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Posttranslational Protein Transport in Yeast Reconstituted with a Purified Complex of Sec Proteins and Kar2p

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Summary

We have reproduced the posttranslational mode of protein translocation across the endoplasmic reticulum membrane with reconstituted proteoliposomes containing a purified complex of seven yeast proteins. This Sec complex includes a heterotrimeric Sec61p complex, homologous to that in mammals, as well as all other membrane proteins found in genetic screens for translocation components. Efficient posttranslational translocation also requires the addition of lumenal Kar2p (BiP) and ATP. The trimeric Sec61p complex also exists as a separate entity that, in contrast with the large Sec complex, is associated with membrane-bound ribosomes. We therefore hypothesize that distinct membrane protein complexes function in co- and posttranslational translocation pathways.

Introduction

In all eukaryotic cells, protein transport across the endoplasmic reticulum (ER) membrane can occur either co- or posttranslationally, depending on characteristics of the transported polypeptide. The cotranslational mode of translocation, prevalent in mammalian systems, is reasonably well understood (for reviews see Rapoport, 1992; Ng and Walter, 1994). The process is initiated by the targeting of a ribosome-bound nascent polypeptide chain to the ER membrane, mediated by the signal recognition particle (SRP) and its membrane receptor (also called docking protein) (Walter and Blobel, 1981; Gilmore et al., 1982; Meyer et al., 1982). The nascent polypeptide is subsequently transported across the membrane, concomitantly with further chain elongation on the membrane-bound ribosome. Transport seems to occur through a protein-conducting channel (Simon and Blobel, 1991; Crowley et al., 1993), the main constituent of which is probably the Sec61p complex. This complex consists of three proteins, a multispanning α subunit and two smaller, single-spanning β and γ subunits (Görlich and Rapoport, 1993; Hartmann et al., 1994). The polypeptide chain contacts Sec61α throughout its transfer across the ER membrane (Görlich et al., 1992; Mothes et al., 1994). During this translocation process, the Sec61p complex is tightly associated with the membrane-bound ribosome (Görlich et al., 1992), providing a tight seal with the membrane (Crowley et al., 1993). It is likely that the nascent chain is transferred directly from the channel in the ribosome into a Sec61p channel in the membrane. The heterotrimeric Sec61p complex, together with the SRP receptor, is sufficient for the translocation of some polypeptides into reconstituted proteoliposomes (Görlich and Rapoport, 1993). However, most polypeptides also require the multispanning translocating chain-associating membrane (TRAM) protein (Görlich and Rapoport, 1993; S. Voigt, B. Jungnickel and T. A. R., unpublished data).

Less is known about the mechanism of posttranslational protein transport and about the components involved in it. One may assume that in this case functions performed by the ribosome in cotranslational translocation are performed by other components. Posttranslational protein transport has been best studied in yeast for the secretory protein prepro-α factor. Microsomes from Saccharomyces cerevisiae, in contrast with those of dog pancreas, can transport this protein with high efficiency in a posttranslational manner (Waters and Blobel, 1986; Rothblatt and Meyer, 1986; Hansen et al., 1986; Garcia and Walter, 1988).

In S. cerevisiae, several translocation components of the ER membrane have been discovered by genetic screening (Deshaies and Schekman, 1987; Rothblatt et al., 1989; Green et al., 1992; Esnault et al., 1993; Feldheim et al., 1993; Kurihara and Silver, 1993; Feldheim and Schekman, 1994). Two of the components, Sec61p itself and SSS1p, are related to constituents of the mammalian Sec61p complex, the α and γ subunits, respectively (Hartmann et al., 1994). The others (Sec62p, Sec63p, Sec71p, and Sec72p) can be coimmunoprecipitated as a complex (Deshaies et al., 1991). Sec61p seems to be in spatial proximity to them, since it can also be coprecipitated after chemical cross-linking. Sec63p, Sec71p, and Sec72p were found to copurify in a multistep protocol (Brodsky and Schekman, 1993) along with Kar2p (BiP), a lumenal chaperone with homology to the DnaK protein of Escherichia coli and other members of the Hsp70 family. Kar2p interacts with a lumenal segment of Sec63p that is related to a portion of DnaJ, a partner of DnaK in E. coli (Sadler et al., 1989). Kar2p also interacts with the incoming polypeptide chain (Sanders et al., 1992). A rapid cessation of translocation was observed in some temperature-sensitive *kar2* (*kar2^{ts}*) mutants upon shifting to restrictive temperatures (Vogel et al., 1990; Sanders et al., 1992).

A role for some of the Sec proteins in posttranslational translocation of prepro-α factor has been demonstrated in vitro. Microsomes derived from *sec62* or *sec63* mutant cells show a defect in translocation (Rothblatt et al., 1989; Deshaies and Schekman, 1989). Also, proteoliposomes reconstituted from unfractionated detergent extracts of membranes from *sec63*, *sec71*, or *sec72* mutants are defective (Brodsky and Schekman, 1993), but the translocation activity can be restored if the wild-type protein complex is supplied during reconstitution.

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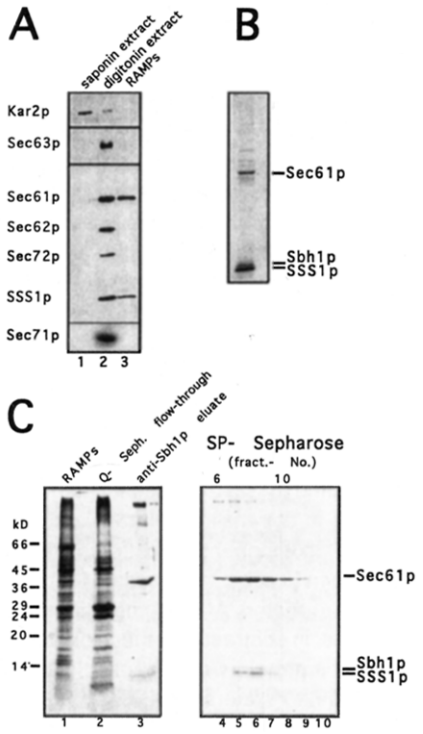

Figure 1. Identification and Purification of a Sec61p Complex from Yeast

(A) Yeast microsomes were extracted sequentially to give a saponin extract, containing lumenal and peripheral membrane proteins, a digitonin extract of solubilized membrane proteins, and a fraction containing RAMPs. Aliquots corresponding to 10 eq of microsomes were analyzed by SDS–PAGE and immunoblotting with antibodies against the indicated proteins.

(B) The Sec61p complex was purified from the RAMP fraction by ion exchange chromatography. The protein pattern in the final preparation (corresponding to about 3,000 eq) was analyzed by SDS–PAGE and staining with Coomassie blue.

