

Sec61p is Adjacent to Nascent Type I and Type II Signal-Anchor Proteins during Their Membrane Insertion

Stephen High,* Søren S.L. Andersen,* Dirk Görlich,‡ Enno Hartmann,‡ Siegfried Prehn,§
Tom A. Rapoport,‡ and Bernhard Dobberstein*

*European Molecular Biology Laboratory, 6900 Heidelberg; ‡Max-Delbrück-Center for Molecular Medicine, 1115 Berlin-Buch; and §Institute of Biochemistry, Humboldt-University, 1040 Berlin, Federal Republic of Germany

Abstract. We have identified membrane components which are adjacent to type I and type II signal-anchor proteins during their insertion into the membrane of the ER. Using two different cross-linking approaches a 37–38-kD nonglycosylated protein, previously identified as P37 (High, S., D. Görlich, M. Wiedmann, T. A. Rapoport, and B. Dobberstein. 1991. *J. Cell Biol.* 113:35–44), was found adjacent to all the membrane inserted nascent chains used in this study. On the basis of immunoprecipitation, this ER protein was shown to be identical to the recently identified

mammalian Sec61 protein. Thus, Sec61p is the principal cross-linking partner of both type I and type II signal-anchor proteins during their membrane insertion (this work), and of secretory proteins during their translocation (Görlich, D., S. Prehn, E. Hartmann, K.-U. Kalies, and T. A. Rapoport. 1992. *Cell*. 71:489–503). We propose that membrane proteins of both orientations, and secretory proteins employ the same ER translocation sites, and that Sec61p is a core component of these sites.

NASCENT membrane proteins are directed to the ER by virtue of a hydrophobic signal sequence (Blobel and Dobberstein, 1975). Two kinds of such ER-targeting signals have been identified: those which are cleaved from the nascent chain by signal peptidase upon membrane insertion (cleavable) and those which remain a part of the membrane inserted protein (uncleaved) (Wickner and Lodish, 1985; High and Dobberstein, 1992b). In the case of membrane proteins with uncleaved signal sequences, the sequence serves both to target the protein to the ER and, subsequently, to anchor the protein in the membrane. Such proteins are referred to as signal-anchor proteins (Lipp and Dobberstein, 1988) to distinguish them from proteins with cleavable signal sequences. Cleavable signal sequences are usually present at the NH₂ terminus of the nascent chain, and membrane proteins bearing such a signal also contain a stop-transfer sequence which functions to prevent complete translocation of the nascent chain across the membrane and anchor the protein in the lipid bilayer (Rapoport and Wiedmann, 1985).

Single spanning membrane proteins with a cleavable signal sequence always have their NH₂-terminus extracyto-

plasmic (type I orientation). In contrast, signal-anchor proteins which span the membrane once can have a type I or a type II (NH₂-terminus cytoplasmic) orientation. The orientation which a signal-anchor protein assumes in the membrane is influenced by the type of charged residues which flank the hydrophobic core of the signal-anchor sequence (Haeuptle et al., 1989; Hartmann et al., 1989; von Heijne, 1988). The mechanism by which different signal-anchor proteins can assume different orientations in the membrane is not known (see High et al., 1991b; High and Dobberstein, 1992a).

All types of signal sequences are recognized by a cytosolic component, the signal recognition particle (SRP)¹ (High et al., 1991b). SRP functions to target nascent chains bearing the appropriate signals to the ER (Rapoport, 1990). The process of targeting requires the presence of a membrane bound receptor for the nascent chain/ribosome/SRP complex, the docking protein (SRP receptor), and GTP (Connolly and Gilmore, 1989; Connolly et al., 1991; High et al., 1991a). After the GTP dependent release of the nascent chain from the 54-kD subunit of SRP it is free to interact with components of the putative membrane insertion machinery (High et al., 1991a).

To identify proteins of the ER which may be involved in

Address correspondence to Dr. High at Department of Biochemistry and Molecular Biology, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom.

Prof. Dobberstein's present address is Zentrum für Molekulare Biologie Heidelberg, Im Neuenheimer Feld 282, Postfach 106249, 6900 Heidelberg, Federal Republic of Germany.

1. *Abbreviations used in this paper:* DSS, disuccinimidyl suberate; Ii, invariant chain of MHC class II molecules; PPL, preprolactin; SRP, signal recognition particle; SRP54, 54-kD subunit of signal recognition particle; TRAM protein, translocating chain-associating membrane protein.

the membrane insertion of nascent signal-anchor proteins we have used a photocross-linking approach (High et al., 1991b). Photocross-linking allows proteins which are adjacent to membrane inserting nascent chains to be detected, it was used to identify the next neighbors of the secreted protein preprolactin (PPL) during its translocation across the ER membrane (Krieg et al., 1989; Wiedmann et al., 1987). To stabilize the normally transient interactions occurring during translocation and membrane insertion, it is necessary to generate translocation intermediates of the nascent chains. These are made by using artificially truncated mRNAs which, upon translation, produce stable ribosome/nascent chain complexes which jam in the translocation machinery (Gilmore et al., 1991). By using different lengths of such truncated nascent chains it has been possible to probe the environment of a protein during its translocation across the membrane of the ER (Krieg et al., 1989; Wiedmann et al., 1989).

Photocross-linking analysis with signal-anchor proteins showed that a 37-kD nonglycosylated protein (P37) was the major cross-linking partner of a type I signal-anchor membrane protein (High et al., 1991b). Translocation intermediates generated with long nascent chains of PPL were also found to interact with a nonglycosylated protein, of estimated 40 kD mol wt, which was identified as the mammalian homologue of Sec61p (Görlich et al., 1992b). This was in contrast to the photocross-linking analysis with short nascent chains of PPL where 34–39 kD glycoproteins, principally the TRAM protein, are the major cross-linking partners (Görlich et al., 1992a; Krieg et al., 1989; Wiedmann et al., 1987). Both type I and type II signal-anchor proteins were also photocross-linked to glycoproteins in the range of 35 kD (High et al., 1991b; Thrift et al., 1991) although these were sometimes a minor component (High et al., 1991b). By using a homobifunctional cross-linking reagent, Kellaris et al. (1991) were able to identify a 34-kD nonglycosylated integral membrane protein (imp34) as the major cross-linking partner of two proteins with cleavable signal sequences (vesicular stomatitis virus G protein and PPL) during membrane insertion. Thus, two major groups of ER proteins are found to be next to nascent secretory and membrane proteins during membrane translocation or membrane insertion. These are nonglycosylated proteins of 34–40 kD (Görlich et al., 1992b; High et al., 1991b; Kellaris et al., 1991) and glycoproteins of 34–39 kD (Görlich et al., 1992a; High et al., 1991b; Krieg et al., 1989; Thrift et al., 1991; Wiedmann et al., 1987). To date, the ER protein which is cross-linked has depended upon both the type of nascent chain used and the method of cross-linking (for recent review see High, 1992).

