4 Discussion

4.1 Movement of Expansion Segment 27

The cryo-EM structure of the non-translating 80S ribosome from *S. cerevisiae* was solved by cryo-EM and single particle reconstruction at a resolution of 24 Å.

Due to additional proteins and rRNA insertion elements, the eukaryotic ribosome is much larger than the prokaryotic ribosome (Gerbi, 1996). Interestingly, the large ribosomal subunit showed a 150 Å long RNA-typical helical structure that was identified as expansion segment 27 (ES27) one of the insertion elements that contributes to the larger size of the eukaryotic ribosome. ES27 was visible in several cryo-EM structures (Beckmann et al., 1997), (Beckmann and Helmers, unpublished). In these cryo-EM structures, ES27 was facing away from the tunnel exit in the large ribosomal subunit through which the nascent chain emerges from the ribosome (outward position). Interestingly, in the structure of the non-translating 80S ribosome, ES27 was rotated by over 90º towards the tunnel exit with its tip reaching all the way over the tunnel exit (inward position).

Cotranslational protein transport across the ER membrane requires the close coordination of signal sequence recognition, RNC targeting to the ER membrane and translocation of nascent chain across the ER membrane. The targeting and translocation machinery has to ensure, that only RNCs bearing a nascent chain with a signal sequence coding for cotranslational protein transport get targeted to the translocation channels in the ER membrane.

We propose a model in which ES27 controls the access to the area around the exit tunnel at the large ribosomal subunit of the 80S ribosome and therefore to the emerging nascent chain. We suggest that the rotating rRNA structure may play a role in coordinating access of non-ribosomal factors such as chaperones, modifying enzymes, SRP or the PCC to the

tunnel exit site and thereby to the emerging nascent chain. It is tempting to speculate that the location of ES27 on the large ribosomal subunit could indicate the functional state of the ribosome. With a nascent chain emerging from the ribosome, the rotation of ES27 towards the outward position would allow the access of the ribosome to components of the targeting and translocation machinery of the cell. With ES27 in the inward position, unspecific interactions of the ribosomes with components of this machinery would be prevented by sterical hindrance. However, it can not be excluded that the movement of ES27 is controlled by the components that interact with the area around the exit tunnel of the large ribosomal subunit rather than by the functional state of the ribosome itself. In this model, the rotation of ES27 would be induced by the interactions of ligands with the ribosome. A direct interaction of ES27 with these ligands would provide a sampling mechanism for active ligands that would coordinate the interactions between the ligands and the ribosome. In addition, the segment may interact directly with the nascent chain or with nascent chain-interacting proteins, thereby keeping it in a conformational state that would facilitate the recognition and binding by components of the targeting and translocation machinery.

In any case, the function of ES27 is essential and conserved; In *Tetrahymena* deletion of this insertion is lethal but can be complemented with the corresponding insert from other species (Gomez-Lorenzo et al., 2000). After the L1 protuberance (Gomez-Lorenzo et al., 2000), the ES27 helix is the second example of an extremely dynamic rRNA segment in the periphery of the 80S ribosome. Further biochemical and structural characterization will be necessary to fully understand the role of ES27 in the targeting and translocation process.

4.2 The Protein Conduction Channel Associated with the Translating 80S Ribosome

The structure of the protein conduction channel in association with the translating 80S ribosome was solved by cryo-EM and 3D-reconstruction to a resolution of 15.4 Å. The complex had been assembled from purified *in vitro* assembled yeast ribosome-nascent chain complexes (RNCs) containing a signal sequence in the nascent chain and the purified protein conducting channel (Sec61p complex), both from *S. cerevisiae*.

Using an excess of Sec61p complex and the detergent DeoxyBigCHAP (DBC), it was possible to acquire a fraction of 75-80% active RNC-Sec61p complexes with a nascent chain protected in protease protection experiments. Thus, by using RNCs with the solubilized Sec61 complex in DBC we successfully reconstituted an active RNC-channel complex with nascent chain fully inserted into the channel. This result is in complete agreement with previous studies that had shown that the Sec61p channel interacts with the ribosome in detergent solution (Beckmann et al., 1997; Prinz et al., 2000a) and that this interaction can take place without the SRP/SR targeting system of the cell (Jungnickel and Rapoport, 1995). It had furthermore been shown that the mammalian Sec61p complex as well as the heptameric complex from yeast show translocation activity in detergent solutions (Matlack et al., 1997; Mothes et al., 1998).

