# Efficient secretion of small proteins in mammalian cells relies on Sec62-dependent posttranslational translocation

**Asvin K. K. Lakkaraju[a,*,†], Ratheeshkumar Thankappan[a,*], Camille Mary[a,‡], Jennifer L. Garrison[b,§], Jack Taunton[b], and Katharina Strub[a]**
[a]Department of Cell Biology, Sciences III, University of Geneva, CH-1211 Geneva 4, Switzerland; [b]Howard Hughes Medical Institute, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158

**ABSTRACT Mammalian cells secrete a large number of small proteins, but their mode of translocation into the endoplasmic reticulum is not fully understood. Cotranslational translocation was expected to be inefficient due to the small time window for signal sequence recognition by the signal recognition particle (SRP). Impairing the SRP pathway and reducing cellular levels of the translocon component Sec62 by RNA interference, we found an alternate, Sec62-dependent translocation path in mammalian cells required for the efficient translocation of small proteins with N-terminal signal sequences. The Sec62-dependent translocation occurs posttranslationally via the Sec61 translocon and requires ATP. We classified preproteins into three groups: 1) those that comprise ≤100 amino acids are strongly dependent on Sec62 for efficient translocation; 2) those in the size range of 120–160 amino acids use the SRP pathway, albeit inefficiently, and therefore rely on Sec62 for efficient translocation; and 3) those larger than 160 amino acids depend on the SRP pathway to preserve a transient translocation competence independent of Sec62. Thus, unlike in yeast, the Sec62-dependent translocation pathway in mammalian cells serves mainly as a fail-safe mechanism to ensure efficient secretion of small proteins and provides cells with an opportunity to regulate secretion of small proteins independent of the SRP pathway.**

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*These authors contributed equally.

Present addresses: †Global Health Institute, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland; ‡Hôpital Cantonal Universitaire, CH-1211 Geneva 4, Switzerland; §Howard Hughes Medical Institute, Laboratory of Neural Circuits and Behavior, The Rockefeller University, New York, NY 10065.

Address correspondence to: Katharina Strub (Katharina.Strub@unige.ch).

Abbreviations used: ATP, adenosine triphosphate; Cal, calnexin; CT08, cotransin 08; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, hemagglutinin; Luc, luciferase; PolyUb, polyubiquitination; SRα, SRP receptor alpha subunit; SRβ, SRP receptor beta subunit; SRP, signal recognition particle; SS, signal sequence; TRAM, translocating chain-associating membrane protein; TRAPα, translocon-associated protein subunit alpha; VSV, vesicular stomatitis virus; WB, Western blot.

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## INTRODUCTION

The secretory pathway ensures that the newly synthesized proteins are properly targeted to their final destination to sustain cell structure and function. The first step in this pathway is the entry of proteins with N-terminal signal sequences into the endoplasmic reticulum (ER). The preproteins can enter the ER either cotranslationally or posttranslationally (for a review, see Rapoport, 2007; Cross *et al*., 2009). In the cotranslational pathway the signal sequence emerging from the ribosome is recognized by the signal recognition particle (SRP), resulting in an arrest of nascent chain elongation, followed by the transfer of the complex to the protein-conducting channel, the Sec61 complex, via the SRP receptor (SR; for a review, see Saraogi and Shan, 2011). The elongation arrest function, which is mediated by the SRP subunit SRP14, is essential for efficient translocation in mammals and yeast (Mason *et al*., 2000; Lakkaraju *et al*., 2008), indicating that nascent chains should not exceed a maximal length to remain translocation competent.

The posttranslational pathway for signal sequence–bearing proteins is abundantly active in yeast and bacteria. In yeast,

ATP-dependent posttranslational translocation was demonstrated using cell-free translation/translocation assays (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters *et al.*, 1986). Proteins with signal sequences of relatively lower hydrophobicity are preferentially translocated via the posttranslational pathway (Ng *et al.*, 1996). It requires a complex of four membrane proteins—the Sec62/Sec63 complex, comprising Sec62, Sec63, Sec71 (also named Sec66), and Sec72—in addition to Sec61. Sec62 and Sec63 are essential genes (Deshaies and Schekman, 1989; Deshaies *et al.*, 1991; Rothblatt *et al.*, 1989; Green *et al.*, 1992; Feldheim *et al.*, 1993; Kurihara and Silver, 1993; Panzner *et al.*, 1995). Sec63 plays a role in cotranslational and posttranslational translocation (Brodsky *et al.*, 1995), whereas Sec62 has been specifically associated with SRP-independent translocation in yeast (Deshaies and Schekman, 1989; Ng *et al.*, 1996). Signal sequence recognition most likely occurs by Sec61. However, it may be enhanced or facilitated by Sec62 and Sec71, since all three proteins are in close proximity to the signal sequence at the same time (Dünnwald *et al.*, 1999; Plath *et al.*, 2004).

Homologues of the yeast Sec62 and Sec63 proteins were identified in mammals, and these proteins were found to form complexes with each other and with Sec61 (Daimon *et al.*, 1997; Meyer *et al.*, 2000; Tyedmers *et al.*, 2000). In addition, mammalian Sec62 can bind to ribosomes near the exit tunnel, suggesting that it gained a function as compared with the yeast homologue (Müller *et al.*, 2010).

Mammalian cells secrete a large number of small proteins (Frith *et al.*, 2006) with N-terminal signal sequences, most of which are involved in regulatory dynamics of the cell. These small proteins are expected to be inefficiently recognized by SRP, since the signal sequence may be exposed only briefly, or not at all, before protein synthesis terminates. Cell-free translation/translocation experiments demonstrated that small secretory proteins (<9 kDa) might enter canine microsomes posttranslationally in the presence of ATP and cytosolic factors (Müller and Zimmermann, 1987, 1988). Recently posttranslational translocation was elegantly demonstrated in mammalian cells using biotinylation of the small reporter protein *Hyalophora cecropia* preprocecropin A (ppcecA) as a readout for completion of its biosynthesis in the cytoplasm before translocation. In addition, the chaperone responsible for maintaining small proteins competent for translocation has been identified as calmodulin (Shao and Hegde, 2011a). Another recent study revealed a role of the cytoplasmic ATPase TRC-40 in posttranslational translocation of ppcecA and of two small mammalian proteins, apelin and statherin, into mammalian microsomes (Johnson *et al.*, 2012). TRC-40 was previously identified as a component involved in membrane insertion of tail-anchor proteins (for a review, see Rabu *et al.*, 2009; Shao and Hegde, 2011b). Moreover, a role of Sec62 in posttranslational translocation of ppcecA was indicated by in vitro translocation assays using Sec62-depleted permeabilized mammalian cells as a source of membranes (Lang *et al.*, 2012).

In our study, we reduced cellular levels of components of the SRP pathway and of Sec62 in mammalian cells using RNA interference and studied the effects on the translocation efficiencies of mammalian reporter preproteins of different chain lengths. We identified and characterized a Sec62-dependent posttranslational entry path into the ER of mammalian cells. The Sec62-dependent entry path was required for efficient translocation of all preproteins with chain lengths shorter than 160 amino acids, whereas it failed to ensure efficient translocation of longer preproteins. Sec62-dependent translocation therefore guarantees efficient secretion of small preproteins independent of the SRP pathway.

## RESULTS

### SRP-independent translocation of preproteins into the ER of mammalian cells

We used RNA interference to reduce cellular levels of the endogenous SRP subunit h14 or of the α subunit of SR (SRα) in HeLa cells to impair the SRP pathway (*Materials and Methods*). Where indicated, the h14-knockdown ($14^{k.d.}$) cells were complemented with the expression of the fusion proteins G14 (green fluorescent protein [GFP]–tagged h14) and the mutated version G14A5 (GFP-tagged h14A5). A reduction in cellular h14 levels leads to reduced levels of SRP (Lakkaraju *et al.*, 2007). Expression of h14A5 restores normal SRP levels, but $SRP^{A5}$ lacks elongation arrest activity, and these cells are therefore impaired in the translocation of preprolactin and of other endogenous reporter proteins (Lakkaraju *et al.*, 2008). The knockdown of h14 and SRα did not affect the cellular levels of other ER translocation-associated proteins examined, such as Sec61α and β, TRAPα, Sec62, and TRAM (Figure 1A).

To identify suitable reporter proteins for studying translocation, we sorted the preproteins in the Secreted Protein Database (Chen *et al.*, 2005) according to size (Supplemental Table S1). We selected preproinsulin and pre-CC2 (pCC2) chemokine as reporters (Table 1, pInf, pCC2f), which have chain lengths of 152 and 141 amino acids, respectively. Preproinsulin has been shown to be a bona fide SRP substrate in cell-free assays (Okun *et al.*, 1990). Translocation of both reporters was assayed in $h14^{k.d.}$ and $SR\alpha^{k.d.}$ cells by pulse labeling newly synthesized proteins during 5 min in the presence of proteasome inhibitor (Figure 1, B and C, top). We failed to observe the accumulation of nontranslocated preproinsulin and pCC2 in cells with an impaired SRP pathway, indicating that translocation might occur via an SRP-independent pathway in vivo. As a positive control, we used preprolactin, which has been shown to depend on SRP for translocation in mammalian cells (Lakkaraju *et al.*, 2008). As expected, preprolactin was inefficiently translocated in SRP- and SRα-depleted cells (Figure 1D and Table 1). The $SR\alpha^{k.d.}$ had a stronger negative effect on translocation, consistent with SRα being a rate-limiting factor in the SRP pathway (Lakkaraju *et al.*, 2008). Translocation of pInf and pCC2f was also assayed by monitoring the accumulation of nontranslocated pPL during 8 h in the presence of proteasome inhibitor (MG132, 10 μM) by Western blotting (Figure 1, B and C, bottom). These experiments also failed to reveal translocation defects. On addition of CT08, a selective Sec61 translocon inhibitor (Garrison *et al.*, 2005; Maifeld *et al.*, 2011), to HeLa cells expressing pInf, we clearly observed the accumulation of preproinsulin, demonstrating that preprotein accumulation was detectable and preproinsulin translocation uses the Sec61 channel for ER entry (Figure 1E).

