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Distinct Isoforms of the Cofactor BAG-1 Differentially Affect Hsc70 Chaperone Function*

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In the mammalian cytosol and nucleus the activity of the molecular chaperone Hsc70 is regulated by chaperone cofactors that modulate ATP binding and hydrolysis by Hsc70. Among such cofactors is the anti-apoptotic protein BAG-1. Remarkably, BAG-1 is expressed as multiple isoforms, which are distinguished by their amino termini. We investigated whether distinct isoforms differ with respect to their Hsc70-regulating activity. By comparing the mainly cytosolic isoforms BAG-1M and BAG-1S, opposite effects of the two isoforms were observed in chaperone-assisted folding reactions. Whereas BAG-1M was found to inhibit the Hsc70-mediated refolding of nonnative polypeptide substrates, the BAG-1S isoform stimulated Hsc70 chaperone activity. The opposite effects are not due to differences in the regulation of the ATPase activity of Hsc70 by the two isoforms. Both isoforms stimulated ATP hydrolysis by Hsc70 in an Hsp40-dependent manner through an acceleration of ADP-ATP exchange. Our results reveal that the different amino termini of the distinct BAG-1 isoforms determine the outcome of an Hsc70-mediated folding event, most likely by transiently interacting with the polypeptide substrate. Employing isoforms of a cofactor with different substrate binding properties appears to provide the means to influence the chaperone function of Hsc70 in addition to modulating its ATPase cycle.

Hsc70, a constitutively expressed member of the ubiquitous and highly conserved Hsp70 family of molecular chaperones, is involved in a variety of cellular processes including the regulation of signal transduction pathways and apoptosis (1–5). During these processes Hsc70 recognizes and stabilizes nonnative polypeptide substrates in a manner regulated by ATP binding and hydrolysis. In recent years several Hsc70-associated cofactors have been identified that modulate the Hsc70 ATPase cycle and, thus, alter the substrate binding affinity of the chaperone protein (2, 3, 6). One important group of cofactors is the Hsp40 protein family. Members of this family stimulate ATP hydrolysis, driving Hsc70 into the ADP-bound form with high affinity for the substrate (2, 3). Another cofactor shown to accelerate the ATPase cycle of Hsc70 is the BAG-1 protein (7–10). Notably, BAG-1 is expressed as distinct isoforms, which arise from a common transcript through the use of alternative in-frame translational start sites (11–13). Although all isoforms share a central ubiquitin-like domain and a carboxyl-terminal domain, which is sufficient for Hsc70 binding and regulation, the isoforms differ considerably in the length of their amino termini (see Fig. 1*A*). This is reflected in a different number of a hexapeptide motif (TRSEE*X*, in one letter code) present in the amino termini. Whereas the longer human isoforms BAG-1L and BAG-1M display 10 TRSEE*X* repeats, the human BAG-1S isoform possesses only four repeats (see Fig. 1*A*). In addition, the extended amino terminus of BAG-1L comprises a putative nuclear localization signal. BAG-1L was indeed shown to localize predominantly to the nucleus, whereas BAG-1M and BAG-1S were mainly found in the cytosol (11–13). Interestingly, oncogenic transformation results in an altered ratio of the distinct isoforms (12, 14). Moreover, the M isoform has been detected in human but not in mouse cells, where only the longer (L-) and shorter (S-) isoforms seem to be generated (12).

Several studies have now revealed that the distinct BAG-1 isoforms differentially affect cellular processes (see Table I). For example a stimulation of the transactivation activity of the androgen receptor was observed for BAG-1L but not for BAG-1M and BAG-1S (15). In another study BAG-1L and BAG-1M were shown to inhibit the DNA binding and transactivation activity of the glucocorticoid receptor, whereas BAG-1S did not affect this activity (16). It remains unclear, however, whether the observed functional diversity is related to differences in the Hsc70-regulating activity of the distinct BAG-1 isoforms. As a first step toward answering this question we decided to compare the mainly cytosolic isoforms BAG-1M and BAG-1S with regard to their activity in the regulation of the Hsc70 ATPase cycle and in Hsc70-mediated refolding reactions. We show that human BAG-1M as well as human and murine BAG-1S all stimulate the ATPase activity of Hsc70 in an Hsp40-dependent manner and in this way induce the release of substrates from Hsc70. Yet BAG-1M and BAG-1S affect the Hsc70-mediated refolding of denatured polypeptide substrates in opposite ways. Whereas BAG-1M inhibits the refolding reaction, BAG-1S displays a stimulating activity. The different amino termini of the BAG-1 isoforms apparently determine the effect of the cofactor on Hsc70-mediated protein folding, most likely due to transient interactions with the polypeptide substrate.

EXPERIMENTAL PROCEDURES

Recombinant Protein Production—Rat Hsc70 was recombinantly expressed in baculovirus-infected Sf9 insect cells, and human Hsp40 was expressed in *Escherichia coli*. Purification was performed as described previously (7). Recombinant human BAG-1M was cloned and expressed in baculovirus-infected Sf9 cells as described (7). Cells were lysed in buffer A (20 mM MOPS,[1] pH 7.2, 50 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol) containing Complete protease inhibitor (Roche Molecular Biochemicals) using a French pressure cell. The lysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant fraction was

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[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

TABLE I
Cellular functions of BAG-1 isoforms

Observation/cellular function	Involvement of BAG-1 isoforms	Reference No.
Binding to Bcl-2 and protection from apoptosis	BAG-1S	27, 28
Binding to and activation of the kinase Raf-1	BAG-1S	29
Binding to and enhanced protection from apoptosis by the hepatocyte growth factor receptor and the platelet-derived growth factor receptor	BAG-1S	30
Suppression of Siah-mediated inhibition of cell growth	BAG-1S	31
Inhibition of the DNA binding and transactivation activity of the glucocorticoid receptor; protection from hormone-induced apoptosis	BAG-1L, BAG-1M, BAG-1S no effect	16, 32
Stimulation of the transactivation activity of the androgen receptor	BAG-1L, BAG-1M no effect, BAG-1S no effect	15
Binding to and inhibition of the transactivation activity of the retinoic acid receptor; inhibition of retinoic acid-induced apoptosis	BAG-1S	33
Accelerated cell motility of gastric cancer cells	BAG-1S	34
Binding to DNA and stimulation of transcription	BAG-1M, BAG-1S no effect	35

