Bag-1M Accelerates Nucleotide Release for Human Hsc70 and Hsp70 and Can Act Concentration-dependent as Positive and Negative Cofactor* 

The cytosol of mammalian cells contains several Hsp70 chaperones and an arsenal of cochaperones, including the anti-apoptotic Bag-1M protein, which regulate the activities of Hsp70s by controlling their ATPase cycles. To elucidate the regulatory function of Bag-1M, we determined its influence on nucleotide exchange, substrate release, ATPase rate, and chaperone activity of the housekeeping Hsc70 and stress-inducible Hsp70 homologs of humans. Bag-1M and a C-terminal fragment of it are potent nucleotide exchange factors as they stimulate the ADP dissociation rate of Hsc70 and Hsp70 up to 900-fold. The N-terminal domain of Bag-1M decreased the affinity of Bag-1M for Hsc70/Hsp70 by 4-fold, indicating a modulating role of the N terminus in Bag-1M action as nucleotide exchange factor.

Surprisingly, under physiological conditions, i.e. low Bag-1M concentrations and presence of Pi, Bag-1M activates the chaperone action of Hsc70/Hsp70 in luciferase refolding. Bag-1M accelerated ATP-triggered substrate release by Hsc70/Hsp70. We propose that Bag-1M acts as substrate discharging factor for Hsc70 and Hsp70.

The Hsp70 proteins are involved in folding processes throughout the entire life span of proteins. The molecular basis of their functions is the transient interaction with substrates through ATP-controlled cycles (1, 2). In the ATP state Hsp70s have a low affinity for substrates, and in the ADP state the affinity for substrates is high (3, 4). ATP hydrolysis was shown to be stimulated synergistically by the simultaneous interaction with a substrate and a cochaperone of the DnaJ family (5–8). Under physiological conditions nucleotide exchange is rate-limiting for substrate release (3, 9, 10). For this functional cycle the regulation by cochaperones is an important issue. How do they act in vivo?

In order to resolve these contradictions we investigated the influence of Bag-1M on nucleotide exchange, substrate release, ATPase, and chaperone activity of two cytosolic and nuclear Hsp70 homologs of human cells, Hsc70 and Hsp70, under identical conditions.

MATERIALS AND METHODS

Protein Expressions and Purification—Hsc70, Hsp70, Bag-1M, and Bag-1M-(151–274) were recombinantly expressed in E. coli Escherichia coli cells. Hsc70 and Hsp70 were purified according to the DnaK protocol (20). Bag-1M was purified as described (13); Bag-1M-(151–274) was purified via DEAE-cellulose and SP-Sepharose columns (Amerham Pharmacia Biotech). Hsp70 was expressed in E. coli and purified according to the DnaJ protocol (21).

ATPase Assay—Steady-state ATP hydrolysis rates were determined as described for DnaK (22–24). Reactions were performed at 30 °C in mixtures containing buffer HKM (25 mM HEPES-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl2), 1 μM Hsc70 or Hsp70, 2 μM Hdj-1, 0–17.5 μM Bag-1M, 250 μM ATP, and 0.1 μM of [α-32P]ATP (Amerham Pharmacia Biotech). Quantification was performed as described (25).

Luciferase Refolding—Hsc70 (1 μM) and cochaperones (Hdj-1, 0.2 μM; Bag-1M as indicated) were incubated in HKM buffer supplemented with 5 mM dithiothreitol and 2 mM ATP for 5 min at 20 °C. Firefly luciferase (70 μM in 1 mM glycyglycine, pH 7.4; Roche Molecular Biochemicals) was added to 0.1 μM and denatured for 20 min at 42 °C. The refolding reaction (20 μl) was started by shifting back the temperature to 30 °C and addition of 5% rabbit reticulocyte lysate (Promega) and 2.5 mM ATP. At the indicated times, 1-μl aliquots were diluted into 125 μl of assay buffer and analyzed for bioluminescence activity in a Biolumat (Berthold, Bad Wildbad, Germany) as described (25). The enzymatic activity of native luciferase diluted in refolding buffer containing Hsc70 (1 μM) and Hdj-1 (0.2 μM) and incubated at 30 °C was set to 100%.
were used. Hsc70/Hsp70 (0.5 μM) and MABA-ADP (0.5 μM) in HKM buffer were preincubated for 30 min at 30 °C and mixed 1:1 in a stopped-flow device (SX-18M Applied Photophysics, Surrey, UK) with a solution of 250 μM ADP + Bag-1M (0–90 μM). Fluorescence (excitation, 360 nm; cut-off filter at 420 nm) was measured for 1–200 s at 30 °C, and the time-dependent decrease was fitted to a single exponential decay. Where indicated 10 mM Pi, was added. ATP association was measured by mixing 0.25 μM MABA-ATP with different amounts of Hsc70/Hsp70. For the determination of ATP dissociation, MABA-ATP was mixed with Hsc70/Hsp70 for 30 s and subsequently mixed with unlabeled ATP + Bag-1M in double-mixing experiments.

