HspBP1, a homologue of the yeast Fes1 and Sls1 proteins, is an Hsc70 nucleotide exchange factor

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Abstract The yeast *FES1* and *SLS1* genes encode conserved nucleotide exchange factors that act on the cytoplasmic and endoplasmic reticulum luminal Hsp70s, Ssa1p and BiP, respectively. We report here that mammalian HspBP1 is homologous to Fes1p and that HspBP1 promotes nucleotide dissociation from both Ssa1p and mammalian Hsc70. In contrast, Fes1p inefficiently strips nucleotide from mammalian Hsc70, and unlike HspBP1 does not inhibit chaperone-mediated protein refolding in vitro. Together, our data indicate that HspBP1 is a member of this new class of nucleotide exchange factors that exhibit varying degrees of compartment and species specificity.

Key words: HspBP1; Fes1p; Sls1p; Hsc70; Hsp70; Nucleotide exchange

1. Introduction

The 70-kDa heat shock proteins (Hsp70s) and the constitutive Hsp70 homologues (Hsc70s) are a ubiquitous family of molecular chaperones known to facilitate protein synthesis, folding, transport and degradation [1–4]. Hsp70s are composed of a highly conserved N-terminal 44-kDa ATPase domain, an 18-kDa peptide-binding domain, and a C-terminal 10-kDa variable 'lid' domain. In the ATP-bound state, Hsp70s display fast on and off rates of peptide binding, whereas in the ADP-bound state these constants are slowed [5,6]. The modulation of the affinity for polypeptide substrates is triggered by a conformational change in the lid that is induced by ATP hydrolysis [7].

The ATPase activity of Hsp70s is weak but can be stimulated by specific members of the Hsp40/DnaJ family, which share a common 70-amino acid J-domain required for binding to and activation of Hsp70s [8,9]. In *Escherichia coli* the ATPase activity of the Hsp70 family member, DnaK, is stimulated by the DnaJ chaperone, which promotes stable binding of DnaK to polypeptides [10–12]. In addition, the GrpE protein acts as a nucleotide exchange factor (NEF) that promotes ADP release from DnaK [13–15]. The preferred rebinding of ATP catalyzes dissociation of DnaK–polypeptide substrate complexes. Cycles of polypeptide association and dissociation prevent aggregation and may facilitate protein folding.

Whereas members of the DnaJ family are ubiquitous [9], homologues of GrpE are found only in prokaryotes, archea, mitochondria and chloroplasts. However, several unrelated factors have been reported to enhance Hsp70/Hsc70 nucleotide exchange in the eukaryotic cytoplasm. These include the antiapoptotic Bag-1 protein [14,16–19] and its homologues [19,20], and the Sls1 and Fes1 proteins that are homologous, reside in the lumen of the endoplasmic reticulum (ER) and in the cytoplasm, respectively, and act preferentially on cognate ER and cytoplasmic Hsp70 chaperones in yeast, BiP and Ssa1p [21,22].

In a search for mammalian homologues of Fes1p/Sls1p, we found that Fes1p has 25% identity and 38% similarity to HspBP1. HspBP1 is expressed in all mammalian tissues, interacts with the ATPase domain of cytosolic Hsp70, and inhibits its ATPase and refolding activities [23]. We also found that purified HspBP1 is a NEF. Thus, Fes1p, Sls1p, and HspBP1 together represent a newly defined family of eukaryotic NEFs.

2. Materials and methods

2.1. Protein purification

Glutathione *S*-transferase (GST)-tagged Fes1p was purified as described previously [22]. Ssa1p, prepared as published [24], was a kind gift of Christine M. Smith (University of Pittsburgh). HspBP1 was purified from a BL21/DE3 *E. coli* strain bearing the pET28a-HspBP1 plasmid [23]. A 50-ml culture in Luria broth containing kanamycin at a final concentration of 50 μg/ml was grown overnight at 26°C and diluted into 2 l of the same medium. After 2 h at 26°C, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and the cells were grown for an additional 4 h before they were harvested, washed sequentially in water and then in sonication buffer (50 mM HEPES–KOH pH 7.4, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol, 0.5% Nonidet P40), and snap-frozen in liquid nitrogen and stored at −80°C. The cells were thawed, protease inhibitors (phenylmethylsulfonyl fluoride: 1 mM; pepstatin A: 1 μg/ml; leupeptin: 1 μg/ml) and 100 mg of lysozyme were added, and after a 30-min incubation on ice, the cells were broken by sonication for 30 s six times with a 1-min incubation on ice between each disruption. The broken cells were centrifuged at 13 000 rpm in a Sorvall SA600 rotor for 15 min, and the resulting supernatant was centrifuged at 20 000 rpm in a Sorvall SA600 for 20 min to obtain a cleared lysate. This lysate was loaded onto a 10-ml Ni-NTA–agarose column (Amersham Pharmacia Biotech) equilibrated in sonication buffer. The column was washed sequentially with 30 ml of the following: (1) sonication buffer; (2) sonication buffer containing 1 M NaCl; (3) sonication buffer containing 5 mM ATP; and (4) sonication buffer containing 5% glycerol.

