Ribosome Binding to and Dissociation from Translocation Sites of the Endoplasmic Reticulum Membrane^D

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We have addressed how ribosome-nascent chain complexes (RNCs), associated with the signal recognition particle (SRP), can be targeted to Sec61 translocation channels of the endoplasmic reticulum (ER) membrane when all binding sites are occupied by nontranslating ribosomes. These competing ribosomes are known to be bound with high affinity to tetramers of the Sec61 complex. We found that the membrane binding of RNC–SRP complexes does not require or cause the dissociation of prebound nontranslating ribosomes, a process that is extremely slow. SRP and its receptor target RNCs to a free population of Sec61 complex, which associates with nontranslating ribosomes only weakly and is conformationally different from the population of ribosome-bound Sec61 complex. Taking into account recent structural data, we propose a model in which SRP and its receptor target RNCs to a Sec61 subpopulation of monomeric or dimeric state. This could explain how RNC–SRP complexes can overcome the competition by nontranslating ribosomes.

INTRODUCTION

Cotranslational translocation is the major pathway in mammals by which proteins are transported across or integrated into the endoplasmic reticulum (ER) membrane. The synthesis of these proteins starts with a free ribosome in the cytosol. Once a hydrophobic signal sequence or transmembrane (TM) segment has emerged from the ribosome, the signal recognition particle (SRP) binds, forming a ribosome-nascent chain (RNC)–SRP complex (for reviews, see Walter and Johnson, 1994; Egea *et al.*, 2005). This complex binds to the ER membrane by interactions between SRP and the SRP receptor (SR) and between the ribosome and the Sec61 complex, a heterotrimeric membrane protein that forms a protein-conducting channel (Van den Berg *et al.*, 2004). The nascent polypeptide chain forms a loop when inserting into the channel, with the signal or TM sequence intercalated into the walls of the channel and the following mature region in the pore proper (for reviews, see Rapoport *et al.*, 2004; Osborne *et al.*, 2005). For secretory proteins, this part of the elongating chain is moved through the channel into the ER lumen, and the signal sequence is eventually cleaved off. In membrane proteins, TM sequences are partitioned sideways through the walls of the channel into the lipid phase, leaving some parts of the polypeptide chain in the cytosol and others in the ER lumen. For both classes of proteins, the translating

ribosome stays bound to the channel during the entire translocation process (Mothes *et al.*, 1997).

Previous work has shown that the Sec61 complex can bind not only RNCs but also nontranslating ribosomes with nanomolar affinity (Borgese *et al.*, 1974; Kalies *et al.*, 1994; Prinz *et al.*, 2000a). Ribosomes remain bound to ER membranes after nascent chain release (Adelman *et al.*, 1973) and do not easily dissociate from the membrane (Borgese *et al.*, 1973; Potter and Nicchitta, 2002). Given that the concentration of free ribosomes in a secretory cell is ~0.3 μ M, 95% of the Sec61 binding sites should be occupied (Blobel and Potter, 1967; Ramsey and Steele, 1976; Barle *et al.*, 1999). Indeed, electron microscopy pictures show that the ER is densely populated with membrane-bound ribosomes, and isolated rough microsomes have a high ribosome content (Adelman *et al.*, 1973; Kalies *et al.*, 1994). The occupation of ribosome binding sites raises the question of how ribosomes that carry nascent chains destined for translocation can efficiently be targeted to Sec61. Experiments *in vitro* have shown that SRP is required for the binding of RNCs in the presence, but not absence, of competing ribosomes (Neuhof *et al.*, 1998; Raden and Gilmore, 1998). SRP promotes RNC targeting both when competing ribosomes are present during the binding reaction and when they are prebound to the membrane. Together, these results suggest that the major role of SRP may be to provide RNC complexes a selective advantage in membrane targeting so that they can overcome the competition by ribosomes not engaged in translocation (Neuhof *et al.*, 1998; Raden and Gilmore, 1998). However, how exactly SRP binding facilitates RNC targeting to Sec61 is unclear.

Electron cryomicroscopy shows that membrane-bound ribosomes are associated with Sec61-tetramers (Menetret *et al.*, 2005). Smaller oligomers, such as trimers (Beckmann *et al.*, 2001) and dimers (Mitra *et al.*, 2005), were seen when purified yeast Sec61p or the bacterial homologue SecY was added to RNCs in detergent. The differing results might reflect the ability of the Sec61–SecY complex to change its oligomeric state underneath the ribosome. Indeed, freeze-fracture experiments suggest that oligomers can form upon

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Abbreviations used: ER, endoplasmic reticulum; luc, firefly luciferase; ppl, preprolactin; RNC, ribosome-nascent chain complex; SRP, signal recognition particle; SR, signal recognition particle receptor.

addition of ribosomes to proteoliposomes containing purified Sec61 complex (Hanein *et al.*, 1996). Whether oligomers are required for translocation is unclear, because the x-ray structure of the archaeobacterial Sec61 homologue SecYE β and cross-linking experiments suggest that the polypeptide chain moves through the center of a Sec61–SecY molecule (Van den Berg *et al.*, 2004; Cannon *et al.*, 2005).

