Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper

Kenneth Thress, Jaewhan Song1, Richard I. Morimoto1 and Sally Kornbluth2

Department of Pharmacology and Cancer Biology, C370 LSRC, Research Drive, Duke University Medical Center, Durham, NC 27710 and 1Rice Institute for Biomedical Research, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 North Campus Drive, Evanston, IL 60208, USA

2Corresponding author

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...
sequester an as yet unidentified direct inducer of mitochondrial cytochrome c release (Thress et al., 1999). Upon binding of Reaper, Scythe released this factor(s), leading to mitochondrial cytochrome c release, caspase activation and full apoptosis. This series of events was recapitulated in a semi-purified system in that immuno-precipitates of Scythe, when washed extensively and incubated with Reaper, released a factor(s) capable of initiating cytochrome c release directly from purified mitochondria.

In experiments reported here, we show that the apparent similarities between BAG-1 and Scythe (the presence of an N-terminal ubiquitin domain, anti-apoptotic activity) are likely to be more than superficial. Indeed, we show that Scythe, like BAG-1, is a direct inhibitor of Hsp70 protein folding activity. Moreover, a BAG domain in Scythe mediates this inhibition. However, while the physiological means of reversing BAG-1-mediated Hsp70 inhibition are not known, we have found that Reaper can relieve Scythe-mediated repression of Hsp70. These data provide the first evidence for reversibility of Hsp70 inhibition by a co-chaperone ligand.

Results

Scythe bears structural similarity to BAG family proteins
As both Scythe and BAG-1 are anti-apoptotic when overexpressed, and share, along with other BAG family proteins, an N-terminal ubiquitin-like domain, we were interested in the possibility that Scythe might also contain a BAG domain. Clustal alignments of BAG family members and both human and Xenopus Scythe proteins revealed candidate C-terminal BAG domains present in Scythe molecules from both species (Figure 1). While the overall similarity of the BAG domain across different proteins is ~30%, four strictly conserved residues, found in all BAG family members, are also conserved in Scythe.

Scythe binds to the ATPase domain of Hsc70/Hsp70 in a BAG domain-dependent fashion
As described above, BAG-1 protein is unusual in its ability to inhibit the protein folding activity of Hsc70/Hsp70 family members; this inhibitory activity depends upon the presence of the BAG domain, which provides a direct binding site for Hsc70/Hsp70 proteins. The presence of a putative BAG domain on Scythe prompted us to examine whether it too might bind Hsc70/Hsp70 proteins. Xenopus egg extract was supplemented with radiolabeled, in vitro translated Hsc70 protein and incubated with Sepharose beads linked to either glutathione S-transferase (GST) or GST fused to the C-terminal half of Scythe (Scythe C312). After pelleting and extensive washing, these resins were examined by SDS–PAGE for the presence of bound Hsc70. As shown in Figure 2A, Hsc70 bound specifically to the Scythe resin. Similarly, Sepharose-linked GST–Hsc70 efficiently bound endogenous Xenopus Scythe from egg extracts, at levels comparable to those obtained using GST–Reaper as bait (Figure 2B). To demonstrate that endogenous Scythe and Hsc70 proteins were able to interact, we immunoprecipitated Hsc70 from Xenopus egg extracts and immunoblotted the samples with anti-Scythe sera. As shown in Figure 2C, endogenous Hsc70 and Scythe proteins co-immunoprecipitated, while Scythe did not associate with a control antibody (anti-Wee1) or protein A–Sepharose alone.
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 ΔC; removal of the C-terminal 81 amino acids of Scythe), but not the N-terminal ubiquitin domain (hScythe ΔN) 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 ΔC 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, Stc (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 Scythe.
data strongly suggest that Scythe has the previously unanticipated ability to act in a BAG-1-like manner to lacking the BAG domain (Scythe-mediated refolding of denatured completely inhibited (Figure 3). Importantly, the refolding (Figure 3). However, when full-length Scythe was added as a Reaper ligand, we wished to determine whether 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 refolding. To test this, we added increasing amounts of Reaper to the Scythe±Hsc70 binding was observed when we added excess recombinant Scythe to 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.

**Reaper inhibits the physical association of Scythe and Hsp70**

Since a Scythe mutant unable to physically bind Hsp70 (ΔC 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).

