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Role of the Cytoplasmic Segments of Sec61α in the Ribosome-binding and Translocation-promoting Activities of the Sec61 Complex

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Abstract. The Sec61 complex performs a dual function in protein translocation across the RER, serving as both the high affinity ribosome receptor and the translocation channel. To define regions of the Sec61 complex that are involved in ribosome binding and translocation promotion, ribosome-stripped microsomes were subjected to limited digestions using proteases with different cleavage specificities. Protein immunoblot analysis using antibodies specific for the NH_2 and COOH terminus of Sec61α was used to map the location of proteolysis cleavage sites. We observed a striking correlation between the loss of binding activity for nontranslating ribosomes and the digestion of the COOH-terminal tail or cytoplasmic loop 8 of Sec61α. The proteolyzed microsomes were assayed for SRP-independent translocation activity to determine whether high affinity binding of the ribosome to the Sec61 complex is a prerequisite for nascent chain transport. Microsomes that do not bind nontranslating ribosomes at physiological ionic strength remain active in SRP-independent translocation, indicating that the ribosome binding and translocation promotion activities of the Sec61 complex do not strictly correlate. Translocation-promoting activity was most severely inhibited by cleavage of cytosolic loop 6, indicating that this segment is a critical determinant for this function of the Sec61 complex.

Key words: endoplasmic reticulum • protein targeting • protein translocation • translocon structure • protein topology

Introduction

Proteins that are translocated across or integrated into the ER are cotranslationally recognized by the 54-kD subunit of the signal recognition particle (SRP)[1] when the NH_2-terminal signal sequence emerges from the exit site on the large ribosomal subunit (for review see Walter and Johnson, 1994). Targeting of the SRP–ribosome nascent chain complex to the RER is mediated by the interaction between the SRP and the SRP receptor (SR), a heterodimeric GTPase localized to the RER. A GTPase cycle involving SRP54 and SRα initiates the release of the signal sequence from SRP, and results in the attachment of the ribosome–nascent chain complex (RNC) to the translocation channel (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1997).

The nascent polypeptide is subsequently transported across the ER membrane through a protein-lined aqueous pore in the membrane (Gilmore and Blobel, 1985; Simon and Blobel, 1991; Crowley et al., 1993). The central core of this protein translocation channel is the Sec61 complex, a heterotrimeric integral membrane protein consisting of Sec61α, Sec61β, and Sec61γ (Görlich et al., 1992; Görlich and Rapoport, 1993). The Sec61 complex oligomerizes within the plane of the membrane to form a quasi-pentagonal 85–100-Å ring surrounding a 20–40-Å pore (Hanein et al., 1996; Hamman et al., 1997). Nascent polypeptides are thought to traverse the membrane via the central pore in the Sec61 oligomer, as proteins undergoing transport are in continuous contact with Sec61α once the ribosome–nascent chain complex engages the translocation channel (Mothes et al., 1994). Of the 10 transmembrane (TM) segments of *Saccharomyces cerevisiae* Sec61α, TM2 and TM7 are the targets for photoreactive cross-linking agents when

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[1]*Abbreviations used in this paper:* C_X-PK-RM, chymotrypsin-digested PK-RM; ECL, enhanced chemiluminescence; NAC, nascent chain–associated complex; OST, oligosaccharyltransferase; PIC, protease inhibitor cocktail; PK-RM, puromycin high salt–washed RM; RM, rough microsomes; RNC, ribosome nascent chain complex; SR, signal recognition particle receptor; SRP, signal recognition particle; TEA, triethanolamine acetate, pH 7.5; TM, transmembrane; T_X-PK-RM, trypsin-digested PK-RM; Th_X-PK-RM, thermolysin-digested PK-RM; V_X-PK-RM, endoproteinase Glu-C–digested PK-RM.

a nascent prepro-α-factor chain is targeted to the yeast SEC complex (Plath et al., 1998).

In addition to serving as the conduit for nascent polypeptide transport across the RER, the Sec61 complex functions as a high affinity ribosome receptor (Görlich et al., 1992; Kalies et al., 1994). Ultrastructural studies of complexes between the *S. cerevisiae* ribosome and the Sec61 complex have revealed that the exit site for the nascent polypeptide on the large ribosomal subunit is aligned with the translocation channel by a single point of contact between the Sec61 oligomer and the ribosome (Beckmann et al., 1997). It is not known which segments of Sec61α are responsible for the high affinity binding of the ribosome to the translocation channel, nor is it certain that Sec61β and Sec61γ do not contribute to the affinity between the ribosome and the Sec61 complex.

Ribosome–nascent chain complexes can bind to unoccupied Sec61 complexes in an SRP-independent reaction that is thought to be driven by the affinity between the ribosome and the Sec61 complex (Jungnickel and Rapoport, 1995; Lauring et al., 1995a; Raden and Gilmore, 1998). This artificial targeting reaction is readily observed when elongation-arrested RNCs are incubated with ribosome-stripped microsomes that contain an excess of Sec61 complexes relative to added RNCs and 80S ribosomes. Although SRP-independent binding of the RNCs to the Sec61 complex is not signal sequence–dependent (Lauring et al., 1995b), the subsequent transport of polypeptides targeted by this mechanism is greatly facilitated by the recognition of the signal sequence by Sec61α (Jungnickel and Rapoport, 1995). This signal sequence recognition activity of the Sec61 complex may provide a proofreading mechanism to enhance the fidelity of protein translocation across the RER.

We have used limited proteolysis to sever cytoplasmically exposed segments of RER membrane proteins. The protease-digested microsomes were assayed for SRP-independent translocation activity and for the ability to bind nontranslating ribosomes or ribosome–nascent chain complexes to determine which cytoplasmic segments of the Sec61 complex contribute to the various functions of the Sec61 complex. We have obtained evidence that SRP-independent translocation is not obligatorily dependent upon high affinity binding of the ribosome to the Sec61 complex. Cytoplasmic segments of the Sec61 complex that are important for high affinity ribosome binding map to COOH-terminal cytoplasmic segments of Sec61α.

Materials and Methods

Preparation of Rough Microsomes (RM), SRP, the SRα Fragment, and Protease-digested PK-RM

Rough microsomes (RM) and SRP were isolated from canine pancreas as described by Walter et al. (1981). The 52-kD SRα fragment was prepared as described previously (Nicchitta and Blobel, 1989). Puromycin high salt–extracted rough microsomes (PK-RM) were prepared from RM as described previously (Raden and Gilmore, 1998), except that PK-RM were washed once by centrifugation rather than twice with 50 mM triethanolamine acetate, pH 7.5 (TEA), 600 mM potassium acetate, 12 mM magnesium acetate, 1 mM DTT, and 1 mM EDTA. The PK-RM were resuspended in membrane buffer (50 mM TEA, 250 mM sucrose, 1 mM DTT) at a concentration of 2 eq/μl (eq as defined in Walter et al., 1981).

Aliquots of the PK-RM (500 μl) were digested at a concentration of 2 eq/μl for 1 h. Trypsin (0–30 μg/ml) and chymotrypsin (0–200 μg/ml) digestions were done on ice and terminated with 1 mM PMSF, followed by a 15-min incubation on ice and adjustment to 10 μg/ml of aprotinin. Digestion with endoproteinase Glu-C (200 μg/ml) was for 1 h at 37°C, and was terminated with 1 mM 3,4-dichloroisocoumarin. Thermolysin digestions (0–50 μg/ml), which were done in the presence of 1 mM $CaCl_2$ at either 25°C or on ice, were terminated by the addition of 2 mM EDTA. The protease-digested PK-RM were adjusted to 550 mM potassium acetate and centrifuged for 30 min at 100,000 g in a Beckman type 50 rotor. The membranes were resuspended at a concentration of 0.1 eq/μl in membrane buffer, and centrifuged for 30 min at 100,000 g in a Beckman type 50 rotor. After repeating the preceding resuspension and centrifugation steps, the protease-digested PK-RM were adjusted to a concentration of 1 eq/μl in membrane buffer, and stored at −80°C.

Isolation of Ribosomes and ^{125}I-Labeling of Ribosomes

Ribosomes were isolated from wheat germ cytosol (Raden and Gilmore, 1998) or from canine RM (Collins and Gilmore, 1991) as described previously. The canine ribosomes were resuspended in TKMD (50 mM TEA, 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT), applied to a 13-ml 10–30% sucrose gradient in TKMD, and centrifuged for 4 h at 200,000 g_{av} in an SW40 rotor. The ribosomes were collected using a density gradient fractionator (ISCO), and were quantified using an extinction coefficient at 260 nm of E = 6.14×10^6.