In mammalian cells, fully synthesized translocation-incompetent preproteins are rapidly polyubiquitinated and degraded (Hessa *et al.*, 2011). If preproinsulin is capable of using an alternate translocation pathway in SRP-depleted cells, it should not be polyubiquitinated. We monitored the polyubiquitination status of preproinsulin as compared with preprolactin in cells treated for 8 h with MG132. Cell extracts were immunoblotted and preprolactin and polyubiquitination revealed with anti-FLAG and anti-hemagglutinin (HA) antibodies, respectively. When the SRP pathway was impaired (Figure 1F, left, A5), we observed preprolactin accumulation. The upper band, PL*, represents phosphorylated forms of the protein in the secretory pathway (Kim and Brooks, 1993). pPLf, was strongly polyubiquitinated in $SRP^{A5}$ but not in wild-type cells (Figure 1F, left). In contrast, polyubiquitination of pInf was not detectable even when the samples were only probed with antibodies against ubiquitin (Figure 1F, right), which increased the sensitivity of detection. CT08

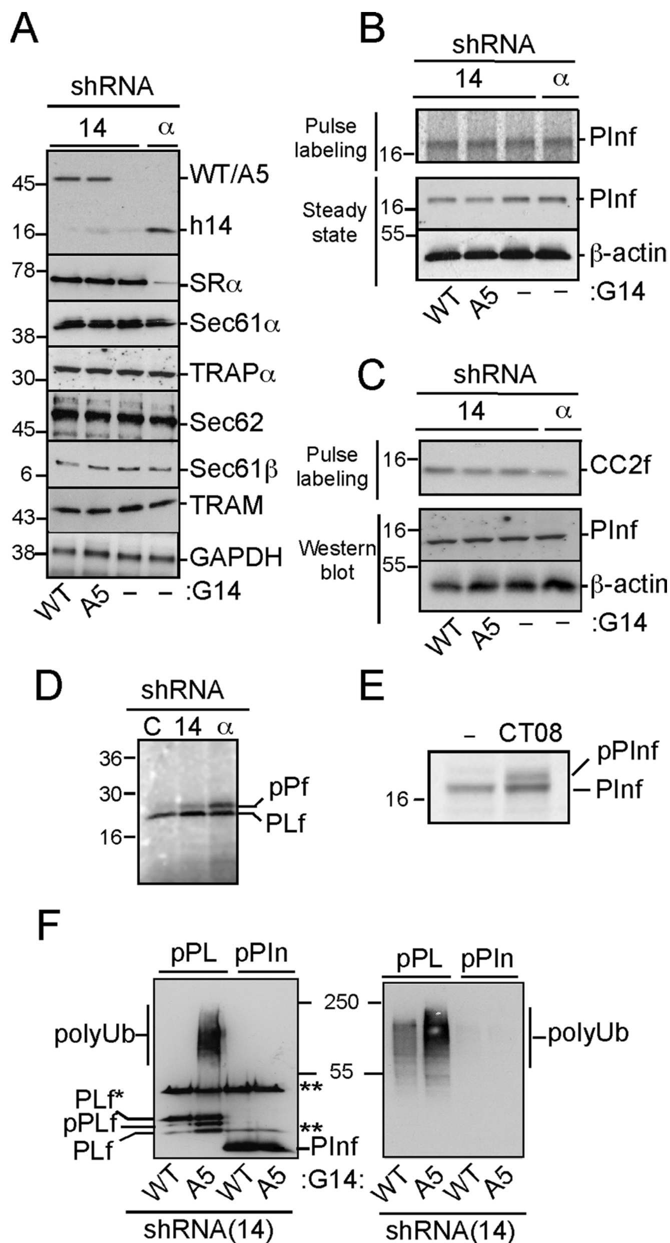


FIGURE 1: Cells with an impaired SRP pathway translocate preproinsulin and pre-CC chemokine 2 efficiently. (A) Cellular levels of different SRP and membrane components after shRNA(14) and shRNA(SRα) expression in HeLa cells. Cell extracts were obtained at 72 h posttransfection. (B, C) Translocation of the reporter proteins indicated was assessed by pulse-labeling experiments (top) and by Western blotting of total cell extracts after treatment with MG132 (10 μm) for 8 h (bottom). In pulse-labeling experiments, cells were treated with 10 μM MG132 during depletion of cold amino acids and during labeling. (B, C) pInf and pCC2f in h14$^{k.d.}$ or SRα$^{k.d.}$ HeLa cells. Wild type and A5: h14$^{k.d.}$ cells expressing the fusion proteins G14 and G14A5, respectively. (D) Pulse labeling of preprolactin in h14$^{k.d.}$ and SRα$^{k.d.}$ HeLa cells as well as control cells expressing shRNA(luc). (E) pInf translocation in wild-type HeLa cells and cells treated with the Sec61 translocon inhibitor CT08. (F) Polyubiquitination of preprolactin and preproinsulin in h14$^{k.d.}$ cells and complemented as in B. Cells also expressed HA-tagged ubiquitin. Immunoprecipitation of the reporter proteins was done with anti-FLAG antibodies. Left and right, Western blots using both anti-FLAG and anti-HA or only anti-HA antibodies, respectively. PLf*, phosphorylated forms of prolactin in transit for secretion; **light and heavy chains of the antibodies.

treatment of cells for 8 h is toxic, and polyubiquitination of preproinsulin could therefore not be assayed in these cells. Polyubiquitination of pInf in translocation-defective cells are shown later in 14$^{k.d.}$/Sec62$^{k.d.}$ cells.

These results were consistent with the translocation of preproinsulin via an alternative pathway.

## Sec62 is necessary in the SRP-independent translocation pathway

As mentioned before, homologues of the yeast Sec62 and Sec63 are conserved in mammalian cells, whereas homologues of Sec71 and Sec72 are absent. Because Sec63 plays a role in cotranslational and posttranslational translocation in trypanosomes and yeast (Brodsky *et al.*, 1995; Goldshmidt *et al.*, 2008), we decided to investigate the role of Sec62 in protein translocation in mammalian cells.

Two different short hairpin RNAs (shRNAs) efficiently reduced the cellular levels of Sec62 starting at 72 h posttransfection (Figure 2A, left and right). In one experiment, we simultaneously reduced cellular levels of h14 (Figure 2A, right). Sec62$^{k.d.}$ significantly slowed down cell proliferation after 72 h (Figure 2B), although an increase in cell death was not apparent. All subsequent experiments were done at 72 h.

We previously observed that reduced SRP levels specifically interfered with protein transport through the Golgi (Lakkaraju *et al.*, 2007). This defect could be revealed by the accumulation of the viral vesicular stomatitis virus G (VSV-G) protein in the Golgi. We observed an even distribution of VSV-G along the secretory pathway in the Sec62$^{k.d.}$ and control cells (Figure 2C). Most importantly, the cellular levels of other translocation-associated proteins, such as Sec63, TRAM, TRAPα, Sec61α, the receptor complex SRα and SRβ, and the ER chaperons calnexin and BiP, remained unchanged at this time point (Figure 2D) in Sec62$^{k.d.}$ and Sec62$^{k.d.}$/h14$^{k.d.}$ cells. Despite Sec63 and Sec62 forming a complex, the knockdown of Sec62 did not affect cellular levels of Sec63 at 72 h after transfection.

In the translocation assays, we expressed shRNA(14) and shRNA(62.3) individually (Figure 3A, lanes 2 and 3) or together (Figure 3A, lanes 4–7), and control cells expressed shRNA(Luc) (lane 1). To detect translocation defects, we treated cells for 8 h with proteasome inhibitor and assessed the presence of nontranslocated precursors by Western blotting. Cellular levels of endogenous and expressed proteins were monitored in the same extracts (Figure 3B). We previously showed that preprolactin accumulation correlated with reduced secretion of the phosphorylated, secreted form of prolactin PL* (Kim and Brooks, 1993) and with inefficient translocation in pulse-labeling experiments in cells with an impaired SRP pathway (Lakkaraju *et al.*, 2008). Monitoring precursor accumulation is therefore a reliable, qualitative readout for translocation defects. However, since the precursor accumulates over 8 h, whereas PL* is continuously secreted outside the cell, the translocation efficiency cannot be quantified in these experiments.

