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Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system

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In a cell-free system based on *Xenopus* egg extracts, Bcl-2 blocks apoptotic activity by preventing cytochrome *c* release from mitochondria. We now describe in detail the crucial role of cytochrome *c* in this system. The mitochondrial fraction, when incubated with cytosol, releases cytochrome *c*. Cytochrome *c* in turn induces the activation of protease(s) resembling caspase-3 (CPP32), leading to downstream apoptotic events, including the cleavage of fodrin and lamin B_1. CPP32-like protease activity plays an essential role in this system, as the caspase inhibitor, Ac-DEVD-CHO, strongly inhibited fodrin and lamin B_1 cleavage, as well as nuclear morphology changes. Cytochrome *c* preparations from various vertebrate species, but not from *Saccharomyces cerevisiae*, were able to initiate all signs of apoptosis. Cytochrome *c* by itself was unable to process the precursor form of CPP32; the presence of cytosol was required. The electron transport activity of cytochrome *c* is not required for its pro-apoptotic function, as Cu- and Zn-substituted cytochrome *c* had strong pro-apoptotic activity, despite being redox-inactive. However, certain structural features of the molecule were required for this activity. Thus, in the *Xenopus* cell-free system, cytosol-dependent mitochondrial release of cytochrome *c* induces apoptosis by activating CPP32-like caspases, via unknown cytosolic factors.

Keywords: apoptosis/caspases/cytochrome *c*/mitochondria/*Xenopus*

Introduction

Cell death commonly takes place via apoptosis, a process whereby the cell becomes re-packaged to enable its clearance by phagocytes without the leakage of cell contents (Kerr, 1971; Wyllie *et al.*, 1980). Apoptosis involves a stereotypical set of morphological changes, which include cell shrinkage, chromatin condensation and fragmentation of the nucleus and cytoplasm.

The ICE/CED-3 family of cysteine proteases, now christened 'caspases' (Alnemri *et al.*, 1996) plays an important role in apoptosis. This family, of which 10 human and several non-human genes are now identified (Alnemri *et al.*, 1996; Nagata, 1997), is related to the *Caenorhabditis elegans ced-3* death gene (Yuan *et al.*, 1993). The caspases (reviewed by Martin and Green, 1995; Fraser and Evan, 1996; Henkart, 1996; Nagata, 1997) all contain a cysteine at their active site, and all cleave after aspartate residues. Furthermore, they are present in cells in the inactive proform, with the active tetramer being formed by removal of the prodomain and cleavage between the large and small subunits. There is convincing evidence that caspases play a crucial role in apoptosis: their ectopic expression induces apoptosis; they become activated during the apoptotic process; they cleave apoptotic substrates *in vitro*; their inhibition blocks downstream events in apoptosis; and their deletion can cause abnormalities in cell death in certain tissues.

Caspase activity is responsible, either directly or indirectly, for the cleavage of >17 cellular proteins which are characteristically proteolyzed during apoptosis, including nuclear proteins such as poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase and lamins, as well as the cytoskeletal protein fodrin (reviewed by Patel *et al.*, 1996; Nagata, 1997). In some cases, the caspases most likely responsible for the cleavages have been identified. For example, caspase-6 can cleave nuclear lamins (Takahashi *et al.*, 1996) and caspases-2, -3, -7 and -9 can cleave PARP (Nagata, 1997). More recently, caspase-3 was found to activate the novel protein complex, DNA fragmentation factor (DFF), which initiates DNA fragmentation in co-incubated nuclei (Liu *et al.*, 1997). It remains unclear which proteolytic events effect the cell blebbing, condensation and fragmentation characteristic of apoptosis.

We have described previously a cell-free system based on *Xenopus* egg extracts, in which apoptosis requires an organelle fraction enriched in mitochondria (Newmeyer *et al.*, 1994). Moreover, this apoptosis is inhibited by Bcl-2 (Newmeyer *et al.*, 1994) via its ability to block cytochrome *c* release from mitochondria (Kluck *et al.*, 1997). This finding was confirmed also in a mammalian cell-free system and in whole cells receiving apoptotic stimuli (Kluck *et al.*, 1997; Yang *et al.*, 1997). Here we describe more fully a series of apoptotic events in the *Xenopus* cell-free system, with particular regard to the central role of cytochrome *c*.

Results

Apoptotic changes induced by mitochondria include CPP32-like caspase activation and proteolysis of lamin B_1 and fodrin

We have shown previously that the apoptotic activity in *Xenopus* egg extracts requires a dense organelle fraction

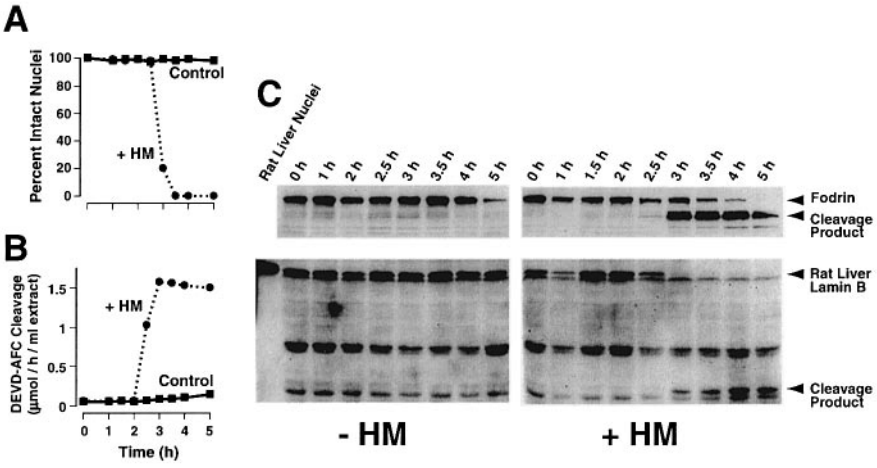

Fig. 1. The presence of the mitochondrial fraction induces CPP32-like protease activation and cleavage of fodrin and lamin B_1. Reconstituted extracts containing cytosol, light membrane (LM) and rat liver nuclei were incubated for 5 h in the presence or absence of the heavy membrane (HM) fraction (1.5% v/v) which is enriched for mitochondria. (**A**) At the indicated times aliquots were assessed for nuclear morphology by Hoechst staining. (**B**) Extracts were sampled over time and analyzed for the ability to cleave the fluorigenic peptide substrate z-DEVD-AFC. (**C**) Extract aliquots were also analyzed by SDS–PAGE and Western blot for fodrin and lamin B_1 cleavage as indicated. This experiment is representative of three experiments. Extra protein bands seen in the lamin B_1 blots are *Xenopus* proteins which cross-react with the anti-lamin B_1 antibody, but which are not cleaved.

containing mitochondria (Newmeyer *et al.*, 1994). This is seen in Figure 1A, where addition of the mitochondrial (heavy membrane, HM) fraction to reconstituted egg extracts resulted in the apoptotic fragmentation of exogenously added rat liver nuclei after 3 h of incubation. To investigate the role of proteolysis in this system, we examined the cleavage of known caspase substrates. Figure 1B shows that, 2.5 h after the addition of HM, a DEVD-specific caspase activity appears, as detected by the cleavage of a fluorimetric substrate, z-DEVD-AFC. Approximately 30 min later, nuclei began to display apoptotic morphology (Figure 1A). Immunoblotting (Figure 1C) detected, at about the same time as these morphological changes, the cleavage of fodrin (a cytoskeletal protein endogenous to the *Xenopus* extract) and lamin B_1 (present in the rat liver nuclei added to the extract). Similar proteolytic events were observed in crude unfractionated extracts which underwent apoptosis (not shown). The ability of extracts to cleave the ICE substrate, z-YVAD-AFC, remained low despite the development of apoptosis (not shown). Thus, with apoptosis, *Xenopus* egg extracts activate caspases resembling the mammalian CPP32 subfamily (DEVDases), but not the ICE-related subfamily (YVADases). The apoptotic pathway in this model further mimics mammalian systems with respect to the cleavage of cellular proteins, such as fodrin and lamin B_1.

