Mitochondrial Hsp70 cannot replace BiP in driving protein translocation into the yeast endoplasmic reticulum

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Abstract To determine whether mitochondrial hsp70 (mHsp70) could substitute for the endoplasmic retuculum (ER) Hsp70 (BiP) during protein translocation, we assembled ER-derived reconstituted proteoliposomes supplemented with either protein. We found that only BiP restored translocation in *kar2* mutant vesicles and stimulated translocation ∼3-fold in wild type proteoliposomes. mHsp70 associated poorly with both a BiP binding (DnaJ) domain of Sec63p and an ER precursor, and its ATPase activity was poorly enhanced upon incubation with the DnaJ domain. In contrast, BiP bound to the Sec63p-DnaJ domain in an ATP-dependent manner and its ATPase activity was stimulated significantly by this polypeptide. We conclude that mHsp70 is unable to support protein translocation into the ER because it fails to associate productively with Sec63p and a precursor.

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Key words: Protein translocation; Heat shock protein; BiP; mHsp70; Endoplasmic reticulum

1. Introduction

Molecular chaperones facilitate the translocation of polypeptides across the endoplasmic reticulum (ER) and mitochondrial membranes [1]. Hsp70 chaperones preferentially bind and release hydrophobic amino acid motifs concomitant with ATP binding and hydrolysis [2], thereby ensuring that unfolded proteins do not aggregate during translocation. Hsp70s in the ER, such as BiP (Kar2p), and mHsp70 in the mitochondrial matrix, also help nascent polypeptides fold [3–5].

Yeast cells containing temperature sensitive mutations in the gene encoding BiP, *KAR2*, accumulate untranslocated precursor proteins [6], and ER-derived microsomes from *kar2* strains are translocation-defective [7]. BiP interacts both with preproteins and with an ER membrane protein, Sec63p, that is also required for translocation and contains a domain homologous to DnaJ molecular chaperones [7–9]; in many other cases, DnaJ chaperones interact with Hsp70s. BiP may function as a motor or ratchet to drive preproteins into the ER during cycles of ATP hydrolysis and is positioned near the translocation pore by Sec63p [10]. BiP is also required for preprotein transfer to the pore after it interacts with a receptor complex on the cytosolic face of the ER [11].

mHsp70 possesses many of these attributes and is ∼50% identical to BiP [3,4,12]. Mutations in the gene encoding mHsp70, *SSC1*, compromise mitochondrial protein transloca-

tion [13,14]. mHsp70 is bound to the mitochondrial inner membrane through a membrane-associated protein, Tim44, and the mHsp70-Tim44 association, as observed for the BiP-Sec63p pair [8,9], is regulated by ATP binding and hydrolysis [15–17]. Furthermore, the domain in Tim44 to which mHsp70 binds contains a region with limited homology to the Sec63p DnaJ domain [16].

A cytosolic Hsp70 required for translocation into mitochondria and the ER lumen is Ssa1p. Because Ssa1p is thought to simply maintain preproteins in an unfolded conformation prior to translocation and, unlike BiP and mHsp70, does not stably associate with the translocation machinery [1,18], it was not too surprising that Ssa1p was unable to substitute functionally for BiP [19]; in fact, mutations in *SSA1* prevent the import of only one ER-targeted preprotein in vivo [20]. In contrast, both BiP and mHsp70 play vital roles in the translocation of many preproteins and actively ratchet or pull preproteins across the membrane [1,18]. BiP [11,18] and mHsp70 [21] may also regulate the translocation machinery in their respective organelles. Thus, we sought to determine whether mHsp70 could functionally substitute for BiP during post-translational translocation into the yeast ER.

2. Materials and methods

2.1. Yeast strains
Microsomes were prepared from either the wild type strain MS10 (*MATa ade2-101 leu2-3,112 ura3-52*) or the *kar2* mutant MS137 (*MATa ade2-101 leu2-3,112 ura3-52, kar2-159*) [6,7].

2.2. In vitro assays
Yeast microsomes, reconstituted proteoliposomes, and measurements of pre-proalpha factor (ppαF) translocation were performed as described [8,19]. The purifications of yeast BiP [8,19], mHsp70 [22], and mGrpE [23] have been described. Steady-state Hsp70 AT-Pase activity was assayed as described [9] and was calculated after the spontaneous hydrolysis of [α-^{32}P]ATP in Hsp70-free reactions was subtracted. The interaction between BiP or mHsp70 and the DnaJ domain of Sec63p was assayed by the method of Corsi and Schekman [9], and chemical crosslinking and immunoprecipitations were performed as described [7].

