Apoptosis Induction by Caspase-8 Is Amplified through the Mitochondrial Release of Cytochrome c*

(Received for publication, April 6, 1998)

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Apoptosis often involves the release of cytochrome c from mitochondria, leading to caspase activation. However, in apoptosis mediated by CD95 (Fas/APO-1), caspase-8 (FLICE/MACH/Mch5) is immediately activated and, in principle, could process other caspases directly. To investigate whether caspase-8 could also act through mitochondria, we added active caspase-8 to a Xenopus cell-free system requiring these organelles. Caspase-8 rapidly promoted the apoptotic program, culminating in fragmentation of chromatin and the nuclear membrane. In extracts devoid of mitochondria, caspase-8 produced DNA degradation, but left nuclear membranes intact. Thus, mitochondria were required for complete engagement of the apoptotic machinery. In the absence of mitochondria, high concentrations of caspase-8 were required to activate downstream caspases. However, when mitochondria were present, the effects of low concentrations of caspase-8 were vastly amplified through cytochrome c-dependent caspase activation. Caspase-8 promoted cytochrome c release indirectly, by cleaving at least one cytosolic substrate. Bcl-2 blocked apoptosis only at the lowest caspase-8 concentrations, potentially explaining why CD95-induced apoptosis can often evade inhibition by Bcl-2.

In many cell types, apoptosis can be induced by cross-linking of the CD95 (Fas/APO-1) receptor, through engagement of the receptor either by its natural ligand or by agonistic antibodies (1–5). Engagement of CD95 leads to the formation, at the receptor’s cytoplasmic side, of a protein complex known as the DISC (death-inducing signaling complex). This complex consists of CD95, FADD (MORT1), and pro-caspase-8 (FLICE/MACH/Mch5) (3, 5). Once caspase-8 is recruited in zymogen form to the DISC, it is processed and released from the complex in active form to cleave substrates in the cell interior. Caspase-8 can process other caspases in vitro, including caspase-3 (CPP32/Yama/apopain) (1, 6). Thus, it is commonly hypothesized that mature caspase-8 could, by itself, activate the downstream “effector” caspases, thus directly linking CD95 with the core apoptotic machinery. This attractive idea may explain why Bcl-2, which functions at mitochondria and the endoplasmic reticulum (7, 8) often fails to protect against CD95-mediated cell killing (9–11). However, the observation that Bcl-2 family members can, in certain cases, inhibit CD95-mediated apoptosis (9, 12–18) suggests that caspase-8 may not always act through a direct caspase cascade or that Bcl-2 can somehow inhibit caspase activation in a manner distinct from its effects on cytochrome c release.

To investigate whether caspase-8 can trigger apoptosis via a pathway involving mitochondria, we used a Xenopus cell-free apoptosis system dependent on these organelles (19). In the Xenopus system, endogenous cytosolic factors promote the time- and temperature-dependent release of cytochrome c from mitochondria. Cytochrome c, in concert with other cytosolic factors, then causes the activation of caspases (“DEVDases”)* similar to caspase-3, leading to downstream apoptotic events. The Bcl-2 protein blocks caspase activation in this system, and in at least some forms of apoptosis in mammalian cells, by preventing cytochrome c translocation (8, 20–24). We now show that caspase-8 can activate caspases through two pathways: mitochondria-dependent and -independent. In the absence of mitochondria, activation of a caspase cascade by caspase-8 produces only a partial apoptotic phenotype in nuclei added to the extract. In contrast, the mitochondria-dependent pathway, which involves the release of cytochrome c from mitochondria into the cytosol, triggers full nuclear apoptosis. Moreover, engagement of the mitochondria-dependent pathway is more efficient than the mitochondria-independent pathway, as it can be activated even by small amounts of caspase-8. Thus, mitochondria provide an efficient means of amplifying the apoptotic signal transduced by caspase-8. Caspase-8 can activate the mitochondria-dependent pathway even when the Bcl-2 protein is present; this helps explain the failure of Bcl-2 to inhibit CD95-dependent apoptosis consistently.