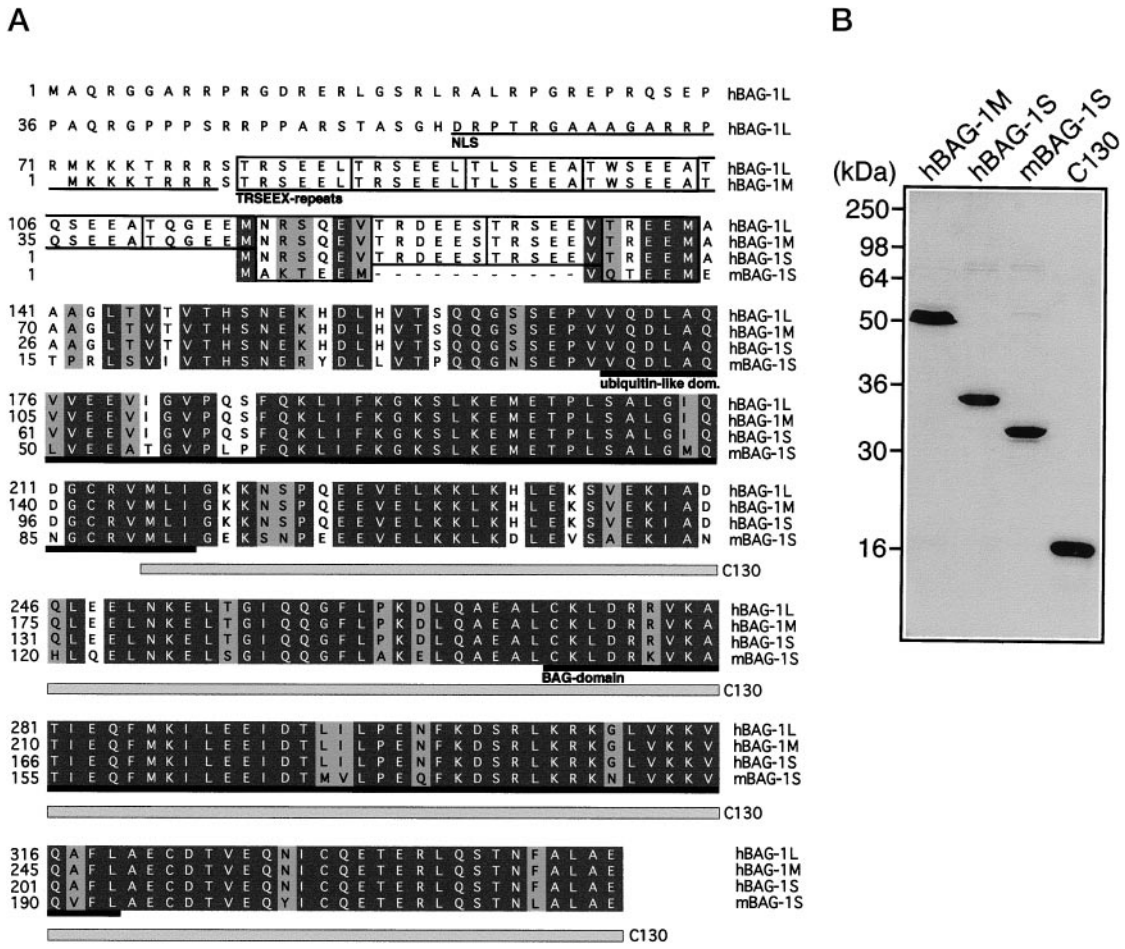

FIG. 1. **Primary structure of BAG-1 isoforms and purified recombinant proteins.** *A,* the amino acid sequence of the murine BAG-1S isoform (*mBAG-1S*) was aligned with the human BAG-1 isoforms (*hBAG-1L*, *hBAG-1M*, and *hBAG-1S*). Identical and similar residues are *shaded dark* and *light gray*, respectively. All isoforms contain a carboxyl-terminal BAG domain and a central ubiquitin-like domain (*black bars*). The amino-terminal extensions of the isoforms differ in length and comprise 10 (human BAG-1L and -M), four (human BAG-1S), and two (murine BAG-1S) repeats of the hexapeptide motif TRSEE*X*, respectively (*boxed*). The human L isoform possesses a putative nuclear localization signal (*underlined*). The deletion fragment C130 used in this study comprises the carboxyl-terminal 130 amino acids of human BAG-1. *B*, purified recombinant human BAG-1M, human BAG-1S, mouse BAG-1S, and the carboxyl-terminal fragment of human BAG-1 (C130) were analyzed by SDS-PAGE. 2 μg of each protein were loaded and visualized by Coomassie staining. A protein band migrating slightly faster than mouse BAG-1S in the corresponding sample represents a degradation product as determined by immunoblotting using an anti-BAG-1 antibody (not shown).

loaded on a DEAE-Sepharose column (Amersham Pharmacia Biotech) equilibrated in lysis buffer. The column was washed with buffer A, and bound protein was eluted by a linear gradient of 50–250 mM KCl in buffer A. Fractions were analyzed by SDS-PAGE and Coomassie staining. BAG-1M-containing fractions were pooled, and the same volume of 20 mM MOPS, pH 7.2, 1 mM β-mercaptoethanol containing Complete protease inhibitor was added. The diluted fractions were loaded on a Bio-Gel HT hydroxyapatite column (Bio-Rad) equilibrated in buffer B (50 mM potassium phosphate, pH 7.0, 1 mM β-mercaptoethanol) containing protease inhibitors. After washing with buffer B, a linear gradient of 50–500 mM potassium phosphate was used to elute bound protein. BAG-1M-containing fractions were pooled, 10 volumes of 20 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride were added, and the diluted fractions were loaded on a Source 30Q column (Amersham Pharmacia Biotech). For equilibration and washing of the 30Q column buffer C (20 mM MOPS, pH 7.2, 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) was used. BAG-1M was eluted from the column with a linear gradient of 20–200 mM KCl.

