# GrpE-like regulation of the Hsc70 chaperone by the anti-apoptotic protein BAG-1

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The BAG-1 protein appears to inhibit cell death by binding to Bcl-2, the Raf-1 protein kinase, and certain growth factor receptors, but the mechanism of inhibition remains enigmatic. BAG-1 also interacts with several steroid hormone receptors which require the molecular chaperones Hsc70 and Hsp90 for activation. Here we show that BAG-1 is a regulator of the Hsc70 chaperone. BAG-1 binds to the ATPase domain of Hsc70 and, in cooperation with Hsp40, stimulates Hsc70's steady-state ATP hydrolysis activity ~40-fold. Similar to the action of the GrpE protein on bacterial Hsp70, BAG-1 accelerates the release of ADP from Hsc70. Thus, BAG-1 regulates the Hsc70 ATPase in a manner contrary to the Hsc70-interacting protein Hip, which stabilizes the ADP-bound state. Intriguingly, BAG-1 and Hip compete in binding to the ATPase domain of Hsc70. Our results reveal an unexpected diversity in the regulation of Hsc70 and raise the possibility that the observed anti-apoptotic function of BAG-1 may be exerted through a modulation of the chaperone activity of Hsc70 on specific protein folding and maturation pathways.

*Keywords*: apoptosis/BAG-1/GrpE/Hsc70/molecular chaperone

## Introduction

Molecular chaperones of the Hsp70 family play a key role in the folding, translocation and degradation of proteins in the eukaryotic cell due to their capacity to bind and stabilize non-native protein conformations (Craig *et al.*, 1994; Hartl, 1996; Rüdiger *et al.*, 1997). Substrate binding involves the recognition of extended hydrophobic peptide segments by the carboxy-terminal peptide-binding domain of the chaperone and is regulated by cycles of ATP binding and hydrolysis mediated by Hsp70's amino-terminal ATPase domain. The ATP-bound form of Hsp70 binds and releases peptides rapidly, whereas the ADP-bound form binds and releases them slowly (Palleros *et al.*, 1991; Schmid *et al.*, 1994; Szabo *et al.*, 1994). In bacteria, cycling of the Hsp70 homologue DnaK between different nucleotide states is regulated by the chaperone cofactors DnaJ and GrpE (Georgopoulos *et al.*, 1994; McCarty *et al.*, 1995; Hartl, 1996). DnaJ stimulates the DnaK ATPase activity, and transfer of DnaK into the ADP-bound state stabilizes its interaction with the polypeptide substrate. In contrast, binding of GrpE to the ATPase domain of DnaK triggers the release of ADP and hence accelerates substrate dissociation upon ATP rebinding. Homologues of the DnaJ protein have been identified in various compartments of the eukaryotic cell where they regulate distinct members of the Hsp70 family in a DnaJ-like manner (Hartl, 1996). The presence of structural homologues of GrpE, however, appears to be limited to compartments of prokaryotic origin only, i.e. mitochondria and chloroplasts (Stuart *et al.*, 1994). We have recently identified the rat Hip protein as a chaperone cofactor of the cytosolic and nuclear heat shock cognate Hsc70 (Höhfeld *et al.*, 1995). The functional characterization of Hip indicated a mechanism for the regulation of Hsp70 family members in the eukaryotic cell that is distinct from the GrpE-mediated dissociation of chaperone/substrate complexes observed in prokaryotes (Ziegelhoffer *et al.*, 1996; Frydman and Höhfeld, 1997). Hip interacts with the ATPase domain of Hsc70 after an initial activation of Hsc70's ATPase activity by the eukaryotic DnaJ homologue Hsp40. In contrast to GrpE, Hip appears to stabilize the ADP-bound form of Hsc70 and may thus prolong the interaction of Hsc70 with a polypeptide substrate (Höhfeld *et al.*, 1995).

The mouse BAG-1 protein was initially identified as a binding partner of Bcl-2, a key inhibitor of apoptotic cell death (Takayama *et al.*, 1995; Reed, 1997). BAG-1 enhances the cell death-inhibiting activity of Bcl-2. In addition, overexpression of BAG-1 prolongs the survival of fibroblast cells challenged by apoptotic stimuli, thus identifying BAG-1 as an anti-apoptotic molecule on its own (Takayama *et al.*, 1995). An involvement of BAG-1 in the regulation of apoptosis is supported further by the interaction of BAG-1 with hepatocyte and platelet-derived growth factor receptors in a manner enhancing their anti-apoptotic functions (Bardelli *et al.*, 1996). Furthermore, BAG-1 interacts with and activates the Raf-1 protein kinase (Wang *et al.*, 1996). Hence, BAG-1 binds to multiple signalling molecules and supports their activation through an as yet unknown mechanism. A human homologue of the BAG-1 protein was recently isolated as a binding partner of several steroid hormone receptors (Zeiner and Gehring, 1995). Steroid hormone receptors as well as the Raf-1 kinase are known to require an association with molecular chaperones for their activation (Stancato *et al.*, 1993; Bohen and Yamamoto, 1994; Schneider *et al.*, 1996; Frydman and Höhfeld, 1997; van der Straten *et al.*, 1997). This prompted us to consider that BAG-1 may exert its cellular activity by participating in molecular chaperone pathways.

