Caspases Induce Cytochrome c Release from Mitochondria by Activating Cytosolic Factors*

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We investigated the ability of caspases (cysteine proteases with aspartic acid specificity) to induce cytochrome c release from mitochondria. When Jurkat cells were induced to undergo apoptosis by Fas receptor ligation, cytochrome c was released from mitochondria, an event that was prevented by the caspase inhibitor, zVAD-fmk (zVal-Ala-Asp-CH2F). Purified caspase-8 triggered rapid cytochrome c release from isolated mitochondria in vitro. The effect was indirect, as the presence of cytosol was required, suggesting that caspase-8 cleaves and activates a cytosolic substrate, which in turn is able to induce cytochrome c release from mitochondria. The cytochrome c releasing activity was not blocked by caspase inhibition, but was antagonized by Bcl-2 or Bcl-xL. Caspase-8 and caspase-3 cleaved Bid, a proapoptotic Bcl-2 family member, which gains cytochrome c releasing activity in response to caspase cleavage. However, caspase-6 and caspase-7 did not cleave Bid, although they initiated cytochrome c release from mitochondria in the presence of cytosol. Thus, effector caspases may cleave and activate another cytosolic substrate (other than Bid), which then promotes cytochrome c release from mitochondria. Mitochondria significantly amplified the caspase-8 initiated DEVD-specific cleavage activity. Our data suggest that cytochrome c release, initiated by the action of caspases on a cytosolic substrate, may act to amplify a caspase cascade during apoptosis.

Genetic and biochemical data indicate that a family of cysteine proteases with aspartate specificity, known as caspases, play a pivotal role in the regulation and execution of apoptotic cell death (reviewed in Refs. 1–6). Caspases are expressed as inactive proenzymes in living cells and become activated by proteolytic processing at internal aspartate residues when cells receive an apoptosis-inducing signal (3). Proteolytic cleavage results in the removal of an amino-terminal prodomain and the generation of a small and a large active subunit, which forms a heterodimer. At present, 14 mammalian caspase family members have been described. Some, including caspase-8, -9, and -10, contain large prodomains and are initiators of cell death. Other family members, such as caspase-3, -6, and -7, carry small prodomains and are mostly involved in the execution of cell death. When caspases are activated, they cleave a number of key substrates, resulting in their activation or inactivation, which orchestrate the morphological and biochemical features of apoptosis (7–11).

Two pathways of caspase activation during apoptosis have been described. The first one involves apoptosis mediated by death receptors, such as Fas or tumor necrosis factor receptors (reviewed in Ref. 12). When Fas ligand binds to the Fas receptor, the adaptor molecule FADD/Mort-1 becomes recruited to the receptor (13–15), allowing binding and autoactivation of procaspase-8 (16–22). When caspase-8 is activated, it can process effector caspases (caspase-3, -6, and -7), inducing a cascade of caspases (23–29).

In the second pathway, diverse proapoptotic signals converge at the mitochondrial level, provoking the translocation of cytochrome c from mitochondria to cytoplasm (30–37). Once cytochrome c is in the cytoplasm, it binds to Apaf-1, a mammalian CED-4 homologue, which then permits recruitment of procaspase-9 (38, 39). Oligomerization results in autoactivation of procaspase-9 (40). Active caspase-9 then cleaves and activates procaspase-3. In the mitochondrial pathway, the complex of cytochrome c, Apaf-1, and caspase-9, called the “apoptosome,” is a critical activator of the effector caspases. Both pathways induce a cascade of caspases, which amplifies the apoptotic signal to ensure fast and irreversible cell death. Cytochrome c release has been documented for apoptosis induced by chemotherapeutic drugs, UV irradiation, growth factor withdrawal, and ligation of Fas and tumor necrosis factor receptors (31–37). Cell survival-promoting molecules Bcl-2 and Bcl-xL, localized at outer mitochondrial membranes, prevent translocation of cytochrome c from mitochondria (30, 35, 36, 41, 42).

