The Sec61 complex is essential for the insertion of proteins into the membrane of the endoplasmic reticulum

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Abstract Cross-linking studies have implicated Sec61α as the principal component adjacent to newly synthesised membrane proteins during insertion into the endoplasmic reticulum. Using proteoliposomes which have been reconstituted from purified components of the endoplasmic reticulum [Görlich, D and Rapoport, T.A., Cell 75 (1993) 615–630] we have found that the Sec61 complex, consisting of three subunits, is essential for the insertion of single-spanning membrane proteins. This is true for signal-anchor proteins of both orientations, and for proteins with a cleavable signal sequence. These results support the view that Sec61α is a major component of the ER translocation site and promotes both the insertion of membrane proteins and the translocation of secretory proteins.

Key words: Membrane protein biosynthesis; Endoplasmic reticulum; Reconstitution

1. Introduction

The signal sequences which direct membrane proteins to the endoplasmic reticulum (ER) are well defined and of two types: cleavable signal sequences which are proteolytically removed from the nascent protein during insertion, and signal-anchor sequences which act to target the protein to the membrane and subsequently function as the transmembrane anchor of the protein [1]. Proteins which span the membrane once and have a cleavable signal sequence usually have the NH_2-terminus extracytoplasmic (denoted a type I orientation). In contrast, signal-anchor proteins which span the membrane once can have either a type I or a type II (NH_2-terminus cytoplasmic) orientation. The principal factor affecting the orientation which a signal-anchor protein assumes in the membrane appears to be the distribution of charged residues which flank the hydrophobic core of the signal-anchor sequence [2–4].

In order to understand the mechanism by which signal-anchor proteins are inserted into the membrane of the ER in the correct orientation we have analysed the ER components which interact with signal-anchor proteins during their membrane insertion. Signal-anchor proteins show a signal-recognition particle dependent targeting to the ER membrane [5] via a process which utilises GTP hydrolysis to drive the vectorial targeting of newly synthesised proteins to the ER membrane via an interaction with the docking protein (SRP receptor)[6]. After

targeting and the release of signal-anchor sequences from the 54 kDa subunit of SRP the nascent membrane proteins are able to interact with putative components of the membrane insertion machinery [5]. We have used a photocross-linking approach to identify these components and found that the major photocross-linking partner of nascent type I and type II signal-anchor proteins is an integral membrane protein denoted Sec61α [7]. Other groups have identified non-glycosylated proteins of between 34 and 39 kDa as being adjacent to nascent membrane proteins during insertion into the ER [8,9]. Although formal identification of these proteins remains to be made it seems likely that these proteins are Sec61α.

Sec61p was first identified in *Saccharomyces cerevisiae* [10] and found to be a major cross-linking partner of secretory proteins artificially trapped during the translocation process (denoted 'translocation intermediates') [11,12]. Sec61α, the mammalian homologue of Sec61p, was isolated via its tight association with ribosomes and shown to interact with nascent secretory proteins during translocation across the ER membrane [13]. A second protein, TRAM, was also shown to interact with nascent secretory proteins [14], in particular the NH_2-terminal region of the cleavable signal sequence [15,16]. The translocation of a region of a polypeptide is a prerequisite for its insertion into the ER membrane and a role for Sec61p in membrane protein insertion was suggested by the observation that mutations in the gene can cause defects in the insertion of some membrane proteins [17,18]. Immunoprecipitation showed that the mammalian homologue, Sec61α, is the major cross-linking partner of signal-anchor proteins when the interactions of the nascent chains were analysed both by photocross-linking and by the use of homobifunctional cross-linking reagents [7]. Mammalian Sec61α is part of a protein complex which has at least two other subunits, Sec61β and Sec61γ [19]. Since the available data suggest Sec61α plays a key role in mediating the insertion of signal-anchor proteins into the membrane of the ER we have tested this proposal using reconstituted proteoliposomes prepared from purified components [20].