To verify that the proteoliposomes contained the desired Hsp70, reconstituted vesicles were trypsin-treated and fractionated [19]. Proteins in the enriched vesicles were precipitated with trichloroacetic acid and resolved by SDS-PAGE. Antibodies to yeast BiP [8] and mHsp70 [22] were used for immunoblots and detected either by ECL or ^{125}I-labeled protein A (Amersham).

3. Results

ER-derived proteoliposomes post-translationally translocate the yeast mating pre-pheromone, ppαF [8,19], and translocation is measured by the appearance of signal sequence-

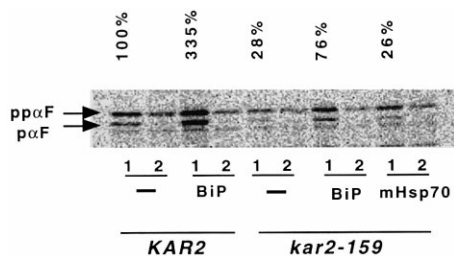*Corresponding author. Fax: (1) (412) 624-4759.
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Fig. 1. mHsp70 is unable to restore translocation in *kar2-159* reconstituted vesicles. The presence of pαF in trypsin-treated samples (lane 1) indicates protein import, and addition of trypsin after Triton X-100 treatment (lane 2) degrades the membrane-enclosed pαF. Values indicate the amounts of translocated pαF relative to *KAR2* (wild type) vesicles.

cleaved ppαF, pro-alpha factor (pαF), that is protected from added trypsin.

To determine whether mHsp70 could support translocation, we prepared reconstituted vesicles from wild type microsomes or from those containing the *kar2-159* mutation [7] and included yeast BiP or mHsp70 such that it comprised 5% of the total protein [8,19]. As before [8,19], we found that *kar2-159* proteoliposomes translocated ppαF ∼30% as efficiently as wild type vesicles and that inclusion of BiP partially restored activity (Fig. 1). In contrast, the introduction of mHsp70 into the *kar2-159* vesicles failed to restore activity. Because BiP increased the efficiency of ppαF translocation into wild type proteoliposomes ∼3-fold (Fig. 1) [8], we prepared wild type vesicles supplemented instead with mHsp70; however, we observed that mHsp70 was unable to stimulate ppαF import (Fig. 2). Addition of up to 2-fold higher concentrations of mHsp70 also failed to rescue the *kar2-159* translocation defect (data not shown).

It was possible that mHsp70 could not substitute for BiP because the yeast ER lacks a GrpE homologue. Mitochondrial GrpE, mGrpE, interacts with mHsp70 and is required for translocation [24,25]. Therefore, both mHsp70 and mGrpE were included in the vesicles but we found that translocation was inhibited by ∼50% (Fig. 2). To further explore this phenomenon, vesicles were supplemented only with mGrpE to 10% of the total vesicular protein but translocation remained proficient (data not shown).

Corsi and Schekman constructed a GST-Sec63p/DnaJ domain fusion protein to demonstrate a specific, ATP-dependent interaction between the DnaJ domain of Sec63p and BiP [9]. Using this fusion protein, we also found that purified BiP binds to the Sec63p DnaJ domain in an ATP-dependent manner (Fig. 3). When we instead assayed for an mHsp70-Sec63p DnaJ domain interaction, we observed that ∼10-fold less mHsp70 bound and that the association was nucleotide-independent (Fig. 3).

Table 1
Steady-state ATPase activities

Protein	ATPase activity (nmol/min/mg)	
	0 mM K$^+$	20 mM K$^+$
MHsp70	1.20 ± 0.05	2.55 ± 0.10
MHsp70+mGrpE	2.00 ± 0.10	5.30 ± 2.20
MHsp70+Mdjlp	7.25 ± 0.30	9.95 ± 0.40
MHsp70+mGrpE+Mdjlp	36.4 ± 1.75	59.3 ± 2.05

Steady-state ATPase activities were measured as in Fig. 4 and represent mean values from one experiment performed in triplicate, ± S.D.
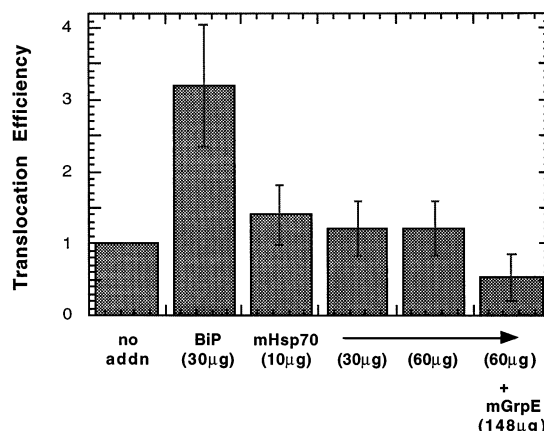

Fig. 2. mHsp70 is unable to stimulate ppαF translocation into wild type reconstituted vesicles. Proteoliposomes were untreated or supplemented with BiP or mHsp70. In one set of experiments, an 8-fold molar excess of mGrpE was also included. Translocation was assayed and the amounts of trypsin-protected pαF were normalized to that in the untreated vesicles ('no addn'). Values represent the means of three independent experiments, ± S.D. *P* values calculated from a *t*-test for the following experiments relative to 'BiP (30 μg)' were 0.02 (mHsp70 (10 μg)), 0.01 (mHsp70 (30 μg)), 0.01 (mHsp70 (60 μg)), and 0.05 (+mGrpE), indicating that the observed differences were statistically significant.

