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BAG-1 modulates the chaperone activity of Hsp70/Hsc70

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The 70 kDa heat shock family of molecular chaperones is essential to a variety of cellular processes, yet it is unclear how these proteins are regulated *in vivo*. We present evidence that the protein BAG-1 is a potential modulator of the molecular chaperones, Hsp70 and Hsc70. BAG-1 binds to the ATPase domain of Hsp70 and Hsc70, without requirement for their carboxy-terminal peptide-binding domain, and can be co-immunoprecipitated with Hsp/Hsc70 from cell lysates. Purified BAG-1 and Hsp/Hsc70 efficiently form heteromeric complexes *in vitro*. BAG-1 inhibits Hsp/Hsc70-mediated *in vitro* refolding of an unfolded protein substrate, whereas BAG-1 mutants that fail to bind Hsp/Hsc70 do not affect chaperone activity. The binding of BAG-1 to one of its known cellular targets, Bcl-2, in cell lysates was found to be dependent on ATP, consistent with the possible involvement of Hsp/Hsc70 in complex formation. Overexpression of BAG-1 also protected certain cell lines from heat shock-induced cell death. The identification of Hsp/Hsc70 as a partner protein for BAG-1 may explain the diverse interactions observed between BAG-1 and several other proteins, including Raf-1, steroid hormone receptors and certain tyrosine kinase growth factor receptors. The inhibitory effects of BAG-1 on Hsp/Hsc70 chaperone activity suggest that BAG-1 represents a novel type of chaperone regulatory proteins and thus suggest a link between cell signaling, cell death and the stress response.

Keywords: BAG-1/Bcl-2/chaperone/Hsc70/Hsp70

Introduction

BAG-1 is a novel multifunctional protein that was first identified based on its ability to bind the anti-apoptotic protein Bcl-2 and to promote cell survival (Takayama *et al.*, 1995). Since its initial discovery as a Bcl-2-binding protein, however, BAG-1 has been reported to interact with and modulate the activities of other proteins. For example, BAG-1 can bind to the catalytic domain of the serine/threonine-specific protein kinase Raf-1, based on yeast two-hybrid experiments, *in vitro* protein-binding assays and co-immunoprecipitation experiments. Moreover, BAG-1 can stimulate Raf-1 kinase activity through a Ras-independent mechanism (Wang *et al.*, 1996a). Raf-1 also binds to Bcl-2 and cooperates in the suppression of apoptosis (Wang *et al.*, 1994, 1996b); therefore, the interaction of BAG-1 with Raf-1 has been hypothesized to provide a mechanism for local action of this kinase in the vicinity of Bcl-2. Recently, however, BAG-1 was also reported to bind the plasma membrane-associated tyrosine kinase growth factor receptors for hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF), enhancing the ability of these receptors to deliver signals for cell survival in the apparent absence of effects on signal transduction pathways linked to mitogenesis such as the Ras/Raf/MEK cascade (Bardelli *et al.*, 1996). BAG-1 can also bind to several steroid hormone receptors (Zeiner and Gehring, 1995), though the functional significance of this interaction remains unknown.

These observations suggest a general facilitatory role for BAG-1 in signal transduction pathways involved in the regulation of cell survival and possibly in the control of other cellular processes as well. How BAG-1 accomplishes these disparate biochemical functions, however, is unclear. Using interaction cloning techniques, we have identified the molecular chaperones, Hsp70 and Hsc70, as BAG-1-binding proteins. The identification of Hsp/Hsc70 as an accessory protein of BAG-1 suggests a possible role for chaperone interactions in regulating the activities of BAG-1. Furthermore, BAG-1 exhibits a novel activity, inasmuch as it functions as a potent inhibitor of chaperone-mediated folding reactions *in vitro*. Hitherto, no inhibitors of chaperone activity have been described, making BAG-1 the first such protein to be identified.

Results

BAG-1 interacts with the ATPase domain of Hsc70

During ligand blotting experiments using recombinant BAG-1 protein as a probe, we noted an ~70 kDa BAG-1-binding protein in extracts prepared from many different types of cells derived from several species (unpublished data). We therefore attempted to identify cDNAs encoding this evolutionarily conserved protein by screening a λgt-11 expression cDNA library using GST–BAG-1 protein as a probe, as well as by yeast two-hybrid methods (Takayama *et al.*, 1995; Takayama and Reed, 1996). Both approaches resulted in the cloning of cDNAs encoding Hsc70, with the overlapping region common to these cDNAs corresponding to amino acids 186–377 of the ATP-binding domain of human Hsc70 (Figure 1A). Indeed, two of the cDNA clones encoded only the ATPase domain of Hsc70

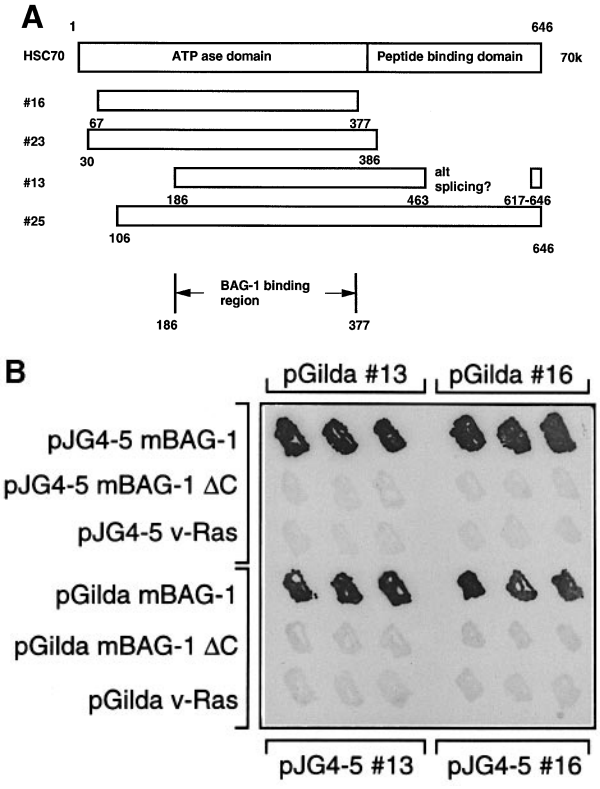


Fig. 1. Interaction cloning results for BAG-1. (**A**) The results of the interaction cloning experiments are presented schematically, showing the portions of human Hsc70 encoded by the cDNAs which were obtained either by screening a λgt-11 human fetal brain cDNA library using GST–BAG-1 protein as a probe (clones #16 and #23) or by yeast two-hybrid screening of a human T cell cDNA library using LexA–BAG-1 as a bait (clones #13 and #25). The deduced minimal region of Hsc70 that should be necessary for BAG-1 binding is indicated and corresponds to the ATPase domain. (**B**) EGY48 strain yeast were transformed with 1 μg each of the indicated LexA DNA-binding domain (pGilda) and transactivation domain (pJG4.5) plasmids and plated initially on medium containing glucose to repress the *Gal1* promoters in the two-hybrid plasmids and leucine to permit growth of auxotrophs in the absence of two-hybrid interactions. Three independent colonies were chosen randomly from each transformation, patched to medium containing glucose and leucine, and then replica plated onto medium containing galactose (for induction of the two-hybrid plasmids) and leucine. Two-hybrid-based interactions were detected by β-galactosidase filter assays. Data shown represent color development after 1 h incubation with X-gal. The BAG-1 (ΔC) mutant is missing the last 47 amino acids of the murine BAG-1 protein. v-Ras is presented as a typical example of a negative control protein. The Hsc70 partial cDNA clones #13 and #16 are depicted in (A).

