

# Role of the Cytoplasmic Segments of Sec61 $\alpha$ in the Ribosome-binding and Translocation-promoting Activities of the Sec61 Complex

David Raden, Weiqun Song, and Reid Gilmore

Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0103

**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<sub>2</sub> and COOH terminus of Sec61 $\alpha$  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 Sec61 $\alpha$ . 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)<sup>1</sup> when the NH<sub>2</sub>-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 in-

volving SRP54 and SR $\alpha$  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 Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  (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 Sec61 $\alpha$  once the ribosome-nascent chain complex engages the translocation channel (Mothes et al., 1994). Of the 10 transmembrane (TM) segments of *Saccharomyces cerevisiae* Sec61 $\alpha$ , TM2 and TM7 are the targets for photoreactive cross-linking agents when

Address correspondence to Reid Gilmore, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655-0103. Tel.: (508) 856-5894. Fax: (508) 856-6231. E-mail: reid.gilmore@umassmed.edu

<sup>1</sup>Abbreviations used in this paper: C<sub>X</sub>-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<sub>X</sub>-PK-RM, trypsin-digested PK-RM; Th<sub>X</sub>-PK-RM, thermolysin-digested PK-RM; V<sub>X</sub>-PK-RM, endoprotease Glu-C-digested PK-RM.

a nascent prepro- $\alpha$ -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 Sec61 $\alpha$  are responsible for the high affinity binding of the ribosome to the translocation channel, nor is it certain that Sec61 $\beta$  and Sec61 $\gamma$  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 Sec61 $\alpha$  (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 Sec61 $\alpha$ .

## Materials and Methods

### Preparation of Rough Microsomes (RM), SRP, the SR $\alpha$ 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 SR $\alpha$  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/ $\mu$ l (eq as defined in Walter et al., 1981).

Aliquots of the PK-RM (500  $\mu$ l) were digested at a concentration of 2 eq/ $\mu$ l for 1 h. Trypsin (0–30  $\mu$ g/ml) and chymotrypsin (0–200  $\mu$ g/ml) digestions were done on ice and terminated with 1 mM PMSF, followed by a 15-min incubation on ice and adjustment to 10  $\mu$ g/ml of aprotinin. Digestion with endoproteinase Glu-C (200  $\mu$ g/ml) was for 1 h at 37°C, and was terminated with 1 mM 3,4-dichloroisocoumarin. Thermolysin digestions (0–50  $\mu$ g/ml), which were done in the presence of 1 mM CaCl<sub>2</sub> 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/ $\mu$ l 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/ $\mu$ l in membrane buffer, and stored at –80°C.

### Isolation of Ribosomes and <sup>125</sup>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*<sub>av</sub> 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  $E = 6.14 \times 10^6$ .

The canine ribosomes were labeled with iodine-125 by incubating 26 pmol of ribosomes with 450  $\mu$ Ci of <sup>125</sup>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 <sup>125</sup>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<sub>2</sub> in an SW40 rotor for 3 h at 200,000 *g*<sub>av</sub>.

### Glycerol Gradient Centrifugation and Superose 12 Chromatography

20 eq of protease-digested PK-RM was mixed with 180  $\mu$ l 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<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM DTT, 1 $\times$  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- $\mu$ l 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<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM DTT, 1 $\times$  PIC, 0.125% digitonin, and 25  $\mu$ g/ml egg yolk phosphatidylcholine. The gradients were centrifuged for 18 h at 85,000 *g*<sub>av</sub> 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<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM DTT, 1 $\times$  PIC, 0.125% digitonin, and 25  $\mu$ g/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 <sup>125</sup>I-labeled ribosomes (typically 0.18 pmol) with 0–6.7 pmol of unlabeled canine ribosomes. The ribosomes were incubated with T<sub>X</sub>-PK-RM, C<sub>X</sub>-PK-RM, V<sub>X</sub>-PK-RM, or TH<sub>X</sub>-PK-RM for 20 min on ice in TKMD. The 30- $\mu$ l sample was applied to a 1.3-ml Sepharose CL-2B column equilibrated in TKMD. The column was eluted with 270  $\mu$ l 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- $\mu$ l fractions of the eluate were collected, and the amount of <sup>125</sup>I-labeled ribosomes in each fraction was determined using a Beckman  $\gamma$ -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 <sup>125</sup>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 NH<sub>2</sub>-terminal 86 residues of preprolactin (pPL86) and the NH<sub>2</sub>-terminal 77 residues of firefly luciferase (fLuc77) were isolated from preparative scale transcriptions as described previously (Rapiejko and Gilmore, 1994). SRP-dependent translocation of preprolactin was assayed using 25- $\mu$ l reticulocyte lysate translations that contained [<sup>35</sup>S]methionine, endogenous SRP, 1.2 eq of protease-digested PK-RM, and 200 fmol of the SR $\alpha$  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 [<sup>35</sup>S]methionine as described previously (Gilmore et al., 1991). Further protein synthesis was blocked by adding cycloheximide to a final concentration of 250  $\mu$ M. The translation products (10  $\mu$ l, 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 =  $100 \times 1.33 \times \text{PL56} / ((1.33 \times \text{PL56}) + \text{pPL86})$ .

