

Requirements for the Membrane Insertion of Signal-anchor Type Proteins

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Abstract. Proteins which are inserted and anchored in the membrane of the ER by an uncleaved signal-anchor sequence can assume two final orientations. Type I signal-anchor proteins translocate the NH₂ terminus across the membrane while type II signal-anchor proteins translocate the COOH terminus. We investigated the requirements for cytosolic protein components and nucleotides for the membrane targeting and insertion of single-spanning type I signal-anchor proteins. Besides the ribosome, signal recognition particle (SRP), GTP, and rough microsomes (RMs) no other components were found to be re-

quired. The GTP analogue GMPPNP could substitute for GTP in supporting the membrane insertion of IMC-CAT. By using a photocrosslinking assay we show that for secreted, type I and type II signal-anchor proteins the presence of both GTP and RMs is required for the release of the nascent chain from the 54-kD subunit of SRP. For two of the proteins studied the release of the nascent chain from SRP54 was accompanied by a new interaction with components of the ER. We conclude that the GTP-dependent release of the nascent chain from SRP54 occurs in an identical manner for each of the proteins studied.

THE known signals which are capable of directing both secreted and membrane proteins to the ER are of two types. The first are NH₂-terminal cleaved signal sequences which are present on both secreted and membrane proteins (Walter and Lingappa, 1986). These signals have a targeting role only and in the absence of other signals the protein will be translocated across the membrane into the lumen of the ER and the signal sequence cleaved off. Membrane proteins of this type possess a second "stop transfer" sequence which prevents complete translocation and stably anchors the protein in the membrane (Rapoport and Wiedmann, 1985).

A number of single-spanning membrane proteins which lack a cleaved signal sequence have been described. In these proteins a single sequence serves to both target the protein to the ER and to anchor the protein in the membrane (Spiess and Lodish, 1986; Zerial et al., 1986). This type of signal has been called a signal-anchor (SA)¹ sequence to distinguish it from the cleaved NH₂-terminal signals (Lipp and Dobberstein, 1988).

For a single-spanning membrane protein two orientations are possible. Those with the NH₂-terminus extracytoplasmic are denoted type I while those with the NH₂-terminus cytoplasmic are denoted as type II (Lipp and Dobberstein, 1986a; Holland and Drickamer, 1986). Examples of SA membrane proteins of both orientations are known. Glycophorin C assumes a type I orientation (High and Tanner,

1987) while the transferrin receptor (TR) and invariant chain of MHC class II molecules are known to assume a type II orientation (Zerial et al., 1986; Lipp and Dobberstein, 1986a). All known membrane proteins with a cleaved NH₂-terminal signal sequence show a type I orientation.

Both cleaved NH₂-terminal signals (Walter and Lingappa, 1986) and SA sequences (Lipp and Dobberstein, 1986b; Holland and Drickamer, 1986; Hull et al., 1988) are known to be dependent on signal recognition particle (SRP) for their correct targeting to and insertion into the ER. The requirement for SRP implies that the membrane-bound receptor for SRP, the docking protein (DP) or SRP receptor (Meyer et al., 1982; Gilmore et al., 1982) is also involved in the targeting of all these proteins to the ER membrane. The high affinity binding to rough microsomes of the nascent chain/ribosome complexes of both secreted (Connolly and Gilmore, 1986) and type II SA membrane proteins (Wilson et al., 1988) has been shown to require GTP.

The aim of this study was to determine the necessary cytoplasmic components and energy sources for the membrane insertion of type I SA membrane proteins and to compare these to the requirements for the membrane insertion of type II SA membrane proteins and the translocation of secreted proteins. To this end we used an assay in which translocation is separated from translation and which therefore allows the translocation of secreted proteins or partial translocation and integration of membrane proteins to be studied independently of protein synthesis (Perara et al., 1986; Connolly and Gilmore, 1986). By using the SRP-mediated arrest of translation (Walter and Blobel, 1981) we were able to generate a stable nascent chain/ribosome/SRP complex which was used

1. *Abbreviations used in this paper:* DP, docking protein; PPL, prolactin; SA, signal-anchor; SRP, signal recognition particle; SRP54, 54-kD subunit of SRP; SSR, signal sequence receptor; TR, transferrin receptor.

to study the requirements of membrane insertion. We extended the analysis to include a photocrosslinking assay. This allowed us to directly analyze the proteins closely associated with a nascent chain under different conditions.

Materials and Methods

Materials

T7 RNA polymerase, restriction endonucleases, ATP, GTP, and all GTP analogues were from Boehringer Mannheim GmbH (Mannheim, Germany). AMPPNP was from Pharmacia, LKB GmbH (Freiburg, Germany). ³⁵S-Met was supplied by Amersham Buchler GmbH (Braunschweig, Germany) and proteinase K by Merck (Darmstadt, Germany). pGEM 3 was obtained from Promega Biotech (Madison, WI). Cycloheximide, emetine, and 7-methyl-guanosine were from Sigma Chemical Co. (St. Louis, MO). TDBA, 4-(3-trifluoromethyl-diazirino) benzoic acid, was a gift from Dr. Josef Brunner, Swiss Federal Institute of Technology, Zürich, Switzerland.