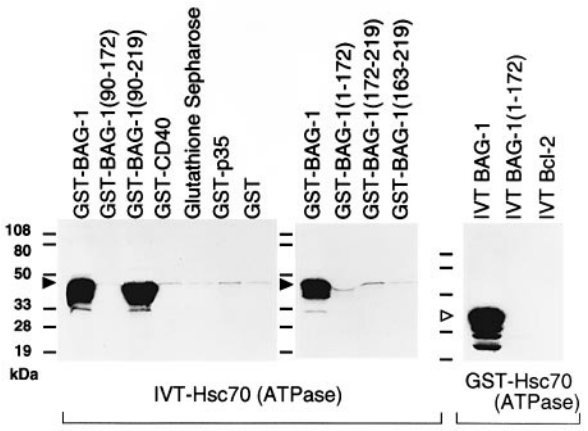


Fig. 2. BAG-1 binds to the ATPase domain of Hsp/Hsc70. BAG-1 interactions with the ATPase domain of Hsc70 were tested by *in vitro* binding assays where BAG-1 or various BAG-1 mutants were expressed as GST fusion proteins (left and middle panels) or *in vitro* translated (IVT) in the presence of [^{35}S]L-methionine (right panel) and incubated with either ^{35}S-labeled, IVT Hsc70 [ATPase domain (residues 1–386)] or with GST–Hsc70(ATPase), respectively. GST fusions representing the cytosolic domain of CD40 or the baculovirus p35 protein were included as controls.

(clones #16 and #23), and were devoid of sequences corresponding to the peptide-binding domain which can interact with proteins in non-native conformations.

The interaction of these fragments of Hsc70 with BAG-1 was readily demonstrable by two-hybrid assays, regardless of whether Hsc70 and BAG-1 were expressed as fusion proteins with an appended DNA-binding domain or with an attached transactivation domain (Figure 1B). In contrast, neither Hsc70 nor BAG-1 interacted in two-hybrid assays with a number of control proteins, including Ras, lamin-B or the cytosolic domains of Fas (CD95) and CD40 (Figure 1B and data not presented). Moreover, certain deletion mutants of BAG-1 failed to display interactions with Hsc70 in two-hybrid assays, such as a carboxy-terminal truncation mutant missing the last 47 amino acids of BAG-1 (Figure 1B), further implying a specific interaction between these two proteins.

To confirm the interaction of BAG-1 with Hsc70 by an independent method, a GST–BAG-1 fusion protein was produced in bacteria and examined for its ability to bind the *in vitro* translated ^{35}S-labeled ATPase domain of Hsc70 (Figure 2, left panel). Hsc70(ATPase) bound to GST–BAG-1 but not to several control GST proteins and not to the glutathione–Sepharose beads used to recover GST fusion proteins. Conversely, when the ATPase domain of Hsc70 was produced as a GST fusion protein and assayed for binding to various ^{35}S-labeled *in vitro* translated proteins, interaction with only BAG-1 was detected. (Figure 2, right panel).

The BAG-1 protein contains a domain with similarity to ubiquitin (residues 48–89 in the 219 amino acid long murine BAG-1 protein). Previous studies have shown that the first 89 amino acids of BAG-1 are expendable for its binding to Bcl-2 and to the kinase Raf-1 (Wang *et al.*, 1996a and unpublished data). Similarly, a GST–BAG-1 mutant lacking residues 1–89 also bound to [^{35}S]Hsc70-(ATP-binding domain) with efficiency comparable with the full-length BAG-1 (Figure 2). In contrast, a mutant of BAG-1 containing only residues 90–172 failed to interact with Hsc70(ATP-binding domain), implying that the region of BAG-1 from 172 to 219 is essential for Hsc70 binding. A C-terminal truncation mutant of BAG-1 lacking only the last 47 amino acids of BAG-1, GST–BAG-1(1–172), also was unable to bind Hsc70(ATP-binding domain), further demonstrating the importance of the region from 172 to the C-terminus. Though important for BAG-1–Hsc70 interactions, the last 48 amino acids of BAG-1 were insufficient for Hsc70(ATPase) binding (Figure 2), implying that additional residues upstream of amino acid 172 are necessary for binding or for proper folding of the domain in BAG-1 which interacts with Hsc70(ATPase). Taken together, these *in vitro* binding studies confirm the ability of BAG-1 to interact with the N-terminal ATPase

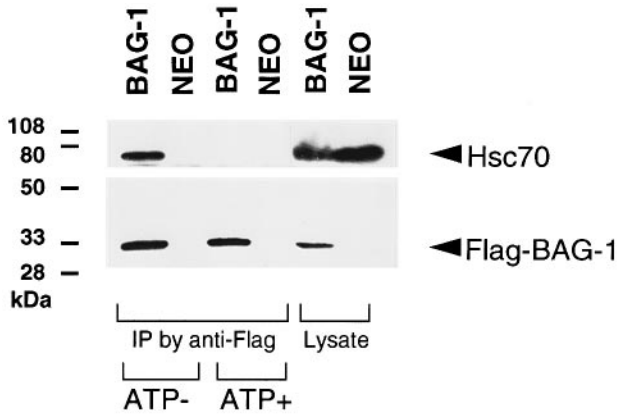


Fig. 3. Hsp/Hsc70 co-immunoprecipitates with BAG-1 in mammalian cells. Cell lysates were prepared with or without addition of 10 mM ATP from FLAG-BAG-1-expressing or Neo control 293 cells transfectants and subjected to immunoprecipitation using anti-FLAG tag antibody followed by SDS–PAGE/immunoblotting using either anti-Hsp70/Hsc70 or anti-FLAG antibodies (top and bottom portions of the blot, respectively). Lysates (one-tenth input) were also loaded directly in the gel, without immunoprecipitation (far right; last two lanes) as a control.

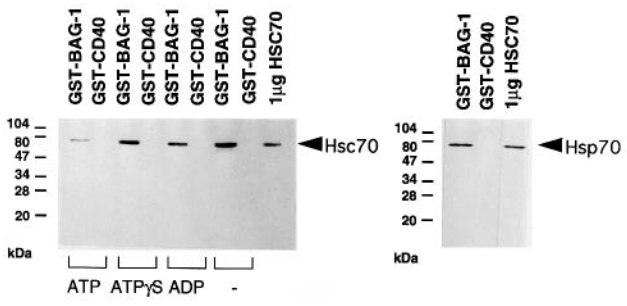


Fig. 4. BAG-1 interaction with Hsc70 is regulated by ATP hydrolysis. Equivalent amounts of purified GST–BAG-1 or GST–CD40 cytosolic domain (negative control) immobilized on glutathione–Sepharose were incubated with either purified Hsp70 or Hsc70 (5 μg) in the presence of 10 mM ATP, ADP or ATPγS as indicated. After washing the beads extensively, associated Hsp/Hsc70 was detected by immunoblotting. One μg of purified Hsc70 or Hsp70 was loaded directly in the gels as a control.