The factor of 1.33 corrects for the loss of the NH<sub>2</sub>-terminal methionine residue from pPL86 upon signal sequence cleavage.

Binding of RNCs bearing <sup>35</sup>S-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  $\mu$ g/ml of protease K as previously described (Connolly et al., 1989).

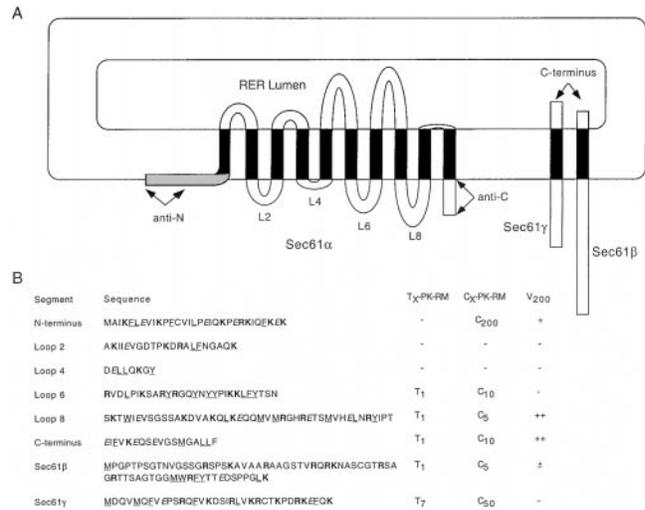
## Protein Immunoblots

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 SR $\beta$  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 NH<sub>2</sub> terminus of Sec61 $\alpha$  and Sec61 $\beta$  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 Sec61 $\alpha$  (KEQSEVGSMSGALLF) using standard procedures.

## Results

### Sensitivity of Sec61 $\alpha$ to Proteolysis

Sec61 $\alpha$  is integrated in the ER with a topology that places four loops (L2, L4, L6, and L8) plus the NH<sub>2</sub>- and COOH-terminal tails on the cytoplasmic face of the membrane (Fig. 1 A). The  $\beta$  and  $\gamma$  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 Sec61 $\alpha$  and, to a lesser extent, to Sec61 $\beta$  (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 Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  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 luminal tails and loops. Sec61 $\alpha$  segments that were used as antigens for the production of anti-NH<sub>2</sub>- and anti-COOH-terminal antibodies are labeled. (B) The amino acid sequences of the NH<sub>2</sub>- and COOH-terminal domains and the cytosolic loops of Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  are shown. Potential protease cleavage sites are indicated in bold (trypsin), italics (endoproteinase Glu-C), or by underlining (chymotrypsin). Digestion of Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  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<sub>X</sub>-PK-RM, C<sub>X</sub>-PK-RM, V<sub>X</sub>-PK-RM, or Th<sub>X</sub>-PK-RM (where the subscript X denotes the concentration of protease, in  $\mu$ g/ml, used for the digestion). Protein immunoblot analysis, using antibodies specific for the NH<sub>2</sub>- and COOH-terminal tails of Sec61 $\alpha$ , was used to determine which cytoplasmic segments of Sec61 $\alpha$  are accessible to proteases. Protease cleavage sites in Sec61 $\alpha$  were mapped to cytoplasmic loops 6 and 8 by comparison to COOH-terminal Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$ , followed by loop 6. The NH<sub>2</sub>-terminal tail, which is proposed to be an amphipathic  $\alpha$ -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

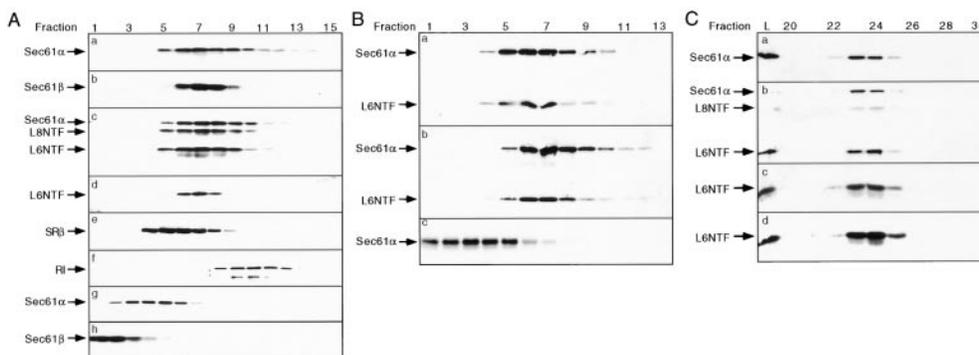
As visualized by electron microscopy, the purified Sec61 complex forms oligomers that are composed of three to four Sec61 heterotrimers (Hanein et al., 1996). Before assaying the protease-digested PK-RM for Sec61-dependent translocation and ribosome binding activities, it was important to determine whether 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 (Cope land 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<sub>0</sub>-PK-RM membranes were analyzed, protein immunoblot analysis showed that Sec61 $\alpha$  (a) was resolved from SR $\beta$  (e), a subunit of the 100-kD SRP receptor, and from ribophorin I (f), a subunit of the oligosaccharyltransferase (OST). As expected, Sec61 $\beta$  (b) and Sec61 $\gamma$  (not shown) cosedimented with Sec61 $\alpha$ . 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 Sec61 $\alpha$  (fractions 6–9) relative to the OST would be consistent with a Sec61 oligomer composed of three to four 70-kD Sec61 heterotrimers. Sec61 $\alpha$  subunits severed in loop 8 (L8NTF) and loop 6 (L6NTF) cosedimented precisely with intact Sec61 $\alpha$  when the T<sub>1</sub>-PK-RM were analyzed (Fig. 2 A, c). The sedimentation rate of Sec61 $\alpha$  was also not altered by quantitative cleavage of Sec61 $\alpha$  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, Sec61 $\beta$  (h) sedimented far less rap-