Caspase-8 can directly cleave and activate caspase-3, serving as a major substrate (17, 18, 21, 26, 29, 38). Thus, mitochondria may not be required for receptor-mediated cell death. Nevertheless, electron transport is inactivated in Fas-induced cell death, and cytochrome c is released from mitochondria (30, 32, 41, 42). In addition, Bcl-2 and Bcl-xL, localized to the outer mitochondrial membrane (43), are able to block these events in some cases, arguing for a role of mitochondria in Fas-mediated cell death (41, 42, 44–46). To search for signals regulating cytochrome c release, caspase-8 and other caspases were tested for their ability to provoke cytochrome c release from mitochondria.

EXPERIMENTAL PROCEDURES

Reagents—Anti-cytochrome c antibody (7H8.2.C12) was from Pharmingen (San Diego, CA); anti-Fas monoclonal antibody CH-11 was from Kamiya Biomedical Company (Seattle, WA). Anti-actin antibody was from ICN Biomedicals, Inc. (clone 14) (Aurora, OH). Anti-caspase-3

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1 The abbreviations used are: Apaf-1, apoptotic protease activating factor-1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
antibodies were from Transduction Laboratories (Lexington, KY). Anti-Fas antibodies were from ICN Biomedicals, Inc. Anti-protein kinase C-δ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-2 antibodies (clone 124) were from DAKO (Mississauga, Canada). Complete mixture of protease inhibitors was purchased from Roche Molecular Biochemicals. Protein concentrations were determined with the Bio-Rad assay kit (Hercules, CA). zVAD-fmk (zVal-Ala-Asp-CH2F) was from Kamiya Biomedical Company (Seattle, WA). Colorimetric substrate DEVD-pNA was from Biomol (Plymouth, PA). Anti-mouse HRP-linked antibodies were from Amersham Pharmacia Biotech.

**Immunoblotting—**Jurkat cells were collected by centrifugation at 200 × g for 5 min at 4 °C and washed twice with ice-cold PBS 7.4. The cell pellet was resuspended in 500 μl of extraction buffer containing 250 mM sucrose, 20 mM Hepes-KOH, pH 7.0, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, and a mixture of protease inhibitors (Roche Molecular Biochemicals). After 20 min on ice, cells were homogenized with a glass Dounce homogenizer (B pestle/50 strokes). Cell homogenates were spun at 14,000 × g for 15 min at 4 °C in a microcentrifuge, and supernatants were stored at −70 °C until gel electrophoresis. 20–50 μg of cytosolic extracts were loaded onto each lane of an 8, 12, or 15% SDS-polyacrylamide gel, separated and transferred to Hybond-ECL nitrocellulose membrane (0.45 μm) (Amersham Pharmacia Biotech) at 150 mA overnight in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). Nonspecific binding was blocked by incubation with 3% bovine serum albumin, 3% nonfat milk powder, and 0.1% Tween-20 in PBS for 3 h at room temperature. Anti-cytomegalovirus (CMV) antibodies were diluted at 1:1000, anti-caspase-3 at 1:2000, anti-Fodrin at 1:2000, anti-protein kinase C-δ at 1:6 200, anti-caspase-8 at 1:5, anti-Actin at 1:4000, and anti-Bid at 1:1000 in PBS, 3% nonfat milk powder, and 0.1% Tween-20. The rest of the procedure was done as described previously (37).

**Isolation of Mouse Liver Mitochondria—**The liver of a 6-week-old Balb/c mouse was minced on ice, resuspended in 10 ml of ice-cold Buffer A (200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4, 0.1% bovine serum albumin, mixture of protease inhibitors), and homogenized with a glass Dounce homogenizer and a tight Teflon pestle. Homogenates were centrifuged at 600 × g for 5 min at 4 °C. Supernatants were then recentrifuged at 3500 × g for 15 min at 4 °C. After centrifugation, supernatants and floating lipid layers were aspirated, and the mitochondrial pellet was resuspended in Buffer A and centrifuged at 1500 × g for 5 min at 4 °C. Supernatants were recentrifuged at 5500 × g for 10 min. The last two steps were repeated twice. Mitochondrial pellets were resuspended in 1 ml of Buffer B (200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, pH 7.4, 10 mM Hepes-KOH, pH 7.4, 0.1% bovine serum albumin). Mitochondria were prepared fresh for each experiment and used within 4 h.

