

Reaper-induced apoptosis in a vertebrate system

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The reaper protein of *Drosophila melanogaster* has been shown to be a central regulator of apoptosis in that organism. However, it has not been shown to function in any vertebrate nor have the cellular components required for its action been defined. In this report we show that reaper can induce rapid apoptosis *in vitro* using an apoptotic reconstitution system derived from *Xenopus* eggs. Moreover, we show that a subcellular fraction enriched in mitochondria is required for this process and that reaper, acting in conjunction with cytosolic factors, can trigger mitochondrial cytochrome c release. Bcl-2 antagonizes these effects, but high levels of reaper can overcome the Bcl-2 block. These results demonstrate that reaper can function in a vertebrate context, suggesting that reaper-responsive factors are conserved elements of the apoptotic program.

Keywords: apoptosis/reaper/*Xenopus*

Introduction

Apoptosis is a dramatic form of cell death used by multicellular organisms to rid themselves of superfluous or potentially harmful cells. While apoptosis plays a critical role in development, tissue homeostasis and immune regulation, dysregulation of this important cellular process may contribute to the development of numerous pathologies, including cancer, neurodegeneration and diabetes.

Although the morphological hallmarks of apoptosis have been extensively documented (plasma membrane blebbing, DNA condensation, cleavage and nuclear fragmentation), the molecular participants in the process are only beginning to be defined (Bellamy *et al.*, 1995). Proteins belonging to the interleukin 1 β -converting enzyme (ICE) family of proteases (also known as caspases) have been widely implicated in execution of the apoptotic program in response to a diversity of stimuli (reviewed in Yuan, 1995; Chinnaiyan and Dixit, 1996). Modulators of the apoptotic process, believed to act upstream of the caspases, include pro- and anti-apoptotic members of the Bcl-2 family of proteins (e.g. bad, bax, bak, bcl-Xs and bcl-xl) (Nunez and Clarke, 1994; Reed, 1994) and

'adaptor' proteins such as FADD, TRADD and RAIDD, which appear to couple cell surface receptors to downstream caspase activation (Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995; Duan and Dixit, 1997).

In a screen to identify novel regulators of the apoptotic process in *Drosophila melanogaster*, White and co-workers identified a small open reading frame encoding a 65 amino acid protein, which they named reaper (White *et al.*, 1994). During fly development induction of reaper mRNA consistently preceded the onset of morphological apoptosis by 1–2 h and ectopic induction of reaper caused massive cell death both in cultured cells and in whole flies (Pronk *et al.*, 1996; White *et al.*, 1996). A chromosomal deletion that includes the reaper gene prevented almost all programmed cell deaths during fly development and suppressed apoptosis in response to a number of different external stimuli. These findings suggest that reaper might be a key regulator of cell death in *Drosophila*. Although reaper participates in diverse apoptotic events, it does not appear to be part of the execution machinery, because apoptosis can occur in reaper mutant embryos when they are irradiated with very high levels of X-rays. Reaper-induced apoptosis is, however, prevented by caspase inhibitors (including peptide inhibitors and the viral inhibitor p35), consistent with the notion that reaper acts upstream of apoptotic proteases in cell death pathways (Pronk *et al.*, 1996; White *et al.*, 1996).

Apoptotic regulators such as caspases and Bcl-2 have been well conserved from *Caenorhabditis elegans* to man, suggesting that the underlying mechanism of apoptotic cell death has been conserved through evolution. However, a vertebrate reaper homolog has not yet been found. Reaper has not been shown to be relevant to apoptosis in higher eukaryotes and has only been demonstrated to induce apoptosis upon ectopic expression in insect cells (Pronk *et al.*, 1996; White *et al.*, 1996; Vucic *et al.*, 1997). It therefore remains an open question as to whether reaper represents an insect-specific inducer of the apoptotic program or whether similar pathways exist in other eukaryotes.

Recently a cell-free extract of *Xenopus* eggs which can support apoptosis was described (Newmeyer *et al.*, 1994). To induce egg laying, female frogs are normally primed with pregnant mare serum gonadotropin (PMSG) and then injected several days later with human chorionic gonadotropin (hCG) to induce egg laying. This regimen allows the preparation of egg extracts that form functional nuclei around added chromatin. Extending the interval between PMSG and hCG administration to several weeks produces extracts with apoptotic activities, most likely reflecting the *in vivo* process of oocyte atresia, wherein oocytes which have matured but have not been laid are apoptotically resorbed. Prolonged incubation (>4 h) of these extracts results in apoptotic nuclear fragmentation and caspase activation.

The development of apoptotic events in *Xenopus* egg extracts requires the presence of a heavy membrane (HM) fraction enriched in mitochondria. In response to factors present in *Xenopus* egg cytosol, mitochondria release a pro-apoptotic protein, cytochrome c (Kluck *et al.*, 1997a,b). Normally, in non-apoptotic cells cytochrome c resides in the intermembrane space of mitochondria, where it participates in the electron transport chain. However, after the cell receives an appropriate apoptotic stimulus cytochrome c is released from the mitochondria. Once this protein is present in the cytosol it induces activation of proteases such as caspase-3 (CPP32), which in turn effect downstream apoptotic events, such as internucleosomal DNA cleavage (Liu *et al.*, 1997). The Bcl-2 protein, through binding to the outer mitochondrial membrane, inhibits release of cytochrome c from mitochondria, thereby preventing caspase activation and downstream apoptotic events (Kluck *et al.*, 1997a; Yang *et al.*, 1997).

In *Xenopus* extracts a lag period occurs prior to release of cytochrome c and the manifestation of overt apoptotic events. This suggests that important signaling events occur upstream of cytochrome c release. Recent studies, in fact, have shown that interaction of tyrosine-phosphorylated proteins and SH2 domains are important for upstream signaling in *Xenopus* extracts (Farschon *et al.*, 1997). In particular, the adaptor protein Crk, which contains SH2 and SH3 domains, is required for apoptosis in this system (Evans *et al.*, 1997). However, the full sequence of signaling events leading to the release of cytochrome c from mitochondria is still unknown.

In this report we demonstrate that the *Drosophila* reaper protein can induce or accelerate apoptosis in *Xenopus* egg extracts. In addition, we show that reaper acts in conjunction with cytoplasmic factors to trigger mitochondrial cytochrome c release. These results provide the first demonstration that reaper can function in a vertebrate context and strongly indicate that reaper acts upstream of mitochondria. These data suggest that reaper-responsive factors are conserved elements of the apoptotic program and that the advantages of the biochemically tractable *Xenopus* system can be applied to elucidate the molecular mechanism of reaper-induced cell death.

