The β Subunit of the Sec61 Complex Facilitates Cotranslational Protein Transport and Interacts with the Signal Peptidase during Translocation

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Abstract. The Sec61 complex is the central component of the protein translocation apparatus of the ER membrane. We have addressed the role of the β subunit (Sec61β) during cotranslational protein translocation. With a reconstituted system, we show that a Sec61 complex lacking Sec61β is essentially inactive when elongation and membrane targeting of a nascent chain occur at the same time. The translocation process is perturbed at a step where the nascent chain would be inserted into the translocation channel. However, if sufficient time is given for the interaction of the nascent polypeptide with the mutant Sec61 complex, translocation is almost normal. Thus Sec61β kinetically facilitates cotranslational translocation, but is not essential for it.

Using chemical cross-linking we show that Sec61β not only interacts with subunits of the Sec61 complex but also with the 25-kD subunit of the signal peptidase complex (SPC25), thus demonstrating for the first time a tight interaction between the SPC and the Sec61 complex. Interestingly, the cross-links between Sec61β and SPC25 and between Sec61β and Sec61α depend on the presence of membrane-bound ribosomes, suggesting that these interactions are induced when translocation is initiated. We propose that the SPC is transiently recruited to the translocation site, thus enhancing its activity.

In higher eukaryotes, most proteins are transported across the ER membrane in a cotranslational manner. The translating ribosome binds tightly to the ER membrane (Connolly et al., 1989; Crowley et al., 1993, 1994; Kalies et al., 1994), and the growing nascent polypeptide chain is transferred directly from the channel in the ribosome into a channel in the membrane (Blobel and Dobberstein, 1975; Connolly et al., 1989; Simon and Blobel, 1991; Görlich et al., 1992*b*; Görlich and Rapoport, 1993).

The cotranslational translocation pathway in mammals can be reproduced with reconstituted proteoliposomes containing only three membrane protein components: the SRP receptor complex, the translocating chain–associating membrane (TRAM)[1] protein, and the Sec61 complex (Görlich and Rapoport, 1993). The signal recognition particle (SRP) receptor is required to target a ribosome–nascent chain complex to the ER membrane. The function of the TRAM protein is still unclear; it is only required for the translocation of a subset of proteins (Görlich et al., 1992*a*; Voigt et al., 1996). The Sec61 complex represents the essential core of the translocation machinery in the ER membrane. It consists of three subunits, an α subunit (Sec61α) that spans the membrane 10 times, and β and γ subunits (Sec61β and Sec61γ, respectively) that each span the membrane a single time.

The Sec61 complex is evolutionarily highly conserved and is proposed to carry out at least three different functions. First, it is the major constituent of the protein conducting membrane channel. Cross-linking experiments have shown that its α subunit is in continuous proximity of nascent polypeptide chains passing through the membrane (Mothes et al., 1994). Recent electron microscopic data demonstrate that the Sec61 complex can form cylindrical oligomers that presumably represent the channels (Hanein et al., 1996; Beckmann et al., 1997). Second, the Sec61 complex is tightly associated with membrane-bound ribosomes and is likely to be the ribosome receptor (Görlich et al., 1992*b*; Kalies et al., 1994; Jungnickel and Rapoport, 1995). Third, the Sec61 complex is involved in a signal sequence recognition event that takes place inside the membrane (Jungnickel and Rapoport, 1995).

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1. *Abbreviations used in this paper*: BMH, bis-maleimidohexane; PK-RM, puromycin and high salt–treated rough microsomes; RM, rough microsomes; SPC, signal peptidase complex; SPC25, the 25-kD subunit of the signal peptidase complex; SRP, signal recognition particle; TRAM, translocating chain–associating membrane.

The role of the Sec61 complex subunits for its different functions is not yet clear. Whereas the presence of a multispanning α subunit in a channel-forming protein complex may not be surprising, the role of the two small single-spanning polypeptide chains remains mysterious. A particular enigma is the β subunit. In *Saccharomyces cerevisiae*, the simultaneous deletion of the β subunits of the two homologous Sec61 complexes (Sec61p and the Ssh1p complex) is not lethal; the cells only show a growth defect at elevated temperatures (Finke et al., 1996). Thus, despite the fact that the protein is evolutionarily highly conserved, it does not appear to be absolutely required in vivo. Post-translational protein transport across yeast ER membranes in vitro is reduced, but not completely prevented, when the β subunits are lacking. At which point the transport process is inhibited, and whether the β subunits play any role in the cotranslational mode of protein transport has not yet been investigated.

The function of the β subunit of the Sec61 complex may not necessarily be restricted to the actual translocation process. The transport of proteins across the ER membrane must be intimately coupled to their modification and folding, and it seems possible that the β subunit could be involved in interactions between the translocation channel and modifying enzymes or chaperones. A physical association of these proteins with the channel may enhance the efficiency of their function. A good example is the signal peptidase, an abundant enzyme whose active site is in the lumen of the ER. Although it can cleave the signal peptide of even completed polypeptide chains, its efficiency is probably much higher when it can act on polypeptide chains that are just emerging from the translocation channel into the lumen of the ER. One may therefore predict its physical association with channel constituents. The signal peptidase complex is composed of five membrane protein subunits (Evans et al., 1986), two with an active site for enzymatic activity, one with a lumenal domain of unknown function, and two (12 and 25 kD) with cytosolic domains. The function of the latter is particularly unclear because they cannot contribute to the enzymatic activity on the lumenal side of the membrane, and are not essential for the viability of yeast cells (Lively and Walsh, 1983; Fang et al., 1996; Kalies and Hartmann, 1996; Mullins et al., 1996). They would be especially good candidates to serve as linkers to the translocation channel.