(C) The Sec61p complex was purified from the RAMP fraction by a procedure involving immunoaffinity chromatography. The RAMPs were first applied to a Q–Sepharose column and the flowthrough material (Q-Seph. flow-through) was bound to an antibody column containing immobilized antibodies against Sbh1p. After elution (anti-Sbh1p eluate), the proteins were bound to SP–Sepharose and eluted in 12 fractions with a salt gradient. Shown are the peak fractions (corresponding to about 8,000 eq) after SDS–PAGE and staining with Coomassie blue. It should be noted that in this preparation the percentage of Sec61p in the RAMP fraction was exceptionally low.

We now report that the posttranslational translocation pathway in yeast can be reproduced with reconstituted proteoliposomes containing a purified complex of seven proteins. This Sec complex contains a heterotrimeric Sec61p complex, like the mammalian one, consisting of Sec61p, SSS1p, and a novel protein (Sbh1p, a homolog of the mammalian Sec61β). In addition, it contains the four other membrane proteins found in genetic screens for translocation components. Efficient posttranslational protein transport into proteoliposomes also requires the presence of lumenal Kar2p and ATP. The heterotrimeric Sec61p complex also exists as a separate entity in yeast cells that, in contrast with the large Sec complex, is associated with membrane-bound ribosomes, suggesting that

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S.cere. Sbh1                      MSSPTPPGGQRTLQKR    16
A.thal. Sec61ß                    MVGSGAPQRGSAAATAS   17
C.fami. Sec61ß  MPGPTPSGTNVGSSGRSPSKAVAARAAGSTVR      32
                                        * .     . .

                          -------------------------
S.cere. Sbh1    KQGSSQKVAASAPKKNTNSNNSILKIYSDEAT      48
A.thal. Sec61ß  MRRRKPTSGAGGGGASGGAAGSMLQFYTDDAP    49
C.fami. Sec61ß  QRKNASCGTRSAGRTTSAGTGGMWRFYTEDSP    64
                  ..   ........ ..*.....

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S.cere. Sbh1    GLRVDPLVVLFLAVGFIFSVVALHVISKVAGKLF    82
A.thal. Sec61ß  GLKISPNVVLIMSIGFIAFVAVLHVMGKLYFVK    82
C.fami. Sec61ß  GLKVGPVPVLVMSLLFIASVFMLHIWGK-YTRS    96
                **...*  **  ... **  *  **. .* .
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Figure 2. Comparison of the Amino Acid Sequences of Sbh1p and Other Sec61β Proteins

Shown are the sequences of Sbh1 from S. cerevisiae, of Sec61β from Arabidopsis thaliana (ATSEC61B; accession number Z26753), and Sec61β from canis familiaris (Hartmann et al., 1994). Broken lines above the sequence of Sbh1p indicate directly determined amino acid sequences. The lines below the sequences indicate membrane anchors. Identical and similar residues among the proteins are indicated by stars and dots, respectively.

like in mammals it is involved in cotranslational translocation.

Results

Association of Yeast Sec Proteins with Membrane-Bound Ribosomes

First, motivated by results in mammals, we tested the association of Sec61p with membrane-bound ribosomes after solubilization of rough microsomes from S. cerevisiae by detergent (Görlich et al., 1992).

A crude microsome fraction was prepared from yeast cells harvested during exponential growth, and the distribution of Sec61p after subsequent fractionation steps was followed by immunoblotting with specific antibodies (Figure 1A). The microsomes were first treated with saponin to remove peripheral and lumenal proteins from the ER (saponin extract; Figure 1A, lane 1). The membrane proteins were then solubilized with digitonin and the ribosomes were sedimented by centrifugation, yielding a supernatant referred to as the digitonin extract (Figure 1A, lane 2). The ribosomal pellet was treated with puromycin/high salt to release ribosome-associated membrane proteins (RAMPs; Figure 1A, lane 3). With this procedure, 20%–30% of Sec61p was found in the RAMP fraction.

We also tested the behavior of the other Sec proteins in the same fractionation procedure using specific antibodies in immunoblots (Figure 1A). Whereas SSS1p showed the same distribution as Sec61p, no significant association with membrane-bound ribosomes was found for Sec63p, Sec62p, Sec72p, and Sec71p. As expected, most of the lumenal Kar2p was detected in the saponin extract.

Purification of the Ribosome-Associated Heterotrimeric Sec61p Complex

To purify the putative ribosome-associated Sec61p complex, the RAMP fraction was subjected to ion exchange

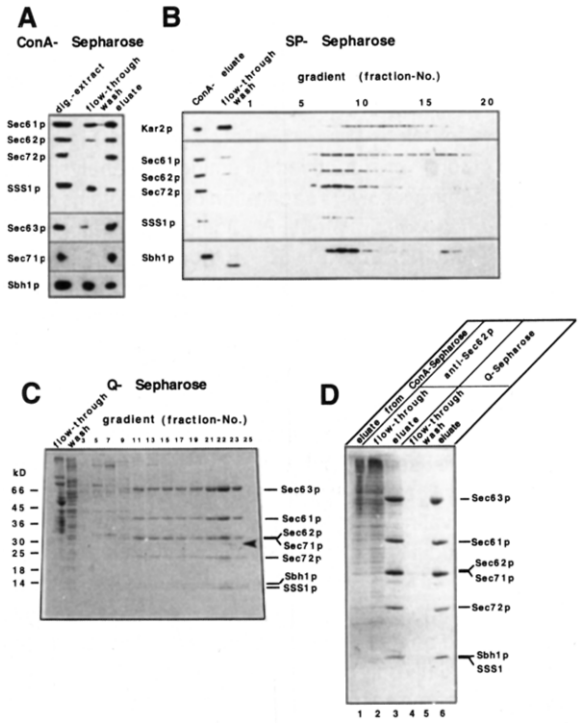

Figure 3. Purification of a Sec Complex

(A) A ribosome-depleted digitonin extract of yeast microsomes (digitonin extract) was passed through a concanavalin A (ConA)–Sepharose column. After a wash, the bound material was eluted with α-methylmannoside. All fractions were analyzed by SDS–PAGE and immunoblotting.

(B) The eluate from the concanavalin A–Sepharose column (ConA-eluate) was submitted to chromatography on SP–Sepharose. Detection of the proteins was carried out after SDS–PAGE by immunoblotting. A small protein in the flowthrough fraction cross-reacts with the Sbh1p antibodies.

(C) The Sec proteins in the peak fractions 7–9 of the SP–Sepharose column (part B) were submitted to chromatography on Q–Sepharose. Protein corresponding to 8,000 eq was analyzed by SDS–PAGE and staining with Coomassie blue. Identification of the proteins was carried out by determination of partial amino acid sequences (PTNYEYDEA for Sec63p; VTLEYNANS for Sec72p; ARASEKGEEKKQ for SSS1p; ETSIYXEL and PFESFLPETII for Sec61p) or by amino acid analysis and immunoblotting after separation of the proteins by reversed phase HPLC with an Aquapore300 C8-column and an acetonitrile gradient. The arrow indicates the position of a underglycosylated form of Sec71p, identified by immunoblotting with specific antibodies (data not shown).

