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Polypeptide translocation machinery of the yeast endoplasmic reticulum

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Abstract. Proteins enter the secretory pathway by two general routes. In one, the complete polypeptide is made in the cytoplasm and held in an incompletely folded state by chaperoning adenosine triphosphatases (ATPases) such as hsp70. In *Saccharomyces cerevisiae*, fully synthesized secretory precursors engage the endoplasmic reticulum (ER) membrane by interaction with a set of Sec proteins comprising the polypeptide translocation apparatus (Sec61p, Sec62p, Sec63p, Sec71p, Sec72p). Productive interaction requires displacement of hsp70 from the precursor, a reaction that is facilitated by Ydj1p, a homologue of the *Escherichia coli* DnaJ protein. Both DnaJ and Ydj1p regulate chaperone activity by stimulating the ATPase activity of their respective hsp70 partners (*E. coli* DnaK and *S. cerevisiae* Ssa1p, resepectively). In the ER lumen, another hsp70 chaperone, BiP, binds ATP and interacts with the ER membrane via its contact with a peptide loop of Sec63p. This loop represents yet another DnaJ homologue in that it contains a region of ~70 residue similarity to the 'J box', the most conserved region of the DnaJ family of proteins. In the presence of ATP, under conditions in which BiP can bind to Sec63p, the secretory precursor passes from the cytosol into the lumen through a membrane channel formed by Sec61p. A second route to the membrane pore that is used by many other secretory precursors, particularly in mammalian cells, requires that the polypeptide engage the ER membrane as the nascent chain emerges from the ribosome. Such cotranslational translocation bypasses the need for certain Sec proteins, instead utilizing an alternate set of cytosolic and membrane factors that allows the nascent chain to be inserted directly into the Sec61p channel.
Key words. Polypeptide translocation; Sec proteins; hsp70; BiP; DnaJ.

Introduction

The movement of proteins across the membrane of the endoplasmic reticulum (ER), the first step of the secretory pathway, is made possible by the presence of a proteinaceous pore that acts as a gateway across the lipid bilayer. Passage of secretory precursor proteins through this gateway is mediated by the orchestrated action of a number of proteins that support distinct phases of the translocation process: docking of the secretory precursor at the membrane, traversing the lipid bilayer and exiting on the lumenal side – or, in the case of transmembrane proteins, attaining their correct position in the bilayer. The signal sequence, usually an amino-terminal extension of soluble secretory proteins, directs the precursor to the docking machinery. This targeting event is followed by translocation of the precursor across the membrane and into the lumen, where the signal sequence is cleaved by a specific processing protease. In contrast to soluble secretory proteins, the signal sequence of integral membrane proteins is generally located in an internal segment of the protein and is not cleaved off after the protein is directed to the membrane (see ref. 1 for review). Translocation of precursors may occur either cotranslationally, in which the polypeptide is threaded through the pore as it spools off the ribosome, or post-translationally, in which synthesis is completed before the polypeptide engages the pore. Both co- and post-translational pathways face an identical challenge: to ensure that the precursor polypeptide is maintained in an extended/unfolded form that is able to pass through the confines of a membrane pore [2]. In the case of cotranslational translocation, the requirement for an extended conformation is met by virtue of the fact that stretches of polypeptide emerging from the ribosome are immediately channelled into the pore. In post-translational translocation, the solution to the conformational challenge is to invoke the assistance of the 70-kD heat shock proteins (hsp70s), which are able to modulate the folding state of the precursor polypeptide [3]. Recent years have seen substantial progress in unravelling the means by which proteins cross a lipid bilayer, and more specifically, in the role of chaperone molecules such as the hsp70s in the process. In this review we examine advances in defining the mechanism of translocation, with particular focus on the involvement of the hsp70 proteins and their DnaJ partners in translocation into the ER of *Saccharomyces cerevisiae*.

