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Transfer of the Ribosome-Nascent Chain Complex to the Translocon in Cotranslational Translocation: A Thesis

Ying Jiang
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TRANSFER OF THE RIBOSOME-NASCENT CHAIN COMPLEX TO
THE TRANSLOCON IN COTRANSLATIONAL TRANSLOCATION

A Thesis Presented

By

Ying Jiang

Submitted to the faculty of the University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR PHARMACOLOGY

August 2007
TRANSFER OF THE RIBOSOME-NASCENT CHAIN COMPLEX TO THE TRANSLOCON IN COTRANSLATIONAL TRANSLOCATION

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Ying Jiang

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Dedicated to my loving and wonderful parents,

Haolin Jiang and Jinmei Zhang.
ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my mentor, Dr. Reid Gilmore. His insightful advice and constant encouragement guided me through my graduate research. I was always inspired by his intelligence and dedication to the work.

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ABSTRACT

Cotranslational translocation is initiated by targeting of a ribosome-bound nascent polypeptide chain (RNC) to the endoplasmic reticulum (ER) membrane. The targeting reaction is coordinated by the signal recognition particle (SRP) through its interaction with the RNC and the membrane-bound SRP receptor (SR). A vacant translocon is a prerequisite for the subsequent nascent chain release from SRP-SR-RNC complex. It has been proposed that the protease-accessible cytosolic domains of the Sec61p complex play an important role in posttargeting steps by providing the binding site for the ribosome or interacting with the SR to initiate the signal sequence releasing. In this study, we have investigated the detailed mechanism that allows transfer of the ribosome-nascent chain (RNC) from the SRP-SR complex to the translocon using yeast S. cerevisiae as the model system.

Point mutations in cytoplasmic loops six (L6) and eight (L8) of yeast Sec61p cause reductions in growth rates and defects in translocation of nascent polypeptides that utilize the cotranslational translocation pathway. Sec61 heterotrimers isolated from the L8 sec61 mutants have a greatly reduced affinity for 80S ribosomes. Cytoplasmic accumulation of protein precursors demonstrates that the initial contact between the large ribosomal subunit and the Sec61 complex is important for efficient insertion of a nascent polypeptide into the translocation pore. In contrast, point mutations in L6 of Sec61p inhibit cotranslational translocation without significantly reducing the ribosome binding activity, indicating that the L6 and L8 sec61 mutants
impact different steps in the cotranslational translocation pathway.

An interaction between the signal recognition particle receptor (SR) and the Sec61 complex has been proposed to facilitate transfer of the ribosome-nascent chain (RNC) complex to an unoccupied translocon. The slow growth and cotranslational translocation defects caused by deletion of the transmembrane span of yeast SR\(\beta\) (srp102p\(\Delta\)TMD) are exaggerated upon disruption of the SSH1 gene, which encodes the pore subunit of a cotranslational translocation channel. Disruption of the SBH2 gene, which encodes the \(\beta\)-subunit of the Ssh1p complex, likewise causes a synthetic growth defect when combined with srp102p\(\Delta\)TMD. The in vivo kinetics of translocon gating by RNCs were slow and inefficient in the ssh1\(\Delta\) srp102p\(\Delta\)TMD mutant. A critical role for translocon \(\beta\)-subunits in SR recognition is supported by the observation that deletion of both translocon \(\beta\)-subunits causes a block in the cotranslational targeting pathway that resembles elimination of either subunit of the SR, and could be partially suppressed by expression of carboxy-terminal Sbh2p fragments.
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>RER</td>
<td>rough ER</td>
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<tr>
<td>RM</td>
<td>rough microsome</td>
</tr>
<tr>
<td>PK-RM</td>
<td>puromycin-high salt-treated RM</td>
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<tr>
<td>RNC</td>
<td>ribosome-nascent chain complex</td>
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<tr>
<td>SRP</td>
<td>signal recognition particle</td>
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<tr>
<td>SR</td>
<td>SRP receptor</td>
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<td>TM</td>
<td>transmembrane</td>
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<td>ubiquitin translocation assay</td>
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<td>Ub</td>
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<td>Endo H</td>
<td>endoglycosidase H</td>
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<td>5-FOA</td>
<td>5-fluoorotic acid</td>
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<td>Tm</td>
<td>tunicamycin</td>
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<tr>
<td>CH</td>
<td>cycloheximide</td>
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<tr>
<td>DPAPB</td>
<td>dipeptidylaminopeptidase B</td>
</tr>
<tr>
<td>CPY</td>
<td>carboxypeptidase Y</td>
</tr>
<tr>
<td>PGK1</td>
<td>3-phosphoglycerate kinase</td>
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CHAPTER I

Introduction

Almost all proteins in the cell are synthesized by ribosomes in the cytosol (a few proteins are synthesized in mitochondria or chloroplasts). For those secreted proteins or membrane proteins that do not stay in the cytosol, they have to be transported to their final destinations to carry out their functions. Translocation across or integration into the endoplasmic reticulum (ER) membrane in eukaryotes or the plasma membrane in prokaryotes is the first event in the intracellular protein trafficking for many proteins. Only the proteins bearing a signal sequence are chosen for the ER transport.

ER protein translocation can occur either prior to or after protein translation termination. These two distinct processes are defined as two translocation pathways, cotranslational and posttranslational translocation. Although efficient protein translocation occurs cotranslationally in higher eukaryotes, the posttranslational one is essential in yeast or bacteria. The core component of the translocation apparatus is the Sec61p complex in mammals and yeast or SecYEG complex in bacteria, which forms the protein-conducting channel in both pathways. For different pathways, additional components are required for efficient translocation of nascent polypeptide.
The signal sequence

The existence of “signal sequence” for protein transport is well established in both eukaryotes and prokaryotes (Gierasch, 1989; Walter and Johnson, 1994b). Proteins carrying a signal sequence are selected by cytosolic proteins (e.g. signal recognition particle (SRP), chaperones) and delivered to the eukaryotic endoplasmic reticulum (ER) or prokaryotic plasma membrane for subsequent translocation. In many cases, the signal sequence is removed by signal peptidases from the mature polypeptide.

Whereas cleavable signal sequence are located at the amino-terminus of the protein, uncleaved signals for secreted proteins or transmembrane segments of membrane proteins can also be located in the middle of a protein or at its carboxyl-terminus (Kutay et al., 1995; Walter and Johnson, 1994b). A signal sequence can be divided into three distinct regions: an amino-terminus positively charged region, a central hydrophobic region and a carboxyl-terminus polar region (von Heijne, 1990). Comparison of all known signal sequences reveals that the central hydrophobic core is the characteristic feature of signal sequences, despite this, they show no sequence conservation.

The signal sequence not only directs the passenger protein to the membrane, but also determines the entry of a passenger protein by using different pathways. Studies in yeast revealed that replacement of the amino-terminus portion of DPAP B (a cotranslational substrate) with the signal sequence of CPY (a posttranslational substrate) leads the fusion protein to the posttranslational pathway and vice versa (Ng
et al., 1996). Furthermore, the fate of the substrate is found to be determined by the hydrophobicity of its signal sequence, the more hydrophobic signal sequence more likely commits the substrate to the cotranslational pathway (Ng et al., 1996).

Since the amino acid composition of signal sequences are highly variable, how is the fidelity of signal sequence-mediated protein transport carried out? The hydrophobicity of signal sequences is not the only answer. Mutated signal sequences with increased hydrophobicity can fail to direct passenger protein to the cotranslational pathway (Matoba and Ogrydziak, 1998). Thus, other features, like structure, may play a role in its function.

The Channel

Proteins transport across the ER membrane through an aqueous environment was first suggested by electrophysiological studies. Release of the nascent chain by puromycin treatment from the dog pancreatic rough microsome (RM) was found to increase the membrane conductance. Subsequent detachment of ribosome from the RM by increasing salt concentration leads to the channel closing (Simon and Blobel, 1991). These findings suggest that ER membrane has an aqueous channel that will be closed after translation termination. The existence of an aqueous channel in the prokaryotic plasma membrane was proved by using the same approaches. Moreover, the signal sequence is indicated to be sufficient for opening the channel (Simon and Blobel, 1992). Taken together, it was proposed that a protein-conducting channel for polypeptide transport exists in the eukaryotic ER membrane or prokaryotic plasma
membrane and that this channel is opened by the signal sequence and closed after translation is completed. An additional evidence to support the existence of an aqueous protein-conducting channel in the ER membrane was provided by fluorescence quenching experiments (Crowley et al., 1994; Crowley et al., 1993). Environmentally-sensitive fluorescent probes were incorporated into the signal sequence of preprolactin during translation. The fluorescence lifetimes of the probes indicated the signal sequence was in an aqueous environment during the translocation reaction.

It is now known that this channel, also called the translocon, is mainly formed by an evolutionarily conserved heterotrimeric complex, called the Sec61p complex in eukaryotes and SecYEG complex in eubacteria and the Sec YEβ complex in archaea.

**The Sec61p/SecYEG/SecYEβ complex**

Sec61p/SecYEG/SecYEβ complex consists of three subunits, the α subunit (Sec61α in mammals, Sec61p in yeast, SecY in eubacteria and archae); the β subunit (Sec61β in mammals, Sbh1p in yeast, SecG in eubacteria, and Secβ in archae); and the γ subunit (Sec61γ in mammals, Sss1p in yeast; SecE in bacteria and archae).

The α subunit, the largest one, is encoded by an essential gene. Sec61p in yeast was first discovered by genetic screens for ER translocation defects (Deshiaies and Schekman, 1987). Temperature sensitive sec61 mutants accumulate precursor forms of all the substrates tested (Deshiaies and Schekman, 1987; Stirling et al., 1992). Therefore, Sec61p was indicated to play a crucial role in the translocation of secretory
and membrane proteins into the endoplasmic reticulum. Mammalian Sec61α was identified by its significant sequence similarity with Sec61p in yeast (about 56% sequence identity) and SecY in bacteria (Görlich et al., 1992). Furthermore, all three proteins share the identical membrane topology. Each of them was predicted to span membrane ten times with both N-terminus, C-terminus and even-numbered loops (L2, L4, L6, L8) facing the cytosol (Görlich et al., 1992; Wilkinson et al., 1996a).

The γ subunit, the smallest subunit in the Sec61p complex, is also encoded by an essential gene. It was first identified in S. cerevisiae in a genetic screen for suppressors of a translocation-deficient mutant of Sec61 (sec61-2), and termed Sss1 (Sec Six-one Supresser 1) (Esnault et al., 1993). Like Sec61p, Sss1p and its homologues are localized on the ER membrane and appear to be conserved during the evolution. Both mammalian Sec61γ and yeast Sss1p contain one C-terminus transmembrane domain and have one N-terminus cytoplasmic domain. Moreover, mammalian Sec61γ can functionally replace Sss1p in S. cerevisiae (Hartmann et al., 1994), which strongly suggested the functional conservation among the γ subunits. The γ subunit in most bacteria is a single-span membrane protein. In E.coli, however, it contains three transmembrane domains. Only the third transmembrane domain is required for its function and also the conserved region is limited to this transmembrane domain (Schatz et al., 1991).

Unlike the other two subunits, the β subunit is not required for cell viability in yeast (Sbh1p) and bacteria (SecG) (Finke et al., 1996; Nishiyama et al., 1994). However, disruption of Sec61β is lethal in drosophila (Valcarcel et al., 1999).
Whereas mammalian Sec61β is structurally related to Sbh1p, SecG shows no obvious sequence and structure similarity to the β subunit of the eukaryotic translocon. Both Sec61β and Sbh1p are predicted to be C-terminus anchored proteins with one transmembrane span. Sequence homology between Sec61β and Sbh1p is mostly concentrated in the transmembrane (TM) domain and the sequence directly preceding the TM span (Görlich and Rapoport, 1993; Panzner et al., 1995). SecG was suggested to span membrane twice in most bacteria. In vitro reconstitution experiments have shown that the β subunit, although not essential, strongly stimulates the translocation efficiency of both eukaryotic and prokaryotic translocons (Kalies et al., 1998; Nishiyama et al., 1993).

The initial evidence suggesting that the Sec61 complex forms the channel is from cross-linking experiments. Photoreactive probes incorporated into nascent chains are exclusively crosslinked to Sec61α after emerging from the ribosome, which suggests that Sec61α is the key component to form the channel (Mothes et al., 1994). Furthermore, when purified mammalian and yeast Sec61p complexes were observed by electron microscopy, the complexes form ring-like oligomer structures with a diameter of about 85 Å and a central pore of 20 Å. Similar oligomeric structures are also present in native membranes and reconstituted proteoliposomes (Hanein et al., 1996). Based on the shape and size of the oligomer, 3-4 Sec61 hetrotrimers were suggested to assemble together, and the channel was proposed to be formed by the interface between the Sec61p hetrotrimers. The diameter of the central pore (20 Å) is smaller than the estimated size of a functional translocon (40 Å-60 Å) in fluorescent
quenching experiments (Hamman et al., 1997). At the time, it was thought that the channels visualized by electron microscopy were in the closed conformation.

The X-ray structure of SecYEβ from the archaea *M. jannaschii* has been resolved recently at 3.2Å resolution (Van den Berg et al., 2004). Consistent with the predicted secondary structure, twelve transmembrane segments are observed in the purified and crystallized SecYEβ complex. SecE and Secβ each contains one TM domain with the N-terminal domain in the cytosol. SecY has ten TM domains with both N and C-termini in the cytosol. The ten TM segments of SecY form two pseudo-symmetric halves, TM1-5 and TM6-10, clamped by SecE, the γ subunit (Fig 1.1A). This SecYEβ structure has a diameter of about 40Å. The interior of SecY forms an hourglass-like shape with a diameter of 20-25Å at the cytosolic side (Fig 1.1B). Surrounded by the other two subunits, SecY is suggested to open laterally towards the lipid on one side. Based on this structure, a single copy of SecYEβ is proposed to function as the channel. A segment of the second TM span (TM2a) was proposed to serve as a plug for the channel (Fig 1.1A, B), to maintain the ion permeability barrier of the membrane in the closed state and to be displaced by the nascent polypeptide during translocation. This “plug” hypothesis has been challenged by some recent biochemical studies. The introduction of destabilizing point mutations or the complete deletion of the “plug” domain showed little or no effect on growth rate and translocation efficiency in yeast (Junne et al., 2006). The TM2a segment may play a role in stabilizing the Sec61p structure instead of sealing the channel (Junne et al., 2006). Regardless of whether TM2a serves as a “plug” or
not, now, more and more evidences support the idea that although more than one Sec61p complex assembles an oligomer to mediate protein translocation, actually only one hetrotrimer forms the channel (Osborne and Rapoport, 2007).

Figure 1.1. Architecture of the translocation channel. (A) Top view from the cytosol. Transmembrane segments 1–5 (TM1–TM5) and 6–10 (TM6–TM10) of the α-subunit are shown in blue and red, respectively. The β- and γ-subunits are shown in gray. Movement of the plug (yellow) towards the γ-subunit is indicated by the double-headed blue arrow. The loop between TM5 and TM6 could serve as a hinge to open the α-subunit at the front. (B) Cross-sectional view of the closed channel from the side, with the hydrophobic pore-ring residues shown in green. This figure and legend are taken from (Rapoport et al., 2004).
Whether the channel itself can maintain the ion permeability barrier is still under debate. Fluorescence quenching experiments have shown that the ribosome is tightly bound to the cytosolic side of the channel and seals the channel during the cotranslational translocation (Crowley et al., 1994). For those channels not occupied by ribosomes, an ER lumenal protein BiP (Kar2p in yeast) was found to be sufficient to prevent quencher movement across the membrane in the presence of ATP or ADP, indicating that the channel may be sealed by BiP (Kar2p) from the lumenal side prior to translocation initiation or after translocation termination (Hamman et al., 1998).

Overview of Cotranslational translocation

The cotranslational translocation process is well studied in the mammalian system, and the mechanism is evolutionally conserved in different organisms. This process is initiated by targeting of a ribosome-bound nascent polypeptide chain (RNC) to the ER membrane. Nascent polypeptides that are translocated by the cotranslational pathway have a more hydrophobic N-terminus signal sequence, which is recognized by the signal recognition particle (SRP). Once the signal sequence of nascent polypeptide emerges from the ribosome, SRP directly binds to the signal sequence and the ribosome to “arrest” or retard protein elongation (Halic et al., 2004) (Fig 1.2a). In the next step, the ribosome-nascent chain-SRP complex (RNC-SRP) is recruited to the ER membrane through the interaction between SRP and the SRP receptor (SR), which is located on the ER membrane (Fig 1.2b). This docking reaction is followed by transferring of the RNC from SRP-SR to the translocon and
the release of SRP from the SR in a GTP-dependent manner (Connolly et al., 1991; Rapiejko and Gilmore, 1997) (Fig 1.2c). Free SRP is allowed to begin a new targeting cycle.

After the signal sequence is inserted into the channel, translation of the nascent polypeptide is resumed. The ribosome seals off the channel from the cytosolic side to prevent cytosolic exposure of the nascent polypeptide. Therefore the elongating polypeptide can only be extended into the ER lumen (Fig 1.2d).

Other than the Sec61 channel, SRP and SRP receptor are the essential components for the cotranslational protein translocation reaction. In vitro ER translocation activity can be reproduced with reconstituted proteoliposomes containing only two ER membrane complexes: Sec61p complex and SRP receptor (SR) (Görlich and Rapoport, 1993). For some substrates, the translocating chain-associating membrane (TRAM) protein is also required.
Figure 1.2. A simplified scheme for co-translational translocation pathway.

(a) SRP binds to the signal sequence and ribosome and retards the polypeptide elongation.

(b) The SRP-ribosome-nascent chain complex (SRP-RNC) is targeted to the ER membrane via interaction with the SRP receptor (SR).

(c) Transfer RNC from SR to the channel in a GTP-dependent manner.

(d) Translation resumes and the nascent chain is translocated into the ER. The thick portion in the polypeptide chain indicates the hydrophobic core of the signal sequence.
The signal recognition particle (SRP)

The signal recognition particle (SRP) is a cytosolic ribonucleoprotein complex found in all organisms. An 11S ribonucleoprotein was first purified from a salt extract of dog pancreas microsomal membranes and shown to be required for efficient protein translocation across ER membrane (Walter and Blobel, 1980). SRP coordinates targeting of signal sequence-bearing proteins to the prokaryotic plasma membrane or the eukaryotic ER membrane. The SRP-dependent translocation mechanism is evolutionarily conserved in all living cells.

SRP facilitates cotranslational protein translocation by two main activities. First, after binding the hydrophobic signal, it interacts with the ribosome to arrest or retard polypeptide elongation (Siegel and Walter, 1985; Siegel and Walter, 1986). Second, SRP helps docking of the ribosome-nascent chain complex (RNC) to the membrane through interaction with the SRP receptor (SR).

In mammals, SRP consists of six protein subunits (SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72) and one 300-nucleotide 7S RNA molecule (SRP RNA) (Walter and Blobel, 1980; Walter and Blobel, 1982). Archaebacterial SRP contains two proteins subunits, which are homologous to SRP54 and SRP19 respectively, and one 7S RNA molecule. Archaebacterial SRP RNA is similar in size and secondary structure to its eukaryotic counterpart (Zwieb and Eichler, 2002). Bacterial SRP contains a 110- nucleotide 4.5S RNA molecule and one Ffh (SRP fifty four homologue) protein (Herskovits et al., 2000).

SRP can be divided into two distinct domains, defined as the S domain and Alu
domain (Gundelfinger et al., 1983). The Alu domain consists of 5’ and 3’ terminal segments of the SRP RNA molecule (Alu fragment) and two small subunits, the SRP9/SRP14 heterodimer, which binds to the 5’ end of the SRP RNA. The SRP Alu domain is responsible for the peptide elongation arrest activity (Halic et al., 2004). All the ribosome contact sites formed by the Alu domain overlap with those formed by elongation factor 2 (eEF2) (Gomez-Lorenzo et al., 2000; Halic et al., 2004). This structural finding suggests that the Alu domain arrests peptide elongation by competing with eEF2 for the elongation factor binding site in the ribosome.

The S domain contains the middle half (nucleotide 100-250) of the SRP RNA molecule (S fragment) and four protein subunits (SRP19, SRP54 and SRP68/SRP72 heterodimer). It functions in signal sequence recognition and the SRP-SR interaction. SRP19 is required for SRP assembly (Walter and Blobel, 1983). Formation of the initial SRP19-RNA intermediate promotes SRP54 binding (Rose and Weeks, 2001).