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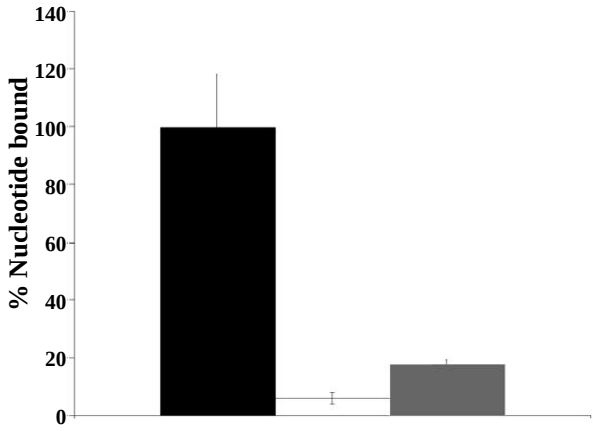

Fig. 1. HspBP1 and Fes1p block ATP binding to Ssa1p. Ssa1p was incubated with [α-^{32}P]ATP for 10 min at 30°C in the presence of buffer (black bars) or a three-fold molar excess of GST-Fes1p (white bars) or HspBP1 (gray bars). Free nucleotide was removed by gel filtration on microspin G-50 columns and the amount of bound nucleotide was quantified. Data represent the means of three independent reactions, ±S.D.

The HspBP1 protein was eluted with a 15×15 ml imidazole gradient (10–250 mM) in sonication buffer supplemented with 5% glycerol, and 1-ml fractions were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie brilliant blue staining. Peak fractions were pooled and diluted four-fold into buffer 88 (20 mM HEPES pH 6.8, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate) and loaded onto a 10-ml Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated in buffer 88. The column was extensively washed with buffer 88 before proteins were eluted with a 15×15 ml gradient of potassium acetate (0.2–2 M) in buffer 88 and 1-ml fractions were collected and analyzed by SDS–PAGE and Coomassie brilliant blue staining. Peak fractions were pooled, dialyzed against 10 mM HEPES–KOH pH 7.0, 50 mM NaCl, and 10 mM dithiothreitol, and kept in small aliquots at 4°C. HspBP1 was >95% pure as assessed by SDS–PAGE and Coomassie brilliant blue staining.

2.2. Nucleotide binding, nucleotide exchange and ATPase assays

For nucleotide-binding assays, Ssa1p (2 μg) was incubated in complex buffer (25 mM HEPES–KOH pH 7.5, 100 mM KCl, 11 mM magnesium acetate) containing 25 μM ATP and 5 μCi of [α-^{32}P]ATP (3000 mCi/mmol; NEN) for 10 min at 30°C. When indicated GST-Fes1p (6 μg) or HspBP1 (3.75 μg) were also present in the reaction. The final concentrations of the proteins in this reaction were: Ssa1p: ~1.5 μM, and GST-Fes1p or HspBP1: ~4.5 μM. Ssa1p-bound nucleotide was then purified from free nucleotide on G-50 microspin columns (Amersham Pharmacia Biotech) and the eluate was mixed with 5 μl of stop buffer (36 mM ATP, 2 M LiCl, 4 M formic acid). The amount of nucleotide remaining was measured by scintillation counting and analyzed by thin layer chromatography (TLC) as described [22].

Nucleotide exchange assays were performed exactly as published [22], and where indicated HspBP1 or GST-Fes1p was added at a 3:1 molar ratio to Ssa1p.

