Pro-caspase-8 Is Predominantly Localized in Mitochondria and Released into Cytoplasm upon Apoptotic Stimulation*

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The recruitment and cleavage of pro-caspase-8 to produce the active form of caspase-8 is a critical biochemical event in death receptor-mediated apoptosis. However, the source of pro-caspase-8 available for activation by apoptotic triggers is unknown. In human fibroblasts and mouse clonal striatal cells, confocal microscopy revealed that pro-caspase-8 immunofluorescence was co-localized with cytochrome c in mitochondria and was also distributed diffusely in some nuclei. Biochemical analysis of subcellular fractions indicated that pro-caspase-8 was enriched in mitochondria and in nuclei. Pro-caspase-8 was found in the intermembrane space, inner membrane, and matrix of mitochondria after limited digestion of mitochondrial fractions, and this distribution was confirmed by immunogold electron microscopy. Pro-caspase-8 and cytochrome c were released from isolated mitochondria that were treated with an inhibitor of the ADP/ATP carrier atractyloside, which opens the mitochondria permeability transition pore. Release was blocked by the mitochondria permeability transition pore inhibitor cyclosporin A (CsA). After clonal striatal cells were exposed for 6 h to an apoptotic inducer tumor necrosis factor-α (TNF-α), mitochondria immunoreactive for cytochrome c and pro-caspase-8 became clustered at perinuclear sites. Pro-caspase-8 and cytochrome c levels decreased in mitochondrial fractions and increased, along with pro-caspase-8 cleavage products, in the cytoplasm of the TNF-α-treated striatal cells. CsA blocked the TNF-α-induced release of pro-caspase 8 but not cytochrome c. Internucleosomal DNA fragmentation started at 6 h and peaked 12 h after TNF-α treatment. These results suggest that pro-caspase-8 is predominantly localized in mitochondria and is released upon apoptotic stimulation through a CsA-sensitive mechanism.

Apoptosis (programmed cell death) originally referred to an active form of cell death with stereotypic morphological characteristics occurring during development (1). A broad range of pathological conditions can induce apoptosis (2, 3). Unbalanced cell proliferation and apoptosis may play a role in pathogenesis of certain types of tumors and neurodegenerative diseases (4, 5). A family of novel cysteine proteases, named caspase, plays an essential role in most, if not all, forms of apoptosis (6–8). Caspases are produced as pro-enzymes and become activated by proteolytic cleavage at internal aspartate residues upon apoptotic stimulation (9). Two categories of caspases important for apoptosis have been recognized: the initiators and executors. The initiator caspases, which include caspase-8, -9, and -10, are activated in the earlier phase of apoptosis; the executor caspases, which include caspase-3, -6, and -7, are activated by initiator caspases and are responsible for dismantling cells (10, 11). Caspase-3 activation, a convergent event in apoptosis, is triggered by a variety of apoptotic stimuli. Caspase-3 cleaves many cytoskeletal proteins, such as fodrin, and proteins involved in DNA repair and fragmentation, such as poly(ADP-ribose) polymerase and DNA fragmentation factor 45 (10, 12, 13). Two pathways lead to the activation of caspase-3 through release of cytochrome c and cleavage of pro-caspase-9 and through ligation of death receptors by tumor necrosis factor (TNF) and Fas ligand. In the pathway stimulated by TNF and Fas ligand, pro-caspase-8 is recruited and activated by the adapter molecules FADD/MORT1 (14–17). Caspase-8 then directly activates pro-caspase-3 and cleaves BID, a member of Bcl-2 family proteins (18–20).

The role of mitochondria in apoptosis was first appreciated in a cell-free system when nuclear apoptotic events were induced only by cytoplasmic fractions enriched in mitochondria (21). A large body of evidence now suggests that many apoptotic stimuli affect the mitochondrial permeability transition pore (MPTP) and cause the release of pro-apoptotic molecules such as cytochrome c and Apaf-1 from mitochondria (22–26). Cytochrome c and Apaf-1 activate pro-caspase-9 to initiate an apoptotic cascade. Recent studies indicate that caspase-2, -3, and -9 are also released from mitochondria during apoptosis (23, 27–29). These findings suggest that mitochondria may be more broadly involved in apoptosis than previously thought. The subcellular distribution of pro-caspase-8 is not clear. Since knowledge about the localization of pro-caspase-8 could be important in understanding its role in apoptosis, we evaluated the subcellular localization of pro-caspase-8 in human fibroblasts and in a mouse clonal striatal cell line. The results suggest that

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† The abbreviations used are: TNF, tumor necrosis factor; FADD, Fas-associated death domain; MPTP, mitochondrial permeability transition pore; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine; CFS, cell-free system; Atr, atractyloside; CaA, cyclosporin A; MORT, mediator of receptor-induced toxicity.
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Pro-caspase-8 is predominantly found in the intermembrane space and inner membrane of mitochondria and can be released upon apoptotic stimulation.

