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Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane

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ABSTRACT | We have reproduced the process of protein transport across and of protein integration into the mammalian endoplasmic reticulum membrane by the use of proteoliposomes reconstituted from pure phospholipids and purified membrane proteins. The transport of some proteins requires only two membrane protein complexes: the signal recognition particle receptor, needed for targeting of a nascent chain to the membrane, and a novel complex, the Sec61p complex, that consists of Sec61p and two smaller polypeptides. The translocation of other proteins also needs the presence of the translocating chain-associating membrane (TRAM) protein. The integration of two membrane proteins of different topologies into the membrane does not require additional components. These results indicate a surprising simplicity of the basic translocation machinery. They suggest that the Sec61p complex binds the ribosome during translocation and forms the postulated protein-conducting channel.

Introduction

A large class of proteins is transported across or integrated into the endoplasmic reticulum (ER) membrane. The process can be divided into a targeting phase, which is fairly well characterized for the system in mammals, and the translocation phase (for review see [33]). Targeting of a nascent, growing polypeptide to the ER membrane occurs in general after the interaction of its signal sequence with the 54 kd polypeptide component of the cytosolic signal recognition particle (SRP) [45,22,21]. Continued elongation of the polypeptide chain is delayed or even arrested until the entire complex, consisting of the ribosome, nascent chain, and SRP, has bound to the SRP receptor (docking protein) [10,27]. The SRP receptor is an integral protein of the ER membrane, consisting of two subunits [42]. In a GTP-dependent reaction, the signal sequence is subsequently displaced from the SRP and inserted into the membrane [4]. Simultaneously, the ribosome becomes anchored to the membrane, presumably by binding to one or more ribosome receptor proteins.

The actual membrane transfer of a polypeptide, following the targeting process, is assumed to occur through a protein-conducting channel, a view that is supported by electrophysiological data [38]. Several candidates for constituents of the putative channel have been proposed. Of particular importance is Sec61p, a multispanning membrane protein of the ER. It was initially found in Saccharomyces cerevisiae via genetic screening for translocation defects and is encoded by an essential gene [7,34,41]. Homologs of Sec61p are now known to be present in mammals as well as in other vertebrates [13, unpublished data]. Furthermore, Sec61p has sequence homology to SecYp [13], a key component of the protein export apparatus in bacteria. Several of the putative membrane-spanning segments of Sec61p contain hydrophilic or even charged amino acid residues, suggesting that they may contribute to a hydrophilic interior of the membrane channel. Sec8lp is adjacent to polypeptide chains passing across the membrane of the mammalian or yeast ER [13,29,35]. In mammals, it is tightly associated with membrane-bound ribosomes [13], suggesting that the nascent chain is transferred directly from the ribosome into a protein-conducting channel that includes Sec61p. However, its requirement for protein translocation in mammals has, until now, not been demonstrated.

Another component of the translocation apparatus may be the translocating chain-associating membrane (TRAM) protein [12]. After membrane insertion of the nascent chain, the TRAM protein was found to be a principal cross-linking partner of different secretory proteins. Further components assumed to be involved in the process include putative ribosome receptors of 34 kd [43,17] and 180 kd [36,37], a membrane protein of 30 kd with affinity for SRP (mp30) [42], an unidentified ATP-binding membrane protein [20] and lumenal proteins, in particular BiP [44]. However, the function of these components, if any, is either disputed or not yet demonstrated. Not all components located at the translocation site need to be essential for the translocation process, as demonstrated for the signal peptidase, the oligosaccharyl transferase, and the translocon-associated protein (TRAP) complex [12,28] (previously called the signal sequence receptor [SSR] [14].

Relatively little is known about the integration of membrane proteins except that they are also targeted to the ER membrane by the SRP and the SRP receptor and that they are translocated in proximity to Sec61p [15,16]. It is generally assumed, however, that the protein-conducting channel is, at some point, opened laterally to release hydrophobic membrane-spanning segments (stop-transfer sequences) into the phospholipid bilayer. The existence of receptors for stop-transfer sequences has been postulated [24].

Many of the open questions, such as which components of the ER membrane are really essential for translocation, which components are involved in ribosome binding, and whether stop-transfer receptorsexist, could be addressed directly if one were able to reconstitute the translocation apparatus of the ER membrane into proteoliposomes with purified membrane proteins. This objective came in reach with the demonstration that translocation-competent vesicles can be reconstituted from an unfractionated detergent extract of dog pancreatic microsomes [30]. Also, some translocation components have been purified, in particular the SRP receptor [42,28] and the TRAM protein [12]. When tested in a crude reconstitution system,

the SRP receptor was found to be essential for protein translocation [28] and the TRAM protein was either required or only stimulatory, depending on the translocation substrate [12].

For the system in Escherichia coli, it has already been reported that translocation of the model protein proOmpA can be reconstituted with a small number of components [2,1]: the integral membrane proteins SecYp and SecEp, Band I (an unidentified protein), SecAp (a peripheral membrane protein with ATPase activity), and the cytosolic chaperone SecBp.

We now report on the ab initio reconstitution of the translocation apparatus of the mammalian ER membrane. To this end, we have purified Sec61p in a functional state. It turns out that Sec61p is tightly associated with two smaller polypeptides of about 14 and 8 kd. This Sec61p complex and the SRP receptor are required for the translocation of all proteins tested. Surprisingly, some proteins are translocated with these two components alone. Some proteins do require the additional presence of the TRAM protein, which can also have a stimulatory effect in cases in which it is not essential. The correct insertion of two membrane proteins of different topology into the membrane did not require any additional translocation component. Although there may exist further factors that increase the efficiencies of the transport or insertion processes, our results define a minimal translocation apparatus. Neither the previously proposed ribosome receptors, nor unidentified stop-transfer receptors or ATP-binding components, nor lumenal proteins seem to be essential. Based on present and previous results, one may suggest the following functions for the components of the basic translocation apparatus: the SRP receptor is needed for membrane targeting of a nascent chain; the Sec61p complex is required for its stable membrane insertion, binds the ribosome during translocation, and forms the postulated protein-conducting channel; and the TRAM protein may be needed to guide some nascent chains into this channel.

Results

Sec61p is a Constituent of a Complex

As a precondition to the goal of establishing a reconstitution system consisting of purified, defined components, we set out to purify Sec61p under nondenaturing conditions. To this end, we exploited its tight association with membrane-bound ribosomes after solubilization of dog pancreatic microsomes with detergent [13] (Fig.1A). The purification was followed by immunoblotting with specific antibodies [13] and, at later stages, by determination of the N-terminal amino acid sequence (data not shown).

Rough microsomes were extracted first with digitonin at low ionic strength to remove lipids, lumenal proteins, and some membrane proteins (preextract) and subsequently at high salt concentration to solubilize most membrane proteins. Some membrane proteins, including Sec61p, remained bound to the ribosomes (proteins referred to as ribosome-associated membrane proteins [RAMPS]). These were released from the ribosomes by puromycin at high ionic strength [13]. The yield of Sec61p in the RAMP fraction was in the range of 30%-95% for different batches of microsomes. This variability may reflect differences in the percentage of translocation sites that are occupied by functional membrane-bound ribosomes. Further purification of Sec61p was achieved by ion exchange chromatography. In the final chromatographic step on an S-Sepharose column, Sec61p eluted as a rather sharp peak, together with two smaller polypeptides of about 14 and 8 kd (Fig.1A; closed triangles). These polypeptides seem to form a defined complex that is referred to as the Sec61p complex (the subunits are called Sec61α [for the actual Sec61p], Sec61β, and Sec61y). Most other proteins could be quantitatively removed. A minor proportion of a protein of about 10 kd (referred to as RAMP4) was still present in the Sec61p-containing fractions (about 4%) although the majority of it eluted much earlier from the column as a sharp peak (see arrows).

The existence and identity of the Sec61p complex could be confirmed by employing an entirely different purification protocol (Fig.1B). Antibodies were raised against a peptide corresponding to the N-terminal sequence of Sec61β and were used for immunoaffinity purification of the Sec61p complex. A detergent extract, prepared from microsomes stripped of ribosomes by puromycin and high salt (PK-RM), was passed through a column containing immobilized, affinity-purified antibodies, and the bound antigen waseluted by addition of the peptide against which the antibodies were raised. The material was further purified and concentrated by ion exchange chromatography. The final preparation contains the three subunits of the Sec61p complex at the same quantitative proportions as those found after its purification on the basis of its ribosome association (Fig.1A,1B). Both preparations were equally active in translocation tests (see below). Immunoblotting experiments indicate that the ratioof Sec61α and Sec61β in the purified complex is nearly the same as in intact membranes (see Fig.3B) suggesting that the majority of these polypeptides are in association with each other. The Sec61p complex is stable in steroid-containing detergents such as digitonin, (N,N-bis-(3+gluconamidopropyl)-cholamide (BigCHAP), (N,N-bis-(3-o-gluconamidopropyl)-deoxycholamide (deoxy-BigCHAP), cholate, and 3-((3cholamidopropyl) dimethylammonio)-l-propansulfonate (CHAPS) but can be dissociated with other detergents, such as Nonidet P-40 (NP-40) or sucrose monolaurate (data not shown).

The Sec61p Complex is Essential for Protein Translocation

Next, we tested whether the purified Sec61p complex is functional and whether it is essential for protein translocation (Fig.2). Proteoliposomes were produced from detergent extracts that were depleted of or replenished with the Sec61p complex. Depletion of Sec61p complex was achieved by a two-step procedure. The first depletion step made use of the fact that much of the Sec61p complex can be removed after solubilization of rough microsomes simply by sedimentation of the ribosomes [13]. For these experiments, a batch of rough microsomes was selected that had 95% of the Sec61p associated with the ribosomes. In the second step, the residual Sec61p complex in the supernatant fraction was adsorbed to immobilized antibodies against Sec61β. The resulting extract contained undetectably low levels of Sec6lα and Sec61β, whereas the concentrations of most other membrane proteins, including the SRP receptor and the TRAM protein, remained unaffected (see Fig.2A,2B, lanes 3 and 4). It should be noted, however, that during

the first depletion step, not only the concentration of the Sec61p complex, but also that of all other RAMPS, was reduced.

The proteoliposomes were then tested for their competence to translocate the secretory protein preprolactin synthesized in vitro. After translation, protease was added to assay for translocated material that was protected from degradation by the phospholipid bilayer (Fig. 2C). Proteoliposomes produced after the first depletion step showed a much reduced but still detectable activity (Fig. 2C, lane 3) whereas proteoliposomes produced from the doubly depleted extract were completely inactive (lane 4). If the purified Sec61p complex was added to the depleted extract, the translocation activity of the resulting vesicles was restored (Fig. 2C, lanes 5, 6, and 7). The levels of activity approached those obtained with proteoliposomes produced from an unfractionated extract of ribosomestripped microsomes (Fig. 2C, lane 2 versus lane 7) similar to those of native membranes (lane 8). One may therefore conclude that the purified Sec61p complex is functional and that it is essential for protein translocation. These results also suggest that the Sec61p complex is the only essential component of the RAMP fraction.