In our previous study, P37 was found adjacent to a type I signal-anchor protein (IMC-CAT) but not a type II signal anchor protein (the transferrin receptor) (High et al., 1991b). This suggested that either the interaction with P37 was specific for type I signal-anchor proteins, or that cross-linking to P37 was dependent upon the position of the modified lysine residues within the nascent chain. Using a different type II signal-anchor protein (Ii) we show here that the latter is the case and that P37 is adjacent to both type I and type II signal-anchor proteins. The ER protein Sec61p has been shown to be adjacent to translocating secretory proteins in both yeast (Müsch et al., 1992; Sanders et al., 1992) and mammalian systems (Görlich et al., 1992b), and impli-

cated in the translocation of secretory proteins across the ER membrane (Rothblatt et al., 1989). The properties of P37 (High et al., 1991b) were similar to those of mammalian Sec61p (Görlich et al., 1992b) raising the possibility that this protein could also play a role in membrane protein insertion. We now show that P37 is identical with mammalian Sec61p and that Sec61p is therefore adjacent to both type I and type II signal-anchor proteins during their membrane insertion. On the basis of these results, we propose that Sec61p plays a central role in both the insertion of proteins into the membrane of the ER, and their complete translocation across it.

Materials and Methods

Materials

T7 RNA polymerase and restriction enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany). [³⁵S]methionine was from Amersham Buchler GmbH (Braunschweig, Germany). Cycloheximide and 7-methylguanosine 5'-monophosphate were supplied by Sigma Chemical Co. (St. Louis, MO). Entensify fluorography solutions were supplied by New England Nuclear (Boston, MA). Disuccinimidyl suberate (DSS) was obtained from Pierce Chemical Co. (Rockford, IL) and the *N*-hydroxy-succinimide ester of 4-(3-trifluoromethyl-diazirino) benzoic acid (TDBA) was a gift from Dr. Josef Brunner, Swiss Federal Institute of Technology, Zürich, Switzerland.

Transcription, Translation and Photocross-linking

The plasmid used for the transcription of IMC-CAT and the complementary oligonucleotide used to obtain a COOH-terminal truncated nascent chain (IMC-CAT₁₀₃) have been previously described (High et al., 1991b). For production of the IMC-CAT₁₅₀ COOH-terminally truncated nascent chain the complementary oligonucleotide 5'AGGGAAATAGGCCAGGTTTTC-ACC3' was included at 40 μg ml⁻¹ in the wheat germ cell free translation system (Haeuptle and Dobberstein, 1986). For the invariant chain (Ii) the coding region was cleaved from the pDSSII plasmid (Lipp and Dobberstein, 1986a) as a SmaI/HincII fragment and subcloned into pGEM4. The resulting plasmid (pGEM4Ii) was linearized with Bam HI for transcription of full length mRNA or NcoI for production of mRNA coding for the NH₂-terminal 103 amino acids (Ii₁₀₃). Transcription of pGEM4Ii was with SP6 RNA polymerase. To obtain Ii₁₂₃ which encodes the NH₂-terminal 123 amino acids of Ii the complementary oligonucleotide 5'TGGAGCAGGTGC-ATCACATG3' was included at 40 μg ml⁻¹ together with the full-length Ii transcript in the wheat germ cell free translation system (Haeuptle and Dobberstein, 1986). Cell free translation in a wheat germ lysate, in the presence of 3.75 pmol of ε-TDBA-Lys-tRNA per 25 μl of translation mixture, was performed as previously described (High et al., 1991b) except that the microsomal membranes were added after the addition of 7-methylguanosine 5'-monophosphate but before the inhibition of protein synthesis by cycloheximide or emetine rather than after inhibition as was previously the case. This allows membrane inserted nascent chains of different lengths, dependent upon the truncated mRNA used, to be synthesized (Krieg et al., 1989).

Cross-linking with Disuccinimidyl Suberate, Immunoprecipitation and Sample Analysis

Carbonate extraction of the photocross-linking products has been described elsewhere (High et al., 1991b). For cross-linking with DSS, the membrane bound components were isolated by centrifugation and resuspended in membrane pellet (MP) buffer as described by Görlich et al. (1990). Cross-linking with DSS was as described (Kellaris et al., 1991). The immunoprecipitation of photocross-linking products with anti-translocating chain-associating membrane (TRAM) protein antibodies and relevant controls were carried out as follows: after photocross-linking the membrane bound components were isolated by centrifugation through a cushion (0.25 M sucrose, 0.5 M KCl, 5 mM MgCl₂ and 50 mM Hepes-KOH pH 7.9) 5 min, 100,000 g and 4°C. The pellet was resuspended in MP buffer (0.25 M sucrose, 80 mM KCl, 3 mM MgCl₂ and 10 mM Hepes-KOH pH 7.5) and then 4 vol of IP dilution buffer (10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1 mM EDTA and 1% Triton X-100) and phenylmethylsulphonyl fluoride to a final concentration of 0.2 mg ml⁻¹ were added followed by the relevant

antibodies or antisera. Samples were incubated at 4°C overnight, protein A Sepharose was then added and the incubation continued for 2 h and samples processed as previously described (Lipp and Dobberstein, 1986a). Immunoprecipitation with anti-Sec61p antibodies and relevant controls were carried out as described above, except that after suspension in MP buffer SDS was added to 1% (vol/vol) and samples heated at 95°C for 5 min before the addition of 4 vol of IP dilution buffer. The anti-Sec61p antibodies had been coupled to Sepharose beads before addition (Görlach et al., 1992b). All samples were analyzed on 10–15% SDS polyacrylamide gels which were subjected to fluorography with Entensify as directed by the manufacturer (New England Nuclear, Boston, MA).