Here, we have addressed the mechanism by which SRP allows RNCs to bind to membranes that are presaturated with nontranslating ribosomes. Our results show that the RNC–SRP complex binds to a Sec61 population that has an only weak intrinsic affinity for nontranslating ribosomes. This provides an explanation for how polypeptide chains can access translocation sites despite the presence of competing ribosomes.

MATERIALS AND METHODS

Purification of Microsomes and Reconstitutions

Rough microsomes (RMs), puromycin high salt-extracted (PK)-RMs, and SRP were prepared from dog pancreas as described in Walter and Blobel (1983a, b) and Neuhof *et al.* (1998), respectively. The Sec61 complex and SR were purified from PK-RMs and reconstituted into proteoliposomes as described in Gorlich and Rapoport (1993). Reconstitution of ribosome-bound and free Sec61 was performed after solubilization of RMs in 1% deoxy-BIGCHAP (Calbiochem, San Diego, CA), with the endogenous lipids for free Sec61 and a synthetic lipid mixture (Gorlich and Rapoport, 1993) for ribosome-bound Sec61.

Synthesis and Isolation of RNCs

mRNAs coding for the N-terminal 86 amino acids of bovine prolactin (ppl86) or for the N-terminal 120 amino acids of firefly (*Photinus pyralis*) luciferase (luc120) were translated for 20 min in the presence of [³⁵S]methionine in a wheat germ extract (at 28°C) or in reticulocyte lysate (at 30°C), as described previously (Jungnickel and Rapoport, 1995). The total ribosome concentration in the *in vitro* translation mixture was ~0.3 μ M. Translation was stopped by the addition of 2 μ M edeine. The translation reaction was used directly for targeting, or the RNCs were isolated by sedimentation through a sucrose cushion [500 mM sucrose, 50 mM HEPES/KOH, pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)₂, 1 mM dithiothreitol (DTT)] in a TLA 100 rotor (Beckman Coulter, Fullerton, CA) at 100,000 rpm for 30 min at 4°C. The samples were resuspended in the original volume of RBB buffer [50 mM HEPES/KOH, pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)₂, 2 mg/ml bovine serum albumin (lipid-free), 1 mM DTT].

Targeting Reactions

For a standard targeting reaction, 20 μ l of a translation mixture (0.3 μ M ribosomes/RNCs) or the same volume of purified ribosomes (0.3 μ M) was used to presaturate 1 equivalent (eq; Walter and Blobel, 1983a) of PK-RMs for 30 min on ice. One equivalent of PK-RMs contained approximately 3 pmol of Sec61. After dilution in 200 μ l of RBB buffer, PK-RMs were sedimented by centrifugation (7 $\times$ 20-mm tubes; 55,000 rpm for 5 min at 4°C) in a TLS-55 rotor (Beckman Coulter) and resuspended in membrane buffer [50 mM HEPES/KOH, pH 7.5, 150 mM KOAc, 10 mM Mg(OAc)₂, 250 mM sucrose, 1 mM DTT]. For each reaction, 0.2 eq of these PK-RMs were incubated in a total volume of 5 μ l for 15 min on ice and for 5 min at 28°C with 0.5 μ l of ppl86 wheat germ *in vitro* translation, which had been preincubated with 50 fmol of purified canine SRP or an equal amount of buffer for 5 min at 28°C. When rabbit reticulocyte lysate was used, no SRP was added, because it contains sufficient amounts of functional SRP (Meyer *et al.*, 1982). To assess protease protection of the nascent chain, samples were treated with 0.5 mg/ml proteinase K for 1 h on ice, and the protease was inactivated with 10 mM phenylmethylsulfonylfluoride (PMSF) for 10 min on ice. The samples were dissolved in urea-containing sample buffer (Knop and Schiebel, 1998) and analyzed by SDS-PAGE and autoradiography. To determine cosedimentation of ribosomes with membranes, the samples were diluted in 200 μ l of ice-cold membrane buffer, and the membranes were sedimented (7 $\times$ 20-mm tubes; 55,000 rpm for 5 min at 4°C) in a TLS-55 rotor (Beckman Coulter). The pellets were analyzed by SDS-PAGE and autoradiography or by scintillation counting of both supernatant and pellet.