Next, we investigated at which step the translocation process is blocked in the absence of Sec61p. A short fragment of preprolactin containing 86 amino acids (pPL86mer) was synthesized in an in vitro system, and its transfer from the SRP into the ER membrane was monitored by cross-linking, using nascent chains with photoreactive lysine derivatives incorporated into the signal sequence [48] (Fig.2D). In the absence of membranes, cross-linking of the signal sequence occurs to the 54 kd subunit of the SRP (SRP54) (Fig.2D, lane 1) [22,21]. In the presence of control membranes (Fig.2D, lane 8) or of proteoliposomes containing all membrane components (lane 2), the cross-linking to SRP54 is greatly diminished. Instead, cross-links to the TRAM protein and to Sec61α appear [12,13]. This indicates that the nascent chain is transferred from the SRP into the membrane. Proteoliposomes depleted of the Sec61p complex do not show these effects (Figure 2D, lanes 3 and 4) but their activity is restored by replenishment with the purified Sec61p complex (lanes 5, 6, and 7). We conclude that the Sec61p complex is required for the stable insertion of the nascent preprolactin chain into the membrane. It should be noted that an interaction of the complex of ribosome, nascent chain, and SRP with the SRP receptor in the membrane does occur in the absence of Sec61p (see below).

Purification of the SRP Receptor, the TRAM Protein, and the Signal Peptidase

As a next step for the establishment of a reconstitution system, the SRP receptor complex, the TRAM protein, and the signal peptidase complex were purified.

The SRP receptor was obtained from a detergent extract of microsomes by immunoaffinity chromatography using antibodies directed against a peptide corresponding to an internal sequence of the a subunit. The final preparation consisted essentially of only the two subunits (Fig.3A, lane 1), as described by others [42,28]. Some of the minor bands probably represent breakdown products.

The signal peptidase and the TRAM protein were purified by chromatography on concanavalin A-Sepharose (both are glycoproteins) followed by chromatography on two succesive ion exchange columns [12]. The final preparation of the signal peptidase complex is seen to consist of five or six bands (Fig. 3, lane 2) as reported previously [9]. The preparation of the TRAM protein resulted in one major band (Fig. 3, lane 3) and sometimes a proteolytic fragment, lacking the intact C-terminus [12]. Signal peptidase was present in minor quantities (see below).

To assess the purity of the isolated translocation components, we reconstituted the SRP receptor, the Sec61p complex, and the TRAM protein into proteoliposomes and compared these with native microsomes by immunoblotting with antibodies against various ER proteins (Fig.3B). The amounts of SRP receptor, Sec61p, and TRAM protein were adjusted to be equal to or in a slight excess (2-fold) over those present in native membranes. With this reference, only signal peptidase and RAMP4 could be detected with certainty in the proteoliposomes (3% and 2%-5%, respectively, compared with their content in microsomes). All other proteins were found to be present in amounts of at least a factor of 1000 lower than in microsomes. In particular, neither of the proposed ribosome receptors (p34 and p180) nor BiP could be detected in the purified preparations.

Cross-contamination of the preparations of the SRP receptor, the Sec61p complex, and the TRAM protein was also excluded by immunoblot experiments (data not shown).

SRP Receptor and Sec61p Complex Are Sufficient for Translocation of Preprolactin

Since the SRP receptor and the Sec61p complex are known to be essential for translocation of preprolactin [28, this paper], we first tested whether these components alone are sufficient for this process. Proteoliposomes were produced with a constant amount of purified Sec61p complex and increasing amounts of SRP receptor or with SRP receptor alone, together with a mixture of phospholipids (phosphatidylcholine, phosphatidylethanol-amine, phosphatidylserine, and phosphatidylinositol) corresponding approximately to that in native microsomes (Fig. 4A). The proteoliposomes were then tested in a wheat germ translation system in which preprolactin was synthesized in the presence of SRP. After translation, half of the sample was treated with high concentrations of protease to assay for translocated and thus membrane-protected material (Fig. 4B, right panel). Vesicles containing SRP receptor or Sec61p complex alone did not show translocation activity (Fig. 4B, lanes 11 and 17). However, almost 50% of the synthesized preprolactin molecules were translocated when both membrane components were present at their optimum ratio (Fig. 4B, lanes 13 and 14). This activity is close to that obtained with native microsomes (68%; Fig. 4B, lane 18). Control experiments demonstrated that the translocated material could be degraded by the protease after lysis of the vesicles with detergent (data not shown).

The SRP receptor alone was able partially to release the elongation arrest exerted by SRP (Fig. 4B, lane 8 versus lane 1), in agreement with previous reports [10]. This clearly indicates that the complex of ribosome, nascent

chain, and SRP can interact with the SRP receptor in the absence of Sec61p. However, a stimulation of the release of the elongation arrest was found if both the SRP receptor and the Sec61p complex were present (Fig. 4B, lanes 3-7). It seems that more SRP receptor is required for maximum release activity than for optimum translocation (note that translocation is inhibited at high levels of SRP receptor; see Fig. 4B, lane 16). The optimum molar ratio of SRP receptor to Sec61p complex varied among different preparations of these components but was generally in the range of 1: 10 to 1:40 (in microsomes, the ratio is estimated to be 1:5 to 1:10). We conclude from these data that SRP receptor and Sec61p complex alone suffice for translocation of preprolactin.

Translocation with Proteoliposomes Containing the TRAM Protein and Signal Peptidase

Next, we examined the influence of the TRAM protein and of the signal peptidase on translocation of preprolactin. Proteoliposomes were produced with the SRP receptor, Sec61p complex, TRAM protein, and signal peptidase or with single omissions, in turn, of each of these four components. The polypeptide composition of these vesicles is shown in Fig. 5A.

When proteoliposomes were tested for translocation of preprolactin, there was again a strict dependence on the presence in the vesicles of both the SRP receptor and the Sec61p complex (Fig. 5B, top; lane 9 versus lanes 10 and 11). The TRAM protein could not replace Sec61p (Fig.5B, lane 11) but had a stimulatory effect (by a factor of 1.5 to 3 in various experiments with different preparations; Fig. 5B, lane 9 versus lane 12) in agreement with our previous results with a crude reconstitution system [12]. The translocation activity approached two thirds of that with native microsomes (34.4% versus 49.1%; Fig. 5B, lane 9 versus lane 14). The fact that in the absence of the TRAM protein the level of translocation was lower than in the experiment shown in Fig. 4 may perhaps be explained by a lower concentration of the Sec61p complex. The signal peptidase had no influence on translocation (Fig. 5B, lane 9 versus lane 13) but, of course, caused signal peptide cleavage. A small amount of processed prolactin was observed even without addition of signal peptidase (about 10%; Fig. 5B, lane 13) owing to the contamination of the TRAM preparation. The processing of preprolactin to prolactin was dependent on the presence of both the SRP receptor and the Sec61p complex (Fig. 5B, lanes 3 and 4). The processed molecules were protected almost quantitatively from the attack by proteinase K (e.g., Fig. 5B, lane 2 versus lane 9). Thus, translocation and signal peptide cleavage are coupled as in intact microsomes, providing further evidence for the faithful translocation of preprolactin and prolactin into proteoliposomes reconstituted from purified membrane components.

The translocation of the secretory protein prepro-α-factor was dependent not only on the SRP receptor and the Sec61p complex but also on the TRAM protein (Fig. 5B, bottom; lane 9 versus lanes lo-12), as reported previously for a crude reconstitution system [12]. Even in the presence of all three components, however, translocation was relatively inefficient (15%-20% of control membranes; Fig. 5B, lane 9 versus lane 14). As expected, in contrast with native microsomes, core glycosylation was not observed with the reconstituted system (Fig. 5B, lane 14). Again, the signal peptidase had an influence only on signal peptide cleavage, not on the translocation activity (Fig. 5B, lane 9 versus lane 13). In the case of prepro-α-factor, some signal peptide cleavage, but no translocation, occurred even in the absence of Sec61p (Fig. 5B, bottom, lane 4). The processing was dependent on the SRP receptor (Fig. 5B, lane 2 versus lane 3). One explanation may be that membrane-targeted prepro-α-factor chains can be cleaved by incorrectly oriented signal peptidase that exposes its catalytic center to the cytosol. Prepro-α-factor may be a better substrate than preprolactin for the signal peptidase.

To assess further the efficiency of translocation into reconstituted proteoliposomes, vesicles containing fixed proportions of SRP receptor, Sec61p complex, TRAM protein, and signal peptidase complex were added in increasing concentrations to a wheat germ system programmed with preprolactin mRNA (Fig. 5C). Translocation reached a maximum with approximately 300-400 nM Sec61p complex in the assay (Fig. 5C, lanes 12-14). At this point, the concentration of the SRP receptor is estimated to be about 30 nM. These values appear to be in a reasonable range, since the concentrations of ribosomes (determined by their absorption at 260 nm) and of SRP are estimated to be 500 nM and 60 nM, respectively. However, the efficiency of the reconstituted proteoliposomes is still lower than that of native membranes by a factor of about 4-5: translocation with the latter is about 2-fold higher (84% versus 46%; Fig. 5C, lane 16 versus lane 13), despite the fact that they contain less Sec61p complex (160 nM versus 400 nM). On the other hand, reconstituted vesicles containing purified components are no less active in translocation of preprolactin than those reconstituted from an unfractionated membrane extract. Furthermore, proteoliposomes produced from a mixture of the purified components and all membrane proteins of an unfractionated extract did not show increased translocation activity for preprolactin (data not shown). It should be noted that the translocation efficiency in the wheat germ system was higher by a factor of about 2 compared with the reticulocyte lysate system (data not shown).

Integration of Membrane Proteins

Next, we tested the integration of two model membrane proteins of different topologies. The G protein of the vesiclular stomatitis virus (VSV) is a type I membrane protein with a cleavable signal sequence, a lumenal domain with attached carbohydrate chains, a single membrane-spanning region of 18 amino acids, and a cytosolic domain of 29 residues [19]. The asialoglycoprotein receptor is a type II signal-anchor membrane protein. Its N-terminal domain of 38 amino acids is located in the cytosol and is followed by a single membrane-spanning segment that functions also as signal sequence [40]. The C-terminal domain is located in the lumen.