The canine ribosomes were labeled with iodine-125 by incubating 26 pmol of ribosomes with 450 μCi of ^{125}I-Bolton-Hunter reagent (Amersham Pharmacia Biotech) for 2 h on ice. Radiolabeling was terminated by adjusting the sample to 10 mM Tris-Cl, pH 7.5. The ^{125}I-labeled ribosomes were separated from unincorporated radiolabel by centrifugation on a 5–20% sucrose gradient in 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$ in an SW40 rotor for 3 h at 200,000 g_{av}.

Glycerol Gradient Centrifugation and Superose 12 Chromatography

20 eq of protease-digested PK-RM was mixed with 180 μl of a detergent high salt buffer to obtain the following final conditions: 20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1× PIC (protease inhibitor cocktail as defined in Walter et al., 1981), and 1% digitonin. After a 20-min incubation on ice, the detergent extracts were clarified by centrifugation for 5 min at 30 psi in an airfuge.

A 150-μl sample of the clarified detergent extract was applied to a 5-ml 8–30% glycerol gradient in 20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1× PIC, 0.125% digitonin, and 25 μg/ml egg yolk phosphatidylcholine. The gradients were centrifuged for 18 h at 85,000 g_{av} in an SW50.1 rotor and separated into 13–15 fractions using a density gradient fractionator (ISCO). Clarified detergent extracts were applied to a 23.6-ml Superose 12 column equilibrated in 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1× PIC, 0.125% digitonin, and 25 μg/ml egg yolk phosphatidylcholine. Fractions of 0.5 ml were collected as the column was eluted with equilibration buffer.

Ribosome Binding Assays of Protease-digested PK-RM

Ribosome binding assays were performed by mixing a constant amount of ^{125}I-labeled ribosomes (typically 0.18 pmol) with 0–6.7 pmol of unlabeled canine ribosomes. The ribosomes were incubated with T_X-PK-RM, C_X-PK-RM, V_X-PK-RM, or TH_X-PK-RM for 20 min on ice in TKMD. The 30-μl sample was applied to a 1.3-ml Sepharose CL-2B column equilibrated in TKMD. The column was eluted with 270 μl of TKMD, followed by 1.5 ml of 50 mM TEA, 500 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT. The ionic strength of the elution buffer was raised to reduce nonspecific absorption of unbound ribosomes to the Sepharose beads. 100-μl fractions of the eluate were collected, and the amount of ^{125}I-labeled ribosomes in each fraction was determined using a Beckman γ-counter. The elution positions of membrane-bound ribosomes (0.3–0.6 ml) and unbound ribosomes (0.6–1.5 ml) were determined in control experiments wherein ^{125}I-labeled ribosomes were fractionated after incubation in the presence or absence of PK-RM. The binding data was fit to the following equation using a nonlinear least squares program: $R_B = (B_M \cdot R_F)/(K_d + R_F)$, where R_B and R_F are the concentrations of bound and free ribosomes, respectively; B_m is the number of binding sites; and K_d is the dissociation constant.

SRP-dependent and SRP-independent Translocation Assays

A full-length mRNA encoding preprolactin (pPL) and truncated mRNAs encoding the NH₂-terminal 86 residues of preprolactin (pPL86) and the NH₂-terminal 77 residues of firefly luciferase (ffLuc77) were isolated from preparative scale transcriptions as described previously (Rapiejko and Gilmore, 1994). SRP-dependent translocation of preprolactin was assayed using 25-μl reticulocyte lysate translations that contained [³⁵S]methionine, endogenous SRP, 1.2 eq of protease-digested PK-RM, and 200 fmol of the SRα 52-kD fragment. Translocated prolactin was resolved from preprolactin by PAGE in SDS.

To assay SRP-independent translocation, the truncated pPL86 mRNA transcript was translated at 25°C for 15 min in a wheat germ system that contained [³⁵S]methionine as described previously (Gilmore et al., 1991). Further protein synthesis was blocked by adding cycloheximide to a final concentration of 250 μM. The translation products (10 μl, unless stated otherwise) were incubated with the protease-digested PK-RM (10 eq, unless specified otherwise) for 5 min at 25°C. Translocation of pPL86 was induced by releasing the peptidyl tRNA from the ribosome by incubation with 25 mM EDTA for 10 min at 25°C (Connolly et al., 1989; Raden and Gilmore, 1998). The assays were prepared for SDS-PAGE as previously described (Connolly et al., 1989), and were subjected to electrophoresis using Tris-tricine gels (Schägger and von Jagow, 1987). The percent translocation of pPL86 was calculated after quantification of PL56 and pPL86 using the following formula: percent translocation = $100 \times 1.33 \times$ PL56/$((1.33 \times$ PL56$) +$ pPL86$)$.

The factor of 1.33 corrects for the loss of the NH₂-terminal methionine residue from pPL86 upon signal sequence cleavage.

Binding of RNCs bearing ³⁵S-labeled pPL86 to the protease-digested PK-RM was assayed by centrifugal flotation on discontinuous sucrose gradients as previously described (Lauring et al., 1995b), or by gel filtration chromatography on Sepharose CL-2B columns, as described above, to separate membrane bound RNCs from unbound RNCs. The percentage of RNCs that were membrane bound was quantified with a PhosphorImager after the pPL86 in each fraction was resolved by SDS-PAGE using Tris-tricine gels. Insertion of pPL86 into the Sec61 translocation channel was assayed by resistance to a 1-h digestion on ice with 400 μg/ml of proteinase K as previously described (Connolly et al., 1989).

Protein Immunoblots

The procedure for protein immunoblots using enhanced chemiluminescence has been described (Raden and Gilmore, 1998). Multiple film exposures were obtained to insure that the ECL signal was linear with respect to the quantity of antigen. Western blots were quantified with a Fluor-s multi-imager (Bio-Rad Laboratories) and Multi-Analyst software. Mouse mAbs specific for ribophorin I and SRβ were provided by Dr. Gert Kreibich (NYU School of Medicine, New York, NY) and Dr. Peter Walter (University of California San Francisco, San Francisco, CA), respectively. Rabbit antisera raised against the NH₂ terminus of Sec61α and Sec61β was provided by Dr. Christopher Nicchitta (Duke University Medical Center, Durham, NC) and Dr. Tom Rapoport (Harvard University, Cambridge, MA), respectively. An antibody was raised in rabbits against the COOH terminus of Sec61α (KEQSEVGSMGALLF) using standard procedures.

Results

Sensitivity of Sec61α to Proteolysis

Sec61α is integrated in the ER with a topology that places four loops (L2, L4, L6, and L8) plus the NH₂- and COOH-terminal tails on the cytoplasmic face of the membrane (Fig. 1 A). The β and γ subunits of the Sec61 complex are integrated by single TM spans located near the COOH terminus. Membrane-bound ribosomes effectively block access of proteases to Sec61α and, to a lesser extent, to Sec61β (Kalies et al., 1994). Ribosome-stripped microsomes (PK-RM) were digested with proteases to sever cytoplasmic segments of the Sec61 complex. Trypsin, chymo-
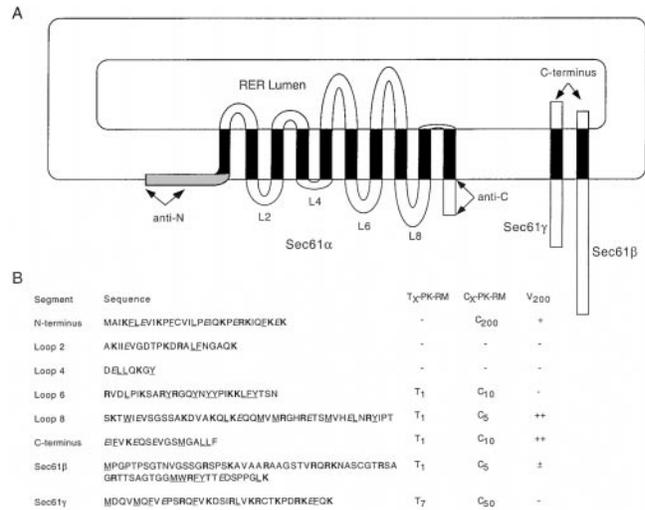

Figure 1. The predicted membrane topology of the Sec61 complex. (A) The topology diagrams for Sec61α, Sec61β, and Sec61γ are adapted from those shown in previous publications (Görlich et al., 1992; Wilkinson et al., 1996). The diagrams show the relative predicted sizes of the cytosolic and lumenal tails and loops. Sec61α segments that were used as antigens for the production of anti–NH₂- and anti–COOH-terminal antibodies are labeled. (B) The amino acid sequences of the NH₂- and COOH-terminal domains and the cytosolic loops of Sec61α, Sec61β, and Sec61γ are shown. Potential protease cleavage sites are indicated in bold (trypsin), italics (endoproteinase Glu-C), or by underlining (chymotrypsin). Digestion of Sec61α, Sec61β, and Sec61γ in PK-RM by these proteases is summarized in the chart to the right. For trypsin and chymotrypsin, the subscript indicates the minimum concentration of protease tested that yielded 50% digestion of a given segment (see Materials and Methods for protease concentrations). Additional symbols designate the following: lack of detectable cleavage (−); less than 50% cleavage (±); ~50% cleavage (+); and digestion to completion (++).