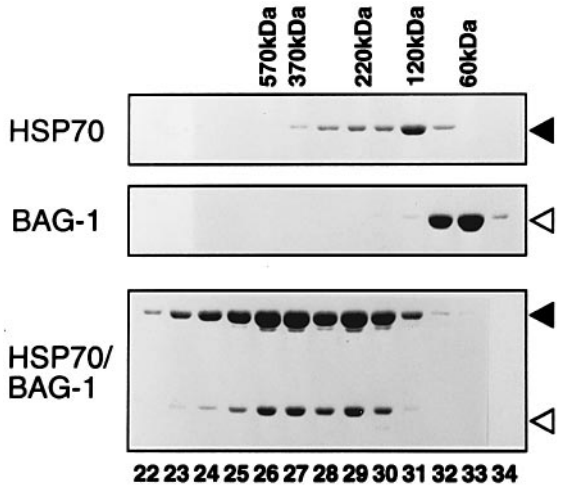


Fig. 5. Gel-sieve chromatographic analysis of BAG-1–Hsp70 complexes. Purified BAG-1, Hsp70 or the combination of BAG-1 and Hsc70 were subjected to gel-sieve chromatography and the eluted fractions were analyzed for the presence of BAG-1 and Hsp70 by SDS–PAGE with Coomassie staining. The elution positions of molecular weight standard proteins are indicated. Dark arrows indicate Hsp70, while open arrows denote BAG-1.

domain of Hsc70, independently of its C-terminal peptide-binding domain.

Evidence for BAG-1 interactions with Hsc70 in cells

Co-immunoprecipitation assays were performed to explore whether BAG-1 associated with the full-length Hsc70 protein in lysates of mammalian cells. For these experiments, a cDNA encoding a FLAG epitope-tagged BAG-1 protein or the parental plasmid was stably transfected into 293 human kidney epithelial cells. Immunoprecipitations were performed using the anti-FLAG monoclonal antibody M2, and the resulting immune complexes were subjected to SDS–PAGE/immunoblotting using the anti-Hsp/Hsc70 antibody 3a3 or the anti-FLAG monoclonal antibody M2. As shown in Figure 3, Hsc70 was recovered in association with the anti-FLAG immune complexes prepared from the FLAG-BAG-1-expressing 293 cells but not from the control 293-Neo cells.

Previous investigations of Hsc/Hsp70 family proteins have shown that ATP binding induces a conformational change in the ATPase domain (Ha and McKay, 1995; Fung *et al*., 1996). Addition of 10 mM ATP to cell lysates prior to immunoprecipitation reduced the association of Hsc70 with BAG-1 (Figure 3), suggesting a conformational dependence of this interaction. Similar co-immunoprecipitation results were obtained using monoclonal antibodies directed against the BAG-1 protein (Takayama *et al.*, 1996) rather than relying upon epitope tags and when employing cell lines (such as the Jurkat T-cell leukemia line) that contain relatively high levels of endogenous BAG-1 protein (data not presented). Thus, gene transfer-mediated overexpression of epitope-tagged BAG-1 is not required for demonstrating interactions with Hsc70 *in vivo*.

BAG-1 binds directly to Hsp70 and Hsc70

The association of BAG-1 with Hsc70 could be direct or mediated by accessory proteins. To distinguish between these possibilities, purified GST–BAG-1 was tested for binding *in vitro* to recombinant, purified Hsc70 and Hsp70. For these experiments, 5 μg of purified Hsc70 or Hsp70 was incubated with 10 μg of GST–BAG-1 immobilized on glutathione–Sepharose, and the BAG-1-associated Hsc/Hsp70 proteins were detected by immunoblotting using an anti-Hsp/Hsc70 antibody (Figure 4). Both Hsc70 and Hsp70 bound to GST–BAG-1, with ~20% of the input chaperone recovered in association with BAG-1, but not to a control GST fusion protein (CD40). The addition of ATP to these binding assays significantly reduced binding of Hsc70 to GST–BAG-1, whereas ADP and non-hydrolyzable ATPγS had less effect.

Molecular sieve chromatography was employed as an additional means of evaluating the BAG-1–Hsp70 interaction. When analyzed by itself, Hsp70 eluted from a Sepharose-6 column with a peak corresponding to Hsp70 homodimers, and BAG-1 eluted from the column with a peak corresponding to BAG-1 homodimers (Figure 5). When BAG-1 and Hsp70 were co-incubated in equimolar quantities and chromatographed, all of the BAG-1 and

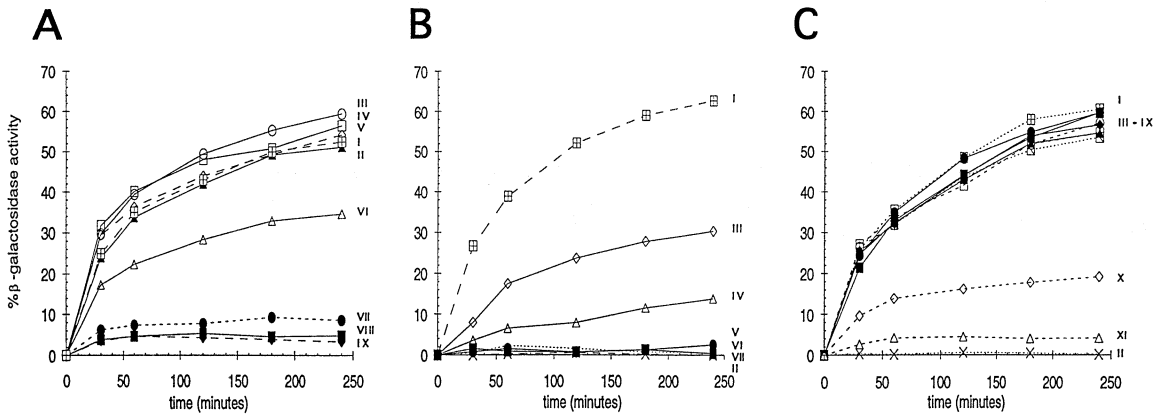


Fig. 6. BAG-1 inhibits Hsc70- and Hsp70-mediated refolding of denatured β-galactosidase. (**A**) BAG-1-mediated inhibition of Hsc70-dependent refolding was examined by diluting denatured β-galactosidase (3.4 μM final) into refolding buffer containing 1.6 μM Hsc70 and 3.2 μM Hdj-1 without (I) or with increasing concentrations of full-length BAG-1: 0.05 (III), 0.1 (IV), 0.2 (V), 0.4 (VI), 0.8 (VII), 1.6 (VIII), 3.2 μM BAG-1 (IX). As a control for inhibition of refolding, denatured β-galactosidase was diluted into refolding buffer containing Hsc70, Hdj-1 and 3.2 μM BSA (II). (**B**) The effect of BAG-1 on Hsp70-mediated β-galactosidase refolding was examined in a similar fashion by adding increasing concentrations of BAG-1 to a refolding reaction containing 1.6 μM Hsp70 and 3.2 μM hdj-1 (I): 0.2 (III), 0.4 (IV), 0.8 (V), 1.6 (VI), 3.2 μM BAG-1 (VII). As a control for spontaneous refolding, denatured β-galactosidase was diluted into refolding buffer containing 1.6 μM BSA (II). (**C**) The effects of BAG-1 ΔC (contains amino acids 1–172) and BAG-1 ΔN (contains amino acids 90–219) deletion mutants on chaperone-mediated refolding were examined: 0.4 μM BAG-1 ΔC mutant (III), 0.8 μM BAG-1 ΔC mutant (IV), 1.6 μM BAG-1 ΔC mutant (V), 3.2 μM BAG-1 ΔC mutant (VI), 0.4 μM BAG-1 ΔN mutant (VII), 0.8 μM BAG-1 ΔN mutant (VIII), 1.6 μM BAG-1 ΔN mutant (X), 3.2 μM BAG-1 ΔN mutant (XI). Denatured β-galactosidase was diluted into refolding buffer containing either Hsc70 and Hdj-1 (I) or Hsc70, Hdj-1 and 3.2 μM (IX) as positive controls and 1.6 μM BSA (II) as a negative control and β-galactosidase assays were performed as described (Freeman and Morimoto, 1996).