**Preparation of Cytosolic Extracts—**Jurkat cells were grown for 3 days in culture in RPMI medium containing 10% fetal bovine serum. The cells were harvested by centrifugation at 200 × g for 10 min at 4 °C, washed twice with PBS, and resuspended in 700 μl of ice-cold buffer (20 mM Hepes-KOH, pH 7.0, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells were disrupted by Dounce homogenization (B pestle/50 strokes). Nuclei were removed by centrifugation at 1000 × g for 10 min at 4 °C in a microcentrifuge. Supernatants were then further centrifuged at 100,000 × g for 1 h. Alternatively, supernatants were centrifuged for 15 min at 14,000 × g in a microcentrifuge. The resulting supernatants were stored at −70 °C and used for in vitro apoptosis assays.

**Isolation of Recombinant Bcl-xL Protein and Caspases—**Bcl21(DE3) cells were transformed with the pET29b-BclxLAC plasmid. Bacteria were grown in 2× TY medium containing 10 μg/ml kanamycin and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside overnight at 30 °C. Bacterial pellets were lysed in 150 ml of buffer (PBS, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, containing protease inhibitors). After being frozen and thawed once, the bacterial lysate was condensed. The cell suspension was condensed. The cell suspension was then loaded on a 2-ml nickel-chelating column. The column was washed with 50–90 ml of Buffer D (50 mM Tris-Cl, pH 8.0, 500 mM NaCl) and then with 10 ml of Buffer C. Protein was eluted by an imidazole gradient (0–200 mM) in Buffer C, pH 8.0.

**Cell-free Apoptosis System—**Standard reactions were carried out in a 50-μl reaction volume with reaction buffer (20 mM Hepes-KOH, pH 7.2, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, 10 mM succinate, 2 mM ATP, 1 mM dATP, 10 mM phosphoethanolamine, 50 μg/ml creatine kinase plus complete mixture of protease inhibitors), 20–50 μg of cytosolic extract, and 5 μg of isolated mitochondrial extract. The reaction mix was incubated at 37 °C for various time periods, and mitochondria were removed by centrifugation at 14,000 × g in a microcentrifuge for 10 min. Supernatants were further used for SDS-PAGE and immunoblotting or for DEVD cleavage assays.

**Measurement of Caspase Activity—**Cytosolic protein (20–100 μg) was diluted in extraction buffer at a final volume of 200 μl in a microtiter plate 100 μM DEVD-pNA substrate (Biomol). Samples were incubated at 37 °C, and absorbance at 405 nm was measured using a SpectraMax microtiter plate reader at 30 min, 1 h, and 2 h.

**RESULTS**

**Caspase Release from Mitochondria Is Prevented by Caspase Inhibition in Fas-induced Apoptosis—**To examine whether most Fas signaling may go through a mitochondrial pathway, Jurkat T-lymphocytes were treated with agonistic anti-Fas antibodies (CH-11), and cytosolic extracts, lacking mitochondria, were prepared at various times and analyzed by immunoblotting. Caspase c accumulated in cytosolic extracts at 2 h after exposure to anti-Fas antibodies, a time when caspase-8 was activated (detected by the 18-kDa active caspase-8 fragment) (Fig. 1, a and b). Following caspase-8

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**FIG. 1.** Cytochrome c is released from mitochondria in Fas-induced apoptosis, which is inhibited by zVAD-fmk. Jurkat T-lymphocytes were treated with anti-Fas antibodies (CH 11, 250 ng/ml) for the indicated times, and cytosolic extracts were prepared. When caspase inhibitors were used, cells were preincubated with 100 μM zVAD-fmk for 1 h and then treated with anti-Fas antibodies. Cytosolic extracts (50 μg) were separated by SDS-PAGE and immunoblotted as described under “Experimental Procedures.” Actin was used as a protein loading control. The percentage of cell death was determined by light microscopy of eosin & hematoxylin-stained cytosins. Cells were scored as apoptotic when the cytoplasm was shrunken and chromatin was condensed. Cyt. c, cytochrome c; casp., caspase; PKC protein kinase C.
activation and cytochrome c release, procaspase-3 became processed at 4 h (recognized by the loss of its pro-form) (Fig. 1). Cleavage of the caspase substrates fodrin and protein kinase C-8 was detectable at 2 h (Fig. 1, d and e). zVAD-fmk treatment prevented cytochrome c release, procaspase-8, and pro-caspase-3 proteolytic processing, substrate cleavage, and cell death (Fig. 1), suggesting that caspase-8 may initiate cytochrome c release in this system.

