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Scythe: a novel reaper-binding apoptotic regulator

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Reaper is a central regulator of apoptosis in *Drosophila melanogaster.* With no obvious catalytic activity or homology to other known apoptotic regulators, reaper's mechanism of action has been obscure. We recently reported that recombinant *Drosophila* reaper protein induced rapid mitochondrial cytochrome *c* release, caspase activation and apoptotic nuclear fragmentation in extracts of *Xenopus* eggs. We now report the purification of a 150 kDa reaper-interacting protein from *Xenopus* egg extracts, which we have named Scythe. Scythe is highly conserved among vertebrates and contains a ubiquitin-like domain near its N-terminus. Immunodepletion of Scythe from extracts completely prevented reaper-induced apoptosis without affecting apoptosis triggered by activated caspases. Moreover, a truncated variant of Scythe lacking the N-terminal domain induced apoptosis even in the absence of reaper. These data suggest that Scythe is a novel apoptotic regulator that is an essential component in the pathway of reaper-induced apoptosis.

Keywords: apoptosis/reaper/Scythe/*Xenopus*

Introduction

Apoptosis is a form of cell death which eliminates superfluous or damaged cells without disturbing overall tissue architecture. Apoptotic elimination of cells is a common feature of metazoan development; in many cell lineages, a significant proportion of the cells initially generated are removed by apoptosis before embryonic development is complete (Ellis *et al*., 1991; Steller, 1995). Moreover, in the adult organism, apoptosis contributes to tissue homeostasis, immune function and prevention of a host of pathologies.

Apoptotic cell death generally involves activation of a family of proteases known as caspases (Chinnaiyan and Dixit, 1996). These aspartate-directed cysteine proteases are synthesized as inactive zymogens. Once activated, they are thought to undermine the structural integrity of the cell through cleavage of key structural protein substrates. Several additional modulators of the apoptotic process have been identified, including pro- and anti-apoptotic bcl-2 family members, and proteins interacting directly with caspases or with the zymogenic pro-caspases (e.g. FADD, TRADD, Apaf-1, IAPs: Nunez and Clarke, 1994; Reed, 1994; Chinnaiyan *et al*., 1995; Hsu *et al*., 1995; Deveraux *et al*., 1997; Seshagiri and Miller, 1997; Zou *et al*., 1997).

The signaling events which favor activation of particular apoptotic regulators are currently a focus of intense investigation. In several systems, transmission of a pro-apoptotic signal results in release of cytochrome *c* from the intermembrane space of mitochondria to the cytoplasm (Liu *et al*., 1996; Kluck *et al*., 1997a). Cytoplasmic cytochrome *c* then serves as a co-factor for caspase activation, leading ultimately to cell death (Zou *et al*., 1997). Bcl-2 family members can prevent both cytochrome *c* release (Kluck *et al*., 1997b; Yang *et al*., 1997) and subsequent apoptosis (Hu *et al*., 1998; Rosse *et al*., 1998) in different contexts. Thus, the apoptotic process is vulnerable to regulation at many levels.

In a screen to identify novel apoptotic regulators in *Drosophila*, White *et al*. (1994) identified a 65 amino acid protein which they named reaper. Transcriptional induction of reaper consistently precedes the onset of programmed cell death in flies, and deletion of reaper prevents all programmed cell deaths. Furthermore, ectopic expression of reaper in lepidopteran cells promotes rapid apoptosis (Pronk *et al*., 1996; White *et al*., 1996). These findings established reaper as a key regulator of apoptosis in flies, and genetic data places reaper upstream of caspase activation, although the molecular intermediates between reaper and caspase activation have not been elucidated.

To date, no reaper homologs have been discovered in vertebrate species. However, we recently reported that recombinant *Drosophila* reaper protein induces rapid apoptosis upon addition to cell-free extracts prepared from *Xenopus* eggs (Evans *et al*., 1997). Addition of reaper triggered many hallmark events of apoptosis including mitochondrial cytochrome *c* release, caspase activation, nuclear fragmentation and the characteristic DNA 'laddering' seen in apoptotic cells of diverse origin. Furthermore, at high stoichiometric ratios of bcl-2 to reaper, these processes were inhibitable (Evans *et al*., 1997). These findings suggested that reaper-responsive pathways were conserved between arthropods and vertebrates.

Reaper-induced mitochondrial cytochrome *c* release required the presence of cytosol, suggesting that intermediary factors acted between reaper and the mitochondria. In order to identify such factors, we have purified proteins from *Xenopus* egg extracts that interact physically with reaper. We report here a reaper-interacting molecule, Scythe, which is required for both mitochondrial cytochrome *c* release and phenotypic apoptosis in response to reaper. Moreover, we show that a C-terminal fragment of Scythe can act as an independent inducer of apoptosis. Collectively, these data establish Scythe as a critical mediator of reaper-induced apoptosis.

Results

Identification of a reaper-interacting protein

Since reaper is a small protein without obvious catalytic activity, we hypothesized that it might act through direct interaction with downstream apoptotic effectors. In order to isolate such reaper interactors, we used GST–reaper protein linked to glutathione–Sepharose as a 'bait' to retrieve interacting proteins from *Xenopus* egg extracts. After incubation in egg extracts, these GST–reaper beads (or the control bait, GST beads) were pelleted and washed extensively. For preliminary identification, all proteins which remained bound to GST or to GST–reaper were modified chemically using a succinimide ester of biotin and then resolved by SDS–PAGE. After transfer to nitrocellulose, the biotinylated proteins were visualized by staining with horseradish peroxidase (HRP)–streptavidin. As shown in Figure 1A, a prominent doublet of 148/150 kDa interacted specifically with GST–reaper.

We scaled up our purification protocol to obtain Coomassie Blue-stainable levels of the 148/150 kDa proteins for microsequencing. Starting with 700 µg of GST–reaper and 15 ml of *Xenopus* egg extract (40 mg/ml total protein), we obtained ~2–3 pmol of the reaper-binding proteins. After SDS–PAGE, proteins in these bands were subjected to tryptic and Lys-C digestion, and eluted peptides were resolved by HPLC. Mass spectrometric analysis indicated that the proteins present in the closely spaced doublet were very highly related, possibly representing closely related isoforms or post-translationally modified variants of each other. Sequencing of peptides derived from the upper band of the doublet revealed it to be highly related to a previously sequenced human open reading frame (ORF), called BAT3 (HLA-B-associated transcript 3), identified in a chromosomal walk through the HLA-B region of the MHC III locus (DDBJ/EMBL/GenBank accession No. M33519; Spies *et al*., 1989; Banerji *et al*., 1990). We have named the protein encoded by this transcript 'Scythe.' Overall, Scythe is not markedly homologous to any other proteins in the DDBJ/EMBL/GenBank database. However, the N-terminal 80 amino acids bear 37% identity and 54% similarity to the human ubiquitin protein.

