BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release

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Molecular chaperones influence the process of protein folding and, under conditions of stress, recognize non-native proteins to ensure that misfolded proteins neither appear nor accumulate. BAG-1, identified as an Hsp70 associated protein, was shown to have the unique properties of a negative regulator of Hsp70. Here, we demonstrate that BAG-1 inhibits the *in vitro* protein refolding activity of Hsp70 by forming stable ternary complexes with non-native substrates that do not release even in the presence of nucleotide and the co-chaperone, Hdj-1. However, the substrate in the BAG-1-containing ternary complex does not aggregate and remains in a soluble intermediate folded state, indistinguishable from the refolding-competent substrate–Hsp70 complex. BAG-1 neither inhibits the Hsp70 ATPase, nor has the properties of a nucleotide exchange factor; instead, it stimulates ATPase activity, similar to that observed for Hdj-1, but with opposite consequences. In the presence of BAG-1, the conformation of Hsp70 is altered such that the substrate binding domain becomes less accessible to protease digestion, even in the presence of nucleotide and Hdj-1. These results suggest a mechanistic basis for BAG-1 as a negative regulator of the Hsp70–Hdj-1 chaperone cycle.
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Introduction

The Hsp70 chaperones have a central role in protein synthesis, translocation, folding, and assembly and disassembly of multimeric complexes (Ungewickell, 1985; Munro and Pelham, 1986; Chirico *et al.*, 1988; Murakami *et al.*, 1988; Beckmann *et al.*, 1990; Skowrya *et al.*, 1990; Langer *et al.*, 1992; Nelson *et al.*, 1992; Shi and Thomas, 1992). In response to stress, they prevent the accumulation of protein aggregates by stabilizing unfolded intermediates, which are subsequently refolded to the native state or are degraded (Chiang *et al.*, 1989; Freeman *et al.*, 1995; Levy *et al.*, 1995; Minami *et al.*, 1996; Bercovich *et al.*, 1997). Hsp70 interacts with stretches of hydrophobic amino acids

that are transiently exposed in early folding intermediates of polypeptides (Blond-Elguindi *et al.*, 1993; Fourie *et al.*, 1994; Knarr *et al.*, 1995; Rüdiger *et al.*, 1997a,b). Refolding to the native state requires ATP and the co-chaperone Hdj-1 (Hsp40), which stimulates Hsp70 nucleotide hydrolysis, and couples nucleotide binding and hydrolysis to release of the substrate in a folded state (Freeman *et al.*, 1995; Levy *et al.*, 1995; Minami *et al.*, 1996).

A difficult feature of Hsp70 interactions has been to reconcile how apparently distinctive activities of Hsp70 are regulated. Hsp70 alone is sufficient to prevent a denatured substrate from aggregating, however, conversion of a soluble intermediate to the native folded state requires both Hsp70 in addition to the co-chaperone Hdj-1 and ATP (Freeman *et al.*, 1995; Levy *et al.*, 1995; Minami *et al.*, 1996). Given the propensity of Hsp70 to release non-native substrates in the presence of nucleotide, it is intriguing that Hsp70 can form stable complexes either with substrates or other chaperones/co-chaperones given that the cellular concentration of ATP greatly exceeds the levels required to disrupt Hsp70 complexes *in vitro* (Liberek *et al.*, 1991; Palleros *et al.*, 1991; Smith *et al.*, 1993; Höhfeld *et al.*, 1995; Minami *et al.*, 1996; Takayama *et al.*, 1997). Two classes of modulators of Hsp70 function have been identified: (i) chaperone enhancers, such as Hip, which stimulate Hsp70 chaperone activity and the assembly of Hsp70 into macromolecular chaperone-containing complexes while Hdj-1 stimulates the Hsp70 ATPase (Freeman *et al.*, 1995; Höhfeld *et al.*, 1995); and (ii) chaperone inhibitors, as exemplified by BAG-1 which was initially characterized as a regulator of receptor activity and Bcl2-dependent apoptosis (Takayama *et al.*, 1995; Bardelli *et al.*, 1996; Wang *et al.*, 1996; Zeiner *et al.*, 1997). BAG-1 associates *in vivo* and *in vitro* with Hsp70, and is comprised of at least two domains; a C-terminal region of BAG-1 which binds to the Hsp70 ATPase domain and an N-terminal ubiquitin-like domain (Takayama *et al.*, 1997; Zeiner *et al.*, 1997).