In the present paper, we have analyzed the role of the β subunit of the Sec61 complex in the cotranslational translocation pathway in mammals. Using a reconstituted system, we demonstrate that the β subunit kinetically facilitates, but is not essential for, cotranslational translocation. The protein does not play a role in the interaction of the ribosome with the Sec61 complex, but rather in the insertion of the nascent chain into the translocation site. Using a bifunctional cross-linker, we also provide evidence that it interacts specifically with the 25-kD subunit of the signal peptidase complex (SPC25), thus demonstrating for the first time a tight interactions between the SPC and the Sec61 complex. Cross-linking is only observed in the presence of membrane-bound ribosomes. These data thus suggest that, upon ribosome binding to the Sec61 complex, an interaction between the cytosolic domains of the Sec61β and SPC25 serves to recruit the signal peptidase complex to the translocation site. Together with the observation that cross-linking between the α and β subunits of the Sec61 complex is also dependent on translocation, these data provide first evidence for structural changes in the translocation apparatus upon initiation of cotranslational translocation.

Materials and Methods

Preparation of Reconstituted Proteoliposomes

Microsomes treated with puromycin and high salt (PK-RM) (Görlich and Rapoport, 1993) were solubilized at a concentration of 2 eq/μl in SB (50 mM Hepes/KOH, pH 7.6, 15% glycerin, 400 mM potassium acetate, 10 mM magnesium acetate, and 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 5 μg/ml chymostatin as protease inhibitors) containing 1.5% decylsucrose for 30 min on ice. After centrifugation for 10 min at 14,000 rpm in a microfuge, 0.7% deoxyBigChap was added to the supernatant. 500 μl detergent extract was mixed with 100 μl of an immunoaffinity resin that contained 0.2 mg/ml affinity-purified antibodies directed against the NH_2 terminus of Sec61β, covalently coupled to protein A–Sepharose (Görlich and Rapoport, 1993). The column was previously equilibrated with SB containing 1.5% decylsucrose and 0.7% deoxyBigChap. The incubation was done for 18 h in an overhead shaker in a cold room. Every 6 h the resin was replaced with a new one. The non-bound material was collected and proteoliposomes were produced by incubation with SM2-biobeads at 4°C (Bio Rad Laboratories, Hercules, CA) (Görlich and Rapoport, 1993). Proteoliposomes containing the purified Sec61 complex were prepared as described previously (Kalies et al., 1994).

The signal peptidase complex (SPC) was purified as reported (Görlich and Rapoport, 1993). Proteoliposomes were produced by mixing 20 μl of the SPC preparation (200 eq) with 20 μl of a phospholipid mixture (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in a ratio of 100:25:3:12.5; total concentration 5 mg/ml). 40 μg SM2 biobeads equilibrated in SB were added and vesicles were prepared as described (Görlich and Rapoport, 1993).

Binding of Ribosomes to Proteoliposomes

The purification, radioactive labeling, and binding of ribosomes to reconstituted proteoliposomes were done as described (Kalies et al., 1994). The ribosome binding was carried out at 26°C.

Cross-linking with Bis-maleimidohexane

A 25 mM stock solution of bis-maleimidohexane (BMH) in dimethylformamid was prepared. Microsomes or proteoliposomes were incubated with increasing amounts of BMH for 30 min at 0°C in a cross-link buffer containing 50 mM Hepes/KOH, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, and protease inhibitors. The reaction was quenched by addition of 100 mM β-mercaptoethanol and the samples were analyzed by SDS-PAGE and immunoblotting, using various antibodies.

Transcription, Translation, and Translocation

Transcripts coding for preprolactin were produced by in vitro transcription with T7 RNA polymerase of the plasmid pGEMBP1. For full-length transcripts, the plasmid was cut with PstI and in the case of the transcripts coding for the 86-mer with PvuII.

Translation of the full-length protein was carried out in the wheat germ system in the presence of 40 nM SRP, [^{35}S]methionine, and membranes at 26°C for 30 min. After 15 min, 4 μM edeine was added to prevent further initiation. In a parallel experiment translation was started in the absence of membranes for 15 min at 26°C. 4 μM edeine and membranes were then added. The samples were incubated on ice for 10 min followed by an incubation at 26°C for additional 15 min. Half of the sample was precipitated with 15% TCA. The other half was treated with 0.5 mg/ml proteinase K for 30 min on ice, before precipitation with TCA. The pellets were washed with acetone and dissolved in SDS sample buffer. After SDS-PAGE, the gels were dried and analyzed in a quantitative manner with a PhosphorImager (Bas 1000; Fuji, Tokyo, Japan).

Translation of the 86-mer of preprolactin was done in the reticulocyte lysate system in the presence of [^{35}S]methionine at 24°C for 20 min. For

the mock translation, the mRNA was omitted. 2 mM cycloheximide and 5 eq membranes were added to 300 μl translation mix and the incubation was continued at 0°C for 10 min and at 25°C for 5 min. After centrifugation in a microfuge at 14,000 rpm for 5 min the samples were layered on top of a sucrose cushion (400 μl of 50 mM Hepes/KOH, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, and 500 mM sucrose) and were centrifuged at 75,000 rpm for 20 min (rotor TLA 100.3; Beckman Instruments, Inc., Fullerton, CA). Finally the pellets were resuspended in 65 μl cross-link buffer.