trypsin, endoproteinase Glu-C, and thermolysin-digested PK-RM are designated, respectively, as T_X-PK-RM, C_X-PK-RM, V_X-PK-RM, or Th_X-PK-RM (where the subscript X denotes the concentration of protease, in μg/ml, used for the digestion). Protein immunoblot analysis, using antibodies specific for the NH₂- and COOH-terminal tails of Sec61α, was used to determine which cytoplasmic segments of Sec61α are accessible to proteases. Protease cleavage sites in Sec61α were mapped to cytoplasmic loops 6 and 8 by comparison to COOH-terminal Sec61α truncation products (Song et al., 2000). The amino acid sequences of the cytoplasmic segments of the Sec61 complex are shown in Fig. 1 B, together with potential cleavage sites for trypsin, chymotrypsin, and endoproteinase Glu-C. Although each cytoplasmic segment of Sec61α contains predicted cleavage sites for at least two of the proteases tested, we observed a remarkable difference in the sensitivity of these regions to protease digestion (Fig. 1 B). Loop 8 and the COOH terminus were the most protease-sensitive regions of Sec61α, followed by loop 6. The NH₂-terminal tail, which is proposed to be an amphipathic α-helix aligned with the membrane surface (Wilkinson et al., 1996), was far less sensitive to digestion. Loops 2 and 4 were completely resistant to proteases under all conditions tested.

Oligomeric Stability of the Protease-digested Sec61 Complex

As visualized by electron microscopy, the purified Sec61 complex forms oligomers that are composed of three to four Sec61 heterotrimers (Hanein et al., 1996). Before assaying the protease-digested PK-RM for Sec61-dependent translocation and ribosome binding activities, it was important to determine whether the oligomeric state of the channel had been altered by protease digestion. Sedimentation velocity measurements of protein–detergent complexes have been used to distinguish between monomeric and oligomeric forms of integral membrane proteins (Copeland et al., 1986; Doms and Helenius, 1986; Hebert and Carruthers, 1991). The undigested and protease-digested PK-RM were solubilized with the nonionic detergent digitonin so that the sedimentation velocity of the intact and protease-digested Sec61 complexes could be compared using digitonin high salt glycerol gradients (Fig. 2 A). When the undigested T_0-PK-RM membranes were analyzed, protein immunoblot analysis showed that Sec61α (a) was resolved from SRβ (e), a subunit of the 100-kD SRP receptor, and from ribophorin I (f), a subunit of the oligosaccharyltransferase (OST). As expected, Sec61β (b) and Sec61γ (not shown) cosedimented with Sec61α. The OST serves as a useful internal sedimentation marker (peak in 9–11, ~11S) corresponding to a protein molecular mass of ~300 kD (Kelleher and Gilmore, 1997). The less rapid sedimentation of Sec61α (fractions 6–9) relative to the OST would be consistent with a Sec61 oligomer composed of three to four 70-kD Sec61 heterotrimers. Sec61α subunits severed in loop 8 (L8NTF) and loop 6 (L6NTF) cosedimented precisely with intact Sec61α when the T_1-PK-RM were analyzed (Fig. 2 A, c). The sedimentation rate of Sec61α was also not altered by quantitative cleavage of Sec61α in loops 6, 8, and the COOH terminus by trypsin (d). When the intact Sec61 complex was solubilized with Triton X-100, and then resolved on the digitonin high salt glycerol gradient, Sec61β (h) sedimented far less rapidly and was well resolved from Sec61α (g). Resolution of Sec61α and Sec61β is consistent with a Triton X-100–induced dissociation of the Sec61 oligomer into individual subunits. Because Sec61α accounts for 75% of the protein molecular mass of the Sec61 complex, the Triton X-100–treated Sec61α subunit provides an approximate sedimentation marker for a Sec61 heterotrimer. Aliquots of the intact and protease-digested Sec61 complexes were mixed before glycerol gradient centrifugation (Fig. 2 B). The intact Sec61α subunits, which were derived from the undigested PK-RM, cosedimented with the NH_2-terminal 22-kD fragment, which was derived from the C_{50}-PK-RM (a) or from the Th_{25} PK-RM (b). As observed in A, the protease-digested Sec61 complexes were well resolved from the Triton X-100–treated Sec61α (c).

A dissociation of the translocation channel into Sec61 heterotrimers would result in a simultaneous decrease in mass and an alteration in shape. To insure that a compensatory shape change did not mask the conversion of an oligomeric ring into heterotrimers, we analyzed the intact and protease-digested Sec61 complexes by gel filtration chromatography in a digitonin high salt buffer (Fig. 2 C). Detergent-solubilized Sec61 complexes, from undigested membranes (sample a), eluted in the same fractions as the NH_2-terminal fragments of Sec61α that were derived by trypsin digestion in loops 6 or 8 (samples b-d), indicating that the Stokes radius of the particle was not altered. Taken together with the glycerol gradient centrifugation data, the gel filtration chromatography experiments demonstrate that the Sec61 oligomer does not dissociate into heterotrimers or isolated subunits when Sec61α is severed in cytoplasmic loops 6 and 8, and the COOH terminus.

Ribosome Binding Is Abrogated by Proteolysis of Sec61α

Now that we have defined which segments of the Sec61 complex are susceptible to protease digestion and have shown that Sec61 oligomers remain intact, we assayed the
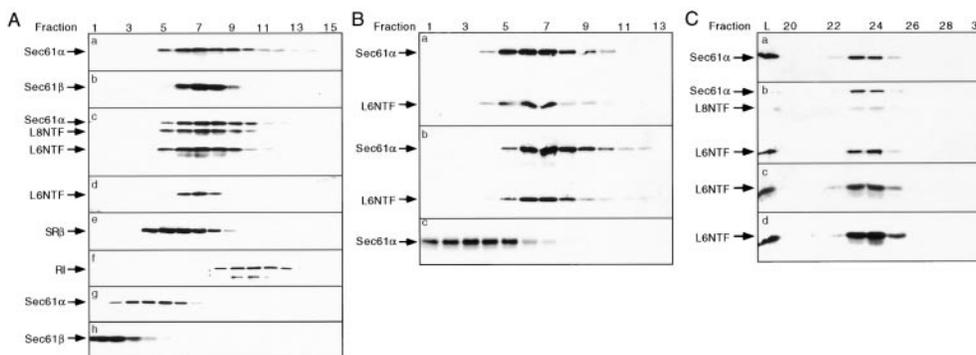

Figure 2. Oligomeric structure of the protease-digested Sec61 complex. (A) Glycerol gradient centrifugation of digitonin high salt extracts of T_X-PK-RM (a–f) or Triton X-100 high salt extracts of T_0-PK-RM (g and h). The following samples were analyzed: (a, b, and e–h) T_0-PK-RM, (c) T_1-PK-RM, and (d) T_{20}-PK-RM. (B) Glycerol gradient centrifugation of detergent high salt extracts of the following samples: (a) T_0-PK-RM mixed with C_{50}-PK-RM; (b) T_0-PK-RM mixed with Th_{25}-PK-RM; and (c) T_0-PK-RM. The protease-digested PK-RM were solubilized in a digitonin high salt buffer (a and b) or in a Triton X-100 high salt buffer (c). In A and B, all detergent extracts were analyzed on digitonin high salt glycerol gradients. (C) Superose 12 chromatography of digitonin high salt extracts of T_X-PK-RM. The following samples were analyzed: (a) T_0-PK-RM, (b) T_1-PK-RM, (c) T_7-PK-RM, and (d) T_{20}-PK-RM. Aliquots of the solubilized T_X-PK-RM (L in C) and each fraction from the glycerol gradients (A and B) and selected fractions from the Superose 12 columns (C) were analyzed on protein immunoblots using antibodies to the NH_2 terminus of Sec61α (all samples except A; b, e, f, and h), Sec61β (A, b and h), SRβ (A, e) or ribophorin I (A, f). L8NTF and L6NTF designate NH_2-terminal fragments of Sec61α severed in cytoplasmic loops 8 and 6, respectively.