**Experimental Procedures**

**Cell Culture**—Human fibroblasts obtained from Coriell Cell Repositories were cultured in minimum Eagle’s medium (Life Technologies, Gaithersburg, MD) supplemented with 15% fetal bovine, 100 units/ml penicillin G/streptomycin, 0.5 μg/ml amphotericin B, 2 mM glucose, 2× final concentration minimum Eagle’s medium vitamins, nonessential and essential amino acids (all from Life Technologies) at 37°C with 5% CO₂. Clonal mouse striatal cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 4.5 glucose, and 50 units/ml penicillin G/streptomycin (30).

**Immunofluorescence and Confocal Microscopy**—Clonal striatal cells or human fibroblasts were cultured on poly-l-lysine-coated microslips for 24 h. Immunohistochemistry was performed as described previously (31-33).

To study the cellular localization of pro-caspase-8, cells were incubated with the rabbit polyclonal antibodies against pro-caspase-8 (SK-441, Ref. 32) or with the mouse monoclonal antibody against cytochrome c (PharMingen, San Diego, CA) for 24 h at 4°C. Microslips were washed and incubated with fluorescein-conjugated goat anti-rabbit IgG antibodies (or donkey against mouse IgG) (Molecular Probes, Eugene) by the effects of TNF-α on localization of pro-caspase-8 and cytochrome c, clonal striatal cells were treated with TNF-α (20 ng/ml) for 6 h and then processed for immunostaining as described above. The nuclei of these cells were stained with propidium iodide. Immunostained cells were examined with a confocal microscope (Bio-Rad 1024) and merged in Adobe Photoshop.

**Immunogold and Electron Microscopy**—Clonal striatal cells were cultured in 60-mm dishes, fixed, and incubated with polyclonal antibodies against pro-caspase-8 as described above and then with gold-conjugated secondary antibody for 2 h at room temperature. Cells were post-fixed in 2.5% glutaraldehyde, incubated in 1% osmium tetroxide and 1% uranyl acetate, dehydrated in increasing grades of alcohol, and embedded in an ethanol-soluble epoxy (LX112, LADD). Embedded cells were sectioned (Ultracut E, Reichert-Jung) and examined with a JEOL 100CX electron microscope.

**Isolation of Mitochondria, Mitochondrial Fractions, and Nuclei**—Purification of mitochondria was performed using a Percoll gradient procedure described by Gasmier et al. (34) with minor modifications. Clonal striatal cells were harvested and rinsed in Hank’s balanced salt solution twice. Cells were suspended in 0.5 ml of buffer A containing 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, 1 mM dithiothreitol, 100 mM MgCl₂, 5 mM pyruvate, 2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μg/ml pepstatin A, 50 μg of antipain, 1 mM dithiothreitol, 10 mM HEPES-NaOH (pH 7.4). The suspension was equally divided into several test tubes determined by the needs of the experiments. Release of pro-caspase-8 and cytochrome c was induced by the ADP/ATP carrier inhibitor atractyloside (Atr, 5 or 10 μM, Sigma). Atractyloside was dissolved in CFS and incubated with mitochondria for 1 h at room temperature. Separation of mitochondria and supernatant was achieved by centrifugation (10,000 × g, 15 min). The supernatant was then further cleared by centrifugation at 100,000 × g for 1 h. To assess whether release of pro-caspase-8 is through MPT, cyclosporin A (CsA, 25 μM, Sigma) was added together with Atr. After drug treatment, mitochondria suspensions were centrifuged at 10,000 × g to pellet mitochondria. The supernatants were centrifuged again at 100,000 × g for 1 h. The resultant supernatant and cytochrome c measured were used as Western blot analysis in mitochondrial and supernatant fractions.

**Determination of Pro-caspase-8 Release from Isolated Mitochondria**—Protein concentrations in all fractions were determined by the BCA kit. Levels of pro- and active caspase-8 and cytochrome c were measured with a Bio-Rad i-Chemi system. The resultant supernatants were used as Western blot, and the pellet fractions were used as inner membrane fraction. Both outer and inner membrane fractions were solubilized with a solution containing 50 mM Tris-HCl, 0.5% Nonidet P-40, and 1 mM calcium chloride (TNC, 34). Protein concentrations in all fractions were determined by the BCA kit.