SRP54 is the main functional subunit in S domain. It is universally conserved (Koch et al., 2003) and responsible for binding the signal sequence and the SRP receptor (Connolly and Gilmore, 1989; Siegel and Walter, 1988). In vitro, mammalian SRP can be functionally replaced by bacterial SRP, which consists of Ffh and the 4.5S RNA (Powers and Walter, 1997), suggesting an essential role for SRP54 in SRP function. SRP54 comprises an N-terminal domain (N domain), a GTPase domain (G domain) and a C-terminal methionine-rich domain (M domain). The N domain, a four-helix bundle, associates with two ribosome proteins (rpL25, rpL35) near the peptide exit site (Pool et al., 2002). These binding sites for SRP overlap
with those for the Sec61p channel on the ribosome (Beckmann et al., 2001), suggesting that the exposure of these binding sites is required for RNC docking onto the channel. Recent studies suggested that the SRP-SR association meditates the rearrangement of SRP relative to the ribosome and exposes the ribosomal channel binding sites (Halic et al., 2006). The G domains of SRP54 and the \( \alpha \) subunit of SRP receptor (FtsY in bacteria) define the SRP subfamily of GTPases. Unlike most GTPases, GTPase of SRP54 has a relatively low affinity for GDP or GTP and a rapid GTPase hydrolysis activity (eukaryote SRP54 has very low GTPase activity, Ffh is more than 10-fold higher). The N domain and the G domains of the SRP GTPases are tightly coupled, hence are referred as the NG domain. The NG domains of SRP54 and SR\( \alpha \) mediate the SRP-SR interaction. The M domain mainly functions in the signal sequence recognition. It can be crosslinked to the signal peptide (Lütcke et al., 1992; Zopf et al., 1990) and its structure shows that it forms a deep hydrophobic groove which may provide the binding site for the hydrophobic signal sequence (Batey et al., 2000).

The SRP68/SRP72 heterodimer is described as a brace connecting the core of the S domain and the hinge (hinge 1) between the S domain and the Alu domain (Halic et al., 2004). Based on the SRP-RNC structure, SRP68/SRP72 heterodimer is suggested to facilitate the SRP9/SRP14 meditated elongation arrest after signal sequence recognition by the S domain (Fig 1.3). In this model, the conformation change of SRP68/SRP72 is proposed to stabilize SRP in a bent shape, resulting in the proximity between SRP9/SRP14 and the elongation-factor binding site in the
inter-subunit space of the ribosome. In agreement with this idea, SRP that lacks SRP68/SRP72 loses the elongation arrest activity (Siegel and Walter, 1985).

Figure 1.3. Signal sequence-dependent SRP-ribosome interaction. On signal sequence binding by SRP54, a kinked conformation of SRP involving possibly SRP68/72 and a rotation around hinge 1 is stabilized. As a result, SRP interacts with the ribosome, stretching from the peptide exit (S domain) to the elongation-factor-binding site in the intersubunit space (Alu domain), where it causes elongation arrest by competition with elongation factors. The 40S small ribosomal subunit is shown in yellow, 60S large ribosomal subunit in blue, the signal sequence (signal) shown in green and SRP in red (pink for SRP68/72 hetrodimer). Exit, peptide tunnel exit; EFS, elongation-factor-binding site. This figure and legend are taken from (Halic et al., 2004).
The SRP receptor (SR)

The SRP receptor (SR) in mammals contains two subunits, SRα and SRβ. Both SR subunits are essential for cotranslational translocation. Historically, SRα was identified as an ER membrane component required for protein translocation by a proteolysis experiment. Mild protease digestion (trypsin, elastase) and high salt treatment together was able to remove an active factor from the ER membrane and block in vitro protein translocation (Meyer and Dobberstein, 1980b; Walter et al., 1979; Warren and Dobberstein, 1978). This inhibitory process is reversible, as translocation can be restored by reducing the salt concentration to a physiological level (Meyer and Dobberstein, 1980b). Later, a 52 kD protein fragment released by elastase /high salt treatment from RM was isolated and was shown to be derived from a 72 kD protein termed SRα (Meyer and Dobberstein, 1980a; Meyer et al., 1982)(Gilmore et al 1982a, b).

SRα is not an integral ER membrane protein, but is instead anchored to the membrane by interacting with the transmembrane protein SRβ (Young et al., 1995). The first 140 residues of SRα is sufficient for membrane binding and this binding is SRP-independent (Young et al., 1995). Other than the N-terminus membrane binding domain, SRα or its E.coli homologue FtsY contains a NG domain like all the other SRP subfamily of GTPases (Montoya et al., 1997). Similar to SRP54, the SRα GTPase has a relatively low GTP binding affinity. While isolated, SRα is in an “empty site” conformation.

SRβ was first claimed to be a proteolytic fragment of SRα (Hortsch et al., 1985).
A 30 kD protein co-purifies with SRα in about equimolar amounts. These two proteins are tightly associated with each other and can be chemically cross-linked. Subsequently, this 30 kD protein was reported as an additional subunit of SRP receptor, SRβ (Tajima et al., 1986). SRβ is an ER integral membrane protein that contains a standard transmembrane domain and a cytosolic GTPase domain. Unlike SRα, the GTPase domain of SRβ is structurally distinct from the SRP subgroup of GTPases. Although SRβ has strong binding affinity for GTP (Bacher et al., 1999), the function of this GTPase is still unclear. A functional requirement for GTP hydrolysis by SRβ during the protein translocation reaction is under debate. The intrinsic nucleotide exchange activity of purified SRβ is undetectable (Legate and Andrews, 2003). Mutations in the GTPase domain of SRβ which inactivate the SR are mostly accompanied by dissociation of SRα and SRβ (Ogg et al., 1998). Therefore, the GTPase domain of SRβ is likely to be involved in SRα-SRβ dimer formation.

In contrast to the GTPase domain, the transmembrane domain (TM) of SRβ is not critical to cotranslational translocation. Although deletion of the TM domain of SRβ in yeast shows some growth defects, no translocation defect was detected. ΔTMD SRβ can still recruit SRα to the ER membrane for proper function (Ogg et al., 1998). This may suggest that SRβ associates with additional components of the ER membrane, most likely translocon.

The 1.7 crystal structure of GTP-bound SRβ without the TM span in complex with the SRX domain (first 158 residues of SRα) in S. cerevisiae has been resolved
(Schwartz and Blobel, 2003) (Fig 1.4). Based on sequence similarity and the crystal structure, SRβ is closely related to the ADP-ribosylation factor (Arf) subfamily of GTPases which are involved in the regulation of vesicle transport (Miller et al., 1995) (Jackson et al., 2000; Schwartz and Blobel, 2003). SRβ consists of a mixed six-stranded β sheet and five surrounding helices. A GTP molecule is present in the GTP-binding pocket of SRβ, and this bound GTP is buried in the SRβ-SRX interface so that it appears to be non-exchangeable and inaccessible to a potential GAP for SRβ. Although the sequence identity is low, the structure of SRβ-GTP:SRX heterodimer is conserved between yeast and mammals (Schlenker et al., 2006; Schwartz and Blobel, 2003). Once formed, this SRα/SRβ heterodimer is stable. SRα can only be released from SRβ by carbonate extraction at pH 12.5 (Miller et al., 1995). The in vivo dissociation of SRα/SRβ dimer has not been observed. Nucleotide-free SRβ has only been crystallized as a homodimer, with two GTPase domains intertwined with each other (Schwartz et al., 2006). This malfolded SRβ homodimer is not able to interact with SRα. The existence of an apo form of SRβ in vivo is still obscure.
Figure 1.4. Structure of the SRβ-GTP:SRX Complex  (A) Structure of the SRβ-GTP:SRX complex from yeast, with the β subunit in cyan and the SRX domain of the α subunit in magenta. The GTP nucleotide is drawn in ball-and-stick representation. Residues 64–72 of SRβ (colored yellow) form the main interaction site with SRα, and this region encompasses switch 1. Secondary structure elements as well as C and N termini are labeled. Unstructured loop regions are colored gray.  (B) Same as (A), but rotated around the horizontal axis counterclockwise by 90°. The figure and legend are taken from (Schwartz and Blobel, 2003).
An SR\(\beta\) homologue is not found in eubacteria or archaebacteria. The SR\(\alpha\) homologue in *E. coli*, FtsY, does not contain a TM domain. Interestingly, about 30% of FtsY cofractionates with the bacterial inner membrane, and membrane localization of FtsY is required for its proper function (Valent et al., 1998; Zelazny et al., 1997). Substitution of the 198-residue-long N-terminal region of FtsY with an unrelated transmembrane polypeptide does not impair its function (Zelazny et al., 1997). FtsY is localized to the membrane through protein-protein or protein-lipid contact. It has been proposed that SecY (Sec61p homologue in bacteria) provides the binding site for FtsY on the membrane (Angelini et al., 2006; Angelini et al., 2005).

**GTP cycle**

The three GTPases involved in the cotranslational protein translocation reaction are SRP54 (Ffh), SR\(\alpha\) (FtsY) and SR\(\beta\). The GTPase activities of SRP54 and SR\(\alpha\) are well known to contribute to the targeting process. During this process, SRP54 and SR\(\alpha\) cooperate to transfer the RNC to the channel in a GTP-dependent manner (Fig 1.5). Whereas the GTP cycle of SRP54 and SR\(\alpha\) is distinct from that of other GTPases, it is highly conserved in different organisms.

Unlike other GTPases, the GTPase domains of SRP54 and SR\(\alpha\) have a relatively low affinity for guanine nucleotide. They are in the empty site conformations prior to targeting initiation (Rapiejko and Gilmore, 1997) (Fig 1.5c, h). After the RNC-SRP complex is directed to the ER membrane through contact with SR\(\alpha\), two GTP molecules are loaded into SRP54 and SR\(\alpha\) (Fig 1.5d, e). The resulting
conformational change stabilizes the SRP54-SRα complex and destabilizes the SRP54-signal sequence interaction. Thus, cooperative binding of GTP to SRP54 and SRα initiates the signal sequence transfer from SRP to the Sec61p channel (Connolly and Gilmore, 1989; High et al., 1991) (Fig 1.5f). Moreover, a functional Sec61p channel is also critical for release of the signal sequence from SRP54. Inactivation of Sec61p complex by proteolysis causes the accumulation of the membrane-bound RNC-SRP intermediates, which suggested that the Sec61 complex may regulate the GTPase cycle of SRP54-SRα at the stage before the signal sequence dissociation (Song et al., 2000).

After the signal sequence dissociates from SRP54, SRP54 still remains bound to SRα. The two GTPases are paired to form a composite active site at the interface between SRP54 (Ffh) and SRα (FtsY) and serve as reciprocal GTPase activating proteins (GAP) (Egea et al., 2004; Focia et al., 2004). The subsequent release of SRP54 from SRα is initiated by GTP hydrolysis (Fig 1.5g, g’). SRP54 and SRα return to their empty site forms for a new targeting cycle.

Although SRβ has been reported to be a GTPase and bind GTP during the translocation reaction (Bacher et al., 1999), it is not clear whether SRβ has a role in addition to serving as a membrane-binding site for SRα. The existence of a GTPase cycle of SRβ is still obscure.
Figure 1.5. The GTPase cycle of SRP54 and SRα. The empty site form of SRP54 (h) binds to the signal sequence of the RNC (a), forming a complex. (b) that is then recognized by the empty site form of SRα (c). SRP54 and SRα in the SR–SRP–RNC complex (d) cooperatively bind GTP (e). GTP binding to the SRP–SR complex promotes signal sequence transfer from SRP54 to Sec61α (f). GTP hydrolysis by SRP54 and SRα permits dissociation of SRP from SR (g). By virtue of their low affinity for GDP, SRP54 and SRα return to their empty site conformations (c and h). For simplicity, neither the TRAM protein nor SRP subunits other than SRP54 are shown. The figure and legend are taken from (Rapiejko and Gilmore, 1997).
The Ssh1p complex

A homologue of Sec61p, Ssh1p (Sec sixty one homolog 1), was identified in S. cerevisiae in the genome-sequencing project (Feldmann et al., 1994). Ssh1p shares about 30% sequence identity with both mammalian Sec61α and yeast Sec61p.

Like its homologues, Ssh1p is localized to the ER membrane (Finke et al., 1996). It forms a heterotrimeric complex with Sbh2p and Sss1p. Sbh2p was identified as a homologue of Sbh1p in yeast. Although the β subunits of Sec61 complex show limited similarity between different species (Panzner et al., 1995), Sbh2p shares about 50% sequence identity with Sbh1p. Furthermore, Sbh2p can associate with Sec61p in the absence of their original partners (in sbh1Δ ssh1Δ strain) (Finke et al., 1996). Like Sbh1p, Sbh2p is not essential for yeast cell growth and ER protein translocation (Finke et al., 1996; Panzner et al., 1995). Sss1p is present in both the Sec61p and Ssh1p complexes (Finke et al., 1996).

The Ssh1p complex is similar, but not functionally equivalent to the Sec61p complex. In contrast to Sec61p, Ssh1p is not essential for cell viability and Ssh1p expression cannot rescue the lethal phenotype of a sec61 null strain. Disruption of SSH1 only causes mild growth and protein translocation defects (Finke et al., 1996; Wittke et al., 2002). The Ssh1p complex does not associate with the Sec62/Sec63 complex (Wittke et al., 2002). Since the Sec62/Sec63 complex is critical for the post-translational translocation pathway, the Ssh1p complex is proposed not to function in the post-translational pathway. In agreement with this idea, the Ssh1p complex was reported to interact with the signal sequences of substrate proteins of the
co-translational translocation pathway (Kar2p and invertase (Suc2p)) but not substrates of the using posttranslational pathway (Mfα1 and carboxypeptidase Y (CPY)) (Wittke et al., 2002).

The Ssh1p complex also interacts with the membrane-bound ribosome (Finke et al., 1996). After reconstitution into proteoliposomes, the purified Ssh1p complex binds ribosomes with an affinity similar to the Sec61p complex (Prinz et al., 2000b). Ribosome-binding activity is consistent with the high sequence similarity between the cytosolic loops of Sec61p and Ssh1p (~45%). Ribosome-depleted membranes purified from an SSH1 knock-out strain do not show obvious reduction ribosome binding sites relative to membranes purified from a wild type strain. Thus, Ssh1p was proposed to be a minor ribosome receptor in the yeast ER membrane.

Unlike the Sec61p complex that is involved in both the cotranslational and posttranslational translocation pathways, the Ssh1 complex is considered to be a dedicated translocon for the cotranslational translocation pathway. The reason why yeast cells have a second co-translational translocation system is still unclear. One of the possibilities is that the existence of Ssh1p complex may allow yeast cells to regulate co or post pathways separately and to balance the number of translocons in different pathways. In agreement with this idea, depletion of free Sec61p complex by overexpressing Sec62/Sec63 complex causes a severe growth defect in an ssh1 null strain but not in a wild type strain (Wittke et al., 2002). Another possibility would be that the Ssh1p complex has other roles in the cotranslational pathway which are not fully satisfied by the Sec61p complex. Although SSH1 is not required for yeast cell
viability, the slight growth defect caused by disruption of Ssh1p cannot be completely complemented by overexpression of Sec61p. Thus, the existence of a subset of Ssh1p-dependent substrates was suggested (Wittke et al., 2002).

Other than *S. cerevisiae*, *S. pombe* also contains two Sec61-related genes (Broughton et al., 1997). The sequence identity between these two genes is about 30%. A gene which is almost identical to Sec61α (~95% identity) was also reported in mammalian cells (Görlich et al., 1992). The relationship between two Sec61α homologues in different cells is not clear.

**Overview of Posttranslational translocation in yeast**

The mechanism of posttranslational transport across the ER membrane is different from cotranslational transport because the ribosome does not play a role (Rapoport et al., 1999). An additional membrane partner, the Sec62/Sec63 complex, is required to associate with the Sec61p channel to form a seven-component SEC complex. In vitro reconstituted proteoliposome experiments have shown that the lumenal ATPase Kar2p and ATP are also required for efficient protein translocation through the SEC complex (Panzner et al., 1995).

The fully synthesized polypeptide chain is released from ribosome and presented to the ER membrane in a complex with cytosolic heat shock proteins (Hsp70s; which are the Ssa proteins (Ssa1, Ssa2, Ssa3 and Ssa4) in the yeast *S. cerevisiae*) (Fig 1.6a). Ssa proteins maintain polypeptide chains in an unfolded conformation and prevent precursor aggregation (Chirico et al., 1988). Like other Hsp70s, the Ssa proteins
cooperate with their cofactors, the J protein Ydj1p, for proper function (Becker et al., 1996; McClellan and Brodsky, 2000). Ydj1p interacts with the Ssa proteins and stimulates the ATP activity, which allows the precursor polypeptide to be released from the chaperone in an ATP hydrolysis-dependent manner before translocation can occur (Zimmermann, 1998) (Fig 1.6b). In the next step the polypeptide chain is inserted into the SEC complex (Plath et al., 1998). The initial peptide insertion is mediated by the interaction between the signal sequence and the SEC complex, which is ATP-independent. The signal sequence primarily contacts TM2 and TM7 of Sec61p. Sec62p and Sec71p may also contribute to the signal sequence recognition (Plath et al., 1998) (Fig 1.6c). When a portion of the peptide chain enters the lumenal side of the membrane, multiple Kar2p molecules associate with the nascent polypeptide following an interaction with the J domain of the Sec63p (Fig 1.6d). Kar2p then acts as a ratchet to prevent passive backward movements of the peptide through the channel (Panzner et al., 1995). Subsequently, dissociation of Kar2p from the polypeptide results in a free polypeptide in the ER lumen (Fig 1.6e).
Figure 1.6. A simplified scheme for posttranslational translocation pathway in yeast.

(a) Cytosolic chaperones (Hsp70) bind to fully synthesized polypeptides and keep them from folding.  
(b) The chaperone-nascent chain complex is targeted to the big SEC complex.  
(c) After cytosolic chaperones are released from the polypeptide, the signal sequence is inserted into the channel and lumenal Kar2p binds the translocated peptide chain.  
(d) Kar2p molecules bind to the incoming polypeptide and provide the driving force by acting as a ratchet to prevent the polypeptide backsliding from the channel.  
(e) Kar2p (BiP) is released from the translocated polypeptide.  The thick portion in the polypeptide chain indicates the hydrophobic core of the signal sequence.
The Sec62/Sbc63 complex and Kar2p

The Sec62/Sec63 complex associates with Sec61p complex to form a heptameric SEC complex. Using prepro-α factor as translocation substrate, the intact SEC complex, but neither of the two subcomplexes shows significant posttranslational translocation activity (Panzner et al., 1995). The association between the Sec61p complex and the Sec62/Sec63 complex is critical for post-translational protein transport.

The tetrameric Sec62/Sec63 complex consists of four subunits, Sec62p, Sec63p, Sec71p and Sec72p, which were identified in genetic screen for translocation components in yeast (Deshaies and Schekman, 1989; Green et al., 1992). The essential Sec62p and Sec63p proteins contain two and three transmembrane domains respectively. In contrast, Sec71p and Sec72p are not required for cell viability. Sec71p, a single-span membrane protein, is the only glycoprotein in the Sec62p/Sec63p complex. Although disruption of Sec71p only causes a growth defect at an elevated temperature (37°C), precursor proteins accumulate at the permissive temperature (Feldheim et al., 1993). Sec72p is a peripheral membrane protein localized to the cytosolic side of ER membrane through an association with Sec71p (Fang and Green, 1994). Although a sec72 null strain lacks a growth defect, subset of secretory protein precursors accumulate in the cytosol (Feldheim and Schekman, 1994).

Sec62p is suggested to stabilize the SEC complex by providing multiple binding sites for Sec61p and Sec63p (Wittke et al., 2000). Furthermore, Sec62p is found to
contribute to signal sequence recognition together with Sec61p during post-translational translocation (Plath et al., 1998; Plath et al., 2004). The function of Sec63p is well investigated by studying its interaction with Kar2p. Kar2p is an ER lumenal Hsp70 molecular chaperone. Like other Hsp70s, Kar2p consists of two major domains, an N-terminal ATPase domain and a C-terminal peptide-binding domain (Gething, 1999). To bind peptides, the cooperation with its cofactor, the J protein, is required. Sec63p contains a lumenal J domain, which is a homologue of the DnaJ protein in bacteria. The recruitment of Kar2p to the translocation sites by the J domain of Sec63p facilitates posttranslational protein translocation by providing the driving force in an ATP-dependent manner (Corsi and Schekman, 1997).