idly and was well resolved from Sec61 $\alpha$  (g). Resolution of Sec61 $\alpha$  and Sec61 $\beta$  is consistent with a Triton X-100-induced dissociation of the Sec61 oligomer into individual subunits. Because Sec61 $\alpha$  accounts for 75% of the protein molecular mass of the Sec61 complex, the Triton X-100-treated Sec61 $\alpha$  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 Sec61 $\alpha$  subunits, which were derived from the undigested PK-RM, cosedimented with the NH<sub>2</sub>-terminal 22-kD fragment, which was derived from the C<sub>50</sub>-PK-RM (a) or from the Th<sub>25</sub> PK-RM (b). As observed in A, the protease-digested Sec61 complexes were well resolved from the Triton X-100-treated Sec61 $\alpha$  (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<sub>2</sub>-terminal fragments of Sec61 $\alpha$  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 Sec61 $\alpha$  is severed in cytoplasmic loops 6 and 8, and the COOH terminus.

### Ribosome Binding Is Abrogated by Proteolysis of Sec61 $\alpha$

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



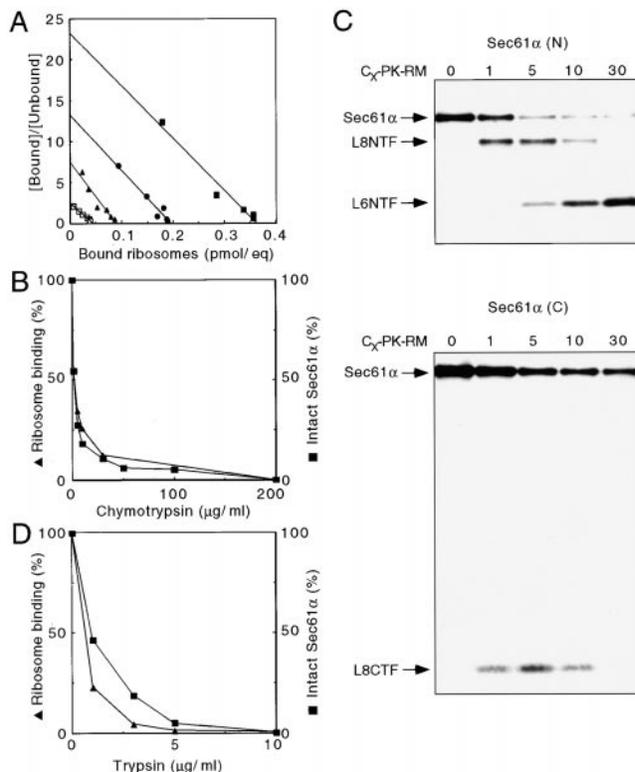
**Figure 2.** Oligomeric structure of the protease-digested Sec61 complex. (A) Glycerol gradient centrifugation of digitonin high salt extracts of T<sub>X</sub>-PK-RM (a–f) or Triton X-100 high salt extracts of T<sub>0</sub>-PK-RM (g and h). The following samples were analyzed: (a, b, and e–h) T<sub>0</sub>-PK-RM, (c) T<sub>1</sub>-PK-RM, and (d) T<sub>20</sub>-PK-RM. (B) Glycerol gradient centrifugation of detergent high salt extracts of the following samples: (a) T<sub>0</sub>-

PK-RM mixed with C<sub>50</sub>-PK-RM; (b) T<sub>0</sub>-PK-RM mixed with Th<sub>25</sub>-PK-RM; and (c) T<sub>0</sub>-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<sub>X</sub>-PK-RM. The following samples were analyzed: (a) T<sub>0</sub>-PK-RM, (b) T<sub>1</sub>-PK-RM, (c) T<sub>7</sub>-PK-RM, and (d) T<sub>20</sub>-PK-RM. Aliquots of the solubilized T<sub>X</sub>-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<sub>2</sub> terminus of Sec61 $\alpha$  (all samples except A; b, e, f, and h), Sec61 $\beta$  (A, b and h), SR $\beta$  (A, e) or ribophorin I (A, f). L8NTF and L6NTF designate NH<sub>2</sub>-terminal fragments of Sec61 $\alpha$  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  $^{125}\text{I}$ -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 Sec61 $\alpha$  in the chymotrypsin-digested PK-RM (Fig. 3 B). The inhibition of ribosome binding activity by chymotrypsin digestion correlated quite well with digestion of Sec61 $\alpha$ . The reduction in ribosome binding activity observed for the  $C_1$ -PK-RM was of particular interest because low concentrations of chymotrypsin sever Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  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  $^{125}\text{I}$ -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 Sec61 $\alpha$  (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 Sec61 $\alpha$  that are respectively designated as Sec61 $\alpha$ (N) and Sec61 $\alpha$ (C). L8NTF and L6NTF designate NH $_2$ -terminal fragments of Sec61 $\alpha$  severed in cytoplasmic loops 8 and 6, respectively; L8CTF designates a COOH-terminal fragment of Sec61 $\alpha$  severed in loop 8.

