

Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper

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Protein folding mediated by the Hsp70 family of molecular chaperones requires both ATP and the co-chaperone Hdj-1. BAG-1 was recently identified as a bcl-2-interacting, anti-apoptotic protein that binds to the ATPase domain of Hsp70 and prevents the release of the substrate. While this suggested that cells had the potential to modulate Hsp70-mediated protein folding, physiological regulators of BAG-1 have yet to be identified. We report here that the apoptotic regulator Scythe, originally isolated through binding to the potent apoptotic inducer Reaper, shares limited sequence identity with BAG-1 and inhibits Hsp70-mediated protein refolding. Scythe-mediated inhibition of Hsp70 is reversed by Reaper, providing evidence for the regulated reversible inhibition of chaperone activity. As Scythe functions downstream of Reaper in apoptotic induction, these findings suggest that Scythe/Reaper may signal apoptosis, in part through regulating the folding and activity of apoptotic signaling molecules.

Keywords: Hsp70 inhibition/Scythe/Reaper

Introduction

The Hsp70 chaperone proteins facilitate proper protein folding, prevent protein aggregation and assist in the assembly of multi-protein complexes. In this way, Hsp70 family members monitor and counteract the accumulation of potentially harmful misfolded polypeptides, particularly following exposure of the cell to stressful conditions (Hartl, 1996). Comprised of both constitutive and induced members, Hsp70 family members share the ability to recognize exposed hydrophobic patches on non-native proteins and promote their re-folding (Rassow *et al.*, 1995; Rudiger *et al.*, 1997). However, this protein folding requires, in addition to Hsp70/Hsc70, both ATP and a 'co-chaperone'. The most intensively studied of these co-chaperones, Hdj-1, enhances ATP hydrolysis and concomitant release of the folded protein substrate (Hohfeld *et al.*, 1995; Minami *et al.*, 1996).

In vitro, purified Hsc70 releases non-native substrates in the presence of ATP. However, it was recently reported that Hsp70 can associate *in vivo* with BAG-1, a protein that prevents release of folded protein substrates, even in

the presence of Hdj-1 and ATP (Hohfeld and Jentsch, 1997; Takayama *et al.*, 1997; Demand *et al.*, 1998; Stuart *et al.*, 1998; Nollen *et al.*, 2000). Indeed, BAG-1, the first reported negative regulator of Hsp70 function, forms ternary complexes with Hsp70, Hdj-1 and the substrate, maintaining the substrate in a partially folded, yet soluble state (Bimston *et al.*, 1998; Luders *et al.*, 2000a,b). In effect, without inhibiting Hsp70-mediated nucleotide hydrolysis, BAG-1 uncouples ATP hydrolysis from release of the folded substrate (Bimston *et al.*, 1998).

Although its biochemical role in modulating Hsp70 function is clear, the precise biological function of BAG-1 is not known. Originally isolated as a bcl-2-interacting protein, BAG-1 was subsequently shown to associate with other signaling molecules, including Raf-1, the intracellular domain of the PDGF receptor and a number of steroid hormone receptors (Takayama *et al.*, 1995; Zeiner and Gehring, 1995; Bardelli *et al.*, 1996; Wang *et al.*, 1996; Song *et al.*, 2001). Intriguingly, under some circumstances, overexpression of BAG-1 was reported to have anti-apoptotic activity (Takayama *et al.*, 1995). Structurally, BAG-1 does not have any particularly striking features; however, as originally described by Reed and colleagues, BAG family proteins do share a conserved C-terminal ~50 amino acid motif dubbed the 'BAG' domain (Takayama *et al.*, 1999). In addition, BAG-1 and several of its relatives have an N-terminal domain with a high degree of homology to ubiquitin. The functional significance of this homology is not yet clear.

We recently isolated an apoptotic regulator called Scythe, whose N-terminus, like that of BAG-1, bears marked homology to ubiquitin (Thress *et al.*, 1998). Scythe acts downstream of Reaper, a small (65 amino acid) protein that was identified in a screen for apoptotic regulators in the fly, *Drosophila melanogaster*. Genetic evidence has implicated Reaper as an important mediator of apoptosis both during development and following DNA damage (White *et al.*, 1994, 1996). Although Reaper homologs have not yet been identified in other systems, fly Reaper can induce apoptosis in human cells and can trigger biochemical hallmarks of apoptosis (mitochondrial cytochrome *c* release, caspase activation) in cell-free extracts prepared from *Xenopus* (Evans *et al.*, 1997; McCarthy and Dixit, 1998). These data suggest that Reaper-responsive pathways are highly conserved.