General Methods

Carbonate extractions and immunoprecipitation of samples before electrophoresis were as previously described (Wiedmann et al., 1987b; Haeupfle et al., 1989). Preparation of SRP was as described by Walter and Blobel (1983).

Plasmid Constructs

IMC-CAT consists of portions of invariant chain, multiple colony stimulating factor, and chloramphenicol transferase and is derived from the LMC-CAT construct (Haeupfle et al., 1989). An EcoRI fragment carrying IMC-CAT was subcloned from pDS5 into pGEM 3 under the T7 promoter. The TR was the EcoRI fragment described by Zerial et al. (1986) recloned into pGEM 1 under the T7 promoter. Preprolactin (PPL) in pSP64 (Siegel and Walter, 1988) was the gift of Peter Walter, Department of Biochemistry and Biophysics (UCSF, California).

In Vitro Transcription and Translation

Plasmids were linearized with HindIII (IMC-CAT), PvuII (PPL), and NdeI (TR) and transcribed as described by the manufacturer (Promega Biotech). The resulting mRNA was translated in a wheat germ cell-free system (Stueber et al., 1984) containing 20 nM SRP. Following incubation at 25°C for 15 min 7-methylguanosine 5'-monophosphate was added to a final concentration of 2 mM and the reaction mixture was incubated for a further 10 min at 25°C. Cycloheximide was added to a final concentration of 0.25 mM, emetine to 1 mM when used. The mixture was then either added to rough microsomes and assayed for membrane insertion directly or was subjected to centrifugation through a high salt/sucrose cushion (see below).

Apyrase Treatment

The products of a 25 μ l cell-free translation were incubated with 4 U of apyrase at 25°C for 15 min before use in a membrane insertion assay. For membranes 0.3 OD₂₈₀ U of microsomes received 2 U of apyrase and were incubated at 25°C for 15 min before use.

Purification of Nascent Chain/Ribosome/SRP Complex

At the end of cell-free translation the mixture was made 0.5 M with respect to potassium acetate and incubated on ice for 5 min. The mixture was layered over a 0.5 M sucrose cushion containing: 0.5 M KOAc, 30 mM Hepes, pH 7.9, 2.8 mM Mg(OAc)₂, 0.25 mM cycloheximide, 1 mM emetine, and 1 mM dithiothreitol. The mixture was spun at 50,000 rpm (166,320 g) for 1 h at 4°C in a centrifuge (model TL100; Beckman Instruments Inc., Palo Alto, CA). The resulting pellet was resuspended in translocation buffer using 9.5 μ l per 25 μ l of translation mixture loaded. TL buffer consisted of 105 mM KOAc, 4 mM Mg(OAc)₂, 80 mM Hepes, pH 7.9, 2.6 mM emetine, and 0.66 mM cycloheximide.

Reconstituting Membrane Insertion

To reconstitute membrane insertion 9.5 μ l of the resuspended nascent

chain/ribosome/SRP complex was added to 10 μ l of wheat germ compensation buffer (20 mM Hepes, pH 7.6, 50 mM KCl, 1 mM Mg(OAc)₂, 0.1% 2-mercaptoethanol) or 10 μ l of wheat germ lysate. 2 μ l of rough microsomes (0.06 OD₂₈₀ U) in 250 mM sucrose, 50 mM triethanolamine.HCl, pH 7.5, 50 mM KOAc, 2 mM Mg(OAc)₂, and 1 mM DTT were added. When used, nucleotides were added to final concentrations ranging between 100 μ M and 2 mM as indicated in the figure legend and the final volume was adjusted to 25 μ l with distilled water. All reactions involving IMC-CAT were in the presence of 30 μ M acceptor tripeptide, benzoyl-Asn-Leu-Thr-N-methylamide, to block glycosylation of asparagine residues unless otherwise specified.

After incubation with membranes at 25°C for 20 min EDTA was added to a final concentration of 25 mM to dissociate the ribosome from the nascent chain. Membrane insertion was then assayed by adding proteinase K to 300 μ g ml⁻¹. Proteolysis was for 10 min at 25°C and was terminated by addition of PMSF to 10 mM. Membranes were recovered by centrifugation at 26,000 g for 15 min and proteins in the pellet analyzed by electrophoresis on a 22% acrylamide gel containing 6 M urea (Haeupfle et al., 1986). Fluorography was done with Enhance as directed by the manufacturer (New England Nuclear, Boston, MA).

