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Apoptosis is a program of cellular suicide which results in the removal of damaged or superfluous cells without causing overall changes in tissue architecture. Apoptotic cellular destruction is carried out by a family of proteases known as the caspases. Under many circumstances, apoptotic caspase activation is preceded by release of cytochrome c from the intermembrane space of the mitochondria to the cytosol. Once released, cytochrome c serves as a co-factor in activation of one of the caspases (caspase 9) by a molecule known as Apaf-1.

During this funding period, we have made two key findings with respect to regulation of apoptosis: We have identified a novel Apaf-1/pro-caspase 9 interactor known as AFG3L2. We hypothesize that this protein will modulate activation of caspase 9 by cytochrome c. In addition, we have shown that the oncogenic protein Bcr/abl requires kinase activity to protect cells from apoptotic destruction after mitochondrial cytochrome c release. We have also identified a potential cytochrome c interactor whole binding is regulated by Bcr/abl. Collectively, these results should lead to insights into the mechanism of apoptotic cellular destruction.

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Sally Kronk 7/27/00

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Introduction

(reproduced here from last year's report, as the general background/goals have not changed):

Apoptosis is a program of cellular suicide which eliminates harmful or superfluous cells without damaging neighboring tissue. Research into the biochemistry of apoptosis promises to broadly impact the treatment of cancer. Specifically, the inability of breast cancer cells to undergo appropriate cell death may contribute to both tumor formation and resistance to chemotherapetic and radiologic treatments.

The key cell executioners in the apoptotic program are a family of proteases known as caspases[1]. To some extent apoptotic signaling can be understood as a series of events leading to caspase activation. Caspases are synthesized as pro-enzymes which must be cleaved prior to proteolytic activation. Numerous lines of evidence support the notion that the pro-domains of caspases, removed upon proteolytic activation, serve as binding sites for critical regulatory proteins which determine whether or not activation of the pro-caspases will occur [2-4]. In our research, we aim to utilize an in vitro apoptotic system based on Xenopus egg extracts to explore the role of caspase pro-domain-interacting proteins in regulation of the cell death program. Our goal has been to identify and purify these binding proteins in the hopes of identifying novel apoptotic regulators. Ultimately, we will assess the roles of these regulators in both our in vitro system and in breast cancer cells.

Body of Report:

The second year of our proposed work has focused on **Technical Objective II**, scale up and purification of pro-domain binding proteins of interest. We have also revisited **task I of Technical objective 1**, production of recombinant GST-pro-domain-binding proteins, as detailed below:

Technical objective II was aimed at definitive identification of pro-domain binding proteins of interest through purification/microsequencing (**task 1**) and PCR cloning (**task 2**). In parallel experiments using the pro-caspase 9 interactor, Apaf-1 as a bait in a two-hybrid screen, we serendipitously encountered a clone that fit the criteria for the sort of apoptosome (Apaf-1/pro-caspase 9[5])-binding factors we proposed to identify through **task 1**. Hence, we proceeded to full-length pcr cloning of this factor (**task 2**) and have now confirmed through biochemical pull-downs, its direct interaction with Apaf-1 (Fig. 1, appendix). This novel apoptosome-interacting factor, named AFG3L2 is a member of the AAA class of ATPases[6]. Although this class of proteins has been

implicated in diverse cellular processes, most intriguingly, a member of this family from C. elegans, MAC-1, was isolated as an anti-apoptotic protein associated with the worm homolog of Apaf-1, Ced 4[7]. In the two-hybrid screen, using Apaf-1 as a bait, the last 107 amino acids of AFG3L2, were isolated multiple times. To verify the binding biochemically, this sequence was produced as a fusion protein with Glutathione-S-transferase (GST) and examined for its ability to bind radiolabeled, in vitro translated Apaf-1. As shown in Fig. 1, this fragment bound directly to Apaf-1, but not to GST alone. As proposed in **Technical objective II, task 4**, we then proceeded to clone the full-length AFG3L2 and examine its binding to the Apaf-1 protein and full-length active caspase 9 (lacking the pro-domain). As shown in Fig. 2, Apaf-1, but not active caspase 9, could bind to AFG3L2. Intriguingly, preliminary results have indicted an overexpression of AFG3L2 in several tumor types, including breast cancer. It is possible that AFG3L2 is an apoptotic regulator whose overexpression contributes to carcinogenesis. As defined in our original proposal, functional experiments with this clone will form the basis of experiments outlined for tasks 4-7.

Problems with the GST-prodomain fusions

As outlined in our original grant proposal and last year's report, we have been working with a series of GST-prodomain fusion proteins, isolating potential binding proteins of interest (**Technical objective I**). We have had difficulty in the scale-up and putification of potentially interesting binding partners, largely because the baits do not perform reliably/reproducibly. Therefore, we decided to re-visit task 1 and redesign our pro-domain baits. N-terminal GST-fusions may have proven problemmatic for two reasons: GST protein tends to dimerize, which might interfere with interactions of pro-domains with their binding partners (which can participate in multimerization of the pro-caspases) and pro-domains, which are normally at the extreme N-termini of the caspases might not behave well with an N-terminal tag. Therefore, we redesigned all of our constructs, cloning the pro-domains into IMPACT vectors from New England Biolabs. These produce proteins with a tag at the C-terminus (intein) which can be self-cleaved by addition of DTT. Furthermore, we have engineered in a myc tag at the C-terminal jucntion between the pro-domain and the intein, allowing us to cleanly immunoprecipitate our proteins of interest using anti-myc monoclonal antibodies. We have now successfully produced baits from these proteins and are repeating our binding/scale up experiments to see if we can better obtain microsequencable pro-domain binding partners.

Bcr/abl and the inhibition of apoptosis

As reported last year and described originally in **Technical objective I, task 3**, we found that the oncogenic protein Bcr/abl could prevent apoptosis in our

Xenopus egg extracts after the release of cytochrome c from the mitochondria. Our data firmly indicated that addition of active Bcr/abl could prevent activation of pro-caspase 9 in response to cytochrome c. These results have now been confirmed. However, as of last year's report, we did not have recombinant, kinase inactive Bcr/abl, allowing us to determine whether kinase activity was required for the protective effects of Bcr/abl. We have now produced this mutant and have shown that only kinase active Bcr/abl can prevent apoptosis, as measured by caspase 3 activation in response to cytochrome c addition (Fig. 3). Intriguingly, we have recently observed that cytochrome c linked to agarose can precipitate a protein from egg extracts supplemented with kinase active, but not kinase inactive, Bcr/abl (Fig. 4). This associated protein is a potential mediator of the protective effects of Bcr/abl; thus, its purification and identification will be pursued.

Reaper-induced apoptosis

Also originally described in Technical objective I, task 3, we wished to look at caspase pro-domain interacting proteins in the presence and absence of the potent apoptotic inducer, Reaper. Work published from our laboratory last year demonstrated that Reaper acts upstream of a novel apoptotic regulator, Scythe and that apoptosis proceeds through mitochondrial cytochrome c release and pro-caspase 9 activation[8]. In this past year, we have reported that Scythe sequesters a cytochrome c-releasing factor which is released upon binding of Reaper[9]. A reprint describing this work is appended.

Key research acomplishments

- Identification of AFG3L2 as an Apaf-1 interactor
- Cloning of full-length AFG3L2
- Further characterization of Bcr/abl protection from apoptosis, including identification of a possible cytochrome c-interacting factor modulated by Bcr/abl
- Engineering of new pro-domain fusion constructs

Reportable outcomes: The Scythe work, which grew out of the Reaper characterization in technical objective I has now grown into a distinct project (no overlap) only tangentially related to our original application. Hence, the groundwork laid in the research funded by the IDEA award has led to a novel project recently funded by the NIH. This work is described in full in the appended manuscript.

