Bax directly induces release of cytochrome c from isolated mitochondria

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ABSTRACT Bax is a pro-apoptotic member of the Bel-2 protein family that resides in the outer mitochondrial membrane. It is controversial whether Bax promotes cell death directly through its putative function as a channel protein versus indirectly by inhibiting cellular regulators of the cell death proteases (caspases). We show here that addition of submicromolar amounts of recombinant Bax protein to isolated mitochondria can induce cytochrome c (Cyt c) release, whereas a peptide representing the Bax BH3 domain was inactive. When placed into purified cytosol, neither mitochondria nor Bax individually induced proteolytic processing and activation of caspases. In contrast, the combination of Bax and mitochondria triggered release of Cyt c from mitochondria and induced caspase activation in cytosol. Supernatants from Bax-treated mitochondria also induced caspase processing and activation. Recombinant Bcl-XL protein abrogated Bax-induced release of Cyt c from isolated mitochondria and prevented caspase activation. In contrast, the broad-specificity caspase inhibitor benzoxycarbonyl-valinyl-alaninyl-aspartyl-(0-methyl)-fluoromethylketone (zVAD-fmk) and the caspase-inhibiting protein X-IAP had no effect on Bax-induced release of Cyt c from mitochondria in vitro but prevented the subsequent activation of caspases in cytosolic extracts. Unlike Ca2+, a classical inducer of mitochondrial permeability transition, Bax did not induce swelling of mitochondria in vitro. Because the organellar swelling caused by permeability transition causes outer membrane rupture, the findings, therefore, dissociate these two events, implying that Bax uses an alternative mechanism for triggering release of Cyt c from mitochondria.

Bcl-2 family proteins play a pivotal role in controlling cell life and death, with some members such as Bcl-2 and Bcl-XL inhibiting apoptosis and others such as Bax inducing cell death (1). Many Bcl-2 family proteins are anchored in the outer membrane of mitochondria by a C-terminal hydrophobic stretch of amino acids (2, 3). Moreover, Bcl-2 family proteins can have profound influences on mitochondrial alterations associated with apoptosis. For example, during apoptosis, several mitochondrial events typically occur, including loss of the electrochemical gradient (∆Ψ), release of Cyt c, and activation of caspases, whereas Bax induces these changes (5, 6, 9–11).

How Bax induces and Bcl-2 inhibits these mitochondrial alterations is currently controversial, but two prevailing theories have been advanced (reviewed in ref. 11). One view relates to the structural similarity of some Bcl-2 family proteins to the pore-forming domains of certain bacterial toxins, such as diphtheria toxin and the colicins that form channels in biomembranes for transport of proteins and ions, respectively (12). In this regard, both anti-apoptotic (Bel-2, Bel-XL) and pro-apoptotic (Bax) members of the Bcl-2 family have been demonstrated to form ion channels in synthetic membranes in vitro (13–16). Moreover, under some in vitro circumstances, Bcl-2 can evidently prevent the formation of Bax channels in liposomes. The dissipation of the mitochondrial ∆Ψ during apoptosis has been attributed to the opening of the mitochondrial megapore, a cyclosporine-inhibitable high-conductance channel that appears to be comprised of multiple proteins, including inner and outer membrane proteins that come into contact at the junctional complexes of this organelle (17, 18). Conceivably, Bcl-2 and Bax could participate in the formation or regulation of this large channel. Alternatively, because some studies have suggested that release of Cyt c from mitochondria precedes loss of ∆Ψ (5, 6), it is possible that Bax creates pores in the outer membrane which are large enough to allow escape of Cyt c, resulting secondarily in megapore opening from either failed electron chain transport or because of Cyt c-mediated activation of caspases which then cleave mitochondrial proteins (19).

An opposing view has been deduced from studies of the cell death proteins discovered in the nematode Caenorhabditis elegans. The worm homolog of Bel-2 (CED-9) has been shown to interact with a caspase (CED-3) via a bridging protein CED-4 that binds simultaneously to CED-9 and CED-3 (20–22). CED-9 prevents CED-4 from inducing proteolytic processing and activation of CED-3 (23). Bcl-XL can also bind to the worm CED-4 protein and alter its location in cells, pulling it from the cytosol to the intracellular membranes where Bcl-2 family proteins often reside (21, 22). However, heterodimerization of Bax-like proteins with Bcl-XL abrogates binding of Bcl-XL to CED-4, presumably freeing it to activate caspases (22). Because caspases have been shown to induce mitochondrial permeability transition (PT) (19), it has been argued that Bax may indirectly induce release of Cyt c and loss of mitochondrial ∆Ψ by interfering with the suppressive effects of Bcl-XL and similar anti-apoptotic Bcl-2 family proteins on CED-4-like proteins, thus allowing caspase activation to occur and thereby secondarily triggering megapore opening.