Using a cDNA probe encoding the human Scythe protein for low stringency hybridization of a *Xenopus* library, we isolated a candidate *Xenopus* homolog of Scythe (Figure 1B). Sequences from 11 different tryptic and Lys-C peptides, derived from both the lower and upper bands of the 148/150 kDa doublet, were found to be identical to sequences encoded by the *Xenopus* Scythe clone (see Figure 1B). Overall, *Xenopus* Scythe is 57% identical and 62% similar to human Scythe.

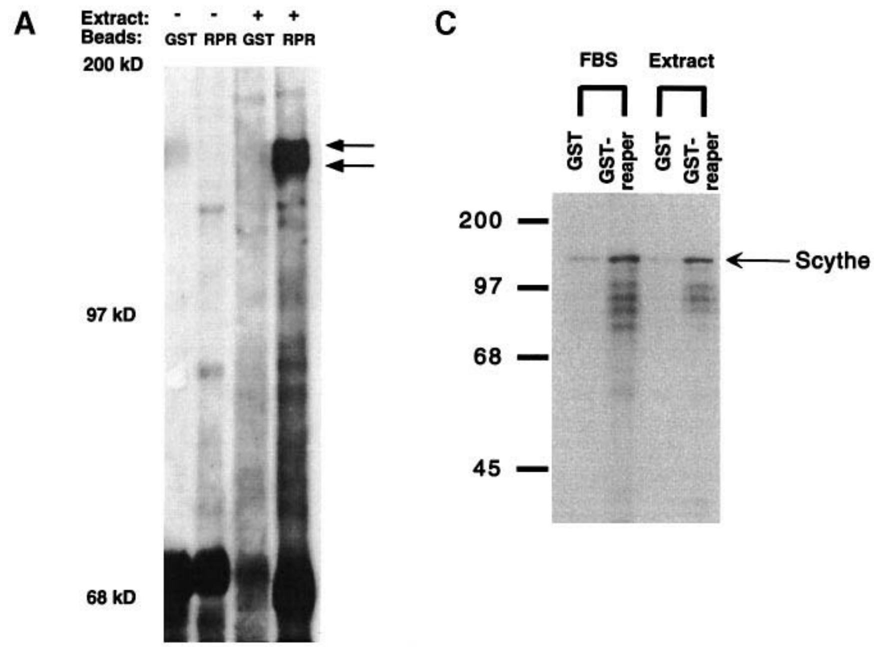
To confirm that Scythe could indeed bind to reaper, we transcribed and translated *Xenopus* Scythe *in vitro*, added it to egg extracts, and incubated these extracts with GST beads or GST–reaper beads. The *in vitro* translated radiolabeled Scythe protein bound tightly to the GST–reaper protein, but not to GST (Figure 1C); the reaper–Scythe interaction was maintained even after washing in buffers containing 1 M NaCl (data not shown). Scythe could also bind directly to reaper in the absence of egg extract, although we observed some background binding of Scythe to GST under these conditions (Figure 1C). Taken together, these data suggest that Scythe is a *bona fide* reaper-interacting protein.

The C-terminal 312 amino acids of Scythe can trigger apoptosis

Since reaper induces apoptosis in *Xenopus* egg extracts, we were interested in the possibility that overproduction of Scythe in these extracts might also trigger apoptosis. However, when we added baculovirus-produced full-length Scythe (final concentration, 600 ng/µl) to extracts containing nuclei, the nuclear morphology of synthetic nuclei formed around sperm chromatin templates was unaltered. In addition, exogenous Scythe did not induce detectable caspase activation (data not shown). Given these data, we reasoned that reaper binding might alter the conformation of Scythe, allowing downstream pro-apoptotic effectors to interact with normally inaccessible domains of Scythe. Consistent with this hypothesis, we found that a bacterially expressed protein consisting of GST fused to the 312 C-terminal amino acids of Scythe (ScytheC312) was a potent inducer of apoptosis; upon addition to *Xenopus* egg extracts, 600 ng/µl recombinant ScytheC312 induced apoptotic nuclear fragmentation and DEVDase activation with a time course very similar to that previously reported for reaper-induced apoptosis in these extracts (Figure 2A and B) (Evans *et al*., 1997). The photomicrographs shown are highly representative in that apoptotic nuclear fragmentation was synchronous within a given sample, proceeding to completion within 10 min, even at concentrations of nuclei as high as 1000/µl. A titration of ScytheC312 protein added to the extract is shown in Figure 2C; note that 600 ng/µl Scythe protein is roughly equivalent to the concentration of Scythe protein found endogenously in the extract (data not shown). The specificity of the ScytheC312 effect is highlighted by the fact that further truncation of the C-terminal portion of Scythe to include only the C-terminal 235 amino acids (ScytheC235) led to a loss of apoptotic activity. Moreover, a Scythe fragment derived from the N-terminal 435 amino acids of the protein (ScytheN435) also lacked the ability to induce either morphological apoptosis or caspase activation (Figure 2A and B). Interestingly, *in vitro* translated, ^{35}S-labeled ScytheC312 protein could bind recombinant reaper, while neither ScytheN435 nor ScytheC235 retained this ability (Figure 3). Taken together, these data suggest that either addition of reaper or removal of the N-terminal 824 amino acids can activate the pro-apoptotic activity of Scythe and that the biologically active fragment of Scythe interacts physically with reaper.

Mitochondrial cytochrome c release in response to ScytheC312 requires accessory cytosolic factors

Since reaper requires cooperating cytosolic factors to trigger mitochondrial cytochrome *c* release, we hypothesized that Scythe might be a cytochrome *c*-releasing factor. Indeed, addition of ScytheC312 to crude egg extracts accelerated release of cytochrome *c* from the mitochondria relative to controls (Figure 4A). ScytheC312 was also able to trigger cytochrome *c* release when added to a mixture of isolated cytosol and mitochondria (Figure 4B). Unlike ScytheC312, Sf9-produced full-length Scythe did not induce mitochondrial cytochrome *c* release in either crude extract or isolated cytosol (Figure 4A and B); in