Using recombinant Reaper as an affinity resin, Scythe was purified as a high-affinity Reaper interactor (Thress *et al.*, 1998). As immunodepletion of Scythe from *Xenopus* egg cell-free extracts prevented both Reaper-induced cytochrome *c* release and caspase activation, it appeared that Scythe acted downstream of Reaper in the pathway of apoptotic induction. Further studies revealed that Scythe was actually a negative regulator of apoptosis, acting to

To determine whether Scythe–Hsc70 interactions could be observed between human proteins in intact cells, full-length myc-tagged human Scythe transfected into 293T cells was immunoprecipitated from cell lysates with anti-myc antibody and examined for the presence of bound Hsc70 protein. As anticipated, human Scythe and endogenous Hsc70 could, like their *Xenopus* counterparts, be co-precipitated. Importantly, as reported for BAG-1 protein, deletion of the BAG domain (hScythe Δ C; removal of the C-terminal 81 amino acids of Scythe), but not the N-terminal ubiquitin domain (hScythe Δ N) from Scythe completely abrogated binding to Hsc70 (Figure 2D), despite equal levels of expression of mutant and wild-type proteins (Figure 2E). Collectively, these data indicate that Scythe binds specifically to Hsc70/Hsp70 and that this binding is mediated by Scythe's C-terminal BAG domain.

As Scythe, like BAG-1 protein, associates with Hsc70 through its BAG domain, we wished to determine whether Scythe also behaved like BAG-1 in its ability to interact specifically with the ATPase domain of Hsc70/Hsp70. Accordingly, we conducted a series of binding studies using His-tagged Scythe produced in baculovirus vectors and bacterially produced GST-tagged Hsp70 proteins. As was seen in the 293T lysates, full-length Hsp70 bound efficiently to full-length Scythe, while Scythe Δ C was greatly impaired in its ability to bind Hsp70 (Figure 2F). Importantly, full-length Scythe was also able to bind to the isolated ATPase domain of Hsp70, while deletion of the BAG domain from Scythe completely abrogated this association. These data are consistent with previously reported findings demonstrating that human Scythe can bind to a short sequence within the ATPase domain of the Hsp70-like protein, Stch (Kaye *et al.*, 2000).

Scythe functions as a negative regulator of Hsp70 chaperone activity

Among the group of co-chaperones/Hsp70 interactors, BAG-1 is the only protein reported to negatively regulate the protein folding ability of Hsc70/Hsp70 proteins (Takayama *et al.*, 1997; Bimston *et al.*, 1998; Nollen *et al.*, 2000). Given the parallels between Scythe and BAG-1, we wished to determine whether Scythe might be a functional relative of BAG-1, able to negatively modulate the protein folding ability of Hsp70. Typically, *in vitro* protein folding assays examine the ability of Hsp70 chaperones to refold denatured test substrates. These assays appear to reflect faithfully the modulation of Hsp70 activity by both activators and inhibitors, even though the test substrates are not the true *in vivo* targets of