By using isolated mitochondria and recombinant Bax protein, we present evidence that Bax can directly induce Cyt c release.

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This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: Cyt c, cytochrome c; PT, permeability transition; zVAD-fmk, benzoxycarbonyl-valinyl-alaninyl-aspartyl-(0-methyl)-fluoromethylketone; AFC, 7-amino-4-trifluoromethyl-coumarin; DEVD, Asp-Glu-Val-Asp; CsA, cyclosporin A.

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release from mitochondria without apparent requirement for caspases. Moreover, this Bax-mediated release of Cyt c is not accompanied by permeability transition and the attendant mitochondrial swelling that is known to rupture the outer membrane.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins. Murine Bax or human Bel-XL were expressed recombinantly as described (24). X-IAP protein was prepared as described (25). Proteins (0.2–0.4 mg/ml) were dialyzed into 20 mM Hepes (pH 7.5), 10 mM KCl, 20 mM MgCl₂, and 1 mM EDTA before use in all experiments.

Isolation of Mitochondria. Female rats were killed by decapitation, and mitochondria were purified from the liver by differential centrifugation and separation on a sucrose gradient (26). Mitochondria were suspended at 10 mg protein/ml in MSB (400 mM mannitol/50 mM Tris-HCl, pH 7.2/5 mg/ml BSA/10 mM KCl/400 mM mannitol) and kept on ice for up to 4 hr.

Cyt c Release Assays. Mitochondria (50 μg protein) were incubated with recombinant proteins (1 μM Bax; 1–12 μM Bel-XL; 0.2 μM X-IAP) or 50 μM HPLC-purified Bax-BH₃ peptide (NH₃-KKLESLKRIGDELDS-amide) (residues 61–76 of human Bax) (Chiron) with or without 10 μM CsA (Novartis, Basel, Switzerland) or 10 μM benzoylcyonaroyl-valinyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone (zVAD-fmk; Enzyme Systems Products, Livermore, CA) in 50 μl of MSB at 30°C for 1 hr. Mitochondria were pelleted by centrifugation at 4,000 x g for 5 min, solubilized in RIPA buffer, and analyzed by immunoblotting with antibodies to F₁-β-ATPase (gift of W. Neupert, Munich, Germany) or Bax (27). The resulting supernatants were electrophoresed for caspase assay activities or analyzed by SDS/PAGE immunoblotting with either 10–20% gradient gels or 15% gels with high Tris (75 mM). Proteins were transferred to nitrocellulose (pore size, 0.1 μm), and the blots were incubated with mAb 7H8.2C12 (PharMingen, or gift of R. Jemmerson, University of Minnesota, Minneapolis, MN) (28, 29) followed by ECL-based detection (30). Similar results were obtained by using a variety of buffer conditions (31, 32).

Assay for Mitochondrial Permeability Transition. The in vitro swelling of mitochondria caused by induction of PT was assayed by using mitochondria resuspended in CFS (220 mM mannitol/65 mM sucrose/2 mM NaCl/5 mM KH₂PO₄/2 mM MgCl₂/10 mM Hepes-NaOH, pH 7.4/5 mM succinate/2 mM ATP/50 mM creatine phosphokinase/10 mM phosphocreatine) with 2 μM rotenone (9, 33–35). Mitochondria were treated with 1 μM Bax or 150 μM Ca²⁺, in the presence or absence of 10 μM CsA, and the absorbance variation caused by swelling was measured by using a double-beam spectrophotometer at 520 nm.

Assay for Mitochondria-Dependent Caspase Activation. Cytosolic extracts were prepared from 293 embryonic kidney cells as described (25). Supernatants (10 μl) from Bax-treated or control mitochondria were incubated with 10 μl of cytosolic extract at 30°C for 1 hr. Aliquots were utilized for caspase assays, initiating caspase processing and activation by addition of 1 mM dATP (7). Alternatively, purified mitochondrial (50 μg protein in 5 μl MSB) were incubated in 30 μl of cytosolic extract, with or without various recombinant proteins and compounds as described above. After incubation at 30°C for 1 hr, mitochondria were removed by centrifugation, and the resulting supernatants were analyzed for caspase activity or used for immunoblot assays with polyclonal antisera to caspase-3 (25).