4.2.1 3D-Reconstruction of the RNC-Sec61p Complex

The 3D-structure of the RNC-Sec61p complex was solved at a resolution of 15.4 Å. The structure showed a clearly visible mass in the inter-subunit canyon that could be identified as a tRNA in the P-site of the 80S ribosome. The presence of the tRNA density in our map indicated the presence of a nascent chain in the structure and served as a prerequisite to permit the interpretation of the structures on the basis of different functional states.

4.2.2 The Connection between the Ribosome and the Sec61p Complex

Fluorescence quenching studies had implicated the seal between the Sec61p complex and the ribosome to be responsible for maintaining the ion barrier during cotranslational protein transport across the ER (Crowley et al., 1994; Hamman et al., 1997). Surprisingly, the connection between the ribosome and the Sec61p complex in our structure was not circumferentially sealed but left a gap of about 15 Å. This agrees well with protease protection experiments showing that emerging nascent chains are accessible to protease when they are either too short to interact productively with the channel or form extended cytosolic loops (Jungnickel and Rapoport, 1995; Hegde and Lingappa, 1996). It also agrees with the finding that nascent chains such as the pre-prolactin 86mer and the substrate used in this study are protected from digestion. These nascent chains form a loop reaching directly from the tunnel exit into the channel, with the space between channel surface and ribosome being too small for the protease to enter (Shaw et al., 1988; Jungnickel and Rapoport, 1995).

However, with a lateral opening of 15 Å between the ribosome and the Sec61p complex, it is unlikely that the ribosome-channel connection can function as an ion-tight seal maintaining the ion permeability barrier of the ER membrane as suggested on the basis of fluorescence quenching experiments (reviewed in Johnson and van Waes, 1999). It is possible, however, that lipids or additional proteins lead to the observed seal formation in native mammalian membranes instead of the PCC itself. On the other hand, a seal formed by the ribosome-membrane junction may be ineffective in light of the existence of additional tunnels in the large ribosomal subunit, which are also present in prokaryotic and archaebacterial ribosomes (Frank et al., 1995; Ban et al., 2000 and Gabashvili et al., 2001). Since these tunnels connect the conduit for the nascent chain with the cytosolic environment, they may allow ion flow between the cytosol and the ER lumen, even if a seal-forming junction between ribosome and membrane would be present.

While a 3D reconstruction of the non-translating 80S ribosome in association with the Sec61p complex had found one connection between the ribosome and the Sec61p complex, our structure of the translating ribosome with the Sec61p complex shows four connections between the ribosome and the Sec61p complex. It is interesting to find ribosomal proteins most likely involved in all ribosome-channel connections. The isolated rRNA of the large ribosomal subunit was shown to be sufficient to bind to the channel with high affinity, even across different species (Prinz et al., 2000). However, in contrast to other parts of the ribosomal periphery, the entire region near the tunnel exit, including rRNA as well as proteins, appears to be relatively conserved. At a resolution between 25-30 Å the mammalian ribosome has a very similar appearance in this region (Morgan et al., 2000) and four ribosome-channel connections have been observed in similar positions (Menetret et al., 2000). This region also appears similar when comparing the 60S subunit of the yeast ribosome with the archeabacterial 50S subunit (Nissen et al., 2000), an important difference being a shorter rRNA helix 59 in *H. marismortui*. This helix in the bacterial *E. coli* ribosome (Gabashvili et al., 2000) is comparable in length to the yeast ribosome, but a rpL19-like protein is missing (L19e in *Archea*). Thus, despite the fact that there are some differences, the overall spatial arrangement of the ribosome-channel interaction is conserved in prokaryotic, archeabacterial, and eukaryotic cells and involves both RNA and proteins of the large ribosomal subunit.