Initially, the J domain of Sec63p interacts with ATP-bound Kar2p and stimulates its ATPase activity. Activated Kar2p binds a variety of substrates through its peptide-binding pocket (Misselwitz et al., 1998). Upon ATP hydrolysis, the peptide-binding pocket of Kar2p is closed, which enhances Kar2p binding to the translocating polypeptide. It was proposed that Kar2p acts as a ratchet to prevent translocating substrate backwards sliding through the channel (Matlack et al., 1999). Once the translocating peptide moves into the lumen, another BiP molecule can bind to it by the same mechanism until the translocation completes. Eventually, Kar2p dissociates from the substrate following nucleotide exchange to release the polypeptide in the ER lumen (Misselwitz et al., 1998).

Whereas Sec62p is only required for post-translational translocation, Sec63p and Kar2p were proposed to function in both pathways (Brodsky et al., 1995; Nicchitta
and Blobel, 1993). Depletion of Sec63p or introduction of mutations in Kar2p causes severe translocation defects in both DPAPB and CPY transport in vivo (Young et al., 2001). Consistent with this theory, homologues of Sec63p and Kar2p (BiP in mammals) were discovered in mammals. Mammalian Sec63 also contains an ER lumenal J domain (Skowronek et al., 1999). In mammals, cotranslational translocation is the predominant pathway. A subset of low molecular weight proteins (usually fewer than 70 residues) can be transported posttranslationally (Zimmerman and Walter, 1990). The existence of an abundant SEC complex specifically involved in a mammalian posttranslational pathway appears unlikely. Although the protein abundance of mammalian Sec63 is similar to that of Sec61α, only a small populations of both proteins were found to associate with each other (Meyer et al., 2000). Homologues of Sec62p, but not Sec71p and Sec72p, were also discovered in mammals, but the role of mammalian Sec62p is unclear.

**Overview of post-translational translocation in eubacteria**

In bacteria, while most inner membrane proteins are cotranslationally targeted to SecYEG by the Ffh-4.5S RNA complex (SRP homolog), periplasmic proteins, outer membrane proteins and secretory proteins are targeted by a posttranslational pathway. The posttranslational targeting pathway is mediated by the cytoplasmic chaperone SecB, and the peripheral ATPase SecA. ATP and the membrane potential are also required (for a review see (de Keyzer et al., 2003)). ATP hydrolysis by SecA and membrane potential together provide the driving force for protein translocation from
the cytoplasmic side of the membrane (Schiebel et al., 1991).

The SecB homotetramer mainly recognizes the mature parts of the newly synthesized polypeptides and maintains them in an unfolded, translocation-competent conformation (Randall and Hardy, 1995; Xu et al., 2000). The SecB-polypeptide complex is directed to the plasma membrane through the interaction between SecB and membrane-bound SecA protein, which interacts with the SecYEG channel and phospholipids (Hartl et al., 1990). Upon peptide binding, a conformational change in the polypeptide binding site of SecB occurs, which results in the transfer of the precursor from SecB to SecA. SecA selectively transports secretory proteins by binding to both the signal sequence and their mature domains (Lill et al., 1990). The exchange of ADP for ATP in the ATPase domain of SecA is stimulates by polypeptide binding (Lill et al., 1989). The resulting conformational change leads to dissociation of SecA from SecB and translocation of approximately 20 amino acids of the polypeptide into the channel (Economou and Wickner, 1994). Upon ATP hydrolysis, the polypeptide is released from SecA. Subsequently, SecA is able to start a new cycle and this process is repeated until translocation of the protein is completed. Thus, it was proposed that SecA pushes the polypeptide through the SecY translocon in a stepwise manner.

Recent studies suggested that SecA exists in equilibrium between monomeric and dimeric states (Or et al., 2002). The monomer is considered to be the active form during translocation (Duong, 2003). Dissociation of the dimer is suggested to be catalyzed by the association with the substrate and the SecY complex.
Homologues of SecA in eukaryotes have only been found in chloroplasts (CPSecA) (Yuan et al., 1994). The mechanism of CPSecA-mediated protein transport in chloroplast is similar to that in bacteria, which also requires ATP and the membrane electrochemical potential.

**Enclosed work**

In this study, we have investigated the detailed mechanism that allows transfer of the ribosome-nascent chain complex (RNC) from the SRP-SR complex to the translocon using the yeast *S. cerevisiae* as the model system. There are at least two steps in this translocon-dependent transfer process which are corresponding to the recognition of an unoccupied translocon and docking of the RNC onto the channel.

Proteolysis of canine Sec61α Loop6 and Loop8 eliminates the ribosome binding affinity. Nonetheless, the ribosome-binding site on the translocation channel had not mapped with precision. Because L6 and L8 have a net positive charge it was not clear whether specific residues, as opposed to the overall charge distribution, were important for the ribosome binding affinity of the Sec61 complex. Point mutations were introduced into cytoplasmic loops six (L6) and eight (L8) of yeast Sec61p. We found that these mutations cause reductions in growth rate and selectively interfere with the cotranslational translocation pathway. Sec61 heterotrimers isolated from the L8 sec61 mutants have a greatly reduced affinity for 80S ribosomes. In contrast, point mutations in L6 of Sec61p inhibit cotranslational translocation without significantly reducing the ribosome binding activity. These findings indicate that the
L8 sec61 mutants impact the ribosome docking step but L6 mutants may interfere with other steps in this process.

Another intriguing question in cotranslational translocation is how SR helps the RNC find an unoccupied channel. Without a transmembrane domain, bacterial SR (FtsY) partially associates with the plasma membrane via a protein-translocon interaction. The evidence to support the SR-translocon interaction in eukaryotes is still missing. Which cytosolic segments on the translocons serve as the marker of vacant channel is unclear. In this study, a sensitized yeast strain expressing soluble SR was constructed by deletion of the transmembrane domain of SRβ to study how the SRP receptor helps the RNC to find an unoccupied translocon. Growth rate and cotranslational translocation defects caused by SRβΔTM are accentuated by the ssh1Δ mutant and suppressed by increased expression of the soluble SR or the Ssh1p translocon, suggesting an SR-Ssh1 complex interaction. Furthermore, synthetic growth defects were caused by mutations in the Ssh1p complex (ssh1EE or sbh2Δ) in the SRβΔTM strain but not by mutations in the Sec61p complex (sec61EE or sbh1Δ), indicating that soluble SR prefers the Ssh1p complex to the Sec61 complex.

Ubiquitin translocation assays (UTA) were utilized to study the in vivo kinetics of the RNC transfer process. Analysis of Dap2 reporter cleavage in the ssh1Δ srp102ΔTM mutant or the sbh1Δ sbh2Δ mutant revealed an extremely slow and inefficient delivery of Dap2-RNCs to the translocation channel. Taken together, we proposed that the soluble SR delivers an RNC to the Ssh1p translocon more efficiently than to Sec61p translocon and that the cytoplasmic domains of translocon β subunits may
play an important role in serving as a marker for an unoccupied channel.

Isolation and characterization of the sec61 L6 mutants were done by Dr. Zhiliang Cheng. Ribosome binding assays were performed by Dr. Elisabet C. Mandon.
CHAPTER II

Identification of cytoplasmic residues of Sec61p involved in ribosome binding and cotranslational translocation

Introduction

Translocation of proteins across the rough endoplasmic reticulum can occur by cotranslational or posttranslational pathways. The signal sequence of a protein that is translocated by the cotranslational pathway is recognized by the signal recognition particle (SRP) as the nascent chain emerges from the polypeptide exit site on the large ribosomal subunit (Halic et al., 2004; Walter and Johnson, 1994a). Targeting to the RER is mediated by the interaction between the SRP-ribosome nascent chain (SRP-RNC) complex and the SRP receptor (SR) (Mandon et al., 2003), which initiates a GTPase cycle that culminates in attachment of the RNC to the protein translocation channel (Song et al., 2000). In S. cerevisiae, proteins that are translocated by the posttranslational pathway are not targeted to the Sec61 translocation channel by SRP, but are instead delivered to the Sec complex by cytosolic Hsp70 proteins (as reviewed in (Corsi and Schekman, 1996). Translocons that mediate cotranslational translocation are oligomers formed from 3-4 copies of a Sec61 heterotrimer (Beckmann et al., 2001; Morgan et al., 2002) that is in turn composed of Sec61p, Sbh1p and Sss1p (Panzner et al., 1995). The Sec complex is composed of a Sec61 translocon plus the Sec62/Sec63 complex (Deshaies et al., 1991;
Panzner et al., 1995). Yeast Ssh1p, a distantly related homologue of Sec61p, assembles with Sbh2p and Sss1p to form an auxiliary translocon that is specific for the cotranslational pathway (Finke et al., 1996; Wittke et al., 2002). Ssh1p translocons are not incorporated into the Sec complex (Finke et al., 1996), hence overexpression of Ssh1p cannot compensate for loss of Sec61p.

The relative contributions of the co- and posttranslational pathways to precursor transport across the RER have been extensively investigated in *S. cerevisiae*. Partitioning of nascent polypeptides between the targeting pathways is governed by the relative hydrophobicity of the signal sequence (Ng et al., 1996), with SRP selecting more hydrophobic signals for the cotranslational pathway. Although the cotranslational pathway is the predominant pathway in vertebrate organisms, SRP and the SR are dispensable in *S. cerevisiae* (Hann and Walter, 1991; Ogg et al., 1992). The predicted topology of yeast Sec61p in the ER (Wilkinson et al., 1996b) has now been refined by the structural determination of the archaebacterial translocation channel SecYEβ (Van den Berg et al., 2004). The N and C-terminus of Sec61p and the even numbered loops (L2, L4, L6 and L8) that separate the 10 membrane spans face the cytoplasm. Proteolytic mapping experiments of canine Sec61α indicated that L6 and L8 are highly exposed on the cytoplasmic surface of the Sec61 complex (Song et al., 2000). Proteolysis of canine Sec61α in L6 and L8 inhibits SRP-dependent translocation activity (Song et al., 2000) and eliminates ribosome binding to the translocon (Raden et al., 2000). Nonetheless, the detailed mechanism that allows transfer of the RNC from the GTP-bound conformation of the SRP-SR
complex to the translocon is not well understood. The ribosome-binding site on the translocation channel had not mapped with precision. Because L6 and L8 have a net positive charge it was not clear whether specific residues, as opposed to the overall charge distribution, were important for the ribosome binding affinity of the Sec61 complex. Here, we have identified residues in L6 and L8 of Sec61p that are critical for the cotranslational translocation pathway, and defined segments of Sec61p that interact with the ribosome and possibly interact with the SRP receptor.

**Results**

**Mutagenesis of cytosolic loops of Sec61p**

A sequence comparison of L6 of Sec61 from diverse eukaryotes reveals a high degree of amino acid identity particularly in the segments that are proximal to transmembrane spans 6 and 7 (Fig. 2.1A). A seven-residue loop, which connects two β-strands in the *M. jannashii* SecY structure (Van den Berg et al., 2004), contains several highly conserved polar residues (K273, R275 and Q277). These three residues, together with G276 and K284 were selected for site directed mutagenesis in *S. cerevisiae* Sec61p. The haploid BWY12 was chosen as a starting strain to analyze yeast sec61 mutants using a plasmid shuffle procedure. In BWY12, a HIS3-marked disruption of the essential SEC61 gene is rescued by the URA3 marked CEN plasmid pBW7 that encodes Sec61p. We disrupted the non-essential SSH1 gene to provide a sensitized genetic background for the analysis of the Sec61p mutants. Although the initial description of an ssh1Δ strain noted a minor decrease in growth rate (Finke et
al., 1996), a more recent study reported that a yeast strain lacking Ssh1p rapidly acquires a petite phenotype when grown on a fermentable carbon source, and displays severe defects in protein translocation and dislocation when maintained on a non-fermentable carbon source (Wilkinson et al., 2001). As shown below, the growth phenotype of our ssh1Δ strain (RGY401) was consistent with the initial report (Finke et al., 1996), hence this strain was suitable for the analysis of L6 and L8 sec61 mutants. For example, when RGY401 cells are grown on glucose containing media (YPD or SD), petite cells (ρ−) arise at a low frequency (~0.3%/cell division).

RGY401 (ssh1Δ) and RGY402 (SSH1) were transformed with LEU2 marked plasmids encoding sec61 point mutants, and plated on media containing 5-FOA to select against retention of pBW7 (Fig. 2.1B). Positive and negative controls for the screen are based upon the observations that Ssh1p is nonessential (SEC61ssh1Δ is viable), and that expression of Ssh1p cannot suppress a sec61 null (sec61R275*SSH1 is not viable). Amino acid substitutions at R275 cause a growth rate defect in the absence, but not in the presence, of Ssh1p. Differences in growth rate were evaluated by plating serial dilutions of cells onto YPD (Fig. 2.1C) or YPEG plates. With the exception of lysine (sec61R275Kssh1Δ), amino acid substitutions at R275 cause obvious reductions in growth rate at 30°C that are accentuated at 37°C, and not
Figure 2.1. Point mutations in L6 of Sec61p.  (A) Secondary structure of L6 (*M. jannaschii* SecY) and sequence alignment between eukaryotic and *M. jannaschii* L6 segments. Identities are boxed and asterisks indicate residues subjected to mutagenesis. (B) Yeast strains RGY401 (*ssh1Δ*) and RGY402 (*SSH1*) that had been transformed with plasmids expressing wild type or mutant (R275*, R275S, R275L or R275G) alleles of Sec61p were streaked on 5-FOA plates and allowed to grow for 2 d at 30°C. Sec61R275* has a termination codon at position 275. (C, D) Growth rates of L6 *sec61* mutants were compared by serial dilution analysis (C) as described in the Materials and Methods and used to assign the L6 *sec61ssh1Δ* mutants to a growth phenotype category (D).
apparent at 18°C (not shown). Reductions in the growth rates of the mutants relative to RGY401 or RGY402 were slightly less obvious on YPEG plates (not shown). The effect of L6 point mutations are summarized in Fig. 2.1D. Substitutions that reverse the charge (R275D or R275E) or substitute an aliphatic or aromatic amino acid for arginine cause a severe growth defect. Less severe growth defects were caused by substitutions of polar (R275S, R275T) or positively charged amino acids (R275H). A wider variety of substitutions were tolerated at K273 and G276. The triple charge-reversal mutant (sec61K273D,R275D,K284Dssh1Δ) designated sec61L6DDD has a more severe growth defect than sec61R275Dssh1Δ (not shown).

Several conserved residues between R389 and E407 were selected for mutagenesis based upon a sequence comparison of the L8 region of eukaryotic Sec61 (Fig. 2.2A). The structure of M. jannashii SecY indicates that four of these residues (G404, K405, R406 and E407) are located in the tip of the L8 loop between two α-helices that project into the cytoplasm from the membrane surface. Point mutations in L8 did not cause a growth rate defect in strains that express Ssh1p (Fig. 2.2B). Serial dilution experiments (e.g. Fig. 2.2C) demonstrated that mutations at K405, R406 and to a lesser extent K396 cause growth rate defects (Fig. 2.2D). Substitutions at the other tested residues had little or no effect including a two-residue deletion (R389ΔD390Δ). A double mutant (L6L8EE) that combined two severe L6 and L8 mutations (R275E and R406E) was suppressed by expression of Ssh1p (Fig. 2.2B).
Figure 2.2. Point mutations in L8 of Sec61p.  (A) Secondary structure of L8 (M. jannaschii SecY) and sequence alignment between eukaryotic and M. jannaschii L8 segments.  Identities are boxed and asterisks indicate residues subjected to mutagenesis.  (B) Yeast strains RGY401 (ssh1Δ) and RGY402 (SSH1) that had been transformed with plasmids expressing wild type SEC61, or mutant alleles (R406*, R406E, L6L8EE (R275E, R406E) or K396D) of Sec61p were streaked onto 5-FOA plates and allowed to grow for 2 d at 30°C.  (C, D) Serial dilution experiments were performed as described in Fig. 2.1C and used to assign the L8 sec61ssh1Δ mutants to a growth phenotype category (D).
Decreased growth rates correlate with protein translocation defects

RGY401 derivatives expressing L6 or L8 sec6l mutants lose respiratory competence at a 3-10 fold higher frequency (~1-3% per generation) than the parental ssh1Δ strain. RGY401 and its derivatives were maintained on SEG media to select against the accumulation of ρ− cells. When growth rates were determined after shifting cells into YPD media, the ssh1Δ mutant shows a 10-20% decrease in growth rate relative to the wild type strain (Fig. 2.3A), which is consistent with the initial description of an ssh1Δ mutant (Finke et al., 1996). The sec61R275E ssh1Δ strain and the sec61R406E ssh1Δ strain showed a 2.5-fold decrease in growth rate at 30°C relative to the ssh1Δ strain (Fig. 2.3A).

The sec6l L6 and L8 mutants were tested for defects in translocation of the SRP-dependent substrate dipeptidylaminopeptidase B (DPAPB) and the SRP-independent substrate carboxypeptidase Y (CPY). To facilitate detection of DPAPB, selected RGY401 derivatives were transformed with a low copy plasmid that encodes DPAPB-HA (Ng et al., 1996). Wild type and mutant cultures were pulse labeled with 35S amino acids 4 h after cells were shifted into SD media (Fig. 2.3B). Integration of the type II membrane protein DPAPB into the RER is accompanied by the addition of 7 to 8 N-linked oligosaccharides. Unglycosylated DPAPB-HA synthesized by tunicamycin treated cells (wt +TM) serves as a mobility marker for the non-translocated precursor (p-DPAPB-HA). The pulse labeling experiments revealed a reduction in DPAPB translocation in the ssh1Δ mutant (Fig. 2.3B) that was greatest 4 h after transfer into SD media (Fig. 2.3C). Importantly, the percentage of
non-translocated DPAPB (15-20% at 4 h) was 4-fold lower than previously reported for an ssh1Δ strain (Wilkinson et al., 2001). A more significant defect in DPAPB translocation was detected in L8 sec61ssh1Δ strains (Fig. 2.3B). Expression of Ssh1p suppresses the translocation defect caused by point mutations in L8, consistent with the lack of a growth defect. While DPAPB integration in wild type cells was efficient at all time points after shift to SD media (Fig. 2.3C, filled squares), transport defects for the L6 (triangles) and L8 (open squares) sec61ssh1Δ mutants reached a peak 4 h after cells were transferred into the SD media and declined thereafter.

Non-glycosylated CPY obtained by labeling cells in the presence of tunicamycin was used as a mobility marker for prepro-CPY (Fig. 2.3D). As expected, there was little or no production of the Golgi (p2) or mature vacuolar forms of CPY during the 7 min pulse-labeling period. Translocation of CPY was similar in the wild type and the ssh1Δ strain, consistent with the observation that the Ssh1p heterotrimer is not incorporated into the Sec complex. Although point mutations in L8 do not cause a translocation defect when expressed in an SSH1 strain, there was a substantial reduction in CPY translocation when the sec61 mutants were tested in the ssh1Δ strain. Endo H digestion experiments confirmed that the protein designated as ppCPY was the precursor, and not comigrating mature CPY (data not shown). Conceivably, a defect in N-linked glycosylation could cause the accumulation of non-glycosylated p1CPY. To address this possibility, spheroplasts prepared from the
Figure 2.3. Translocation defects in sec61 mutants are suppressed by expression of Ssh1p.  (A) Wild type yeast (RGY402, solid squares) and ssh1Δ mutants expressing wild type Sec61p (solid circles), sec61R275E (open squares) or sec61R406E (triangles) were grown to mid log phase at 30°C in SEG media.  The cultures were diluted into YPD media at 0 h and allowed to grow for 8-12 h at 30°C.  (B, D) Wild type and mutant yeast cultures were pulse-labeled for 7 min at 30°C after 4 h of growth in SD media at 30°C.  One sample of wild type cells was treated with tunicamycin (wt + TM) for 30 min prior to pulse-labeling.  DPAPB-HA (B) and CPY (D) immunoprecipitates were resolved by PAGE in SDS.  The ER (p1), Golgi (p2) and precursor (ppCPY) forms of CPY and the glycosylated (D) and nonglycosylated (p-D) forms of DPAPB-HA are labeled.  Translocation of CPY or integration of DPAPB-HA was quantified with a BioRad FX Molecular Imager.  (C) Wild type yeast (RGY402, filled squares) and ssh1Δ mutants expressing wild type Sec61p (filled circles), sec61R275E (triangles) or sec61R406E (open squares) were pulse-labeled to evaluate integration of DPAPB-HA as described above after 1, 2, 4, 8 or 24 h of growth in SD media.  As needed, cell cultures were diluted with fresh SD media to maintain an A600 of less than 0.8.  (E) Pulse-labeled sec61L6DDD spheroplasts were osmotically lysed and centrifuged at 0.5Kg to remove unbroken cells.  Spheroplast lysates were incubated on ice with trypsin (100 µg/ml) as indicated.  The lane designated 15-TX contained trypsin plus Triton X-100.  Trypsin was inactivated with PMSF prior to immunoprecipitation.
sec61L6DDD mutant were pulse labeled for 7 min prior to osmotic lysis. As shown in Fig. 2.3E, the majority of p1CPY was trypsin-resistant in the absence of detergent, unlike ppCPY which was accessible to the protease. As observed for DPAPB integration (Fig. 2.3C), the maximal defect in CPY translocation was observed 4 h after transfer of cells into SD media (not shown). Suppression of a CPY transport defect in the SSH1 strain is unlikely to occur by transport of CPY through an Ssh1p translocon (Wittke et al., 2002), suggesting that reduced translocation of CPY in the L8 sec61 mutants arises by an indirect mechanism.