A bovine Hsc70–[α-^{32}P]ATP complex was obtained as published for the Ssa1p–[α-^{32}P]ATP complex preparation [24], except that 10 μg of purified bovine Hsc70 was used (Stressgen). Nucleotide exchange assays with this complex were performed as described above.

Single-turnover ATPase assays were performed essentially as described [22,24] with a three-fold molar excess of GST-Fes1p or HspBP1 to Ssa1p.

2.3. Firefly luciferase refolding assays

Renaturation of heat-denatured firefly luciferase in rabbit reticulocyte lysates was performed essentially as described [23]. Where indicated 20 μM of HspBP1 or GST-Fes1p were added to the reaction.

3. Results

3.1. Fes1p and HspBP1 inhibit nucleotide binding to Ssa1p

As a first step toward investigating the biochemical function of HspBP1, we performed a nucleotide-binding assay with Ssa1p. The experimental procedure was similar to the one used to study the effect of HspBP1 on ATP binding to Hsp70 [23]. Ssa1p was incubated with ATP in the presence or absence of either purified GST-Fes1p or HspBP1 protein, and free and chaperone-bound nucleotides were resolved by gel filtration on G-50 microspin columns. The amount of bound nucleotide was quantified by scintillation counting and by TLC followed by phosphorimager analysis. As shown in Fig. 1, both Fes1p and HspBP1 inhibited ATP binding to

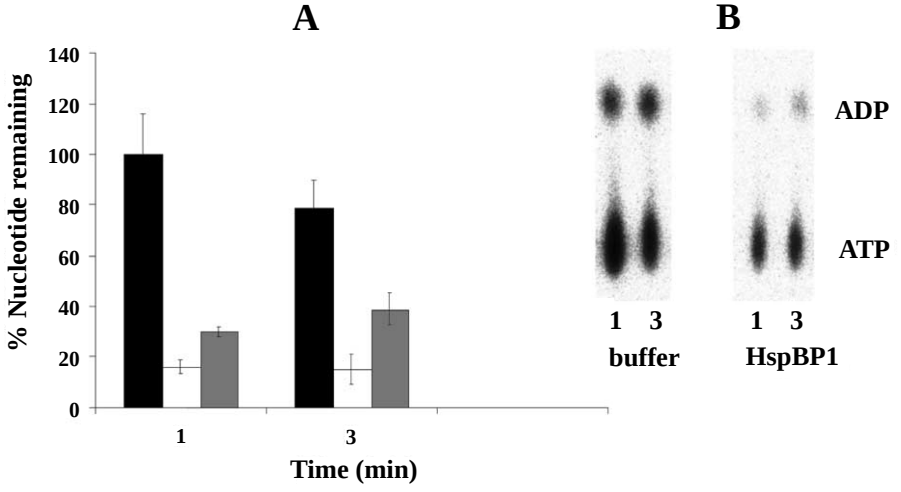

Fig. 2. HspBP1 is a NEF for Ssa1p. Ssa1p–[α-^{32}P]ATP complex was incubated with buffer (black bars) or a three fold-molar excess of GST-Fes1p (white bars) or HspBP1 (gray bars) at 30°C. At the indicated times an aliquot was taken and free nucleotide was removed as in Fig. 1. The reaction in the eluate was quenched and the amount of Ssa1p-bound nucleotide was (A) determined by scintillation counting or (B) analyzed by TLC as described [22,24]. Data represent the means from three independent reactions, ±S.D.