Results

P37 is Photocross-linked to Type I and Type II Signal-Anchor Proteins

To determine ER proteins which are involved in the insertion of nascent proteins into the membrane of the ER we have used a photocross-linking approach. This approach has been successfully used to identify a small number of ER proteins which are found in the immediate vicinity of membrane proteins (High et al., 1991b; Thrift et al., 1991) and secretory proteins (Krieg et al., 1989; Wiedmann et al., 1987) during their insertion into or translocation across the membrane of the ER.

In our previous work we found that the major photocross-linking partner of the type I signal-anchor protein IMC-CAT was a previously undescribed 37-kD nonglycosylated protein, denoted P37 (High et al., 1991b). P37 was not found as a major photocross-linked partner of a type II signal-anchor protein the transferrin receptor (High et al., 1991b). This result had at least two possible interpretations: either different ER proteins were in proximity to the nascent chains of type I and type II signal-anchor proteins, or, different regions of the nascent chains were close to different ER proteins. In the first case, the different proteins could mediate the different orientations of the type I and type II signal-anchor proteins. In the second case, the exact position of the lysine residues within the nascent chain from which photocross-linking occurred would determine which ER protein was photocross-linked. To distinguish between these two possibilities we repeated the photocross-linking experiments with a different type II signal-anchor protein, the invariant chain of MHC class II molecules (Ii) (Lipp and Dobberstein, 1986b).

As in the previous study, a stable nascent chain/ribosome/SRP complex was generated by translation in the presence of canine SRP and the absence of microsomes. Upon the addition of microsomes the SRP arrest was relieved and chain extension allowed to continue to the end of the truncated mRNA (Krieg et al., 1989). In the case of IMC-CAT₁₀₃ and Ii₁₀₃ the truncations were chosen to be the same length as the major SRP arrested species (High et al., 1991b) and no further synthesis occurred upon the addition of membranes. In contrast, for IMC-CAT₁₅₀ elongation resumed after the addition of membranes to give a 150 amino acid NH₂-terminal fragment. The position of the lysine residues within these various nascent chains is shown in Fig. 1 a. The nascent chains remain attached to the ribosome as peptidyl-tRNA and, upon insertion into the membrane, form translocation intermediates which are thought to interact with the membrane insertion machinery. An outline of the proposed

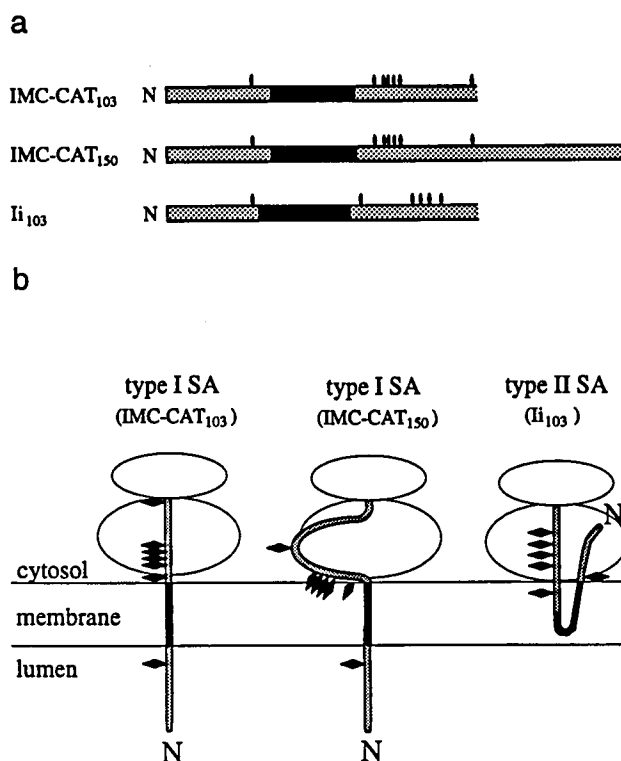


Figure 1. (a) shows the position of lysine residues (black diamonds) present in the nascent chains of COOH-terminally truncated IMC-CAT (IMC-CAT₁₀₃ and IMC-CAT₁₅₀) and Ii (Ii₁₀₃). Black boxes indicate the hydrophobic core of the signal-anchor sequences. Lysine residues are present at positions 27, 69, 72, 73, 75, 77, and 101 of IMC-CAT₁₀₃ and IMC-CAT₁₅₀ and positions 27, 64, 80, 83, 86, and 90 of Ii₁₀₃. (b) Shows putative translocation intermediates of type I (IMC-CAT) and type II (Ii) signal-anchor proteins (see High et al., 1991b). While the NH₂ terminus of IMC-CAT is translocated (High et al., 1991a,b) the NH₂ terminus of type II proteins such as Ii remains on the cytoplasmic side of the membrane (High et al., 1991b). The estimated position of the lysine residues (see Krieg et al., 1989) is indicated by the black diamonds.

translocation intermediates is shown in Fig. 1 b (after Krieg et al., 1989).

Photocross-linking analysis of IMC-CAT₁₀₃, Ii₁₀₃ and IMC-CAT₁₅₀ was carried out to determine the major protein components which interact with the nascent chains during membrane insertion (Fig. 2). Two categories of photocross-linked partners were observed all of which were dependent upon UV irradiation (cf Fig. 2, lanes 7–9 and 10–12). A portion of the nascent chain was found photocross-linked to SRP54 because the release of the SRP from the nascent chain upon the addition of membranes is often incomplete (High et al., 1991a). Part of the SRP54 photocross-linked products stayed associated with the membranes when they were isolated by centrifugation through a high salt/sucrose cushion (Fig. 2, lanes 10–12). These were, however, removed from the membranes by subsequent extraction of the high salt pellet with sodium carbonate solution (Fig. 2, lanes 13–15). The photocross-linking products between SRP54 and the various nascent chains were all immunoprecipitated by antibodies specific for SRP54 (High et al., 1991b; data not shown) and present in the supernatant after extraction with sodium carbonate solution (Fig. 2, lanes 16–18).