Mitochondria-induced apoptosis is blocked by caspase inhibitors

To determine whether CPP32-like protease activity was responsible for the apoptotic changes described above, specific inhibitors were added to extracts. A peptide inhibitor relatively specific for the CPP32 subfamily of cysteine proteases, Ac-DEVD-CHO (1 µM), prevented nuclear changes and fodrin cleavage; while Ac-YVAD-CHO, which preferentially inhibits ICE, achieved only partial inhibition at a higher concentration (100 µM) (Figure 2). These findings show that one or more caspases are responsible for the apoptotic events in this system. Furthermore, as the CPP32 inhibitor was most effective, it is likely that *Xenopus* CPP32 (Yaoita and Nakajima, 1997) and/or other DEVD-specific caspases, rather than ICE-like caspases, are the major enzymes involved.

Bcl-2, previously shown to inhibit apoptotic nuclear morphology in this system (Newmeyer *et al.*, 1994), also inhibits fodrin cleavage (Figure 2C) and DEVDase activation (Kluck *et al.*, 1997) occurring in the presence of mitochondria.

A soluble mitochondrial factor induces apoptosis

When mitochondria were incubated with cytosol and then removed (see Materials and methods), the resulting cytosol induced apoptosis rapidly when added in small amounts to mitochondria-depleted extracts (Figure 3A). Thus, the co-incubation of cytosol and HM produces a soluble factor that induces apoptotic events. This factor, termed heavy membrane cytosolic factor (HMCF) can account for the activity of the mitochondrial fraction. The production of this factor was a time- and temperature-dependent process, and did not occur if cytosol was replaced by buffer (not shown). As seen for HM (Figure 2B), the effects of HMCF were inhibited by low concentrations of Ac-DEVD-CHO and less so by Ac-YVAD-CHO (Figure 3B). GST-CrmA had little effect (Figure 3B; immunoblots of fodrin and lamin cleavage not shown). Thus, as with HM, HMCF-induced apoptosis is also mediated by CPP32-like proteases. However, Bcl-2 did not inhibit the effects of HMCF (Figure 3C).

HMCF preparations contain cytochrome c

Initial steps were undertaken to characterize HMCF. Proteinase K treatment and heating at 95°C for 5 min both rendered HMCF inactive, while lipase treatment did not affect activity, and lipid extraction of HMCF did not recover activity (not shown), suggesting that HMCF is at least partly proteinaceous. Size-exclusion chromatography identified a narrow peak of HMCF activity at ~15 kDa

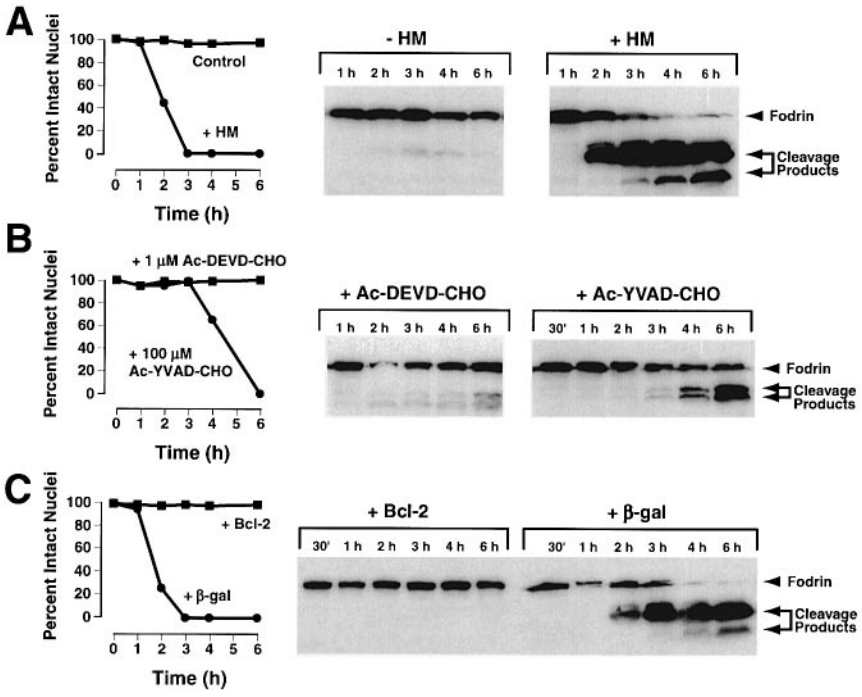

Fig. 2. Caspase inhibitors and Bcl-2 inhibit mitochondrial-induced apoptosis. (**A**) Reconstituted extracts were incubated with or without HM (1.5% v/v) for 6 h. (**B** and **C**) Extracts containing HM were incubated in the presence of Ac-DEVD-CHO (1 μM), Ac-YVAD-CHO (100 μM) and *Sf*9 lysate (2% v/v) expressing either Bcl-2 or β-galactosidase (β-gal) as indicated. At the indicated times, extracts were sampled and assessed for nuclear morphology (left panels) and fodrin cleavage (right panels). This experiment is representative of three experiments.

(Figure 4B, fraction number 17). While this work was in progress, it was reported that the mitochondrial protein, cytochrome *c*, was responsible for activating apoptotic proteases in a human cell-free system (Liu *et al.*, 1996). To determine whether cytochrome *c* could account for HMCF activity, we probed immunoblots of cytosol and HMCF preparations with anti-cytochrome *c* antibodies and found significant cytochrome *c* content in HMCF, but negligible or non-detectable levels in fresh cytosol (Figure 4A). In addition, Western blot (Figure 4B inset) and spectrophotometric (not shown) analyses indicated the presence of cytochrome *c* only in the 15 kDa active peak (fractions 16 and 17). The failure of the smaller amount of cytochrome *c* present in fraction 16 to activate DEVD-cleaving activity in the 4 h assay (Figure 4B), can be attributed to the inhibitory effect of salts contributed by lyophilizing buffer B; not shown). Thus cytochrome *c* was released from mitochondria following incubation in cytosol.

Cytochrome c activates the Xenopus apoptotic pathway

To determine whether cytochrome *c* could be responsible for the pro-apoptotic activity of HMCF, we tested its ability to substitute for the HM fraction. Addition of 0.2 μM horse heart cytochrome *c* (HHCc, Sigma) to HM-depleted extracts resulted in the rapid development of nuclear apoptotic morphology and fodrin cleavage (Figure 5A). The apoptotic effects of cytochrome *c* were inhibited by Ac-DEVD-CHO (1 μM), Ac-YVAD-CHO (100 μM) and z-VAD-fmk (10 μM) (Figure 5B, C and D), but not by Bcl-2 (Kluck *et al.*, 1997). Thus HHCc by itself mimicked all apoptotic effects of HMCF.