The translocation of the lumenal domain of the G protein of VSV was dependent on the presence of the SRP receptor, the Sec61p complex, and the TRAM protein in the vesicles (Fig. 6A; Fig. 6B, lane 11 versus lanes 8-10). The protease-protected material (G-C) was slightly smaller than the nonproteolyzed material (G; Fig. 6, lane 11 versus lane S), as expected following the removal of the C-terminal 29 amino acids. The material precisely comigrated with the proteolyzed, nonglycosylated form of the G protein inserted into native microsomes (Fig. 6B,

lane 12, lower band). Since the mobility shift of the G protein after protease treatment is rather small, the experiment was repeated with trypsin, and the proteolyzed and nonproteolyzed samples were run in parallel through an SDS gel (Fig. 6C). The difference in size is now obvious (Fig. 6C, lane 3 versus lane 4) and corresponds exactly to that found with unglycosylated G protein in native microsomes (lane 5 versus lane 6). All translocated G protein molecules seem to be correctly inserted into the membrane, since they have all lost their cytoplasmic tails following proteolysis.

The elongation arrest exerted by SRP was released whenever SRP receptor was present in the proteoliposomes (Fig. 6B, lane 2 versus lanes 3-5).

The membrane insertion of the asialoglycoprotein receptor was also dependent on the presence of the SRP receptor and the Sec61p complex (Fig. 7B, lane 11 versus lanes 8-9). The TRAM protein had a significant stimulatory effect (Fig. 7B, lane 11 versus lane 10). In the presence of protease, the membrane-protected protein fragment was clearly some 4.5 kd smaller than the untreated protein (Fig. 7B, lane 11 versus lane 5) and comigrated exactly with the proteolyzed, unglycosylated asialogyloprotein receptor inserted into native microsomes (lane 12, lower band, versus lane 11). These data indicate that the protein was correctly inserted into the membrane with the N-terminal 38 amino acids remaining in the cytosol. Molecules that had been completely transferred across the membrane could not be detected.

In conclusion, these results clearly demonstrate that the integration of the two membrane proteins thus tested is dependent upon the same translocation components that are required for the transport of secretory proteins.

Discussion

We have reconstituted the protein translocation apparatus of the mammalian ER membrane into proteoliposomes from pure phospholipids and purified membrane proteins. Our results indicate that the basic translocation machinery is surprisingly simple. The translocation of all proteins tested depends on just two membrane protein complexes: the SRP receptor complex, consisting of two subunits [42], and a novel complex (Sec61p complex) that contains Sec61p (now called Sec61α) and two additional small polypeptides of 14 and 8 kd (Sec61β and Sec61γ, respectively). Some proteins, like preprolactin, are translocated with these components alone. Others, however, like prepro-α-factor and the VSV G protein, additionally require the TRAM protein. In cases in which the TRAM protein is not essential, it does exert a stimulatory effect. We have also demonstrated that two membrane proteins of different topology, the type I VSV G protein and the type II asialoglycoprotein receptor, are correctly inserted into the membrane with only the SRP receptor, the Sec61p complex, and the TRAM protein being present in the phospholipid bilayer. Indeed, these three translocation components alone allow the translocation of all proteins tested (preprolactin, prepro-α-factor, VSV G protein, asialoglycoprotein receptor [this paper], immunoglobulin light and heavy chains, growth hormone, invertase, and interleukin II (B. Jungnickel, D.G., and T.A.R., unpublished data). The three components would appear to constitute the minimum apparatus required for the transport of any protein across the membrane. Our data do not exclude, however, the possibility that further stimulatory factors exist that in vivo may even be essential. They may affect different translocation substrates to varying degrees. For example, it is possible that the low efficiency of translocation of prepro-α-factor observed with the minimum apparatus can be increased by glycosylation of the nascent chain or by its interaction with lumenal proteins. It is also possible that only one round of translocation occurred in the reconstituted system. Further components may be needed for the recycling of the translocation components or for the efficient release of nascent chains at the lumenal side of the ER membrane, as suggested for SecDp and SecFp of E. coli [26]. Indeed, we cannot strictly exclude the possibility that both the secretory and membrane proteins remained at the translocation site after termination of translation, although a similar reservation would also hold for native microsomes.

The simple structure of the basic translocation machinery of the ER membrane correlates with that of the corresponding system in E. coli. Reconstitution experiments have indicated that only one integral component of the inner membrane of E. coli is essential, a complex, related to the Sec61p complex, that comprises SecYp, SecEp, and Band I [2,1]. The posttranslational targeting in the E. coli system, however, requires other components (SecAp, SecBp) than the SRP-dependent cotranslational targeting in the ER system.

The Sec61p complex is a genuine complex consisting of three subunits that could be isolated in the same stoichiometric ratio following two entirely different protocols. The ratio of the α and β subunits in the purified complex corresponds to that in intact microsomes, and the neighborhood of the two subunits could be demonstrated by cross-linking with a bifunctional reagent (K.-U. Kalies, D.G., and T.A.R., unpublished data). The complex is functional in a translocation system.

We consider it likely that protein translocation is faithfully reproduced by our reconstitution system. The transported proteins were not only protected by the phospholipid bilayer against the attack of proteases, but also their signal peptides were cleaved by the signal peptidase at the lumenal side of the membrane. For both membrane proteins tested, the correct domains were translocated. Polypeptides passing through the membrane of the proteoliposomes met the same protein environment as in intact microsomes, as demonstrated by photo cross-linking using different translocation intermediates of preprolactin (data not shown). The translocation of all proteins tested depended on the SRP receptor, as had previously been observed for microsomes. Finally, the absolute requirement for Sec61p in our system correlates with the observation that in S. cerevisiae, mutations within the corresponding gene affect the translocation of exported proteins [41].

With preprolactin, almost 50% of all synthesized protein molecules were transported. Such a high translocation rate was obtained in spite of only a moderate excess of translocation components, compared with microsomes. Even though the system is clearly less efficient than that of native ER membranes, for preprolactin it is still as efficient as that produced from an unfractionated detergent extract of microsomes. It is conceivable that the reduced activity is caused by a partial inactivation of translocation components during their solubilization, purification, and reconstitution, or by their inefficient

assembly in vesicles, or by both. In contrast with their integration in vivo, the three membrane components may insert in the wrong orientation or into separate vesicles, reducing the probability of correct assembly. Incorrectly assembled translocation sites may even compete with the function of those correctly assembled. This is indicated by the fact that an excess of SRP receptor inhibited translocation, presumably by causing membrane binding of nascent chains interacting with SRP without subsequent translocation. Taking these considerations into account, it is actually surprising to achieve such a relatively high efficiency of translocation. It suggests the existence of cooperative effects in the assembly of the translocation components, although in a solubilized state an interaction among the SRP receptor, the Sec61p complex, and the TRAM protein has not been observed.

Can we exclude that minor contaminants contribute or even cause the activities ascribed to the SRP receptor, the Sec61p complex, or the TRAM protein? With the exception of the signal peptidase complex and a polypeptide of about 10 kd (referred to as RAMP4) that represented minor contaminants of the TRAM protein and Sec61p preparations, respectively, all other ER components tested for were almost entirely removed (reduced by a factor of at least 1000 in comparison with native microsomes). While it may be conceivable that a protein of such a low abundance could act in a catalytic manner during the translocation process, one can definitely exclude its stoichiometric participation in each translocation site. However, we consider even the former possibility unlikely on the basis of our results with the signal peptidase, an enzyme that probably acts in a catalytic manner: 3% contamination with signal peptidase caused only about 10% processing of preprolactin, and it would appear unlikely that a component that causes 50% translocation should have escaped our detection.

In agreement with our previous results, the signal peptidase has no influence on the translocation per se [12], and the residual amounts of the enzyme in the TRAM protein preparation therefore pose no problem. On the other hand, we cannot completely exclude a function for RAMP4 in translocation. It is an integral membrane protein and is associated with ribosomes to a similar extent as the Sec61p complex (data not shown). It remained a contaminant of all preparations of the Sec61p complex, regardless of the purification procedure. Further addition of RAMP4 did not lead to an increased translocation activity of proteoliposomes (data not shown). The majority of RAMP4 could be clearly separated from the Sec61p complex. On the basis of its low abundance in the purified Sec61p complex preparations, we assume that RAMP4 can have at best a catalytic or regulatory function in the translocation process.

Our results indicate that the basic translocation machinery of the ER membrane does not include a number of components hitherto implicated, such as putative ribosome receptors of 34 and 180 kd, a membrane protein of 30 kd with affinity for SRP (mp30), and additional ATP-binding membrane proteins and stop-transfer sequence receptors. Also, proteins similar to the Sec62/63p complex, which has been implicated in early phases of the translocation process in yeast [8,29,35] do not seem to be involved.

Arguments against the putative ribosome receptors have been raised previously [32,3,13]. We now present evidence that these proteins are in fact not needed for translocation. Almost 50% of all synthesized preprolactin molecules can be translocated through membranes that do not contain these proteins. In the case of p180, not even traces could be detected. Consistent with our conclusion, microsomes from which p180 was completely removed by proteolysis have an unreduced activity ([49], K.-U. Kalies, D.G., and T.A.R., unpublished data). Our results do not agree with those of Savitz and Meyer [37] who have recently reported that p180 is required for translocation in a crude reconstituted system. Perhaps in these experiments the yield rather than the activity of the proteoliposomes was affected by the presence of p180.

Furthermore, components of the ER lumen do not seem to be part of the basic translocation apparatus. For example, BiP, one of the most abundant lumenal proteins, could be lowered in its concentration by a factor of at least 10,000 without causing a block in translocation. On the other hand, in yeast, genetic experiments have indicated a function of Kar2p (BiP) in protein translocation [44]. It is possible that lumenal proteins are essential only for the posttranslational mode of translocation; they may determine directionality of transport by binding to the incoming protein. A similar model has been proposed for mitochondrial protein import [18,25]. In the case of cotranslational transport, the nascent chain may traverse the membrane in a similar manner as within the ribosomal channel; the latter would simply be extended by a tight coupling with the membrane channel. Here, lumenal proteins may not be absolutely required, although they may increase the efficiency of the process, depending on properties of the translocation substrate. In any case, we consider it unlikely that proteins are transported in an active manner by a pump, as also proposed by Simon et al. [39].

Our results do not agree with those of Nicchitta and Blobel [31], who recently reported that lumenal proteins are absolutely required for unidirectional translocation. It seems possible that in their hands the alkaline pH employed to release lumenal proteins from the microsomes impaired the function of translocation components of the membrane. The partial restoration of translocation activity observed following the readdition of lumenal proteins may be due to a protective effect of the chaperones.

So far, a specific role for the phospholipids in the process of protein translocation has not been found. The composition of the phospholipid bilayer can be varied and acidic phospholipids omitted without loss of translocation activity (data not shown).