Substrate Release—To determine protein substrate release 3H-labeled RCLMA was used. 10 μM Hsp70 or Hsc70 and 0.3 μM 3H-RCLMA ± 20 μM Bag-1M were preincubated in 20 μL of T buffer (25 mM Tris, pH 7.8, 200 mM KCl, 5% glycerol, 0.05% Tween 20) for 1 h at 30 °C, ± 50 μM unlabeled RCLMA and ± 2.5 mM ATP were added, incubated for 1 min at 30 °C, and separated via a Superdex 200 gel filtration column (Amersham Pharmacia Biotech). To determine peptide substrate koff, dansyl chloride (Molecular Probes)-labeled NR peptide (NR-LLLTG) was used (D-NR). Hsp70 (5 μM) and D-NR (2 μM) were preincubated in HKM buffer for 30 min at 30 °C and mixed 1:1 in a stopped-flow device (SX-18M Applied Photophysics, Surrey, UK) with 250 μM unlabeled NR ± 0.5 mM ATP ± 5 μM Bag-1M. Fluorescence (excitation, 354 nm; cut-off filter at 385 nm) was measured for 1–1000 s at 30 °C. To determine the rate constants a single or double exponential decay function was fitted to the time-dependent decrease in fluorescence.

Partial Proteolytic Digest—To determine the domain structure of Bag-1M a partial proteolytic digest with trypsin was performed. 18 μg of Bag-1M were incubated with 0.01 μg of trypsin at 30 °C for 0–30 min. Reaction was stopped by the addition of SDS sample buffer and incubation at 95 °C for 5 min. For analysis a 15% SDS gel was used.

RESULTS

Association and Dissociation of Fluorescent Nucleotide Analogs—We wanted to investigate the effects of Bag-1M on nucleotide exchange of Hsc70 and Hsp70 by directly measuring nucleotide release. As nucleotide exchange is a rapid process for Hsc70, we intended to use the fluorescent ADP and ATP analogs MABA-ADP/ATP and stopped-flow instrumentation. These analogs were shown previously to have similar kinetic properties as their authentic counterparts in case of the E. coli homolog DnaK (10). To verify the suitability of MABA-ADP and MABA-ATP for measuring Hsc70 and Hsp70 nucleotide exchange kinetics, we determined the mutual exchange rates of MABA-ADP versus ADP and MABA-ATP versus ATP. ADP and ATP exchange rates were independently measured using the intrinsic fluorescence of the single tryptophan that was shown to change in response to ATP binding (26, 27). The measured rates were very similar, and the fluorescent analogs were therefore suitable for the nucleotide exchange experiments. The MABA-ADP koff values determined for Hsc70 at 30 °C (0.24 s⁻¹) were approximately 10-fold higher than published values for ADP release at 25 °C (Table I (28)). Hsp70 had nearly identical MABA-ADP dissociation rates as Hsc70. For both chaperones, the addition of physiological concentrations of Pi (10 mM) decreased the dissociation rate by 5-fold (Table I).

Nucleotide Exchange in the Presence of Bag-1M—We used MABA-ADP to determine directly the effects of Bag-1M on nucleotide release from Hsc70 and Hsp70. As evidenced by the differential decrease in fluorescence signal, Bag-1M stimulated the off-rate of MABA-ADP for Hsc70 (Fig. 1A) and Hsp70 (not shown) in the absence of Pi. Titrating the Bag-1M concentration and plotting the measured off-rates against the Bag-1M concentration resulted in a hyperbolic curve with an apparent Kd and a maximal rate of 1.8 μM and 23 s⁻¹ for Hsp70 and 3.9 μM and 24 s⁻¹ for Hsp70, respectively (Fig. 1, B and C). This clearly demonstrates that Bag-1M is a nucleotide exchange factor for both cytosolic Hsp70 proteins investigated, stimulating the off-rate of MABA-ADP by up to 100-fold.