Time Course Experiments

To follow the time course of protein insertion a membrane insertion assay was incubated at 25°C and aliquots removed at various times. Controls showed that incubation with membranes at 37°C gave identical results (data not shown). The aliquots were immediately made 25 mM with respect to EDTA and the samples then frozen on liquid nitrogen. When all time points had been taken samples were thawed on ice and proteinase K added to 300 μ g ml⁻¹. Proteolysis was for 1 h at 0°C and the samples were further processed as described above.

Photocrosslinking

For the photocrosslinking analysis we used truncations of the IMC-CAT and TR mRNAs which were chosen to be the same size as the major SRP-arrested species. This gave a homogenous population of nascent chains which is optimal for clear results upon photocrosslinking (see Krieg et al., 1989). The truncations were achieved by linearization of the TR transcript at the NdeI site within the coding region (expected product 151 amino acids) and for IMC-CAT by inclusion of the complementary oligonucleotide 5' CCCATATCACCAGCTCACCG3' at 40 μ g ml⁻¹ in the wheat germ cell-free translation system (Haeupfle et al., 1986) (expected product 103 amino acids). For PPL the SRP arrest is especially pronounced and results in essentially only a single species of 70 amino acids being produced (Walter and Lingappa, 1986). A PvuII truncated PPL transcript was used (expected product 86 amino acids in absence of SRP) to minimize the amount of any full-length preprolactin synthesized and so prevent photocrosslinks of this molecular weight from being obscured.

After cell-free translation of the transcripts in the presence of ϵ -TDBA-Lys-tRNA (Wiedmann et al., 1987a) the nascent chain/ribosome/SRP complexes were isolated by centrifugation through a high salt/sucrose cushion as described above. The resulting pellet was resuspended in 20 μ l of TL buffer per 25 μ l of translation mixture loaded onto the cushion. 20 μ l of this resuspended mixture was incubated in the presence of 0.06 OD₂₈₀ U of rough microsomes and in the absence of nucleotides or the presence of 500 μ M nucleotides as indicated. When IMC-CAT was used the acceptor tripeptide was present to prevent glycosylation of the nascent chain (see above). After incubation for 5 min at 25°C the samples were placed on ice and subjected to UV irradiation as described by Wiedmann et al. (1987a). The samples were extracted with alkaline sodium carbonate solution and after centrifugation the resulting proteins in the supernatant and membrane pellet were recovered (Fujiki et al., 1982; Wiedmann et al., 1987b). These proteins were analyzed by electrophoresis on 10–15% SDS-polyacrylamide gels which were subjected to fluorography as described above.

Results

The NH₂ Terminus of IMC-CAT Can Be Translocated Independently of Protein Synthesis

To study the membrane insertion of a type I SA protein we used a model protein which can be N-glycosylated in the NH₂-terminal domain and identified by antibodies against the hydrophilic NH₂-terminal portion. LMC-CAT is a sig-

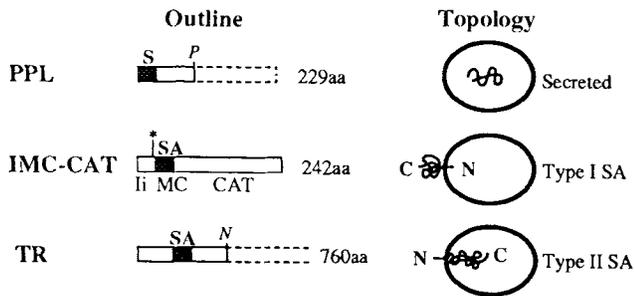


Figure 1. Outline and membrane topology in microsomal membranes for the proteins used in this study. (*PPL*) preprolactin; *IMC-CAT*; (*TR*) transferrin receptor. The cleaved NH₂-terminal signal sequence (*S*) and hydrophobic core of the SA sequences are indicated. For *IMC-CAT* the regions derived from invariant chain (*li*), multiple colony stimulating factor (*MC*) and chloroamphenicol transferase (*CAT*) are indicated along with the glycosylation site on the NH₂-terminal domain (*). Where truncated products were used the shortened product is shown by a solid line while the complete protein is indicated by dashed line. The truncations were generated by cutting the transcription template with PvuII (*P*) or NdeI (*N*) as discussed in the Materials and Methods section.

nal anchor membrane protein which adopts a type I orientation in the membrane (Haeuptle et al., 1989). We replaced the NH₂ terminus of this protein with 30 amino acids from the NH₂ terminus of invariant chain and tested whether the NH₂ terminus of the resulting *IMC-CAT* could be translocated across microsomal membranes posttranslationally. The *IMC-CAT* construct contains only a single site for the addition of N-linked carbohydrate at residue 32 (see Fig. 1). Therefore the addition of N-linked sugar indicates that the NH₂-terminus of *IMC-CAT* has been translocated across the membrane and is accessible to the lumenally disposed glycosylation apparatus (Kornfeld and Kornfeld, 1985).