Here, we identify BAG-1 as a component of the Hsc70 chaperone system. Following Hsp40-mediated stimulation of Hsc70's ATP hydrolysis activity, BAG-1 stimulates the release of ADP from Hsc70. Hence, BAG-1 fulfils a GrpE-like function in the eukaryotic cell. BAG-1 binds
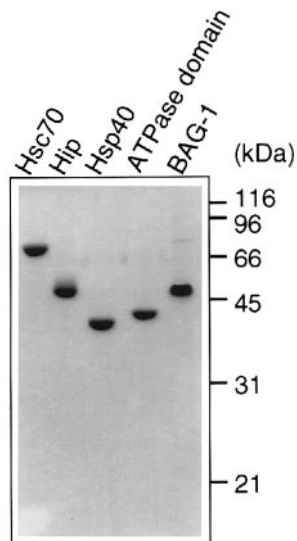
**Fig. 1.** Purified recombinant Hsc70, Hip, Hsp40, the ATPase domain of Hsc70, and BAG-1 were analysed by SDS–PAGE. 2 µg of each protein was loaded and visualized by Coomassie staining.

to the ATPase domain of Hsc70 in a manner preventing an interaction of the chaperone with the regulatory cofactor Hip. The existence of competing cofactors that conversely affect the stability of the ADP state of the chaperone reveals a versatility in the regulation of Hsc70 not previously anticipated.

## Results

### Purification of human BAG-1 and of components of the mammalian Hsc70 chaperone system

To investigate a potential cooperation of BAG-1 with components of the Hsc70 chaperone system, Hsc70, the Hsc70-interacting protein Hip, Hsp40 and the ATPase domain of Hsc70 (amino-acids 1–386) were purified after recombinant expression (Figure 1). For expression of BAG-1, the human *bag-1* cDNA was amplified by the polymerase chain reaction using cDNA of neuronal SY5Y cells as template. [Although the human BAG-1 homologue was initially termed RAP46 (Zeiner and Gehring, 1995), we will refer to it as BAG-1 throughout this study.] For initial interaction assays a fusion protein comprising the bacterial maltose binding protein (MBP) and full-length human BAG-1 was constructed, expressed in *Escherichia coli*, and purified (MBP–BAG) (data not shown). However, for functional studies an unmodified form of human BAG-1 was expressed in *Spodoptera frugiperda* (Sf9) cells following infection with a recombinant baculovirus and was purified to homogeneity (Figure 1).

### BAG-1 interacts with the ATPase domain of Hsc70

Hsc70 binds to a multitude of polypeptide substrates through its carboxy-terminal peptide binding domain (Rüdiger *et al.*, 1997). To eliminate the possibility that BAG-1 is recognized as a substrate, we used the amino-terminal ATPase domain of Hsc70 in interaction assays (Rüdiger *et al.*, 1997). ATPase domain binding is a hallmark of the Hsp70-regulating factors GrpE from bacteria and the eukaryotic Hip protein (Buchberger *et al.*, 1994; Höhfeld *et al.*, 1995; Harrison *et al.*, 1997). BAG-1/
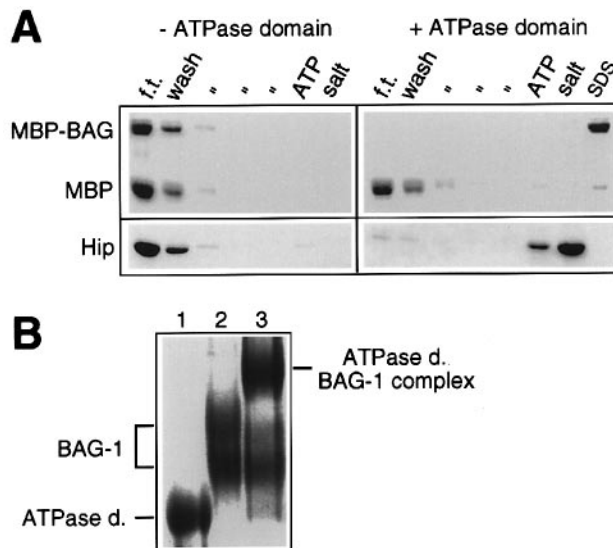


**Fig. 2.** BAG-1 interacts with the ATPase domain of Hsc70. (**A**) A purified fusion protein of BAG-1 and maltose binding protein (MBP–BAG) was incubated with Sepharose beads (–ATPase domain) and ATPase domain–Sepharose (+ATPase domain), respectively, in the presence of 0.1 mM ADP. After collecting the flow-through fraction (f.t.), beads were washed (wash) and subsequently eluted with buffer containing 1 mM ATP (ATP) or 250 mM NaCl (salt) and with SDS–PAGE sample buffer (SDS). (**B**) 10 µg each of the ATPase domain (1) and of unmodified BAG-1, purified after expression in insect cells (2), or a mixture of both proteins (3), preincubated for 30 min at 4°C in the absence of nucleotide, were subjected to blue native electrophoresis. At a 1.5-fold molar excess of BAG-1, the ATPase domain and ~60% of BAG-1 were shifted into a slower-migrating ATPase domain/BAG-1 complex.