Surprisingly, higher concentrations of Bag-1M were needed to reach similar stimulatory effects in the presence of physiological concentrations of Pi (10 mM). Under these conditions we were not able to reach saturation with the Bag-1M concentrations used, and the data could not be fitted by a hyperbolic function. Due to the 5-fold decreased basal koff for ADP in the presence of Pi, the highest stimulation factors reached were

| Table I |
| Association and dissociation rates of MABA-ADP and MABA-ATP |
| Dissociation of MABA-ADP was determined by rapidly mixing complexes of MABA-ADP and Hsc70 or Hsp70 with an excess of unlabeled ADP in a stopped-flow device. For the determination of the MABA-ATP dissociation rate, MABA-ATP was first mixed with the nucleotide-free Hsc70/Hsp70 for 30 s and subsequently with an excess of unlabeled ATP in double-mixing experiments. Association of MABA-ATP was measured by mixing MABA-ATP or unlabeled ATP with different concentrations of nucleotide-free Hsc70 or Hsp70. Single and double exponential equations were fitted to the change of fluorescence.

| Hsp70 | Hsc70 |
|---|---|
| k_{off} (s⁻¹) | 0.24 | 0.3 |
| ADP | 0.046 | 0.05 |
| | 0.2 | 0.78 |
| ATP | 0.01 | 0.06 |
| k_{off} (s⁻¹) | 4.9 × 10⁵ | 1.1 × 10⁶ |
| k_{ATP} (M⁻¹ s⁻¹) | 0.2² | ND² |

² Measured by tryptophan fluorescence at 18 °C, k_{off} for MABA-ATP was 6.9 × 10⁵ M⁻¹ s⁻¹ at 30 °C.

² ND, not determined.

![Fig. 1. Stimulation of ADP release from Hsc70/Hsp70 by Bag-1M. A, dissociation of the MABA-ADP-Hsc70 complex after addition of an excess of unlabeled ADP in the absence (■) and presence of Bag-1M (0.5 μM) (○). B and C, the k_{off} values of MABA-ADP for Hsp70 (B) and Hsc70 (C) were determined at various Bag-1M concentrations, and the rates were plotted against the Bag-1M concentration. D, k_{off} values for MABA-ADP and Hsc70 in the presence of increasing Bag-1M-(151–274) concentrations. ■, Hsc70 − Pi; ○, Hsc70 + Pi; ●, Hsp70 − Pi; ▲, Hsp70 + Pi.](http://www.jbc.org/)}
Regulation of Hsc70 and Hsp70 Activity by Bag-1M

690- and 460-fold over the basal rate for Hsc70 and Hsp70, respectively. In order to compare these data with the previously studied E. coli DnaK system (29), we performed a similar titration using the E. coli GrpE to measure DnaK-MABA-ADP dissociation. The data obtained were almost identical to those published (29) demonstrating that P_i does not affect the GrpE-stimulated ADP dissociation (not shown).

We also investigated the association and dissociation of ATP using either MABA-ATP or tryptophan fluorescence. For both proteins the association and dissociation of ATP occurred in a two-step process as previously published for Hsc70 (26). MABA-ATP associated with Hsp70 with a 2-fold higher rate than with Hsc70. ATP association measured via tryptophan fluorescence yielded within the error range identical values (Table I). MABA-ATP dissociated from Hsp70 also in a two-step mechanism with the faster rate being 0.78 s^{-1} which is four times faster than the dissociation rate for MABA-ATP from Hsc70 (Table I). The slower rate, which most likely corresponds to an isomerization step that precedes dissociation, was for Hsp70 (0.06 s^{-1}) even six times faster than for Hsc70 (0.01 s^{-1}). The presence of Bag-1M even at high concentrations did not increase the apparent association and dissociation rates of MABA-ATP for either Hsp70 or Hsc70.

The potent nucleotide exchange factor Bag-1M therefore differs from GrpE in as much as P_i influences the concentration necessary for half-maximal stimulation, and that Bag-1M discriminates between bound ADP and bound ATP.