To obtain the cDNA sequence corresponding to the human BAG-1S

isoform (amino acids 45–274 of human BAG-1M) plasmid pVL-*bag-1* (7) was used as template in a polymerase chain reaction with primers containing *Eco*RI and *Bam*HI restriction sites for subcloning into vector pVL1392 (Pharmingen). The cDNA of murine BAG-1S was cloned from total RNA of mouse NIH3T3 cells by employing the Access reverse transcription-polymerase chain reaction system (Promega). RNA was purified with the RNeasy total RNA kit (Qiagen). To generate recombinant baculovirus the digested reverse transcription-polymerase chain reaction fragment was inserted into the *Eco*RI and *Bam*HI restriction sites of plasmid pVL1392. The resulting constructs pVL-*hbag-1s* and pVL-*mbag-1s* were used for co-transfection of Sf9 cells, and recombinant baculoviruses were generated. After infection of insect cells with recombinant viruses and expression of human and mouse BAG-1S for 4 days, cells were harvested and lysed in a French pressure cell. Both proteins were purified by chromatography on DEAE-Sepharose (Amersham Pharmacia Biotech), Bio-Gel HT hydroxyapatite (Bio-Rad), and Source 30Q (Amersham Pharmacia Biotech) resins. Purification of human BAG-1S was performed as described above for the human BAG-1M isoform. The mouse BAG-1S isoform was purified similarly except for binding to the hydroxyapatite resin, which was performed at 15 mM potassium phosphate followed by elution with a gradient of 15–500 mM potassium phosphate. Pooled fractions were diluted 10-fold with 20 mM triethanolamine, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol containing protease inhibitors and loaded on a Source 30Q column equilibrated in buffer D (20 mM triethanolamine, pH 8.0, 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol). Mouse BAG-1S was eluted using a gradient of 20–250 mM KCl in buffer D.

The cDNA sequence coding for the carboxyl-terminal fragment of human BAG-1 (C130, amino acids 145–274 of human BAG-1M) was polymerase chain reaction-amplified from plasmid pVL-*bag-1* (7) and subcloned into *Nde*I/*Sma*I-digested plasmid pTYB2 (New England Biolabs) using an *Nde*I restriction site overlapping the ATG start codon. In the resultant construct, the cDNA sequence of the BAG-1 fragment is fused in-frame to the coding region of a self-cutting intein/chitin binding domain. For the expression and purification of the carboxyl-terminal fusion protein, the IMPACT T7 kit (New England Biolabs) was employed according to a standard procedure described in the manufacturer's instructions. Briefly, cells were lysed in a French pressure cell in lysis buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 2 mM EDTA) containing Complete protease inhibitor. The cleared lysate was mixed with a chitin affinity resin for 1 h at 4 °C, and after binding of the fusion protein, the resin was washed extensively with lysis buffer and finally with cleavage buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 0.1 mM EDTA). The affinity matrix was equilibrated in cleavage buffer containing 40 mM dithiothreitol and incubated overnight at 4 °C. The reducing agent induced intein-mediated self-cleavage of the fusion protein, resulting in the release of the BAG-1 fragment. For elution the resin was washed with 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol. The obtained BAG-1 fragment differed from the native sequence by one additional glycine residue at the carboxyl terminus.

After purification of the BAG-1 isoforms and the deletion fragment, proteins were concentrated by ultrafiltration, and the buffer was exchanged to 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol. Protein concentrations were determined using the Bio-Rad Bradford reagent with purified IgG (Sigma) as the standard and by measuring absorbance at 205 nm (17). Molar concentrations of purified proteins were calculated for monomeric proteins. Purity of the protein preparations was determined by scanning and quantification of the Coomassie-stained SDS-PAGE gel.

ATPase and Nucleotide Release Assays—ATPase activity was measured as described (18). Hsc70 was incubated at 30 °C with cofactors as indicated in 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 70 μCi/ml [α-^{32}P]ATP. After 10-fold dilutions of aliquots into 20 mM MOPS-KOH, pH 7.2, 20 mM EDTA and thin layer chromatography, ADP formation was quantified using the Bio-imaging Analyzer (Fuji). ADP release from Hsc70-^{32}P-nucleotide complexes in the presence of human BAG-1S was investigated as described for the M isoform (7).

Gel Filtration Analysis of Protein Complexes—3 μM Hsc70, 2 μM Hsp40, and 6 μM BAG-1 as indicated were preincubated in complex formation buffer (20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 2 mM ATP at room temperature for 5 min. 1 μM luciferase was added, and the mixture was incubated for 15 min at 42 °C. After denaturation of luciferase, one volume of ice-cold glycerol/EDTA buffer (20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, 10% glycerol) was added. Alternatively, BAG-1 (6 μM) or just complex formation buffer were added, and the samples were incubated at 30 °C for 15 min before mixing with glycerol/EDTA buffer. The samples were centrifuged at 4 °C and 100,000 × *g* for 30 min, and the supernatant fractions were subjected to gel filtration analysis using a Superose 12 column (Amersham Pharmacia Biotech) as described previously (19).

