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Review

Posttranslational Protein Translocation Across the Membrane of the Endoplasmic Reticulum

Tom A. Rapoport*, Kent E.S. Matlack, Kathrin Plath, Benjamin Misselwitz and Oliver Staeck

Howard Hughes Medical Institute and Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115-6091, USA

* Corresponding author

Posttranslational protein translocation across the membrane of the endoplasmic reticulum is mediated by the Sec complex. This complex includes a transmembrane channel formed by multiple copies of the Sec61 protein. Translocation of a polypeptide begins when the signal sequence binds at a specific site within the channel. Binding results in the insertion of the substrate into the channel, possibly as a loop with a small segment exposed to the lumen. While bound, the signal sequence is in contact with both protein components of the channel and the lipid of the membrane. Subsequent movement of the polypeptide through the channel occurs when BiP molecules interact transiently with a luminal domain of the Sec complex, hydrolyze ATP, and bind to the substrate. Bound BiP promotes translocation by preventing the substrate from diffusing backwards through the channel, and thus acts as a molecular ratchet.

Key words: BiP / Endoplasmic reticulum / Protein translocation / Sec61 / Signal sequence.

Introduction

Proteins are translocated across the membrane of the endoplasmic reticulum (ER) through a channel whose central component is the heterotrimeric Sec61p complex (for review, see Matlack *et al*., 1998). Transport can occur either during or after the translation of the substrate ('co-' or 'posttranslational'). In the cotranslational pathway, directionality of transport is determined by the binding of the translating ribosome to the Sec61p channel. The channels in the ribosome and the membrane are aligned and the lumenal end of the channel is therefore the only exit site for the elongating polypeptide chain (Crowley *et al*., 1993; Mothes *et al*., 1994; Beckmann *et al*., 1997). Posttranslational transport must be fundamentally different since no ribosome is present. Other aspects of the two pathways are similar, however, in particular the way the signal sequence of a substrate is recognized once it reaches the channel. In this review, we will summarize recent studies that have elucidated the mechanism of posttranslational translocation in yeast.

Components Required for Posttranslational Translocation

Posttranslational translocation has been studied most extensively in *S. cerevisiae*. The process can be reproduced with reconstituted proteoliposomes containing a purified seven-component membrane protein complex, the Sec complex, and soluble Kar2p (the name for BiP in yeast) in the lumen (Panzner *et al*., 1995). The Sec complex consists of two sub-complexes, the heterotrimeric Sec61p complex and the tetrameric Sec62/63p complex (Deshaies *et al*., 1991; Panzner *et al*., 1995). The Sec61p complex consists of

i) a large subunit with multiple (10) membrane spanning segments (Sec61p) (Deshaies and Schekman, 1987), homologous both to Sec61α in mammals (Görlich *et al*., 1992) and to the bacterial SecY protein (Ito, 1984)

ii) a small, single-spanning subunit (Sss1p) homologous to the mammalian protein Sec61γ and to the bacterial protein SecE (Esnault *et al*., 1993; Hartmann *et al*., 1994), and

iii) an intermediate sized, single-spanning component (Sbh1p) (Finke *et al*., 1996), that is similar to the mammalian protein Sec61β, but does not share any homology to SecG, the third component of the bacterial SecYEG complex.

The Sec62/63p complex consists of Sec62p, Sec63p, Sec71p, and Sec72p (Deshaies and Schekman, 1989, 1990; Rothblatt *et al*., 1989; Sadler *et al*., 1989; Feldheim *et al*., 1992, 1993; Green *et al*., 1992). Sec62p and Sec63p, in contrast to Sec71p and Sec72p, are essential for the viability of yeast cells. Sec71p, Sec62p, and Sec63p span the membrane one, two, and three times, respectively, while Sec72p does not span the membrane, but is tightly associated with its cytoplasmic side. The precise functions of all these components, with the exception of a lumenal domain of Sec63p, the J-domain (see below), remain unclear. For example, Sec62p and Sec63p have sizable cytosolic domains that have an essential, but unknown, function (Deshaies and Schekman, 1990; Feldheim *et al*., 1992). Surprisingly, with the exception of Sec62p, all components (and also Kar2p) have a role late

in karyogamy (Ng and Walter, 1996), perhaps in the fusion of the two nuclei, but the mechanism of their involvement is not understood. Homologs of Sec62p and Sec63p have been found in mammals, but a function in posttranslational translocation has not yet been demonstrated for them (Woollatt *et al*., 1999; and unpublished results).