Hsc70 proteins eluted in complexes >120 kDa, thus confirming the efficient formation of BAG-1–Hsp70 complexes. The elution characteristics of the BAG-1–Hsp70 complexes were consistent with a minimal stoichiometry of 2:2 (heterotetramer), though larger complexes were also present. Dimer formation by BAG-1 alone was confirmed by dynamic light-scattering experiments (not shown).

BAG-1 modulates the in vitro chaperone activity of Hsp70

Unlike other members of the chaperone family (Hsp90, cyclophilin 40, p23) which maintain unfolded proteins in a transitional state amenable to subsequent refolding, Hsp/Hsc70 can also complete the process of refolding non-native proteins to their native structures through a ATP and co-chaperone (Hdj-1)-dependent mechanism (Freeman and Morimoto, 1996; Freeman *et al.*, 1996). We therefore evaluated the effects of purified GST–BAG-1 on the ability of Hsc70 and Hsp70 to assist in the refolding of denatured β-galactosidase. In the absence of molecular chaperones, denatured β-galactosidase aggregates and does not regain enzymatic activity. In contrast, in the presence of ATP and Hdj-1, denatured, enzymatically inactive β-galactosidase can be refolded by Hsp/Hsc70 with recovery of ~50% of the enzymatically active protein. When BAG-1 was added to the assay, Hsc70-mediated refolding of denatured β-galactosidase was inhibited in a concentration-dependent manner, with complete inhibition achieved at a 1:1 molar ratio (Figure 6A). Essentially identical results were noted for BAG-1 inhibition of Hsp70-mediated refolding of β-galactosidase (Figure 6B)

The specificity of the inhibitory effect of BAG-1 on the chaperoning activities of Hsc70 and Hsp70 was confirmed by use of a GST–BAG-1 (ΔC) mutant deleted for the C-terminal 47 residues of BAG-1. This mutant form of BAG-1 failed to bind to Hsc/Hsp70 and did not affect the refolding assay. In contrast, a GST–BAG-1 (ΔN) mutant retaining Hsp/Hsc70 binding ability was nearly as active as the full-length BAG-1 in inhibiting Hsc/Hsp70-mediated refolding of denatured β-galactosidase (Figure 6C and data not shown). Thus, BAG-1 binding to Hsp/Hsc70 correlates with BAG-1-mediated suppression of Hsp/Hsc70 chaperone activity.

BAG-1 stably associates with the chaperone–substrate complex

Hsp70 family members bind to non-native proteins via the C-terminal peptide-binding domain. To explore the effect of BAG-1 on interactions of Hsp70 with a denatured protein substrate, Hsp70 was incubated with the permanently unfolded substrate ^{125}I-labeled reduced carboxymethylated α-lactalbumin (RCMLA). The formation of Hsp70–RCMLA heteromeric complexes in the presence or absence of purified GST–BAG-1 was monitored by native polyacrylamide gel electrophoresis. Hsp70, but not BAG-1 or bovine serum albumin (BSA), forms a complex with RCMLA, resulting in the appearance of a slower migrating band (Figure 7A). In contrast, when GST–BAG-1 was added at a 1:1 molar ratio with Hsp70, a 'super-shifted' complex was detected, consistent with the idea that BAG-1 stably associates with the Hsp70–RCMLA complex.

At higher BAG-1:Hsp70 ratios, typically little or no complexes with the [^{125}I]RCMLA substrate were detected (see Figure 7A, lane 6), implying that BAG-1 either prevented the formation of Hsp70–RCMLA complexes or promoted their rapid disassembly under these conditions. We cannot exclude the rather unlikely possibility, however, that, at higher BAG-1 concentrations, the size of the trimeric BAG-1–Hsp70–RCMLA complexes impairs their ability to enter gels.

The specificity of the interaction of BAG-1 with Hsp70–RCLMA complexes was confirmed by experiments

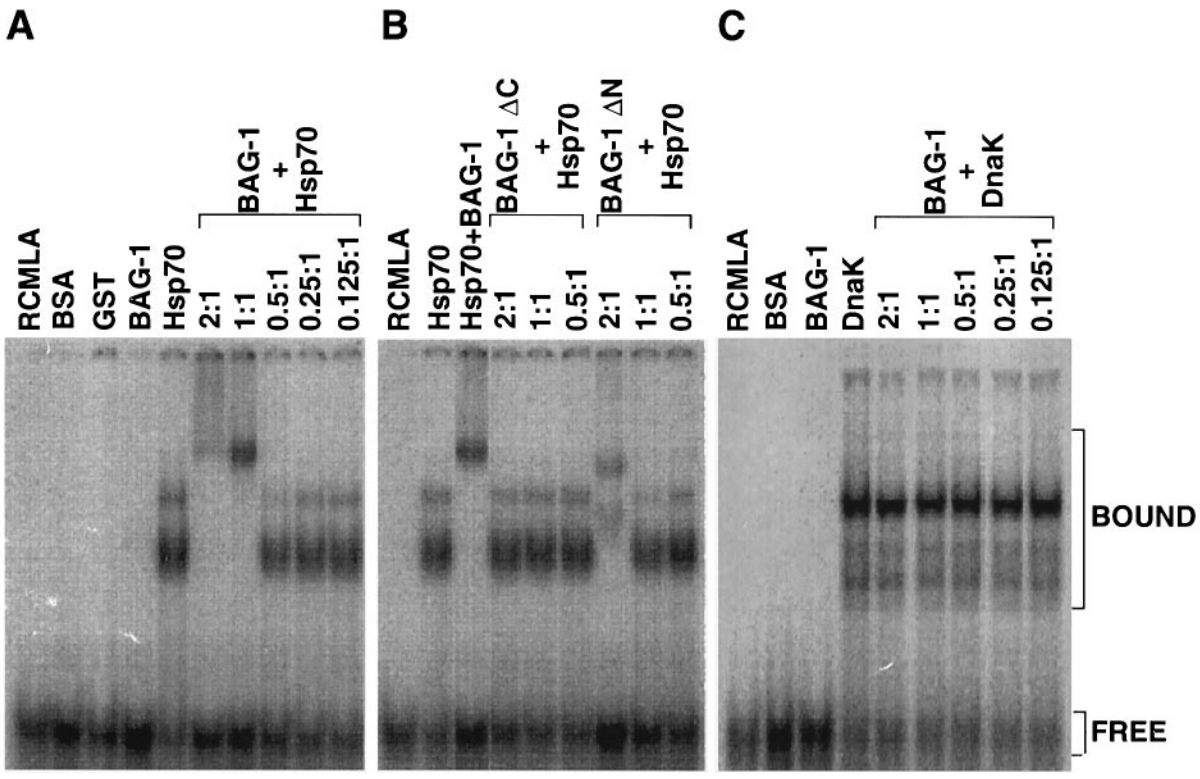


Fig. 7. Differential effects of BAG-1 on Hsp70 and DNaK interactions with a denatured protein substrate. ^{125}I-labeled RCMLA was incubated with 14 μM Hsp70 alone or in combination with various molar ratios of (**A**) wild-type GST–BAG-1 or (**B**) GST–BAG-1 mutants lacking either the first 89 amino acids, GST–BAG-1 (ΔN), or the last 47 amino acids, GST–BAG-1 (ΔC), of the murine BAG-1 protein. In (**C**), 14 μM bacterial DNaK was employed instead of Hsp70. The samples were analyzed by non-denaturing polyacrylamide gel electrophoresis, followed by autoradiography. As controls, BSA or GST proteins were incubated with RCLMA, demonstrating no binding. Alternatively, RCLMA was added without other proteins. The positions of free and bound RCLMA are indicated.

employing deletion mutants of BAG-1. As shown in Figure 7B, the BAG-1 (ΔN) protein, which binds Hsc/Hsp70 and inhibits refolding, produced a super-shifted complex, whereas BAG-1 (ΔC) did not. These data strongly suggest that BAG-1 binds to chaperone–RCMLA heteromers and is a component of these super-shifted complexes.