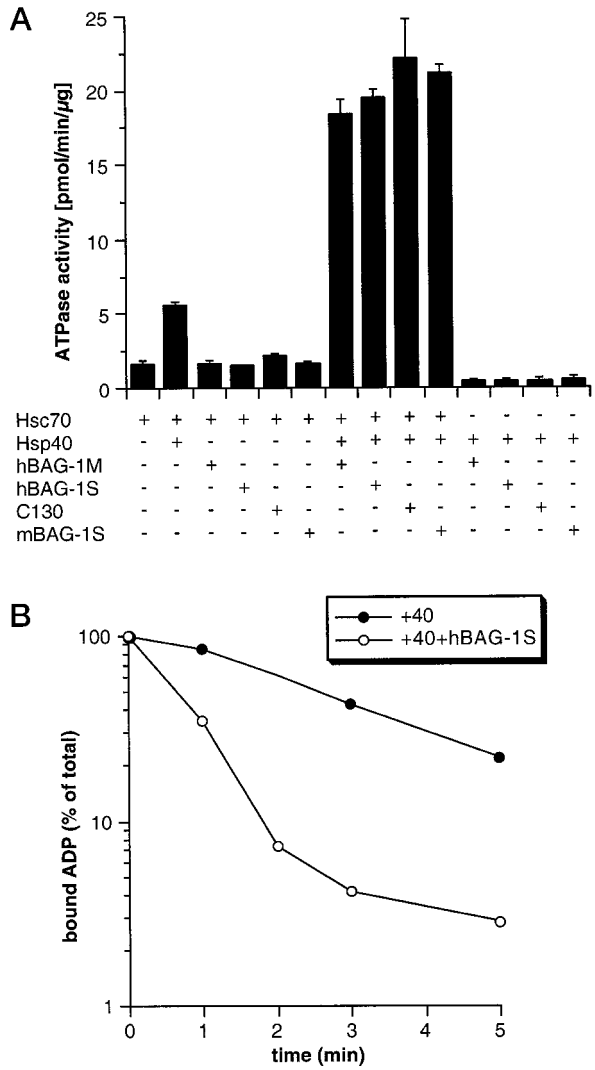

FIG. 2. **Distinct BAG-1 isoforms all stimulate Hsc70 ATPase activity in an Hsp40-dependent manner.** *A*, ATPase activities were determined at 30 °C in the presence of [α-^{32}P]ATP for the indicated combinations of 3 μM Hsc70, Hsp40, and the different BAG-1 isoforms. Aliquots were taken at different time points, and formed ADP was quantified after thin layer chromatography. Results shown are means of three independent experiments. *Error bars* represent S.D. *B*, the human BAG-1S isoform accelerates ADP release from Hsc70 in the presence of Hsp40. Hsc70-^{32}P-nucleotide complexes were formed at 30 °C by incubating 10 μM Hsc70 in the presence of 200 μM [α-^{32}P]ATP. Complexes were separated from free nucleotide by gel filtration and further incubated at room temperature in the presence of 50 μM non-labeled ATP and either 3 μM Hsp40 alone (*40*) or 3 μM Hsp40 and 3 μM human BAG-1S (*40 + BAG-1S*). At different time points, Hsc70-^{32}P-nucleotide complexes were re-isolated at 4 °C using gel filtration spin columns, and bound ADP was quantified as in *A*.

Refolding Assays—Refolding of heat-denatured firefly luciferase in the absence of rabbit reticulocyte lysate was performed as described (19). 100% activity corresponds to the enzyme activity of native luciferase that was diluted into refolding buffer A (10 mM MOPS-KOH, pH 7.2, 50 mM KCl, 3 mM $MgCl_2$, 2 mM dithiothreitol) containing 1 mg/ml bovine serum albumin. For the refolding of chemically denatured β-galactosidase, a recently published assay was employed (8). Refolding of 3.4 nM guanidine hydrochloride-denatured β-galactosidase in the presence of 1.6 μM Hsc70, 3.2 μM Hsp40, and 3.2 μM indicated proteins was assayed with the Galacto-Light system (Tropix). At various time points an aliquot of each sample was mixed with reaction buffer in a luminometer tube and incubated for 10 min at room temperature. The tube was placed in the luminometer and, after injection of accelerator solu-