4.2.3 Structure and Function of the Protein Conducting Channel

The Sec61p complex is an oligomeric assembly of three subunits, Sec61α (10 transmembrane helices), β (1 transmembrane helix), and γ (1 transmembrane helix). It has long been debated how many Sec61 trimers form the active aqueous channel that is used during cotranslational protein translation across the ER membrane. To estimate the oligomeric Sec61p complex stoichiometry, we have determined the number of transmembrane helices of known transmembrane proteins that could be fitted into the Sec61p complex electron density. The outline of the Sec61p complex offered space for

about 35 helices, indicating that three Sec61 trimer of 12 transmembrane helices each form an active Sec61p complex. For the homologous SecYEG complex it had been reported that an average area of 199 $Å^2$ per helix is occupied in the plane of the membrane (Collinson et al., 2001). Assuming a similar occupation for the Sec61p complex, the density of our structure could encompass any number of transmembrane helices between 30 and 39. This calculation would support the initial estimate of three Sec61 trimers per active Sec61p complex. However, neither our structure nor the structure of the mammalian Sec61p complex show a threefold symmetry (Menetret et al., 2000). It is possible though, that the lack of a threefold rotational symmetry may be a feature of the channel itself or could be induced by the asymmetric interaction with the ribosome. Supporting this model is the fact that a crosslinking study had found interactions between two Sec61β subunits leading to Sec61β homo dimers (Kalies et al., 1998). This indicates that the oligomeric assembly of the Sec61 trimer into the Sec61p complex is not necessarily symmetric.

In contrast to the structure of the non-translating ribosome with the Sec61p complex, the structure of the active ribosome with the Sec61p complex did not show the central pore in the Sec61p complex (Beckmann et al., 1997). This indicates that the interaction with the signal sequence does not necessarily lead to a widely open channel conformation. Instead, the active Sec61p complex appears compact independent of the presence of a signal sequence. Even at higher contour levels, the Sec61p complex shows no central pore, which is different from the previous structure of the empty ribosome-channel complex in Triton X-100 detergent solution (Beckmann et al., 1997). The open conformation in Triton X-100 may be induced by this strong detergent and may explain the accessibility of the nascent chain to protease under these conditions as observed in our protease protection assays. The slightly flattened and more elongated shape of the inactive channel could indicate that gating by the signal sequence leads to a small rotation of the membrane helices towards a position more perpendicular to the plane of the membrane. This would represent an iris-like movement reminiscent of the conformational change suggested for the gating of gap junctions (Unwin and Zampighi, 1980). Our results do not support conclusions from fluorescence quenching experiments

with native mammalian ER membranes suggesting a pore of 9-15 Å diameter in the inactive Sec61p complex (Hamman et al., 1998) and one of 40-60 Å diameter in the translocating one (Hamman et al., 1997). However, Sec61p complexes in native membranes are known to be associated with additional proteins in different oligomeric states (Wang and Dobberstein, 1999; Menetret et al., 2000). In a recent cryo-EM study of mainly mammalian ribosome-channel and mixed ribosome-translocon complexes (Menetret et al., 2000), the Sec61p complexes are shown with open conformations.

However, the contour levels chosen in that study appear to be too high. At slightly lower, more realistic contour levels, the Sec61p complexes of that study show closed conformations as well and are in good agreement with the structures of this study. Taken together, in all studies of the eukaryotic PCC using either 2D (Hanein et al., 1996) or 3D reconstructions (Beckmann et al., 1997; Menetret et al., 2000) the center of the Sec61p complex is aligned with the nascent chain tunnel exit of the ribosome showing either an indentation or a central pore. This strongly suggests that the central region of the oligomeric Sec61p complex is less dense and most likely more flexible than the remainder of the complex. These properties would potentially allow it to function as the conduit for nascent chains during cotranslational protein transport. However, due to resolution limits of our structure that did not allow for the identification of an electron density representing the nascent chain, further structural studies are necessary to determine the precise spatial arrangement of all Sec61 subunits and their involvement during cotranslational protein translocation across the ER membrane.

The 3D structure of the translating ribosome with the attached Sec61p complex showed the active Sec61p complex in a compact closed conformation with a gap between active channel and the translating ribosome. The compact appearance would indicate that the gating of the channel by the signal sequence leads to an opening just large enough to be completely occupied by the inserted nascent polypeptide chain, which would prevent the flow of ions across the Sec61p complex during protein translocation.