In separate experiments, titrations of HMCF or cytochrome *c* showed similar behavior in their activation of DEVDases (Figure 4C). Both reagents were inactive below a certain threshold concentration. Furthermore, at concentrations just above this threshold, there was a distinct time-lag before the onset of DEVDase activation, as well as a diminished rate of caspase activation. This suggests that cytochrome *c* is involved somehow in an enzymatic process leading to caspase activation. The similar titration behavior of HMCF and cytochrome *c* also suggests that the activity of HMCF preparations can be entirely accounted for by their content of cytochrome *c*.

CPP32 cleavage in Xenopus extracts

To investigate further the relationship of the proteolytic activity seen in *Xenopus* extracts to that seen in mammalian systems, we examined whether the precursor form of CPP32 could be processed in the *Xenopus* system. *In vitro*-translated [^{35}S]proCPP32 was incubated in *Xenopus* HM-depleted extract alone, or supplemented with HM, HMCF, or cytochrome *c* (Figure 6). The activation of DEVDase was monitored in parallel aliquots not containing [^{35}S]CPP32 (Figure 6B). In all extracts in which endogenous DEVDase was activated, full-length CPP32 was cleaved to fragments corresponding to the active 12 and 17 kDa subunits, as well as to the 24 kDa intermediate cleavage product (Martin *et al.*, 1996). This provides further evidence of significant conservation of the apoptotic pathway between amphibians and mammals.

[^{35}S]CPP32 was not cleaved in the presence of cytochrome *c* if cytosol was replaced either by an ultrafiltrate of cytosol (i.e. the fraction of cytosol passing through an ultrafiltration membrane with a molecular weight cut-off of ~10 kDa; Figure 6A, lane 6) or by buffer (Figure 6A, lane 7). Thus, cytochrome *c* cannot by itself

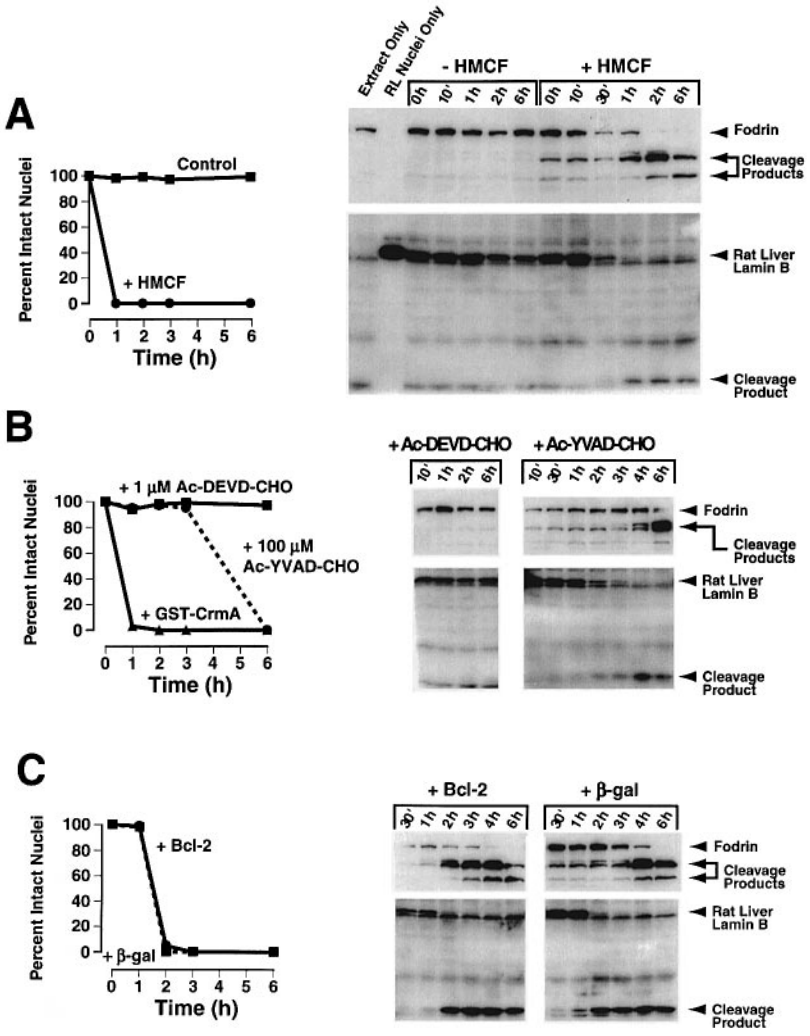

Fig. 3. A soluble mitochondrial factor (HMCF) induces apoptotic changes which are inhibited by caspase inhibitors but not by Bcl-2. (**A**) Reconstituted extracts were incubated with or without HMCF (10:100 v/v) for 6 h. (**B** and **C**) Extracts containing HMCF were also incubated in the presence of Ac-DEVD-CHO (1 μM), Ac-YVAD-CHO (100 μM) and *Sf*9 lysate (2% v/v) expressing either Bcl-2 or β-gal as indicated. At the indicated times extracts were assessed for nuclear morphology (left panels) and for fodrin and lamin B_1 cleavage (right panels). This experiment is representative of two experiments.

induce the activation of pro-CPP32. Instead, it cooperates with one or more unidentified cytosolic macromolecules larger than ~10 kDa to institute caspase activation.

Molecular features of cytochrome c required for caspase activation

In this system, cytochrome *c* release from mitochondria appears to be responsible for the activation of CPP32-like proteases, leading to the cleavage of protein substrates and characteristic effects on nuclei. Additional experiments provided some initial insight as to how this well-studied molecule might act, once released from mitochondria.

Cytochrome *c* preparations obtained from bovine, horse, pigeon and tuna heart (Sigma) were equivalent in their ability to induce apoptosis in mitochondria-depleted *Xenopus* extracts (Table I), again reflecting the evolutionary conservation of the apoptotic pathway. Heat treatment (100°C, 1 h) of bovine heart cytochrome *c* eliminated its activity (Table I), as did heat treatment of HMCF (above). Enzyme-degraded HHCc (Sigma), which retains the heme portion of the molecule and possesses significant peroxidase activity, had no pro-apoptotic activity.

Cytochrome *c* from *Saccharomyces cerevisiae* (~9:1 mix of isoforms 1 and 2) did not induce apoptosis in *Xenopus* extracts, even at 100×the concentration at which cytochromes *c* from other species were active (Table I). Furthermore, when mitochondria isolated from *S.cerevisiae* were incubated in *Xenopus* extracts, they released yeast cytochrome *c* but did not induce apoptotic changes (R.M.Kluck, D.R.Green, M.Yaffe, E.Margoliash, and D.D.Newmeyer, in preparation). In contrast, mitochondria isolated from CEM and HeLa cells both released cytochrome *c* and induced apoptotic changes (not shown). Together, the results in Table I indicate that subtle structural features of the cytochrome *c* molecule, and not merely the presence of heme, are responsible for its activity.