As compared with preprolactin, the results of the translocation assays were different for the two reporter proteins pInf and pCC2f. For pInf and pCC2f, a translocation defect was only detectable when both h14 and Sec62 were simultaneously depleted (Figure 3A, lane 4). Furthermore, the plasmid-driven expression of either Sec62 or G14 in the double-knockdown cells (Figure 3A, lanes 5 and 7) restored translocation. These results confirmed that the observed defects in the knockdown cells were the consequence of reduced Sec62 and h14 levels, respectively. In addition, Sec62 was required for efficient translocation of the small preproteins in the absence of the SRP pathway. Moreover, expression of G14A5 failed to restore efficient translocation in Sec62$^{k.d.}$ cells, indicating

| | Name | Length (amino acids) | SS (amino acids) | $SR\alpha^{k.d.}$ T[%] | $Sec62^{k.d.}$ T[%] |
|---|---|---|---|---|---|
| | | In vitro expression | | | |
| Pre-CC2 with 3f-tag | pCC2f | 141 | 23 | | |
| Preproinsulin with 3f-tag | pInf | 152 | 24 | 96 | 67 |
| Preresistin with h-tag | pRh-126 | 126 | 18 | | 85 |
| Truncated preresistin with h-tag | pRh-100 | 100 | 18 | | 58 |
| Pre–lysozyme C with h-tag | pLCh-164 | 164 | 18 | 67 | 92 |
| Truncated prelysozyme with h-tag | pLCh-100 | 100 | 18 | 96 | 55 |
| Preprolactin with 3f-tag | pPLf | 274 | 30 | 56 | 96 |
| Truncated preprolactin with 3f-tag | pPLf-194 | 194 | 30 | 55 | 95 |
| | pPLf-163 | 163 | 30 | 75 | 97 |
| | pPLf-140 | 140 | 30 | 94 | 79 |
| | | In vitro expression | | | |
| Preproinsulin (rat) | pIn | 110 | 24 | | |
| Pre-CC motif chemokine 2 | pCC2 | 141 | 23 | | |
| Preresistin | pRes | 108 | 18 | | |
| Prelysozyme C | pLC | 148 | 18 | | |
| Preprolactin (bovine) | pPL | 229 | 30 | | |
| Truncated preprolactin | pPL-190 | 190 | 30 | | |
| | pPL-140 | 140 | 30 | | |

3f-tag, MDYKDHDGDYKDHDIDYKDDDDK; h-tag, YPYDVPDYAHHHHHH; SS, signal sequence; T[%], translocation efficiency, quantification from the in vivo experiments shown in Figures 1 and 3.

TABLE 1: Protein expression constructs and translocation efficiency.

that the elongation arrest function became important at reduced Sec62 levels (Figure 3A, lane 6). In contrast, the translocation of preprolactin was always inefficient when the SRP pathway was impaired (Figure 3A, lanes 2, 4, and 6), and, consequently, expression of Sec62 alone could not restore efficient translocation in the double-knockdown cells (Figure 3A, lane 7). Of note, reduced levels of Sec62 did not impair preprolactin translocation (Figure 3A, lane 3). The quite unexpected slight increase of PL* in translocation-defective cells is most likely due to a delay in protein transit through the secretory pathway caused by an incomplete clearance of incorrectly folded proteins from the ER in MG132-treated cells and a gradual decrease in membrane components and modifying enzymes. Consistent with this interpretation, the ER form prolactin, which is undetectable in untreated cells (see later discussion), became detectable in MG132-treated control cells (Figure 3A, lane 1).

In the same experimental setup, we also assayed the polyubiquitination status of pInf. As before, we found a strong correlation between translocation defects and polyubiquitination of pInf (Figure 3C). In double-knockdown cells without and with the expression of G14A5, pInf accumulated (Figure 3C, middle) and was strongly polyubiquitinated. In contrast, in cells in which the SRP pathway was restored by the expression of G14, polyubiquitination of pInf almost disappeared, as expected if the translocation is efficient.

The unchanged cellular levels of many ER-associated proteins in single- and double knockdown cells, the efficient translocation of preprolactin in $Sec62^{k.d.}$ cells, and the rescue of insulin and CCL2 translocation in double-knockdown cells by the plasmid-driven expression of Sec62 were consistent with the direct involvement of Sec62 in an SRP-independent translocation pathway. In addition, the results demonstrated that the small reporter protein could employ both pathways.

### Chain length influences Sec62 dependence

To examine the role of preprotein chain length for Sec62-dependent translocation, we studied translocation in $Sec62^{k.d.}$ cells quantitatively by pulse-labeling experiments. In addition to pInf, we used epitope-tagged pre–lysozyme C, resistin, and two truncated versions thereof (Table 1; the number indicates chain length). The cells were labeled for 5 min with [$^{35}$S]methionine/cysteine and treated with MG132 during starvation and labeling. The labeled reporter proteins were separated from the total extract by immunoprecipitation using epitope-specific antibodies.

In $Sec62^{k.d.}$ cells, the translocation efficiencies of the shortest reporters, pLCh-100 and pRh-100, were strongly reduced (Figure 4A). Significant but lesser translocation defects were observed for pRh-126 and pInf (152 amino acids), whereas translocation defects were undetectable for pLCh-164. The amounts of labeled protein in the immunoprecipitates cannot be compared between different samples because translocation-competent and translocation-defective cells contain different amounts of unlabeled protein. However, the relative abundances of precursor and translocated substrates in the same sample is a quantitative readout of the translocation efficiency, since both forms were precipitated in the presence of the same amount of unlabeled protein.

We also monitored the translocation efficiencies of pLCh-100 and pLCh-164 in cells with reduced SRα levels (Figure 4B). We observed the inverse effects on translocation as compared with $Sec62^{k.d.}$ cells. pLCh-100 translocated efficiently, whereas pLCh-164 translocated inefficiently. These results suggested that preproteins

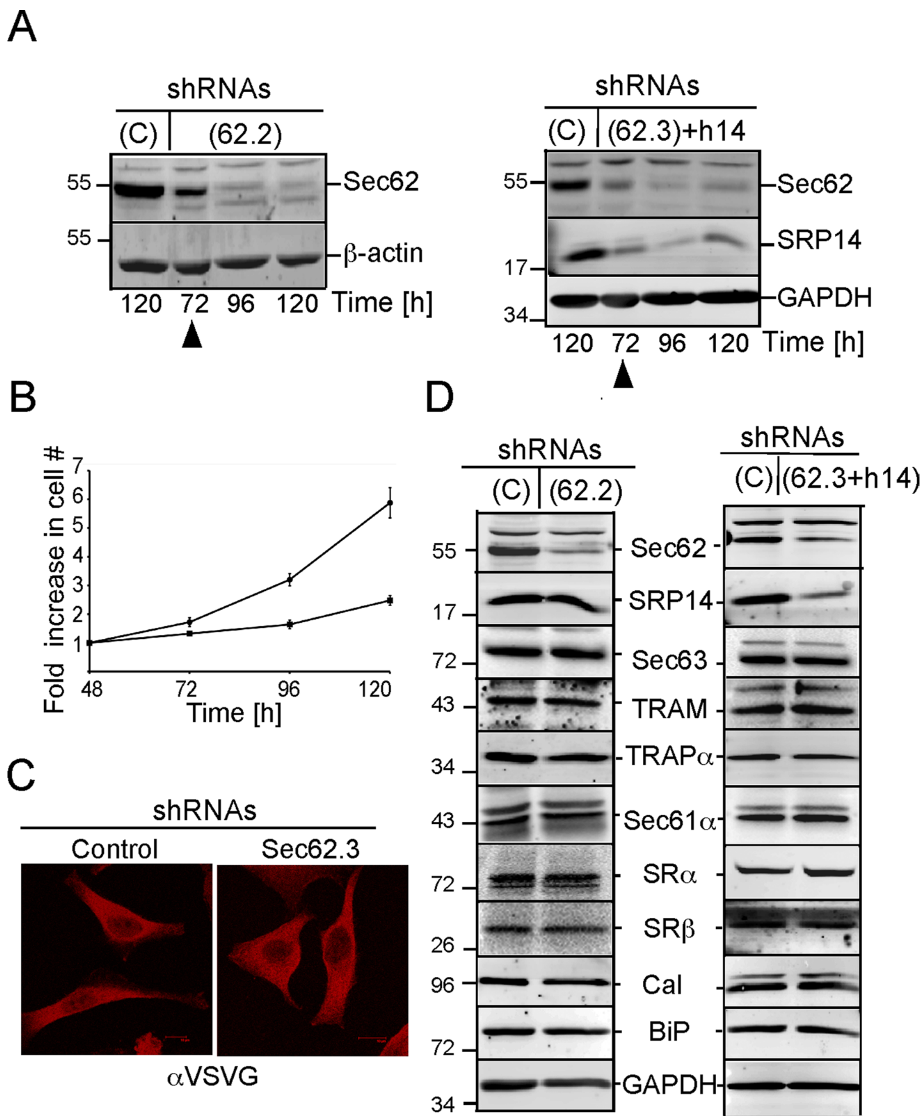


FIGURE 2: Characterization of Sec62 knockdown in HeLa cells. (A) Western blots of extracts from HeLa cells expressing shRNA 62.2 (left) and shRNAs 62.3 and 14 (right). Membranes were probed with antibodies against the proteins indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin, loading controls. 62.2 and 62.3, two different shRNAs targeting Sec62 mRNA. Arrow, time point of all following experiments. (B) HeLa cells expressing shRNAs (62.3) and the negative control (Luc) were analyzed for cell growth by counting the number of live cells manually every 24 h starting from 48 h posttransfection. Error bars, SD. (C) G protein expression after infection of Sec62$^{k.d.}$ and control HeLa cells with vesicular stomatitis virus. The cells were permeabilized with Triton to reveal the G protein in the secretory pathway. Scale bar, 10 μm. (D) Western blots of cell extracts from cells expressing shRNA 62.2 (left) and shRNAs Sec62.3 and 14 (right) probed with antibodies against the proteins indicated. Cal, calnexin. The quantified values varied between 90 and 100% in single-knockdown and between 86 and 115% in double-knockdown experiments when normalized to GAPDH and compared with control cells.

comprising more than ~160 amino acids required the SRP pathway for efficient translocation, since they could not be translocated efficiently by the Sec62-dependent pathway.