Results

In order to determine whether *Drosophila* reaper could induce or accelerate apoptosis in *Xenopus* egg extracts, we produced recombinant reaper protein fused to the C-terminus of glutathione S-transferase (GST-reaper). GST-reaper protein (final concentration 600 ng/ μ l) was added to egg extracts along with sperm chromatin (for formation of synthetic nuclei). Although there was some extract-to-extract variability in the timing of apoptotic events, between 70 and 120 min after reaper addition, membrane blebs formed on the surface of the nuclei, followed by visible fragmentation of the chromatin and packaging of the DNA into small membrane-enclosed vesicles. Complete fragmentation of the nuclei was observed within 90–140 min in extracts supplemented with reaper protein, while nuclei formed in extracts lacking exogenously added reaper were stable for >4 h (Figure 1A and B). Nuclear fragmentation in the extract was highly synchronous, with all nuclei beginning fragment-

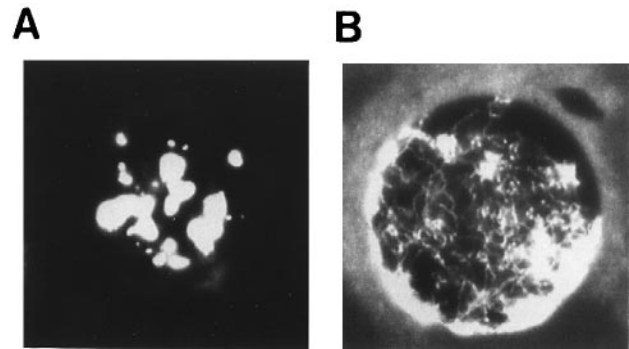


Fig. 1. Reaper-induced apoptosis in *Xenopus* egg extracts. GST-reaper protein (final concentration 600 ng/ μ l), or an equivalent concentration of GST protein alone, was added to extracts of *Xenopus* eggs in the presence of sperm chromatin (1000 nuclei/ μ l) and an ATP regenerating system. (A) Photomicrograph of a representative nucleus induced to enter apoptosis by addition of recombinant GST-reaper protein. All nuclei in the extract behaved similarly, entering apoptosis within 10 min of each other. (B) Photomicrograph of a representative stable nucleus formed *in vitro* by incubation of sperm chromatin in egg extract. This morphology was not altered by the addition of GST protein alone.

ation within 10 min of each other (even at nuclear concentrations >1000 per μ l extract). The nuclear fragmentation induced by reaper was strongly reminiscent of apoptotic nuclear fragmentation seen *in vivo* and was morphologically identical to that previously described in egg extracts. This nuclear fragmentation occurred in cycloheximide-treated extracts, arguing that translation was not required for reaper action.

Nuclear fragmentation induced in response to reaper was accompanied by cleavage of the DNA into fragments, producing a laddering pattern characteristic of that seen in apoptotic cells when resolved on agarose gels. Furthermore, these nuclear events were inhibited by addition of 10 μ M Ac-YVAD-cmk or zVAD-FMK, peptide inhibitors of caspases (data not shown).

Reaper induces caspase activation *in vitro*

To directly demonstrate reaper-induced activation of caspases in the extract, we added trace amounts of various ³⁵S-labeled substrates of these proteases to the extract. The substrates used were poly(ADP) ribose polymerase (PARP) (Lazebnik *et al.*, 1994), the zymogens pro-caspase 3 (Yama/CPP32) (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), pro-caspase 1 (ICE), and pro-caspase 7 (ICE-LAP3) (Duan *et al.*, 1996) (members of the caspase family which are themselves cleaved to form active proteases from inactive precursors) and baculovirus p35, which acts as a competitive inhibitor of caspases (at higher concentrations than used here) while being cleaved by them (Bump *et al.*, 1995; Xue and Horvitz, 1995). Aliquots of extract containing these radiolabeled substrates were withdrawn every 10 min during a 3 h room temperature incubation and were then resolved by SDS-PAGE and processed for autoradiography. With the sole exception of pro-caspase 1, all of these substrates were cleaved to their characteristic apoptotic fragments in extracts to which reaper had been added, but not in control extracts (Figure 2). Routinely, cleavage of all these proteins preceded the initial stages of nuclear fragmentation by ~10 min. It is unclear whether the failure to cleave pro-caspase 1 reflects

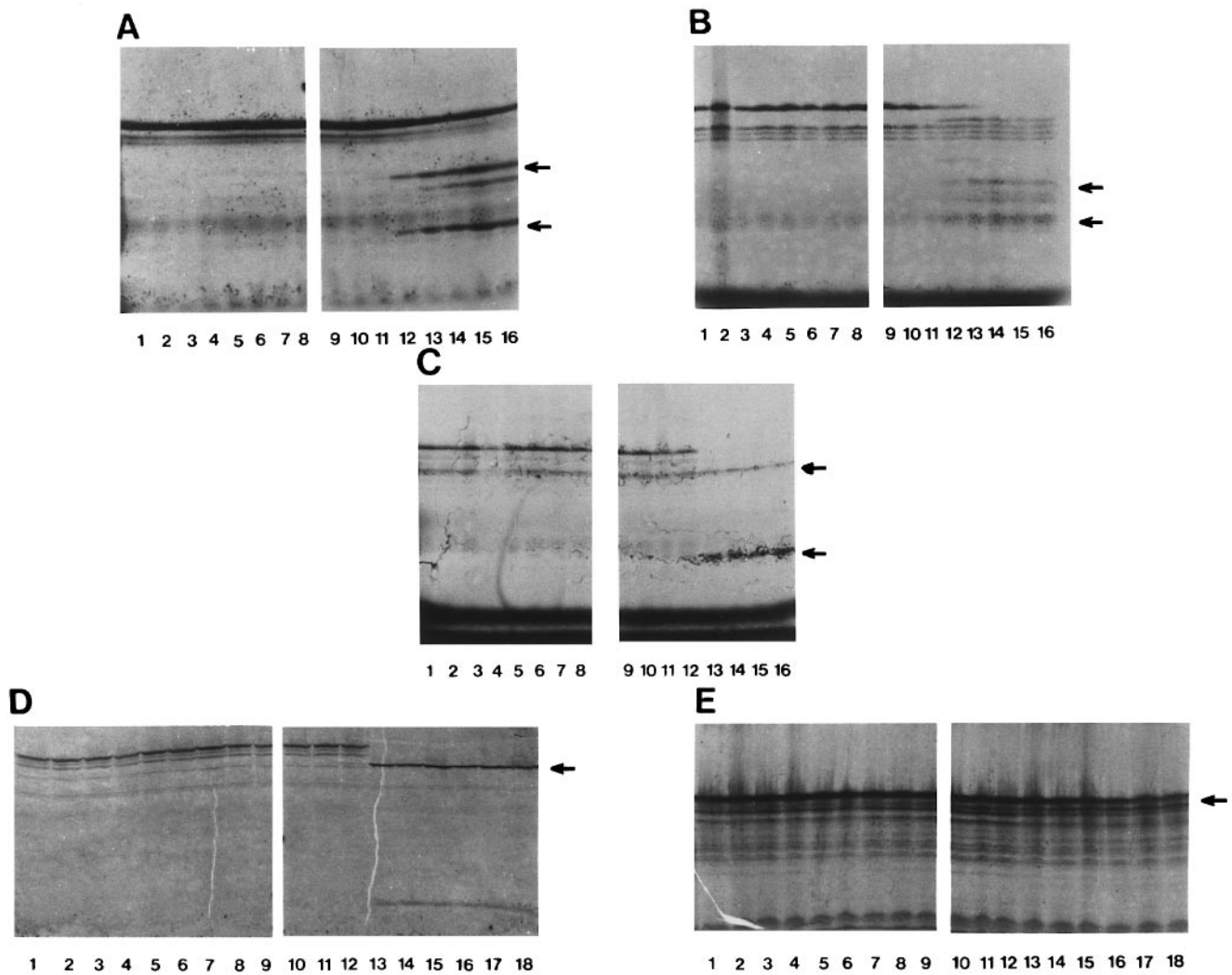


Fig. 2. Reaper induces proteolytic cleavage of apoptotic substrates. ^{35}S -labeled pro-caspases 1, 3 or 7 (ICE, YAMA and ICE-LAP-3 respectively) or caspase substrates (baculovirus p35 and PARP) were added to extracts in the presence (right hand panel in every pair, lanes 9–16, A–C, and 10–18, D and E) or absence (left hand panel in every pair, lanes 1–8, A–C, and 1–10, D and E) of recombinant GST–reaper. Beginning 30 min after the start of room temperature incubation (lanes 1 and 9, A–C, and 1 and 10, D and E), samples were withdrawn at 10 min intervals, resolved by SDS–PAGE and processed for autoradiography. The position of signature apoptotic cleavage fragments are denoted by arrows. (A) Pro-caspase 7; (B) pro-caspase 3; (C) baculovirus p35; (D) PARP; (E) pro-caspase 1.

