Caspase-2 Induces Apoptosis by Releasing Proapoptotic Proteins From Mitochondria*

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Abbreviations used: Caspase-9 DN, caspase-9 dominant negative; AIF, apoptosis-inducing factor; Smac/Diablo, second mitochondria-derived activator of caspases protein; Apaf-1, apoptotic protease activating factor-1; Cox, cytochrome oxidase; NGF, nerve growth factor; IAP, inhibitor of apoptosis protein; CRADD, caspase and RIP adaptor with death domain; PAGE, polyacrylamide gel electrophoresis; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp- (OMe) fluoromethyl ketone; VDVAD-afc, VDVAD-7-Amino-4-trifluoromethyl coumarin.
ABSTRACT

Caspase-2 is one of the earliest identified caspases, but the mechanism of caspase-2-induced apoptosis remains unknown. We show here that caspase-2 engages the mitochondria-dependent apoptotic pathway by inducing the release of cytochrome c (Cyt c) and other mitochondrial apoptogenic factors into the cell cytoplasm. In support of these observations we found that Bcl-2 and Bcl-xL can block caspase-2- and CRADD/RAIDD-induced cell death. Unlike caspase-8, which can process all known caspase zymogens directly, caspase-2 is completely inactive towards other caspase zymogens. However, like caspase-8, physiological levels of purified caspase-2 can cleave cytosolic Bid protein, which in turn can trigger the release of Cyt c from isolated mitochondria. Interestingly, caspase-2 can also induce directly the release of Cyt c, AIF (apoptosis inducing factor) and Smac/Diablo from isolated mitochondria independent of Bid or other cytosolic factors. The caspase-2-released Cyt c is sufficient to activate the Apaf-caspase-9 apoptosome in vitro. Combined our data suggest that caspase-2 is a direct effector of the mitochondrial apoptotic pathway.
INTRODUCTION

Apoptosis is a form of cellular suicide that is essential for development and tissue homeostasis of all metazoan organisms. Caspases, a family of cysteine-dependent aspartate-directed proteases, play critical roles in initiation and execution of apoptosis (1). Determining the cellular processes, which lead to activation of caspases during apoptosis, and identifying the relevant intracellular caspase substrates and regulators have been the subjects of intensive investigation in the last several years.

Two relatively well-characterized apoptotic pathways have been identified. The first pathway is mediated by death receptors, such as Fas or Tumor Necrosis Factor (TNF) receptor (2). Specific adaptor proteins such as FADD/MORT1 (3,4) or CRADD/RAIDD (5,6) bind to the ligand-bound receptor complexes. Interaction between the adaptor molecules and the prodomain of initiator caspases 2, 8 or 10 triggers sequestration-mediated autoactivation of these caspases (7-9). The activated initiator caspases in turn cleave and activate the downstream effector caspases 3, 6 and 7, inducing a cascade of caspase activation (10,11). In the second pathway, Cyt c is released from mitochondria to the cytoplasm in cells exposed to chemotherapeutic drugs, UV irradiation, growth factor withdrawal, or ligation of Fas and TNF receptors (12-16). Released Cyt c binds to Apaf-1 (apoptotic protease activating factor-1) and promotes its oligomerization (17,18). Recruitment of procaspase 9 to this active apoptosome results in its autoactivation, and subsequent activation of caspase-3 by the active caspase-9-Apaf-1 complex (18-20). In both pathways activation of effector caspases by initiator caspases amplifies the apoptotic signal to ensure fast and irreversible cell death. In addition, there appears to be a cross talk between the death receptor and the mitochondrial apoptotic pathways. Caspase-8, an initiator caspase in the death receptor pathway can connect death receptors to the core apoptotic
machinery by directly cleaving downstream executioner caspases (11). Alternatively, it may
activate the caspase cascade indirectly by cleaving Bid, a proapoptotic Bcl-2 family member.
The cleaved Bid fragment translocates from the cytosol to the outer mitochondrial membrane
resulting in disruption of the outer mitochondrial membrane and release of Cyt c from the
mitochondrial inter membrane space (21,22). Engagement of the mitochondria-dependent
pathway is more efficient than the mitochondria-independent pathway, as it only requires a small
amount of active caspase-8 (23). Recent reports suggest that induction of Cyt c release from
mitochondria is not only restricted to caspase-8. Effector caspases, caspase-3, -6, and -7 can also
facilitate rapid Cyt c release from the mitochondria when other cytosolic factors are present (24).

Because of the critical role of mitochondria in apoptosis, it has been the subject of
intensive research recently. Proapoptotic factors such as Cyt c (13,17), procaspases 2, 3, 9
(25,26), apoptosis-inducing factor (AIF) (27), and second mitochondria-derived activator of
caspases protein Smac (also known as Diablo) (28-31) are safely sequestered within the
mitochondrial inter membrane space in non-apoptotic cells. Upon apoptotic challenge, however,
a rapid release of these factors through the outer mitochondrial membrane into the cytoplasm
signals the initiation of the apoptotic process. The mechanism of release of these mitochondrial
apoptotic factors into the cytoplasm remains unclear.

Caspase-2, initially described as Nedd-2/Ich-1, has been identified as a gene related to
the C. elegans cell death gene ced-3 and mammalian interleukin-1β-converting enzyme (ICE,
caspase-1) (32). Two distinct caspase-2 mRNA species derived from alternative splicing encode
two proteins, caspase-2L and caspase-2S. Overexpression of caspase-2L induces cell death,
whereas overexpression of caspase-2S can antagonize cell death. Caspase-2L is the dominant
isoform that is expressed in most tissue (32). The murine caspase-2 is highly expressed in mouse
embryonic brain and down regulated in adult brain (33). Mice carrying a null mutation for
caspase-2 develop normally without severe phenotypic abnormalities. However, in caspase-2
knockout mice, sympathetic neuron and motor neurons are more sensitive to death than neurons
of wild type mice when deprived of NGF. In contrast, B lymphoblasts are resistant to apoptosis
mediated by granzyme B and perforin. In addition, germ cells and oocytes are found to be
resistant to cell death after treatment with chemotherapeutic drugs (34). Thus, for caspase-2
knockout mice, defects in apoptosis are cell-type and stimulus-dependent.