We also included truncated versions of preprolactin (Table 1) to assess whether a strongly SRP-dependent preprotein could become SRP-independent upon shortening of the polypeptide chain. Indeed, we found that pPLf-140 translocation was efficient in SR$\alpha^{k.d.}$ cells (Figure 4B) but was reduced in Sec62$^{k.d.}$ cells (Figure 4A). The longer polypeptides, pPLf-163 and pPLf-194, had the inverse phenotype: The translocation was moderately and strongly impaired in SR$\alpha^{k.d.}$ cells, respectively, and unchanged in Sec62-depleted cells. Of note, pPLf-163 and pLCh-164 were less affected by the knockdown of Sec62 than preprolactin (Table 1). The same preproteins were also analyzed after treatment with proteasome inhibitor for 8 h in SRP$^{A5}$ cells (Figure 4C). Consistent with the pulse-labeling experiments, we observed accumulation of precursor proteins for pPLf-194 and pPLf-163. The fact that we failed to detect pInf accumulation in Sec62$^{k.d.}$ cells (Figure 3A) might be explained by an insufficient protection of the nontranslocated preproinsulin. To examine pRh-100 accumulation in Sec62$^{k.d.}$ cells, we therefore used a higher concentration of the protease inhibitor ALLN (50 μM) to protect preresistin from degradation. We clearly observed the accumulation of nontranslocated pRh-100 in Sec62$^{k.d.}$ cells (Figure 4D), consistent with the observed translocation defect in pulse-labeling experiments.

Analysis of polyubiquitination of preprolactin proteins of different chain lengths showed the expected correlation between SRP-dependent translocation (Figure 4E, left) and polyubiquitination (Figure 4E, right). The longer proteins, pPLf-163 and -194, were strongly polyubiquitinated in SRP$^{A5}$ cells, whereas the shorter protein, pPLf-140, was only weakly polyubiquitinated, consistent with the interpretation that pPLf-140, but not pPLf-164 and -194, could use the Sec62-dependent pathway efficiently.

In summary, chain length is an important criterion for the efficient use of the Sec62-dependent translocation pathway. Furthermore, the dependence on Sec62 for efficient translocation increased as chain length decreased. Conversely, preproteins comprising more than ~160 amino acids could not use the Sec62 pathway efficaciously and depended entirely on SRP for efficient translocation.

### Sec62-dependent translocation occurs posttranslationally

If Sec62-dependent translocation represents an alternate pathway for preproteins that fail to be recognized by SRP during translation, it is likely to occur posttranslationally. We took advantage of an assay developed to study tail-anchor protein insertion using permeabilized HeLa cells (Henderson *et al.*, 2007; Rabu *et al.*, 2008). In short, the preprotein was synthesized in reticulocyte lysate and recovered from the supernatant after pelleting the ribosomes by centrifugation. The preprotein was then incubated with permeabilized HeLa cells and cytosol. In the presence of ATP, we saw the appearance of a shorter product, presumably proinsulin, indicating that preproinsulin was translocated posttranslationally (Figure 5A, left).

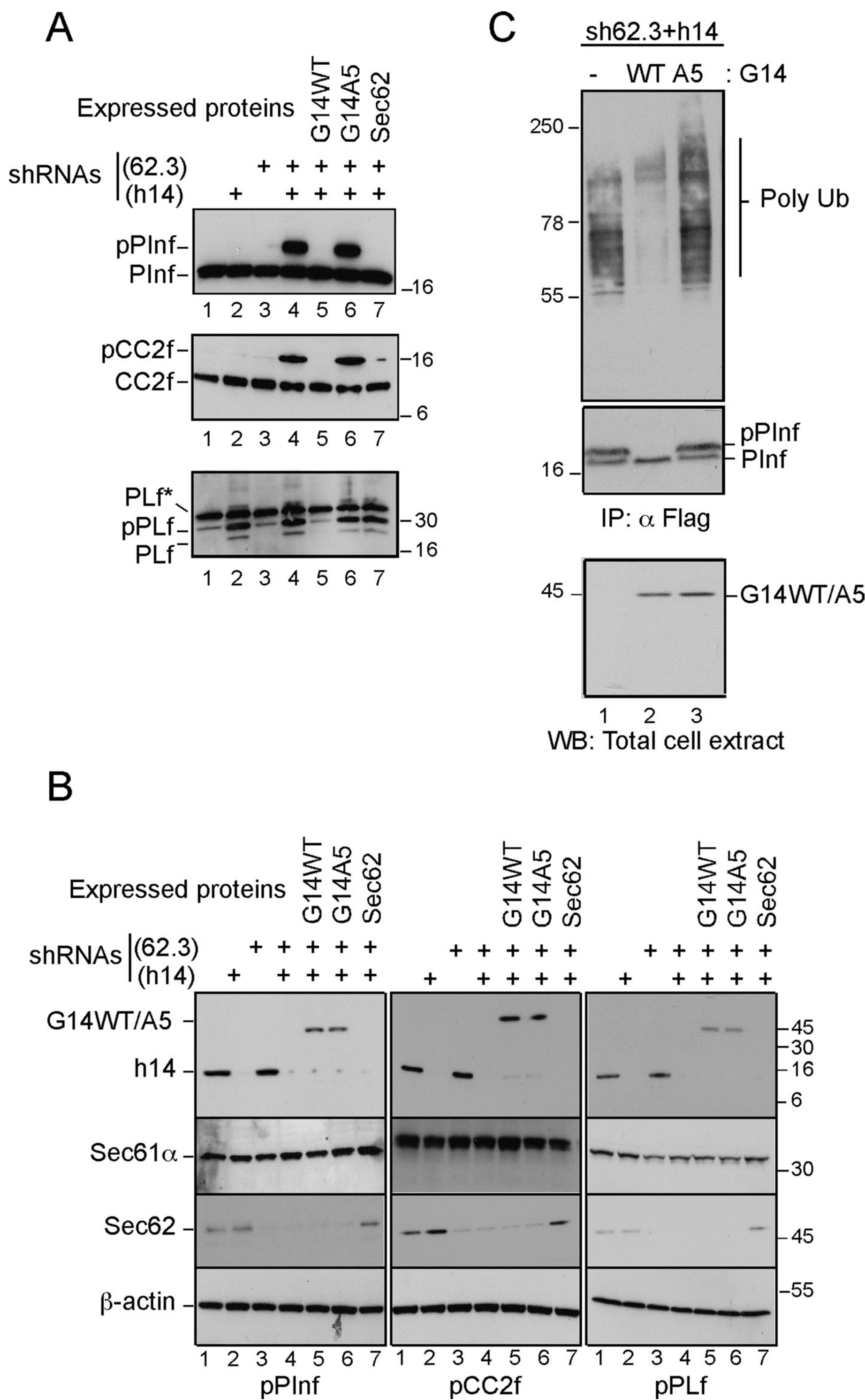


FIGURE 3: SRP-independent translocation depends on Sec62. (A) Protein translocation of preproinsulin (pPInf), pre-CC2 (pCC2f), and preprolactin (pPLf) monitored in cells depleted of either of h14 and Sec62 individually (lanes 2 and 3) or of h14 and Sec62 simultaneously (lanes 4–7) after treatment with MG132 (10 μM) for 8 h. Lane 1, shRNA(Luc). The double-knockdown cells were complemented by the expression of G14 (lane 5), G14A5 (lane 6), and Sec62 (lane 7). Reporter proteins were revealed with anti-FLAG antibody. PLf*, phosphorylated prolactin in transit for secretion. (B) Total cell extracts from the experiment shown in A revealed by Western blotting for the proteins indicated. (C) Polyubiquitination of nontranslocated pInf. Western blots of total cell extracts prepared from HeLa cells expressing shRNAs 62.3 and 14 and, when indicated, G14 and G14A5 after treatment with MG132 for 8 h. Cells also expressed HA-tagged ubiquitin. Insulin was immunoprecipitated and revealed with anti-FLAG, polyubiquitination of insulin was revealed with anti-HA, and fusion proteins in cell extracts were revealed with anti-GFP antibodies.

CT08 treatment inhibited formation of the shorter product, confirming that it represented the translocated proinsulin (Figure 5B). Another SRP-independent substrate, pPLf-140, also translocated in vitro, whereas the SRP-dependent preprotein, pPLf-190, did not (Figure 5A, right). Furthermore, signal sequence processing occurred with membranes prepared from SRα $^{k.d.}$ cells and with cytosol depleted of h14 (Figure 5, C and D), demonstrating that translocation was independent of SRP, as expected for posttranslational translocation. We then examined whether posttranslational translocation of lysozyme C, resistin, and pre-CC2 (Figure 5E) was Sec62 dependent using permeabilized Sec62$^{k.d.}$ cells in the assay. Translocation was abolished for all three preproteins studied, demonstrating that the observed posttranslational translocation was Sec62 dependent. Of note, authentic pLC, comprising 148 amino acids (Figure 5E and Table 1), was translocated posttranslationally, whereas the h-tagged (h-tag, YPYDVPDYAHHHHHH) protein pLCh-164 was SRP dependent for translocation in vivo (Figure 4, A and B). The results of the in vitro translocation assays are consistent with the conclusion that the Sec62-dependent translocation, which we investigated in mammalian cells, occurs posttranslationally.