Oxidation/reduction requirements for cytochrome c induction of apoptosis

The role of cytochrome *c* in oxidative phosphorylation within the intermembrane space of mitochondria is dependent on its redox activity, i.e. its ability to accept an electron from cytochrome *c* reductase and pass it on to cytochrome *c* oxidase. To determine whether the apoptotic effects of

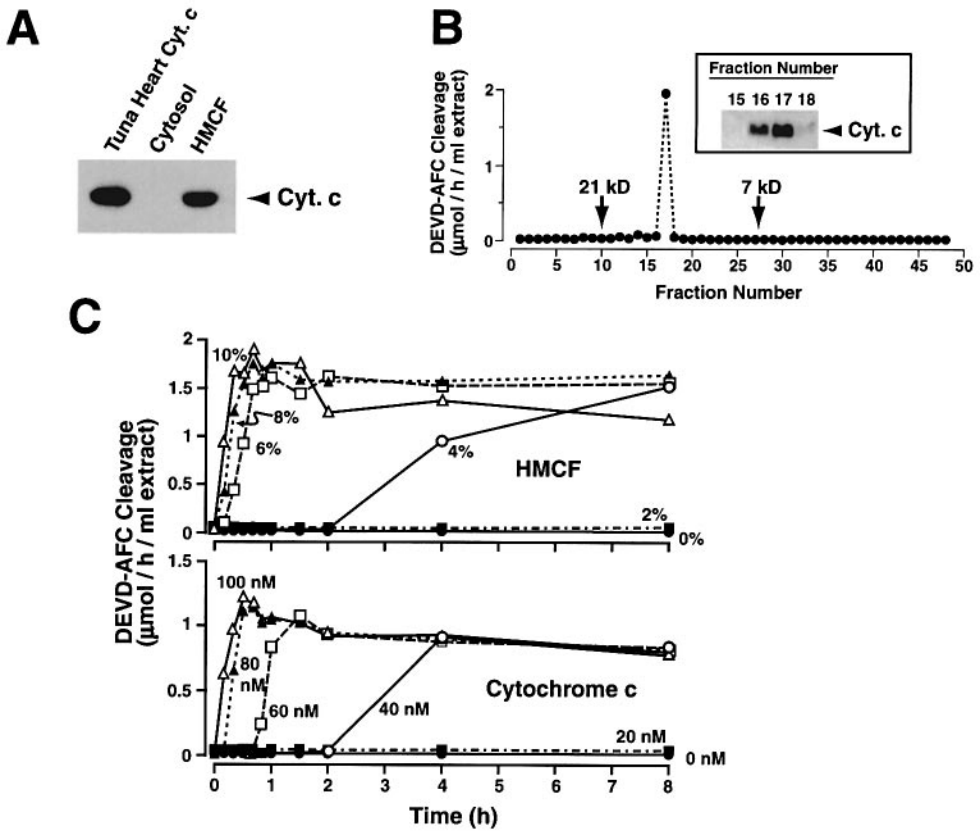

Fig. 4. Cytochrome *c* is present in HMCF preparations and mimics the apoptotic effects of HMCF. (**A**) Western blot analysis of cytosol before (Cytosol) and after (HMCF) incubation with HM (12% v/v) for 4 h. Tuna heart cytochrome *c* was loaded in lane 1. (**B**) HMCF was size fractionated and the fractions tested for HMCF activity as determined by the ability to activate DEVDases in fresh cytosol (see Materials and methods). The elution of size markers, trypsin inhibitor (21 kDa) and aprotinin (7 kDa), is indicated. Inset shows the presence of cytochrome *c* in fractions 16 and 17 only, as analyzed by Western blot, and this was corroborated by absorption spectroscopy (not shown). (**C**) Cytosol was incubated with the indicated concentrations of HMCF (top panel) or cytochrome *c* (bottom panel) and the cytosol mix then analyzed for the ability to cleave z-DEVD-AFC at the indicated times. Experiments shown are representative of two or more experiments.

Fe-HHCc were redox-dependent, Cu- and Zn-substituted cytochrome *c* (Cu-HHCc, Zn-HHCc), which have no redox activity (Findlay *et al.*, 1977; Anni *et al.*, 1995; Zhou *et al.*, 1995), were tested for pro-apoptotic activity in the cell-free system (Figure 7). Both cytochrome *c* derivatives were able to induce apoptotic changes (although with ~50% reduced potency), indicating that redox activity is not a requirement for cytochrome *c* induction of apoptosis.

A second approach to determine whether oxidation–reduction status affected the ability of Fe-HHCc to initiate apoptosis was undertaken by oxidizing the extracts with $K_3Fe(CN)_6$. A careful titration showed that the addition of 6 mM $K_3Fe(CN)_6$ to reconstituted extracts caused Fe-HHCc to be oxidized, as seen by its loss of absorbance at 550 nm. While Fe-HHCc added to normal (reduced) extracts rapidly activated DEVDases, as measured by the ability to cleave the DEVD substrate, it failed to do so when added to oxidized extracts [containing either 6 mM or 12 mM $K_3Fe(CN)_6$]. However, it was found that Cu- and Zn-HHCc, which cannot be oxidized to the 3+ form, were also inactive in oxidized extracts (not shown). Assuming that $K_3Fe(CN)_6$ acts only as an oxidant, this suggests that effectors downstream of cytochrome *c* require reducing conditions.

To examine the effect of oxidation on one of the downstream events, DEVDase activity, $K_3Fe(CN)_6$ was added to mixtures of apoptotic cytosol (which contains active DEVDases) and DEVD substrate. A titration of $K_3Fe(CN)_6$ produced a maximal 40% reduction in the rate of substrate cleavage (not shown), indicating that at least one of the DEVDases active in apoptotic *Xenopus* extracts prefers reducing conditions. However, as DEVDase activity was not completely inhibited, this cannot account for the ability of $K_3Fe(CN)_6$ to abolish the activation of DEVDases by Fe-, Cu- and Zn-HHCc (see above). Thus, it is likely that some step in the activation of DEVDases initiated by cytochrome *c* requires reducing conditions.

Zn-HHCc is degraded on exposure to oxygen and ambient light, with a half-time of 2 h. Degradation of a 10 µM solution of Zn-HHCc, by 15 h exposure to room light at 22°C, resulted in greatly attenuated spectral characteristics (not shown), and rendered Zn-HHCc unable to induce apoptosis (Figure 7). As controls, Fe-HHCc was exposed to light in the same manner, either by itself or in a mixture with Zn-HHCc, and was found to be unaltered in its apoptotic effects (not shown). These experiments show that cytochrome *c* itself, rather than a contaminant present in cytochrome *c* preparations, is the molecule responsible for inducing caspase activation.