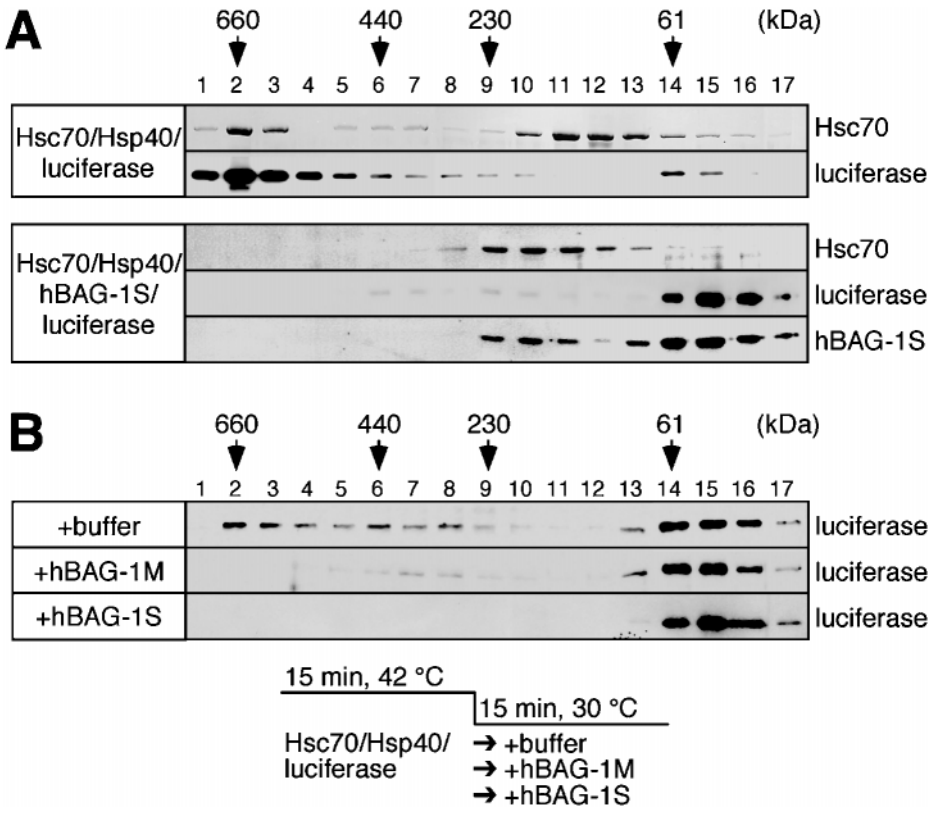

FIG. 3. **BAG-1 can dissociate Hsc70/substrate complexes.** *A*, luciferase (1 μM) was denatured in 2 mM ATP at 42 °C in the presence of Hsc70 and Hsp40 alone (3 μM and 2 μM, respectively) or Hsc70/Hsp40 and human BAG-1S (6 μM) as labeled on the left side of the *panels*. After centrifugation at 100,000 × *g*, the soluble fraction was analyzed by gel filtration on a Superose 12 column. Fractions were analyzed by Western blots. On the right side of the *panels*, the detected proteins are indicated. *B*, after an initial incubation of Hsc70, Hsp40, and luciferase (concentrations as in *A*) at 42 °C in 2 mM ATP for 15 min to form Hsc70-luciferase complexes, human BAG-1M (6 μM), human BAG-1S (6 μM), or buffer was added, and samples were shifted to 30 °C and further incubated for 15 min. Analysis of soluble protein complexes was as in *A*.

tion, light emission was counted for 10 s. The enzymatic activity of native β-galactosidase in refolding buffer B (25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol) was set to 100%.

Aggregation Assays—Aggregation of luciferase during heat denaturation was assayed as before (19) except for the centrifugation step, which was performed at 20,800 × *g*. The β-galactosidase aggregation assay was performed as described previously (20). After denaturation of β-galactosidase for 30 min at 30 °C in denaturation buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 6 M guanidine hydrochloride) denatured β-galactosidase was diluted 1:125 to a final concentration of 3.4 nM into refolding buffer B containing 1.6 μM Hsc70, 3.2 μM Hsp40, and other proteins (3.2 μM) as indicated. The reaction mixtures were incubated for 2 h at 37 °C, placed on ice, and centrifuged at 4 °C for 20 min at 20,800 × *g*. Supernatant and pellet fractions were separated and analyzed by SDS-PAGE and immunoblotting using a monoclonal anti-β-galactosidase antibody (Promega). Soluble β-galactosidase detected in the supernatant fraction was quantified relative to the total amount of β-galactosidase detected in both fractions by employing a LAS-1000 CCD camera and Image Gauge analysis software (Fuji).

RESULTS

Different BAG-1 Isoforms All Stimulate the ATPase Activity of Hsc70 in an Hsp40-dependent Manner and Induce Release of an Hsc70-bound Substrate—To test whether isoforms of BAG-1 that differ in their amino termini behave similarly in the regulation of Hsc70 function, we expressed and purified recombinant human BAG-1M, BAG-1S, and mouse BAG-1S (Fig. 1, *A* and *B*). All protein preparations were at least 93% pure. In addition, a carboxyl-terminal fragment of human BAG-1, C130, was expressed and affinity-purified to homogeneity (>98% pure) (Fig. 1, *A* and *B*).

The ATPase activity of Hsc70 was measured in the presence of the various BAG-1 proteins. As we have shown previously, Hsc70 ATPase activity is strongly stimulated by human BAG-1M in the presence of Hsp40 (7, 18). When we replaced the human M isoform with either the shorter S isoform or a carboxyl-terminal fragment of human BAG-1, C130, we observed the same strong stimulation of Hsc70 ATPase activity (Fig. 2*A*). This effect was dependent on the presence of Hsp40, as addition of BAG-1 isoforms or the fragment alone did not significantly affect the low basal ATPase activity of Hsc70 (Fig. 2*A*). The human M isoform increases the Hsc70 ATP hydrolysis rate by accelerating nucleotide exchange in the presence of Hsp40 (7). Similarly, the BAG-1S isoform stimulated ADP release from Hsc70 when added together with Hsp40 (Fig. 2*B*). It appears that accelerating nucleotide exchange on Hsc70 is a common function of the distinct human BAG-1 isoforms.

Compared with its human counterpart, the murine BAG-1S isoform possesses an amino terminus of slightly different primary structure. The amino acid similarity in this part of the molecules is not as high as in the ubiquitin-like and carboxyl-terminal domains, and the murine protein displays only two copies of the repeat motif TRSEE*X* compared with four repeats in human BAG-1S (Fig. 1*A*). Therefore we also tested the mouse protein for its ability to stimulate the chaperone ATPase activity in conjunction with Hsp40. Similar to the human isoforms, the mouse BAG-1S protein strongly stimulated the rate of ATP hydrolysis by Hsc70 in a manner dependent on the presence of Hsp40 (Fig. 2*A*). Apparently, the structural differences observed within the amino termini of the BAG-1 isoforms do not affect the regulation of the Hsc70 ATPase cycle by the cofactors.

We have recently shown that BAG-1M can dissociate Hsc70-substrate complexes based on its nucleotide exchange activity (19). A stimulation of substrate release from Hsc70 was also observed for the shorter human BAG-1S isoform. When firefly luciferase was heat-denatured in the presence of Hsc70 and Hsp40, formation of a high molecular mass chaperone-substrate complex was observed (about 600–700 kDa) (Fig. 3*A*) (19). In the presence of human BAG-1S, however, the complex was no longer detectable, and luciferase eluted from the gel filtration column as an uncomplexed form (Fig. 3*A*). To verify that accelerated substrate release as opposed to an inhibition of substrate binding is responsible for this observation, an additional experiment was performed. In this case, BAG-1M or

BAG-1S was added after formation of the chaperone-substrate complex, and samples were incubated for 15 min under refolding conditions. Whereas a significant amount of the high molecular mass complex was still detectable in the absence of the BAG-1 isoforms under these conditions, the complex was completely dissociated in the presence of the cofactors (Fig. 3*B*). This is consistent with an accelerated release of substrates from Hsc70 triggered by the distinct BAG-1 isoforms. Taken together, the BAG-1M and BAG-1S isoforms both stimulate the ATPase activity of Hsc70 by facilitating nucleotide exchange and, in this way, promote the dissociation of an Hsc70-substrate complex.