ATPase domain interaction was analysed by affinity chromatography on the bovine Hsc70 ATPase domain immobilized on Sepharose beads. When the MBP–BAG fusion protein was incubated with the affinity resin in the presence of ADP, the fusion protein was quantitatively retained, while MBP itself did not bind to the ATPase domain (Figure 2A). Under these conditions the Hsc70-interacting protein Hip also efficiently interacted with the immobilized ATPase domain (Figure 2A). However, Hip was partially eluted from the affinity resin by addition of ATP, and interaction was completely disrupted in the presence of increased salt concentrations (250 mM NaCl). In contrast, interaction of BAG-1 with the ATPase domain was salt-resistant and apparently independent of the nucleotide state of the domain (Figure 2A). Complex formation between BAG-1 and Hsc70's ATPase domain was also analysed by blue native gel electrophoresis. Incubation of a 1.5-fold molar excess of baculovirus-derived BAG-1 with the purified ATPase domain before native gel electrophoresis led to the formation of a BAG-1/ATPase domain complex that migrated with reduced mobility in the gel (Figure 2B). The same result was obtained with full-length Hsc70 (data not shown). BAG-1 is thus a novel Hsc70 partner protein, interacting with the chaperone through Hsc70's ATPase domain.

### BAG-1 stimulates the steady-state rate of ATP hydrolysis by Hsc70 in an Hsp40-dependent manner

Partner proteins of Hsp70 family members play key roles in the regulation of the ATPase activity of the chaperone,
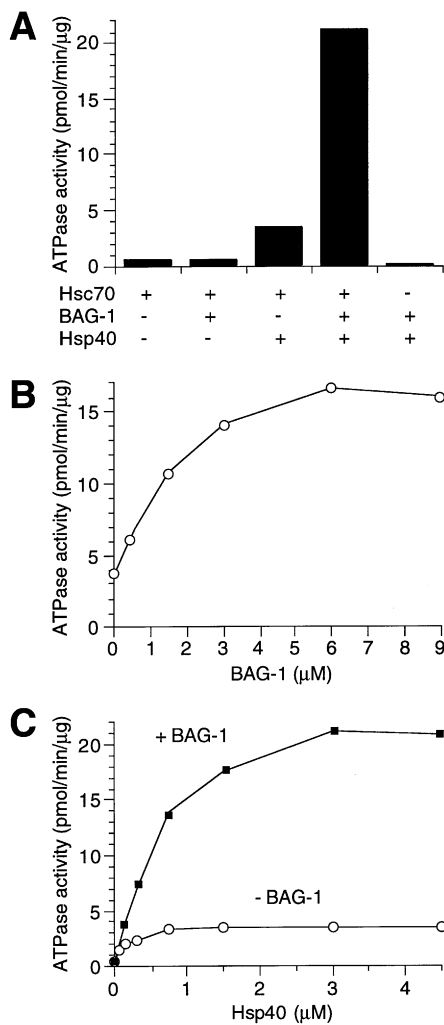
**Fig. 3.** BAG-1 stimulates the ATPase activity of Hsc70 in the presence of Hsp40. (**A**) Hsc70 (3 μM) was incubated at 30°C with BAG-1 (6 μM) and Hsp40 (3 μM) as indicated, and the amount of hydrolysed ATP was determined. Bars represent averaged values from four independent experiments. (**B**) Increasing concentrations of BAG-1 were incubated with 3 μM Hsc70 and 1.5 μM Hsp40, and Hsc70 ATP-hydrolytic activity was assayed. Values were averaged from six samples taken during the linear course of the reaction in two independent experiments. (**C**) Increasing concentrations of Hsp40 were incubated with 3 μM Hsc70 in the absence (–BAG-1) and presence of 6 μM BAG-1 (+BAG-1), respectively, and Hsc70 ATPase activity was determined.

thus modulating the intrinsic peptide binding affinity of Hsp70 (Hartl, 1996; Rüdiger *et al.*, 1997). Incited by the observed interaction of BAG-1 with the ATPase domain of Hsc70, we tested whether BAG-1 can modulate Hsc70's ATPase activity. Hsc70 displays only a very low basal rate of ATP hydrolysis that can be stimulated ~7-fold by the eukaryotic DnaJ homologue Hsp40 (Figure 3A). BAG-1 did not significantly alter the basal rate of ATP hydrolysis by Hsc70. Yet, addition of BAG-1 to Hsc70 co-incubated with Hsp40 led to an ~ 40-fold increase of the steady-state ATPase activity (Figure 3A). BAG-1 exerted its ATPase-stimulating activity at stoichiometric concentrations with respect to Hsc70, with maximal stimulation at a 2-fold molar excess of BAG-1 (Figure 3B). Cooperation of Hsp40 and BAG-1 in the stimulation of Hsc70's ATPase activity was established further by titra-

tion experiments (Figure 3C). In the absence of BAG-1, stimulation of the ATPase activity by Hsp40 reached a plateau at substoichiometric Hsp40 concentrations (at a ratio of Hsc70 to Hsp40 of 6:1). When BAG-1 was present at a 2-fold molar excess over Hsc70, however, a higher plateau was reached at higher Hsp40 levels (Figure 3C). Apparently, BAG-1 accelerates a step in the Hsc70 reaction cycle that becomes otherwise rate-limiting at substoichiometric concentrations of Hsp40.