Caspase Assay. Caspase activity was assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) from DEVD containing synthetic peptides using continuous-reading instruments as described (25, 36). Typically, 1 μl of final cytosol-containing solution was added to 99 μl caspase buffer [50 mM Hepes/0.1 M NaCl/1 mM EDTA/0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/10% sucrose/5 mM DTT] and reactions were initiated by addition of 100 μM Ac-DEVD-AFC (Enzyme Systems Products).

Transfections and Subcellular Fractions. 293T cells were seeded into 10-cm dishes and transfected with pcDNA3 control, pcDNA Bax (5 μg), pGFP (3 μg), or various combinations of these plasmids (normalized for total 25 μg DNA) by a CaPO₄ precipitation method (37). Transfection efficiency was 20–50%, as monitored by green fluorescent protein (GFP) fluorescence. Cells were lysed after 24 hr in HEB (50 mM Pipes/50 mM KCl/5 mM EGTA/2 mM MgCl₂/10 μM Cytochalasin/1 mM DTT) for 20 min on ice. The samples were adjusted to 220 mM mannitol and 68 mM sucrose (final concentration) and were dounced briefly before pelleting organelles by centrifugation at 16,000 g for 0.5 hr. The resulting supernatants (cytosol) and pellets were mixed with RIPA buffer and analyzed by SDS/PAGE immunoblotting (25 μg per lane) as described above.
analyzed by SDS
jected to SDS
pelleted by centrifugation, and the resulting supernatants were sub-
failed to induce release of Cyt
peptide, both, or neither of these reagents. Cyt
Bcl-XL as determined by surface plasmon resonance (BIA-
DEVD-AFC as a substrate. As shown in Fig. 2,
release was measured 0.5 hr later by spectrofluorimetric assays with
mitochondria were then incubated with 1 M Bax or diluent control (C) were added directly to cytosol. After incubation at 30°C for 0.5 hr, caspase activity was measured by
mitochondria were pelleted by centrifugation and caspase activity was measured in the resulting supernatant. In C, an aliquot of cytosolic fractions was subjected to SDS/PAGE immunoblot assay by using anti-Cyt c antibody. As an additional control (Left), cytosol was treated with 10 M Bax. Addition of either Bax or mitochondria alone was sufficient for triggering activity of this DEVD-cleaving caspase (Fig. 2C).
Bax Does Not Induce Mitochondrial Swelling. The swelling of mitochondria is a colloidosmotic process that is observed during induction of PT in vitro (17, 33). To determine whether Bax triggers opening of the mitochondrial megapore leading to PT, mitochondria were exposed to Bax protein under conditions previously shown to be conducive for swelling induced by Ca2+ and other activators of mitochondrial PT. As expected, addition of Ca2+ to isolated mitochondria induced rapid swelling, which was measured as a decrease in OD at 520 nm (Fig. 3). This Ca2+-induced swelling of mitochondria was completely abrogated by prior addition of cyclosporin A (CsA), which is known to block mitochondrial PT by inhibiting a mitochondrial cyclophilin which regulates the megapore (17, 33–35). In contrast to Ca2+, Bax did not induce mitochondrial swelling under these same conditions. Electron microscopy representing the BH3 domain of Bax, because mitochondria isolated from rat liver might have anti-apoptotic Bcl-2 family proteins integrated into their outer membranes that perhaps could be neutralized with BH3 peptides (40). In contrast to recombinant Bax protein, addition of 50 M BH3 peptide failed to induce release of Cyt c release from mitochondria in vitro (Fig. 1D). At these or lower concentrations, however, the same BH3 peptide completely prevented binding of Bax to Bel-XL, as determined by surface plasmon resonance (BLA-
core) experiments (24), thus confirming its ability to bind Bcl-2 family proteins.

**Bax-Treated Mitochondria Release Factors that Activate Caspases in Vitro.** Cyt c has been shown to induce processing and activation of caspase-3 and some other caspases that cleave the peptide DEVD (7, 25). To investigate whether Bax-treated mitochondria could release factors that induce activation of DEVD-cleaving caspases, isolated mitochondria were treated in vitro for 1 hr with either Bax protein or diluent control, followed by removal of the mitochondria by centrifugation and collection of the resulting supernatants. These supernatants and control supernatants were then added to cytosolic fractions derived from human cell lines, and caspase activity was measured 0.5 hr later by spectrofluorimetric assays with DEVD-AFC as a substrate. As shown in Fig. 2A, Bax by itself failed to induce increases in caspase activity in cytosols.