Nucleotide Exchange in the Presence of the Bag-1M C-terminal Fragment—To identify the domain within Bag-1M that is responsible for the observed stimulation of the ADP dissociation by Hsc70 and Hsp70, we attempted to determine the domain structure of Bag-1M biochemically using partial proteolytic digestion. Tryptic digestion of Bag-1M produced a very stable 20-kDa fragment consisting of the C-terminal 124 residues (151–274) as determined by N-terminal sequencing and molecular weight (Fig. 2). We cloned and purified the corresponding fragment (Bag-1M-(151–274)) and investigated its effect on ADP dissociation by Hsc70/Hsp70. Previous work used sequence homology criteria to derive a Bag-1 fragment that was slightly longer (Bag-1M-(129–274)) (14). The recently published crystal structure of a Bag-1 fragment in complex with Hsc70 as well as the recently published NMR structure of Bag-1 used fragments that were almost identical to our fragment (30, 31).

Bag-1M-(151–274) catalyzed the ADP release by both Hsp70 proteins even more efficiently than the full-length Bag-1M protein. This was particularly apparent in the presence of P_i as demonstrated in Fig. 1D for Hsp70. A hyperbolic function could be fitted to the data yielding K_d values of 1 and 17 μM and a maximal rate of 20 and 42 s^{-1} (83- and 913-fold stimulation over basal dissociation rate) in the absence and presence of P_i, respectively. Comparison of the K_d values with the value for full-length Bag-1M in absence of P_i (3.9 μM) revealed that the apparent affinity of the Bag-1M fragment was almost 4-fold higher. If the same were valid in the presence of P_i the K_d for the full-length Bag-1M protein would be in the order of 70 μM. This would explain why these data points (see Fig. 1B) seem to follow a linear dependence. The titration curves for Hsp70 were very similar (not shown).

Taken together, the Bag-1 fragment stimulates nucleotide release by Hsp70 and Hsc70 even more efficiently than full-length Bag-1M.

Influence of Bag-1M on the Steady-state ATPase Activity of Hsc70 and Hsp70—After having analyzed the influence of Bag-1M on nucleotide association and dissociation, we investigated its effect on the ATPase cycle. Measurement of the intrinsic ATPase rates yielded 2.48·10^{-3} s^{-1} and 4.5·10^{-4} s^{-1} for Hsc70 and Hsp70, respectively. This is much slower than the ADP dissociation and the ATP association rates indicating that for both proteins ATP hydrolysis is rate-limiting for the ATPase cycle consistent with earlier publications for Hsc70 (28). Under these conditions Bag-1M should not have any effect on the steady-state ATPase rate. The DnaJ-protein Hdj-1 stimulated both Hsp70 proteins up to a maximal steady-state ATPase rate of 0.008 and 0.003 s^{-1} for Hsc70 and Hsp70, respectively. Under single turnover conditions increasing concentrations of Hdj-1 stimulated the ATPase activity of both Hsp70 proteins to much higher rates of more than 0.03 s^{-1} (not shown), demonstrating that in the presence of Hdj-1 ATP hydrolysis is not more rate-limiting for the ATPase cycle. Therefore, in the presence of Hdj-1, acceleration of nucleotide exchange by Bag-1M should stimulate the overall steady-state ATPase rate. We therefore determined the steady-state ATPase rates for Hsc70 and Hsp70 in the presence of high concentrations of Hdj-1 (2 μM) and titrated the Bag-1M concentration (1–15 μM).

In the case of Hsc70, Bag-1M stimulated the ATPase rate as

![Figure 2: Tryptic digest of Bag-1M](A) showing the time course of the digestion of 18 μg of Bag-1M with 10 ng of trypsin. B, position of the trypsin cuts in Bag-1M as revealed by Edman degradation of the indicated fragment; I, Arg-7; 2, Ser-9; 3, Ser-12; 4, Ser-18; 5, Ser-48; 6, Asn-151.