Translation of the *IMC-CAT* transcript in the wheat germ cell-free system gives a product of 30 kD (Fig. 2, lane 1). When SRP is present during the translation there is an arrest of elongation and an accumulation of peptides, the majority ranging from 8 to 14 kD (Fig. 2, lane 2). This is consistent with the SRP-mediated targeting of *IMC-CAT*. In the absence of SRP no arrest occurs and no membrane insertion is detected (data not shown). Cycloheximide was added to the SRP-arrested translation to prevent further chain elongation, rough microsomes were added, and membrane insertion assayed by analysis of the protection of the nascent chain from protease.

After addition of membranes and protease digestion, three protected fragments of 10, 13.2, and 13.5 kD are seen together with a smaller 4.5-kD fragment (Fig. 2, lane 4). When an acceptor tripeptide which inhibits N-linked glycosylation is present during the experiment only the 10-kD fragment of the larger species is seen (Fig. 2, lane 5). Thus the 10-kD fragment represents membrane inserted *IMC-CAT* where the NH₂ terminus is unglycosylated. The 13.5-kD fragment represents membrane inserted *IMC-CAT* which is glycosylated at the NH₂ terminus. The 13.2-kD fragment is probably due to partial processing of the high mannose chain which is known to occur in the ER.

The protection of the 10-kD fragment from protease was dependent on an intact membrane since disruption of the lipid bilayer with 0.5% NP40 before the protease treatment

allowed the digestion of the fragment. The smaller fragment of 4.5 kD is a limit digest product of *IMC-CAT* seen in the presence of NP40 (Fig. 2, lane 6) and also in the absence of membranes (data not shown). Treatment with puromycin and EDTA before the addition of membranes abolished the membrane insertion (data not shown). Since treatment with puromycin and EDTA disrupts the association of the nascent chain with the ribosome this result suggests that an intact nascent chain/ribosome/SRP complex is required for membrane insertion to occur.

Membrane Insertion Requires Nucleotides

Having shown that *IMC-CAT* could insert into the membrane and translocate its NH₂-terminal domain independently of protein synthesis we tested the nucleotide requirements of this process using the total cell-free translation system. Apyrase had been successfully used to deplete nucleotides from other translocation assay systems (Zimmermann et al., 1988). When the SRP-arrested translation reaction was treated with apyrase before the addition of rough microsomes membrane insertion was abolished (compare Fig. 3, lanes 1 and 2 with lanes 3 and 4). Pretreatment of the microsomes with apyrase had little effect (compare Fig. 3,

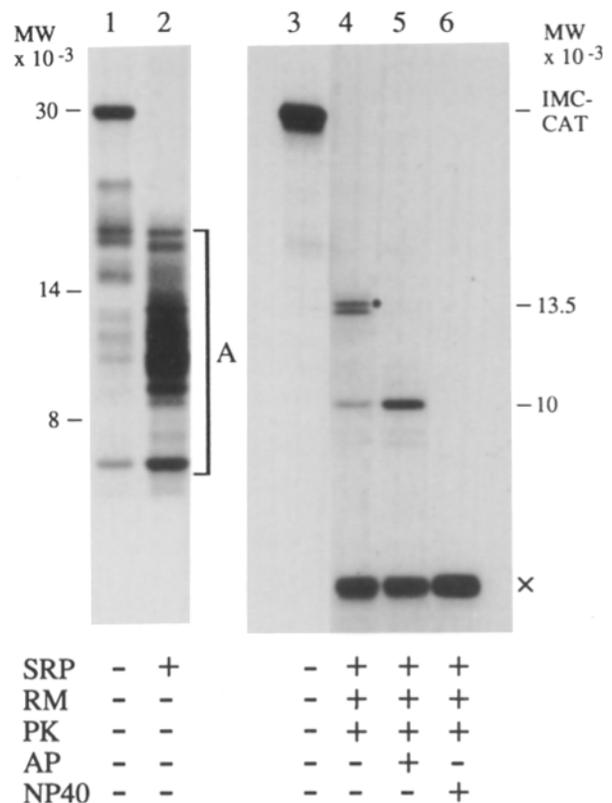


Figure 2. Posttranslational membrane insertion of *IMC-CAT*. Lanes 1 and 3 show the translation product of the *IMC-CAT* transcript. Lane 2 shows the arrest of translation (A) caused by the presence of SRP. Lanes 4 and 5 show the protease resistant fragments present after addition of membranes (*RM*) in the absence and presence of acceptor peptide (*AP*). Lane 6 shows the fragments present after digestion in the presence of membranes and 0.5% NP40. PK denotes addition of proteinase K. The glycosylated form of the protease protected fragment of *IMC-CAT* (*) and the limit digest product (X) are indicated.