4.2.4 Binary Model for Cotranslational Protein Translocation and Membrane Protein Integration

Based on these findings we propose a binary model of how the ribosome-PCC complex could function in cotranslational translocation and membrane protein insertion. First, we suggest that the conserved tunnel in the large ribosomal subunit represents an important functional domain of the ribosome which exclusively allows the folding of alpha-helical secondary structures. The tunnel dimensions are such that α-helix folding is possible while β-sheet formation is sterically impossible or at least problematic. The introduction of a folding hierarchy with priority for α-helix formation would have general implications for protein folding by providing folding seeds along the sequence and by considerably reducing the number of folding possibilities. In our context, it has been shown that helicality, besides hydrophobicity, is obligatory for productive signal sequence interaction with the signal recognition particle (SRP) and with the PCC (reviewed in Keenan et al., 2001; Plath et al., 1998). Probing of the nascent chain for hydrophobic helices by the channel would lead to the insertion and capture of any sufficiently hydrophobic segment in such a way that the inserted polypeptide is tightly accommodated.

Depending upon its orientation in the channel, the hydrophobic segment can expose its C-terminal end either to the cytosolic or to the lumenal side of the ER membrane. In the latter case, it would result in a loop formation of the remaining nascent chain in the channel (Figure 4.1). Importantly, insertion would not lead to further opening of the pore to any predefined size. As a result, the following nascent chain would either accumulate on the cytosolic side or, if it has been co-inserted in a loop, translocate across the ER membrane.