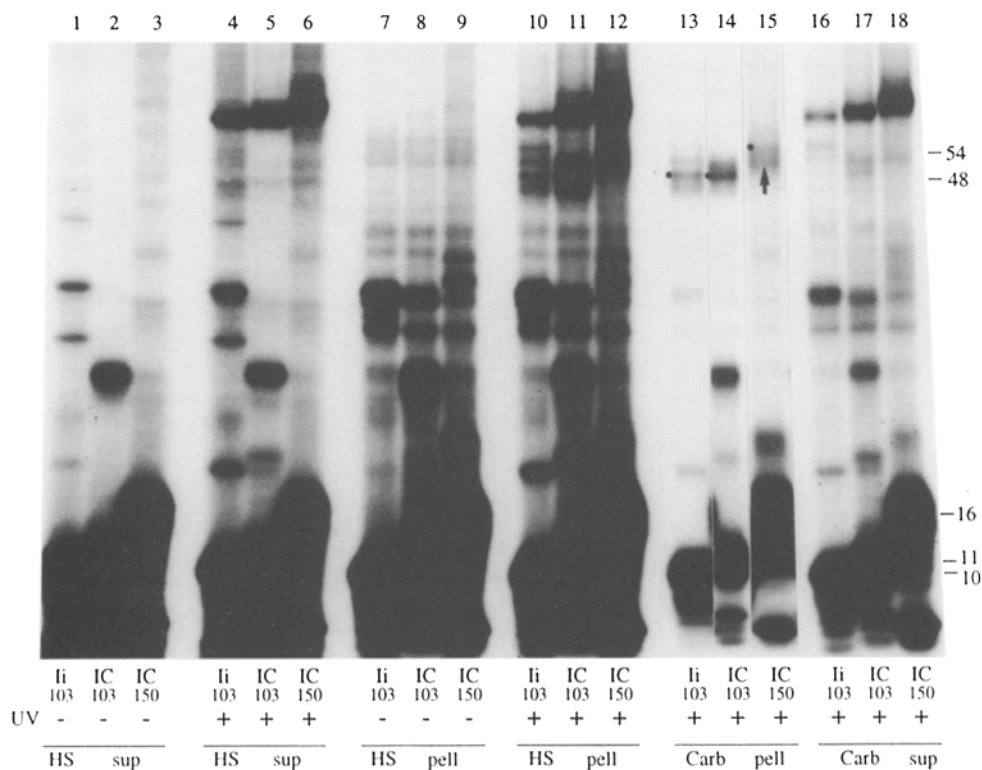


Figure 2. Photocross-linking of nascent signal-anchor membrane proteins to ER membrane components. Nascent chain/ribosome/SRP complexes were allowed to interact with microsomal membranes and subsequently UV irradiated to induce cross-linking (+UV). Control samples were not irradiated (-UV). The nascent chains used were Li₁₀₃ (Li₁₀₃), IMC-CAT₁₀₃ (IC₁₀₃) and IMC-CAT₁₅₀ (IC₁₅₀). Membrane associated components were separated by centrifugation through a high salt/sucrose cushion to give a supernatant containing unbound components (HS sup) and a pellet containing membrane bound material (HS pell). Both were analyzed directly, and in addition, the membrane pellet was subjected to extraction with sodium carbonate solution to give a membrane pellet containing integral membrane proteins (Carb pell) and a supernatant containing periph-

erally bound and luminal proteins (Carb sup). Molecular masses (kD) of the nascent chains and photocross-linking products are indicated. Photocross-linking products between the various nascent chains and a protein of 37-38 kD (black circle) and a protein of 35 kD (arrow) are also shown.

The second group of photocross-linking products were only seen upon the addition of microsomes (data not shown) and were resistant to carbonate extraction (Fig. 2, lanes 13-15). The 11-kD nascent chain of the type I signal-anchor protein IMC-CAT₁₀₃ was photocross-linked to P37, a 37-kD nonglycosylated protein (Fig. 2, lane 14), as previously shown (High et al., 1991b). The 103 amino acid truncation of the type II signal-anchor protein Li (Li₁₀₃) gave an almost identical result (cf, Fig. 2, lanes 13 and 14) despite being in the opposite orientation (Fig. 1 b; Lipp and Dobberstein, 1986b). The apparent molecular weight of Li₁₀₃ is 10 kD, the major photocross-linking product is 48 kD (Fig. 2, lane 13) giving an estimated size of 38 kD for the photocross-linked component. Fainter bands of 51-52 kD, which remained in the membrane pellet obtained after extraction with sodium carbonate solution, were observed with IMC-CAT₁₀₃ and Li₁₀₃ (Fig. 2, lanes 13 and 14). Because these products are not immunoprecipitated with an antiserum specific for the nascent chains (see Fig. 4, lanes 2 and 6) we conclude that these bands do not represent photocross-linking products.

The extension of the truncated nascent chain of IMC-CAT to 150 amino acids (IMC-CAT₁₅₀) will allow only an increase in the length of the nontranslocated COOH terminus (High et al., 1991b). This longer IMC-CAT nascent chain resulted in two major products being visible as opposed to the one major product seen with IMC-CAT₁₀₃ (cf Fig. 2, lanes 14 and 15). Both of these products were dependent upon UV irradiation and were immunoprecipitated by an antibody specific for the nascent chain (Fig. 4, lane 10). The

apparent molecular weights of the two products were 51 (Fig. 2, lane 15, arrow) and 54-kD (Fig. 2, lane 15, black circle) giving an estimate of 35 and 38 kD for the interacting components after subtracting the 16-kD contribution of the nascent chain. A longer truncation of Li encoding the NH₂-terminal 123 amino acids (Li₁₂₃) was also used for photocross-linking analysis. A major photocross-linking product to a protein of 38 kD was observed only when microsomes were added (data not shown). Thus, we find the P37 protein close to all the membrane proteins we have studied with the exception of the transferrin receptor (High et al., 1991b).