B

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1    MAANEKMEVT VKTLDSQTRT FTVETEISVK DFKAHISSDV GISPEKQRLI YQGRVLQEDK KLKEYNVDGK VIHLVERAPP QTQPSTGGPS TSSSTSPTSS
101  NAAPVPGAPE RNGNSYVMVG TFNLPHVMSG LVRQQRPSVS TVNGNDGSTL DVHINLDQQL PVQSEPRVRL VLAQHILQDI QRILDRLEGQ AVNEQAAEPM
201  DTAESEGEAS SRETLPQTTQ NTDGQSNTTP TSHPSPSEYV EVLQSLSRVE ERLAPFMQRY REILSSATSD AYENQEEREQ SQRIINLVGE SLRLLGNALV
301  AVSDLRCNLS SASPRHLHVV RPMSHYSGPM LLQQAAIPIQ INVGTTVTAT GNGTHAGHMP SDGNAAQPPS TNTSEPQRPN TENQPPSNGE RPASDAPPTS
401  VPHPHPRVIR ITHQTVEPVM MMHMNIQDSA SGGPTTIPPP TAGHGGSAHI HMPGLPPEFM QAISHQITQQ AMAAASGQQI PGFQAPPRFV FTRPAAPSFQ
501  FQPGTATTPP GPGGATTTVP GATVGPAGNA SLAQMISGLV GQLLMHPVVV AQGGSSTSSS TSSSTFTSTS SSASSSSSTD TTSTTTTSST ANPTVSSVPS
601  SQPPPGTDQH LSQLLGSLLG TASSGMSNIT MGSPSITVTV PGMPAFLQGV TDILQATQTV PVSTAPTQSA SQAPPPSSPP PPPAHSSPPP AAAPESLPPE
701  FFTSVVQGVL SSMLGSLSAA DQSGTESIAA FIQRLSGTHN IFQPDAEGPG GFFGDLLTLI CHNFSLVDMV MLLHGHSQPL QNLQPQLRSF FLQEYLHQVD
801  PTPNNIQMAS RNLTNGLEEY IRESFASVTV RDDVDITRTN IEFLQDQFNR ITTHILHCAD STFGQRLLEM CNQSLFEWLA LNLYCLRGDQ NALTSVINER
901  IRRLSLDVSP VLVSWVTSVL SLRLQVLLGQ MPVTEGEIQR HVRRVGDAPQ VPEPSSQEQP METMPVDCQN GAASPVLATH SGGVLFLPPQ SSVPTICPDS
1001 DHPTQEDGGS EQWAASVPPE WVPVIRQDMQ NQRKIKQQPP LSDAYLSGMP AKRRKTMQGE GPHLSLSEAV SRAMKATGAK PESSAECVRR ELDNSEAQGR
1101 YREQLCQDIQ KTLQDNESYS AQRFPNTQRA FRGDP*
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Fig. 1. (**A**) A protein doublet of 148/150 kDa interacts specifically with reaper (RPR) beads. Recombinant GST–reaper protein on glutathione–Sepharose beads (‘RPR beads’) was incubated with *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed twice with egg lysis buffer (ELB), twice with NaBicarb buffer, resuspended in NaBicarb and incubated with a succinimide ester of biotin for 1 h at room temperature. The beads were then pelleted, washed twice with ELB, resuspended in SDS sample buffer and processed for a Western blot using an HRP-linked streptavidin antibody. (**B**) Predicted amino acid sequence of Scythe ORF. Eleven tryptic and Lys-C peptides that were found in the two different Scythe isoforms (148 and 150 kDa) are indicated as follows: single underlining = 150 kDa; double underlining = 148 kDa; dotted underlining = 148 + 150 kDa. (**C**) *In vitro* transcribed/translated Scythe protein interacts with reaper. ^{35}S-Labeled Scythe protein was incubated with GST or GST–reaper beads in the presence of either heat-inactivated FBS or *Xenopus* egg extract for 30 min at room temperature. Beads were then washed three times with ELB, resolved by SDS–PAGE and processed for autoradiography.

several experiments, we observed some suppression of cytochrome *c* release by the full-length Scythe protein. In contrast to the results obtained in the presence of cytosol, ScytheC312 did not promote cytochrome *c* release from isolated mitochondria in buffer (in the absence of other cytosolic proteins), even in the presence of recombinant reaper (Figure 4C). These data suggest that other accessory cytosolic factors are required to promote cytochrome *c* release.

Scythe is required for reaper-induced apoptosis

To evaluate the role of Scythe in reaper-induced apoptosis, we wished to deplete endogenous Scythe from extracts and determine whether the depleted extracts retained the ability to induce apoptosis in response to reaper. For immunodepletion, we produced several antisera directed against Scythe. Antisera directed against a peptide consisting of the 40 C-terminal amino acids of Scythe (anti-peptide sera) and antisera directed against ScytheC312 both recognized a 150 kDa doublet on immunoblots of *Xenopus* egg extracts (data not shown). Using the anti-ScytheC312 sera coupled to protein A–Sepharose, we performed three successive rounds of immunoprecipitation of Scythe from aliquots of egg extract. As shown in Figure 5A, these extracts were fully depleted of Scythe, as indicated by Western blotting with the anti-peptide antisera (this gel was not of sufficient resolution to separate the doublet). Similar depletions with pre-immune sera did not detectably remove any Scythe protein from the extract. Depletion of Scythe prevented reaper-induced DEVDase