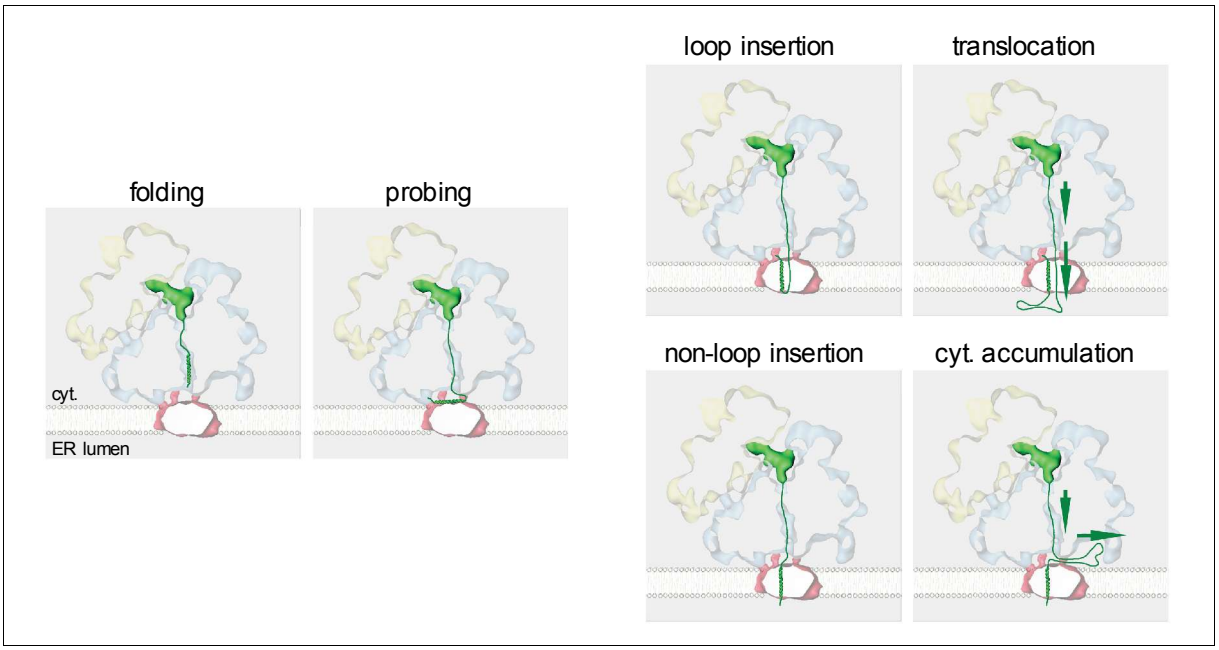

Figure 4.1: Binary model of cotranslational protein transport. The model is based on the finding that a gap exists between the RNC and the Sec61p complex and that the translating Sec61p complex has a compact conformation and can hence provide a seal to maintain the ion permeability barrier of the ER membrane: **(1)** The tunnel in the large ribosomal subunit facilitates folding of alpha-helical segments. **(2)** The emerging segment is probed by the Sec61p complex before insertion. Hydrophobicity, helicality, and the nature of the flanking regions (i.e. positive charges) determine if and in what orientation the segment is inserted. **(3)** Insertion can occur in two different orientations with the channel just opening wide enough to accommodate the inserted nascent polypeptide. In case of *loop insertion* the following nascent chain is guided through the membrane and translocation is possible. **(4)** In case of *non-loop insertion,* the following nascent chain cannot translocate, accumulating on the cytosolic side of the membrane. Because of the sealed channel and the existence of the gap between ribosome and the Sec61p complex, such cytosolic domains can easily exit into the cytosol at any time without compromising the ion permeability barrier.

Nascent chain translocation after loop insertion and cytosolic accumulation after non-loop insertion are the only two principally different functional states of the RNC-Sec61p complex. A simple secretory protein would experience only one loop insertion of the signal sequence after targeting by SRP and translocate. For a polytopic membrane protein, the states would alternate with every new hydrophobic transmembrane domain. This model is of course simplified and offers space for additional regulation (i.e. stop transfer sequences) and exceptions.

This would have two major implications:

1. After ribosome targeting to the Sec61p complex, further events are almost exclusively dependent on the presence and nature of hydrophobic segments and their interaction with the channel, especially their preferred orientation for channel insertion.

2. The channel itself would have the property of a seal, preventing ion flow across the membrane independent of the presence of the ribosome.

This would require that the accessibility of the pore to hydrated ions in the presence of a translocating chain would not be larger than 6 Å in diameter (reviewed in Johnson and vanWaes, 1999). How could the channel provide this flexibility? One possibility is that the overall arrangement of transmembrane helices and subunits is flexible and can be adjusted accordingly. At the same time, as observed for SRP (reviewed in Keenan et al., 2001), the spatial arrangement of amino acid side chains in the center of the Sec61p complex forming the central pore could behave like bristles to provide an appropriate environment for nascent chain conductance and could possibly accommodate the large number of different nascent chains that are translocated across the Sec61p complex during cotranslational protein transport.

Using immuno-purified RNCs for a molecular analysis of the RNC-PCC complex we find conserved rRNA segments as well as ribosomal proteins involved in forming the ribosome-channel junction. We observe the translocating channel in a compact conformation and a gap between channel and ribosome, which leads to the proposal of a simple binary model for cotranslational translocation. The model needs to postulate only two principally different functional states of the RNC-PCC complex in order to explain the translocation of secretory proteins or insertion of almost all kinds of membrane proteins: translation preceded by a loop insertion resulting in translocation across the membrane (e.g. secretory proteins), or translation preceded by a channel insertion without loop insertion resulting in accumulation in the cytosol (e.g. cytosolic domains of membrane proteins or signal anchor proteins). For polytopic membrane proteins these states would

be alternating. Thus, the sealing properties of the translocating channel and a gap between channel and ribosome, as we and others observe it, would significantly reduce the regulatory and spatial requirements for the RNC-channel complex processes such as release of cytosolic domains and partitioning of transmembrane domains into the bilayer. Furthermore, seal-like behavior of the Sec61 complex may also maintain the permeability barrier of the membrane in posttranslational or retrograde protein translocation.

4.3 The GTPase Cycle of the SRP Receptor β-Subunit

Based on biochemical and structural data, a recent study had suggested that SRβ requires a GAP and GEF to complete its GTP switch cycle (Schwartz and Blobel, 2003). Without a GEF, the nucleotide exchange in most GTPases occurs very slowly under physiological conditions (reviewed in Jackson and Casanova, 2000) In agreement with this, we could not detect any intrinsic nucleotide exchange activity of purified SRβ, which indicates that SRβ relies on an extrinsic GEF.