2. Materials and methods

2.1. Materials
Restriction enzymes were from Boehringer Mannheim, T7 RNA polymerase from Promega and RNAsin from Pharmacia. [^{35}S]Methionine was from Amersham and cycloheximide and 7-methyl-guanosine 5'-monophosphate were supplied by Sigma. Wheat germ lysate, SRP and washed rough microsomes were prepared by published methods [21–23].

2.2. Preparation of proteoliposomes
Proteoliposomes were reconstituted from purified protein compo-

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nents and commercially available phospholipids as previously described [20]. The composition of the proteoliposomes was checked by Western blotting [20]. A total of three different sets of proteoliposome preparations were used for the experiments described (denoted batch A, batch B and batch C in Table 1).

2.3. Transcription and translation

The model membrane proteins used in this study were glycophorin C (Gly C), the invariant chain of the MHC class II complex (Ii), the influenza (Japan strain) haemagglutinin glycoprotein (HA) and glycophorin A (Gly A). Plasmids encoding the model proteins were transcribed using T7 RNA polymerase as described by the manufacturer (Promega) and the resulting RNA was translated in a wheat germ translation system in the presence of [^{35}S]methionine and supplemented with purified signal recognition particle (SRP) and canine pancreatic microsomes or proteoliposomes [21]. The membrane insertion of Gly C and HA was performed by translating the encoding RNAs in a 25 μl reaction volume for 30 min (Gly C) or 45 min (HA) at 26°C in the presence of 25 nM SRP and 1.5 μl of microsomes, proteoliposomes or buffer. For Gly A, a 50 μl reaction volume containing 2 μl of microsomes, proteoliposomes or buffer was translated for 1 h as described above. In all three cases, cycloheximide was added to 2 mM and samples analysed as described below. In the case of Ii, an SRP arrested fragment was generated by incubating the encoding RNA in a wheat germ translation system supplemented with 25 nM SRP but lacking any membranes. After 10 min 7-methyl-guanosine 5'-monophosphate was added to a final concentration of 4 mM and the incubation continued for a further 5 min. 1.5 μl of microsomes, proteoliposomes or buffer were then added, the samples incubated for 10 min and cycloheximide added to 2 mM. The use of N-ethylmaleimide (NEM) to inactivate rough microsomes and acceptor tripeptide to inhibit asparagine linked glycosylation followed published protocols [24,25].

2.4. Membrane insertion assay

After protein synthesis, 10% of the final translation volume was removed to assess total protein synthesis. The remainder of the samples was layered over a 150 μl cushion (50 mM HEPES-KOH, pH. 7.9, 5 mM Mg(OAc)$_2$, 500 mM KOAc and 250 mM sucrose) in Beckman TLA100 tubes. These were spun for 10 min at 100,000 × g and 4°C. Western blot analysis for constituent proteins confirmed that both the

microsomes and the various proteoliposome preparations were all pelleted under these conditions (data not shown). The supernatant and cushion was removed and discarded and the pellets were resuspended in 50 μl of buffer (50 mM HEPES-KOH, pH. 7.9, 5 mM Mg(OAc)$_2$, 100 mM KOAc and 250 mM sucrose). For Gly C, Ii and HA, 20% of the resuspended pellet was TCA precipitated to assess the amount of membrane associated material while 70% was incubated with 0.44 mg/ml proteinase K for 30 min on ice to determine the amount of protease protected material. For Gly A, 45% of the resuspended pellet was TCA precipitated and 45% incubated with 0.5 mg/ml chymotrypsin at 26°C for 1 h. After digestion PMSF was added to 1.5 mg/ml and samples TCA precipitated. The TCA precipitated material from each step of the assay was solubilised in gel loading buffer and samples were analysed on 12% SDS-polyacrylamide gels. In the case of the proteolysed Gly A samples, hot sample buffer was added directly to the incubation after digestion. Dried gels were exposed directly to Kodak X-Omat film for visualisation. For quantitative analysis the gels were analysed using a BAS2000 phosphorimager.