Dissociation Experiments

Wheat germ ribosomes were purified from wheat germ extract (Erickson and Blobel, 1983) by sedimentation through a 2-ml sucrose cushion [50 mM HEPES/KOH, pH 7.5, 0.5 M sucrose, 150 mM KOAc, 5 mM Mg(OAc)₂] for 45 min at 100,000 rpm (TLA 100.3; Beckman Coulter). Ribosomes were resuspended in 0.4 ml of RBB. This was repeated two more times. Purified ribosomes

were radiolabeled with ³⁵S-labeling reagent (t-Boc-[³⁵S]methionine-N-hydroxy-succinimidyl ester; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) following the manufacturer's instructions, except that 50 mM HEPES/KOH, pH 7.8, 150 mM KOAc, 5 mM Mg(OAc)₂ was used as buffer, and labeling was performed for 1 h on ice. The radiolabeled ribosomes were purified by gel filtration through a P-30 MicroBio Spin column (Bio-Rad, Hercules, CA) and by sedimentation through a sucrose cushion as described above. Purified ribosomes were resuspended in RBB and analyzed by sucrose gradient centrifugation. For measuring dissociation kinetics, 0.4 pmol of radiolabeled ribosomes per eq of PK-RMs was bound for 30 min on ice in a total volume of 4 μ l, diluted 100 $\times$ in prewarmed RBB buffer, and incubated at 28°C. Samples containing 0.2 eq of PK-RMs were sedimented as described above. When 100 μ M aurintricarboxylic acid (ATA) or 50 μ g/ml chymotrypsin was used to test dissociation, dilution was omitted and the samples were sedimented directly (ATA) or after inhibiting chymotrypsin with 10 mM PMSF for 2 min on ice. To investigate dissociation of prebound ribosomes during RNC targeting, radiolabeled ribosomes were used for PK-RM presaturation, and ppl86 was translated in the presence of unlabeled methionine. The targeting reaction was performed, and the membranes were sedimented as described above. Error bars depict the SD from the means of at least two experiments.

Cross-linking and Antibody Binding Experiments

RMs (12 eq) were treated with 1 mM bismaleimidohexane (BMH) in a final volume of 35 μ l of buffer [50 mM HEPES/KOH, pH 7.5, 100 mM KOAc, 5 mM Mg(OAc)₂, 250 mM sucrose] for 30 min on ice. BMH was quenched with 100 mM β -mercaptoethanol for 5 min on ice, followed by addition of 1.5% digitonin (Sigma-Aldrich, St. Louis, MO) for solubilization and sucrose gradient centrifugation. For antibody binding experiments, 8 eq of RMs or ribosome-saturated PK-RMs were incubated with 2 μ g of affinity-purified antibody against the C terminus of Sec61 α (rabbit; immunogenic peptide CKEQSEVGSMGALLF) or normal rabbit control IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h on ice. Each sample was diluted with 0.2 ml of ice-cold membrane buffer, and the membranes were sedimented (7 $\times$ 20-mm tubes; 55,000 rpm for 5 min at 4°C) in a TLS-55 rotor (Beckman Coulter). The membranes were resuspended in 0.2 ml of membrane buffer, sedimented again, solubilized, and analyzed by sucrose gradient centrifugation.

Solubilization of Membranes, Sucrose Gradient Centrifugation, and Immunoblotting

Membranes (5 eq) were solubilized with 1.5% digitonin (Sigma-Aldrich) in a total volume of 50 μ l in RBB buffer for 30 min on ice. The extract was cleared by centrifugation (14,000 rpm for 1 min; Eppendorf tabletop centrifuge) and loaded on top of a 10–40% sucrose step gradient in 50 mM HEPES/KOH, pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)₂, 1 mM DTT, 0.5% digitonin. The step size was 5% sucrose. Centrifugation was performed for 105 min at 55,000 rpm and 4°C in a TLS-55 rotor (11 $\times$ 34-mm tubes; Beckman Coulter). The gradient was fractionated from top to bottom, and fractions were precipitated according to Wessel and Flugge, 1984. The precipitates were denatured in urea-containing sample buffer for 10 min at 65°C, followed by SDS-PAGE, and immunoblotting with affinity-purified antibodies to Sec61 α , Sec61 β , and SR α (Gorlich and Rapoport, 1993).

RESULTS

Testing the Membrane Dissociation of Prebound Ribosomes

To study the membrane targeting of RNCs, mRNA coding for ppl86, lacking a stop codon, was translated in a wheat germ extract, generating stalled ribosomes with nascent chains associated as peptidyl-tRNAs (Gilmore *et al.*, 1991; Jungnickel and Rapoport, 1995). These RNCs could bind to RMs that were stripped of ribosomes by puromycin/high salt treatment (PK-RMs; Supplemental Figure 1A). As reported previously, the preincubation of PK-RMs with an excess of nontranslating ribosomes significantly reduced RNC binding, unless SRP was present (Supplemental Figure 1A; Neuhof *et al.*, 1998; Raden and Gilmore, 1998). Similar results were obtained with proteoliposomes containing purified Sec61 complex and SR (Supplemental Figure 1B; Neuhof *et al.*, 1998). These data confirm that SRP binding gives RNCs an advantage in membrane targeting over nontranslating ribosomes and that this effect can be reproduced with just the Sec61 complex and SR present in the membrane.