**Proteinase K Digestion of Isolated Mitochondria**—A protocol reported by Samali et al. (36) with modifications was used. Mitochondria were incubated in H-medium containing 0.2 μg/ml proteinase K or in H-medium containing 1% Nonidet P-40 plus proteinase K (0.2 μg/ml) for 3 min at room temperature. Control mitochondria were incubated with H-medium only or H-medium with 1% Nonidet P-40. Proteolysis was terminated by adding an equal volume of TNC buffer containing 15 μg phenylmethylsulfonyl fluoride, 2% SDS, 20 μl of loading buffer, and boiling for 5 min. Samples were analyzed immediately by immunoblotting on an immunoblotting gel.

**Determination of Pro-caspase-8 Release from Isolated Mitochondria**—We followed the protocol of Susin et al. (29) with minor modifications. A crude mitochondrial fraction, prepared as described above, was suspended in a cell-free system (CFS) containing 220 mM mannitol, 65 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 2 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μg/ml pepstatin A, 50 μg of antipain, 1 mM dithiothreitol, 10 mM HEPES-NaOH (pH 7.4). The suspension was equally divided into several test tubes determined by the needs of the experiments. Release of pro-caspase-8 and cytochrome c was induced by the ADP/ATP carrier inhibitor atractyloside (Atr, 5 or 10 μM, Sigma). Atractyloside was dissolved in CFS and incubated with mitochondria for 1 h at room temperature. Separation of mitochondria and supernatant was achieved by centrifugation (10,000 × g, 15 min). The supernatant was then further cleared by centrifugation at 100,000 × g for 1 h. To assess whether release of pro-caspase-8 is through MPT, cyclosporin A (CsA, 25 μM, Sigma) was added together with Atr. After drug treatment, mitochondria suspensions were centrifuged at 10,000 × g to pellet mitochondria. The supernatants were centrifuged again at 100,000 × g for 1 h. The resultant supernatant and cytochrome c measured were used as Western blot analysis in mitochondrial and supernatant fractions.

**Western Blot Analysis**—Rabbit polyclonal antibodies against pro-caspase-8 (SK-441) or active caspase-8 (SK-440) (33) or monoclonal antibodies against cytochrome c, HSP70 (StressGen Biotechnologies Corp., Victoria, BC, Canada) or cytochrome oxidase IV (Molecular Probes) were applied at 4°C overnight. Membranes were washed three times for 5 min each with Tris-HCl-buffered saline with 0.1% Tween 20, and immunoreactivity was visualized with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and visualized by autoradiography. Autoradiograms were analyzed by densitometry with image analysis software (Sigma Scan Pro 4.0) after capturing the digital images with Hewlett Packard ScanJet 4C/T according to the manufacturer’s protocol. The total density and area of each band were measured. The total values of pro-caspase-8, cytochrome c, actin, or cytochrome oxidase were calculated for 2 min on ice, and centrifuged at 150,000 × g for 1 h at 4°C. The resultant supernatants were used as the matrix fraction, and the pellet fractions were used as inner membrane fraction. Both outer and inner membrane fractions were solubilized with a solution containing 50 μM Tris-HCl, 0.5% Nonidet P-40, and 1 mM calcium chloride (TNC, 34). Protein concentrations in all fractions were determined by the BCA kit.

For purification of nuclei, human fibroblasts were lysed in a buffer containing 0.1% Triton X-100, 20 mM Tricine-NaOH, 250 mM KC1, 5 mM MgCl₂ (pH 7.8), and protease inhibitors tablets (Roche Molecular Biochemicals). Crude homogenate was centrifuged at 2000 × g at 4°C for 10 min to remove supernatant (S1) and a crude nuclear pellet (P1). The nuclear pellet was purified using iodixanol step gradients according to manufacturer’s instructions (Optiprep, Accurate Chemicals, Westbury, NY).

**Proteinase K Digestion of Isolated Mitochondria**—A protocol reported by Samali et al. (36) with modifications was used. Mitochondria were incubated in H-medium containing 0.2 μg/ml proteinase K or in H-medium containing 1% Nonidet P-40 plus proteinase K (0.2 μg/ml) for 3 min at room temperature. Control mitochondria were incubated with H-medium only or H-medium with 1% Nonidet P-40. Proteolysis was terminated by adding an equal volume of TNC buffer containing 15 μg phenylmethylsulfonyl fluoride, 2% SDS, 20 μl of loading buffer, and boiling for 5 min. Samples were analyzed immediately by immunoblotting on an immunoblotting gel.
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The mitochondrial localization of pro-caspase-8 was further examined in mitochondrial fractions. Western blot analysis showed that pro-caspase-8 was detected in the intermembrane space, inner membrane, and matrix fractions (Fig. 3B). In several independent experiments, outer membrane always contained little or no pro-caspase-8, whereas inner membrane contained the highest levels of pro-caspase-8. Both intermembrane space and matrix contained intermediate levels of pro-caspase-8. Cytochrome oxidase and HSP70 were examined for the purpose of monitoring separation of outer and inner membranes. Consistent with the known distribution of these proteins, cytochrome oxidase was found only in the inner membrane fraction, whereas HSP70 was enriched in the outer membrane and in the matrix fraction (Fig. 3B).