![Figure 3: Effect of Hdj-1 and Bag-1M on the ATPase activity of Hsc70 and Hsp70](B) showing the time course of the digestion of 18 μg of Bag-1M with 10 ng of trypsin. B, position of the trypsin cuts in Bag-1M as revealed by Edman degradation of the indicated fragment; I, Arg-7; 2, Ser-9; 3, Ser-12; 4, Ser-18; 5, Ser-48; 6, Asn-151.
Regulation of Hsc70 and Hsp70 Activity by Bag-1M

The activity of Bag-1M to stimulate nucleotide dissociation from Hsc70 and Hsp70 conceivably allows ATP to bind to the nucleotide-free form of the chaperone and, consequently, is expected to trigger the release of any bound substrate. However, based on native gel electrophoresis data, Bag-1M was proposed to uncouple nucleotide exchange and substrate release (17). We used gel filtration to determine the amount of complex of Hsc70 or Hsp70 with tritiated, reduced carboxymethylated α-lactalbumin ([3H]RCMLA) as substrate after addition of ATP in the absence or presence of Bag-1M. Unlabeled RCMLA was added together with ATP to prevent rebinding of the tritiated substrate. 1 min after the addition of ATP the amount of Hsc70- or Hsp70-bound [3H]RCMLA was significantly reduced (Fig. 4). This reduction was enhanced and not prevented by Bag-1M. However, when the quench RCMLA was omitted a significant amount of [3H]RCMLA was found in complex with Hsc70 and Hsp70, indicating rebinding of the released substrate.

To resolve the influence of Bag-1M on the dissociation of the Hsc70-substrate complex kinetically, we used the fluorescent labeled peptide substrate D-NR (dansyl-NRLLTGC). This peptide bound to Hsp70-ADP with high affinity, and binding was detectable by an increase of the dansyl fluorescence at 526 nm. Mixing the complex of D-NR and Hsp70-ADP with an excess of unlabeled peptide led to a decrease in fluorescence at a rate of 3.9×10^{-3} s^{-1} which is consistent with substrate dissociation rates measured for peptides and other Hsp70 homologs in the ADP state (3, 4, 32, 33). The addition of ATP to the Hsp70-ADP-D-NR complex accelerated the dissociation of D-NR to 0.43 s\(^{-1}\) which is similar to the ADP dissociation rate, indicating that nucleotide exchange is rate-limiting for substrate release under these conditions. Addition of Bag-1M in the absence of ATP did not influence the dissociation rate of the peptide. However, in the presence of ATP, Bag-1M accelerated the dissociation of the peptide significantly to 2.48 s\(^{-1}\) (Fig. 5). This value is identical to the dissociation rate of the Hsp70-ADP-D-NR complex in the presence of ATP when nucleotide-free preparations of Hsp70 are used (Table II). This clearly demonstrates that Bag-1M accelerates substrate release by accelerating nucleotide exchange. Our data suggest that Bag-1M acts as a substrate unloading factor for Hsc70 and Hsp70.

Effects of Bag-1M on the Chaperone Activity of Hsc70 and Hsp70—To analyze the regulatory influence of Hdj-1 and Bag-1M on the chaperone activity of Hsc70 and Hsp70, we measured refolding of heat-denatured firefly luciferase. For Hsc70 and Hsp70 we first titrated Hdj-1 and Bag-1M to determine the optimal ratios for luciferase refolding (not shown). For both

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**Fig. 4.** Bag-1M does not inhibit substrate dissociation. A, gel filtration chromatograms; Hsp70 + [3H]RCMLA, solid line; Hsp70 + [[3H]RCMLA + Bag-1M, dashed line; Hsp70 + [3H]RCMLA + Bag-1M + ATP + unlabeled RCMLA, dotted line; peak 1, Hsp70-[3H]RCMLA-Bag-1M complex; peak 2, Hsp70-[3H]RCMLA complex; peak 3, free [3H]RCMLA. B, the amount of Hsc70- and Hsp70-bound [3H]RCMLA depends on preincubation with ATP, Bag-1M, and/or excess of unlabeled RCMLA. Hsc70 and Hsp70 (10 μM) were incubated with [3H]RCMLA (0.3 μM) for 1 h. ATP (2.5 mM), Bag-1M (20 μM), and unlabeled RCMLA (50 μM) were added as indicated and incubated for 1 min at 30 °C, and Hsc70- and Hsp70-bound [3H]RCMLA were separated from free [3H]RCMLA by gel filtration on a Superdex 200 HR10/30 column (Amersham Pharmacia Biotech).