The simplest possibility by which SRP could confer an advantage to RNCs is that RNC–SRP complexes bind more strongly to free binding sites that are generated by the

occasional dissociation of nontranslating ribosomes. To test whether the dissociation rate of nontranslating ribosomes is consistent with such a model, we labeled ribosomes with a reagent that attaches ^{35}S to amino groups. These ribosomes sedimented at the expected 80S position in a sucrose gradient (Supplemental Figure 2) and bound to PK-RMs with a binding constant of 50 nM, consistent with previous results (our unpublished data; Prinz *et al.*, 2000a). To test ribosome dissociation from the membrane, radiolabeled ribosomes were incubated with PK-RMs, the samples were diluted to prevent rebinding, and the membranes were sedimented at different times. The dissociation of the prebound ribosomes was found to be extremely slow, with <10% dissociating over 2 h at 28°C (Figure 1A). Control experiments showed that dissociation was not affected by the addition of a 40-fold excess of nontranslating ribosomes (our unpublished data), indicating that the rebinding of dissociated ribosomes is indeed negligible. Together, these results indicate that RNC-SRP complexes do not bind to sites spontaneously vacated by the dissociation of nontranslating ribosomes.

Next, we tested whether the binding of RNC-SRP complexes actively induces the dissociation of prebound nontranslating ribosomes. PK-RMs were preincubated with saturating amounts of radiolabeled ribosomes and reisolated. When subjected to a second round of sedimentation, >90% of the radioactivity was associated with the membranes (Figure 1B, $t = 0$). No significant dissociation of ribosomes was observed upon incubation with buffer or an excess of unlabeled ppl86, in the absence or presence of SRP. The ppl86-SRP complexes were not limiting in the reaction, because increasing their amounts up to 10-fold gave similar results (our unpublished data). To demonstrate that the PK-RMs were indeed saturated with nontranslating ribosomes, the preincubation was carried out with unlabeled ribosomes; this prevented binding of labeled ribosomes (Figure 1B, presat.). Targeting of ppl86 to these membranes, as measured by protease protection of the radiolabeled nascent chains, was also prevented (Figure 1C, lane 4), unless SRP was present (Figure 1C, lane 6).

The lack of ribosome dissociation was confirmed in experiments testing whether ppl86 can displace prebound ribosomes that carry a short, radiolabeled fragment of a cytosolic protein (luc120). PK-RMs were saturated with an excess of RNCs carrying luc120 and reisolated by sedimentation (Figure 1D, lane 4). When these membranes were incubated with ppl86, synthesized *in vitro* in the absence of [^{35}S]methionine, all the labeled luc120 stayed with the membrane during sedimentation, regardless of whether SRP was present (Figure 1D, lanes 10 and 12). Controls showed that no luc120 sedimented in the absence of membranes (Figure 1D, lanes 6 and 8). When the membranes were incubated with ^{35}S -labeled ppl86 and SRP and sedimented, the majority of ppl86 chains were targeted to the membrane, but labeled luc120 did not dissociate (Figure 1D, lane 24 vs. 23). In the absence of SRP, the majority of ppl86 remained in the supernatant (Figure 1D, lane 22 vs. 21), indicating that the ribosome binding sites were indeed occupied. The sedimentation of some ppl86 is likely due to aggregation, because it occurred in the absence of membranes as well (Figure 1D, lanes 18 and 20). Together, these results indicate that the binding of RNC-SRP complexes to ribosome-saturated membranes does not lead to significant dissociation of prebound ribosomes. It thus seems that the RNC-SRP complexes associate with free sites to which nontranslating ribosomes cannot bind. These binding sites must contain the Sec61 complex, because the ppl86 chain is protected from proteases and thus located in the channel (Gorlich and Rapoport, 1993).