A larger collection of the L6 and L8 sec61 mutants were assayed for defects in translocation of DPAPB-HA, CPY and Gas1p (Fig. 2.4). Between 30 and 50% of the DPAPB was not integrated in each of the sec61ssh1Δ mutants that were tested. Deficiencies in CPY translocation showed significantly greater variation, with some substitutions (e.g. R275F and R275V) causing only minor defects relative to the parental ssh1Δ strain. A second SRP-independent substrate (Gas1p) was analyzed to determine if the L6 and L8 sec61 mutants have defects in translocation of other substrates that utilize the posttranslational translocation pathway. The percentage of Gas1p that was not translocated during the 7 min pulse was much lower than observed for CPY. Taken together with the genetic evidence presented in the preceding figures, these data suggest that mutations in L6 and L8 preferentially interfere with the SRP-dependent translocation pathway.
Figure 2.4. Differential effect of Sec61p mutations upon SRP-dependent and SRP-independent translocation pathways. Integration of DPAPB-HA and translocation of CPY and Gas1p was evaluated by pulse labeling of wild type and mutant yeast strains that were grown for 4 h in SD media at 30°C. Pulse labeling and immunoprecipitation of proteins was conducted as in Fig. 2.3.
Impact of sec61 mutations on protein dislocation and precursor accumulation

A mutation that reduces folding of Sec61p should inhibit all protein transport pathways that are mediated by the translocon due to a reduction in the cellular content of the Sec61 heterotrimer. Identical amounts of total protein extracts of yeast cells were resolved by PAGE in SDS for a subsequent protein immunoblot using antibodies specific for Sec61p (Fig. 2.5A). Similar amounts of Sec61p were expressed in the wild type and L6 and L8 sec61 mutants. Migration differences between lanes are explained by increases in the number of acidic residues in the mutant proteins. Thus, the translocation defects are not explained by a reduction in the cellular content of Sec61p.

Dislocation of unfolded proteins from the ER lumen back into the cytosol for degradation by the proteasome is thought to occur through the Sec61 complex (Wiertz et al., 1996). Degradation of the well characterized degradation substrate CPY*HA was monitored using a cycloheximide-chase procedure (Spear and Ng, 2003), as the apparent rate of dislocation determined using this method should not be perturbed by the kinetic delay in CPY*HA translocation (Fig. 2.5B). The p1 form of CPY*HA was degraded rapidly with a calculated half life of less than 30 min in all strains, suggesting that mutations in L6 and L8 of Sec61p do not interfere with the dislocation pathway. Mutations in gene products that are required for CPY*HA dislocation typically increase the half time of degradation to roughly 1 h (Spear and Ng, 2003). Cytoplasmic precursors (pre-Kar2p, prepro-CPY and prepro-α factor) that are
Fig 2.5. Transport pathways affected by Sec61 mutations. (A) Equal amounts of total protein (25 µg) were resolved by PAGE in SDS for protein immunoblot analysis using a C-terminal specific antibody to Sec61p. (B) Degradation of CPY<sup>∗</sup><sub>HA</sub> in L6 and L8 sec61 mutants. Cell extracts prepared at 30 min intervals after cycloheximide addition were resolved by PAGE in SDS. Non-translocated ppCPY<sup>∗</sup><sub>HA</sub> and translocated p1CPY<sup>∗</sup><sub>HA</sub> were detected using anti-HA antibodies. Protease digestion experiments confirmed that p1CPY<sup>∗</sup><sub>HA</sub> but not ppCPY<sup>∗</sup><sub>HA</sub>, was in a membrane-enclosed compartment (not shown). The apparent half-life (t<sub>1/2</sub>) of p1CPY<sup>∗</sup><sub>HA</sub> determined according to a first order decay process is plotted below representative time courses.
translocated through the Sec complex are readily detected by protein immunoblot analysis when sec62 or lhs1 mutants are analyzed at a semi-permissive temperature (Baxter et al., 1996; Hamilton and Flynn, 1996).

Protein immunoblot analysis of total cell extracts prepared from the L6 and L8 sec61 mutants revealed a single immunoreactive species for CPY (Fig. 2.6A). Mature CPY comigrates with prepro-CPY due to cleavage of the propeptide in the vacuole. Deglycosylation of mature CPY with Endo H resolved prepro-CPY from deglycosylated mature CPY. Prepro-CPY was only faintly visible in the Endo H digested lanes demonstrating that the majority of the CPY precursor detected in a 7 min pulse-labeling experiment is subsequently translocated into the ER.

Additional evidence supporting a minor kinetic delay in transport of SRP-independent precursors was obtained by pulse-chase analysis of Gas1p biosynthesis (Fig. 2.6B). Although the Gas1p precursor was detected after the 7 min pulse, the majority of the precursor was translocated into the ER during the subsequent 10-min chase (Fig. 2.6B). These results suggest that there is a reduction in transport rate for precursors that utilize the Sec complex.

Protein immunoblots showed that the non-translocated DPAPB-HA precursor accumulates in the sec61 mutants after 4 h of growth in SD media (Fig. 2.7A). Cellular accumulation of pDPAPB-HA was elevated 2 to 3-fold relative to the ssh1Δ mutant and reached a maximal value 6-8 h after the sec61 mutants were transferred into SD media (not shown). We next asked whether the non-translocated DPAPB
Fig 2.6. Kinetic delay in posttranslational translocation pathway. (A) Total cell extracts were prepared for PAGE in SDS with or without prior digestion by Endo H. Deglycosylated mature CPY (dgm) is resolved from vacuolar CPY (m) and non-translocated preproCPY (p). The asterisk designates an incomplete Endo H digestion product. (B) Yeast cultures were pulse-labeled for 7 min and chased for 10, 20 or 30 min. The non-translocated precursor (p-Gas1), the translocated ER-form (Gas1) and the mature form (m-Gas1) of Gas1p are labeled.
was soluble or membrane-associated. Differential centrifugation of spheroplast lysates achieved a partial resolution of the pDPAPB-HA from DPAPB-HA (Fig. 2.7B). As expected, DPAPB-HA was recovered in the P13 fraction that contains vacuoles. RER membranes, as detected using antibodies to the oligosaccharyltransferase subunit Ost1p, were enriched in the P0.5 and P13 fractions (not shown). The precursor (pDPAPB-HA) was not in the cytosol fraction (S100) but instead sedimented at low and intermediate speeds. Subsequent centrifugation of the P13 fraction on a sucrose step gradient demonstrated that the precursor was membrane associated since it did not sediment through a 1.6 M sucrose cushion (Fig. 2.7C). In contrast to mature DPAPB-HA, the precursor was insoluble in the non-ionic detergent digitonin (Fig. 2.7C), suggesting that it is incorporated into a membrane-associated aggregate. These results suggest that pDPAPB-HA molecules that are not translocated by the SRP-dependent pathway rapidly adopt a translocation incompetent conformation.

Defects in Ribosome Binding

Microsomal membranes that were isolated from the ssh1Δ strain as well as several L6 and L8 sec61ssh1Δ mutants were treated with puromycin and high salt to remove endogenous membrane bound ribosomes. The resulting ribosome-stripped microsomes (PK-RM) were assayed for ribosome-binding activity in a physiological ionic strength buffer (Fig. 2.8A and 2.8B). PK-RM prepared from the ssh1Δ strain bind ribosomes in a saturable manner (Fig. 2.8A, filled circles) with a binding affinity
Fig 2.7. DPAPB precursor accumulation in *sec61* mutants. (A) Protein immunoblot detection of p-DPAPB-HA and mature DPAPB-HA in total cell extracts resolved by PAGE in SDS. Protein immunoblots were quantified by densitometry. (B) Differential centrifugation of spheroplast lysates prepared from the *sec61L6DDDssh1A* mutant. Total lysates (T), supernatant (S) and pellet (P) fractions were obtained after centrifugation at 0.5 Kg, 13 Kg and 100 Kg. (C) The P13 fraction (T) was resuspended in buffer A (50 mM Hepes pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)$_2$, 1 mM DTT) adjusted to 250 mM sucrose and applied to a sucrose step gradient in buffer A with 1.6 M and 2 M sucrose layers. Following centrifugation for 1 h at 100 Kg, the gradient was resolved into the following fractions: (1) 0.25 sample load plus 0.25/1.6 M interface, (2) 1.6M sucrose layer plus 1.6/2 M interface, (3) 2 M sucrose layer, (4) pellet. The P13 fraction (T) was solubilized in 3% digitonin, 500 mM KOAc and centrifuged at 100 Kg for 1 h to obtain supernatant (S) and pellet (P) fractions.
(K_d=5.5±0.5 nM) that is in good agreement with previous reports (Prinz et al., 2000a; Prinz et al., 2000b). The negative reciprocal of the slope of a Scatchard plot is proportional to the K_d, so a decrease in slope corresponds to a decrease in binding affinity. Mutagenesis of R275 to aliphatic or acidic residues (Fig. 2.8A) caused a minor reduction in apparent ribosome binding affinity (R275L, K_d=13.1±0.3 nM; R275E, K_d=15.7±3.2 nM; R275V K_d=20.7±3.5 nM). The ribosome binding affinity of the triple mutant (L6DDD) was similar (K_d=17±2.6 nM), suggesting that basic residues in L6 are not the primary determinants for the ribosome-Sec61p interaction. Point mutations in L8 (Fig. 2.8B) that caused mild growth defects also reduced the ribosome binding affinity by 2-3 fold (K396D, K_d=18.2±1.7 nM; R406H, K_d=11.3±1.5 nM). Less conservative substitutions at R406 caused a more significant decrease in ribosome binding affinity (R406D, K_d=37.4±10.6 nM; R406W, K_d=54.2±9.8 nM; RRL6L8EE, K_d=38.2±7.7 nM). The reduction in ribosome binding affinity caused by several L8 sec61 mutations was accompanied by an apparent increase in ribosome binding sites, suggesting that the residual binding activity might be non-specific. To address this possibility, wild type and mutant Sec61 heterotrimeric were purified from yeast strains expressing an affinity tagged derivative (His_6-FLAG-Sbh1p) of the Sbh1p subunit of the Sec61 complex. The ribosome binding affinity of purified Sec61 translocons was determined after reconstitution into liposomes (Fig. 2.8C). Proteoliposomes prepared with wild type Sec61p and a loop 6 mutant had similar binding affinities for the 80S ribosome (wild type K_d=6.5±1.7 nM; R275E, K_d=2.3±0.4 nM). In contrast, proteoliposomes prepared using sec61
R406E or sec61 L6L8EE had a dramatically reduced capacity and affinity for the ribosome (Fig. 2.8C) even though the proteoliposomes contained comparable amounts of Sec61p (not shown). The Sec61p-ribosome interaction was also monitored in detergent solution using a cosedimentation assay (Prinz et al., 2000a). Wild type and mutant Sec61p heterotrimers, as detected using anti-FLAG sera (Fig. 2.8D) or antibody to Sec61p (not shown), were recovered in the supernatant fraction in the absence of added ribosomes. Purified wild type Sec61p heterotrimers and two different L6 mutants (R275E and R275L) quantitatively co-sedimented with ribosomes in this assay (Fig. 2.8D). Cosedimentation of the L8 mutant (R406E) and the L6L8 double mutant (RRL6L8EE) with the ribosome was undetectable using anti-FLAG sera (Fig. 2.8D) or antibody to Sec61p (not shown). The identity of the protein or proteins responsible for the residual ribosome binding activity of PK-RM isolated from the L8 mutants is not known.
Figure 2.8. Binding of ribosomes to yeast PK-RM and Sec61 proteoliposomes.

(A-C) Scatchard plots of ribosome binding to PK-RM (A, B) or Sec61p proteoliposomes (C) isolated from wild type (SEC61ssh1Δ) or L6 (A, C) and L8 (B, C) sec61ssh1Δ mutants. (D) Sec61 heterotrimers (150-300 fmol) purified from wild type and selected L6 and L8 mutants were incubated in the presence or absence of 900 fmol of yeast ribosomes prior to centrifugation to obtain supernatant (S) and pellet (P) fractions. Following PAGE in SDS, Sbh1p was detected using anti-FLAG antibodies.
Discussion

Isolation of a novel class of sec61 mutants

Alleles of sec61 that selectively interfere with the cotranslational translocation pathway have not been described previously, in part because expression of Ssh1p suppresses the growth and translocation defects. Two temperature sensitive sec61 alleles (sec61-2 and sec61-3) encode unstable proteins that are degraded at the restrictive temperature (Sommer and Jentsch, 1993), hence these mutants do not display selective defects in translocation or dislocation at the restrictive temperature (Plemper et al., 1997; Stirling et al., 1992). A screen for cold-sensitive sec61 mutants yielded several strains (sec61-8, sec61-10, sec61-110) that were primarily defective in transport of substrates that utilize the posttranslational translocation pathway (Pilon et al., 1998).

Cytosolic loops of Sec61p are critical for cotranslational translocation

Mutagenesis of yeast Sec61p can be interpreted in the context of the recently solved X-ray structure of M. jannashii SecYEG because the length, and to a lesser extent, the sequence of L6 and L8 are well conserved between the archae and eukaryotic translocation channels. The amphipathic H2 α-helix in SecE (γ-subunit, homologous to Sss1p) defines the interface between the membrane and the cytosol (Van den Berg et al., 2004). L6 and L8 of SecY project ~20Å into the cytosol from the membrane surface (Fig. 2.9A). Four (K273, R275, G276 and Q277) of the five residues in L6 that were selected for mutagenesis are located at the tip of the loop between two β-strands, while the fifth residue (K284) is located near the polar head
group region of the membrane bilayer (Fig. 2.9B). Examination of the corresponding residues (R239 and K241) in *M. jannashii* SecYEG reveals that the positively charged side chains of K273 and R275 are exposed and oriented towards the cytosol. In contrast, the side chain on A243 (Q277 in Sec61p) is oriented towards the membrane surface, which likely explains why point mutations at this site do not cause growth defects. Point mutations at G276 (G242 in *M. jannashii*) that cause growth defects in *S. cerevisiae* might do so by introducing a negative charge (G275E) or by reducing the flexibility of L6 (e.g. G276P).

Four of the eight residues selected for mutagenesis in L8 of Sec61 are located in the tip of a loop that connects two α-helical segments (Fig. 2.9B). The importance of K405 and R406 in Sec61p is readily explained by the orientation and location of the corresponding side chains (F359 and K360) in *M. jannashii* SecY (Fig. 2.9B). Interestingly, replacement of K405 with phenylalanine (as in *M. jannashii* SecY) did not cause growth or translocation defects (not shown) indicating that basic or bulky hydrophobic residues are tolerated at this site. The top view of SecYEG shows that the side chains of four residues in L8 (R389, D390, K393, E407) that did not cause growth defects upon mutagenesis (Fig. 2.9C, yellow side chains) are closer to the membrane surface and directed away from the proposed translocation pore in the SecY subunit (Van den Berg et al., 2004). When viewed from the top, the critical residues in L6 and L8 are in three separate clusters separated by 15-20 Å (Fig. 2.9D).
Figure 2.9. Point mutations in L6 and L8 define a contact surface for cytoplasmic ligands of the Sec61 complex. (A) A ribbon diagram of SecYEG complex showing the three subunits (SecY, green; SecE, cyan and SecG, magenta) as viewed from within the plane of the membrane. The L6 (blue) and L8 (white) regions in SecY are highlighted. The SecY residue that aligns with a Sec61 residue subjected to mutagenesis is designated by a colored side chain; mutagenesis of red, but not yellow, side chains caused growth defects. (B) An expanded view of panel A showing that the critical residues in Sec61p are located at the tips of L6 and L8. (C) A top-view of the SecYEG complex. The subunits, loops and mutagenized residues are colored as in A. The dimerization interface for the SecYEG complex is formed by the TM span of SecE (cyan chain). The asterisk designates the proposed translocation pore in SecYEG that is plugged by the short TM2a helix. (D) An expanded top-view of the L6 and L8 regions. SecE is hidden to simplify the image. The figure was created with MacPyMOL software using SEC YEG structure (PDB 1RHZ).
Point mutations in L6 and L8 of Sec61p interfere with RNC transfer to the translocation channel

How might single amino acid substitutions in L6 and L8 of Sec61p interfere with translocation of SRP-dependent substrates? The non-additive nature of the translocation defects displayed by the RRL6L8EE sec61 mutant suggests that the R275E and R406E mutations affect different steps in a single pathway, not parallel pathways, leading to cotranslational translocation of SRP-dependent substrates.

Attachment of a ribosome-nascent chain complex to the translocation channel is a multi-step process that is regulated by the SRP and SR GTPases and is dependent upon critical interactions between Sec61p, the ribosome and the signal sequence (Jungnickel and Rapoport, 1995; Song et al., 2000). There are at least two steps in this reaction pathway that are likely dependent upon cytoplasmic segments of the Sec61 complex. The two steps correspond to recognition of an unoccupied translocon by a post-targeting intermediate, and docking of the ribosome onto the channel. A stable post-targeting intermediate (SR-SRP-RNC complex) is formed when SRP-RNCs are incubated with microsomes or proteoliposomes that lack a functional Sec61 complex (Song et al., 2000). The binding sites for SRP54 on the large ribosomal subunit overlap with the Sec61 binding sites, hence SRP must dissociate from the ribosome prior to Sec61 attachment (Halic et al., 2004). Previously we proposed that a direct interaction between the post-targeting intermediate and a vacant Sec61 complex facilitates transfer of the RNC to the translocation channel following dissociation of SRP54 from the signal sequence.
Cytosolic loops of Sec61p would be the optimal marker for an unoccupied translocon, as these segments will be occluded upon attachment of a ribosome to the translocation channel (Morgan et al., 2002). Residues in L6 of Sec61p are excellent candidates for such a recognition determinant, as point mutations in L6 (e.g. R275E) interfere with the cotranslational protein translocation pathway without causing a significant reduction in ribosome binding affinity. Although current models for the cotranslational translocation pathway typically depict an interaction between the SR and the Sec61 complex, biochemical evidence to support this conjecture is scant.

An analysis of RNC-translocon interactions (Jungnickel and Rapoport, 1995) has indicated that the initial binding of an RNC to the Sec61 complex is sensitive to salt, and precedes signal sequence insertion into the translocation pore. Point mutations that reduce the affinity between the translocation channel and the ribosome should reduce the efficiency of RNC attachment to the translocon by destabilizing this intermediate. RNCs can bind to protease-inactivated Sec61 complexes that lack detectable affinity for non-translating ribosomes (Raden et al., 2000), hence signal sequence insertion into the translocation pore is not obligatorily dependent upon intimate ribosome-channel contact. This may explain why certain point mutations in L8 (R406E) do not cause a complete block in the cotranslational translocation pathway. Three-dimensional EM reconstructions of the ribosome-Sec61 complex and the RNC-Sec61 complex have revealed the presence of a 15Å gap between the channel and the ribosome which is bridged by four stalk-like connections (Beckmann
et al., 2001; Morgan et al., 2002). Four connections per translocon would be consistent with the presence of three to four Sec61 heterotrimers per channel and this would imply that a single structural element in Sec61p forms the stalk-like connections. Notably, the diameter of the ribosome-channel connections observed by electron microscopy (~20Å (Morgan et al. 2002)) is very similar to the diameter of the SecY domain formed by the L6 and L8 loops (Fig. 2.9C). Contact points on the ribosome for the Sec61 complex correspond to several large subunit proteins (L25, L26 and L35) and specific 25S rRNA segments (Beckmann et al., 2001; Morgan et al., 2002). Inhibition of ribosome binding to the mammalian Sec61 complex by the canine 28S rRNA, but not by the 18S rRNA, supports the conclusion that specific protein-rRNA contacts contribute to the evolutionarily conserved binding of the ribosome to Sec61p/SecY (Prinz et al., 2000a). Here, we observed that point mutations in surface exposed residues in L8 cause dramatic reductions in ribosome-binding activity, suggesting that salt bridges between the basic side chains on Sec61p and the phosphodiester backbone of the 25S rRNA are critical for ribosome attachment.