Immunoblotting, Immunoprecipitation, and Antibodies

Immunoblotting was carried out as described (Görlich et al., 1992*b*). Before immunoprecipitation, the samples were denatured in SDS sample buffer omitting reducing reagents. The samples were then mixed with 10 vol of SB containing 1% Triton X-100, and incubated for 20 min on ice. Affinity-purified antibodies against Sec61β coupled to protein A–Sepharose were added. After shaking in a cold room for 3 h, the antibody resin was washed with 1% Triton X-100 in SB. Finally, the material bound to the column was analyzed by SDS-PAGE and Western blotting.

Polyclonal antibodies directed against the following synthetic peptides were used: against the NH_2 terminus of Sec61β (Görlich et al., 1992*b*); against the COOH terminus of Sec61α (Görlich and Rapoport, 1993); against the position 137 to 150 of the SRP-receptor α (docking protein α); against the COOH terminus of the TRAM protein (Görlich et al., 1992*a*); against the NH_2 terminus of Sec61γ (DQVMQFVEPSRQC); and against the COOH terminus of SPC25 (Kalies and Hartmann, 1996).

Results

Sec61β Facilitates Cotranslational Protein Translocation

We used reconstituted proteoliposomes, immunodepleted of the β subunit of the Sec61 complex, to investigate the role of the protein in cotranslational protein translocation. Mammalian PK-RM, were solubilized in a detergent mixture that leads to the dissociation of the Sec61 complex into its subunits. The detergent extract was incubated either with antibodies directed against Sec61β that had been immobilized on protein A–Sepharose or, to generate a mock-depleted extract, with protein A–Sepharose alone. The efficiency of immunodepletion was tested by Western blotting, using a radioactively labeled secondary antibody and a PhosphorImager. In a typical experiment, the depleted proteoliposomes contained <0.5% of the original amount of Sec61β, whereas all other proteins tested remained almost unaffected (Fig. 1 *A*). The only exception was Sec61α, the concentration of which was reduced to 50–70% in the worst case. Apparently, under the conditions used, the α and the β subunits are not totally dissociated.

We then tested the reconstituted proteoliposomes for their ability to translocate proteins synthesized in a wheat germ translation system. Microsomes (PK-RM) served as a control. Transcripts coding for full-length preprolactin were translated at 26°C in the presence of microsomes or proteoliposomes, the concentration of which was normalized for their Sec61α content (Fig. 1 *A*). PK-RM and mock-depleted proteoliposomes had the same translocation activity (Fig. 1 *B*, lanes *6* and *7*), whereas the depleted proteoliposomes were totally inactive (lane *8*). In the absence of Sec61β, processed prolactin was produced (Fig. 1 *B*, lane *4*) that, however, was accessible to the action of proteinase K; it therefore presumably represents material generated by signal peptidase that was incorporated into the reconstituted membrane in the inverse orientation.

Since Sec61β is not essential in yeast, we wondered if the mammalian Sec61 complex lacking this component may show in vitro translocation activity under less stringent conditions than used before. We therefore performed the translocation reaction such that more time would be allowed for the membrane binding of the ribosome–nascent chain complex. Translation of the full-length transcript coding for preprolactin was initially carried out in the presence of SRP but absence of membranes. This leads to a translational arrest when the polypeptide chain reaches a length of ~70 residues. The membranes were then added at 0°C, conditions that allow efficient membrane binding of the ribosome–nascent chain complexes but no chain elongation. The samples were then warmed up to 26°C to continue translation and concomitant translocation. With this protocol, proteoliposomes lacking Sec61β were active in translocation, although their activity was lower by a factor of three compared with the wild-type

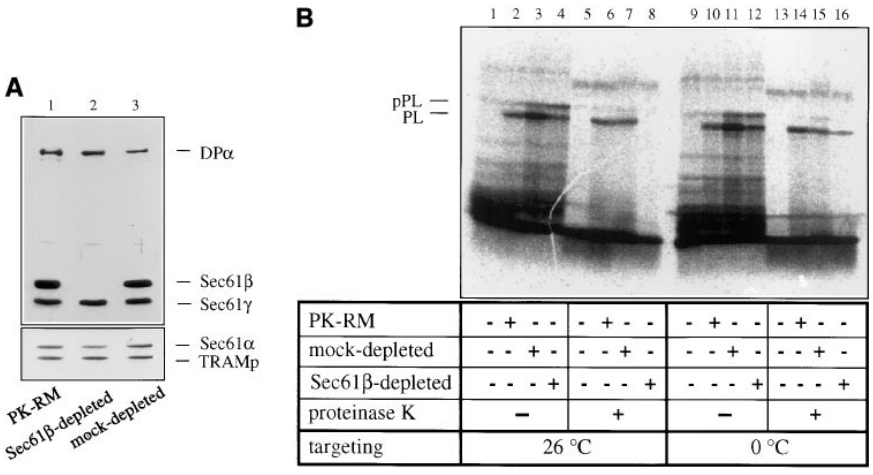

Figure 1. Sec61β facilitates cotranslational protein translocation. (*A*) To produce proteoliposomes lacking Sec61β ribosome-free membranes (*PK-RM*) were solubilized under conditions where the Sec61 complex dissociates into its subunits. The detergent extract was immunodepleted with an antibody column directed against Sec61β or was incubated with protein A–Sepharose to generate a mock-depleted extract. After reconstitution the proteoliposomes and PK-RM were analyzed by SDS-PAGE and Western blotting using a radioactively labeled secondary antibody and a PhosphorImager system for quantitation. (*B*) The membranes and proteoliposomes analyzed in *A* were tested for their competence to translocate polypeptides. Preprolactin mRNA was translated in the wheat germ system in the presence of SRP and microsomes or proteoliposomes, respectively, at 26°C (lanes *2–8*). In a parallel experiment translation was started in the absence of microsomes. After addition of membranes or proteoliposomes, respectively, the targeting reaction was carried out at 0°C followed by an elongation at 26°C (lanes *10–16*). Half of the sample was treated with proteinase K to assay for material that is translocated into the vesicles and is therefore protected against the added protease. *DPα*, α subunit of the SRP receptor; *pPL*, preprolactin; *PL*, prolactin.