Searching the sequence database, we have identified a sequence similarity between the β-subunits of the two homologous trimeric PCCs in yeast and the conserved Sec7 domain that is present in all ARF-GEFs. The residues in contact with ARF1 in the ARF1-Sec7 complex form a binding groove made up of two regions forming a functional module: the α-helix H and the loop connecting the preceding helices F and G of the all-α-helical Sec7 domain (Goldberg, 1998). This module is not a folded domain but is rather held in shape by the remainder of the Sec7 domain, which forms the surrounding architectural scaffold. Therefore it appears that the SRβ binding groove of Sbh1p/Sbh2p is presented in a different structural context. Sbh1p and Sbh2p are very small proteins and not related to the Sec7 domain except for the functional module. The cytosolic domains of Sbh1p and Sbh2p consist of only about 60 residues and thus represent the smallest functional GEFs to date. We note that not all residues that are important for

Sec7 function are conserved in Sbh1p/Sbh2p. The conserved glutamic acid in the F-loop (Glu923) is missing in Sbh1p/Sbh2p. Therefore the guanine nucleotide exchange of SRβ will also differ from ARF-GEFs in some detail.

Using a fluorescence nucleotide exchange assay, we show that recombinant proteins comprising the cytosolic domains of Sbh1p and Sbh2p indeed function as the GEFs for SRβ. Despite the small size of their cytosolic domains, Sbh1p and Sbh2p are able to efficiently promote the exchange of nucleotide for SRβ. We find that Sbh1p is also functional as the GEF for SRβ when assembled into the detergent-solubilized trimeric Sec61p complex isolated from yeast. A direct interaction between the trimeric Sec61p complex and SRβ is supported by the fact that a protein interaction screen has found the trimeric Sec61p complex and its homologue, the Ssh1p complex, to be in the proximity of SRβ (Wittke et al., 2000). The calculated reaction rates at which the trimeric Sec61p complex, Sbh1p, and Sbh2p promoted nucleotide exchange are very similar, which indicates that both β-subunits promote the nucleotide exchange equally well, independent of their assembly state with the other subunits of the trimeric complexes.

However, when Sbh1p was assembled into the detergent-solubilized heptameric complex isolated from yeast, it did not express its GEF activity. This suggests that the cytosolic domain of Sbh1p in this complex might not be accessible to SRβ. The β-subunit of the SR is a eukaryotic feature; the SRα homologue in bacteria directly associates with the membrane (reviewed in Keenan et al., 2001). Interestingly, there is no bacterial homologue for Sbh1p. It is tempting to speculate that in higher organisms, the SRβ subunit evolved in concert with its GEF, not merely to provide a membrane anchor for SRα but also to provide an additional regulatory step to the translocation process; the interaction between these two proteins in the eukaryotic ER membrane, one a component of the targeting machinery (SR), the other a component of the trimeric PCCs, suggests that these proteins might link the two processes in a controllable fashion.

The association of the SR subunits is controlled by the nucleotide bound state of SRβ (Schwartz and Blobel, 2003). With SRβ in its GDP bound form, the SR subunits

dissociate. The β-subunits of the trimeric PCCs, acting as the GEFs for SRβ, reload SRβ with GTP and therefore control the reassociation of the SR subunits. It is conceivable that the β-subunits of the occupied PCC are inaccessible to SRβ. This would prevent the nucleotide exchange and the hetero-dimerization with SRα. As a result the targeting machinery could not be linked to the occupied PCC. Sbh1p can be crosslinked to Spc25p, a subunit of the signal peptidase complex (Kalies et al., 1998). Inaccessibility of the β-subunits of the PCCs may therefore not only be caused by the RNC complex binding to the PCCs, but also by the lateral recruitment within the plane of the ER membrane of other integral ER membrane proteins, such as the SPC, the oligo-saccharyl-transferase, or the additional subunits recruited to form the heptameric complex which is involved in posttranslational transport.

It had been reported that the ribosome decreases the affinity of SRβ for nucleotide (Bacher et al., 1999) Another study has found that SRβ can be cross linked to a 21 kDa ribosomal protein dependent on its nucleotide state. From these results it had been concluded that the ribosome might stabilize an empty state of SRβ and therefore could function as its GEF (Fulga et al., 2001). However, using either purified non-translating or translating ribosomes in our assay had no effect on the nucleotide exchange of SRβ.

Our data shows a specific function for the β-subunits of the two homologous PCCs and allows for a refined model of the cotranslational targeting process that had previously been suggested (Schwartz and Blobel, 2003) (Figure 4.2).

