Binding of Ribosomes to the Rough Endoplasmic Reticulum Mediated by the Sec61p-Complex

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Abstract. The cotranslational translocation of proteins across the ER membrane involves the tight binding of translating ribosomes to the membrane, presumably to ribosome receptors. The identity of the latter has been controversial. One putative receptor candidate is Sec61α, a multi-spanning membrane protein that is associated with two additional membrane proteins (Sec61β and γ) to form the Sec61p-complex. Other receptors of 34 and 180 kD have also been proposed on the basis of their ability to bind at low salt concentration ribosomes lacking nascent chains. We now show that the Sec61p-complex has also binding activity but that, at low salt conditions, it accounts for only one third of the total binding sites in proteoliposomes reconstituted from a detergent extract of ER membranes. Under these conditions, the assay has also limited specificity with respect to ribosomes. However, if the ribosome-binding assay is performed at physio-

logical salt concentration, most of the unspecific binding is lost; the Sec61p-complex then accounts for the majority of specific ribosome-binding sites in reconstituted ER membranes.

To study the membrane interaction of ribosomes participating in protein translocation, native rough microsomes were treated with proteases. The amount of membrane-bound ribosomes is only slightly reduced by protease treatment, consistent with the protease-resistance of Sec61α which is shielded by these ribosomes. In contrast, p34 and p180 can be readily degraded, indicating that they are not essential for the membrane anchoring of ribosomes in protease-treated microsomes.

These data provide further evidence that the Sec61p-complex is responsible for the membrane-anchoring of ribosomes during translocation and make it unlikely that p34 or p180 are essential for this process.

M ANY proteins are targeted to the mammalian ER membrane by means of the signal recognition particle (SRP)[1] and its membrane receptor (SRP-receptor or docking protein) (for review see Rapoport, 1992). They are subsequently transported across the membrane, presumably through a protein-conducting channel. The main component of this channel may be Sec61p (now named Sec61α), a multi-spanning membrane protein, originally discovered in *S. cerevisiae* by genetic screening for translocation defects (Deshaies and Schekman, 1987; Stirling et al., 1992). Sec61α is adjacent to polypeptide chains passing through the ER membrane of yeast and mammals, it is essen-

tial for protein translocation in both systems and it has sequence similarity with SecYp, a key component of the protein export apparatus of bacteria (Görlich et al., 1992b). The mammalian Sec61α is part of a complex (Sec61p-complex) that includes two additional polypeptides of about 14 and 8 kD molecular mass (β- and γ-subunits) (Görlich and Rapoport, 1993; Hartmann et al., 1994). Another component of the protein-conducting channel of the mammalian ER may be the 'translocating chain-associating membrane (TRAM)" protein, a multi-spanning membrane protein, which seems to contact the signal sequence of nascent polypeptide chains during early phases of their translocation through the membrane (Görlich et al., 1992a). The reconstitution of the mammalian translocation process from purified components indicates that the transport of some polypeptides requires only two integral membrane protein complexes, the SRP receptor and the Sec61p-complex, whereas others additionally require the presence of the TRAM protein (Görlich and Rapoport, 1993).

Many proteins are transported across the mammalian ER membrane during their synthesis on membrane-bound ribosomes. A tight ribosome-membrane junction is thought to be required for the direct transfer of the nascent chain from the channel in the ribosome into the protein-conducting channel

1. *Abbreviations used in this paper:* eq., equivalents; PK-RM, puromycin/high salt-stripped rough microsomes; RM, rough microsomes; SLR, S-labeling reagent; SRP, signal recognition particle; TRAM protein, translocating chain-associating membrane protein.

in the membrane. The translocating, nascent polypeptide chain does not provide the sole linkage between the ribosome and the membrane since it can be released from the ribosome by puromycin at physiological salt concentrations, without detachment of the ribosome from the membrane; detachment requires both puromycin treatment and high salt concentrations (Adelman et al., 1973). It is therefore assumed that the ribosome is also anchored to the membrane by one or more ribosome receptor proteins.

Ribosomes may also bind to ER membranes independent of ongoing translation or translocation in a mode that is sensitive to high salt concentrations alone. This interaction may also be relevant for the translocation process as it may precede the tighter binding of ribosomes that is found after the nascent chain has completed its targeting to the membrane (Connolly and Gilmore, 1986). It is also possible that ribosomes are "stored" at the membrane in a less tightly bound state until they are recruited to translocation sites or are required for other cellular processes.

Previous attempts to identify ribosome receptors have concentrated on the salt-sensitive binding of ribosomes lacking nascent polypeptide chains. As demonstrated by Borgese et al. (1974), microsomes contain a saturable number of protease-sensitive binding sites for such ribosomes. The binding constant is in a reasonable order of magnitude, and rough microsomes contain more binding sites than smooth ones. It has been generally assumed that the membrane protein(s) responsible for the binding of ribosomes in this assay may be identical with the one(s) binding ribosomes engaged in translocation, but the existence of distinct ribosome receptors has not yet been excluded.

On the basis of the ribosome-binding assay of Borgese et al. (1974), performed at low salt conditions (25 mM), putative ribosome receptors of 34 kD (p34) (Tazawa et al., 1991; Ichimura et al., 1992) and 180 kD (p180) (Savitz and Meyer, 1990, 1993) have been proposed. However, the role of these proteins has been questioned. The majority of ribosome-binding activity was found in a fraction of membrane proteins that did not contain p180 (Collins and Gilmore, 1991; Nunnari et al., 1991). On the other hand, Savitz and Meyer (1993) have reported that p180 does in fact account for all the binding activity and that it is essential for protein translocation in reconstituted proteoliposomes. Arguments against p34 being responsible for the membrane-binding of translocationally engaged ribosomes have also been raised (Görlich et al., 1992b).

We have studied previously the binding of ribosomes engaged in translocation and have obtained evidence for an interaction of the Sec61p-complex with membrane-bound ribosomes (Görlich et al., 1992b). Sec61p was among the membrane proteins that remained tightly associated with ribosomes after solubilization of rough microsomes. This interaction was induced by the targeting of a nascent chain to the ER membrane and it was not exclusively mediated by the nascent chain. Rather, the conditions needed to dissociate the Sec61p-ribosome complex were the same as those required for the release of ribosomes from native membranes, i.e., they required both puromycin and high salt. If ribosomes lacking nascent chains were added to microsomes before solubilization, an interaction of ribosomes with Sec61p was also seen but it appeared to be weaker than that including a nascent chain (Görlich et al., 1992b). These data suggested

that the Sec61p-complex may be generally involved in the membrane binding of ribosomes. However, since in these experiments the interaction of Sec61p with ribosomes was determined after solubilization of the membranes, it might not have fully reflected the physiological situation. On the other hand, the fact that some proteins can be transported into reconstituted proteoliposomes containing only the SRP receptor and the Sec61p-complex (Görlich and Rapoport, 1993) suggests that the latter is involved in the membrane binding of translating ribosomes.

In the present paper, we provide further evidence that the Sec61p-complex is a membrane receptor both for ribosomes lacking nascent chains and for ribosomes engaged in protein translocation. Using reconstituted proteoliposomes, we demonstrate that under physiological salt concentrations, the Sec61p-complex accounts for the majority of membrane binding sites for ribosomes lacking nascent chains. Treatment of native rough microsomes with externally added proteases indicates that the amount of membrane-bound ribosomes is only slightly reduced, consistent with the protease-resistance of Sec61α which is shielded by these ribosomes. In contrast, p34 and p180 can be readily degraded, indicating that they are not essential for the anchoring of ribosomes to the protease-treated microsomes. Proteolytic degradation of p180 also does not reduce the translocation activity of microsomes. These data indicate that the Sec61p-complex, but not p34 or p180, is essential for the membrane binding of ribosomes during translocation.