To exclude the possibility that BAG-1 merely competes with RCMLA for binding to the peptide-binding site of the 70 kDa chaperone, the effects of BAG-1 were tested on the binding of RCMLA by either a truncation mutant of Hsp70 consisting only of the peptide-binding domain [residues 386–640] and therefore lacking the ATPase domain needed for BAG-1 binding (not shown), or by DnaK, a bacterial homolog of the eukaryotic Hsp/Hsc70 family. The Hsp70 [386–640]–RCMLA (not shown) and DnaK–RCMLA complexes (Figure 7C) were unaffected by addition of GST–BAG-1, thus confirming the specificity of the functional interaction of BAG-1 with Hsc70 and Hsp70 and providing additional evidence that BAG-1 is not recognized as a denatured substrate by the 70 kDa chaperones.

When taken together with the data demonstrating inhibition of Hsp/Hsc70 chaperone activity by BAG-1 (Figure 6), the finding that BAG-1 binds to Hsp/Hsc70–substrate complexes suggests that the interaction between chaperone and substrate is affected by BAG-1 such that chaperone folding activity is inhibited.

ATP-dependent interaction of BAG-1 with Bcl-2 suggests a role for chaperone proteins

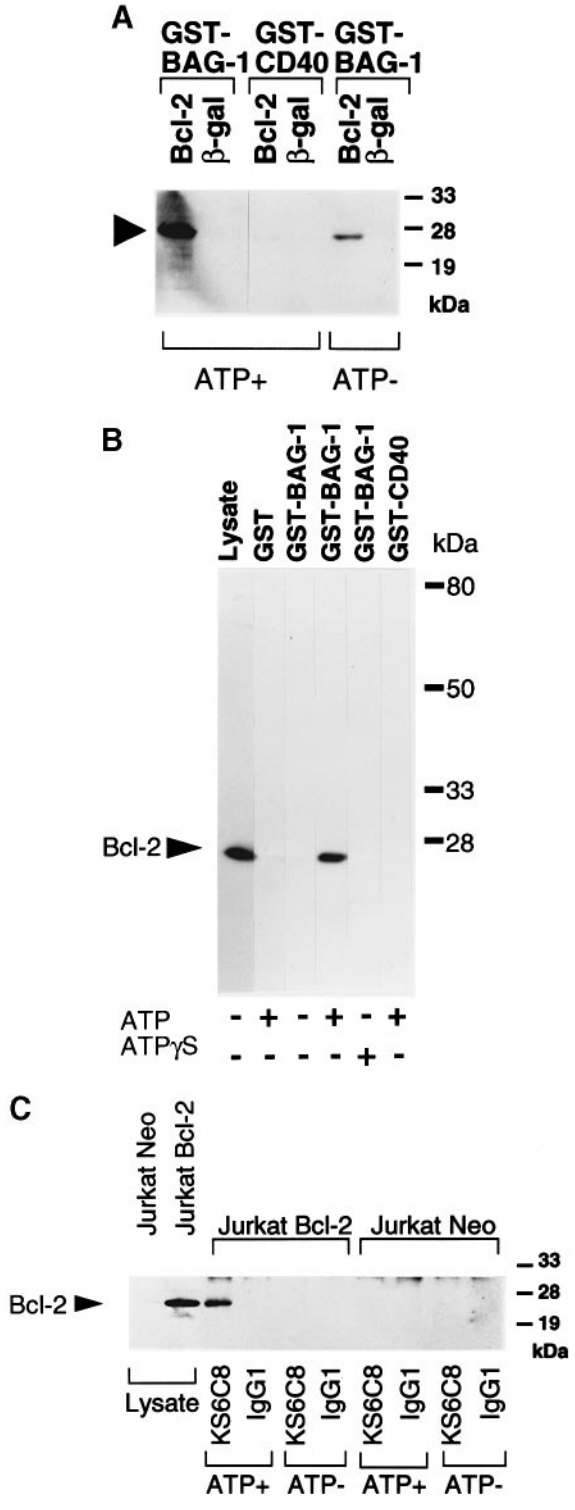
Attempts to demonstrate binding of BAG-1 to Bcl-2 using purified recombinant proteins have been unsuccessful, suggesting the need for an additional protein or proteins (unpublished observations). These findings prompted us to explore the possibility that the 70 kDa chaperones might regulate interactions between Bcl-2 and BAG-1. The ability of Hsp70 and Hsc70 to alter the conformations of proteins and to promote assembly of protein complexes is dependent on ATP hydrolysis (reviewed in Pratt and Welsh, 1994). We therefore examined the ATP dependence of Bcl-2 interactions with BAG-1, using cell lysates that contain chaperone components including Hsc70.

When purified GST–BAG-1 was incubated with cell lysates derived from insect *Sf*9 cells infected with a recombinant baculovirus encoding Bcl-2, >10-fold more Bcl-2 bound to GST–BAG-1 when 10 mM ATP was added compared with extracts without ATP supplementation (Figure 8A). In contrast, Bcl-2 did not bind to GST–CD40 or other control GST fusion proteins, regardless of ATP levels; nor did other control proteins such as β-galactosidase bind to GST–BAG-1 (Figure 8A and data not shown). Treating cell lysates with apyrase to consume endogenous ATP completely abolished the small amount of Bcl-2 binding to GST–BAG-1 that occurred in the absence of added ATP (data not presented).

Similarly, binding of GST–BAG-1 to Bcl-2 protein in

mammalian cell lysates also was increased markedly by addition of ATP. Furthermore, a requirement for ATP hydrolysis or nucleotide-specific conformational changes was suggested by use of non-hydrolyzable ATPγS, which failed to promote BAG-1 interactions with Bcl-2 in either mammalian or insect cell lysates (Figure 8B and data not shown).

In addition to experiments with GST–BAG-1 fusion protein, the ability of Bcl-2 to co-immunoprecipitate with endogenous BAG-1 was greatly enhanced if 10 mM ATP was included in the cell lysis buffer. Figure 8C, for example, shows the results derived from Jurkat T cells that had been stably transfected with either a Bcl-2-encoding plasmid (Jurkat-Bcl-2) or the same parental plasmid without an insert (Jurkat-Neo). When the endogenous BAG-1 protein was immunoprecipitated from these cells using a monoclonal antibody specific for the human BAG-1 protein (KS6C8), far more Bcl-2 protein was recovered in associated with BAG-1 when ATP was added to the lysates. Similar results were also obtained using lysates prepared from other mammalian cells (not shown). Since neither BAG-1 nor Bcl-2 have intrinsic ATP-binding or ATP hydrolysis activities (unpublished observations), we conclude that some additional protein(s) with ATP-binding capability modulates the interaction of Bcl-2 with BAG-1. Inasmuch as requirements for ATP and for ATP hydrolysis are highly characteristic of Hsp/Hsc70-mediated protein complex assembly, these results indirectly suggest a role for the Hsp/Hsc70 chaperone system in regulating BAG-1–Bcl-2 interactions in cells.