Discussion

Using the *Xenopus* cell-free system, we have determined several sequential steps in the apoptotic pathway (Figure

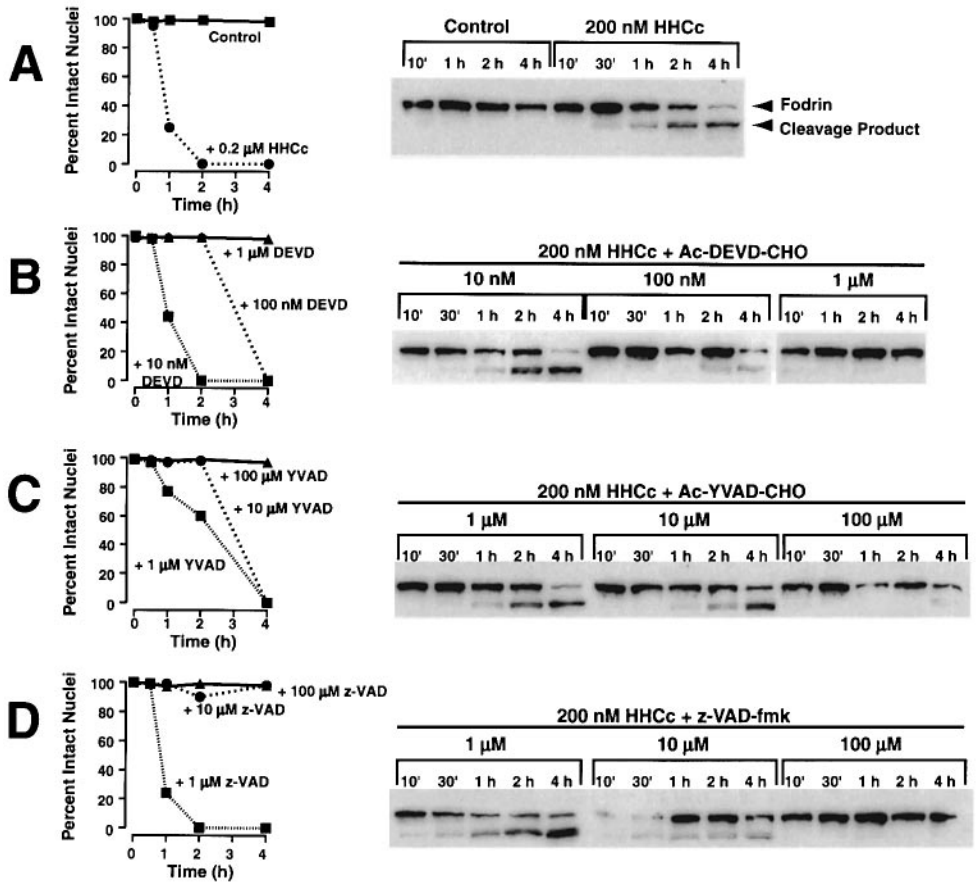

Fig. 5. Cytochrome c-initiated apoptosis is inhibited by protease inhibitors. (**A**) Reconstituted extracts were incubated with or without horse heart cytochrome *c* (HHCc, 0.2 μM) for 4 h. (**B**, **C** and **D**) Extracts containing HHCc were co-incubated with the caspase inhibitors Ac-DEVD-CHO, Ac-YVAD-CHO or z-VAD-fmk at the indicated concentrations. At the indicated times, extracts were assessed for nuclear morphology (left panels) and for fodrin cleavage (right panels). The experiment shown is representative of three experiments.

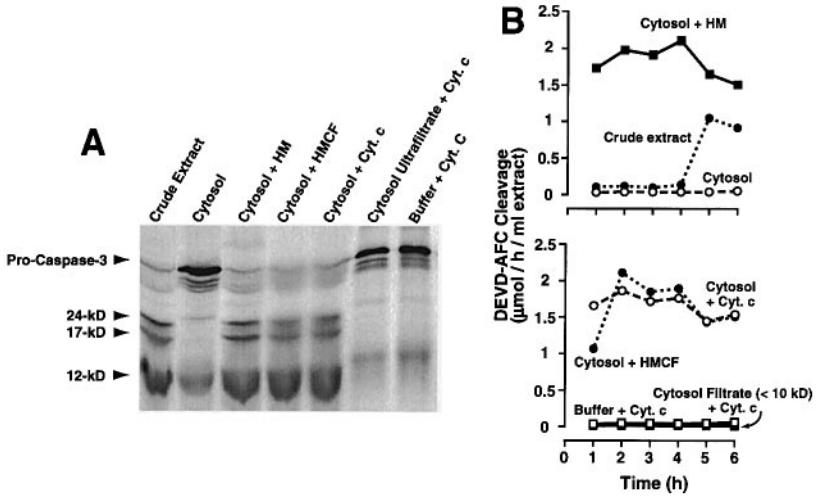

Fig. 6. CPP32 cleavage occurs during apoptosis in *Xenopus* extracts and requires cytosol. (**A**) [^{35}S]methionine-labeled CPP32 was incubated in crude extract (which contains mitochondria), cytosol, cytosol ultrafiltrate (<10 kDa) or buffer A, to which HM (10% v/v), HMCF (10% v/v) or HHCc (0.1 μM) was added as indicated. At 6 h, extracts were analyzed by SDS–PAGE and autoradiography for cleavage of [^{35}S]methionine-labeled CPP32 (see Materials and methods). (**B**) Parallel incubations not containing [^{35}S]methionine-labeled CPP32 were monitored for apoptotic DEVDase activity. Experiment is representative of two experiments.

8). First, interaction of the mitochondrial fraction with cytosol releases cytochrome *c*. Once the cytochrome *c* concentration in cytosol rises above a certain threshold, interaction with at least one other cytoplasmic factor initiates the activation of DEVD-specific caspases. These activated proteases then either directly or indirectly cleave cytoplasmic and nuclear protein targets, culminating in apoptotic nuclear morphology. Bcl-2 inhibits apoptosis by blocking cytochrome *c* release, but not the effects of cytochrome *c* (Kluck *et al.*, 1997; Yang *et al.*, 1997),

whereas Ac-DEVD-CHO, Ac-YVAD-CHO and z-VAD-fmk all inhibit the caspases downstream of cytochrome *c* (Figure 5B–D).

Cytochrome *c* is released from mitochondria when the mitochondrial fraction is incubated with cytosol, but not when incubated with buffers. The release occurs in the presence or absence of the fraction containing endoplasmic reticulum, and is the step in the apoptotic pathway inhibited by Bcl-2 in this system (Kluck *et al.*, 1997) and in mammalian systems (Kluck *et al.*, 1997; Yang *et al.*, 1997). The *Xenopus* extracts appear to be primed to release cytochrome *c*, as no further stimulus is required for apoptosis. Whether this priming is a feature of the cytosol, the mitochondria or both, is yet to be determined, but it is possibly related to the hormone regimen given to the frogs to induce egg laying (Newmeyer *et al.*, 1994) and to oocyte atresia (Tilly *et al.*, 1992). Of the many signals which might impinge on mitochondria, SH2 domain interactions appear to be important, as free phosphotyrosine delays apoptosis (Newmeyer *et al.*, 1994), SH2 domains from various signalling molecules inhibit apoptosis (Farschon *et al.*, 1997) and immunodepletion of the adaptor protein c-Crk prevents apoptosis in *Xenopus* egg extracts (Evans *et al.*, 1997).