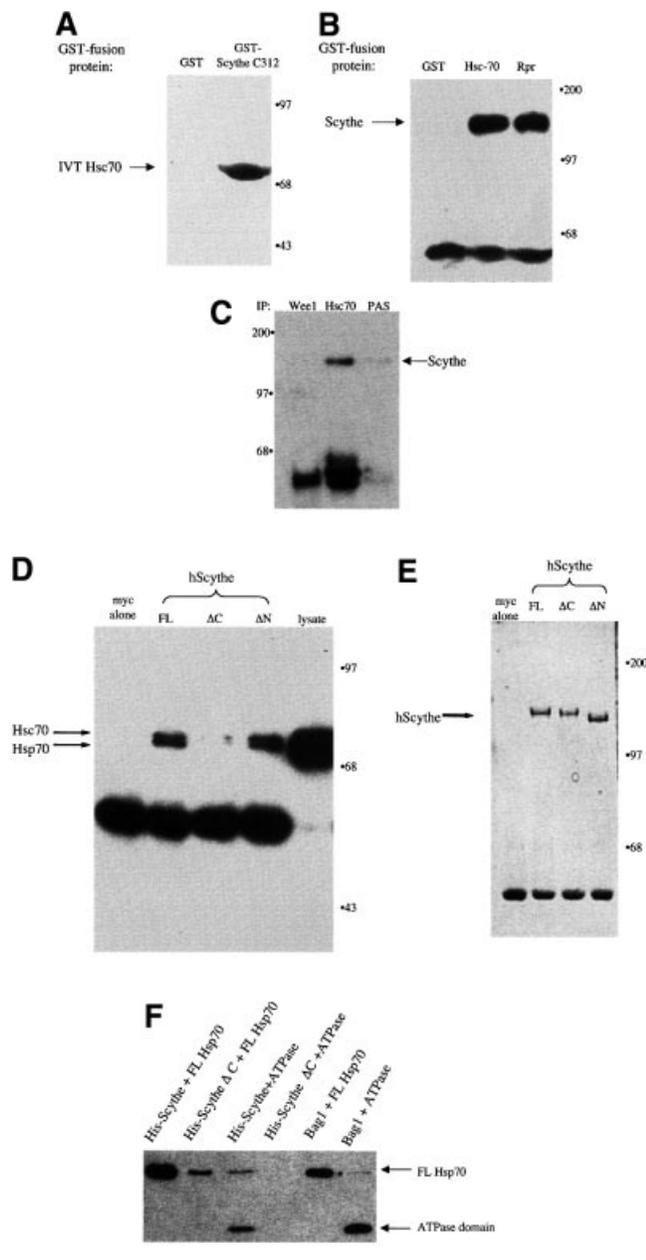


Fig. 2. Scythe binds Hsp70/Hsc70 in a BAG domain-dependent fashion. (A) *In vitro* translated *Xenopus* Hsc70 (IVT Hsc70) was added to extracts and incubated for 30 min at 4°C. GST or GST–Scythe C312 immobilized on glutathione–Sepharose beads was added to the extract and incubated for an additional 30 min. The beads were then washed three times with egg lysis buffer (ELB), resolved by SDS–PAGE and bands were visualized by autoradiography. (B) GST or the indicated GST fusion protein was immobilized on glutathione–Sepharose beads and incubated in the presence of *Xenopus* egg extract for 1 h at 4°C. The beads were then washed three times with ELB, resolved by SDS–PAGE and processed for western blotting with an anti-Scythe polyclonal antibody. (C) Antibodies against *Xenopus* Wee1 or Hsc70 were coupled to Protein A–Sepharose (PAS) beads and then incubated in *Xenopus* egg extract for 1 h at 4°C. Immunoprecipitates were washed three times with ELB, resolved by SDS–PAGE and processed for immunoblotting with an anti-Scythe polyclonal antibody. (D) 293T cells were transfected with 3 μ g of either the indicated myc-tagged human Scythe construct (hScythe) or the parental myc plasmid alone (myc alone). Thirty-six hours after transfection, cells were lysed, centrifuged, and the supernatants incubated with a monoclonal myc antibody for 1 h at 4°C. PAS beads were then added and, after an additional 1 h incubation, the beads were pelleted, washed three times in lysis buffer, and bound proteins were resolved by SDS–PAGE. After western transfer, the blots were probed with an anti-Hsp70/Hsc70 monoclonal antibody. (E) The identical samples processed in (D) were run on a parallel SDS gel and proteins were stained with Coomassie Brilliant Blue. (F) His-tagged Scythe, His-tagged Scythe lacking the BAG domain (His-Scythe Δ C) or His-tagged BAG-1 was incubated in the presence of either full-length Hsp70 (FL Hsp70) or the ATPase domain of Hsp70 (ATPase) for 1 h at 4°C. His-tagged proteins were recovered using a nickel resin and bound proteins were separated by SDS–PAGE. After western transfer, the blots were probed with an anti-Hsp70 monoclonal antibody.

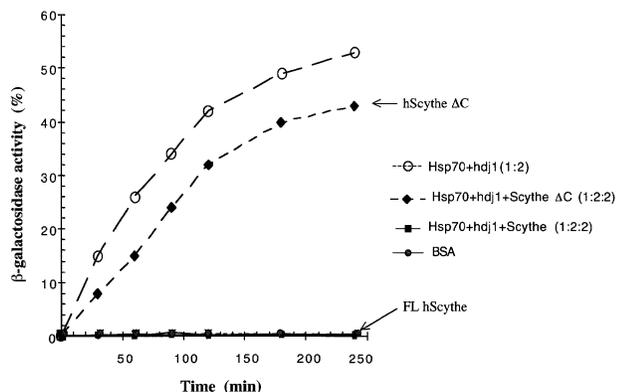


Fig. 3. Scythe functions as a negative regulator of Hsp70 chaperone activity. The inhibitory effect of full-length human Scythe (FL hScythe, 3.2 μ M) and a truncated Scythe mutant lacking the 'BAG' domain (Scythe Δ C, 3.2 μ M) on Hsp70-dependent refolding was examined by the percentage recovered activity of unfolded β -galactosidase (3.2 nM) diluted into a refolding buffer containing ATP (1 mM), Hsp70 (1.6 μ M) and Hdj-1 (3.2 μ M). As a control for spontaneous refolding, denatured β -galactosidase (3.2 nM) was diluted into refolding buffer containing 3.2 μ M bovine serum albumin (BSA).

the modulator-Hsp70 complex (Takayama *et al.*, 1997; Nollen *et al.*, 2000). Accordingly, we asked whether Scythe could alter the ability of Hsp70 to refold denatured β -galactosidase. Following denaturation in 6 M guanidine HCl, recombinant β -galactosidase was incubated in refolding buffer with purified recombinant Hsp70, ATP and the co-chaperone Hdj-1 in the presence or absence of baculovirus-produced Scythe protein. After various times of incubation, samples were assayed for β -galactosidase activity using the colorimetric substrate *o*-nitrophenyl- β -D-galactopyranoside. As reported previously, recombinant Hsp70/Hdj-1 allowed refolding of denatured β -galactosidase, with 50% recovery of enzymatic activity (Figure 3). However, when full-length Scythe was added to the assay at a 1:2 molar ratio with Hsp70, the Hsp70-mediated refolding of denatured β -galactosidase was completely inhibited (Figure 3). Importantly, the refolding assay was not appreciably inhibited by Scythe protein lacking the BAG domain (Scythe Δ C) (Figure 3). These data strongly suggest that Scythe has the previously unanticipated ability to act in a BAG-1-like manner to inhibit Hsp70-mediated protein refolding.