Conclusions: We have identified a potentially interesting and novel apoptosome interactor . Preliminarily, this gene appears to be overexpressed in breast cancer cells. Since cancers can arise from a failure of apoptosis and chemotherapy is

not effective in cells resistant to apoptosis, we hypothesize that AFG3L2 may be an apoptotic suppressor amplified in tumors. This requires further experimentation to substantiate. We are on our way to understanding how Bcr/abl, an important oncogenic protein in human leukemias, protects cells from apoptosis and will continue this research in the next year. A fuller understanding of mechanisms of apoptosis should allow us to understand how these processes are inappropriately regulated in tumor cells.

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Apaf 1 binds to the C-terminus of AFG3L2

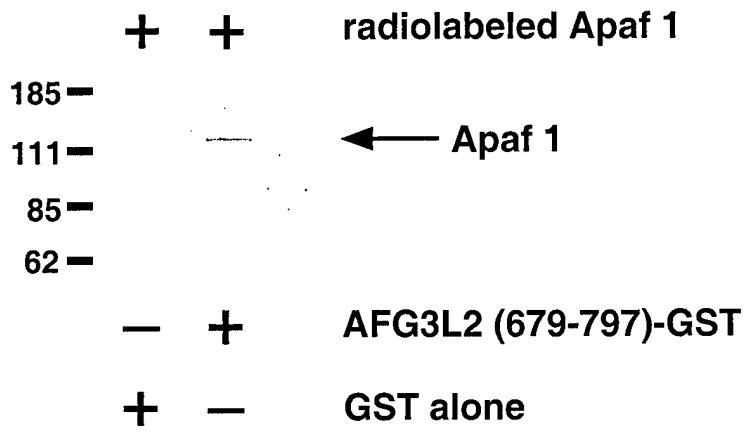

Figure 1

Recombinant, bacterially-produced AFG3L2 (amino acids 679-797) or GST alone were coupled to glutathione sepharose beads and incubated with radiolabeled Apaf 1 in PBST plus 1% casamino acids in for 1 hour at four degrees. The beads were washed and the bound protein visualized with SDS-PAGE and autoradiography.

Full-length AFG3L2 binds to Apaf 1-his, but not to Caspase 9-his

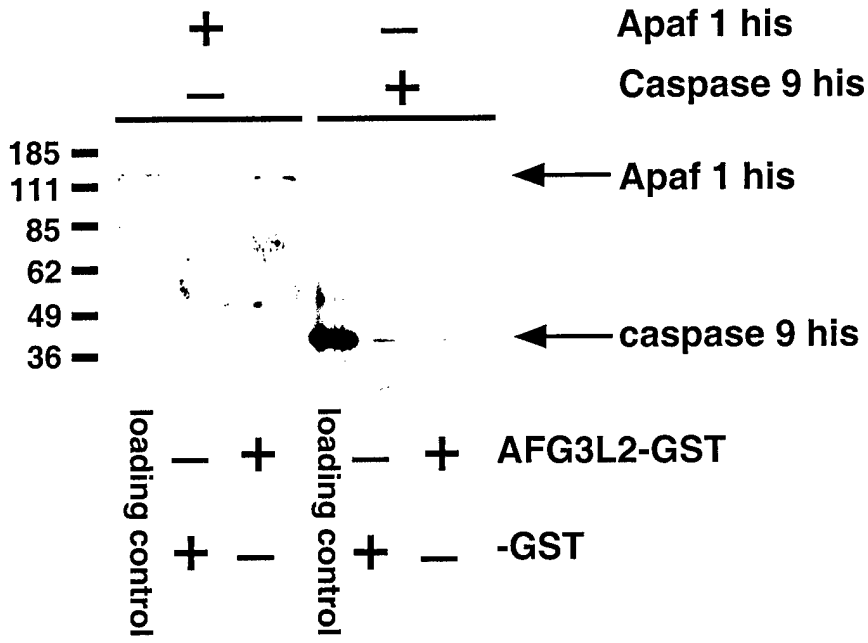

Figure 2

Baculovirus-expressed Apaf 1-his or caspase 9-his were incubated in lysates made from bacteria expressing either full-length AFG3L2-GST or -GST alone for one hour at four degrees celcius. Glutathione sepharose beads were then added to lysates and incubated for 30 minutes at four degrees. The beads were washed and the bound protein visualized by Western blotting with an anti-penta-his antibody.

Kinase activity is required for BCR-Abl-mediated inhibition of cytochrome c-dependent caspase 3 activation

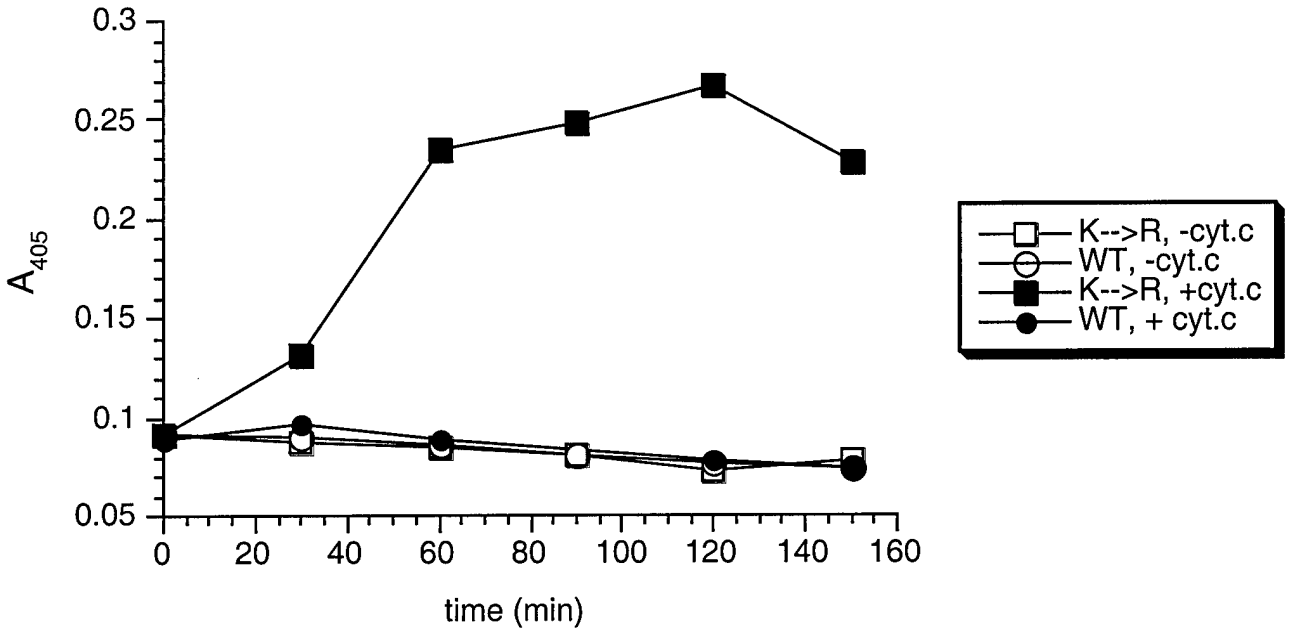

Figure 3

Purified Xenopus cytosol was incubated with biochemically purified wild type BCR-Abl (WT) or kinase dead BCR-Abl (K-->R) for 20 minutes at room temperature. Cytochrome c was then added to 0.9 ng/μl and caspase 3 activity was measured over time using a colorimetric caspase 3 substrate (Biomol).