In this study, we examine the interactions between BAG-1 and Hsp70, and demonstrate that BAG-1 forms a ternary complex with Hsp70 and a non-native or partially folded substrate. Unlike other chaperone–co-chaperone interactions which enhance the folding activities of chaperones, BAG-1 association with the ATPase domain uncouples the ATP-dependent release of the Hsp70-associated substrate.

Results

BAG-1 forms a stable ternary complex of Hsp70, BAG-1 and non-native substrate

The inhibitory effects of BAG-1 on Hsp70-dependent chaperone activity were demonstrated previously using chemically denatured β -galactosidase (β -gal) or luciferase

(Gebauer *et al.*, 1997; Takayama *et al.*, 1997). An explanation for the inability of the Hsp70–BAG-1 complex to assist in protein refolding is that BAG-1 interferes with the substrate recognition or binding properties of Hsp70. To address this, we incubated either the permanently unfolded substrate 125 I-reduced carboxymethylated lactalbumin, [125 I]RCMLA, or denatured β -gal in the presence of Hsp70 alone or together with BAG-1. Using a gel-sieving chromatographic assay to detect association, [125 I]RCMLA formed a complex with Hsp70 which was further retarded in elution profile following addition of BAG-1 (Figure 1A). Complex formation between [125 I]RCMLA, Hsp70 and BAG-1 was also detected by

native polyacrylamide gel electrophoresis (Figure 2) in which free [125 I]RCMLA had a faster mobility than the substrate bound to either Hsp70, or Hsp70 and BAG-1. These results establish that an unfolded polypeptide can associate with the Hsp70–BAG-1 complex. However, because [125 I]RCMLA is permanently unfolded, we also examined whether another substrate, denatured β -gal, associated with Hsp70–BAG-1. Neither native nor denatured β -gal associates with BAG-1 alone in biochemical assays for complex formation (Figure 1C) whereas, only denatured β -gal forms a stable complex with Hsp70 (Figure 1D, lanes 3 and 4) and with Hsp70–BAG-1 (lanes 7 and 8). These results show that only the denatured β -gal interacts with the chaperone or chaperone complex and furthermore that BAG-1 is a component of the ternary complex with Hsp70 bound to denatured β -gal.

Taken together, our results reveal that BAG-1 inhibits refolding activity without interfering with the ability of Hsp70 to associate with unfolded polypeptides. Because non-native substrates bound to Hsp70 alone are released in the presence of nucleotide, we examined the effects of ATP on complexes containing BAG-1 bound to Hsp70 and non-native substrate (Liberek *et al.*, 1991; Palleros *et al.*, 1991).

Permanently denatured [125 I]RCMLA or unfolded β -gal was incubated with either Hsp70, or Hsp70 and BAG-1 in the presence or absence of ATP. In the absence of ATP, Hsp70 forms a stable complex with [125 I]RCMLA, as detected in the native gel assay (Figure 2, lane 2), that rapidly dissociates upon addition of ATP (Figure 2, lanes 4–9). In striking contrast, [125 I]RCMLA in the ternary Hsp70–BAG-1 complex is unaffected by ATP (Figure 2, lanes 10–15). Similar conclusions are drawn from the effect of ATP on the complexes of unfolded β -gal, Hsp70 and BAG-1 (Figure 3). GST–Hsp70 forms a stable complex with denatured β -gal (Figure 3, lane 5) that is dissociated upon addition of ATP (Figure 3, lane 8). However, in the presence of BAG-1 (Figure 3, lane 9), ATP does not cause release of the substrate. We conclude

Fig. 1. Analysis of complexes formed between Hsp70, BAG-1 and non-native substrate. (A) Detection of complexes between Hsp70, BAG-1 and RCMLA by gel sieving chromatography. Reaction mixtures containing 14 μ M GST-BAG-1 + 14 μ M Hsp70 + 2.8 μ M [125 I]RCMLA ($\circ$), 14 μ M Hsp70 + 2.8 μ M [125 I]RCMLA ($\square$), or 2.8 μ M [125 I]RCMLA alone ($\diamond$) were incubated and loaded on the Superdex G-200 column. Radioactivity in each fraction was measured by γ -counting. Complexes were also detected *in vitro* by incubation of GST-Hsp70 with BAG-1 and denatured β -gal, and detected by affinity chromatography, SDS-PAGE and Western blot analysis. (B) SDS-PAGE of purified proteins. GST-Hsp70, β -gal and BAG-1 (lanes 1–3, respectively), or Hsp70, GST-BAG-1 and β -gal (lane 4) were resolved by 12% SDS-PAGE followed by Western blotting using anti- β -gal, anti-Hsp70, or anti-BAG-1 antibodies. Partial degradation products of GST-Hsp70 is indicated by * (lane 1). (C) BAG-1 associates with neither native nor non-native β -gal. Chemically denatured or native β -gal (124 nM final concentration) was incubated with 1 μ M GST-BAG-1 (total, T; lanes 1 and 3). Proteins bound to glutathione–Sephadex beads were pelleted, eluted and resolved by 12% SDS-PAGE, followed by Western blotting using mixtures of anti- β -gal, anti-Hsp70 (5A5) and anti-BAG-1 antibodies. (bound, B; lanes 2 and 4). (D) Non-native β -galactosidase forms a stable complex with Hsp70–BAG-1. Chemically denatured or native β -gal (124 nM final concentration) was incubated with 1 μ M GST-Hsp70 and/or 1 μ M BAG-1 (total, T; lanes 1, 3, 5 and 7). Protein complexes bound to glutathione–Sephadex beads were analyzed as described in Figure 1C (bound, B; lanes 2, 4, 6 and 8). Partial degradation products of GST-Hsp70 are indicated by *.