Our results lead to a greatly simplified picture of the translocation system of the mammalian ER membrane. One essential component is the Sec61p complex, and it alone may form the putative protein-conducting channel. The a subunit may represent the main constituent, as indicated by size and structure, its evolutionary conservation, and its vicinity to translocating nascent chains. Whether the channel contains more than one copy of the Sec61p complex is as yet unknown. The functions of the β and γ subunits are also not yet clear. They may be involved in the regulation of the opening and closing of the channel, in signal sequence recognition, or in ribosome binding. How and when the Sec61p channel opens laterally to release membrane-spanning segments of membrane proteins into the phospholipid bilayer also remains to be investigated.

We have previously shown that Sec61p is tightly associated with membrane-bound ribosomes, an interaction that is induced by the targeting of a nascent chain and most likely terminated by the dissociation of the ribosome into its two subunits [13]. The linkage was shown to be direct and not primarily via the nascent chain. Since we have now shown that the translocation of some proteins does not require additional proteins other than the SRP receptor, one may conclude that the Sec61p complex is responsible for the binding of the ribosome during translocation. The interaction of the ribosome with the ER membrane is known to be remarkably stable. Crowley et al. [6] have recently shown that short translocating nascent chains carrying fluorescent probes are shielded from quenching by iodide ions added to the cytosolic compartment. It therefore seems likely that the ribosome makes numerous contacts with the Sec61p complex, ensuring a tight linkage between this and the protein-conducting channel in the ribosome.

The SRP receptor is involved in the targeting process. In all experiments, the optimum ratio between the SRP receptor and the Sec61p complex indicated a large molar excess of the latter, in agreement with the conditions in microsomes (at least 1:5). This indicates that the SRP receptor may not be part of the translocation site after the targeting phase. Also, some proteins may bypass the SRP receptor altogether [47]. On the other hand, the SRP-dependent targeting process requires the presence of the Sec61p complex. In its absence, an interaction of the complex of ribosome, nascent chain, and SRP with the SRP receptor does occur, as measured by the release of the inhibition of translation. The nascent chain, however, does not stably insert into the membrane.

The function of the TRAM protein is unclear at present, and we do not yet understand why some translocation substrates require it and others do not. Nevertheless, since the TRAM protein is clearly essential in many cases and since it is a neighbor of translocating chains even if it is not required for their translocation, it must be regarded as a constituent of the basic machinery. Our current working hypothesis is that at the beginning of the translocation phase, the Sec61p translocation channel can be expanded by the inclusion of the TRAM protein to allow the insertion of the nascent chain in a loop structure. Presumably after signal peptide cleavage, the TRAM protein would disengage, and Sec61p alone would constitute a narrower channel. Some translocation substrates may be guided into the final channel directly.

The establishment of a defined reconstitution system with the components of the basic translocation machinery now paves the way for a detailed understanding of the molecular mechanism by which proteins are transported across the ER membrane.

Experimental Procedures

Detergents and Lipids

Digitonin from Merck was further purified as follows: 30 g of digitonin was dissolved in 500 ml of boiling double-distilledw ater. The mixture was kept at 4°C for 36 hr, and the precipitate was then removed by centrifugation at 15,000 x g for 15 min. The clear solution was adjusted to pH 8.0 with 1 M Tris base and was passed sequentially through 10 ml Q-Sepharose FF (Pharmacia) and 1 ml S-Sepharose FF (Pharmacia) columns at a flow rate of 40 ml/hr. The colorless flowthrough is defined as a 5% stock solution.

BigCHAP (lot number 810017) and deoxyBigCHAP (lot number 034391) were obtained from Calbiochem. Both substances contain an impurity, as judged from thin-layer chromatography followed by staining with sulfuric acid, that seems to be important for efficient reconstitutions. Other batches of BigCHAP from the same company did not contain this contaminant (instead they contained others) and were inactive in reconstitution experiments. The active batch of Big-CHAP is no longer available, but one can use deoxyBigCHAP or cholate instead.

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were purchased from Sigma as chromatographically purified substances (catalog numbers P7763, P8923, P8518, and P2517, respectively). To prepare a lipid stock solution, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol solutions (each 10 mg/ml) were mixed in the ratio 100:25:3:12.5. Half a milliliter of the mixture (5 mg of lipid) was added to a 2 ml Eppendorf tube containing 5 mg of deoxyBigCHAP (50 µl of a 10% stock solution). Dithiothreitol (DIT) (10 mM) was added to prevent oxidation. The organic solvent was removed overnight in a Speedvac under high vacuum without heating. The residue was dissolved in 250 µl of 50 mM HEPES-KOH (pH 7.8) and 15% glycerol, with shaking and mild sonication in a waterbath to give a 20 mg/ml phospholipid stock solution. Recent experiments have shown that deoxyBigCHAP can be replaced by 4 mg of cholate, but in this case acidic phospholipids should be omitted.

Purlficatlon of the Sec61p Complex via Its Rlbosome Associatlon

Dog pancreatic rough microsomes (RM) (30,000 equivalents [eq]; for definition see Walter et al. [45,46]) were sedimented by centrifugation in a tabletop ultracentrifuge (Beckman) using a TLA 100.4 rotor for 20 min at 100,000 rpm. The membranes were resuspended at a concentration of 1 eq/µl in a buffer containing 20 mM HEPES-KOH (pH 7.8), 5 mM magnesium acetate, 5 mM DTT, 10 µg/ml leupeptin, 5 µg/ml chymostatin, and 15% w/v glycerol, and digitonin was added to a final concentration of 0.4%. The mixture was centrifuged for 40 min at 100,000 rpm. The supernatant is referred to as preextract. The pellet was resuspended in extraction buffer (same buffer as before, except that potassium acetate was added to 400 mM, magnesium acetate was added to 12 mM, and the digitonin concentration was increased to 4%) and, after 10 min on ice, the mixture was centrifuged for 60 min at 100,000 rpm at 2°C. The supernatant is referred to as the RM extract. The ribosome pellet was washed once in extraction buffer and then resuspended at a concentration of 1 eq/µl in puromycin buffer (1 mM puromycin, 100 mM HEPES-KOH [pH 7.8], 17.5 mM magnesium acetate, 1000 mM potassium acetate, 5 mM DTT, 10 µg/ml leupeptin, 5 µg/ml chymostatin, 15% w/v glycerol, 3% digitonin, and 0.2 mM GTP). The mixture was incubated for 60 min on ice followed by 30 min at 30°C and then centrifuged in a TLA 100.4 rotor for 90 min at 100,000 rpm at 25°C to remove ribosomal subunits. The supernatant is referred to as the RAMP fraction. It was dialyzed against 200 vol of 10 mM HEPES (pH 7.8) 20% w/v sucrose, 1 mM

magnesium acetate, 5 mM DTT, and 0.02% digitonin overnight at 4°C. The precipitate formed was removed by centrifugation for 10 min at 100,000 rpm at 2°C, and the supernatant was applied to a 15 ml G-Sepharose FF column, equilibrated in 10 mM HEPES-KOH (pH 7.8) 10% w/v glycerol, 5 mM DTT, and 4% digitonin. The flowthrough fractions were pooled and applied to a 2 ml S-Sepharose FF column, equilibrated in 50 mM HEPES-KOH (pH 7.8) 10% w/v glycerol, 5 mM DTT, and 2.5% digitonin. The column was washed with the same buffer, except that 150 mM potassium acetate was added, and elution was carried out with an 80 ml linear salt gradient (150 mM to 500 mM potassium acetate in 50 mM HEPES-KOH [pH 7.8], 15% glycerol, 5 mM DIT, and 2.5% digitonin). Fractions of 4 ml (each 40 min) were collected, and aliquots of 50 µl were precipitated as follows. The samples were diluted to 500 µl with 1% NP-40, and, after thorough mixing, trichloroacetic acid (20% final concentration) was added. Alter centrifugation at 4°C, the pellet was washed with cold acetone and taken up in SDS sample buffer. This precipitation procedure is needed for recovery of Sec61γ. Analysis of the proteins was carried out by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by staining with Coomassie blue. About 10-20 nmol (700-1400 µg) of Sec61p complex was obtained (corresponding to an overall yield of about 15%-30%).

Purlflcation of the Sec61p Complex by Immunoaffinity Chromatography

This purification procedure started from ribosome-depleted microsomes (PK-RM), following puromycin/high salt treatment as follows: rough microsomes (30,000 eq) were resuspended in 40 ml of buffer consisting of 1 mM puromycin, 500 mM potassium acetate, 5 mM magnesium acetate, 0.2 mM GTP, 5 mM DTT, 10 µg/ml leupeptin, 5 µg/ml chymostatin, and 5 µg/ml aprotinin. After incubation for 20 min at 25°C, the membranes were pelleted in a TLA 100.4 rotor for 15 min at 100,000 rpm. The pellet was resuspended in a buffer containing 2 M sucrose, 50 mM HEPES-KOH (pH 7.8) 500 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 10 ug/ml leupeptin, and 5 µg/ml chymostatin (12 ml final volume). A portion of the suspension (1.5 ml) was overlayered in polycarbonate tubes for the TLA 100.4 rotor with 1 ml of a solution containing 1.5 M sucrose, 50 mM HEPES-KOH (pH 7.8) 500 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 10 µg/ml leupeptin, and 5 µg/ml chymostatin, followed by 0.5 ml of the same buffer but lacking sucrose. The membranes were floated by centrifugation for 1 hr at 100,000 rpm at 25°C, and the ribosome-free membranes were collected from the top of the 1.5 M sucrose cushion. The membranes were diluted with 1 vol of 50 mM HEPES-KOH (pH 7.8) and 5 mM DTT and were sedimented for 20 min at 100,000 rpm. Finally, they were taken up at a concentration of 2 eq/µl in the solubilization buffer (50 mM HEPES-KOH [pH 7.8],500 mM potassium acetate, 5 mM magnesium acetate, 5 mM β-mercaptoethanol, 10 µg/ml leupeptin, 5 µg/ml chymostatin, and 15% w/v glycerol). If used as control microsomes in the translocation assays, the PK-RM were washed three times in the solubilization buffer to remove puromycin. They were then resuspended at 2 eq/µl in 50 mM HEPES-KOH (pH 7.8). 250 mM sucrose, 150 mM potassium acetate, and 5 mM DTT.