A Cytochrome C Interacting Protein is Regulated by BCR-Abl

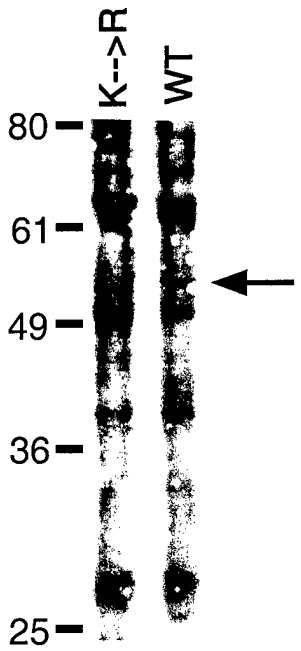

Figure 4

Purified Xenopus cytosol was treated with biochemically purified wild-type BCR-ABl (WT) or kinase dead BCR-Abl (K-->R) for 30 minutes at room temperature, then the cytosol and BCR-ABl were mixed with 15 µl cytochrome c-agarose beads (Sigma) for one hour at room temperature. The beads were washed and biotinylated. The binding proteins were visualized by SDS-PAGE and a horse radish peroxidase/streptavidin reagent (Amersham)

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Reaper-induced dissociation of a Scythe-sequestered cytochrome *c*-releasing activity

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Reaper is a potent apoptotic inducer critical for programmed cell death in the fly *Drosophila melanogaster*. While Reaper homologs from other species have not yet been reported, ectopic expression of Reaper in cells of vertebrate origin can also trigger apoptosis, suggesting that Reaper-responsive pathways are likely to be conserved. We recently reported that Reaper-induced mitochondrial cytochrome *c* release and caspase activation in a cell-free extract of *Xenopus* eggs requires the presence of a 150 kDa Reaper-binding protein, Scythe. We now show that Reaper binding to Scythe causes Scythe to release a sequestered apoptotic inducer. Upon release, the Scythe-sequestered factor(s) is sufficient to induce cytochrome *c* release from purified mitochondria. Moreover, addition of excess Scythe to egg extracts impedes Reaper-induced apoptosis, most likely through rebinding of the released factors. In addition to Reaper, Scythe binds two other *Drosophila* apoptotic regulators: Grim and Hid. Surprisingly, however, the region of Reaper which is detectably homologous to Grim and Hid is dispensable for Scythe binding.
Keywords: apoptosis/cytochrome *c*-releasing activity/Reaper/Scythe

Introduction

Apoptosis is a program of cellular suicide, which leads to the elimination of excess or damaged cells while leaving neighboring cells unperturbed. Apoptosis is critical for organismal homeostasis in the adult and is an integral part of the developmental program in all metazoans (Vaux *et al.*, 1994; Steller, 1995).

With a few exceptions, cellular death by apoptosis is executed by a family of aspartate-directed cysteine proteases known as the caspases (Chinnaiyan and Dixit, 1996). These enzymes, responsible for cleaving a battery of proteins during apoptotic cellular execution, are synthesized as inactive zymogens. Activation of pro-caspases can be triggered by binding of regulatory proteins to their pro-domains (most likely inducing pro-caspase oligomerization) or through cleavage *in trans* by already activated caspases (Muzio *et al.*, 1998; Yang *et al.*, 1998). In many instances, apoptotic pathways leading to caspase activation proceed via signaling-induced release of cytochrome *c* from the intermembrane space of the mitochondria to the cytosol. In the cytosol, cytochrome *c* serves as a cofactor, with the protein Apaf-1, to activate pro-caspase 9. Active caspase 9 then activates other caspases, most notably one of the prominent effector caspases, caspase 3 (Liu *et al.*, 1996; Kluck *et al.*, 1997; Zou *et al.*, 1997).

The activation of caspases and, ultimately, apoptosis can be blocked by members of several different protein families. Those characterized most well are the IAP (inhibitor of apoptosis) proteins and anti-apoptotic members of the Bcl-2 family (Adams and Cory, 1998; Deveraux and Reed, 1999). A growing number of proteins in the Bcl-2 family can modulate apoptosis either positively or negatively. At least in vertebrate cells, it appears that the primary locus of Bcl-2 family action is the mitochondrion. Bcl-2 and its relative Bcl-xL can suppress mitochondrial cytochrome *c* release, while several of the pro-apoptotic Bcl-2 family members, including Bid, Bax and Bak, can accelerate its release (Li *et al.*, 1998; Luo *et al.*, 1998; Desagher *et al.*, 1999; Griffiths *et al.*, 1999; Gross *et al.*, 1999). Interestingly, it was recently reported that Bcl-2 family members can bind to the mitochondrial voltage-dependent anion channel to modulate cytochrome *c* release (Shimizu *et al.*, 1999).

IAPs were first described as baculoviral proteins involved in the suppression of virally induced host cell death (Crook *et al.*, 1993; Birnbaum *et al.*, 1994; Clem and Miller, 1994). Subsequently, it has been shown that cellular IAPs exist in a number of species examined, and human IAPs Xiap, c-Iap1 and c-Iap2 can all prevent pro-caspase activation. Baculoviral IAP repeat (BIR) domains present in all of the IAPs are necessary, and in some cases sufficient, to suppress caspase activation and apoptosis (Roy *et al.*, 1997; Deveraux *et al.*, 1998; Takahashi *et al.*, 1998). The precise molecular mechanism of this suppression is not yet understood.

Genetic analysis in several organisms has successfully identified novel apoptotic regulators which, acting in conjunction with proteins such as IAPs, caspases and Bcl-2 family members, are critical for implementation of the cell death program. In an extensive analysis of chromosomal deletion mutants in the fly *Drosophila melanogaster*, Steller and colleagues identified a chromosomal region containing a number of genes critical for programmed cell death occurring during embryonic development (White *et al.*, 1994). Three genes in this region encode Reaper, Hid and Grim proteins, which are potent cell death inducers (Grether *et al.*, 1995; Chen *et al.*, 1996b; White *et al.*, 1996). In their absence, cell death is abrogated, while ectopic expression of these genes promotes apoptotic death not only in fly cells, but in human cells as well (Claveria *et al.*, 1998; McCarthy and Dixit, 1998; Haining *et al.*, 1999).

In an effort to understand the mechanism of action of

Reaper protein, we produced recombinant Reaper and examined its effects in cell-free extracts prepared from *Xenopus* eggs. While these extracts will spontaneously release mitochondrial cytochrome *c* and activate endogenous caspases after prolonged incubation at room temperature (~4.5–7 h), Reaper addition greatly accelerated this process, triggering mitochondrial cytochrome *c* release, caspase activation and fragmentation of added nuclei within ~1.5–2 h (Newmeyer *et al.*, 1994; Evans *et al.*, 1997a,b).

As Reaper is a very small protein (65 amino acids) with no significant homology to known signaling molecules and no evident catalytic function, we searched for Reaper-interacting molecules potentially required for Reaper-induced apoptosis. In doing so, we identified a 150 kDa Reaper-binding protein, which was named Scythe (Thress *et al.*, 1998). While the primary amino acid sequence of Scythe did not provide any clues as to its mechanism of action, immunodepletion of Scythe from *Xenopus* egg extracts eliminated Reaper-induced cytochrome *c* release, caspase activation and the induction of apoptotic nuclear fragmentation. Moreover, a truncated variant of Scythe (Scythe C312), consisting of the C-terminal 312 amino acids of Scythe fused to glutathione *S*-transferase (GST), induced apoptosis in the egg extracts very effectively, even in the absence of Reaper. Scythe C312 protein induced mitochondrial cytochrome *c* release in *Xenopus* egg extracts, but could not trigger cytochrome *c* release from purified mitochondria, indicating a requirement for accessory cytosolic factors. Collectively, these experiments led to the conclusion that Scythe was a novel apoptotic regulator required in the pathway of Reaper-induced apoptosis, but that additional factors were required to promote Reaper-induced cytochrome *c* release and consequent caspase activation.

