

Figure 6. Bcl2 Overexpression Antagonized Bid-Induced Cytochrome c Release

Mitochondria-enriched pellets from human leukemia HL-60/neo and HL-60/Bcl2 cells were prepared as described in Yang et al., 1997.
(A) Mitochondria-enriched pellets from HL-60/neo and HL-60/Bcl2 were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-Bcl2 antibody as described in Experimental Procedures. The filter was exposed to film for 30 s.
(B) Aliquots of 3 μl mitochondria-enriched pellets from HL-60/neo cells (lanes 1–4) or from HL-60/Bcl2 cells (lanes 5–8) were incubated with aliquots of 2 μl caspase-8 plus the indicated amount of recombinant Bid for 30 min at 30°C. After incubation, the samples were centrifuged at 12,000 $\times$ g for 5 min at 4°C. The resulting supernatants (top panel) and pellets (bottom panel) were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody, and the filter was exposed to film for 3 s.
(C) Aliquots of 2 μl of in vitro–translated, ^{35}S-methionine-labeled, and affinity-purified Bid were incubated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) aliquots of 2 μl caspase-8 in the presence of aliquots of 6 μl mitochondria-enriched pellets from HL-60/neo (lanes 1–4) or HL-60/Bcl-2 (lanes 5–8). After 30 min incubation at 30°C, the samples were centrifuged at 12,000 $\times$ g for 5 min at 4°C. The resulting supernatants (lanes 1, 2, 5, and 6) and pellets (lanes 3, 4, 7, and 8) were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter that was subsequently exposed to X-ray film for 16 hr at room temperature.

from mitochondria of Bcl2 cells (lane 5). However, the inhibitory effect of Bcl2 was overcome by a 10-fold higher concentration of Bid (lane 8). Consistent with the data shown in Figure 5, COOH-terminal part of the cleaved Bid associated with mitochondria from both neo and Bcl2 cells (Figure 6C, lanes 4 and 8).

Bid Is Cleaved during Fas-Induced Apoptosis

To demonstrate that Bid is indeed cleaved in vivo during Fas activation, we treated Jurkat cells with an agonistic antibody against Fas at different time points and analyzed Bid, caspase-3, and its substrate DFF45 by Western blot analysis. As shown in Figure 7A, Bid exists as an ~26 kDa protein in growing cells (lane 1). After a 1 hr treatment with anti-Fas antibody, a smaller cleaved fragment of Bid appeared (Figure 7A, lane 2, lower panel). This fragment was further cleaved into a slightly smaller fragment after a 4 hr treatment (lanes 4 and 5). This is likely due to the activation of a caspase(s) that cleaves Bid after Asp-74 at the IEAD site, a putative cleavage site for the downstream caspases such as caspase-6. The NH_2-terminal cleaved fragment was not recognized by this anti-Bid antibody. The amount of the 26 kDa Bid was significantly decreased after a 2 hr treatment and became nondetectable after a 4 hr treatment (lanes 3–5). The cleavage of Bid was completely prevented when a caspase inhibitor, z-VAD-fmk, was added together with the anti-Fas antibody (lane 6). The cleavage of Bid during Fas-induced apoptosis occurred earlier than the cleavage of caspase-3 and its substrate DFF45 (Figures 7B and 7C). The cleaved caspase-3 and DFF45 started after a 2 hr treatment with anti-Fas antibody, and their precursors disappeared after a 6 hr treatment.