Cotranslational translocation

The co- and post-translational pathways use both common and distinct machinery to effect the passage of a polypeptide across the ER membrane. While both pathways utilize the translocation pore as a means of cross-

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ing the bilayer, the cytosolic accessory factors required for each pathway are unique. A pivotal component of the cotranslational path is the ribonucleoprotein complex known as the signal recognition particle (SRP), which recognizes the signal sequence of secretory proteins as the nascent chain emerges from the ribosome (see refs 4–6 for reviews). Fidelity of signal sequence recognition by SRP is ensured by NAC (nascent polypeptide-associated complex), a heterodimeric protein that prevents proteins lacking signal sequences from binding SRP [7]. The binding of the signal sequence by SRP serves two functions. First, translation of the nascent polypeptide is arrested. This forestalls premature folding of the polypeptide chain while SRP carries out its second function, that of targeting the ribosome·nascent chain complex to the translocation site at the ER membrane. The interaction of the cytosolic SRP·ribosome·nascent chain complex with the membranous SRP receptor (SR) allows SRP to act as an intermediary to link the cytosolic machinery of translation to the membranous machinery of translocation. Upon interaction of SRP with SR, the signal sequence binds to the translocation machinery at the ER membrane. Translation reinitiates, and cotranslational translocation proceeds on guanosine triphosphate (GTP) hydrolysis by SRP or SR [8], releasing SRP and SR for another round of signal sequence targeting. SRP-mediated translocation accounts for the bulk of translocation in mammalian cells, in which the cotranslational pathway is used almost exclusively. Homologues of the components of SRP have been identified in *S. cerevisiae*, but none are essential for viability ([9]; see ref. 5 for review). Translocation in *S. cerevisiae* in the absence of SRP function still proceeds, albeit less efficiently, via an SRP-independent post-translational translocation pathway that coexists with the cotranslational pathway.

Post-translational translocation

Post-translational translocation also requires a set of accessory cytosolic factors. Since translocation occurs after the completion of protein synthesis, the cell must provide a means of preventing polypeptides from folding prematurely, in order to maintain them in an extended, import-competent conformation. Two early studies of post-translational translocation into the mammalian ER first suggested that hsp70 molecules might be involved in maintaining import competence [10, 11]. The ability of hsp70 proteins to bind a range of polypeptide substrates makes them ideally suited to carry out this cytosolic chaperoning function. The preferred binding motif for hsp70s is short stretches of alternating hydrophobic and basic residues, but some acidic residues may also be tolerated [12, 13]. The adenosine diphosphate (ADP)-bound form of an hsp70 molecule favors stable association with a polypeptide substrate [14, 15], and the substrate-binding event stimulates ADP→ATP (adenosine triphosphate) exchange on the hsp70. The subsequent binding of ATP causes a conformational change that results in release of the bound substrate [16], and hydrolysis of ATP by hsp70 then returns the molecule to its stable substrate-binding mode so the cycle can resume. This immediately suggests a mechanism by which cytosolic hsp70s could function in maintaining translocation competence in a polypeptide substrate: as the precursor protein is translated, hsp70 molecules could bind to and 'coat' the nascent polypeptide emerging from the ribosome, preventing premature folding. Nucleotide-dependent binding and dissociation of the hsp70s would allow the substrate to be presented to the translocation machinery in a translocation-competent conformation.

Cytosolic factors

Ssa1p/Ssa2p. Yeast cytosol contains a family of four hsp70s – two that are constitutively expressed (Ssa1p/Ssa2p) as well as two that are synthesized only under conditions of cellular stress (Ssa3p/Ssa4) [17]. The involvement of Ssa1p/Ssa2p in ER translocation was demonstrated by three independent approaches. Deshaies et al. [18] showed that depletion of Ssa1p (placed under regulatable control) in an *ssa1ssa2ssa4* deletion strain resulted in the cytosolic accumulation of untranslocated precursor proteins. The authors also showed that the precursor protein pre-pro-alpha factor (ppαF), translated in vitro in a wheat germ lysate, required Ssa1p and a second unidentified factor for efficient translocation into yeast ER microsomal vesicles. Chirico et al. [19] analysed a translocation-stimulating activity from yeast cytosol and found that it was composed of two distinct fractions – one contained an NEM-sensitive component, while the second, n-ethylmaleimide (NEM)-insensitive fraction contained Ssa1p and Ssa2p. A substantial clue to the mechanism of Ssa1p/Ssa2p action came from the observation that urea could substitute for hsp70 function: precursor proteins denatured in urea no longer required Ssa1p/Ssa2p for translocation competence [19], suggesting that the primary role of the cytosolic hsp70s in the translocation reaction was to maintain the precursor protein in a partially unfolded, import-competent state.