The lumenal component required for posttranslational translocation, Kar2p (BiP), is a member of the Hsp70 family of ATPases. Like all other Hsp70 proteins, Kar2p needs to collaborate with a J-protein partner. J-proteins (named after the *E. coli* protein DnaJ, which cooperates with the Hsp70 family member DnaK) are defined by a J-domain, a folded segment of about 70 residues. The J-partner for Kar2p in translocation is a lumenal domain of Sec63p (the 'J-domain') (Sadler *et al*., 1989). An interaction between Kar2p and Sec63p has been demonstrated both *in vivo* and *in vitro* (Sanders *et al*., 1992; Brodsky and Schekman, 1993; Scidmore *et al*., 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack *et al*., 1997, 1999). For example, translocation is perturbed both *in vivo* and *in vitro* by a mutation in the J-domain of Sec63p (sec63-1 mutant) (Rothblatt *et al*., 1989; Matlack *et al*., 1997), and the mutation can be suppressed *in vivo* by mutations in Kar2p (Scidmore *et al*., 1993).

Posttranslational Translocation Begins with Signal Sequence Recognition

Posttranslational translocation proceeds in at least two distinct steps: a substrate first binds to the channel and is then moved through it (Figure 1). The initial binding of a substrate to the Sec complex involves signal sequence recognition and does not require Kar2p or ATP (Sanz and Meyer, 1989; Lyman and Schekman, 1997; Matlack *et al*., 1997; Plath *et al*., 1998). These two components are required only during the second phase, when the actual translocation process that moves the substrate through the channel occurs.

We have studied signal sequence recognition with a systematic photo-crosslinking approach (Plath *et al*., 1998). Specifically, we have analyzed how the signal sequence of the secretory protein prepro-α-factor interacts with the Sec complex during the first step of posttranslational protein transport. Prepro-α-factor was bound to the Sec complex or intact membranes under conditions that prevented subsequent translocation. Each substrate used contained a single photoactivatable crosslinking group at a defined position. In a large collection of different prepro-α-factor substrates the position was systematically moved through the the sequence of the molecule. Sec61p was found to be the major protein crosslinked to positions within the signal sequence (Plath *et al*., 1998). Interestingly, the crosslinking pattern was periodic for positions within the hydrophobic core of the signal sequence (residues 9–17); several distinct forms of crosslinked product were produced, distinguishable by their mobility in SDS gels. We used mutants in Sec61p, which had either unique cleavage sites for the protease factor Xa in cytoplasmic and lumenal loops (factor Xa mutants), or were synthesized as two separate fragments (split mutants), to map the crosslinking sites to specific transmembrane domains (Wilkinson *et al*., 1996, 1997). TM domains 2 and 7 were primarily responsible for the interaction with the hydrophobic core of the signal sequence. Weaker crosslinks were also seen with TM domain 1. There was an excellent correlation between crosslinking to TM domain 2 or 7 and the mobility of the crosslinked band. These results therefore showed that the signal sequence adopts a helical structure that is intercalated between particular TM domains of Sec61p. The bound signal sequence is likely oriented perpendicular to the plane of the membrane because it contacts the two TM domains of Sec61p over a considerable distance through the membrane. Since the signal sequence is precisely positioned, these data provided evidence that it is recognized by a protein-protein interaction, rather than by partitioning into the lipid phase.

The translocation substrate could also be crosslinked to lipid. Crosslinks between prepro-α-factor and lipid occurred throughout the entire signal sequence, up to the site where it would normally be cleaved (Plath *et al*., 1998). These crosslinks could be co-immunoprecipitated with