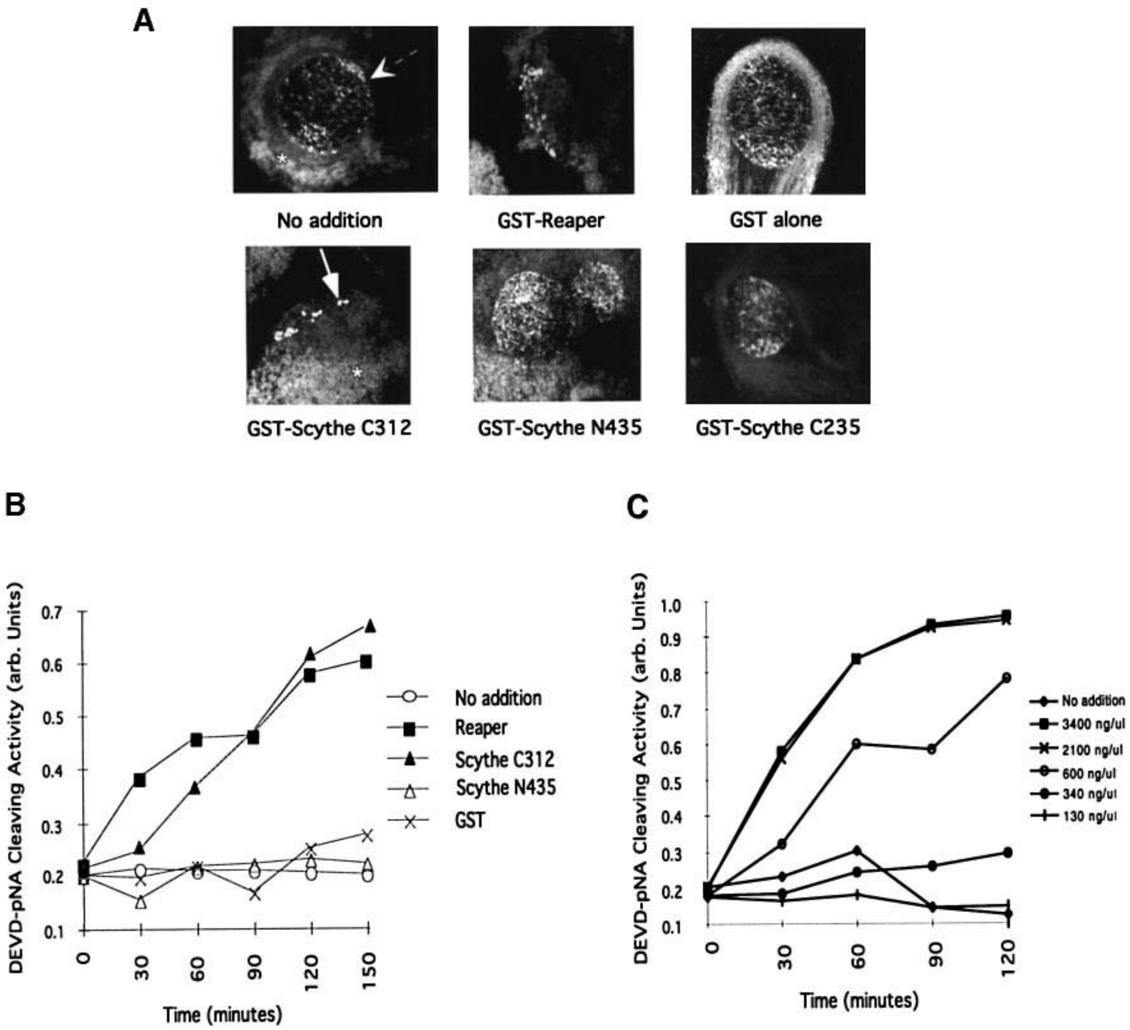

Fig. 2. ScytheC312 induces morphological characteristics of apoptosis and DEVDase activation. The indicated GST fusion proteins (final concentration 600 ng/μl) or an equivalent amount of GST protein alone were added to *Xenopus* egg extract in the presence of sperm chromatin to form synthetic nuclei in the extracts (1000 nuclei/μl) and an ATP regeneration system. (**A**) Photomicrographs of representative nuclei upon staining with the DNA intercalating dye, Hoescht 33258, 90 min after protein addition: dotted arrow = uncondensed interphase chromatin contained within an intact nuclear envelope, solid arrow = condensed, apoptotically fragmented chromatin, * = background staining of membranes present in the extract. (**B**) At the indicated times, 2 μl of extract were collected for a DEVD-pNA cleavage assay. (**C**) The indicated amounts of ScytheC312 were added to extracts and, at the indicated times, 2 μl of extract were collected for a DEVD-pNA cleavage assay.

activation, reaper-induced mitochondrial cytochrome *c* release (Figure 5B and C) and reaper-induced apoptotic nuclear fragmentation (data not shown). However, DEVDase activity was still induced in depleted extracts upon addition of recombinant caspase 8, at concentrations of caspase 8 which exhibited no intrinsic DEVDase activity (Figure 5D). In addition, Scythe-depleted extracts manifested all of the characteristic morphological changes of apoptosis upon addition of caspase 8, again showing that these extracts were still responsive to previously activated caspases (data not shown). Moreover, ScytheC312 was still able to induce apoptosis in extracts immunodepleted of full-length Scythe, indicating that Scythe-responsive factors were still active in the extract (Figure 6). These data demonstrate that Scythe is an essential intermediate in the reaper-induced apoptotic pathway.

ScytheC312-interacting factors are required for reaper-induced apoptosis

The ability of ScytheC312 to induce apoptosis in extracts depleted of full-length Scythe suggests that pro-apoptotic factors engaged by ScytheC312 remain in the extract following Scythe removal. If such factors are *bona fide* signaling components in reaper-induced apoptosis, then their removal should block reaper-induced apoptosis even in the presence of Scythe. To explore this issue, we coupled ScytheC312 or ScytheN435 to Sepharose beads to produce a resin capable of depleting Scythe-interacting factors from extracts. These Scythe 'beads' were incubated in extracts and then removed by gentle centrifugation. The depleted extracts were then incubated with reaper protein. We found that depletion of ScytheC312-interacting factors from the extracts blocked reaper-induced DEVDase activation, though recombinant caspase 8 was still effective

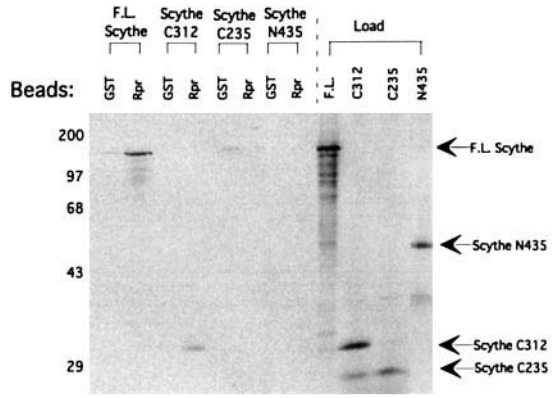

Fig. 3. ScytheC312, but not ScytheN435 or C235, interacts with reaper. ^{35}S-Labeled full-length (F.L.) Scythe, ScytheC312, C235 and N435 proteins were incubated with GST or GST–reaper beads in the presence of *Xenopus* egg extract for 60 min at room temperature. Beads were then washed three times with ELB, resolved by SDS–PAGE and processed for autoradiography. If 100% of the input ^{35}S-labeled proteins were bound to reaper, the intensity of the signal would be equivalent to that seen in the control 'Load' lanes. Note that 100% recovery is unlikely due to competition from endogenous Scythe present in the extract.