### Short reporter proteins can be coimmunoprecipitated with Sec62

To strengthen further the argument that Sec62 or a complex comprising Sec62 is involved in the translocation of short proteins, we examined whether a translocation intermediate comprising Rh-100 and Sec62 could be detected by immunoprecipitation experiments. Such an intermediate is expected to be short-lived, and to increase chances of its detection, we treated cells with cycloheximide, which, at the concentration used, slows nascent chain elongation fourfold (Lakkaraju *et al.*, 2008). We argued that this delay might keep the translocon occupied for longer time periods and thereby reduce the availability of Sec61 for posttranslational translocation.

We transfected cells with the reporter plasmid expressing pRh-100 and treated them with cycloheximide for 1 h before immunoprecipitating the reporter protein from cell extracts using the epitope-specific anti–h-tag antibody. The immunoprecipitates were displayed by SDS–PAGE, and the upper and lower parts of the membrane revealed with anti-Sec62 and with anti–h-tag antibody, respectively. We found that antibodies against the h-tag of pRh-100 pulled down specifically Sec62 (Figure 6A, left). In

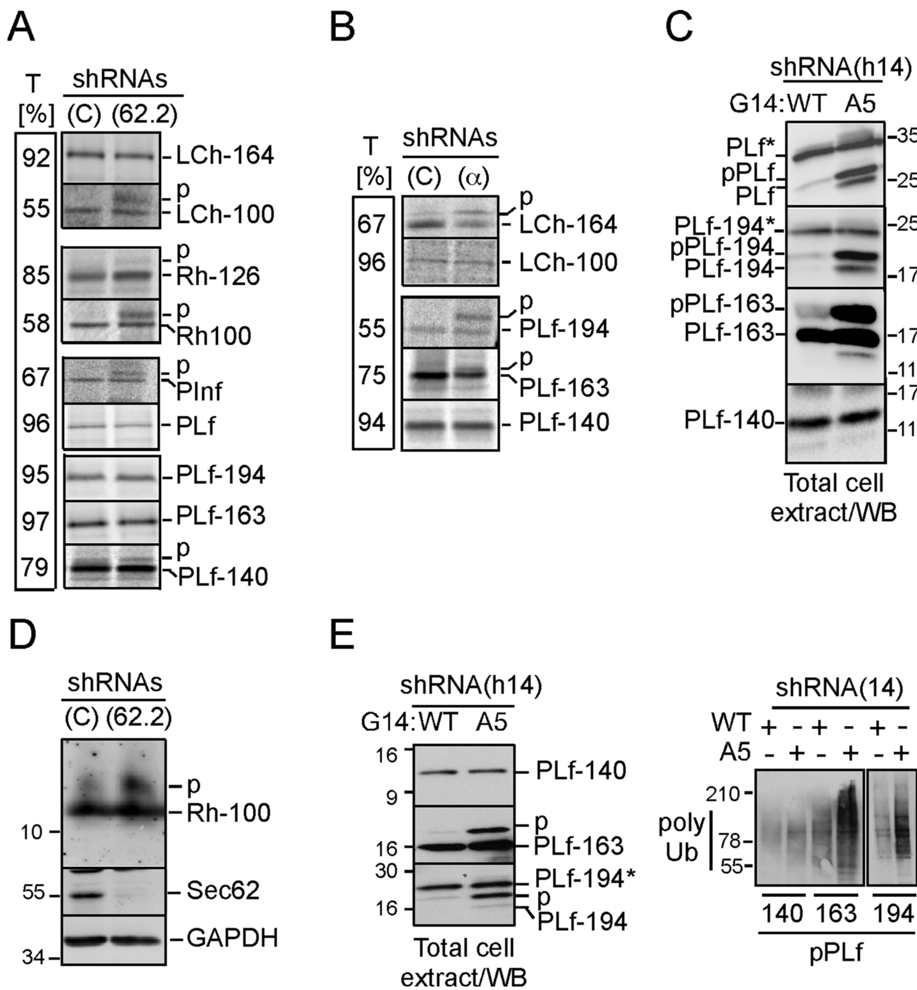


FIGURE 4: Sec62-dependent translocation is efficient for preproteins comprising <160 amino acids. Translocation efficiencies (T[%]) of different reporter proteins in HeLa cells with reduced cellular levels of Sec62 (A) and SRα (B). C, shRNA(Luc). Pulse-labeled proteins were immunoprecipitated with anti-FLAG antibodies, displayed by SDS–PAGE, and visualized by phosphoimager. Nontranslocated preproteins were stabilized with MG132 (10 µM) before and during labeling. Proteins are named according to their chain length (see Table 1). p, preprotein. (C) SRP-dependent translocation of preprolactin proteins of decreasing chain length in HEK 293T cells. Cells expressed shRNA (14) and, as indicated, G14 and G14A5. Preprotein accumulation was monitored by Western blotting of cell extracts harvested after treatment with MG132 (10 µM) for 8 h. To resolve the signal sequence–cleaved and uncleaved forms, the proteins were displayed on different gels. pPLf and pPLf-194, 12%; pPLf-163 and pPLf-140, 15%. (D) Preresistin accumulation in Sec62$^{k.d.}$ cells. Western blots of cell extracts with anti-His antibodies. Cells were treated with the protease inhibitor ALLN (50 µM) for 8 h before harvesting. (E) Polyubiquitination of preprolactin proteins of different chain length in HeLa cells expressed shRNA 14 and, as indicated, G14 and G14A5. Cells also expressed HA-tagged ubiquitin. Left, total cell extracts probed with anti-FLAG antibodies to reveal the reporter proteins. Right, reporter proteins were immunoprecipitated with anti-FLAG antibodies and displayed by SDS–PAGE, and the reporter proteins and ubiquitination were revealed by Western blotting with anti-FLAG and anti-HA antibodies, respectively.

the negative controls, anti-FLAG antibodies, and cell extracts without pRh-100, Sec62 was absent in the immunoprecipitates. We presumed that Sec62 was coprecipitated with pRh-100. However, the precursor and mature forms could not be resolved in this gel. In addition, we expected only a very small fraction of the protein to be in the precursor form in the absence of proteasome inhibitor (see total cell extracts, Figure 6A, right). In parallel experiments we used preprolactin as a reporter protein. We could not detect any Sec62 coprecipitating with preprolactin, suggesting that translocation intermediates of preprolactin do not include Sec62 (Figure 6B).

## DISCUSSION

Our studies provide evidence for an SRP-independent but Sec62-dependent posttranslational entry path of signal sequence–bearing proteins into the ER of mammalian cells. The Sec62-dependent posttranslational translocation pathway ensures efficient secretion of preproteins shorter than around 160 amino acids in cells with an impaired SRP pathway. Hence it provides cells with a fail-safe mechanism for efficient translocation of preproteins that are targeted inefficiently by the SRP due to their small size. Around 360 secretory proteins are found in the human proteome, comprising fewer than ~160 amino acid residues (Supplemental Table S1). Many of these proteins are secreted by specialized cells in response to physiological stimuli requiring a fast adaptation of the cell's secretory capacity. Sec62-dependent translocation provides cells with the opportunity to regulate secretion of small proteins independent of the housekeeping SRP pathway.

The smallest preproteins we studied, pRh-100 and pLCh-100, are most likely primarily dependent on the Sec62 pathway for translocation, as suggested by the almost 50% reduction in translocation in Sec62$^{k.d.}$ cells (Figure 4A). Similarly, SRP-dependent translocation of preprolactin is reduced to ~50% in cells with an impaired SRP pathway (Table 1). Slightly larger preproteins, comprising up to 160 amino acid residues, can apparently use both pathways. For example, preproinsulin is translocated either SRP or Sec62 dependently in vitro and in vivo (Figures 4 and 5E; Okun *et al*., 1990). However, their efficient translocation is only guaranteed by the posttranslational Sec62-dependent pathway in vivo. Because the translocation efficiencies of pRh-126, pPL-140, and pInf (152 amino acids) were not strictly correlated with chain length in Sec62-depleted cells, factors other than size, such as signal sequence recognition by SRP, might influence the choice. Preproinsulin has a twofold-lower affinity for SRP than does preprolactin (Flanagan *et al*., 2003) and might therefore use the SRP pathway less efficaciously than preprolactin. This could explain its lower translocation efficiency in Sec62-depleted cells as compared with pPLf-140.