protease-digested PK-RM for ribosome binding activity to investigate the role of the cytoplasmic domains of the Sec61 complex. Ribosome binding to the protease-digested PK-RM was assayed by incubating the membranes with a fixed amount of ^{125}I-labeled ribosomes and increasing amounts of unlabeled ribosomes (Fig. 3 A). A physiological ionic strength buffer (150 mM potassium acetates) was used in these assays to minimize nonspecific binding of ribosomes to other RER membrane proteins (Kalies et al., 1994). Nonlinear least squares analysis of the binding data indicated that the undigested PK-RM bind 0.36 pmol of ribosomes/eq with a binding affinity ($K_d \sim 18$ nM), which is a value comparable to previous reports (Kalies et al., 1994). The ribosome binding data for the C_0-PK-RM, C_1-PK-RM, C_5-PK-RM, and C_{30}-PK-RM are graphically displayed as Scatchard plots in Fig. 3 A. Notably, the number of ribosome binding sites per eq of the C_X-PK-RM (X-intercept value) was substantially reduced by proteolysis of the microsomes. The slope of a Scatchard plot is the negative reciprocal of the binding affinity (K_d). The observation that the Scatchard plots are linear rather than curved indicates that proteolysis of the Sec61 complex does not lead to a mixed population of high affinity and low affinity ribosome binding sites. The roughly parallel slopes of the Scatchard plots reveals that the residual binding sites present in the protease-digested PK-RM have a binding affinity that is not significantly lower (less than twofold) than the sites detected in the undigested PK-RM. We compared the number of ribosome binding sites/eq of protease-digested PK-RM to the percentage of intact Sec61α in the chymotrypsin-digested PK-RM (Fig. 3 B). The inhibition of ribosome binding activity by chymotrypsin digestion correlated quite well with digestion of Sec61α. The reduction in ribosome binding activity observed for the C_1-PK-RM was of particular interest because low concentrations of chymotrypsin sever Sec61α uniquely within cytoplasmic loop 8, yielding an NH$_2$-terminal fragment (L8NTF) and a COOH-terminal fragment (L8CTF; Fig. 3 C). More extensive digestion of the PK-RM (e.g., C_{30}-PK-RM) cleaves Sec61α within loops 6 and 8 and causes a loss of COOH-terminal immunoreactivity.

The number of ribosome binding sites was reduced fourfold when 55% of the Sec61α was digested in the T_1-PK-RM (Fig. 3 D). Conceivably, the more dramatic effect of trypsin digestion on the ribosome binding activity of the Sec61 complex could be explained by the liberation of cytoplasmic Sec61α segments, which are crucial for ribosome binding. One limitation of mapping protease cleavage sites by protein immunoblot analysis is that we cannot determine whether the Sec61α subunits have single or multiple cleavage sites within loops 6 and 8. Two cleavages within a single loop would release a soluble tryptic peptide. The V_{200}-PK-RM did not bind ribosomes (not shown).

Previous studies that predated the identification of the Sec61 complex as the ribosome receptor had shown that trypsin digestion of ribosome-stripped microsomes inhibits the subsequent rebinding of 80S ribosomes (Borgese et al., 1974; Hortsch et al., 1986). In these earlier studies, ribosome binding to the membranes was assayed under low ionic strength conditions that permit ribosome binding to the p180 protein (Savitz and Meyer, 1990, 1993) as well as
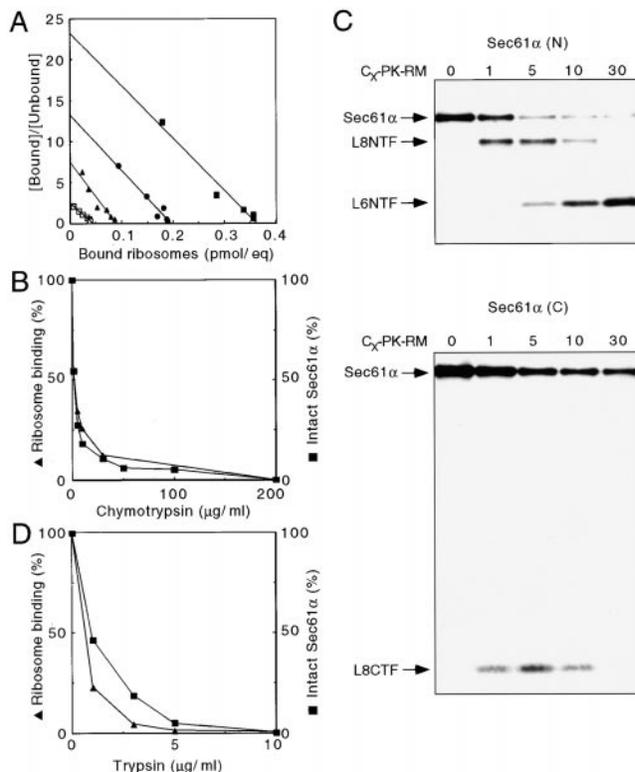

Figure 3. Ribosome binding to trypsin and chymotrypsin-digested PK-RM. (A, B, and D) Aliquots (5 eq) of the C_X-PK-RM and T_X-PK-RM were incubated with 0.18 pmol of ^{125}I-labeled canine ribosomes and between 0.0 and 6.5 pmol of unlabeled ribosomes in 50 mM TEA, 150 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT (TKMD). Membrane-bound ribosomes were separated from unbound ribosomes by gel filtration chromatography as described in Materials and Methods. (A) The ribosome binding affinity and the number of ribosome binding sites per eq of the protease-digested PK-RM were calculated as described in Materials and Methods. The results are displayed as Scatchard plots for the C_0-PK-RM (squares), C_1-PK-RM (circles), C_5-PK-RM (triangles), or C_{30}-PK-RM (open squares). Ribosome binding activities of the C_X-PK-RM (B) and T_X-PK-RM (D) are expressed as a percentage (triangles) of that displayed by undigested PK-RM (360 fmol/eq). The percentage of intact Sec61α (squares) in the T_X-PK-RM and C_X-PK-RM was quantified by scanning protein immunoblots. Aliquots of the T_X-PK-RM (not shown) and C_X-PK-RM (C) were subjected to protein immunoblot analysis using antibodies specific for the NH$_2$ terminus or COOH terminus of Sec61α that are respectively designated as Sec61α(N) and Sec61α(C). L8NTF and L6NTF designate NH$_2$-terminal fragments of Sec61α severed in cytoplasmic loops 8 and 6, respectively; L8CTF designates a COOH-terminal fragment of Sec61α severed in loop 8.

the Sec61 complex (Kalies et al., 1994). Because p180 is very sensitive to trypsin digestion (Kalies et al., 1994), we reasoned that the T_X-PK-RM could be assayed for ribosome binding activity in hypotonic buffers (50 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl$_2$) without interference from p180. The T_5-PK-RM that lack intact Sec61α do not bind ribosomes in a hypotonic buffer (not shown). The latter result confirms the previous reports concerning the trypsin sensitivity of ribosome binding sites in mammalian RER (Borgese et al., 1974; Hortsch et al., 1986).

RNC Interactions with Protease-digested Sec61 Complexes

Two distinct interactions are thought to be responsible for attachment of an RNC to the RER (Adelman et al., 1973; Gilmore and Blobel, 1985). One interaction is the ionic strength–sensitive binding of the ribosome to the Sec61 complex (Kalies et al., 1994), which was assayed in the preceding experiments. Unlike nontranslating ribosomes that are readily detached from microsomes by 0.5 M potassium acetate, RNCs remain tightly bound to the membrane in high salt (Gilmore and Blobel, 1985). This ionic strength–insensitive interaction between the RNC and the membrane is dependent upon the presence of the nascent polypeptide, and occurs upon signal sequence insertion into the translocation channel (Jungnickel and Rapoport, 1995).

To determine whether RNC binding to the protease-digested PK-RM was reduced, we took advantage of the observation that RNCs will bind to vacant translocation channels in an SRP-independent reaction (Jungnickel and Rapoport, 1995; Lauring et al., 1995a; Raden and Gilmore, 1998). A substrate for the RNC binding experiments was prepared by translating a truncated mRNA encoding the NH_2-terminal 86 residues of preprolactin (pPL86) in a wheat germ system in the absence of SRP. RNCs that are bound to translocation channels can be separated from unbound RNCs by centrifugal flotation through a sucrose density gradient in either low (150 mM potassium acetate) or high salt (500 mM potassium acetate; Lauring et al., 1995b). The membrane-bound RNCs are recovered at the interface between the low density top and middle fractions (T and M), whereas the unbound RNCs remain in the high density bottom (B) fraction (Fig. 4 A, T_0-PK-RM). When the trypsin-digested PK-RM were assayed using a low salt sucrose gradient, the RNCs did not remain with the membrane vesicles, but were instead recovered exclusively in the bottom fraction (Fig. 4 A). Clearly, the interaction between the RNC and the Sec61 channel is unstable when Sec61α subunits are severed.

