Protein translocation: **Is Hsp70 pulling my chain?**

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Hsp70 proteins in the lumen of the endoplasmic reticulum and in the mitochondrial matrix are thought to drive the translocation of proteins into each organelle. Recent experiments aimed at distinguishing between two models for Hsp70 function appear to reach opposite conclusions.

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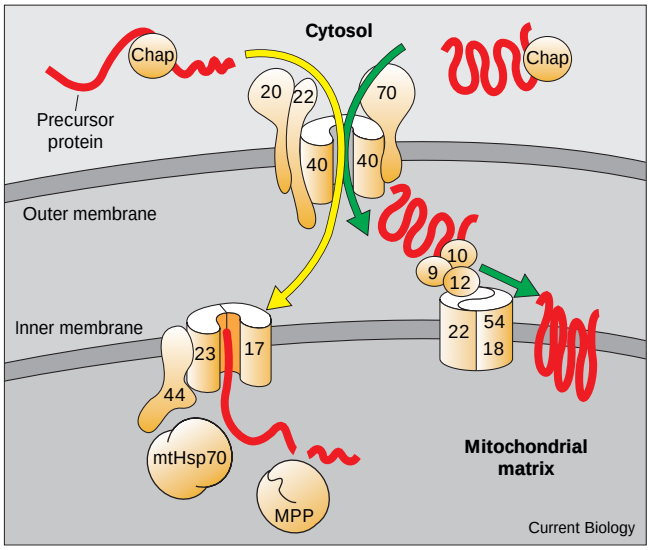
Many proteins synthesized by cytoplasmic ribosomes are targeted to specific subcellular compartments called organelles. Proteins destined for these compartments must be transported through, or integrated into, the membrane (or membranes) that surround the organelle. A major question, one which has generated some controversy, is what powers protein translocation through the bilayer. Recent experiments [1,2] aimed at determining the driving force for protein translocation appear to have reached opposite conclusions about the part played by Hsp70s in the process. How can the different findings be reconciled?

Extensive studies over the past two decades have shown that organisms have evolved complicated molecular machineries to carry out protein sorting at various membranes [3–5]. In each case, translocation is mediated by a multiprotein complex, located in the membrane, termed a translocon or translocase, the core components of which form a protein-conducting pore in the membrane. The mitochondrion, for example, imports proteins made in the cytosol using translocases found in each of the two mitochondrial membranes: a 'TOM' complex in the outer membrane and at least two separate 'TIM' complexes in the inner membrane (Figure 1). One TIM translocon mediates insertion of polytopic proteins into the inner membrane, whereas the other, the Tim23–Tim17 complex, is required for translocation of proteins across the inner membrane into the matrix. In addition to Tim23 and Tim17, two other proteins, Tim44 and mtHsp70, are essential for protein import into the matrix.

In the endoplasmic reticulum (ER) membrane of mammalian cells, the translocon is composed of at least four membrane proteins, a protein known as TRAM and the heterotrimeric Sec61 complex [3,4]. Translocation at the mammalian ER membrane occurs co-translationally, but in yeast, protein translocation into the ER can also proceed post-translationally. In addition to the yeast Sec61 complex, other proteins, such as Sec63 and BiP, are required for post-translational translocation into the ER (Figure 2).

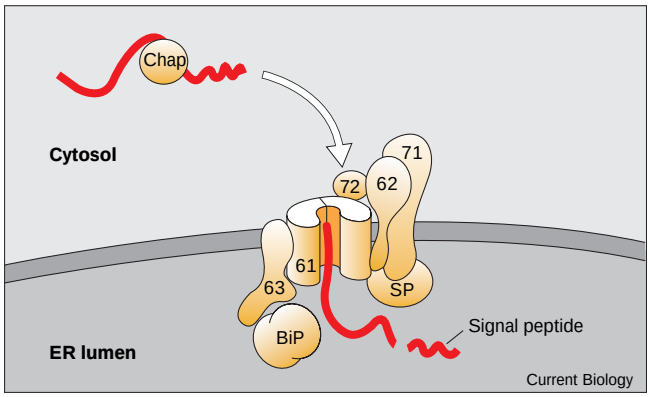
Molecular chaperones, such as members of the heat shock protein 70 (Hsp70) family, are involved in a number of cellular processes, including protein folding, the disassembly of oligomeric protein structures, protein degradation and protein translocation [6]. Hsp70s have a two-domain structure: a carboxy-terminal domain that binds short regions of