A Free Sec61 Population Preferentially Accessible to RNC-SRP Complexes

To test whether PK-RMs saturated with nontranslating ribosomes contain a free Sec61 population, they were solubilized in the detergent digitonin, and the extract was subjected to sucrose gradient centrifugation. Fractions were analyzed by immunoblotting for the α -subunit of the Sec61 complex and of SR. Most of the Sec61 complex sedimented in heavy fractions, where the ribosomes are found (Supplemental Figure 2), as shown by Coomassie staining of the ribosomal proteins (our unpublished data). However, ~30% of Sec61 and all of the SR were found at the top of the gradient (Figure 2A). When untreated PK-RMs were used, Sec61 α and SR were found exclusively on top of the gradient (Figure 2A). Control experiments showed that the ribosome-saturated PK-RMs used in Figure 2A did not bind RNCs carrying ppl86 unless SRP was present (Figure 2B, lane 4 vs. 6). They also did not bind radiolabeled nontranslating ribosomes, confirming that they were indeed saturated with ribosomes (Figure 2C, right bar). These results show that a surprisingly high percentage of free Sec61 is present in membranes that fail to bind ribosomes. Indeed, at the chosen ribosome concentration (300 nM) and the apparent binding constant determined by us and others (6–50 nM; Borgese *et al.*, 1974; Prinz *et al.*, 2000a, b), one would have expected a much lower percentage of free Sec61 complex (<5–10%). A high percentage of free Sec61 was also seen when ribosome-saturated proteoliposomes containing the Sec61 complex were solubilized and analyzed by sucrose gradient centrifugation (Figure 2D). The additional presence of SR had no effect.

Next, we probed for the presence of a free Sec61 population in intact membranes. PK-RMs were treated with buffer or saturated with ribosomes under the same conditions as described above. The membranes were then incubated with antibodies against the cytosolic C-terminus of Sec61 α , reisolated by sedimentation, washed, and solubilized in digitonin. The ribosome-associated Sec61 was separated from the ribosome-free Sec61 by sucrose gradient centrifugation. The Sec61-associated IgG was detected by immunoblotting with a secondary antibody (Figure 3A). The antibodies comigrated only with the free Sec61 fraction and not with ribosome-bound Sec61, as expected, because ribosome binding sterically shields Sec61 (Menetret *et al.*, 2005). Ribosome-saturated PK-RMs bound a significant amount of Sec61 α antibodies, and these antibodies were found at the top of the gradient, comigrating with the free Sec61 population (Figure 3A). When the membranes were preincubated with the same amount of control antibodies, no IgG could be detected (Figure 3B). Similar results were obtained with RMs (Figure 3, C and D). Control experiments with PKRMs in the absence of ribosomes showed that a significantly larger amount of free Sec61 complex could be detected by Sec61 α antibodies (Figure 3, E and F). These results show that a free Sec61 population exists in intact, ribosome-saturated membranes.

The existence of a free Sec61 population in ribosome-saturated membranes suggested that it either cannot bind ribosomes at all or only with low affinity. When PK-RMs were preincubated with ribosomes at a concentration of 0.3 μM (Figure 4A), which is sufficient to saturate the high-affinity sites and prevent RNC targeting in the absence of SRP (Figure 2, B and C), the fraction of free Sec61 was ~30%. When the ribosome concentration was increased to 1.6 μM , this fraction decreased to ~5% (Figure 4A). Untreated RMs contained ~30% free Sec61, a percentage that decreased to <5% upon preincubation with 4 μM nontranslating ribosomes (Figure 4B). These data suggest that Sec61 can form both high- and

Figure 1. Testing the dissociation of ribosomes from the membrane. (A) PK-RMs were incubated with radiolabeled ribosomes and diluted 100× with buffer. Dissociation of the ribosomes at 28°C was followed by sedimentation of the membranes at different times and measuring the radioactivity in the pellet. (B) PK-RMs were preincubated with saturating amounts of purified, radiolabeled ribosomes, and reisolated. Ribosome dissociation from these membranes was analyzed by a second sedimentation, either immediately ($t = 0$) or after incubation with a translation mix containing RNCs carrying unlabeled ppl86, in the absence or presence of SRP, or after incubation with buffer only. In one sample (presat.), the preincubation was done with unlabeled, instead of labeled, ribosomes. The membranes were reisolated and tested for ribosome binding by incubation with radiolabeled ribosomes and sedimentation. (C) PK-RMs were presaturated with unlabeled, nontranslating ribosomes and incubated with RNCs carrying radiolabeled ppl86 under the same conditions as in B. Insertion of the nascent chain into the channel was tested by treatment with proteinase K (PK). The asterisk indicates the nascent chain fragment protected by the ribosome alone. tRNA shows the position of nonhydrolyzed ppl86-peptidyl-tRNA. (D) PK-RMs were preincubated with an excess of translation mix containing RNCs carrying radiolabeled luc120 and reisolated (lane 4). A control was performed without membranes (lane 2). The membranes with bound luc120 were then incubated with a translation mix containing RNCs carrying either unlabeled (lanes 5–12) or labeled ppl86 (lanes 17–24), or with a translation mix lacking mRNA (mock; lanes 13–16). After sedimentation of the membranes, the samples were analyzed by SDS-PAGE and autoradiography. Controls were performed in the absence of membranes (lanes 5–8, 13, 14, and 17–20).

low-affinity ribosome binding sites in the membrane. We estimate the K_D^{app} of the low-affinity sites to be in the micromolar range. Control experiments showed that the RMs used in Figure 4B do not bind radiolabeled ribosomes at physiological

concentrations ($\sim 0.3 \mu\text{M}$) or RNCs carrying ppl86 (Figure 4C, white bars). This indicates that the high-affinity sites are saturated and that the presence of a nascent chain does not suffice for ribosome binding to the low-affinity sites.