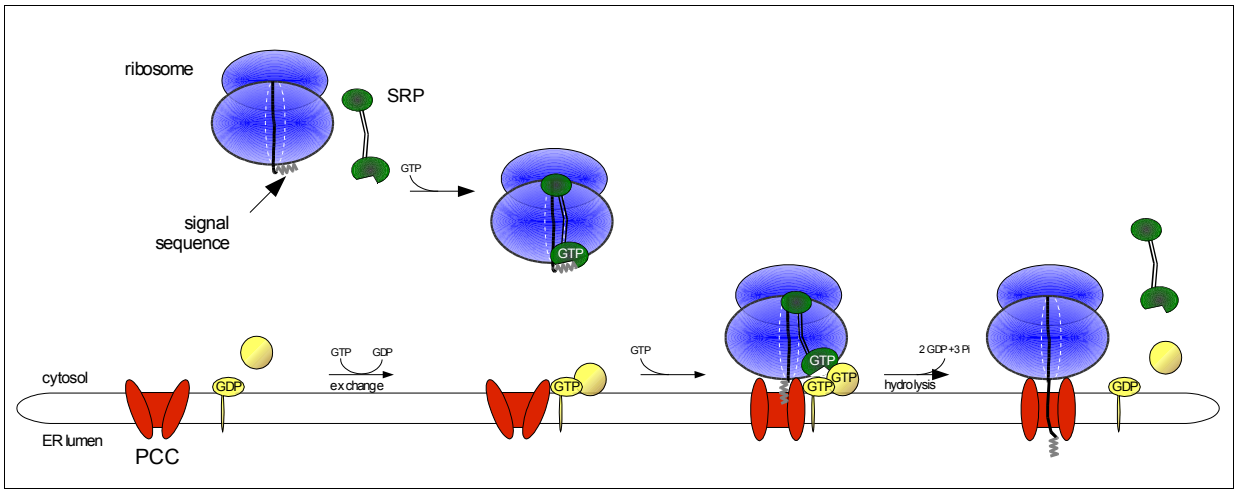

Figure 4.2: Model for SRP-SR mediated cotranslational protein targeting to the ER membrane. SRP 54 binds to the signal sequence emerging from the translating ribosome, resulting in an RNC-SRP complex and GTP-binding to SRP54. The trimeric Sec61p complex functions as the GEF for SRβ, loading it with GTP, thereby reassociating the two SR subunits. SRP54 and SRα form a GTP stabilized complex, targeting the RNC-SRP complex to the ER membrane. With all three GTPases in their GTP bound state, the signal sequence is transferred to the Sec61p complex. GTP hydrolysis of SRβ dissociates the SR subunits, and the mutual GTP hydrolysis of SRP54 and SRα resolves the SRP-SRα interaction.

In a first step, the SRP recognizes the signal sequence and binds to the RNC complex. The interaction between the ribosome and the SRP results in the binding of GTP by SRP54 (Bacher et al., 1996). Sbh1p assembled into the trimeric Sec61p complex functions as the GEF for SRβ, loading it with GTP. This nucleotide exchange reaction triggers the assembly of the SR subunits in the proximity of the trimeric Sec61p complex. Only with the SR in its assembled state can the RNC-SRP complex be targeted to the ER membrane where SRP54 and SRα form a GTP stabilized complex. Next, the trimeric Sec61p complex replaces the SRP at the exit site of the ribosome, the signal sequence is transferred to the trimeric Sec61p complex, and translocation starts. GTP hydrolysis of SRβ would lead to the dissociation of the SR subunits (Schwartz and Blobel, 2003). In a last step, SRP54 and SRα act as mutual GAPs, thereby resolving the SRP-SRα interaction.

The precise timing and coordination of the events during the signal sequence transfer still remain poorly understood. Signal sequence transfer to the trimeric Sec61p complex requires GTP binding by SRP54, SRα, and SRβ but not necessarily GTP hydrolysis (Song et al., 2000). The events triggering the GTP hydrolysis of the three GTPases and the release of the signal sequence remain to be elucidated. Further biochemical characterization will be necessary to completely understand the GTPase cycle of SRβ.