Mitochondria Amplify Caspase-8-induced DEVD-specific Cleavage Activity—We had previously observed that the activation of caspase-3 by caspase-8 in vitro proceeds identically in the presence or absence of cytosol (29). This raised the question of whether the release of cytochrome c following Fas ligation can contribute to caspase activation. To test the possible role of mitochondria in cell death mediated by Fas or tumor necrosis factor receptors, purified caspase-8 and cytosol were incubated with increasing amounts of mitochondria for various times, and the resulting caspase activity was measured by conversion of the colorimetric substrate, DEVD-pNA. As shown in Fig. 2, caspase-8-induced cleavage activity was low and reached an early plateau after 60 min, when either no or a low amount (1%; v/v) of mitochondria were present. In contrast, the caspase-8-mediated DEVD-specific cleavage activity was significantly elevated in the presence of 5, 10, and 15% (v/v) mitochondrial fraction. Thus, mitochondria enhance caspase-8-induced DEVD-specific cleavage activity, most likely by release of cytochrome c and activation of the Apaf-1-procaspase-9 complex.

Caspase-8-induced Cytochrome c Release from Mitochondria Requires Cytosol—To examine whether caspase-8 would induce cytochrome c release from mitochondria, cytosolic Jurkat T-lymphocyte cell extracts, purified caspase-8 and mitochondria were incubated in vitro. When cytosol was incubated with mitochondria alone, no cytochrome c release was observed, indicating that the mitochondrial preparation was not releasing cytochrome c nonspecifically (Fig. 3A). Similarly, when caspase-8 was incubated with the mitochondrial fraction alone, no cytochrome c release was detectable by immunoblotting, indicating that caspase-8 had no direct effect on mitochondria (Fig. 3B). However, when caspase-8, cytosol, and mitochondria were incubated together, cytochrome c was released from mitochondria within 20 min (Fig. 3C, lane 4), and reached maximum release at 90 min (Fig. 3C, lane 7). These results indicate that caspase-8 is capable of mediating cytochrome c release in the presence of cytosolic extract, presumably by proteolytically activating one or several cytosolic cytochrome c releasing factors.

Ultrafiltration of Cytosol Removes the Caspase-8-induced Cytochrome c Releasing Activity—To further characterize the caspase-8-induced cytochrome c releasing activity, cytosolic extract from Jurkat cells was filtered to remove proteins that were larger than 10 kDa, prior to incubation with caspase-8, and mitochondria. Fig. 4A shows a control experiment, in which unfiltered cytosol and mitochondria without caspase-8 were incubated. As shown above (Fig. 3A), no cytochrome c release was observed under these conditions. On the other hand, incubation of caspase-8 with unfiltered cytosolic extract and mitochondria induced rapid cytochrome c release, detectable after 10 min at 37 °C (Fig. 4B). However, when cytosol was first filtered, caspase-8-induced cytochrome c release from mitochondria was abolished (Fig. 4C). These data indicate that the caspase-8-induced cytochrome c releasing activity resides in the cytosol and is likely a protein larger than 10 kDa.