Treatments which disrupt translocation intermediates by removing the ribosome (EDTA or puromycin and high salt) prevent the ability of IMC-CAT₁₀₃ to be photocross-linked to P37 (High et al., 1991b). Under these conditions, although the nascent chain remains inserted in the membrane (i.e., resistant to extraction with sodium carbonate solution), it is no longer in the proximity of the P37 component. The treatment of the Li₁₀₃ and IMC-CAT₁₅₀ translocation intermediates with puromycin and high salt before activation of the photocross-linking group also abolished photocross-linking to P37 (data not shown). Thus, in each case, the nascent chain must be present within the membrane in the context of the ribosome, and therefore as a bona fide translocation intermediate, in order to interact with P37.

P37 is the Mammalian Homologue of Sec61p

Analysis of the photocross-linking products for concanavalin A Sepharose binding (High et al., 1991b; data not shown)

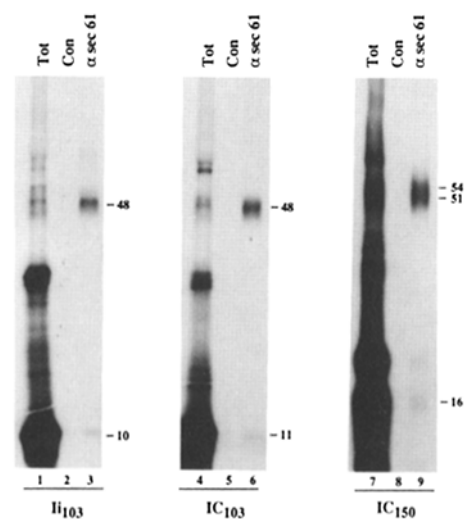


Figure 3. Immunoprecipitation of photocross-linking products with anti-Sec61 antibodies. After photocross-linking of Ii₁₀₃ (Ii₁₀₃), IMC-CAT₁₀₃ (IC₁₀₃) and IMC-CAT₁₅₀ (IC₁₅₀) to ER membrane components, the membranes were isolated by centrifugation through a high salt/sucrose cushion. The products were then analyzed directly (Tot), or after immunoprecipitation in the presence of 0.2% SDS with a control antiserum (Con), or Sepharose coupled antibodies recognizing Sec61p (αSec61). Immunoprecipitations were carried out from 2.5-fold (lanes 8 and 9) or fivefold (lanes 2, 3, 5, and 6) more material than used for the total samples. Molecular masses (kD) of the nascent chains and photocross-linking products are indicated.

showed that the P37 protein was not glycosylated. The apparent size of the protein, together with the absence of glycosylation, resembled the recently identified mammalian Sec61p (Görlich et al., 1992b). For this reason, we used antibodies specific for Sec61p to immunoprecipitate the photocross-linking products obtained with IMC-CAT₁₀₃, Ii₁₀₃ and IMC-CAT₁₅₀. In each case, the major photocross-linking product between the nascent chains and the P37 protein was efficiently immunoprecipitated (Fig. 3, lanes 3, 6, and 9). The immunoprecipitated products showed a broad band centered around a cross-link to a 37–38-kD protein as previously seen by Görlich et al. (1992b). This suggests that either the Sec61p is heterologous, or that the nascent chain might be photocross-linked to several different sites within the protein. In the latter case these different photocross-linking adducts may have different mobilities upon gel electrophoresis. In the case of IMC-CAT₁₅₀ both the 35 and 38 kD photocross-linked proteins were immunoprecipitated, once again as broad bands. It seems unlikely that this is due to proteolysis of the photocross-linking product because this was not observed with the other nascent chains. One possibility is that the 35-kD protein is a second homologue of Sec61p which is recognized by the antibodies used for immunoprecipitation. Incorrectly spliced cDNA clones of a second Sec61p homologue have been reported (Görlich et al., 1992b). Alternatively, the two photocross-linking products may represent different sites of photocross-linking between IMC-CAT₁₅₀ and Sec61p. We conclude that Sec61p contacts IMC-CAT₁₀₃, Ii₁₀₃ and IMC-CAT₁₅₀ during their membrane

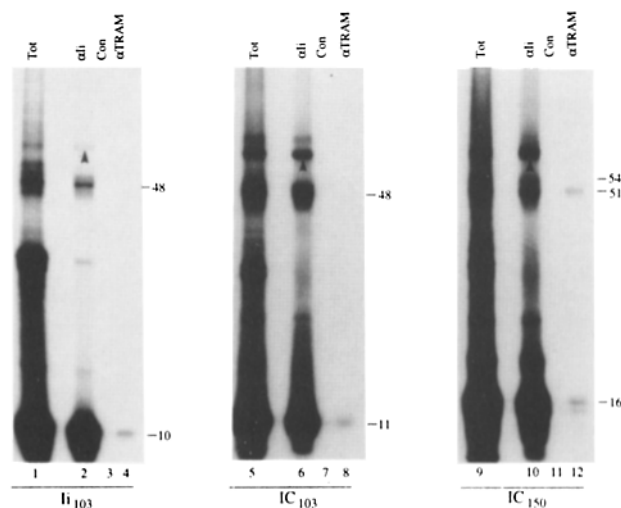


Figure 4. Immunoprecipitation of photocross-linking products with anti-TRAM antibodies. After photocross-linking of Ii₁₀₃ (Ii₁₀₃), IMC-CAT₁₀₃ (IC₁₀₃) and IMC-CAT₁₅₀ (IC₁₅₀) to ER membrane components, the membranes were isolated by centrifugation through a high salt/sucrose cushion. The products were then analyzed directly (Tot) or after immunoprecipitation, in the absence of SDS, with an antiserum recognizing the NH₂ terminus of both Ii and IMC-CAT (αIi), a control antiserum (Con), or antibodies recognizing the translocating chain associating membrane protein (αTRAM). Molecular masses (kD) of the nascent chains and photocross-linking products are indicated. Residual photocross-linking products of the nascent chains and SRP54 are indicated by arrow heads. The samples shown in lanes 1 to 4 were run on a different gel from those in lanes 5 to 12.

insertion, and that the previously identified P37 component (High et al., 1991b) is identical to mammalian Sec61p (Görlich et al., 1992b).