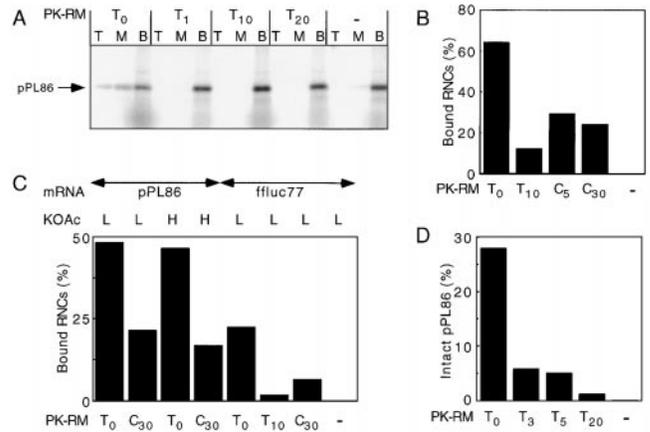
the Sec61 complex (Kalies et al., 1994). Because p180 is very sensitive to trypsin digestion (Kalies et al., 1994), we reasoned that the  $T_X$ -PK-RM could be assayed for ribosome binding activity in hypotonic buffers (50 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl $_2$ ) without interference from p180. The  $T_5$ -PK-RM that lack intact Sec61 $\alpha$  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<sub>2</sub>-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<sub>0</sub>-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 Sec61 $\alpha$  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<sub>0</sub>-PK-RM in the void volume of the gel filtration column that was equilibrated in 150 mM potassium acetate (Fig. 4 B). The C<sub>5</sub>-PK-RM, which had shown a threefold reduction in the binding of nontranslating ribosomes, displayed a twofold decrease in RNC binding. The C<sub>30</sub>-PK-RM and the T<sub>10</sub>-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 (~80%) of the pPL86 RNCs that bind to the protease-digested PK-RM (C<sub>30</sub>-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<sub>10</sub>-PK-RM and C<sub>30</sub>-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<sub>X</sub>-PK-RM or C<sub>X</sub>-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<sub>X</sub>-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 (~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<sub>10</sub>-PK-RM) or 3.5-fold reduced (C<sub>30</sub>-PK-RM) relative to intact microsomes (Fig. 4 C).

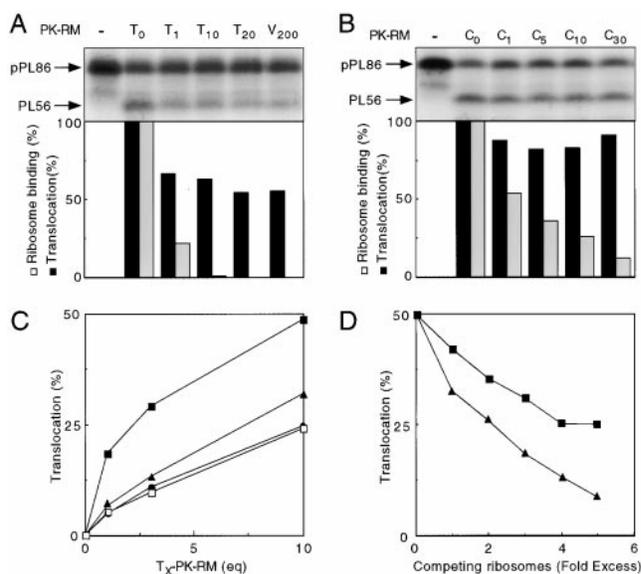
When the nascent polypeptide is inserted into the translocation channel, it resides in an environment that is inaccessible to proteases (Connolly et al., 1989). Intimate contact between the cytosolic domains of the Sec61 complex and the ribosome is thought to be responsible for maintaining a tight seal 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<sub>0</sub>-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 Sec61 $\alpha$  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<sub>1</sub>-PK-RM or C<sub>5</sub>-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  $\mu$ l 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 Sec61 $\alpha$  reduced SRP-independent translocation of pPL86 by, at most, two-fold (Fig. 5 A, solid bars). Even more striking, chymotrypsin digestion of Sec61 $\alpha$  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<sub>1</sub>-PK-RM, T<sub>10</sub>-PK-RM, and T<sub>30</sub>-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<sub>10</sub>-PK-RM and T<sub>30</sub>-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<sub>x</sub>-PK-RM, C<sub>x</sub>-PK-RM, or V<sub>200</sub>-PK-RM were assayed for SRP-independent translocation of pPL86 as described in Materials and Methods. (A and B) The assays contained 10  $\mu$ l of translation products and 10 eq of T<sub>x</sub>-PK-RM, V<sub>200</sub>-PK-RM (A), or C<sub>x</sub>-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<sub>0</sub>-PK-RM or C<sub>0</sub>-PK-RM). The ribosome binding activity (shaded bars) of the T<sub>x</sub>-PK-RM, C<sub>x</sub>-PK-RM, or V<sub>200</sub>-PK-RM is taken from Fig. 3. (C) Aliquots (10  $\mu$ l) of the pPL86 translation products were incubated with 0, 1, 3, or 10 eq of T<sub>0</sub>-PK-RM (filled squares), T<sub>1</sub>-PK-RM (triangles), T<sub>10</sub>-PK-RM (circles), or T<sub>30</sub>-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<sub>0</sub>-PK-RM (triangles) or T<sub>3</sub>-PK-RM (squares). 4.5 pmol corresponds to 1 $\times$  competing ribosomes. The assays contained 5 eq of T<sub>0</sub>-PK-RM (~1.5 pmol of Sec61 oligomers) or 15 eq of T<sub>3</sub>-PK-RM (~4.5 pmol of Sec61 oligomers). Greater amounts of T<sub>3</sub>-PK-RM were assayed to obtain comparable extents of translocation in the absence of competing ribosomes.