Discussion

Bid Bridges the Activation of Cell Surface Death Receptors and Mitochondria

Activation of Fas on the cell membrane by Fas ligand or agonist antibody results in the activation of caspase-8, the apical caspase in this pathway (Boldin et al., 1996; Muzio et al., 1997). In the presence of high concentrations of caspase-8, the downstream caspases were activated independent of mitochondria and the activation was insensitive to Bcl-x_L (Kuwana et al., 1998; Scaffidi et al., 1998). When mitochondria were present, however, the effect of low concentrations of caspase-8 was amplified through cytochrome c–dependent caspase activation, an event blocked by Bcl-x_L (Kuwana et al., 1998; Medema et al., 1998; Scaffidi et al., 1998; Srinivasan et al., 1998). Cytochrome c release from mitochondria induced by Fas activation can also be blocked by caspase-8 inhibitors such as z-VAD-fmk or CrmA (Krippner et al., 1996; Vander Heiden et al., 1997; Scaffidi et al., 1998). Caspase-8 therefore functions upstream of mitochondria in the Fas pathway. Activated caspase-8 has been proposed to relay the cell membrane death signal to mitochondria through a cytosolic mediator (Krippner et al., 1996). We have now identified and purified such a mediator that directly triggers cytochrome c release in purified mitochondria after cleavage by caspase-8. This protein is Bid, a member of BH3 domain–containing proteins (Wang et al., 1996).

Bid contains a single BH3 domain, a domain also shared by several other proapoptosis members of this family such as Bad and Bax (Oltvai et al., 1993; Zha et al., 1996). Interestingly, Bid lacks other homologous domains found in the Bcl2 family of proteins such as

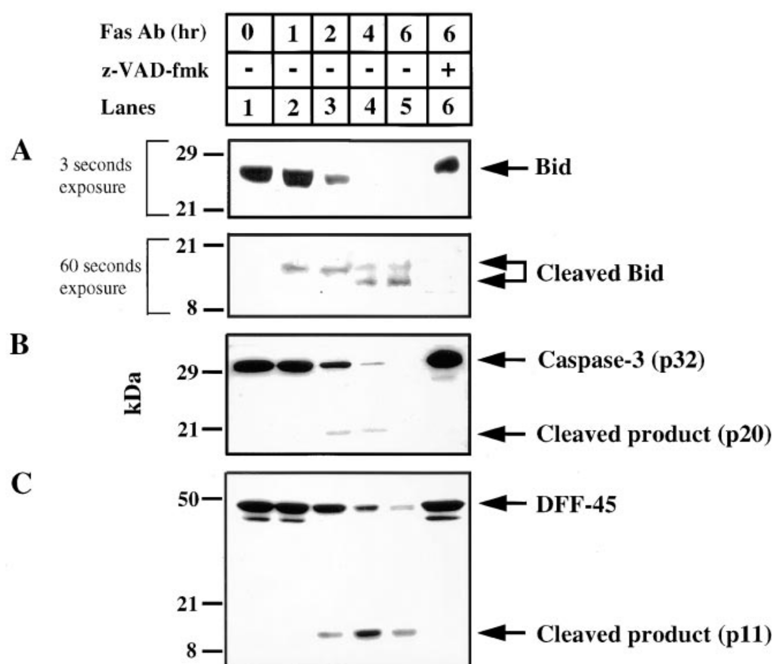

Figure 7. Cleavage of Bid In Vivo during Fas Activaion

Human lymphoblastoid T-cell Jurkat cells (3×10^6) in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate were treated with 500 ng/ml monoclonal anti-human Fas antibody (clone CH11, Upstate Biotech.) for the indicated time points. In lane 6, z-VAD-fmk was added to a final concentration of 20 μM together with the anti-Fas antibody. The cells were collected by centrifugation at 1,000 × g for 10 min at 4°C. After washing twice with ice-cold PBS, the cell pellets were lysed in 300 μl of 1× SDS-PAGE loading buffer. Aliquots of 30 μg of Jurkat whole cell lysate were loaded to three 15% SDS-PAGE gels and transferred to nitrocellulose filters.

(A) The first filter was probed with a rabbit polyclonal anti-Bid antibody (1:2000 dilution) as described in Experimental Procedures. The filter was exposed to X-ray film for 3 s to visualize the full-length Bid (upper panel) and further exposed to another film for 60 s to visualize the cleaved Bid (lower panel).

(B) The second filter was probed with a mouse monoclonal antibody against caspase-3 (Signal Transduction Lab.), and the antigen-antibody complexes were detected by an ECL method. The filter was exposed to X-ray film for 5 min.

(C) The third filter was probed with a rabbit polyclonal antibody against DFF-45 as described in Liu et al., 1997, and the antigen-antibody complexes were detected by an ECL method. The filter was exposed to X-ray film for 3 s.