Ydj1p. The identity of the NEM-sensitive component of the cytosolic fraction is not yet known. However, a candidate for the second stimulatory factor is the cytosolic DnaJ homologue Ydj1p. DnaJ homologues are often physically and functionally coupled with hsp70 partners, from the archetypical DnaJ·DnaK pair in *Escherichia coli* to similar pairings in yeast and higher eukaryotes [20]. Ydj1p, like its counterpart in *E. coli*, is able to stimulate the ATPase activity of its hsp70 part-

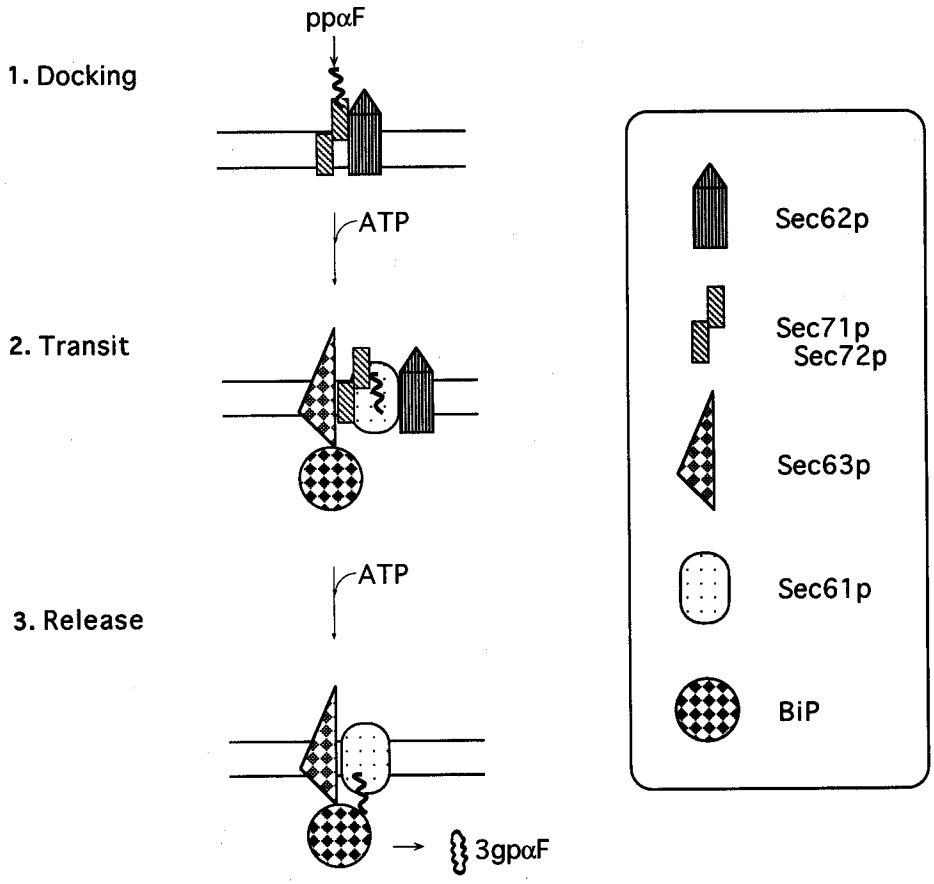

Figure 1. Model of post-translational translocation across the ER membrane (see text for details).