### BAG-1 accelerates the recycling of Hsc70 by promoting the release of ADP from the chaperone
The observed Hsp40-dependent stimulation of Hsc70's steady-state ATPase activity by BAG-1 was reminiscent of the regulatory function exerted by GrpE in the prokaryotic Hsp70 reaction cycle. We therefore investigated whether BAG-1, similar to bacterial GrpE, accelerates recycling of Hsc70 to the ATP-bound conformation. For this purpose the nucleotide-bound state of Hsc70 under steady-state conditions was analysed. In the absence of Hsp40, Hsc70 was predominantly in the ATP-bound state due to the low intrinsic rate of ATP hydrolysis (Figure 4A). The ATP/ADP ratio was largely unaffected by addition of BAG-1, suggesting that the cofactor does not stimulate the ATP hydrolysis step in the Hsc70 reaction cycle. In the presence of Hsp40, the ADP-bound state of Hsc70 became populated, consistent with the hydrolysis-stimulating activity of Hsp40. Further addition of BAG-1 allowed Hsc70 to return efficiently to the ATP-bound form (Figure 4A). Apparently, BAG-1 promotes the recycling of Hsc70 in the presence of Hsp40. Single turnover experiments revealed a stimulation of the release of bound ADP as the molecular cause for the Hsc70-recycling activity of BAG-1 (Figure 4B). BAG-1 thus fulfils a GrpE-like function in the eukaryotic cell, acting as an ADP/ATP exchange factor in the Hsc70 reaction cycle.

### BAG-1 regulates Hsc70 in a manner contrary to Hip
We have previously characterized the Hip protein as a factor which participates in the regulation of Hsc70 (Höhfeld *et al.*, 1995; Irmer and Höhfeld, 1997). In contrast to BAG-1, Hip appears to stabilize the ADP-bound state of Hsc70. To provide a comparative analysis of BAG-1 and Hip function in the regulation of Hsc70, we investigated the effect of Hip, purified after recombinant expression in insect cells, on the Hsc70 ATPase. When increasing amounts of Hip were added to Hsc70 co-incubated with Hsp40, Hip partially inhibited the steady-state ATPase activity of Hsc70 (Figure 5A). Hip-mediated inhibition of the ATPase activity was most pronounced at substoichiometric Hsp40 concentrations, reaching ~50% inhibition at a 10:1 molar ratio of Hsc70 to Hsp40 in the presence of three to four molecules of the Hip oligomer (Figure 5A). In contrast to BAG-1, the Hip protein apparently slows down the ATPase cycle of Hsc70 in conjunction with Hsp40. Analysis of the nucleotide state of Hsc70 in single turnover experiments revealed a stabilization of Hsc70's ADP-bound state by the Hip protein (Figure 5B). BAG-1 and Hip thus conversely affect the stability of the ADP-bound state of the chaperone protein (Figure 6).
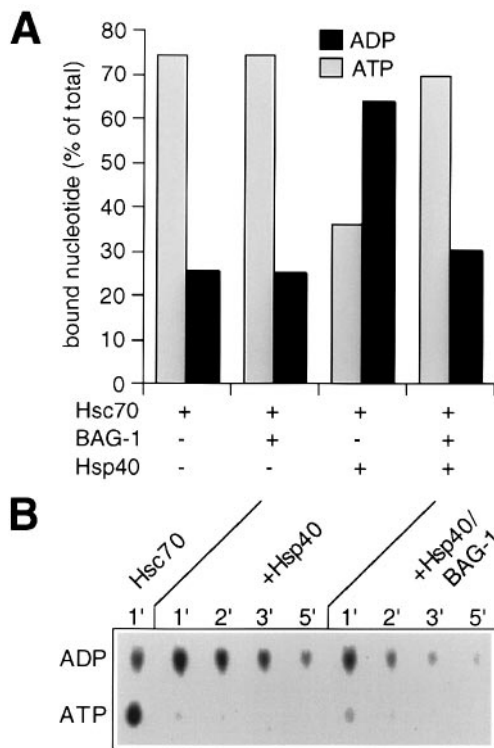
**Fig. 4.** BAG-1 promotes the recycling of Hsc70 by accelerating the release of bound ADP. (**A**) Hsc70-bound nucleotide under steady-state conditions was analysed after incubation of 1 µM Hsc70 in the presence of 200 µM [α-$^{32}$P]ATP for 10 min at 30°C. When indicated, 1 µM BAG-1 and 0.5 µM Hsp40 were added. Free nucleotide was removed by size-exclusion chromatography at 4°C, and bound nucleotide determined by autoradiography after thin-layer chromatography of eluted protein fractions. BAG-1 and Hsp40 alone did not bind nucleotide. Given values were averaged from two independent experiments. (**B**) Hsc70–$^{32}$P-nucleotide complexes were formed by incubation of 10 µM Hsc70 with 200 µM [α-$^{32}$P]ATP for 10 min at 30°C. Complexes were isolated by size-exclusion chromatography at 4°C and incubated further at room temperature with 100 µM non-labelled ATP in the presence of 1.5 µM Hsp40 and 3 µM BAG-1 as indicated. At different time points complexes were re-isolated at 4°C on gel filtration spin columns. Bound nucleotide was analysed by thin-layer chromatography. For quantification of the amount of bound ADP, see Figure 6.

### *BAG-1 and Hip compete in binding to the ATPase domain of Hsc70*

BAG-1 and Hip both modulate Hsc70's ATPase activity through an association with the ATPase domain of the chaperone (Figure 2). We therefore investigated whether both proteins compete in binding to the ATPase domain. Hip was incubated with the immobilized ATPase domain under conditions that resulted in quantitative binding of the chaperone cofactor (Figure 7A). Accordingly, Hip was not detectable in the supernatant fraction after removal of the affinity resin by centrifugation. The presence of increasing amounts of BAG-1 during incubation interfered with the binding of Hip to the immobilized ATPase domain, and Hip was found in the supernatant fraction (Figure 7A). Notably, BAG-1 and Hip do not interact directly (Figure 7B). It thus appears that BAG-1 binds to the ATPase domain of Hsc70 in a manner excluding Hip/ATPase domain interaction.