So far the mechanism by which caspase-2 can induce activation of the effector caspases
is not fully understood. CRADD/RAIDD, an adaptor molecular for FAS/TNFR, recruits pro-
caspase-2 to the death receptor pathway (5,6). It has been suggested that the prodomain of
caspase-2 interacts with the CARD domain of CRADD/RAIDD (5,6), allowing the dimerization
and autoactivation of procaspase-2. However, events subsequent to caspase-2 activation remain
largely unknown. To understand how caspase-2 triggers the apoptotic pathway, we examined
the possibility that caspase-2 employs the mitochondrial pathway to activate the caspase cascade.
Our results indicate that physiological amounts of recombinant caspase-2 protein could directly
provoke release of Cyt c and other proapoptotic factors from the mitochondria independent of
any cytosolic protein and also through cleavage of Bid. The released proapoptotic factors trigger
activation of the downstream caspases, thus setting into motion the apoptotic cascade.
EXPERIMENTAL PROCEDURES

Reagents—Anti-Cyt c antibody (7H8.2 c12, 6H2.B4) was obtained from BD PharMingen (San Diego, CA). Anti-caspase 2 antibody (directed towards amino acid 225-401) was purchased from Transduction Laboratories (Lexington, KY). Anti-AIF and anti-Bid was purchased from Santa Cruz Biotechnology Inc. Anti-caspase 3 antibody was purchased from BD PharMingen. Anti human cytochrome oxidase subunit II (A-6404) was purchased from Molecular Probes. Anti-Smac monoclonal antibody was raised against mature Smac. VDVAD-AFC was purchased from Enzyme System. Anti-citrate synthase polyclonal antibody is a gift from Yuri Lazebnik. Protease inhibitors were purchased from Roche Molecular Biochemicals. Protein concentrations were determined by the Bio-Rad assay kit (Hercules, CA).

Mammalian and Bacterial Expression Vectors—For apoptosis assays, we used the mammalian double expression vector pRSC-LacZ, which allows expression of lacZ under the Rous sarcoma virus promoter, and the recombinant proteins (caspase-2 or CRADD) under the cytomegalovirus promoter. Constructs encoding CRADD, caspase-2, caspase-9-DN, Bcl-2, Bcl-xL, p35, and XIAP have been described before (5), (19,35), 36]. Bid wild type, cleavage site D59 to E mutant, double mutant (D59 and D75 to E) genes were excised from constructs generously given by Dr. J. Yuan (Harvard Medical School, Boston, MA) and then subcloned into T7pcDNA3 vector. For bacterial expression, cDNA was cloned in pET 21 or pET 28 (Invitrogen). Recombinant proteins with C-terminal or N-terminal His6 tags were expressed in BL-21 (DE3) bacteria and purified on a Ni2+-affinity resin by standard affinity-purification procedures as described previously (18).

Apoptosis Assays—MCF-7 cells were transiently cotransfected with pRSC-lacZ constructs in the presence or absence of different apoptosis inhibitors. The cells were stained
with β-galactosidase 30-36 h after transfection or 20 µM z-VAD-fmk treatment and examined for morphological signs of apoptosis (36). Normal and apoptotic blue cells were counted by phase contrast microscopy. The graphs depict the mean percentage of stained apoptotic cells as a fraction of the total number of blue cells after subtracting the percentage of apoptotic cell in the empty vector-transfected control cells (means ± S.D.). Data provided represents the average of at least three individual experiments (n ≥ 3 and S.D. ± 1-4%).

**Cleavage Assay**—Wild type caspases, Bid and Bid mutants were in-vitro translated in the presence of [35S] methionine in rabbit reticulocyte lysate with a T7-RNA polymerase-coupled TNT kit (Promega), using the pRSC-LacZ, T7pcDNA3, or pET 28 constructs as templates according to the manufacturer’s recommendations. Equal amounts of the translation reactions were diluted in 10 µl ICE buffer (25 mM HEPES, 1 mM EDTA, 5 mM DTT, and 0.1% 3-[3-cholamidopropyl) dimethylamino]-1-propanesulfonate (PH 7.5)) and incubated with purified recombinent caspase-2 and caspase-8, respectively at 30°C for 1 hour. The reactions were fractionated by 10% SDS-PAGE and analyzed by autoradiography.

**Transfection, Immunodepletion, and Immunoblot Analyses**—These were done as previously described (36).

**Mitochondrial Preparation and Cytosolic Extracts**—HeLa cells were collected by centrifugation at 600 xg for 10 min. The cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS, PH 7.4) and resuspended with five volume of buffer A (250 mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM phenylmethylsulfonyl fluoride, pH 7.5). The cells were homogenized in a glass Dounce homogenizer until ~60% of the cells became Trypan blue positive (60 strokes for HeLa cells). The homogenates were centrifuged twice at 750 xg for 10 min at 4°C. Supernatants were
centrifuged at 10,000 xg for 15 min at 4°C, and the resulting mitochondrial pellets were re-
suspended in buffer A (13). In some cases mitochondria were further purified on Percol gradient
(Pharmacia) as per the manufacturer’s recommendations. The supernatants of the 10,000 xg spin
were further centrifuged at 100,000 xg for 1 hour at 4°C, and the resulting supernatants
(designated S-100) were frozen as aliquots at -80°C for subsequent experiments. In some
experiments Percol gradient-purified mouse liver mitochondria were used (37).

**In Vitro Assay of Cyt c Release**—A 5 µl aliquot of mitochondria (2 µg/µl) was incubated
with 10 µg of cytosolic extract (S-100) and recombinant caspase-2 or caspase-8, in a final
volume of 25 µl buffer A at 37°C for 1 hour. The reaction mixture was then centrifuged at
12,000 xg for 10 min at 4°C to pellet the mitochondria. Supernatants and the pellets were
subjected to SDS-PAGE and immunoblotting. Similar procedures were used to assay the release
of Smac/Diablo and AIF.
RESULTS

The Mitochondrial Pathway Is Required for Caspase-2- and CRADD-Induced Apoptosis and Caspase-3 Processing—To gain a better understanding of how caspase-2 and its adaptor molecule CRADD/RAIDD engage the death pathway, we investigated their apoptotic and procaspase-3 processing ability in the presence of various apoptosis inhibitors. Among the inhibitors used, Bcl-2 and Bcl-xL inhibit apoptosis by blocking release of Cyt c and other proapoptotic factors from the mitochondria, while caspase-9-DN (active site cysteine 287 to alanine) interferes with formation of a functional Apaf-1-caspase-9 complex by a dominant negative mechanism. As shown in Figure 1A, CRADD- and caspase-2-induced apoptosis in MCF7-Fas cells was efficiently blocked by Bcl-2, Bcl-xL and caspase-9-DN (Fig. 1A), suggesting that they require the participation of the mitochondria and the Apaf-1-caspase-9 complex to trigger activation of the apoptotic pathway.