T<sub>x</sub>-PK-RM, the fold reduction in translocation activity is considerably less than the observed reduction in the ribosome binding or RNC binding activities.

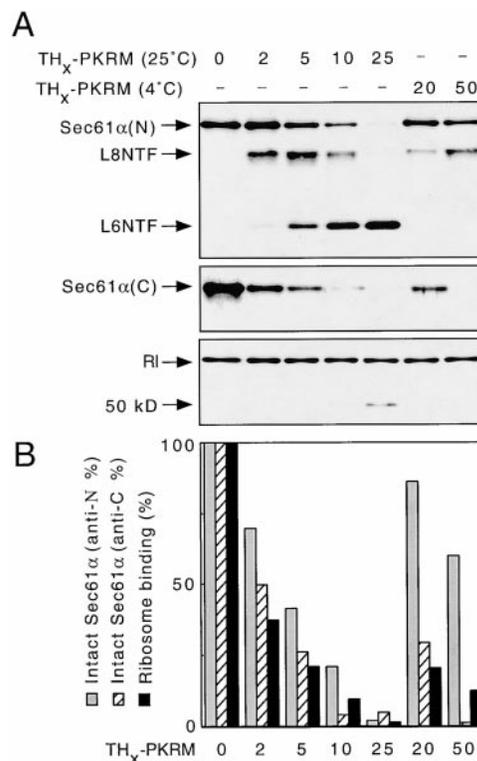
We next used a sensitive competition assay to determine whether the protease-digested Sec61 complexes retain residual binding determinants for 80S ribosomes that were not detected using the classical ribosome binding assay shown in Fig. 3. Nontranslating 80S ribosomes compete with RNCs for binding to 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<sub>3</sub>-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<sub>3</sub>PK-RM were assayed, we observed that the 80S ribosomes were twofold less effective as inhibitors of SRP-independent translocation across the T<sub>3</sub>-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 non-translating ribosomes.

### Thermolysin Dissection of Sec61 $\alpha$

Given the dramatic reduction in ribosome binding activity caused by cleavage of Sec61 $\alpha$  at multiple sites, we incubated the PK-RM with thermolysin on ice or at 25°C to achieve more selective digestion of Sec61 $\alpha$  (Fig. 6 A). The protein immunoblots using the NH<sub>2</sub>-terminal-specific antibody to Sec61 $\alpha$  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 Sec61 $\alpha$ , respectively. A more rapid loss of Sec61 $\alpha$  immunoreactivity was observed when the blots were probed with the COOH-terminal-specific antibody (Fig. 6 A). Smaller immunoreactive fragments of Sec61 $\alpha$  were not detected with either antibody. Proteolysis within the 14-residue COOH-terminal tail should abolish immunoreactivity without substantially altering the gel mobility of Sec61 $\alpha$ . When thermolysin digestions were performed on ice, the NH<sub>2</sub>-terminal antibody revealed limited digestion of Sec61 $\alpha$  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<sub>20</sub>-PK-RM and Th<sub>50</sub>-PK-RM (Fig. 6 A). Control immunoblots using the antibody to ribophorin I (Fig. 6 A) showed that differences in Sec61 $\alpha$  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 Sec61 $\alpha$  that was recognized by the COOH-terminal antibody, and the intact-sized Sec61 $\alpha$  that was recognized by the NH<sub>2</sub>-terminal antibody (Fig. 6 B). The difference between the NH<sub>2</sub>- and COOH-terminal values indicates the percentage of Sec61 $\alpha$  that was selectively cleaved in the COOH-terminal tail.