(D) Purification of the Sec complex was carried out by immunoaffinity chromatography. A digitonin extract of microsomes was submitted to chromatography on concanavalin A–Sepharose, and the bound material was eluted with α-methylmannoside (eluate from ConA-Sepharose). It was then applied to an immunoaffinity column containing antibodies against Sec62p (anti-Sec62p), and the bound proteins were eluted with the peptide against which the antibodies are directed. Finally, the material was bound to Q–Sepharose and, after a wash, eluted with salt. The pattern of proteins corresponding to 2,000 eq was analyzed by SDS–PAGE and staining with Coomassie blue.

chromatography on Q– and SP–Sepharose columns, using conditions similar to those previously employed for the purification of the mammalian Sec61p complex (Görlich and Rapoport, 1993). The final preparation contained as major bands Sec61p, SSS1p, and a third protein (Figure 1B), migrating slightly above SSS1p in SDS gels. On the basis of partial amino acid sequences, it was identified as a homolog of the β subunits of the mammalian and plant Sec61p complexes; it was therefore named Sbh1p (Sec61β homolog 1). The complete sequence of Sbh1p could be derived from an hitherto unidentified gene in the data base (Figure 2). Homology among the Sec61β sequences is most pronounced in the membrane-spanning domains and in the regions directly preceding them.

To prove that Sbh1p is a constituent of the yeast Sec61p complex, we purified the complex with antibodies raised against an N-terminal peptide of Sbh1p (Figure 1C). The purified material (Figure 1C, lanes 4–10) contained essentially only the three subunits of the Sec61p complex. These results show that there exists a ribosome-associated Sec61p complex in S. cerevisiae, similar to that in mammals.

Purification of a Sec Complex

Next, we analyzed the Sec61p population that is not associated with ribosomes after solubilization of the microsomes. We found that when the digitonin extract was passed through a concanavalin A–Sepharose column, about half of the nonglycoprotein Sec61p remained bound to the lectin (Figure 3A). SSS1p and Sbh1p behaved similarly, suggesting that some of the Sec61p complex is associated with one or more glycoproteins. Sec72p, Sec63p, Sec62p, and Sec71p were found almost exclusively in the glycoprotein fraction. With the exception of Sec71p, which is glycosylated (Feldheim et al., 1993; Kurihara and Silver, 1993), all these proteins must be bound to the column indirectly. Kar2p, a nonglycosylated protein, was found both in the flowthrough (data not shown) and in the bound fraction (see Figure 3B).

The population of Sec61p, Sbh1p, and SSS1p that does not bind to the concanavalin A–Sepharose column is heterotrimeric Sec61p complex not bound to ribosomes, since these proteins could be purified together from the flowthrough fraction, using antibodies against Sbh1p and ion exchange chromatography (data not shown).

To analyze the lectin-bound fraction of the Sec proteins, we used ion exchange chromatography in subsequent purification steps. On SP–Sepharose, most of the Sec61p molecules eluted at about 300 mM salt (Figure 3B, fractions 7–9), distinctly earlier than the trimeric Sec61p complex would elute (about 500 mM). SSS1p and Sbh1p coeluted with Sec61p. Some of these proteins were found in later fractions, suggesting that some dissociation of a larger Sec complex had occurred. Almost all of Sec62p and Sec72p (as well as Sec71p and Sec63p; data not shown) coeluted with Sec61p in the early fractions. Kar2p did not comigrate; it smeared broadly through the gradient.

The material eluting early from the SP–Sepharose column was further purified on Q–Sepharose (Figure 3C). Unlike the trimeric Sec61p complex, which does not bind to this ion exchange column, all Sec proteins were retained and could only be eluted at high salt concentration in a rather broad peak (see Figure 3C, lanes 11–23). Only low amounts were found in the flowthrough fractions (immu-

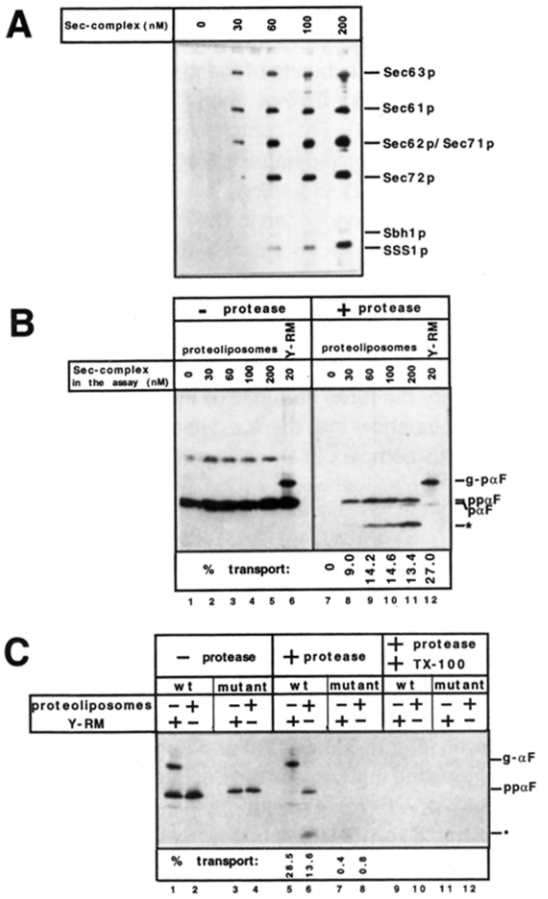

Figure 4. Translocation of Prepro-α Factor into Reconstituted Proteoliposomes Containing the Purified Sec Complex

(A) The protein pattern of proteoliposomes containing a constant amount of lipid and different amounts of purified Sec complex was analyzed by SDS–PAGE and silver staining.

(B) Tests for posttranslational translocation of radiolabeled prepro-α factor were carried out with the vesicles analyzed in part (A) or with native yeast microsomes (Y-RM) in the presence of ATP and an energy regenerating system. After incubation, the samples were either analyzed directly (minus protease) or after treatment with proteinase K (plus protease) by SDS–PAGE and fluorography. The concentration of Sec complex in the translocation assay was estimated from the amount of Sec62p added to the reconstitution samples, as determined by quantitative analysis of the amino acid composition after HPLC. The percentage of incorporation of Sec complex into the vesicles was close to 100%. The amount of Sec62p in Y-RM was determined by quantitative immunoblotting. Percent transport is defined as the ratio of radioactivity in protease-protected prepro-α factor and the input radioactivity in prepro-α factor, both determined by analysis with a phosphorimager. The radioactivity in the smaller protease-resistant fragment, indicated by a star, was not taken into account (for its possible origin, see text). g-pαF, ppαF, and pαF correspond to glycosylated pro-α factor, prepro-α factor and pro-α factor, respectively.