For immunopurification of the Sec61p complex, 30,000 eq PK-RM were adjusted to 3% digitonin in solubilization buffer in a final volume of 40 ml. After a 20 min incubation on ice, the mixture was centrifuged for 30 min at 100,000 rpm at 2°C. The supernatant (PK-RM extract) was applied at 10ml/hr in a cold room to an 8 ml immunoaffinity column that contained 2 mg/ml affinity-purified antibodies directed against the N-terminus of Sec61β. The column was washed with the equilibraton buffer (50 mM HEPES-KOH [pH 7.8], 500 mM potassium acetate, 5 mM 8-mercaptoethanol, 1 µg/ml leupeptin, 0.5 µg/ml chymostatin, 15% w/v glycerol, and 0.5% digitonin). Elution of the Sec61p complex was performed at room temperature and at a flow rate of 4 ml/hr with 1 mg/ml of the peptide against which the antibodies were raised in 50 mM HEPES, 150 mM potassium acetate, 15% glycerol, and 0.5% digitonin. Fractions of 2 ml were collected and analyzed by SDS-PAGE and by staining with Coomassie blue. The two peak fractions were combined, diluted 4-fold in 10 mM HEPES-KOH (pH 7.8) 5 mM DTT, and 5% digitonin, and were then passed through a 2 ml Q-Sepharose FFcolumn equilibrated in 50 mM HEPES-KOH (pH 7.8) and 3% digitonin. Concentration and detergent exchange to BigCHAP or to deoxyBigCHAP were performed on a 0.5 ml S-Sepharose FF column, as described below. The final yield of Sec61p complex was about 10-20 nmol (about 15%-30% overall yield).

Purification of the TRAM Protein and of the Signal Peptidase Complex

An RM extract (30,000 eq; see above) was applied overnight in a cold room to a 6 ml concanavalin A-Sepharose (Pharmacia) column. The column was washed with 100 ml of equilibration buffer (50 mM HEPES-KOH [pH 7.8], 500 mM potassium acetate, 5 mM DTT, 1 µg/ml leupeptin, 0.5 µg/ml chymostatin, 15% w/v glycerol, and 0.5% digitonin) followed by 20 ml of a buffer containing 50 mM HEPES-KOH (pH 7.8) 5 mM DTT, 15% glycerol, and 0.5% digitonin. Elution of the glycoproteins was carried out at room temperature with 50 ml of 1 M α-methylmannoside in 50 mM HEPES-KOH (pH 7.8) 5 mM DTT, and 4% digitonin at a flow rate of 5 ml/hr. The eluate was collected on ice, diluted with 50 ml of 10 mM HEPES-KOH (pH 7.8) and 4% digitonin, and then passed through a 10 ml Q-Sepharose FF column. The flowthrough fractions were applied to a 2 ml S-Sepharose FF column equilibrated in 50 mM HEPES-KOH (pH 7.8) 2 mM DTT, and 1% digitonin. The column was washed with equilibration buffer and was eluted with 100 ml of a linear salt gradient (50-450 mM potassium acetate in 50 mM HEPES-KOH [pH 7.8], 15% glycerol, 2 mM DTT, and 2.5% digitonin). Fractions of 5 ml (each 40 min) were collected, and 50 µl aliquots were precipitated with 95% acetone. The pelleted material was washed with methanol and analyzed by SDS-PAGE and by staining with Coomassie blue. The signal peptidase eluted as a broad peak between 100 and 200 mM potassium acetate, the TRAM protein between 270 and 350 mM. The peak fractions were combined and diluted 3-fold in 10 mM HEPES-KOH (pH 7.8), 5 mM DTT, and 0.5% digitonin, and the detergent was exchanged to deoxyBigCHAP by chromatography on a 0.5 ml S-Sepharose FF column. Signal peptidase (8 nmol) and TRAM protein (10-30 nmol) were obtained (about 20%-60% overall yield).

Purification of the SRP Receptor

The SRP receptor was purified by immunoaffinity chromatography [28] starting with the flowthrough fraction after chromatography on concanavalin A-Sepharose of an RM extract made with β-mercaptoethanol instead of DTT. Alternatively, the extract was made with PK-RM, and the flowthrough fractions of a Sec61β antibody column were used. The material (corresponding to 30,000 eq) was applied at 10 ml/hr at 2°C to a 2.5 ml column that contained 2 mg/ml affinity-purified antibodies raised against a peptide of the α subunit of the SRP receptor. The column was washed with 50 ml of equilibration buffer (50 mM HEPES-KOH [pH 7.8],500 mM potassium acetate, 5 mM β-mercaptoethanol, 1 μg/ml leupeptin, 0.5 μg/ml chymostatin, 15% w/v glycerol, and 0.5% digitonin). Elution of the complex of the two subunits of the SRP receptor was carried out at room temperature and at a flow rate of 2 ml/hr with 1 mg/ml of the peptide against which the antibodies were raised in 50 mM HEPES-KOH (pH 7.8), 750 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM GTP, 15% glycerol, and 0.5% digitonin. Fractions of 0.5 ml were collected and analyzed by SDS-PAGE and by staining with Coomassie blue. The peak fractions (1.5 ml) were combined and diluted 10-fold in 10 mM HEPES-KOH (pH 7.8) 0.5% digitonin, and 5 mM DTT, and the detergent was exchanged to deoxyBigCHAP by chromatography on a 0.5 ml S-Sepharose FF column, as detailed below. About 2 nmol of SRP receptor was obtained (about 40% overall yield).

Exchange of Detergent

For reconstitution studies, the detergent was exchanged from digitonin to BigCHAP or deoxyBigCHAP. The peak fractions of the Sec61p complex, the TRAM protein, the signal peptidase, and the SRP receptor preparations were diluted and bound to 0.5 ml S-Sepharose FF columns equilibrated with 50 mM HEPES-KOH (pH 7.8). 5 mM DTT, and 0.3% digitonin. The columns were washed two times with 5 ml of 50 mM HEPES-KOH (pH 7.8) 15% glycerol, and 5 mM DTT containing either 0.5% BigCHAP or 0.4% deoxyBigCHAP and were eluted with 50 mM HEPES-KOH (pH 7.8) 750 mM potassium acetate, 5 mM DTT, 15% glycerol, and either 0.5% BigCHAP or 0.3% deoxyBigCHAP. Fractions of 100 μl were collected, and 1 μl aliquots were analyzed by SDS-PAGE and by staining with Coomassie blue. The peak fractions were pooled and frozen in small aliquots. The final protein concentration was between 0.5 and 2 mg/ml. In recent experiments, elution from the S-Sepharose column was carried out with 0.3% cholate at 400 mM salt, following a brief wash with detergent-free buffer.

Reconstitution of Proteoliposomes from Membrane Extracts

PK-RM or RM were adjusted to a concentration of 1 eq/μl in 50 mM HEPES-KOH (pH 7.8) 350 mM potassium acetate, 12 mM magnesium acetate, 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml chymostatin, 15% glycerol, and 3% w/v BigCHAP (Calbiochem lot number 810017). After a 20 min incubation on ice, particles larger than 20 S were sedimented in a tabletop ultracentrifuge. A 1 ml portion of the extract obtained from RM was incubated overnight in an overhead shaker in a cold room with 150 mg of Sepharose containing immobilized, affinity-purified antibodies against Sec61β. Of this Sec61p complex-depleted extract, 200 μ1 was replenished with 140, 200, or 300 eq of purified Sec61p complex. Proteoliposomes were produced from 200 μl aliquots of the detergent extracts by incubation overnight in a cold room with 200 mg of Biobeads SM2 (Bio-Rad) that had been previously equilibrated with 50 mM HEPES-KOH (pH 7.8) 300 mM potassium acetate, 5 mM DTT, and 15% glycerol. The fluid phase was separated from the beads, diluted with 5 vol of ice-cold distilled water, and submitted to centrifugation in a TLA 100.3 rotor in microtest tubes for 20 min at 75,000 rpm. The proteoliposomes were finally resuspended at a concentration corresponding to 3 eq/μl.

Reconstitution with Purified Components

In pilot experiments, the optimum lipid to protein ratio was determined separately for each membrane protein component and for each preparation. The optimum is defined as the lowest amount of phospholipid that results in a quantitative incorporation of the corresponding protein into vesicles (a clear vesicle pellet without aggregated protein). Approximately 1 μg of phospholipid for 1 pmol of each component was needed. In reconstitution assays containing different components, the total amount of phospholipid corresponded to the sum of those needed for each component separately.

Vesicles were formed by using 0.5 mg of Biobeads SM2, previously equilibrated in 40 mM HEPES-KOH (pH 7.8) and 15% glycerol per microliter of mixed components. After incubation at 4°C with shaking overnight, the fluid phase was separated from the beads and diluted with 5 vol of ice-cold distilled water. The liposomes were sedimented in a TLA 100.3 rotor in microtest tubes for 15 min at 75,000 rpm at 2°C. The pellet was resuspended in a buffer containing 50 mM HEPES-KOH (pH 7.8) 250 mM sucrose, 5 mM DTT, and 100 mM potassium acetate (which facilitates resuspension). Of this suspension, 1 μl was used per 10 μl of translation mixture. The final concentrations of the components are given in the figure legends.

Antibodies

Antibodies against the N- and the C-termini of Sec61α [13], the C-terminus of the TRAM protein [12], the 22 kd subunit of the signal peptidase [13], and the N-terminus of TRAPβ (previously SSRβ [11]) have been described. Antibodies directed against p180, mp30, and BiP were gifts of D. Meyer, P. Walter, and R. Zimmermann, respectively.

Antibodies were newly made against the following peptides: a peptide of the α subunit of the SRP receptor corresponding to positions 137-150 in the amino acid sequence [23] plus a C-terminal cysteine (KKFEDSEKAKKPVRC), a peptide corresponding to the N-terminal 9 amino acids of Sec61β plus a C-terminal cysteine (PGPTPSGTNC), a peptide corresponding to the N-terminal 10 amino acids of RAMP4 plus a C-terminal cysteine (VAKQRIRMANC), a peptide corresponding to the C-terminal 10 amino acids of ribophorin I [5] plus an N-terminal cysteine (CTKIDHILDAL), and a peptide corresponding to the N-terminal 10 amino acids of canine p34 (unpublished data) plus a C-terminal cysteine (TKAGSKGGNLC). These peptides were coupled to

keyhole limpet hemocyanin that had been activated with sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). To obtain antibodies with a high titer but low affinity for use in immunoaffinity chromatography (peptide elution), 2 mg of coupled peptide for each immunization was mixed with 200 µg of adjuvant peptide (Sigma) and incomplete Freund's adjuvant and injected into rabbit. All antibodies were affinity purified on columns (Sulfolinkgel; Pierce) to which the peptides were coupled. The purified antibodies reacted only with the expected proteins in immunoblots (data not shown). Immobilization of the antibodies has been previously described [13].