Caspase-8-induced Cytochrome c Releasing Factor Is Not Inhibited by zVAD-fmk—Other caspase family members are known caspase-8 substrates (18, 21, 23–29). Therefore, it is conceivable that caspase-8 activates another caspase, which then acts directly on mitochondria to release cytochrome c. To test this possibility, cytosol was treated either simultaneously with caspase-8 and caspase inhibitor, zVAD-fmk, or first with caspase-8, allowing proteolytic processing of the putative substrates, and then with zVAD-fmk (to inhibit possible downstream caspases), and mitochondria were added. Fig. 5a, lanes 1–4, shows cytochrome c release in response to cytosol and caspase-8, when no caspase inhibitor was added. When caspase-8, cytosol and zVAD-fmk were incubated together from the beginning, no cytochrome c release from mitochondria was observed, confirming that the inhibitor was effective in blocking caspase activity (Fig. 5b, lanes 1–4). Interestingly, when cytosol was pretreated with caspase-8, and then zVAD-fmk and mitochondria were added, cytochrome c release proceeded with similar kinetics as in control experiments, without zVAD-fmk (Fig. 5b, lanes 5–8). Thus, the cytosolic target of caspase-8, which triggers the release of cytochrome c from mitochondria, is unlikely to be a caspase.

Bcl-2/Bcl-xL Inhibit the Caspase-8-induced Cytochrome c Releasing Activity—Bcl-2 and Bcl-xL prevent cytochrome c release from mitochondria during apoptosis induced by different...
stimuli (30, 34–36, 41, 42). To test the effect of Bcl-2 on caspase-8-induced cytochrome c release, mitochondria were isolated from CEM-neo or CEM-Bcl-2 cells, which over-express Bcl-2. Fig. 6A, a, shows the amount of Bcl-2 overexpression in CEM-Bcl-2 cells. Mitochondria from both cell lines were incubated with caspase-8 and cytosolic extracts, and cytochrome c release was measured by immunoblotting. As shown in Fig. 6A, b, cytochrome c was rapidly released from the mitochondria of CEM-neo cells, starting at 10 min of incubation with active caspase-8. In contrast, cytochrome c release from CEM-Bcl-2 mitochondria was considerably delayed, occurring only at 40 min (Fig. 6A, b). Similarly, when increasing amounts of purified, recombinant Bcl-xL protein were added to cytosol containing caspase-8 and mitochondria, cytochrome c release was inhibited (Fig. 6C). In contrast, increasing amounts of proapoptotic Bax protein had a moderate additive effect on the caspase-8-induced cytochrome c releasing activity. Taken together, these results indicate that the cytochrome c releasing activity can be inhibited by cell death inhibitors Bcl-2 or Bcl-xL.

**Bid Cleavage by Caspase-3 and Caspase-8**—Recent reports identified Bid, a proapoptotic Bcl-2 family member, as a cytosolic protein that triggers cytochrome c release from mitochondria after proteolytic processing by caspase-8 (61–64). Therefore, we tested the ability of caspase-3, caspase-6, caspase-7, and caspase-8 to cleave Bid protein. Recombinant, active site-titrated caspases were used. Cytosolic extracts from Jurkat cells were incubated with the caspases for various time periods, and the cleavage of Bid was determined by immunoblotting (Fig. 7). Caspase-8 cleaved Bid most effectively, which was detectable at 5 min and was completed at 15 min. Similarly, caspase-3 caused Bid cleavage, but with slower kinetics than caspase-8. In contrast, no cleavage of Bid was detectable by caspase-6 and caspase-7.

Because caspase-8 can cleave and activate caspase-3, we also examined how effectively Bid would be cleaved in comparison to caspase-3. Interestingly, Bid and caspase-3 were processed by caspase-8 with similar rates (Fig. 7).

**Cytosolic Cytochrome c Release by Effector Caspases Requires Cytosolic**—We then tested whether effector caspases (caspase-3, caspase-6, and caspase-7) would also promote cytochrome c translocation from mitochondria in vitro. Three different concentrations of purified active caspases (20, 50, and 100 nM), normally found in cells, were tested for their ability to initiate release of cytochrome c in the presence or absence of cytosol. Fig. 8A shows that all caspases provoked cytochrome c release from isolated mitochondria in vitro. However, this effect was dependent on the presence of cytosol, indicating that none of the caspases had a direct effect on mitochondria at physiological concentrations. Caspase-3 and caspase-8 were more effective among the tested caspases to induce cytochrome c release, with a maximum release at 30 min and at a concentration of 20 nM. Similarly, caspase-6 was able to initiate cytochrome c release. Caspase-7 was the least efficient among the tested caspases in promoting cytochrome c release, which was only detectable at 60 min and concentrations of 50 and 100 nM.

