Caspase-2 Permeabilizes the Outer Mitochondrial Membrane and Disrupts the Binding of Cytochrome c to Anionic Phospholipids

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Mari Enoksson†, John D. Robertson‡, Pengli Bu§, Andrey Kropotov‡, Boris Zhivotovsky‡, Vladimir Gogvadze‡, and Sten Orrenius¶

From the †Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden and the ‡Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, Kansas City, Kansas 66160

Caspases are cysteine proteases that play a central role in the execution of apoptosis. Recent evidence indicates that caspase-2 is activated early in response to genotoxic stress and can function as an upstream modulator of the mitochondrial apoptotic pathway. In particular, we have shown previously that fully processed caspase-2 can permeabilize the outer mitochondrial membrane and cause cytochrome c and Smac/DIABLO release from these organelles. Using permeabilized cells, isolated mitochondria, and protein-free liposomes, we now report that this effect is direct and depends neither on the presence or cleavage of other proteins nor on a specific phospholipid composition of the liposomal membrane. Interestingly, caspase-2 was also shown to disrupt the interaction of cytochrome c with anionic phospholipids, notably cardiolipin, and thereby enhance the release of the hemoprotein caused by treatment of mitochondria with digitonin or the proapoptotic protein Bax. Combined, our data suggest that caspase-2 possesses an unparalleled ability to engage the mitochondrial apoptotic pathway by permeabilizing the outer mitochondrial membrane and/or by breaching the association of cytochrome c with the inner mitochondrial membrane.

Apoptosis is executed by a family of cysteine proteases known as caspases. In vertebrates, these enzymes are present constitutively as zymogens and become activated in response to numerous apoptotic stimuli (1). Once activated, caspases cleave proteins after aspartate residues and thereby bring about many of the biochemical and morphological features characteristic of apoptosis. Of the two primary caspase activation pathways (death receptor-mediated and mitochondrially mediated), the mitochondrial pathway is most commonly associated with apoptosis triggered by cytoxic stress and involves the activation of caspase-9 within the Apaf-1-containing apoptosome complex (2). Until recently, the dominant viewpoint has been that caspase-9 activation represents the apex of the caspase cascade during stress-induced apoptosis. However, this hypothesis is currently being challenged by evidence suggesting that caspase-2 may be critical for the engagement of the mitochondrial pathway, and hence caspase-9 activation, during DNA damage-induced apoptosis (3–5).

Although it was the first mammalian apoptotic caspase to be identified (6, 7) and is the evolutionarily best conserved member of this family (8), the role of caspase-2 in apoptotic cell death remains contentious. Like caspase-9 it contains a caspase recruitment domain (CARD), but recent evidence indicates that caspase-2 activation occurs in an Apaf-1-independent complex, which also includes the death domain (DD)-containing protein PIDD and the adaptor protein RAIDD (9, 10). Additional findings have shown that caspase-2 retains a unique ability to engage directly the mitochondrial apoptotic pathway (4, 11), an effect that appears to require processing of the zymogen but not the associated catalytic activity (12).

The aim of the current study was to determine more precisely the molecular requirements for caspase-2-mediated engagement of the mitochondrial apoptotic pathway. The results indicate that processed recombinant human caspase-2 triggers a rapid and concentration-dependent release of cytochrome c from isolated mitochondria as well as liposomes. This occurs independently of other proteins and does not require a specific phospholipid profile within liposomes. In addition, our results demonstrate that caspase-2 has the capacity to detach cytochrome c from the inner mitochondrial membrane by compromising its association with anionic phospholipids.

EXPERIMENTAL PROCEDURES

Expression and Purification of Caspase-2 Proteins—Bacterial expression plasmids containing active, catalytically inactive (C303A) (11), or uncleavable (D316G) recombinant caspase-2 were expressed in Escherichia coli strain BL21(DE3) as C- or N-terminal His6-tagged proteins using pET-21a or pET-28a vector (Novagen) and purified by standard Ni2+-affinity chromatography.

Site-directed Mutagenesis—Active caspase-2 was used as a template to introduce a D316G point mutation using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation was confirmed by sequence analysis.

Cell Culture—Jurkat T-lymphocytes were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2% (v/v) glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified air/CO2 (19:1) atmosphere at 37 °C. Cells were maintained in a logarithmic growth phase for all experiments.

Digitonin-permeabilized Cells—Jurkat T-lymphocytes (106) were washed in phosphate-buffered saline and resuspended in 100 μl KCl sucrose buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH2PO4, 5 mM succinate, 1 mM EGTA, 5 mM Tris, pH 7.4). Following a 2-min stabilization period, cells were permeabilized with 5 μg of digitonin, and 5 μM rotenone was added to maintain pyridine nucleotides in a reduced form.