ner [21]. Ydj1p containing a temperature sensitive (ts) mutation (*ydj1-151p*) is defective in stimulation of the ATPase activity of Ssa1p in vitro, and a *ydj1-151* strain accumulates the secretory precursor ppαF at the restrictive temperature in vivo [22], suggesting that regulation of Ssa1p function by Ydj1p is essential for the translocation process. A pool of Ydj1p is farnesylated, creating a subset of the protein that is peripherally attached to the ER membrane [23]. As a membrane-bound protein, Ydj1p could target an Ssa1p-coated precursor protein to the membrane site of the translocation apparatus, upon which the precursor could be released from Ssa1p in a Ydj1p-mediated reaction, allowing it to engage the docking machinery and to subsequently be translocated through the lipid bilayer. Ydj1p may also have a more direct role in binding the precursor protein and presenting it to the translocation machinery, as some evidence suggests that DnaJ can itself serve as a chaperone [24].

Membrane machinery

Translocation across the membrane may be modeled as a three-step process: (1) docking of the precursor at the translocation site, (2) transit through the membrane and (3) release into the lumen (see fig. 1). Genetic and biochemical analyses have defined a set of membrane proteins that constitute the minimum translocon: Sec62p, Sec71p, Sec72p, Sec63p and the Sec61p complex, plus a stimulatory lumenal factor, BiP [25–27]. A variety of experimental approaches over the last several years have begun to define the precise functions of these components in the translocation reaction.

Sec62p, Sec71p, Sec72p. Sec62p is an integral membrane protein [28] that appears to be involved early in the translocation process. Sanz and Meyer [29] have shown that translocation is divided by energy requirements into two steps – ATP-independent association of the precursor with the membrane, followed by ATP-dependent passage through the membrane. Intriguingly, Müsch et al. [30] have found that a ribosome-tethered molecule of the precursor protein ppαF will cross-link to Sec62p in the absence of ATP, but in the presence of ATP, Sec61p (the translocation pore) becomes the major cross-linking partner of ppαF. This suggests that the Sec62p·precursor interaction is dissolved in an ATP-dependent reaction that results in the precursor being transferred to Sec61p. Further evidence for an early role for Sec62p comes from the observation that a translocation-defective allele of *sec62* (*sec62-1*) [31] impairs the ability of ppαF to interact with Sec61p [32].

Two other factors likely to be involved early in the translocation reaction are Sec71p and Sec72p (previ-

ously known as Sec66p and Sec67p) [33]. The *SEC72* gene is not essential, and *SEC71* is essential only at elevated temperatures, but deletion or mutation of either gene results in the accumulation of a subset of precursor proteins whose translocation is impaired in the absence of these gene products [34–36]. Feldheim and Schekman [35] used chimeric proteins to demonstrate that the translocation defect manifested in *Δsec72* is associated with the signal sequence portion of the secretory precursor rather than with the mature region. This observation, in conjunction with the fact that *Δsec71* and *Δsec72* strains show translocation defects for several of the same secretory precursors, suggests that Sec71p and Sec72p are involved in some facet of signal sequence recognition.

Sec62p, Sec71p and Sec72p seem to function primarily in post-translational translocation rather than in cotranslational translocation. Mutation or deletion of the *sec62*, *sec71* or *sec72* genes [26, 34–36] has a much more significant effect on the maturation of ppαF and prepro-carboxypeptidase Y, precursors that are able to translocate post-translationally [18, 19] than on that of invertase, which translocates chiefly cotranslationally [37]. The proposed role of Sec62p, Sec71p and Sec72p as a signal sequence receptor complex in post-translational translocation is likely fulfilled in cotranslational translocation by the binding of the signal sequence to SRP and the subsequent interaction of SRP with its receptor (SR) at the ER membrane [6].