Materials and Methods

Buffers

MB: 50 mM Hepes/KOH pH 7.6, 1 mM DTT; LSB: 50 mM Hepes/KOH pH 7.6, 25 mM KCl, 5 mM MgCl$_2$, 1 mM DTT; MSB: 50 mM Hepes/KOH pH 7.6, 150 mM KCl, 5 mM MgCl$_2$, 1 mM DTT; HSB: 50 mM Hepes/KOH pH 7.6, 500 mM KCl, 10 mM MgCl$_2$, 1 mM DTT; LSB-C: 50 mM Hepes/KOH pH 7.6, 25 mM CsCl, 5 mM MgCl$_2$, 1 mM DTT; HSB-C: 50 mM Hepes/KOH pH 7.6, 500 mM CsCl, 10 mM MgCl$_2$, 1 mM DTT; SB: 50 mM Hepes/KOH pH 7.6, 400 mM potassium acetate, 10 mM magnesium acetate, 15% glycerin, 5 mM β-mercaptoethanol, 1.2% deoxyBigChap.

Binding of Ribosomes and DNA to Membranes

Dog pancreatic ribosomes and microsomes, stripped of ribosomes by treatment with puromycin and high salt (PK-RM), were prepared as previously described (Görlich et al., 1992b).

Ribosomes were radiolabeled with ^{35}S-labeling reagent (SLR; Amersham) as follows: 200 μl SLR were dried down in a SpeedVac. The residue was dissolved and mixed with 1,600 equivalents (eq.) (150 A$_{260}$-units) ribosomes from dog pancreas in a final volume of 800 μl of 0.25 M sucrose in 50 mM Hepes/KOH pH 7.6, 25 mM KCl, 5 mM MgCl$_2$. After an incubation on ice for 60 min, the reaction was quenched by addition of 80 μl of 1 M glycine/HCl pH 7.6. The labeled ribosomes were centrifuged twice through a 1.5-ml cushion consisting of 0.5 M sucrose in LSB containing the following protease inhibitor cocktail: 10 μg/ml leupeptin, 5 μg/ml chymostatin and 2 μg/ml pepstatin. This cocktail was also used in other experiments (see below). Centrifugation was carried out for 1 h at 100,000 rpm at 2°C in a Beckman (Beckman Instruments, Inc., Palo Alto, CA) table-top ultracentrifuge (rotor TLA 100.3). The ribosomes were finally resuspended in 0.25 M sucrose in LSB. When tested in sucrose gradient centrifugation, the labeled material ran at 80 S (data not shown).

Plasmid DNA (pUC19) was cut with NciI, and the ends were filled in by incubation with Klenow enzyme in the presence of [^{32}P]α-dCTP. Excess of the radioactive dCTP was removed by precipitation of the DNA with ethanol. The DNA-pellet was extensively washed with 70% ethanol, dissolved in water and reprecipitated with ethanol. The final preparation did not contain unincorporated dCTP, as determined by thin layer chromatogra-

phy. mRNA coding for full-length preprolactin was produced by in vitro transcription as described below.

The binding of labeled ribosomes or DNA to PK-RM or to reconstituted proteoliposomes was carried out in a final volume of 30 μl in LSB or MSB containing 0.25 M sucrose. After incubation on ice for 30 min, the samples were thoroughly mixed with 170 μl of 2.3 M sucrose in LSB or MSB, and layered in micro test tubes under a discontinuous sucrose gradient consisting of 0.8 ml of 1.9 M sucrose in LSB or MSB, and 0.2 ml of 0.25 M sucrose in LSB or MSB. After centrifugation for 2 h at 75,000 rpm at 5°C (rotor TLA 100.3), the floated material was collected by removing the upper 800 μl. The radioactivity in this and the remaining fraction was determined after dilution with 1 ml water and mixing with a scintillation cocktail in a liquid scintillation counter.

The inhibition of ribosome binding was determined after preincubation of the microsomes with plasmid DNA, mRNA, or other inhibitors for 10 min at 0°C.

Treatment of Microsomes with Proteases

Rough microsomes (RM) were diluted with 0.25 M sucrose in LSB to a concentration of 0.5 eq. (for definition see Walter et al. [1981]) per ml. Proteases were added as indicated in the figures and the samples were incubated on ice for 1 h. Proteolysis was stopped by addition of either 15% TCA or of appropriate inhibitors.

Elastase (1 mg/ml) was pretreated with 0.3 mg/ml aprotinin at 0°C for 20 min before addition to the microsomes.

Transcription, Translation, and Translocation

Transcripts coding for preprolactin were produced by transcription with T7 RNA polymerase of the plasmid pGEMBP1, previously cut with PstI. Transcripts for prepro-α-factor were produced by transcription with SP6 polymerase of the plasmid pSP65αFWT linearized with SalI.

Proteolysis of RM with 5 μg/ml elastase or 15 μg/ml V8 protease was stopped by addition of 0.5 mM PMSF. The membranes were sedimented twice through a high-salt cushion consisting of 600 μl of 400 mM sucrose in HSB containing protease inhibitors and 0.1 mM PMSF in the cushion of the first sedimentation step. After centrifugation for 30 min at 75,000 rpm and 2°C (rotor TLA 100.3, micro test tubes), the pellets were resuspended in 250 mM sucrose in MB.

Translation of the transcripts was carried out in the wheat germ system in the presence of 50 nM SRP and [^{35}S]methionine at 24°C for 3 min. Edein was then added to a final concentration of 5 μM, and the samples were incubated for 20 min on ice in the presence of 1.5 eq. of microsomes per 10 μl of translation mixture. Thereafter, they were warmed up to 26°C and the incubation was continued for 20 min. Half of the sample was precipitated with 15% TCA, the other half was treated with 500 μg/ml proteinase K at 0°C for 30 min, before precipitation with 15% TCA. The pellets were washed with methanol containing 5 mM PMSF and dissolved in SDS sample buffer. The samples were analyzed by SDS-PAGE using a 12.5% polyacrylamide gel, followed by fluorography.

Flotation of Protease-treated Microsomes

After protease treatment of 300 eq. RM, 1 mM PMSF and the protease inhibitor cocktail were added and the microsomes were sedimented through a sucrose cushion consisting of 1 ml 0.4 M sucrose in LSB. Centrifugation was carried out for 30 min at 100,000 rpm at 5°C (rotor TLA 100.3). The membranes were resuspended in 110 μl of 0.5 M sucrose in HSB-C containing protease inhibitors and mixed with 300 μl of 2.2 M sucrose in the same buffer. 200 μl of the sample were layered under a sucrose gradient consisting of 0.5 ml of 1.5 M sucrose in HSB-C and 0.2 ml of 0.25 M sucrose in LSB. After centrifugation (3 h, 120,000 rpm, 5°C, TLA 120.2), the upper 700 μl were removed and diluted with 2 ml 50 mM Hepes/KOH pH 7.6. The lower 200 μl were used to resuspend the sedimented ribosomes. This fraction was diluted with 1.5 ml of 50 mM Hepes/KOH pH 7.6. Both the floated membranes and the resuspended ribosomes were submitted to centrifugation for 1 h at 100,000 rpm and 2°C (rotor TLA 100.3). The pellets were resuspended in 150 μl of 0.25 M sucrose in MB.