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**Fig. 5.** Bag-1M stimulates substrate release by acceleration of nucleotide exchange. Hsp70-ADP-bound D-NR was mixed with ATP in the absence or presence of ATP + Bag-1M. The solid lines represent the single (basal) and double exponential curve fits resulting in dissociation rates of 3.9×10^{-3} s^{-1} (basal), 3.85×10^{-3} s^{-1} (+Bag-1), 0.42/0.044 s^{-1} (+ATP), and 2.48/0.23 s^{-1} (+ATP+Bag-1M).
systems, Hsp70/Hdj-1 and Hsc70/Hdj-1, we then titrated Bag-1M in the absence and in the presence of Pi and measured the initial rate of luciferase refolding.

In the absence of Pi, Hsp70 and Hsc70 in combination with Hdj-1 refolded up to 60% of denatured luciferase within 1 h (Fig. 6A). Addition of increasing concentrations of Bag-1M or Bag-1M-(151–274) decreased significantly the rate and the yield of luciferase refolding as previously observed by different laboratories (13, 15–17) (Fig. 6, B and C, closed symbols).

In the presence of physiological concentrations of Pi, the refolding rates and yields for both chaperones were reduced by 50–60%. Under these conditions substoichiometric concentrations of Bag-1M (up to 1 μM), which in the absence of Pi inhibited refolding by 40–70%, did not inhibit luciferase refolding (Fig. 6, B and C, open symbols). The Hsc70/Hdj-1 system was even stimulated by Bag-1M to a refolding activity that was almost as high as in the absence of Pi (Fig. 6B).

We conclude that under physiological concentrations of Pi, Bag-1M can positively affect the chaperone activity of Hsc70 and Hsp70.

The concentration dependence of the regulatory effects of Bag-1M on the chaperone activity of Hsc70 parallels the concentration dependence of the GrpE effect on the DnaK/DnaJ-mediated refolding of luciferase, especially when the 10-fold lower ADP dissociation rate of DnaK is considered (29). To analyze this parallelism between the Hsc70/Hdj-1/Bag-1M and the DnaK/DnaJ/GrpE systems more closely, we wanted to compare the luciferase refolding rate with the time the unfolded substrate is on average bound by the respective Hsp70 chaperone. As ADP dissociation is the rate-limiting step for substrate release, the time a substrate is bound by the Hsp70s is directly proportional to the half-life of the Hsp70-ADP complex.

We therefore calculated for each Bag-1M concentration (≥1 μM) used in the luciferase refolding assay the half-life of the Hsp70-ADP complex using the fit parameter determined in Fig. 1B. Similarly, we calculated for each GrpE concentration used in the luciferase refolding assays published by Stein and co-workers (29) the ADP half-life using the fit parameter from their GrpE titration of the ADP dissociation. We then plotted the luciferase refolding rates normalized to the highest value for each Hsp70 system against the calculated ADP half-lives.

The data points describe for both Hsp70 systems a similar rate dependence of the GrpE effect on the DnaK/DnaJ-ADP dissociation and the apparent Kd values for the interaction of Bag-1M and Hsp70 and Hsc70 (1.8 and 3.9 μM). Compared with these values the prokaryotic DnaK/GrpE system reaches a 5-fold higher maximal ADP dissociation rate but has also a 5–10-fold higher apparent Kd value for nucleotide exchange (20 μM (29)). Interestingly, in the presence of Pi, the Kd value for the interaction of Bag-1M with Hsc70 and Hsp70 was drastically increased and even significantly higher than for the prokaryotic system, which was not affected by Pi. In addition, contrary to GrpE, Bag-1M did not affect ATP dissociation. This discrepancy between the prokaryotic and the eukaryotic nucleotide exchange factor indicates that Bag-1M binds in a different way to Hsc70 than GrpE to DnaK. One explanation is that the ATPase domain of Bag-1M only binds to and stabilizes the open conformation of the ATPase domain of Hsc70. The key process, which then determines the affinity for Bag-1M, is the rate of transition from the closed to the open conformation, which would depend on the nucleotide, being highest for bound ADP, intermediate for ADP + Pi, and lowest for ATP. In contrast to Bag-1M, GrpE is proposed to bind to all nucleotide states of DnaK but then shifts the equilibrium to the open state. Both alternatives are in good agreement with the recently published structure of the Bag-domain/Hsc70-ATPase domain complex (31). In this structure the Bag-domain has a number of contacts to subdomain IIB but very little contact to subdomain IB on the other side of the nucleotide binding cleft, and it is hard to imagine how Bag-1 should effect the opening of the nucleotide binding cleft. In contrast to this, in the GrpE/DnaK structure (34), GrpE makes extensive contacts to both subdomains of the ATPase domain and seems to insert into the nucleotide binding cleft to break open two salt bridges and a van-der-Waals bridge as recently suggested (35).