Fig. 2. BAG-1 prevents ATP-dependent substrate release. [125 I]RCMLA (2.8 μ M) was incubated with 14 μ M Hsp70 or 14 μ M Hsp70 + 28 μ M BAG-1 in the presence or absence of ATP. Aliquots were removed at 20 and 40 s, and 1, 2, 5 and 30 min, and immediately loaded onto a 6% 1 $\times$ TBE gel (lanes 4–9 and 10–15, respectively). Due to these conditions, the 30 min samples have a retarded migration (lanes 9 and 15). The results were analyzed by PhosphorImager analysis (Molecular Dynamics).

Fig. 3. Formation of stable GST-Hsp70-BAG-1- β -gal complexes with or without ATP. GST-Hsp70, BAG-1 and β -gal was immunoblotted on different lanes as controls (lanes 1–3). 1 μ M GST-Hsp70 and 1 μ M BAG-1 were incubated in the absence or presence of ATP (lanes 4 and 7). Chemically denatured β -gal (124 nM final concentration) was incubated with 1 μ M GST-Hsp70 alone, or 1 μ M GST-Hsp70 + 1 μ M BAG-1 in the absence (lanes 5–6) or presence (lanes 8–9) of ATP. Protein complexes pulled down by glutathione-Sepharose beads were analyzed as described in Figure 1C. Partial degradation products of GST-Hsp70 are indicated by *.

from these studies that BAG-1 functions by preventing the nucleotide-regulated release of substrate-bound Hsp70.

Biochemical properties of the non-native substrate in the Hsp70-BAG-1 ternary complex

What is the biochemical state of the non-native substrate in the Hsp70-BAG-1 ternary complex? The dynamics of the ATPase cycle and release of the non-native substrate have been shown to be important for the acquisition of a soluble native state (Liberek *et al.*, 1991; Palleros *et al.*, 1991; Freeman *et al.*, 1995; Minami *et al.*, 1996). The inhibitory effect of BAG-1, therefore, might affect the state of the substrate bound to Hsp70. Using a combination of assays that provide information on the folded state of the chaperone-associated substrate, we examined the solubility of the β -gal intermediate by fractionation into soluble or insoluble fraction (Freeman and Morimoto, 1996; Freeman *et al.*, 1996).

Upon dilution of unfolded β -gal in the presence of

Fig. 4. Analysis of the folded state of the non-native substrate in the presence of Hsp70 and/or BAG-1. (A) Chemically denatured β -gal (64 nM final concentration) was incubated with indicated proteins (3.2 μ M of BSA, Hdj-1, BAG-1, Hsp70, or Hsp70 + BAG-1). Soluble (s) or aggregated (p) proteins were separated by centrifugation and resolved by 12% SDS-PAGE followed by Western blotting using anti- β -gal antibodies. (B) Chemically denatured β -gal (64 nM final concentration) was incubated with indicated proteins (3.2 μ M of BSA, Hdj-1, BAG-1, Hsp70, or Hsp70 + BAG-1). Following removal and quenching of one half of the reaction in loading buffer (0 min), chymotrypsin was added for partial proteolysis (15 min). Samples were resolved by 12% SDS-PAGE followed by Western blotting with anti- β -gal antibodies. (C) Chemically denatured β -gal (3.2 nM final concentration) was incubated with 1.6 μ M Hsp70 + 3.2 μ M Hdj-1, 1.6 μ M Hsp70 + 3.2 μ M Hdj-1 + 1.6 μ M BAG-1, or 3.2 μ M BSA. β -gal activity was measured at 30, 60, 120, 180 and 240 min.