BH1, BH2, BH4, and COOH-terminal membrane anchoring domain. It is able to interact with both pro- and antiapoptosis members of this family (Wang et al., 1996). Overexpression of Bid potentiates apoptosis induced by serum withdrawal (Wang et al., 1996). Such an observation is consistent with its role as a mediator of apoptotic signals. Recently, a BH3-containing proapoptosis protein, EGL-1, has been isolated from *C. elegans* (Conradt and Horvitz, 1998). EGL-1 has been shown to function upstream of CED-9, a *C. elegans* homolog of Bcl2, to trigger apoptosis in this organism. Like Bid, EGL-1 contains a single BH3 domain that mediates its interaction with CED-9 but lacks other recognizable homology with the Bcl2 family of proteins. EGL-1 and Bid may therefore represent upstream apoptotic signaling molecules that relay the signal to CED-9 and Bcl2, respectively.

How Does Bid Cause Cytochrome c Release?

Bid exists in the cytosolic fraction of living cells as an inactive precursor that becomes activated upon cleavage by caspase-8. The cleavage occurs at aspartic acid 59 after the LQTD site, a site conserved between human and mouse Bid (Wang et al., 1996). After cleavage, the COOH fragment of Bid translocates onto mitochondria, an event that is independent of its BH3 domain (Figure 5). The activated Bid by itself is sufficient to induce complete release of cytochrome c from mitochondria (Figure 4C). Recently, Bax, another proapoptotic member of the Bcl2 family of proteins has been shown to induce cytochrome c release both in vivo and in vitro (Jurgensmeier et al., 1998; Rosse et al., 1998). However, Bid is a much more potent cytochrome c releasing factor than Bax. Bax only releases up to 20% of the total mitochondrial cytochrome c even at a high concentration (200 nM) (Jurgensmeier et al., 1998). Bid, on the other hand, was able to release close to 100% of mitochondrial cytochrome c at a 500-fold lower concentration (~0.2–2 nM) (Figure 3). Further, the minimum Bax that was needed to trigger cytochrome c release is about 0.1 μM. In contrast, 0.2 nM of Bid caused complete cytochrome c release (Figure 6B).

Bid initiates the release of cytochrome c apparently without evoking gross mitochondrial swelling and permeability transition, two events that have been proposed to cause cytochrome c release (Budihardjo and Wang, data not shown; Susin et al., 1997; Vander Heiden et al., 1997; Pastorino et al., 1998). Such a finding indicated that loss of mitochondrial membrane potential is not the cause for Bid-mediated cytochrome c release. The BH3 domain of Bid is important for its function, and such a domain dimerizes with other Bcl2 family members (Wang et al., 1996). Thus, it is possible that Bid will form a complex with a proapoptotic member of the family like Bax to trigger cytochrome release from mitochondria. Alternatively, Bid may inactivate an antiapoptotic member of the Bcl2 family that plays an active role in preventing cytochrome c release. Interestingly, a BH3 mutant (G94E) of Bid that is defective in cytochrome c release (Figure 3A) and is unable to interact with Bcl2 or Bax (Wang et al., 1996) associates with mitochondria to the same extent as wild-type Bid (Figure 5C). This finding indicates that Bid may interact with a yet unidentified target on the outer membrane of mitochondria. However, this protein–protein (or protein–lipid) interaction is not sufficient to trigger cytochrome c release, an event that also requires the interaction with

another protein through its BH3 domain. Although the molecular mechnism of cytochrome c release is still obscure, the reconstitution of this reaction with the recombinant, constitutively active Bid (aa 60–195) will allow us to further study this process in detail.

Does Bid Mediate Cytochrome c Release in Response to Stimuli Other than Caspase-8?