Chromatography on a Sepharose CL-2B gel filtration column was used as a second method to separate membrane bound and unbound RNCs. More than 60% of the pPL86 RNCs coeluted with the undigested T_0-PK-RM in the void volume of the gel filtration column that was equilibrated in 150 mM potassium acetate (Fig. 4 B). The C_5-PK-RM, which had shown a threefold reduction in the binding of nontranslating ribosomes, displayed a twofold decrease in RNC binding. The C_{30}-PK-RM and the T_{10}-PK-RM, which had a 10-fold or greater defect in ribosome binding (Fig. 3), bound 2.7-fold and 5-fold less RNCs than the undigested PK-RM. RNCs that bind to intact Sec61 complexes resist extraction with 0.5 M potassium acetate, which is consistent with nascent chain insertion into the translocation channel (Fig. 4 C). The majority ($\sim$80%) of the pPL86 RNCs that bind to the protease-digested PK-RM (C_{30}-PK-RM) were also insensitive to high salt extraction (Fig. 4 C). To determine whether binding of RNCs to the Sec61 complex was signal sequence–independent, we incubated intact and protease-digested microsomes (T_{10}-PK-RM and C_{30}-PK-RM) with RNCs that were assembled by translation of a truncated firefly luciferase
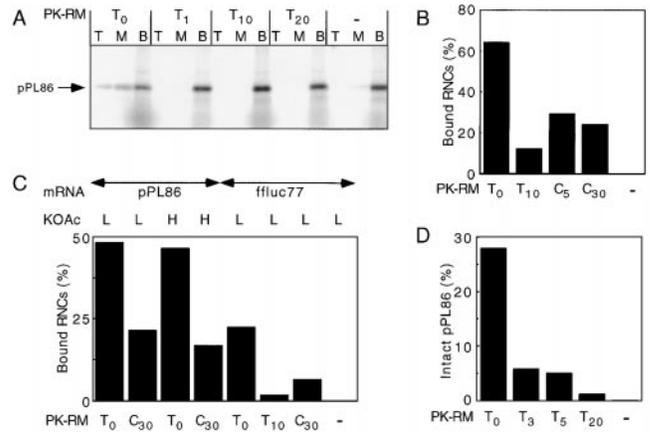

Figure 4. Proteolysis-induced changes in the ribosome–membrane junction. The pPL86 and ffLuc77 mRNAs were translated in the absence of SRP to assemble RNCs. The translation products were incubated with the T_X-PK-RM or C_X-PK-RM. The individual assays were chilled on ice, and binding of RNCs to the protease-digested PK-RM was assayed as follows. (A) Binding of RNCs to T_X-PK-RM was assayed by adjusting samples to 2.1 M sucrose and applying them as the bottom layer of a three-step discontinuous sucrose gradient (see Materials and Methods). After centrifugation, membrane-bound pPL86 was recovered in the top (T) and middle (M) fractions, whereas unbound pPL86 remained in the bottom (B) fraction. (B) Membrane-bound RNCs were separated from unbound RNCs by gel filtration chromatography columns equilibrated in TKMD. The percentage of the pPL86 that coeluted with the microsomes was quantified after SDS-PAGE. (C) Membrane-bound RNCs were separated from unbound RNCs by gel filtration chromatography columns equilibrated in TKMD (L) or in TKMD adjusted to 500 mM potassium acetate (H). Eluate fractions, containing pPL86 or ffLuc77-RNCs, were spotted onto Whatmann 3MM filter paper, precipitated in cold 10% TCA, and boiled in 5% TCA before scintillation counting. In B and C, the percentage of RNCs that elute in the void volume of the column in the absence of PK-RM ($\sim$7% of RNCs) has been subtracted as background. (D) The samples were digested with proteinase K on ice as described in Materials and Methods. The protease-resistant pPL86 is expressed as a percentage of the pPL86 in undigested control samples.

mRNA (ffLuc77). As reported previously (Lauring et al., 1995b), translocation channels in the PK-RM will bind RNCs that lack a signal sequence (Fig. 4 C). Binding of the ffLuc77 RNCs to the protease-digested PK-RM was either scarcely above background (T_{10}-PK-RM) or 3.5-fold reduced (C_{30}-PK-RM) relative to intact microsomes (Fig. 4 C).

When the nascent polypeptide is inserted into the translocation channel, it resides in an environment that is inaccessible to proteases (Connolly et al., 1989). Intimate contact between the cytosolic domains of the Sec61 complex and the ribosome is thought to be responsible for maintaining a tight seal between the ribosome and the membrane surface during translocation of proteins across the RER (Crowley et al., 1993). The interaction between the protease-digested translocation channel and the RNC was analyzed by testing whether a nascent polypeptide was sensitive to proteolysis (Fig. 4 D). Nascent pPL86 was

quantitatively digested by proteinase K in the absence of the microsomes. In the experiment shown here, 28% of the nascent pPL86 chains were protected from proteinase K digestion when we assayed the T_0-PK-RM. The percentage of protease-inaccessible pPL86 is similar to the typical efficiency (35–50%) for SRP-independent translocation reactions (see Fig. 5). A far smaller proportion (1–6%) of the nascent polypeptides was protected from proteinase K upon attachment of RNCs to the trypsin-digested Sec61 complexes (Fig. 4 D). We can conclude that the ribosome–Sec61 junction is substantially altered when loops 6 and 8 of Sec61α are severed.

SRP-independent Translocation across Protease-digested PK-RM

Based upon the results described above, we anticipated that the extensively digested PK-RM would be inactive in an SRP-independent translocation assay, whereas the microsomes that retained significant ribosome binding activity (e.g., T_1-PK-RM or C_5-PK-RM) would show SRP-independent translocation defects that were proportional to the fold reduction in ribosome binding or RNC binding activities. To simplify the interpretation of SRP-independent translocation assays, we used the TRAM protein–independent substrate pPL86 for these experiments (Görlich and Rapoport, 1993). The ribosome–pPL86 complexes were incubated with the protease-digested PK-RM for 5 min to allow RNC binding to the Sec61 complex. RNCs that had engaged the translocation channel were detected by releasing the nascent polypeptide with EDTA, which permits translocation of pPL86 into the ER lumen, where it is processed to PL56 by cleavage of the signal sequence. All of the protease-digested PK-RM were assayed for SRP-independent translocation activity. Representative assays using 10 eq of the protease-digested PK-RM (~3 pmol of Sec61 oligomers) and 10 μl of wheat germ translation products (4.5 pmol of ribosomes, ~250 fmol of pPL86 RNCs) are shown in Fig. 5 (A and B). Surprisingly, trypsin or endoproteinase Glu-C digestion of Sec61α reduced SRP-independent translocation of pPL86 by, at most, twofold (Fig. 5 A, solid bars). Even more striking, chymotrypsin digestion of Sec61α caused no more than a 20% reduction in SRP-independent translocation activity (Fig. 5 B, solid bars). Binding of nontranslating ribosomes to the protease-digested membranes was more severely inhibited than the SRP-independent translocation activity (Fig. 5, A and B, shaded bars). Control experiments demonstrated that the processed PL56 was sequestered within microsomal vesicles.

The trypsin-digested membranes (T_1-PK-RM, T_{10}-PK-RM, and T_{30}-PK-RM) were assayed for SRP-independent translocation activity under conditions where 80S ribosomes were present in excess relative to RNC binding sites in the undigested PK-RM (Fig. 5 C). Translocation of pPL86 across the protease-severed Sec61 channels was proportional to the quantity of added microsomes. When limiting amounts of protease-digested PK-RM were assayed (1 eq), the extensively digested PK-RM (T_{10}-PK-RM and T_{30}-PK-RM) were threefold less active than undigested PK-RM in SRP-independent translocation of pPL86. Thus, even when the RNCs are in excess relative to the
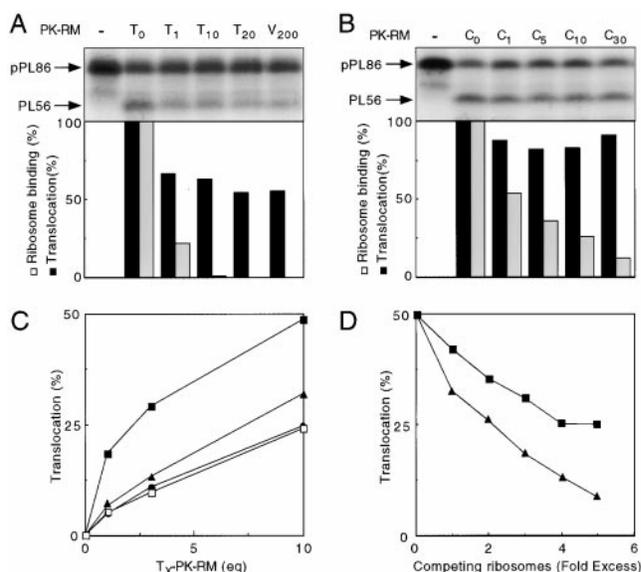