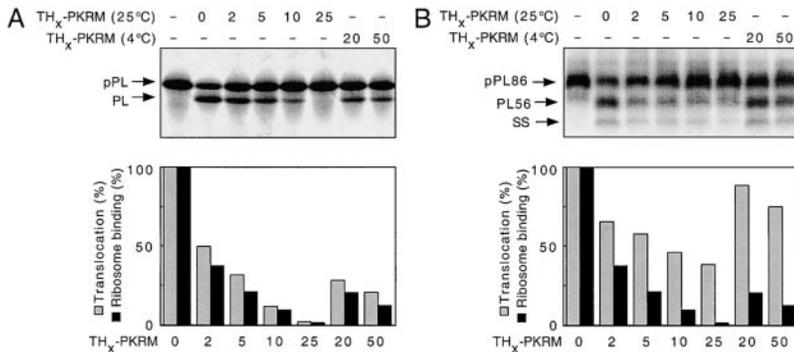
The thermolysin-digested PK-RM were assayed for ribosome binding activity (Fig. 6 B). The quantity of ribosome binding sites (solid bars) was compared with the amount of intact Sec61 $\alpha$  recognized by the NH<sub>2</sub>-terminal (shaded bars) and COOH-terminal (diagonal bars) antibodies. A comparison of the ribosome binding activities of the Th<sub>2</sub>-PK-RM, Th<sub>20</sub>-PK-RM, and Th<sub>50</sub>-PK-RM revealed the critical importance of loop 8 and the COOH-terminal tail of Sec61 $\alpha$ . 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 Sec61 $\alpha$  and ribophorin I. Antibodies specific for the NH<sub>2</sub> terminus or COOH terminus of Sec61 $\alpha$  are designated as Sec61 $\alpha$  (N) and Sec61 $\alpha$  (C), respectively. L8NTF and L6NTF designate NH<sub>2</sub>-terminal fragments of Sec61 $\alpha$  severed in cytoplasmic loops 8 and 6, respectively. (B) The protein immunoblots shown in A were quantified to calculate the percentage of intact-sized Sec61 $\alpha$  that are recognized by antibodies to the NH<sub>2</sub> terminus (shaded bars), or intact Sec61 $\alpha$  that are recognized by the antibody to the COOH terminus (hatched bars). Binding of 80S ribosomes to the Th<sub>x</sub>-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 Sec61 $\alpha$  is evident upon comparison of Th<sub>2</sub>-PK-RM and Th<sub>50</sub>-PK-RM. Although the extent of digestion within loop 8 was comparable, Th<sub>50</sub>-PK-RM lack the COOH-terminal tail of Sec61 $\alpha$ , and display three-fold fewer ribosome binding sites. The latter result demonstrates that an intact Sec61 $\alpha$  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 SR $\alpha$  fragment to reconstitute SRP receptor activity, as protein immunoblots had shown that the Th<sub>x</sub>-PK-RM lack intact SR $\alpha$  (not shown). Translocat-



**Figure 7.** Translocation activity of thermolysin-digested PK-RM. (A) The Th<sub>X</sub>-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<sub>X</sub>-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<sub>X</sub>-PK-RM (shaded bars), and ribosome binding to the Th<sub>X</sub>-PK-RM (solid bars) is expressed relative to that obtained with undigested PK-RM. (B) The Th<sub>X</sub>-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<sub>X</sub>-PK-RM (shaded bars) and ribosome binding to the Th<sub>X</sub>-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<sub>25</sub>-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 Sec61 $\alpha$

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 endoprotease 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 endoprotease Glu-C. Instead, we observed that the COOH-terminal half of Sec61 $\alpha$  was

much more sensitive to proteolysis than the NH<sub>2</sub>-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<sub>2</sub> terminus is consistent with the hypothesis that this segment of canine Sec61 $\alpha$  is embedded on the membrane surface as an amphipathic  $\alpha$ -helix as has been proposed for *S. cerevisiae* Sec61p (Wilkinson et al., 1996). However, we cannot exclude the possibility that the NH<sub>2</sub> 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 Sec61 $\alpha$ . More than 50% of Sec61 $\gamma$  remained intact in protease-digested membranes that lacked detectable ribosome binding (e.g., T<sub>5</sub>-PK-RM and V<sub>200</sub>-PK-RM), demonstrating that Sec61 $\gamma$  is not sufficient for ribosome binding activity. The extreme sensitivity of Sec61 $\beta$  to thermolysin digestion (not shown) yielded the Th<sub>2</sub>-PK-RM that lack intact Sec61 $\beta$  yet retain considerable ribosome binding activity. Kalies et al. (1994) had also concluded that Sec61 $\beta$  was dispensable for ribosome binding to the Sec61 complex. However, we cannot exclude the possibility that Sec61 $\beta$  or Sec61 $\gamma$  help stabilize the association between the ribosome and Sec61 $\alpha$ .