The localization of pro-caspase-8 inside mitochondria was also examined with immunogold electron microscopy. Numerous gold particles were found inside mitochondria, but none were seen on the outer mitochondrial membrane (Fig. 4). Gold particles were absent when primary antibody was omitted (data not shown).

Release of Pro-caspase-8 from Isolated Mitochondria—To assess whether mitochondria release pro-caspase-8, crude mitochondria were suspended in the cell-free system in the presence or absence of Atr (5 or 10 mM), which opens the MPTP. After incubation, mitochondria and supernatant were separated by centrifugation. Pro-caspase-8 was modestly decreased in mitochondria and robustly detected in the supernatant after Atr treatment. Quantitative analysis of three independent experiments showed pro-caspase-8 levels in mitochondrial fractions decreased to 86.2 ± 3% (5 mM) or 79.4 ± 3.8% (10 mM) of control (nontreated mitochondria) and in the supernatants increased to 267.7 ± 103 (5 mM) or 351.7 ± 73 (10 mM) of control. Concurrently, cytochrome c was markedly decreased in the mitochondrial fraction and strongly detected in the supernatant after Atr treatment. In mitochondrial fractions, cytochrome c decreased to 57.3% ± 15.1% (5 mM) or 34.8 ± 8.9% (10 mM) of control (nontreated mitochondria), whereas in the supernatants the levels were 246.3 ± 51.2% and 534 ± 199%, respectively (Fig. 5, top).

To evaluate whether mitochondria release pro-caspase-8 through the MPTP, mitochondria were treated with Atr in the presence or absence of the MPTP inhibitor CsA (25 μM). Quan-
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Fig. 2. Subcellular localization of pro-caspase-8. A, pro-caspase-8 in crude mitochondria (Mit). Clonal striatal cells were processed to obtain cytosol and crude mitochondria as described under “Experimental Procedures.” A is a representative immunoblot from three independent experiments. Pro-caspase-8 was highly enriched in the mitochondria but low in the cytosol. Cytochrome c was high in the mitochondrial fraction but low in the cytosol. B, pro-caspase-8 in purified mitochondria. Crude mitochondria were purified with a Percoll gradient as described under “Experimental Procedures.” B is a representative immunoblot from three independent experiments. Pro-caspase-8 was enriched in the mitochondria but was low in the cytosol. Cytochrome oxidase was only detected in the mitochondrial fraction but not in the cytosolic fraction. Cytochrome c was low in mitochondria after Percoll gradient purification; therefore cytochrome oxidase was used as a mitochondrial marker in these studies. C, pro-caspase-8 in purified nuclei. Human fibroblasts were processed to obtain crude homogenate, S1, P1, and N fractions as described under “Experimental Procedures.” C is a representative immunoblot from three independent experiments. CH is crude homogenate; S1 is supernatant resulting from 2000 × g centrifugation of crude homogenate. P1 is the pellet resulting from 2000 × g centrifugation. N is the purified nuclear fraction obtained from P1 by iodixanol step gradients. Pro-caspase-8 is present in purified nuclei and in the P1 fraction from which nuclei were purified. 20 μg of protein were loaded in each lane in all experiments.

Fig. 3. Mitochondrial localization of pro-caspase-8. A, proteinase K degradation of pro-caspase-8 in crude mitochondria. Crude mitochondria were isolated as described under “Experimental Procedures.” Mitochondria were incubated in CFS in the presence or absence of proteinase K (Pro K) or proteinase K plus Nonidet P-40. A is a representative immunoblot from four independent experiments. Proteinase K alone partially degraded HSP70 but had no effect on pro-caspase-8 and cytochrome c. In the presence of Nonidet P-40, proteinase K degraded pro-caspase-8, HSP70, and cytochrome c. B, pro-caspase-8 distribution in mitochondrial fractions. Mitochondria fractions were prepared as described under “Experimental Procedures.” B is a representative immunoblot from four independent experiments. Pro-caspase-8 was found in the matrix and intermembrane space (IMS) and at its highest levels in the inner membrane fraction (IM). HSP70 was enriched in both outer membrane (OM) and matrix fractions. Cytochrome oxidase was only found in the inner membrane fraction. 20 μg of protein were loaded in each lane.