Isolation of Rat Liver Mitochondria—The liver of a male Sprague-Dawley rat was homogenized in 1 ml KCl sucrose buffer using a Dounce homogenizer. The homogenate was centrifuged at 9000 × g for 10 min at 4 °C. The mitochondrial pellet was resuspended in 1 ml KCl sucrose buffer and recentrifuged.

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Dawley rat was minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a tight-fitting glass-Teflon motor-pellet by centrifugation at 100,000 g for 20 min, and the supernatant was subsequently separated from the pellet by centrifugation at 5,500 g for 15 min, and resuspended in MSH buffer at a protein concentration of 80–100 mg/ml.

Caspase-2-induced Cytochrome c Release—Isolated rat liver mitochondria (1 mg/ml) were incubated in the presence or absence of recombinant caspase-2 in a final volume of 100 μl. Incubations were performed either in buffer A, consisting of 140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH2PO4, 5 mM sucinate, 1 mM EGTA, pH 7.4, or buffer B (where indicated), consisting of 150 mM KCl, 1 mM KH2PO4, 5 mM succinate, 1 mM EGTA, 5 mM Tris, pH 7.4. For caspase-2-induced cytochrome c release from Jurkat T-lymphocytes, cells (10⁶) were washed in phosphate-buffered saline, resuspended in 100 μl buffer A, and permeabilized with digitonin (5 μg). Following a 1-min stabilization period, the indicated amount of recombinant caspase-2 was added.

Preparation of Liposomes—Lyophilized diloeyl phosphatidylcholine (PC),¹ phosphatidylserine (PS), and bovine heart cardiolipin (CL) (all from Sigma) were resuspended in chloroform to concentrations of 10–50 mg/ml. Chloroform-loaded liposomes consisting of PC (80%) and PS (20%), PC (80%), and CL (20%) or PC (100%) were prepared using published protocols (13). Briefly, liposomes of uniform size were prepared in sucrose buffer (256 mM sucrose, 5 mM Tris, pH 7.4) using a lipid extruder equipped with 0.1-μm pore-sized polycarbonate filter (Avanti Polar Lipids, Alabaster, AL). Liposomes prepared for pore-forming experiments were made in sucrose buffer supplemented with 5 mg/ml 20-kDa dextran-fluorescein isothiocyanate (FITC) (Sigma) or 50–200 μg/ml bovine heart cytochrome c (Sigma). After five washes in KCl buffer, the liposome pellets were resuspended in sucrose buffer and stored for a maximum of 1 week at 4 °C. Dextran-FITC liposomes were used within 24 h.

Caspase-2-induced Release from Liposomes—Liposomes (100 μg/ml) filled with cytochrome c or 20-kDa dextran-FITC were incubated with various amounts of caspase-2, mutant caspase-2, or caspase-3 in KCl buffer for 20 min at 22 °C. After centrifugation at 100,000 g for 45 min, the amount of cytochrome c or dextran-FITC in the supernatant and pellet was evaluated. Cytochrome c release was monitored by Western blotting, whereas dextran release was monitored in duplicates by a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using an excitation wavelength of 485 nm and monitoring wavelength of 538 nm.

RESULTS AND DISCUSSION

Caspase-2 Causes Membrane Permeabilization of Dextran- or Cytochrome c-containing Liposomes—To test this premise and to determine more precisely the molecular requirements for caspase-2-induced membrane permeabilization, experiments isolated liver mitochondria as well as permeabilized Jurkat T-lymphocytes (12). This effect appears to require processing of the zymogen but not the associated catalytic activity. To further examine the ability of caspase-2 to permeabilize mitochondria and stimulate cytochrome c release, we used wild-type (C-2), catalytically inactive (C303A), and uncleavable (D316G) recombinant caspase-2 proteins to treat permeabilized Jurkat cells, isolated liver mitochondria, or liposomes that had been loaded with either FITC-coupled 20-kDa dextran or bovine heart cytochrome c. Importantly, catalytically inactive caspase-2 (C303A) possessed no VDVADase activity (12), whereas uncleavable caspase-2 (D316G) retained 20% activity relative to that of wild type, which is consistent with data reported previously (14).

When recombinant wild-type caspase-2 was added to permeabilized Jurkat cells (Fig. 1A) or isolated liver mitochondria (Fig. 1B), cytochrome c was released in a rapid and concentration-dependent manner. In contrast, neither catalytically inactive nor uncleavable caspase-2 was able to induce any cytochrome c release (Fig. 1C). Furthermore, only wild-type caspase-2 caused a significant decline in the rate of uncoupled respiration (VCCP) when mitochondria were incubated with m-chlorophenylhydrazone (CCCP) (Fig. 1D), an effect that was most likely due to the prominence of cytochrome c release in the presence of caspase-2 (Fig. 1C). These findings, combined with our earlier results demonstrating that the apoptotic activity of caspase-2 is dispensable for membrane permeabilization and/or the release of cytochrome c, suggested that fully processed caspase-2 was targeting directly the outer mitochondrial membrane, or contact sites between the inner and outer mitochondrial membranes, to cause the release of proapoptotic proteins.