(C) Tests for posttranslational translocation of radiolabeled wild-type prepro-α factor or of a mutant protein, carrying a defect in its signal sequence, were carried out with proteoliposomes containing the purified Sec complex (final concentration of Sec complex 100 nM) or with native microsomes (Y-RM; 20 nM Sec62p). The samples were either analyzed directly by SDS–PAGE and fluorography (minus protease) or first treated with proteinase K in the absence (plus protease) or presence of 1% Triton X-100 (plus protease, plus TX-100).

noblots not shown). The Sec proteins represented the major bands in the final preparation. Some of them were identified by determination of N-terminal amino acid sequences (Sec63p, Sec72p, and SSS1p) or of sequences of internal peptides (Sec61p) (see legend to Figure 3), and the others were identified by amino acid analysis and immunoblotting following separation of the proteins on reversed phase high pressure liquid chromatography (HPLC) (data not shown). The minor band just below Sec62p/Sec71p (arrow) is an underglycosylated form of Sec71p, since it reacted with the corresponding antibodies (data not shown). Several other minor bands in the preparation probably represent impurities.

Since the Sec complex purified by ion exchange chromatography resulted in material that still contained some impurities, we used antibodies against Sec62p in an immunoaffinity purification protocol (Figure 3D). The eluate from the concanavalin A–Sepharose was incubated with immobilized antibodies against Sec62p and, after washing, the bound material was released with the antigenic peptide (Figure 3D, lane 3). The material in the eluate was finally concentrated on Q–Sepharose (Figure 3D, lane 6). It consisted essentially only of the known Sec proteins in approximately the same relative amounts as in the preparation obtained by ion exchange chromatography. Kar2p was virtually absent; by immunoblot analysis, the molar ratio of Kar2p to Sec61p was estimated to be lower than 1:50. The immunoaffinity procedure was employed in all subsequent experiments.

Taken together, these results demonstrate that yeast cells contain in addition to the trimeric Sec61p complex a large Sec complex that is not associated with ribosomes and that contains both the constituents of the Sec61p complex (Sec61p, Sbh1p, and SSS1p) and all other membrane proteins implicated in protein translocation on genetic grounds (Sec62p, Sec63p, Sec71p, and Sec72p). Rough estimates obtained from different preparations indicate that about 20%–30% of the total Sec61p population is contained in the ribosome-associated trimeric Sec61p complex, 30%–50% is in the nonassociated trimeric Sec61p complex, and 20%–30% is in the large Sec complex.

Reconstitution of Posttranslational Translocation of Prepro-α Factor

To test the possibility that the large Sec complex is involved in the posttranslational mode of protein translocation, proteoliposomes reconstituted with the purified complex and a mixture of phospholipids were tested for posttranslational translocation of prepro-α factor. The test protein was synthesized in vitro in the reticulocyte lysate system in the presence of [^{35}S]methionine. To exclude cotranslational translocation, cycloheximide was added and the ribosomes were removed by centrifugation prior to the addition of the proteoliposomes. The amount of protease-protected prepro-α factor was dependent on the amount of Sec complex in the vesicles (Figure 4A shows the protein pattern of the proteoliposomes, Figure 4B the translocation tests), as well as on the time of incubation (see Figure 6D). Small amounts of signal-cleaved pro-α factor

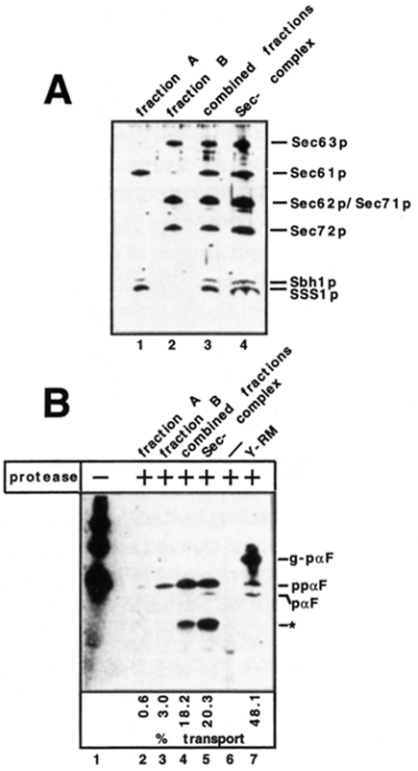

Figure 5. The Intact Sec Complex Is Required for Posttranslational Protein Transport

(A) The protein pattern of proteoliposomes containing subcomplexes of the Sec complex separately or in combination was analyzed by SDS–PAGE and silver staining. For comparison, proteoliposomes containing the intact Sec complex were also analyzed. Fraction A contains the constituents of the trimeric Sec61p complex, and fraction B contains the other Sec proteins.

(B) Tests for posttranslational translocation of prepro–α factor were carried out with the vesicles analyzed in (A) and with native microsomes (Y-RM). The sample shown in lane 1 was analyzed directly by SDS–PAGE and fluorography, and all others were first treated with proteinase K. The final concentration in the assay of the undissociated Sec complex (lane 5) was 100 nM in terms of Sec62p. Quantitation of the transport reaction was done with a phosphorimager. g-pαF, ppαF, and pαF correspond to glycosylated pro–α factor, prepro–α factor, and pro–α factor, respectively. The star indicates this small protease-resistant fragment of prepro–α factor.

were also often seen, particularly at high concentrations of the Sec complex (Figure 4B), indicating that our preparations of the Sec complex still contain some signal peptidase. The polypeptides of the signal peptidase complex could not be detected in the stained gels (see Figure 4A), but experience with the mammalian system indicates that even minute amounts of signal peptidase may cause significant signal peptide cleavage (Görlich and Rapoport, 1993). In addition to the bands corresponding to prepro–α factor and pro–α factor, a smaller protease-protected fragment was consistently observed (Figure 4B; labeled by a star). Preliminary evidence suggests that it may be produced from a population of prepro–α factor that is first translocated into the lumen of the vesicles and then partially reexported by molecules of the Sec complex that are wrongly oriented in the reconstituted membrane (data not shown). Neither of the protected polypeptide species were seen in samples containing protein-free liposomes (Figure 4B, lane 7) or lacking membranes (see Figure 5B, lane 6). All protected species were degraded by the protease if the membranes were solubilized with detergent (see Figure 4C, lane 10 versus lane 6) or if the protease was present during a freeze–thaw cycle (data not shown). With native microsomes, the protease-protected material consisted essentially only of the glycosylated form of pro–α factor (Figure 4B, lane 12; Figure 4C, lane 5).

To test whether the reaction depends on prepro–α factor having a functional signal sequence, as expected for faithful translocation, we used a mutant that contains a defective signal sequence (Figure 4C). The transport of the mutant protein was clearly much less efficient than that of the wild-type protein (Figure 4C, lanes 7 and 8 versus lanes 5 and 6). The appearance of the smaller protease-protected fragment was also dependent on a functional signal sequence.