Translation, Cross-Linking, and Translocation

Cross-linking of the pPL 86mer and immunoprecipitation of the products with antibodies against the TRAM protein and the Sec61p complex, as well as binding of the products to concanavalin A-Sepharose, have been described [12,13]. Translation was carried out either in the reticulocyte lysate system (Promega) or in the wheat germ system in the presence of 60 nM SRP as described [11]. Translocation was assayed after translation in the presence of membranes by adding to half of the sample on ice an equal volume of the protease in 50 mM HEPES, 250 mM sucrose, and 150 mM potassium acetate. After incubation for 30 min at 0°C, the reaction was stopped either by precipitation with trichloroacetic acid (wheat germ system) or by addition of 5 mM phenylmethylsulfonyl fluoride (reticulocyte lysate system). Transcripts coding for preprolactin and for the prepro-a-factor were produced as described [13]. RNA coding for VSV G (Indiana type) was synthesized with T7 polymerase from a BamHI-linearized plasmid, provided by R. Gilmore and M. Rose. RNA coding for the human asialoglycoprotein receptor H1 subunit was synthesized with SP6 polymerase from the plasmid pSA1 and linearized with EcoRI (a gift of M. Spiess).

The translation products were analyzed by SDS-PAGE followed by fluorography. Quantitative estimates were obtained from a duplicate gel by analysis with a Fuji Phosphoimager BASZOOO.

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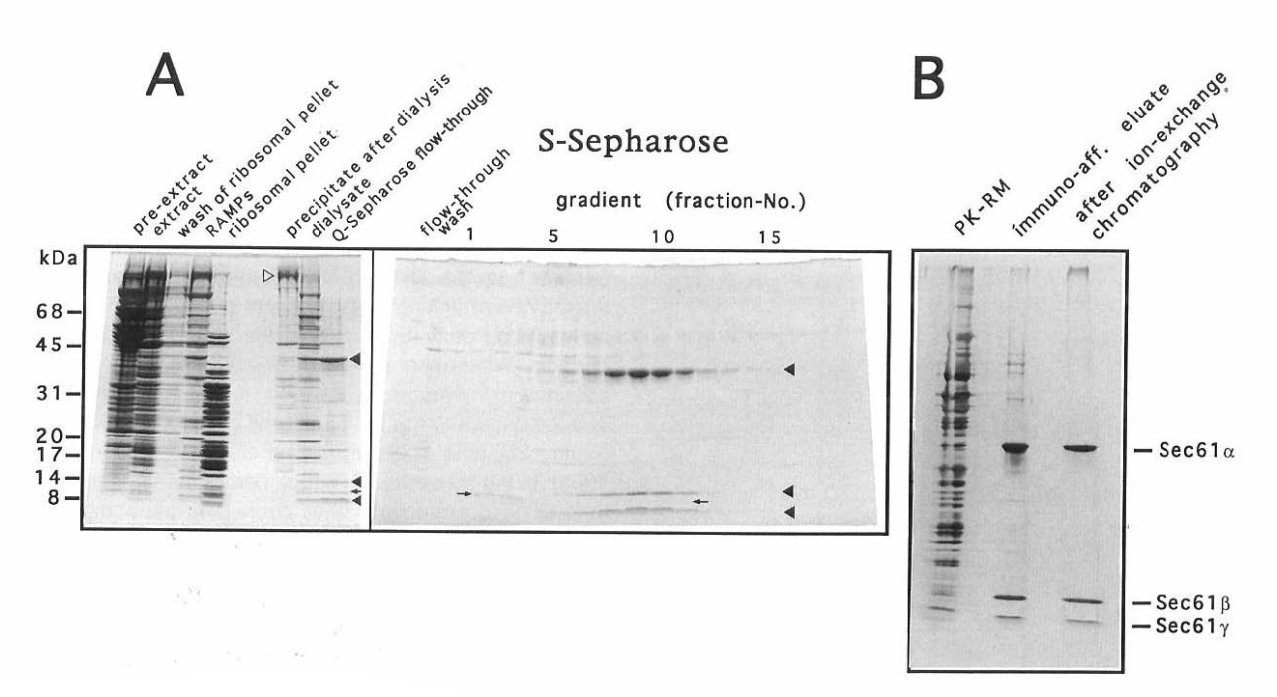

Figure 1. Purification of the Sec61p Complex.

(**A**) The Sec61p complex was purified on the basis of its ribosome association. Rough dog pancreatic microsomes were treated with detergent at a low salt concentration to yield preextract and membrane remnants. The latter were then solubilized at high salt and subsequently centrifuged to give a supernatant (extract) and a ribosomal pellet containing the RAMPS. The pellet was washed with buffer, and the RAMPS were then released from the ribosomes by puromycinlhigh salt. After dialysis of the RAMP fraction, during which a precipitate was formed that predominantly contained the putative ribosome receptor p180, the nonprecipitated material was passed through a Q-Sepharose column. The proteins in the flowthrough fractions were subsequently chromatographed on S-Sepharose. The figure shows the protein pattern at the different stages of the purification procedure, visualized by SDS-PAGE (7.5%-17.5% linear polyacrylamide gel) and by staining with Coomassie blue. Material corresponding to the following quantities of microsomes was loaded per lane: up to the ribosomal pellet, 5 eq; precipitate after dialysis and dialysate, 10 eq; Cl-Sepharose flowthrough, 30 eq; S-Sepharose fractions, 200 eq. The open triangle indicates the position of p180, the closed triangles that of the subunits of the Sec61p complex, and the arrow that of RAMP4 (see text).
(**B**) The Sec61p complex was purified by immunoaffinity chromatography. Microsomes stripped of ribosomes (PK-RM) were solubilized in detergent, and the extract was applied to a column containing immobilized, affinity-purified antibodies directed against Sec61β. After extensive washing, the bound material was eluted with the peptide against which the antibodies were raised (immuno-aff. eluate). Residual impurities were removed by passing the eluate through Q-Sepharose, followed by concentration on S-Sepharose (this fraction is labeled after ion-exchange chromatography). The figure shows the protein pattern after SDS-PAGE (7.5%-17.5% polyacrylamide gel) and visualization by silver staining.

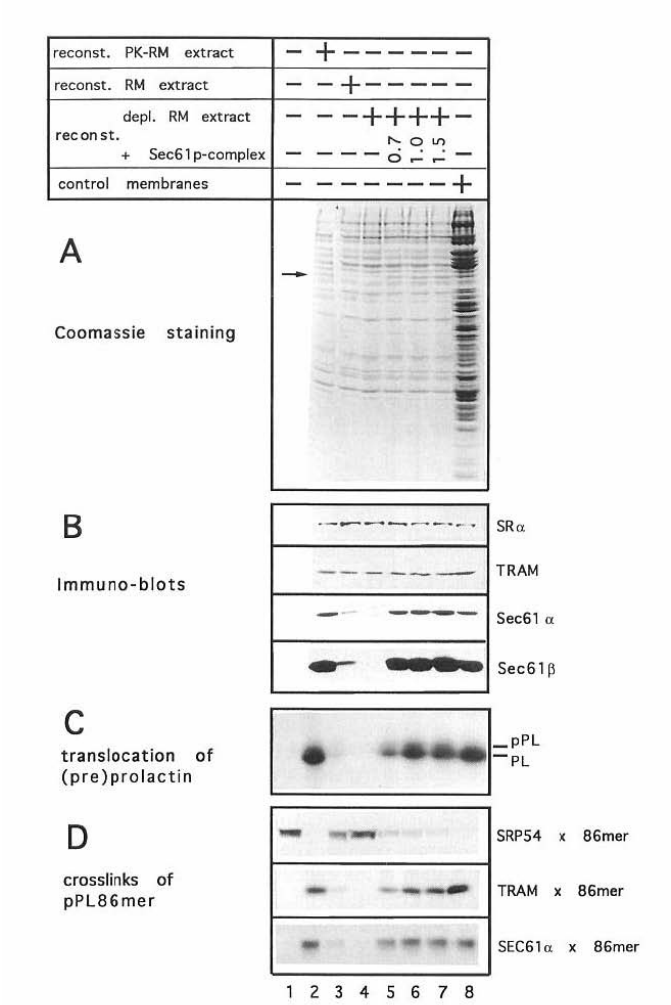

Figure 2. The Sec61p Complex Is Essential for Translocation of Preprolactin into Reconstituted Proteoliposomes.

Proteoliposomes were produced from extracts containing different amounts of Sec61p complex. A detergent (BigCHAP) extract containing the full complement of Sec61p complex was obtained from ribosome-stripped microsomes (PK-RM) (lanes 2). An extract depleted of the Sec61p complex (by about 9.5%) was produced by solubilization of rough microsomes (RM) and sedimentation of the ribosomes, along with most of the Sec61p complex (lanes 3). Residual amounts were adsorbed to immobilized antibodies directed against Sec61β (lanes 4). To a doubly depleted extract, Sec61p complex, purified on the basis of its ribosome association, was added (0.7, 1.0, or 1.5 eq/eq extract; 200 eq total [lanes 5-7]). PK-RM served as control membranes (lanes 8).

(**A**) Overall protein composition of the membranes as analyzed by SDS-PAGE and by staining with Coomassie blue. The arrow indicates the position of Sec61α.

(**B**) Determination by immunoblotting of the amounts of SRP receptor a subunit (SRα), TRAM protein, Sec61α, and Sec61β in the vesicles.

(**C**) The translocation of preprolactin and prolactin into the vesicles was tested. Synthesis of preprolactin was carried out for 30 min at 30°C in a reticulocyte lysate system, and the material protected from proteolysis by 500 μg/ml proteinase K was analyzed by SDS-PAGE and fluorography.

(**D**) The transfer of a fragment of preprolactin from the SRP into the vesicles was tested by photo cross-linking. A fragment of 86 amino acids (pPL88mer) was synthesised in the wheat germ system in the presence of SRP and modified lysyl-tRNA, containing in the side chain of the amino acid a carbene-producing photoreactive group, as described [48]. Vesicles were added as indicated, and the samples were irradiated. Analysis of the products was carried out either directly by SDS-PAGE (cross-links to SRP54). or after binding to concanavalin A (cross-links to the TRAM protein), or following immunoprecipitation with a mixture of antibodies directed against the N- and C-termini of Sec61α (cross-links to Sec61α). The exposure times of the X-ray films were 3 hr for SRP54 cross-links, 24 hr for TRAM cross-links, and 72 hr for Sec61p cross-links.