In this report, we demonstrate that Scythe sequesters positive regulators of apoptosis that, when not bound by Scythe, can trigger cytochrome *c* release from purified mitochondria in the absence of other cytosolic components. Importantly, we show that this cytochrome *c*-releasing activity is liberated when Reaper binds to Scythe, providing a mechanistic explanation for the Scythe requirement in Reaper-induced apoptosis.

Results

Excess Scythe inhibits Reaper-induced apoptosis

We previously reported that Scythe C312 could induce mitochondrial cytochrome *c* release and consequent caspase activation upon addition to egg extracts. According to this scenario, Scythe C312 was an 'activated' Reaper-independent Scythe variant, mimicking a conformation characteristic of Reaper-bound Scythe. Indeed, addition of excess recombinant Scythe on its own never triggered apoptosis, suggesting that this excess Scythe could not adopt an activated C312-like pro-apoptotic conformation in the absence of Reaper. Nonetheless, we reasoned that co-addition of Reaper along with excess full-length Scythe might produce a particularly robust apoptotic response, perhaps leading to swifter or greater caspase activation than that induced by Reaper alone. To test this, we added 300 ng/μl exogenous Scythe protein (~5-fold that found endogenously) to *Xenopus* egg extracts along with recombinant Reaper and monitored apoptotic progression. Surprisingly, we found that addition of excess full-length Scythe suppressed, rather than accelerated, Reaper-induced caspase activation (Figure 1) and morphological apoptosis (data not shown). These data raised the possibility that Scythe might have intrinsic anti-apoptotic activity that could be antagonized by Reaper. Intriguingly, we found that spontaneous apoptosis following prolonged incubation of egg extracts at room temperature was also inhibited by addition of excess Scythe, reinforcing the hypothesis that Scythe is inherently anti-apoptotic (data not shown). Importantly, excess Scythe was not able to inhibit apoptosis induced by the addition of low levels of recombinant caspase 8 (not shown), demonstrating that excess Scythe did not destroy the competence of the extract to undergo apoptosis.

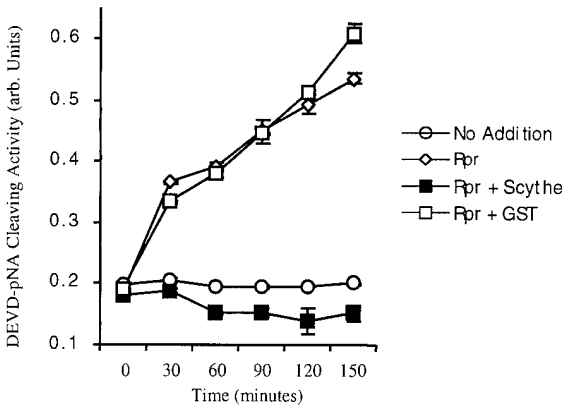

Fig. 1. Recombinant Scythe inhibits Reaper-induced caspase activity. Recombinant Reaper (Rpr) protein alone (300 ng/μl) or Reaper in combination with equivalent amounts of either recombinant Scythe or GST proteins was added to *Xenopus* egg extracts. At the indicated times, 2 μl aliquots of extract were analyzed for caspase activity using a DEVD-pNA cleavage assay.

A Scythe-sequestered apoptotic inducer is released by Reaper

Since immunodepletion of Scythe from egg extracts prevents Reaper-induced apoptosis, but Scythe itself appeared to be anti-apoptotic, we suspected that a pro-apoptotic factor required for Reaper-induced apoptosis might be bound to, and co-depleted with, Scythe. A model for at least one pathway of Reaper-induced apoptosis, based on this hypothesis, is shown in Figure 2A. According to this model, endogenous Scythe in the extract sequesters a pro-apoptotic factor ('X'). Upon binding of Reaper to Scythe, 'X' is released, thereby triggering mitochondrial cytochrome *c* release and consequent caspase activation. This model would account for the inability of full-length Scythe to induce apoptosis, as well as the observation that excess Scythe could inhibit Reaper-induced apoptosis; after Reaper-induced release of 'X' from a subpopulation of Scythe, excess Scythe would simply resequester 'X'. This hypothesis predicts that immunoprecipitates of Scythe should contain a pro-apoptotic factor susceptible to release from the precipitate following addition of Reaper protein.

To test this hypothesis, we linked either anti-Scythe or pre-immune sera to protein A–Sepharose, incubated these 'beads' in crude *Xenopus* egg extract, and then pelleted and washed the beads. The washed precipitates were then

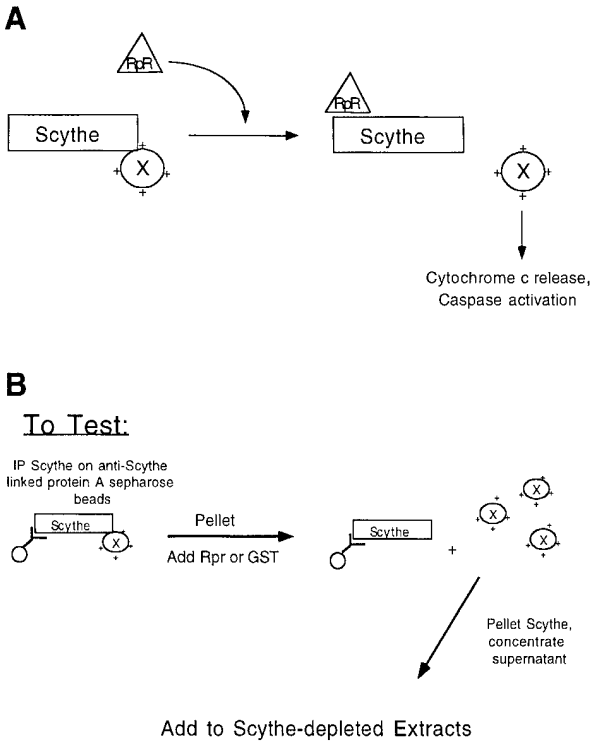

Fig. 2. A possible model for Rpr-induced apotosis via Scythe. (**A**) In this model, Scythe is normally bound to and sequesters a pro-apoptotic activity, here denoted as factor 'X'. Upon Reaper (Rpr) addition to the extract, Rpr binds to Scythe, thus causing the release of factor 'X' from Scythe. Factor 'X' is then free to induce cytochrome *c* release and subsequent caspase activation. (**B**) To test the above model, endogenous Scythe (bound to factor 'X') is immunoprecipitated using Scythe antibody linked to protein A–Sepharose beads. The beads are then pelleted, washed, and incubated with recombinant Reaper protein (Rpr) to induce release of factor 'X' into the supernatant. The beads are spun out to remove Scythe and remaining Scythe-associated proteins, and the factor 'X'-containing supernatant is concentrated and added to Scythe-depleted extracts.