Extensive mutagenesis of *E. coli* SecY has shown that R357 (R406 in Sec61p) is a crucial residue for the translocation activity of SecYEG (Mori and Ito, 2001). Suppression of the translocation defect of the *E. coli* SecY R357E mutant by "superactive alleles of SecA" has been interpreted as evidence that a functional SecA binding site maps to the C5 region (L8) of SecY. However, other SecY point mutations (A363T) in L8 selectively interfere with the Ffh/FtsY dependent integration
of inner membrane proteins (Newitt and Bernstein, 1998). Clearly, this region of the translocation channel is an evolutionarily conserved segment that is critical for interaction with cytosolic effectors of the translocation pathway.

**Secondary defects in posttranslational translocation**

Kinetic delays in transport of the SRP-independent substrates CPY and Gas1p were observed when the *sec61* mutants were grown in rich media. Expression of Ssh1p eliminates the posttranslational transport defects caused by the *sec61* mutants suggesting that cytosolic accumulation of SRP-dependent substrates interferes with one or more steps in the posttranslational targeting pathway. Accumulation of non-translocated precursors in the cytosol may reduce the effective concentration of Hsp70 chaperones that deliver precursors like preproCPY to the Sec complex. Posttranslational translocation via the Sec complex of substrates that are normally transported by a cotranslational pathway could also cause kinetic delays in transport of posttranslational substrates by increasing precursor flux through the Sec complex.

**Shared phenotypes with SRP pathway mutants**

A comparison of the phenotypes of the L6 and L8 *sec61* mutants with those described for SRP targeting pathway mutants is informative. The 4-5 fold decrease in growth rate that is caused by repressing expression of SRP54 or SRα in *S. cerevisiae* (Hann and Walter, 1991; Ogg et al., 1992) is more severe than the 2-3 fold reductions in growth rate that are caused by point mutations in the cytosolic loops of Sec61p. The simplest interpretation of this difference is that point mutations in L6 and L8 do not eliminate the SRP-dependent targeting of RNCs to the RER, but
instead interfere with the efficient transfer and attachment of the RNC to the translocation channel. The rate at which the L6 and L8 sec61 mutants acquire a petite phenotype is less pronounced than the rapid and complete conversion of srp54Δ strains to a ρ- phenotype (Hann and Walter, 1991). Although the mechanistic link between a defect in cotranslational protein translocation and subsequent loss of mitochondrial respiration remains undefined, the morphologies of the cortical ER and the mitochondria are grossly perturbed when temperature sensitive SRα mutants are shifted to the restrictive temperature (Prinz et al., 2000c). A third characteristic of the L6 and L8 sec61 mutants is the transient nature of the translocation defect. Gene product depletion experiments using the GAL1/GAL10 promoter have shown that repression of SRP54 or SRα synthesis is accompanied by a severe, yet transient defect in translocation of SRP-dependent substrates (Hann and Walter, 1991; Ogg et al., 1992). Adaptation of yeast cells to the elimination of the SRP-dependent targeting pathway occurs by induction of cytosolic chaperones and reductions in the protein synthesis rate (Mutka and Walter, 2001). The L6 and L8 sec61 mutants described here likely adapt by a comparable mechanism.
CHAPTER III

An interaction between the SRP receptor and the translocon is critical for the cotranslational protein translocation

Introduction

The signal recognition particle (SRP) dependent targeting pathway allows rapid and efficient delivery of the ribosome-nascent chain complex (RNC) to the protein translocation channel. Formation of the SRP-RNC complex occurs when the signal sequence emerges from the polypeptide exit site on the large ribosomal subunit (reviewed in (Walter and Johnson, 1994a)). Targeting of the SRP-RNC to the SRP receptor initiates the SRP54-SRα GTPase cycle that results in dissociation of SRP54 from the signal sequence and transfer of the RNC to the Sec61 heterotrimer (Rapiejko and Gilmore, 1997). Dissociation of SRP from the signal sequence is blocked by inactivation or absence of the translocon (Song et al., 2000), suggesting that the SR may locate an unoccupied translocon by interacting with the Sec61 complex. Dissociation of SRP from the RNC is a prerequisite for RNC attachment to the translocon, because the SRP binding site on the large ribosomal subunit overlaps the Sec61 binding site (Halic et al., 2004). With the exception of the SR-translocon interaction, each of these interactions (SRP-RNC, SRP-SR, SRP-SR-RNC, and RNC-translocon) has been characterized at the biochemical and structural levels (Beckmann et al., 2001; Egea et al., 2004; Focia et al., 2004; Halic et al., 2006;
The yeast *S. cerevisiae* has distinct cotranslational and posttranslational protein translocation channels. The Sec61 heterotrimer (Sec61p, Sbh1p and Sss1p) is the major cotranslational protein translocation channel, and also assembles with the Sec62-Sec63 complex to form the Sec complex (Deshaies et al., 1991; Panzner et al., 1995). The Sec complex lacks ribosome-binding activity and serves as the posttranslational translocation channel. The non-essential Ssh1p heterotrimer (Ssh1p, Sbh2p and Sss1p) does not assemble with the Sec62-Sec63 complex (Finke et al., 1996), but instead serves as an auxiliary cotranslational translocation channel (Prinz et al., 2000b; Wittke et al., 2002).

Unlike the eukaryotic SR that is anchored to the membrane by the N-terminal transmembrane (TM) span of SRβ, the single-subunit *E. coli* SR (FtsY) is present in both membrane-bound and soluble forms in vivo. Membrane-bound FtsY interacts directly with the *E. coli* translocation channel (SecYEG) (Angelini et al., 2005) to form a carbonate-resistant complex that is stabilized by blocking the GTPase activity of FtsY (Angelini et al., 2006). A different mode of interaction has been observed between the GTPase domain of yeast SRβ and the yeast translocon β-subunits (Sbh1p or Sbh2p). The *E. coli*-expressed cytosolic domains of Sbh1p or Sbh2p stimulate dissociation of GDP from yeast SRβ (Helmers et al., 2003). As GTP binding to SRβ is required for SRα-SRβ dimerization (Legate et al., 2000; Schwartz and Blobel, 2003), the nucleotide exchange activity of the translocon β-subunits would appear to be upstream of an interaction between the SR and the Sec61 complex that would
facilitate delivery of the RNC to an unoccupied translocon.

In yeast, both SRα (Srp101p) and SRβ (Srp102p) are necessary for the
cotranslational targeting pathway (Ogg et al., 1998; Ogg et al., 1992). The
observation that the GTPase domain, but not the transmembrane span, of Srp102p is
required for SRP receptor to function in the cotranslational translocation pathway
(Ogg et al., 1998) is explained by the GTP-dependent interaction between SRα and
SRβ (Legate et al., 2000; Schwartz and Blobel, 2003) and the presence of an
uncharacterized binding site on the ER membrane for the soluble SR (Srp101p+
srp102ΔTMD). Here we have tested the hypothesis that the SR interacts with the
Sec61p and Ssh1p complex to identify unoccupied cotranslational channels. Analysis
of yeast strains that express the soluble SR indicates that Ssh1 heterotrimer is a
preferred targeting site for the SRP-RNC. Analysis of yeast strains that display
synthetic slow-growth phenotypes indicate that translocon β-subunits are essential for
the rapid and efficient gating of the translocon by RNCs that are targeted by the
SRP-dependent targeting pathway.
RESULTS

Genetic Interactions between the soluble SR and the Ssh1 translocon

A haploid yeast strain (YJY101) was constructed to test the hypothesis that the translocation channel provides the ER binding site for the soluble SRP receptor. In YJY101, chromosomal disruptions of the SEC61 and SRP102 genes are covered by a URA3 marked plasmid encoding Sec61p and Srp102p. Disruption of the SSH1 gene in YJY101 yielded an additional starting strain (YJY102) that lacks the Ssh1p translocon. A plasmid shuffle procedure was used to replace the URA3 marked SEC61 SRP102 plasmid with pairs of plasmids encoding intact or soluble (srp102ΔTMD) forms of Srp102p and wild type or mutant alleles of Sec61p and Ssh1p.

Yeast strains that lack the Ssh1p translocon have a minor growth defect on rich (YPD) media (Chapter II, Fig. 2.1B). Deletion of the transmembrane domain of Srp102p causes a growth rate defect at 30°C (Ogg et al., 1998), which is accentuated at 37°C. Combining the ssh1Δ and srp102ΔTMD mutations caused a severe synthetic growth defect at 30 and 37°C (Fig 3.1A). SEC61 is an essential gene so we tested whether point mutations in Sec61p (Cheng et al., 2005) cause a synthetic growth defect in the srp102ΔTMD strain. The sec61R275E R406E mutant (sec61EE) was chosen because these charge-reversal substitutions in cytoplasmic loops 6 and 8 selectively interfere with the cotranslational translocation pathway. Expression of Ssh1p suppresses the slow growth and defective translocation phenotypes of the sec61EE mutant (Cheng et al., 2005). The growth rate defect caused by the
A

30°C

SEC61 SSH1 p[SRP102]
SEC61 ssh1Δ p[SRP102]
SEC61 SSH1 p[srp102ΔTMD]
SEC61 ssh1Δ p[srp102ΔTMD]
sec61EE SSH1 p[srp102ΔTMD]
SEC61 ssh1EE p[srp102ΔTMD]

37°C

B

30°C

SSH1 p[srp102ΔTMD]
ssh1Δ p[srp102ΔTMD]
SSH1 p[srp102ΔTMD] 2μ[SSH1*]
SSH1 2μ[SRP101 srp102ΔTMD]
ssh1Δ 2μ[SRP101 srp102ΔTMD]
SSH1 2μ[srp102ΔTMD]
ssh1Δ 2μ[srp102ΔTMD]

37°C

C

Growth at 30°C
a. SEC61 SSH1 p[SRP102]
b. SEC61 ssh1Δ p[SRP102]
c. SEC61 SSH1 2μ[SRP101 srp102ΔTMD]
d. SEC61 SSH1 2μ[SSH1*] p[srp102ΔTMD]
e. SEC61 SSH1 p[srp102ΔTMD]
f. SEC61 ssh1Δ p[srp102ΔTMD]
Figure 3.1. Genetic interactions between the SR and the Ssh1 complex.  (A) Yeast strains expressing wild type or soluble (ΔTMD) forms of Srp102p and wild type or mutant alleles of Sec61 (sec61EE) or Ssh1 (ssh1EE, or ssh1Δ) were grown to mid-log phase in SEG media at 30°C.  Growth rates were compared by serial dilution analysis on YPD plates at both 30°C and 37°C as described in Materials and Methods. Plates were photographed after 2d (30°C) or 3d (37°C) of growth.  (B) Yeast strains (SSH1 or ssh1Δ) expressing wild type Sec61p and srp102ΔTMD from low copy plasmids were transformed with high copy plasmids (2µ) encoding srp102ΔTMD, soluble SR (Srp101p + srp102ΔTMD) or the Ssh1p complex (Ssh1p + Sbh2p + Sss1p).  Growth rates of yeast strains on YPD media were compared by serial dilution analysis at 30° or 37°C as described above.  (C) Growth rates of selected yeast strains expressing intact or soluble (ΔTMD) forms of Srp102p in liquid YPD media at 30°C.  All strains express wild type Sec61p.
srp102ΔTMD mutation is not accentuated by the sec61EE mutation but is instead partially suppressed (Fig. 3.1A). Sequence conservation between Sec61p and Ssh1p allowed construction of the corresponding ssh1EE mutant (ssh1R278ER411E).

Although the ssh1EE mutant grows at a wild-type rate when intact Srp102p is present (not shown), the ssh1EE srp102ΔTMD mutant grows very slowly at 30°C and is inviable at 37°C (Fig. 1A). Synthetic genetic phenotypes can be explained by inactivating lesions in parallel pathways or by partial impairment of interacting components within a linear pathway (Huffaker et al., 1987). The synthetic interactions observed here are examples of the latter case where a partially defective SR interacts weakly with the Sec61 heterotrimer, or interacts weakly or unproductively with a defective ssh1EE heterotrimer.

If the soluble SR interacts with the Ssh1 translocon, increased expression of either the Ssh1 heterotrimer or the soluble SR should suppress the growth defect of the srp102ΔTMD mutant. Since the Ssh1 translocon is a heterotrimer that shares one subunit (Sss1p) with the Sec61 complex, the Ssh1 heterotrimer (Ssh1p, Sbh2p plus Sss1p) was expressed from a single high copy plasmid (Fig. 3.1B, 2µ[SSH1*]).

Overexpression of the Ssh1 complex reduces the growth defect of the srp102ΔTMD mutant at 30°C and suppresses the temperature-sensitive phenotype. The six-hour cell-division time for the srp102ΔTMD mutant in liquid media at 30°C is reduced to 4 hours when the Ssh1 complex is overexpressed (Fig. 3.1C). Increasing the cellular content of the soluble SR (Srp101p plus srp102ΔTMD) alleviates the slow growth phenotype (Fig. 3.1B), and reduces the cell division time to 3.5 h in liquid media (Fig.
3.1C). Overexpression of the soluble SR is ineffective in the ssh1Δ mutant, supporting the hypothesis that the soluble SR interacts with the Ssh1 translocon. Overexpression of srp102ΔTMD did not increase the growth rate indicating that the soluble SR, not just the GTP-bound form of SRβ, that is the growth-rate limiting factor.

The very slow growth rate of ssh1Δ srp102ΔTMD mutant in liquid media (>8h/generation) is comparable to the growth rates of srp102Δ, srp101Δ or srp54Δ mutants (Hann and Walter, 1991; Ogg et al., 1998; Ogg et al., 1992). The uniform pink to red colony color of the ssh1Δ and srp102ΔTMD strains (Fig. 3.1A and 3.1B) demonstrates that the single mutants are not petite (ρ-). Unlike the srp102Δ, srp101Δ or srp54Δ mutants, the ssh1Δ srp102ΔTMD mutant is viable on media containing glycerol and ethanol as sole carbon sources (not shown). However, the ssh1Δ srp102ΔTMD mutants lose respiration competence with a 10-fold increased frequency (~3%/generation) relative to an ssh1Δ strain or an srp102ΔTM strain (Table I) when grown on synthetic defined media containing dextrose (SD media). To prevent the accumulation of ρ- mutants, all yeast strains were maintained on synthetic minimal media containing ethanol and glycerol (SEG media).

Table I  Petite percentage per generation in wild type and mutant cells.
Translocation defects of the ssh1Δ srp102ΔTMD mutant

Yeast strains were shifted from SEG media into SD media and grown for 4 or 24 h prior to being pulse-labeled for 7 min with 35S amino acids. The vacuolar membrane protein dipeptidylaminopeptidase B (DPAPB, i.e. Dap2p) was used as a reporter because DPAPB integration is mediated by the SRP-dependent targeting pathway (Ng et al., 1996). Integration of DPAPB into the ER was detected by the reduced gel mobility caused by N-linked glycosylation. DPAPB integration was very efficient in wild type and ssh1Δ cells after 4 or 24h of growth in SD media (Fig. 3.2A, 3.2B). Two major differences were observed when DPAPB integration was analyzed in cells that express the soluble SR. First, we observed a 3-5 fold reduced incorporation of radiolabel into DPAPB-specific products after 4 h of growth in SD media (Fig. 3.2A). Secondly, less than 50% of the DPAPB products are integrated into the membrane in the srp102ΔTMD mutant. The translocation defect of the srp102ΔTMD strain was enhanced in cells that lack the Ssh1 complex, but not in cells that express the sec61EE or ssh1EE alleles (Fig. 3.2A). Translocation defects were not previously observed in the srp102ΔTMD mutant (Ogg et al., 1998).
Figure 3.2. Cotranslational translocation defects of yeast that express srp102ΔTMD.

(A-D) Wild-type and mutant yeast cells were grown to mid-log phase at 30°C in SEG media. The cultures were diluted into SD media and allowed to grow for 4h (A, C, D) or 24 h (B) at 30°C. Wild type or mutant cells (4 A600) were collected and pulse-labeled for 7 min. DPAPB immunoprecipitates was resolved by SDS-PAGE. Glycosylated (DPAPB) and non-glycosylated (pDPAPB) forms of DPAPB were quantified with a BioRad FX molecular imager to calculate the % precursor. (A, B) Total incorporation of Tran-^{35}S-label (pDPAPB + DPAPB) in each strain is expressed relative to the wild type strain.
discrepancy is explained by an adaptation process that occurs upon continued growth of the srp102ΔTMD strain in SD media (Fig. 3.2B). After 24h, incorporation of $^{35}$S amino acids into DPAPB was usually proportional to the growth rate of the strain in liquid media, and pDPAPB accumulation had decreased several-fold.

Additional pulse-labeling experiments were conducted after 4 h of culture in SD media to investigate how increased expression of the Ssh1 complex (Fig. 3.2C) or the soluble SR (Fig. 3.2D) impacts the cotranslational translocation pathway. The non-translocated precursor in the ssh1Δsrp102ΔTMD mutant is reduced in a dosage dependent manner by expression of the Ssh1 complex from low copy and high copy plasmids (Fig. 3.2C). Increased expression of the soluble SR heterodimer, but not srp102ΔTMD alone, significantly increases DPAPB integration, provided that cells express the Ssh1 complex (Fig. 3.2D).

**In vivo kinetics of Dap2 integration**

The in vivo kinetics of DPAPB integration can be analyzed using the Dap2 series of ubiquitin translocation assay (UTA) reporters (Cheng and Gilmore, 2006). The Dap2 reporters consist of the N-terminal cytosolic and transmembrane domains of DPAPB (45 residues) followed by a variable length spacer segment (49-265 residues), ubiquitin (Ub; 76 residues), a cleavage site for an Ub-specific protease (UBP), a linker (42 residues) and HA-epitope tagged Ura3p (Fig. 3.3A). Rapid folding of the ubiquitin domain in the cytosol allows cleavage by UBP and release of the cleaved (U-HA) reporter segment (Fig. 3.3B). However, if Dap2-RNCs gate the translocon before the Ub domain emerges from the large ribosomal subunit, the intact reporter
Figure 3.3. The scheme of ubiquitin translocation assay (UTA). (A) Dap2 reporters consist of (i) the N-terminal cytoplasmic domain of Dap2p (gray, Dap2p_{1-29}) followed by the TM span (black, Dap2p_{30-45}), (ii) 49- to 265-residue spacer segments (cyan) derived from Dap2p, (iii) an Ub domain (red), (iv) a 42-residue linker (blue) with a processing site (arrowhead) for an Ub-specific protease, and (v) a Ura3 reporter domain followed by a triple-HA epitope tag (yellow). Sites for N-linked glycosylation (Y-shaped symbols) are indicated. (B) Cleavage of the Dap2 reporter defines the \textit{in vivo} kinetics of translocon gating.
will be integrated into the ER. Mutations in SRP54 (Johnsson and Varshavsky, 1994), SR or Sec61α (Cheng and Gilmore, 2006) enhance UTA reporter cleavage by retarding reaction steps that precede translocon gating (Fig. 3.3B). For example, the SRP-SR dependent targeting pathway can be acutely blocked by shifting the temperature sensitive srp102(K51I) mutant from the permissive to restrictive temperature (Cheng and Gilmore, 2006; Ogg et al., 1998) which allows very efficient cleavage of the Dap265 reporter.