To determine whether overexpression of caspase-2 and CRADD can induce processing of procaspase-3, 293T cells were transiently transfected with CRADD and caspase-2 expression plasmids together with or without expression plasmids encoding Bcl-xL or caspase-9-DN. The cell lysates were immunoblotted with antibody against the processed caspase-3-p20 subunit. As expected, overexpression of caspase-2 and CRADD induced processing/activation of procaspase-3 (Fig. 1B, lane 2 and 3). This processing was completely blocked when 4-fold excess of caspase-9-DN or Bcl-xL was co-expressed together with CRADD or caspase-2 (Fig. 1B, lanes 5,6 and lanes 8,9). These observations are consistent with the result in Fig. 1A, confirming the importance of the mitochondrial pathway in activation of the effector caspases by CRADD and caspase-2.
Comparison of the Protease Activities of Caspase-2 and Caspase-8 Toward Other Caspase Zymogens—Caspase-8 can process and activate most known caspase zymogens (procaspases) (11). To compare the relative activities of caspase-2 and caspase-8 toward other known caspase zymogens, recombinant caspase-2 and caspase-8 were expressed in bacteria and then affinity-purified. Expression of caspase-2 and –8 zymogens in bacteria allowed autoactivation of the two zymogens to generate the two processed subunits of the mature caspase-2 and caspase-8 (here referred to as caspase-2 and caspase-8) (Fig. 2A). Equal amounts of caspase-2 and caspase-8 were incubated with a panel of in vitro-translated [35S] methionine-labeled caspase zymogens and the reactions were then analyzed by SDS-PAGE and autoradiography. Unlike caspase-8, caspase-2 was not able to process zymogens of effector caspases 3, 6 or 7, or initiator caspases 8, 9 or 10. However, caspase-2 was able to process its own zymogen and the baculovirus IAP inhibitor, p35 (Fig. 2B and C). This suggests that caspase-2 could execute the apoptotic program independent of other effector or initiator caspases, or by activating another unknown caspase. Alternatively, caspase-2 could activate the caspase cascade indirectly via the mitochondrial pathway by causing release of Cyt c and other apoptotic factors into the cytoplasm.

Caspase-2 Induces Cyt c Release from the Mitochondria—To examine whether caspase-2 induces Cyt c release from the mitochondria, purified mitochondria from HeLa cells were incubated with purified caspase-2 in vitro with or without cytosolic extracts. Caspase-8 was used at the same time as a control. When mitochondria were incubated with cytosol alone, almost no Cyt c release was observed in the supernatant, indicating that the mitochondrial preparation was not releasing Cyt c nonspecifically (Fig. 3A, lane 2). However, when mitochondria were incubated with cytosol and increasing amounts of caspase-2, an increase in
the amount of Cyt c in the supernatant was detected by immunoblotting (Fig. 3A, lanes 8-12). The same results were obtained with caspase-8 (Fig. 3A, lanes 3-7). The amount of Cyt c released into the supernatant by a fixed amount of caspase-2 or caspase-8 was also increased by increasing the amount of the S-100 cytosol (Fig. 3B). This suggests that both caspases are capable of inducing Cyt c release, presumably by proteolytically activating a cytosolic factor(s). Surprisingly, when the S-100 cytosol was omitted from the reaction mixture, caspase-2, but not caspase-8, was still able to induce Cyt c release (Fig. 3A and B, compare lanes 13 and 14), suggesting that the presumable cytosolic factor(s) is dispensable for stimulation of mitochondria by caspase-2, but not by caspase-8.

Caspase-2 Can Cleave Bid—Bid is a proapoptotic Bcl-2 family member that triggers Cyt c release from the mitochondria after proteolytic cleavage by caspase-8 (21,22). Careful examination of the amino acid sequence of Bid identified two potential caspase cleavage sites that match the preferred cleavage sites for caspase-2 (38). We examined the ability of caspase-2, compared to caspase-8, to cleave Bid. In vitro translated [$^{35}\text{S}$]-labeled wild type Bid (WT), Bid D59E mutant (D59E), or Bid D59E/D75E double mutant (DM) were incubated with recombinant caspase-2 or caspase-8. As shown in Figure 4A, both caspase-2 and caspase-8 were able to cleave WT Bid, but not the D59E or DM mutants, into two small fragments almost to the same extent. Based on these results, caspase-2 can efficiently cleave Bid at Asp59, suggesting that caspase-2 induces Cyt c release by proteolytically activating Bid.

To determine whether Bid is necessary for caspase-2- or caspase-8-induced Cyt c release from the mitochondria, endogenous Bid was immunodepleted from HeLa cell S-100 extracts before incubating with caspase-2 or –8 and mitochondria. As expected, depletion of Bid from the S100 extracts totally abolished the Cyt c releasing ability of caspase-8 (Fig. 4B, compare
However, depletion of Bid decreased, but did not totally inhibit the Cyt c releasing ability of caspase-2 (Fig. 4B, compare lane 4 with lane 8). This suggests that Bid may not be critical for caspase-2-induced Cyt c release but its presence could enhance this caspase-2 activity.