Reaper relieves Scythe-mediated inhibition of Hsp70

Although BAG-1 protein clearly inhibits Hsp70's protein folding activity, its mode of regulation has yet to be elucidated. It has been postulated that BAG-1-mediated repression of Hsp70 must be reversible by as yet undiscovered ligands. As Scythe was originally identified as a Reaper ligand, we wished to determine whether Reaper might reverse Scythe's antagonistic effect on Hsp70-mediated protein folding. To test this, we added increasing amounts of Reaper to the *in vitro* β -galactosidase refolding assay. As shown in Figure 4A, Reaper was able to relieve Scythe's inhibition of Hsp70 refolding, with 80% reversal at a 1:4 (Scythe:Reaper) molar ratio. It is unclear whether this molar ratio reflects true complex stoichiometries because a significant fraction

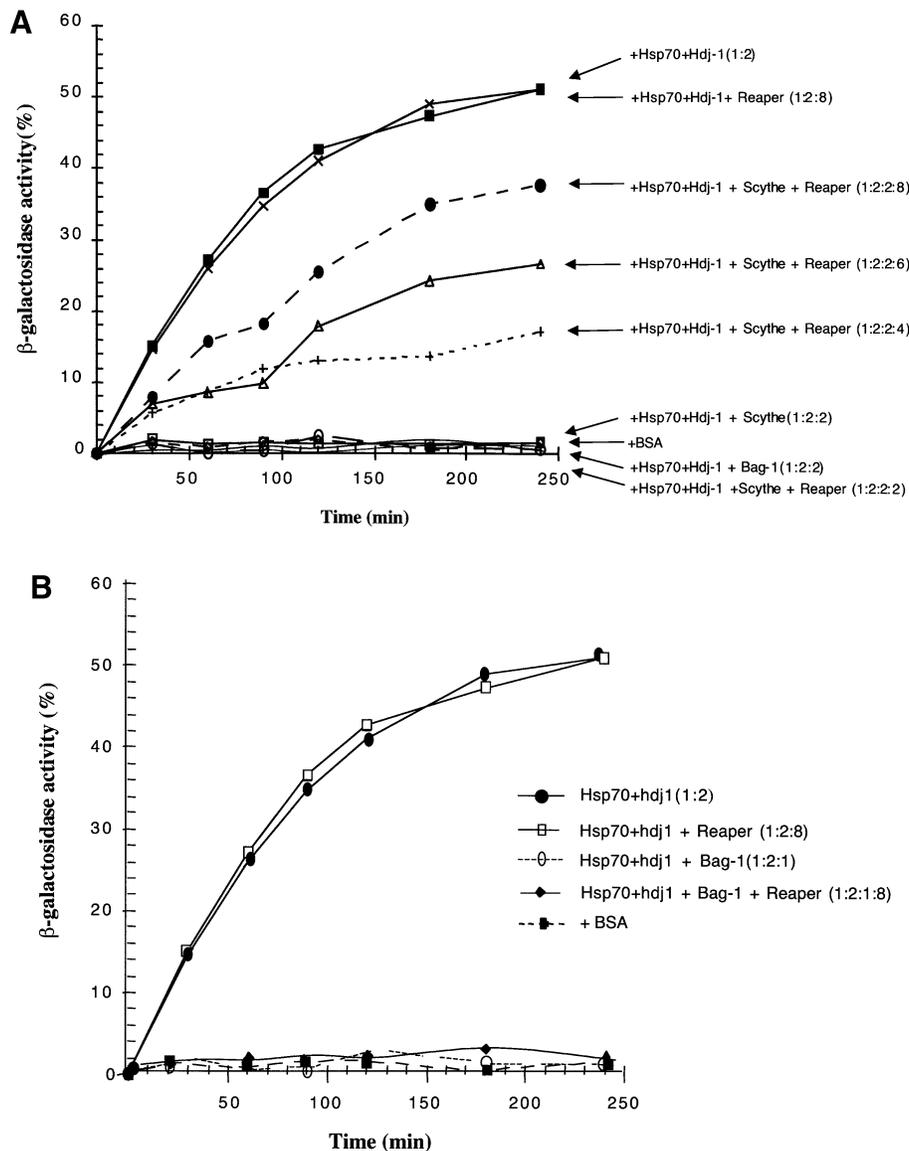
of bacterially produced Reaper protein may be insoluble/non-functional. Nonetheless, similar amounts of Reaper had no effect on BAG-1-mediated inhibition of Hsp70, indicating that the reversal was specific for Scythe (Figure 4B). These data demonstrate that Reaper binding provides an effective means of reversing Scythe's inhibition of Hsp70 function.