Investigative analysis of results from three independent experiments showed that CsA blocked the Atr-induced decrease in pro-caspase-8 levels in mitochondria (from 86.9 ± 9.7% of control to 113.7 ± 22% in the presence of CsA). CsA inhibited the Atr-induced increase in pro-caspase-8 levels in the supernatants (from 693.6 ± 206 to 109.8 ± 34.8% of control in the presence of CsA). CsA also inhibited the Atr-induced increase in cytochrome c in the supernatants (from 470.8 ± 170.5% of controls to 77.4 ± 22% in the presence of CsA) and prevented the Atr-induced decrease in cytochrome c in mitochondria (from 65.9 ± 5 to 99.2 ± 16.3 in the presence of CsA). In addition, after Atr treatment, the pro-caspase-8 cleavage product (p10) was also detected in the supernatant and was inhibited by CsA (Fig. 5, bottom).

TNF-α Effects on Localization of Mitochondria and Pro-caspase-8—The effects of the apoptotic inducer TNF-α on mitochondria localization were determined by immunofluorescence and confocal microscopy. In untreated clonal striatal cells, cytochrome c and pro-caspase-8 immunoreactive mitochondria were distributed relatively evenly throughout the cytoplasm (Fig. 6, top panels, green label). Treatment with TNF-α for 6 h induced marked clustering, fusion, and peri-nuclear localization of mitochondria labeled for cytochrome c or pro-caspase-8. These changes appeared in cells with or without nuclear fragmentation, a feature of apoptosis. In some of the cells including those that were shrunken with fragmented nuclei, labeling for mitochondria with cytochrome c or pro-caspase-8 was severely reduced or absent, and diffuse staining for cytochrome c (Fig. 6, left middle and lower panels), or pro-caspase-8 was evident in the cytoplasm (Fig. 6, right middle and lower panels).

Release of Pro-caspase-8 from Mitochondria in Intact Cells—To evaluate mitochondrial release of pro-caspase-8 in intact cells under apoptotic conditions, clonal striatal cells were treated with TNF-α (10 or 20 ng/ml), for 6 h and then mitochondrial and cytosolic fractions were prepared. TNF-α caused a reduction in levels of pro-caspase-8 in mitochondria, whereas cytosolic levels of pro-caspase-8 levels were increased (Fig. 7A). Quantitative analysis of three independent experiments showed that in the mitochondrial fractions, pro-caspase-8 levels decreased to 78.6 ± 5.2% (10 ng/ml) or 69.4 ± 0.64% (20 ng/ml) of control (vehicle-treated cells), whereas in the cytosol, the levels were 140.4 ± 23.7% and 331.9 ± 34.5% of control, respectively. Cytochrome c levels in the mitochondrial fractions were decreased to 28 ± 9.2% (10 ng/ml) or 18.1 ± 3.1% (20 ng/ml) of control (vehicle-treated cells), whereas in the cytosol, cytochrome c increased to 603.2 ± 362.5% (10 ng/ml) or 516.6 ± 330.5% (20 ng/ml) of control. In the mitochondrial fraction, an active form of caspase-8 (p10) was detected in all cells but tended to rise in cells treated with TNF-α. Active caspase-8 (p10 and p20) was almost undetectable in cytosolic fractions of control cells but readily detected in TNF-α-treated cells.

To evaluate whether apoptosis was induced in clonal striatal cells after TNF-α administration, low molecular weight DNA was extracted, and internucleosomal DNA fragmentation was examined. Internucleosomal DNA fragmentation was barely...
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FIG. 4. Immunogold electron micrographs of pro-caspase-8 localization. Clonal striatal cells were immunostained using polyclonal antibody against pro-caspase-8 and gold-conjugated secondary antibody. Two representative mitochondria are shown. Gold particles (arrowheads) appear inside mitochondria. No gold particles appear in association with mitochondrial outer membranes (arrowheads). Scale bar = 1 μm.

detectable at 6 h after TNF-α administration and clearly appeared 12–24 h after TNF-α treatment (Fig. 7B). Since release of pro-caspase-8 and cytochrome c occurred by 6 h, these results suggested that mitochondrial release of pro-caspase-8 and cytochrome c preceded the execution of apoptosis.

To evaluate whether the release of pro-caspase-8 in intact cells is dependent on MPTP, the effect of CsA on TNF-α-induced pro-caspase-8 release was examined in clonal striatal cells. These studies showed that pro-caspase-8 and cytochrome c levels robustly increased in the cytosol and decreased in mitochondria. Coadministration of CsA markedly attenuated TNF-α-induced increases in the level of pro-caspase-8 in the cytosol (Fig. 7C). Treatment with CsA alone had no effect on either cytosolic or mitochondrial levels of pro-caspase-8 and cytochrome c (data not shown). Quantitative analysis of three independent experiments showed that CsA attenuated the TNF-α-induced decrease in pro-caspase-8 levels in mitochondria from 63.7 ± 9.8% (TNF-α 20 ng/ml) to 82.3 ± 9.1% (TNF-α + CsA) of control (vehicle-treated cells). CsA inhibited the increase in pro-caspase-8 levels in the cytosol from 209.5 ± 47.8% (TNF-α) to 102.6 ± 29.6% (TNF-α + CsA) of control (vehicle-treated cells). However, CsA was ineffective in blocking the effect of TNF-α on cytochrome c. In the mitochondrial fractions, cytochrome c levels were 57.7 ± 11.2% in TNF-α-treated cells and 44.6 ± 11.7% of control (vehicle-treated cells) in TNF-α plus CsA-treated cells. In the cytosol, the levels of cytochrome c were 784.5 ± 267.2% and 644.4 ± 104.1%, respectively.