Sec61p. The central component of the yeast translocon is Sec61p, the translocation pore. Sec61p is a hydrophobic integral membrane protein of 52 kD with 8 to 10 predicted membrane-spanning domains [38]. Sec61p is associated with two smaller polypeptides of 14kD and 8 kD (Sbh1p and Sss1p) in a heterotrimeric complex that is the homologueous counterpart of the mammalian Sec61p complex, in which Sec61α, β and γ correspond to the yeast Sec61p, Sbh1p and Sss1p; respectively [27]. Sec61p (Sec61α) likely forms the membrane conduit through which secretory precursors pass. In yeast, a precursor protein artificially blocked in the process of traversing the ER membrane can be chemically cross-linked to Sec61p [32], and in the mammalian ER Sec61p is the major cross-linking partner of nascent chains emerging from the ribosome [39]. In mammalian cells Sec61p is also associated with ribosomes during translation, suggesting that the association may serve to direct the emerging polypeptide into the membrane pore during cotranslational translocation [40, 41]. Panzner et al. [27] have found that the Sec61p complex in yeast exists in two distinct populations: one in which the heterotrimeric Sec61p complex is associated with Sec62p, Sec63p, Sec71p and Sec72p, and the other in which the heterotrimeric complex is associated with membrane-bound ribosomes. The authors speculate that this partitioning of the Sec61p complex could reflect a functional segregation of the pore proteins between post-translational and cotranslational pathways, an intriguing possibility that remains to be tested experimentally.

Sec63p, BiP (Kar2p). Sec63p is an integral membrane protein containing three predicted transmembrane segments that define cytoplasmic and lumenal domains. Within a 120-amino acid loop protruding into the lumen is a 70-amino acid region that is 43% identical to the 'DnaJ box', the most highly conserved region of the DnaJ family of proteins [42–44]. The recurring evolutionary motif of hsp70·DnaJ pairings suggests that the function of the J box in Sec63p may act as a protein anchor to provide a means of linking the membranous translocation machinery to a second hsp70 involved in translocation – the lumenal hsp70 homologue Kar2p (BiP).

Sec63p and BiP have been both genetically and biochemically implicated in translocation: *sec63-1*, a ts mutation in *sec63* that maps to the conserved J box, is defective for translocation both in vitro and in vivo [26]. A group of ts alleles of *kar2* are defective for translocation in vitro [32] and accumulate untranslocated precursors in vivo [45], a phenotype that is reproduced by the cellular depletion of BiP [45, 46]. Several lines of evidence indicate that BiP and Sec63p interact. Scidmore et al. [47] have noted allele-specific synthetic lethality between *sec63* alleles and *kar2* alleles. Synthetic lethality is a phenomenon in which the phenotype of a pairwise combination of two mutant alleles is more severe than that of either allele alone, often indicating that the gene products interact or act at a common step in a biological process [48]. Brodsky and Schekman [49] provided direct biochemical evidence for a physical interaction between BiP and Sec63p: a complex containing BiP, Sec63p, Sec71p and Sec72p can be isolated from yeast microsomal membranes and copurifies over several column steps. However, if the complex is purified from the *sec63-1* strain, in which a conserved residue of the J box has been mutated (A179→T) [50], BiP is released from the complex, implying that BiP interacts with Sec63p via the lumenal J box and that the *sec63-1* mutation uncouples BiP from Sec63p. Interestingly, purification in the presence of the nonhydrolysable analogue ATPγS also releases BiP from the complex, suggesting that the interaction of BiP with Sec63p is regulated by ATP.