EXPERIMENTAL PROCEDURES

Extract preparation, fractionation, and use were as described previously (8, 19, 24, 25), except as indicated in the figure legends. Cytosolic content of cytochrome c was measured by immunoblotting as described (8, 26).

DEVDase activity was quantitated as described (8, 24), except that the colorimetric substrate DEVD-p-nitroanilide was used. At each time point, 2-μl aliquots were collected and mixed with 40 μM DEVD-p-nitroanilide in egg lysis buffer (250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 50 μg/ml cycloheximide, 1 mM dithiothreitol, and 5 μg/ml cytochalasin B). Assay mixtures were incubated for 2 h at 22 °C prior to measurement of absorbance at 405 nm using a SpectraMax 96-well spectrophotometer.

DNA extraction was performed as follows. Rat liver nuclei (800,000)
Fig. 1. Caspase-8 induces apoptosis in Xenopus egg extracts. Extracts containing synthetic nuclei formed around sperm chromatin templates (500 nuclei/μl) were supplemented with egg lysis buffer alone (A and C) or with 16 μg/ml recombinant active caspase-8 (B and D). After ~60 min, nuclei in extracts containing caspase-8 underwent synchronous apoptotic fragmentation, as observed by staining with Hoechst 33258 (B) or with the lipophilic dye 3,3′-dihexyloxacarbocyanine (D). Nuclei in extracts supplemented with buffer alone were stable for >3.5 h (A and C, stained with Hoechst 33258 or 3,3′-dihexyloxacarbocyanine, respectively).

were added to 200 μl of extract and incubated for 1 h. Caspase-8 or egg lysis buffer was then added, and the mixture was incubated at 22 °C. At various times, 25-μl samples were withdrawn into 250 μl of DNA extraction buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS, and 200 μg/ml proteinase K) and incubated for 2 h at 50 °C. DNA was extracted with phenol/chloroform and ethanol-precipitated. The DNA was resuspended in water and resolved on a 1% Tris borate/EDTA (TBE)-agarose gel. The gel was washed in TBE containing ethidium bromide and 300 μg/ml ribonuclease A for 1 h and then destained for 1 h in TBE.

RESULTS

Morphological Apoptosis Induced by Caspase-8 in the Xenopus Cell-free System—To investigate whether caspase-8 might act via a pathway involving mitochondria, we employed a cell-free apoptosis system, based on Xenopus egg extracts, in which the apoptotic process is dependent on the release of cytochrome c from mitochondria (8, 19, 24). In these extracts, apoptotic nuclear morphology, including chromatin condensation and nuclear fragmentation, normally appears spontaneously after 2.5–4 h of incubation. (Although the onset of apoptotic events occurs at various times in different extracts, the sequence of events is highly reproducible (39).) However, when active recombinant caspase-8 was added, apoptosis was accelerated strikingly, with nuclear morphological changes occurring ~1 h after caspase-8 addition (Fig. 1). In this and all subsequent experiments, the photomicrographs shown are highly representative in that nuclei behaved synchronously and uniformly in a given extract. We conclude from these results that Xenopus egg extracts contain elements that respond to active caspase-8, giving rise to the same apoptotic phenotype that spontaneously occurs in these extracts, although much more rapidly.

In complete extracts containing mitochondria, caspase-8 caused fragmentation of the nuclear membrane. When caspase-8 was added to extracts lacking mitochondria (i.e., reconstituted from the cytosol and light membranes) (19), chromatin condensation and the eventual loss of nuclear DNA (as measured by staining with the DNA-binding fluorochrome Hoechst 33258) were observed (Fig. 2B). However, the nuclear membrane structure appeared largely intact, as observed either by phase-contrast microscopy or by fluorescence microscopy after staining of nuclear membranes with the fluorescent lipophilic dye 3,3′-dihexyloxacarbocyanine (Fig. 2E). To show that the extracts lacking mitochondria are capable of supporting complete nuclear disintegration, we added purified equine cytochrome c. This resulted in complete nuclear vesiculation and DNA fragmentation within 60 min (Fig. 2, C and F). Furthermore, the addition of active recombinant caspase-3 also produced complete nuclear disintegration, even in the absence of mitochondria (data not shown.) On the other hand, internucleosomal cleavage of DNA was produced by caspase-8 both in the presence and absence of mitochondria (Fig. 3).