To release membrane-bound ribosomes from flotated, protease-treated RM, two samples of material, each corresponding to 100 eq. microsomes, were mixed with 0.5 ml 1 mM puromycin and 0.2 mM GTP in LSB containing 0.25 M sucrose. After incubation for 1 h at 0°C followed by 20 min at 30°C, the membranes were sedimented (30 min, 75,000 rpm, 2°C, TLA 100.3, micro test tubes). One of the samples was resuspended in 30 μl 0.5 M

sucrose in LSB-C. It was then mixed with 170 μl of 2.4 M sucrose in LSB-C and layered under a sucrose gradient consisting of 0.5 ml 2.0 M sucrose in LSB-C and 150 μl MB. The other sample was taken up in 30 μl 0.5 M sucrose in HSB-C and mixed with 170 μl 2.0 M sucrose in HSB-C. It was then layered under a sucrose gradient consisting of 0.5 ml 1.5 M sucrose in HSB-C and 150 μl MB. Both samples were submitted to centrifugation for 3 h at 120,000 rpm and 5°C (rotor TLA 120.2). The upper 750 μl were removed, diluted with 2 ml water and precipitated with 15% TCA. The sedimented ribosomes were resuspended in the remaining 100 μl of the sucrose gradient and diluted with 1 ml water before precipitation with 15% TCA. The pellets after TCA-precipitation were washed with methanol and dissolved in SDS sample buffer.

Analysis of Ribosome-associated Membrane Proteins

The association of membrane proteins with ribosomes was tested as described previously (Görlich et al., 1992b), except that 5 eq. microsomes were solubilized in 75 μl 50 mM Hepes/KOH pH 7.6, 400 mM potassium acetate, 10 mM magnesium acetate, 15% glycerin, 1.5% digitonin, 5 mM β-mercaptoethanol and protease inhibitors. After incubation for 30 min at 0°C, the ribosomes were centrifuged through a 100-μl cushion of 1.5 M sucrose in the same buffer, except that it contained only 0.1% digitonin. Membrane proteins in the ribosome pellet were analyzed after SDS-PAGE by immunoblotting.

Preparation of Proteoliposomes from Detergent Extracts of Microsomes

PK-RM were solubilized at a concentration of 1 eq./μl by incubation for 30 min at 0°C in SB containing protease inhibitors. After centrifugation for 15 min at 14,000 rpm in a microfuge, 500 μl of the supernatant were added to 100 μl of an immuno-affinity resin that contained 0.2 mg affinity-purified antibodies directed against the NH$_2$-terminus of Sec61β, covalently coupled to protein A–Sepharose (Görlich and Rapoport, 1993). The column was equilibrated with SB. After shaking the column in the cold room overnight, the fluid phase was collected. 200 μl of the Sec61p-depleted extract were either first replenished with purified Sec61p-complex (see below) or mixed directly with 20 μl of a mixture of phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in the ratio of 100:25:3:12.5; total concentration 5 mg/ml). Proteoliposomes were produced by incubation of this mixture with 300 mg Biobeads SM2 (Biorad Labs., Hercules, CA) which had been equilibrated with SB lacking detergent. After incubation for 15 h in the cold, the fluid phase was removed, diluted with 0.8 ml 50 mM Hepes/KOH pH 7.6 and centrifuged (30 min, 75,000 rpm, 2°C, TLA 100.3, micro test tubes). The pellet was resuspended in 250 mM sucrose in MB.

Preparation of Proteoliposomes Containing the Purified Sec61p-Complex

The Sec61p-complex was purified either from rough microsomes, on the basis of its tight association with ribosomes after solubilization, or by an immunoisolation procedure, starting with ribosome-stripped microsomes (Görlich and Rapoport, 1993). Both preparations gave identical results. Proteoliposomes were produced by mixing 20 μl of the Sec61p-complex (200 eq.) with 20 μl of a phospholipid mixture (see above). 50 mg Biobeads SM2 (Biorad Labs.) equilibrated in SB lacking detergent were added. Further processing of the vesicles was carried out as described above.

Immunoblotting and Antibodies

Immunoblotting with various antibodies was performed, as previously described (Görlich et al., 1992b).

The following polyclonal antibodies, directed against synthetic peptides, were used after immunoaffinity purification: against the COOH terminus of Sec61α (Görlich et al., 1992b), the NH$_2$ terminus of Sec61β (Görlich and Rapoport, 1993), the NH$_2$ terminus of p34 (Görlich and Rapoport, 1993), the position 137 to 150 of the α-subunit of the SRP-receptor (Görlich and Rapoport, 1993), and against the position 1 to 63 of Sec61β (cytosolic domain). The polyclonal antibodies against p180 have been described by Savitz and Meyer (1993) and were a kind gift of Dr. D. I. Meyer. The affinity-purified polyclonal antibodies against the ribosomal protein S26 and against p34 were kind gifts of Drs. J. Stahl and S. High, respectively.

Results

Binding of Ribosomes under Low Salt Conditions

We first tested the role of the Sec61p-complex in the binding of ribosomes lacking nascent chains employing, as in previous studies, low salt concentrations in the binding test. Dog pancreatic microsomes, stripped of ribosomes by treatment with PK-RM, were incubated at 25 mM KCl with different amounts of radioactively labeled canine ribosomes, and submitted to flotation in a sucrose gradient to separate the bound from the unbound fraction (Borgese et al., 1974). Scatchard
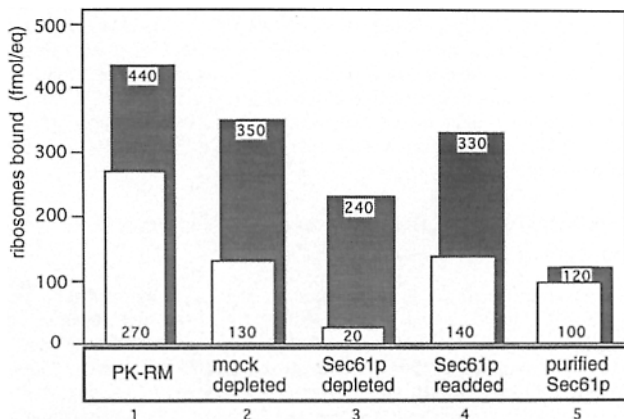

Figure 1. Ribosome binding to microsomes and proteoliposomes. The binding of ribosomes to microsomes, stripped of ribosomes by puromycin/high salt treatment (*PK-RM*), or to proteoliposomes was measured at low salt (25 mM; *filled columns*) or at physiological salt concentrations (150 mM; *empty columns*). Proteoliposomes were produced from an unfractionated detergent extract of PK-RM that was mock-depleted by incubation with protein A-Sepharose (*mock-depleted vesicles*). Proteoliposomes, depleted of the Sec61p-complex, were produced from an extract that was incubated with Sec61β-antibodies coupled to protein A-Sepharose. The residual amounts of Sec61α and of Sec61β were estimated to be ∼10% and <0.5%, respectively, using quantitative immunoblotting with specific antibodies and ^{35}S-labeled anti-rabbit immunoglobulins. Proteoliposomes, replenished with the Sec61p-complex, were produced from a depleted extract by addition of purified Sec61p-complex (purified by the immunoisolation procedure, see Görlich and Rapoport, 1993). The latter was also reconstituted alone in proteoliposomes using a phospholipid mixture corresponding approximately to that in native ER membranes. In each case, the membranes were incubated with radiolabeled ribosomes and increasing amounts of unlabeled ribosomes, the bound and free fractions were separated by flotation of the membranes, and the radioactivity in these fractions was determined. Scatchard plot analysis with at least six data points was used to estimate the number of binding sites (*columns*) and the apparent binding constants. For the latter, the following values were obtained at low and physiological salt concentrations, respectively: (*PK-RM*) 17 and 9 nM; (*mock-depleted*) 7 and 21 nM; (*Sec61p-depleted*) 15 and 16 nM; (*Sec61p-readded*) 8 and 15 nM; (*purified Sec61p*) 6 and 4 nM. The Scatchard plots were generally linear with correlation coefficients above 0.93, except for the experiment with Sec61p-depleted vesicles at 150 mM KCl (0.85). The estimated binding sites in separate experiments were almost identical (less than 20 fmol/eq. difference). The amounts of Sec61-complex in PK-RM and in mock-depleted vesicles, as well as the amounts of purified Sec61p employed, were adjusted to identical values on the basis of quantitative immunoblots.