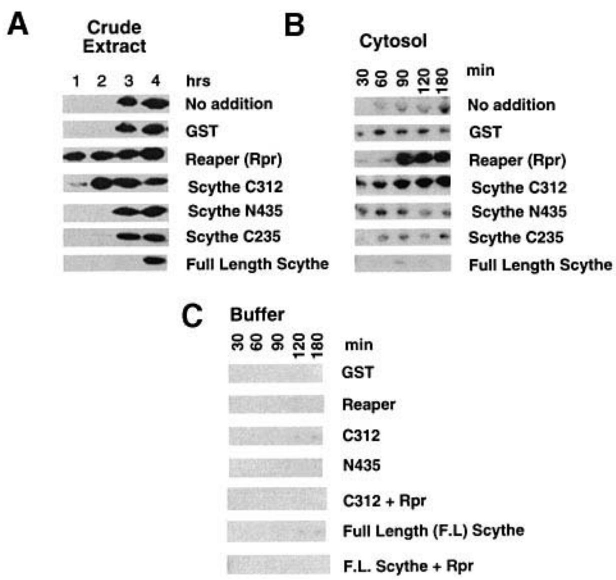

Fig. 4. ScytheC312 accelerates cytochrome *c* release from mitochondria. Recombinant GST protein or the indicated GST fusion proteins were added to either (**A**) crude egg extract, (**B**) mitochondria and egg cytosol, or (**C**) buffer alone. At the indicated times, the samples were filtered through a 0.1 μM microfilter to remove particulate components, including mitochondria. Aliquots (10 μl) of protein filtrate were separated by SDS–PAGE and processed for Western blot with an anti-cytochrome *c* monoclonal antibody. Note that there is extract to extract variability in the absolute timing of cytochrome *c* release. The cytosol in (B) is not from the same batch of *Xenopus* eggs as the crude extract used in (A). Hence, the absolute time course of cytochrome *c* release is slightly different in these two panels.

at inducing DEVDase activity and apoptotic nuclear fragmentation in these extracts (Figure 7 and data not shown). In contrast, depletion of ScytheN435-interacting factors had no effect (Figure 7). These data show that factors that act downstream of ScytheC312 are critical for reaper-induced apoptosis.

Collectively, our data suggest that reaper activation of Scythe promotes a conformational change which can be mimicked by truncation of Scythe. Pro-apoptotic factors do not appear to bind to Scythe prior to reaper addition (since they remain in the extract after immunodepletion of endogenous Scythe), but the conformational change leads to their engagement and, ultimately, to mitochondrial cytochrome *c* release and cell death.

Discussion

This report describes the isolation and characterization of a novel apoptotic regulator, Scythe, which binds tightly to reaper, a central regulator of programmed cell death in *Drosophila*. The ability of recombinant reaper to induce apoptosis in *Xenopus* egg extracts provided the first evidence that reaper could engage the apoptotic machinery in vertebrate cells. Here we show that Scythe is a critical component of this reaper-responsive machinery.

Sequence features of Scythe

The primary structure of the Scythe protein is rather unremarkable. Only the N-terminal 80 amino acids, which are 54% similar to ubiquitin, bear homology to other reported protein sequences. Recently, it was reported that sentrin, a protein of 100 amino acids with 48% similarity to ubiquitin, can interact in the yeast two-hybrid system with the intracellular domains of two potent apoptotic regulators, Fas/APO-/CD95 and the tumor necrosis factor (TNF) receptor (Okura *et al*., 1996). While it remains to be seen whether the N-terminal region of Scythe serves to link Scythe to other apoptotic regulators, we found that depletion of extracts on a resin linked to the N-terminus of Scythe did not disrupt the ability of reaper to induce apoptosis. Moreover, the reaper-binding site on Scythe lies within the C-terminal 312 amino acids of Scythe.

We noted within the primary sequence of Scythe at least one potential caspase cleavage site, DDVD, beginning at amino acid 832. Although Scythe could be cleaved at multiple sites *in vitro*, we were unable to detect any cleavage of the endogenous Scythe protein in reaper-treated egg extracts with high levels of DEVDase activity (data not shown). However, it may be that other factors in the full extract protect Scythe from cleavage.

The Scythe protein bound to reaper migrates as a 148/150 kDa doublet on SDS–polyacrylamide gels. Because *Xenopus* is pseudo-tetraploid, we suspect that these bands represent closely related, though not identical gene products. Indeed, all of the peptide sequences obtained from both protein species were identical to sequences encoded by our cloned Scythe cDNA. *In vitro* translation of this Scythe-encoding cDNA does not produce two forms of the protein, nor do additional forms appear after incubation of the translated product in *Xenopus* egg extracts. This suggests the possibility that the electrophoretic mobility shift may not have been due to post-translational modification.

Truncation of Scythe mimics binding by reaper

We found that recombinant full-length Scythe produced in baculovirus-infected Sf9 cells retained reaper-binding ability (data not shown), but did not induce detectable caspase activation or nuclear fragmentation upon addition to *Xenopus* egg extracts. There are at least two possible interpretations of these data (in addition to the trivial