Competition of BAG-1 and Hip in binding to the ATPase domain was also revealed when the steady-state
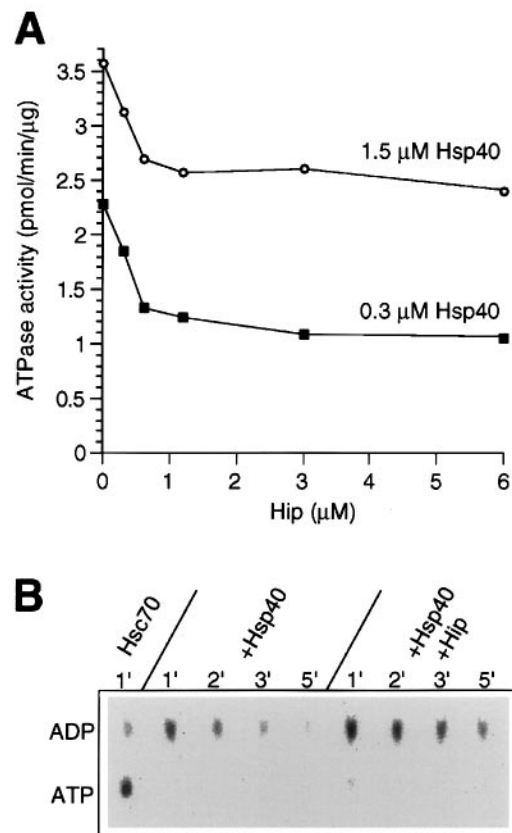


**Fig. 5.** Hip partially inhibits the steady-state ATPase activity of Hsc70 by stabilizing the ADP-bound state of the chaperone. (**A**) Hsc70 (3 µM) was incubated at 30°C with 1.5 (○) and 0.3 µM Hsp40 (■), respectively, in the presence of increasing amounts of Hip oligomer, and the steady-state rate of ATP hydrolysis was determined. Values were averaged from six samples taken during the linear course of the reaction. (**B**) The effect of Hip on the release of ADP from Hsc70 was analysed as described in the legend of Figure 4B, except for the addition of 3 µM Hip oligomer when indicated. For quantification, see Figure 6.
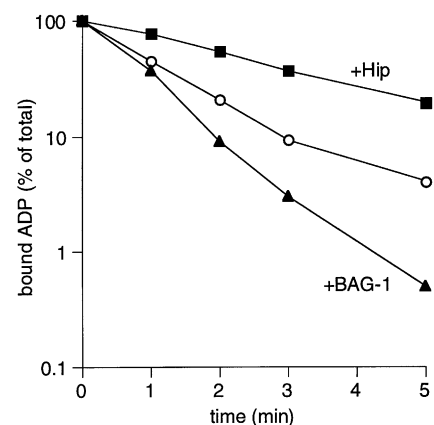


**Fig. 6.** BAG-1 and Hip conversely affect the stability of the ADP bound state of Hsc70. Kinetics of ADP release from Hsc70 were determined in single turnover experiments as described in Figures 4B and 5B. Hsc70–$^{32}$P-nucleotide complexes were incubated with 1.5 µM Hsp40 (○), 1.5 µM Hsp40 and 3 µM BAG-1 (+BAG-1, ▲), or 1.5 µM Hsp40 and 3 µM Hip oligomer (+Hip, ■). Data were quantified using a phosphorimager system and corrected for the amount of re-isolated Hsc70. The amount of total nucleotide bound to Hsc70 after initial size-exclusion chromatography was set to 100%. Values were averaged from two independent experiments.
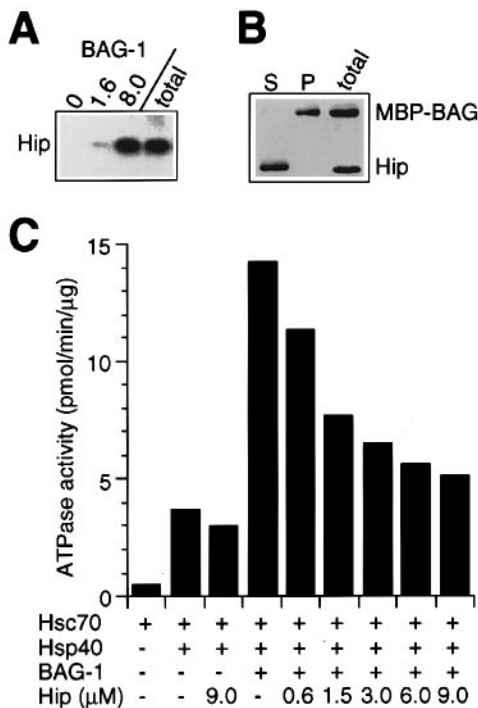
**Fig. 7.** (**A**) BAG-1 competes with Hip in binding to the ATPase domain of Hsc70. Hip was incubated with ATPase domain–Sepharose, equilibrated in ADP, in the absence of BAG-1 and in the presence of a 1.6- and 8-fold molar excess of BAG-1, respectively. Unbound Hip was detected in the supernatant fraction by immunoblotting after removal of the ATPase domain–Sepharose by centrifugation. (**B**) BAG-1 does not interact with the Hip protein. MBP–BAG was immobilized on amylose beads and incubated with an equal amount of purified Hip at 4°C for 1 h. Beads were separated by centrifugation, and the supernatant (S) and pellet fractions (P) were analysed by SDS–PAGE followed by Coomassie blue staining. Hip was detected exclusively in the supernatant fraction. (**C**) Competition between BAG-1 and Hip counteracts the ATPase-stimulating activity of BAG-1. The ATPase activity of Hsc70 (3 μM) was determined in the presence of 1.5 μM Hsp40, 3 μM BAG-1 and different concentrations of Hip as indicated.