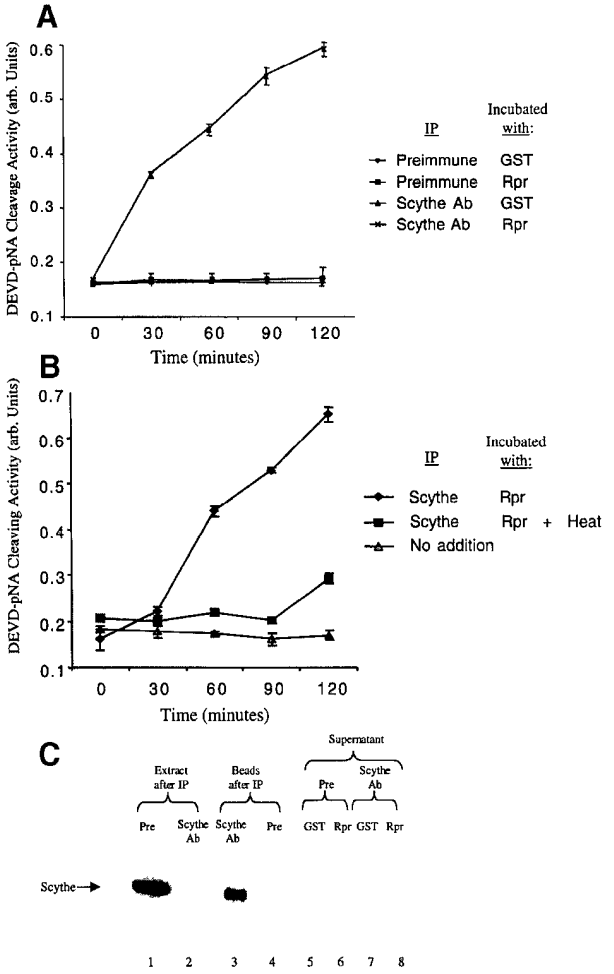

Fig. 3. Reaper-induced release of a pro-apoptotic activity from Scythe. (**A**) Scythe antisera or pre-immune sera linked to protein A–Sepharose beads were incubated with *Xenopus* egg extracts for 1 h at 4°C. After immunoprecipitation, the beads were washed and resuspended in ELB. The washed beads were then incubated with either recombinant GST or GST–Reaper (Rpr) for 30 min at room temperature. The beads were spun out and the supernatant concentrated ~10-fold by centrifugation in microcon 10s for 20 min at 4°C. The resulting samples were added 1:10 to Scythe-depleted egg extract and, at the indicated times, 2 µl aliquots of extract were processed for DEVD-pNA cleavage activity. (**B**) The activity released by Reaper is heat labile. Immunoprecipitates from protein A-linked Scythe antibody (Scythe Ab) were incubated with recombinant Reaper (Rpr) protein as described above. After concentration, the supernatants were either added directly to Scythe-depleted extracts or first incubated at 80°C for 10 min prior to addition to extracts. (**C**) Apoptotically active supernatant does not contain Scythe protein. Equivalent amounts of the indicated samples were separated by SDS–PAGE, processed for immunoblotting using anti-Scythe sera, and Scythe protein visualized via a chemiluminescence detection kit (Amersham). Pre, samples immunoprecipitated with pre-immune 'beads'; Scythe Ab, samples immunoprecipitated with anti-Scythe 'beads'; GST, supernatant treated with GST protein; Rpr, supernatant treated with Rpr protein. Shown in lanes 1 and 2 are proteins remaining in the extract after immunoprecipitation with pre-immune or immune sera. Lanes 3 and 4 show Scythe present in the immune but not pre-immune precipitate. As seen in lanes 5–8, none of the 'released' supernatants contain detectable Scythe protein.

incubated with either GST protein or with GST–Reaper in order to initiate the release of the presumptive pro-apoptotic factor(s) 'X' into the supernatant (Figure 2B). After removal of the bead-bound material (including Scythe and Reaper) by centrifugation, the residual supernatant was concentrated and added to a crude *Xenopus* egg extract that had been entirely immunodepleted of endogenous Scythe protein. In agreement with the proposed model, both caspase activation (Figure 3A) and mitochondrial cytochrome *c* release (data not shown) were induced by material released from the Scythe immunoprecipitate by Reaper. GST alone did not induce the release of such an activity from the Scythe immunoprecipitate. In addition, Reaper did not induce the release of pro-apoptotic factors from the pre-immune beads (indicating, as expected, that that any Reaper carried over into the supernatant did not induce apoptosis in the Scythe-depleted extract), nor did GST induce the release of such activity from the Scythe immunoprecipitate. The activity released by Reaper appeared to be heat labile, as incubation of the supernatant at 80°C for 10 min inactivated the pro-apoptotic factor(s) in the supernatant (Figure 3B). Moreover, the apoptotically active supernatant obtained when GST–Reaper was added to Scythe precipitates did not contain any detectable Scythe protein (Figure 3C), so it is very unlikely that Scythe–Reaper complexes carried over into the Scythe-depleted extract (see also below).

As described in Figure 1, recombinant Scythe, when present in excess, can inhibit Reaper-induced apoptosis. One potential explanation for this observation is that

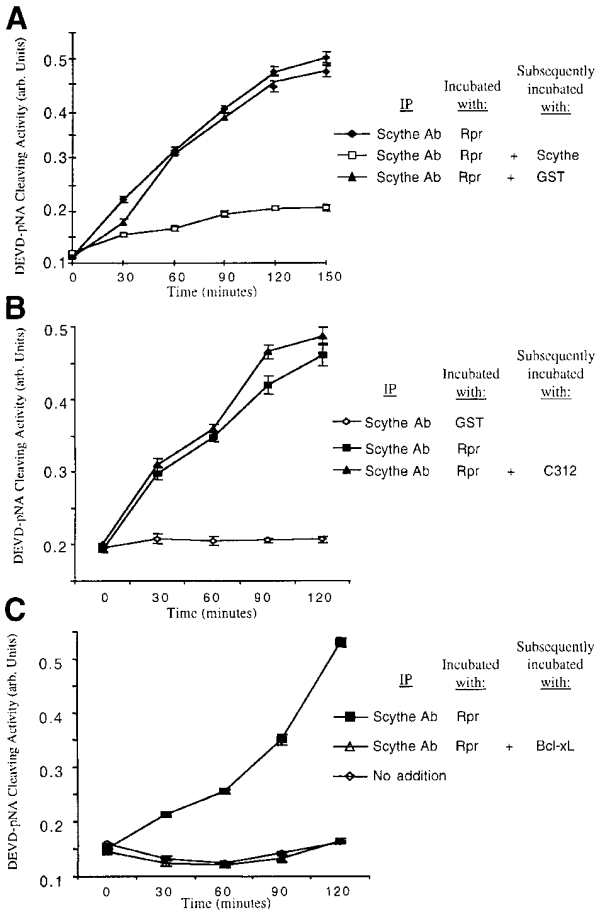

Fig. 4. Recombinant Scythe, but not Scythe C312, resequesters the pro-apoptotic activity. (**A**) Immunoprecipitates from protein A-linked Scythe antibody (Scythe Ab) were incubated with recombinant Reaper (Rpr) protein as described above. After concentration, the supernatants were either left untreated or incubated with equivalent amounts of either recombinant Scythe or GST protein for 30 min at 4°C. The resulting samples were added 1:10 to Scythe-depleted egg extract and at the indicated times 2 µl aliquots of extract were processed for DEVD-pNA cleavage activity. (**B**) An assay identical to (A) was carried out, but instead of incubating the released, concentrated supernatant with recombinant Scythe, the samples were incubated with recombinant Scythe C312 for 30 min at 4°C. (**C**) Recombinant Bcl-xL inhibits released activity. The released, concentrated supernatant was supplemented with recombinant Bcl-xL and added to Scythe-depleted extracts, and at the indicated times 2 µl aliquots of extract were processed for DEVD-pNA cleavage

the exogenously added Scythe can resequester the pro-apoptotic factor(s) released upon Reaper addition. To address this issue, we repeated the release experiments described above, but prior to adding the released proteins to the Scythe-depleted extract, we supplemented the supernatant with either recombinant full-length Scythe or GST protein. As shown in Figure 4A, incubation with Scythe, but not GST, prevented the released supernatant from inducing caspase activation. Collectively, these experiments suggest that Reaper can trigger the release of a pro-apoptotic activity that, once liberated, can initiate cell death through cytochrome *c* release and caspase activation. In contrast to full-length Scythe, the truncated C312 Scythe protein did not suppress the activity of the supernatant released from the Scythe precipitates (Figure 4B).