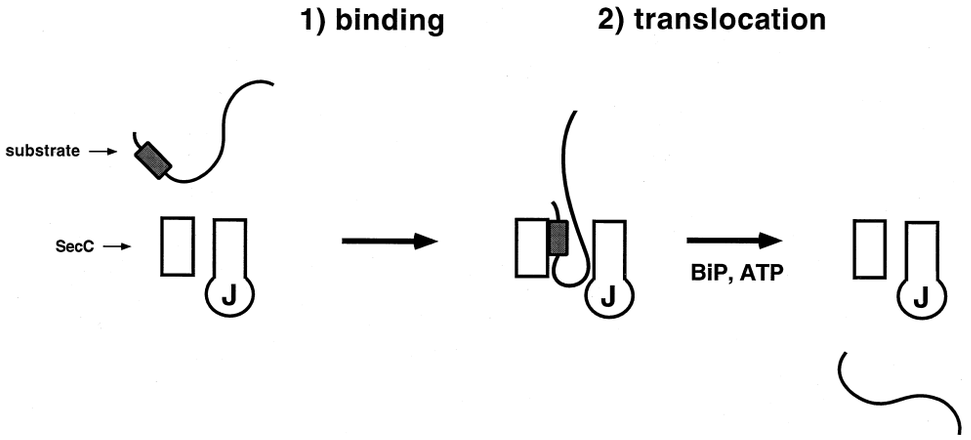

Fig. 1 Scheme Showing the Two Phases of Posttranslational Protein Translocation.

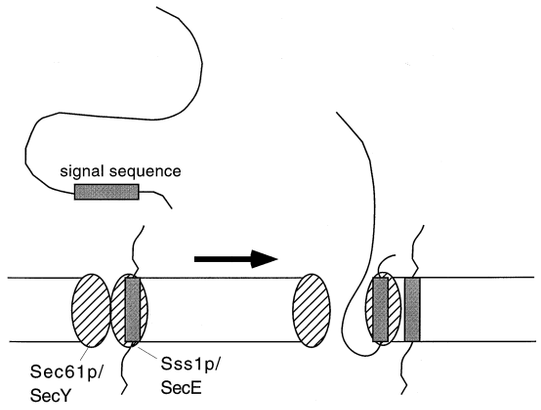

Fig. 2 Scheme Showing the Hypothesis That the Signal Sequence Replaces the Smallest Subunit of the Sec61p/SecYEG Complex from Its Binding Site at the Largest Subunit, thus Triggering Channel Opening.

the Sec complex, indicating that they originate from substrate molecules that are bound to the channel. The signal sequence binding site is therefore apparently located at an interface between the Sec61p channel and lipid. No lipid crosslinks were seen with residues in the mature region of prepro-α-factor, although a non-periodic pattern of Sec61p crosslinks remained up to at least residue 29. These data suggest that the polypeptide chain is inserted into the channel in a loop structure, with the C-terminal part of the hairpin in an environment that excludes lipid, likely the central pore (see Figure 2).

Two different types of crosslinking probes showed some interesting differences. With probes on lysine residues, crosslinking to Sec62p and Sec71p was observed for positions within the hydrophobic core of the signal sequence, whereas no such crosslinks appeared with phenylalanine-based probes. Presumably, Sec62p and Sec71p are more distant from the actual signal sequence binding site and can only be reached by the longer and more flexible side chains of the lysine probes. Another difference between the two probes was that the signal sequence helix was rotated by one position relative to TM domains 2 and 7. Presumably, the binding pocket is flexible, allowing signal sequences with different side chains to be accomodated with small energetic differences. These results provide an explanation for why signal sequences can be different in primary structure yet still be recognized by the same receptor.

Signal sequence recognition in the cotranslational pathway is similar to that in the posttranslational mode. Photocrosslinking experiments with ribosome-bound prepro-α-factor nascent chains inserted into mammalian ER membranes again showed both a periodic pattern of Sec61p crosslinks for residues within the hydrophobic core of the signal sequence and continuous lipid crosslinking throughout the same region (Mothes *et al*., 1998; Plath *et al*., 1998). Although the crosslinking sites could not be mapped within Sec61p, it seems likely that the same TM domains are involved. The signal sequence of preprolactin occupies a similar binding site at an interface between channel and lipid, and reaches it through the interior of the channel (Mothes *et al*., 1998). Interestingly, with both prepro-α-factor and preprolactin crosslinks to the TRAM protein occurred but were restricted to residues located on one side of the helix formed by the signal sequence. This corresponds approximately to the position where Sec62/71p-crosslinks were observed in the posttranslational system from yeast. This may be the site where trans-membrane domains of membrane proteins leave the channel and enter the lipid.