Figure 1



The mitochondrial protein import machinery. Mitochondrial precursor proteins are kept in a loosely-folded, import-competent conformation by their interaction with cytosolic chaperones (Chap) before they interact with the TOM – translocase of the outer membrane – machinery. The import signal is recognized by one of several receptors and then translocated through a pore consisting mainly of Tom40 subunits, but assisted by additional TOM proteins (not shown). Once through the TOM complex, the pathway diverges. On the left is the pathway for proteins destined for the matrix. The precursor protein is inserted into a TIM – translocase of the inner membrane – complex consisting of Tim23 and Tim17, which are thought to form a protein-translocating channel. mtHsp70 interacts with Tim44 and drives the translocation of the precursor into the matrix where the amino-terminal import signal is removed by the processing protease, MPP. On the right is the pathway for import of polytopic inner membrane proteins. The imported protein is bound by the Tim9–Tim10 complex in the intermembrane space and shuttled to the Tim54–Tim22–Tim18 translocon ([11,12] and our unpublished data), which inserts the protein into the inner membrane.

Figure 2



The post-translational translocation machinery of the yeast ER. The precursor protein is kept in a loosely-folded, translocation-competent conformation by its interaction with cytosolic chaperones (Chap). Targeting of the preprotein to the ER is thought to involve Sec62, Sec71 and Sec72. The core components of the translocon consist of Sec61, Sbh1 and Sss1, which associate to form a protein-translocating channel in the membrane (horseshoe-shaped structure). In the yeast ER lumen, the resident Hsp70 BiP is recruited to the *trans* side of the translocon by binding to Sec63. SP, signal peptidase.

polypeptide, and a conserved amino-terminal ATPase domain. Studies with purified Hsp70s have indicated that they undergo cycles of peptide binding and release: in the presence of ATP, they bind weakly and reversibly to substrate; ATP hydrolysis causes a conformational change that induces tight substrate binding; exchange of ATP for ADP promotes substrate release, completing the cycle. The cycle is controlled in the cell by co-chaperones. All Hsp70s interact with a particular member of the DnaJ family. DnaJ proteins all have a highly-conserved J domain, which is essential for binding to Hsp70 and the stimulation of its ATPase activity. The exchange of ADP for ATP on Hsp70 often requires another co-chaperone, in this case a member of the GrpE family.

Hsp70 chaperones play key roles in protein translocation [3–5]. Many studies have shown that tightly-folded protein domains cannot be imported into mitochondria or translocated post-translationally into the ER. The cytosolic Hsp70 proteins help maintain proteins in a loosely-folded and non-aggregated conformation before they are transported into organelles. In addition, Hsp70 proteins located in the ER lumen — the protein also known as BiP mentioned above — or the mitochondrial matrix — mtHsp70 — are thought to drive the translocation reaction. BiP is recruited to the ER translocon by the transmembrane protein, Sec63, whereas mitochondrial mtHsp70 associates with the inner membrane translocon component, Tim44. Sec63 and Tim44 both have J domains that mediate the specific interactions with their Hsp70 partners.

Two models have been proposed to describe how Hsp70 molecules inside the ER or a mitochondrion can drive the unidirectional movement of proteins through the translocon [7]. In the 'trapping' or 'Brownian ratchet' model (Figure 3a), a protein in the translocon is free to diffuse by Brownian motion in either direction. When a sufficient amount of the protein extends out of the *trans* face of the translocon, the resident Hsp70 — BiP in the ER lumen and mtHsp70 in the mitochondrial matrix — binds to the translocating protein. This binding prevents diffusion backwards in the pore and thus traps the protein on the *trans* side of the membrane. Diffusion in the forward direction, followed by the binding of additional Hsp70 molecules, drives the translocation of the protein completely through the pore. In this model, the J domain-containing partner, Sec63 or Tim44, solely functions to localize the Hsp70 protein near the translocation site and to facilitate Hsp70 binding to the substrate.

In the alternative model, Hsp70 functions as a kind of 'translocation motor' (Figure 3b). In this model, Hsp70 interacts with the incoming polypeptide while bound to its cognate J domain-containing co-chaperone. An ATP-dependent conformational change in Hsp70 then 'pulls' the polypeptide through the translocon. As the translocon and the membrane are massive, a conformational extension of DnaJ-bound BiP or mtHsp70 will extend the substrate binding site further away from the translocon — thereby 'pulling the chain'.