BAG-1 Isoforms Modulate the Refolding Activity of Hsc70 in Opposite Ways—How do the distinct BAG-1 isoforms affect Hsc70-mediated protein folding? To answer this question, firefly luciferase was heat-denatured in the presence of Hsc70, Hsp40, and different BAG-1 isoforms, and effects of the isoforms on the refolding reaction were analyzed. In agreement with previous findings (19), the human BAG-1M isoform inhibited the Hsc70/Hsp40-mediated reactivation of luciferase (Fig. 4*A*). Surprisingly, however, under the same conditions the shorter human S isoform had the opposite effect and strongly stimulated the refolding capacity of the Hsc70/Hsp40 system in a concentration-dependent manner (Fig. 4, *A* and *B*). When we examined the mouse BAG-1S isoform, a stimulating activity was again observed, and the amount of reactivated luciferase was comparable with that obtained in the presence of human BAG-1S (Fig. 4*A*). It was conceivable that the stimulating activity of the small isoforms was due to contaminating proteins following expression of the isoforms in insect cells. In fact, minor amounts of polypeptides in the 70-kDa size range were detectable in the BAG-1S preparations (Fig. 1*B*). However, the refolding activity of Hsc70/Hsp40 was also stimulated by the BAG-1 fragment C130, which was derived from a corresponding fusion protein expressed in *E. coli* and was affinity-purified to homogeneity (Fig. 4*A*, see also Fig. 1*B*). We therefore conclude that the stimulating activity represents an authentic function of the BAG-1S isoforms and that the amino terminus of the human BAG-1M isoform negatively affects the reactivation of heat-denatured luciferase by Hsc70 and Hsp40.

We extended our analysis using β-galactosidase as an additional substrate protein. Consistent with our results in the luciferase refolding assay, reactivation of chemically denatured β-galactosidase by Hsc70 and Hsp40 was blocked in the presence of human BAG-1M, whereas either the human or the mouse BAG-1S isoform exerted a significant stimulation of the refolding reaction (Fig. 5). Interestingly, however, the yield of refolded β-galactosidase was not increased by the addition of the C130 fragment, which stimulated the refolding of luciferase. In the case of β-galactosidase, the amino terminus of the BAG-1S isoform is apparently necessary to mediate a stimulating activity, whereas the extended amino terminus of BAG-1M inhibits the reactivation reaction. Again, distinct isoforms of BAG-1 differentially affect Hsc70-mediated protein folding.

BAG-1 Isoforms Differentially Affect the Stabilization of Nonnative Polypeptide Substrates—We next investigated the capacity of the Hsc70/Hsp40 system to prevent the aggregation of denatured polypeptide substrates in the absence and presence of BAG-1 isoforms, respectively. Luciferase was heat-denatured in the presence of different combinations of Hsc70 and cofactors, and the amount of soluble luciferase was assessed. Similarly, the aggregation of β-galactosidase was analyzed after dilution of chemically denatured β-galactosidase into buffer containing Hsc70 and cofactors in different combinations.

Hsc70 in conjunction with Hsp40 stabilized denatured luciferase and β-galactosidase, and about 80% and 35% of the total amount of the substrates, respectively, remained soluble in this situation. Addition of BAG-1M, however, strongly impaired the stabilizing activity of Hsc70 (Fig. 6, *A* and *B*). This is in agreement with the inhibitory function of BAG-1M observed in the refolding experiments (see above). In contrast, upon the addition of the human and murine BAG-1S isoforms or the C130 fragment to an Hsc70/Hsp40-containing reaction, luciferase or β-galactosidase were efficiently stabilized (Fig. 6*A*). These results again reveal a differential regulation of Hsc70 chaperone function by the BAG-1M and BAG-1S isoforms.

When we incubated the denatured substrates in the presence of each BAG-1 isoform alone, human BAG-1M stabilized a significant portion of both denatured luciferase and β-galactosidase (60–65% soluble; Fig. 6, *A* and *B*). In the case of the BAG-1S isoforms and the C130 fragment, however, both sub-

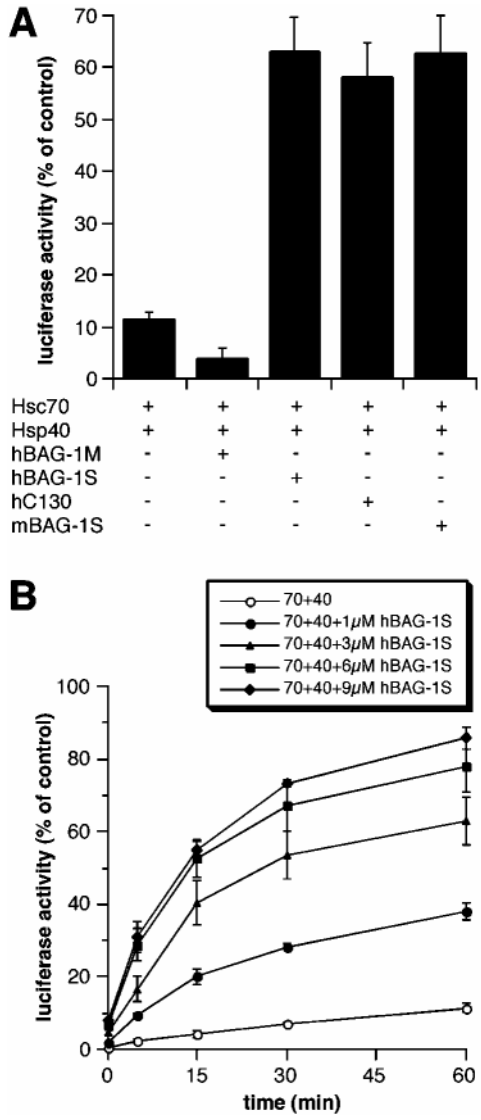

FIG. 4. **Depending on the isoform, BAG-1 either inhibits or stimulates refolding of heat-denatured luciferase.** *A*, luciferase was heat-inactivated at 42 °C in the presence of 3 μM Hsc70, 2 μM Hsp40, and 3 μM each of indicated proteins. The amount of reactivated luciferase was assessed by measuring enzyme activity after refolding at 30 °C for 60 min. Data represents the means of three experiments. S.D. are shown by *error bars*. *B*, refolding of luciferase was analyzed as in *A* in the presence of 3 μM Hsc70 (*70*), 2 μM Hsp40 (*40*), and different concentrations of human BAG-1S. At various time points, aliquots of the reactions were assayed for luciferase activity. Mean values of three experiments ± S.D. are plotted.