Cleavage of the Dap2 reporters was analyzed after 4 h of growth in SD media (Fig. 3.4). The intact glycosylated reporters (e.g. g49) as well as the cleaved U-HA domain were recovered by immunoprecipitation with anti-HA monoclonal antibody (Fig. 3.4A). Dap2 cleavage decreases dramatically as the spacer length is increased (Fig. 3.4A, left panel, quantified in 3.4B) indicating that most Dap2-RNCs gate the translocon after 170, but before 224, residues of the reporter emerge from the large ribosomal subunit (N-terminal 45 residues + 103 residues spacer + 76 residue Ub domain = 224 residues) in wild type yeast. Further increases in spacer length have little impact upon Dap2 reporter cleavage in wild type cells (Fig. 3.4B, squares). The difference between the plateau value for Dap2 reporter cleavage, and the pDPAPB detected in a pulse labeling experiment (Fig. 3.2A) indicates the fraction of pDPAPB precursors that are translocated by a posttranslational pathway. Dap2 reported cleavage was higher in the srp102ΔTMD strain, regardless of spacer length (Fig. 3.4A, middle panel). Quantification revealed a wider translocon gating window and an elevated plateau value (Fig. 3.4B, triangles). Elimination of
Figure 3.4. Gating defects in the ssh1Δ srp102ΔTMD mutant before adaptation.  (A) In vivo cleavage of the Dap2 reporter in wild type, srp102ΔTMD (abbreviated SRβΔTM) or ssh1Δ srp102ΔTMD strains after 4 h of growth in SD media.  Labels designate the intact glycosylated (e.g., g49), intact non-glycosylated (arrowheads) and cleaved (U-HA) reporter domains. (B) Spacer-length dependence of Dap2 reporter cleavage (% cytosolic Ura3-HA) for the wild type (squares), ssh1Δ (circles), srp102ΔTMD (triangles) or ssh1Δ srp102ΔTMD (diamonds) yeast strains after 4 h of growth in SD media.  Data points are averages of two experiments for each strain.
the Ssh1 translocon raises the plateau value for Dap2 cleavage due to a reduced membrane content of cotranslational translocons (Fig. 3.4B, circles). Analysis of Dap2 reporter cleavage in the double mutant (ssh1Δ srp102ΔTMD) revealed an extremely slow and inefficient delivery of Dap2-RNCs to the Sec61 complex (Fig. 3.4B, diamonds) diagnostic of a severe impairment of the cotranslational targeting pathway. After extended culture in SD media Dap2 reporter cleavage in wild type (not shown) and ssh1Δ cells (Fig. 3.5B, circles) is essentially identical (Cheng and Gilmore, 2006). Dap2 reporter cleavage was also greatly reduced in the srp102ΔTMD mutant (Fig. 3.5A, left panel). Quantification revealed a modest elevation in plateau value relative to a wild type cell, but apparently normal translocon gating kinetics (Fig. 3.5B, triangles). More efficient targeting of the Dap2 reporter in the srp102ΔTMD mutant could be explained by a slower protein synthesis elongation rate. However, elongation rates for Dap2p in wild type (~7.5 residues/sec) and srp102ΔTMD mutant yeast cells (~7 residues/sec) are similar (Fig. 3.6). After adaptation, Dap2 reporter cleavage was reduced in the ssh1Δsrp102ΔTMD mutant (Fig. 3.5A, middle panel; Fig. 3.5B, filled diamonds). Because protein synthesis elongation rates were slower in the ssh1Δ srp102ΔTMD mutant (~5 residues/sec; Fig. 3.6), we further reduced the elongation rate by treating cells with cycloheximide (CH). A low concentration of cycloheximide reduced cleavage considerably (Fig. 3.5B, open diamonds), hence we can conclude that more time is required for the soluble SR to deliver an RNC to the Sec61 complex in cells that lack Ssh1 translocons.
Figure 3.5. Slow targeting of Dap2-RNCs in the *ssh1Δ srp102ΔTMD* mutant after adaptation.  (A) In vivo cleavage of the Dap2 reporters in the absence or presence of CH (cycloheximide) after 24h of growth in SD media.  (B) Spacer-length dependence of Dap2 reporter cleavage (% cytosolic Ura3-HA) for the *ssh1Δ* (circles), *srp102ΔTMD* (triangles) or *ssh1Δ srp102ΔTMD* (filled diamonds, -CH; open diamonds, +CH) yeast strains after 24 h of growth in SD media.  Data points are averages of two experiments for each strain.
Figure 3.6. Protein synthesis elongation rates. Yeast strains were transformed with pDN317 (Ng et al., 1996) that encodes DPAPB-HA under control of the glyceraldehyde 3-phosphate dehydrogenase promoter. Cells were pulse labeled at 30°C as described in Materials and Methods. At frequent time points (30 s to 5 min), cells (4 A₆₀₀) were removed and the labeling reaction terminated by adding an equal volume of chilled 20 mM NaN₃ plus unlabeled cysteine and methionine to 0.6 mg/ml, followed by freezing in liquid nitrogen. Total incorporation of Tran-³⁵S-label into protein was determined by TCA precipitation. Radiolabeling of full-length DPAPB-HA was determined by immunoprecipitation, followed by SDS-PAGE and detection with a BioRad FX molecular imager. (A) Time course of incorporation of Tran-³⁵S-label into total protein (circles) and into DPAPB-HA (squares) by the srp102ΔTMD yeast strain. Extrapolation of the linear part of the incorporation curves to the abscissa yielded a lag time for incorporation of Tran-³⁵S-label into total protein or into DPAPB-HA incorporation. The difference between these two lag times equals half the time required to synthesize DPAPB-HA (Horwitz et al., 1969). (B) The protein synthesis rate (residues·s⁻¹) was calculated by dividing the residues in DPAPB-HA by the synthesis time.
Translocon β-subunits are critical for the cotranslational translocation pathway

Additional strains were constructed to determine whether the lack of a translocon β-subunit (either Sbh1p or Sbh2p) causes a synthetic growth defect in cells that express the soluble SR (Fig. 3.7A). While lack of a single translocon β-subunit does not cause a growth defect in cell that express wild type SR (Finke et al., 1996), disruption of the SBH2 gene, but not the SBH1 gene, causes a growth defect in the srp102ΔTMD background that is slightly less severe than that caused by disruption of SSH1. The sbh2Δsrp102ΔTM strain has a relatively faster protein synthesis rate (~7.0 residues/sec, Fig 3.6) than the ssh1Δsrp102ΔTM strain. Both mutant strains lost the mitochondria respiration competence with a similar frequency (~3%/generation), which are ~ 30 fold higher than srp102ΔTM (0.1%/generation) and sbh1Δsrp102ΔTM strains (0.1%/generation) (Table I).

To determine whether the observed synthetic defects in ssh1Δsrp102ΔTM and sbh2Δsrp102ΔTM was caused by a reduced number of total translocon, identical amount of total protein extracts were resolved by SDS-PAGE and subsequently immunobloted with antibodies against a cytosolic control protein (PGK1) and Sec61p (Fig 3.7B). Interestingly, disruption of Ssh1p or Sbh2p in srp102ΔTM strain causes an increase in the cellular content of Sec61p. Increased Sec61p expression level in cells lacking an intact Ssh1p complex suggests the importance of Ssh1p complex and a possible adaptation mechanism. Conversely, disruption of Sbh1p in srp102ΔTM slightly reduced the Sec61p expression level, suggesting a complex-stabilizing role for Sbh1p.
Fig 3.7 Genetic interactions between translocon β-subunits and srp102ΔTMD.  (A) Growth rates of ssh1Δ, sbh1Δ or sbh2Δ yeast strains expressing the soluble SR (srp102ΔTMD) and wild type Sec61p were determined by serial dilution analysis at 30 or 37°C as in Fig. 3.1.  (B) Protein immunoblot detection of Sec61p in wild type and mutant strains.  PGK1 is utilized as a loading control.
Figure 3.8. Expression of Sbh1p or Sbh2p fragments in the sbh2Δsrp102ΔTM strain cannot rescue its growth rate defect. (A) Diagrams of intact Sbh2p and N-terminal deletion mutants. The segment labeled Secβ is homologous to the C-terminal segment of *M. jannaschii* Secβ (residues 21-52) that was resolved in the SecYEβ structure. (B,C) Growth rates of yeast strains were determined by serial dilution analysis at 30 or 37°C as in Fig. 3.1. The SEC61sbh2Δsrp102ΔTMD strain was transformed with a low copy plasmid encoding intact Sbh2 (B), low or high (2µ) copy plasmids encoding intact Sbh1p (B) or low copy plasmids expressing C-terminal fragments of Sbh2p (C).
The high sequence identity between Sbh1p and Sbh2p (>50%) raised the possibility that Sbh1p could assemble with Shh1p. Sec61p-Sbh2p-Sss1p heterotrimers are found to be assembled in an \textit{sbh1Δssh1Δ} mutant in which both original partners for Sec61p and Sbh2p are missing (Finke et al., 1996). Overexpression of Sbh1p from a low copy or high copy plasmid did not suppress the synthetic slow growth phenotype of the \textit{sbh2Δsrp102ΔTMD} mutant at 30°C or 37°C (Fig. 3.8B). Sbh2 deletion mutants lacking as few as 11 N-terminal residues are unable to suppress the synthetic slow growth phenotype of the \textit{sbh2Δsrp102ΔTMD} mutant (Fig. 3.8C).

The synthetic growth defect of the \textit{sbh2Δsrp102ΔTMD} mutant suggests that translocon \textit{β}-subunits may be important for a functional interaction between the SR and the translocon. Single (\textit{sbh1Δ} or \textit{sbh2Δ}) and double (\textit{sbh1Δ sbh2Δ}) mutants were constructed to explore a potential role for translocon \textit{β}-subunits in the cotranslational translocation pathway. Although the \textit{sbh1Δsbh2Δ} mutant grows at a wild type rate at 30°C, the strain is nearly inviable at 37°C ((Finke et al., 1996), Fig. 3.11A). Pulse-labeling experiments showed that translocation of CPY and DPAPB are inefficient in the double mutant (Fig. 3.9A and 3.9B). Low amounts of prepro-CPY were detected in the \textit{sbh1Δ} mutant (Fig. 3.9B), which can be explained by the reduced stability of Sec61 complex (data not shown). Our pulse-labeling results agree with a previous report (Finke et al., 1996) concerning the synergistic effect of translocon \textit{β}-subunit deletions on yeast translocation pathways.

Non-translocated precursor (pDPAPB) was detected by protein
Figure 3.9. Translocation defects in \(sbh1\Delta sbh2\Delta\) mutants. Integration of DPAPB (A) and translocation of CPY (B) were evaluated by 7-min pulse labeling of wild type and mutant yeast strains (\(sbh1\Delta, sbh2\Delta\) and \(sbh1\Delta sbh2\Delta\)) at 30°C. The glycosylated (DPAPB) and non-glycosylated (pDPAPB) forms of DPAPB and the ER (p1CPY) and precursor (ppCPY) forms of CPY are labeled. Asterisks designate incompletely trimmed forms of glycosylated DPAPB and CPY. Protein immunoblot detection of DPAPB-HA (C) or CPY (D) in wild type and \(sbh1\Delta sbh2\Delta\) strains. Total cell extracts were prepared for SDS-PAGE with or without prior digestion by Endo H. Precursor and mature forms of DPAPB-HA are labeled. Deglycosylated mature CPY (dgCPY) is resolved from vacuolar CPY (m) and hypoglycosylated CPY (-1,-2).
Fig 3.10 Degradation of precursor DPAPB in \textit{sbh1}\textDelta sbh2\textDelta mutant. \textit{sbh1}\textDelta sbh2\textDelta double mutant cells were pulse-labeled for 7 min before chasing. The chase was initiated by adding unlabeled cysteine and methionine to a final concentration of 0.6 mg/ml. 4A600nm cells were collected at 10 min intervals and prepared for DPAPB immunoprecipitation. The percentage of mature DPAPB (DPAPB) and precursor DPAPB (pDPAPB) were quantified by a BioRad FX molecular Imager. The stability of DPAPB (squares) and pDPAPB (circles) relative to zero time point (T0) was plotted as a function of chase time.
immunoblotting in extracts prepared from the \textit{sbh1Δsbh2Δ} mutant (Fig. 3.9C), but the precursor was less abundant than mature DPAPB. A pulse-chase experiment indicates that untranslocated pDPAPB is degraded within 30 min by the \textit{sbh1Δsbh2Δ} mutant (Fig. 3.10). Cell extracts prepared from wild-type and \textit{sbh1Δsbh2Δ} mutant were digested with endoglycosidase H to remove N-linked oligosaccharides prior to protein immunoblot analysis using antibodies specific for CPY. The untranslocated precursor (pp-CPY), if present, would comigrate with vacuolar CPY (e.g. mCPY) prior to Endo H digestion. The nontranslocated precursor CPY precursor was not detected in extracts prepared from \textit{sbh1Δsbh2Δ} mutant. Mature CPY synthesized by \textit{sbh1Δsbh2Δ} mutant migrates as a glycoform doublet due to incomplete N-glycosylation. The reduced electrophoretic mobility of p1CPY and DPAPB synthesized by the \textit{sbh1Δsbh2Δ} mutant (Fig. 3.9A and 3.9B, *) is caused by delayed trimming of glucose residues from the N-linked oligosaccharides. The defects in transfer and processing of N-linked oligosaccharides are probably indirect consequences of the chronic translocation defect.

Eukaryotic translocon β-subunits are C-tail anchored membrane proteins that expose 55-60 residues on the cytosolic side of the membrane. \textit{M. jannaschii} Secβ has a smaller cytosolic segment than Sbh1p or Sbh2p, and most of this region of Secβ is disordered in the SecYEβ crystal structure (Van den Berg et al., 2004). Carboxyl-terminal fragments of Sbh2 (Fig. 3.8A) were expressed in the \textit{sbh1Δsbh2Δ} mutant to identify regions of Sbh2 that are important for function (Fig. 3.11A). Partial suppression of the temperature-sensitive growth defect occurred upon
Figure 3.11. Effect of N-terminal deletion on Sbh2p. (A,B) Plasmids encoding full length or N-terminal deletion alleles of Sbh2 were transformed into the \textit{sbh1}Δ\textit{sbh2}Δ strain. (A) Growth rates of wild type and mutant strains were compared by serial dilution analysis as described in Fig.3.1. Integration of DPAPB (B, upper panel) and translocation of CPY (B, bottom panel) were evaluated by pulse labeling as described in Fig 3.2.
Figure 3.12. In vivo cleavage of Dap2 reporters in *sbh1Δ sbh2Δ* mutant. (A) Labels designate the intact glycosylated (e.g. g265), intact non-glycosylated (arrowheads) and cleaved (U-HA) reporter domains. (B) Spacer-length dependence of Dap2 reporter cleavage in wild type (squares), *srp101Δ* (diamonds), *sbh1Δ sbh2Δ* (circles) and the *sbh1Δ sbh2Δ* mutants expressing sbh2Δ37 (triangles). Data points are averages of two experiments, one of which is shown in panel A.
expression of a minimal Sbh2 fragment (sbh2Δ54), suggesting that the temperature sensitive growth phenotype might be explained by reduced stability of β-subunit deficient Ssh1 translocon at 37°C. Expression of Sbh2p fragments that lack the first 11 or 37 residues strongly suppress the temperature sensitive growth defect of the sbh1Δsbh2Δ mutant (Fig. 3.11A). Translocation of CPY and integration of DPAPB was analyzed in cells expressing the Sbh2p deletion mutants (Fig. 3.11B). Expression of sbh2Δ11 restored DPAPB integration and CPY translocation to a level that was similar to the sbh1Δ mutant. Sbh2 fragments that lacked large segments of the cytosolic domain (sbh2Δ37 and sbh2Δ54) partially suppressed the translocation defects of the sbh1Δsbh2Δ mutant.

Cleavage of the Dap2 UTA reporters was evaluated in the sbh1Δsbh2Δ mutant to determine whether inefficient DPAPB integration is explained by slower translocon gating kinetics (e.g. wide gating window), or is instead explained by a reduced number of fully-functional translocons (e.g. elevated plateau value). Remarkably, even after adaptation, the translocon-gating kinetics for Dap2 RNCs in the sbh1Δsbh2Δ mutant were very slow with no apparent plateau value (Fig. 3.12B, circles). Expression of the sbh2Δ37 fragment in the sbh1Δsbh2Δ mutant increased the percentage of Dap2 RNCs that engage the cotranslational pathway (Fig. 3.12B, triangles), but did not restore wild-type translocon gating kinetics.

Yeast strains that do not express SRP54 or either subunit of the SR adapt to the loss of the SRP-dependent targeting pathway (Ogg et al., 1998). Analysis of Dap2 reporter cleavage in the srp102Δ mutant (not shown) or srp101Δ mutant (Fig. 3.12B,
diamonds) mutants yielded similar, but unexpected results. Yeast cells that adapt to loss of the SR translocate 35-40% of Dap2 reporters by a cotranslational pathway. Remarkably, the fraction of Dap2 precursors that are translocated by a cotranslational pathway in \textit{srp101}Δ and \textit{sbh1}Δ\textit{sbh2}Δ mutant expressing sbh2Δ37 fragment are similar.

**Cell morphology in yeast that express soluble SR or lack translocon β-subunits.**

The \textit{srp102}Δ\textit{TM} mutant, as well as double mutants that do not express Ssh1p, Sbh1p or Sbh2p, have relatively mild but persistent translocation defects following adaptation (Fig. 3.2, and data not shown). Wild type and selected mutant strains were visualized by differential interference contrast (DIC) microscopy to compare the morphology of the cells (Fig. 3.13). Prior to microscopy the cells were stained with the lipophilic vital dye FM 4-64 to stain the endocytic compartment and incubated for 3 h to allow delivery of the dye to the vacuole. Yeast cells that express the soluble SR either alone (Fig. 3.13B) or in combination with the \textit{sbh1} disruption (Fig. 3.13C) are round rather than ovoid, and in many cases contain enlarged vacuoles relative to wild type cells (Fig. 3.13A). The \textit{ssh1}Δ\textit{srp102}Δ\textit{TM}D cells (Fig. 3.13C) are round, vary greatly in size and have non-uniform vacuoles. The majority of \textit{sbh2}Δ\textit{srp102}Δ\textit{TM} mutant cells was enlarged and contained clusters of vacuoles that occupy the majority of the cell volume (Fig. 3.13E). The \textit{sbh1}Δ\textit{sbh2}Δ cells have a wild type morphology when grown at 30°C (Fig. 3.13F). One hour (Fig. 3.13G) or two hours (Fig. 3.13H) after \textit{sbh1}Δ\textit{sbh2}Δ cultures are shifted to 37°C, the cells are
Figure 3.13. Vacuolar morphology in selected mutants. Wild-type (A), \textit{srp102}Δ\textit{TMD} (B), \textit{sbh1}Δ\textit{srp102}Δ\textit{TMD} (C), \textit{ssh1}Δ\textit{srp102}Δ\textit{TMD} (D), \textit{sbh2}Δ\textit{srp102}Δ\textit{TMD} (E), and \textit{sbh1}Δ\textit{sbh2}Δ (F) strains were grown in SEG media at 30°C and collected by centrifugation. The yeast cells were resuspended in YPD media and labeled with FM 4-64 (see Materials and Methods) and incubated for an additional 3 h at 30°C.

(G-J) Wild type (I, J) and \textit{sbh1}Δ\textit{sbh2}Δ (G, H) strains were shifted to 37°C for an additional 1 h (G, I) or 2 h (H, J) before stained with FM 4-64. The cells were viewed under a fluorescence microscope using DIC optics (left) or filters for FM 4-64.
enlarged and contain numerous refractile structures that are visible by DIC. Staining with FM 4-64 revealed fragmented vacuoles as well as brightly stained vesicles that are likely endosomes. FM 4-64 staining of wild types cells cultured at 37°C did not reveal fragmented vacuoles (Fig 3.13I, J).
DISCUSSION

Interaction between the SR and the Ssh1p complex

Cell fractionation and protein purification experiments indicate that the yeast ER contains three roughly equal-sized pools of Sec61p that correspond to the heptameric Sec complex as well as ribosome-bound and unbound Sec61 heterotrimers (Panzner et al., 1995). Here, we have investigated interactions between the SRP-SR-RNC complex and the translocon that facilitate rapid and efficient transfer of the RNC to vacant Sec61p or Ssh1p heterotrimers. In wild type cells, the majority of Dap2 RNCs gate the translocon within 30 sec after initiation of translation (Cheng and Gilmore, 2006). When the SR is anchored to the membrane by the N-terminal span of SRβ, the search for a vacant translocon is restricted to the two-dimensional surface of the ER membrane, and is not compromised by loss of a single translocon β-subunit (Sbh1p or Sbh2p) or the Ssh1p translocon. The SR in srp102ΔTMD cells is present in both membrane-bound and soluble pools (Ogg et al., 1998). Prior to adaptation, srp102ΔTMD cells have a significant defect in the cotranslational targeting pathway that is explained by a delay in the kinetics and a reduced efficiency of translocon gating by RNCs, consistent with the view that a three dimensional search for a vacant translocon by the SRP-SR-RNC complex will require additional time. After adaptation, the apparently normal translocon gating kinetics of the srp102ΔTMD strain appear to be explained by a slight decrease in protein synthesis elongation rate, and an overall reduction in protein synthesis capacity.