In many cases, stable interactions between Hsp70 and DnaJ chaperones cannot be observed, but transient interactions between an Hsp70 and its cognate DnaJ may be surmised if the DnaJ homologue stimulates the Hsp70's ATPase activity. When BiP was incubated with the GST-Sec63p/DnaJ domain fusion protein ('63J'), a rate enhancement of ∼4-fold was noted previously [9]. We found that 63J stimulated the ATPase activity of BiP ∼10-fold (Fig. 4A); by comparison, 63J promoted only a 0.4-fold increase in ATPase activity of mHsp70.

Some chaperone activities are potassium-dependent [26]. Therefore, we repeated the experiments in Fig. 4A using buffer containing 20 mM K$^+$ [9]. As anticipated [26], the basal ATPase activities of mHsp70 and BiP were somewhat higher (Fig. 4B); nevertheless, we again concluded that 63J interacted productively only with BiP.

To verify that the mHsp70 used for these experiments was active and able to associate productively with mGrpE and its cognate DnaJ, Mdj1p, we measured its steady-state ATPase activity in either the absence or presence of Mdj1p and
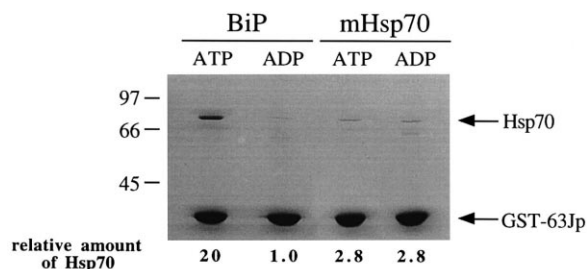

Fig. 3. mHsp70 is unable to associate productively with the Sec63p DnaJ domain. BiP or mHsp70 was incubated with the GST-Sec63p DnaJ peptide in the presence of 1 mM ATP or ADP and glutathione-agarose as described [9]. Molecular weight marker positions (×10^{-3}) are indicated. Values below the figure state the relative amounts of precipitated Hsp70.
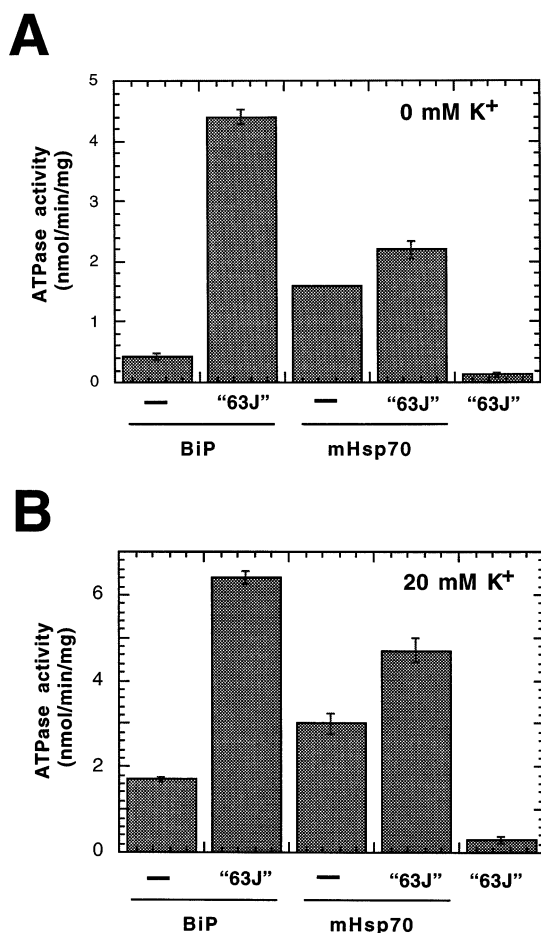
A

B

Fig. 4. The GST-Sec63p DnaJ polypeptide enhances the ATPase activity of BiP. '63J': reactions in the presence of the GST-Sec63p DnaJ domain fusion protein. Values represent the means from two independent experiments, each conducted in duplicate, ± S.D.

mGrpE. As determined previously, mGrpE and Mdj1p elicited a ~ 2-fold and ~ 7-fold enhancement of mHsp70 ATPase activity, respectively, and the presence of both co-chaperones stimulated the ATPase activity of mHsp70 by > 25-fold (Table 1; [27]); as observed for BiP (Fig. 4), the basal activity of mHsp70 was higher and DnaJ homologue-mediated activation was lower in the presence of 20 mM potassium (Table 1).