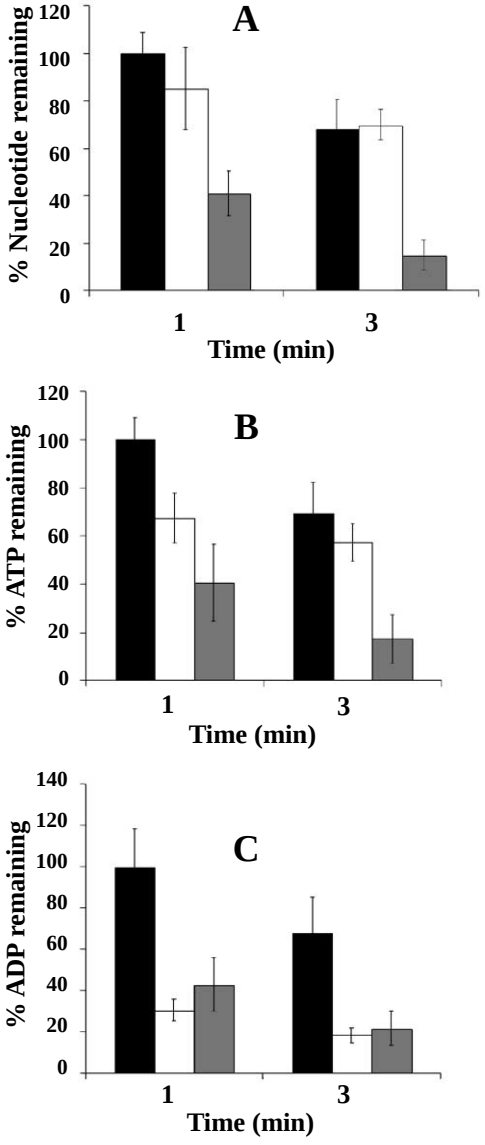

Fig. 3. Effects of HspBP1 and Fes1p on mammalian Hsp70–nucleotide complexes. Reactions were assembled as in Fig. 2, except purified bovine Hsc70–[α-^{32}P]ATP was used for the nucleotide exchange assays; black bars: buffer; white bars: GST-Fes1p; gray bars: HspBP1. The amount of bound nucleotide was quantified by (A) scintillation counting (total nucleotide remaining) or (B,C) quantitation of phosphorimaged TLC plates (percent ATP and ADP remaining, respectively). Data represent the means from three independent reactions, ± S.D.

Ssa1p, indicating that HspBP1 acts on both Ssa1p and mammalian Hsp70.

3.2. HspBP1 is a NEF

The observed inhibition of nucleotide binding to Ssa1p (Fig. 1) might have arisen from Fes1p or HspBP1-induced nucleotide dissociation. Indeed, we previously showed that Fes1p stimulates nucleotide dissociation from Ssa1p [22], and Fes1p and HspBP1 exhibit 38% similarity and 25% identity (our unpublished data). We consequently performed nucleotide exchange assays using purified Ssa1p–[α-^{32}P]ATP complex [22], which was then incubated at 30°C in the presence of buffer or in buffer containing GST-tagged Fes1p or HspBP1. Aliquots were removed over time and Ssa1p-bound and free nucleotides were resolved by gel filtration chromatography [22]. As shown in Fig. 2A, Fes1p and HspBP1 promoted nucleotide dissociation from Ssa1p. HspBP1 was less efficient in these assays, possibly because its affinity for Ssa1p is lower than for Hsc70, but the effect of HspBP1 on Ssa1p is significantly greater than we previously observed when examining the effect of GrpE or Sls1p on Ssa1p [22]. As observed for Fes1p and Sls1p with their respective Hsp70s [21,22], HspBP1 promoted the dissociation of both ATP and ADP from Ssa1p (Fig. 2B).

Because HspBP1 acted promiscuously with yeast Ssa1p, we next wished to examine whether HspBP1 and Fes1p could strip nucleotide from purified mammalian Hsc70. To this end, an Hsc70–[α-^{32}P]ATP complex was prepared and incubated with buffer, GST-Fes1p or HspBP1, and aliquots were removed over time and nucleotide-bound chaperone was purified from free nucleotide by gel filtration chromatography. HspBP1 was able to stimulate both ATP and ADP dissociation from Hsc70, whereas Fes1p appeared relatively inactive (Fig. 3). These combined data indicate that the mammalian Fes1p homologue, HspBP1, is a NEF that can act on both yeast and mammalian Hsp70s, but that the yeast NEF associates preferentially with the cytoplasmic yeast Hsp70, Ssa1p.

3.3. HspBP1 inhibits Ssa1p-mediated ATP hydrolysis

We previously showed that Fes1p inhibits Ssa1p ATPase activity in single turnover assays, most likely by stripping ATP before it can be hydrolyzed [22]. Similar effects were observed with other NEFs and their cognate Hsp70s [25]. To determine whether HspBP1 would inhibit the single turnover ATPase activity of Ssa1p, as would be expected from the data presented in Fig. 2, we performed single turnover assays using the Ssa1p–[α-^{32}P]ATP complex incubated in the presence of buffer or a three-fold molar excess of GST-Fes1p or HspBP1. As shown in Fig. 4, HspBP1 inhibited Ssa1p ATPase activity; as a control, we found that Fes1p also inhibited Ssa1p-mediated ATP hydrolysis, as previously published [22]. These data further support the conclusion that HspBP1 is a NEF.