complex (Fig. 1 *B*, lane *16* vs. lane *15*). Thus, the depleted proteoliposomes are capable to translocate polypeptides if given enough time in the membrane targeting reaction. The results also indicate that the Sec61 complex lacking its β subunit has not been irreversibly denatured during the prolonged immunodepletion procedure. These data suggest that the ribosome–nascent chain complex was targeted to the membrane and thus brought in contact with the signal peptidase, but that a subsequent translocation step was perturbed. We also found that a fragment of preprolactin of 86 amino acids could be efficiently targeted to reconstituted proteoliposomes and reached a protease-protected state even if Sec61β was lacking (data not shown), supporting the conclusion that insertion of the nascent chain into the Sec61β-depleted translocation site can occur if no chain elongation is going on.

It should be noted that in the absence of membranes, almost no full-length preprolactin could be observed (Fig. 1 *B*, lanes *1* and *9*), indicating that under the conditions used, SRP produced a tight translational arrest. Both the microsomes and the two types of proteoliposomes were able to release the translational arrest (Fig. 1 *B*, lanes *2–4* and *10–12*), indicating that this reaction is not dependent on the presence of Sec61β.

Sec61β Is Not Required for Ribosome Binding

Our data suggested that in the absence of Sec61β, the binding of the ribosome–nascent chain complex to the ER membrane is less efficient. This could be due to either a defect in the interaction of the mutated Sec61 complex with the ribosome, or to a perturbed insertion of the nascent chain into the translocation site. We therefore analyzed whether the β subunit plays a role in ribosome binding. Depleted and mock-depleted proteoliposomes were incubated at physiological salt concentrations with radioactively labeled ribosomes and increasing amounts of unlabeled ribosomes, both lacking nascent polypeptide chains. Under these conditions, the ribosomes interact mainly with the Sec61 complex (Kalies et al., 1994). To separate the unbound from the bound fraction, the membranes were floated in a sucrose gradient. Scatchard plot analysis was used to estimate the number of binding sites and the apparent dissociation constants (Fig. 2). Both the depleted and mock-depleted proteoliposomes were found to bind ribosomes with approximately the same binding constant. Also, the number of binding sites was about the same. The measured parameters are in good agreement with published data for the binding of ribosomes to PK-RM and proteoliposomes (Kalies et al., 1994), although the dissociation constants seem to be somewhat higher at 26° than at 0°C. Thus, steps other than the interaction between SRP and its membrane receptor or the binding of ribosomes to the Sec61 complex must be responsible for the less efficient insertion of the nascent polypeptide chains into proteoliposomes lacking Sec61β.

Sec61β Interacts with the 25-kD Subunit of the Signal Peptidase Complex

To further analyze the function of Sec61β, we investigated its molecular environment in the membrane by chemical cross-linking. Rough microsomes (RM) were treated with

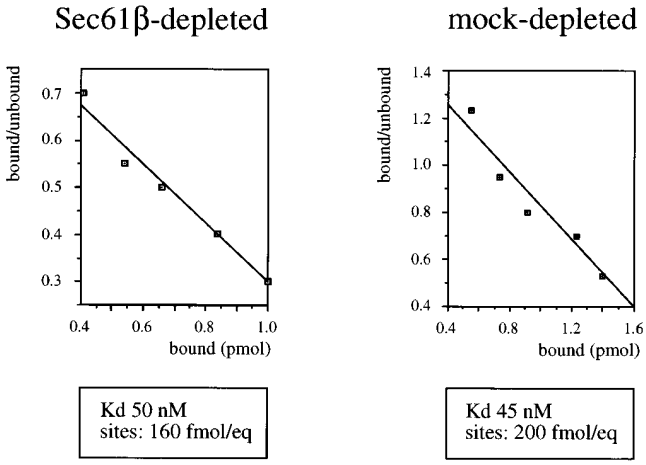

Figure 2. Binding of ribosomes to reconstituted proteoliposomes. Sec61β- and mock-depleted proteoliposomes were incubated with radioactively labeled ribosomes and increasing amounts of unlabeled ribosomes at physiological salt concentrations and 26°C. To separate the bound from the unbound fraction the samples were submitted to flotation in a sucrose gradient. The number of binding sites and the apparent dissociation constants were estimated by Scatchard plot analysis. K_d, dissociation constant; *eq*, membrane equivalant (Walter and Blobel, 1983).

increasing amounts of BMH, a bifunctional cross-linking reagent that reacts with sulfhydryl (SH) groups. The proteins were subsequently separated by SDS-PAGE and analyzed by immunoblotting with antibodies against Sec61β (Fig. 3, lanes *2–6*). Three cross-linked products were detected with the antibodies (Fig. 3, lanes *2–6* vs. lane *1*). The apparent molecular weights of the cross-linked proteins were estimated to be 12, 23, and 38 kD, assuming an apparent molecular weight for Sec61β of 13 kD.