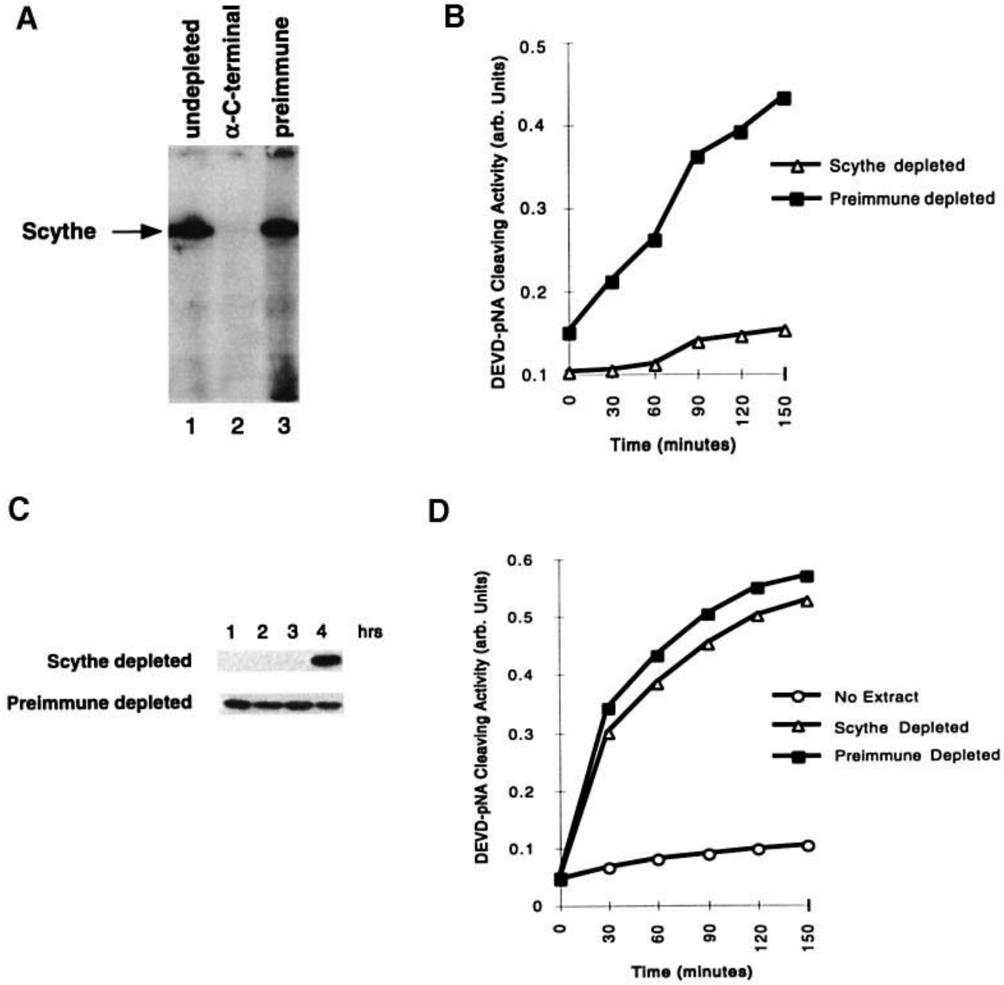

Fig. 5. Depletion of Scythe inhibits reaper-induced DEVDase activation and mitochondrial cytochrome *c* release. (**A**) Scythe was immunodepleted from 100 μl of crude extract using anti-ScytheC312 sera linked to protein A–Sepharose. After three successive rounds of immunodepletion, 10 μl aliquots of extract were resolved by SDS–PAGE and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 40 amino acids of the *Xenopus* Scythe protein. Lane 1, undepleted extract; lane 2, extract depleted with anti-Scythe C312 sera; lane 3, extract depleted with pre-immune sera. (**B**) Recombinant reaper protein (600 ng/μl) was added to either extract depleted of endogenous Scythe protein or extracts similarly treated with pre-immune sera. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (**C**) Recombinant reaper protein (600 ng/μl) was added to either Scythe-depleted or pre-immune-depleted *Xenopus* egg extracts. At the indicated times, the samples were filtered through a 0.1 μM microfilter to remove particulate components, including mitochondria. Aliquots (10 μl) of cytosolic protein were separated by SDS–PAGE and processed for Western blot with an anti-cytochrome *c* monoclonal antibody. (**D**) Recombinant, active caspase 8 (lacking the pro-domain; 400 ng/μl) was added to buffer (no extract), extract depleted of endogenous Scythe protein or extracts similarly treated with pre-immune sera. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity.

possibility that the full-length recombinant protein does not fold properly). Conceivably, insect cells produce an anti-apoptotic factor that associates with Scythe and blocks its action (although a stoichiometric inhibitor would have to be present at very high levels). Alternatively, full-length Scythe may not be pro-apoptotic *per se*. This idea is consistent with the fact that the extracts contain abundant Scythe yet require reaper addition to become rapidly apoptotic. Moreover, the observation that full-length Scythe had some suppressive effects on cytochrome *c* release suggests that full-length Scythe may have some anti-apoptotic activity prior to engagement by reaper. Reaper binding might alter the conformation of Scythe, allowing it to activate downstream pro-apoptotic effectors. Indeed, ScytheC312 (the C-terminal 312 amino acids) induced apoptosis in the absence of reaper. Depletion of ScytheC312-interacting factors prevented reaper-induced apoptosis, strongly supporting the notion that the pro-apoptotic pathway engaged by reaper is identical to that engaged by ScytheC312. We propose that reaper binding allows an otherwise masked Scythe C-terminus to interact with downstream apoptotic regulators. Whether the C-terminus is normally masked by an N-terminal portion of Scythe or by other cellular factors merits future investigation. Alternatively, if Scythe has intrinsic anti-apoptotic activity, then Scythe C312 may be acting as a trans-dominant inhibitor of the endogenous Scythe protein.

Scythe is an indirect inducer of mitochondrial cytochrome c release

The requirement for accessory factors in order for reaper to induce mitochondrial cytochrome *c* release provided the impetus to search for factors which might lie between reaper and the mitochondria. Since immunodepletion of Scythe from cytosol prevented reaper-induced mitochondrial cytochrome *c* release, Scythe is undoubtedly one

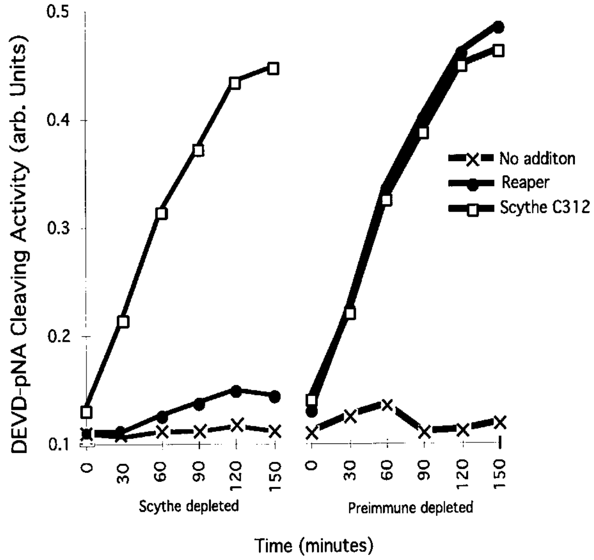

Fig. 6. The C-terminal 312 amino acids of Scythe are capable of inducing DEVDase activation in extracts depleted of endogenous Scythe protein. Recombinant reaper protein (600 ng/µl), an equivalent amount of recombinant ScytheC312 protein, or buffer was added to either Scythe-depleted or pre-immune-depleted extracts and, at the indicated times, 2 µl aliquots of extract were processed for DEVD-pNA cleavage activity.

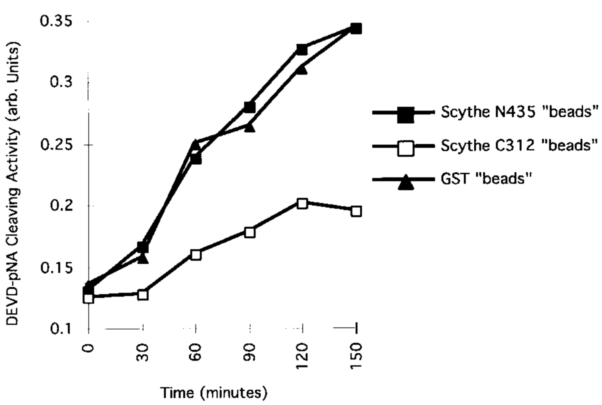

Fig. 7. Depletion of ScytheC312-interacting factors inhibits reaper-induced DEVDase activation. Recombinant reaper was added to *Xenopus* egg extract that had been depleted with the indicated 'beads'. At the indicated times, 2 µl aliquots of extract were collected for a DEVD-pNA cleavage assay.

such factor. However, Scythe is unlikely to be a direct cytochrome *c*-releasing factor, because neither ScytheC312 nor full-length Scythe bound to recombinant reaper could induce cytochrome *c* release in the absence of cytosolic factors. Since depletion of extracts using a ScytheC312 resin abrogated reaper-induced apoptosis, it is likely that at least one of the factors required for reaper-induced cytochrome *c* release can physically interact with the C-terminal region of Scythe.