3. Results

3.1. Type I signal-anchor protein insertion

The insertion of the type I signal-anchor protein, glycophorin C (Fig. 1), into reconstituted proteoliposomes was tested. Total protein synthesis was unaffected by the presence of different proteoliposomes, microsomes or buffer in the translation reaction (Fig. 2, panel a). Only in the presence of active microsomes was any glycosylated material observed (Fig. 2, panel a, lane 7). The inclusion of a tripeptide comprising the consensus site for asparagine linked oligosaccharide addition prevented this glycosylation (Fig. 2, panel a, lane 8). When the membrane fraction was isolated, the amount of Gly C associated with this fraction was clearly dependent upon the composition of the proteoliposomes. In the absence of the Sec61 complex (Fig. 2, panel b, lanes 3 and 4) background levels of protein were observed (c.f. no membranes, Fig. 2, panel b, lane 5). Since the
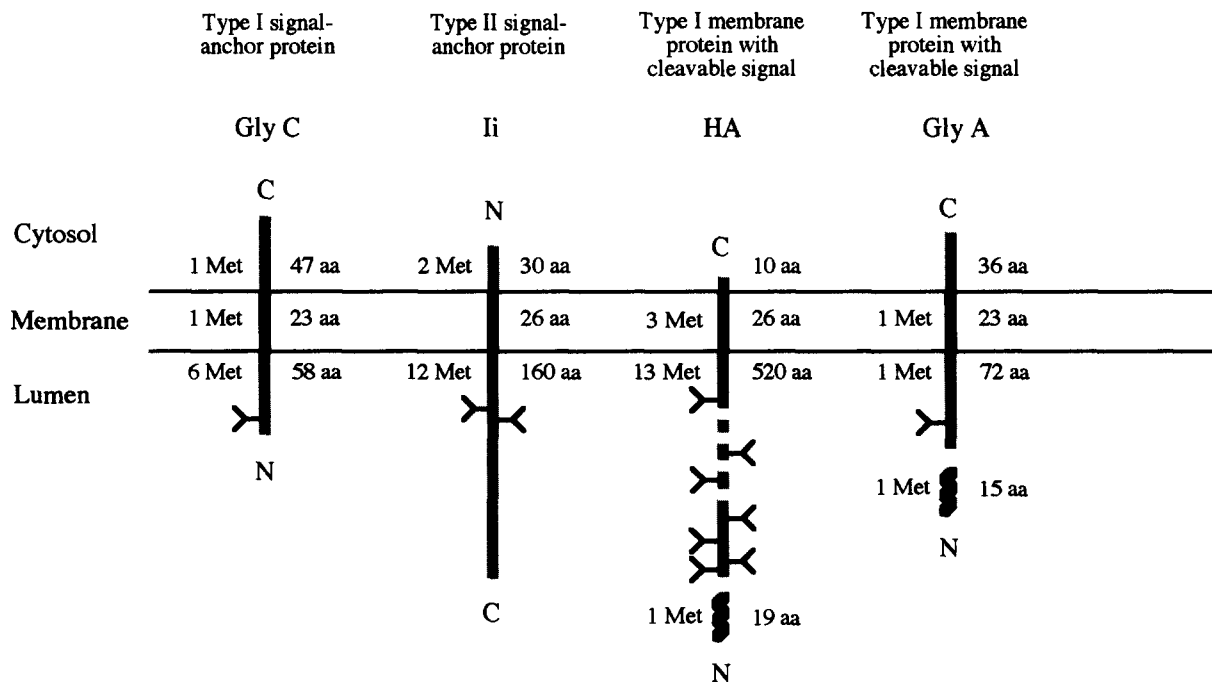