These results suggested that, in the absence of mitochondria, caspase-8 could activate some, but not all, of the downstream effectors (“executioners”) of apoptosis. We hypothesized that the activity of additional executioner caspases would be required to obtain the full apoptotic phenotype in nuclei. Indeed, Fig. 4 shows that caspase-6 (Mch2a), while having no proapoptotic activity of its own (panel B), could synergize with caspase-8 to produce full nuclear apoptosis in extracts lacking mitochondria (panel C). Similar results were obtained with exogenously added rat liver nuclei (data not shown.)

The occurrence of DNA degradation and other cleavage events in the nucleus during apoptosis implies that apoptotic executioners somehow gain access to the nuclear interior. Therefore, we asked whether the nuclear morphological features induced by caspase-8 depended on active nuclear protein import. To answer this, we used wheat germ agglutinin (WGA), a lectin that blocks nuclear import by interacting with O-linked glycoproteins in the nuclear pore complex (27–29). Inhibition of nuclear protein import was confirmed by the addition of a fluorescently labeled nuclear import substrate (human serum albumin linked to the SV40 T-antigen nuclear localization se-
Caspase-8 promotes the translocation of cytochrome c

Fig. 3. Caspase-8 induces DNA fragmentation in the presence or absence of mitochondria. Rat liver nuclei (4000/µl extract) were added to crude extracts containing mitochondria (left panel) or to extracts depleted of dense organelles (right panel). Caspase-8 (plus lanes; final concentration of 16 µg/ml or egg lysis buffer (minus lanes) was added to extracts at a 1:25 (v/v) dilution. At 0, 30, or 90 min (as indicated), aliquots of extract containing nuclei were taken, and DNA was extracted from the samples as described under “Experimental Procedures.” Extracted DNA was resolved on a 1% agarose gel and visualized by ethidium bromide staining.

Fig. 4. Addition of caspase-6 with caspase-8 restores the full apoptotic nuclear morphology in extracts devoid of mitochondria. Caspase-8 alone (16 µg/ml; A), caspase-6 alone (200 µg/ml; B), or caspase-8 and -6 together (C) were added to extracts containing nuclei, but lacking mitochondria. After 60 min of incubation, samples were stained with Hoechst 33258 and visualized by fluorescence microscopy.

Fig. 5. Active nuclear import is required for caspase-8-induced apoptosis. Synthetic nuclei were formed either in extracts lacking mitochondria (reconstituted from the cytosol and light membranes) (A and B) or in crude extracts containing mitochondria (C–E). After 45 min of nuclear formation, samples were incubated for 15 min with 1 mg/ml WGA to block nuclear import (B and E) or with buffer alone (A and D). After 15 min of incubation, samples were then supplemented with 16 µg/ml caspase-8 (A, B, D, and E) and stained 60 min later with Hoechst 33258 for visualization by fluorescence microscopy. WGA treatment (B and E) prevented caspase-8-induced DNA degradation in extracts lacking mitochondria (compare A and B) and in unfractionated extracts containing mitochondria (compare D and E). WGA treatment (E) blocked nuclear growth (compare E with C) because nuclear protein import is required for this process.

The effect of mitochondria was even more evident at low concentrations of caspase-8. In the absence of mitochondria, as the caspase-8 concentration was titrated down, DEVDase activation was progressively slowed, eventually becoming undetectable at a caspase-8 concentration of 100 ng/ml (Figs. 6 and 7). On the other hand, when mitochondria were present, DEVDase activation was strikingly potentiated even at caspase-8 concentrations as low as 20–50 ng/ml (Figs. 6–8). However, as the caspase-8 concentration was reduced below 250 ng/ml, the lag period prior to DEVDase activation was prolonged (Fig. 8). This suggests that, at low concentrations of caspase-8, the cleavage of one or more critical caspase-8 substrates became rate-limiting.