Caspase-2 Can Directly Release Apoptogenic Factors From the Mitochondria—Cyt c, AIF and Smac are normally localized in the inter membrane space of the mitochondria (27,30,31). It has been demonstrated by two-color immunofluorescence detection that AIF is released shortly before Cyt c from the mitochondria upon staurosporine stimulation (39). The released AIF translocates from the mitochondria to the nucleus where it induces caspase-independent apoptosis by causing chromatin condensation and large-scale DNA fragmentation in the nucleus. Smac is also released with Cyt c into the cytoplasm during apoptosis and functions to potentiate caspase activation by inhibiting IAPs (28-31). To further confirm the ability of caspase-2 to directly release apoptogenic factors from the mitochondria, increasing amount of purified caspase-2 was incubated with mitochondria in-vitro and the release of Cyt c, AIF and Smac was determined. As shown in Fig. 5A, Cyt c, Smac and AIF were released from isolated Hela cell mitochondria into the supernatant in a dose-dependent manner by purified caspase-2. The direct release of AIF and Smac from the mitochondria into the cytosol by caspases has not been reported before. Cytochrome oxidase, an integral inner mitochondrial membrane protein, and citrate synthase, a soluble mitochondrial matrix protein, were not released by caspase-2 (Fig. 5A) indicating that the inner mitochondrial membrane remains intact after caspase-2 treatment. Combined, the above data suggest that caspase-2 specifically release soluble mitochondrial inter membrane space proteins only. Similar results were obtained with Percoll gradient-highly purified mouse liver mitochondria (Fig. 5B), indicating that the effect of caspase-2 is not due to
contaminating cytosolic factors or a specific effect on HeLa cell mitochondria. To further rule out the possibility that the direct effect of caspase-2 on the mitochondria is mediated by contamination of the mitochondrial preparation with cytosolic Bid and/or caspase-8 we analyzed the mitochondrial and corresponding cytosolic fractions by western blot analysis with Bid and caspase-8 specific antibodies. As shown in Fig. 5C, no Bid or caspase-8 bands were detected in the mitochondrial fraction. Interestingly, the proapoptotic Bcl-2 protein Bax was entirely present in the mitochondrial preparation (Fig. 5C, lane 1). The role of Bax in the direct release of the mitochondrial apoptotic factors by caspase-2 is currently unknown. Taken together, our results suggest that caspase-2 could stimulate both the Apaf-1-caspase-9 pathway and the caspase-independent AIF apoptotic pathway by directly releasing Cyt c, Smac and AIF from the mitochondria.

The Released Cyt c Can Activate the Downstream Caspase Cascade—Cyt c released from the mitochondria by caspase-2 could induce activation of Apaf-1 and caspase-9, which in turn could activate the downstream caspases. To test this hypothesis we reconstituted an in vitro procaspsase-3 processing system. In this system, we incubated T7-tagged procaspsase-3 C-A with purified Apaf-1 and procaspsase-9 in the presence or absence of dATP and Cyt c. As shown in Fig. 5D, only after addition of increasing amounts of Cyt c, procaspsase-3 was processed. Caspase-2 alone, or together with Apaf-1 and procaspsase-9 could not promote processing of procaspsase-3 (Fig. 5D, lanes 1-5). To determine whether Cyt c released from the mitochondria by caspase-2 could also induce procaspsase-3 processing in this system, we incubated increasing amounts of caspase-2 with purified mitochondria and Apaf-1, procaspsase-9 and dATP. As expected, caspase-2 induced processing of procaspsase-3 in a dose dependent manner, only in the
presence of mitochondria (Fig. 5E, lanes 4-6). These results demonstrate that caspase-2 can activate the downstream caspases via the mitochondrial amplification pathway.

**Active Site Mutant Caspase-2 Is Unable to Induce Cyt c Release from Mitochondria**—If caspase-2 protease activity is critical for its ability to induce Cyt c release from mitochondria, then we expect an active site mutant of caspase-2 to be inactive with respect to Cyt c release. To test this hypothesis, we expressed caspase-2 C-A (active site cysteine 303 to alanine) in bacteria and affinity purified it. Unlike the WT caspase-2 zymogen, the inactive C-A mutant did not undergo processing in bacteria (Fig. 6A). When the same amount of caspase-2 and caspase-2 C-A were incubated with purified mitochondria, only the WT caspase-2 was able to induce Cyt c release in a dose-dependent manner from the mitochondria into the supernatants (Fig. 6B, lanes 1-6 compared with lanes 7-12). The recombinant purified caspase-2 C-A used in the above experiment was not able to cleave the caspase-2 substrate VDVAD-AFC (Fig. 6C). Taken together, these observations suggest that the protease activity of caspase-2 is necessary for caspase-2 to directly induce Cyt c release from the mitochondria.

**Activated Caspase-2 Can Process Endogenous Procaspase-2 to Amplify the Apoptotic Signal**—Procaspase-2 has been found in the cytoplasm, nucleus, Golgi complex, and mitochondria (40,41). It has been shown that upon induction of apoptosis and PT (permeability transition) pore opening, caspase-2 is released from purified mitochondria and become activated (26). To determine whether active caspase-2 can process the endogenous procaspase-2 found in the mitochondria and cytoplasm, we incubated recombinant caspase-2 with purified mitochondria and cytoplasmic extract from three different cell lines, MCF-7, HEK293T, and HeLa cells. Endogenous mitochondrial and cytosolic procaspase-2 was cleaved to generate a major band of apparent molecular masses of 35 kDa (Fig. 7, lanes 3,4,7,8,11, and 12). Some of
the exogenous recombinant caspase-2 is seen as a p30 band that is smaller than the processed fragment of the endogenous proenzyme (Fig. 7, compare lanes 13 with the other lanes). Our data indicate that activated caspase-2 can directly process the endogenous mitochondrial and cytosolic procaspase-2. The ability of exogenous caspase-2 to process the endogenous mitochondrial procaspases-2, which is present in the inter membrane space (26), suggests that activated caspase-2 can translocate across the outer mitochondrial membrane. This suggests that, in addition to its ability to release mitochondrial inter membrane space proteins from the mitochondria, activated cytoplasmic caspase-2 could also activate mitochondrial procaspase-2 during apoptosis to further amplify the apoptotic cascade.
DISCUSSION

Two alternatively spliced caspase-2 isoforms, caspase-2L and caspase-2S, with opposing effects on cell death have been identified previously (32). In this study, we focused on the predominant proapoptotic caspase-2L isoform (referred to here as caspase-2) that is widely expressed in most tissues. Caspase-2 is activated in many cell types in response to various apoptotic stimuli, including growth factor withdrawal, DNA damaging agents, TNF-α, Fas ligation, and antigen receptor ligation (42-45). In most of these cases procaspase-2 is directly cleaved by the effector caspase-3 suggesting a positive feedback amplification loop or a possible down-stream involvement of this caspase in the apoptotic cascade (41,46,47). However, because procaspase-2 has characteristics of an initiator caspase, including a large N-terminal prodomain with a CARD and the ability to interact through this CARD with the TNF-R1-associated adaptor protein CRADD (5,6), we postulate that caspase-2 is an initiator caspase acting upstream of the effector caspases. Supporting this notion, we have previously shown that caspase-2 activation occurs upstream of caspase-3 activation in the B-cell receptor signaling pathway leading to apoptosis (45). Moreover, recent studies demonstrated that caspase-2 is activated within 10-15 min after infection with Salmonella or induction of anoikis followed by activation of the downstream effector caspases 3, 6 and 7 (48,49). These studies demonstrated also that cytochrome c release occurs shortly after activation of caspase-2 (48,49), providing additional evidence that activated caspase-2 acts upstream of the mitochondria and could contribute to the release of cytochrome c by acting directly or indirectly on the mitochondria. However, it was not evident from these studies whether the observed activation of the effector caspases, which followed activation of caspase-2 was dependent, or not, on cytochrome c release. In the present study, using a cell-free system we provided evidence that caspase-2 plays a role as an initiator
caspase in the proteolytic caspase cascade by triggering the release of apoptogenic factors
including Cyt c from the mitochondria directly and indirectly through cleavage of Bid. We show
that the released Cyt c is sufficient to activate the Apaf-1-caspase-9 complex, which in turns
activates the effector caspases.