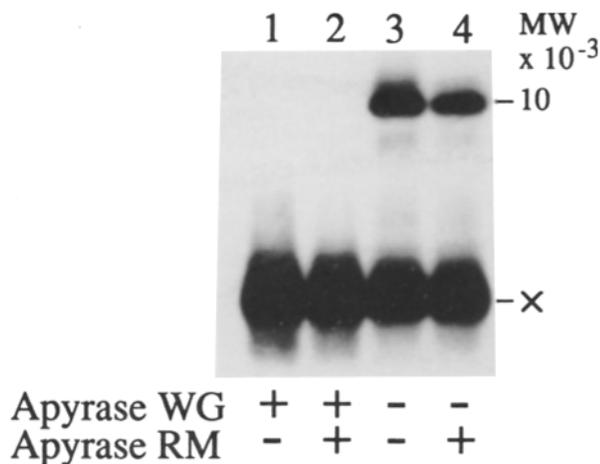


Figure 3. Apyrase treatment abolishes membrane insertion. After addition of cycloheximide to the translation reactions samples were treated with apyrase (*Apyrase WG*) or mock treated as indicated. Membranes which had been apyrase treated (*Apyrase RM*) or mock treated were then added and membrane insertion assayed by protease protection. The limit digest product is indicated (X).

lanes 3 and 4). The result firmly establishes the dependence of membrane insertion on nucleotide triphosphates.

Purification of Nascent Chain/Ribosome/SRP Complex and Reconstitution of Membrane Insertion

To test if cytosolic components other than SRP were required for posttranslational membrane insertion we isolated the nascent chain/ribosome/SRP complex of IMC-CAT. After translation the reaction mixture was subjected to a high-salt extraction, to disrupt electrostatic interactions, and the nascent chain/ribosome/SRP complex was isolated by centrifugation through a high salt/sucrose cushion. The resulting pellet contained nearly all of the radioactively labeled nascent chains but was depleted of the bulk of cytosolic proteins as determined by Coomassie staining (data not shown).

Since nucleotides were known to be required for membrane insertion (see Fig. 3) both ATP and GTP were included in the mixture for trial reconstitution of membrane insertion using the isolated complex. Two major protease protected species were observed, with a predominant 6.5-kD fragment as well as the 10-kD fragment (Fig. 4 a, lane 1). This pattern was also seen when HeLa cytosol was added in place of wheat germ lysate (data not shown). When wheat germ lysate was added back the pattern of protease protected fragments was identical to that seen in the earlier assay (Figs. 2 and 3) with a 10-kD fragment predominating (Fig. 4 a, lane 3).

To further characterize the two major fragments we used an antibody specific for the NH₂-terminal invariant chain-derived region of IMC-CAT (Lipp and Dobberstein, 1986a). Both the 6.5- and 10-kD fragments are immunoprecipitated with this antibody (Fig. 4 a, lanes 2 and 4) and therefore include the NH₂-terminal region. Both the 6.5- and 10-kD fragments can be glycosylated *in vitro* by microsomes giving an additional glycosylated fragment of 10 and 13.5 kD, respectively (see Fig. 4 b, lanes 2 and 4). Since the only glycosylation site present on IMC-CAT is at the NH₂-terminus this domain must be luminal in both fragments.

Both the 6.5- and 10-kD fragments are resistant to alkaline carbonate extraction (data not shown), a procedure known to remove luminal and peripheral proteins (Fujiki et al., 1982), and therefore behave as integral membrane proteins. Thus cytosolic factors can somehow alter the accessibility of the membrane-inserted nascent chain to protease. They do not however seem to greatly increase the amount of IMC-CAT inserted into the membrane. Since the efficiency of membrane insertion we achieve in our assay, as judged by the proportion of synthesized nascent chains which are protease protected, is low (usually <10%, data not shown) we cannot rule out that other cytosolic factors may play a role under more efficient conditions such as cotranslational translocation.

Analysis of Nucleotide Requirement Using the Purified System

By using apyrase treatment we had shown a nucleotide dependence for the membrane insertion of IMC-CAT. To determine the type of nucleotide required we used the isolated SRP-arrested complex. We found that the membrane insertion of IMC-CAT, as judged by the appearance of the 6.5-kD protease-protected fragment, showed an absolute requirement for GTP or the nonhydrolyzable analogue GMPPNP (Fig. 5, lanes 3 and 7). ATP alone showed no stimulation of membrane insertion above background and the analogue AMPPNP was also ineffective (Fig. 5, lanes 2 and 6). Combinations of GTP and ATP or GMPPNP and AMPPNP were no more efficient than GTP or GMPPNP alone.

The ability of the nonhydrolyzed analogue GMPPNP to substitute for GTP is surprising since in other systems the hydrolysis of GTP is often used to drive reactions. It was possible that GMPPNP promoted membrane insertion less efficiently than GTP but over the long incubation time used both nucleotides allowed membrane insertion to reach completion. To resolve this question we decided to look at a time course of membrane insertion in the presence of GTP and GTP analogues. While GMPPNP has been shown to function in translocation systems (Connolly and Gilmore, 1986; Wilson et al., 1988) GTP γ S was known to inhibit other GTP-dependent processes (reviewed by Bourne, 1988). We decided to make use of the GTP analogues: GMPPNP, GMPPCP, and GTP γ S and determine their efficiency in promoting membrane insertion.