Is there a vertebrate reaper?

Efforts by our group and others have failed to identify a vertebrate reaper homolog using standard molecular cloning techniques. Given our findings, we hypothesize that *Drosophila* reaper triggers apoptosis in *Xenopus* egg extracts by mimicking an endogenous vertebrate Scythe-activating factor. By analogy to reaper, such a Scythe-activating factor might be transcriptionally induced in response to external stimuli or in response to developmental cues. Therefore, using Scythe as a 'bait' to search for reaper-like factors in extracts from appropriately staged or irradiated embryos may provide a means to isolate reaper-like factors which may not be well conserved at the primary sequence level. It will be equally interesting to determine whether there are Scythe-related proteins acting downstream of reaper in *Drosophila*. It is theoretically possible that reaper accesses an apoptotic pathway in *Xenopus* egg extracts which is distinct from that used in flies.

Using recombinant reaper, we have uncovered a novel component of a reaper-responsive apoptotic signaling pathway which also has the ability, upon truncation, to trigger apoptosis independently. Taken together, our data suggest that Scythe is a critical link between reaper and downstream factors required for mitochondrial cytochrome *c* release. We anticipate that Scythe will provide a foothold not only into the isolation of vertebrate reaper-like factors, but also into the pathway leading from reaper to the mitochondria and apoptotic cell death.

Materials and methods

Preparation of Xenopus egg extracts

For induction of egg laying, mature female frogs were injected with 100 U of pregnant mare serum gonadotropin (PMSG) (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (HCG; USB). At 14–20 h after injection with HCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine (pH 7.8), washed three times in modified Ringer's solution (MMR) (1 M NaCl, 20 mM KCl, 10 mM $MgSO_4$, 25 mM $CaCl_2$, 5 mM HEPES pH 7.8, 0.8 mM EDTA) and then washed in egg lysis buffer [ELB; 250 mM sucrose, 2.5 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 50 mM KCl, 10 mM HEPES] pH 7.4. 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. For nuclear formation, extracts were supplemented with demembranated sperm chromatin (1000 nuclei/µl) and an ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP and 50 µg/ml creatine phosphokinase). In some experiments, extracts were treated with recombinant GST–reaper protein, GST–Scythe protein and/or baculovirus-expressed His-tagged full-length Scythe protein (all at a final concentration of 600 ng/µl). For assessment of apoptotic nuclear morphology after Scythe or reaper addition, samples were withdrawn at regular intervals during room temperature incubation and visualized by fluorescence microscopy following staining with Hoechst 33258 and formaldehyde fixation. In assays measuring caspase cleavage of ^{35}S-labeled Scythe, extracts were supplemented with 1/10 volume of rabbit reticulocyte lysate containing ^{35}S-labeled Scythe in the presence or absence of recombinant reaper protein. Samples were then diluted with 2× sample buffer, resolved by SDS–PAGE and processed for autoradiography.

Protein biotinylation

GST or GST–reaper protein coupled to glutathione–Sepharose beads were washed three times with ELB. The GST–protein beads were then blocked by incubation with 10 mg/ml bovine serum albumin (BSA; fraction V) in ELB for 30 min at 4°C. The bead–protein complex was then pelleted and washed twice with ELB. Crude extract was then added at 10 times the volume of beads and rotated at 4°C for 90 min. The beads were then pelleted, washed twice in ELB, twice in sodium bicarbonate buffer (NaBicarb), resuspended in 1 ml of NaBicarb and incubated with 30 µl of biotinylation reagent (Amersham Biotinylation module) for 1 h at room temperature. The beads were again pelleted, washed three times with ELB and diluted with 2× sample buffer. The samples were then resolved by SDS–PAGE, transferred to PVDF immobilon, probed with HRP-linked streptavidin and visualized through an ECL chemiluminescence detection system.

Protein sequencing of Scythe

Starting with 15 ml of crude *Xenopus* extract and ~700 µg of GST–reaper protein as 'bait', ~2–3 pmol of the 148/150 kDa doublet were obtained using the bead-binding protocol outline above. After the proteins were separated on an SDS–PAGE gel, they were electroblotted onto a PVDF membrane (ProBlott, Applied Biosytem) and visualized by staining with 0.1% Coomassie Blue in 50% methanol. After excision of the 148/150 kDa bands from the membrane, the bands were reduced and alkylated with isopropylacetamide followed by digestion in 20 µl of 0.05 M ammonium bicarbonate containing 0.5% Zwitergent 3–16 (Calbiochem) with 0.2 µg of trypsin (Frozen Promega Modified) or Lysine-C (Wako) at 37°C for 17 h as described previously (Krutzsch and Inman, 1993; Lui *et al*., 1996). The solution was then injected directly onto a 0.32×150 mm C18 capillary column. Peptides generated from *in situ* digests were separated on a C18 0.32×100 mm capillary column (LC Packing, Inc.). The HPLC consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 UV detector equipped with a Z-shaped flow cell (LC Packing, Inc.). A 30 cm length of 0.025 mm i.d. glass capillary was connected to the outlet of the Z-shaped cell inside the detector housing to minimize the delay volume. The total delay volume was 0.45 µl which corresponded to a delay of 6 s for a flow rate of 3.5 µl/min. The short delay greatly facilitated hand collections of the HPLC fractions (Henzel and Stults, 1995). Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.08% TFA. The peptides were eluted with a linear gradient of 0–80% B in 60 min, detected at 195 nm and hand collected into 0.5 ml Eppendorf tubes.