BAG-1 overexpression protects some but not all cells from heat shock-induced cell death

If BAG-1 modulates the activity of Hsp70 and Hsc70 *in vivo*, then we reasoned that BAG-1 overexpression might alter the ability of heat shock to induce cell death. In this regard, heat shock proteins are thought to play cytoprotective roles in cells during times of stress, including thermal stress (Kabakov and Gabai, 1994; Polla *et al.*, 1996), though they may also promote apoptosis in some circumstances (Galea-Lauri *et al.*, 1996; Ishiyama *et al.*, 1996). For this reason, we established stable transfectants of human 293 kidney epithelial cells and GM701 immortalized human fibroblasts using the control plasmid pcI-Neo or the BAG-1-producing plasmid pcI-FLAG-BAG-1. Immunoblot analysis confirmed the expression of the FLAG-tagged BAG-1 protein in both 293 and GM701 cells but not in the Neo control transfectants (not shown). When challenged with 20 min of heat shock at 48°C, the BAG-1-overexpressing GM701 cells displayed enhanced survival compared with control transfectants (Figure 9). In contrast, BAG-1 did not confer increased resistance to heat shock-induced cell death in 293 cells (Figure 9), despite expressing comparable levels of the FLAG-BAG-1

Fig. 8. Interaction of BAG-1 with Bcl-2 displays ATP dependence. (**A**) Lysates from *Sf*9 insect cells that had been infected with either Bcl-2- or β-galactosidase-producing recombinant baculoviruses (Wang *et al.*, 1996a) were incubated directly or after adding 10 mM ATP with either GST–BAG-1 or GST–CD40 immobilized on glutathione–Sepharose. BAG-1-associated Bcl-2 protein was detected by immunoblotting. (**B**) Lysates were prepared from the human B cell lymphoma cell line RS11846 (contains high levels of Bcl-2 due to t[14;18] translocation) and incubated with GST fusion proteins immobilized on glutathione–Sepharose with or without 10 mM ATP or ATPγS. BAG-1-associated Bcl-2 was detected by immunoblotting. Lysate from cells was also run directly in the gel (first lane) as a control. (**C**) Lysates were prepared with or without 10 mM ATP from Jurkat T-cells which had been stably transfected with either Bcl-2 or Neo control expression plasmids (Takayama *et al.*, 1995) and immunoprecipitations were performed using either the IgG1 anti-BAG-1 monoclonal KS6C8 (Takayama *et al.*, 1996) or an IgG1 control antibody. Immune complexes were analyzed by SDS–PAGE/immunoblot assay using anti-Bcl-2 polyclonal antiserum. Lysates from cells (one-tenth input) were also run directly in the gel as a control (far left/first two lanes).

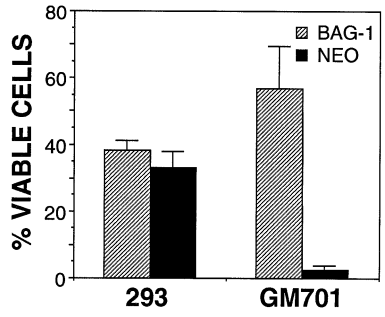


Fig. 9. BAG-1 protects GM701 but not 293 cells from heat shock-induced cell death. 293-NEO, 293-BAG-1, GM701-NEO and GM701-BAG-1 transfectants were subjected to heat shock (48°C) for 20 min. After returning cells to 37°C culture for 2 days, the percentage of viable cells was determined by trypan blue dye exclusion assay (mean ± SE; $n = 3$). Data contrast BAG-1 (hatched bars) and NEO control (dark bars) cells. The starting cell viability was >95% for all cultures.

protein. Similar results were obtained using a variety of temperatures and lengths of heat exposure time (not shown). We conclude, therefore, that BAG-1 can contribute to protection against heat shock-induced cell death in some but not all types of cells. Though many potential explanations exist, one possible reason for the different results is that 293 cells contain very high levels of Hsp70 as these cells are E1a transformed and E1a is a direct transactivator of the human Hsp70 promoter.

Discussion

The molecular chaperones Hsp70 and Hsc70 participate in many important cellular processes, including protein folding and refolding, assembly and disassembly of multiprotein complexes and protein translocation across biological membranes (reviewed in Rassow *et al.*, 1995; Hartl, 1996). Hsp70/Hsc70 and their homologs have also been implicated in the control of diverse biochemical events, including the activation of transcription factors, proteolysis and protein phosphorylation. We present evidence here that BAG-1 specifically binds to Hsp70 and Hsc70 and appears to modulate the function of these molecular chaperones, based on *in vitro* refolding assays. The precise mechanism by which BAG-1 regulates the activity of Hsp/Hsc70, however, remains to be determined. However, the finding that BAG-1 binds to Hsp/Hsc70–substrate complexes suggests a possible *in vivo* function for BAG-1 in the regulation of the ability of Hsp/Hsc70 to modify the structure and therefore function of potential substrates (e.g. Bcl-2, Raf-1, receptors, etc.). Moreover, it seems likely that BAG-1 interactions with Hsp70 and Hsc70 will cause a net inhibition of some chaperone-dependent events in cells, while enhancing others, depending on the specific substrate involved. The observation that BAG-1 can protect some cell lines but not others from heat shock-induced apoptosis implies that its net effects on the *in vivo* functions of heat shock proteins and stress responses may be dependent on the cellular context.

While the chaperone function of Hsp/Hsc70 is dependent on Hdj-1, it remains unclear how Hsp/Hsc70 activities are regulated *in vivo*. In addition to BAG-1, the Hip protein also interacts with the ATPase domains of Hsp70 and Hsc70. Hip is thought to function by enhancing Hsp/Hsc70-mediated refolding measured *in vitro*, presumably by inducing a conformational change in the ATPase domain which influences events in the peptide-binding domain (Höhfeld *et al.*, 1995; Prapapanich *et al.*, 1996). It is tempting to speculate that BAG-1 and Hip could compete for binding to the ATPase domains of Hsp70 and Hsc70, having opposing effects on the chaperone activity of these proteins. This and other issues concerning the mechanism by which BAG-1 inhibits Hsp/Hsc70 chaperone function *in vitro* are currently under investigation.

One potential implication of the finding that BAG-1 interacts with Hsp/Hsc70 is that BAG-1 may provide a bridge between the molecular chaperones (best known for their roles in stress responses) and proteins such as Bcl-2, Raf-1, HGF-R, PDGF-R and steroid hormone receptors which are involved in cell survival and growth regulation. The association between BAG-1 and Hsp/Hsc70 may afford BAG-1 the opportunity to adopt different conformations, analogous to the role of molecular chaperones with steroid receptors, thus enhancing interactions between BAG-1 and different partner proteins. A hint of this possibility comes from the observations that the interaction of BAG-1 with Bcl-2 is regulated by ATP and (evidently) by ATP hydrolysis in cell lysates. Alternatively, Raf-1 and steroid hormone receptors are constitutively associated with Hsp90 in cells (Stancato *et al.*, 1993; Wartmann and Davis, 1994), and inducible interactions between Hsp90 and Hsp/Hsc70 are well documented (Hutchison *et al.*, 1994; Jakob and Buchner, 1994; Pratt and Welsh, 1994). Thus, it is possible that BAG-1-induced alterations in the activity of Hsp/Hsc70 promote the association of BAG-1–Hsp/Hsc70 complexes with other proteins such as Raf-1 and steroid hormone receptors that are bound by Hsp90. In this regard, preliminary experiments indicate that the direct interaction *in vitro* of purified BAG-1 with chaperones is limited to Hsc/Hsp70 and does not include Hsp40 or Hsp90 (unpublished observations). Attempts currently are under way to explore whether the interaction of BAG-1 with Raf-1 and other Hsp90-binding proteins is direct or mediated by bridging chaperones.