The results of a time course experiment are shown in Fig. 6. The relative efficiency of membrane insertion in the presence of GTP and GMPPNP is 100 and 82%, respectively, after 15 min and therefore the detectable difference between these two nucleotides is small. At shorter time points, such as 5 min GMPPNP (61%) and GTP (82%) still show similar results while GMPPCP is significantly less efficient (13%). The analogue GTP γ S showed no stimulation above background with membrane insertion being less than 1% of the control value after 15 min (data not shown). Commercial preparations of GTP γ S are usually contaminated with GDP (up to 10%) and in other translocation assays it has been shown that GDP is a potent inhibitor of translocation (Connolly and Gilmore, 1986). We found that the inclusion of low concentrations of GDP strongly inhibited the membrane insertion of IMC-CAT (data not shown) and conclude that the failure of GTP γ S to stimulate membrane insertion in our assay is an artifact caused by the presence of contaminating

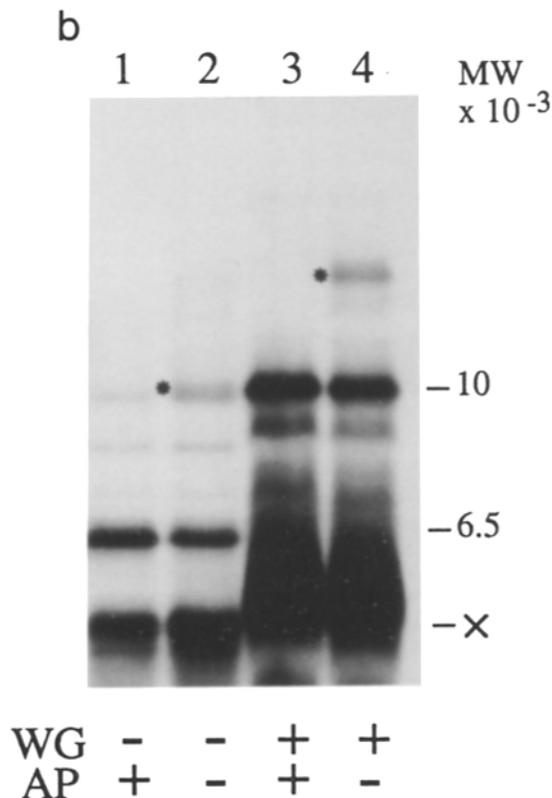
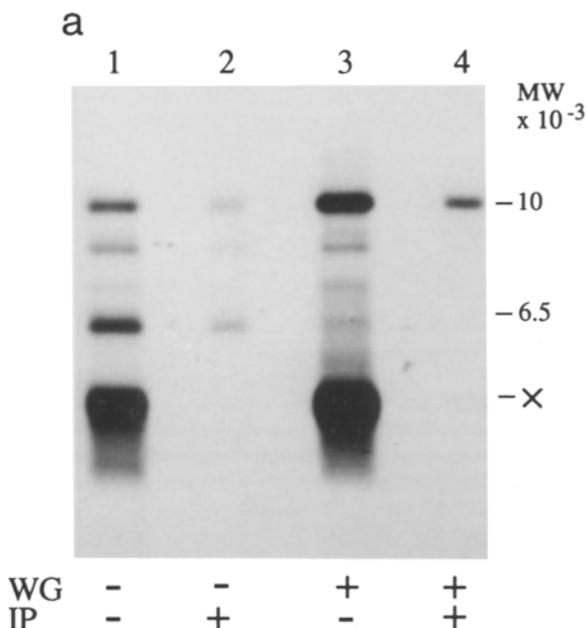


Figure 4. (a) Immunoprecipitation of protease protected fragments. The IMC-CAT/ribosome/SRP complex was incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of wheat germ lysate (WG) with rough microsomes and acceptor tripeptide. After proteolysis the total products (lanes 1 and 3) and products immunoprecipitated (IP) with the NH₂-terminal-specific anti-invariant chain serum (lanes 2 and 4) were analyzed. The limit digest product is indicated (X). (b) Glycosylation of protease protected fragments of IMC-CAT. The IMC-CAT/ribosome/SRP complex was incubated with membranes in the presence or absence of wheat germ lysate (WG) and acceptor peptide (AP) as indicated and then subjected to proteolysis. The glycosylated forms of the 6.5-kD fragment (lane 2) and the 10-kD fragment (lane 4) are indicated (*). The limit digest product (X) was present in excess in lanes 3 and 4 and smeared up the gel to give a broad band visible just below the 6.5-kD marker.