An aliquot (0.2 µl) of each of the isolated HPLC fractions was applied to a pre-made spot of matrix (0.5 µl of 20 mg/ml α-cyano-4-hydroxycinammic acid plus 5 mg/ml nitrocellulose in 50% acetone/50% 2-propanol) on the target plate (Shevchenko *et al*., 1996). Ions were formed by matrix-assisted laser desorption/ionization with a 337 nm nitrogen laser. Spectra were acquired with a PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer, operated in liner delayed extraction mode. Subsequently, fragment ions for selected precursor masses were obtained from postsource decay (PSD) experiments (Kaufmann *et al*., 1994). To enhance the ion abundances at low mass, collision gas (air) was introduced to the collision cell during the acquisition of the lower portion of the fragment ion spectrum. Each peptide mass and its associated fragment ion masses was used to search an in-house sequence database with an enhanced version of the FRAGFIT program (Henzel *et al*., 1993). The program was modified to permit potential methionine oxidation and partial proteolytic cleavage. Furthermore, experimentally determined PSD fragment ion masses can be compared with theoretical fragment ions (b and y ions) from the database entries (Clauser *et al*., 1995). The latter approach provides a high degree of searching specificity without the need for spectral interpretation. Peptide fractions were sequenced on a model 494CL PE Applied Biosystems sequencer using 6 mm microcartridges and equipped with an on-line parathyroid (PTH) analyzer (model 140D). Peaks were integrated with Justice innovation software using Nelson Analytical 760 interfaces. Sequence intepretation was perfromed on a DEC Alpha computer (Henzel *et al*., 1987).

cDNA cloning of Scythe

A 535 bp *Sac*I–*Eco*RI fragment of the human BAT3 cDNA (a generous gift from Dr Thomas Spies) was labeled with [α-^{32}P]dCTP using the Random Primed DNA labeling kit (Boehringer Mannheim). This cDNA fragment was then used to screen a λZAP *Xenopus* library (gift from Dr Bruce Mayer) by hybridizing duplicate filters at 37°C overnight. The filters were washed twice with 2× SSC/0.1% SDS at 37°C for 30 min and twice more with 1× SSC/0.1% SDS at 42°C for 30 min. Of the 5×10^5 plaques screened, six positive clones were identified. After rescue, three of these clones were found to be ~3.8 kb in length. These three clones were sequenced and found to represent the full-length *Xenopus* cDNA homologs of BAT3.

Preparation of GST–Scythe protein

Three separate truncations of recombinant *Xenopus* Scythe protein were constructed; the C-terminal 312 amino acids, C-terminal 235 amino acids and N-terminal 435 amino acids. cDNA encoding these truncations were PCR amplified using the following primers: C-terminal 312 amino acids, 5′-gat cgg atc cag ctt tgc ctc cgt tac tgt c-3′ and 5′-gat caa gct ttt agg ggt ccc ccc tga a-3′; C-terminal 235 amino acids, 5′-gat cgg atc cat tcg aag gct ctc tct tga g-3′ and 5′-gat caa gct ttt agg ggt ccc ccc tga a-3′; N-terminal 435 amino acids, 5′-gat cgg atc cat ggc agc taa tgag aaa-3′ and 5′-gat caa gct ttt aag gtc cac cag atg c-3′. PCR fragments were cloned into the expression vector Gex KG, a derivative of Gex 2T (Pharmacia) containing additional polylinker sites and a polyglycine insert, and transformed into the Topp 1 bacterial strain (Stratagene). Recombinant protein was produced as previously described (Evans *et al*., 1997).

Baculovirus production of full-length Scythe protein

Full-length *Xenopus* Scythe protein was produced using the Bac-to-Bac Baculovirus Expression System (Gibco). Briefly, full-length Scythe was PCR amplified, digested with *Nco*I and *Xba*I, and ligated into the pFastBac vector which had been cut previously with the same enzymes. The resulting donor plasmid encoding an N-terminal 6× His tag preceding full-length Scythe was transformed into DH10Bac *Eschericha coli* cells. These *E.coli* cells containing recombinant bacmid were cultured and recombinant bacmid DNA was recovered using a standard miniprep protocol. Sf-9 insect cells were transfected with the bacmid DNA using CellFECTIN reagent (Gibco), incubated for 48 h at 27°C, and the resulting recombinant baculovirus particles were harvested. Subsequently, Sf-9 cells (2×10^6 cells/ml) were infected with baculovirus for 48 h, washed twice in phosphate-buffered saline (PBS) and lysed by dounce homogenization in HBS [10 mM HEPES pH 7.5, 20 mM β-glycerolphosphate, 150 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml each of pepstatin, chymostatin and leupeptin]. The lysate was then centrifuged at 4°C for 10 min at 10 000 r.p.m., and the supernatant was incubated with 1 ml of Ni-NTA agarose (Qiagen) for 30 min at 4°C. The beads were washed in 50 volumes of HBS and eluted with HBS containing 200 mM imidazole in five fractions of 500 µl each.

Cytochrome c release assays

To fractionate the crude egg extract into cytosolic and membranous components, the crude extract was centrifuged further at 55 000 r.p.m. (200 000 *g*) in a Beckman TLS-55 rotor for the TL-100 centrifuge for 1 h. The cytosolic and heavy membrane fractions (enriched in mitochondria) were removed, and the cytosolic fraction was re-centrifuged at 55 000 r.p.m. for an additional 25 min. The mitochondrial fraction was purified further by centrifugation of the heavy membrane through a Percoll gradient consisting of 42, 37, 30 and 25% Percoll in mitochondria isolation buffer (1 M sucrose, 100 mM ADP, 2.5 M KCl, 1 M DTT, 1 M succinate, 1 M HEPES–KOH pH 7.5, 0.5 M EGTA, 1.5 M mannitol) for 25 min at 25 000 r.p.m. with no brake in the TLS-55 rotor. The isolated heavy membrane fraction containing mitochondria was diluted 1:10 into cytosol or ELB containing an ATP-regenerating cocktail (10 mM phosphocreatine, 2 mM ATP and 50 µg/ml creatine phosphokinase). At various time points, the cytochrome *c* content was analyzed after filtering 25 µl of the mixture through a 0.1 µm ultrafree-MC filter (Millipore). Aliquots of 10 µl of cytosolic protein were then separated by SDS–PAGE and immunoblotted with an anti-cytochrome *c* monoclonal antibody (Pharmingen), HRP-linked anti-mouse sera and an ECL chemiluminescence detection system (Amersham).

DEVDase assays

To measure caspase activity, 3 µl of each sample were incubated with 90 µl of assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric substrate Ac-DEVD-pNA (final concentration, 200 µM; Biomol Caspase-3 assay system) at 37°C. At various time points, absorbance was measured at 405 nm in a LabSystems MultiSkan MS microtiter plate reader.

Immunodepletion assays

Protein A–Sepharose beads were washed in ELB and pre-incubated with 10 mg/ml BSA in ELB for 40 min at 4°C. The beads were washed twice more with ELB, and 10 µl of Sepharose beads were incubated with 100 µl of pre-immune or anti-Scythe antisera at 4°C for 70 min. The beads were washed again with ELB and then incubated with 100 µl of either the crude *Xenopus* egg extract or isolated cytosol. After 1 h at 4°C, the antibody–bead complexes were pelleted, the supernatant was transferred to a fresh microfuge tube and the depletion process was repeated, using fresh beads, twice more. This depleted extract was then assayed for the ability to induce apoptotic nuclear fragmentation, cytochrome *c* release or DEVDase activation.

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