It will be interesting to see whether other death signals such as excessive DNA damage and growth factor deprivation also exert their apoptotic effects on mitochondria through Bid. Overexpression of Bid potentiates cell death induced by IL-3 withdrawal, indicating the involvement of Bid in this pathway (Wang et al., 1996). It is possible that Bid could be activated by other forms of posttranslational modification such as phosphorylation. Alternatively, Bid may be regulated by proteins such as 14–3–3, which regulate the activity of another BH3-containing protein, Bad (Zha et al., 1996). Moreover, Bid might be activated by caspases other than caspase-8 to form an amplification loop for caspase activation. Conversely, there could be other BH3-containing pro-apoptotic proteins such as Bad and Bax that migrate from cytosol to mitochondria upon apoptotic stimuli and interact with the same mitochondrial target as Bid (Wolter et al., 1997; Zha et al., 1996). Now with the identification of Bid as a cytochrome c releasing factor, all these possibilities become testable hypotheses.

Experimental Procedures

We obtained dATP and other nucleotides from Pharmacia, radioactive materials from Amersham, and molecular weight standards for SDS-PAGE and gel filtration chromatography from Bio-Rad. Protein concentrations were determined by the Bradford method, and general molecular biology methods were used as described in Sambrook et al., 1989. Mouse monoclonal anti-cytochrome c antibody (clone 7H8.2C11) was a generous gift from Dr. Ronald Jemmerson (University of Minnesota).

Preparation of Mitochondria

Mouse liver mitochondria were isolated as described by Ellerby et al., 1996. In brief, the mouse livers were removed after sacrifice and dounce homogenized in ice-cold mitochondria isolation buffer (MIB) containing 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, and 0.1% (w/v) BSA (pH 7.2) supplemented with the protease inhibitors of leupeptin (1 μg/ml), pepstatin A (1 μg/ml), antipain (50 μg/ml), and PMSF (0.1 mM). Unbroken cells and nuclei were pelleted by centrifugation at 600 × g for 5 min at 4°C. The supernatants were further centrifuged at 10,000 × g for 10 min at 4°C to pellet the mitochondria. The mitochondria pellet was resuspended in 4 ml MIB and loaded onto a continuous Percoll gradient consisted of 30% (v/v) Percoll (Sigma), 225 mM mannitol, 25 mM HEPES, 0.5 mM EGTA, and 0.1% (w/v) BSA (pH 7.2). The suspension/gradient was centrifuged at 40,000 × g for 1 hr. The mitochondria were removed from the brownish band at 1.10 g/ml with a transfer pipette. The mitochondrial pellets were washed with MIB by centrifuging for 10 min at 6300 × g at 4°C. The mitochondria were then resuspended gently in mitochondria resuspension buffer containing 400 mM mannitol, 10 mM KH_2PO_4, and 50 mM Tris-HCl (pH 7.2) with 5 mg/ml BSA and stored on ice for up to 4 hr.

Mitochondria-enriched pellets from HL-60/neo and HL-60/bcl2 cell lines were prepared as described in Yang et al., 1997.

In Vitro Assay for Cytochrome c Release

The full-length caspase-8 in pET28 (Novagen) was described in Srinivasula et al., 1996. The expression plasmid was transformed into bacteria BL21 (DE3) cells (Novagen), and the recombinant caspase was prepared as in Liu et al., 1997, and stored in multiple aliquots at −80°C. A 3 μl aliquot of mouse liver mitochondria was incubated with the indicated protein fraction in the presence or absence of a 2 μl aliquot of recombinant caspase-8 in a final volume of 25 μl buffer B (220 mM mannitol, 68 mM sucrose, 20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF) at 30° for 30 min. At the end of incubation, the reaction mixture was centrifuged at 12,000 × g for 5 min at 4°C to pellet the mitochondria. Eight microliters of 4× SDS sample buffer was added to the resulting supernatants. The mitochondria pellets were resuspended in equal volume (33 μl) of 1× SDS sample buffer. The samples were boiled for 3 min and subjected to 15% SDS-PAGE. The gel was transferred to a nitrocellulose filter and probed with a mouse monoclonal anti-cytochrome c antibody (from Dr. R. Jemmerson) or a mouse monoclonal anti-cytochrome c-oxidase antibody (Molecular Probes). Western blot analysis for cytochrome c was performed as described previously (Liu et al., 1996b). Antigen/antibody complexes were detected using a horseradish peroxidase conjugated goat anti-mouse (for both cytochrome c and cytochrome c oxidase) immunoglobulin G using enhanced chemiluminescence Western blotting detection reagents (Amersham).