Our results suggest that recognition of the signal sequence and its insertion into the channel are one and the same process in both the co- and posttranslational translocational pathways. In the cotranslational system insertion of short nascent chains can be demonstrated by their protection against proteolysis (Mothes *et al*., 1998). Insertion requires a functional signal sequence, and the channel opens toward the lumen as a result (Jungnickel and Rapoport, 1995; Crowley *et al*., 1994). We therefore assume that in both co- and post-translational translocation, an interaction of the signal sequence with Sec61p opens the channel. Opening of the channel for ions by an interaction with a signal peptide has also been reported for the related bacterial system (Simon and Blobel, 1992).

Genetic experiments in *E. coli* have provided evidence that signal sequence recognition by the homologous SecY protein requires the same TM domains as in yeast (for review, see Ito, 1995). Mutants in SecY that suppress signal sequence mutations (prlA mutants) map primarily into regions of TM domain 7 and the loop between TM domains 1 and 2. Moreover, other genetic experiments indicate that TM domains 1, 2, 7, and 10 (the latter was not found in our crosslinking experiments) may also be responsible for the interaction between SecY and SecE (Flower *et al*., 1995). Crosslinking and genetic experiments in yeast also suggest an interaction of the region around TM domain 7 in Sec61p with Sss1p (Wilkinson *et al*., 1997).

On the basis of these data, we have postulated that the signal sequence and Sss1p/SecE bind to the same or overlapping regions in Sec61p/SecY (Plath *et al*., 1998). Specifically, we have proposed that Sss1p/SecE acts as a surrogate signal sequence. When the signal sequence of a substrate arrives, it would replace Sss1p/SecE in its binding site on Sec61p/SecY and thus open the channel for polypeptide transport (see scheme in Figure 2).

Sss1p/SecE indeed look similar to signal sequences since their essential regions consist of no more than a TM domain and a few surrounding residues. As indicated by extensive mutagenesis of *E. coli* SecE (Murphy and Beckwith, 1994), the hydrophobic segment is important, but not the precise amino acid sequence within it, similar to the properties required for a functional signal sequence. However, several residues of the cytoplasmic domain preceding the hydrophobic domain of SecE were found to be important, suggesting that interactions with a cytoplasmic loop of SecY outside the lipid bilayer may also be involved.

Our hypothesis may provide an explanation for the puzzle of why such a small polypeptide chain as Sss1p/SecE is found in all organisms, is essential for their viability, and is always kept as a separate entity from the multi-spanning Sec61p/SecY component. It is indeed striking that all other components involved in protein translocation, such as the β-subunit of the Sec61p complex, the four components of the Sec62/63p complex, TRAM, Kar2p (BiP), SecG, SecA, and SecD/F/ydj1 are present either in eukaryotes or in prokaryotes, but not in both. The basic machinery of translocation may thus consist only of a large channel-forming subunit that spans the membrane multiple times (Sec61p/SecY), and a small single-spanning polypeptide (Sss1p/SecE) that serves as a surrogate signal sequence. Other components may have their effects by acting upon this complex.

According to our hypothesis, prl mutants in SecY would have a weakened interaction with SecE, allowing the channel to be more easily opened. A weakened interaction between SecY and SecE has indeed recently been found in co-immunoprecipitation experiments (Duong and Wickner, 1999).

Moving the Substrate through the Channel; Kar2p Acts as a Ratchet

The second phase of posttranslational translocation is the actual movement of the polypeptide through the channel (Figure 1). We have recently provided evidence that Kar2p acts as a molecular ratchet during this phase of translocation (Matlack *et al*., 1999), a mechanism proposed earlier by Simon *et al*. (1992) and Schneider *et al*. (1994). In a ratcheting mechanism, Kar2p would bind to a segment of the polypeptide substrate as it emerged on the lumenal side of the membrane, and prevent its diffusion back into the channel without diminishing its ability to move inward. Each time inward movement exposed a new segment in the lumen another Kar2p molecule would bind.

Many of the experiments that led to this model were based on the establishment of a system in which translocation could be observed in detergent solution, in the absence of membranes (Matlack *et al*., 1997). To establish such a system, we first tested whether a translocation substrate bound to the Sec complex would remain associated in detergent solution. To this end, *in vitro* synthesized prepro-α-factor or proOmpA was incubated with proteoliposomes that contained the purified Sec complex but lacked Kar2p or ATP. After solubilization in digitonin, we found that a large percentage of the substrate remained bound to the Sec complex. Binding required a functional signal sequence in the substrate and the presence of both sub-complexes of the Sec complex.