A requirement for ATP and ATP hydrolysis is highly characteristic of Hsp/Hsc70-mediated protein complex assembly, and thus indirectly suggests a role for the Hsp/Hsc70 chaperone system in the regulation of BAG-1–Bcl-2 interactions. The opposite effect that ATP has as an inducer of BAG-1–Hsp70(Hsc70) complex disassembly is also very typical of chaperone system-mediated protein complex assembly reactions (Pratt and Welsh, 1994; Smith *et al.*, 1995). In this regard, preliminary experiments suggest that BAG-1 does not possess intrinsic ATP-binding or ATPase activity, indicating that the ATP dependence of its interaction with Bcl-2 is mediated by other proteins, presumably Hsp/Hsc70 and/or the other chaperone proteins with which Hsp/Hsc70 can associate. Conversely, Hsp70 does not bind directly to Bcl-2 *in vitro* (using purified proteins), implying that Hsp70 requires other proteins if it is to interact functionally or physically with Bcl-2 (unpublished observations). By analogy to other protein complexes that Hsp/Hsc70 have been shown to regulate (Hutchinson *et al.*, 1994; Pratt and Welsh, 1994; Smith *et al.*, 1995), therefore, we envisage that BAG-1 brings Hsp/Hsc70 along with it as it enters protein complexes that

contain Bcl-2. Having entered the complexes, however, the chaperones remain only transiently associated, and are quickly released (concomitant with a cycle of ATP binding and hydrolysis) after exerting their effects on protein assembly or conformation. As predicted from other systems (Hutchinson *et al.*, 1994; Pratt and Welsh, 1994; Smith *et al.*, 1995), transient association of Hsp70/Hsc70 with Bcl-2-containing protein complexes presumably would explain why we have been unable to co-immunoprecipitate Bcl-2 with Hsp70 or Hsc70 (data not shown). Moreover, by analogy to progesterone receptors which transiently associate with at least 11 different chaperone proteins during the process of cycling from steroid hormone binding-competent (active) to incompetent (inactive) states (Pratt and Welsh, 1994; Smith *et al.*, 1995), the ensemble of proteins required for Bcl-2–BAG-1 interactions may be large.

The findings presented here may bear on several cellular and biochemical phenomena in which either BAG-1 or heat shock proteins have been implicated. For example, the demonstration here that BAG-1 forms a homodimer in solution suggests a potential explanation for its ability to induce Raf-1 kinase activation, in that homodimerization recently has been shown to be sufficient for Raf-1 activation (Farrar *et al.*, 1996; Luo *et al.*, 1996). Alternatively, the constitutive association of Raf-1 with Hsp90 in cells (Stancato *et al.*, 1993; Wartmann and Davis, 1994) may permit protein complexes containing BAG-1 and Hsp/Hsc70 to alter the conformation of Raf-1 indirectly, through effects of Hsp/Hsc70 on Hsp90, thus resulting in kinase activation. BAG-1 binding to Hsp/Hsc70 may also account for its association with steroid hormone receptors, which are known to bind Hsp/Hsc70 indirectly through interactions with Hsp90 (Hutchinson *et al.*, 1994; Jakob and Buchner, 1994; Pratt and Welsh, 1994). Interesting effects of heat shock or heat shock proteins on apoptosis have also been described, with heat shock reportedly cooperating with Bcl-2 in the maintenance of cell survival under some circumstances and protecting mitochondria from induction of permeability transition (Strasser and Anderson, 1995; Dix *et al.*, 1996; Polla *et al.*, 1996).

The interaction of BAG-1 with Hsc/Hsp70 described here also suggests a potential explanation for the observations from gene transfection studies showing that BAG-1 cooperates with Bcl-2 in suppressing apoptosis. In this regard, Bcl-2 and its closely related homolog Bcl-X_L (which binds to BAG-1) also have structural and functional similarity to the pore-forming domains of certain bacterial toxins which are thought to be 'molten globules' that switch back and forth from membrane-integrated to unintegrated states through profound changes in protein conformation (Muchmore *et al.*, 1996; Minn *et al.*, 1997; Schendel *et al.*, 1997). The interaction of BAG-1 with the Hsp/Hsc70 chaperone system, therefore, may create opportunities for altering the conformation of Bcl-2 family proteins in ways that influence their probability of forming channels by integrating into mitochondrial and other intracellular membranes where these apoptosis-regulating proteins reside, particularly since Hsp/Hsc70 family proteins are thought to recognize the molten globule state of proteins (Hightower, 1991). Moreover, since it has been speculated that Bcl-2 may form channels that transport proteins through membranes (Reed, 1997), a role for Hsp/Hsc70 in temporarily unfolding proteins during the transport process can also be imagined, especially given the strong evidence that these chaperones participate in protein translocation across the membranes of mitochondria and nuclear envelope where Bcl-2 resides (Jakob and Buchner, 1994; Rassow *et al.*, 1995). Finally, because Bcl-2 has been reported to interact with several other proteins, including CED-4, Raf-1, calcineurin and other members of the Bcl-2 family (Reed, 1997), chaperone-mediated conformational changes in Bcl-2 could potentially alter its affinity for these proteins.

Further investigations of the interaction of BAG-1 with Hsp/Hsc70, therefore, are likely to provide additional insights into the molecular mechanisms that regulate the activities of the Bcl-2, Raf-1, steroid hormone receptors and other proteins in cells, and may improve our understanding of how these proteins exert their effects on cell survival, proliferation and differentiation.

Materials and methods

Interaction cloning methods

Ligand blotting and two-hybrid cDNA library screens were performed as described previously (Golemis, 1994; Rassow *et al.*, 1995; Takayama *et al.*, 1995; Takayama and Reed, 1996). A GST–BAG-1 fusion protein was employed for λ phage library screening of a fetal human brain cDNA library (Clontech, Inc.). Detection was accomplished with an anti-GST monoclonal antibody 7E5, as described (Takayama *et al.*, 1995; Takayama and Reed, 1996). From a total of ~10^6 λgt-11 clones, three produced proteins that bound to GST–BAG-1 but not to GST, and all three of these contained Hsc70 cDNAs (clone #16 obtained twice and clone #23 once). From the two-hybrid screen of ~1.6×10^7 cDNAs from a Jurkat T-cell cDNA library, 23 positives were obtained after mating tests using LexA–BAG-1 and three negative control LexA bait proteins (Bax, Fas, Ras). Two of these contained Hsc70 cDNAs (clones #13 and #25).