Fig. 1. The orientation of model membrane proteins in the ER membrane. The orientation of the model membrane proteins used in this study is shown. The number of amino acids exposed on the cytosolic and luminal sides of the ER membrane is indicated as well as the predicted length of the transmembrane domain (shown in black). The proteins, HA and Gly A have cleavable signal sequences (black zig-zag) while Gly C and Ii have signal-anchor sequences. The number of the methionine residues which carry the radioactive label are shown for each region, and the number and approximate position of sites modified by asparagine linked carbohydrate side chains are indicated by the branched structure.
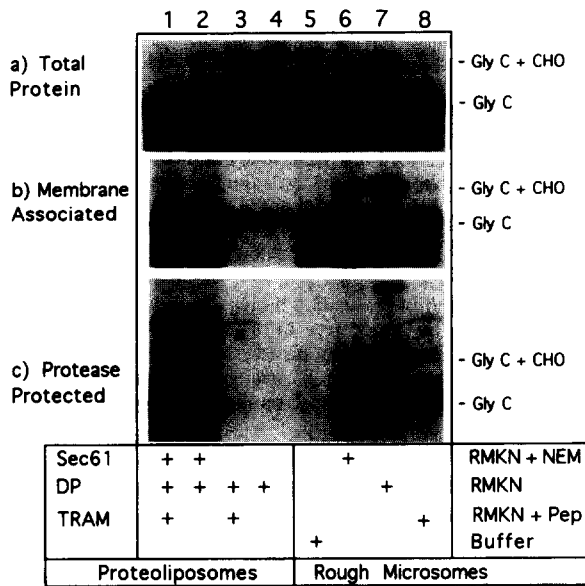
Fig. 2. The insertion of glycophorin C into proteoliposomes. The insertion of glycophorin C (Gly C) into proteoliposomes containing the indicated combinations of the Sec61 complex (Sec61), the docking protein complex (DP), and the TRAM protein (TRAM) was assayed. Controls were nuclease treated, washed, rough microsomes (RMKN), RMKN which had been inactivated by NEM treatment (RMKN + NEM), RMKN plus a tripeptide inhibitor of asparagine linked glycosylation (RMKN + Pep) and the RMKN storage buffer alone (Buffer). Gly C shows the position of the unglycosylated protein and Gly C + CHO shows the position of the glycosylated protein (1 glycosylation site). Panel a shows total protein synthesis, panel b shows the membrane associated material, i.e. the material found in the pellet after centrifugation as described in materials and methods, and panel c shows the protease protected material. The filled arrow heads show the position of Gly C (lanes 1, 2, 3 and 8) and Gly C + CHO (lane 7) which was resistant to proteolytic digestion.

reconstituted proteoliposomes lack the enzymes necessary to glycosylate asparagine residues [20] this modification is not observed and the mobility of the protein is identical to the non-glycosylated protein produced in the presence of canine pancreatic microsomes (Fig. 2, panel b, lane 8). In the control microsomes most of the membrane associated protein is glycosylated (Fig. 2, panel b, lane 7). Canine pancreatic microsomes which had been treated with NEM [24] to prevent membrane insertion had a significant fraction of Gly C associated with the membrane pellet. The samples were treated with proteinase K to determine what fraction of the membrane associated material was actually inserted into the membrane. Only proteoliposomes containing the Sec61 complex showed significant protease protected material (Fig. 2, panel c, lanes 1 and 2), the size of this product was identical to that obtained in microsomes where asparagine linked glycosylation had been inhibited (Fig. 2, panel c, lane 8). The presence of the TRAM protein in addition to the docking protein complex and the Sec61 complex did not affect the efficiency of Gly C insertion (Table 1). The orientation of Gly C in microsomes was confirmed by the observation that all the protease protected material was glycosylated at the single site present in the NH$_2$-terminus (Fig. 2, panel c, lane 7). Since no glycosylation occurs with proteoliposomes, the predicted sizes of the protease protected NH$_2$ and COOH domains of Gly C are not dramatically different (see Fig. 1.) However, six of the total of eight methionine