Reaper inhibits the physical association of Scythe and Hsp70

Since a Scythe mutant unable to physically bind Hsp70 (Δ C Scythe) could not inhibit Hsp70-mediated protein refolding, we postulated that Reaper might act by rendering Scythe unable to bind Hsp70. To examine this issue, we conducted a series of protein binding studies using recombinant Reaper, Scythe and Hsp70 proteins. His-tagged Scythe was incubated with Hsp70 in the presence of increasing amounts of recombinant Reaper. As shown in Figure 5A, Reaper effectively inhibited the Scythe-Hsp70 interaction, while having a considerably less substantial effect on the BAG-1-Hsp70 association. These results are consistent with Reaper's ability to reverse the functional effects of Scythe, but not BAG-1, on Hsp70-mediated protein re-folding. A similar reversal of Scythe-Hsc70 binding was observed when we added recombinant GST-Reaper protein, but not GST alone, to Scythe immunoprecipitates from 293 cells (Figure 5B). Collectively, these data demonstrate that Reaper binding to Scythe both displaces Hsc70 and reverses Scythe-mediated inhibition of Hsp70 function.

The BAG domain is required for sequestration of Scythe-associated cytochrome c-releasing activity

As described above, addition of recombinant Reaper to cell-free extracts of *Xenopus* eggs triggers a number of biochemical hallmarks of apoptosis, including mitochondrial cytochrome *c* release, caspase activation, and internucleosomal DNA cleavage and fragmentation of added nuclei. As we reported previously, addition of excess recombinant Scythe to *Xenopus* egg extracts inhibits Reaper-induced apoptosis. We therefore speculated that the exogenously added Scythe protein could re-sequester cytochrome *c*-releasing factors dissociated from endogenous Scythe by Reaper. In agreement with this interpretation, the material released from Scythe immunoprecipitates by Reaper could no longer induce cytochrome *c* release if first incubated with recombinant Scythe protein (Thress *et al.*, 1999). In order to determine whether the ability of Scythe to interact with Hsc70 is important for its ability to sequester cytochrome *c*-releasing factor(s), egg extracts were supplemented with Reaper in combination with either wild-type human Scythe or Scythe unable to bind Hsc70 (Δ C Scythe). Under these conditions, the wild-type protein, but not the Δ C mutant Scythe, markedly dampened caspase activation and mitochondrial cytochrome *c* release in response to Reaper addition (Figure 6A and B). Importantly, while Δ C Scythe was unable to bind Hsc70 and inhibit Reaper-induced caspase activation, the Δ C and wild-type Scythe proteins bound Reaper to a similar extent (Figure 6C). Furthermore, the BAG domain of Scythe seems to be sufficient for these effects, as GST protein fused to the isolated BAG domain from Scythe (Scythe BAG) was as



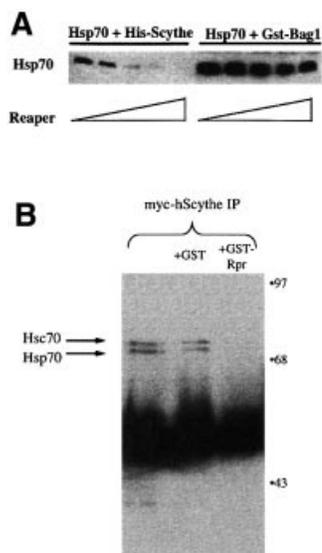


Fig. 5. Reaper specifically inhibits the physical association of Scythe and Hsp70. (A) His-Scythe (1 μM) or GST-BAG-1 (1 μM) was incubated with Hsp70 (1 μM) in refolding buffer. After complex formation, increasing concentrations (0, 2, 4, 8, 10 μM) of Reaper were added. Bound proteins were precipitated with either Ni²⁺-agarose (His-Scythe) or glutathione-Sepharose (GST-BAG-1), washed, resolved by SDS-PAGE and processed for western blotting using Hsp70 monoclonal antibody 5a5. (B) 293T cells were transfected with 5 μg of myc-tagged human Scythe (myc-hScythe). Thirty-six hours after transfection, cells were lysed and centrifuged, and supernatants were incubated with recombinant GST or GST-Reaper (GST-Rpr) for 30 min at 4°C. Subsequently, the lysates were incubated with a monoclonal myc antibody for 1 h at 4°C. PAS beads were then added and, after an additional 1 h incubation, the beads were pelleted, washed three times in lysis buffer, and bound proteins were resolved by SDS-PAGE. After western transfer, the blots were probed with an anti-Hsc70 monoclonal antibody.

Hsp70, in accordance with the hypothetical scheme originally proposed for BAG-1. Interestingly, Reaper binds to Scythe at a site distinct from the BAG domain (between amino acids 235 and 312 of Scythe); therefore, the displacement of Hsc70 from Scythe is not the result of competitive binding of Reaper to the same site (C.Holley, K.Thress and S.Kornbluth, unpublished observations). Rather, we hypothesize that Reaper promotes a conformational change in Scythe, leading to dissociation of Hsp70.