A 36-kD glycoprotein, the translocating nascent chain associating membrane (TRAM) protein, has been shown to be the major photocross-linking partner of short PPL nascent chains (Görlich et al., 1992a). We also used antibodies specific for the TRAM protein to analyze the photocross-linking products obtained with the three nascent chains. The products obtained with IMC-CAT₁₀₃ and Ii₁₀₃ showed no reactivity with the anti-TRAM antibodies (Fig. 4, lanes 4 and 8). The 54-kD product obtained with IMC-CAT₁₅₀ was also not recognized (Fig. 4, lane 12). However, a 51-kD product is weakly immunoprecipitated by the anti-TRAM antibodies under conditions where the photocross-linking product between a PPL fragment and the TRAM protein is quantitatively immunoprecipitated (Görlich et al., 1992a). This low efficiency of immunoprecipitation is consistent with the 51-kD product representing photocross-linking of the nascent chain to at least two proteins of similar apparent molecular weight. Our results show that the bulk of this product represents photocross-linking to a Sec61p related protein, while a minor portion represents photocross-linking to the TRAM protein.

Sec61p Is the Major Cross-linked ER Component when a Homobifunctional Reagent Is Used

The homobifunctional amine reactive cross-linking reagent

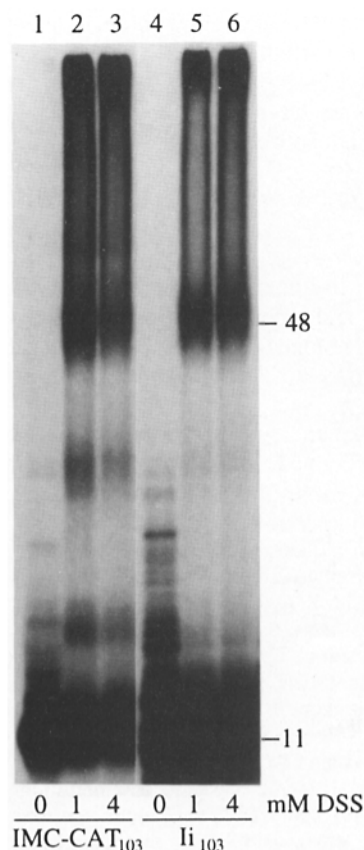


Figure 5. Cross-linking of IMC-CAT₁₀₃ and Ii₁₀₃ to ER membrane components using DSS. Translocation intermediates of the IMC-CAT₁₀₃ and Ii₁₀₃ nascent chains were incubated with 0, 1, or 4 mM DSS as indicated, and the products extracted with sodium carbonate solution. The resulting membrane pellets were analyzed by SDS PAGE. Molecular masses (kD) are indicated.

DSS has been used to identify a 34-kD nonglycosylated protein (imp34) which is adjacent to the translocation intermediates of two proteins with cleavable signal sequences (Kellaris et al., 1991). We used the same cross-linking reagent to confirm the proximity of the nascent membrane proteins to Sec61p. IMC-CAT₁₀₃ and Ii₁₀₃ were cross-linked to adjacent components with DSS, and the membrane pellets obtained after extraction with sodium carbonate solution were analyzed. A major product of 48 kD was observed with both nascent chains (Fig. 5, lanes 2, 3, 5 and 6). The products were only seen when DSS was included in the reaction (see Fig. 5, lanes 1 and 4) and above 1 mM DSS the yield of cross-linked products was not concentration dependent.

The DSS-dependent cross-linking products obtained with IMC-CAT₁₀₃ and Ii₁₀₃ were analyzed by immunoprecipitation with antibodies specific for Sec61p and TRAM. In both cases, the 48-kD cross-linking product was efficiently immunoprecipitated by anti-Sec61p antibodies (Fig. 6, lanes 2 and 5), but not with anti-TRAM antibodies (Fig. 6, lanes 3 and 6). Thus, using two different cross-linking approaches, photocross-linking and a homobifunctional cross-linking reagent, nascent type I and type II signal-anchor proteins are found adjacent to Sec61p.

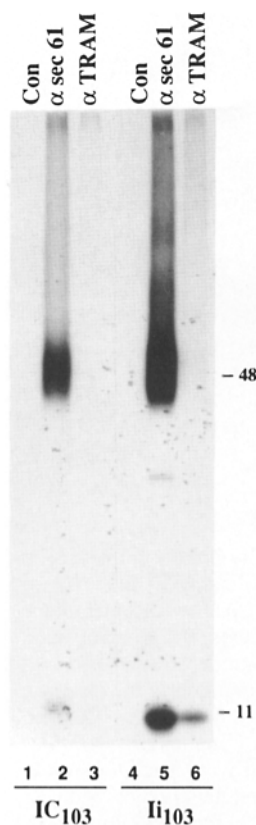


Figure 6. Immunoprecipitation of DSS cross-linked products. IMC-CAT₁₀₃ and Ii₁₀₃ were cross-linked to ER membrane components with 1 mM DSS. Samples were immunoprecipitated in the presence (lanes 1, 2, 4, and 5) or absence (lanes 3 and 6) of 0.2% SDS with a control antiserum (Con), sepharose coupled antibodies recognizing Sec61p (αSec61), or antibodies recognizing the translocating chain associating membrane protein (αTRAM). Nascent chain specific antiserum (αIi) no longer recognized the nascent chains after modification with DSS (data not shown).

Discussion

Proteins which span the membrane once, and have an uncleaved signal-anchor sequence, can insert into the ER membrane in one of two orientations: with the COOH terminus cytoplasmic (type I) or NH₂ terminus cytoplasmic (type II). While there is evidence that the insertion of proteins into the ER membrane is a protein mediated process, the mechanism by which these two different orientations are generated is not known (see High and Dobberstein, 1992a). The insertion of signal-anchor proteins in one of two possible orientations could be mediated by the same ER membrane insertion machinery or alternatively a different machinery could be responsible for each orientation. To distinguish between these possibilities, we have previously used one example each of a type I and a type II signal-anchor protein and analyzed the requirements of the membrane insertion process (High et al., 1991a) and the ER proteins in their immediate proximity during this process (High et al., 1991b). The membrane integration of both type I and type II signal-anchor proteins showed an absolute requirement for GTP (High et al., 1991a) as had been previously found for the first stages in the translocation of secretory proteins across the ER (Connolly and Gilmore, 1986). This GTP requirement was to allow the release of the nascent chain from the SRP54 (Connolly and Gilmore, 1989; Connolly et al., 1991; High et al., 1991a) showing that the SRP dependent targeting step was the same in each case.