**Effect of Bag-1M on the ATPase Cycle**—In the presence of Hdj-1 increasing concentrations of Bag-1M had significantly different effects on Hsc70 and Hsp70. Whereas low concentrations of Bag-1M already stimulated the ATPase activity of Hsc70, very high concentrations of Bag-1M were necessary to stimulate the ATPase rate of Hsp70. This was surprising as the individual steps of the ATPase cycle (stimulation of the ATPase activity by Hdj-1 under single turnover conditions and stimulation of nucleotide exchange by Bag-1M) revealed only small differences between the two Hsp70 homologs. However, these small differences of the individual steps may add up when the whole cycle is analyzed. Alternatively, the cochaperones may influence each other when they act together at the same time. These results demonstrate an astonishing complexity of the regulation of the ATPase cycle of Hsp70s. Our results reconcile the apparently contradicting data of previous publications (13, 14, 17).

**Effect of Bag-1M on Substrate Release**—For DnaK it was unstable, and we demonstrate that the refolding reaction is thereby inhibited significantly. Under these conditions, Bag-1 at concentrations substoichiometric to Hsc70/Hsp70 stimulates the refolding reaction of Hsc70/Hdj-1 and at least does not inhibit the refolding reaction of Hsp70/Hdj-1. We propose that the Bag-1 effect on the chaperone activity of Hsc70/Hsp70 is caused by the acceleration of substrate release through the stimulation of nucleotide exchange.

**Comparison of Bag-1M and GrpE and Implications for the Nucleotide Exchange Mechanism**—By using fluorescent labeled nucleotides we show that Bag-1M stimulates the ADP dissociation rate of Hsc70 and Hsp70. We determined the maximal stimulated ADP dissociation rates in the absence of Pi (24 s⁻¹) and the apparent Kd values for the interaction of Bag-1M and Hsp70 and Hsc70 (1.8 and 3.9 μM). Compared with these values the prokaryotic DnaK/GrpE system reaches a 5-fold higher maximal ADP dissociation rate but has also a 5–10-fold higher apparent Kd value for nucleotide exchange (20 μM (29)). Interestingly, in the presence of Pi, the Kd value for the interaction of Bag-1M with Hsc70 and Hsp70 was drastically increased and even significantly higher than for the prokaryotic system, which was not affected by Pi. In addition, contrary to GrpE, Bag-1M did not affect ATP dissociation. This discrepancy between the prokaryotic and the eukaryotic nucleotide exchange factor indicates that Bag-1M binds in a different way to Hsc70 than GrpE to DnaK. One explanation is that the ATPase domain of Bag-1M only binds to and stabilizes the open conformation of the ATPase domain of Hsc70. The key process, which then determines the affinity for Bag-1M, is the rate of transition from the closed to the open conformation, which would depend on the nucleotide, being highest for bound ADP, intermediate for ADP + Pi, and lowest for ATP. In contrast to Bag-1M, GrpE is proposed to bind to all nucleotide states of DnaK but then shifts the equilibrium to the open state. Both alternatives are in good agreement with the recently published structure of the Bag-domain/Hsc70-ATPase domain complex (31). In this structure the Bag-domain has a number of contacts to subdomain IIB but very little contact to subdomain IB on the other side of the nucleotide binding cleft, and it is hard to imagine how Bag-1 should effect the opening of the nucleotide binding cleft. In contrast to this, in the GrpE/DnaK structure (34), GrpE makes extensive contacts to both subdomains of the ATPase domain and seems to insert into the nucleotide binding cleft to break open two salt bridges and a van-der-Waals bridge as recently suggested (35).