As Fig. 7 shows, the mitochondria-dependent pathway activated by caspase-8 involved the release of cytochrome c from mitochondria. In this experiment, caspase-8 was added at 100 ng/ml, a concentration too low to promote activation of DEVDases in the cytosol alone (Fig. 7B). However, in the complete extract (containing mitochondria), caspase-8 caused the appearance of cytochrome c in the soluble fraction within 1 h (and in experiments not shown, within 10 min) after addition. In the absence of caspase-8, cytochrome c release (mediated by endogenous cytosolic factors) (8) occurred much later, after ~4 h of incubation. We conclude from the data shown in Figs. 6 and 7 that caspase-8, at high concentrations, can activate downstream effector caspases through two mechanisms, one involving the mitochondrial release of cytochrome c and the other involving only cytosolic components. However, at lower caspase-8 concentrations, the mitochondrial pathway is used predominantly, if not exclusively.

Inhibition of Caspase Activation by Bcl-2 Can Be Overcome by Caspase-8—Previously, Bcl-2 was shown to block apoptosis in the Xenopus system and in mammalian cells by preventing the efflux of cytochrome c from mitochondria (8, 21, 22). We assessed the ability of Bcl-2 to inhibit apoptosis induced by caspase-8. Fig. 8 shows that Bcl-2 was able to inhibit DEVDase...
activation only at the lowest concentrations of caspase-8, although, as shown previously (8), Bcl-2 did inhibit the spontaneous DEVDase activation mediated by cytochrome c translocation in response to endogenous cytosolic factors. Note that at intermediate caspase-8 concentrations (~250 ng/ml), Bcl-2 largely failed to inhibit DEVDase activation, even though at these concentrations of caspase-8, the mitochondrial, rather than the cytosolic, pathway was predominant (Fig. 5). Thus, sufficient amounts of caspase-8 could somehow bypass or inactivate the inhibitory effect of Bcl-2 on cytochrome c translocation. In another study, we found similarly that the Drosophila pro-apoptotic protein Reaper could induce or accelerate the release of cytochrome c from mitochondria and that high concentrations of Reaper could overcome the antagonistic effect of Bcl-2 (26). It is presently unknown whether Reaper and caspase-8 use similar mechanisms to promote cytochrome c translocation. However, cytochrome c release produced by both Reaper and caspase-8 required the presence of cytosolic factors. To determine whether the cytochrome c release activity of caspase-8 was due to cleavage of a cytosolic or a mitochondrial substrate, we performed the experiment shown in Fig. 9. The cytosol was treated with caspase-8 or buffer, followed by the addition of mitochondria in the presence of zVAD-fmk (100 μM) to prevent further caspase-8 activity. At various times afterward, the presence of cytosolic cytochrome c was assayed by immunoblotting. Fig. 9 shows that pretreatment of the cytosol with caspase-8 produced a marked acceleration of cytochrome c translocation. In contrast, pretreatment of mitochondria with caspase-8 prior to the addition of the cytosol produced no acceleration of cytochrome c release (data not shown). Thus, caspase-8 functions indirectly by cleaving a cytosolic substrate, which then interacts with mitochondria and possibly other cytosolic factors to promote cytochrome c release. Efforts are now underway to purify these cytosolic factors.

**DISCUSSION**

Our results show that the addition of caspase-8 to Xenopus egg extracts induces the rapid efflux of cytochrome c from mitochondria. Because cytochrome c induces the activation of downstream caspases, in a manner sufficient to produce the complete set of apoptotic events occurring in the Xenopus extracts and in other systems (20, 21, 24, 30), this mitochondrial pathway can potently amplify the effects of small amounts of caspase-8. In a particular cell type, whether caspase-8 can trigger apoptosis through a direct (i.e. cytochrome c-independent) caspase cascade would, according to our results, depend on
the concentration of activated caspase-8 present in those cells. However, even in cells with high levels of caspase-8, our results suggest that, for the complete apoptotic phenotype, including disruption of the nuclear envelope, the release of cytochrome c from mitochondria would be required.