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 Sec61 $\alpha$  is the ribosome-binding site. In support of the hypothesis that the most protease-accessible regions of Sec61 $\alpha$  might correspond to the ribosome binding site, we observed that Sec61 complexes that lack intact Sec61 $\alpha$  subunits do not bind nontranslating ribosomes. A comparison of 12 eukaryotic Sec61 $\alpha$  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 Sec61 $\alpha$  is one of the least conserved cytoplasmic segments of the Sec61 complex, an important role for the COOH terminus of Sec61 $\alpha$  in ribosome binding was unexpected. The results obtained with the C<sub>1</sub>-PK-RM and the Th<sub>2</sub>-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 Sec61 $\alpha$ , by thermolysin on ice (Th<sub>50</sub>-PK-RM), showed that this segment of Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  subunits. One unexpected result was the observation that ribosome binding to the protease-digested PK-RM requires more than one intact Sec61 $\alpha$  subunit per translocation channel. Consider an example of membranes that retain ~50% intact Sec61 $\alpha$  (e.g., C<sub>1</sub>-PK-RM or Th<sub>2</sub>-PK-RM). A random 50% digestion of tetrameric translocation channels would yield a binomial distribution of channels that contain zero to four intact Sec61 $\alpha$  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<sub>1</sub>-PK-RM or Th<sub>2</sub>-PK-RM (40–50% of that present in PK-RM) is much greater than the 6% of complexes that retain four intact Sec61 $\alpha$  subunits, and is much less than the 94% of complexes that retain at least one intact Sec61 $\alpha$  subunit. Instead, our results are best explained by a model that requires multivalent contact between the ribosome and two or three Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  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 Sec61 $\alpha$  with thermolysin. Cleavage of the COOH terminus of Sec61 $\alpha$  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 Sec61 $\alpha$ . 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 luminal 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 Sec61 $\alpha$ . 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 Sec61 $\alpha$ 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<sub>25</sub>-PK-RM, digestion of Sec61 $\alpha$  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 Sec61 $\alpha$  (Song et al., 2000). The SRP-independent pathway was not as severely inhibited by proteolysis of Sec61 $\alpha$ ; 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 Sec61 $\alpha$ . 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<sub>2</sub>- and COOH-terminal segments of Sec61p were tested for the ability to suppress a *sec61* null allele. With the exception of NH<sub>2</sub>-terminal segments truncated within loops 6 or 7, coexpression of the complementary fragment yielded a functional Sec61p. Thus, an intact loop 6 in Sec61 $\alpha$  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 Sec61 $\alpha$  will require further ultrastructural and molecular genetic analysis, the results described here show that the COOH-terminal half of Sec61 $\alpha$  should be the focus for further scrutiny.

We thank Gert Kreibich, Christopher Nicchitta, Tom Rapoport, and Peter Walter for providing antibodies to ribophorin I, Sec61 $\alpha$ , and Sec61 $\beta$ , and SR $\beta$ , respectively. We thank Kennan Kellaris (Georgetown University, Washington, DC) for raising antisera to the COOH terminus of Sec61 $\alpha$  while she was a postdoctoral fellow in this lab.

This work was supported by National Institutes of Health grant PHS GM 35687.

Submitted: 12 November 1999

Revised: 31 May 2000

Accepted: 31 May 2000

### **References**

- Adelman, M.R., D.D. Sabatini, and G. Blobel. 1973. Ribosome-membrane interaction. Nondestructive disassembly of rat liver rough microsomes into ribosomal and membrane components. *J. Cell Biol.* 56:206-229.
- Akiyama, Y., and K. Ito. 1987. Topology analysis of the SecY protein, an integral membrane protein involved in protein export in *Escherichia coli*. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3465-3470.
- Beckmann, R., D. Bubeck, R. Grassucci, P. Penczek, A. Verschoor, G. Blobel, and J. Frank. 1997. Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. *Science*. 278:2123-2126.
- Borgese, N., W. Mok, G. Kreibich, and D.D. Sabatini. 1974. Ribosomal-membrane interaction: *in vitro* binding of ribosomes to microsomal membranes. *J. Mol. Biol.* 88:559-580.
- Collins, P., and R. Gilmore. 1991. Ribosome binding to the endoplasmic reticulum: a 180-kD protein identified by cross-linking to membrane bound ribosomes is not required for ribosome binding activity. *J. Cell Biol.* 114:639-649.
- Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. *Cell*. 57:599-610.