With apoptosis, nearly all of the cytochrome *c* contained in mitochondria is released into the cytosol (T.Kuwana and D.D.Newmeyer, unpublished data; Yang *et al.*, 1997). This loss of cytochrome *c* may underlie the observed decrease in oxygen consumption following anti-CD95 treatment of Jurkat cells (Krippner *et al.*, 1996). Cytochrome *c* is transcribed from a nuclear gene and synthesized in the cytoplasm as apocytochrome *c*. The apoprotein, on entry into the mitochondrial intermembrane space, gains a heme group, to become the fully folded holocytochrome *c* (Dumont *et al.*, 1991). This globular, positively charged, polar protein can no longer pass through the outer mitochondrial membrane and is thought to become electrostatically attached to the inner membrane, perhaps via negatively charged phospholipids such as cardiolipin (reviewed by Jordi and De Kruijff, 1996). It is interesting to conjecture how cytochrome *c* may be released from mitochondria during apoptosis, based on our limited knowledge of the Bcl-2 family. As discussed above, Bcl-2 blocks apoptosis by inhibiting the release of cytochrome *c* from mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997). Bcl-x_L is structurally related to the bacterial pore-forming colicin proteins (Muchmore *et al.*, 1996), and Bcl-2 is thought to have a similar structure (Vance *et al.*, 1996). Bcl-x_L can form pores in artificial membranes (Minn *et al.*, 1997). Thus, the Bcl-2 family may directly modulate the permeability of the outer mitochondrial membrane to ions or proteins. The result may be to alter the ionic environment of the intermembrane space, or to allow cytochrome *c* efflux, or both.

A different apoptotic cell-free system has been described, in which mitochondria are mixed with nuclei in buffer, in the absence of cytosol (Susin *et al.*, 1996; Zamzami *et al.*, 1996). In that system, Bcl-2 blocks the mitochondrial release of a 50 kDa protease called apoptosis-inducing factor (AIF). This factor is released

Table I. Pro-apoptotic activity of cytochrome *c* variants added to *Xenopus* cytosolic extracts

Cyt. *c* variant	Concentration (μM)	Pro-apoptotic activity
Bovine heart	0.1	+
Horse heart	0.1	+
Pigeon heart	0.1	+
Tuna heart	0.1	+
S.cerevisiae[a]	10	–
Heat-denatured bovine heart[b]	1	–
Enzyme-degraded horse heart[c]	10	–

[a]Reagent (a mix of the 2 yeast isomers, iso-1- and iso-2- cytochrome *c*) was supplied by Sigma and by F.Sherman.
[b]100°C for 1 h.
[c]Also called microperoxidase MP-11 (Sigma C4532). Proposed structure is the heme portion with amino acids 11–21 still attached.

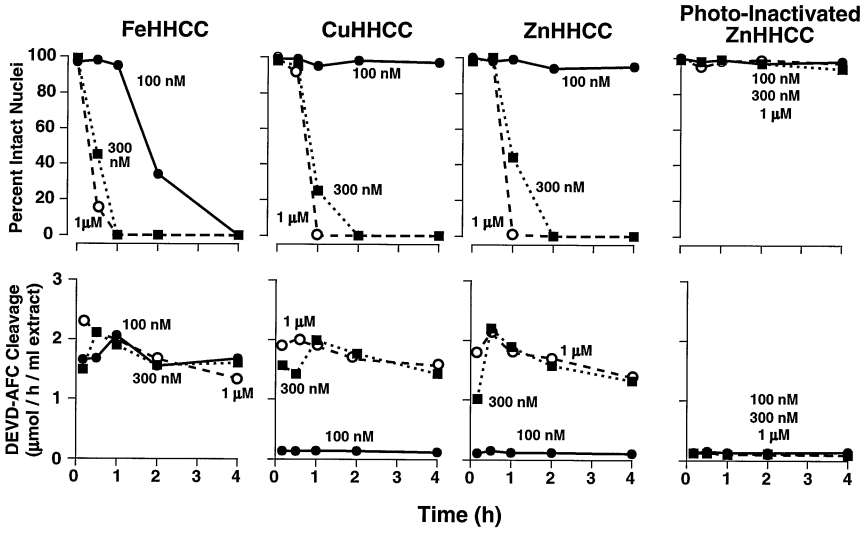

Fig. 7. Redox activity is not required for the apoptotic effects of cytochrome *c*. Mitochondria-depleted extracts were incubated for 4 h in the presence of Fe-HHCc, Cu-HHCc, Zn-HHCc or photo-inactivated Zn-HHCc at the indicated concentrations. At various times, aliquots were assessed for (top) percent intact nuclei and for (bottom) DEVDase activity. Experiment is representative of three experiments.

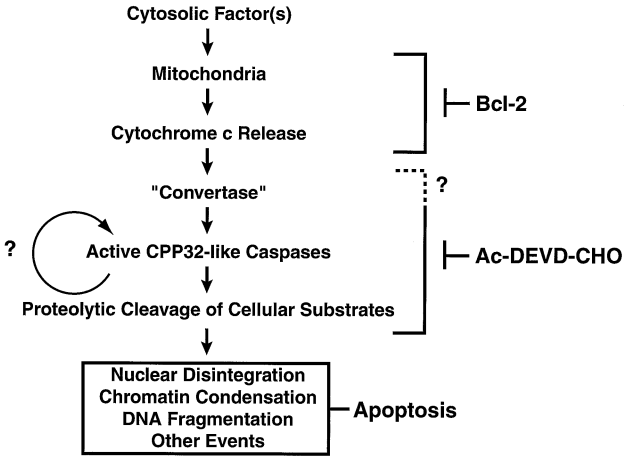

Fig. 8. Pathway by which apoptosis may proceed in the *Xenopus* cell-free system.

from isolated mitochondria following treatment with agents known to cause a phenomenon called the permeability transition (PT). The PT involves the opening of pores in the inner mitochondrial membrane that allow passage of solutes <1500 Da. AIF, which is apparently not a caspase, directly induces nuclear changes reminiscent of apoptosis, and is inhibited by zVAD-fmk, but not by Ac-DEVD-CHO (Susin *et al.*, 1996). However, AIF does not seem to be important in the *Xenopus* system, as the pro-apoptotic activity released from mitochondria (HMCF) is fully inhibitable by Ac-DEVD-CHO (Figure 3B), and cytochrome *c* alone can substitute for HMCF (Figures 4C and 5). Moreover, in the *Xenopus* cell-free system, no evidence of PT was seen in isolated mitochondria during apoptosis (Kluck *et al.*, 1997). Studies with HL60 cells treated with staurosporine (Yang *et al.*, 1997) and with CEM and HeLa cells treated with staurosporine or UV-B light (E.Bossy-Wetzel, D.D.Newmeyer and D.R.Green, in preparation) show that a reduction in mitochondrial membrane potential ($\Delta\Psi_m$) occurs late in the apoptotic process, well after the release of cytochrome *c*. Furthermore, in the latter work, the loss of $\Delta\Psi_m$ is blocked by zVAD-fmk, while cytochrome *c* release is not. These results argue that mitochondrial membrane depolarization and the PT are, at least in some instances of apoptosis, byproducts of caspase activation and therefore late events in the apoptotic pathway. Furthermore, cytochrome *c* release can occur in the absence of the PT.

Both the polypeptide chain and the heme prosthetic group of cytochrome *c* are required to activate caspases. Cytochrome *c* that is released into the cytosol contains a heme group, as indicated by spectral analysis (not shown). Apocytochrome *c* is not likely to be involved in apoptosis in the cell-free system, for several reasons: cycloheximide present in the extracts precludes its synthesis; it is not present in mitochondria (Dumont *et al.*, 1991); and it does not exhibit apoptotic effects (Yang *et al.*, 1997). Experimental modifications of cytochrome *c*, such as partial digestion to form 'microperoxidase', heat denaturation and light-induced denaturation (in the case of Zn-cytochrome c) eliminated pro-apoptotic activity. After accumulation in cytosol, the holoprotein appears to be stable for at least several hours (Kluck *et al.*, 1997).