the fact that caspase 1 is not activated during reaper-induced apoptosis or was due to some incompatibility between the heterologous components in this assay (*Xenopus* extract, *Drosophila* reaper protein and human caspase 1). However, it is striking that reaper can engage the *Xenopus* apoptotic machinery, triggering endogenous protease activation.

Mitochondria are required for reaper-induced apoptosis

Ultracentrifugation of crude egg extracts at 250 000 g produces cytosolic and microsomal membrane fractions which are cleanly separated from mitochondria, ribosomes and other dense organelles. As noted previously, naturally occurring *in vitro* apoptosis in these extracts requires a subcellular fraction containing mitochondria. Therefore, although extracts reconstituted by mixing the light membrane and cytosolic fractions are fully competent to form functional nuclei around added sperm chromatin templates, they never enter apoptosis, even upon prolonged room

temperature (e.g. >8 h) incubation. Similarly, we found that reconstituted extracts lacking mitochondria did not respond to reaper. These extracts failed to undergo microscopically visible nuclear fragmentation and did not initiate proteolytic cleavage of caspase substrates after reaper addition, as exemplified using pro-caspase 3 as substrate (Figure 3A). However, when the reconstituted cytosolic and membrane fractions were supplemented with a purified subcellular fraction containing mitochondria the ability to respond to reaper was restored, resulting in caspase activation (Figure 3B). Thus, reaper-induced apoptosis in these extracts requires the mitochondrial fraction.

Reaper accelerates development of latent apoptotic activities

Although reconstituted egg extracts lacking mitochondria cannot sustain full-blown apoptosis, we recently reported that incubation of these extracts for 2.5 h at room temperature allows development of latent apoptotic activities which are manifested upon transfer of a 1/10 volume

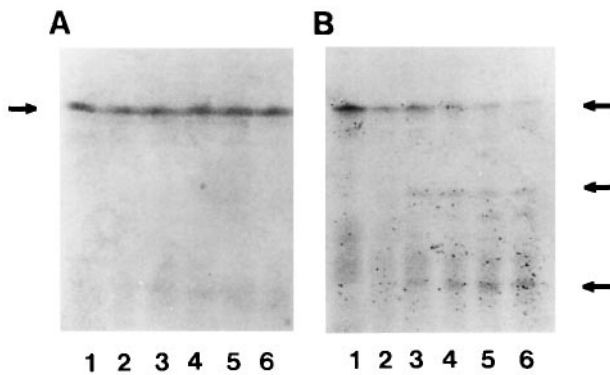


Fig. 3. Reaper requires a mitochondrial component for caspase activation. **(A)** ^{35}S -labeled pro-caspase 3 (YAMA) and recombinant GST-reaper protein were added to fractionated egg extracts lacking mitochondria. Samples were withdrawn at 0, 0.5, 1, 1.5, 2 and 2.5 h (lanes 1–6 respectively) and processed for SDS–PAGE and autoradiography. Pro-caspase 3 remains uncleaved during this 2.5 h incubation. **(B)** Addition of mitochondria to fractionated extract results in cleavage of ^{35}S -labeled pro-caspase 3. Radiolabeled pro-caspase 3 and GST-reaper protein were added to extracts supplemented with purified mitochondria. Samples were withdrawn at 0, 0.5, 1, 1.5, 2 and 2.5 h (lanes 1–6) and processed as in (A). Specific caspase 3 cleavage products appear at 1.5 h of room temperature incubation and are denoted by arrows.

of the preincubated reconstituted extract into crude extract containing nuclei and mitochondria (schematically depicted in Figure 4A; Evans *et al.*, 1997). This results in a marked acceleration of caspase activation and apoptotic nuclear fragmentation (which occurs within 60–90 min, instead of the typical 4 h), suggesting that the mitochondrial fraction of the crude extract allows execution of the apoptotic program in response to latent activities developed during the 2.5 h preincubation of membrane and cytosol. We therefore refer to the reconstituted light membrane and cytosolic fractions, where latent apoptotic activities develop, as the ‘latent extract’ and the crude extract containing mitochondria and nuclei as the ‘execution extract’. Preincubation of the latent extract for <2.5 h does not result in acceleration of apoptosis upon dilution into the execution extract.

Although we found that reaper was unable to induce apoptosis in the absence of mitochondria, we postulated that reaper might accelerate the development of apoptotic activities in the latent extract. To determine if this was the case, we added reaper (600 ng/ μl) to latent extracts and incubated them for various times at room temperature. Interestingly, we found that reaper substantially reduced the time required to develop latent apoptotic activities, as evidenced by its ability to shorten the required preincubation time of the latent extract from 2.5 h to 30 min. (Figure 4C and D). However, latent extracts incubated for the full 2.5 h induced apoptosis 70–80 min after transfer into crude extracts in the presence or absence of added reaper, suggesting that maximal levels of latent apoptotic activity develop in 2.5 h and that downstream components in the execution extract then become rate limiting (Figure 4B). It should be noted that the amounts of reaper present in the execution extract after transfer of latent extracts containing reaper (equivalent to 1/10 the amount used in the experiments shown in Figures 1–3) did not accelerate apoptosis when added directly to the execution extract.

These experiments suggest that reaper participates in events upstream of the step requiring mitochondria, during the ‘latent’ period of the apoptotic process *in vitro*.