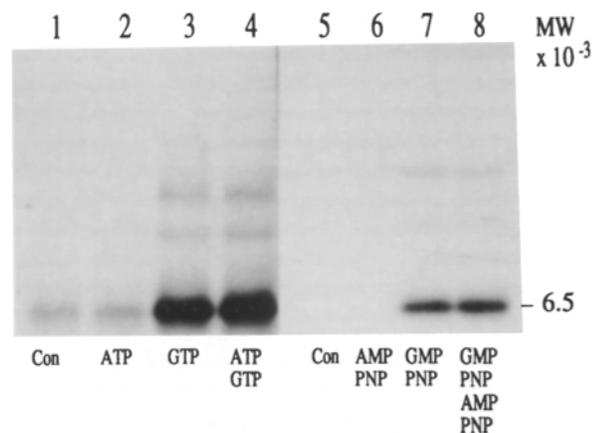


Figure 5. Nucleotide dependence of IMC-CAT membrane insertion. The IMC-CAT/ribosome/SRP complex was incubated in WG compensation buffer with rough microsomes and acceptor tripeptide. Samples in lanes 1 and 5 contained no added nucleotides (Con). The nucleotide concentrations of the other samples were: lane 2, 500 μM ATP; lane 3, 500 μM GTP; lane 4, 500 μM ATP; and 500 μM GTP; lane 6, 2 mM AMP-PNP; lane 7, 2 mM GMP-PNP; lane 8, 1 mM AMP-PNP; and 1 mM GMPPNP. Samples in lanes 2, 3, and 4 also contained 10 mM creatine phosphate and 80 μg ml⁻¹ creatine phosphokinase as an energy-regenerating system.

GDP. The reduced efficiency of GMPPNP and in particular GMPPCP in promoting membrane insertion (relative to GTP) probably reflects the lower affinity of these analogues for some GTP-binding sites (Scherer et al., 1989).

The Role of GTP in Membrane Insertion

We find that GTP or the nonhydrolyzed analogue GMPPNP are required for the membrane insertion of IMC-CAT. It has previously been shown that GTP or GMPPNP are also necessary for the translocation of secreted proteins (Connolly and Gilmore, 1986) and the membrane integration of single-spanning type II SA proteins (Wilson et al., 1988). Two proteins have been identified to date which are candidates for controlling this GTP-dependent step of protein translocation. Both the 54-kD subunit of SRP (SRP54) (Römisch et al., 1989; Bernstein et al., 1989) and the α subunit of DP (Connolly and Gilmore, 1989) contain a conserved consensus GTP-binding motif. To date only DPα has been shown to bind GTP, and it was further shown that the DP-dependent release of SRP from the nascent chain required GTP (Connolly and Gilmore, 1989). Using photocrosslinking it has previously been shown that the signal sequence of prolactin interacts with the SRP54 (Kurzchalia et al., 1986; Wiedmann et al., 1987a). We used this approach to determine more closely the GTP-dependent step