Cytochrome *c* is highly conserved in terms of its sequence and respiratory function (Margoliash and Schejter, 1996). Some of this conservation also applies to its apoptotic function, as horse, bovine, tuna and pigeon heart cytochrome *c* induced apoptosis in the *Xenopus* system with equal efficacy (Table I). Mitochondria isolated from human cells also released cytochrome *c* and induced apoptosis when incubated in *Xenopus* egg cytosol (not shown).

An exception to this conservation was found when purified *S.cerevisiae* iso-1 and iso-2 cytochromes *c* did not activate apoptosis in the *Xenopus* system (Table I). Isolated *S.cerevisiae* mitochondria released cytochrome *c*, but did not induce apoptosis when incubated in *Xenopus* extracts (R.M.Kluck, D.R.Green, M.Yaffe, E.Margoliash and D.D.Newmeyer, in preparation). The yeast cytochrome *c* isoforms both contain an ε-*N*-trimethylated lysine residue at position 72 (Paik *et al.*, 1989), a region of the molecule thought critical for interaction with all demonstrated binding partners (Margoliash and Schejter, 1996). The consequences of such sequence differences are being investigated.

The redox function of cytochrome *c* is essential for its function in oxidative phosphorylation, but is shown here not to be required for its pro-apoptotic activity. Cu- and Zn-substituted cytochrome *c*, which are structurally similar to Fe-cytochrome *c*, but redox-inert (Findlay *et al.*, 1977; Anni *et al.*, 1995; Zhou *et al.*, 1995), are strong pro-apoptotic agents (Figure 7). A somewhat diminished potency compared with Fe-cytochrome *c* may indicate that redox activity can contribute to apoptotic activity; however, there are other possible explanations for the reduced potency, including an altered conformation.

At least one unknown cytosolic element interacts with cytochrome *c* to activate caspases. This is evidenced by the inability of cytochrome *c* to cleave CPP32 in the absence of cytosol (Figure 5, lanes 6 and 7). We refer to this cytosolic element as the 'convertase'. Such a cytosolic component is also required for cytochrome *c* induction of apoptosis in cell-free systems based on mammalian cell extracts (S.J.Martin, R.M.Kluck, C.A.Casiano, D.D. Newmeyer, J.C.Reed, D.D.Nicholson, E.S.Alnemri and D.R.Green, in preparation; Liu *et al.*, 1996). Of interest is the possibility that CED-4 may be related to the convertase, as mutation experiments in *C.elegans* suggest that CED-4 acts between CED-9 and CED-3 (the Bcl-2 and caspase homologs respectively) (Shaham and Horvitz, 1996).

In the *Xenopus* cell-free system, the addition of either HMCF or cytochrome *c* to fresh cytosol rapidly activated DEVDases, leading to the cleavage of fodrin and lamin B_1 and to apoptotic nuclear morphology. All apoptotic features were dependent on CPP32-like caspase activity, as they were all preferentially blocked by Ac-DEVD-CHO (Figures 2, 3 and 5; Cosulich *et al.*, 1996; Farschon *et al.*, 1997). cDNA clones of *Xenopus* XCPP32 and XICE (counterparts, respectively, of mammalian CPP32 and ICE) have recently been obtained (Yaoita and Nakajima, 1997). The increased XCPP32 mRNA expression and low XICE mRNA expression in the regressing tadpole tail, together with the potent inhibitory effect of Ac-DEVD-CHO reported in that study, are consistent with XCPP32 being a dominant caspase active in *Xenopus* tissues.

The ability of extracts to cleave z-YVAD-AFC did not increase with apoptosis, but initial YVADase activity was higher than initial DEVDase activity (not shown), suggesting that some ICE-like caspase activity is already present in the *Xenopus* extracts. As these extracts still require the presence of mitochondria, it is possible that ICE-like caspases facilitate the release of cytochrome *c*. In cells, caspase-8 (MACHα/Flice) is directly activated by CD95/Fas and p55 tumor necrosis factor receptor signalling (Boldin *et al.*, 1996; Muzio *et al.*, 1996), although it has not been determined whether this protease acts by stimulating cytochrome *c* release. However, in the *Xenopus* cell-free system (not shown) and in CEM cells treated with UV or staurosporine (Kluck *et al.*, 1997; E.Bossy-Wetzel, D.D.Newmeyer and D.R.Green, in preparation), the caspase inhibitor, z-VAD-fmk (100 μM) did not block cytochrome *c* release. This argues that the endogenous YVADase activity is not required for apoptosis in the *Xenopus* extracts. We cannot exclude, however, that YVADase activity occurring prior to or during extract preparation contributes to the apoptotic phenotype.

Consequent to DEVDase activation is the cleavage of the cytoplasmic cytoskeletal protein fodrin and the nuclear structural protein lamin B_1 (Figure 1). Cleavage of these proteins is inhibited either by Bcl-2 (if cytochrome *c* is not already released; Figure 2; Kluck *et al.*, 1997) or by Ac-DEVD-CHO (even after cytochrome *c* is present in cytosol; Figures 3 and 5). Other proteins characteristically cleaved in apoptotic cells, PARP and DNA-dependent protein kinase, are also cleaved with apoptosis in *Xenopus* egg extracts (Cosulich *et al.*, 1996; Le Romancer *et al.*, 1996).

The *Xenopus* cell-free system was the first used to highlight the requirement for mitochondria in apoptosis (Newmeyer *et al.*, 1994). This requirement is now explained by the release of cytochrome *c* from mitochondria, in agreement with findings in a mammalian cell-free system (Liu *et al.*, 1996). In the present study, we have delineated other events in apoptosis. In particular, we have shown that cytosolic factors are required upstream of mitochondria to initiate cytochrome *c* release and that other cytosolic factors (the convertase) are required downstream of mitochondria for cytochrome *c* to activate caspases. In addition, we have shown that the pro-apoptotic effect of cytochrome *c* requires certain structural features of the molecule, but not its electron transfer capacity. It will now be of interest to fill in the remaining gaps in the apoptotic pathway. In particular, this system should be useful for identifying the factors and mechanisms involved in the translocation and pro-apoptotic function of cytochrome *c*.

Materials and methods

Materials

Ac-DEVD-CHO and Ac-YVAD-CHO were obtained from Bachem (King of Prussia, PA). z-VAD-fmk was obtained from Kamiya Biochemical (Seattle, WA). *Sf*9 insect cell lysates containing baculovirus-expressed Bcl-2 or β-galactosidase were produced as described (Newmeyer *et al.*, 1994). Cytochromes *c* from bovine, horse, pigeon and tuna heart (>95% purity) were obtained from Sigma (St Louis, MO).

Preparation of egg extracts

Crude and fractionated *Xenopus* egg extracts, and rat liver nuclei were prepared as described (Newmeyer *et al.*, 1994). HM-depleted (reconstituted) extract preparations were prepared by the combination of cytosol (supplemented with an ATP regenerating system of 10 mM phosphocreatine, 2 mM ATP and 150 mg/ml creatine phosphokinase), 10% v/v light membrane (predominantly endoplasmic reticulum) and 4×10^3 rat liver nuclei per μl. In some experiments, a simplified system derived from only cytosol (with ATP regenerating system) was used. These reconstituted extracts, which never underwent apoptotic changes, were then induced to undergo apoptosis by incubation with the mitochondria-enriched heavy membrane fraction (HM), HMCF or cytochrome *c*.