When Kar2p and ATP were added to the detergent-solubilized complex of substrate and Sec complex, release of the substrate from the Sec complex occurred (Matlack *et al*., 1997). The reaction required ATP hydrolysis by Kar2p and an intact J-domain on Sec63p. To determine whether release of the substrate required its movement through the channel, and therefore reflected translocation, we employed a substrate with a bulky tRNA attached to its C-terminus. This substrate was not released. Kar2p and ATP did, however, induce its movement into the channel to the point that its C-terminal region could be crosslinked to Sec61p. This is the predicted result if translocation proceded until the tRNA reached the channel. When the tRNA was removed by treatment with puromycin, release occurred. Kar2p and ATP thus induce release of a bound substrate by moving it through the channel and releasing it from the lumenal side of the Sec complex. These results led to the surprising conclusion that posttranslational protein translocation can occur with purified components in the absence of a lipid bilayer, and provided evidence for the existence of a channel with a limited pore size.

To address the validity of the ratcheting model, we first used the soluble system to demonstrate that Kar2p is transferred to the substrate during the translocation reaction (Matlack *et al*., 1999). A fully translocated prepro-α-factor molecule (165 amino acids) had as many as seven Kar2p molecules bound to it, indicating that a translocated substrate can be very densely covered with Kar2p. The interaction was transient, however, Kar2p molecules starting to dissociate even while translocation was still in progress.

We next asked if Kar2p bound to the substrate could perform the central function of a ratchet, preventing backwards movements of the substrate. We developed a back-sliding assay with the following rational. If a substrate with a bulky group attached to its C-terminus is imported to its maximum extent into proteoliposomes, protease treatment should give a defined fragment, corresponding to the piece of the polypeptide chain inside the vesicles (Figure 3, scheme I). If the substrate slips backwards upon dissociation of Kar2p, protease treatment should result in a heterogeneous mixture of fragments that would be undetectable (Figure 3, scheme II). Thus, the loss of the specific fragment should be a measure of back-sliding. With this assay, we found that the dissociation of Kar2p from the substrate by depletion of ATP led to significant back-sliding. When the tRNA-associated substrate was first imported to the maximum extent and then an antibody to the C-terminus of the substrate was added in the presence of ATP, it gained access to segments of the substrate that had previously been translocated into or through the channel (Figure 3, scheme III). Thus, transient backwards movements of the substrate occur even under the conditions of normal translocation. Taken together, these results support a ratcheting mechanism: transient backwards movements of the substrate do occur during translocation and are minimized by the binding of BiP at the lumenal side of the membrane.

If Kar2p acts as a ratchet during translocation, then the length of time that it spends bound to a substrate is crucial; a BiP mutant with an increased rate of dissociation from the substrate should be less effective in preventing backwards movements, and thus should be less efficient

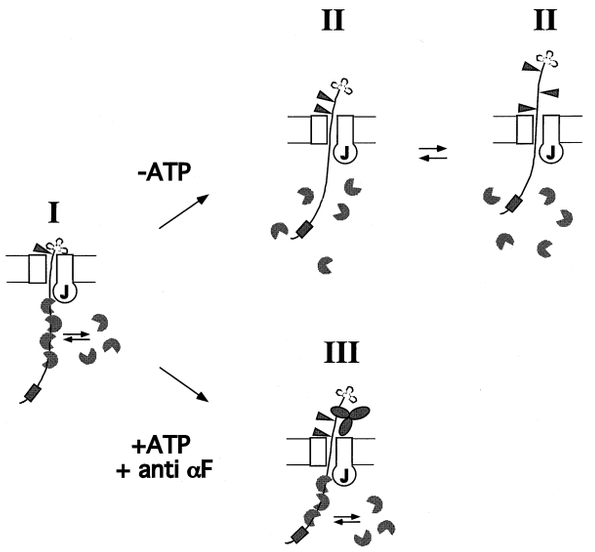

Fig. 3 Concept of the Back-Sliding Assay.
A substrate with an attached tRNA is first imported into proteoliposomes containing the Sec complex, BiP, and ATP, resulting in a unique stalled position (scheme I). A single fragment, slightly shorter than the intact polypeptide, will be generated by proteolytic cleavage (arrowhead). Removal of ATP results in loss of BiP from the substrate, allowing its diffusion back through the channel (schemes II). Heterogeneity of positions of substrate molecules within the channel will prevent one proteolyic fragment from dominating. If back-sliding occurs in the presence of ATP, an antibody to the C-terminal domain of the substrate prepro-α-factor (anti αF) will bind and promote the disappearence of the unique proteolytic fragment (scheme III).

at promoting translocation. We showed that a truncation mutant, containing both the ATPase and peptide binding domains of Kar2p but lacking the C-terminal lid domain, indeed had an increased dissociation rate and behaved like a ratcheting mutant.