In the same experiment, we tested whether cytosolic Bid was cleaved. Fig. 8B shows that although all caspases induced cytochrome c release from isolated mitochondria, Bid was only cleaved by caspase-3 and caspase-8. In addition, Bid was less effectively processed by caspase-3 than by caspase-8, although both caspases released cytochrome c with similar, rapid kinetics. Neither caspase-6 nor caspase-7 cleaved Bid (Figs. 7 and 8B), despite their ability to induce cytochrome c release (Fig. 8A). Thus, cytosol may contain a caspase substrate, which differs from Bid and which, in response to cleavage by effector caspases, releases cytochrome c.

**DISCUSSION**

Binding of Fas ligand to the Fas receptor results in autoprocessing and activation of caspase-8 (13–22). Active caspase-8 directly engages the caspase cascade by activating and cleaving caspase-3 (29). Alternatively, caspase-8 can activate other caspases indirectly, by inducing release of cytochrome c from mitochondria. Once in the cytoplasm, cytochrome c binds and activates Apaf-1 and procaspase-9 (38, 39). Active caspase-9 then cleaves and activates procaspase-3. In this study, we examined the role of mitochondria in Fas-mediated cell death. We have found that cytochrome c is released from mitochondria in Jurkat cells undergoing cell death by Fas activation (Fig. 1). Unlike cell death induced by other stimuli (37), the cytochrome...
Release by Fas signaling was effectively prevented by caspase inhibitors, rendering caspase-8 and other caspase family members likely candidates for this process. This is in agreement with other reports, describing inactivation of electron transport and subsequent cytochrome c loss from mitochondria in Fas-mediated cell death (30, 32, 41, 42). At 12 h, some cytochrome c accumulated in zVAD-fmk plus anti-Fas treated cells (Fig. 1a). An explanation might be that long-term Fas-receptor ligation may activate other signaling molecules with cytochrome c releasing activity and that are not blocked by caspase inhibitors. A candidate for such a signaling molecule might be Daxx, which is implicated in Fas-mediated apoptosis (47).

Several cell-free systems of apoptosis revealed that mitochondria play a pivotal role in the regulation of cell death (48, 49). We show here that purified caspase-8 induces rapid cytochrome c release from mitochondria in vitro (Fig. 2). However, the effect was indirect, because a cytosolic extract was required (Fig. 3C), suggesting that caspase-8 cleaves and activates one or several cytosolic targets, provoking cytochrome c release. Similar results were obtained in a Xenopus cell-free system, in which caspase-8 induced cytochrome c release indirectly by cleaving at least one cytosolic factor (50). Recent reports identified Bid as cytosolic protein that induces cytochrome c release in response to cleavage by caspase-8. Bid is a proapoptotic member of the Bcl-2 family that interacts with Bcl-2 and Bax (61). Bid is normally present in the cytosol as inactive protein and undergoes caspase cleavage in response to Fas or tumor necrosis factor receptor signaling (62–64). The carboxyl-terminal fragment of Bid translocates then to mitochondrial membranes and triggers cytochrome c release. Here, we show that Bid is cleaved rapidly by caspase-8 (Fig. 7). In addition,
Caspase-induced Cytochrome c Release

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Bax, another proapoptotic member of the Bcl-2 family, can directly induce cytochrome c release from mitochondria (51, 52). Bax protein resides in the cytoplasm in living cells and translocates to mitochondria when cells receive an apoptotic signal (53, 54). Thus, one can speculate that another cytosolic caspase target could be either a Bax-binding protein, which keeps it in an inactive state in the cytoplasm, or Bax itself. There is precedent for Bcl-2 family members being caspase substrates. Caspase-3 can cleave Bcl-xL, converting it into a less good substrate for caspase-3 (63, 64).

There is precedent for Bcl-2 family members being caspase targets. Caspase-3 can cleave Bcl-xL, converting it into a less good substrate for caspase-3 (63, 64).

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