Figure 2. Membranes saturated with nontranslating ribosomes contain a free Sec61 population. (A) PK-RMs were preincubated with buffer (top) or with saturating concentrations of unlabeled nontranslating ribosomes (bottom) and reisolated. The membranes were solubilized in digitonin and the extract subjected to sucrose gradient centrifugation. Fractions were collected and analyzed by immunoblotting for Sec61 α and SR α . The sedimentation position of the ribosomes is indicated. (B) The ribosome-saturated membranes used in A were tested for the binding of RNCs carrying radiolabeled ppl86 in the absence or presence of SRP (lanes 4 and 6). Controls were performed without membranes (lanes 1 and 2) and with untreated PK-RMs (lanes 3 and 5). (C) Ribosome-saturated or untreated PK-RMs used in A were incubated with purified, radiolabeled ribosomes. The membranes were sedimented and the radioactivity associated with them was measured. (D) Proteoliposomes containing purified Sec61 complex alone or Sec61 complex and SR were incubated with an excess of nontranslating ribosomes. The membranes were solubilized, and the extract was subjected to sucrose gradient centrifugation.

To test whether RNC-SRP complexes can be targeted to the free Sec61 population, we determined the kinetics of targeting by using membranes that contain different amounts of free channel (Figure 4B). These membranes were

incubated with RNCs in the presence of SRP for different times; the samples were placed on ice, which stops further membrane targeting (our unpublished data); and treated with protease. The rate of targeting, as measured by the

Figure 3. A free Sec61 population detected by antibodies in native membranes. (A) PK-RMs were presaturated with nontranslating ribosomes and reisolated. The membranes were then incubated with affinity-purified antibody against the cytosolic C terminus of Sec61 α , reisolated by sedimentation, washed, and solubilized in digitonin. The extract was subjected to sucrose gradient centrifugation, and fractions were analyzed by immunoblotting for IgG heavy chain (HC) and Sec61 β . (B) As in A, but using an identical amount of rabbit control IgG instead of Sec61 α antibodies. (C and D) As in A and B, respectively, but with RMs. (E and F) As in A and B, respectively, but with untreated PK-RMs. The sedimentation position of the ribosomes is indicated.

appearance of protease-protected ppl86 over time (Figure 4D, quantitation in E), correlated with the amount of free Sec61 in the membrane (Figure 4B). Targeting was very fast with PK-RMs (Figure 4, D and E) that have a high content of free Sec61 (Figure 4B); it was considerably slower with RMs, and very slow with RMs that had been preincubated with high concentrations of nontranslating ribosomes (Figure 4, B, D, and E). These results indicate that RNC-SRP complexes are targeted to a free Sec61 population that has an only weak affinity for nontranslating ribosomes or RNCs in the absence of SRP.

Interconvertible Sec61 Populations

Next, we tested whether the high- and low-affinity binding sites provided by Sec61 are interconvertible. A detergent extract of RMs was separated by sucrose gradient centrifugation (Figure 5A), and the ribosome-bound and free Sec61 populations were separately reconstituted into proteoliposomes. When proteoliposomes derived from the free Sec61 population were incubated with nontranslating ribosomes, solubilized, and reanalyzed by sucrose gradient centrifugation, again both free and ribosome-bound fractions were observed (Figure 5B). Likewise, when proteoliposomes derived from the ribosome-bound fraction were solubilized and analyzed by sucrose gradient centrifugation, both Sec61 populations occurred (Figure 5C). Thus, the two Sec61 populations seem to be interconvertible.

Interconversion is also suggested by experiments in which we tested whether ribosome-bound Sec61 can give rise to free Sec61. Chymotrypsin digestion can be used to selectively cleave Sec61α molecules that are not covered by ribo-

somes (Kalies *et al.*, 1994); cleavage occurs in the loops between transmembrane segments 6 and 7 and 8 and 9 (Raden *et al.*, 2000; Song *et al.*, 2000). Although only the free Sec61 population was degraded by chymotrypsin (our unpublished data), this led to the dissociation of ~60% of the prebound ribosomes (Figure 5D), suggesting that Sec61 molecules that previously were beneath a ribosome became accessible to the protease. Dissociation was not due to the disassembly of ribosomes by chymotrypsin, because RMs treated in the same way retained RNCs whose nascent chains were labeled by translation in the presence of [³⁵S]methionine (our unpublished data). These data support a model in which Sec61 complexes can move from the ribosome-associated to the free population by lateral diffusion in the plane of the membrane.