Figure 5. SRP-independent translocation activity of protease-digested PK-RM. The T_X-PK-RM, C_X-PK-RM, or V_{200}-PK-RM were assayed for SRP-independent translocation of pPL86 as described in Materials and Methods. (A and B) The assays contained 10 μl of translation products and 10 eq of T_X-PK-RM, V_{200}-PK-RM (A), or C_X-PK-RM (B). The percent translocation (solid bars) was calculated by quantifying pPL86 and PL56 after resolution by PAGE in SDS, and is expressed relative to that observed with undigested membranes (T_0-PK-RM or C_0-PK-RM). The ribosome binding activity (shaded bars) of the T_X-PK-RM, C_X-PK-RM, or V_{200}-PK-RM is taken from Fig. 3. (C) Aliquots (10 μl) of the pPL86 translation products were incubated with 0, 1, 3, or 10 eq of T_0-PK-RM (filled squares), T_1-PK-RM (triangles), T_{10}-PK-RM (circles), or T_{30}-PK-RM (open squares). (D) Competition between wheat germ 80S ribosomes and RNCs for binding to intact and protease-digested translocation channels. The translation products (4.5 pmol of 80S ribosomes, ~250 fmol of pPL86-RNCs) were mixed with 0–22.5 pmol of purified 80S ribosomes and T_0-PK-RM (triangles) or T_3-PK-RM (squares). 4.5 pmol corresponds to 1× competing ribosomes. The assays contained 5 eq of T_0-PK-RM (~1.5 pmol of Sec61 oligomers) or 15 eq of T_3-PK-RM (~4.5 pmol of Sec61 oligomers). Greater amounts of T_3-PK-RM were assayed to obtain comparable extents of translocation in the absence of competing ribosomes.

T_X-PK-RM, the fold reduction in translocation activity is considerably less than the observed reduction in the ribosome binding or RNC binding activities.

We next used a sensitive competition assay to determine whether the protease-digested Sec61 complexes retain residual binding determinants for 80S ribosomes that were not detected using the classical ribosome binding assay shown in Fig. 3. Nontranslating 80S ribosomes compete with RNCs for binding to the Sec61 complex, hence, they act as competitive inhibitors of the SRP-independent targeting pathway (Lauring et al., 1995; Neuhof et al., 1998; Raden and Gilmore, 1998). We reasoned that the T_3-PK-RM, which retain ~5% of the high affinity ribosome binding sites detectable in intact PK-RM (Fig. 3 D), might be less sensitive to inhibition by 80S ribosomes when assayed for SRP-independent translocation activity. To allow a direct comparison of the effect of competing ribosomes, the com-

petition assays were adjusted to obtain comparable translocation activity in the absence of the competitor. As observed previously, the addition of 80S ribosomes caused a concentration-dependent decrease in SRP-independent translocation across the undigested PK-RM (Fig. 5 D, triangles). When the protease-digested T_3PK-RM were assayed, we observed that the 80S ribosomes were twofold less effective as inhibitors of SRP-independent translocation across the T_3-PK-RM (Fig. 5 D, squares). Nonetheless, the 80S ribosomes did interfere with RNC binding to the Sec61 complex, suggesting that the protease-severed Sec61 complexes do retain residual affinity for the nontranslating ribosomes.

Thermolysin Dissection of Sec61α

Given the dramatic reduction in ribosome binding activity caused by cleavage of Sec61α at multiple sites, we incubated the PK-RM with thermolysin on ice or at 25°C to achieve more selective digestion of Sec61α (Fig. 6 A). The protein immunoblots using the NH_2-terminal–specific antibody to Sec61α revealed proteolytic fragments for the 25°C digestions that were similar to those obtained with chymotrypsin (compare Fig. 6 A with Fig. 3 C). Proteolysis within loops 8 and 6 yielded 30- and 22-kD immunoreactive fragments of Sec61α, respectively. A more rapid loss of Sec61α immunoreactivity was observed when the blots were probed with the COOH-terminal–specific antibody (Fig. 6 A). Smaller immunoreactive fragments of Sec61α were not detected with either antibody. Proteolysis within the 14-residue COOH-terminal tail should abolish immunoreactivity without substantially altering the gel mobility of Sec61α. When thermolysin digestions were performed on ice, the NH_2-terminal antibody revealed limited digestion of Sec61α within loop 8. Selective cleavage of the COOH-terminal tail was readily apparent, as shown by the loss of COOH-terminal immunoreactivity for the Th_{20}-PK-RM and Th_{50}-PK-RM (Fig. 6 A). Control immunoblots using the antibody to ribophorin I (Fig. 6 A) showed that differences in Sec61α COOH-terminal immunoreactivity could not be explained by differential recovery of the protease-digested membranes during preparative procedures. Quantification of the protein immunoblots disclosed the percentage of intact Sec61α that was recognized by the COOH-terminal antibody, and the intact-sized Sec61α that was recognized by the NH_2-terminal antibody (Fig. 6 B). The difference between the NH_2- and COOH-terminal values indicates the percentage of Sec61α that was selectively cleaved in the COOH-terminal tail.

The thermolysin-digested PK-RM were assayed for ribosome binding activity (Fig. 6 B). The quantity of ribosome binding sites (solid bars) was compared with the amount of intact Sec61α recognized by the NH_2-terminal (shaded bars) and COOH-terminal (diagonal bars) antibodies. A comparison of the ribosome binding activities of the Th_2-PK-RM, Th_{20}-PK-RM, and Th_{50}-PK-RM revealed the critical importance of loop 8 and the COOH-terminal tail of Sec61α. The loss of ribosome binding activity by these three membrane preparations cannot be ascribed to digestion of loop 6, which remained intact, but instead must be dependent upon digestion of either loop 8, the COOH terminus or both segments. The importance of the
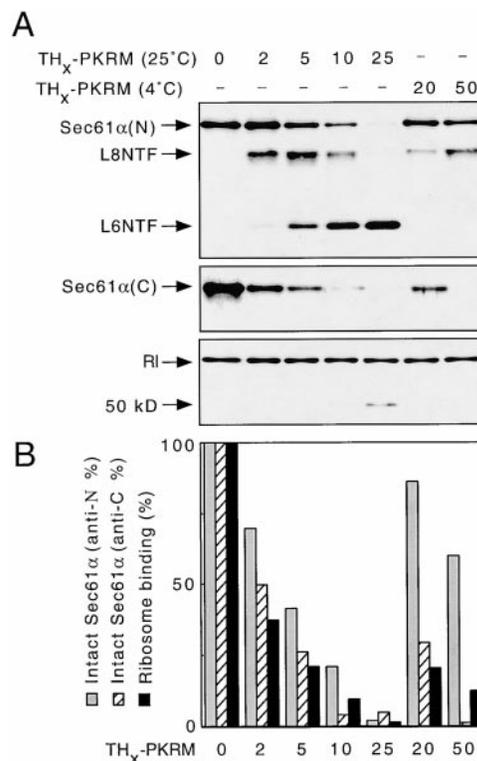

Figure 6. Ribosome binding activity of thermolysin-digested PK-RM. (A) PK-RM were digested at 25°C or on ice with thermolysin as described in Materials and Methods. Aliquots of the protease-digested PK-RM were analyzed on protein immunoblots probed with antibodies to Sec61α and ribophorin I. Antibodies specific for the NH_2 terminus or COOH terminus of Sec61α are designated as Sec61α (N) and Sec61α (C), respectively. L8NTF and L6NTF designate NH_2-terminal fragments of Sec61α severed in cytoplasmic loops 8 and 6, respectively. (B) The protein immunoblots shown in A were quantified to calculate the percentage of intact-sized Sec61α that are recognized by antibodies to the NH_2 terminus (shaded bars), or intact Sec61α that are recognized by the antibody to the COOH terminus (hatched bars). Binding of 80S ribosomes to the Th_X-PK-RM was assayed as described in Materials and Methods, and is expressed as a percentage (solid bars) of that displayed by undigested PK-RM (360 fmol/eq).

COOH-terminal tail of Sec61α is evident upon comparison of Th_2-PK-RM and Th_{50}-PK-RM. Although the extent of digestion within loop 8 was comparable, Th_{50}-PK-RM lack the COOH-terminal tail of Sec61α, and display threefold fewer ribosome binding sites. The latter result demonstrates that an intact Sec61α COOH terminus is important for the interaction between an 80S ribosome and the translocation channel.