**Fig. 8.** Model of Hsc70 reaction cycles in the presence of Hsp40, Hip and BAG-1. (**A**) The ATP-bound form of Hsc70 is characterized by rapid association and dissociation of an unfolded polypeptide substrate (U) or a folding intermediate (I). Upon Hsp40-stimulated conversion of bound ATP to ADP, the interaction of Hsc70 with the substrate is stabilized. In the presence of Hsp40 alone, subsequent ADP/ATP exchange occurs spontaneously, resulting in the release of the polypeptide substrate. (**B**) Following conversion to the ADP-bound form, the Hip protein can bind efficiently to Hsc70. Binding of Hip slows down the release of ADP, which in turn may prolong Hsc70/substrate interaction. (**C**) In contrast, BAG-1 accelerates ADP release, and thus stimulates the recycling of Hsc70.

ATPase activity of Hsc70 was measured in the presence of both cofactors (Figure 7C). In a reaction mixture containing Hsc70, Hsp40 and BAG-1, increasing Hip concentrations counteracted the stimulation of the Hsc70 ATPase by BAG-1. Thus, BAG-1 and Hip do not seem to cooperate during a single Hsc70 reaction cycle.

## Discussion

In this study, we identify the anti-apoptotic BAG-1 protein as a GrpE-like cofactor of Hsc70. Modulation of the ATP-binding and hydrolysis properties of the chaperone protein by regulatory cofactors is crucial for the binding and release of non-native polypeptide substrates (Hartl, 1996; Frydman and Höhfeld, 1997; Rüdiger *et al.*, 1997). Efficient interaction of Hsc70 with a polypeptide substrate depends on the conversion of bound ATP to ADP (Figure 8). Only the ADP-bound form confers stable substrate binding (Palleros *et al.*, 1991; Sadis and Hightower, 1992; Minami *et al.*, 1996). Due to the low intrinsic ATPase activity of Hsc70, stimulation of ATP hydrolysis by the DnaJ homologue Hsp40 is necessary to generate efficiently the ADP-bound form of the chaperone protein (Figure 8A). Hence, Hsp40 facilitates the binding of non-native
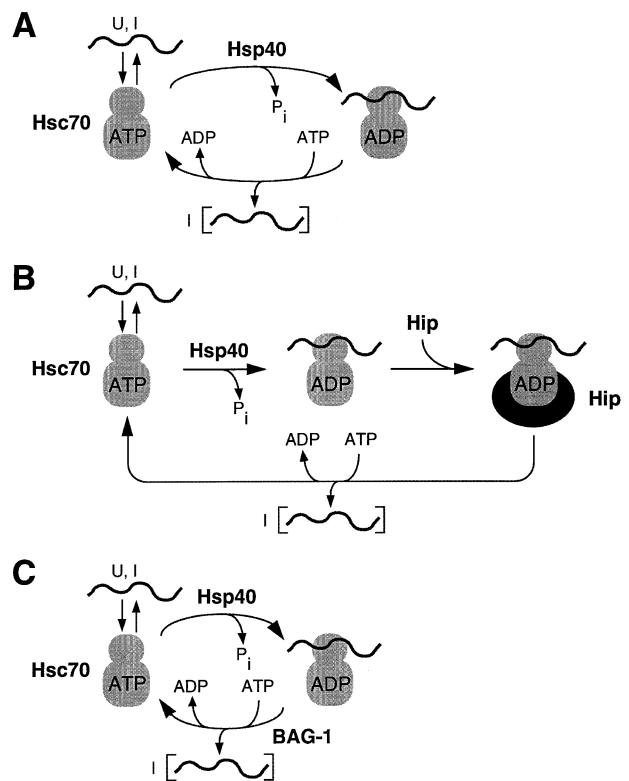
polypeptides to Hsc70 (Frydman *et al.*, 1994; Minami *et al.*, 1996). However, ADP release occurs at a significant rate when Hsc70 cooperates with Hsp40 (Figure 6). In the presence of Hsp40 alone, Hsc70 can apparently return to the ATP-bound state, which results in the release of a polypeptide substrate. A minimal Hsc70 reaction cycle may thus involve only Hsp40 or another eukaryotic DnaJ homologue (Figure 8A). This is consistent with the observation that Hsc70, in conjunction with Hsp40 alone, is able to promote the refolding of denatured proteins *in vitro* (Freeman *et al.*, 1995; Freeman and Morimoto, 1996; Minami *et al.*, 1996).