Scythe-Hsp70 and apoptotic regulation

How does Scythe-mediated inhibition of Hsp70 protein folding relate to the ability of Scythe/Reaper to regulate apoptosis? Building on the model originally proposed for BAG-1 (Figure 7), we hypothesize that the cytochrome *c*-releasing factor(s) sequestered by Scythe (denoted as 'X' in Figure 7) is held in a soluble, partially folded state through binding to Hsc70-Scythe complexes. Although we do not yet know whether cytochrome *c*-releasing factors form direct contacts with both Scythe and Hsp70, Scythe, like BAG-1, binds to the ATPase domain of Hsp70 (Figure 2F), making the substrate-binding domain of Hsp70 potentially available for binding 'X' or similar molecules. Moreover, if Scythe functions mechanistically like BAG-1, it is likely that the contact between Hsc70/Hsp70 and 'X' is direct. Upon binding to Reaper, Scythe-Hsp70 complexes dissociate; according to our

model, Hsp70 thus relieved of its inhibition then goes on to fold 'X', leading to cytochrome *c* release, caspase activation, etc.

Superficially, Hsc70 seems to fit the description of 'X' itself, i.e. a protein bound to Scythe and dissociated by addition of Reaper. However, multiple experiments have failed to reveal any direct cytochrome *c*-releasing activity of recombinant Hsc70 protein (data not shown). Given the abundance of Hsp70 family members in the cell, this is not surprising. Nonetheless, the ability of Scythe to interact with Hsc70 appears to be important for its ability to sequester cytochrome *c*-releasing factor(s); a Scythe molecule able to interact with Reaper (data not shown), but lacking the BAG domain, could not behave like excess wild-type Scythe in preventing Reaper-induced apoptosis.

Although we have not yet definitively identified the Scythe-associated cytochrome *c*-releasing factors, the only factors thus far demonstrated to have direct cytochrome *c*-releasing activity are pro-apoptotic members of the bcl-2 family (Desagher *et al.*, 1999; Gross *et al.*, 1999; Shimizu *et al.*, 1999). That 'X' may indeed be a bcl-2 family member is supported by several observations. First, the cytochrome *c*-releasing activity of 'X' released from Scythe can be abrogated by incubation with recombinant bcl-xL, an anti-apoptotic bcl-2 family member that can act through heterodimerization with its pro-apoptotic counterparts (Zha *et al.*, 1997). Secondly, we have recently identified a relatively well conserved BH3 domain in the C-terminus of Scythe (M.Olson and S.Kornbluth, unpublished observations). BH domains (bcl-2-like heterodimerization domains), of which there are four types (BH₁-BH₄), are contiguous sequences shown to be critical for the association of bcl-2 family members (reviewed in Gross *et al.*, 1999). Although BH3-containing proteins may, themselves, be cytochrome *c*-releasing factors, Scythe alone does not seem to have such an activity. We speculate, therefore, that the BH3 domain of Scythe is a docking site for a heterodimerized pro-apoptotic bcl-2 family member. This possibility is currently under investigation. However, since bcl-2 family members may form higher order multimers (e.g. for formation of pores in mitochondrial membranes), the role of Scythe-bound Hsc70 may, in this context, be to assemble higher order complexes, rather than to properly fold monomeric 'X' (Adams and Cory, 1998; Lewis *et al.*, 1998).

Although our studies have thus far been confined to analysis of a *Drosophila* protein (Reaper) in a vertebrate system (*Xenopus* egg extracts), we note that the function of Scythe and its BAG domain may ultimately prove to be important in the context of fly apoptosis. As has recently been described, the *Drosophila* genome contains an apparent Scythe homolog (Adams *et al.*, 2000; Jasny, 2000). Moreover, in preliminary studies we have found that *in vitro* translated fly Scythe can bind to fly Reaper (K.Thress and S.Kornbluth, unpublished).

Regulation of cellular signaling through modulation of Hsp70/Hsc70

Although Hsp70 family chaperones are abundant in the cell, proteins like BAG-1 and Scythe may confer substrate specificity on these proteins, promoting protein folding/assembly of bound substrates in a regulated manner. Held in an inactive or sequestered state, Hsc70 substrates bound