Caspase-2 is similar to caspase-8 in its ability to cleave Bid. However, unlike caspase-8,
caspase-2 cannot process and activate the effector caspases directly. A recent study, utilized a
series of cell lines stably transfected with a caspase-2 antisense construct to analyze the role of
caspase-2 in the death receptor pathway, provided evidence that down-regulation of caspase-2
significantly prevents Bid cleavage and abolish Cyt c release and caspase-3 activation after Fas
stimulation (50). Another study demonstrated that ectopically expressed caspase-2 could trigger
the translocation of Bid to mitochondria and release of Cyt c (51). Together with our data, the
above evidence suggests that one way by which caspase-2 could activate the effector caspases
after death receptor ligation or other apoptotic stimuli is through cleavage of Bid. Since
translocation of Bid to the mitochondria is triggered by its cleavage and subsequent
myristoylation at glycine 60 (52), the cleaved Bid could in turn translocate from the cytoplasm to
the mitochondria causing disruption of the outer mitochondrial membrane and release of a
number of mitochondrial apoptogenic factors (53,54). Among these factors, Cyt c activates the
Apaf-1-caspase-9 apoptosome, while Smac inhibits IAPs and promotes further caspase
activation. AIF on the other hand could translocate to the nucleus and induce large scale DNA
cleavage and caspase-independent apoptosis (55).

Caspase-2 can also release Cyt c and other apoptotic factors from the mitochondria
independent of Bid or other cytosolic factors. Three models have been proposed for the release
of Cyt c from the mitochondria during apoptosis (56). One mechanism involves mitochondria
swelling and physical rupturing of the outer mitochondrial membrane. However, this mechanism was challenged by electron microscopic studies, which revealed that apoptotic cells do not always show swelling of the mitochondria (57-59). Furthermore, the pro-apoptotic protein Bid (21) and Bax (60-62) can release Cyt c from isolated mitochondria in the absence of detectable mitochondrial swelling. The second mechanism involves formation of large ion channel in the mitochondrial membrane. This mechanism explains the activity of some Bcl-2 family members such as Bcl-2, Bcl-xL, Bid, and Bax, which can form ion channels in synthetic lipid membrane in vitro and regulate efflux of Cyt c (63-65). More recently a third mechanism based on the mitochondria permeability transition (PT) pore was proposed to explain release of Cyt c from the mitochondria. PT pore is formed at the site of contact between mitochondrial inner and outer membranes and consists of the mitochondrial voltage-dependent anion channel (VDAC, also called porin), adenine nucleotide translocator (ANT), cyclophilin D, and other proteins (66). Through direct interaction with VDAC, Bax and Bak have been shown to induce Cyt c release by accelerating the opening of VDAC. Bcl-xL on the other hand prevents opening of the VDAC channel (67). However, tBid (c-terminal truncated Bid) or Bik can cause Cyt c release and cell death independent of PT pore opening (68). Thus, whether any of these mechanisms could explain the direct effect of caspase-2 on the mitochondria remains to be determined.

Nevertheless, the absence of Cyt c release from isolated mitochondria by inactive mutant of caspase-2 suggests that the caspase activity of caspase-2 is required for its direct Cyt c-releasing activity. Exogenous mature caspase-2 can cleave the endogenous mitochondria procaspsase-2 indicating that mature caspase-2 could freely translocate through the outer mitochondrial membrane. This raised the possibility that caspase-2 could regulate PT pore opening by cleaving VDAC. However, we find that caspase-2 does not bind to VDAC or cleave
it into small fragments (Data not shown). We postulate that caspase-2 might cleave an outer mitochondrial membrane protein, perhaps a member of the Bcl-2 family, causing the release of Cyt c and other mitochondrial apoptotic factors. Supporting this hypothesis, our data clearly show that Bcl-2 or Bcl-xL can protect cells from apoptosis induced by overexpression of caspase-2, indicating that caspase-2 regulates the integrity of the outer mitochondrial membrane. Other preliminary data (not shown) also showed complete inhibition of Cyt c release when caspase-2 was incubated with mitochondria along with Bcl-xL protein.

In conclusion, our data demonstrate that caspase-2 can induce the release of several mitochondrial apoptotic proteins, indicating that caspase-2 induces apoptosis via the mitochondrial apoptotic pathway. However, the mechanism of by which caspase-2 exerts its direct effect on the mitochondria remains to be elucidated.
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REFERENCES

1. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A.,
   Wong, W. W., and Yuan, J. (1996) Cell 87(2), 171
2. Nagata, S. (1997) Cell 88(3), 355-65
3. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81(4), 505-12
4. Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach,
   D. (1995) J Biol Chem 270(14), 7795-8
5. Ahmad, M., Srinivasula, S. M., Wang, L., Talanian, R. V., Litwack, G., Fernandes-
   Alnemri, T., and Alnemri, E. S. (1997) Cancer Res 57(4), 615-9
6. Duan, H., and Dixit, V. M. (1997) Nature 385(6611), 86-9.
7. Yang, X., Chang, H. Y., and Baltimore, D. (1998) Mol Cell 1(2), 319-25.
8. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J
   Biol Chem 273(5), 2926-30.
9. Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998) J Biol Chem 273(8),
   4345-9.
10. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) J Biol Chem 272(5), 2952-6.
11. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S.
    (1996) Proc Natl Acad Sci U S A 93(25), 14486-91
12. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) Science
    275(5303), 1132-6
13. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P.,
    and Wang, X. (1997) Science 275(5303), 1129-32
14. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) Embo J 17(1), 37-49
15. Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z. M., Saxena, S., Weichselbaum, R., Nalin, C., and Kufe, D. (1997) Proc Natl Acad Sci U S A 94(13), 6939-42

16. Adachi, S., Cross, A. R., Babior, B. M., and Gottlieb, R. A. (1997) J Biol Chem 272(35), 21878-82

17. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86(1), 147-57

18. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Mol Cell 1(7), 949-57

19. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91(4), 479-89

20. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) Cell 90(3), 405-13

21. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94(4), 481-90.

22. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cell 94(4), 491-501

23. Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D., and Kornbluth, S. (1998) J Biol Chem 273(26), 16589-94

24. Bossy-Wetzel, E., and Green, D. R. (1999) J Biol Chem 274(25), 17484-90

25. Mancini, M., Nicholson, D. W., Roy, S., Thornberry, N. A., Peterson, E. P., Casciola-Rosen, L. A., and Rosen, A. (1998) J Cell Biol 140(6), 1485-95

26. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M. C., Alzari, P. M., and Kroemer, G. (1999) J Exp Med 189(2), 381-94

27. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397(6718), 441-6
28. Chai, J., Du, C., Wu, J. W., Kyin, S., Wang, X., and Shi, Y. (2000) *Nature* **406**(6798), 855-62

29. Srinivasula, S. M., Datta, P., Fan, X. J., Fernandes-Alnemri, T., Huang, Z., and Alnemri, E. S. (2000) *J Biol Chem*

30. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) *Cell* **102**(1), 43-53

31. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) *Cell* **102**(1), 33-42.

32. Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78**(5), 739-50

33. Kumar, S., Kinoshita, M., Noda, M., Copeland, N. G., and Jenkins, N. A. (1994) *Genes Dev* **8**(14), 1613-26

34. Bergeron, L., Perez, G. I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K. E., Flaws, J. A., Salter, J. C., Hara, H., Moskowitz, M. A., Li, E., Greenberg, A., Tilly, J. L., and Yuan, J. (1998) *Genes Dev* **12**(9), 1304-14

35. MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S. (1997) *J Biol Chem* **272**(41), 25417-20

36. Srinivasula, S. M., Ahmad, M., Lin, J. H., Poyet, J. L., Fernandes-Alnemri, T., Tsichlis, P. N., and Alnemri, E. S. (1999) *J Biol Chem* **274**(25), 17946-54

37. Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R., and Bredesen, D. E. (1997) *J Neurosci* **17**(16), 6165-78.

38. Thornberry, N. A., Rosen, A., and Nicholson, D. W. (1997) *Adv Pharmacol* **41**, 155-77
39. Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N.,
    Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000) *Faseb J*
    **14**(5), 729-39.

40. Colussi, P. A., Harvey, N. L., and Kumar, S. (1998) *J Biol Chem* **273**(38), 24535-42.

41. Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-
    Rosen, L. A., and Rosen, A. (2000) *J Cell Biol* **149**(3), 603-12.

42. Kumar, S., and Harvey, N. L. (1995) *FEBS Lett* **375**(3), 169-73.

43. Harvey, N. L., Butt, A. J., and Kumar, S. (1997) *J Biol Chem* **272**(20), 13134-9.

44. Stefanis, L., Troy, C. M., Qi, H., Shelanski, M. L., and Greene, L. A. (1998) *J Neurosci*
    **18**(22), 9204-15.

45. Chen, W., Wang, H. G., Srinivasula, S. M., Alnemri, E. S., and Cooper, N. R. (1999) *J
    Immunol* **163**(5), 2483-91.

46. Slee, E. A., Adrain, C., and Martin, S. J. (1999) *Cell Death Differ* **6**(11), 1067-74.

47. Li, H., Bergeron, L., Cryns, V., Pasternack, M. S., Zhu, H., Shi, L., Greenberg, A., and
    Yuan, J. (1997) *J Biol Chem* **272**(34), 21010-7

48. Grossmann, J., Walther, K., Artinger, M., Kiessling, S., and Scholmerich, J. (2001) *Cell
    Growth Differ* **12**(3), 147-55.

49. Jesenberger, V., Procyk, K. J., Yuan, J., Reipert, S., and Baccarini, M. (2000) *J Exp Med*
    **192**(7), 1035-46.

50. Droin, N., Bichat, F., Rebe, C., Wotawa, A., Sordet, O., Hammann, A., Bertrand, R., and
    Solary, E. (2001) *Blood* **97**(6), 1835-44.

51. Paroni, G., Henderson, C., Schneider, C., and Brancolini, C. (2001) *J Biol Chem* **276**(24),
    21907-15.
52. Zha, J., Weiler, S., Oh, K. J., Wei, M. C., and Korsmeyer, S. J. (2000) *Science* **290**(5497), 1761-5.

53. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999) *J Biol Chem* **274**(2), 1156-63

54. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) *J Cell Biol* **144**(5), 891-901

55. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G. (2000) *J Exp Med* **192**(4), 571-580

56. Tsujimoto, Y., and Shimizu, S. (2000) *FEBS Lett* **466**(1), 6-10.

57. Skulachev, V. P. (1996) *FEBS Lett* **397**(1), 7-10

58. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997) *Cell* **91**(5), 627-37

59. Martinou, I., Desagher, S., Eskes, R., Antonsson, B., Andre, E., Fakan, S., and Martinou, J. C. (1999) *J Cell Biol* **144**(5), 883-9

60. Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J. C. (1998) *J Cell Biol* **143**(1), 217-24

61. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc Natl Acad Sci U S A* **95**(9), 4997-5002

62. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. (1999) *J Biol Chem* **274**(4), 2225-33

63. Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997) *Nature* **385**(6614), 353-7
64. Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R., and Martinou, J. C. (1997) Science 277(5324), 370-2
65. Schendel, S. L., Azimov, R., Pawlowski, K., Godzik, A., Kagan, B. L., and Reed, J. C. (1999) J Biol Chem 274(31), 21932-6
66. Bernardi, P., Broekemeier, K. M., and Pfeiffer, D. R. (1994) J Bioenerg Biomembr 26(5), 509-17
67. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) Nature 399(6735), 483-7
68. Shimizu, S., and Tsujimoto, Y. (2000) Proc Natl Acad Sci U S A 97(2), 577-82
FIGURE LEGENDS

FIG. 1. Inhibition of caspase-2 and CRADD induced apoptosis in human cells.
A, MCF-7 cells were transiently transfected with the indicated pRSC-lacZ constructs and a 4-fold excess of an empty vector or constructs encoding Bcl-2, Bcl-xL, Caspase-9-DN, XIAP or 20 µM z-VAD-fmk treatment. 30 hour after transfection, cells were stained with β-galactosidase and examined for morphological signs of apoptosis. The graphs show the percentage of round blue apoptotic cells relative to total blue cells under each condition after subtracting the percentage of apoptotic cells in the empty vector-transfected cells (mean ± S.D.) (n≥3). This is representative of three independent experiments. B, human embryonic kidney 293T cells were transiently cotransfected with the indicated T7-tagged expression plasmids and a 4-fold excess of an empty vector or constructs encoding caspase-9-DN or Bcl-xL. Cells were collected 30 h after transfection, and the cell lysates were analyzed by Western blot analysis using polyclonal antibody against human Caspase-3 p20 subunit.