bovine serum albumin (BSA), Hdj-1 or BAG-1, the β -gal aggregated and was recovered entirely in the precipitate (Figure 4A). In contrast, in the presence of Hsp70 alone or Hsp70 and BAG-1, nearly 40 to 50% of the non-native β -gal remained in the soluble fraction (Figure 4A). The conformation of the unfolded substrate was also assessed using proteolysis to probe protein structure (Figure 4B). Upon the dilution of the denatured substrate into buffer alone or BSA, the unfolded β -gal is completely digested by limited concentrations of chymotrypsin, whereas in the presence of Hsp70 or BAG-1-Hsp70, we detected a set of protease resistant fragments of β -gal, indicating that the soluble β -gal adopted an intermediate folded state with a substantial amount of tertiary structure. Furthermore, these data reveal that BAG-1 does not interfere either with the ability of Hsp70 to associate with the unfolded polypeptide or to be maintained in a soluble and

Fig. 5. Effects of Hdj-1 or BAG-1 on Hsp70 ATPase activity. (A) Time dependence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (30 nM) hydrolysis using single turnover was measured in the presence of 0.5 μM Hsp70, 0.5 μM Hsp70 + 1 μM BAG-1, 0.5 μM Hsp70 + 1 μM Hdj-1 or 0.5 μM Hsp70 + 1 μM BAG-1 + 1 μM Hdj-1. The data were quantified by PhosphorImager analysis after thin-layer chromatography and averaged from three separate experiments. (B) Dissociation rates of ADP from Hsp70 in the presence of BAG-1 and/or Hdj-1. The release of nucleotide, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, or $[\gamma\text{-}^{32}\text{P}]\text{ADP}$, bound to 2 μM Hsp70 under steady-state conditions was measured by the addition of 100 μM of ATP with or without 4 μM BAG-1 and/or 4 μM Hdj-1 at 25°C. The complex of Hsp70 and nucleotide was purified from free nucleotide and Pi by gel filtration on Sephadex G-50. The total nucleotide bound to Hsp70 after first gel filtration on Sephadex G-50 was set to 100%. The data was quantified by PhosphorImager analysis after thin-layer chromatography and averaged from three independent experiments.

partially folded state. We conclude that the substrate is maintained in a soluble and partially folded state, and that the effect of BAG-1 is to form a stable complex with Hsp70 while maintaining the substrate in a soluble non-native state. Yet, despite the maintenance of the substrate in a folding competent state, the effect of BAG-1 is inhibitory in the refolding of the non-native β -gal to the native state (Figure 4C).

In summary, the state of the substrate bound to BAG-1–Hsp70 complex is partially folded, and the conformation of β -gal associated with Hsp70 is indistinguishable in the presence or absence of BAG-1.

BAG-1 enhances the Hsp70 ATPase activity and does not function as a nucleotide exchange factor

The inability of the ternary BAG-1–Hsp70 complex to be dissociated by ATP could occur either by BAG-1 rendering Hsp70 insensitive to ATP or by alteration of the ATPase activity. Wild-type Hsc/Hsp70 purified from heat shocked HeLa cells hydrolyzes ATP with a turnover rate of 0.14 per minute, comparable to that described for the rat Hsc70 ATPase (Ha and McKay, 1994). Permanently denatured substrate, RCMLA, stimulates this rate 3.8-fold, while addition of BAG-1 in 1:1 or 1:2 molar ratio of Hsp70 stimulates the rate of hydrolysis by 3.5- or 5.1-fold (Table I). These results reveal that BAG-1 enhances the Hsp70 ATPase.

A more accurate measure of ATPase activity was obtained using single turnover assays. The turnover rate of Hsp70 is 0.23 per minute, which is comparable to that described for the bovine Hsc70 ATPase. In the presence of BAG-1 or Hdj-1 this value is enhanced 2-fold (Table I; Figure 5A), with an additive effect when BAG-1 and Hdj-1 are present simultaneously. As BAG-1 has been

Table I. Turnover rate of Hsp70 with Bag-1, Hdj-1, RCMLA, or Bag-1 and Hdj-1

	K_{cat}^a (min^{-1})
Hsp70 ^b	0.14
Hsp70:RCMLA	0.5
Hsp70:BAG-1 (1:1)	0.5
Hsp70::BAG-1 (1:2)	0.7
	K_{cat}^c (min^{-1})
Hsp70 ^d	0.2 $\pm$ 0.03
Hsp70:BAG-1 (1:2)	0.3 $\pm$ 0.01
Hsp70:Hdj-1 (1:2)	0.3 $\pm$ 0.02
Hsp70:Hdj-1:BAG-1 (1:2:2)	0.4 $\pm$ 0.04

^aATPase hydrolysis rate under steady state.