- Connolly, T., P. Collins, and R. Gilmore. 1989. Access of proteinase K to partially translocated nascent polypeptides in intact and detergent-solubilized membranes. *J. Cell Biol.* 108:299–307.
- Copeland, C.S., R.W. Doms, E.M. Bolzau, R.G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.
- Crowley, K.S., S. Liao, V.E. Worrell, G.D. Reinhart, and A.E. Johnson. 1994. Secretory proteins move through the endoplasmic reticulum via an aqueous, gated pore. *Cell.* 78:461–471.
- Crowley, K.S., G.D. Reinhart, and A.E. Johnson. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. *Cell.* 73:1101–1115.
- Doms, R.W., and A. Helenius. 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* 60:833–839.
- Gilmore, R., and G. Blobel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell.* 42:497–505.
- Gilmore, R., P. Collins, J. Johnson, K. Kellaris, and P. Rapiejko. 1991. Transcription of full length and truncated mRNA transcripts to study protein translocation across the endoplasmic reticulum. *Methods Cell Biol.* 34:223–239.
- Görlich, D., and T.A. Rapoport. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the ER membrane. *Cell.* 75:615–630.
- Görlich, D., S. Prehn, E. Hartmann, K.-U. Kalies, and T.A. Rapoport. 1992. A mammalian homologue of Sec61p and SecYp is associated with ribosomes and nascent polypeptides during translocation. *Cell.* 71:489–503.
- Hamman, B.D., J.-C. Chen, E.E. Johnson, and A.E. Johnson. 1997. The aqueous pore through the translocon has a diameter of 40–60 Å during cotranslational protein translocation at the ER membrane. *Cell.* 89:535–544.
- Hamman, B.D., L.M. Hendershot, and A.E. Johnson. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocation pore before and early in translocation. *Cell.* 92:747–758.
- Hanein, D., K.E.S. Matlack, B. Jungnickel, K. Plath, K.-U. Kalies, K.R. Miller, T.A. Rapoport, and C.W. Akey. 1996. Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. *Cell.* 87:721–732.
- Hebert, D.N., and A. Carruthers. 1991. Cholate-solubilized erythrocyte glucose transporters exist as a mixture of homodimers and homotetramers. *Biochemistry.* 30:4654–4658.
- Hortsch, M., D. Avossa, and D.I. Meyer. 1986. Characterization of secretory protein translocation: ribosome-membrane interaction in endoplasmic reticulum. *J. Cell Biol.* 103:241–253.
- Ichimura, T., T. Ohsumi, Y. Shindo, T. Ohwada, H. Yagame, Y. Monose, S. Omata, and H. Sugano. 1992. Isolation and some properties of a 34-kD membrane protein that may be essential for ribosome binding in rat liver rough microsomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 296:7–10.
- Jungnickel, B., and T.A. Rapoport. 1995. A posttranslational signal sequence recognition event in the endoplasmic reticulum membrane. *Cell.* 82:261–270.
- Kalies, K.-U., D. Görlich, and T.A. Rapoport. 1994. Binding of ribosomes to the rough endoplasmic reticulum is mediated by the Sec61p complex. *J. Cell Biol.* 126:925–934.
- Kelleher, D.J., and R. Gilmore. 1997. DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. *Proc. Natl. Acad. Sci. USA.* 94:4994–4999.
- Lauring, B., G. Kreibich, and M. Wiedmann. 1995a. The intrinsic ability of ribosomes to bind to endoplasmic reticulum membranes is regulated by signal recognition particle and nascent-polypeptide-associated complex. *Proc. Natl. Acad. Sci. USA.* 92:9435–9439.
- Lauring, B., H. Sakai, G. Kreibich, and M. Wiedmann. 1995b. Nascent polypeptide-associated complex protein prevents mistargeting of nascent chains to the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 92:5411–5415.
- Moes, W., S. Prehn, and T.A. Rapoport. 1994. Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3973–3982.
- Neuhof, A., M.M. Rolls, B. Jungnickel, K.-U. Kalies, and T.A. Rapoport. 1998. Binding of signal recognition particle gives ribosome/nascent chain complexes a competitive advantage in endoplasmic reticulum membrane interaction. *Mol. Biol. Cell.* 9:103–115.
- Nicchitta, C.V., and G. Blobel. 1989. Nascent secretory chain binding and translocation are distinct processes: differentiation by chemical alkylation. *J. Cell Biol.* 108:789–795.
- Nicchitta, C.V., and T. Zheng. 1997. Regulation of ribosome-membrane junction at early stages of presecretory protein translocation across the endoplasmic reticulum. *J. Cell Biol.* 139:1697–1708.
- Plath, K., W. Mothes, B.M. Wilkinson, C.J. Stirling, and T.A. Rapoport. 1998. Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell.* 94:795–807.
- Raden, D., and R. Gilmore. 1998. Signal recognition particle-dependent targeting of ribosomes to the rough endoplasmic reticulum in the absence and presence of the nascent polypeptide associated complex. *Mol. Biol. Cell.* 9:117–130.
- Rapiejko, P.J., and R. Gilmore. 1994. Signal sequence recognition and targeting of ribosomes to the endoplasmic reticulum by the signal recognition particle do not require GTP. *Mol. Biol. Cell.* 5:887–897.
- Rapiejko, P.J., and R. Gilmore. 1997. Empty site forms of the SRP54 and SRPα GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum. *Cell.* 89:703–713.
- Savitz, A.J., and D.I. Meyer. 1990. Identification of a ribosome receptor in the rough endoplasmic reticulum. *Nature.* 346:540–544.
- Savitz, A.J., and D.I. Meyer. 1993. 180-kD ribosome receptor is essential for both ribosome binding and protein translocation. *J. Cell Biol.* 120:853–863.
- Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl-sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100 kDa. *Anal. Biochem.* 166:368–379.
- Simon, S.M., and G. Blobel. 1991. A protein-conducting channel in the endoplasmic reticulum. *Cell.* 65:371–380.
- Song, W., D. Raden, E. Mandon, and R. Gilmore. 2000. Role of Sec61α in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocation channel. *Cell.* 100:333–343.
- Voigt, S., B. Jungnickel, E. Hartmann, and T.A. Rapoport. 1996. Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum. *J. Cell Biol.* 134:25–35.
- Walter, P., and A.E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10: 87–119.
- Walter, P., I. Ibrahim, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* 91:545–550.
- Wilkinson, B.M., A.J. Critchley, and C.J. Stirling. 1996. Determination of the transmembrane topology of yeast Sec61p; an essential component of the ER translocation complex. *J. Biol. Chem.* 271:25590–25597.
- Wilkinson, B.M., Y. Esnault, R.A. Craven, F. Skiba, J. Fieschi, F. Képès, and C.J. Stirling. 1997. Molecular architecture of the ER translocase probed by chemical crosslinking of Sss1p to complementary fragments of Sec61p. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4549–4559.