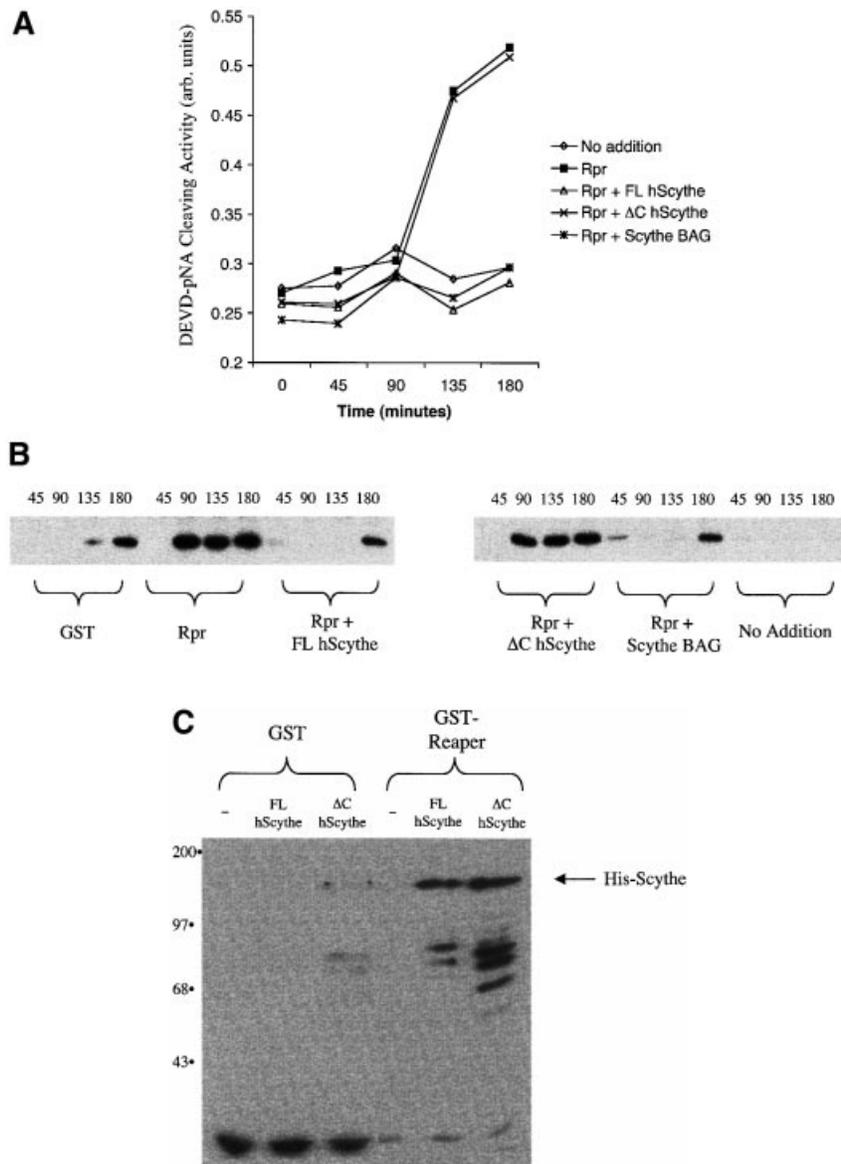


Fig. 6. BAG-deficient human Scythe is unable to protect against Reaper-induced apoptosis. **(A)** Recombinant Reaper (Rpr) protein (300 ng/ μ l) was added to *Xenopus* egg extracts in combination with equivalent amounts of recombinant full-length human Scythe (FL hScythe), BAG-deficient human Scythe (Δ C hScythe) or just the BAG domain of human Scythe (Scythe BAG). At the indicated times, 2 μ l aliquots of extract were analyzed for caspase activity by cleavage of the artificial caspase substrate, DEVD-pNA. Following cleavage, released pNA was measured spectrophotometrically. **(B)** Samples were processed as in (A), but 15 μ l aliquots were filtered through a 0.1 μ m microfilter to remove particulate components, including mitochondria, and samples were processed for immunoblotting with an anti-cytochrome *c* monoclonal antibody. **(C)** Recombinant GST or GST-Reaper fusion proteins immobilized on glutathione-Sepharose beads were added to *Xenopus* egg extract and incubated at 4°C in the presence of equivalent amounts of either His-tagged full-length human Scythe (FL hScythe) or BAG-deficient Scythe (Δ C hScythe) proteins. After 1 h, the beads were pelleted, washed three times with ELB, resuspended in SDS sample buffer, and processed for immunoblotting using a monoclonal penta-His antibody.

to Scythe/BAG-like proteins could be tightly controlled by binding of specific ligands able to dissociate Scythe-Hsc70 or BAG-1-Hsc70 complexes. That these reactions would have the requisite specificity is highlighted by the fact that Reaper was able to reverse Scythe-mediated inhibition of Hsp70 protein folding, while having no effect on similar inhibition by BAG-1. As alluded to earlier, BAG-1 binds a number of cellular signaling molecules; it is not known whether Scythe is similarly diverse in its binding partners. Moreover, it is not clear whether distinct ligands might have differential effects on different BAG-substrate or Scythe-substrate complexes. Nonetheless, regulatory networks of ligand-

co-chaperone-Hsc70 proteins as exemplified by Reaper-Scythe-Hsc70 offer a novel means to regulate the activity of cell signaling molecules critical for cell proliferation, cell death or cellular responses to stress.