To investigate whether the intact, large Sec complex is required for the posttranslational transport reaction, we dissociated the complex into two subcomplexes and tested these separately or together. Dissociation of the Sec complex was achieved after its binding to concanavalin A–Sepharose by treatment with Triton X-100. The components of the trimeric Sec61p complex were released (fraction A; see protein pattern in Figure 5A, lane 1), whereas the other Sec proteins remained bound and were eluted only after incubation with α-methylmannoside (fraction B; lane 2). Fraction A reconstituted into proteoliposomes alone was essentially inactive (Figure 5B, lane 2), and fraction B showed only low activity (lane 3), the latter being presumably caused by residual amounts of the components of the trimeric Sec61p complex (Figure 5A, lane 2). However, when both fractions were recombined in proteoliposomes (Figure 5A, lane 3), translocation activity was restored to a level almost as high as with the original, nondissociated Sec complex (Figure 5B, lane 4 versus lane 5). We conclude that only the intact Sec complex has the capacity to mediate posttranslational protein transport.

Dependence on ATP and Kar2p

Since ATP is known to be required for posttranslational protein transport into native yeast microsomes (Waters and Blobel, 1986; Hansen et al., 1986; Rothblatt and Meyer, 1986), we tested its effect on the transport into proteoliposomes. After in vitro synthesis of prepro–α factor, ATP was removed by gel filtration and by reaction with hexokinase and glucose (Figure 6A, lanes 1–4). Whereas the translocation into microsomes was completely abolished by ATP depletion (Figure 6A, lane 2) and restored by readdition of ATP (lane 6), the reaction with proteoliposomes remained unaffected (lane 3 versus lane 7). Similar results were obtained if the depletion of ATP was carried out by apyrase or if Mg^{2+} was removed by addition of EDTA (data not shown). It therefore appears that the translocation into proteoliposomes is independent of MgATP or re-

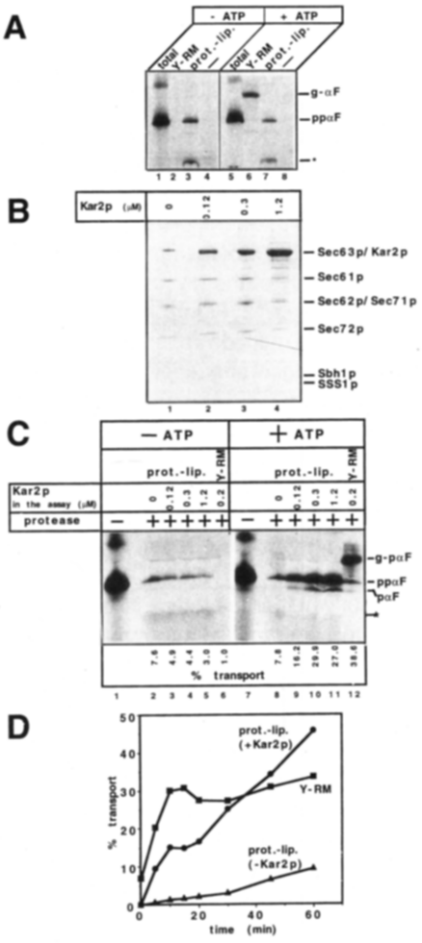

Figure 6. Effects of ATP and Kar2p on the Posttranslational Transport of Prepro–α Factor into Proteoliposomes

(A) The effect of ATP depletion (minus ATP) and ATP readdition (plus ATP) on the posttranslational transport of prepro–α factor was tested with proteoliposomes containing only the Sec complex (final concentration in the assay 100 nM) or with native microsomes (Y-RM; 20 nM Sec62p). ATP depletion of in vitro synthesized prepro–α factor was carried out by two sequential steps of gel filtration. The sample denoted minus ATP was further incubated with 1 mg/ml yeast hexokinase and 20 mM glucose for 10 min at room temperature, before addition of the proteoliposomes. The sample denoted by plus ATP received 1 mM ATP, 1 mg/ml creatine kinase, and 10 mM creatine phosphate (final concentrations). After incubation with the vesicles, all samples were treated with proteinase K and analyzed by SDS–PAGE and fluorography.

(B) The protein pattern of proteoliposomes containing the Sec complex and increasing amounts of Kar2p was analyzed by SDS–PAGE and Coomassie staining. Purified, His-tagged Kar2p was added during the reconstitution reaction as well as during dilution of the resulting vesicles. The latter were washed with buffer before use.

(C) Tests for posttranslational translocation of prepro–α factor were carried out with the proteoliposomes analyzed in (B) or with native microsomes (Y-RM), both in the absence or presence of ATP (minus ATP and plus ATP, respectively). In vitro synthesized prepro–α factor was submitted to two rounds of gel filtration and either used directly (minus ATP) or first complemented with ATP and an ATP-regenerating system (plus ATP) before addition of the vesicles. The final concentration of Sec complex was estimated to be 60 nM in terms of Sec62p. The concentrations of Kar2p in the translocation assay were calculated from those in the reconstitution mixture. These values were in reasonable agreement with those obtained by immunoblotting of the vesicles, using as a standard the preparation of purified Kar2p; its concentration was determined by quantitative analysis of the amino acid composition. The Kar2p concentration for the sample of Y-RM was estimated by immunoblotting. g-pαF, ppαF, and pαF correspond to glycosylated pro–α factor, prepro–α factor, and pro–α factor, respectively. A star indicates this small protease-resistant fragment of prepro–α factor.

(D) Kinetics of the transport reaction with native microsomes (Y-RM; 20 nM Sec62p) and proteoliposomes containing either both the Sec complex (90 nM) and Kar2p (1.2 μM) (prot.-lip. [plus Kar2p]) or only the Sec complex (prot.-lip. [minus Kar2p]). All reactions were carried out in the presence of ATP.

quires much lower concentrations than the reaction with native microsomes.

Next, we tested the effect of Kar2p in our reconstituted system. A His-tagged version of Kar2p, previously shown to be active in translocation tests (Brodsky and Schekman, 1993), was purified from an overproducing E. coli strain. Kar2p was added in increasing concentrations during the reconstitution of proteoliposomes from phospholipids and Sec complex (the protein pattern of the resulting vesicles is shown in Figure 6B; several of the minor bands are degradation products of Kar2p [immunoblot not shown]). If the vesicles were tested in the presence of ATP, posttranslational translocation of prepro–α factor was stimulated at high concentrations of Kar2p by a factor of about 3–5 (Figure 6C, lane 11 versus lane 8). Signal sequence cleavage was also stimulated. A prolonged exposure of the translocated polypeptide to signal peptidase in the lumen, caused by its binding to Kar2p, may explain the increase in the level of signal-cleaved and protease-protected pro–α factor. In the absence of ATP, Kar2p had a slightly inhibitory effect (Figure 6C, lanes 2–5). It may be due to the presence of some Kar2p on the outside of the vesicles, since its direct addition to the translocation assay also inhibited the transport reaction regardless of whether or not ATP was present (data not shown). Thus, Kar2p exerts its stimulatory effect only if present at the right location, i.e., in the lumen of the vesicles. The effect of added ATP implies that it can get into the proteoliposomes, probably because these are leaky to small molecules (data not shown).