To identify the cross-linked proteins, microsomes were treated with two different concentrations of BMH and dissolved in SDS-containing buffer to dissociate noncovalent chemical bonds. The extract was subjected to immunoprecipitation with Sec61β antibodies and the precipitated material was analyzed by Western blotting using different antibodies. Fig. 3 shows the immunoblots with antibodies directed against Sec61β (Fig. 3, lanes *7–10*), Sec61α (Fig. 3, lanes *11–14*) and SPC25 (lanes *15–18*). The product containing the 38-kD protein could be immunoprecipitated with Sec61β antibodies (Fig. 3, lane *10*) and was recognized by the Sec61α antibody (lane *14*), indicating that it is generated by cross-linking between the α and β subunits of the Sec61 complex. The product containing a protein of ~23 kD could be immunoprecipitated with Sec61β antibodies (Fig. 3, lane *9*) and reacted with antibodies against SPC25 (lane *17*) and is thus generated by cross-linking between these two proteins. Neither Sec61α nor SPC25 were coprecipitated with Sec61β if BMH was omitted (Fig. 3, lanes *12* and *16*), and both antibodies recognized single bands in untreated RM (lanes *11* and *15*).

The product containing the protein of ~12 kD did not react with any of the antibodies tested. Considering its size, we suspected that it may represent a product generated by cross-linking of two β subunits of the Sec61 complex. To test this assumption, purified Sec61 complex was

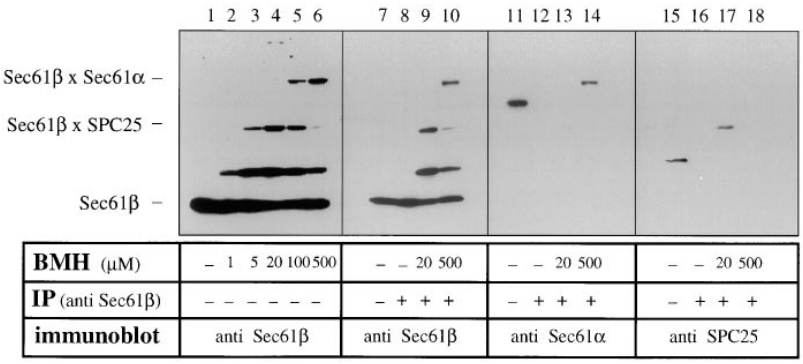

Figure 3. Analysis of the environment of Sec61β by chemical cross-linking. RM were treated with increasing amounts of bis-maleimidohexane (*BMH*) and were subsequently analyzed by SDS-PAGE and immunoblotting using antibodies directed against the β subunit of the Sec61 complex (lanes *1–6*). To identify the cross-linked polypeptides, RM were treated with BMH as indicated. The samples were subsequently immunoprecipitated under denaturing conditions with an anti-Sec61β column. The precipitated material was analyzed by Western blotting using antibodies directed against Sec61β (lanes *7–10*), Sec61α (lanes *11–14*) or the 25-kD subunit of the signal peptidase complex (*SPC25*) (lanes *15–18*), respectively. Lanes *1*, *7*, *11*, and *15* show the results for untreated RM.

reconstituted into proteoliposomes and subjected to cross-linking with BMH. When analyzed by SDS-PAGE and immunoblotting with Sec61β antibodies, a cross-linked product containing a 12-kD protein was again observed (Fig. 4 *A*, lanes *10–12*), indicating that the cross-linking partner is indeed a constituent of the Sec61 complex. The smaller cross-linked product in Fig. 4 *A*, lanes *11* and *12* (marked with an *asterisk*) is probably generated by cross-linking between Sec61β and Sec61γ, the smallest subunit of the Sec61 complex. The appearance of this cross-linked product in native microsomes was variable among different experiments.

Ribosome-dependent Structural Changes of the Translocation Site

We were concerned that the membrane-bound ribosomes may prevent full access of the bifunctional cross-linker to Sec61β so that only a subset of its interacting partners could be detected. However, to our surprise, when PK-RM