plot analysis was used to estimate the number of binding sites and the apparent binding constant. Both values (Fig. 1, *filled column 1*, and legend) were in good agreement with previous estimates. Next, we tested the binding of ribosomes to the purified Sec61p-complex, reconstituted into proteoliposomes with a phospholipid mixture corresponding approximately to that of native ER membranes. Scatchard plot analysis demonstrated that the Sec61p-containing proteoliposomes bound ribosomes with high affinity (Fig. 1, legend) but contained only 120 fmol/eq. binding sites, ∼25% of the number in microsomes (calculated on the basis of the Sec61α-content; *filled column 5*). Since reconstitution per se might have reduced the number of binding sites, we tested proteoliposomes which were reconstituted from an unfractionated detergent extract of PK-RM and therefore contained the majority of integral membrane proteins of microsomes (*filled column 2*). The number of binding sites was indeed somewhat reduced but remained much higher than that in proteoliposomes containing the same amount of purified Sec61p-complex. To confirm the low contribution of the Sec61p-complex to the total number of binding sites, proteoliposomes were produced from a detergent extract of PK-RM which was immunodepleted with immobilized antibodies against Sec61β. Although the depleted proteoliposomes contained only ∼10% of the original amounts of Sec61α and undetectably low levels of Sec61β, the ribosome binding sites were reduced by only 30% (in absolute terms by 110 fmol/eq.; *filled column 3*). If the purified Sec61p-complex was added back to the depleted detergent extract, the number of binding sites in the resulting proteoliposomes was restored to almost the original level (*filled column 4*). The difference between the number of ribosome-binding sites in Sec61p-containing and -depleted vesicles corresponds well to the value obtained with the purified protein complex. In summary, these data indicate that the Sec61p-complex is able to bind ribosomes with high affinity but that it is not the only binding partner under the conditions used; the majority of binding sites appear to be contributed by other membrane proteins.

At low salt concentrations, the binding assay had also limited specificity with respect to ribosomes. Plasmid DNA was found to bind to microsomes with similar characteristics as ribosomes; both the number of binding sites and the apparent binding constant were in a similar range (Fig. 2 *A*). Furthermore, at low salt, preincubation of microsomes with plasmid DNA or mRNA reduced the binding of ribosomes to levels almost as low as found after treatment of microsomes with trypsin (Fig. 2 *B, filled columns*), suggesting that the nucleic acids and the ribosomes had the same or overlapping sites of binding. A number of other polyanions, such as sperm DNA, heparin, and aurintricarboxylic acid also acted as potent inhibitors of ribosome binding under low salt conditions (data not shown; see also Borgese et al., 1974).

Binding of Ribosomes under Physiological Salt Conditions

Since it seemed possible that at low salt concentrations unspecific electrostatic interactions superpose on the specific ribosome binding, and to approach more physiological conditions, we repeated the ribosome binding tests at 150 mM KCl (Fig. 1, *empty columns*). The increased salt concentra-
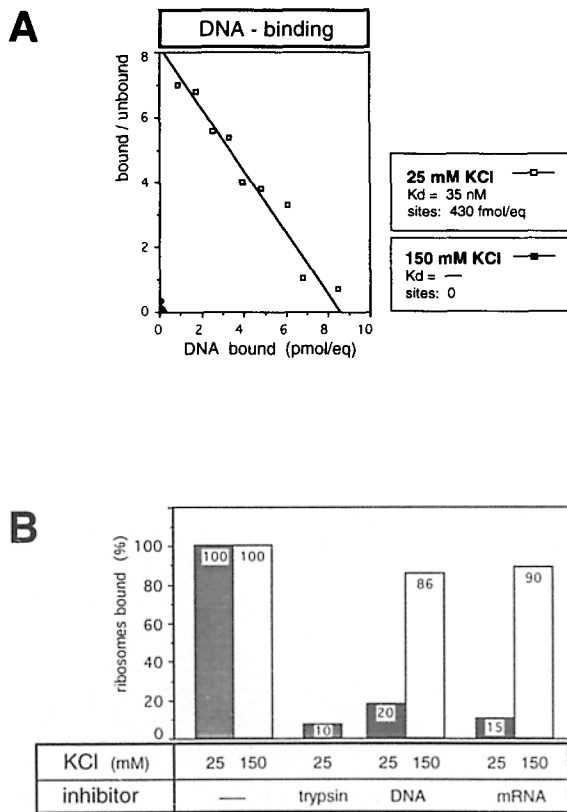
A

B

Figure 2. Binding of DNA and mRNA to microsomes. (*A*) Radiolabeled plasmid DNA was mixed with increasing concentrations of unlabeled DNA and incubated at 25 mM or 150 mM KCl concentration with 20 eq. PK-RM. The membranes were submitted to flotation and the radioactivity in the bound and unbound fractions was determined. The number of binding sites and the binding constants (Kd) were estimated by Scatchard plot analysis. (*B*) Plasmid DNA or preprolactin mRNA were incubated at 25 mM or 150 mM KCl with 20 eq. PK-RM before addition of radiolabeled ribosomes. The assay at 25 mM salt contained 10 pmol DNA or 60 pmol RNA and 12 or 10 pmol ribosomes, respectively, that at 150 mM salt 16 pmol DNA or 18 pmol RNA and 5 or 3 pmol ribosomes, respectively. The radioactivity in the bound fraction as expressed in relation to that found in the absence of inhibitors. As a control, trypsinized microsomes were employed. The trypsin digestion (10 μg/ml) was stopped by addition of 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin, and the membranes were washed twice by centrifugation through a sucrose cushion.

tion reduced the number of binding sites in PK-RM and in mock-depleted proteoliposomes (*columns 1* and *2*), in agreement with previous results (Borgese et al., 1974), although in our system the reduction was less pronounced. Sec61p-depleted proteoliposomes, however, showed a dramatic decrease (*column 3*); only a very small number of ribosome binding sites remained at 150 mM KCl, indicating that few if any proteins other than Sec61p bind under these conditions. On the other hand, proteoliposomes containing the purified Sec61p-complex were largely insensitive to the increase in salt concentration (*column 5*) (experiments with varying concentrations of KCl demonstrated, however, that above 250 mM the binding was reduced to background levels; data not shown). The numbers of ribosome binding sites

in mock-depleted proteoliposomes and in vesicles, first depleted of the Sec61p-complex, and then replenished with it, were almost identical with those found for vesicles containing the same amount of purified Sec61p-complex (*columns 2* and *4* vs *5*). Thus, at physiological salt conditions, the Sec61p-complex accounts for the majority (at least 75%) of all binding sites in unfractionated proteoliposomes. Most of the unspecific membrane binding of polyanions is also lost at 150 mM KCl: the binding of plasmid DNA to microsomes was reduced to background levels (Fig. 2 *A*), and DNA and RNA did not compete with the binding of ribosomes under these conditions (Fig. 2 *B*, *empty columns*).