^bHsp70 purified from heat shocked HeLa cells.

^cATPase hydrolysis rate under single turnover state.

^dRecombinant Hsp70 purified from *E.coli*.

shown to increase the ADP-off rate and have properties similar to GrpE, we compared the ATP hydrolysis rates for DnaK alone, and in the presence of DnaJ and GrpE (Höhfeld *et al.*, 1997). The turnover rate of DnaK is 0.036 per minute (data not shown), which is similar to that observed by other investigators (Karzaï and McMacken, 1996). This intrinsic turnover rate of DnaK is much lower than that of Hsp70. The addition of GrpE increased the DnaK ATPase rate <2-fold, whereas in the presence of both DnaJ and GrpE, the turnover rate increased nearly 50-fold. Thus, comparison of the intrinsic and enhanced single turnover rates of Hsp70 and DnaK reveal important distinctions in ATPase rates.

We next examined whether BAG-1 affected the nucleotide exchange rate of Hsp70. ADP exchange experiments

Fig. 6. Conformation of Hsp70 in the presence of BAG-1, nucleotide, or Hdj-1. Partial digestion of Hsp70 with trypsin in the presence of Hdj-1, or BAG-1. Hsp70 (5 µg), Hsp70 (5 µg) + Hdj-1 (5 µg), or Hsp70 (5 µg) + BAG-1 (7 µg) were digested with trypsin (0.025 µg) in the presence or absence of ATP (2 mM final concentration) at different time points; 1, 5, 10, 20 min. Partially digested Hsp70 was resolved by 10% SDS-PAGE followed by Western blotting using anti-Hsp70 monoclonal antibodies (5A5 and 3A3).

were performed in the presence of BAG-1 or Hdj-1. Hsp70 was incubated with limiting amounts of [γ - 32 P]ATP to allow formation of the Hsp70-ADP complex, and the rate of exchange was measured following addition of 50-fold unlabeled ATP to the Hsp70-ADP complex in the presence of either or both Hdj-1 and BAG-1. The rate of ADP exchange for Hsp70 alone was unaffected by BAG-1 (Figure 5B). Addition of Hdj-1 increased the exchange rate nearly 10-fold at the 5 min time point and BAG-1 did not have any synergistic effect on the ADP off rate in the presence of Hdj-1. These results show that BAG-1 interacts and activates Hsp70 ATPase activity in a manner distinct from Hdj-1.

BAG-1 affects Hsp70 conformation, independent of nucleotide and Hdj-1

The effects of BAG-1 on Hsp70 are distinct from Hdj-1, yet both proteins stimulate the Hsp70 ATPase to similar levels (Figure 5A). Yet, despite the ability of BAG-1 to enhance the Hsp70 ATPase, BAG-1 inhibits the nucleotide-dependent release of the substrate. To understand how BAG-1 effects this change in Hsp70 activity, we compared the conformation of Hsp70 in the presence of nucleotides, Hdj-1 or BAG-1, using partial proteolysis as a tool to detect changes in the native state. The proteolytic products were separated by SDS-PAGE and the Western blots incubated with monoclonal antibodies which recognize the ATPase domain and the substrate binding domains of Hsp70. The proteolytic digestion patterns for Hsp70, or Hsp70 and Hdj-1 yield the appearance of the 45 kDa ATPase domain, and the 18 and 20 kDa substrate binding domains (Figure 6; data not shown). Upon addition of BAG-1, the proteolytic fragmentation pattern changes; the 18/20 kDa fragments from the substrate binding domain fragments are not detected and the 58 kDa proteolytic

fragment intensifies. In the presence of ATP, the overall conclusions are similar. Accessibility of the tryptic cleavage site generating the substrate binding domain is diminished in the presence of ATP, resulting in reduced levels of the C-terminal fragment (Figure 6). Consistent with the previous results, addition of BAG-1 in the presence of ATP led to the stabilization of the 58 kDa fragment and loss of appearance of the substrate binding domain.

We conclude from these experiments that BAG-1 alters the conformation of Hsp70 with effects in both the N- and C-terminal domains. These BAG-1-induced changes in Hsp70 are not reversed by nucleotide or Hdj-1, and provide an explanation for the dominant inhibitory effect of BAG-1 on Hsp70 refolding, despite its effects on ATPase activity.