One important distinction between these two models is how they explain protein unfolding during translocation. In the trapping model, folded domains of a protein on the cytoplasmic side of the translocon are proposed to unfold spontaneously, thus allowing the inward diffusion of the polypeptide chain. The rate of translocation for some proteins may therefore be dependent upon the rate of unfolding. In the translocation motor model, by contrast, unfolding can be actively promoted by the pulling force generated by Hsp70, thus allowing the efficient translocation of proteins that would otherwise unfold very slowly.

In their recent paper, Matlack *et al.* [1] described experiments that strongly support the trapping model. The authors examined the role of BiP in post-translational protein translocation into the ER, using detergent-solubilized or liposome-reconstituted translocation machinery purified from yeast cells. The principal translocation substrate used in these studies was the small, easily translocatable protein known as prepro-α-factor (the precursor form of a yeast mating pheromone). As predicted by the trapping model, BiP bound directly to prepro-α-factor as it came through the translocon, and multiple BiP molecules interacted with a single prepro-α-factor molecule. Furthermore, while wild-type BiP mediated the

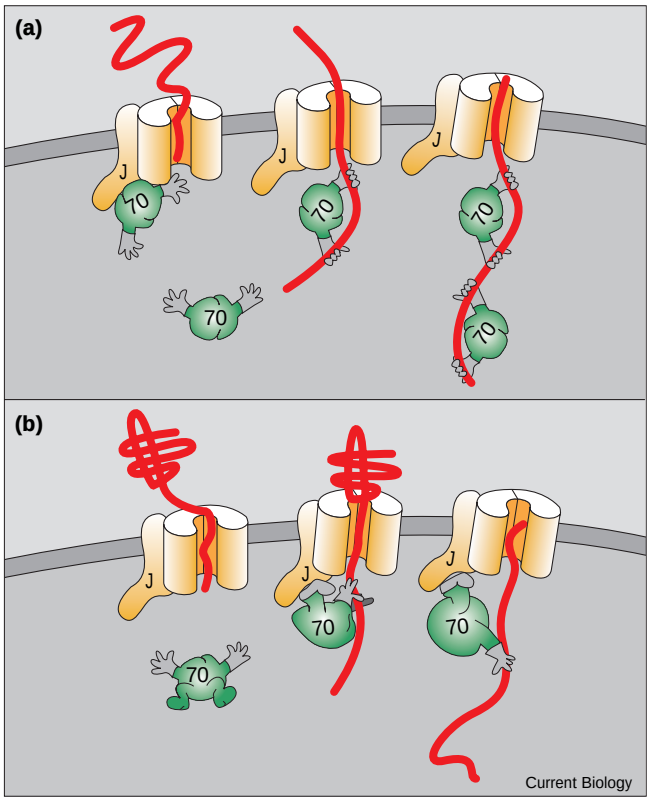
translocation of prepro-α-factor, a mutant BiP that bound weakly to the substrate was defective in translocation.

Satisfying a central requirement of the trapping model, BiP prevented the backsliding of a protein in the translocon. This was shown using a form of prepro-α-factor with a tRNA covalently-linked to its carboxyl terminus, which was firmly held in the translocation channel by BiP. In the absence of ATP, BiP dissociated and allowed the prepro-α-factor–tRNA construct to diffuse back out of the translocon. Perhaps the most intriguing observation made by Matlack *et al.* [1] was that antibodies to prepro-α-factor could substitute for BiP function. When the translocation machinery was reconstituted into membrane vesicles containing antibodies against prepro-α-factor, prepro-α-factor was transported into the antibody-containing vesicles nearly as efficiently as into vesicles containing BiP. Translocation of prepro-α-factor in the reconstituted system therefore does not require an ATP-dependent motor.

Although the results of Matlack *et al.* [1] are enticing, it is possible that their conclusions may be colored by the simplicity of their system. For example, the translocons reconstituted from purified components in this study allow the free movement of the translocating polypeptide in both directions, as well as the diffusion of ATP into the proteoliposomes. In contrast, the complete ER translocon maintains a permeability barrier during protein movement *in vivo*. Given this functional role, it is conceivable that additional proteins are required to create the permeability barrier, and they may also prevent the substrate from moving through the pore freely. In addition, the purified post-translational system lacks any cytosolic chaperones that would trap the preprotein on the *cis* side of the translocon. In either case, BiP may need to exert a pulling force. It is also likely that, while prepro-α-factor requires only the trapping activity of BiP, other substrate proteins — such as more tightly-folded proteins — may require a motor activity for their translocation.