Purification of Caspase-8-Dependent Cytochrome c Releasing Factor from HeLa S-100

All purification steps were carried out at 4°C. All chromatography steps except the Q-Sepharose column (Pharmacia) were carried out using an automated fast protein liquid chromatography (FPLC) station (Pharmacia).

Six hundred milliliters (3 g of protein) of HeLa S-100 from 100 l of suspension-cultured HeLa cells were prepared as described in Liu et al., 1996b, and applied to a Q-Sepharose column (100 ml bed volume) equilibrated with buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF). The column was washed with three column volumes of buffer A and eluted with four column volumes of buffer A containing 0.1 M NaCl and four column volumes of buffer A containing 0.2 M NaCl. The protein peak from the 0.2 M NaCl elution (250 ml, 314 mg protein) was heated at 65°C for 15 min and centrifuged at 20,000 × g for 15 min to precipitate denatured proteins. Ammonium sulfate (30%) was added directly to the supernatant (240 ml, 36 mg protein). After stirring at 4°C for 1 hr, the mixture was centrifuged at 20,000 × g for 15 min. Ammonium sulfate was added to the supernatant to a final concentration of 80% and stirred at 4°C for 1 hr. The mixture was then centrifuged again at 20,000 × g for 15 min at 4°C. The resulting pellet (13 mg protein) was resuspended in 8 ml buffer A, loaded onto a Superdex-200 Hi-Load (26/60) gel filtration column (300 ml) equilibrated with buffer A containing 0.1 M NaCl, and eluted with the same buffer. Fractions of 4 ml were collected (two runs) and assayed for CCRF activity. The active fractions from the gel filtration column (48 ml, 4 mg protein) were pooled and loaded onto a 1 ml hydroxylapatite column (Bio-Rad) equilibrated with buffer A. The column was eluted with a linear gradient of 10 ml buffer A to 50 mM KPO_4 (pH 7.5) followed by 15 ml buffer A containing 50 mM KPO_4 (pH 7.5). Fractions of 1 ml were collected and assayed for CCRF activity. The active fractions (6 ml, 1 mg protein) were pooled, and ammonium sulfate was added directly to these fractions to a final concentration of 1 M. The fractions were then loaded onto a 1 ml Phenyl-superose column (Pharmacia) equilibrated with buffer A containing 1 M ammonium sulfate and eluted with 15 ml buffer A containing 400 mM ammonium sulfate. Fractions of 1 ml were collected and assayed for CCRF activity. The active fractions (6 ml, 286 μg protein) were loaded onto a Superdex-200 (10/30) gel filtration column equilibrated with buffer A containing 0.1 M NaCl and eluted with the same buffer (12 runs). Fractions of 1 ml were collected and assayed for CCRF activity. Pooled active fractions from the gel filtration column (25 ml, 155 μg protein) were loaded directly onto a Mono Q 5/5 column (Pharmacia) equilibrated with buffer A containing 60 mM NaCl. The column was eluted with a 50 ml linear gradient from 60 mM NaCl to 250 mM NaCl, both in buffer A. Fractions of 1 ml were collected and assayed for CCRF activity.

Protein Identification of CCRF by Peptide Mass Fingerprinting

A 10 pmol aliquot of CCRF purified through the Mono Q column step was subjected to SDS polyacrylamide gel electrophoresis. After staining with Coomassie blue R-250, the 26 kDa band was excised from the gel and digested with trypsin as described (Jensen et al., 1996). A 1 μl aliquot of the total digest (total volume 30 μl) was used for peptide mass fingerprinting (Williams and Stone, 1995). The masses of the tryptic peptides were measured with a Voyager DE time-of-flight (TOF) mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). Matrix-assisted laser desorption/ionization (MALDI) was performed with α-cyano-4-hydroxycinnamic acid as the matrix. A precision of at least ±0.02% was achieved by using delayed extraction and calibration of the mass scale with three internal peptide standards. Searching of the mass values against the NCBI database (rev. 03/21/98) was performed with the program MS-Fit available at http://prospector.ucsf.edu/htmlucsf/msfit.htm.