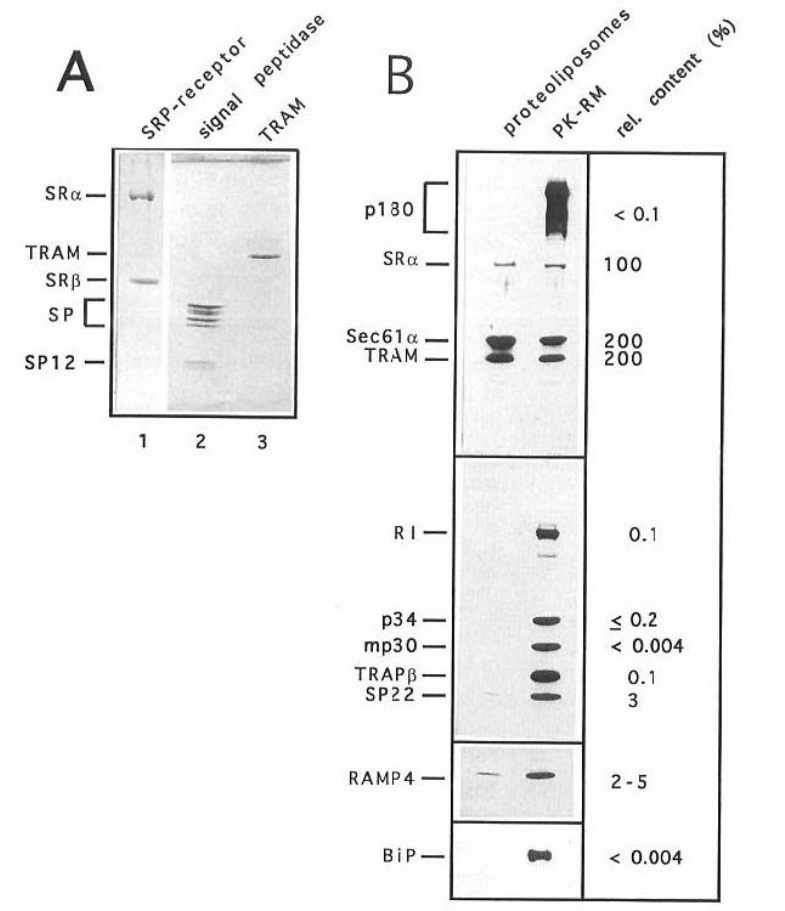

Figure 3. Purification of Translocation Components and Analysis of Their Purity.

(**A**) The SRP receptor was purified by immunoaffinity chromatography (lane 1), and the signal peptidase (lane 2) and the TRAM protein (lane 3) by chromatography on concanavalin A-Sepharose followed by ion exchange chromatography. The protein patterns of the final preparations are shown after SDS-PAGE and staining with Coomassie blue. Abbreviations: SRα and β, α subunit and β subunit of the SRP receptor; SP and SP12, signal peptidase subunits of 18-25 kd and signal peptidase subunit of 12 kd, respectively.

(**B**) The content of ER proteins was determined in rough microsomes and in proteoliposomes produced from pure phospholipids and purified SRP receptor, Sec61p complex, and TRAM protein. The proteins in the vesicles were separated in a 7.5%-17.5% polyacrylamide SDS gel and visualized after blotting with specific antibodies. Bound antibodies were detected with an enhanced chemiluminiscence system. Quantitative estimates were obtained from several experiments by using different exposure times of the X-ray film and films of different sensitivity (Kodak X-OMAT LS and AR), as well as by comparing a constant amount of proteoliposomes with a dilution series of native microsomes (data not shown). The estimated percentage of a protein in the proteoliposomes with respect to its content in microsomes is given (rel. content). Abbreviations: p180, putative ribosome receptor p180; SRα, a subunit of the SRP receptor; RI, ribophorin I; p34, putative ribosome receptor p34; mp30, membrane protein with affinity for SRP; TRAPβ, β subunit of the TRAP complex (previously called SSR); SP22, 22 kd subunit of the signal peptidase complex; BiP, immunoglobulin-binding protein (grp78).

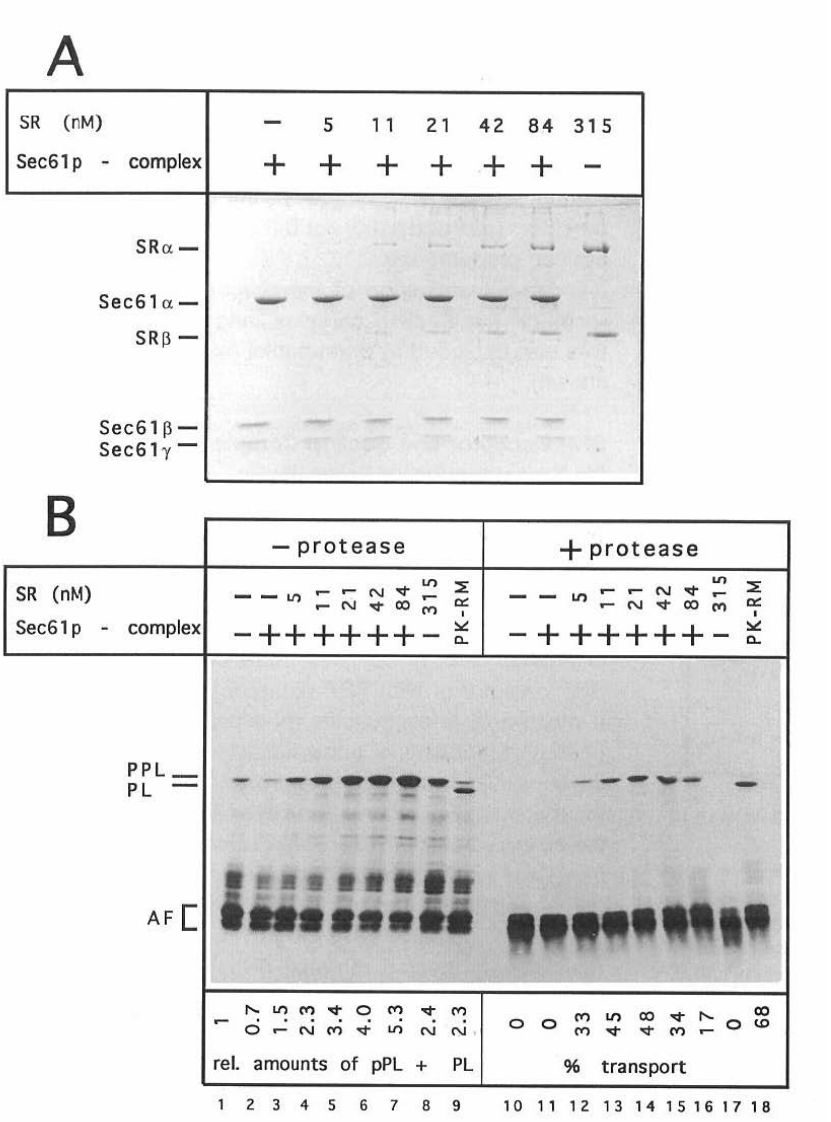

Figure 4. Translocation of Preprolactin into Proteoliposomes Containing Only SRP Receptor and Sec61p Complex.

(**A**) Proteoliposomes were produced with pure phospholipids and with either Sec61p complex, purified on the basis of its ribosome attachment, and increasing amounts of SRP receptor (SR) or with SRP receptor alone. The protein pattern in the vesicles was analyzed by SDS-PAGE (10%-20% polyacrylamide) and by staining with Coomassie blue. The weak staining ofSec61γ is due to its diffuse migration in this gel system.
(**B**) The various types of proteoliposomes were tested for the translocation of preprolactin that was synthesized in a wheat germ system for 15 min at 24°C in the presence of 60 nM SRP. The concentration of SRP receptor is given with respect to the volume of the translation mixture. The concentration of Sec61p complex, where added, was estimated to be 800 nM. Ribosome-stripped microsomes (PK-RM) served as a control (about 160 nM Sec61p complex in the assay). After translation, half of the sample was treated with 500 µg/ml proteinase K (plus protease), while the other half remained untreated (minus protease). The products were separated by SDS-PAGE (12% polyacrylamide) and visualized by fluorography. Quantitatve estimates were obtained from a duplicate gel by analysis with a phosphoimager. Relative amounts of synthesized preprolactin were calculated with reference to those obtained in the absence of membranes (rel. amounts of pPL). Percent transport gives the percentage of preprolactin molecules that are protected from protease. Abbreviations: AF, fragment of preprolactin arrested in elongation by the SRP; pPL, preprolactin; PL, prolactin.

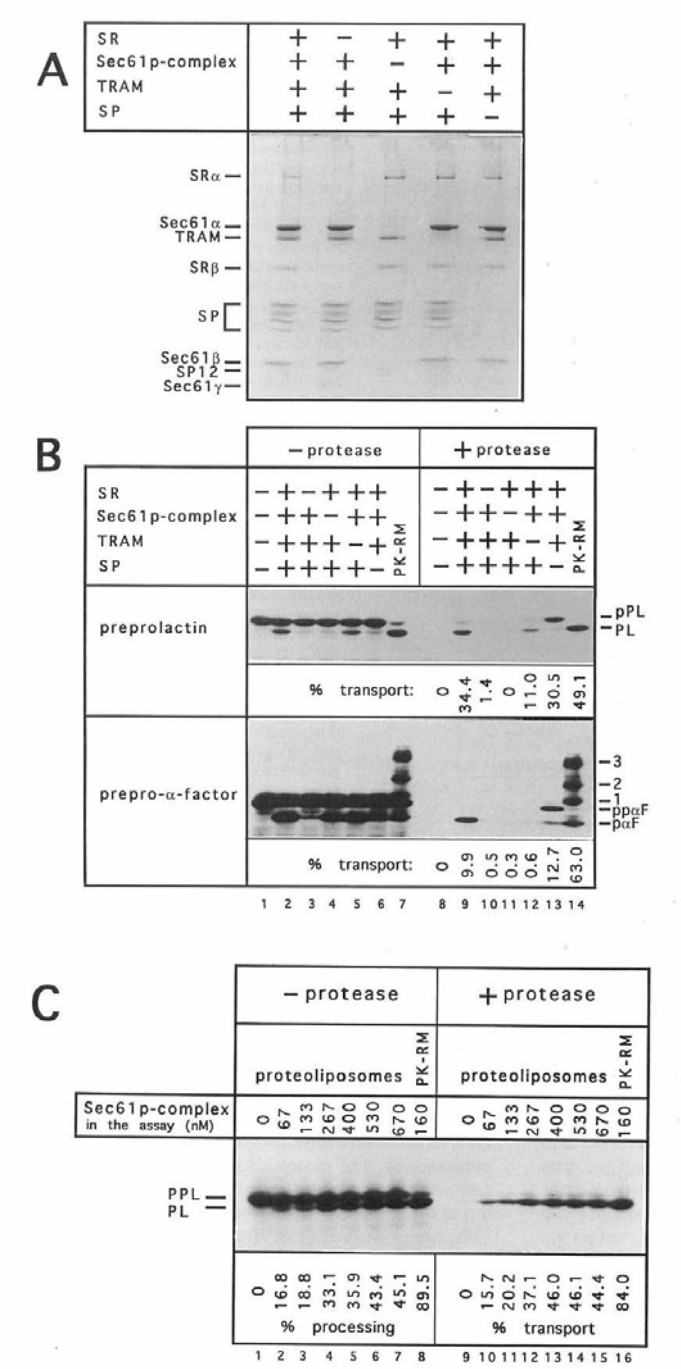

Figure 5. Translocation of Preprolactin and Prepro-α-Factor into Proteoliposomes Reconstituted from Purified Components.