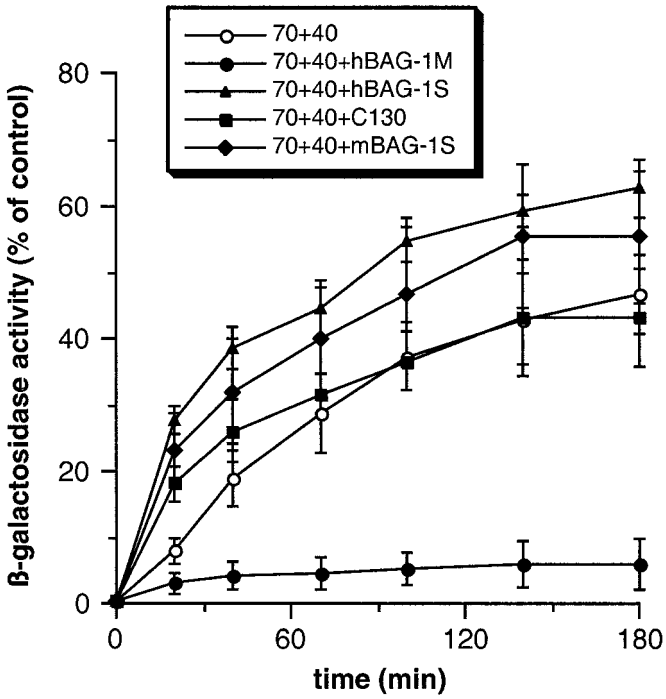

FIG. 5. **BAG-1 isoforms differentially affect the Hsc70-mediated refolding of chemically denatured β-galactosidase.** β-Galactosidase was denatured in guanidine hydrochloride and diluted into buffer containing the indicated combinations of proteins. Concentrations of Hsc70 (*70*), Hsp40 (*40*), and BAG-1 isoforms were 1.6 μM, 3.2 μM, and 3.2 μM, respectively. Refolding of β-galactosidase at 37 °C was followed by measuring enzyme activity at various time points. Values of three experiments were averaged and plotted. S.D. are shown by *error bars*.

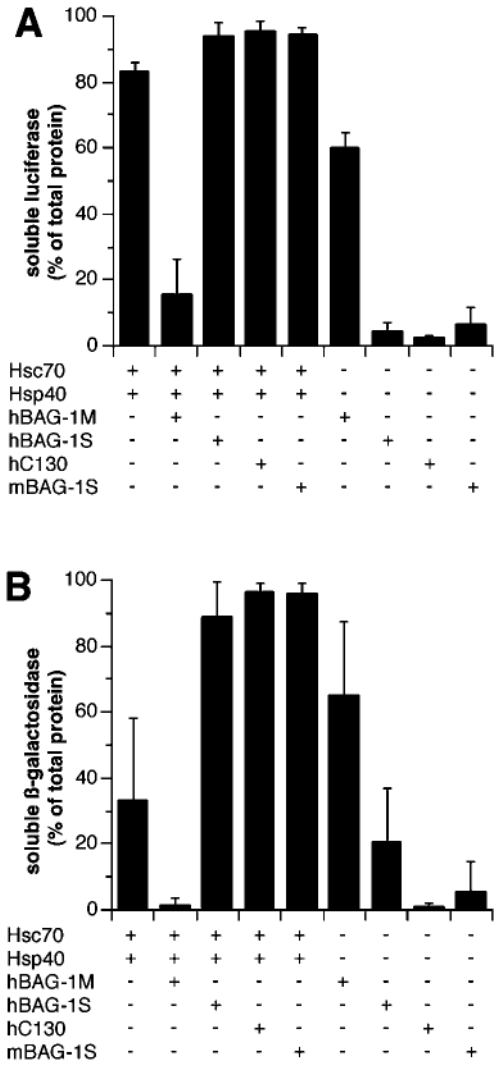

FIG. 6. **BAG-1 isoforms differentially affect the stabilization of denatured polypeptide substrates.** The stability of denatured luciferase (*A*) and of denatured β-galactosidase (*B*) was analyzed in the presence of different combinations of proteins as indicated. Luciferase was incubated at 42 °C for 15 min and chemically denatured β-galactosidase for 2 h at 37 °C as described in Figs. 4 and 5, respectively. The percentage of total protein in the soluble fraction is plotted. Results shown are means of at least three experiments ± S.D.

strate proteins were found mainly in the insoluble pellet fractions (5–20% soluble; Fig. 6, *A* and *B*). Thus it appears that the BAG-1M isoform can interact with a substrate protein to promote stabilization in the absence of Hsc70. For this interaction, the extended amino terminus of the M isoform appears to be essential, as the shorter S isoforms did not stabilize denatured luciferase and only weakly affected denatured β-galactosidase.