Reaper circumvents the requirement for crk in apoptosis

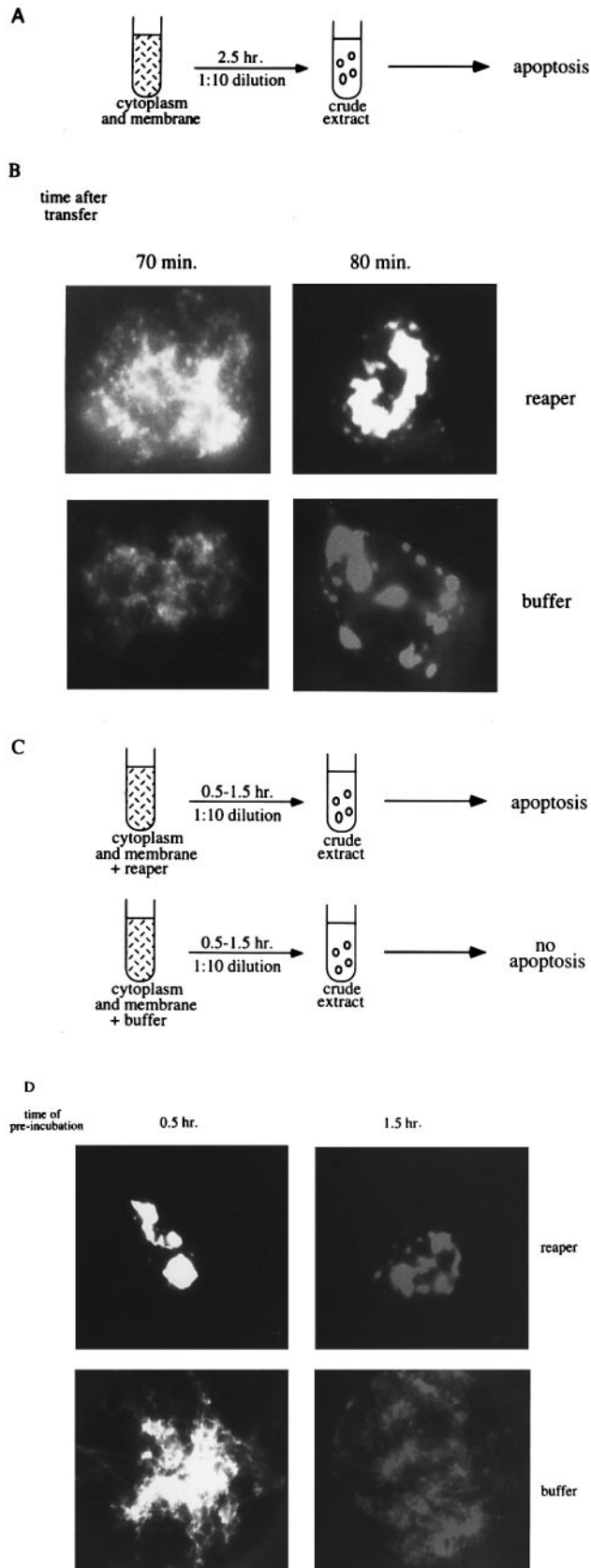
We recently reported that the SH2 and SH3 domain-containing protein crk also acts during this latent stage of apoptosis in the egg extract (Evans *et al.*, 1997). Specifically, addition of dominant-negative variants of crk or immunodepletion of crk from latent extracts prevents acceleration of apoptosis upon dilution into execution extracts. Moreover, direct addition of anti-crk sera to latent extracts also impedes subsequent apoptosis, which can be reversed by addition of recombinant *Xenopus* crk protein. Since we found that reaper protein could accelerate events occurring in the latent extract, we wished to determine whether this acceleration relied upon the presence of crk. As reported previously, when we immunodepleted crk from the latent extract subsequent apoptosis in the execution extract was not accelerated (Figure 5C). However, when we added reaper to latent extracts depleted of crk apoptosis occurred on schedule 60 min after dilution of the preincubated latent extract into the execution extract, demonstrating that reaper-responsive factors promoted acceleration of apoptosis even in the absence of crk (Figure 5D).

Interestingly, we found that GST-reaper protein was able to induce apoptosis in otherwise non-apoptotic extracts (i.e. those which did not spontaneously initiate the apoptotic process even after 6 h). This suggests that reaper engages the apoptotic machinery downstream of the endogenous signaling events responsible for activating the apoptotic process in *Xenopus* egg extracts, consistent with the ability of reaper to circumvent the requirement for crk protein.

Reaper promotes cytochrome c release from mitochondria

The fact that reaper could accelerate events occurring in the latent extract suggested that reaper could modulate factors acting upstream of the requirement for mitochondria. Recently Liu *et al.* (1996) reported that cytochrome c is required for *in vitro* apoptosis initiated by addition of dATP to HeLa cell extract. Moreover, regulated release of cytochrome c from mitochondria accompanies activation of the apoptotic program, both in mammalian cells and in the *Xenopus* cell-free system (Kluck *et al.*, 1997a; Yang *et al.*, 1997). Bcl-2, a potent inhibitor of apoptosis, acts, at least in part, by inhibiting cytochrome c release (Kluck *et al.*, 1997a; Yang *et al.*, 1997). We surmised that the absence of cytochrome c in our reconstituted extracts (lacking mitochondria) might account for the inability of reaper to induce apoptosis. Therefore, we added bovine or equine heart cytochrome c to the reconstituted extracts in the presence and absence of reaper and observed nuclei in these extracts for the appearance of apoptotic changes. Between 70 and 85 min after initiation of room temperature incubation the nuclei in these extracts began to enter apoptosis, as monitored by fluorescence microscopy, regardless of whether reaper was present (data not shown). This is in marked contrast to extracts lacking cytochrome c, which showed no signs of apoptotic nuclear fragmentation,

even 6 h after reaper addition. These results were confirmed at a biochemical level by analysis of baculovirus p35 degradation (Figure 6). Addition of cytochrome c to extracts lacking mitochondria promoted rapid apoptotic



cleavage of p35 regardless of whether or not reaper was present (Figure 6). Even after careful titration we were not able to identify a concentration of cytochrome c which could accelerate apoptosis only in the presence of reaper (data not shown). Interestingly, addition of free cytochrome c to extracts containing mitochondria also greatly accelerated apoptosis, consistent with the idea that release of cytochrome c from mitochondria is a rate limiting step in this process (E.K.Evans *et al.*, unpublished results). Taken together these data suggest that reaper might induce downstream release of cytochrome c from mitochondria, thereby triggering caspase activation.

To determine whether reaper could indeed induce mitochondrial cytochrome c release, we incubated GST-reaper in unfractionated *Xenopus* egg extracts. Aliquots were assayed at various times for cytochrome c release and caspase activity. Although the exact timing of apoptotic events varied between extracts (Farschon *et al.*, 1997), we consistently observed that reaper accelerated both the release of cytochrome c from mitochondria and the consequent activation of DEVD-cleaving caspases, as measured by cleavage of a fluorimetric substrate, DEVD-pNA. In this experiment, release of cytochrome c and DEVDase activation occurred at ~6–7 h incubation in the absence of reaper, but at ~1–2 h in the presence of reaper (Figure 7). In another experiment (not shown) apoptotic events occurred earlier in the control extract, at ~4 h incubation, but reaper nevertheless caused precocious release of cytochrome c, at 1–1.5 h incubation. We conclude that cytochrome c release is a downstream consequence of reaper action.

We wished to investigate whether reaper could induce cytochrome c release through direct interaction with mitochondria or instead required cytosolic macromolecules or light membranes (microsomes). To that end, we mixed mitochondria (HM) either with cytosol or an ultrafiltrate of cytosol, consisting only of molecules smaller than ~10 kDa, and then assayed the kinetics of cytochrome c release in the presence or absence of reaper. As Figure 8

Fig. 4. Reaper accelerates the development of latent apoptotic activities. (A) Schematic diagram illustrating the assay for apoptosis. The cytoplasm and membrane components of the egg extract were incubated together for 2.5 h at room temperature. After this incubation period the cytoplasm and membrane were transferred at 1/10 volume into a fresh crude extract (containing mitochondria). Upon addition of an ATP regenerating mix and sperm chromatin, nuclei formed and underwent apoptosis after 60–90 min at room temperature. (B) Reaper does not accelerate the onset of apoptosis when the latent extract (cytoplasm plus membrane) is preincubated for 2.5 h. Recombinant GST-reaper protein or ELB was added to the latent extract at the start of the 2.5 h incubation. This extract was transferred into an execution extract as described above. Samples were withdrawn at 10 min intervals and viewed under fluorescence microscopy. While nuclei appeared stable at 70 min in extracts supplemented with GST-reaper or buffer alone, both extracts displayed the apoptotic phenotype at 80 min. (C) Schematic diagram demonstrating the experimental design. Latent extract was incubated for 0.5, 1 or 1.5 h in the presence of recombinant reaper protein or buffer alone and transferred at 1/10 volume into an execution extract. (D) Reaper reduces the incubation time for development of latent apoptotic activities. (Top) Apoptotic nuclei in crude execution extract after addition of latent extract (cytoplasm plus membrane) supplemented with reaper and incubated for 0.5 or 1.5 h before transfer. (Bottom) Stable nuclei formed in crude execution extracts after addition of latent extract which had been incubated for 0.5 or 1.5 h. Latent extract incubated for 1 h with reaper but not with buffer alone induced apoptosis in the same manner.