Table 1
The efficiency of membrane protein insertion into proteoliposomes

Protein	Gly C	Ii	HA	Gly A
Proteolip. Batch	A	B	B	C
Sec61, DP, TRAM	275	36	54	134
Sec61, DP	273	32	3	133
DP, TRAM	0	0	0	0
Sec61, TRAM	n.d.	n.d.	n.d.	20
DP	0	0	0	n.d.
Buffer	0	0	0	0
RMKN + NEM	17	2	0	14
RMKN	100	100	100	100

The material protected against proteolytic digestion (Figs. 2 to 5, panel c) was quantified using a Fuji BAS2000 phosphorimager and the accompanying Fuji software package. In each case the value obtained with the rough microsome control (RMKN) was defined as 100% and all the other figures given are relative to this. Comparisons are therefore only valid within the individual columns. The glycosylated product was quantified for the RMKN + NEM and the RMKN samples (i.e. Gly C + CHO of Fig. 2, panel c, lanes 6 and 7; Ii + 2 CHO of Fig. 3, panel c, lanes 6 and 7; HA + 7 CHO of Fig. 4, panel c, lanes 6 and 7; and Gly A + CHO of Fig. 5, panel c, lanes 6 and 7). For all other samples the non-glycosylated product was quantified (i.e. Gly C of Fig. 2, panel c, lanes 1 to 5; Ii of Fig. 3, panel c, lanes 1 to 5; HA of Fig. 4, panel c, lanes 1 to 5; and Gly A of Fig. 5, panel c, lanes 1 to 5).

residues are present in the NH$_2$-terminal domain, one is in the transmembrane domain and one in the COOH-terminal domain. Of the total radiolabelled protein which was membrane associated (Fig. 2, panel b), an estimated 25% was protected against proteolysis both by the rough microsomes (Fig. 2, panel
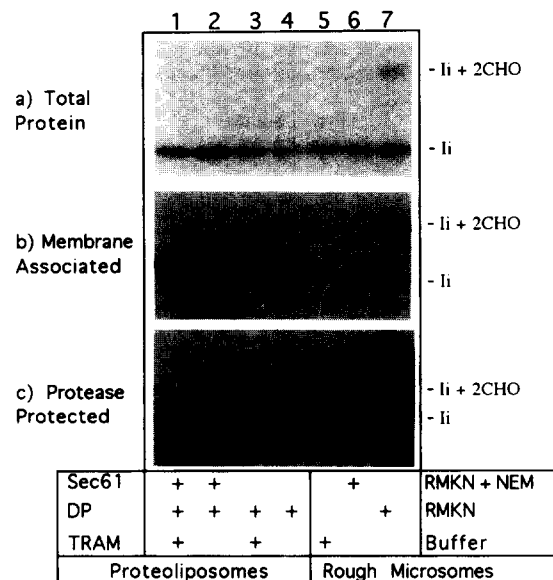

Fig. 3. The insertion of the invariant chain into proteoliposomes. The insertion of the invariant chain (Ii) into proteoliposomes containing the indicated combinations of the Sec61 complex (Sec61), the docking protein complex (DP), and the TRAM protein (TRAM) was assayed. Controls were nuclease treated, washed, rough microsomes (RMKN), RMKN which had been inactivated by NEM treatment (RMKN + NEM) and the RMKN storage buffer alone (Buffer). Ii shows the position of the unglycosylated protein and Ii + 2CHO shows the position of the fully glycosylated protein (2 glycosylation sites). Panel a shows total protein synthesis, panel b shows the membrane associated material, and panel c shows the protease protected material. The filled arrow heads show the position of Ii which was resistant to proteolytic digestion (lanes 1 and 2).