To detect the ER proteins which were adjacent to different nascent chains during their membrane insertion, we used a photocross-linking approach which had previously been suc-

cessfully applied to study the translocation of secretory proteins (Krieg et al., 1989; Wiedmann et al., 1987). The results showed that different proteins were found photocross-linked to the type I and type II signal-anchor proteins used (High et al., 1991b). The P37 component was only found photocross-linked to the type I signal-anchor protein IMC-CAT, suggesting that it may be specific for type I signal-anchor proteins. This result could also be due to the different positions of lysine residues within the type I and type II signal-anchor proteins used or because the different membrane proteins will form different types of translocation intermediates (cf Fig. 1 b) which interact with different proteins.

In the present work we have extended our investigation to include another type II signal-anchor protein and also used translocation intermediates generated by truncations of different lengths. Photocross-linking analysis showed that during their membrane insertion P37 is closely associated with all the nascent chains used in this study, independent of orientation and chain length. The P37 component proved to be identical to mammalian Sec61p recently identified by Görlich et al. (1992b). The use of the homobifunctional reagent DSS for cross-linking yielded identical results to those obtained with the photocross-linking approach, and Sec61p was found to be the major cross-linking partner of both the type I (IMC-CAT₁₀₃) and type II (Ii₁₀₃) signal-anchor proteins used.

In addition to P37, which we show here to be identical to mammalian Sec61p, other studies have identified nonglycosylated proteins which are adjacent to nascent membrane and secretory proteins during membrane insertion or translocation. A 37-kD nonglycosylated protein (imp34) is found cross-linked to two proteins with cleavable signal sequences (Kellaris et al., 1991), and nonglycosylated proteins of 40 kD or less have also been found photocross-linked to nascent secretory and membrane proteins (Krieg et al., 1989; Thrift et al., 1991). Judging from the results presented here, and in other studies (Görlich et al., 1992b; High, S., B. Martoglio, D. Görlich, S. Andersen, A. Ashford, A. Giner, E. Hartmann, S. Prehn, T. Rapoport, B. Dobberstein, and J. Brunner, submitted for publication), it seems certain that these components also represent cross-linking of the nascent chains to mammalian Sec61p.

We observe that the signal-anchor sequence of IMC-CAT remains close to Sec61p even after the following 90 amino acids have been synthesized (IMC-CAT₁₅₀). This suggests that the arrival of a signal-anchor sequence at the membrane insertion site does not promote its immediate insertion into the phospholipid bilayer. Under these circumstances, it seems unlikely that a signal-anchor sequence could be recognized by a stop transfer sequence receptor leading to stable integration into the bilayer. Rather, our data support the proposal that it is the termination of protein translation which triggers release of the nascent chain from the translocation site (High, 1992; Simon and Blobel, 1991; Thrift et al., 1991).

We also detect a weak interaction between the IMC-CAT₁₅₀ and the TRAM protein. This additional cross-linking partner may arise because one of the lysines which is buried in the ribosome in IMC-CAT₁₀₃ becomes exposed in IMC-CAT₁₅₀ (see Fig. 1 b) allowing photocross-linking to the TRAM protein. Alternatively, during the process of chain

extension, the position of the nascent chain within the membrane insertion complex may be altered and, in addition to an existing contact with Sec61p, it could also move close to TRAM. Reconstitution studies have shown that the TRAM protein is essential for the efficient in vitro translocation of some secretory proteins across the ER membrane (Görlich et al., 1992a), its role in the insertion of membrane proteins remains to be tested.

Present knowledge about the role of Sec61p in membrane insertion and translocation has come both from studies in *Saccharomyces cerevisiae* and, more recently, the identification of a mammalian homologue (Görlich et al., 1992b). Several Sec proteins were identified by a genetic selection as being important for efficient ER translocation in *S. cerevisiae* (Deshaies and Schekman, 1987). Among these, the gene products Sec61p, Sec62p, and Sec63p have been shown to be adjacent to each other in the ER membrane (Deshaies et al., 1991). Translocation intermediates of secretory proteins can be cross-linked to Sec61p, and to a lesser extent Sec62p, (Müsch et al., 1992; Sanders et al., 1992) consistent with a role in protein translocation. Furthermore, mutations in the genes encoding the three Sec proteins have been shown to cause defects in the insertion of some membrane proteins (Green et al., 1992; Stirling et al., 1992) and the translocation of secreted proteins (Deshaies and Schekman, 1989; Rothblatt et al., 1989; Sadler et al., 1989).

We show here that mammalian Sec61p is the principal cross-linking partner of both type I and type II signal-anchor proteins during their membrane insertion. In contrast to our earlier work (High et al., 1991b), this suggests that a single ER protein may be able to mediate the membrane insertion of signal-anchor proteins in both a type I and a type II orientation. It has also been shown that specific regions of the nascent secretory protein PPL are in contact with Sec61p during its translocation across the ER membrane (Görlich et al., 1992b; High, S., B. Martoglio, D. Görlich, S. Andersen, A. Ashford, A. Giner, E. Hartmann, S. Prehn, T. Rapoport, B. Dobberstein, and J. Brunner, manuscript submitted). Taking these data together we propose that membrane protein insertion and secretory protein translocation are mediated by the same ER protein complex, and that Sec61p is a core component of this complex.

Thanks to Joen Luirink for his careful reading of the manuscript, and to Oddmund Bakke for supplying the pGEM4li construct.

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Do 199/5-3 and SFB352).

Received for publication 16 June 1992 and in revised form 9 December 1992.