The thermolysin-digested membranes were assayed for SRP-dependent translocation activity using the TRAM-independent substrate preprolactin (Görlich and Rapoport, 1993; Voigt et al., 1996). To assay SRP-dependent translocation activity, preprolactin mRNA was translated in a reticulocyte lysate system in the presence of the thermolysin-digested PK-RM. The assays were further supplemented with the 52-kD SRα fragment to reconstitute SRP receptor activity, as protein immunoblots had shown that the Th_X-PK-RM lack intact SRα (not shown). Transloca-
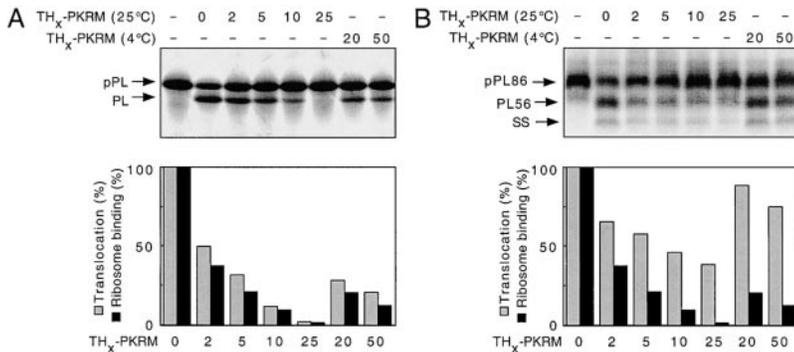
Figure 7. Translocation activity of thermolysin-digested PK-RM. (A) The Th_X-PK-RM were assayed for SRP-dependent translocation of preprolactin. Reticulocyte lysate translations were programmed with a preprolactin mRNA transcript and were supplemented with 1.2 eq of Th_X-PK-RM and 200 fmol of the 52-kD fragment of $SR\alpha$. Preprolactin (pPL) and prolactin (PL) were resolved by SDS-PAGE. SRP-dependent translocation of preprolactin across the Th_X-PK-RM (shaded bars), and ribosome binding to the Th_X-PK-RM (solid bars) is expressed relative to that obtained with undigested PK-RM. (B) The Th_X-PK-RM were assayed for SRP-independent translocation of pPL86. pPL86 was resolved from PL56 and the signal sequence (SS) by SDS-PAGE. SRP-independent translocation of pPL86 across the Th_X-PK-RM (shaded bars) and ribosome binding to the Th_X-PK-RM (solid bars) is expressed relative to that obtained with undigested PK-RM.

tion of preprolactin across the membrane is accompanied by signal sequence cleavage to yield processed prolactin. Quantification of the results (bottom) shows that the SRP-dependent translocation activity correlated quite well with the ribosome binding activity. Translocation of preprolactin was abolished when $Sec61\alpha$ was severed in cytoplasmic loop 6 (e.g., Th_{25}-PK-RM) and was strongly inhibited when $Sec61\alpha$ was cleaved in loop 8 (Fig. 7 A). Similar results were obtained when we assayed SRP-dependent integration of op156, a ribosome-tethered nascent chain derived from bovine opsin (data not shown).

The thermolysin-digested PK-RM were also assayed for SRP-independent translocation activity (Fig. 7 B). Quantification of the data revealed that the ribosome binding and SRP-independent translocation-promotion activities of the Sec61 complex are not strictly linked. The COOH-terminal tail of $Sec61\alpha$ is dispensable for SRP-independent translocation activity. The most severe reduction in the SRP-independent translocation of pPL86 was observed when $Sec61\alpha$ was cleaved in both loops 6 and 8.

Discussion

Proteolysis of Sec61α

The topology of $Sec61\alpha$ in the mammalian ER membrane was initially deduced using hydropathy algorithms (Görlich et al., 1992) and by comparison to the experimentally derived topology model for the homologous *Escherichia coli* SecY protein (Akiyama and Ito, 1987). The good agreement between the predicted topology of canine $Sec61\alpha$ and the experimentally verified topology model for *S. cerevisiae* Sec61p (Wilkinson et al., 1996) lends considerable credence to the model depicted in Fig. 1 A. Here, proteolysis using enzymes with different cleavage specificities was used to identify surface-exposed loops on the cytoplasmic face of $Sec61\alpha$. Based upon the presence of potential cleavage sites for trypsin, chymotrypsin, and endoproteinase Glu-C in each of the four loops and two termini, it was conceivable that each cytoplasmic segment of $Sec61\alpha$ would be susceptible to cleavage by all three proteases, with the exception of loop 6 which lacks a predicted cleavage site for endoproteinase Glu-C. Instead, we observed that the COOH-terminal half of $Sec61\alpha$ was

much more sensitive to proteolysis than the NH_2-terminal tail, whereas loops 2 and 4 were not digested by any protease we tested. Although a lack of digestion within loop 4 can be explained by the short length of this segment, loop 2 is comparable in length to loop 6, so size alone cannot be the explanation. The relative insensitivity of the NH_2 terminus is consistent with the hypothesis that this segment of canine $Sec61\alpha$ is embedded on the membrane surface as an amphipathic α-helix as has been proposed for *S. cerevisiae* Sec61p (Wilkinson et al., 1996). However, we cannot exclude the possibility that the NH_2 terminus of $Sec61\alpha$ is protected by another mechanism. The selective digestion of the COOH-terminal tail by thermolysin on ice indicates that this segment of $Sec61\alpha$ is highly exposed on the surface of the Sec61 oligomer.

Proteolysis of $Sec61\alpha$ did not cause the translocation channel to dissociate into Sec61 heterotrimers or fragments thereof. The maintenance of the oligomeric structure was not unexpected as hydrophobic interactions between the TM spans of $Sec61\alpha$, $Sec61\beta$, and $Sec61\gamma$ are presumably responsible for the stability of the oligomer. Once we established that the oligomeric structure of the Sec61 complex was not compromised by proteolytic digestion of cytoplasmic loops, we assayed the protease-digested PK-RM for ribosome binding activity, SRP-independent translocation of pPL86, and SRP-dependent translocation of preprolactin.

Binding of Nontranslating Ribosomes to the Sec61 Complex

Several RER membrane proteins (Sec61 complex, p180 and p34) bind ribosomes in hypotonic solution when reconstituted into proteoliposomes (Ichimura et al., 1992; Savitz and Meyer, 1993; Kalies et al., 1994). However, Sec61 complex proteoliposomes retain a high affinity for ribosomes in a physiological ionic strength buffer (Kalies et al., 1994). Furthermore, it is now well established that the Sec61 complex is also the ribosome binding site during the protein translocation reaction (Görlich and Rapoport, 1993; Kalies et al., 1994; Hanein et al., 1996; Beckmann et al., 1997). By analyzing ribosome binding to the protease-digested PK-RM in a physiological ionic strength buffer, our assay specifically monitored how proteolysis of the

Sec61 complex altered binding of nontranslating ribosomes to the translocation channel.

Our results support the hypothesis that the ribosome binding activity of the Sec61 complex can be ascribed to Sec61α. More than 50% of Sec61γ remained intact in protease-digested membranes that lacked detectable ribosome binding (e.g., T_5-PK-RM and V_{200}-PK-RM), demonstrating that Sec61γ is not sufficient for ribosome binding activity. The extreme sensitivity of Sec61β to thermolysin digestion (not shown) yielded the Th_2-PK-RM that lack intact Sec61β yet retain considerable ribosome binding activity. Kalies et al. (1994) had also concluded that Sec61β was dispensable for ribosome binding to the Sec61 complex. However, we cannot exclude the possibility that Sec61β or Sec61γ help stabilize the association between the ribosome and Sec61α.

As the ultrastructural evidence indicates that the ribosome is tethered to the Sec61 complex via a single visible junction (Beckmann et al., 1997), it would be logical to suggest that the most exposed surface on Sec61α is the ribosome-binding site. In support of the hypothesis that the most protease-accessible regions of Sec61α might correspond to the ribosome binding site, we observed that Sec61 complexes that lack intact Sec61α subunits do not bind nontranslating ribosomes. A comparison of 12 eukaryotic Sec61α sequences reveals that loops 6 and 8 are highly conserved, particularly with respect to the location and number of charged amino acids. Because the COOH terminus of Sec61α is one of the least conserved cytoplasmic segments of the Sec61 complex, an important role for the COOH terminus of Sec61α in ribosome binding was unexpected. The results obtained with the C_1-PK-RM and the Th_2-PK-RM strongly support the hypothesis that loop 8 is required for ribosome binding to the Sec61 complex. Selective cleavage of the COOH-terminal tail of Sec61α, by thermolysin on ice (Th_{50}-PK-RM), showed that this segment of Sec61α is crucial for the binding of a nontranslating ribosome to the translocation channel.