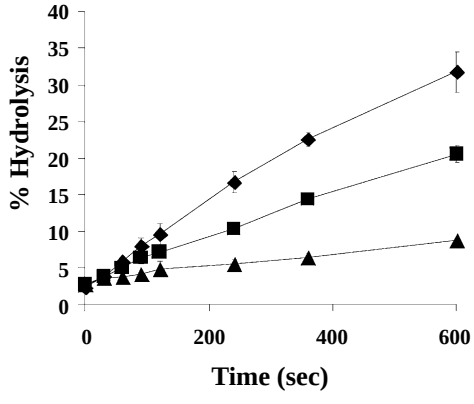

Fig. 4. HspBP1 and Fes1p inhibit Ssa1p ATP hydrolysis. Ssa1p–[α-^{32}P]ATP complex (see Fig. 2) was incubated with buffer (diamonds) or a three-fold molar excess of GST-Fes1p (squares) or HspBP1 (triangles) at 30°C, and single turnover ATPase activity was measured as described [22,24]. At the indicated times aliquots were removed, the reactions were quenched, and triplicate aliquots were analyzed by TLC. Data represent the means from three independent reactions, ± S.D.

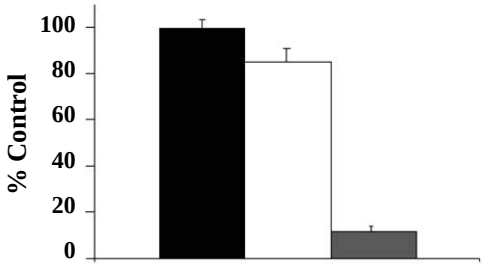

Fig. 5. Only HspBP1 inhibits protein refolding in vitro. Firefly luciferase was denatured, chilled, and then added to rabbit reticulocyte lysate in the presence of buffer (black bars) or 20 μM GST-Fes1p (white bars) or HspBP1 (gray bars). The samples were incubated for 90 min at 26°C and assayed for luciferase activity as described [23]. Data represent the means from three independent experiments, ± S.D.

3.4. *Luciferase refolding is inhibited by HspBP1 but not by Ssa1p*

HspBP1 was previously shown to inhibit the chaperone-mediated refolding of heat-denatured luciferase when added to rabbit reticulocyte lysate or to a defined system containing purified chaperones [23]. Because the effects of Fes1p and HspBP1 on mammalian Hsc70 were different (Fig. 3), we measured luciferase renaturation in reticulocyte lysate in the presence of buffer, Fes1p, or HspBP1. As shown in Fig. 5, whereas HspBP1 inhibited the refolding of luciferase substantially (gray bar), Fes1p was without effect on refolding (white bar), thus supporting the data presented in Fig. 3.

4. Conclusions

The work described in this report establishes that HspBP1 is homologous to the Fes1 protein, and that like Fes1p it acts as a NEF for Hsp70. HspBP1 has also been reported to have homology to Sls1p [26]. Furthermore, although the degree of homology between Fes1p and HspBP1 is not high, HspBP1 induces nucleotide dissociation from yeast Ssa1p (Fig. 2) and inhibits its ATPase activity in single turnover assays (Fig. 4). In contrast, Fes1p was unable to act as a NEF for mammalian Hsp70 (Fig. 3), and did not inhibit the Hsp70-mediated refolding of heat-denatured firefly luciferase (Fig. 5). We suggest that these factors are not functionally interchangeable, but instead bind to their Hsp70 partners to perform unique functions within the cell. Indeed, the luminal ER Fes1p/HspBP1 homologue, Sls1p, is a NEF that activates the luminal Hsp70 BiP to facilitate protein translocation and degradation [21], whereas Fes1p inhibits Ssa1p ATPase activity, and this regulation is restricted to Ssa1p function during protein translation [22]. While the function of HspBP1 in vivo remains unclear, HspBP1 was found to inhibit Hsp70-mediated protein refolding ([23]; Fig. 5). Thus, as for J-domain-containing proteins [8,9], this recently defined class of NEFs can be used differentially to add further complexity and plasticity to Hsp70 function and regulation. Based on our studies, it will next be imperative to map the region(s) of these proteins that dictates their interaction with specific or multiple Hsp70s, and in turn to determine how these interactions impact specific cellular processes.

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