The transition between SRP-dependent and SRP-independent translocation is illustrated by the preproteins pLCh-164 and pPLf-163. They had smaller translocation defects in SRα-depleted cells than the longer pPLf protein variants, suggesting that they can still use to some extent the posttranslational Sec62-dependent pathway, albeit with decreased efficiency. Clearly, the efficient translocation of

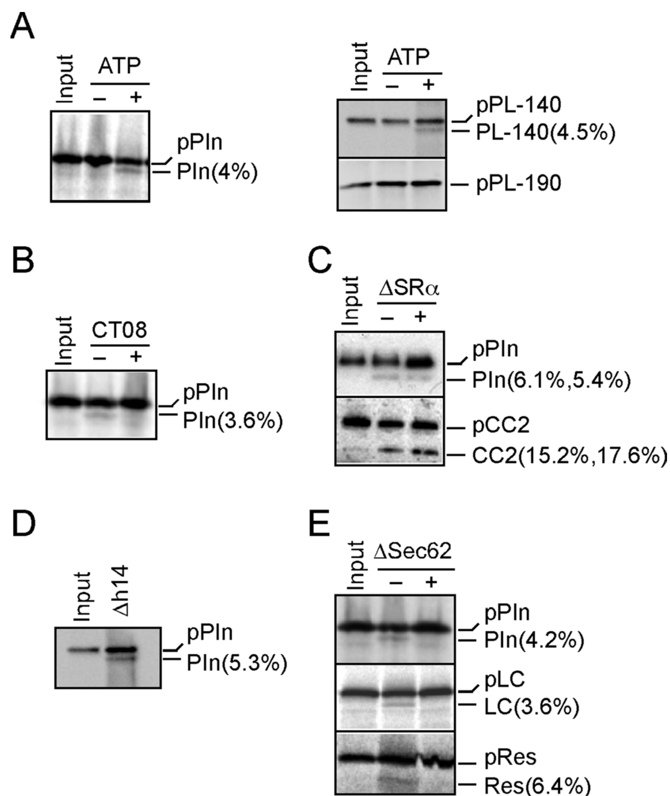


FIGURE 5: Posttranslational translocation in vitro requires Sec62. In vitro synthesized $^{35}S$-labeled preproteins (Input) were incubated with permeabilized HeLa cells, cytosolic extract, and ATP for translocation. (A) pPIn (left) and pPLf-190 and pPLf-140 (right) in the presence or absence of ATP. Translocation was monitored by signal sequence cleavage (lane 3). (B) pPIn translocation with and without CT08. (C) pPIn, pCC2 incubated with permeabilized SRα and control HeLa cells. (D) pPIn translocation with cytosol from h14$^{k.d.}$ cells. (E) pPIn, pLCh, and pRes translocation with permeabilized Sec62$^{k.d.}$ (+) and control (–) HeLa cells. The relative translocation efficiencies (percentages) as compared with the inputs are shown in parentheses.

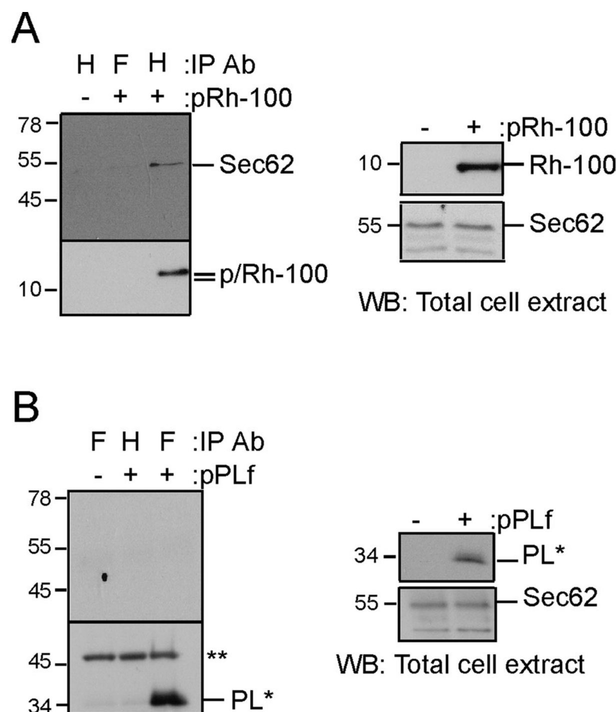


FIGURE 6: Sec62 can be immunoprecipitated with a short reporter protein. HeLa cells were transfected with pRh-100– and pPLf-expressing plasmids, cells were harvested 25 h later after treatment with cycloheximide (3 µg/ml) for 1 h, and protein complexes were precipitated with the antibodies indicated. F, anti-FLAG antibody; H, anti-His antibody. (A) Immunoprecipitates displayed on a 4–20% gradient gel. The upper part of the membrane was revealed with anti-Sec62 and the lower part with anti-His antibodies. pRh-100 and Rh-100 could not be resolved on this gel. Right, total cell extracts displayed on Tris-Tricine gel and 12% PAGE for Rh-100 and Sec62, respectively. (B) As in A, but with preprolactin as a reporter protein. Protein was revealed with anti-FLAG antibody. PL*, phosphorylated form of prolactin; **heavy chain.

the longer preproteins, pPLf and pPLf-194, requires the SRP pathway and is independent of Sec62 (Figures 1D and 4, A–C).

Cotranslational translocation becomes inefficient in the absence of elongation arrest activity in mammalian cells (Lakkaraju *et al.*, 2008), and SRP-dependent targeting is impaired by increasing nascent chain length in biochemical experiments (Flanagan *et al.*, 2003), implying a size limit of the substrate for efficient translocation. The apparent limitation in substrate size for efficient translocation in the SRP and Sec62-dependent pathways provides a rationale for the prevalence of the SRP pathway in mammalian cells, since SRP has the capacity to keep the substrate short. Furthermore, it suggests that the limitation of substrate size for efficient translocation is a characteristic of the translocation apparatus shared by the two pathways. Remarkably, the translocation of small preproteins became strongly dependent on the elongation arrest function of SRP in the absence of the Sec62-dependent pathway (Figure 3A). For these short preproteins, which do not have to be kept short, the elongation arrest function might ensure cotranslational targeting by preventing termination of protein synthesis.

We do not know whether Sec62 binds directly to preproteins during translocation. However, a direct involvement of Sec62 in posttranslational translocation of small preproteins is strongly supported by four arguments: 1) there is no general translocation defect in Sec62$^{k.d.}$ cells, since cotranslational translocation is normal (this study; Lang *et al.*, 2012), and the cellular levels of other known translocation-associated membrane factors are unchanged at the time point of the experiments; 2) the defect is specific, as translocation of small proteins is restored in Sec62$^{k.d.}$ cells by exogenous expression of Sec62; 3) the small preprotein Rh-100, but not preprolactin, was found in a complex with Sec62 in immunoprecipitation experiments; and 4) the homologous protein plays a role in posttranslational translocation in yeast (Deshaies and Schekman, 1989; Ng *et al.*, 1996).

The mechanistic role of Sec62 remains to be studied, as does the link between the Sec62-dependent pathway described here and the two recently identified cytosolic factors that recognize signal sequences of short preproteins such as ppcecA. One of the factors, calmodulin, interacts in a calcium-dependent manner with signal sequences of short nontranslocated precursors to protect them from degradation until they translocate posttranslationally (Shao and Hegde, 2011a). Calmodulin was previously found to bind Sec61 (Erdmann *et al.*, 2011), indicating a pathway of delivery to the membrane. The other factor, TRC-40, also binds ppcecA, as well as two mammalian proteins, and delivers them to the membrane via the TRC-40 receptor WRB (Vilardi *et al.*, 2011), where translocation occurs via Sec61 (Johnson *et al.*, 2012). The Sec62 pathway studied here also delivers preproteins to the Sec61 translocon. It is therefore

conceivable that calmodulin and TRC-40 deliver substrate via Sec62 to the translocon Sec61.

The results in mammalian cells differ from those in yeast in that the posttranslational pathway in yeast is efficient for polypeptides of any size both in vivo and in vitro (Hansen *et al.*, 1986; Waters *et al.*, 1986; Deshaies and Schekman, 1989; Ng and Walter, 1994). The mammalian translocon lacks homologues of yeast Sec71 and Sec72 and contains a different set of ER-associated chaperones (Dudek *et al.*, 2009). The Sec71 and Sec72 proteins are dispensable in yeast, and deletion strains display only conditional translocation defects (Feldheim *et al.*, 1993; Feldheim and Schekman, 1994). Yet other ER-associated factors may influence translocation and explain why mammals handle larger substrates differently from yeast. It also remains to be studied whether selected substrates or specific physiological conditions might increase the efficacy of posttranslational translocation of longer polypeptides in mammalian cells. Another interesting question for future studies is to understand how small membrane proteins cope with inefficient recognition by the SRP. In trypanosomes, membrane proteins with cleavable signal sequences may use either the SRP-dependent or the Sec71-dependent pathway (Goldshmidt *et al.*, 2008).

## MATERIALS AND METHODS

### Cell culture

HeLa cells (CCL-2; American Type Culture Collection, Manassas, VA) and HEK 293T cells were grown in DMEM at 37°C supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO).

### RNA interference, transfections, and complementation assays

Expression of shRNAs and complementing proteins was described in Lakkaraju *et al*. (2008). shRNAs against h14 and luciferase were described previously (Lakkaraju *et al*., 2007). shRNAs against Sec62 were generated by cloning the appropriate sequence complementary to the target sequence: 5′ GAAAGGAGAGGAAGCTTTA (shRNA62.2, position 224 in the coding region) and 5′ GAGCAGACTTAAAGTAGCT 3′ (shRNA62.3, position 2175 in 3′ untranslated region) into pSuper.retro.puro vector (OligoEngine, Seattle, WA). The target sequences for the shRNAs were chosen using the Bioinformatics and Research Computing Web interface at http://jura.wi.mit.edu/bioc/siRNAext/home.php.

To deplete cells of endogenous proteins, we transfected equal numbers of cells with shRNA-expressing plasmids. After 24 h, the cells were selected for puromycin resistance for 24 h (3 µg/ml cell culture medium). For the complementation of $h14^{k.d.}$ in HeLa cells, cells were cotransfected with the plasmids expressing either the fusion protein of GFP and h14 (pG14) or the h14 mutant protein h14A5 (pG14A5; Lakkaraju *et al*., 2008). Cotransfection of the plasmid pCMV6-XL5-Sec62 (OriGene, Rockville, MD) complemented Sec62-depleted cells. If required, the plasmids of the reporter proteins pPLf, pInf, pLCh-164, pRh-126, and pCC2f and their truncated versions were also transfected together with the shRNA-expressing plasmids. The protocol for transfections in HEK 293T cells has been described (Lakkaraju *et al*., 2007).