cDNA Cloning and Site-Directed Mutagenesis of Bid

An aliquot of λExlox HeLa cDNA library (Zou et al., 1997) was PCR amplified by primers 5′-GCT GCC CAG CAT ATG GAC TGT GAG GTC AAC and 5′-CTT CTG GAA GGA TCC GTT CAG TCC ATC CCA TTT CTG designed according to EST clone 52055 encoding full-length human Bid. A 585 bp PCR product containing the coding region of Bid was subcloned in-frame into the NdeI-BamHI sites of bacterial expression vector PET-15b (Novagen). The cleavage site (D59A) and the BH3 domain (G94E) mutations were generated by a PCR-SOEing method using mutagenic oligonucleotides (Ho et al., 1989). Both mutations were confirmed by DNA sequencing and then subcloned in-frame into NdeI-BamHI sites of PET-15b for generating Histidine-tagged fusion proteins.

Production of Recombinant Bid Protein

The full-length coding regions of wild-type Bid, D59A Bid, and G94E Bid were cloned into the BamHI and NdeI sites of pET-15b vector (Novagen). The expression plasmids were transformed into bacteria BL21 (pLys) cells (Novagen). Aliquots of 5 ml overnight culture of bacteria containing wild-type Bid, or D59A Bid, or G94E Bid expression vectors were added into aliquots of 1 l LB broth and cultured for 3 hr by shaking at 220 rpm in 37°C. Isopropyl-1-thio-B-D-galactopyranoside (IPTG) was added to the culture in a final concentration of 1 mM, and shaking continued for another 2 hr. The bacteria were pelleted by centrifugation, and the bacteria pellet was resuspended in 10 volumes of buffer A. The resuspended cells were lysed in buffer A by sonication for 3–4 min. After centrifugation at 10,000 × g for 15 min, the supernatant was loaded onto a 2 ml nickel affinity column (Qiagen). The column was washed with 20 ml buffer A followed by 20 ml buffer A containing 1 M NaCl. After reequilibrating the column with 20 ml buffer A, the bound protein was eluted with buffer A containing 250 mM imidazole. The eluted protein was diluted 10-fold in buffer A and loaded onto a Mono Q column (Pharmacia) equilibrated with buffer A containing 60 mM NaCl. The column was eluted with a 50 ml linear gradient from 60 mM NaCl to 250 mM NaCl, both in buffer A. The COOH-terminal truncation of Bid was generated by PCR amplifying the coding region between glycine 60 and COOH-terminal end of Bid. The PCR product was subcloned into the BamHI and NdeI sites of the pET-15b vector (Novagen) and expressed as the full-length Bid except 1% (w/v) CHAPS (Sigma) was included in buffer A during bacterial pellet resuspension. After being eluted from a 2 ml nickel affinity column, the recombinant protein was loaded onto a Mono S column (Pharmacia) and eluted with a 25 ml linear salt gradient from 50 mM NaCl to 350 mM NaCl, both in buffer A.

Western Blot Analysis

A polyclonal anti-Bid antibody was generated by immunizing rabbits with a recombinant wild-type Bid fusion protein (see above). Immunoblot analysis was performed with a horseradish peroxidase conjugated goat anti-rabbit (Bid), or goat anti-mouse (cytochrome c, cytochrome c oxidase), immunoglobulin G using enhanced chemiluminescence Western blotting detection reagents (Amersham).

In Vitro Translation of Wild-Type and Mutant Recombinant Bid

The full-length coding regions of wild-type Bid, D59A Bid, and G94E Bid were cloned into the BamHI and NdeI sites of pET-15b vector (Novagen). The resulting fusion proteins with six histidines were translated in a TNT T7 transcription/translation kit (Promega) in the presence of ^{35}S-methionine according to the manufacturer's instruction. The translated proteins were passed through 1 ml nickel affinity columns (Qiagen) equilibrated with buffer A. After washing the columns with 10 ml buffer A containing 1 M NaCl, the columns were reequilibrated with buffer A, and the translated proteins were eluted with buffer A containing 250 mM imidazole (Sigma).

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