DISCUSSION

Here we demonstrate that distinct isoforms of the chaperone cofactor BAG-1 differentially affect Hsc70 chaperone function. The mainly cytosolic isoforms BAG-1M and BAG-1S in fact display opposite effects. The longer M isoform acts as an inhibitor of the Hsc70 chaperone system, whereas the S isoform promotes the folding capacity of the chaperone protein. Remarkably, these differences are not caused by distinct regulatory activities of the two isoforms in the modulation of the ATPase cycle of Hsc70. Both BAG-1M and BAG-1S strongly stimulate the ATPase activity of Hsc70 in a manner dependent on Hsp40. This appears to be due to an accelerated exchange of ADP against ATP following the Hsp40-induced hydrolysis step, since both isoforms are able to trigger ADP release from Hsc70 (Fig. 2*B*) (7). An Hsp40-dependent stimulation of the ATPase activity of Hsc70 was also observed for a carboxyl-terminal fragment of BAG-1, which lacks the ubiquitin-like domain and the amino-terminal TRSEE*X* repeat region (Fig. 2*A*). Apparently, the amino-terminal portion of BAG-1 does not influence the regulation of the Hsc70 ATPase cycle by the cofactor. Yet, the distinct amino termini of the different BAG-1 isoforms determine the effect of the cofactor in Hsc70-mediated folding reactions.

How can this be explained? Secondary structure analysis of the BAG-1M amino terminus predicts that the 10 TRSEE*X* repeats form an extended amphipathic α-helix with the negatively charged residues aligned in three rows along the axis of the helix (21). In addition, six positively charged residues are found at the extreme amino terminus of BAG-1M and appear to form a cluster on top of the amphipathic helix (Fig. 1*A*). It is tempting to speculate that this remarkable structural arrangement represents a protein-protein interaction domain. The BAG-1S isoform lacks the positively charged head group and displays an incomplete repeat region (Fig. 1*A*). Conceivably, these differences in the structure of the amino termini may cause altered protein binding properties of the BAG-1 isoforms. The different effects observed for BAG-1M and BAG-1S in Hsc70-mediated protein folding may thus be explained by an altered interaction of the cofactors with the chaperone protein or, alternatively, with the polypeptide substrate. We favor the latter notion because the amino terminus of BAG-1 is dispensable for the interaction of the cofactor with Hsc70 and does not modulate the regulatory function of the cofactor in the ATPase cycle (Fig. 2). The amino termini of the BAG-1 isoforms may represent substrate interaction domains with distinct binding properties. In fact the BAG-1M isoform alone is able to stabilize denatured luciferase and β-galactosidase, whereas the BAG-1S

isoform does not prevent luciferase aggregation during heat treatment and displays only a weak stabilizing activity in the case of β-galactosidase (Fig. 6). Despite the stabilizing activity of BAG-1M we were unable to demonstrate the formation of a complex comprising the chaperone cofactor and the denatured substrate (not shown). The interaction that causes the observed stabilization appears to be rather transient. In any case, our data clearly reveal distinct substrate binding properties of the BAG-1 isoforms determined by their distinct amino termini.

The findings presented here regarding the function of the mouse BAG-1S isoform differ from previously published observations. A recent report described that the protein inhibits the refolding of chemically denatured β-galactosidase mediated by Hsc70 and Hsp40 (20), whereas we observed a stimulating activity of the BAG-1S isoform. We can only speculate about the reasons for these differences. Important aspects might be the source of the recombinant proteins that were used and possibly a modification of the proteins for facilitated purification. To ensure functionality, we expressed the different BAG-1 isoforms in eukaryotic cells without any modifying tags. Leaving the amino terminus unperturbed might be of particular importance in the light of our findings indicating a role in substrate binding. In this regard it is also noteworthy that Nollen *et al.* (22) recently described an inhibiting activity of BAG-1S on the refolding of heat-denatured luciferase *in vivo*. BAG-1S-induced release of luciferase from Hsc70, which results in productive folding *in vitro*, may thus be unproductive in the cellular environment, possibly due to inappropriate interactions with other proteins.

An interaction of a chaperone cofactor with nonnative polypeptide substrates has previously been described for the bacterial Hsp40 homologue DnaJ (23, 24). The cofactor appears to deliver polypeptide substrates to its Hsp70 partner protein. In this way a transfer of the chaperone into the high affinity substrate binding state, which is induced by the Hsp40 homologue, may only occur when a substrate is present in the near vicinity. The BAG-1 isoforms act later in the ATPase and peptide binding cycle of Hsc70 and can trigger the dissociation of the chaperone-substrate complex (Fig. 3) (19). The isoforms may thus bind to the polypeptide substrate following its release from Hsc70. Remarkably, BAG-1M alone stabilizes heat-denatured luciferase but, in the presence of Hsc70 and Hsp40, promotes aggregation (Fig. 6). In the latter case, the polypeptide substrate might be presented to BAG-1M in a more extended conformation, and accordingly, a transient interaction with the cofactor might be insufficient to exert a stabilizing effect while at the same time the cofactor interferes with the action of Hsc70. We cannot exclude that a so-far undetected interaction of the amino terminus of BAG-1M with Hsc70 might occur during this process.

Our results are intriguing with respect to recently observed functional differences between the BAG-1 isoforms in the regulation of signal transduction pathways (see Table I). For example BAG-1L and BAG-1M but not BAG-1S were found to inhibit the transactivation activity of the glucocorticoid receptor (16). For the inhibition the complete TRSEE*X* repeat region of the longer isoforms was required. Since Hsc70 cooperates with the molecular chaperone Hsp90 during the regulation of steroid hormone receptors (2, 6, 25), the distinct effects of the BAG-1 isoforms in receptor activation may be caused by their distinct Hsc70-modulating activities.

By combining the carboxyl-terminal Hsc70 binding domain of BAG-1 with different amino termini, the cell appears to have found means to expand the regulatory function of the cofactor. In this regard it is remarkable that several BAG-1-related proteins have recently been identified (26). All carry a region of about 45 amino acids similar to the Hsc70 binding domain of BAG-1 (BAG-domain; Fig. 1*A*). The BAG domain is often located near or at the carboxyl terminus of these proteins, whereas different sequence motifs and domains extend toward the amino terminus. It is intriguing to speculate about an involvement of these BAG-1-related proteins in inducing functional specificity of the Hsc70 chaperone system. The diversity in Hsc70 regulation achieved by employing distinct BAG-1 isoforms may thus have been further extended through the recruitment of a larger family of BAG-1-related proteins.

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