Proteoliposomes containing Kar2p had a 3- to 4-fold higher initial rate of transport than those lacking it (Figure 6D). In the presence of Kar2p and ATP, the rate of transport into proteoliposomes approached that into native microsomes within a factor of about 2 (Figure 6D). In some experiments, the final transport levels were even higher (see Figure 6D), since the reaction with native microsomes leveled off after about 20 min, whereas that with proteoliposomes continued for at least 60 min.

Translocation of pro-OmpA

We tested the reconstituted system with another translocation substrate, pro-OmpA, a bacterial protein known to be transported posttranslationally into yeast microsomes (Sanz and Meyer, 1989; see also Figure 7, lane 8). Translocation of pro-OmpA was observed with proteoliposomes containing the Sec complex, Kar2p, and ATP (Figure 7, lane 7). In the absence of ATP (data not shown) or of Kar2p (Figure 7, lane 6), no translocated pro-OmpA was seen. Thus, in contrast with the results with prepro–α factor,

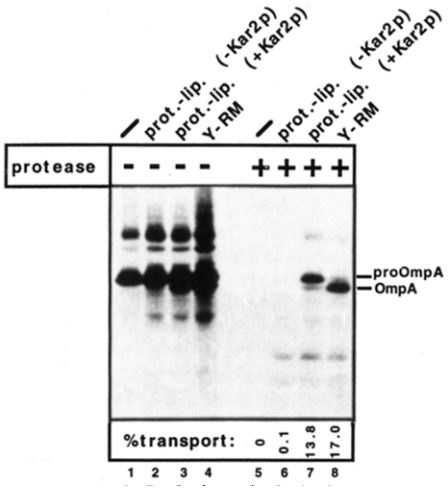

Figure 7. Posttranslational Transport of Pro-OmpA into Proteoliposomes

In vitro synthesized, radiolabeled pro-OmpA was incubated in a posttranslational reaction in the presence of ATP with proteoliposomes containing the Sec complex or the Sec complex plus Kar2p, or with native microsomes (Y-RM). The final concentrations of Sec complex and of Kar2p were estimated to be 100 nM (in terms of Sec62p) and 1.2 μM, respectively. The samples were either analyzed directly by SDS–PAGE and fluorography (minus protease) or were first treated with proteinase K (plus protease).

posttranslational translocation of pro-OmpA seems to be absolutely dependent on lumenal Kar2p and ATP.

Discussion

Our results indicate that the posttranslational translocation pathway into the ER of yeast is mediated by a large Sec complex that contains the components of a heterotrimeric Sec61p complex, similar to that in mammals, and all other known membrane proteins found by genetic screening for translocation components. The trimeric Sec61p complex also exists as a separate entity in yeast cells that we hypothesize to mediate cotranslational protein transport involving membrane-bound ribosomes.

The yeast Sec61p complex consists of Sec61p, SSS1p, and Sbh1p, the newly discovered homolog of the mammalian Sec61β. An interaction between Sec61p and SSS1p had already been suggested by sequence similarities to mammalian Sec61α and Sec61γ, respectively (Hartmann et al., 1994), and by the fact that SSS1p was found to be a suppressor of *sec61^{ts}* mutants (Esnault et al., 1993). Our proposal that this trimeric complex is involved in cotranslational protein transport is based on the observation that it was associated with membrane-bound ribosomes, like the structurally related complex in mammals, for which the involvement in cotranslational translocation has been demonstrated (Görlich and Rapoport, 1993). Direct proof that the trimeric Sec61p complex mediates cotranslational protein transport must await the establishment of an in vitro translocation system from yeast that is dependent on SRP and SRP receptor.

The existence of a larger complex of yeast Sec proteins was not entirely unexpected. Previously, a subcomplex had been isolated (Sec63p complex; Brodsky and Schekman, 1993), and the spatial proximity of several Sec proteins in membranes had been demonstrated (Deshaies et al., 1991). However, it seems that only the choice of mild conditions that we employed to solubilize the microsomes made it possible to isolate the intact Sec complex. Whereas Kar2p has been reported to be a constituent of the Sec63p complex (Brodsky and Schekman, 1993), it is not contained in our Sec complex, probably because different detergents were employed. As judged from staining of SDS gels with silver or Coomassie blue, the Sec complex does not contain stoichiometric constituents other than the identified Sec proteins. Also, the separation of the components by HPLC did not give any indication of an additional abundant protein. Of course, the presence of substoichiometric quantities of polypeptides that could have catalytic functions in translocation cannot be excluded.

Given that the major constituents of the large Sec complex are the genetically identified translocation components (plus the novel protein Sbh1p) and that the complex contains all of them, we believe that this complex represents an intact functional unit that mediates posttranslational transport in vivo. Consistent with this assumption, in our reconstituted system only the entire assembly was active in posttranslational translocation of prepro-α factor; both the trimeric Sec61p complex and a subcomplex that contains the other Sec proteins, obtained by dissociation of the Sec complex in Triton X-100, were found to be essentially inactive. Although it remains to be seen if a similar disassembly can occur in vivo, it is attractive to view the translocation apparatus as a dynamic assembly of "building blocks," the core component being the trimeric Sec61p complex. In the posttranslational pathway, it may associate with Sec62p, Sec63p, Sec71p, and Sec72p, and in the cotranslational pathway, as indicated by the results in the mammalian system, it may cooperate with the SRP receptor and TRAM. The lack of posttranslational transport of prepro-α factor into dog pancreatic microsomes may be explained by the assumption that only the trimeric Sec61p complex is abundant and that the other Sec proteins are either missing or present in low amounts.

From our in vitro data, one would predict that mutations in Sec62p, Sec63p, Sec71p, and Sec72p affect in vivo only proteins transported posttranslationally. Indeed, in most of these mutants, the secretion of invertase, a protein transported exclusively in a cotranslational manner (Hansen and Walter, 1988), is much less disturbed than that of prepro-α factor (Rothblatt et al., 1989; Green et al., 1992; Feldheim et al., 1993; Feldheim and Schekman, 1994). In cases where defects of invertase secretion are seen, these may be caused by indirect effects of the mutations.

It should be noted that our data do not exclude the possibility that a polypeptide chain may be transported by the Sec complex before its synthesis is completed. However, unlike the case with the trimeric Sec61p complex, it appears unlikely that the translating ribosome makes a tight

contact with the large Sec complex, since the latter was not found to be associated with membrane-bound ribosomes.