FIG. 2. Activity of caspase-2 and caspase-8 towards other caspase zymogens.
A, Expression of caspase-2 and caspase-8 in bacteria. His<sub>6</sub>-epitope-tagged caspase-2 and caspase-8 zymogens were expressed in *Escherichia Coli*, purified on Talon Ni<sup>2+</sup>-affinity resin, and then analyzed by SDS-PAGE and Coomassie staining. *Lane M*, molecular mass markers (kDa); *Lane Casp-8*, Talon affinity-purified caspase-8; *Lane Casp-2*, Talon affinity-purified caspase-2. B, *In vitro* processing of caspase zymogens by purified recombinant caspase-2 and caspase-8. <sup>35</sup>S-labeled caspase zymogens were incubated with (+) or without (-) purified caspase-2 or caspase-8 at 37°C for 1 h as indicated. The reactions were stopped by addition of SDS-sample buffer, and the products were analyzed by SDS-PAGE and autoradiography. C, <sup>35</sup>S-
labeled caspase-2 zymogen and baculovirus p35 were incubated with (+) or without (-) purified caspase-2 or caspase-8 and then analyzed as in B.

FIG. 3. Caspase-2 and caspase-8 induce Cyt c release from isolated mitochondria.
A, Freshly isolated HeLa cell mitochondria (10 µg) were incubated with increasing amounts of caspase-2 or –8 in the presence (+) or absence (-) of HeLa cytosol (10 µg) at 37°C for 1h. After incubation, the mitochondria were separated from the supernatants by centrifugation at 12,000 xg for 10 min. The supernatants were subjected to SDS-PAGE (15%), and immunoblotting with anti-Cyt c antibody. The pellets were immunoblotted with anti-cytochrome oxidase (Cox) antibody. B, Freshly isolated HeLa cell mitochondria (10 µg) were incubated with caspase-2 or –8 (50 ng) and increasing amounts of HeLa cytosol at 37°C for 1 h. The reactions were then analyzed as in A.

FIG. 4. Caspase-2 induces Cyt c release by cleaving Bid.
A, Determination of the caspase-2 cleavage site in Bid. 35S-labeled wild type or mutant Bid proteins were incubated with purified caspase-2 or caspase-8 at 37°C for 1 h as indicated. The reactions were stopped by addition of SDS-sample buffer, and the products were analyzed by SDS-PAGE and autoradiography. B, HeLa S-100 extract (40 µg), or HeLa S-100 extract immunodepleted with Bid antibody (40 µg) were incubated with 5 µl of freshly isolated mitochondria (10 µg) in the absence (lanes 2 and 6) or presence of caspase-8 (100 ng) (lanes 3 and 7) or caspase-2 (100 ng) (lanes 4 and 8). After 1 hour of incubation at 37°C, the samples were centrifuged at 12,000 xg for 10 min at 4°C. The resulting supernatants and pellets were subjected to SDS-PAGE and immunoblotting with anti-Cyt c and cytochrome oxidase.
antibodies, respectively. Mitochondria incubated with tBid (lane 9) were used as a positive control.

FIG. 5. Caspase-2 induces Cyt c release from isolated mitochondria and activates the downstream caspases.

A, Dose-respond of Cyt c, AIF and Smac release by caspase-2 from isolated mitochondria. Freshly isolated HeLa cell mitochondria were incubated with increasing amounts of purified recombinant caspase-2 in buffer A at 37°C for 1 hour. The samples were then centrifuged at 12,000 xg for 10 min at 4°C. The resulting supernatants and pellets were fractionated by SDS-PAGE and then the supernatants were western blotted with anti-Cyt c, AIF, Smac, citrate synthase (CS) and cytochrome oxidase antibodies. The corresponding pellets were western blotted with citrate synthase and cytochrome oxidase antibodies. B, Percoll gradient-purified mouse liver mitochondria were incubated with increasing amounts of purified recombinant mature caspase-2 in buffer A at 37°C for 1 hour and then analyzed by western blotting with Cyt c antibody as described above. C, Mitochondrial (M, lane 1) and cytosolic (C, lane 2) fractions were isolated from HeLa cells and the two fractions were fractionated by SDS-PAGE and then western blotted with anti-Bax, cytochrome oxidase, Bid, caspase-8 and β-actin. D, purified recombinant T7-tagged pro-caspase-3 C-A was incubated with caspase-2 (100 ng), Apaf-1, caspase-9, and dATP with (lanes 6-8) or without (lanes 1-5) increasing amounts of Cyt c (1, 10, 50 ng) for 1 h at 37°C as indicated. The reaction products were then analyzed by SDS-PAGE and immunoblotted with anti-T7-tag antibody. E, purified recombinant T7-tagged pro-caspase-3 C-A was incubated with Apaf-1, caspase-9 and dATP in the presence (+) or absence (-) of freshly isolated HeLa cell mitochondria and increasing amounts of caspase-2 (0, 100, 200 ng) at
37°C for 1 h. The reaction products were then analyzed as in D. Lane 1, a control T7-tagged procaspase-3 C-A incubated with Apaf-1, caspase-9, dATP, and Cyt c. Lane 7, a control T7-tagged procaspase-3 C-A incubated with 200 ng caspase-2 treated mitochondria.

FIG. 6. The inactive caspase-2 C-A mutant does not induce Cyt c release from isolated mitochondria.