Assuming that proteolysis of the translocation channel results in a random digestion of Sec61α subunits in a tetramer of Sec61 heterotrimers, the partially digested membranes should contain a mixture of translocation channels that have between zero and four intact Sec61α subunits. One unexpected result was the observation that ribosome binding to the protease-digested PK-RM requires more than one intact Sec61α subunit per translocation channel. Consider an example of membranes that retain ~50% intact Sec61α (e.g., C_1-PK-RM or Th_2-PK-RM). A random 50% digestion of tetrameric translocation channels would yield a binomial distribution of channels that contain zero to four intact Sec61α subunits (6.25% with zero intact, 25% with one intact, 37.5% with two intact, 25% with three intact, and 6.25% with four intact). The number of ribosome binding sites we detect in C_1-PK-RM or Th_2-PK-RM (40–50% of that present in PK-RM) is much greater than the 6% of complexes that retain four intact Sec61α subunits, and is much less than the 94% of complexes that retain at least one intact Sec61α subunit. Instead, our results are best explained by a model that requires multivalent contact between the ribosome and two or three Sec61α subunits per Sec61 oligomer. In this regard, our demonstration that the Sec61 oligomer remains intact following proteolysis was a critical observation. Based upon the minimal protein bridge that tethers a ribosome to the yeast Sec61 oligomer (Beckmann et al., 1997), one might have predicted that three of the Sec61α subunits in a tetrameric translocation channel could be proteolyzed without reducing ribosome binding activity. Our interpretation of this apparent paradox is that physiological salt-insensitive binding of the ribosome to the canine Sec61 complex requires one or more secondary contact points that were not observed in the three-dimensional reconstructions of the *S. cerevisiae* ribosome-Sec61 complex.

The Signal Sequence Contributes to the Specificity and Affinity of RNC Attachment

The three independent methods we used to analyze RNC binding provided evidence that protease-severed Sec61 complexes bind RNCs with a reduced capacity and a reduced affinity relative to intact Sec61 complexes. Nonetheless, RNC binding to the Sec61 complex cannot be directly equated with high affinity ribosome binding activity. Attachment of the pPL86 RNCs to Sec61 complexes that lack high affinity ribosome binding activity is most readily explained by the hypothesis that the signal sequence of the nascent polypeptide is a second ligand that contributes significantly to the specificity and affinity of the interaction between an RNC and the Sec61 complex. RNCs that lack a signal sequence (e.g., ffLuc77) bind poorly to the protease-digested Sec61 complexes. How can we rationalize this conclusion with the previous data showing that 80S ribosomes compete with RNCs for SRP-independent binding to the translocation channel? As noted previously (Raden and Gilmore, 1998), 80S ribosomes do not compete on a 1:1 basis with RNCs for binding to the Sec61 complex. We hypothesize that the signal sequence substantially enhances the affinity of the RNC for the translocation channel by reducing the dissociation rate of the ribosome from the Sec61 complex. When ribosome competition experiments were conducted using the protease-digested PK-RM, we observed that free ribosomes were twofold less effective as competitors of RNC binding, which is consistent with the view that the affinity between the ribosome and the Sec61 complex was reduced by proteolysis. Photo cross-linking studies indicate that the signal sequence of a nascent polypeptide is inserted into the yeast Sec61 complex, so that it contacts TM spans 2 and 7 (Plath et al., 1998). Our results strongly suggest that signal sequence insertion occurs in a region of Sec61α that is inaccessible to proteases, hence, it is distinct from the cytoplasmic loops that contact the ribosome.

SRP-independent Translocation of Polypeptides through Protease-digested Sec61 Complexes

SRP-independent translocation through the Sec61 complex is thought to accurately mimic the RNC binding, nascent chain insertion and transport phases of the translocation reaction. When RNCs are targeted by the SRP-independent pathway, binding of the RNC to the Sec61 complex is signal sequence–independent (Lauring et al., 1995a), and is competitively inhibited by the presence of nontranslating 80S ribosomes (Lauring et al., 1995b; Neuhof et al., 1998; Raden and Gilmore, 1998). All of

the SRP-independent translocation assays described here used RNC preparations that contained the nascent chain–associated complex (NAC). NAC, a ribosome-associated protein, is proposed to be a negative regulator of RNC binding to the Sec61 complex (Lauring et al., 1995a,b). However, this conclusion has been challenged because the endogenous NAC in wheat germ and reticulocyte lysate cytosol does not prevent RNC binding to the Sec61 complex (Neuhof et al., 1998; Raden and Gilmore, 1998), therefore, removal of NAC was not necessary.

When we assayed SRP-independent translocation across the protease-digested PK-RM, we made several unexpected observations. Protease-digested PK-RM that lack binding sites for nontranslating ribosomes remain competent for SRP-independent translocation of pPL86. These results indicate that a functional interaction between a nascent polypeptide and the translocation channel is not strictly dependent upon an initial high affinity binding of the ribosome to Sec61 complex. The most definitive resolution of the ribosome binding and the translocation promotion activities of the Sec61 complex was obtained by limited digestion of Sec61α with thermolysin. Cleavage of the COOH terminus of Sec61α drastically reduced ribosome-binding activity while having a relatively modest effect upon SRP-independent translocation of pPL86.

The interaction between an RNC and the Sec61 complex progresses through several distinct stages as the nascent polypeptide increases in length (Crowley et al., 1994; Jungnickel and Rapoport, 1995; Nicchitta and Zheng, 1997). RNCs bearing preprolactin chains that are shorter than 70 residues are attached in a salt-sensitive manner even though the nascent polypeptide is in contact with Sec61α. Salt-resistant RNC attachment occurs upon further elongation when the signal sequence is inserted into a protease-inaccessible environment in the translocation channel (Connolly et al., 1989; Jungnickel and Rapoport, 1995; Nicchitta and Zheng, 1997). Gating of the lumenal end of the translocation channel also occurs at this stage of nascent chain elongation and transport (Crowley et al., 1994; Hamman et al., 1998). Although the pPL86 nascent chain is of sufficient length to support the more stable interaction between an RNC and the Sec61 complex, we observed that the interaction between an RNC and the translocation channel was substantially weakened by proteolysis of Sec61α. RNCs bearing pPL86 did not remain attached to the protease-digested PK-RM on sucrose flotation gradients in a physiological ionic strength buffer (Fig. 4 A). Gel filtration chromatography, which avoids exposure of the sample to 2 M sucrose and high centrifugal fields, provided evidence that the RNCs were bound to the protease-digested PK-RM (Fig. 4, B and C). Further evidence that the RNC–Sec61 interaction was altered was provided by the finding that the junction between the ribosome and the membrane was not sufficiently tight to prevent access of a macromolecular probe (proteinase K) to the nascent polypeptide.

Regions of Sec61α Implicated in RNC Binding and Protein Translocation

All of the protease-digested PK-RM described here were also assayed for SRP-dependent translocation activity us-

ing the procedure shown in Fig. 7 A (Song et al., 2000). As shown here for the Th$_{25}$-PK-RM, digestion of Sec61α in either cytoplasmic loop 6 or loop 8 leads to a complete block in the SRP-dependent translocation pathway. The restrictive block of the SRP-dependent targeting pathway is most readily explained by the accumulation of an upstream translocation intermediate that precedes transfer of the RNC from SRP54 to Sec61α (Song et al., 2000). The SRP-independent pathway was not as severely inhibited by proteolysis of Sec61α; cleavage within loops 6 and 8 reduced RNC binding and nascent chain translocation. Although the moderate reduction in SRP-independent translocation activity probably reflects the reduced affinity of the translocation channel for the ribosome, our results strongly suggest that the translocation-promoting function of the Sec61 complex resides in a protease-inaccessible region of Sec61α. A molecular genetic dissection of Sec61p has suggested that an intact cytoplasmic loop 6 is crucial for the in vivo function of the Sec61 complex (Wilkinson et al., 1997). Complementary NH$_2$-and COOH-terminal segments of Sec61p were tested for the ability to suppress a *sec61* null allele. With the exception of NH$_2$-terminal segments truncated within loops 6 or 7, coexpression of the complementary fragment yielded a functional Sec61p. Thus, an intact loop 6 in Sec61α appears to be crucial for translocation of proteins across the ER. Our results indicate that loop 8 and the COOH terminus are required for high affinity binding of ribosome to the Sec61 complex. We propose that these two segments cooperate to form a ribosome-binding platform that is responsible for both the primary and secondary contacts between the translocation channel and the ribosome. Whereas a detailed description of the ribosome-binding site in Sec61α will require further ultrastructural and molecular genetic analysis, the results described here show that the COOH-terminal half of Sec61α should be the focus for further scrutiny.

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