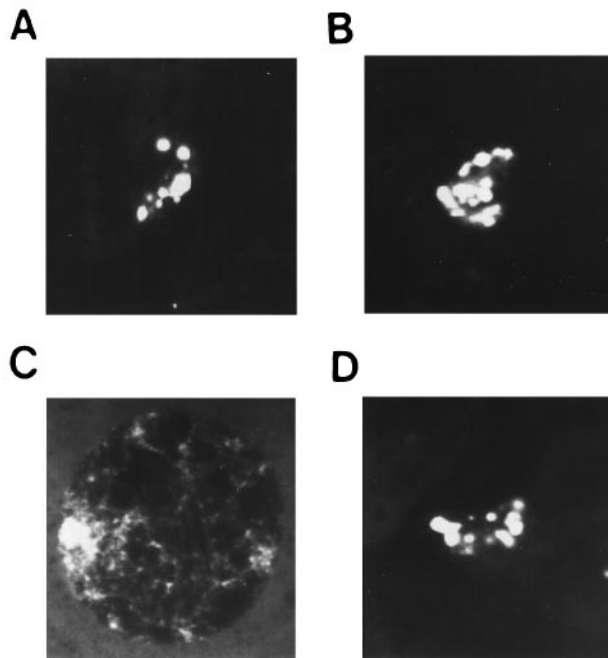


Fig. 5. Reaper circumvents the requirement for crk in induction of apoptosis. (A) The latent extract was immunodepleted with non-immune serum. This depleted extract was recombined with membrane, incubated for 2.5 h at room temperature and transferred into crude execution extract. Apoptotic nuclei were observed at 75 min after transfer. (B) Latent extract immunodepleted with non-immune serum was supplemented with GST-reaper protein and membrane and incubated for 2.5 h. Apoptosis was observed 75 min after transfer into the crude extract. (C) Latent extract immunodepleted with anti-crkl serum was reconstituted with membrane and incubated as described in (A). Apoptosis was not accelerated upon transfer into the crude extract and nuclei remained stable. (D) GST-reaper protein was added to latent extract immunodepleted with anti-crkl serum. This extract was reconstituted with membrane and incubated for 2.5 h. After 75 min in the crude extract apoptotic nuclei were observed.

shows, when mitochondria were incubated with cytosol, addition of GST-reaper caused a significant acceleration of cytochrome c release (~1–2 h in this experiment). (We note that the timing of apoptotic events in mixtures of cytosol and mitochondria vary, depending on the batches of cytosol and mitochondria and the proportions of these components used in the mixture, however, acceleration by reaper is observed reproducibly.) In contrast, when mitochondria were mixed with an ultrafiltrate of cytosol, cytochrome c failed to be released from the mitochondria, whether or not reaper was present. We conclude that reaper cannot by itself induce the mitochondrial efflux of cytochrome c, but instead acts in concert with endogenous cytosolic macromolecules. Light membranes (microsomes), on the other hand, are not required. Interestingly, in the absence of reaper cytochrome c release is consistently slower in crude egg extracts than in cytosol containing mitochondria but no light membranes (compare the controls in the absence of reaper addition in Figures 7 and 8). This is consistent with observations we have recently made suggesting that there is an inhibitor of cytochrome c release present in the light membranes (T.Kuwana and D.D.Newmeyer, unpublished results).

We were interested in determining whether any of the cytosolic factors required for reaper-induced translocation of cytochrome c could be enzymes similar to the known

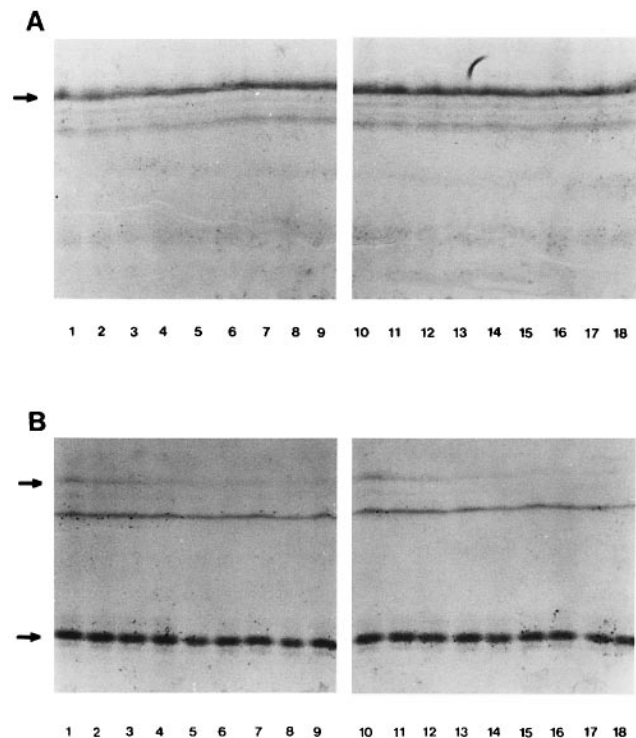


Fig. 6. Reaper does not accelerate cytochrome c-induced caspase activation. ³⁵S-labeled baculovirus p35 protein was added to extracts lacking a subcellular fraction enriched in mitochondria without (lanes 1–9, A and B) or with (lanes 10–18, A and B) added reaper protein. In (B) extracts were supplemented with bovine heart cytochrome c. Starting 20 min after the beginning of room temperature incubation (lanes 1 and 10) samples were withdrawn at 20 min intervals and processed for SDS-PAGE and autoradiography. Although some undegraded p35 can still be seen at the first time point (lanes 1 and 10), a specific p35 cleavage product is present by 20 min in the presence of exogenously added cytochrome c. Arrows indicate the uncleaved protein and a signature apoptotic cleavage product. On several occasions we saw p35 cleavage products appear as early as 5 min after cytochrome c addition.

caspses. If so, one would predict that induction of cytochrome c release by reaper would be delayed or blocked by caspase inhibitors. However, we found that the tetrapeptide aldehyde inhibitors DEVD-CHO and YVAD-CHO, even at relatively high concentration (100 μ M) had no effect on acceleration of cytochrome c translocation promoted by reaper (Figure 9). Similarly, zVAD-FMK, which inhibits a broader spectrum of caspases, produced only a slight delay in reaper-induced cytochrome c release when added at 100 μ M. Thus our data argue against the involvement of known caspases in reaper-mediated release of cytochrome c from mitochondria. However, the slight effect produced by 100 μ M zVAD-FMK could reflect inhibition of an uncharacterized caspase or non-caspase activity.