It should be noted that in absolute terms the number of ribosome binding sites at 150 mM KCl was found to be rather low. PK-RM bound only ∿0.25–0.30 mol ribosomes per mol of Sec61p-molecules (250–300 fmol/eq. ribosomes per 1,000 fmol/eq. Sec61p-complex). Also, the number of binding sites in PK-RM corresponds to only ∿30–40% of the number of ribosomes originally present in a salt-resistant manner (∿750 fmol/eq.). If one assumes that the further reduction of binding sites by ∿50%, observed after solubilization of the microsomes followed by reconstitution without fractionation (Fig. 1, *empty columns 1* vs *2*), is caused by the random orientation of the Sec61p-complex in the reconstituted vesicles, this protein complex may also be the major ribosome binding partner in microsomes.

Membrane-bound Ribosomes in Protease-treated Microsomes

Next, we wished to study the membrane interaction of ribosomes participating in protein translocation. It was reasoned that, on proteolytic digestion of native rough microsomes, there should be a correlation between the amounts of membrane-bound ribosomes and of preserved ribosome receptors. We therefore first tested the sensitivity of the putative ribosome receptors towards externally added proteases. Rough microsomes were treated with chymotrypsin, V8 protease or elastase, and submitted to flotation in a sucrose gradient at high salt concentration to remove proteolytic fragments of proteins as well as loosely bound ribosomes. Microsomes before (total; T) and after flotation (F) were analyzed by immunoblotting with specific antibodies (Fig. 3 *A*). Treatment with even low concentrations of protease led to the degradation of p180. Most proteolytic fragments of p180 were not tightly bound to the membrane and could be removed by flotation (lanes *5*, *7*, *9*, *11*, *13*, and *15*), in agreement with previous results of Savitz and Meyer (1990). The degradation of p34 could be achieved with chymotrypsin at concentrations above 60 μg/ml (lanes *8–11*). The proteolytic removal of Sec61β required still higher concentrations of chymotrypsin and subsequent flotation (lane *11*). Elastase produced a slightly smaller fragment of Sec61β that remained associated with the microsomes. In contrast to the behavior of these proteins, only a slight reduction of the quantity of Sec61α was found at even high concentrations of protease.

Next, we determined the amount of membrane-bound ribosomes remaining in the protease-treated and floated microsomes (Fig. 3 *B*). The salt wash alone reduced the amount of ribosomes associated with the membranes, as determined by their absorption at 260 nm, to ∿45% in comparison with rough microsomes (see mock-treated sample).
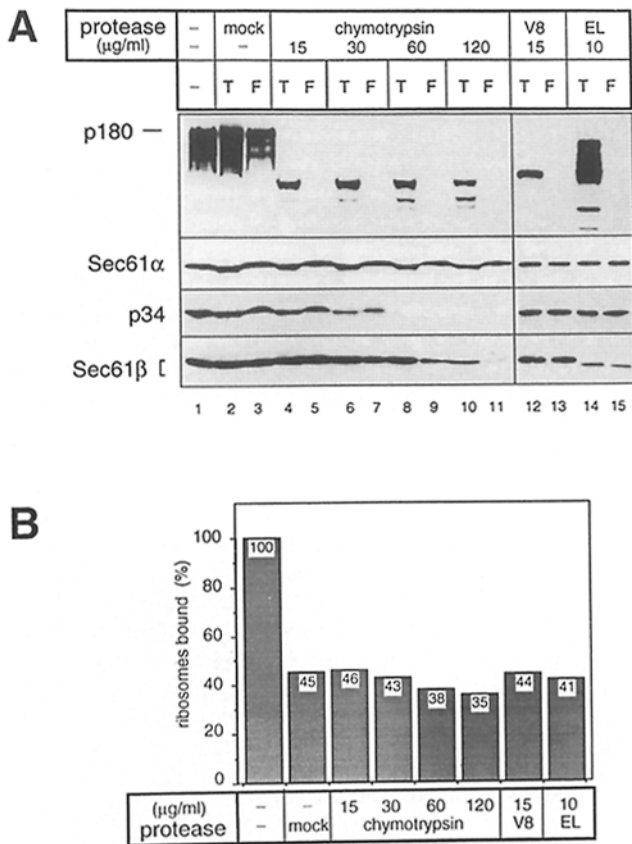
Figure 3. Correlation of the intactness of putative ribosome receptors with the amount of membrane-bound ribosomes after protease treatment of rough microsomes. (*A*) Rough microsomes were incubated with chymotrypsin, V8 protease (*V8*) or elastase (*EL*) at the concentrations indicated, or they were mock-treated. The membranes were then submitted to flotation at high salt conditions. The degradation of the putative ribosome receptors p180, Sec61-complex (Sec61α and β analyzed) and p34 in the microsomes, both before (*total*; *T*) and after their flotation (*F*), was tested by SDS-PAGE followed by immunoblot analysis with specific antibodies. (*B*) The amount of ribosomes in the protease-treated, floated membranes was determined by the absorption at 260 nm and is expressed relative to that in untreated rough microsomes.

The removed ribosomes must have been only loosely associated with the microsomes. The amount of the remaining firmly bound ribosomes was only slightly reduced, even after pretreatment of the microsomes with proteases at high concentrations (Fig. 3 *B*). The comparison with Fig. 3 *A* shows that among the tested proteins, the behavior of Sec61α would be consistent with its assumed role in anchoring the remaining membrane-bound ribosomes to protease-treated microsomes; it shows the same resistance to proteases as the membrane interaction of ribosomes. On the other hand, the other tested proteins can be largely degraded without reducing the amount of bound ribosomes.

A possible caveat to these conclusions is that the remaining ribosomes in protease-treated microsomes may be bound in an unphysiological manner or that the nascent polypeptide chain may provide the sole linkage. We therefore tested if the remaining ribosomes fulfill the criterium used to define a membrane-bound state, i.e., that they can only be released
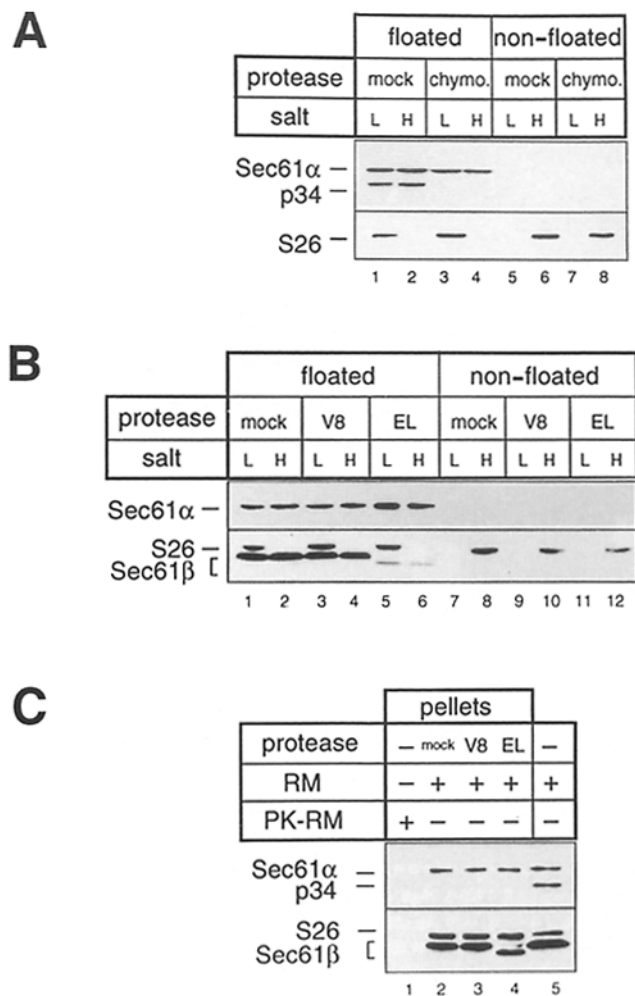