¹The abbreviations used are: PC, phosphatidylcholine; PI, phosphatidylinositol; CL, cardiolipin; FITC, fluorescein isothiocyanate; CCCP, m-chlorophenylhydrazone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
were performed using dextran-containing, protein-free liposomes. Importantly, liposome-based methodology has been used by several groups to faithfully mimic the behavior of mitochondria (15, 16). Here, liposomes of varying composition (100% PC; 20% PI, 80% PC; and 20% CL, 80% PC) containing FITC-coupled 20-kDa dextran were prepared and incubated with different amounts of recombinant caspase-2. As illustrated in Fig. 2A, recombinant caspase-2 induced a similar concentration-dependent release of dextran from all three lipid preparations, whereas caspase-3 and the two caspase-2 mutant proteins stimulated the release of only trace amounts of dextran.

In light of these findings, the next step was to determine whether caspase-2 would trigger a similar release of cytochrome c from liposomes. However, because cytochrome c preferentially binds to anionic phospholipids, notably CL located in the inner mitochondrial membrane (17, 18), we conjectured that the binding of cytochrome c to CL-containing liposomes might be different. Therefore, to ensure equal loading and binding to the lipid membrane among all liposome preparations, different amounts of cytochrome c were used for liposomes containing an anionic phospholipid (PI or CL) as compared with liposomes containing only PC (50 μg/ml and 200 μg/ml of cytochrome c/ml, respectively). Treatment of liposomes with caspase-2 resulted in the same release pattern of cytochrome c as was observed for the dextran experiments. Specifically, low amounts of caspase-2 led to only trace amounts of cytochrome c release, whereas robust cytochrome c release was observed when liposomes were incubated with either 1000 or 1500 ng of caspase-2 (Fig. 2B). Notably, neither caspase-3 nor mutant caspase-2 was able to induce a release of cytochrome c from the liposomes (Fig. 2C), which is consistent with our previous findings with isolated mitochondria and permeabilized cells (12).

Caspase-2 Causes Detachment of Cytochrome c from the Outer Surface of Liposomes—Interestingly, we determined during the aforementioned experiments (Fig. 2) that liposomes with cytochrome c attached to the outer (as well as the inner) surface of the membrane, as a result of fewer washing steps,

![Fig. 2. Caspase-2 (C-2) stimulates a complete release of 20-kDa dextran and cytochrome c (Cyt c) from liposomes. Liposomes filled with FITC-coupled 20-kDa dextran (A) or cytochrome c (B and C) were prepared as described under "Experimental Procedures." Wild-type caspase-2, each of the two mutant (C303A and D316G) caspase-2 proteins, or active caspase-3 (C-3) was added to the liposomes for 20 min at 22 °C. After a 45-min centrifugation period at 100,000 \( \times g \), pellets were resuspended in KCl buffer. The resulting supernatants (Sup.) and pellets were analyzed spectrofluorometrically at 538 nm for FITC fluorescence (A) or by Western blotting for cytochrome c (B and C).](image1)

![Fig. 3. Low levels of caspase-2 (C-2) trigger a detachment of cytochrome c (Cyt c) bound to the outer surface of liposomes. Sucrose-filled liposomes (100 μg/ml) with cytochrome c bound to the outer surface were incubated with either caspase-2 or mutant caspase-2 at the indicated concentrations. Subsequently, supernatant (Sup.) and pellet fractions were collected, separated by SDS-PAGE, and Western blotted.](image2)

![Fig. 4. Low levels of caspase-2 (C-2) augment cytochrome c (Cyt c) release from isolated mitochondria induced by digitonin (dig) or Bax. A, mitochondria (0.1 mg) were incubated for 15 min at 22 °C in the absence or presence of 10 ng of C-2 plus or minus 1.5 μg of recombinant oligomeric Bax as described under "Experimental Procedures." B, same as A except 0.03% digitonin was used instead of oligomeric Bax. At the end of the incubation period, supernatant (Sup.) and pellet fractions were collected, separated by SDS-PAGE, and Western blotted. C, caspase-2-induced changes in succinate-supported respiration after permeabilization of the outer membrane with digitonin. Cont, control.](image3)
would release their cytochrome c when incubated in the presence of lower, non-permeabilizing concentrations of caspase-2 (data not shown). As expected, this observation was especially pronounced in liposomes containing CL and could possibly be explained by caspase-2-mediated detachment of cytochrome c from CL on the outer surface of liposomes.