Discussion

Our results with respect to the regulatory properties of BAG-1 on the Hsp70 chaperone cycle are summarized in the schematic shown in Figure 7. We arbitrarily enter the Hsp70 cycle at the transition between Hsp70 in the substrate-free state and substrate-bound state which is dependent upon substrate, nucleotide and Hdj-1, with the outcome that a folded substrate is released. BAG-1 can either associate with Hsp70 in the substrate-free or substrate-bound state; in either case, the non-native substrate is not released even though Hsp70 is in the ADP state and capable of nucleotide hydrolysis. This is depicted as a stable ternary complex in which BAG-1 is associated with the Hsp70 ATPase domain and the unfolded substrate is retained in the substrate binding domain. The interaction of BAG-1 with Hsp70 alters the conformation of Hsp70 such that the substrate binding domain is not readily accessible to exogenous protease digestion with the consequence that Hsp70 does not release the unfolded substrate in the presence of nucleotide and Hdj-1.

The inhibitory effect of BAG-1 on Hsp70 requires direct interaction with the ATPase domain and uncouples the release of the unfolded substrate from the substrate binding domain. This occurs despite the ability of BAG-1 to enhance the intrinsic ATPase rate of Hsp70. Consequently, BAG-1 converts Hsp70 from a chaperone which interacts transiently with the non-native substrate to enhance folding, to an alternative and perhaps novel state in a stable ternary complex in which the substrate remains bound in an intermediate folded state. We speculate that other, as yet unidentified, proteins may subsequently associate with the BAG-1-Hsp70 ternary complex to dissociate BAG-1, thus allowing the refolding cycle to continue. This possibility is indicated in the scheme shown in Figure 7 by a process in which BAG-1 is released from the Hsp70-substrate complex. Although our experiments demonstrate that two different non-native substrates (RCMLA and denatured β -gal) associate with the Hsp70-BAG-1 complex, we have not addressed whether BAG-1 could alter features of the substrate recognition properties of Hsp70.

BAG-1 and the human ortholog HAP46 (RAP46) have been shown to inhibit Hsp70-dependent *in vitro* refolding of denatured proteins (Gebauer *et al.*, 1997; Takayama *et al.*, 1997). Overexpression of BAG-1 in mammalian

Fig. 7. Schematic for the inhibitory effects of BAG-1 on the Hsp70 chaperone cycle *in vitro*. The Hsp70 (ATP) complex (①) is converted to the Hsp70 (ADP) state which associates with unfolded substrate (②). In the presence of ATP and Hdj-1, the unfolded substrate is released and native substrate is detected (③). Simultaneous addition of BAG-1 and unfolded substrate (④) to Hsp70 (ATP) leads to a ternary complex in which both BAG-1 and substrate are bound to Hsp70 (⑤). In the presence of ATP and Hdj-1, the unfolded substrate remains bound and native substrate is not detected (⑥). We hypothesize that BAG-1 may interact with unknown factors and dissociate from Hsp70 (⑦), thus allowing for bound substrate to be refolded and released in the presence of ATP and Hdj-1 (③).

cells represses Hsp70-dependent *in vivo* reactivation of thermally denatured luciferase (E.A.A.Nollen, J.Song, J.F.Brunsting, H.H.Kampinga and R.I.Morimoto, personal communication). Taken together, we propose that BAG-1 functions as a negative regulator of Hsp70 both *in vitro* and *in vivo*. Although the studies presented here establish that BAG-1 inhibits Hsp70 by forming a stable complex which does not release the substrate, alternative observations suggest a different role for BAG-1 in the Hsp70 cycle. BAG-1 (HAP46) has been suggested to have the activity of a nucleotide exchange factor, analogous to the role of GrpE in the bacterial DnaK/DnaJ cycle (Höfheld and Jentsch, 1997). Our data are inconsistent with this since BAG-1 stimulates the Hsp70 ATPase to the same extent as Hdj-1, but does not enhance the rate of exchange of bound nucleotide. Furthermore, it is difficult to reconcile a role for BAG-1 as a GrpE-like chaperone activity enhancing factor given the inhibitory effects of BAG-1/Hap46 on chaperone activity described here and elsewhere (Gebauer *et al.*, 1997; Takayama *et al.*, 1997). However, we note that murine BAG-1 and human HAP46, while nearly identical in the Hsp70 binding domain and ubiquitin-like domain, differ in the N-terminus, which may account for some of the observed differences.