Identification and quantitation of apoptotic nuclear morphology

Apoptotic nuclear morphology was quantified as described (Newmeyer *et al.*, 1994). Aliquots (3.5 μl) of extract were mixed with 0.8 μl of a 100 μg/ml solution of Hoechst 33258 on a glass slide, a coverslip added and then examined by fluorescence microscopy. In intact nuclei, chromatin was uniformly distributed. With the onset of apoptosis, the chromatin showed characteristic signs of apoptosis i.e. condensation, margination and beading to multiple spherical particles, with subsequent disintegration of the nucleus. All the results presented here were qualitatively reproducible. However, the time at which nuclear destruction began to occur varied between extracts, making it impossible to average the results from separate experiments in a meaningful way; therefore, representative experiments are shown.

Measurement of DEVDase activity

To measure CPP32-like caspase activity, extract aliquots (2 μl) were incubated with the CPP32-specific fluorigenic peptide substrate z-DEVD-AFC (80 μM, Enzyme Systems Products, Dublin, CA) in 100 μl buffer A (250 mM sucrose, 20 mM HEPES–KOH pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT). After 15 min incubation at 22°C, the reaction was stopped by the addition of 1% sodium acetate trihydrate in 175 mM acetic acid (50 μl) and cooling on ice. After dilution with water (1 ml), fluorescence at 400/505 nm was measured with an SLM Aminco Bowman 8000C fluorimeter. Measurements were calibrated against a standard curve of 7 amino-4-trifluoromethyl-coumarin (AFC, Sigma). It was noted that individual egg extract preparations differed 2- to 3-fold in their maximal DEVDase activity (not shown).

Production of cytosol containing HMCF

The soluble factor termed HMCF was produced by incubation of HM (12% v/v) in cytosol (containing the ATP regenerating system) for 4 h at 22°C. HM and any possible membrane fragments were then removed by pelleting (12 000 *g*) then by consecutive microfiltration of the supernatant through 0.22 μm and then 0.1 μm filters (Millipore) at 4°C, according to manufacturer's instructions. The recovered cytosolic filtrate was aliquoted and stored at –80°C.

Treatments of HMCF

To ascertain whether the active component(s) of HMCF were lipid, HMCF was incubated with agarose-bound lipase (Sigma L2764) at 0.5 units per 20 μl for 3 h at 37°C, in the presence or absence of 4 mM $CaCl_2$, prior to removal of lipase by two steps of centrifugation at 14 000 r.p.m. A similar protocol was used to treat HMCF with agarose-bound proteinase K (Sigma) at 0.01 units per 20 μl for 3 h at 37°C. Agarose-bound enzymes had been pre-washed in buffer A and resuspended in minimal quantities of the same before addition to HMCF. Organic extraction of lipids from HMCF was performed by mixing 20 μl HMCF with 70 μl chloroform–methanol (50:50) containing 5 mM HCl. Following phase separation by centrifugation, the organic layer was dried under N_2.

Western blotting

Protein concentration of egg extracts was 30–50 mg/ml, as measured with the Bio-Rad protein microassay. For examination of fodrin and lamin B_1, extract samples (3 μl) were loaded onto a 10% SDS–polyacrylamide gel and electrophoresed at 70 V for 3 h, then transferred (70 mA) to PVDF membranes (Bio-Rad) overnight. Membranes were then blocked in TBST (25 mM Tris–HCl, pH 7.4, 140 mM NaCl, 27 mM KCl, 0.02% Tween 20) containing 5% nonfat dried milk for 1 h. Detection of fodrin was carried out using a monoclonal antibody to fodrin (non-erythroid spectrin; Chemicon, Temecula CA), while lamin B_1 was detected using chicken anti-lamin B_1 antibody kindly provided by S.Kaufmann. After washing in TBST, recognized proteins were detected using either horse-radish peroxidase-labeled anti-mouse or anti-chicken antibodies (Amersham), in combination with enhanced

chemiluminescence (Amersham). Western blot of cytochrome *c* was as described (Kluck *et al.*, 1997).

[^{35}S]methionine-labeled CPP32 experiments

Coupled transcription/translation of pro-CPP32B was carried out in rabbit reticulocyte lysates using the TNT kit (Promega) as described (Martin *et al.*, 1996). [^{35}S]methionine-labeled CPP32 cleavage in *Xenopus* extract was analyzed by SDS–PAGE and autoradiography. Each extract mix was prepared in a volume of 10 µl containing crude extract, cytosol, filtered cytosol (<10 kDa) or buffer A, combined with the ATP regenerating system and (excepting the crude extract mix) 10% v/v light membrane. Each mix was divided into duplicate 5 µl reactions. One reaction mix was monitored for DEVDase activity (using 1 µl aliquots). The parallel reaction mix was used for the analysis of CPP32 cleavage, by the addition of 0.5 µl of the [^{35}S]methionine-labeled CPP32 substrate, and incubation for 6 h, followed by addition of an equal volume of 2× reducing SDS–PAGE buffer, heating to 90°C for 5 min, and SDS–PAGE. Gels were fixed in methanol–acetic acid–water (20:10:70) for 1 h, dried and autoradiographed overnight at –80°C.

Partial purification of HMCF by size fractionation

Size fractionation of HMCF was performed using a Superose 12 gel filtration column (Pharmacia) and automatic fast protein liquid chromatography (FPLC) station (Pharmacia) equilibrated with buffer B (25 mM KCl, 5 mM HEPES–KOH pH 7.4). Cytosol containing HMCF (60 µl, 1.8 mg protein) was loaded onto the column, with a flow rate of 0.4 ml/min, and after an initial 15 min period, fractions were collected every 2 min. Parallel runs with the size markers trypsin inhibitor (21 kDa) and aprotinin (<7 kDa) were also performed. To assess HMCF activity of each FPLC fraction, aliquots (50 µl) were lyophilized and resuspended in 5 µl cytosol and then incubated for 4 h at 22°C. Incubated cytosol samples were then assessed for the ability to cleave z-DEVD-AFC as described above. To analyze cytochrome *c* content, 200 µl of each fraction was lyophilized, reconstituted in 1× SDS–PAGE reducing buffer (40 µl) and subjected to gel electrophoresis and Western blotting as described above.

Comparison of Fe-HHCc to redox-inert Cu- and Zn-HHCc

Cu- and Zn-substituted HHCc were prepared as described previously (Vanderkooi *et al.*, 1976; Findlay *et al.*, 1977), and contained 0.2–3.0% Fe-HHCc contamination as determined by UV spectroscopy. As Zn-HHCc is degraded by light in the presence of oxygen ($t_{1/2}$ ~2 h in room light) these experiments were carried out in a dark room with sampling performed in low light conditions. To examine the pro-apoptotic activity of photo-inactivated Zn-HHCc, a 10 µM stock was exposed to room light for 15 h. As controls, solutions of Fe-HHCc and of a Zn-HHCc and Fe-HHCc mixture were also exposed to room light for 15 h.

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