The ADP-bound form of Hsc70, generated during interaction with Hsp40, can be stabilized by the Hip protein (Höhfeld *et al.*, 1995). Hip recognizes the ADP-bound conformation of the ATPase domain of Hsc70 (Irmer and Höhfeld, 1997), and slows down the release of ADP from Hsc70 (Figure 5 and 6). In this way, Hip may prolong the time window during which Hsc70 interacts stably with a polypeptide substrate (Figure 8B). Notably, Hip does not completely arrest cycling of Hsc70 at the ADP-bound state. In the presence of Hip and Hsp40, the steady-state rate of ATP hydrolysis was reduced by only ~50% (Figure 5A). Apparently, ADP release and

ATP rebinding can still occur spontaneously to a certain extent when Hip and Hsp40 cooperate with Hsc70 (Figure 8B). Down-regulation of the Hsc70 ATPase activity by Hip may be important for the efficient interaction of Hsc70 with other chaperone systems in the eukaryotic cytosol (Frydman and Höhfeld, 1997). Intriguingly, the human homologue of Hip, p48, has been found to associate with the progesterone receptor as part of an Hsc70/Hsp90 chaperone complex (Smith *et al.*, 1995; Prapapanich *et al.*, 1996). Steroid hormone receptors require a sequential interaction with Hsc70 and the Hsp90 chaperone system to reach the high-affinity conformation for hormone binding (Bohen and Yamamoto, 1994; Smith *et al.*, 1995). Thus, Hip-mediated regulation of Hsc70 may be of particular importance for the cooperation of Hsc70 with Hsp90 (Ziegelhoffer *et al.*, 1996; Frydman and Höhfeld, 1997).

In contrast to Hip, the BAG-1 protein promotes the dissociation of ADP from Hsc70 (Figures 6 and 8C). In conjunction with Hsp40, BAG-1 thus dramatically stimulates the steady-state ATPase activity of the chaperone protein (Figure 3). ATPase-stimulating activity has also been reported for non-native proteins and peptides that are recognized by Hsc70 as substrates (Flynn *et al.*, 1991; Palleros *et al.*, 1991; Sadis and Hightower, 1992). However, in contrast to substrate-mediated stimulation, induction of Hsc70's ATPase activity by BAG-1 was extremely efficient and was dependent on an initial activation of the ATP hydrolysis step by Hsp40 (Figure 3). The regulatory function of BAG-1 in the Hsc70 reaction cycle resembles the function of bacterial GrpE which stimulates the steady-state ATPase activity of DnaK in conjunction with DnaJ through an acceleration of ADP release (Szabo *et al.*, 1994; McCarty *et al.*, 1995). Similar to GrpE and Hip, BAG-1 binds to the ATPase domain of an Hsp70 family member (Buchberger *et al.*, 1994; Höhfeld *et al.*, 1995; Harrison *et al.*, 1997). In fact, Hip and BAG-1 compete in binding to Hsc70's ATPase domain. It thus appears unlikely that Hip and BAG-1 interact successively with Hsc70 during one reaction cycle. Rather, association of Hip with the ADP-bound form of Hsc70 prevents an interaction of BAG-1 with the chaperone protein. Only after release of Hip upon ATP rebinding could BAG-1 associate with Hsc70 to destabilize the ADP-bound state in a subsequent round of ATP hydrolysis. We would therefore like to propose that BAG-1-mediated regulation represents an alternative way to modulate Hsc70 chaperone activity in the eukaryotic cell, which does not involve the Hip protein (Figure 8).

Despite the GrpE-like activity of BAG-1, its primary structure does not display regions of significant similarity to bacterial GrpE or the mitochondrial GrpE homologue Mge1p. Notably, the previously identified members of the GrpE family exhibit only a low degree of sequence identity at the amino acid level (21% between rat mitochondrial and bacterial GrpE) (Naylor *et al.*, 1996). It thus appears that functional similarity does not require a strict conservation of the primary structure of these chaperone cofactors. A remarkable structural feature of the BAG-1 protein is the presence of a ubiquitin-like domain including a conserved lysine residue which, in the case of ubiquitin, serves as an acceptor site for the attachment of other ubiquitin molecules (Takayama *et al.*, 1995). Ubiquitin conjugation has been shown to be involved in protein degradation and protein sorting (Hochstrasser, 1996). The putative ubiquitin attachment site in BAG-1 may thus provide the means for a regulated turnover of the chaperone cofactor. Alternatively, covalent modification of BAG-1 by ubiquitin or a ubiquitin-like molecule may serve as a degradation-independent targeting signal.

Our data demonstrate that eukaryotic Hsc70 can be conversely regulated by two competing co-factors, BAG-1 and Hip. A similar versatility has not been observed in the regulation of the bacterial Hsp70 homologue DnaK (Georgopoulos *et al.*, 1994; Rüdiger *et al.*, 1997) and may thus represent an evolutionary adaptation to allow Hsc70 to fulfil its expanded spectrum of functions in the eukaryotic cell. Notably, BAG-1 comprises only ~0.005% of total cellular protein in human HeLa cells (data not shown), and is thus ~10-fold less abundant than Hip (Höhfeld *et al.*, 1995; Prapapanich *et al.*, 1996). BAG-1-mediated regulation of Hsc70 may therefore be restricted to certain pathways of protein biogenesis or be selective for specific substrates. In this context, the observed interaction of BAG-1 with the cell death inhibitor Bcl-2 and certain signalling molecules is intriguing (Takayama *et al.*, 1995; Zeiner and Gehring, 1995; Bardelli *et al.*, 1996; Wang *et al.*, 1996). Although it is possible that these interactions and the described anti-apoptotic activity of BAG-1 are unrelated to its Hsc70-regulating function, a cooperation of Hsc70 and BAG-1 during the maturation and activation of Bcl-2 and certain signalling molecules appears conceivable. The GrpE-like activity of BAG-1 may stimulate the release of BAG-1 binding partners from Hsc70 which may then be recognized by BAG-1 and accompanied during later stages of their maturation. Future studies are required to verify such a pathway and to determine whether the GrpE-like function of BAG-1 is responsible for the anti-apoptotic activity of the Hsc70 chaperone cofactor.