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). Fourteen to twenty hours after injection with HCG, eggs were harvested for extract production. Jelly coats were removed from eggs by incubation with 2% cysteine pH 7.8,

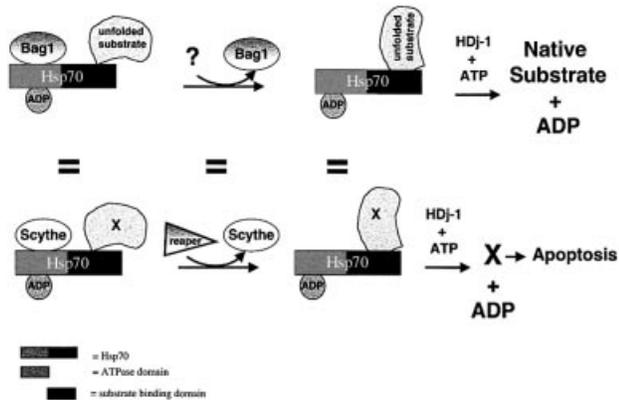


Fig. 7. Model for Reaper/Scythe function. BAG-1 binds to the ATPase domain of Hsp70 and inhibits its ability to mediate protein folding. In the presence of a hypothetical ligand, BAG-1 is released from Hsp70, promoting release of native substrate. In the case of Scythe we hypothesize that a similar series of events occurs; however, Reaper serves as the ligand to trigger Scythe dissociation from the Hsp70 complex. According to this speculative model, 'X' is released in its native form and can then trigger mitochondrial cytochrome *c* release and caspase activation. The figure has been adapted from Bimston *et al.* (1998).

washed three times in modified Ringers solution (1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 25 mM CaCl₂, 5 mM HEPES pH 7.8, 0.8 mM EDTA), and then washed in ELB [250 mM sucrose, 2.5 mM MgCl₂, 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 demembrated 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 CaCl₂, 1 mM MgCl₂, 10 mM K-HEPES pH 7.7) and added at a concentration of 300 ng/µl, unless otherwise indicated.

Preparation of GST fusion proteins

Full-length *Xenopus* Hsc70 was PCR amplified using the following primers: 5'-GATCTCTAGACATGCTCTAAGGGACCAGCAGTT-3' and 5'-GATCCTCGAGTTAGTCAACCTCCTCAATAGT-3'. PCR fragments were cloned into the *Xba*I-*Xho*I sites of 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). Full-length *Drosophila* Reaper and the C-terminal 312 amino acids of Scythe (Scythe C312) fused with GST were produced in a similar manner and constructed as described in Thress *et al.* (1998). GST fusions of human BAG-1 and Hsp70 were produced as previously described (Bimston *et al.*, 1998).

Baculovirus production of human Scythe protein

Full-length human Scythe (FL hScythe) and a truncated hScythe lacking the C-terminal 81 amino acids (hScythe ΔC) were both PCR amplified to possess a C-terminal His₆ tag and cloned into the *Xba*I-*Xho*I sites of the pFastBac vector. Protein was produced using the Bac-to-Bac Baculovirus Expression System (Gibco). Briefly, the resulting hScythe-pFastBac donor plasmids were transformed into DH10Bac *Escherichia coli* cells. *Escherichia coli* 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 resulting recombinant baculovirus particles were harvested. Subsequently, SF-9 cells (2 × 10⁶ cells/ml) were infected with baculovirus for 48 h, washed twice in phosphate-buffered saline 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]. 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 vols of HBS and eluted with HBS containing 200 mM imidazole in five fractions of 500 µl each.

Cell culture and transfections

FL hScythe, hScythe lacking the N-terminal 87 amino acids (hScythe ΔN) or hScythe ΔC was PCR amplified and cloned into a modified pcDNA3.1 mammalian expression vector (Invitrogen) containing an in-frame C-terminal myc tag. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). Cells (5 × 10⁵) were plated onto 60 mm dishes and 24 h later transfected with 3 µg of the appropriate myc-tagged hScythe construct or vector alone DNA using the Fugene transfection reagent (Roche biochemicals) as per manufacturer's instructions.

Immunoprecipitations

Thirty-six hours after transfection, cells were harvested in lysis buffer (10 mM Tris pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2% Tween-20, 10% glycerol) and lysates were incubated with anti-myc monoclonal antibody (Santa Cruz) for 2 h at 4°C. PAS beads were added to lysates, incubated for an additional 1 h, pelleted and washed three times in lysis buffer. Bound proteins were solubilized with sample buffer and resolved by SDS-PAGE. Resolved proteins were transferred to PVDF, blotted with an anti-Hsp70/Hsc70 monoclonal antibody (Affinity Bioreagents), incubated with HRP-linked goat anti-mouse secondary antibody and detected using the ECL system (Amersham).

Protein refolding assays

Protein refolding assays were conducted as previously described (Freeman *et al.*, 1996).

DEVDase assay

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 mM; 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.

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