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shown earlier that nucleotide exchange is the rate-limiting step for substrate release and that GrpE stimulates substrate release by stimulating nucleotide exchange (see also Refs. 29 and 35). We show here that Bag-1M has a similar function for Hsc70 and Hsp70. Bag-1M stimulates nucleotide dissociation and, hence, binding of ATP, which then increases the rate of substrate dissociation. Our data are consistent with Ref. 31 but are difficult to reconcile with a proposed role of Bag-1M in uncoupling the nucleotide status of Hsp70 from substrate release (17). It is possible that the experimental conditions used to establish such a role of Bag-1M as uncoupling factor measured events occurring during repetitive cycles of substrate and nucleotide binding and release during migration in an electric field and in the molecular crowding conditions of an acrylamide gel. Together, these data demonstrate that Bag-1M acts as substrate discharging factor for Hsp70 proteins.

**Negative Modulation of the Bag Domain**—The identified stable proteolytic C-terminal fragment Bag-1M-(151–274) (Bag domain) stimulated nucleotide exchange of Hsc70 and Hsp70 even more efficiently than full-length Bag-1M. Half-maximal stimulation of ADP release by the Bag domain was reached at one-fourth the concentration needed for full-length Bag-1M, indicating a negative influence of the N-terminal part of Bag-1M. This observation sheds a different light onto the three translation initiation variants of Bag-1 (L, M, and S) and the other members of the Bag family (11). Due to the negative influence of the additional domains, the affinity of these variants and homologs of Bag-1 for the cytoplasmic Hsp70s could be significantly different. Furthermore, it is conceivable that under conditions where the additional domain(s) interact with specific target proteins (e.g. proteasome, Bcl-2, or Raf-kinase (36–38)), conformational changes could modulate their negative effect on the binding of the C terminus to Hsp70s. This would lead to a more efficient stimulation of the nucleotide exchange and thereby accelerate substrate release when the Bag protein and the respective Hsp70s are at specific target locations. Bag-1 could then act as a targeting factor.

**Bag-1M Function during Refolding of Heat-denatured Luciferase**—In this study, we could show that there are two factors important for the regulatory action of Bag-1M, the concentration of Bag-1M itself and P$_i$. In the absence of P$_i$, Bag-1M inhibited refolding of heat-denatured luciferase already at substoichiometric concentrations consistent with the earlier publications (13–18). In the presence of physiological concentrations of P$_i$, however, low concentrations of Bag-1M stimulated significantly the refolding of luciferase by Hsc70/Hdj-1. The Hsc70/Hdj-1 system differed in this respect from the Hsp70/Hdj-1 system for which only a small but consistent stimulation of Hsc70/Hdj-1 system differed in this respect from the Hsp70/Hdj-1 system for which only a small but consistent stimulation of luciferase was observed. At high concentrations, Bag-1M inhibited the chaperone activity of both Hsp70 systems. These results imply that the concentration of Bag-1M in the cell is of critical importance for its effect on the chaperone activity of Hsc70 and Hsp70. This circumstance considerably complicates *in vivo* experiments using cell lines that overproduce Bag-1M, as too much Bag-1M may disturb its cellular distribution and normal functions. The *in vivo* concentration of Bag-1M varies in different tissues considerably but is generally substoichiometric to the constitutively expressed Hsc70 which implies a positive regulatory effect on this chaperone (39, 40). However, in some tumor cell lines and upon treatment with interferons Bag-1 protein levels are increased (40–42). The chaperone function of Hsp70 proteins in these cell lines will consequently be affected which might contribute to the pathogenic phenotype. In addition, there are indications that Bag-1M is not evenly distributed in the cytosol, and the local concentration is likely to vary considerably.

**Mechanistic Implications of the Nucleotide Exchange Factor Effect on the Hsp70 Refolding Activity**—We found that the refolding rate of denatured luciferase by the prokaryotic DnaK/DnaJ/GrpE and the eukaryotic Hsc70/Hdj-1/Bag-1M systems are maximal at similar half-lives for the chaperone-ADP complex and therefore at similar half-lives...
for the chaperone-substrate complex.

Based on these results the influence of Bag-1M on the chaperone activity of Hsc70 and of GrpE on the activity of DnaK could be explained in two ways. First, by accelerating substrate release the concentration of free substrate that would be in a folding competent but still aggregation-prone intermediary conformation will be increased. This may increase the refolding rate but at the same time increases the probability of aggregation. Above a threshold concentration of this intermediate, aggregation may predominate and the refolding yield will decrease. Second, by accelerating substrate release the half-life of the Hsp70-substrate complex is decreased. It is possible that a certain half-life of this complex is optimal for refolding.

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