Because mHsp70 cannot interact with Sec63p, mHsp70 may have been unable to support translocation since it was excluded from the proteoliposomes. To test this hypothesis, we purified vesicles either lacking or containing mHsp70 or BiP [19]. By quantitative immunoblotting, we found that 48% as much mHsp70 was present in the vesicles as BiP (Fig. 5).
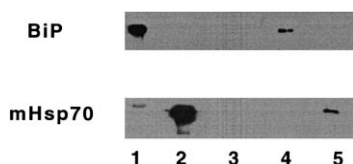

Fig. 5. Hsp70 incorporation into reconstituted vesicles. Vesicles were formed in the absence (lane 3) or presence of BiP (lane 4) or mHsp70 (lane 5); lane 1, 400 ng of BiP and lane 2, 400 ng of mHsp70. Note that the anti-mHsp70 antibody crossreacts weakly with BiP ('mHsp70', lane 1).
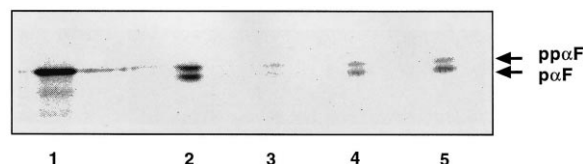

Fig. 6. The interaction of BiP and mHsp70 with a translocating preprotein. PpαF was translocated into vesicles reconstituted with either containing BiP (lanes 2 and 3) or mHsp70 (lanes 4 and 5) to 10% of the total protein and after crosslinking with DSP [7], the samples were immunoprecipitated with anti-BiP antibodies (lanes 2 and 4) or anti-mHsp70 antibodies (lanes 3 and 5) and protein A-Sepharose. Untranslocated ppαF is shown in lane 1, and the positions of ppαF and pαF are depicted to the right of the figure.

It was possible that ppαF could partially translocate into reconstituted vesicles and interact with either mHsp70 and BiP, but that mHsp70 was unable to facilitate complete import. To explore this possibility, ppαF was translocated into reconstituted vesicles containing either BiP or mHsp70 and chemical crosslinking and immunoprecipitations were performed (Fig. 6). We observed that vesicles supplemented with BiP bound ~ 5-fold more ppαF and pαF than those supplemented with mHsp70 (compares lanes 2 and 4); the bound substrate in lane 4 derived from BiP originating from the solubilized ER. We also found that ~ 3-fold more BiP bound to the substrate than mHsp70 during translocation into proteoliposomes supplemented with either chaperone (compare lanes 2 and 5).

4. Discussion

Our results indicate that mHsp70 is unable to support post-translational translocation into the ER because it interacts productively neither with Sec63p nor with a translocating precursor. The reduced association between mHsp70 and ppαF is in part due to the fact that less mHsp70 reconstitutes into vesicles than BiP (Fig. 5). The further reduction in mHsp70-substrate binding may arise either because mHsp70 does not recognize this substrate or because only BiP may be able to 'gate' the translocation channel [28]; the inability of Sec63p to tether mHsp70 at the pore may thus prevent the channel from opening and does not allow ppαF access to the reconstituted mHsp70. Alternatively, lumenal chaperones may only recognize those preproteins targeted to the proper organelle. In this case, the interaction between a chaperone and the precursor may provide a proofreading mechanism to ensure that only properly targeted precursors are translocated. Of note, the affinity between DnaK [29] and mHsp70 [27] and model presequences is high (~ 50 nM).

Our data also support previous observations that the pairing of unique DnaJs with Hsp70s is required during protein translocation [19]. mHsp70 may associate with a domain in Tim44 that contains limited homology to the DnaJ domain in Sec63p [16]; based on our data, it is likely that amino acid sequences required for mHsp70 and BiP to interact with Tim44 and Sec63p, respectively, have diverged to such an extent that promiscuous interactions may be prevented.

mHsp70 may also be unable to support ER translocation because it cannot form secondary contacts with other components of the ER translocation machine, such as the translocation channel (see above). Finally, it is possible that essential lipid contacts between mHsp70 and the membrane are absent

in the reconstituted vesicles. Nevertheless, it is clear from this study that BiP and mHsp70 require unique molecular environments to drive protein translocation in yeast. An investigation of each vital aspect of this environment will be the subject of future studies.

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