Synthetic genetic phenotypes can be explained by inactivating lesions in
parallel pathways or by partial impairment of interacting components within a linear pathway (Huffaker et al., 1987). The synthetic interactions observed in double mutant strains (e.g. ssh1Δ srp102ΔTMD) are most likely examples of the latter case. We favor the model that the Ssh1 translocon and the SR are involved in a linear pathway, because the Ssh1 translocon had been found to selectively interact with SRP-dependent substrates and located in close proximity to the SR on the ER membrane (Wittke et al., 2002).

Synthetic slow growth defects observed in double mutant strains (e.g. ssh1Δ srp102ΔTMD) indicate that the Ssh1p translocon is the preferred interaction site for the soluble SR. Genetic evidence for a direct interaction between the Ssh1p translocon and the soluble SR was provided by the observations that overexpression of either the Ssh1 translocon or the soluble SR largely alleviates the growth and translocation defects of the srp102ΔTMD mutant. Analysis of Dap2 reporter cleavage in the ssh1Δ srp102ΔTMD mutant before adaptation revealed a near complete block in the cotranslational translocation pathway that is comparable to that observed in the srp102(K51I) mutant prior to adaptation (see Fig. 1f in (Cheng and Gilmore, 2006)). In contrast to the srp102ΔTMD mutant and ssh1Δ mutant, the double mutant (srp102ΔTMD ssh1Δ) did not display normal translocon gating kinetics following adaptation. Transfer of RNC to the ER membrane only containing Sec61 translocon is inefficient.

An essential role for the Ssh1p translocon was first observed at the semi-permissive temperature in the yeast sec61-2 mutant (Finke et al., 1996).
interestingly, expression of Ssh1p suppresses the growth and translocation defects caused of the L6 and L8 sec61 mutants (chapter II). Point mutations in cytoplasmic loops 6 and 8 of Sec61p (e.g. R406E) inhibit ribosome binding to Sec61 heterotrimers, hence these mutations block a step downstream from the targeting defect caused by deletion of the SRβ TM span. Sequential defects in the cotranslational translocation pathway likely explain the dramatically different phenotypes of the ssh1EEsrp102ΔTMD and sec61EEsrp102ΔTMD mutants. We propose that the soluble SR delivers the SRP-RNC to the defective ssh1EE translocon in preference to the wild-type Sec61 translocon.

A synthetic slow growth phenotype was also observed upon disruption of the SBH2 gene, but not the SBH1 gene, in cells that express the soluble SR. Although Sbh2p is unstable in an ssh1Δ strain, dimeric Ssh1p translocons (Ssh1p plus Sss1p) are stable without Sbh2p (Finke et al., 1996). There were several remarkable aspects of the synthetic slow growth phenotype of the sbh2Δsrp102ΔTMD stain. The specificity (sbh2Δ vs. sbh1Δ) supports the conclusion that the Ssh1p complex is the preferred interaction site for the soluble SR. The observation that loss of Sbh2p mimics loss of the Ssh1p translocon demonstrates that Sbh2p is critical for the function of the Ssh1p translocon in cells that express the soluble SR. The temperature sensitive growth defect of the sbh2Δsrp102ΔTMD mutant could not be suppressed by expression of C-terminal Sbh2p fragments or overexpression of intact Sbh1p. The longer C-terminal fragments of Sbh2p (e.g. Sbh2pΔ11) are stable in vivo as expression of Sbh2pΔ11 from a low-copy plasmid strongly suppresses the
temperature sensitive growth defect of the $s bh1 \Delta sbh2 \Delta$ mutant. The transmembrane span of Sbh2p provides the targeting information that directs integration of this tail-anchored membrane protein (Stefanovic and Hegde, 2007), and by analogy to the SecYE$\beta$ structure (Van den Berg et al., 2004), packs against TM spans 1 and 4 of Ssh1p.

**Translocon $\beta$-subunits are critical for the cotranslational translocation pathway**

Previous studies concerning the role of translocon $\beta$-subunits have suggested diverse and somewhat contradictory roles for these subunits in the cotranslational and posttranslational translocation pathways. The initial evidence that translocon $\beta$-subunits are not essential in yeast was provided by an analysis of single and double mutant strains (Finke et al., 1996). As observed here for CPY translocation, the $s bh1 \Delta$ mutant analyzed by Finke et al. (1996) had a very mild in vivo defect in prepro-$\alpha$-factor transport that was accentuated upon deletion of Sbh2p. CPY and prepro-$\alpha$-factor are translocated through the heptameric Sec complex, not through the Ssh1p complex (Wittke et al., 2002), so the severe posttranslational translocation defect in the $s bh1 \Delta sbh2 \Delta$ mutant is best explained by an indirect mechanism. When translocon $\beta$-subunits are missing, the reduced efficiency of translocon gating by the heterodimeric Sec61p and Ssh1p cotranslational translocons causes increased protein flux through the heptameric Sec complex and competition for cytosolic chaperones. While cytosolic chaperones levels are upregulated by lesions in the cotranslational translocation pathway, expression of the heptameric Sec complex does not increase (Mutka and Walter, 2001). Although we favor this indirect explanation for the
posttranslational translocation defect, it has been reported that microsomes isolated from \textit{sbh1Δsbh2Δ} mutant transport prepro-\textalpha-factor at a reduced rate in vitro when membranes are sub-saturating (Finke et al., 1996).

Proteoliposomes reconstituted with Sec61\textbeta-depleted canine Sec61p complexes are defective in cotranslational translocation, unless the SRP-RNC targeting step is performed at a reduced temperature (Kalies et al., 1998). Based upon this result, Kalies and colleagues proposed that Sec61\textbeta mediates a post-targeting event in cotranslational translocation that might correspond to signal-sequence insertion into the Sec61p pore. Although mammalian Sec61\textbeta is dispensable for the ribosome binding activity of the Sec61 complex (Kalies et al., 1994), purified recombinant Sec61\textbeta binds ribosomes with an affinity comparable to the Sec61 heterotrimer (Levy et al., 2001). Sec61\textbeta can be crosslinked to SPC25, a subunit of the signal peptidase complex, suggesting that cytoplasmic domain of canine Sec61\textbeta recruits signal peptidase to the translocon when an RNC is bound to Sec61\textalpha (Kalies et al., 1998).

When assayed in vitro, the isolated cytosolic domains of Sbh1p and Sbh2p act as guanine nucleotide exchange factors for the GDP-bound form of yeast SR\textbeta (Helmers et al., 2003). However, the limited sequence alignment between the cytosolic domains of Sbh1p and Sbh2p and the Sec7 GEF domain (Helmers et al., 2003), is not well conserved in vertebrate, plant or insect translocon \textbeta-subunits. The Sec YE\textbeta crystal structure does not provide significant insight into the structure of the cytoplasmic domain of translocon \textbeta-subunits due both to the low conservation
between the archae Secβ and eukaryotic Sec61β (Sbh1p/Sbh2p) and the disordered structure of the *M. jannaschii* Secβ N-termini (Van den Berg et al., 2004). If the GEF activity of Sbh1p and Sbh2p is essential in vivo, one would predict that the *sbh1Δsbh2Δ* mutant would phenocopy *srp102Δ* alleles that are defective in heterodimer formation (Ogg et al., 1998). While our DAP2 reporter experiments demonstrate that *sbh1Δsbh2Δ* cells have distinct translocation kinetics. We propose that the translocation defect in the *sbh1Δsbh2Δ* mutant is not due to the lack of an SRβ GEF activity, but is instead due to a rate-limiting step in recognition of a vacant translocon by the SRP-SR-RNC complex. An interaction between the translocon β-subunit and the SR heterodimer that facilitates recognition of the unoccupied translocon is an attractive explanation for the network of synthetic interactions observed here between the soluble SR, translocon β-subunits and the Ssh1p complex.

**Adaptation of yeast strains to loss of the SRP-targeting pathway**

Several of the double mutants strains described here (e.g. *ssh1Δsrp102ΔTMD*) share features with more conventional SRP pathway mutations, including an increased frequency of the rho- phenotype and a reduction in protein synthesis capacity and protein elongation rate. Yeast cells that lack SRP or the SR (e.g. *srp54Δ, SRAΔ* or *SRβΔ*) undergo an adaptation process that allows growth in the absence of the SRP-targeting pathway. Adaptation has been most thoroughly characterized using cells that express the *srp102(K51I)* ts mutant or a dominant negative allele of SRP54 (Mutka and Walter, 2001). Adaptation to loss of the SRP pathway involves substantial, yet transient, overexpression of cytosolic chaperones, and long-term
repression of gene products required for protein synthesis (Mutka and Walter, 2001). The reduction in protein synthesis capacity and protein elongation rate reduces the substrate load for the translocation channels thereby allowing a bypass of the SRP-targeting pathway (Mutka and Walter, 2001). However, an open question was whether cells that lack the SRP-targeting pathway retain a vestige of a cotranslational translocation pathway, or instead transport all substrates through the heptameric Sec complex. Here we addressed this question using the Dap2 series of UTA reporters. Interestingly, yeast strains that lack the SR transport roughly one third of Dap2 precursors by a cotranslational translocation pathway. Cotranslational translocon gating in srp101Δ cells is slower than in wild type cells as revealed by a wider gating window. An SRP-independent cotranslational pathway has been investigated using the mammalian cell-free system, where it has been shown that RNCs assembled by translation of a termination codon-deficient mRNA can engage translocons on ribosome-stripped membranes (Jungnickel and Rapoport, 1995; Lauring et al., 1995; Raden and Gilmore, 1998; Wiedmann et al., 1994). Ribosome-Sec61 and signal sequence-Sec61 interactions would promote translocon gating by this SRP-independent targeting pathway. Alternatively, an 80S ribosome that was pre-bound to a translocation channel could initiate translation of a mRNA encoding a secretory protein (Potter and Nicchitta, 2000). These SRP-independent targeting mechanisms may allow cotranslational integration of hydrophobic membrane proteins that may not be optimal substrates for the posttranslational translocation pathway due to their potential for aggregation in the cytosol.
CHAPTER IV

Discussion

The ribosome-channel interaction

In this study, we isolated a novel class of sec61 point mutants with substitutions in loop 6 (L6) and loop 8 (L8), which selectively interfere with cotranslational translocation in S. cerevisiae. The phenotypes of these sec61 mutants were only revealed in an ssh1 null background, which may partially explain why this class of sec61 mutants had not been identified previously.

It has been demonstrated that the protease-accessible cytosolic segments (Sec61\(\alpha\) loop 6, Sec61\(\alpha\) loop 8 and Sec61\(\beta\)) of the Sec61 heterotrimer are involved in SRP-dependent translocation and contribute to the ribosome-channel interaction (Raden et al., 2000; Song et al., 2000). Nonetheless, the specific residues in these segments corresponding to the function had not been identified, and the ribosome binding sites on Sec61p had not been mapped with precision.

The X-ray structure of SecYEB\(\beta\) had not been solved when we started this project. Residues in Sec61 loop6 and loop8 were selected for site-directed mutagenesis based on sequence conservation and predicted membrane topology. Given the fact that ribosomal proteins (L25, L35) and ribosomal RNA (25S/28S rRNA) both contribute to the binding of the ribosome to the channel (Beckmann et al., 2001; Morgan et al., 2002), conserved positively charged residues were selected for mutagenesis.
Results from our mutagenesis experiments were interpreted in the context of the recently resolved X-ray crystal structure of archae SecYEβ complex. Point mutations at residues in L6 (K273, R275) or L8 (K405, R406) that caused severe growth rate and cotranslational translocation defects were found to be located in flexible segments at the tips of the loops and to be oriented towards the cytosol. These four critical residues (K273, R275, K405 and R406) together form a cytosolic domain with a diameter of ~20Å at the surface of the translocon, which is very similar to the diameter of the ribosome-channel connections observed by electron microscopy (Morgan et al., 2002). Considering that 3-4 Sec61p complexes form one translocon, our results agree with the idea of four connections between the ribosome and one translocon (Beckmann et al., 2001).

A significant reduction of ribosome-binding affinity was observed in L8 mutants. The charge-reversal substitution of a single residue in L8 (e.g., R406E) totally eliminated the ribosome binding activity of the Sec61 complex. The ribosome-channel interaction is not obligatory for signal sequence insertion into the translocon (Raden et al., 2000), which may explain why sec61R406E does not completely block the cotranslational translocation pathway.

Unlike L8 sec61 mutants, an in vitro defect in ribosome binding activity was not observed for the L6 sec61 mutants. The intact loop 6 in Sec61α was found to be crucial for protein translocation across the ER membrane by segments complementary experiment based on the fact that co-expression of the segment containing TM1-6 and the segment containing TM7-10 of Sec61α can not rescue the sec61 null mutation
What is the role of L6 in the cotranslational translocation pathway? One interpretation is that although L6 of Sec61p does not appear to be critical for binding a non-translating ribosome, it may be important for binding of the ribosome-nascent chain complex in vivo. Since Sec61p is opened by the signal sequence during the translocation reaction, L6 may have a different conformation when bound to an RNC relative to the closed form when bound to a non-translating ribosome. It is reasonable to speculate that the opening of the channel causes a conformational change that may render loop 6 more exposed to the ribosome.

Other than serving as a receptor for the ribosome, a vacant Sec61 channel has also been proposed to interact with SR during the SRP-SR mediated post-targeting process to insure that the signal sequence is directly inserted into a channel upon release from SRP. After RNCs are targeted to the ER membrane via the interaction between SRP and SRP receptor, the subsequent signal sequence release from SRP is translocon dependent. Proteoliposomes containing purified SRP receptor bind to the SRP-RNC complex, but do not promote signal sequence release even in the presence of GTP or Gpp(NH)p (Song et al., 2000). The accumulation of post-targeting intermediates (SR-SRP-RNC) in the absence of translocon indicated that a vacant channel plays an important role in the post-targeting step. Thus, how to find an unoccupied translocon is a crucial question in cotranslational translocation pathway.

We do not exclude the possibility that loop 6 in Sec61p may function as a marker for an unoccupied translocon by providing the contact sites for RNC-SRP-SR and promoting the translocon-dependent signal sequence releasing from SRP. Other than
L6 in Sec61p, the translocon β subunit would also be a good candidate.

**The SR-channel interaction**

Bacterial SR (FtsY), which lacks a transmembrane domain, partially cofractionates with the bacterial inner membrane (Valent et al., 1998). The membrane-bound FtsY was found to directly associate with the SecYEG translocon (Angelini et al., 2005). Eukaryotic SRP receptor is anchored to the ER membrane by the TM domain of SRβ. However, this TM domain is not required for the function of SRβ (Ogg et al., 1998), suggesting the existence of a binding partner for SR on the ER membrane. Conceivably, the Sec61 complex and the Ssh1 complex are the best candidates to provide the contact sites for SR in yeast.

A sensitized yeast strain expressing the soluble SR was constructed by deletion of the transmembrane domain of SRβ to investigate the possible interaction between the SR and translocons. Similar to the phenotypes of other yeast strains with defects in SRP pathway components, the cotranslational translocation defect caused by the *srp102ΔTMD* mutation is transient. After adaptation, apparently normal kinetics for translocon gating were observed in the *srp102ΔTMD* strain relative to wild type cells, indicating that the soluble SR can efficiently transfer RNCs to the translocon through an interaction with ER membrane components.

Since the Sec61 complex is thought to be the predominant translocon in yeast, we had anticipated that the Sec61 complex would play a more significant role than the Ssh1 complex in the SR-meditated post-targeting step in the translocation reaction.
However, our results suggest the opposite conclusion. Disruption of *SSH1* in the *srp102ΔTMD* strain transiently blocks the cotranslational pathway, as shown by the severe defect in the delivery of the RNC to the translocon in assays using the Dap2 UTA reporters. Growth rate and cotranslational translocation defects caused by the *srp102ΔTMD* mutation are suppressed by the increased expression of the soluble SR or the Ssh1p translocon, suggesting an SR-Ssh1 complex interaction. Furthermore, synthetic growth defects were caused by mutations in the Ssh1p complex (*ssh1EE* or *sbh2Δ*) in the *srp102ΔTM* strain but not by mutations in the Sec61p complex (*sec61EE* or *sbh1Δ*), indicating that the soluble SR prefers the Ssh1p complex to the Sec61 complex.

Although the Ssh1 complex was proposed to be an auxiliary translocon, the Ssh1 complex and the Sec61 complex have a similar affinity for non-translating ribosomes (Prinz et al., 2000b) and almost equal abundance in the ER membrane (Finke et al., 1996). Considering that some Sec61p hetrotrimers are incorporated into the heptameric SEC complex, the channels committed to the cotranslational translocation reaction may consist of more Ssh1 complexes than Sec61 complexes. Ssh1p is previously proposed as a minor ribosome receptor on the yeast ER membrane since ribosome-depleted membrane purified from Ssh1 knock-out strain was found to show no obvious reduction of ribosome binding sites relative to that purified from wild type strain (Prinz et al., 2000b). Yet, the unchangeable ribosome binding sites observed in Ssh1 null strain can be explained by the increased Sec61p expression level (Fig).

It is not clear why the Ssh1 complex is the favored partner for the soluble SR
even though an *ssh1* disruption in a wild type background only causes mild growth and translocation defects. One possibility is that when anchored to the ER membrane, the SRP receptor is in the proximity of both the Ssh1 complex and the Sec61 complex (Wittke et al., 2002). Depletion of the Ssh1 complex may be rescued by an increased interaction between the SR and the Sec61 complex. Indeed, expression of the Sec61 heterotrimer is elevated in the *ssh1Δ* mutant. Nonetheless, the detailed mechanism of Ssh1 translocon-mediated cotranslational translocation is still unclear.

Based on the X-ray structure of SecYEβ, a single copy of Sec61 heterotrimer is proposed to serve as the channel, while the native translocon is formed by three to four Sec61 heterotrtrimers (Van den Berg et al., 2004). This model was supported by recent crosslinking experiments, where it was shown that the signal sequence and mature region of a precursor simultaneously contact the SSB site and transport pore of a single SecY molecule (Osborne and Rapoport, 2007). Whether the Ssh1 heterotrimer can assemble with a Sec61 heterotrimer to form a translocon remains unknown. While the Ssh1p heterotrimer has not been found to coimmunoprecipitate with the Sec61 heterotrimer, it can be explained by detergent-mediated conversion of the oligomeric translocons into Sec61p or Ssh1p heterotrimers prior to immunoprecipitation.

Although the interaction between FtsY and the SecYEG complex can be stabilized by blocking the GTPase activity of FtsY (Angelini et al., 2006), a physical interaction between the SR and an eukaryotic translocon has not been detected by
using biochemical approaches like protein crosslinking, co-immunoprecipitation and cosedimentation experiments in the presence or absence of GppNHp (data not shown). A potential dynamic interaction between the SR and the translocon could be explored using the biosensor. We cannot exclude the possibility that an additional membrane or cytosolic component may be required for this interaction.

Yeast cells have two Sec61β homologues, Sbh1p in the Sec61 complex and Sbh2p in the Ssh1 complex. Unlike the other two subunits of the translocon, Sec61β is not essential. However, the \( \text{sbh1} \Delta \text{sbh2} \Delta \) double mutant exhibits a severe cotranslational translocation defect. A synthetic growth rate defect was only observed in the \( \text{sbh2} \Delta \text{srp102} \Delta \text{TMD} \) strain, but not in \( \text{sbh1} \Delta \text{srp102} \Delta \text{TMD} \). This finding not only supports our hypothesis that soluble SR prefers the Ssh1 complex to the Sec61p complex; but also sheds light on the possible role of translocon β subunit in an SR-meditated post-targeting reaction.

While the prokaryotic SR (FtsY) does not have an SRβ homologue, deletion of yeast SRβ causes a complete block in the SRP-dependent pathway. Other than anchoring SRα to the ER membrane, the role of SRβ in the cotranslational reaction is not well understood. The cytosolic GTPase domain, but not the TM domain is required for the function of SRβ (Ogg et al., 1998). Although GTP binding of SRβ was proposed to regulate the release of the signal sequence from SRP54 in the presence of an unoccupied Sec61 channel (Fulga et al., 2001), this report is controversial (Mandon et al., 2003). A different mode of interaction has been observed between the GTPase domain of yeast SRβ and the yeast translocon
β-subunits (Sbh1p or Sbh2p). The *E. coli*-expressed cytosolic domains of Sbh1p or Sbh2p stimulate dissociation of GDP from yeast SRβ (Helmers et al., 2003). Furthermore, opposite to the Sec61 hetrotrimer, the Sec complex was found to lose the nucleotide exchange ability for SRβ (Helmers et al., 2003), which is interpreted by that Sec61 β-subunit is occluded by association with the Sec62/Sec63 complex.