**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 (ΔC Scythe). Under these conditions, the wild-type protein, but not the ΔC mutant Scythe, markedly dampened caspase activation and mitochondrial cytochrome c release in response to Reaper addition (Figure 6A and B). Importantly, while ΔC Scythe was unable to bind Hsc70 and inhibit Reaper-induced caspase activation, the ΔC 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
effective as excess full-length Scythe in abrogating Reaper-induced caspase activation and cytochrome c release (Figure 6A and B). These data suggest that the BAG domain, required for both Hsc70 binding and inhibition of Hsc70/Hsp70-mediated protein folding is also required for effective re-sequestration of the apoptosis-inducing factors released from endogenous Scythe by Reaper.

**Discussion**

Scythe is a Reaper-interacting protein critical for Reaper-induced apoptosis in *Xenopus* egg extracts. In this report, we demonstrate that Scythe is also a modulator of Hsc70/Hsp70, able to inhibit chaperone-mediated protein folding. Reaper, in turn, inhibits this activity of Scythe. These findings raise the intriguing possibility that regulation of protein folding plays an important role in control of apoptosis by Reaper.

**Reaper reversal of Scythe-mediated Hsp70 repression**

Although BAG-1 was previously shown to inhibit Hsp70 function, a ligand able to associate with the BAG-1–Hsp70–substrate complex, dissociate BAG-1 and allow resumption of protein folding was only speculated (Bimston *et al.*, 1998). Clearly, if BAG-1, or molecules like it, are to be considered viable regulators of protein homeostasis, the inhibition of Hsp70 must be reversible. In this report, we have identified Reaper as a ligand capable of reversing Scythe-mediated inhibition of Hsp70. Coincident with this reversal, Scythe is displaced from

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**Fig. 4.** Reaper specifically relieves Scythe-mediated inhibition of Hsp70. (A) The experiment shown in Figure 3 was repeated with 3.2 μM human Scythe in the presence of increasing concentrations of Reaper (3.2–12.8 μM) to examine the reversal of Scythe-mediated inhibition on Hsp70-mediated refolding. (B) The inhibitory effect of BAG-1 (1.6 μM) on refolding was observed in the presence or absence of 12.8 μM Reaper, demonstrating that Reaper-induced reversal is specific for Scythe. As a control for spontaneous refolding, denatured β-galactosidase (3.2 nM) was diluted into refolding buffer containing 3.2 μM BSA.
BAG-1 (Figure 7), we hypothesize that the cytochrome c-releasing factor(s) sequestered by Scythe (denoted as ‘X’ molecules. Moreover, if Scythe functions mechanistically (Figure 2F), making the substrate-binding domain of Scythe–Hsp70 complexes dissociate; according to our we do not yet know whether cytochrome c in Figure 7) is held in a soluble, partially folded state Hsp70 and ‘X’ is direct. Upon binding to Reaper, apoptosis? Building on the model originally proposed for BAG-1, 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

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**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 Ni⁺-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 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. 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.**
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).

Preparation of GST fusion proteins

Full-length Xenopus Hsc70 was PCR amplified using the following primers: 5′-GATCTCTCAGGCAATGGGAGCCGAGCATTT-3′ and 5′-GATCTCCGATTTGTCACCCTCATTGATGTT-3′. PCR fragments were cloned into the XbaI–Xhol 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 Scythe were produced as previously described (Bimston et al., 1998).

Baculovirus production of human Scythe protein

Full-length human Scythe (FL ᵃScythe) and a truncated ᵃScythe lacking the C-terminal 81 amino acids (孵Scythe ΔC) were both PCR amplified to possess a C-terminal His₆ tag and cloned into the XbaI–Xhol sites of the pFastBac vector. Protein was produced using the Bac-to-Bac Baculovirus Expression System (Gibco). Briefly, the resulting ᵃScythe-pFastBac donor plasmids were transformed into DH10Bac Escherichia coli cells. Escherichia coli containing 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 ᵃScythe, ᵃScythe lacking the N-terminal 87 amino acids (孵Scythe AN) or ᵃScythe ΔC was PCR amplified and cloned into a modified pcDNA3 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 × 10⁶) were plated onto 60 mm dishes and 24 h later transducted with 3 μg of the appropriate myc-tagged ᵃScythe 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-myec 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/Scythe monocular 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 μM; Biomol) was measured at 405 nm in a LabSystems MultiSkan MS microtiter plate reader.

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