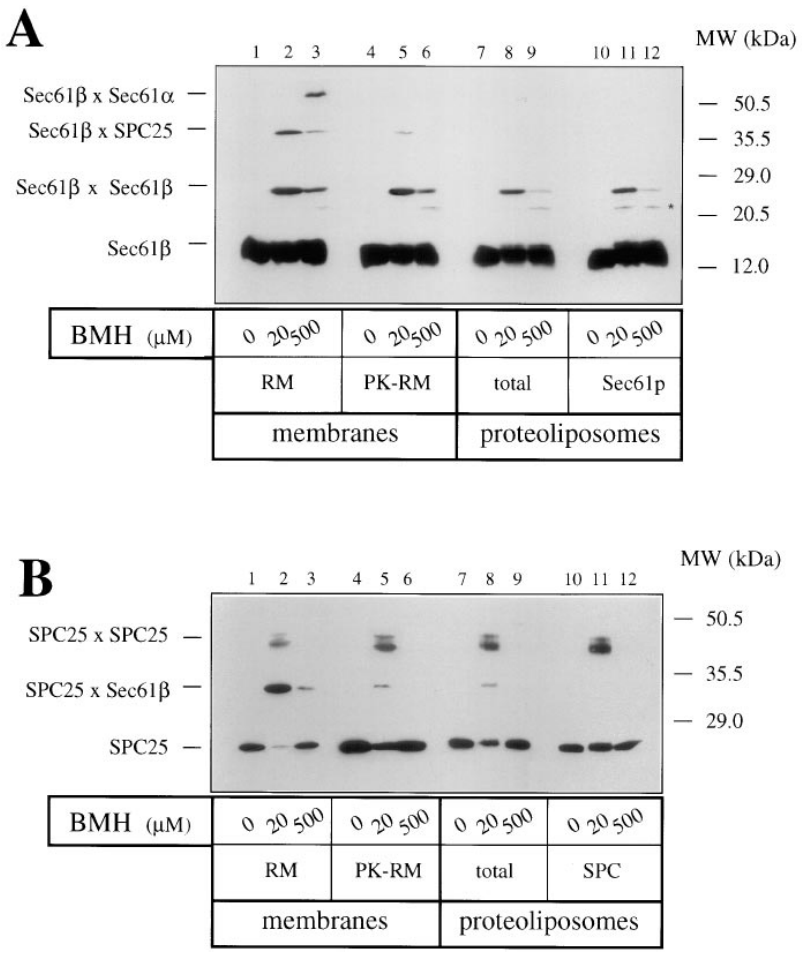

Figure 4. Sec61β is involved in ribosome-dependent structural changes of the translocation site. Rough microsomes (*RM*), ribosome-free membranes (*PK-RM*), proteoliposomes produced from an unfractionated detergent extract of PK-RM (*total*), and proteoliposomes containing the purified Sec61 complex (*Sec61p*) or the purified signal peptidase complex (*SPC*), respectively, were treated with BMH as indicated. After SDS-PAGE the samples were analyzed by immunoblotting with antibodies against Sec61β (*A*) or SPC25 (*B*). The position of a probable cross-link between Sec61β and Sec61γ is marked by an asterisk.

were used in cross-linking experiments, not only were no additional cross-links observed, but those between Sec61α and Sec61β and between Sec61β and SPC25 could no longer be seen (Fig. 4 *A*, lanes *4–6*). Treatment of RM with high salt or puromycin alone did not change these cross-links (data not shown), suggesting that their disappearance requires the dissociation of the ribosomes into subunits. In agreement with this assumption, proteoliposomes reconstituted from a crude detergent extract of microsomes or proteoliposomes containing only the purified Sec61 complex, which both lack membrane-bound ribosomes, also did not give these cross-links (Fig. 4 *A*, lanes *7–12*).

A similar conclusion could be drawn when the cross-linking reaction was analyzed with SPC25 antibodies (Fig. 4 *B*). With RM two cross-linked products were seen, one of ~36 kD between SPC25 and Sec61β, and another of ~46 kD (Fig. 4 *B*, lane *2* vs. lane *1*). With PK-RM or with proteoliposomes reconstituted from a crude detergent extract, the adduct of SPC25 and Sec61β was no longer observed (Fig. 4 *B*, lanes *4–6* and lanes *7–9*, respectively), whereas the 46-kD cross-linked product remained unchanged. The latter was also observed with proteoliposomes containing only the purified signal peptidase complex (Fig. 4 *B*, lane *11*). Two SPC subunits, the nonglycosylated SPC25 and the glycoprotein SPC22/23 carry SH groups and have an appropriate molecular weight to produce this 46-kD cross-link with SPC25. As the molecular weight of the cross-linked product did not change after treatment with *N*-glycosidase F (data not shown), we conclude that it consists of two SPC25.

To exclude that the high salt treatment during the preparation of PK-RM was responsible for the structural alterations identified, ribosomes were detached from the membrane by an independent method. When the reaction with BMH was performed in the presence of 10 mM EDTA under low salt conditions, the cross-link between Sec61β and Sec61α and that between Sec61β and SPC25 could not be seen anymore (Fig. 5, lanes *5* and *4* vs. lanes *2* and *3*). However, the homotypic cross-link between two Sec61β remained unchanged, indicating that the EDTA did not interfere with the reactivity of the BMH. It should be noted that the extent to which the cross-linking intensity was reduced varied in different experiments.

If the ribosome-dependent alteration of the cross-link pattern has a real physiological significance it should be possible to reproduce the cross-link between Sec61β and Sec61α and that between Sec61β and SPC25 by a retargeting of ribosomes carrying nascent polypeptide chains at ribosome free membranes. Ribosome–nascent chain complexes were produced by an in vitro translation of truncated mRNA coding for the first 86 amino acids of preprolactin (86-mer). Ribosome-free membranes (PK-RM) were then added to the translation mix (Fig. 6 *A*, lanes *4–6*). As controls PK-RM and RM were incubated with a translation mix that did not contain any preprolactin mRNA (Fig. 6 *A*, lanes *1–3* and lanes *7–9*). After isolation of the membranes aliquots of each sample were treated with different amounts of BMH. The samples were analyzed by Western blotting with Sec61β antibodies using enhanced chemiluminescence as a detection system (Fig. 6 *A*) or by quantitative immunoblotting using radioactively labeled secondary antibodies and a PhosphorImager (Fig. 6 *B*). The quantitation (Fig. 6 *B*) shows that the incubation of PK-RM with ribosome–nascent chain complexes led to a clear restimulation of the cross-link intensity between Sec61β and Sec61α and between Sec61β and SPC25. Similar results were obtained if EDTA-treated membranes were analyzed (data not shown).