To characterize further the factors released from Scythe

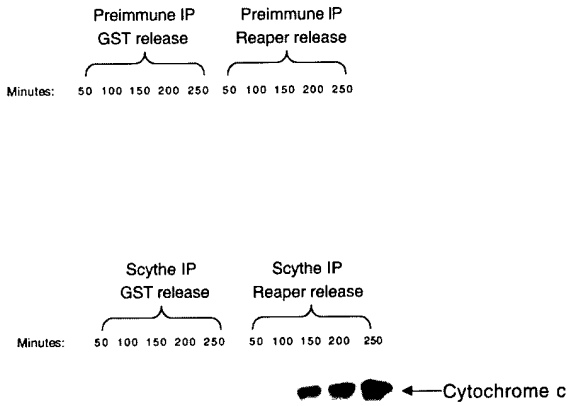

Fig. 5. The pro-apoptotic activity released from Scythe upon Reaper treatment induces direct release of cytochrome *c* from isolated mitochondria. Mitochondria isolated from *Xenopus* egg extracts and further purified by centrifugation through a percoll gradient were diluted 1:10 into ELB containing an ATP-regeneration mix. The indicated supernatants obtained as described above were added 1:10 to the mitochondria at room temperature and at the indicated times 25 µl of the mixture were filtered through a 0.1 µM microfilter. Aliquots of filtrate (10 µl) were separated by SDS–PAGE and processed for Western blotting using an anti-cytochrome *c* monoclonal antibody.

by Reaper, we added recombinant Bcl-xL protein to the released supernatant; this protein very effectively inhibited the induction of caspase activity by the released factor(s) (Figure 4C).

The pro-apoptotic activity released from Scythe is a direct inducer of mitochondrial cytochrome c release

As we reported previously, neither the C312 variant of Scythe nor recombinant Reaper can induce cytochrome *c* release from purified mitochondria in the absence of additional cytosolic components (Thress *et al.*, 1998). Reaper added along with full-length recombinant Scythe is also inactive in this assay, reinforcing the notion that accessory factors are required for Scythe/Reaper-induced cytochrome *c* release. Potentially, the material released from Scythe immunoprecipitates by Reaper addition might contain such factors. We incubated the various released supernatants described above with purified mitochondria. As shown in Figure 5, the supernatant obtained from the Reaper-treated Scythe immunoprecipitates triggered direct cytochrome *c* release from isolated mitochondria, while only low levels of background cytochrome *c* efflux were observed in mitochondria treated with control supernatants or incubated with buffer alone. These data indicate that Scythe sequesters cytochrome *c*-releasing activity which is liberated following binding of Scythe to Reaper.

Scythe is not required downstream of mitochondrial cytochrome c release

Although the experiments described above firmly place Scythe upstream of mitochondrial cytochrome *c* release in the pathway of Reaper-induced apoptosis, they do not preclude the possibility that Scythe plays an additional post-cytochrome *c* role. To address this, we immuno-depleted Scythe from the egg extract and asked whether addition of pure cytochrome *c* to the depleted extract could still induce caspase activation and morphological

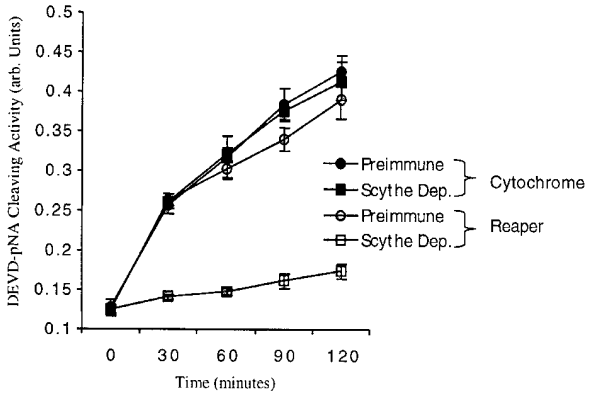

Fig. 6. Scythe acts exclusively upstream of mitochondria. Recombinant Reaper protein (300 ng/μl) or equine heart cytochrome *c* (1 ng/μl) were added to either *Xenopus* egg extract depleted of endogenous Scythe protein (Scythe Dep.) or extracts similarly treated with pre-immune sera (Preimmune). At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity.

apoptosis. As shown in Figure 6, Scythe depletion was unable to interfere, even partially, with cytochrome *c*-induced caspase activation. Thus, Scythe is not required after efflux of cytochrome *c* from the mitochondria.

Excess IAPs neither prevent Reaper-induced cytochrome c release nor disrupt the Reaper–Scythe interaction

It has been reported by several groups that overexpression of IAPs can block Reaper-induced apoptosis (Hay *et al.*, 1995; Vucic *et al.*, 1997a; McCarthy and Dixit, 1998). Moreover, IAPs can bind directly not only to Reaper, but to other critical regulators of *Drosophila* apoptosis: Grim and Hid (Kaiser *et al.*, 1998; Vucic *et al.*, 1998). While IAPs reportedly inhibit caspase activity and pro-caspase activation, their ability to inhibit upstream events, such as mitochondrial cytochrome *c* release, has not been examined (Roy *et al.*, 1997; Deveraux *et al.*, 1998). To test this, we produced the three BIR domains of c-IAP 1, previously reported to be an effective inhibitor of Reaper-induced apoptosis, in bacteria (McCarthy and Dixit, 1998). After purifying the BIR protein, we added it to egg extracts together with Reaper. At concentrations of BIR that very effectively blocked Reaper-induced apoptosis, we observed no inhibition of Reaper-induced cytochrome *c* release (Figure 7A and B). Even when present at a 10-fold molar excess to Reaper, this protein blocked neither Reaper-induced cytochrome *c* release nor binding of Reaper to Scythe (Figure 7C).

Since the first 15 amino acids of Reaper are critical for IAP binding (McCarthy and Dixit, 1998) and excess IAP protein did not interfere with the Scythe–Reaper interaction, we assumed that IAPs and Scythe protein must interact with Reaper at distinct sites. In accordance with this, we found that full-length Reaper protein and a mutant Reaper protein lacking the first 15 amino acids (Rpr 16–65) were both capable of binding Scythe (Figure 8A). Moreover, a fusion protein consisting of GST linked to the first 15 amino acids of Reaper was unable to bind to Scythe (Figure 8A). As has been reported in other systems using similar N-terminal Reaper mutants, Rpr 16–65 could still trigger caspase activation and morphological apoptosis upon addition to *Xenopus* egg extracts, but less efficiently