References

- Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67:835-851.
- Connolly, T., and R. Gilmore. 1986. Formation of a functional ribosome-membrane junction during translocation requires the participation of a GTP-binding protein. *J. Cell Biol.* 103:2253-2261.
- Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. *Cell.* 57:599-610.
- Connolly, T., P. J. Rapiejko, and R. Gilmore. 1991. Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. *Science (Wash. DC)*. 252:1171-1173.
- Deshaies, R. J., S. L. Sanders, D. A. Feldheim, and R. Schekman. 1991. As-

- sembly of yeast SEC proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature (Lond.)*. 349:806-808.
- Deshaies, R. J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633-645.
- Deshaies, R. J., and R. Schekman. 1989. SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. *J. Cell Biol.* 109:2653-2664.
- Gilmore, R., P. Collins, J. Johnson, K. Kellaris, and P. Rapiejko. 1991. Transcription of full-length and truncated mRNA transcripts to study protein translocation across the endoplasmic reticulum. *Methods Cell Biol.* 34: 223-239.
- Görlich, D., E. Hartmann, S. Prehn, and T. A. Rapoport. 1992a. A protein of the mammalian endoplasmic reticulum involved early in polypeptide translocation. *Nature (Lond.)*. 357:47-52.
- Görlich, D., S. Prehn, E. Hartmann, K.-U. Kalies, and T. A. Rapoport. 1992b. A mammalian homologue of Sec61p and SecYp is associated with ribosomes and nascent polypeptides during translocation. *Cell*. 71:489-503.
- Görlich, D., S. Prehn, E. Hartmann, J. Herz, A. Otto, R. Kraft, M. Wiedmann, S. Knespel, B. Dobberstein, and T. A. Rapoport. 1990. The signal-sequence receptor consists of two subunits and is part of a translocation complex in the endoplasmic reticulum as probed by bifunctional reagents. *J. Cell Biol.* 111:2283-2294.
- Green, N., H. Fang, and P. Walter. 1992. Mutants in three novel complementation groups inhibit membrane protein insertion into the soluble protein translocation across the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*. *J. Cell Biol.* 116:597-604.
- Haeuptle, M.-T., N. Flint, N. M. Gough, and B. Dobberstein. 1989. A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. *J. Cell Biol.* 108:1227-1236.
- Haeuptle, M. T., R. Frank, and B. Dobberstein. 1986. Translation arrest by oligodeoxynucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. *Nucleic Acids Res.* 14:1427-1448.
- Hartmann, E., T. A. Rapoport, and H. F. Lodish. 1989. Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl Acad. Sci. USA*. 86:5786-5790.
- High, S. 1992. Membrane protein insertion into the endoplasmic reticulum: another channel tunnel? *Bioessays*. 14:535-540.
- High, S., and B. Dobberstein. 1992a. Mechanisms determining the transmembrane disposition of proteins. *Curr. Opin. Cell Biol.* 4:581-586.
- High, S., and B. Dobberstein. 1992b. Membrane protein insertion into the endoplasmic reticulum: signals, machinery and mechanisms. In *Membrane Biogenesis and Protein Targeting*. New Comprehensive Biochemistry. Vol. 22. W. Neupert and R. Lill, editors. Elsevier Science Publishers, Amsterdam. 105-118.
- High, S., N. Flint, and B. Dobberstein. 1991a. Requirements for the membrane insertion of signal-anchor type proteins. *J. Cell Biol.* 113:25-34.
- High, S., D. Görlich, M. Wiedmann, T. A. Rapoport, and B. Dobberstein. 1991b. The identification of proteins in the proximity of signal-anchor sequences during their targeting to and insertion into the membrane of the ER. *J. Cell Biol.* 113:35-44.
- Kellaris, K. V., S. Bowen, and R. Gilmore. 1991. ER translocation intermediates are adjacent to a nonglycosylated 34-kD integral membrane protein. *J. Cell Biol.* 114:21-33.
- Krieg, U. C., A. E. Johnson, and P. Walter. 1989. Protein translocation across the endoplasmic reticulum membrane: identification by photocross-linking of a 39-kD integral membrane glycoprotein as part of a putative translocation tunnel. *J. Cell Biol.* 109:2033-2043.
- Lipp, J., and B. Dobberstein. 1986a. The membrane spanning segment of invariant chain (I_h) contains a potentially cleavable signal sequence. *Cell*. 46:1103-1112.
- Lipp, J., and B. Dobberstein. 1986b. Signal recognition particle-dependent membrane insertion of mouse invariant chain: a membrane-spanning protein with a cytoplasmically exposed amino terminus. *J. Cell Biol.* 102:2169-2175.
- Lipp, J., and B. Dobberstein. 1988. Signal and membrane anchor function overlap in the type II membrane protein I_hCAT. *J. Cell Biol.* 106:1813-1820.
- Müsch, A., M. Wiedmann, and T. A. Rapoport. 1992. Yeast Sec proteins interact with polypeptides traversing the endoplasmic reticulum membrane. *Cell*. 69:343-352.
- Rapoport, T. A. 1990. Protein transport across the ER membrane. *Trends Biochem. Sci.* 15:355-358.
- Rapoport, T. A., and M. Wiedmann. 1985. Application of the signal hypothesis to the incorporation of integral membrane proteins. *Curr. Top. Membr. Transp.* 24:1-63.
- Rothblatt, J. A., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* 109:2641-2652.
- Sadler, I., A. Chiang, T. Kurihara, J. Rothblatt, J. Way, and P. Silver. 1989. A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to *DnaJ*, and *Escherichia coli* heat shock protein. *J. Cell Biol.* 109:2665-2675.
- Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell*. 69:353-365.
- Simon, S. M., and G. Blobel. 1991. A protein-conducting channel of the endoplasmic reticulum. *Cell*. 65:371-380.
- Stirling, C. J., J. Rothblatt, M. Hosobuchi, R. Deshaies, and R. Schekman. 1992. Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell*. 3:129-142.
- Thrift, R. N., D. W. Andrews, P. Walter, and A. E. Johnson. 1991. A nascent membrane protein is located adjacent to ER membrane proteins throughout its integration and translation. *J. Cell Biol.* 112:809-821.
- von Heijne, G. 1988. Transcending the impenetrable: how proteins come to terms with membranes. *Biochim. Biophys. Acta*. 947:307-333.
- Wickner, W. T., and H. F. Lodish. 1985. Multiple mechanisms of protein insertion into and across membranes. *Science (Wash. DC)*. 230:400-407.
- Wiedmann, M., D. Görlich, E. Hartmann, T. V. Kurzchalia, and T. A. Rapoport. 1989. Photocrosslinking demonstrates proximity of a 34-kDa membrane protein to different portions of preprolactin during translocation through the endoplasmic reticulum. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 257:263-268.
- Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. A signal sequence receptor in the endoplasmic reticulum membrane. *Nature (Lond.)*. 328:830-833.