(**A**) Purified SRP receptor (SR), Sec61p complex (purified on the basis of its ribosome association), TRAM protein, and signal peptidase were mixed as indicated and reconstituted with pure phospholipids into proteoliposomes. The protein pattern in the vesicles is shown after SDS-PAGE and staining with Coomassie blue.
(**B**) The various types of proteoliposomes were tested for the translocation of prolactin, preprolactin, pro-α-factor, and prepro-α-factor that were synthesized in a wheat germ system for 40 min at 26°C in the presence of 60 nM SRP. The products were analyzed with or without protease digestion, as described in Fig.4. Where added, the final concentrations of the components in the translation assay were SR 26 nM, Sec61p complex 330 nM,TRAMprotein 100 nM, and signal peptidase 120 nM. Abbreviations: PK-RM, rough microsomes stripped of ribosomes (about 160 nM Sec61p complex); pPL, preprolactin; PL, prolactin; ppαF, preproa-α-factor; and pαF. pro-α-factor. The glycosylated forms of the α-factor protein are indicated by the number of carbohydrate chains that are attached. Percent transport is defined as the percentage of total radioactivity in the completed molecules that is protease protected (e. g., radioactivity in protease-protected preprolactin plus prolacttn divided by the radioactivity in total preprolactin plus prolactin; the loss of label by signal peptide cleavage has been ignored). (**C**) Proteoliposomes containing all components in the relations mentioned above were tested in increasing concentrations for translocation of preprolactin. The concentrations of the Sec61p complex in the translation mixtures are presented. Percent processing is defined as radioactivity in prolactin divided by that in preprolactin plus prolactin.

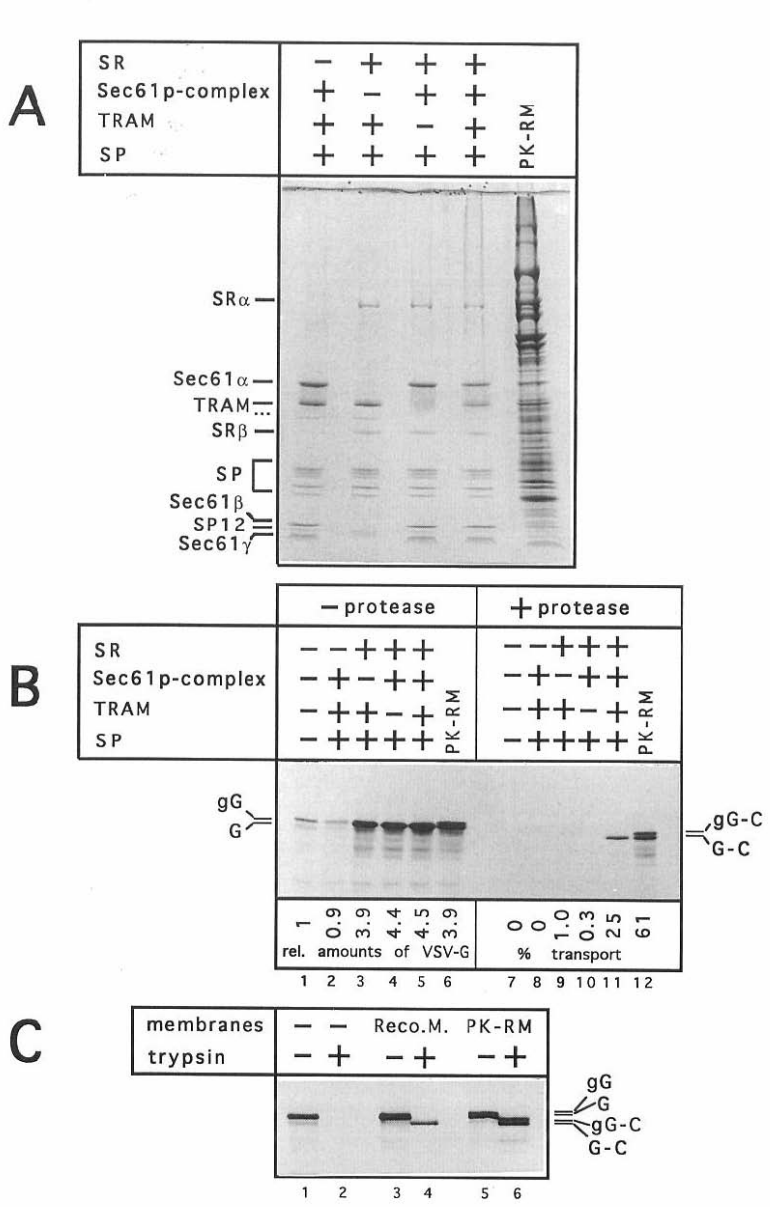

Figure 6. Integration of the VSV G Protein into Proteoliposomes Reconstituted from Purified Membrane Components.

(**A**) Purified SRP receptor (SR), Sec61p complex (purified by immunoaffinity chromatography), TRAM protein, and signal peptidase were mixed as indicated and reconstituted with pure phospholipids into proteoliposomes. The protein pattern in the vesicles is shown after SDS-PAGE (7.5%-17.5% polyacrylamide) and staining with Coomassie blue. The dotted line indicates a proteolytic fragment of the TRAM protein that lacks the C-terminus and was present in the preparation.
(**B**) The various types of proteoliposomes were tested for the translocation of the VSV G protein that was synthesized in a wheat germ system for 30 min at 26°C in the presence of 60 nM SRP. The control with ribosome-stripped microsomes (PK-RM; about 160 nM Sec61p complex in the assay) was performed in the presence of 100 μM of an acceptor peptide (Ac-Asn-Tyr-Thr-NH_2 to inhibit glycosylation and to permit a more accurate comparison with proteoliposomes that lack the capacity for glycosylation (the inhibition was, however, incomplete; the form with two carbohydrate chains was no longer seen but that with one chain was still visible). The products were analyzed with or without protease digestion (500 μg/ml proteinase K) by SDS-PAGE (10% polyacrylamide) followed by fluorography or analysis with a phosphoimager. Abbreviations: G and gG, VSV G protein carrying no or one carbohydrate chain, respectively: G-C and gG-C, protease-protected forms that lack the cytoplasmic C-terminus of the G protein. It should be noted that the mobility shift of the G protein by signal peptide cleavage is not visible in this gel system.
(**C**)The correct integration of the VSV G protein into proteoliposomes reconstituted from purified components (SRP receptor, Sec61p complex, TRAM protein, signal peptidase) was tested as in (B), except that translation was performed for 60 min and 500 μg/ml trypsin was used to assay for protease-protected material (lanes 3 and 4). Controls included translation in the absence of membranes (lanes 1 and 2) or with PK-RM and acceptor peptide being present (lanes 5 and 6).

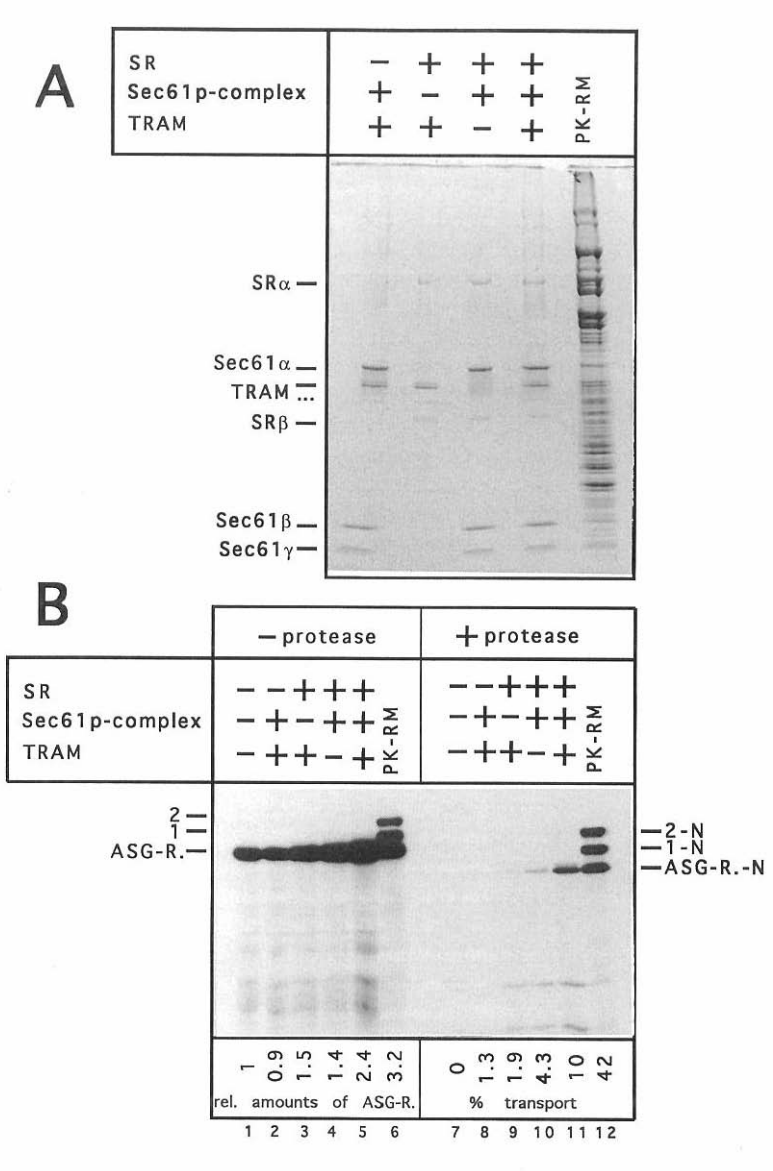

Figure 7. Integration of the Asialoglycoprotein Receptor into Proteoliposomes Reconstituted from Purified Membrane Components.

(**A**) Purified SRP receptor (SR), Sec61p complex (purified by immunoaffinity chromatography), and TRAM protein were mixed as indicated and reconstituted with pure phospholipids into proteoliposomes. The protein pattern in the vesicles is shown after SDS-PAGE (7.5%-17.5% polyacrylamide) and staining with Coomassie blue.
(**B**) The various types of proteoliposomes were tested for the translocation of the asialoglycoprotein receptor that was synthesized in a wheat germ system for 30 min at 26°C in the presence of 60 nM SRP. The products were analyzed with or without protease digestion (500 µg/ml trypsin) by SDS-PAGE (12.5% polyacrylamide), followed by fluorography or analysis with a phosphoimager. Abbreviations: ASG-R., asialoglycoprotein receptor (the numbers indicate glycosylated protein forms with one or two carbohydrate chains); ASG-R.-N, 1-N, and 2-N, protease-protected protein forms that lack the N-terminal cytosolic domains (about 4.5 kd).