To analyze ubiquitination, the pUb-HA plasmid expressing HA-tagged ubiquitin was transfected along with the other plasmids. MG132 (10 µM) was added for 8 h, the cells were lysed, and the pPLf (also the variants of different chain lengths) and pPInF were immunoprecipitated using anti-FLAG antibody (M2; Sigma-Aldrich). HA-tagged Ub was blotted using anti HA–horseradish peroxidase antibodies (11667475001; Roche, Indianapolis, IN). Cotransin 08 (CT08) was synthesized as described previously (Garrison *et al*., 2005).

### Plasmids for in vivo expression of reporter proteins

The cDNAs for Sec62 (SC118087), resistin (SC113077), and lysozyme C (SC125273) were obtained from OriGene. The pre-CC chemokine 2 cDNA (CC2) was obtained from Open Biosystems (Thermo Biosystems, Huntsville, AL). The plasmids pLCh-164 and pLCh-100 were generated by cloning the full-length and truncated versions of lysozyme C into pCDNA3 (Invitrogen, Carlsbad, CA) bearing a His tag. Full-length and truncated versions of preresistin (pRh-126 and pRh-100) were cloned into pCDNA3. The plasmids pCpInf and pCp-CC2f were obtained by cloning their respective cDNAs into pCK-3f vector (Lakkaraju *et al*., 2008), which harbors a triple FLAG tag at its C-terminus. Amplifying the appropriate fragments from the plasmid pPL3F expressing preprolactin (Lakkaraju *et al*., 2008) followed by their insertion into pCK-3f for the expression in mammalian cells generated clones for the expression of truncated preprolactin (pCpPLf-194, pCpPLf-163, pCpPLf-140, pCpPLf-110). The plasmid pCK and the plasmid containing the rat insulin coding sequence pIns-PCR were kind gifts from Didier Picard (University of Geneva, Geneva, Switzerland) and Sandra Wolin (Yale University, New Haven, CT), respectively.

### Analysis of preproteins

To analyze the accumulation of preproteins, we transfected the reporter proteins together with the shRNA-expressing plasmids. At 64 h posttransfection the cells were treated with 10 µM of MG132 for 8 h to monitor preprotein accumulation by Western blot. Preresistin was protected from degradation by treating cells with 50 µM of ALLN (Calbiochem, La Jolla, CA) for 8 h. Cell lysates were collected and equal amounts loaded onto the SDS–PAGE gels, and the proteins were revealed by Western blotting. To analyze the accumulation of preproteins in pulse-labeling experiments, we transfected cells with the reporter plasmids 24 h before the analysis. The cells were initially treated with 10 µM of MG132 or 50 µM of ALLN for resistin for 45 min, followed by starvation in medium lacking cysteine and methionine for 30 min. Subsequently, cells were labeled using [$^{35}$S]cysteine/methionine (200 µCi/ml; Hartmann Analytic, Braunschweig, Germany) for 5 min, followed by lysis and immunoprecipitation.

### Immunoprecipitation

For the radiolabeling experiments, the cells were lysed in buffer containing 0.1 M Tris-HCl, pH 8, and 1% SDS. The viscous lysate was heated at 90°C for 10 min with occasional vortexing until it became less viscous. The lysate was now diluted five times into immunoprecipitation (IP) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8, 1 mM EDTA, and 1× protease inhibitor cocktail; final volume, 1 ml). The labeled proteins were incubated overnight with anti-tag antibodies and immobilized on protein G–Sepharose beads for 4 h. The immunoprecipitates were washed three times for 5 min each, and the beads were suspended in SDS–PAGE sample buffer (two times). The samples were heated at 65°C for 20 min, and, depending on the size of the protein and the preprotein, the samples were migrated on different gels. To monitor the proteins smaller than 140 amino acids, the Tris-tricine gels (12%T [total percentage concentration of both monomers (acrylamide and bisacrylamide)]/3.3%C [percentage concentration of bisacrylamide relative to total monomer concentration]) were used (Schägger and von Jagow, 1987). Proteins longer than or equal to 140 amino acids were migrated on either 12 or 15% Tris-glycine gels.

Cell extracts comprising resistin were treated with NEM (Sigma-Aldrich) in the sample buffer as described (Schneider *et al*., 1994).

The gels were fixed and dried. Visualization and quantification of the gels were done using the Bio-Rad phosphoimager (Bio-Rad, Hercules, CA). The translocation efficiencies of the samples were quantified using the following equation after the background was subtracted: T = 100 × translocated protein/(preprotein + translocated protein). In all the experiments the translocation of the control sample (C) was set as 100%.

In the pulse-labeling experiments, CT08 (1 μM final concentration) was present 60 min before and during labeling of cells. For the in vitro translocation assays, CT08 (1 μM) was added to the cells for 30 min before the membrane isolation and also added during the translocation assay.

For the immunoprecipitations of complexes comprising substrate and Sec62, cells were transfected with the substrate for 24 h, followed by 1-h treatment with cycloheximide (3 μg/ml). The cells were lysed using the lysis buffer: 20 mM Tris HCl, 10% glycerol, 20 mM sodium molybdate, 0.1 mM dithiothreitol, 0.25% Triton X-100, and protease inhibitors. The cell lysate was sonicated three times for 30 s. The lysate was spun down at 10,000 × *g* for 5 min, and the supernatant was precleared using protein G–Sepharose followed by incubation with the antibody overnight and by immobilization on Sepharose beads for 4 h. The immunoprecipitates were migrated on 4–20% gradient gels, and the proteins were detected by Western blot.

### Preparation of semipermeabilized HeLa cells

HeLa cells grown in 10-cm dishes were washed twice with ice-cold phosphate-buffered saline (PBS), followed by the extraction of the cytosolic content using digitonin (0.015%) for 10 min on ice in KHM buffer (110 mM KOAc, 2 mM MgOAc, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.2). The cells were further washed with HEPES buffer and resuspended in KHM buffer. The permeabilized cells served as the source of ER membranes in the in vitro translocation assays as described previously (Rabu *et al*., 2008). Each translocation mix consisted of 15 μl of reticulocyte lysate obtained after ultracentrifugation (140,000 × *g* for 40 min), 20 μl of the suspended semipermeabilized HeLa cells, and 15 μl of cytosol. ATP was added at a final concentration of 1 mM. Recombinant chaperones Hsp40 and Hsc70 were obtained from Stressgen (Enzo Life Sciences, San Diego, CA) and were used at a concentration of 2.5 and 1.5 μM, respectively. The translocation assay was performed at 30°C for 30 min; the membranes were recovered by a brief spin in a tabletop centrifuge and were suspended in the SDS–PAGE sample buffer (2×). The proteins were displayed on Tris-tricine gels and visualized by a Bio-Rad phosphoimager. The low translocation efficiencies are due to inactivation of calmodulin in nuclease-treated reticulocyte lysate (Shao and Hegde, 2011a).

### Antibodies and Western blotting

The anti-Sec62 (dilution 1:500) and the anti-SRα (1:500) antibodies were generous gifts from R. Zimmermann (Saarland University, Saarbrücken, Germany) and Peter Walter (University of California, San Francisco, San Francisco, CA), respectively. Anti-SRβ (1:500) antibody was purchased from Abcam (Cambridge, MA). Anti-6His antibody (Abcam, 1:2000) and anti–glyceraldehyde-3-phosphate dehydrogenase antibody (Abcam, 1:1000) were revealed with the Ettan DIGE imager (GE Healthcare, Piscataway, NJ) using ECL Plex goat anti–rabbit immunoglobulin G (IgG; Cy5) and ECL Plex goat anti–mouse IgG (Cy3; both GE Healthcare) at the dilutions recommended by the supplier. All the other antibodies were described previously (Lakkaraju *et al*., 2008).

### Cell-free transcription and translation

In vitro transcription of pPL, pCC2, and pIn cDNAs was done with SP6 RNA polymerase (Promega, Madison, WI) as described previously (Huck *et al*., 2004). The plasmids pSP-BP4 and pSP-insulin were linearized with *Eco*RI before transcription. The cDNA clones of resistin and lysozyme C (pCMV6-XL4-res and pCMV6-XL4lysoC, respectively) were obtained from OriGene, linearized simultaneously with *Xho*1 and *Xba*1, and transcribed using T7 RNA polymerase (Promega). The synthetic RNAs were purified using a G50–Sepharose column. To produce truncated variants of preprolactin, stop codons were introduced at the desired sites. Proteins were expressed in rabbit reticulocyte lysate (L4960; Promega). The standard translation mix contained 35 μl of reticulocyte lysate, 1 μl of amino acid mix (1 mM) lacking methionine and cysteine, 1 μl of RNase inhibitor (RNAsin 40 U/μl; Invitrogen), 2 μl of [$^{35}$S]cysteine/methionine (10 μCi/ml; Hartmann Analytic), and 1 μl of RNA (1 μg/μl). Translation was allowed to proceed at 30°C for 60 min.

### Immunofluorescence and VSV infection

Immunofluorescence was performed as described previously (Lakkaraju *et al*., 2007). The coverslips were analyzed using the SP2 laser scanning confocal microscope (63×/1.4 numerical aperture, PlanApo, He/Ne laser). VSV infection in HeLa cells was performed as described previously (Le Blanc *et al*., 2005).

### Compilation of small signal sequence–bearing proteins

We downloaded the human Secreted Protein Database (Chen *et al*., 2005) and sorted the proteins according to their sizes. The original database contains >3000 proteins.

### Statistical methods and figure preparation

Error bars in Figure 2B represent the SD: $SD = \sqrt{[\sum (X - M)^2] / (n - 1)}$, where X is the individual data point, M the mean, and n the number of samples. Figures were prepared using Canvas software (ACD Systems, Seattle, WA).