While it is intriguing to consider that BAG-1 may function as a negative regulator of Hsp70, how does one explain observations that BAG-1/Hap46 also interacts with such diverse proteins as Bcl2, human growth factor

receptor, androgen receptor, several steroid receptors, Raf1, retinoic acid receptor and the p53-inducible negative regulator of cell growth protein, Siah (Takayama *et al.*, 1995; Bardelli *et al.*, 1996; Wang *et al.*, 1996; Froesch *et al.*, 1998; Kullmann *et al.*, 1998; Liu *et al.*, 1998; Matsuzawa *et al.*, 1998). The association between BAG-1 and Bcl2 reveals that events governing cell stress and cell death are linked by direct protein–protein interaction of key components of both processes. Association of BAG-1 with Raf1, for example, promotes activation of this kinase. This could occur directly by BAG-1 alone, or indirectly by the ability of BAG-1 to deliver the chaperone activities of Hsp70 to regulate Raf1 function (Wang *et al.*, 1996). In this role, BAG-1 provides an example of a new class of Hsp70 regulatory proteins analogous to, but functionally distinct from, the large class of J-domain proteins such as auxilin, T-antigen or p58 which specify interactions with specific substrates and Hsp70 (Sawai and Butel, 1989; Ungewickell *et al.*, 1995; Jiang *et al.*, 1997; Melville *et al.*, 1997; Srinivasan *et al.*, 1997; Zalvide *et al.*, 1998).

Dual function co-chaperones such as BAG-1 could provide a means by which highly abundant chaperones of the Hsp70 class, which interact with any number of substrates *in vitro*, acquire specificity *in vivo*. Thus, Hsp70 in the cellular environment is surrounded by a constellation of regulatory/specificity proteins, of which BAG-1, Hdj-1 (J-domain proteins) or Hip represent the initial members of what appears to be an even larger class of co-chaperones.

Materials and methods

Protein purification

Construction of plasmids for overexpression of Hsp70, Hdj-1, his-Hsp and GST-BAG-1 has been described (Freeman *et al.*, 1995; Prapapanich *et al.*, 1996; Takayama *et al.*, 1997). Hsc/Hsp70 was purified from heat shocked HeLa cells (Freeman and Morimoto, 1996). The pGEX2T-Hsp70 plasmid was generated by PCR amplification of wild-type Hsp70 DNA, digested with *Bam*HI and *Eco*RI, and inserted into the vector, pGEX2T (Pharmacia-LKB). GST-Hsp70 was purified from transformed BL21/DE3 cells. Crude extracts from IPTG-induced cells were loaded onto a 200 ml DEAE Sepharose column (Pharmacia-LKB) and eluted with a 50–400 mM NaCl gradient over five column volumes. The fractions containing GST-Hsp70 were pooled and recirculated over a 30 ml GST-Sepharose column (Pharmacia-LKB) and sequentially washed with 1 M NaCl, 100 mM NaCl, and eluted with 50 mM glutathione. Fractions containing GST-Hsp70 were pooled, concentrated by ultrafiltration in a Centrprep-10 (Amicon). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce) relative to the standard BSA.

In vitro assays for chaperone activity

Refolding of guanidine-HCl denatured β -gal (Sigma) followed existing protocols (Freeman *et al.*, 1995). Complex formation between chaperones and non-native substrates was analyzed by native gel electrophoresis as described by Freeman *et al.* (1995).

Analysis of chaperone complexes

Recombinant purified GST-Hsp70 (1 μ M) and BAG-1 (1 μ M), or GST-BAG-1 (1 μ M) and Hsp70 (1 μ M) were incubated for 15 min at 30°C in the absence or presence of β -gal (124 nM final concentration) in buffer B (25 mM HEPES pH 7.5, 5 mM MgCl₂, and 50 mM KCl). GST-Sepharose 4B (Pharmacia-LKB) was added at a final concentration of 10% (v/v) and incubated an additional 30 min at 4°C to pull down GST-BAG-1 or GST-Hsp70 complexes. Following centrifugation, the pelleted material was washed with 100 vol. of buffer B, and the bound material eluted with 3 vol. of the same buffer containing 100 mM glutathione. Proteins eluted from the glutathione-Sepharose were resolved by 12% SDS-PAGE followed by Western blotting using anti-Hsp70 monoclonal antibody (5A5), anti- β -gal antibody, and/or anti-BAG-1 antibody (Freeman *et al.*, 1995; Freeman and Morimoto, 1996; Takayama *et al.*, 1997).