Figure 4. Membrane-bound ribosomes in protease-treated microsomes. (*A*) Rough microsomes were incubated with 120 μg/ml chymotrypsin (*chymo.*) or were mock-treated, and then submitted to flotation at high salt concentrations. They were subsequently incubated with puromycin at low salt conditions, sedimented and resuspended at low (*L*) or high (*H*) salt concentration. After a second round of flotation to separate membrane-bound ribosomes (*floated*) from unbound ones (*non-floated*), the proteins in these fractions were separated by SDS-PAGE and analyzed in immunoblots with antibodies to Sec61α and p34, as well as to the ribosomal protein S26. (*B*) The same experiment as in part *A* was carried out, except that rough microsomes were incubated with V8 protease (*V8*; 15 μg/ml) or elastase (*EL*; 10 μg/ml). (*C*) The floated microsomes pretreated with protease (same samples as in part *B*) were solubilized with digitonin at high salt concentration. The ribosomes were sedimented by centrifugation and the association of membrane proteins was tested by SDS-PAGE followed by immunoblot analysis with antibodies to Sec61α, Sec61β, and p34. The presence of ribosomes was verified by antibodies against the ribosomal protein S26. Lane *1* shows a control with PK-RM, and lane *5* shows RM which have not been solubilized.

by puromycin at high salt concentrations. To this end, microsomes that were pretreated with chymotrypsin, V8 protease, or elastase and subsequently floated under high salt conditions (see Fig. 3 *A*), were incubated with puromycin at low salt concentrations, and submitted to a second round of flotation at low or high salt concentrations (L and H) (Fig. 4, *A*

and *B*). The fractions of the floated membranes and of the non-floated free ribosomes were analyzed by immunoblotting for their content of Sec61p-complex and of ribosomes (using an antibody directed against the ribosomal protein S26). It may be seen that after treatment with puromycin and flotation at low salt concentration, the ribosomes remained membrane-bound and could be quantitatively floated with the membranes (Fig. 4 *A*, lanes *1* and *3* vs lanes *5* and *7*; Fig. 4 *B*, lanes *1*, *3*, and *5* vs lanes *7*, *9*, and *11*). At high salt concentrations, however, the ribosomes were released and were found in the pellet (Fig. 4 *A*, lanes *2* and *4* vs lanes *6* and *8*; Fig. 4 *B*, lanes *2*, *4*, and *6* vs lanes *8*, *10*, and *12*).

Further evidence that the protease-treated microsomes retain truly membrane-bound ribosomes is given in Fig. 4 *C*. Microsomes treated or non-treated with protease were submitted to flotation, and then solubilized in detergent at high salt concentration. The ribosomes were subsequently sedimented through a sucrose cushion, and the amounts of membrane proteins and of the ribosomal protein S26 in the ribosome pellet were determined by immunoblotting. The subunits of the Sec61p-complex remained associated with the ribosomes, regardless of whether or not the microsomes had been treated with proteases (Fig. 4 *C*, lane *2* vs *3* and *4*). If ribosome-stripped microsomes (PK-RM) were solubilized, no Sec61p-complex was found in the pellet (lane *1*). Neither p34 (lane *5* vs *2–4*) nor the TRAM protein (data not shown) were found to be associated with the ribosomal pellet. Thus, the membrane-bound ribosomes in protease-treated microsomes behave similarly to those in non-treated ones. In conclusion, these results indicate that Sec61α, but not any of the other tested proteins, is likely to be responsible for anchoring the ribosomes in protease-treated rough microsomes.

Ribosomes Protect Proteins from Proteolysis

The extraordinary resistance of Sec61α in rough microsomes against proteolytic attack suggested that it may be shielded by membrane-bound ribosomes. To test this possibility, microsomes containing or lacking membrane-bound ribosomes (RM or PK-RM) were incubated with different concentrations of chymotrypsin or trypsin, and the degradation of the putative ribosome receptors was followed by immunoblotting with specific antibodies (Fig. 5, *A* and *B*). With both proteases, the presence of membrane-bound ribosomes did not affect the degradation of p180 or p34. On the other hand, Sec61α was clearly more resistant to proteolytic attack in RM when compared with PK-RM. A significant, but less pronounced protection by membrane-bound ribosomes was also found for Sec61β. Sec61γ was resistant to protease treatment of both RM and PK-RM (data not shown). Thus, it seems that membrane-bound ribosomes protect components of the Sec61p-complex, but not p34 or p180, from proteolysis.

If ribosomes lacking nascent chains were added back to PK-RM under low salt concentrations before addition of proteases, there was no difference to the control in the case of trypsin (Fig. 5 *B*, lanes *11–15* vs lanes *6–10*). However, with chymotrypsin the Sec61p-complex was clearly protected (see Fig. 5 *A*, same lane numbers), supporting again our conclusion that the Sec61p-complex is the binding partner in the ribosome-binding test. The other two receptor candidates
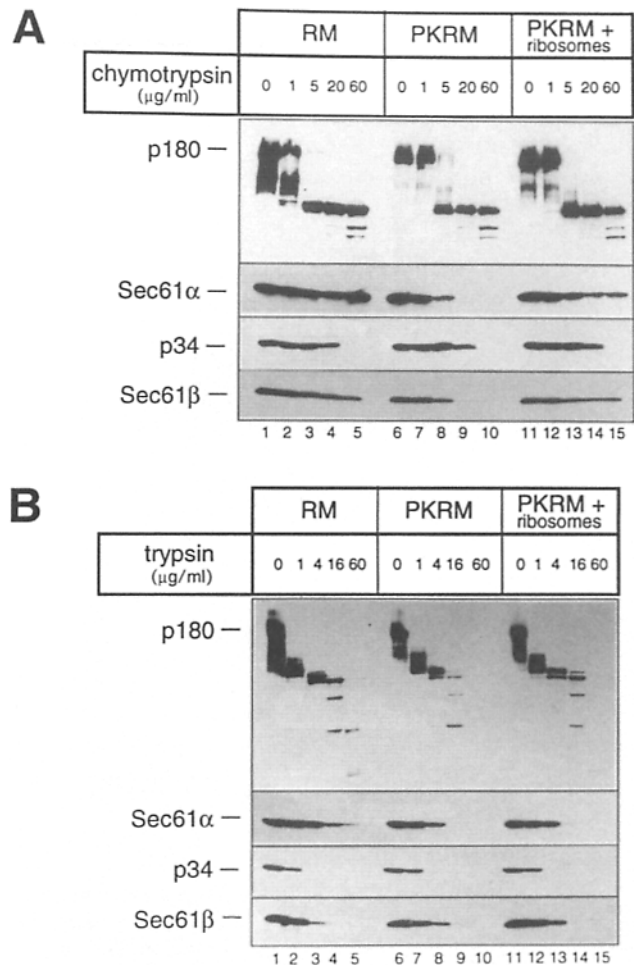

Figure 5. Testing putative ribosome receptors for their accessibility to proteases. Rough microsomes (*RM*) or microsomes stripped of ribosomes (*PK-RM*) were incubated with different concentrations of chymotrypsin (part *A*) or trypsin (part *B*). The proteolytic reaction was stopped by precipitation with 15% TCA. The pellets were washed with 5 mM PMSF in methanol, and the proteins were analyzed by SDS-PAGE and by immunoblotting with specific antibodies. Lanes *11–15* show experiments in which PK-RM were incubated with ribosomes lacking nascent chains (1.5 pmol ribosomes/eq. membranes) before addition of the proteases.

were not shielded from the action of the proteases by the added ribosomes.