Based on the SRβ-SRX (first 158 residues of SRα) structure, the GTP molecule observed in the active site of SRβ is actually buried by the interface between SRX and SRβ (Schwartz and Blobel, 2003), suggesting that SRα/SRβ heterodimer dissociation is a prerequisite for GTP hydrolysis and nucleotide exchange in the GTPase domain of SRβ. Since the in vivo or in vitro dissociation of the SRα/SRβ dimer has not yet to be observed, GTP hydrolysis by SRβ during the translocation reaction appears unlikely.

Therefore, instead of serving as a GEF for SRβ, the β subunit of the translocon is more likely to cooperate with the β subunit of SR to regulate the signal sequence release from SRP to facilitate RNC docking onto the channel. Further studies are required to address whether the interaction between the β subunit of translocon and the β subunit of SR is functionally responsible for the SR recognition by translocon. Although no synthetic defects were observed in *srp102ΔTMD sec61R275E* or *srp102ΔTMD ssh1R278E* strains (data not shown), we cannot exclude the possibility that other residues in the cytosolic loops of Sec61p or Ssh1p may also contribute to the SR recognition.
CHAPTER V

Experimental Procedures

Plasmid and Strain Constructions for the sec61 L6 and L8 mutants

The strains used to express the sec61 L6 and L8 mutants are derived from BWY12 (MATα, trp1-1, ade2, leu2-3,112, ura3, his3-11, can1 sec61::HIS3[pBW7]; provide by C. stirling). The SSH1 gene in BWY12 was disrupted to obtain RGY400. PCR using the plasmid pFA6a-KanMX4 as a template (Wach et al., 1994) was used to generate a DNA fragment containing a kanamycin resistance gene flanked by 5′ (nucleotides -203 to -1) and 3′ (nucleotides 1224 to 1470) regions from the SSH1 gene. Following transformation of BWY12, G418 resistant colonies were selected and disruption of the SSH1 gene was confirmed by PCR. Transformation of RGY400 and BWY12 with pGAL-Kar2GFP (derived from pDN182) yielded RGY401 and RGY402 respectively.

An N-terminal His6-FLAG tag was added to Sbh1p using a two-step PCR-based gene disruption method. The SBH1 gene in RGY400 was disrupted using a linear DNA fragment encoding the hygromycin B resistance gene (HPH) derived from plasmid pAG32 (Goldstein and McCusker, 1999) flanked by 5′ (-198 to -1) and 3′ (249 to 528) SBH1 noncoding regions. Integration of the disruption construct into the SBH1 locus to obtain RGY403 was confirmed by PCR analysis of hygromycin B resistant transformants. RGY403 was transformed with a linear DNA fragment
containing the following segments: (a) the 5′ noncoding region of the SBH1 gene, (b) the Sbh1p coding sequence with a His6-FLAG tag inserted after the initiation codon, (c) the heterologous TRP1 gene from K. lactis (derived by PCR amplification of the plasmid pYM3 (Knop et al., 1999) and (d) the 3′ SBH1 noncoding segment. Integration of the construct into the SBH1 locus to obtain RGY404 was confirmed by PCR analysis of trp+ hygromycin B sensitive transformants. Expression of epitope-tagged Sbh1p was confirmed by protein immunoblotting.

**Cassette Mutagenesis of Sec61**

Restriction sites (PstI to SacI, XhoI to SalI) in the polylinker of pRS315 were removed by sequential rounds of double digestion, filling in with T4 DNA polymerase followed by blunt end ligation and plasmid isolation. The resulting plasmid is designated pRS315ΔRS. Silent unique restriction sites for SalI (bp 810 relative to the ATG initiation codon), SacII (bp 825), SpeI (bp 862) and AatII (bp 901) were introduced into the coding sequence of SEC61 by PCR amplification of the plasmid pBW11 (Wilkinson et al., 1996b) using a QuickChange mutagenesis kit (Stratagene) and synthetic oligonucleotide primers. Digestion of the resulting plasmid with HindIII yielded a 3.2 kb fragment which was cloned into the HindIII site of pRS315ΔRS to obtain the plasmid designated pZCSEC61-L6. Unique restriction sites for BamHI (bp 1111), BglII (bp 1145), XhoI (bp 1163), NcoI (bp 1196), SacI (bp 1241) and PstI (bp 1263) were introduced into plasmid pZCSEC61-L6 by the same
procedure to obtain the plasmid designated pZCSEC61-L6L8. The NcoI site in the L8 coding region causes a substitution (G399A) at a non-conserved residue in Sec61p (see Fig. 2.2A). The G399A mutation does not cause growth or translocation defects (not shown).

Oligonucleotides that were 32-fold degenerate at a single codon (NNG/C on the sense strand, G/CNN on the nonsense strand) were designed to span the gap between unique restriction sites in pZCSEC61-L6 or pZCSEC61-L6L8. The oligonucleotides were annealed and ligated to double-digested pZCSEC61-L6 or pZCSEC61-L6L8 to introduce mutations in L6 or L8 respectively. E. coli (DH5α) was transformed with the resulting plasmid pools, and 40-60 transformants were selected for plasmid isolation and DNA sequencing.

RGY401, RGY402 and RGY404 were transformed with the pZCSEC61-L6 or pZCSEC61-L6L8 derivatives, and Leu+ Trp+ prototrophs were selected on synthetic defined media (SD) plates supplemented with uracil and adenine. Several transformants for each point mutant were streaked onto 5-fluoroorotic acid plates and incubated for 2 d at 30°C to select colonies that had lost pBW7 (SEC61, URA3).

Yeast L6 and L8 sec61 mutants were maintained on SEG media (synthetic minimal media containing 2% ethanol and 3% glycerol) to select against ρ- cells.

**Plasmid and strain constructions for the srp102ΔTM mutants and sbh1, sbh2 null mutants**

A hygromycin B resistance (HPH) gene flanked by 5’ (bp, -512 to -1) and 3’ (bp,
736-941) regions from the SRP102 gene was used to transform the diploid yeast strain BWY100 (MATa/MATα, trp1-1/trp1-1, ade2/ade2, leu2-3,112/leu2-3,112, ura3/ura3, his3-11/his3-11, can1/can1, sec61::HIS3/SEC61; provided by C. Stirling). Hygromycin B resistant colonies were selected, transformed with pJY01 (SEC61, SRP102-HA, URA3), and induced to sporulate. Disruption of the SRP102 gene in the Hph+ His+ Ura+ haploid progeny (YJY101) was confirmed by PCR. The SSH1 gene in YJY101 was disrupted as described above to obtain the strain YJY102.

To construct plasmids containing srp102ΔTMD under control of its own promoter, the coding sequence of amino acids 3-24 of Srp102p were deleted using recombinant PCR. A SacI-EcoRI digested fragment containing the 5' segment (bp, -440 to 6), coding sequence and 3' segment (bp, 73 to 1945) of the SRP102 gene was inserted into SacI-EcoRI digested pRS314 to obtain pJY209 (srp102ΔTMD TRP1). Deletion of the transmembrane region of SRP102 was confirmed by sequencing. A SacI-KpnI fragment encoding srp102ΔTMD was obtained by digestion of pJY209 and ligated into SacI-KpnI digested pRS424 (Christianson et al., 1992) to produce the high copy plasmid pJY210. An EcoRI-BamHI digested DNA fragment (bp, -480 to 3101) from the SRP101 gene was inserted into EcoRI-BamHI digested pJY210 to obtain the high copy plasmid encoding the soluble SR, pJY211.

A SacI-XhoI digested PCR product corresponding to the SSH1 gene (bp, -442 to 2710) was inserted into SacI-XhoI digested pRS315 to construct a plasmid encoding wild type SSH1. Oligonucleotide primers containing point mutations (R278E or R411E) were used in recombinant PCR to produce the ssh1EE mutant. Mutations
were confirmed by sequencing. An Apa1-Sac1 DNA fragment encoding the three subunits of the Ssh1 complex (Ssh1p, Sbh2p and Sss1p) was produced using recombinant PCR using yeast DNA (for SSS1) or plasmid DNA (SSH1 and SBH2) as templates. The final PCR product (ApaI - SSH1 (bp, -442 to 2710) - XhoI - SBH2 (bp, -600 to 810) - BamHI- SSS1 (bp, -330 to 563) -SacI) was cloned into Apa1-Sac1 digested pRS425 to obtain pJY213.

An XhoI-XbaI digested PCR product corresponding to the SBH1 gene (bp, -385 to 685) was ligated into Xhol-XbaI digested pRS316 to obtain pJY301. A SacI-XhoI fragment encoding SBH1 was obtained by digestion of pJY301 and inserted into SacI-XhoI digested pRS426 to produce the high copy plasmid pJY302. To construct plasmids expressing wild type SBH2 or SBH2 truncations, a XhoI-HindIII digested PCR product corresponding to the 5’ UTR of SBH2 (bp, -598 to -1) and HindIII-XbaI digested PCR products containing the encoding sequence of SBH2 wild type or truncations (Δ11, Δ37, Δ54) and 3’ UTR (bp, 1169 to 1671) were ligated and inserted into Xhol-XbaI digested pRS316 to obtain pJY300, pJY311, pJY337, pJY354 respectively.

A plasmid shuffle procedure (Sikorski and Boeke, 1991) was used to replace plasmid-born wild type genes (e.g. SRP102 plus SEC61) with mutant alleles (e.g. srp102ΔTMD) or overexpression plasmids (2µ srp102ΔTMD). For example, the plasmids pJY209 (srp102ΔTMD) and pBW11 (SEC61, LEU2; provided by C. Stirling) were co-transformed into YJY101 or YJY102. The Trp⁺ Leu⁺ Ura⁺ prototrophs were selected on SD media containing 5-fluoroorotic acid (5-FOA), giving rise to YJY1209.
and YJY2209 respectively. Plasmids expressing wild type SSH1, ssh1EE, or the Ssh1 complex were co-transformed with pJY022 (SEC61, srp102ΔTMD, TRP1) into YJY102 and the Trp⁺ Leu⁺ Ura⁻ prototrophs were selected on SD media containing 5-FOA.

Yeast strain ZCY101 (MATα, trp1-1, ade2, leu2-3,112, ura3, his3-11, can1 sec61::HIS3[pBW11]) expressing full-length wild type Sec61p was derived from BWY12. The SBH1 gene in ZCY101 was disrupted as previously described to obtain YJY103. A DNA fragment containing the HPH gene flanked by 5’ (bp, -545 to -1) and 3’ (bp, 268 to 758) regions from the SBH2 gene was generated and used to transform ZCY101 and YJY103, giving rise to sbh2Δ strain YJY104 and sbh1Δ sbh2Δ strain YJY105 respectively. The SBH1 or SBH2 genes were disrupted in YJY1209 to obtain YJY3209 (sbh1Δ) and YJY4209 (sbh2Δ) respectively using the same method.

**Growth curves and frequency of petite phenotype**

For serial dilution experiments, yeast strains were grown in SEG media at 30°C to mid-log phase. After dilution of cells to 0.1 OD at 600 nm, 5 µl aliquots of 10-fold serial dilutions were spotted onto YPD plates that were incubated at 30 or 37°C for 2-3 days. RGY401 (ssh1Δ) did not show a colony sectoring phenotype when grown on YPD plates, in contrast to a previous report (Wilkinson et al., 2001).

For liquid growth rate experiments, yeast cells were first grown in SEG media to mid-log phase at 30°C. The cells were collected, diluted to 0.1 OD at 600 nm in
fresh YPD media, and allowed to grow for 10 hours at 30°C. Aliquots were taken every hour to measure $A_{600}$.

For petite percentage test, yeast cells grown to mid-log phase in YPEG media were harvested by centrifugation and transferred to YPD media for subsequent growth at 30°C. Cells were diluted into fresh media when the $A_{600}$ reached 0.8-1 OD. After about 20 generations of growth, cells were diluted and plated onto YPD plates. After two days, over hundreds of colonies were tested for respiratory competence by replica plating colonies onto YPD and YPEG plates.

**Immunoprecipitation of radiolabeled proteins and protein immunoblots**

Yeast strains bearing pZCSEC61-L6 or pZCSEC61-L6L8 derivatives were transformed with the URA3 marked plasmid pDN317 which encodes DPAPB-HA under control of the glyceraldehyde 3-phosphate dehydrogenase promoter (Ng et al., 1996). DPAPB-HA expression is roughly 10-fold greater than endogenous DPAPB.

After growth at 30°C in SEG media to mid-log phase (0.4 to 0.6 OD at 600 nm) yeast were collected by centrifugation and resuspended in SD media and grown for additional 4 h or 24 h at 30°C. Yeast cells were collected by centrifugation and resuspended in fresh SD media at a density of 4 $A_{600}$/ml and pulse-labeled for 7 min with Tran-35S-label (100 µCi/OD). In pulse-chase experiments, the chase was initiated by adding unlabeled cysteine and methionine to a final concentration of 0.6 mg/ml. Radiolabeling experiments were terminated by the addition of an equal volume of ice-cold 20 mM NaN₃, followed by freezing in liquid nitrogen. Rapid
lysis of cells with glass beads and immunoprecipitation of yeast proteins was done as described (Rothblatt and Schekman, 1989). Immunoprecipitated proteins were resolved by SDS-PAGE and detected with a Bio-Rad FX molecular Imager. Spheroplasts, prepared as described below from cells grown in SD media for 4 h at 30°C, were allowed to recover for 15 min in SD media adjusted to 1.2 M sorbitol prior to pulse-labeling.

Total protein extracts were prepared as described (Arnold and Wittrup, 1994) from cells after 4 h or 24 h of growth at 30°C in SD media. Aliquots of the protein extracts were digested with Endo H (New England Biolabs) prior to CPY immunoblots. The protein concentration in extracts was determined using the Bio-Rad DC protein assay kit (Bio-Rad) so that identical protein amounts (50 μg/lane) were analyzed on Sec61p immunoblots. Proteins were resolved by PAGE in SDS, transferred to PVDF membranes, and incubated with polyclonal or monoclonal antibodies. Peroxidase-labeled second antibodies were visualized using an ECL Western blotting detection kit (Amersham Corp.).

Degradation of CPY*HA, expressed from the plasmid pDN431, was evaluated using a cycloheximide-chase protocol (Spear and Ng, 2003). Cell extracts prepared at 30 min intervals after adjustment of the culture to 100 μg/ml cycloheximide were resolved by SDS-PAGE for protein immunoblot analysis using anti-HA monoclonal antibodies. Densitometric scans of protein immunoblots were used to determine the half-life for p1 CPY*HA.
Protein synthesis rate

Yeast strains were transformed with pDN317 (Ng et al., 1996) that encodes DPAPB-HA under control of the glyceraldehyde 3-phosphate dehydrogenase promoter. Cells were grown for 24 h in SD media and radiolabeled for 5 min with Tran-\(^{35}\)S-label as described above. At frequent time points (30 s to 5 min), cells (4 A\(_{600}\)) were removed and the labeling reaction terminated by adding an equal volume of chilled 20 mM NaN\(_3\) plus unlabeled cysteine and methionine to 0.6mg/ml, followed by freezing in liquid nitrogen. Total incorporation of Tran-\(^{35}\)S-label into protein was determined by TCA precipitation. Radiolabeling of full-length DPAPB-HA was determined by immunoprecipitation, followed by SDS-PAGE and detection with a BioRad FX molecular imager. Extrapolation of the linear part of the incorporation curves to the abscissa yielded a lag time for incorporation of radiolabel into total protein or DPAPB-HA. The difference between these two lag times equals half the time required to synthesize DPAPB-HA (Horwitz et al., 1969). The protein synthesis rate (amino acid residues per second) was calculated by dividing the amino acid length of DPAPB-HA by the synthesis time.

Cell fractionation and purification of Sec61p complexes

Yeast cells (5 gm) grown in YPD media at 25°C to a density of 1.8 OD at 600 nm were collected by centrifugation, chilled to 4°C, adjusted to 10 mM NaN\(_3\) and converted to spheroplasts with Zymolase (ICN) as described (Walworth and Novick, 1987). Spheroplasts were centrifuged for 10 min at 0.5 Kg, and broken by
resuspension in 10 ml of 10 mM triethanolamine-acetate pH 7.2, 0.8 M sorbitol, 1mM EDTA using a serological pipette. Microsomes were isolated from spheroplast lysates as described (Goud et al., 1988). Puromycin high salt-stripped membranes (PK-RM) were prepared from yeast microsomes as described (Görlich and Rapoport, 1993). Spheroplast lysates from sec61L6DDD cells were fractionated as described (Gerrard et al., 2000).

Purification of the Sec61 complex was facilitated by construction of a strain (RGY404) that expresses His6-FLAG-Sbh1p. The plasmid shuffle procedure was repeated to allow purification of the L6 and L8 sec61 mutants from RGY404 derivatives. The Sec61p complex was purified from digitonin-solubilized PK-RM by sequential chromatography on Con-A Sepharose, Ni-NTA agarose, Q-Sepharose fast flow and SP-Sepharose fast flow using chromatography conditions described previously (Panzner et al., 1995) and standard chromatography methods for Ni-NTA agarose. The SEC complexes were resolved from Sec61p heterotrimers by Con-A chromatography. Purification of Sec61 heterotrimers was monitored by coomassie blue staining after SDS-PAGE and by protein immunoblot analysis using anti-FLAG and anti-Sec61p antibodies. Point mutations in Sec61p do not destabilize the Sec61p-Sbh1p-Sss1p heterotrimer. The Sec61p-proteoliposomes were prepared as described (Song et al., 2000).
Ribosome binding to yeast PKRM, Sec61 proteoliposomes or purified Sec61p complex

Ribosomes were isolated from wild type yeast as described (Beckmann et al., 1997). Loosely associated proteins were separated from 80S ribosomes by two sequential centrifugations through a high salt-sucrose cushion followed by sucrose density gradient (10-30%) centrifugation and resuspension in 50 mM triethanolamine-acetate pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)₂. Binding of ¹²⁵I-labeled ribosomes to PK-RM or Sec61p-proteoliposomes was assayed as described previously (Mandon et al., 2003; Raden et al., 2000). Membrane or proteoliposome-bound and unbound ribosomes were separated by gel filtration chromatography (Raden et al., 2000). The cosedimentation assay to measure binding of purified Sec61p heterotrimers to ribosomes in detergent solution was performed as described (Prinz et al., 2000a).

Ubiquitin translocation assay (UTA)

The Dap2 series of UTA reporters (Dap2-49 to -265) have been described previously (Cheng and Gilmore, 2006). Cells expressing Dap2 reporters were radiolabeled with Tran⁻³⁵S-label as described above. The intact reporters and the Ura3-HA fragments were immunoprecipitated with anti-HA monoclonal antibodies. For experiments conducted in the presence of cycloheximide (+Ch), the cells were incubated with SD media containing 0.2 µg/ml cycloheximide for 10 min at 30°C prior to radiolabeling. The distribution of methionine and cysteine residues in the
intact UTA reporter and the Ub-Ura3-HA fragment was determined and the cleavage percentage was calculated as described (Cheng and Gilmore, 2006).

**FM4-64 staining**

All strains were grown at 30°C to 0.8-1.6 OD at A_{600} in SEG media. For wild type and the sbh1Δ sbh2Δ strain, aliquots of cells were shifted to 37°C for an additional 1 or 2 h incubation before staining. Cells were collected by centrifugation and resuspended at 20 A_{600}/ml in YPD media containing 20 μm FM4-64. FM4-64 staining was performed by shaking at 30°C for 15 min as described (Vida and Emr, 1995). The free dye was removed by centrifugation. Cells were resuspended in fresh YPD media at 10 A_{600}/ml and incubated at 30°C for an additional 3 h. Cells were viewed with a QIMAGING Retiga 1300 digital camera mounted on an Olympus BX51 upright microscope through a UplanFI ×100/1.3 oil-immersion objective, using differential interference contrast (DIC) or U-MWIG2 mirror/filter for FM4-64. Images were taken with Qcapture Pro version 5.1.1.14.
CHAPTER VI

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