The inability of caspase-8 to produce nuclear membrane fragmentation in the absence of mitochondria argues that caspase-8, by itself, can induce the activation of only some of the downstream effectors of apoptosis. Based on the similarity in appearance of the nuclei in Fig. 2 (B and E) to those present in cells induced to enter apoptosis in the presence of non-degradable lamins (31), we suspected at first that caspase-8 failed to activate the laminase(s) present in the Xenopus extracts in the absence of mitochondria. Thus, we predicted that recombinant caspase-6, which is the presumed laminase in human cells (32, 33), would restore the complete apoptotic phenotype if added together with caspase-8. Indeed, caspase-6, while having no apoptosis-inducing activity of its own, acted synergistically with caspase-8 to produce complete nuclear fragmentation in Xenopus extracts lacking mitochondria (Fig. 4). Surprisingly, however, immunoblot analysis of the endogenous Xenopus lamin proteins and of 35S-labeled lamins added exogenously to the extract showed that caspase-8 by itself was equally effective at triggering cleavage of these proteins in the presence or absence of mitochondria (data not shown.) This suggests either that caspase-8 can activate, in the absence of mitochondria, an endogenous Xenopus laminase differing in specificity from human caspase-6 or that caspase-8 itself can cleave lamin proteins. Furthermore, it suggests that caspase-6, as well as an endogenous protease activable by cytochrome c, is required to cleave substrates other than the lamins that are critical for vesicularization of the nuclear membrane during apoptosis.

Our observations may help explain why Bcl-2 family members have been reported to block CD95-induced apoptosis in some circumstances, but not others (9–18, 34–36). It is unclear how caspase-8 can overcome the inhibitory effects of Bcl-2 on cytochrome c release. While this manuscript was in preparation, Cheng et al. (37) reported that Bcl-2 is a substrate for caspase-3 in vitro. Bcl-2 is also cleaved, presumably by undetermined caspases, in cells undergoing CD95-induced apoptosis, although it is unclear from these studies whether Bcl-2 cleavage occurs prior to cytochrome c release or after caspase-3 activation. If caspase-8 were to cleave Bcl-2, this might explain how caspase-8 could overcome the anti-apoptotic effects of Bcl-2 in the Xenopus cell-free system, even when caspase-8 is present at levels too low to activate caspase-3 directly. However, we found that when baculovirus-expressed human Bcl-2 was added to a mixture of Xenopus cytosol and mitochondria, it was not cleaved following the addition of recombinant caspase-8 at 500 ng/ml.2 Thus, caspase-8 in this system can apparently promote cytochrome c release despite the presence of intact Bcl-2 on mitochondrial surfaces.

Cheng et al. (37) also found that when the Bcl-2 cleavage product was transfected into cells, it promoted or enhanced

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2 T. Kuwana and D. D. Newmeyer, unpublished data.
apoptosis. One might therefore ask whether caspase-8 functions in the Xenopus cell-free system by cleaving an endogenous Bcl-2 relative, thereby converting it into a Bax-like pro-apoptotic molecule. This remains a formal possibility, as the caspase-8 substrate responsible for inducing cytochrome c release has not yet been identified. However, because this factor is cytosolic, it is unlikely to be a strict homolog of Bcl-2, which is entirely membrane-associated.

In conclusion, our results link a proximal signaling event, the activation of caspase-8 at the plasma membrane, with a distal apoptotic pathway involving cytochrome c release from mitochondria and subsequent activation of executioner caspases. Why should the cell use this indirect pathway when caspase-8 could activate at least some of these downstream caspases simply and directly? Perhaps for three reasons: first, cytochrome c can amplify the effect of small amounts of active caspase-8; second, the participation of mitochondria would allow the cell greater flexibility in controlling the apoptotic process through modulating the events leading to cytochrome c release; and third, the effects on mitochondria (including, but not necessarily limited to, cytochrome c release) may lead to cell death even in the absence of functional executioner caspases, as has been shown for Bax-induced cell killing (38).

Acknowledgments—We thank Reimer Stick and Chris Hutchinson for lamin clones and antibodies.

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