Mechanism of translocation: BiP and Sec63 function

An array of cytosolic, membranous and lumenal factors must cooperate in order to orchestrate the movement of a secretory protein across the ER membrane. The first step of post-translational translocation, the docking of secretory proteins to the ER membrane (fig. 1, step 1) is an ATP-independent process that involves binding of the precursor to a putative signal sequence receptor

complex comprised of Sec62p and likely also Sec71p and Sec72p. Once localized to the translocon site, the precursor is transferred from Sec62p to Sec61p, the pore, in an ATP-dependent reaction [30] (fig. 1, step 2). Müsch et al. [30] have shown that this ATP dependence does not reflect a cytosolic requirement, suggesting that the lumenal ATPase BiP participates in the transfer reaction. In concordance with this, both the hsp70 BiP and its DnaJ cohort Sec63p have been implicated in an early step of translocation: Sanders et al. [32] characterized a mutant allele of *kar2* (*kar2-159*) that was primarily defective in an early step of translocation, as the mutation largely prevented the formation of a precursor·pore complex. The authors also showed that the *sec63-1* J box mutation decreased the ability of an artificially generated membrane-spanning intermediate to form a complex with the pore, implying that the *sec63-1* allele may impair translocation at the same step as *kar2-159*; and that one of the roles of Sec63p is to act with BiP at this transfer step.

BiP and Sec63p have also been implicated in the final step of translocation (fig. 1, step 3), release of the precursor from the pore into the lumen. BiP binds to a precursor protein blocked in transit, suggesting that the hsp70 interacts with transiting polypeptides as they emerge from the pore [32]. Compelling evidence for a role of BiP in the final phase of translocation comes from characterization of a mutant allele of *kar2* (*kar2-203*) that does not allow the precursor to exit from the pore [32], resulting in the formation of a 'stalled' precursor·pore complex. Lyman and Schekman [51] have expanded on this observation by demonstrating that the *sec63-1* allele, which uncouples BiP from its interaction with Sec63p, results in a phenotype identical to that of *kar2-203*: the precursor protein is 'stalled' in the pore, unable to transit into the lumen, suggesting that Sec63p and BiP also act together in this final phase of translocation.

These observations converge on the conclusion that BiP and Sec63p must interact at two distinct points in the translocation cycle (fig. 1, steps 2 and 3) in order to provide the impetus for the precursor to enter the pore, traverse it and exit into the lumen. How might BiP and Sec63p act in this process? The topology of Sec63p provides some clues – with its large cytosolic tail, three transmembrane domains and the lumenal J box [43], Sec63p is ideally suited to link lumenal BiP function to that of the membranous translocon. The observation that Sec63p exists in a complex with Sec61p, Sec62p, Sec71p and Sec72p [25, 49] raises the possibility that Sec63p could serve as a go-between through which BiP could act or be acted upon by the other components of the translocon through a series of allosteric conformational changes, as suggested in the basic tenets of the model presented by Brodsky and Schekman [52].

Such communication between BiP, Sec63p and the translocon components could have a bipartite function – early, in 'activating' the pore to receive precursor (fig. 1, step 2) and later, in facilitating the final phase of precursor transfer across the membrane. The early role of BiP may involve the 'pore-gating' function proposed by Crowley et al. [53], who demonstrated that the mammalian translocation pore opens to the lumen only when the span of the chain engaged by the pore reaches a minimum critical length of ~70 residues. The authors suggested that a lumenal translocation factor such as BiP 'plugs' the pore until the advancing secretory polypeptide somehow triggers its release. The cytosolic domain of Sec63p might serve as a sensor to alert BiP to the binding of a precursor protein at the putative signal sequence receptor complex, upon which an ATP-mediated conformational change originating from BiP in the lumen could open the pore and allow access to the waiting precursor. Alternatively, as suggested by Brodsky and Schekman [52], one role of BiP might be to 're-prime' the translocation apparatus to ready it for a second round of import.