We next tested whether translocation of prepro-α-factor can occur by Brownian ratcheting, in which forward movement of the polypeptide chain occurs by simple diffusion. Specifically, we asked whether translocation would occur if Kar2p is replaced in the lumen by non-physiological binding partners of prepro-α-factor that cannot interact with the channel or hydrolyze ATP. For this we used antibodies directed against different regions of prepro-α-factor. An antibody directed against a domain immediately following the signal sequence could induce translocation, and the reaction was further stimulated by antibodies against more C-terminal domains.

These results demonstrated that a simple Brownian ratchet is sufficient to provide the driving force for translocation. All that is required is the channel-forming Sec complex and a binding partner for the translocation substrate on its lumenal side. Although any binding partner would drive translocation, the physiological partner Kar2p functions as a general and efficient ratchet. Kar2p has several properties that make it superior to other potential ratcheting molecules: 1) because of its interaction with the J-domain of Sec63p it most likely binds immediately at the lumenal end of the channel, where it could most effectively prevent backwards movements of the substrate; 2) it has low sequence specificity, which allows it to bind to many sites on a given polypeptide and would allow it to transport a wide range of substrates, and 3) its interaction with the substrate is transient, being bound long enough to promote translocation, but quickly dissociating to allow subsequent folding or modification reactions. Kar2p is probably also the only ratchet operative during translocation; because it coats a substrate, other molecules would be prevented from participating.

It is possible that the driving force for posttranslational protein translocation is provided entirely by Kar2p acting as a Brownian ratchet since significant levels of translocation were achieved even with antibodies as lumenal binding partners of the substrate. However, we cannot exclude that Kar2p actively 'pulls' on the substrate in addition to its function as a ratchet: after generating force while bound to both the J-domain and the substrate, Kar2p would remain bound only to the substrate, acting as a ratchet to preserve the forward movement caused by the power stroke. It is possible that force generation by Kar2p would be required if the substrate was folded into a stable conformation on the cytosolic side of the membrane or if cytosolic proteins were strongly bound, as has been proposed in the case of mitochondrial protein import (Glick, 1995).

A Specific Model for Posttranslational Translocation

Posttranslational translocation may occur as shown in Figure 4. In the first step, the translocation substrate is bound to the Sec complex by virtue of its signal sequence (shaded box) and is inserted into the channel as discussed above (Figure 4; Matlack *et al*., 1997; Plath *et al*., 1998). Once inserted, the substrate is probably bound as a loop with a small portion in the lumenal space (Shaw *et al*., 1988; Mothes *et al*., 1994; Plath *et al*., 1998). Kar2p does not seem to be required to open the channel or to transfer the polypeptide into it, at least not in the reconstituted system with prepro-a-factor as the substrate. Ratcheting would be initiated by a transient interaction of Kar2p in its ATP form with the J-domain of Sec63p (Figure 4). The J-domain activates Kar2p for peptide binding. It induces rapid hydrolysis of the nucleotide, converting the open peptide binding pocket of BiP-ATP into the closed pocket of BiP-ADP, and resulting in the ADP form bound to the translocating polypeptide chain. J-activated BiP is very short-lived and binds peptides with low specificity (Misselwitz *et al*., 1998), allowing it to bind to essentially any segment of the substrate close to the lumenal end of the channel. Attachment of a BiP molecule would prevent the bound segment of the substrate from re-entering the channel, but would not hinder inward movements (Figure 4). The polypeptide may move back and forth (double arrow), but once enough has moved into the lumen, another BiP molecule would bind by the same mechanism,