**DISCUSSION**

A current model for the activation of caspase-8 in apoptosis after stimulation by Fas and TNF-α involves the recruitment of cytosolic FADD/MORT1 and pro-caspase-8 to form a death-inducing signaling complex (41–44). Pro-caspase-8 has the ability to activate itself. FADD/MORT1 brings into proximity two or more pro-caspase-8 molecules, which accelerates the self-cleavage and enhances caspase-8 activation (44, 45). The subcellular localization of pro-caspase-8 has not been well defined until now, making it unclear how pro-caspase-8 becomes available to FADD/MORT1 during apoptosis. We showed that mitochondria are a major site for pro-caspase-8 localization and regulation during apoptosis. By immunofluorescent staining, immunogold labeling, and Western blot analysis, pro-caspase-8 was located primarily inside mitochondria. Mitochondrial levels of pro-caspase-8 and cytochrome c were unaffected by pro-

**FIG. 5. Mitochondrial release of pro-caspase-8.** Crude mitochondria were prepared as described under “Experimental Procedures.” Mitochondria were suspended in CFS in the presence or absence of Atr (5 or 10 mM). After 1 h of incubation at room temperature, mitochondria and supernatants were separated by centrifugation. Top, Atr-induced release of pro-caspase-8, representative of three independent experiments. In mitochondria (Mit), Atr reduced pro-caspase-8 by densitometry, the signal was decreased to 86.2 ± 3% (5 mM) or 79.4 ± 3.8% (10 mM) of control) and cytochrome c (signal decreased to 57.3 ± 15.1% (5 mM) or 34.8 ± 8.9% (10 mM) of control). In the supernatant (Sup), pro-caspase-8 increased to 267.7 ± 103% (5 mM) or 351 ± 73% (10 mM) of control, and cytochrome c increased to 246.3 ± 51.2% (5 mM) or 534 ± 199% (10 mM) of control. Bottom, blockade of pro-caspase-8 release by cyclosporin A. Mitochondria were suspended in CFS with or without Atr (5 mM). To block MPTP, cyclosporin A (25 μM) was added. After a 1-h drug treatment, mitochondria (Mit) and supernatants (Sup) were separated by centrifugation. Experiment which is representative of three independent experiments. In the supernatant, CsA inhibited the increases in pro-caspase-8 (decreased to 109.8 ± 34.8% (Atr + CsA) from 693.6 ± 206% (Atr) of control) and cytochrome c (decreased to 77.4 ± 22% (Atr + CsA) from 470.5 ± 170.5% (Atr) of control) in the mitochondria, CsA reduced Atr-induced decreases in pro-caspase-8 (increased to 113.7 ± 22% (Atr + CsA) from 86.9 ± 9.7% (Atr) of control by densitometry) and cytochrome c increased to 99.2 ± 16.3% (Atr + CsA) from 65.9 ± 5% (Atr) of control). CsA also reduced pro-caspase-8 cleavage products (p10) induced by Atr in the supernatant. The total protein loaded in each lane was 8 μg for mitochondrial fraction and 18 μg for cytosol fraction.

Proteinase K treatment, again consistent with localization inside mitochondria. The presence of pro-caspase-8 in mitochondria was found in three cell lines (human fibroblasts, monkey COS cells, mouse clonal striatal cells). Zhivotovsky et al. (46) report low levels of pro-caspase-8 in mitochondria of apoptotic Jurkat cells and no pro-caspase 8 in normal Jurkat cells by Western blot. Although only biochemical assays were performed in their study, the discrepancy with our findings is still hard to explain. Other caspasess including pro-caspase-2, -3, -6, and -9 have also been identified in mitochondria (27–29). Similar to pro-caspase-8, caspase-9 is enriched in mitochondria compared with cytosol (27).