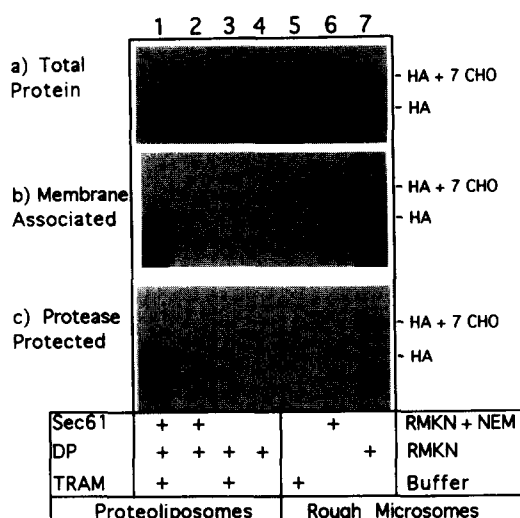| Sec61	+	+			+		RMKN + NEM
DP	+	+	+	+		+	RMKN
TRAM	+	+			+		Buffer
Proteoliposomes					Rough Microsomes		

Fig. 4. The insertion of the haemagglutinin glycoprotein into proteoliposomes. The insertion of the haemagglutinin glycoprotein (HA) into proteoliposomes containing the indicated combinations of the Sec61 complex (Sec61), the docking protein complex (DP), and the TRAM protein (TRAM) was assayed. Controls were nuclease treated, washed, rough microsomes (RMKN), RMKN which had been inactivated by NEM treatment (RMKN + NEM) and the RMKN storage buffer alone (Buffer). HA shows the position of the unglycosylated protein and HA + 7CHO shows the position of the fully glycosylated protein (7 glycosylation sites). Panel a shows total protein synthesis, panel b shows the membrane associated material, and panel c shows the protease protected material.

c, lane 7) and by the functional proteoliposomes (Fig. 2, panel c, lanes 1 and 2) (data not shown). The similar levels of protection confirm that the bulk of the Gly C has inserted into the proteoliposomes in the correct orientation as observed in vivo [26,27]. A small amount of undigested material was observed with both the proteoliposomes and microsomes (Fig. 2, panel c, arrowheads).

3.2. Type II signal-anchor protein insertion

As a model type II signal anchor protein the invariant chain of the MHC class II complex was used [28]. The total protein synthesis was not dramatically affected by the different proteoliposome additions (Fig. 3, panel a). Ii was only found in the membrane fraction when functional microsomes (Fig. 3, panel b, lane 7) or proteoliposomes containing the Sec61 complex were used (Fig. 3, panel b, lanes 1 and 2). In all cases this material was protected against proteolysis with the loss of only 3 kDa representing the N-terminal exposed tail (Fig. 1). Both proteoliposome preparations contained a small amount of undigested Ii (Fig. 3, panel c, arrowheads). As for Gly C, the presence of TRAM in the reconstituted proteoliposomes had no significant effect on the efficiency of Ii insertion (Table 1).

3.3. Insertion of type I proteins with a cleavable signal sequence

Influenza haemaglutinin (HA) is a large type I membrane protein with a cleavable signal sequence [29]. Analysis showed that the presence of the Sec61 complex in the reconstituted proteoliposomes was essential for both membrane association (Fig. 4, panel b, lane 1) and membrane insertion (Fig. 4, panel c, lane 1). In contrast to the two signal-anchor proteins, the TRAM protein was also essential for membrane association

and insertion (Table 1). In the absence of the TRAM protein, almost no HA was recovered in the membrane pellet (Fig. 4, panel b, lane 2) or was protected against protease (Fig. 4, panel c, lane 2).

A second type I membrane protein with a cleavable signal sequence, glycophorin A [30], was analysed to determine if a requirement for TRAM was specific for this class of proteins. Gly A was found to be associated with all proteoliposomes which contained the Sec61 complex, whether or not the TRAM protein was also present (Fig. 5, panel b, lanes 1, 2 and 4). Protease resistant material, indicating insertion, was observed in the proteoliposomes containing the Sec61 complex and DP, or Sec61 complex, DP and TRAM (Fig. 5, pane c, lanes 1 and 2). The amount of protease protected material was not influenced by the presence of the TRAM protein (Table 1). Although a large amount of material was found associated with proteoliposomes containing the Sec61 complex and TRAM but lacking DP (Fig. 5, panel b, lane 4), only a small fraction of this material was protected against proteolysis (Fig. 5, panel c, lane 4).