Solubility, and limited proteolytic digestion of denatured β -gal and Hsp70

The protocols are modifications of those described previously by Freeman and Morimoto (1996), and Freeman *et al.* (1996). For solubility assay, native β -gal (10 mg/ml) was denatured in denaturation buffer A (25 mM HEPES pH 7.5, 5 mM MgCl₂, 50 mM KCl, 5 mM β -mercaptoethanol and 6 M guanidine-HCl) or added to 1 M glycylglycine (pH 7.5, native control) for 30 min at 30°C and immediately placed on ice. An aliquot of the native control or the denatured β -gal (1 μ g/ μ l total volume) was diluted into 124 μ l of buffer B supplemented with 3.2 μ M of the following proteins: BSA, Hdj-1, Hsp70, BAG-1 or Hsp70 + BAG-1, and incubated for 2 h at 37°C. Each reaction mixture was centrifuged for 30 min at 12 000 g. The supernatant was removed and the pellet was resuspended in 150 μ l of 1 $\times$ loading buffer. Resuspended pellet and supernatant were resolved by 12% SDS-PAGE followed by Western blotting using anti- β -gal antibodies. For the limited proteolytic digestion native β -gal was prepared as described in solubility assay followed by dilution into 124 μ l buffer B supplemented with 3.2 μ M of the following proteins: BSA, Hdj-1, Hsp70, BAG-1 or Hsp70 + BAG-1, and incubated for 2 h at 37°C. Following removal and quenching of 12 μ l of the reaction in 5 $\times$ loading buffer (0 min), 1 μ g chymotrypsin was added and the reaction was allowed to incubate for an additional 15 min before quenching. The samples were resolved by 12% SDS-PAGE followed by Western blotting with anti- β -gal antibodies. For the limited proteolysis of Hsp70, Hsp70 (5 μ g), BAG-1 (7 μ g) and/or Hdj-1 (5 μ g) in the absence or presence of ATP (2 mM) was incubated at 37°C for 15 min in buffer B. Proteolytic cleavage was carried out at 37°C by addition of trypsin (0.025 μ g). At specific times, samples were removed and the reactions stopped by addition of 5 $\times$ loading buffer and freezing on dry ice. When the time course was completed, samples were resolved by 10% SDS-polyacrylamide gels followed by Western blotting with the 3A3 and 5A5 anti-Hsp70 monoclonal antibodies (Freeman *et al.*, 1995).

Measurement of steady-state and single-turnover ATPase rates

ATP hydrolysis was determined by measuring the conversion of [α -³²P]ATP (ICN Pharmaceutical, Inc.) to [α -³²P]ADP according to the protocol of Sadis and Hightower (1992). The effect of protein substrate (RCMLA) on the ATPase rate was measured in a 1:20 Hsp70:RCMLA molar ratio prior to incubation at 37°C. The k_{cat} values were determined from the slope of a best-fit line through the data using the Grafit plotting program. The rate of ATP hydrolysis was measured under single turnover condition, with recombinant Hsp70 present in substantial molar excess over ATP according to the modified protocol of Karzai and McMacken (1996). Hsp70 and 0.1 mCi/ml [α -³²P]ATP were preincubated separately at 25°C for 5 min and then mixed; the final concentrations of Hsp70 and [α -³²P]ATP were 0.5 μ M and 30 nM, respectively. Ten microliters of the reaction solution were removed and mixed with 2 μ l of 1 M HCl. Two microliters of quenched reaction were spotted on a thin layer chromatogram. The data were visualized and quantified by PhosphorImager analysis (Molecular Dynamics); hydrolysis in the absence of added Hsp70 was routinely subtracted from the data. The data were fit by linear regression to determine the slope (initial reaction rate). The rates of dissociation of ADP from Hsp70 in the presence of BAG-1 and/or Hdj-1 were determined according to the modified protocol of Ha and McKay (1994). A stoichiometric complex was formed by incubating a mixture of 0.2 mCi/ml 2 μ M [α -³²P]ATP and 2 μ M Hsp70 at 37°C for 30 min. The complex was purified from free nucleotide and Pi by gel filtration on Sephadex G-50 (Pharmacia). The concentrations of the eluted protein were determined using BCA assay (Pierce) relative to BSA. The final concentration of 2 μ M of Hsp70 was used for the assay. The release of nucleotide, [α -³²P]ATP or [α -³²P]ADP, bound to Hsp70 (2 μ M) under single turnover conditions, was measured by the addition of 100 μ M of ATP with or without BAG-1 (4 μ M), and/or Hdj-1 (4 μ M) at 25°C. The complex of Hsp70 and nucleotide was purified from free nucleotide and Pi by gel filtration on Sephadex G-50. The release rate of ADP was determined utilizing an average of three separate experiments for each Hsp70 protein after the background hydrolysis had been subtracted. The data was visualized and quantified by PhosphorImager analysis (Molecular Dynamics).

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