In the second recent paper addressing this issue, Voisine *et al.* [2] report evidence that Hsp70 functions as a translocation motor for at least some proteins. These authors examined a yeast mutant, *ssc1-2*, defective in the peptide-binding domain of the mitochondrial mtHsp70 protein. The properties of the mutant mtHsp70 protein suggest that trapping by mtHsp70 cannot explain the full role of mtHsp70 in protein import. Mitochondria isolated from *ssc1-2* cells could not import tightly-folded precursor proteins, but they were able to import loosely-folded proteins or proteins that had been artificially unfolded with denaturants. Furthermore, the defective mtHsp70 protein does not associate with its J-domain-containing partner, Tim44.

Figure 3



Two models of Hsp70's role in protein translocation. The horseshoe-shaped structures in the lipid bilayer represent the translocon, and the dark wavy line represents the incoming polypeptide. Also depicted are Hsp70 molecules (70): BiP in the ER lumen or mtHsp70 in a mitochondrion. In the trapping model (a), the protein to be imported is inserted into the translocon by the targeting machinery (not shown). After part of the substrate has diffused inwards, the protein is trapped by the tight binding of a lumenal Hsp70 molecule (depicted by the 'hands'). This binding requires ATP hydrolysis and interaction with a DnaJ co-chaperone (not shown). Further inwards diffusion and additional Hsp70 binding continues until translocation of the protein through the pore is complete. Exchange of ADP for ATP promotes dissociation of Hsp70 (not shown). In the translocation motor model (b), the incoming protein is again bound by Hsp70 ('hands'). Hsp70 also associates (by the 'feet') with the J domain of its partner DnaJ co-chaperone (J). Tight binding of Hsp70 to the substrate and to the J domain requires ATP hydrolysis. An ATP-hydrolysis-dependent conformational change of Hsp70 'pulls' the protein through the translocon. It is not yet clear how the system re-sets the putative power-stroke cycle. Perhaps the same Hsp70 remains bound to the DnaJ molecule while a co-chaperone elicits ATP exchange for ADP and initiates a new substrate binding and hydrolysis cycle. Alternatively, Hsp70 binding to the J domain may be weakened after ATP hydrolysis, leading to Hsp70 dissociation from the J domain and binding of a new Hsp70 molecule in its place. If this were to occur, any continued binding of the DnaJ-released Hsp70 molecule to the substrate would constitute a 'trapping' mechanism as in (a).

As the mutant mtHsp70 remained bound to the translocating polypeptide for much longer times than wild-type mtHsp70, Voisine *et al.* [2] reasoned that it should be capable of driving import by trapping. For loosely-folded

protein, this is indeed the case. On the other hand, as the mutant mtHsp70 does not interact with Tim44, it should not be capable of generating the pulling force required to import a tightly-folded substrate. Supporting this view, intragenic suppressors of the *ssc1-2* mutant were isolated and found to restore mtHsp70's abilities both to import tightly-folded substrates and to interact with Tim44. A tightly-folded protein seems to require the translocation motor activity of Hsp70 for its unfolding and subsequent translocation into an isolated mitochondrion.

While the results of Voisine *et al.* [2] provide compelling evidence for the translocation motor model, it is possible that their conclusions may be colored by the complexity of their system. For example, the import substrates — complicated fusion proteins — were synthesized in rabbit reticulocyte lysate and imported into yeast mitochondria. Moreover, specific cytosolic chaperones that normally keep yeast proteins in a loosely-folded, import-competent conformation may be missing in the rabbit lysate. In the absence of these factors, a pulling force may be necessary for translocation of some substrates, but in the cell trapping may be sufficient.

It is tempting to speculate that the trapping and motor functions of Hsp70 are both used during protein translocation. It may be that loosely-folded proteins use only the trapping activity, whereas tightly-folded proteins require a pulling force (and possibly trapping as well). It is important, however, to bear in mind that Hsp70 proteins may display other functions during protein translocation. For example, the ER translocon pore is dynamic, with a pore diameter ranging from about 15–50 Å [8,9], and BiP is responsible for closing the pore when the ribosome leaves the ER translocon at the end of co-translational translocation [9]. BiP may also regulate the opening of the pore, allowing it to accommodate larger — partially folded — substrates. Moreover, the active participation of the translocon components in the movement of proteins through the pore cannot be ruled out. Interestingly, mitochondrial mtHsp70 has also been shown to interact directly with the mitochondrial translocon [10]. Is it therefore possible that the role of Hsp70 has less to do with substrate movement than with translocon regulation? Further experiments are clearly needed before closing the book on the mechanism(s) by which Hsp70 mediates protein translocation.

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