Addition of supernatants derived from control-treated mitochondria caused only a modest elevation in DEVD-cleaving activity. In contrast, supernatants from Bax-treated mitochondria consistently resulted in striking increases in caspase activity. Similar results were obtained when mitochondria were added directly to cytosolic fractions. The combination of Bax plus mitochondria resulted in marked increases in caspase activity, whereas neither Bax nor mitochondria individually stimulated substantial elevations in caspase activity (Fig. 2B). Immunoblot analysis of these same cytosolic extracts demonstrated that the combination of Bax plus mitochondria induced proteolytic processing of caspase-3, whereas addition of neither Bax nor mitochondria alone was sufficient for triggering processing of this DEVD-cleaving caspase (Fig. 2C).
investigated whether CsA affected Bax-induced Cytc. The presence of CsA prevented the release of Cytc completely and also prevented the activation of caspases that is normally induced by addition of Bax protein and mitochondria to cytosolic extracts (Fig. 6). In contrast, the sample in which CsA was dissolved had no effect on Bax-induced Cytc release. Similar results were obtained with a non-immunosuppressive cyclosporine analogue (data not shown). CsA did not prevent the binding of Bax to mitochondria (Fig. 4C), excluding this as a trivial explanation for the observed suppression of Bax-induced Cytc release.

Caspases Are Not Required for Bax-Induced Release of Cytc from Mitochondria. The effects of Bax on Cytc release from mitochondria could be an indirect consequence of Bax-induced activation of caspases that subsequently cleave mitochondrial proteins and liberate Cytc from these organelles (19). To explore this possibility, zVAD-fmk, an irreversible broad-specificity peptidyl inhibitor of caspses, was added to isolated mitochondria prior to Bax. As shown in Fig. 5B, zVAD-fmk did not inhibit Bax-induced release of Cytc from isolated mitochondria. However, when zVAD-fmk was added with mitochondria to cytosolic extracts, Bax-induced processing of caspase-3 was suppressed by zVAD-fmk, and only small amounts of a partially processed form of caspase-3 appeared (Fig. 5). Similar results were obtained when zVAD-fmk was replaced with ~0.2 μM recombinant X-IAP protein (data not shown), an anti-apoptotic protein that directly binds to and inhibits caspses within the Cytc pathway (27). The addition of CsA or CsA plus mitochondria, Bax, or Bcl-XL, with or without zVAD-fmk, did not inhibit Bax-induced Cytc release (Fig. 5A). Thus, the observed suppression of Bax-induced Cytc release by CsA and CsA plus mitochondria was due to the inhibition of the binding of Bax to mitochondria (Fig. 4C), excluding this as a trivial explanation for the observed suppression of Bax-induced Cytc release.

CsA Inhibits Bax-Induced Release of Cytc from Mitochondria. Although Bax did not induce mitochondrial swelling, we investigated whether CsA affected Bax-induced Cytc release. As shown in Fig. 4B, incubation of mitochondria with Bax in the presence of CsA prevented the release of Cytc. CsA also completely prevented the activation of caspases that is normally induced by addition of Bax protein and mitochondria to cytosolic extracts (Fig. 6). In contrast, the sample in which CsA was dissolved had no effect on Bax-induced Cytc release. Similar results were obtained with a non-immunosuppressive cyclosporine analogue (data not shown). CsA did not prevent the binding of Bax to mitochondria (Fig. 4C), excluding this as a trivial explanation for the observed suppression of Bax-induced Cytc release.

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of zVAD-fmk or X-IAP protein to cytosolic extracts prior to Bax and mitochondria also induced caspase activity as measured by DEVD hydrolysis, confirming that these caspase inhibitors were active in these assays (data not shown). Taken together, these data suggest that Bax directly induces release of Cyt c from mitochondria without requirement for caspases. Moreover, the findings indicate that Cyt c release precedes processing of caspase-3.