Plasmid preparation and protein production

The ends of the Hsc70 cDNA clone #16 were modified by PCR using the primers 5′-TGATCTCGAGTTACGGTGCCTG-3′ (reverse) and 5′-CGGCCGCTCTAGAACTAGTG-3′ (forward). After digestion with *Eco*RI and *Xho*I, the resulting fragment was subcloned in-frame into the *Eco*RI and *Xho*I sites of either pGEX-4T-1 for production as a GST fusion protein or into the hemagglutinin (HA) tag-containing pcDNA3 plasmid pShin-HA for *in vitro* translation as described (Wang *et al.*, 1996a). Oligonucleotides 5′-GAGCTCATGGACTACAAAGACGATG-ACGACAAGCTTGATATCG-3′ and 5′-AATTCGATATCAAGCTTGT-CGTCATCGTCTTTGTAGTCCATGAGCTC-3′ encoding the FLAG epitope (MDYKDDDDKLDIQF) within the context of a Kozak translation initiation signal sequence were annealed and subcloned into pcI-Neo (Invitrogen, Inc.) at a blunted *Xho*I and natural *Eco*RI site, creating the plasmid pcI-FLAG. An *Eco*RI–*Xho*I mBAG-1 cDNA was then excised from pGEX-4T-1 (Takayama *et al.*, 1995) and subcloned into the *Eco*RI and *Sal*I sites of pcI-FLAG. BAG-1 mutant cDNAs were created by a PCR-based method, subcloned into the *Eco*RI and *Xho*I sites in pGEX-4T-1, expressed in XL1-blue cells (Stratagene, Inc.) and affinity purified using glutathione–Sepharose, as described (Takayama *et al.*, 1995).

Gel-sieve chromatography

GST–BAG-1 was immobilized on glutathione–Sepharose and cleaved with thrombin to release BAG-1, which was purified to apparent homogeneity by molecular sieve chromatography (Sephacryl S-200, 3.2×90 cm). Purified BAG-1 (60 μg), Hsp70 (400 μg) or both proteins were incubated in 20 mM HEPES pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 0.1% 2-mercaptoethanol on ice for 1 h, and then 50 μl of sample was analyzed by molecular sieve chromatography using a Sepharose-6 (10/30) column (Pharmacia) equilibrated in the same solution at a flow rate of 0.25 ml/min with collection of 0.5 ml fractions. Each fraction (0.2 ml) was precipitated with 10% trichloroacetic acid, washed with acetone, and the protein pellet was resuspended in Laemmli SDS sample buffer and analyzed by SDS–PAGE followed by Coomassie staining.

Protein refolding and ATPase assays

Assays for Hsp70-mediated refolding of denatured β-galactosidase were performed exactly as described (Freeman and Morimoto, 1996), except that GST–BAG-1 or various GST–BAG-1 mutant proteins were added prior to initiation of reactions. Briefly, β-galactosidase (Sigma) was denatured in 6 M guanidine hydrochloride for 30 min at 30°C. The refolding reaction was performed at 37°C in refolding buffer [25mM HEPES pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 1 mM ATP] with 3.4 nM β-galactosidase. The activity of β-galactosidase was measured at various times by mixing 10 μl of each refolding reaction with 10 μl of ONPG, followed by incubation at 37°C for 15 min. Assays were terminated by the addition of 50 μl of 0.5 M sodium carbonate and the absorbance of each sample was measured at $A_{412\ nm}$. The percentage refolding activity is calculated relative to the activity of native β-galactosidase.

ATPase assays were performed as described (Freeman *et al.*, 1995), where ^{32}P release from [α-^{32}P]ATP was measured on polyethyleneimine thin layer sheets at various times after incubation at 37°C using a 1:1 molar ratio of Hsp70 ATP-binding domain and GST–BAG-1 proteins of a BAG-1 from which GST has been cleaved with thrombin. BAG-1 had negligible ATPase activity by itself.

Analysis of RCMLA binding to Hsp70 by non-denaturing gel electrophoresis

The RCMLA binding assay was performed as described previously (Freeman *et al.*, 1995). Briefly, native gel electrophoresis was performed with chaperone (14 mM final) and iodinated RCMLA that were combined at a 5:1 molar ratio in 20 mM HEPES pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl, incubated at 37°C for 30 min and resolved over 6% acrylamide–1× TBE gels at 4°C. GST–BAG-1 protein was added to the reactions at various molar ratios. BSA and GST (14μM) were employed as negative controls.

Protein binding and co-immunoprecipitation assays

*Sf*9 insect cells (2×10^6) were infected for 2–4 days with either Bcl-2- or β-galactosidase-producing recombinant baculoviruses (Wang *et al.*, 1996a) and lysed in HKMEN solution (10 mM HEPES pH 7.2, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40) that contained or lacked 10 mM ATP or ATPγS. Alternatively, lysates were prepared from 10^7 RS11846 lymphoma cells. Lysates were incubated with either GST–BAG-1 or GST–CD40 cytosolic domain (10 μg) immobilized on glutathione–Sepharose (10–20 μl) at 4°C for 1 h, followed by five washes with 1 ml of HKMEN. BAG-1-associated Bcl-2 was detected by SDS–PAGE/immunoblot assay using anti-Bcl-2 polyclonal antiserum with an enhanced chemiluminescence (ECL) detection method. For some experiments, 10 μg of immobilized GST–BAG-1 or GST–CD40 was incubated with either purified Hsp70 or Hsc70 (5 μg) (Freeman and Morimoto, 1996) in 0.2 ml of HKMEN solution containing 10 mM ATP, ADP or ATPγS for 30 min at 30°C. After washing the beads extensively, associated Hsp/Hsc70 was detected by immunoblotting using anti-Hsp/Hsc70 monoclonal antibody 3a3 (Affinity Bioreagents, Inc.).

For experiments with *in vitro* translated proteins, GST fusion proteins (0.5–1 μg immobilized on 1–2 μl of glutathione beads) and 2 μl of TNT lysates containing ^{35}S-labeled *in vitro* translated (IVT) proteins were incubated in 0.1 ml of HKMEN at 4°C for 60 min. The beads were washed three times with 1 ml of HKMEN solution and boiled in 25 μl of Laemmli SDS sample buffer. Use of equivalent amounts of intact GST fusion proteins and successful IVT of all proteins was confirmed by SDS–PAGE analysis using Coomassie staining or autoradiography, respectively.

For co-immunoprecipitation assays, Jurkat T-cells (2×10^7) which had been stably transfected with either Bcl-2 or Neo control expression plasmids (Takayama *et al.*, 1995) were lysed in HKMEN solution containing or lacking 10 mM ATP, and immunoprecipitations were performed using either the IgG1 anti-BAG-1 monoclonal KS6C8 (Takayama, 1996) or an IgG1 control antibody. Alternatively, FLAG-BAG-1-expressing or Neo control 293 cells transfectants (2×10^7) were lysed in HKMEN solution, with or without 10 mM ATP, and immunoprecipitates were prepared using anti-FLAG tag antibody M2 conjugated to agarose (Kodak, Inc.). Immune complexes were analyzed by SDS–PAGE/immunoblot assay using anti-Bcl-2 antiserum, anti-Hsp/Hsc70 monoclonal antibody 3a3 (Affinity Bioreagents) or anti-FLAG monoclonal M2 with ECL-based detection.

Cell transfections and viability assays

293 human kidney epithelial cells and GM701 immortalized fibroblasts were stably transfected by a calcium phosphate precipitation method with pcI-Neo or pcI-FLAG-BAG-1 plasmid DNA followed by selection in medium containing 1.8 mg/ml G418 (Gibco/BRL, Inc.). The resulting transfectants were passaged as polyclonal cell lines. For heat shock-induced cell death, 10^6 cells in 1 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum in Falcon #2085 polystyrene tubes were incubated at 48°C for 5–20 min, then plated at 0.5×10^6 cells per 30 mm dish with 3.5 ml of medium. Cells were returned to culture at 37°C for 2 days, after which adherent cells were recovered by trypsinization and the percentage of viable cells was determined by Trypan blue dye exclusion assay.

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