Protein Translocation with Protease-treated Microsomes

The role of p180 in protein translocation could be tested directly with microsomes that had been incubated with proteases under conditions which lead to degradation and membrane detachment of this protein but leave intact other translocation components. Rough microsomes were treated with V8 protease or elastase and washed repeatedly by sedimentation through a sucrose cushion containing a high salt concentration. These membranes contained no detectable p180 but almost unreduced quantities of Sec61α and β, SRP-receptor α-subunit (SRα), p34 (Fig. 6 *A*), and the TRAM protein (data not shown). When tested in an in vitro translation sys-
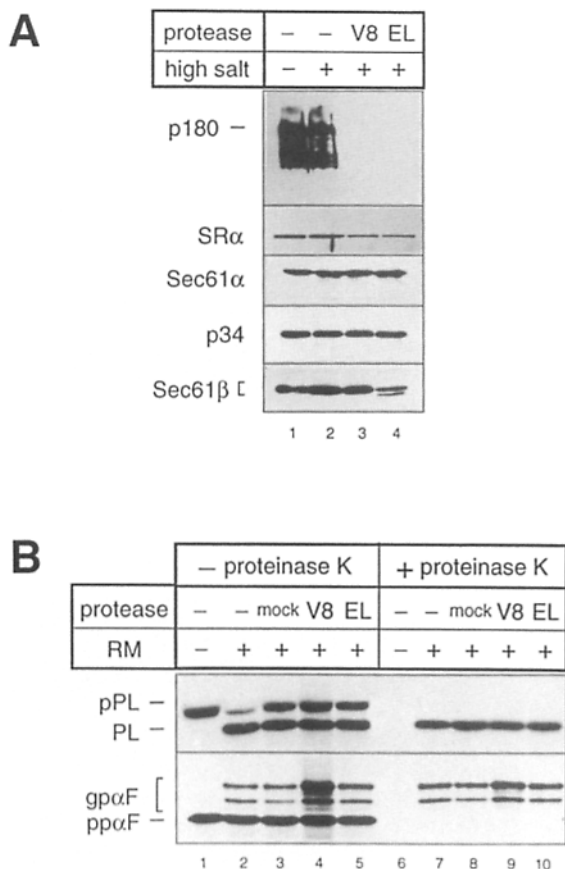
A

protease	–	–	V8	EL
high salt	–	+	+	+

p180 –

SRα

Sec61α

p34

Sec61β [

1 2 3 4

B

		– proteinase K				+ proteinase K			
protease	–	–	mock	V8	EL	–	–	mock	V8	EL
RM	–	+	+	+	+	–	+	+	+	+

pPL –
PL –

gpαF [
ppαF –

1 2 3 4 5 6 7 8 9 10

Figure 6. Microsomes lacking the putative ribosome receptor p180 retain their translocation competence. (*A*) Rough microsomes were incubated with V8 protease (*V8*; 15 µg/ml) or elastase (*EL*; 5 µg/ml) to degrade p180 without affecting known translocation components. They were washed repeatedly to remove proteolytic fragments and were then analyzed for their content of various membrane proteins by SDS-PAGE and immunoblot analysis with specific antibodies. (*B*) The membrane samples analyzed in part *A* were tested for their competence to translocate preprolactin or prepro-α-factor. Preprolactin or prepro-α-factor mRNAs were translated in the presence of the membrane vesicles and thereafter half of the sample was treated with proteinase K to assay for material that is protected against the protease by the phospholipid bilayer. The proteins were separated in an SDS-gel and visualized by fluorography. *pPL*, preprolactin; *PL*, prolactin; *ppαF*, prepro-α-factor; *gpαF*, glycosylated forms of pro-α-factor.

tem programmed with either preprolactin or prepro-α-factor mRNA, the protease-treated microsomes showed the same activity of protein translocation as mock-treated ones (Fig. 6 *B*). Thus, the presence of p180 is not essential for translocation in vitro.

Discussion

The data presented here support the notion that the Sec61p-complex is involved in the binding of ribosomes to the ER membrane. We initially studied the role of the Sec61p-complex in the binding of ribosomes lacking nascent chains, using the low salt concentrations (25 mM) generally employed in previous studies on ribosome receptors. The

Sec61p-complex indeed showed strong ribosome binding with all the characteristics reported before. However, other membrane proteins accounted for the majority (70–75%) of ribosome binding sites in proteoliposomes reconstituted from an unfractionated detergent extract of microsomes. Under low salt conditions, the binding assay also had limited specificity with respect to ribosomes; for example, plasmid DNA was bound with similar characteristics, and DNA and various other polyanions competed with ribosomes for the binding to microsomes. Presumably, the low salt concentration favors electrostatic interactions between polyanions and several membrane proteins, including the Sec61p-complex and the proposed ribosome receptors p34 and p180, and some of these interactions may be unspecific.

To reduce unspecific interactions and to approach physiological conditions, we therefore used elevated salt concentrations (150 mM) in the traditional ribosome-binding assay. It turned out that most of the unspecific binding of polyanions was lost under these conditions. Furthermore, the Sec61p-complex now accounted for the majority (at least 75%) of all ribosome-binding sites in reconstituted proteoliposomes. It therefore seems that most membrane proteins which bind ribosomes at low salt concentrations do not play a role in ribosome binding under physiological salt conditions. Our conclusion is based on the binding of ribosomes to proteoliposomes produced from detergent extracts of microsomes which had been specifically depleted of or replenished with the Sec61p-complex, as well as on studies with proteoliposomes containing the purified complex alone. The agreement between the two experiments also indicates that the purified Sec61p-complex retained all its ribosome-binding activity.

Presumably, at physiological salt concentrations, the Sec61p-complex also accounts for the majority of ribosome-binding sites in ribosome-stripped microsomes. Microsomes contain twice as many binding sites as unfractionated, reconstituted proteoliposomes, but this would be expected if the Sec61p-complex were randomly inserted in both orientations into proteoliposomes. However, the absolute number of ribosome-binding sites in stripped microsomes is significantly lower than that of Sec61p-molecules contained in them, and it is also lower than the number of ribosomes originally present in rough microsomes. It therefore appears that only a portion of the Sec61p molecules is active in ribosome rebinding. Possibly, the puromycin/high salt treatment may have inactivated some molecules. It should be noted that we cannot exclude that the difference in the number of binding sites between native and reconstituted microsomes is due to additional, unidentified ribosome receptors, the activity of which may be lost during reconstitution. Such receptors should not include p180 and p34 which have been shown to bind at low salt concentration ribosomes in reconstituted proteoliposomes (Savitz and Meyer, 1990; Tazawa et al., 1991).