An interconversion of the ribosome-bound and free Sec61 populations implies that the ribosome-Sec61 interaction is dynamic. This was indeed confirmed in experiments in which we tested the effect of ATA on the dissociation of ribosomes from membranes. ATA is an inhibitor of ribosome binding to ER membranes that leaves ribosomes intact (Borgese *et al.*, 1974; Fresno *et al.*, 1976) and is expected to prevent the reformation of ribosome-Sec61 bonds. The ribosome makes several different connections with Sec61, and if they can break and reform, one would expect that the normally slow dissociation (Figure 1A) is accelerated by ATA. Indeed, when added to PK-RMs containing prebound radiolabeled ribosomes, ATA greatly stimulated the dissociation of ribosomes from the membrane, particularly at elevated temperatures (Figure 5E). A minor fraction of ribosomes seems to be resistant to ATA, suggesting some heterogeneity

Figure 5. Free and ribosome-bound Sec61 populations are interconvertible. (A) RMs were solubilized in deoxy-BIGCHAP, and the detergent extract was separated by sucrose gradient centrifugation (also using deoxy-BIGCHAP). Fractions were analyzed by immunoblotting for Sec61 α and SR α . (B) The fractions containing free Sec61 complex in A were pooled and reconstituted into proteoliposomes overnight. These proteoliposomes were incubated with an excess of nontranslating ribosomes and solubilized. The extract was separated by sucrose gradient centrifugation as described above, and fractions were analyzed for Sec61 α and SR α . (C) The ribosome-bound fractions of Sec61 in A were reconstituted and analyzed as in B without addition of ribosomes. (D) PK-RMs were preincubated with purified, radiolabeled ribosomes and reisolated. The membranes were incubated with either buffer or 50 μ g/ml chymotrypsin on ice. Proteolysis was stopped at different times, and the membranes were sedimented. The radioactivity in both supernatant and pellet was measured. (E) PK-RMs were preincubated with purified, radiolabeled ribosomes and reisolated. The membranes were incubated with buffer or 100 μ M ATA at 0 or 28°C, as indicated. The dissociation of labeled ribosomes was determined by sedimentation of the membranes at different times.

among the tightly bound ribosomes. ATA did not dissociate RNCs, because treated RMs retained nascent chains labeled by translation in the presence of [35 S]methionine (our unpublished data). These results suggest that there are multiple connections between a ribosome and the high-affinity binding site provided by Sec61; these connections continu-

ously break and reform but together prevent the ribosome from complete detachment.

We used cross-linking to determine whether there are differences between the ribosome-bound and free Sec61 populations in their conformation or oligomeric state. Intact RMs were treated with BMH, a reagent that results in cross-

Figure 6. Sec61 β –Sec61 β cross-links formed in ribosome-bound Sec61 are largely reduced in free Sec61. (A) RMs were treated with 1 mM BMH for 30 min on ice. The reaction was stopped, and the membranes were solubilized. The extract was separated on a sucrose gradient and analyzed by immunoblotting for Sec61 α and Sec61 β . The cross-links between Sec61 α and Sec61 β and between Sec61 β and Sec61 β are indicated (α/β and β/β , respectively). (B) As in A, but in the absence of BMH. (C) Quantitation of the experiment in A. The intensities of the bands corresponding to Sec61 α , the α/β cross-link (α/β), and the β/β cross-link (β/β) in both the free (black bars) and ribosome-bound (gray bars) fractions were determined by densitometry.

links between Sec61 α and Sec61 β as well as between two Sec61 β molecules (Kalies *et al.*, 1998). The membranes were then solubilized in digitonin and subjected to sucrose gradient centrifugation. Cross-links of ~ 30 kDa, corresponding to Sec61 β –Sec61 β cross-links, were essentially confined to the ribosome-bound fraction (Figure 6, A and B; quantitation in Figure 6C). These cross-links migrate at the same position as the Sec61 β –Sec61 β cross-links seen in Sec61-proteoliposomes (our unpublished data) and contain two Sec61 β molecules covalently linked through the single cysteine in their cytoplasmic tails. Cross-links of ~ 50 kDa, which were detected with a mixture of Sec61 α and Sec61 β antibodies, correspond to cross-links between Sec61 β and Sec61 α and were seen at equal intensity in both the free and ribosome-bound fraction (Figure 6, A and C). The Sec61 β –Sec61 β cross-linking data suggest that there is a pronounced conformational difference between ribosome-bound and free Sec61. It is possible that ribosome binding leads to a rearrangement of Sec61 complexes within a tetrameric assembly. However, given that the rather long and flexible cytoplasmic tail of Sec61 β should give rise to cross-links over a wide range of conformations within a Sec61 tetramer and that Sec61 β does not contribute major interactions with the ribosome (Kalies *et al.*, 1998) and is not essential for its function, we favor the idea that ribosome-bound Sec61 is in a higher oligomeric state than free Sec61.