In the latter part of the translocation reaction (fig. 1, step 3), BiP cooperates with Sec63p in driving the transfer of the polypeptide across the membrane and into the lumen. The two primary models for hsp70 function in this reaction, as reviewed by Glick [54], propose that hsp70s work either as a Brownian ratchet or as molecular motors. The ratchet model supposes that the random thermal oscillations of Brownian motion cause the transiting polypeptide to slide back and forth in the translocation channel. Binding of a lumenal hsp70 such as BiP to the end of the polypeptide flickering in and out of the pore could trap that portion of the polypeptide on the lumenal side. As more of the polypeptide chain 'flickers' into the lumen, successive binding of other hsp70 molecules would then increasingly prevent retrograde movement and ultimately deposit the entire precursor into the lumen. The role of Sec63p in this model would primarily be to serve as a beacon to localize BiP to the site of the translocation machinery in the membrane. In contrast, the molecular motor model proposes that an hsp70 molecule actively reels the transiting polypeptide into the lumen. In this scenario, hsp70s generate a pulling force by being anchored in the ADP-bound form to a membrane component (i.e. Sec63p) while simultaneously binding a portion of the emerging precursor protein. A conformational change induced by ADP→ATP exchange on hsp70 would allow force to be transmitted to the transiting polypeptide, pulling it into the lumen. ATP hydrolysis by the hsp70 would allow it to rebind the membrane component and engage the polypeptide chain in another cycle of binding.

Since there are hsp70s placed on either side of the membrane (Ssa1p/Ssa2p and BiP), both of these models

of lumenal hsp70 action must invoke some means of preventing the precursor from being caught in a 'tug of war' between the opposing hsp70s. Brodsky et al. [55] have shown that BiP and Ssa1p will not function interchangeably on opposite sides of the membrane, suggesting that despite their significant homologuey (63% identity), each molecule carries out unique functions. Coordinated forward movement of the precursor through the pore might be accomplished by the regulated release of precursor by Ssa1p/Ydj1p or by a series of binding reactions with increasingly graded affinity (i.e. strength of the precursor·Ssa1p binding < precursor·Sec62p binding < precursor·BiP binding).

The common feature of both both the Brownian ratchet and the molecular motor models is that lumenal hsp70 provides the energetic asymmetry necessary to prevent precursor backsliding and to deposit the transiting polypeptide into the lumen. Surprisingly, a yeast reconstituted system that lacks BiP and ATP still supports a low level of apparently faithful translocation, but addition of BiP and ATP stimulates translocation several-fold to a final level comparable to that achieved with native ER microsomal vesicles [27]. The authors suggest that the BiP-independent translocation represents a basal level of 'passive' transport, or that there is enough energy 'stored' in the system to support a single round of translocation – implying perhaps that the directionality provided by BiP is an important, but not absolutely essential, feature of translocation. It is possible that lumenal modifications such as glycosylation may also play a role in preventing retrograde precursor movement [56]. Regardless, it is clear that efficient translocation in yeast requires both BiP and ATP. The role of BiP in mammalian translocation, however, is unclear. Although one report states that lumenal proteins are required for the completion of translocation [57], a purified reconstituted mammalian system does not require BiP [58]. The apparent mechanistic variance between yeast and mammalian translocation might be traced to a choice of lifestyle: while yeasts use both co- and post-translational pathways, translocation in mammalian cells is almost exclusively cotranslational [6]. In a cotranslational reaction, the ribosome might provide a 'pushing' force as it spools nascent polypeptide into the pore, obviating the need for BiP to supply a 'pulling' force. There might be more pieces to the puzzle, however – Brodsky et al. [37] recently demonstrated that mutant alleles of *kar2* and *sec63* are defective for cotranslational translocation in yeast as well as for post-translational translocation, suggesting that their roles may be more complex than currently supposed.

A well-defined role for BiP in addition to its service as a member of the translocon is derived from a requisite of the translocation process: since proteins must be partially unfolded in order to cross the membrane, they must be folded again upon entry into the lumen. Hsp70s are thought to promote correct folding of their substrates by the binding and regulated, ATP-dependent release of exposed hydrophobic surfaces, thus preventing nonspecific hydrophobic aggregation. Several studies have implicated BiP in the normal folding pathways of secretory proteins [59–62], supporting the notion of hsp70-mediated folding in the ER. A lumenal DnaJ homologue, Scj1p [63], may cooperate with BiP in the folding process, providing one more link in the chain of hsp70·DnaJ interactions essential for the journey of a secretory protein from cytosol to lumen.

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