## Materials and methods

### Protein expression and purification

For construction of MBP-BAG, *bag-1* cDNA was amplified by PCR using random-primed cDNA of human neuronal SY5Y cells as template. PCR primers contained restriction sites for subcloning of the *bag-1* gene into vector pMAL-c2 (New England Biolabs). The pMAL–*bag-1* construct was introduced into *Escherichia coli* TG1 cells, and expression and affinity purification was performed according to the protocol of the manufacturer (New England Biolabs). To obtain non-tagged BAG-1 protein, the PCR-amplified *bag-1* cDNA was subcloned into vector pVL1392 (PharMingen) for expression in Sf9 insect cells after infection with recombinant baculovirus. In a similar manner, expression of rat Hip was induced in Sf9 cells infected with baculovirus carrying the plasmid pVL1393-*hip* which encodes full-length Hip. Sf9 cells were infected and maintained as described by the manufacturer (PharMingen). After growth of infected cells for ~60 h, BAG-1 and Hip, respectively, were purified by chromatography on DEAE–Sepharose (Pharmacia), Bio-Gel HT hydroxyapatite (Bio-Rad) and Source 30Q material (Pharmacia). Before final concentration and dialysis, the Hip preparation was passed over an ATP–agarose column (Sigma) at 500 mM KCl to remove associated Hsc70. Hsc70 was purified from Sf9 cells infected with a recombinant baculovirus carrying the plasmid pVL1393-*hsc70*. The plasmid contains a PCR-amplified cDNA fragment encoding full-length rat Hsc70 (Sorger and Pelham, 1987). Following infection of Sf9 cells and growth for ~60 h, cells were lysed using a French pressure cell, and Hsc70 protein was purified as described (Schlossman *et al.*, 1984). Human Hsp40 was expressed from the plasmid pET–Hsp40 in *E.coli* BL21 (DE3) cells and purified as described by Minami *et al.* (1996). BL21 (DE3) cells were also used to express the ATPase domain of bovine Hsc70 from a corresponding pET plasmid, and subsequent

purification of the ATPase domain was performed according to Willbanks *et al.* (1994). Protein concentrations were determined using Bradford assay reagent (Bio-Rad) with bovine γ-globulin as the standard. Molar concentrations of Hip were calculated for a pentameric complex (J.Höhfeld, unpublished data), and Hsp40 concentrations are given considering the dimeric structure of the DnaJ homologue.

### Hsc70 ATPase domain interaction assays

Interaction of MBP–BAG and Hip with the immobilized ATPase domain of bovine Hsc70 was analysed as described (Irmer and Höhfeld, 1997). Complexes of non-tagged human BAG-1 with the ATPase domain of bovine Hsc70 and full-length rat Hsc70 were analysed using a 5–18% blue native polyacrylamide gel system (Schägger *et al.*, 1994). 10 μg of the indicated purified proteins were incubated in 20 μl cathode buffer lacking dye for 30 min at 4°C, followed by the addition of 5 μl cathode buffer containing 15% glycerol and gel electrophoresis at 4°C. Competition between BAG-1 and Hip in binding to the ATPase domain was investigated using a previously established interaction assay (Irmer and Höhfeld, 1997) by incubation of 5 μl ATPase domain–Sepharose with 2 μg of purified Hip in the absence of BAG-1 and in the presence of a 1.6- and 8-fold molar excess of BAG-1 over the Hip oligomer, respectively. Incubation was performed for 2 h at 4°C before removal of the ATPase domain–Sepharose by centrifugation at 5000 *g* for 5 min.

### Analysis of Hsc70 ATPase activity

Rates of ATP hydrolysis were determined as described by Liberek *et al.* (1991). The nucleotide-bound state of Hsc70 under steady-state conditions was analysed according to Höhfeld *et al.* (1995). The effect of BAG-1, Hip and Hsp40 on Hsc70–$^{32}$P-nucleotide complexes in single turnover experiments was investigated as described (Minami *et al.*, 1996), except for using micro-spin G50 columns (Pharmacia) to rapidly isolate Hsc70–nucleotide complexes during the time course of ADP release.

### Analysis of Hip/BAG-1 interaction

10 μg of MBP–BAG was incubated with 40 μl amylose resin in 200 μl 20 mM MOPS–KOH, pH 7.2, 50 mM KCl, 2 mM MgCl$_2$, 1 mM β-mercaptoethanol (buffer A) at 4°C for 3 h. The resin was washed twice with 1 ml buffer A before addition of 200 μl buffer A containing 10 μg Hip protein. After incubation at 4°C for 1 h, the reaction mixture was centrifuged at 5000 *g* for 2 min. The supernatant fraction was TCA-precipitated for SDS–PAGE analysis, whereas the amylose resin was washed twice with buffer A before addition of SDS-sample buffer.

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