Efficient posttranslational transport of prepro–α factor requires proteoliposomes containing not only the Sec complex but also lumenal Kar2p and ATP. In the case of pro-OmpA, translocation was totally dependent on the latter two components. These results are consistent with others that showed an important role in protein translocation for Kar2p both in vivo and in vitro (Vogel et al., 1990; Sanders et al., 1992). However, to our surprise, we have observed some translocation of prepro–α factor even in the absence of added Kar2p and ATP. We believe that this represents faithful translocation because of the following. First, a significant proportion of prepro–α factor was rendered protected against the action of protease in a process that was dependent on time and temperature (the latter not shown). Second, the reaction required the intact Sec complex in the vesicles. Third, it was dependent on a functional signal sequence in the translocation substrate. Fourth, as with native microsomes (Sanz and Meyer, 1989), it was inhibited by N-ethylmaleimide (data not shown). Several explanations for the occurrence of a base level of translocation of prepro–α factor without energy provision may be offered and need to be explored. One is that single-round translocation occurs with energy somehow "stored" in the system; another is that a base level of transport can occur in some passive manner.

The function of Kar2p in posttranslational translocation may be analogous to that proposed for the homolog of Kar2p in the matrix of mitochondria, mtHsp70, for protein import into this organelle. In this case, Hsp70 binds the incoming polypeptide chain and prevents back translocation (Schneider et al., 1994). It appears to act together with the inner membrane protein MIM44 as a molecular ratchet that utilizes the hydrolysis of ATP. Such a model had been proposed before on the basis of theoretical considerations (Simon et al., 1992). In the ER system, the partner of Kar2p appears to be Sec63p (Brodsky and Schekman, 1993). By analogy to the mitochondrial system (Kang et al., 1990), one may also assume that Kar2p can pull a polypeptide through the membrane causing it to unfold at the cytoplasmic side of the membrane. This may explain why Kar2p is essential for the translocation of some proteins, like pro-OmpA. The functions of BiP/Kar2p do not seem to be essential for the cotranslational mode of protein transport (Görlich et al., 1992; Görlich and Rapoport, 1993), in agreement with the fact that a Sec63p-like protein is not involved.

The precise functions of the different components of the Sec complex in posttranslational protein transport have not been determined as yet, but some are likely to replace those of the membrane-bound ribosome in cotranslational translocation. The establishment of a reconstituted system now offers the opportunity for a detailed analysis.

Experimental Procedures

Detergents and Lipids

Digitonin (Merck) was purified as described (Görlich and Rapoport, 1993). Saponin was purified by treating a concentrated solution with a mixed bed ion exchanger (TMD-8) and with QAE–Sephadex. The final solution had an A_{280} of about 10 and a conductivity below 20 μS/cm.

A 20 mg/ml stock solution of a mixture of phosphatidylcholine and phosphatidylethanolamine in a ratio of 4:1 was prepared in 3% deoxy-BigCHAP, as described (Görlich and Rapoport, 1993).

Antibodies

Antibodies were raised against peptides corresponding to the C-termini of the following proteins plus additional N-terminal cysteines: Sec61p (CLVPGFSDLM), Sec62p (CNKKKAINEKAEQN), Sec71p (CELKINNDGRLVN), and Sec72p (CTARNMAEYNGE). The peptide of Sbh1p corresponded to the positions 3–16 plus an N-terminal cysteine (CPTPPGGQRTLQKRK) and that of SSS1p to the N-terminus plus C-terminal cysteine and alanine (ARASEKGEEKKQSCA). Antibodies against Kar2p and Sec63p were gifts of Dr. R. Schekman. Affinity purification and immobilization of antibodies were carried out as described (Görlich et al., 1992; Görlich and Rapoport, 1993).

Preparation of Membranes from Yeast Cells

200 g of yeast cells (S. cerevisiae wild-type strain RSY521), harvested at exponential growth phase, were homogenized with glass beads in a Dyno-Mill (W. A. Bachofen) with 50 ml of a buffer containing 250 mM HEPES–KOH (pH 7.5), 25 mM potassium acetate, 5 mM magnesium acetate, 5 mM EDTA, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50% glycerol, 10 μg/ml leupeptin, and 5 μg/ml chymostatin. A postmitochondrial supernatant was submitted to centrifugation for 35 min at 25,000 rpm and 2°C in a Ti45 rotor of a Beckman ultracentrifuge. The resulting membrane pellet was resuspended in 50 mM HEPES–KOH (pH 7.5), 10% glycerol, 2 mM DTT, 1 μg/ml leupeptin, 0.5 μg/ml chymostatin to a final concentration of 1–2 equivalents (eq; 1 eq is defined as 1 μl of a membrane suspension with $A_{280\ nm}$ of 50) per microliter. The yield of membranes was about 150,000 eq. They were frozen in liquid nitrogen and stored at −80°C.

Differential Extraction of the Membranes

All steps were carried out at 2°C. Membranes corresponding to about 100,000 eq were resuspended in a final volume of 190 ml of 50 mM HEPES–KOH (pH 7.5), 2.5% saponin, 800 mM potassium acetate, 16 mM magnesium acetate, 10% glycerol, 7 mM β-mercaptoethanol, 5 μg/ml leupeptin, 2.5 μg/ml chymostatin. After centrifugation for 80 min at 45,000 rpm in a Ti45 rotor of a Beckman ultracentrifuge (sediments particles larger than about 100 S), the supernatant (saponin extract) was removed and the pellet fraction was resuspended in about 50 ml of 50 mM HEPES–KOH (pH 7.5), 3% digitonin, 400 mM potassium acetate, 8 mM magnesium acetate, 10% glycerol, 7 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml chymostatin. After centrifugation for 90 min at 60,000 rpm in a Ti70 rotor of a Beckman ultracentrifuge (sediments particles larger than 30 S), the supernatant (digitonin extract) was removed and the pellet was extracted a second time. After resuspension, treatment with puromycin at high salt concentration and subsequent centrifugation (see Görlich and Rapoport, 1993) yielded a supernatant that contained the RAMPs.

Purification of the Sec61p Complex

Purification of the complex by conventional ion exchange chromatography was carried out essentially as described for the mammalian Sec61p complex (Görlich and Rapoport, 1993).

For the purification procedure involving immunoaffinity chromatography, RAMPs (50,000 eq) were dialyzed against 100 vol of 15 mM HEPES–KOH (pH 7.5), 20% sucrose, 1 mM magnesium acetate, 7 mM β-mercaptoethanol, 0.025% digitonin. After centrifugation for 30 min at 90,000 rpm in a 100.4 rotor in a Beckman tabletop ultracentrifuge, the dialyzate was passed through a 10 ml Q–Sepharose fast flow column. The material in the flowthrough fraction was bound to a 2 ml column containing about 4 mg of immobilized antibodies against Sbh1p. After washing with D buffer (50 mM HEPES–KOH [pH 7.5], 1% digitonin, 10% glycerol, 7 mM β-mercaptoethanol, 5 μg/ml leupeptin, 2.5 μg/ml chymostatin) containing 500 mM potassium acetate, bound material was eluted in 30 min at room temperature with 1 mg/ml of the peptide against which the antibodies were raised. The eluate was diluted 5-fold with D buffer and applied to a SP–Sepharose fast flow column. Elution was performed with a gradient from 100 mM to 700 mM potassium acetate in D buffer.