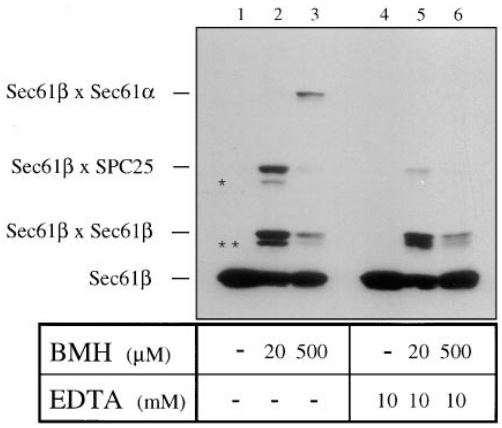

Figure 5. Structural changes of the translocation site in EDTA-treated microsomes. RM were incubated with 10 mM EDTA before BMH was added as indicated. The samples were analyzed by SDS-PAGE and immunoblotting with anti-Sec61β antibodies.

Taken together, these data provide evidence that the β subunit of the Sec61 complex is involved in ribosome-dependent conformational changes of the translocation channel and that it specifically interacts with the signal peptidase during cotranslational translocation.

Discussion

In this paper, we have studied the role of the β subunit of the Sec61 complex during cotranslational protein translocation into the mammalian ER. We have found that the translocation competence of reconstituted proteoliposomes immunodepleted of Sec61β is greatly reduced when tested under conditions in which membrane targeting and translation occur at the same time. However, if ribosomes carrying short nascent chains are first targeted to the membrane before translation is continued, i.e., if enough time is given for their membrane insertion, the depleted proteoliposomes are only marginally reduced in their activity. These results can be explained by the assumption that elongation of a nascent chain and its insertion into the translocation channel are kinetically competing processes: if the membrane insertion is too slow, elongation of the nascent chain would continue in the cytosol and its folding would prevent a later interaction of the signal sequence with the translocation apparatus. We therefore infer that the β subunit is required for a rapid insertion of the ribosome-bound nascent chain into the translocation sites in the ER membrane. The observation that the β subunit is not essential is in agreement with the fact that the deletion of the two β subunits in yeast cells is not lethal and leads to a growth phenotype only at elevated temperatures. Since microsomes isolated from the mutant had a reduced activity for posttranslational protein transport, these and the present results indicate that the β subunit plays a role in both pathways, but is not absolutely required in either one.

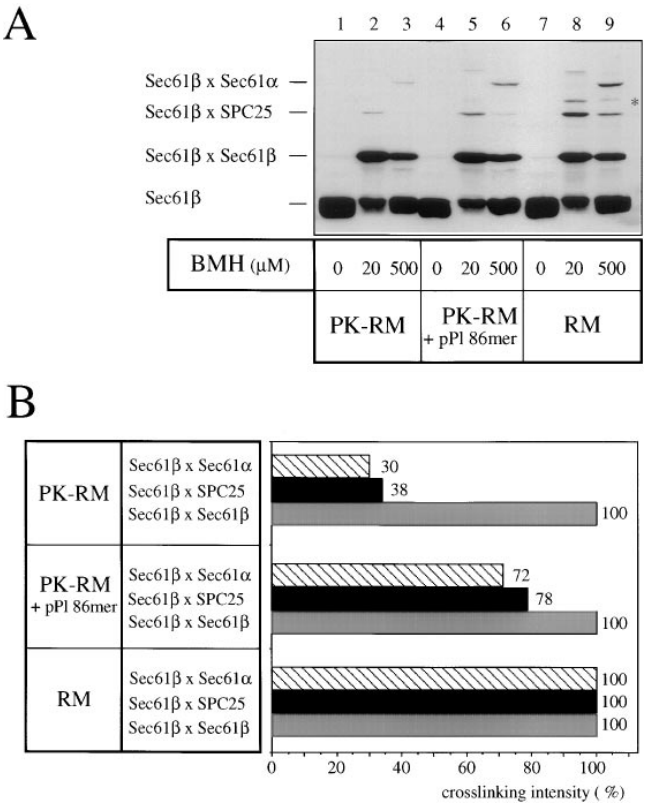

Figure 6. Structural changes of the translocation site are induced by the targeting of ribosomes carrying the preprolactin 86-mer (*pPl 86mer*). (*A*) Translation was carried out in the reticulocyte lysate system in the presence (lanes *4–6*) or absence (lanes *1–3* and *7–9*) of mRNA coding for the pPl 86-mer. After addition of PK-RM (lanes *1–6*) or rough microsomes (lanes *7–9*), respectively, the targeting reaction was carried out at 0°C followed by an incubation at 26°C. The membranes were collected by a centrifugation through a sucrose cushion, separated into three aliquots and treated with bis-maleimidohexane (*BMH*) as indicated. The samples were analyzed by immunoblotting with antibodies against Sec61β using as a detection system enhanced chemiluminescence (*A*) or radioactively labeled secondary antibodies (*B*), respectively. The unknown cross-link marked with an asterisk was not observed in other experiments. (*B*) A PhosphorImager system was used for quantitation of the cross-linking intensities. The cross-linking intensities obtained for RM were defined as 100%.