Bcl-2 protein was previously shown to inhibit release of cytochrome c from mitochondria, thus explaining one way in which Bcl-2 might inhibit apoptosis in a variety of cellular systems. However, in lepidopteran cells reaper-induced apoptosis was reported to be unaffected by Bcl-2 expression (Vucic *et al.*, 1997). We therefore investigated whether reaper-induced caspase activation in *Xenopus* extracts could be inhibited by Bcl-2 and whether Bcl-2 inhibition was dependent on the concentration of reaper.

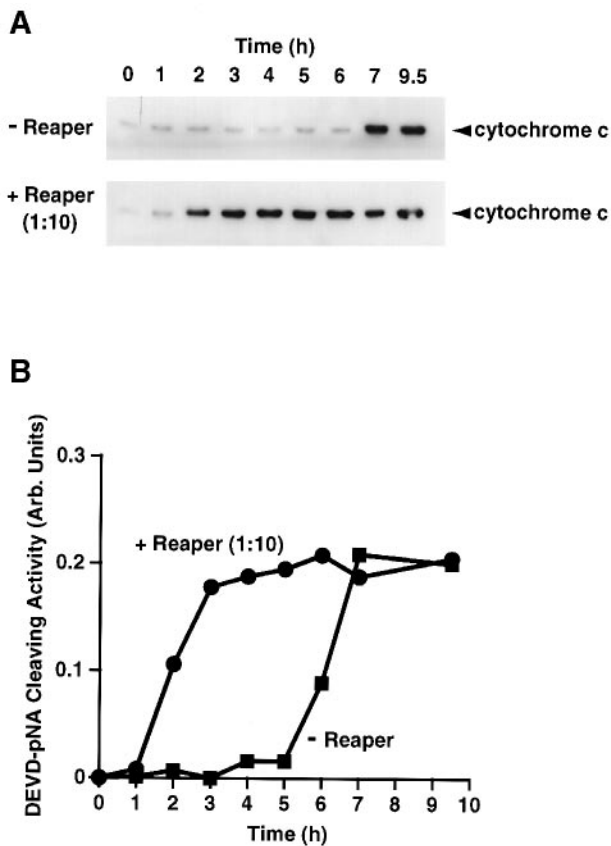


Fig. 7. Reaper accelerates cytochrome c release from mitochondria and DEVDase activation. Recombinant reaper protein or buffer were added to the crude extract at a 1:10 (v/v) dilution (600 ng/ μ l final concentration). At the indicated times 15 μ l samples were removed for measurement of cytochrome c release by Western blotting (A) and 2 μ l were collected for DEVD-pNA cleavage assay (B). The former samples were filtered through centrifugal 0.1 μ m microfilter units to remove particulate components, including mitochondria, prior to analysis. Cytochrome c release (A) appeared at 2 h in the presence of reaper, compared with 7 h in the control extract. In the same extract reaper similarly accelerated DEVD-cleaving activity (B).

To answer these questions, either buffer or increasing concentrations of GST-reaper were added to unfractionated extracts, along with lysates from baculovirus-infected Sf9 cells expressing either Bcl-2 protein or, as a control, β -galactosidase. Figure 10 shows that the timing of caspase activation depended on the levels of reaper present in the extract. When the GST-reaper preparation was diluted to 1:1000 in the extract (starting with a 6 mg/ml stock of bacterially produced GST-reaper) no effect of reaper was seen. However, when the final concentration of reaper was increased the onset of caspase activation occurred progressively earlier. Each time the concentration of GST-reaper was increased 3-fold, DEVDase activation occurred \sim 1 h sooner. At concentrations of reaper (dilutions of 1:300 or 1:100) that were just sufficient to accelerate DEVDase activation measurably, Bcl-2 inhibited these effects markedly. However, at higher reaper concentrations (dilutions of 1:30 or 1:10) inhibition by Bcl-2 was reduced or eliminated.

It was possible that at high concentrations of reaper the mitochondrial events activated by reaper occurred before Bcl-2 was able to integrate completely into the outer mitochondrial membrane. To address this possibility, we

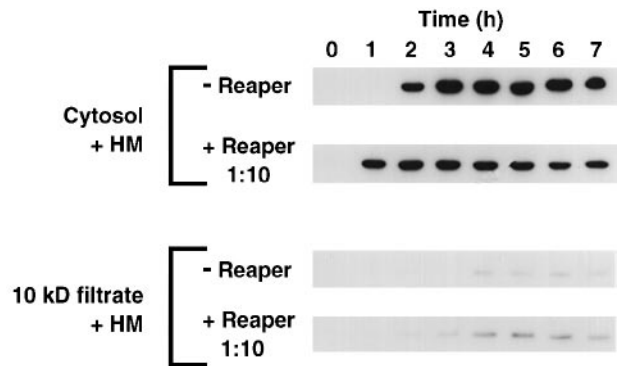


Fig. 8. Reaper requires the cooperation of cytosolic macromolecules to accelerate cytochrome c release from mitochondria. Reaper or XB buffer was added at a 1:10 (v/v) dilution to a reconstituted system consisting of 170 μ l cytosol or cytosol ultrafiltrate (prepared using a Millipore centrifugal microfiltration unit with a 10 kDa cut-off membrane) and 14 μ l heavy membrane (HM) preparation. At each time point 15 μ l of the mixture were filtered through a 0.1 μ m centrifugal microfiltration unit for Western blotting with a monoclonal antibody to cytochrome c (PharMingen). Note that in the presence of cytosol cytochrome c release from mitochondria reached a maximum at 3 h; when reaper was added, cytochrome c release was complete in 1 h. When mitochondria were incubated in the cytosol ultrafiltrate lacking macromolecules $>$ 10 kDa there was no significant cytochrome c release in either the presence or the absence of reaper.

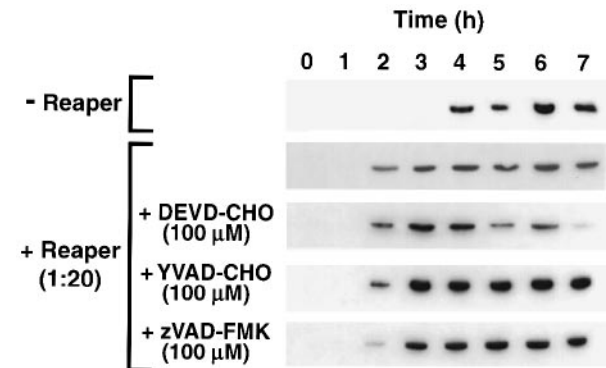


Fig. 9. Known caspase activities are not involved in reaper-induced acceleration of cytochrome c translocation. Reaper protein was added to *Xenopus* egg extract at a 1:20 dilution, in either the absence or the presence of the caspase inhibitors DEVD-CHO, YVAD-CHO and zVAD-FMK (each at 100 μ M). After incubation for the indicated times release of cytochrome c into the soluble fraction was analyzed as in Figure 8.

preincubated extracts for 1 h in the presence of Bcl-2 prior to addition of reaper, a time which allows near maximal association of Bcl-2 with mitochondria (R.M.Kluck and D.D.Newmeyer, unpublished results). Still, we found that Bcl-2 was unable to inhibit the effects of high reaper concentrations (not shown). Thus the failure of Bcl-2 to reverse the effects of high levels of reaper was not simply because Bcl-2 lacked sufficient time to integrate into the mitochondrial membrane. We conclude that while Bcl-2 can antagonize the effects of reaper on mitochondria, high concentrations of reaper can overcome the Bcl-2 block.