DISCUSSION

Our results suggest a model in which Sec61 can form two classes of ribosome binding sites, which differ in their affinity for ribosomes (Figure 7). We find that RNCs, carrying nascent secretory chains, are targeted to the low-affinity binding sites by the SRP system. This allows RNC–SRP complexes to bind Sec61 even in the presence of a large excess of competing ribosomes. High-affinity binding sites seem to be generated by ring-shaped tetramers of mammalian Sec61, which form at least seven connections with the ribosome (Menetret *et al.*, 2005). Although these connections can each break and reform, as suggested by the ATA experiments, collectively they keep the ribosome firmly bound to the membrane, explaining the extremely slow dissociation rate in the absence of ATA. Interestingly, the Sec61-oligomers in RMs are not disassembled upon removal of the ribosomes by treatment with puromycin/high salt (Hanein *et al.*, 1996), which may explain why PK-RMs retain high-affinity sites for ribosomes. The ribosome-bound, tetrameric Sec61 population seems to be interconvertible with a free

Sec61 population that binds ribosomes and RNCs lacking SRP only poorly. This Sec61 population is conformationally different because it does not give rise to Sec61 β –Sec61 β cross-links as the ribosome-bound tetramers do. A possible interpretation is that this Sec61 population is in a lower oligomeric state, likely corresponding to monomers or dimers. Freeze-fracture experiments with proteoliposomes support the idea that the formation of Sec61-oligomers may be induced by ribosome binding (Hanein *et al.*, 1996). Different quaternary states of the bacterial homologue SecYEG in the membrane have also been described, and their relative abundance may change during translocation (Bessonneau *et al.*, 2002; Scheuring *et al.*, 2005).

Why can free Sec61 efficiently associate with RNCs in the presence of SRP? SRP blocks the membrane binding site of the ribosome; therefore, SR needs to induce a conformational change in SRP that exposes a Sec61 interaction site on the ribosome (Fulga *et al.*, 2001; Pool *et al.*, 2002; Halic *et al.*, 2004). Recent electron microscopy experiments show that the addition of a soluble domain of SR to an RNC–SRP complex does not completely release SRP from the ribosome. Rather, only a domain of SRP becomes disordered, exposing an interaction surface on the ribosome that could accommodate a maximum of two Sec61 complexes (Halic *et al.*, 2006). This fits well with our model, in which the RNC–SRP complex would bind to a Sec61 monomer or dimer. The membrane binding of an RNC–SRP complex could thus be mediated by both ribosome–Sec61 and SRP–SR interactions, explaining why nontranslating ribosomes would not be able to bind to the free Sec61 population. Once the nascent chain has inserted into the channel, the ribosome–Sec61 interaction is stabilized, and both SRP and SR dissociate (Jungnickel and Rapoport, 1995). When all Sec61 binding sites on the ribosome are exposed, a tetramer of Sec61 complexes could form underneath the ribosome, further stabilizing the translocating RNC at the ER membrane.

The proposed model explains why the membrane targeting of RNC–SRP complexes does not lead to the immediate dissociation of prebound nontranslating ribosomes, even if these occupy all high-affinity binding sites. Under physiological conditions, there is always a significant percentage of free Sec61 complex that can be accessed by RNC–SRP complexes. The free pool cannot be easily depleted by the low-affinity binding of nontranslating ribosomes or RNCs without SRP. This would guarantee that RNCs with nascent chains destined for translocation always find a binding site on the ER membrane.

Figure 7. Model for the role of SRP in targeting ribosomes to translocation sites. ER membranes contain a pool of tetrameric Sec61 complex (indicated by two neighboring gray ovals) that has a high affinity for ribosomes and is interconvertible with a pool of free Sec61 that has a low affinity for ribosomes or RNCs (gray oval) and may consist of Sec61 monomers or dimers. Ribosomes carrying a nascent chain with a signal or transmembrane sequence can interact with SRP. These complexes can bind to the SR and subsequently to free Sec61. The nascent chain is inserted into the Sec61 channel and stabilizes the complex. At a later stage of translocation, a tetramer of Sec61 complexes may form underneath the ribosome.

The eventual dissociation of ribosomes from the membrane might occur through the disassembly of tetrameric Sec61 complex into monomers or dimers underneath a bound ribosome, which would weaken the interaction. It is possible that dissociation requires additional factors (Blobel, 1976). Free binding sites could also be generated by the membrane dissociation of ribosomes that translate a growing cytosolic polypeptide chain (Potter and Nicchitta, 2000). Ribosome detachment does not seem to be mechanistically linked to termination of translation and could occur with significant delay (Borgese *et al.*, 1973; Potter and Nicchitta, 2002). The proposed mechanism is not in contradiction with the proposal that large ribosomal subunits can reinitiate translation on the membrane (Potter *et al.*, 2001), but these sites would be distinct from those used by newly arriving RNC-SRP complexes.

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