4. Discussion

The results presented above show that the Sec61 complex is required for the correct insertion of all types of single spanning membrane proteins. A role for Sec61α in this process had been proposed on the basis that it is the major cross-linking partner of type I and type II signal-anchor proteins [7]. These results are in agreement with those of Görlich and Rapoport [20] who

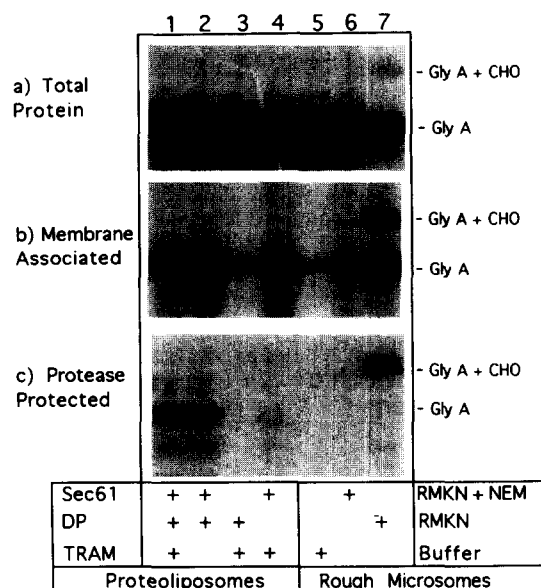| Sec61	+	+		+		+		RMKN + NEM
DP	+	+	+				+	RMKN
TRAM	+		+	+		+		Buffer
Proteoliposomes						Rough Microsomes		

Fig. 5. The insertion of glycophorin A into proteoliposomes. The insertion of glycophorin A (Gly A) into proteoliposomes containing the indicated combinations of the Sec61 complex (Sec61), the docking protein complex (DP), and the TRAM protein (TRAM) was assayed. Controls were nuclease treated, washed, rough microsomes (RMKN), RMKN which had been inactivated by NEM treatment (RMKN + NEM) and the RMKN storage buffer alone (Buffer). Gly A shows the position of the unglycosylated protein and Gly A + CHO shows the position of the glycosylated protein (1 glycosylation site). Panel a shows total protein synthesis, panel b shows the membrane associated material, and panel c shows the protease protected material. The filled arrow heads show the position of Gly A which was resistant to proteolytic digestion (lanes 1 and 2).

showed that the Sec61 complex was required for the insertion of a type I membrane protein with a cleavable signal sequence (vesicular stomatitis virus glycoprotein) and a type II signal anchor protein (asialoglycoprotein receptor). Our results show that the Sec61 complex is necessary and sufficient for the correct insertion of type I signal-anchor proteins suggesting that no additional proteins are required to promote the translocation of the NH_2-terminus of these proteins [4,7]. This observation is in contrast to the situation in *E. coli* where the translocation of a comparable NH_2-terminal domain has been shown to occur independently of the *sec* machinery normally responsible for protein translocation [31].

Our results also show that a requirement for the TRAM protein is not restricted to any particular class of membrane proteins. In this paper we show examples of type I signal-anchor proteins, type II signal anchor proteins and type I membrane proteins with a cleavable signal sequence which are all efficiently inserted into proteoliposomes in the absence of TRAM. In other cases the insertion of type I membrane proteins with a cleavable signal sequence shows an absolute dependency on TRAM [20 and this study] while the insertion of a type II signal-anchor protein is stimulated over two fold [20]. These results mirror the effect of TRAM on the efficiency of secretory protein translocation into proteoliposomes which varies widely depending on the secretory protein studied [20]. Our observation that type I and type II signal-anchor proteins can be correctly inserted into the membrane in the absence of TRAM suggests that this protein does not play a universal role in orienting signal-anchor sequences during membrane insertion [32]. A direct role for the Sec61 complex in mediating the insertion of all classes of single-spanning membrane proteins at the ER has now been established. The mechanism by which this occurs remains to be elucidated.

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