DISCUSSION

Elevations in Bax protein levels are induced in several clinically relevant settings where cell death occurs, including tumor cells during responses to chemotherapy and radiation (38, 41), neurons following cerebral ischemia (27), and cardiomyocytes following acute myocardial infarction (42). However, the mechanisms by which Bax promotes apoptosis have been difficult to determine. In many other areas of cell biology and metabolism, in vitro reconstitution of complicated pathways has greatly assisted analysis of the cause-and-effect relations among the various components. Here we describe a step toward this goal, demonstrating that addition of recombinant purified Bax protein to isolated mitochondria can induce Cyt c release.

A critical issue in understanding the mechanism by which Bax induces dissipation of the mitochondrial ΔΨ and caspase activation in cells is whether these events are direct effects of Bax, perhaps related to its putative function as a channel protein, versus indirect consequences of Bax-mediated effects on anti-apoptotic members of the Bcl-2 family that might prevent caspase activation by inhibiting CED-4-like proteins. The data presented here suggest that Bax can directly induce release of Cyt c, without apparent requirement for caspases. Of course, the caveat must be raised that undiscovered caspases may exist that are not inhibited by zVAD-fmk, even at the high concentrations employed here (100 μM). Our data, therefore, cast doubts on models that envision Bax as merely a transdominant inhibitor of anti-apoptotic Bcl-2 family proteins that triggers the release of CED-4-like proteins upon heterodimerizing with Bcl-2 or Bcl-XL.

If Bax does not rely on caspases for inducing release of Cyt c from mitochondria, then how does it accomplish this? One possibility is that recombinant Bax protein heterodimerizes with anti-apoptotic proteins such as Bcl-2 and Bcl-XL that might be present endogenously in the outer membranes of mitochondria isolated from rat liver. Previous studies have demonstrated the dependence of the BH3 domain of Bax for its homo- or heterodimerization with itself and other anti-apoptotic Bcl-2 family proteins, respectively (43). Moreover, expression in mammalian cells of Bak fragments that contain only one CsA-binding protein (10, 17, 47, 48).

If CsA on Bax function complex is the F_{0}F_{1}-ATPase/proton-pump of the inner membrane, which is required for optimal Bax-induced cell death (46). Regardless of the actual mechanism, these findings with CsA illustrate an advantage of using a cell-free system for evaluating the effects of Bax on mitochondrial function in that this approach permits some experiments that are impossible in cells. For example, micromolar concentrations of CsA are required to inhibit the megapore of mitochondria but generally only nanomolar concentrations of this drug are tolerated by cells. Moreover, CsA affects other cellular proteins, complicating interpretation of the results derived from intact cells. In contrast, mitochondria contain only one CsA-binding protein (10, 17, 47, 48).

The data reported here are consistent with evidence that gene transfer-mediated overexpression of Bax in whole cells can induce mitochondrial ΔΨ even in the presence of the broad-specificity caspase inhibitor zVAD-fmk (49), suggesting that Bax can have effects on mitochondria even when caspases are inhibited. However, our findings go further to demonstrate that Bax directly induces release of Cyt c from mitochondria without necessarily triggering mitochondrial PT. Thus, we can deduce that Bax-induced release of Cyt c does not necessarily occur as a secondary consequence of PT-induced rupture of the mitochondrial outer membrane. This finding does not discount the possibility that stimuli which use the megapore as their primary target, such as elevated cytosolic Ca^{2+}, can induce Cyt c release by a PT-dependent mechanism. Thus, more than one mechanism for releasing Cyt c probably exists, with the Bax-mediated route occurring without concomitant swelling of mitochondria and subsequent rupture of the outer membrane but other stimuli relying upon it.

Though it could be argued that Bax-induced release of Cyt c from mitochondria should have secondarily induced mitochondrial PT because of failed electron chain transport and subsequent generation of reactive oxygen species, we noted that Bax typically induced release of only ~20% of the total Cyt c from mitochondria. Thus, under these conditions, presumably sufficient Cyt c remains associated with the inner membrane to sustain electron chain transport. However, this small proportion of Cyt c released is evidently capable of potently triggering caspase activation. Of note, it has been reported that even with isolated mitoplasts in which the outer membrane has been selectively solubilized, Cyt c remains tightly bound to the inner membrane through its interactions with proteins such as Cyt c oxidase, typically requiring high salt concentrations to efficiently release it (50). Thus, by releasing only a small proportion of the total Cyt c initially, Bax may favor induction of a cell death process that involves rapid activation of caspases causing apoptosis, as opposed to the
oxidative stress type of cell death that generally befalls mitochondrial PT and which can lead to necrosis.

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