To further investigate this possibility, experiments were performed with sucrose-loaded liposomes that had cytochrome c bound to the outer surface only (Fig. 3). These liposomes were incubated with cytochrome c (250 µg/ml) and thereafter washed gently with KCl buffer. This had the effect of decreasing the amount of loosely, non-specifically bound cytochrome c to the outer surface while allowing some retention of cytochrome c binding to the anionic phospholipids, PI and CL (Fig. 3, lanes 1, 3, and 7). Next, concentrations of caspase-2 that were below those required for permeabilization (cf. Fig. 2, A and B) were used to treat these liposomes (Fig. 3). The results indicated that caspase-2 caused the detachment of cytochrome c from liposomes containing either PI or CL (Fig. 3, lanes 4, 5, 8, and 9). Moreover, caspase-2 was slightly more efficient at detaching cytochrome c from the outer surface of CL-containing liposomes (Fig. 3), suggesting that caspase-2 may have a slightly higher affinity for cardiolipin as has been described previously for Bid (19, 20).

**A Low Concentration of Caspase-2 Augments Cytochrome c Release Induced by Digitonin or Bax**—Because caspase-2 seemed to be disrupting cytochrome c binding to CL in liposomes, it was of interest to test this possibility using whole mitochondria. To that end, isolated rat liver mitochondria were incubated with a non-permeabilizing amount (10 ng) of caspase-2 and either recombinant oligomeric Bax or digitonin (Fig. 4, A and B). In both instances, the inclusion of 10 ng of caspase-2 led to significantly more cytochrome c being released, presumably due to caspase-2-mediated dissociation of the interaction between cytochrome c and CL. Importantly, the cytochrome c release data correlated nicely with the functional data obtained indicating that, although 10 ng of caspase-2 alone had no effect on respiration, its inclusion with digitonin resulted in a more severe decline in both state 3 and uncoupled (CCCP) respiration than was observed with digitonin alone (Fig. 4C). The rate of uncoupled respiration was partially restored upon inclusion of cytochrome c in the incubation medium.

**Concluding Remarks**—In the current study, we show that caspase-2 can permeabilize the outer mitochondrial membrane in a direct manner. Studies with liposomes revealed that this process requires neither other proteins nor a particular phospholipid composition of the liposomal membrane. Permeabilization appears to have been due to pore formation as incubation of liposomes with caspase-2 did not result in any apparent change in their size distribution (data not shown). This, combined with our previous finding that AIF was not released by treatment of isolated mitochondria or permeabilized Jurkat cells with caspase-2, whereas cytochrome c and Smac/DIABLO were, argues against a detergent-like effect of caspase-2 being responsible for the release of proteins from mitochondria (12).

Binding of cytochrome c to the inner mitochondrial membrane is known to involve a unique mitochondrial phospholipid, cardiolipin (17). There is also ample evidence that a decreased level of cardiolipin in the mitochondrial membrane, as a result of inhibited synthesis, accelerated break down, or oxidative stress, leads to impaired cytochrome c binding and increases the susceptibility of cells to undergo mitochondrially mediated apoptosis (see (21) for review). Furthermore, cardiolipin was shown to be a mitochondrial target for the proapoptotic Bcl-2 protein, Bid, and to play a critical role in mitochondrial permeabilization/cytochrome c release by tBid/Bax (15, 19, 20, 22). The cardiolipin precursor, phosphatidylglycerol, and other anionic phospholipids (e.g. phosphatidylinositol) can partially substitute for cardiolipin, but their association with cytochrome c is considerably weaker as compared with that of cardiolipin (18). Our results show that caspase-2 has the surprising ability to disrupt the association between cytochrome c and anionic phospholipids, notably CL, thereby making additional cytochrome c available for release into the cytosol/supernatant. This effect probably explains why considerably more cytochrome c is released from mitochondria treated with caspase-2 as compared with digitonin or recombinant oligomeric Bax. An alternate explanation might be that caspase-2 can stimulate unfolding of mitochondrial cristae in a manner resembling that of tBid (20).

Considering the number of pathways reportedly linking cytotoxic stress with mitochondrial engagement (see Ref. 23 for review), it would seem reasonable that the extent of the involvement of caspase-2 is likely to depend on a number of factors, including the nature of the cytotoxic stimulus, the cell type that is being affected, and the intracellular abundance of caspase-2 relative to that of other proapoptotic proteins (e.g. Bax/Bak). Our current data suggest that in some instances caspase-2 may be responsible for direct permeabilization of the outer mitochondrial membrane, while in other instances, caspase-2 may act in conjunction with Bak to amplify cytochrome c release by causing its displacement from cardiolipin. However, additional studies are needed to better understand how these different scenarios play out under physiological conditions.

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