Activation of caspase-8 requires interactions with death domains in proteins that are present in the cytosol (47, 48). Thus sequestering pro-caspase-8 in mitochondria may be a safeguard mechanism. How pro-caspase-8 enters mitochondria remains unclear. There is a mitochondrial matrix-targeting signal at the N terminus of pro-caspase-9, but other mitochondrial
TNF-α is thought to activate caspase-8 by a mechanism involving recruitment of pro-caspase-8 by FADD/MORT1 (14–17). TNF-α and Fas have also been shown to induce release of cytochrome c and activation of caspase-9 and -3 (49–51). In some cell types, these effects can be inhibited by Bcl-2 family proteins (52–53). Consistent with a previous report in L929 cells (54), we found that treatment of clonal striatal cells with TNF-α altered mitochondrial localization, which can affect mitochondrial function. Concurrently, TNF-α treatment reduced pro-caspase-8 in mitochondria and increased pro-caspase-8 and its active cleavage products in the cytoplasm. Levels of cytochrome c were reduced in the mitochondria and increased in the cytoplasm in the same TNF-α-treated cells. These events precede internucleosomal DNA fragmentation, indicating that they occur during the initiation of apoptosis. With both Atr and TNF-α stimulation, the decline in signal on Western blot for pro-caspase-8 in mitochondria fractions was smaller (15–30% decrease) than for cytochrome c (50–80% decrease). However, the actual total protein change for pro-caspase-8 may be significant since mitochondria fractions have much higher concentrations of pro-caspase-8 than cytosol. The role of MPTP in the transport of mitochondrial proteins to the cytoplasm in apoptosis remains unclear. Many stimuli, including withdrawal of nerve growth factor, UV irradiation, calcium concentration surges, oxidative stress, and certain anti-tumor drugs can cause collapse of mitochondrial transmembrane potential and induce MPTP (55–57). When MPTP is induced, proteins and other molecules participating in apoptosis can move out of mitochondria. Despite an apparent size limitation (1500 kDa) for molecules to pass through the MPTP (22), a molecule larger than pro-caspase-8, namely apoptosis-inducing factor, can be released through a CsA-sensitive MPTP (58). In addition to pro-caspase-8, mitochondria release pro-caspases-9, -6, and -3.
(27, 29); the mechanisms of release are not clear.

Isolated mitochondria released pro-caspase-8 when stimulated by Atr, an inhibitor of the ADP/ATP carrier and a stimulator of opening of the MPTP. The release of both pro-caspase-8 and cytochrome c was inhibited by CsA, a widely used MPTP blocker (59, 60), suggesting that release of pro-caspase-8 in isolated mitochondria is mediated by a CsA-sensitive MPTP. In intact clonal striatal cells, CsA inhibited TNF-α-induced release of pro-caspase-8 but not cytochrome c. The discrepancy in effects of CsA on cytochrome c release in isolated mitochondria and intact cells may relate to differences in preparations. The effects of Atr in isolated mitochondria are directly mediated by binding to ADP/ATP carrier, whereas in intact cells, TNF-α could have direct and indirect effects on mitochondria that cause release of pro-caspase-8 and cytochrome c through separate mechanisms. TNF-α can release cytochrome c in several cell types, but few studies have shown that this effect can be blocked by CsA treatment alone (61). Apoptotic death of mouse fibroblasts by TNF-α was inhibited by CsA in combination with the phospholipase A2 inhibitor arachidonic acid, indicating involvement of multiple mechanisms (62). In our study, CsA failed to inhibit TNF-α-induced apoptosis. One potential problem with using CsA is its toxicity in some types of cells (63). We found that clonal striatal cells developed intense internucleosomal DNA fragmentation 12 h after CsA administration alone (data not shown).

The classic model of pro-caspase-8 activation by TNF-α and death receptors involves recruitment of pro-caspase-8 from an unknown compartment by FADD/MORT1 through protein-protein interactions (Fig. 8). We speculate that TNF-α-induced release of pro-caspase-8 from mitochondria could be mediated by different signaling pathways (Fig. 8). One pathway could involve TNF receptors through an as yet unknown mechanism. Another pathway could involve BID. Active caspase-8 cleaves cytoplasmic BID and causes BID to translocate to mitochondria. Like BAX, BID induces release of mitochondrial DNA fragmentation 12 h after CsA administration alone (data not shown).

Mitochondria Contain and Release Pro-caspase-8

Mitochondrial dysfunction has been implicated in certain neurological diseases, especially Huntington’s disease (64). Expanded polyglutamine containing proteins including mutant huntingtin can recruit and activate caspase-8 (65). Preliminary studies in our laboratory show that the localization of mitochondria expressing pro-caspase-8 immunoreactivity is altered by increased expression of mutant huntingtin in the cytoplasm. Alterations in mitochondrial localization can affect the MPTP (54), thereby increasing the possibility for the mitochondrial release of pro-caspase-8 and subsequent activation of pro-caspase-8 by mutant huntingtin. Thus, the capacity for mitochondria to localize and release pro-caspase-8 may have important implications in Huntington’s disease pathogenesis.