Discussion

The results presented here demonstrate that reaper can act in a vertebrate cellular environment, activating the

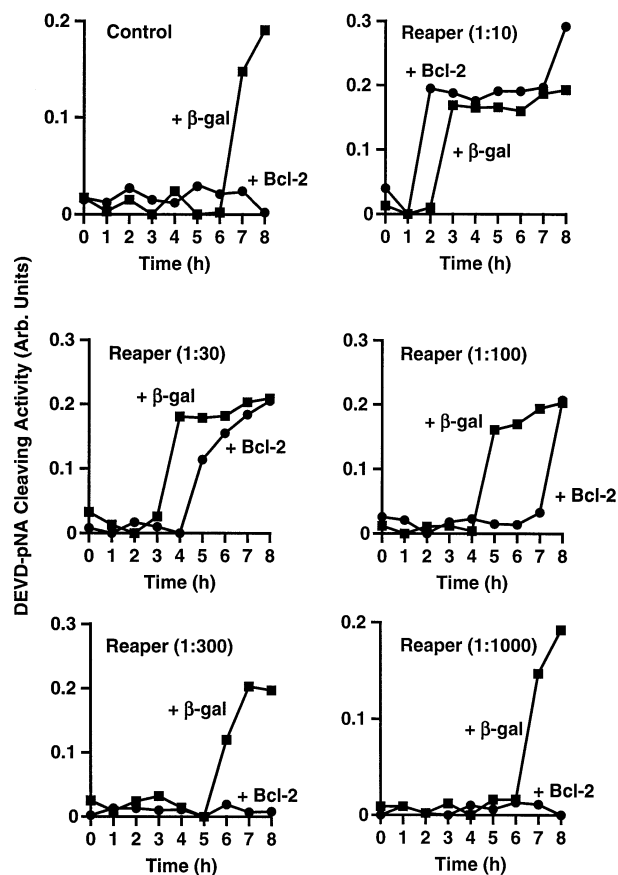


Fig. 10. Bcl-2 antagonizes reaper-induced acceleration of caspase activation, but this inhibition is overcome by high concentrations of reaper. The indicated amounts of reaper were added to a *Xenopus* egg extract in the presence of recombinant baculovirus-expressed Bcl-2 (1:50 v/v) or, as a control, recombinant baculovirus-expressed β -galactosidase (β -gal; 1:50 v/v). A 2 μ l sample from each time point was subjected to DEVD cleavage assay. Note that Bcl-2 inhibited DEVDase activation when reaper was added at dilutions of at least 1:100.

endogenous *Xenopus* death machinery. This clearly indicates that the targets of reaper are not unique to *Drosophila* and are well conserved across a large evolutionary distance. Although a vertebrate reaper molecule has not yet been found, the data presented here strongly suggest that a reaper-like pathway exists in vertebrates.

Reaper and cytochrome c in apoptotic signaling

Addition of reaper to otherwise non-apoptotic egg extracts triggered rapid apoptosis, a finding consistent with the action of reaper in *Drosophila* cells, where rapid death ensues upon transcriptional induction of reaper. Given the short time required for reaper to induce apoptosis in the *Xenopus* egg extract, it is likely that reaper circumvents or accelerates the upstream signaling events important for naturally occurring apoptosis in egg extracts. We have shown that one signaling molecule normally required for apoptosis in egg extracts, the crk proto-oncoprotein, is not essential for reaper-induced apoptosis. This suggests either that reaper acts in a different pathway from crk in apoptotic induction or that reaper accesses the same signaling pathway at a point downstream of crk.

Previous studies have shown that the Bcl-2 protein inhibits caspase activation, and therefore apoptotic cell

death, through blocking release of cytochrome c from mitochondria (Kluck *et al.*, 1997a; Yang *et al.*, 1997). As reaper was found to induce or accelerate translocation of cytochrome c (Figure 7), it was important to determine if Bcl-2 could block reaper function. We found that Bcl-2 did indeed antagonize the ability of reaper to trigger caspase activation, at least at lower concentrations of reaper. That high amounts of reaper overcome the inhibition by Bcl-2 in our experiments and that reaper-induced apoptosis was unaffected by Bcl-2 expression in insect cells (Vucic *et al.*, 1997) may simply have been a matter of stoichiometry. We have also observed (T.Kuwana and D.D.Newmeyer, unpublished results) that Bcl-2 can inhibit reaper-induced apoptosis in reconstituted extracts consisting only of cytosol and mitochondria; the effect of increasing the density of Bcl-2 on mitochondrial membranes is now being investigated. The observation that microsomal membranes are dispensable might also imply that Bcl-2 can inhibit reaper function solely through interactions with mitochondria. However, we cannot exclude the possibility that Bcl-2, in some instances, acts on endoplasmic reticulum membranes to inhibit the effects of reaper. In addition, since a *C.elegans* homolog of Bcl-2, ced-9, appears to act, at least in part, through disruption of interactions between a caspase (ced-3) and its regulator (ced-4), it is possible that Bcl-2 may antagonize reaper-induced apoptosis in part through modulation of caspase-interacting proteins (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997).

The data presented in this report suggest that the critical pro-apoptotic function of reaper in the *Xenopus* system may be to cause cytochrome c efflux from mitochondria. However, it is still unclear whether the mechanism by which reaper activates cytochrome c release is the same one used by endogenous *Xenopus* factors in the absence of reaper. Presently these endogenous protein factors are being purified (T.Kuwana and D.D.Newmeyer, unpublished results). It will be important to determine whether they are sufficient, in cooperation with GST-reaper, to cause cytochrome c translocation *in vitro* or whether distinct cytosolic factors are required.

In this regard, we have recently purified two potential effectors of reaper function which are stably associated with reaper in the egg extract (K.Thress and S.Kornbluth, unpublished observations).

Reaper and the death domain

An intriguing homology has been noted between reaper and a region of the intracellular domain of cell surface receptors which trigger cell death upon ligand binding (e.g. the 'death domains' of Fas and the TNF receptor) (Golstein *et al.*, 1995). Recent discoveries have demonstrated that receptors such as Fas and the TNF receptor engage apoptotic proteases directly through adaptor proteins which interact with both the death domain of the receptor and caspase 8 (FLICE/MACH1) (Boldin *et al.*, 1996; Muzio *et al.*, 1996). We have found that activated FLICE, unlike reaper, can induce apoptosis rapidly in extracts lacking mitochondria (Kuwana *et al.*, unpublished observations). This makes the hypothesis that reaper serves as a direct cytoplasmic activator of FLICE or related proteases unlikely and is consistent with the observations: (i) that apoptosis induced in SF-21 cells by reaper and

FADD (a protein which links FLICE and Fas) exhibit distinct characteristics (Vucic *et al.*, 1997); (ii) that mutations in reaper which parallel inactivating mutations in the TNF receptor death domain do not impair reaper function (Chen *et al.*, 1996).

A vertebrate reaper homolog?