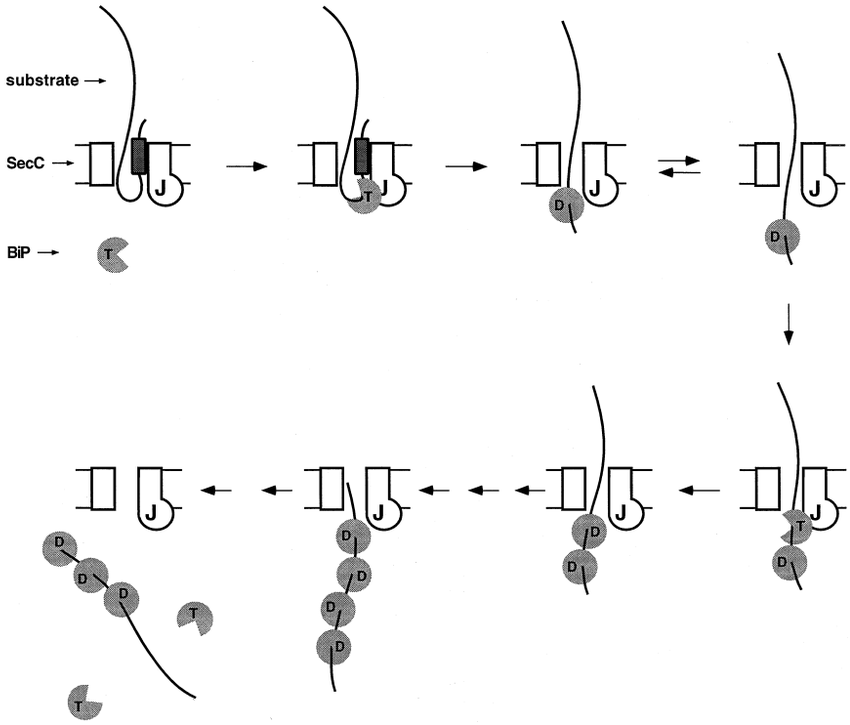

Fig. 4 Scheme Showing the Ratcheting Model of Translocation. 'T' and 'D' stand for ATP and ADP, respectively.

and this process would be repeated until the polypeptide chain is entirely translocated. The presence of multiple BiP molecules on a substrate increases the efficiency of the ratcheting mechanism (Simon *et al*., 1992). BiP would dissociate from the substrate following nucleotide exchange (Misselwitz *et al*., 1998); binding of ATP would re-open the peptide binding pocket and release the substrate.

How Different Is Mitochondrial Protein Import?

Two models have been proposed for mitochondrial protein import. In one, mitochondrial Hsp70 (mtHsp70) would act as a molecular ratchet (Schneider *et al*., 1994), similar to the mechanism described here for BiP in the ER system. In the other, mtHsp70 would act as a force-generating motor (Glick, 1995). More recently, it has been suggested that 'trapping' (ratcheting) is sufficient for the transport of some substrates, while 'pulling' is required for those that are more folded (Voisine *et al*., 1999). On the other hand, it has been demonstrated that at least for the model proteins studied their spontaneous unfolding rate on the mitochondrial surface is fast enough to be consistent with a Brownian ratcheting mechanism (Gaume *et al*., 1998). Nevertheless, it is possible that mitochondrial protein import differs from postranslational protein transport into the ER. The existence of a strong interaction between the translating ribosome and the translocation channel in the parallel cotranslational pathway into the ER may allow transport of polypeptides which would not be able to cross the membrane after completion of their synthesis because of tightly folded domains. In mitochondrial protein import, the lack of tight ribosome binding to the outer mitochondrial membrane may require a more active 'pulling' mechanism to transport such folded polypeptides. It is also striking that Tim44, the membrane partner of Tim44, seems to act similarly to a J-protein, and yet has only marginal sequence similarity (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994). In the case of the mitochondrial system, a stable complex consisting of mtHsp70, Tim44, and peptide has been reported (Horst *et al*., 1996), whereas a complex between Kar2p, its partner J-domain, and peptide could not be detected (Misselwitz *et al*., 1998; 1999). Perhaps, Tim44 has evolved to provide a stronger interaction than a J-protein with the Hsp70 partner. Clearly, more experiments are required to clarify if a ratcheting mechanism is insufficient to explain mitochondrial protein import.

Acknowledgement

We dedicate this review to Walter Neupert, on the occasion of his 60th birthday, in recognition of his pioneering and continuing contributions to the field of translocation. We all look forward to his future results. T.A.R. would like to thank Walter for years of support, insight, and friendship.

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