REFERENCES
1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1974) Br. J. Cancer 29, 239–257
2. Evans, G. V. G. (1993) Cell Biol. Int. 17, 461–476
3. Green, D. R., Bachmann, R. P., Glynn, J. M., and Shi, Y. (1992) Semin. Immunol. 4, 379–388
4. Morrison, J. H., and Hof, P. R. (1997) Science 278, 412–419
5. Andreyev, A. V., Fahy, B., and Fiskum, G. (1998) FEBS Lett. 439, 373–376
6. Alnemri, E. S. (1997) J. Cell. Biochem. 64, 33–42
7. Chinnaiyan, A. M., O’Rourke, K., Lane, B. R., and Dixit, V. M. (1997) Science 275, 1123–1126
8. Kroemer, G., Dalporta, B., and Resche-Rigon, M. (1998) Annu. Rev. Physiol. 60, 619–642
9. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
10. Huang, P. P., Yamaichi, J., and Salvesen, G. (1998) Scand. J. Immunol. 47, 523–531
11. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 312–316
12. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) Cell 89, 175–184
13. Tewari, M., Quan, L. T., O’Rourke, R., Desmyeux, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1998) Cell 81, 801–809
14. Joo, P., Kuo, C. J., Yuan, J., and Blenis, J. (1998) Curr. Biol. 8, 1001–1008
15. Nagata, S., and Golstein, P. (1995) Science 267, 1449–1456
16. Nagata, S. (1995) Cell 88, 355–365
17. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Golove, Y. V., Kovalenko, A. V., and Boldin, M. P. (1998) Annu. Rev. Immunol. 17, 331–367
18. Tang, D., Labit, J. M., and Kild, V. J. (2000) J. Biol. Chem. 275, 3933–3937
19. Yamada, H., Tada-Olikawa, S., Uchida, A., and Kawanishi, S. (1999) Biochem. Biophys. Res. Commun. 265, 130–133
20. Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Breeden, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) J. Biol. Chem. 273, 27084–27090
21. Newmeyer, D. D., Farschon, D. M., and Reed, J. C. (1994) Cell 79, 353–364
22. Crompton, M. (1999) Biochem. J. 341, 233–249
23. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
24. Liu, X., Kim, C. N., Yang J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
25. Reed, J. C. (1999) Cell 91, 559–562
26. Waterhouse, N. N., and Green, D. R. (1999) J. Clin. Immunol. 19, 378–387
27. Krajewska, S., Krajewska, M., Ellerby, L. M., Welsh, K., Xie, Z., Deveraux, Q. L., Salvesen, G. S., Breeden, D. E., Rosenthal, E. R., Fiskum, G., and Reed, J. C. (1999) Proc. Natl. Acad. Sci. 96, 5752–5757
28. 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, 1485–1495
29. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Pre´vost, M-C., Alzari, P. M., and Kroemer, G. (1999) J. Exp. Med. 189, 318–193
30. Wainwright, M. S., Perry, B. J., Won, L., O’Malley, K. L., Wang, W. Y., Ehrlich, M. E., and Heller, A. (1995) J. Neurosci. 15, 676–688
31. Kegel, K. B., Kim, M., Sapp, E., McIntyre, C., Castano, J. G., Aronin, N., and DiPiglia, M. (2000) J. Neurosci. 20, 7268–7278

FIG. 8. Proposed model of death receptor-mediated caspase-8 activation after TNF-α or Fas ligand stimulation. The classic model of caspase-8 activation induced by death receptors involves recruitment of pro-caspase-8 by FADD/MORT1 through protein-protein interactions. Caspase-8 activates its target caspase, caspase-3. In this model, the source of pro-caspase-8 available to FADD/MORT1 is not known (solid arrows). Present results suggest that pro-caspase-8 is predominantly localized in mitochondria and is released upon TNF-α stimulation. We speculate that an additional pathway exists (dashed arrows) in which mitochondria are involved in death receptor-induced caspase-8 activation by releasing pro-caspase-8. It remains unclear, however, if the pro-caspase-8 released from mitochondria directly contributes to caspase-8 activation or replenishes a small pool of cytoplasmic pro-caspase-8. Signals that cause mitochondria to release pro-caspase-8 in response to TNF-α remain to be determined. One mechanism could involve BID, which can be cleaved by caspase-8 and translocate to mitochondria. Like BAX, BID induces release of cytochrome c from mitochondria upon its mitochondrial translocation. The other possible mechanism may involve signals generated directly from activation of death receptors. TNF-α-induced release of cytochrome c can activate caspase-9, thus amplifying activation of caspase-3.