Since there is limited homology between reaper and other known proteins and reaper has been isolated from only one species, molecular cloning techniques have not yet been successful in isolating a vertebrate reaper homolog. Our data demonstrate that reaper-responsive factors are present in *Xenopus* eggs, but this does not prove that a true vertebrate reaper protein exists. Indeed, since transcriptional induction of reaper directly precedes apoptosis during *Drosophila* development, it is unlikely that endogenous reaper protein is present in our non-apoptotic egg extracts. It is possible, however, that a reaper-like protein exists in *Xenopus* egg extracts but, unlike *Drosophila* reaper, it must be activated post-translationally. Since *Drosophila* reaper protein has been shown to oligomerize, it may be productive to search for a *Xenopus* protein appearing later in embryogenesis which forms hetero-oligomers with the *Drosophila* reaper protein. Alternatively, factors isolated from the egg extract through interaction with *Drosophila* reaper may be effective tools to isolate a *Xenopus* reaper-like protein from extracts of *Xenopus* embryos. Regardless of whether an endogenous reaper molecule exists in vertebrates, the fact that *Drosophila* reaper can engage the apoptotic machinery in *Xenopus* suggests that apoptotic signaling elements downstream of reaper are evolutionarily conserved and that *Xenopus* egg extracts will provide an excellent system with which to unravel both general and reaper-specific apoptotic signaling pathways.

Materials and methods

Preparation of GST-reaper fusion protein

Drosophila reaper was PCR amplified using the primers 5'-CCA ACA ACC ATG GCA GTG GC-3' and 5'-TTA CTC GAA GCT TCA TTG GCA TGG-3', using templates kindly provided by Drs Hermann Steller and Robin Wharton, and cloned into the expression vector Gex KG, a derivative of Gex 2T (Pharmacia) containing additional polylinker sites and a poly(glycine) insert. The bacterial strain Topp 1 (Stratagene) was transformed with this construct and recombinant protein was produced according to previously described methods (Evans *et al.*, 1997), except that 10% glycerol was included in the glutathione elution buffer. Control GST protein was expressed from the Topp1 strain as well and prepared in a manner identical to that used for GST-reaper preparation.

Preparation of *Xenopus* egg extracts

To induce egg laying mature female frogs were injected with 100 U pregnant mare serum gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (US Biochemical). Fourteen to twenty hours after injection with hCG eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine, pH 8.0, washed three times in modified Ringer solution (MMR) (1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 25 mM CaCl₂, 5 mM HEPES, pH 7.8, 0.8 mM EDTA) and then washed in egg lysis buffer (ELB) (250 mM sucrose, 2.5 mM MgCl₂, 1.0 mM DTT, 50 mM KCl, 10 mM HEPES, pH 7.7). Eggs were packed by low speed centrifugation at 400 g. Following addition of aprotinin and leupeptin (final concentration 5 µg/ml), cytochalasin B (final concentration 5 µg/ml) and cycloheximide (final concentration 50 µg/ml), eggs were lysed by centrifugation at 10 000 g for 15 min. Recombinant proteins added to extracts were

diluted in XB buffer (50 mM sucrose, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM K-HEPES, pH 7.7)

Preparation of ³⁵S-labeled caspase substrates

cDNA clones encoding caspases 1, 3 and 7 (generous gifts of Dr Vishva Dixit) and baculovirus p35 (kindly provided by Dr Lois Miller) were used to produce ³⁵S-labeled protein by coupled *in vitro* transcription/translation (TNT kit; Promega).

Cytochrome c addition and substrate cleavage assays

In some experiments recombinant GST-reaper protein, bovine heart cytochrome c (Sigma) or equine heart cytochrome c (Sigma) (final concentration 2 ng/µl) and/or ³⁵S-labeled caspase substrates were added directly to this unfractionated extract which had been supplemented with sperm chromatin (1000 nuclei/µl) and an ATP regenerating system (150 mM creatine kinase, 20 mM ATP, 2 mM EGTA and 20 mM MgCl₂). Samples were withdrawn at regular intervals during room temperature incubation and visualized for morphological changes characteristic of apoptosis by fluorescence microscopy following staining with Hoechst 33258 and formaldehyde fixation. In substrate cleavage assays *in vitro* translated ³⁵S-labeled caspase substrates were added at a 1:10 dilution into extracts and 10 µl samples were withdrawn and diluted with 2× sample buffer. The cleavage products were resolved by SDS-PAGE, incubated for 30 min in 1 M sodium salicylate at room temperature, dried and exposed to film for 2–6 days.

Extract fractionation

To remove mitochondrial components and separate the extract into cytoplasmic and membrane components, crude extract was centrifuged at 55 000 r.p.m. (250 000 g) in a Beckman TLS-55 rotor for the TL-100 centrifuge. The cytosolic fraction was removed and recentrifuged at 55 000 r.p.m. for an additional 25 min. Aliquots were frozen in liquid nitrogen and stored at –80°C. The membrane fraction was diluted into 1.5 ml ELB and pelleted through a 0.5 M sucrose cushion for 20 min at 20 000 r.p.m. The isolated membrane was frozen and stored in the same manner as for the cytoplasm described above. The frozen membrane and cytosolic fractions were recombined by diluting the membrane 1:10 into the cytoplasm in the presence of an ATP regenerating system (final concentration 2 mM ATP, 0.05 mg/ml creatine kinase and 20 mM phosphocreatine). Visualization of nuclei and caspase substrate cleavage assays was performed as described above.

Immunodepletion assays

Protein A-Sepharose beads (Sigma) were washed in ELB and preincubated with 10 mg/ml bovine serum albumin (Sigma) in ELB for 20 min at 4°C. The beads were washed twice more with ELB and 10 µl Sepharose beads were incubated with 25 µl non-immune or anti-crck antiserum at 4°C for 45 min. The beads were again washed with ELB and then incubated with 100 µl cytoplasmic fraction. After 1 h at 4°C the antibody-bead complex was pelleted. The supernatant was transferred to a fresh microfuge tube, recombined (10:1) with membrane from frozen stock and supplemented with an ATP regenerating system. This depleted extract was incubated for 2.5 h at room temperature, diluted 1:10 into the crude extract and assayed for the ability to induce apoptotic nuclear fragmentation.

Cytochrome c release assays

For assays in crude extracts the extracts were supplemented with an ATP regenerating system (10 mM phosphocreatine, 2 mM ATP and 150 mg/ml creatine phosphokinase). At various time points cytosolic cytochrome c content was analyzed after filtering the supernatant through a 0.1 µm ultrafree-MC filter (Millipore) to remove particulate components and mitochondria. Aliquots of 7 µl cytosolic protein (~210 µg) were then separated by SDS-PAGE, immunoblotted and probed with an anti-cytochrome c monoclonal antibody (Pharmingen), horseradish peroxidase-linked anti-mouse sera and ECL chemiluminescence detection reagent (Amersham). In assays containing purified cytosol and mitochondria the heavy membrane fraction containing mitochondria (14 µl) was mixed with cytosol (170 µl) and an ATP regenerating cocktail. At each time point 15 µl of the mixture were filtered, as above, and immunoblotted with the monoclonal antibody directed against cytochrome c.

DEVDase assays

To measure caspase activity, 2 µl of each sample was incubated with 40 µl fluorogenic substrate DEVD-pNA (Biomol Research Laboratories Inc.) in ELB. After 15 min incubation at 22°C the reaction was stopped by addition of 1% sodium acetate trihydrate in 175 mM acetic acid and

cooling on ice. Fluorescence was measured at 405 nm with an Aminco Bowman 8000C fluorimeter.

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