A. Expression of caspase-2 and caspase-2 C-A in bacteria. His$_6$-epitope-tagged caspase-2 and caspase-2 C-A mutant were expressed in *Escherichia Coli*, purified on Talon Ni$^{2+}$-affinity resin, and then analyzed by SDS-PAGE and Coomassie staining. *Lane M*, molecular mass markers (kDa); *Lane Casp-2*, Talon affinity-purified caspase-2; *lane Casp-2 C-A*, Talon affinity-purified caspase-2 C-A; Pcasp-2, procaspase-2; LS, large subunit; SS, small subunit. B. Aliquots of freshly isolated HeLa cell mitochondria (10 µg each) were incubated with increasing amounts of recombinant purified caspase-2 (*lanes* 1-6) or caspase-2 C-A (*lanes* 7-12) at 37°C for 1 hour. The reaction mixtures were then centrifuged at 12,000 xg for 10 min at 4°C. The resulting supernatants and pellets were subjected to SDS-PAGE and immunoblotting with anti-Cyt c and cytochrome oxidase antibodies, respectively. C. Enzymatic activities of reaction B were correspondingly analyzed with the penta-peptide substrate VDVAD-AFC at the same time. RFU, relative fluorescent unit.

FIG.7. Endogenous procaspase-2 is cleaved by activated caspase-2 in vitro.

Mitochondrial (M) and cytosolic (C) fractions were isolated from HeLa, MCF-7, and HEK293T cell lines as described under "Experimental Procedures" and incubated with (*lanes*, 3,4,7,8,11, and 12) or without (*lanes* 1,2,5,6,9, and 10) purified caspase-2 protein at 37°C for 1
hour. The reaction mixtures containing the mitochondrial fractions were centrifuged at 12,000 xg for 10 min at 4°C and the pellets were washed in buffer A twice to remove the exogenous caspase-2. All reaction mixtures were then analyzed by SDS-PAGE and immunoblotted with anti-caspase-2 antibody. Lane 13 is the total input of caspase-2 alone.
Figure 1

**A**

![Bar chart showing the percentage of apoptotic cells for different conditions.](chart.png)

- **Caspase-2**
  - Vector
  - Casp-9-DN
  - Bcl-2
  - Bcl-xL
  - XIAP
  - zVAD-fmk

- **CRADD**
  - Vector
  - Casp-9-DN
  - Bcl-2
  - Bcl-xL
  - XIAP
  - zVAD-fmk

**B**

![Western blot showing Casp-3-p20 levels for different conditions.](western_blot.png)

- **Control**
- **Casp-9-DN**
- **Bcl-xL**
Figure 2

Panel A: Gel image showing bands at 46kDa, 30kDa, and 10kDa.

Panel B: Gel images for Effector Caspases (Casp-3, Casp-6, Casp-7) and Initiator Caspases (Casp-8, Casp-9, Casp-10).

Panel C: Gel images for S35-labeled Casp-2 and P35.
Figure 3

A

| Caspase (ng) | 0 | 10 | 20 | 50 | 100 | 200 | 0 | 10 | 20 | 50 | 100 | 100 | 100 |
|--------------|---|----|----|----|-----|-----|---|----|----|----|-----|-----|-----|
| HeLa S-100 (5 μg) | - | + | + | + | + | + | + | + | + | + | + | - | - |
| Lanes        | 1 | 2  | 3  | 4  | 5   | 6   | 7  | 8  | 9  | 10 | 11  | 12  | 13  |

Cyt c  

Cox  

pellet  

supernatant

B

| Caspase (ng) | 0 | 10 | 0.1 | 0.5 | 1 | 5 | 10 | 0.1 | 0.5 | 1 | 5 | 10 |
|--------------|---|----|-----|-----|---|---|----|-----|-----|---|---|----|
| HeLa S-100 (μg) | 0 | 0  | 10  | 0.1 | 0.5 | 1 | 5 | 0.1 | 0.5 | 1 | 5 | 10 |
| Lanes        | 1 | 2  | 3  | 4  | 5  | 6 | 7  | 8  | 9  | 10 | 11 | 12 |

Cyt c  

Cox  

pellet  

supernatant
Figure 4

A

| Lanes | BidWT | BidD59E | BidDM | S^{35}\text{-labeled} |
|-------|-------|---------|-------|-------------------------|
| 1     | Control | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 2     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 3     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 4     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 5     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 6     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 7     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 8     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 9     | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 10    | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 11    | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |
| 12    | Casp-8 | Control | Casp-8 | Control | Casp-2 | S^{35}\text{-labeled} |

B

| Caspase (100 ng) | HeLa S-100 | Bid-depleted S-100 | tBid |
|------------------|-------------|--------------------|------|
| Caspase (100 ng) | -           | -                  | -    |
| Mitochondria (10 μg) | +         | +                  | +    |
| Lanes            | 1           | 2                  | 3    |

Cyt c

supernatant

Cox

pellet
Figure 5

A

| mitochondria | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------|---|---|---|---|---|---|
| Casp-2 (ng)  | + | + | + | + | + | + |

B

| mitochondria | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------|---|---|---|---|---|---|
| Casp-2 (ng)  | + | + | + | + | + | + |

C

1 | 2
---|---
Bax | |
Cox | |
Bid | |
Casp-8 | |
β-actin | |

D

| pcasp-3- | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------|---|---|---|---|---|---|---|---|
| T7casp-3 C-A | + | + | + | + | + | + | + | + |
| Casp-2    | - | + | + | + | + | + | + | + |
| Apaf-1    | - | - | + | + | + | + | + | + |
| Casp-9    | - | - | - | + | + | + | + | + |
| dATP      | - | - | - | - | + | + | + | + |
| Cyt c     | - | - | - | - | - | + | + | + |

E

| pcasp-3- | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|----------|---|---|---|---|---|---|---|
| T7casp-3 C-A | + | + | + | + | + | + | + |
| Apaf-1+dATP | + | + | + | + | + | + | - |
| +Casp-9    | + | + | + | + | + | + | + |
| mitochondria | - | - | + | + | + | + | + |
| Cyt c      | + | - | - | - | - | - | - |
| Casp-2     | - | - | + | + | + | + | + |
Figure 6

A

B

C
Figure 7

The figure shows a gel electrophoresis pattern with bands for different samples: HeLa, MCF7, and 293T. The bands are labeled for Procasp-2 and Cleaved casp-2. The molecular weight markers are indicated at the left side in kilodaltons (kDa): 45.7, 32.5, and 18.
Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria
Yin Guo, Srinivasa M. Srinivasula, Anne Druilhe, Teresa Fernandes-Alnemri and Emad S. Alnemri

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