As the cotranslational translocation process is known in much detail, we have been able to analyze the effect of Sec61β depletion on the various steps. One of the first steps is the interaction of the ribosome–nascent chain–SRP complex with the SRP receptor in the membrane that leads to the release of the translational arrest exerted by SRP. This reaction was not perturbed by the depletion of Sec61β, although previous data showed that, for optimal release, both the SRP receptor and the Sec61 complex are required (Görlich and Rapoport, 1993). We therefore conclude that the β subunit is not essential for this activity of the Sec61 complex. The binding of ribosomes to the ER membrane was also not affected by the absence of Sec61β. Both the number of binding sites and the dissociation constant remained unchanged and were almost identical to the values determined for the reconstituted, purified wild-type Sec61 complex. These results are consistent with the observation that proteolytic degradation of the cytosolic domain of Sec61β in microsomes does not prevent the binding of ribosomes (Kalies et al., 1994). Taken together, our results indicate that even in the absence of Sec61β, efficient release of the SRP arrest and binding of the ribosome–nascent chain complex to the Sec61 complex can occur. This conclusion is further supported by the observation that the membrane targeted nascent chains undergo signal peptide cleavage by signal peptidase located in the wrong orientation in the reconstituted membrane. It therefore appears that a step subsequent to membrane targeting, most likely the step in which the nascent chain is inserted into the Sec61 channel, is perturbed in the absence of the β subunit. Structural changes in the Sec61 channel, which may be required for its opening (Crowley et al., 1994; Jungnickel and Rapoport, 1995), may occur with a reduced rate.

To further analyze the function of Sec61β, we have probed its molecular environment in ER membranes by cross-linking. A bifunctional cross-linker was used that reacts specifically with SH groups and therefore can be expected to give a relative simple cross-linking pattern. As predicted, in rough microsomes Sec61β was found in close proximity to the α subunit of the Sec61 complex. We also found a cross-linked product consisting of two Sec61β molecules, perhaps explained by the occurrence of oligomers of the trimeric Sec61 complex in microsomes (Hanein et al., 1996). Most interestingly, however, a specific cross-linked product containing SPC25 was observed. Conversely, when the cross-linking partners of SPC25 were analyzed, the only partner outside the signal peptidase complex was found to be Sec61β. Remarkably, ≤70% of SPC25 could be cross-linked to Sec61β. We therefore believe that the cross-link between the two proteins indicates their specific interaction. These data provide the first evidence that the signal peptidase physically contacts the protein conducting channel in the membrane. The cross-linking between Sec61β and SPC25 has likely occurred between their cytosolic domains since the only cysteine in Sec61β is in its NH_2 terminus and two appropriately located cysteines exist in the NH_2-terminal domain of SPC25 (Fig. 7), previously shown to be cytosolic (Kalies and Hartmann, 1996). SPC25 has almost no amino acid residues in the lumen of the ER where the active site of the signal peptidase resides (Fig. 7) and its function was therefore obscure. On the basis of our data, we propose that it is involved in an interaction with the Sec61 complex to bring the enzyme close to the translocating polypeptide substrate. However, sequestration in the translocation site may not be absolutely essential for its function because the signal peptidase complex is very abundant and can probably reach its substrates by mere diffusion in the plane of the membrane, explaining why neither SPC25 nor Sec61β are essential for the viability of yeast cells.

Further evidence for our conjecture that Sec61β may be involved in recruiting the signal peptidase complex to the translocation site comes from cross-linking experiments in which ribosome-stripped membranes were used. These membranes gave many cross-link–reduced cross-links between Sec61β and SPC25. After retargeting of ribo-

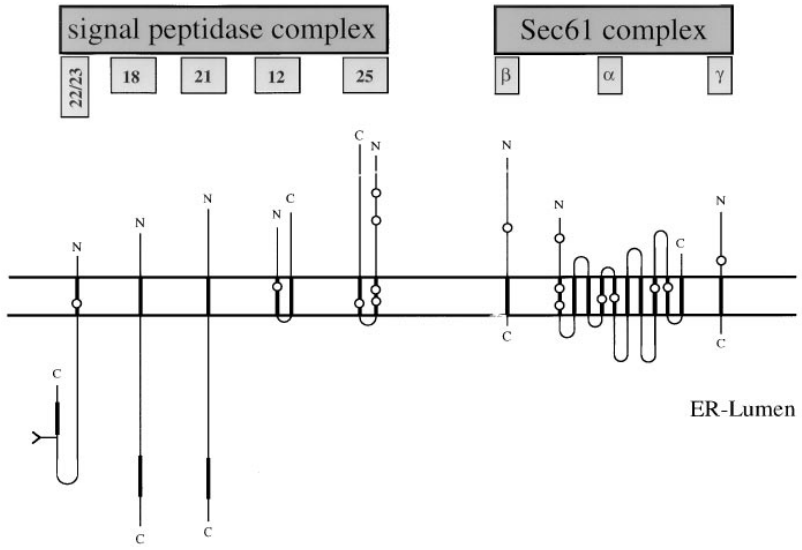

Figure 7. Membrane topology of the signal peptidase complex and the Sec61 complex. The topologies of all subunits are schematically illustrated. The dark boxes indicate hydrophobic segments or membrane spanning segments, respectively. SPC22/23 is drawn in the glycosylated form. The position of SH-groups is marked by white circles.

some–nascent chain complexes at almost ribosome-free membranes, the cross-linking between both proteins was increased, suggesting that their interaction is induced when translocation is initiated by ribosome binding. The cross-linking between Sec61β and Sec61α was also dependent on the presence of membrane-bound ribosomes. Both effects required the dissociation of the ribosome, and not merely the release of the nascent chain from the ribosome, since they occurred with puromycin at high but not at low salt concentrations. The changes in cross-linking pattern upon ribosome removal or retargeting of ribosome–nascent chain complexes could be caused by conformational alterations in an assembled complex or by dissociation of an assembly into subcomplexes. In any case, they provide first evidence for structural changes among known components of the translocation apparatus induced by the onset of cotranslational translocation.

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