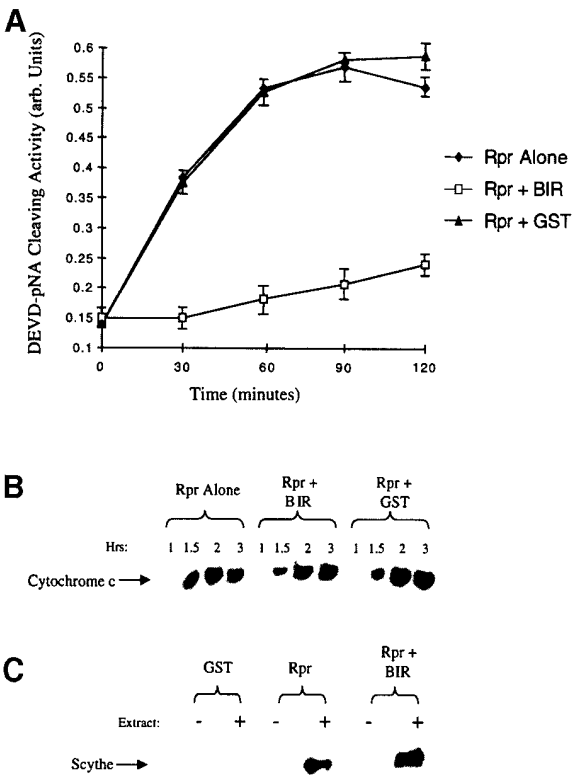

Fig. 7. Excess BIR protein inhibits Rpr-induced apoptosis but not Rpr-induced cytochrome *c* release. (**A**) Recombinant Reaper (Rpr) protein alone (300 ng/μl) or Reaper in combination with equivalent amounts of either recombinant BIR or GST proteins were added to crude *Xenopus* egg extracts. At the indicated times, 2 μl aliquots of extract were processed for DEVD-pNA cleavage activity. (**B**) Samples were processed as in (A), but 15 μl aliquots were filtered through a 0.1 μM microfilter and processed for immunoblotting with an anti-cytochrome *c* monoclonal antibody. (**C**) Recombinant GST, GST–Reaper and GST–Reaper pre-incubated with a 10-fold molar excess of recombinant BIR protein were immobilized on glutathione–Sepharose beads and incubated in the absence (–) or presence (+) of *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 16 amino acids of the *Xenopus* Scythe protein.

than the similarly added wild-type Reaper protein (data not shown) (Chen *et al.*, 1996a; Vucic *et al.*, 1997b).

The Reaper, Grim and Hid proteins are not notably homologous outside of a region of limited homology found at their extreme N termini (corresponding to the first 15 amino acids of Reaper), which appears to be responsible for their shared ability to bind IAPs (Chen *et al.*, 1996b; McCarthy and Dixit, 1998). Surprisingly, despite the fact that Scythe could bind to a region of Reaper with no overt primary sequence homology to Grim and Hid proteins, GST–Hid and GST–Grim proteins bound Scythe nearly as well as did GST–Reaper (Figure 8B). Several control proteins tested, including GST alone, did not bind to Scythe. Therefore, although the primary sequences of these proteins do not reveal an obvious shared motif, Grim, Hid and Reaper can all interact with Scythe.

Discussion

Reaper protein has no evident catalytic activity and only limited homology to other apoptotic regulators, yet it is a

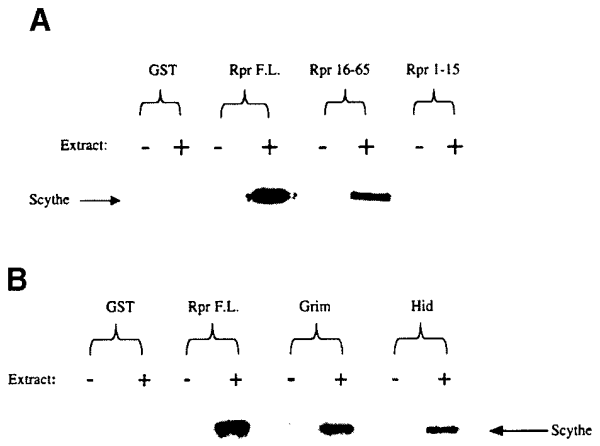

Fig. 8. Scythe interacts with the C-terminal 50 amino acids of Reaper as well as with the *Drosophila* apoptotic regulators Hid and Grim. (**A**) Recombinant GST protein or various GST–Reaper fusion proteins were immobilized on glutathione–Sepharose beads and incubated in the absence (–) or presence (+) of *Xenopus* egg extract for 1 h at 4°C. The beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer and processed for immunoblotting using anti-peptide sera targeted against the C-terminal 16 amino acids of the *Xenopus* Scythe protein. Rpr F.L., full-length Reaper (amino acids 1–65); Rpr 16–65, amino acids 16–65 of Reaper; Rpr 1–15, amino acids 1–15 of Reaper. (**B**) Recombinant GST protein alone or the indicated GST fusion proteins were immobilized on glutathione–Sepharose beads, incubated in the absence (–) or presence (+) of *Xenopus* egg extract for 1 h at 4°C and treated as described in (A).

potent inducer of apoptosis in cells of both lepidopteran and vertebrate origin. In this report, we demonstrate that Reaper acts, at least in part, by inducing the dissociation of a Scythe-bound factor that can trigger direct release of mitochondrial cytochrome *c*.

Scythe sequesters a positive apoptotic regulator

Neither C312 Scythe, Reaper alone nor Reaper added with full-length Scythe can induce direct mitochondrial cytochrome *c* release; the experiments presented here make a strong case that the cytochrome *c*-releasing factor(s) which operates downstream of Reaper is initially bound to Scythe and maintained in an inactive form. While it is not yet clear how Reaper induces the dissociation of these factors from Scythe, it is attractive to speculate that Reaper binding induces a conformational change in Scythe leading to desequestration of the bound factor(s). Alternatively, it is possible that Reaper displaces the factor competitively through binding to the same site on Scythe.

Although we have not yet identified the Scythe-bound factor(s) responsible for the cytochrome *c*-releasing activity, several Bcl-2 family members have been implicated in the direct release of mitochondrial cytochrome *c* (Li *et al.*, 1998; Luo *et al.*, 1998; Desagher *et al.*, 1999). Thus, we consider it quite possible that a Bcl-2 family member is one of the Scythe-bound factors. Although the cytochrome *c*-releasing activity of pro-apoptotic Bcl-2 family members may be activated by caspase cleavage [e.g. bid cleavage by caspase 8 (Li *et al.*, 1998; Luo *et al.*, 1998)], the Scythe-sequestered factor probably does not require caspases for activity because caspase inhibitors do not appear to prevent Reaper-induced cytochrome *c* release (Evans *et al.*, 1997a). While the ability of Bcl-xL protein to inhibit the activity of factor 'X' is consistent with the hypothesis that a positively acting Bcl-2 family member may be sequestered by Scythe, attempts to test this hypothesis by immunoblotting of anti-*Xenopus* Scythe immunoprecipitates with anti-Bcl-2 family sera have been hampered by the lack of cross-reactivity of the available antisera with homologous *Xenopus* proteins. However, preparation of anti-human Scythe antisera should soon facilitate the examination of factors associated with human Scythe. Four specific Scythe-bound proteins can be detected in *Xenopus* anti-Scythe immunoprecipitates by silver staining of SDS–PAGE gels, but their identity remains to be determined (K.Thress and S.Kornbluth, unpublished).

An alternative hypothesis of Scythe function to be considered is that Scythe may be part of an Apaf-1-like complex, which, upon binding Reaper, promotes the processing of a pro-caspase that acts upstream of mitochondrial cytochrome *c* release. High levels of broad-spectrum caspase inhibitors do not appear to prevent Reaper-induced cytochrome *c* release, but this does not rule out the involvement of a caspase insensitive to the inhibitors used in those experiments (Evans *et al.*, 1997a). Since exogenous Scythe can resequester the pro-apoptotic factor(s) released by Scythe, an Apaf-1/pro-caspase-like model for Scythe function would have to postulate that Scythe can re-bind and neutralize the released and activated caspase.