The N-terminal sequence of Sbh1p was determined after purification of the Sec61p complex by immunoaffinity chromatography from a yeast strain that is partially defective in N-acetyltransferase activity (Mullen et al., 1989). The sequence of internal peptides was determined after cleavage of the protein with Lys-C or chymotrypsin.

Purification of the Sec Complex

The procedure employing ion exchange chromatography is as follows. Digitonin extract (50 ml) containing 100,000 eq was incubated with 7 ml of concanavalin A–Sepharose (Pharmacia) for 12 hr in the cold. After washing, the glycoproteins were eluted over a period of 4 hr at 24°C with 50 ml of 1 M methyl-α-D-mannopyranoside (Sigma) in D buffer. The eluate (20 ml) was applied to a 4 ml SP–Sepharose fast flow (Pharmacia) column equilibrated with D buffer. After washing, elution was carried out with 40 ml of a linear gradient from 0–600 mM potassium acetate in D buffer. Fractions containing the Sec complex were applied to a 1 ml Q–Sepharose fast flow (Pharmacia) column, equilibrated with D buffer containing 100 mM potassium acetate. The column was washed with the same buffer containing 400 mM potassium acetate. Elution was carried out with 20 ml of a linear gradient from 400–1000 mM potassium acetate in D buffer. The fractions containing the Sec complex were identified by immunoblotting and Coomassie staining after SDS–PAGE with 14% acrylamide gels.

Purification of the Sec complex by immunoaffinity chromatography was carried out with the material eluted from the concanavalin A column, essentially as described for the Sec61p complex, except that an immunoaffinity column was used which contained 4–8 mg immobilized, affinity-purified antibodies against Sec62p. The eluted material was concentrated on a small Q–Sepharose fast flow column. From 100,000 eq membranes, about 60–100 μg of Sec61p contained in the Sec complex were obtained. This corresponds to a yield of about 10%–15% of the Sec complex.

To exchange the detergent for reconstitution assays, the Sec complex was bound to 300 μl of Q–Sepharose in a spin column. After washing with 2 ml of D buffer containing 400 mM potassium acetate, the detergent was changed with 5 ml of 50 mM HEPES–KOH (pH 7.5), 0.3% deoxy-BigCHAP, 15% glycerol, 2 mM DTT over 1–2 hr. The Sec complex was eluted with 1000 mM potassium acetate in the same buffer.

Dissociation of the Sec Complex

Purified Sec complex (40,000 eq) was bound to 200 μl of concanavalin A–Sepharose, and the components of the Sec61p complex released in 15 min with three times 0.4 ml of a buffer containing 50 mM HEPES–KOH (pH 7.5), 200 mM potassium acetate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 7 mM β-mercaptoethanol, 1 μg/ml leupeptin, 0.5 μg/ml chymostatin. The eluted proteins were bound to 50 μl of SP–Sepharose fast flow, and the detergent was changed to deoxy-BigCHAP as described for the intact Sec complex. To obtain the proteins that remained bound to concanavalin A–Sepharose, the resin was washed with D buffer and elution was carried out for 3 hr with 1.6 ml of 1 M methyl-α-D-mannopyranoside in D buffer. The eluted proteins were bound to 50 μl of Toyopearl DEAE-650M (TosoHaas), and the detergent was exchanged as before.

Purification of Recombinant His-Tagged Kar2p

His-tagged Kar2p was purified from the E. coli strain MR2623 harboring a plasmid coding for Kar2p of S. cerevisiae with six His residues at the N-terminus of the mature protein (a gift of Dr. M. Rose). Expression was induced by isopropyl-thio-β-D-galactoside (IPTG), and the protein was purified from a postribosomal supernatant by chromatography on a Ni–NTA–agarose column (Qiagen). It was eluted with a buffer containing 300 mM imidazole. The protein was then bound to a Q–Sepharose fast flow column and eluted with a buffer containing 50 mM HEPES–KOH (pH 7.5), 1.3 M potassium acetate, 15 mM magnesium acetate, 10% glycerol, 7 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastatinal, and 1 μg/ml pepstatin A. About 70% of the purified Kar2p could be bound to ATP–agarose.

Reconstitution into Proteoliposomes

For a standard reconstitution reaction, 300 μg of phopholipids and Sec complex containing about 7 pmol Sec62p were mixed in a total volume of 50 μl. Kar2p was added, where indicated, in a maximum volume of 2 μl. Potassium acetate and glycerol were adjusted to give final concentrations of 400 mM and 16% (w/v), respectively. Removal of the detergent was carried out with SM-2 beads (Görlich and Rapoport, 1993). For reconstitutions including Kar2p, dilution of the proteoliposomes was carried out with a Kar2p solution in water of the same concentration as used during reconstitution, to balance leakage of the lumenal protein from the vesicles by osmotic shock. Proteoliposomes were collected by centrifugation for 30 min at 70,000 rpm in a Beckman TLA 100.3 rotor and resuspended in 10 μl of buffer R (20 mM HEPES [pH 7.5], 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, and 250 mM sucrose). If Kar2p was coreconstituted, the vesicles were washed once with 200 μl of buffer R lacking sucrose. The vesicles were kept on ice before use in translocation tests.

Translation and Translocation

Transcripts coding for prepro–α factor were produced from the plasmid pSP65-αF (Rothblatt et al., 1987) by in vitro transcription with SP6 polymerase. Transcripts coding for a signal sequence mutant of prepro–α factor were obtained from the plasmid pSP65-αFm2 that carries at position 13 a Pro residue instead of an Ala (Allison and Young, 1988). Transcripts for pro-OmpA were obtained from plasmid pSI053, linearized with HindIII (Yamada et al., 1989). In vitro translation was carried out in the reticulocyte lysate system for 20 min at 30°C. The samples were then incubated for 5 min with 1 mM cycloheximide, and the ribosomes were removed by centrifugation for 10 min at 100,000 rpm in a Beckman TL100 rotor. The supernatant (200 μl) was usually submitted to gel filtration through a NAP5 (Pharmacia) column, equilibrated in buffer R. Material corresponding to the void volume of the column was collected and immediately frozen in liquid nitrogen.

For translocation assays, 10 μl samples of gel-filtered translation product were mixed with 1–2 μl of proteoliposomes and incubated at 22°C for 60 min. Half of the sample was precipitated directly with 20% trichloroacetic acid, and the other half was first incubated at 0°C for 40 min with an equal volume of 1 mg/ml proteinase K in buffer R, before precipitation with trichloroacetic acid. Kinetic experiments were performed in a total volume of 20 μl, and 2 μl samples were removed at the indicated timepoints. Where indicated, 1 mM ATP and an energy-regenerating system containing 10 mM creatine phosphate and 1 mg/ml creatine kinase (final concentrations) were added to the sample of gel-filtered prepro–α factor prior to the addition of proteoliposomes.

Analysis of the samples was performed by SDS–PAGE with 14% acrylamide gels, followed by quantitation with a Fuji phosphorimager BAS2000. The gels were subsequently rehydrated and processed for fluorography.

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