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## REFERENCES


Brodsky JL, Goeckeler J, Schekman R (1995). BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. Proc Natl Acad Sci USA 92, 9643–9646.

Chen Y, Zhang Y, Yin Y, Gao G, Li S, Jiang Y, Gu X, Luo J (2005). SPD—a Web-based secreted protein database. Nucleic Acids Res 33, D169–D173.

Cross BC, Sinning I, Luirink J, High S (2009). Delivering proteins for export from the cytosol. Nat Rev Mol Cell Biol 10, 255–264.

Daimon M, Susa S, Suzuki K, Kato T, Yamatani K, Sasaki H (1997). Identification of a human cDNA homologue to the *Drosophila* translocation protein 1 (Dtrp1). Biochem Biophys Res Commun 230, 100–104.

Deshaies RJ, Sanders SL, Feldheim DA, Schekman R (1991). Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature 349, 806–808.

Deshaies RJ, Schekman R (1989). SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. J Cell Biol 109, 2653–2664.

Dudek J, Benedix J, Cappel S, Greiner M, Jalal C, Muller L, Zimmermann R (2009). Functions and pathologies of BiP and its interaction partners. Cell Mol Life Sci 66, 1556–1569.

Dünnwald M, Varshavsky A, Johnsson N (1999). Detection of transient in vivo interactions between substrate and transporter during protein translocation into the endoplasmic reticulum. Mol Biol Cell 10, 329–344.
Erdmann F *et al*. (2011). Interaction of calmodulin with Sec61alpha limits Ca2+ leakage from the endoplasmic reticulum. EMBO J 30, 17–31.
Feldheim D, Schekman R (1994). Sec72p contributes to the selective recognition of signal peptides by the secretory polypeptide translocation complex. J Cell Biol 126, 935–943.
Feldheim D, Yoshimura K, Admon A, Schekman R (1993). Structural and functional characterization of Sec66p, a new subunit of the polypeptide translocation apparatus in the yeast endoplasmic reticulum. Mol Biol Cell 4, 931–939.
Flanagan JJ, Chen JC, Miao Y, Shao Y, Lin J, Bock PE, Johnson AE (2003). Signal recognition particle binds to ribosome-bound signal sequences with fluorescence-detected subnanomolar affinity that does not diminish as the nascent chain lengthens. J Biol Chem 278, 18628–18637.
Frith MC, Forrest AR, Nourbakhsh E, Pang KC, Kai C, Kawai J, Carninci P, Hayashizaki Y, Bailey TL, Grimmond SM (2006). The abundance of short proteins in the mammalian proteome. PLoS Genet 2, e52.
Garrison JL, Kunkel EJ, Hegde RS, Taunton J (2005). A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum. Nature 436, 285–289.
Goldshmidt H, Sheiner L, Butikofer P, Roditi I, Uliel S, Gunzel M, Engstler M, Michaeli S (2008). Role of protein translocation pathways across the endoplasmic reticulum in *Trypanosoma brucei*. J Biol Chem 283, 32085–32098.
Green N, Fang H, Walter P (1992). Mutants in three novel complementation groups inhibit membrane protein insertion into and soluble protein translocation across the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*. J Cell Biol 116, 597–604.
Hansen W, Garcia PD, Walter P (1986). In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-alpha-factor. Cell 45, 397–406.
Henderson MP, Billen LP, Kim PK, Andrews DW (2007). Cell-free analysis of tail-anchor protein targeting to membranes. Methods 41, 427–438.
Hessa T, Sharma A, Mariappan M, Eshleman HD, Gutierrez E, Hegde RS (2011). Protein targeting and degradation are coupled for elimination of mislocalized proteins. Nature 475, 394–397.
Huck L, Scherrer A, Terzi L, Johnson AE, Bernstein HD, Cusack S, Weichenrieder O, Strub K (2004). Conserved tertiary base pairing ensures proper RNA folding and efficient assembly of the signal recognition particle Alu domain. Nucleic Acids Res 32, 4915–4924.
Johnson N, Vilardi F, Lang S, Leznicki P, Zimmermann R, High S (2012). TRC-40 can deliver short secretory proteins to the Sec61 translocon. J Cell Sci, doi: 10.1242/jcs.102608.
Kim BG, Brooks CL (1993). Isolation and characterization of phosphorylated bovine prolactin. Biochem J 296, 41–47.
Kurihara T, Silver P (1993). Suppression of a sec63 mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus. Mol Biol Cell 4, 919–930.
Lakkaraju AK, Luyet PP, Parone P, Falguieres T, Strub K (2007). Inefficient targeting to the endoplasmic reticulum by the signal recognition particle elicits selective defects in post-ER membrane trafficking. Exp Cell Res 313, 834–847.
Lakkaraju AK, Mary C, Scherrer A, Johnson AE, Strub K (2008). SRP keeps polypeptides translocation-competent by slowing translation to match limiting ER-targeting sites. Cell 133, 440–451.
Lang S *et al*. (2012). Differential effects of Sec61alpha-, Sec62- and Sec63-depletion on transport of polypeptides into the endoplasmic reticulum of mammalian cells. J Cell Sci 125, 1958–1969.
Le Blanc I *et al*. (2005). Endosome-to-cytosol transport of viral nucleocapsids. Nat Cell Biol 7, 653–664.
Maifeld SV, MacKinnon AL, Garrison JL, Sharma A, Kunkel EJ, Hegde RS, Taunton J (2011). Secretory protein profiling reveals TNF-alpha inactivation by selective and promiscuous Sec61 modulators. Chem Biol 18, 1082–1088.
Mason N, Ciufo LF, Brown JD (2000). Elongation arrest is a physiologically important function of signal recognition particle. EMBO J 19, 4164–4174.
Meyer HA, Grau H, Kraft R, Kostka S, Prehn S, Kalies KU, Hartmann E (2000). Mammalian Sec61 is associated with Sec62 and Sec63. J Biol Chem 275, 14550–14557.
Müller G, Zimmermann R (1987). Import of honeybee prepromelittin into the endoplasmic reticulum: structural basis for independence of SRP and docking protein. EMBO J 6, 2099–2107.
Müller G, Zimmermann R (1988). Import of honeybee prepromelittin into the endoplasmic reticulum: energy requirements for membrane insertion. EMBO J 7, 639–648.
Müller L *et al*. (2010). Evolutionary gain of function for the ER membrane protein Sec62 from yeast to humans. Mol Biol Cell 21, 691–703.
Ng DT, Brown JD, Walter P (1996). Signal sequences specify the targeting route to the endoplasmic reticulum membrane. J Cell Biol 134, 269–278.
Ng DT, Walter P (1994). Protein translocation across the endoplasmic reticulum. Curr Opin Cell Biol 6, 510–516.
Okun MM, Eskridge EM, Shields D (1990). Truncations of a secretory protein define minimum lengths required for binding to signal recognition particle and translocation across the endoplasmic reticulum membrane. J Biol Chem 265, 7478–7484.
Panzner S, Dreier L, Hartmann E, Kostka S, Rapoport TA (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell 81, 561–570.
Plath K, Wilkinson BM, Stirling CJ, Rapoport TA (2004). Interactions between Sec complex and prepro-alpha-factor during posttranslational protein transport into the endoplasmic reticulum. Mol Biol Cell 15, 1–10.
Rabu C, Schmid V, Schwappach B, High S (2009). Biogenesis of tail-anchored proteins: the beginning for the end? J Cell Sci 122, 3605–3612.
Rabu C, Wipf P, Brodsky JL, High S (2008). A precursor-specific role for Hsp40/Hsc70 during tail-anchored protein integration at the endoplasmic reticulum. J Biol Chem 283, 27504–27513.
Rapoport TA (2007). Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature 450, 663–669.
Rothblatt JA, Deshaies RJ, Sanders SL, Daum G, Schekman R (1989). Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. J Cell Biol 109, 2641–2652.
Rothblatt JA, Meyer DI (1986). Secretion in yeast: reconstitution of the translocation and glycosylation of alpha-factor and invertase in a homologous cell-free system. Cell 44, 619–628.
Saraogi I, Shan SO (2011). Molecular mechanism of co-translational protein targeting by the signal recognition particle. Traffic 12, 535–542.
Schägger H, von Jagow G (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166, 368–379.
Schneider A, Oppliger W, Jeno P (1994). Purified inner membrane protease I of yeast mitochondria is a heterodimer. J Biol Chem 269, 8635–8638.
Shao S, Hegde RS (2011a). A calmodulin-dependent translocation pathway for small secretory proteins. Cell 147, 1576–1588.
Shao S, Hegde RS (2011b). Membrane protein insertion at the endoplasmic reticulum. Annu Rev Cell Dev Biol 27, 25–56.
Tyedmers J, Lerner M, Bies C, Dudek J, Skowronek MH, Haas IG, Heim N, Nastainczyk W, Volkmer J, Zimmermann R (2000). Homologs of the yeast Sec complex subunits Sec62p and Sec63p are abundant proteins in dog pancreas microsomes. Proc Natl Acad Sci USA 97, 7214–7219.
Vilardi F, Lorenz H, Dobberstein B (2011). WRB is the receptor for TRC40/Asna1-mediated insertion of tail-anchored proteins into the ER membrane. J Cell Sci 124, 1301–1307.
Waters MG, Chirico WJ, Blobel G (1986). Protein translocation across the yeast microsomal membrane is stimulated by a soluble factor. J Cell Biol 103, 2629–2636.