The salt dependence of ribosome binding in our experiments was less pronounced than that observed in some of the previous studies (e.g., Borgese et al., 1974), perhaps because in the system from rats employed in the earlier experiments, ribosomes may have a weaker interaction with membranes at 150 mM salt concentration than in the system from dogs.

The low salt conditions and thus limited specificity of the

ribosome-binding assay may also explain some of the conflicting previous results. However, it remains unclear why Savitz and Meyer (1993) found that all the ribosome-binding activity is caused by p180. These results also contradict those of Collins and Gilmore (1991) and of Nunnari et al. (1991).

To study the membrane interaction of ribosomes participating in protein translocation, we have treated native rough microsomes with various proteases and searched for a correlation between the amount of membrane-bound ribosomes and the intactness of putative ribosome receptors. The amount of membrane-bound ribosomes was found to be only slightly reduced by protease treatment, consistent with the protease-resistance of Sec61α which was found to be shielded by these ribosomes. In contrast, p34 and p180 could be readily degraded, indicating that they are not essential for the anchoring of ribosomes in the protease-treated microsomes. Interestingly, on the same ground, it is also not likely that Sec61β serves such a function; conditions were found under which all the immunoreactive material was removed from the microsomes without significant reduction in the amount of membrane-bound ribosomes. The ribosomes in protease-treated microsomes retained the essential properties expected for membrane-bound ones in that they could only be removed from the membrane by puromycin/high salt treatment (Adelman et al., 1973) and in that they bound the Sec61p-complex after solubilization of the membranes (Görlich et al., 1992b).

It should be noted that we cannot completely exclude that proteolytic fragments of the proteins were produced which did not react anymore with the respective antibodies but remained membrane-bound and retained ribosome-binding activity. Although the antibodies against p180 (Savitz and Meyer, 1993) and Sec61β were raised against the entire protein and the entire cytosolic domain, respectively, and although the absence of p34 in protease-treated microsomes could be confirmed with antibodies raised against the entire protein (provided by S. High; data not shown), it remains possible that remaining membrane-bound proteolytic fragments lacked reactive epitopes. In the case of p180, treatment with elastase gives large proteolytic fragments (up to 160 kD) which can be removed by flotation (Fig. 4 A, lane 14); thus, only small portions, including the membrane anchor of the protein could have remained with the membranes. Savitz and Meyer (1990) have shown that the binding activity of p180 in the ribosome-binding assay is located in a cytosolic domain of 160 kD that can be readily cleaved off from the membrane. Thus, it seems unlikely that the 20-kD fragment remaining in the membrane is responsible for the binding of ribosomes in protease-treated microsomes.

Other evidence also argues against an essential role for p34 or p180 in the binding of ribosomes participating in translocation and support our conclusion that the Sec61p-complex is involved. Zimmerman and Walter (1991) have used trypsinized microsomes that had lost p180 and the cytosolic domain of the α-subunit of the SRP-receptor, and could rescue translocation by complementation with only the latter. We have confirmed here that microsomes specifically lacking p180 after proteolysis with V8 protease or elastase do not have a reduced translocation activity. We have also shown recently that proteoliposomes reconstituted from pure phospholipids and purified membrane proteins are translocation competent even though p180 could not be detected and

the level of p34 was reduced by a factor of at least 1000 compared with that in native microsomes (Görlich and Rapoport, 1993). The divergent results of Savitz and Meyer (1993), who reported that p180 is absolutely required for protein translocation in a reconstituted system, may perhaps be due to experimental conditions in which the yield, rather than the activity of reconstituted proteoliposomes was influenced by p180. Although most data argue against an essential or even stimulatory role for p34 or p180 in translocation or in ribosome-anchoring to the ER membrane, they do not strictly exclude a possible interaction of these proteins with ribosomes. For example, it is conceivable that they would increase the membrane affinity for ribosomes or that they constitute ribosome-binding sites without essential function in translocation. It seems also possible that their observed interaction with ribosomes at low salt concentration reflects an actual RNA-binding activity. Binding of RNA to p180, blotted onto nitrocellulose filters, has indeed been observed (Prescott, C., K. Jürchott, and T. A. Rapoport, unpublished results).

Most data support the hypothesis that the Sec61p-complex is responsible for the binding of ribosomes during protein translocation. The interaction of the ER membrane with ribosomes lacking nascent chains, which may mimick a stage of protein translocation in which the nascent chain is not inserted into the membrane, seems to be mediated by the Sec61p-complex because (a) the Sec61p-complex accounts for the majority of binding sites, (b) Sec61α is protected from the action of proteases by added ribosomes, and (c) the Sec61p-complex cofractionates with ribosomes if microsomes are incubated with ribosomes lacking nascent chains and solubilized with detergent (Görlich et al., 1992b). The membrane binding of ribosomes engaged in protein translocation also seems to involve the Sec61p-complex because (a) the Sec61p-complex is among the membrane proteins most tightly bound to membrane-bound ribosomes after solubilization of rough microsomes (Görlich et al., 1992b), (b) this interaction is induced by the targeting of a nascent chain to the membrane, (c) the interaction is abolished by puromycin/high salt treatment in the same manner as is the membrane-binding of ribosomes, (d) Sec61α, and to a lesser extent, Sec61β, are shielded by membrane-bound ribosomes from the attack by proteases, and (e) Sec61α shows the same remarkable resistance to proteases as membrane-bound ribosomes. Finally, perhaps the best evidence that the Sec61p-complex is sufficient for the binding of ribosomes during translocation comes from reconstitution experiments (Görlich and Rapoport, 1993). Some proteins, like preprolactin, require for their translocation only the presence of the SRP-receptor and of the Sec61p-complex in proteoliposomes. Assuming that the SRP-receptor is only needed for their membrane targeting, it follows that the Sec61p-complex must bind the translating ribosome.

It is not yet clear whether the nascent chain alters the precise mode of interaction of a ribosome with the ER membrane. The initial, salt sensitive ribosome binding to the Sec61p-complex may be strengthened by an additional interaction of the SRP-receptor with SRP that, in turn, is associated with both the ribosome and the nascent chain (Connolly and Gilmore, 1986). Such an additional interaction may explain why the vast excess of ribosomes in an in vitro translation system does not inhibit the membrane-targeting

of ribosomes carrying nascent polypeptides. Alternatively, or in addition, unidentified protein factors may modulate the ribosome-membrane interaction. After membrane insertion, the nascent chain may simply provide an additional link to the ER membrane or might cause a conformational change in the Sec61p-complex or the ribosome or both. In favor of the latter possibility, the Sec61p-ribosome complex is sensitive to solubilization of the membrane with harsh detergents, unless stabilized by a translocating nascent chain (Görlich et al., 1992*b*).

How the ribosome is bound to the Sec61p-complex is still unknown. Crowley et al. (1993) have shown that fluorescent probes, incorporated into short translocating polypeptide chains, are shielded from quenching by iodide ions added to the cytosolic compartment. One may therefore speculate that the translating ribosome makes numerous contacts with the cytosolic loops of Sec61α, ensuring a tight seal. Whereas a direct role for Sec61β in ribosome anchoring seems unlikely, we cannot exclude such a function for Sec61γ. The interacting partner in the ribosome (protein, RNA?) also remains to be identified.

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