The C312 Scythe protein probably acts as a dominant-negative Scythe variant

Data presented in Figure 4B illustrate that the Scythe C312 protein, which can induce apoptosis independently of Reaper, cannot, like full-length Scythe, suppress the activity of pro-apoptotic factors released from Scythe. However, as reported previously, a resin linked to C312 Scythe very effectively depletes *Xenopus* egg extracts of factors required for Reaper-induced cytochrome *c* release and caspase activation (Thress *et al.*, 1998). It is possible that the C312 protein assumes an 'active' conformation that triggers activation of a bound cytochrome *c*-releasing factor. However, we have found that the amount of C312 protein required to induce apoptosis in egg extracts exceeds the level of endogenous Scythe by at least 2-fold (data not shown). Collectively, these data suggest that the C312 protein may act, not as an activated variant of Scythe, but as a dominant interfering Scythe protein. Possibly, both full-length and C312 Scythe can bind to pro-apoptotic factors, but only the full-length Scythe can inhibit their activity.

Two pathways of Reaper-induced apoptosis?

Since excess IAP protein did not appear to block Reaper-induced mitochondrial cytochrome *c* release, while effectively blocking Reaper-induced apoptosis, it is entirely possible that excess IAPs prevent Reaper-induced apoptosis primarily through post-mitochondrial inhibition of pro-caspase activation. Indeed, purified IAP protein very effectively prevents activation of pro-caspases 9 and 3 upon addition of purified cytochrome *c* to the *Xenopus* egg extract (K.Thress and S.Kornbluth, unpublished). What then is the role of Reaper binding to IAPs? The 16–65 Reaper variant is less active than the wild-type

Reaper protein, suggesting that the first 15 amino acids of Reaper may serve a pro-apoptotic function. This is the region of Reaper that also binds IAPs, prompting the speculation that Reaper binding may serve to inactivate an anti-apoptotic function of IAPs in the egg extract, rather than IAPs acting to incapacitate Reaper. Indeed, it has recently been demonstrated that Reaper, Grim and Hid proteins can block the ability of a *Drosophila* IAP to suppress caspase-dependent death of yeast. Moreover, it was shown that the N-terminal region of Hid, which is homologous to Reaper, mediated its IAP-suppressing activity (Wang *et al.*, 1999).

Because the 16–65 protein retains the ability to interact with Scythe, it is likely that Scythe mediates the residual apoptosis-inducing activity of the 16–65 Reaper protein. Interestingly, when this protein is added to egg extracts at 4- to 5-fold higher levels than wild-type Reaper protein, the 16–65 and wild-type proteins induce roughly equivalent levels of caspase activity (data not shown). This suggests that Scythe-dependent pathways, when sufficiently activated, may be able to compensate for the absence of pathways (possibly IAP inhibition) which normally act coordinately with Scythe to mediate Reaper-induced apoptosis.

Scythe in vertebrates

The conservation of Scythe protein across species, coupled with the ability of *Drosophila* Reaper to trigger the dissociation of cytochrome *c*-releasing factors from *Xenopus* Scythe, argues strongly that a similarly acting Scythe ligand must exist in vertebrates. Reaper, Grim and Hid proteins have all been shown to induce apoptosis in human cells (Claveria *et al.*, 1998; McCarthy and Dixit, 1998; Haining *et al.*, 1999). These proteins share the ability to bind IAPs and, as demonstrated here, have the common ability to bind Scythe. We have also found that these proteins can bind to an *in vitro* translated form of the human Scythe protein (data not shown). Whether there will be several distinct Scythe ligands that share primary sequence homology to Grim, Hid or Reaper proteins remains to be determined. It will also be of great interest to determine whether regulated release of Scythe-sequestered cytochrome *c*-releasing factors is important for other, non-Reaper-mediated, pathways of apoptosis.

Materials and methods

Preparation of GST fusion proteins

Two separate truncations of recombinant *Drosophila* Reaper protein were constructed: the N-terminal 15 amino acids (Rpr 1–15) and the C-terminal 50 amino acids (Rpr 16–65). cDNAs encoding these truncations were PCR amplified using the following primers. Rpr 1–15: 5′-GATCGGATCCATGGCAGTGGCATTC-3′; 5′-GATCAAGCTTTC-ACCGCAACAGAGTCGC-3′. Rpr 16–65: 5′-GATCCCATGGAGG-CGGAGCAGAAGGAGCAG-3′; 5′-GATCAAGCTTTCATTGCGATG-GCTTGCGATA-3′. Full-length *Drosophila* Grim and HID were also PCR amplified using the following primers. Grim: 5′-GATC-GGATCCATGGCCATCGCCTATTTC-3′; 5′-GATCAAGCTTTTAGTT-CTCCTTGGAGGTGGCATC-3′. HID: 5′-GATCGGATCCATGGCCG-TGCCCTTTTATTTG-3′; 5′-GATCAAGCTTTCATCGCGCCGCAAA-GAAGCC-3′. cDNA encoding a truncated hIAP-1 protein consisting of the three BIR domains, but lacking the C-terminal RING finger domain, was amplified using the following primers: 5′-GATCGGAT-TCATGAACATAGTAGAAAAC-3′; 5′-GATCAAGCTTTCATGTTCT-TTCTTCTGGTAG-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 described previously (Evans *et al.*, 1997a). Control GST protein was expressed and prepared in a manner identical to that used for all other GST fusion proteins.

Preparation of Xenopus egg extracts

For induction of egg laying, mature female frogs were injected with 100 U of pregnant mare serum gonadotropin (Calbiochem) to induce oocyte maturation, followed by injection (3–28 days later) with human chorionic gonadotropin (HCG; USB). Fourteen to 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 the addition of aprotinin and leupeptin (final concentration 5 mg/ml), cytochalasin B (final concentration 5 mg/ml) and cycloheximide (final concentration 50 mg/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 mg/ml creatine phosphokinase). Recombinant proteins added to extracts were diluted in XB buffer (50 mM sucrose, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM K–HEPES, pH 7.7) and added at a concentration of 300 ng/μl, unless indicated otherwise.

Immunodepletion assays

Protein A–Sepharose beads were washed in ELB and pre-incubated with 10 mg/ml bovine serum albumin 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 the crude *Xenopus* egg extract. 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 and/or caspase activity directed against artificial substrate (DEVDase) activation.

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 *N*-acetyl-DEVD-*p*-nitroanilide (Ac-DEVD-pNA) (final concentration 200 mM; Biomol Caspase-3 assay system) at 37°C. Absorbance was measured at 405 nm at various time points in a LabSystems MultiSkan MS microtiter plate reader. All measurements were repeated in triplicate for each time point and the average was reported.

Scythe release assays

Either anti-Scythe or pre-immune sera linked to protein A–Sepharose beads were used to immunoprecipitate from *Xenopus* egg extract as described above. Following three successive rounds of precipitation, the beads were combined and washed three times in ELB. The beads were resuspended in ELB and incubated with recombinant, soluble GST or GST–Reaper protein (added 1:10, protein:bead volume) for 30 min at room temperature. The beads were then pelleted by centrifugation and the supernatant concentrated in microcon 10s (Amicon) by centrifugation for 20 min at 4°C. Following concentration, the supernatant was added 1:10 to extracts depleted of endogenous Scythe protein, the extracts were incubated at room temperature, and at the indicated times 3 μl aliquots were collected for DEVD-pNA cleavage activity.

Mitochondrial cytrochrome 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 heavy membrane fraction (enriched in mitochondria) was removed and 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 ELB containing an ATP-regenerating cocktail (10 mM phosphocreatine, 2 mM ATP and 50 mg/ml creatine phosphokinase). At various time points, 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 protein were then separated by SDS–PAGE and immunoblotted with an anti-cytochrome *c* monoclonal antibody (Pharmingen), horseradish peroxidase-linked anti-mouse sera and an ECL chemiluminescence detection system (Amersham).

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