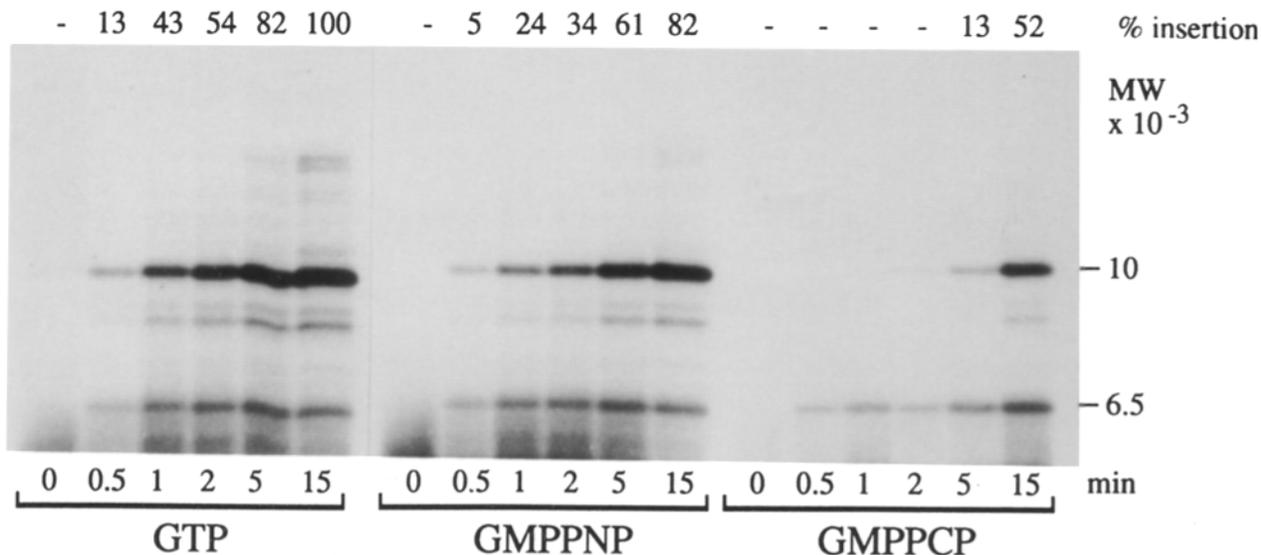


Figure 6. Time course of membrane insertion in the presence of GTP analogues. Incubations in WG compensation buffer contained rough microsomes and acceptor peptide. The indicated nucleotides were present at a final concentration of 500 μ M. The numbers below the lanes indicate the time in minutes for which membrane insertion was allowed to occur. The figures for percent relative membrane insertion (% insertion) are a measure of the intensity of the 10-kD band. The value obtained for 15 min with 500 μ M GTP was taken as 100% and all other values are relative to this. A dash represents a figure of <1% of this value.

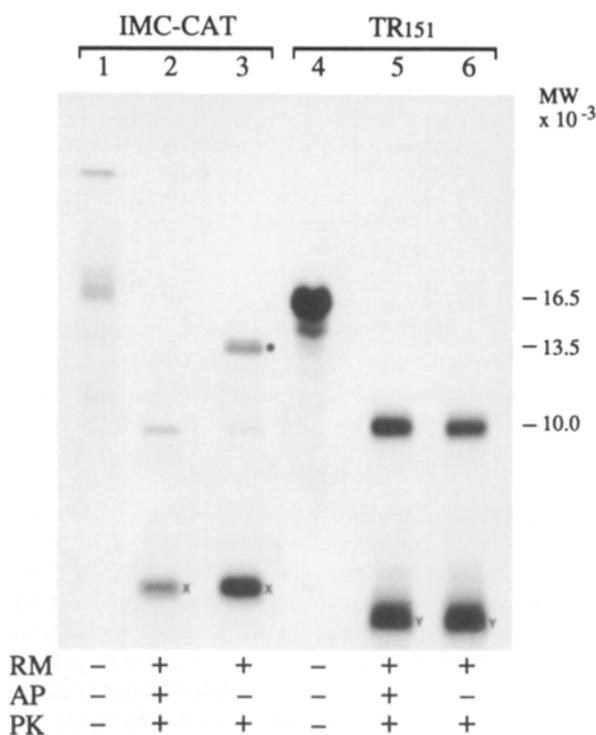


Figure 7. Membrane insertion of the transferrin receptor. IMC-CAT (IC) or transferrin receptor (TR) were synthesized in the presence of SRP and cycloheximide was then added. Membranes (RM) were added in the presence or absence of acceptor peptide (AP) and the proteinase K (PK) protected fragments analyzed. The glycosylated form of the protease protected fragment (*) and the limit digest product of IMC-CAT (X) are indicated. The 4-kD fragment present in the TR151 lanes (Y) was also seen when NP40 was present during proteolysis and was therefore not dependent on an intact membrane (data not shown).

involved in the translocation of secreted proteins and the membrane insertion of type I and type II SA proteins.

We used PPL as a model-secreted protein with a cleaved NH_2 -terminal signal sequence since its translocation has been well characterized (Connolly and Gilmore, 1986). As a model type II SA protein we chose TR a single-spanning membrane protein known to expose the COOH terminus on the luminal side of microsomes (Zerial et al., 1986). Since posttranslational membrane insertion of TR has not been shown previously we first had to establish it could occur. We used a truncated transcript which resulted from cleaving the TR template at an NdeI site within the coding region to give a translation product of 151 amino acids (denoted TR₁₅₁). Truncation with NdeI removes all potential glycosylation sites from the COOH terminus of TR (Schneider et al., 1984). Translation in the presence of SRP yielded a product with an apparent molecular weight of 16.5 kD on 22% acrylamide/6 M urea gels (Fig. 7, lane 4). This is the same size as the major SRP-arrested fragment obtained when the full-length TR transcript is used (data not shown). To test membrane insertion of TR₁₅₁ we added the SRP-arrested complex to membranes in the presence of protein synthesis inhibitors. After protease digestion of the cytoplasmically exposed regions a 10-kD protease protected fragment was observed (Fig. 7, lanes 5). The protected fragment of TR₁₅₁ was not glycosylated (Fig. 7, lane 6) under conditions where the NH_2 -terminus of IMC-CAT was clearly glycosylated (Fig. 7, lane 3). Since the NH_2 -terminal domain of TR₁₅₁ contains two potential sites for addition of N-linked oligosaccharide while the NdeI-truncated COOH-terminus contains none this is consistent with COOH-terminal translocation as expected for a type II SA protein (Zerial et al., 1986). It should be noted that the potential glycosylation sites of the NH_2 -terminal domain of TR have not been shown to be functional either in vitro or in vivo. The nascent

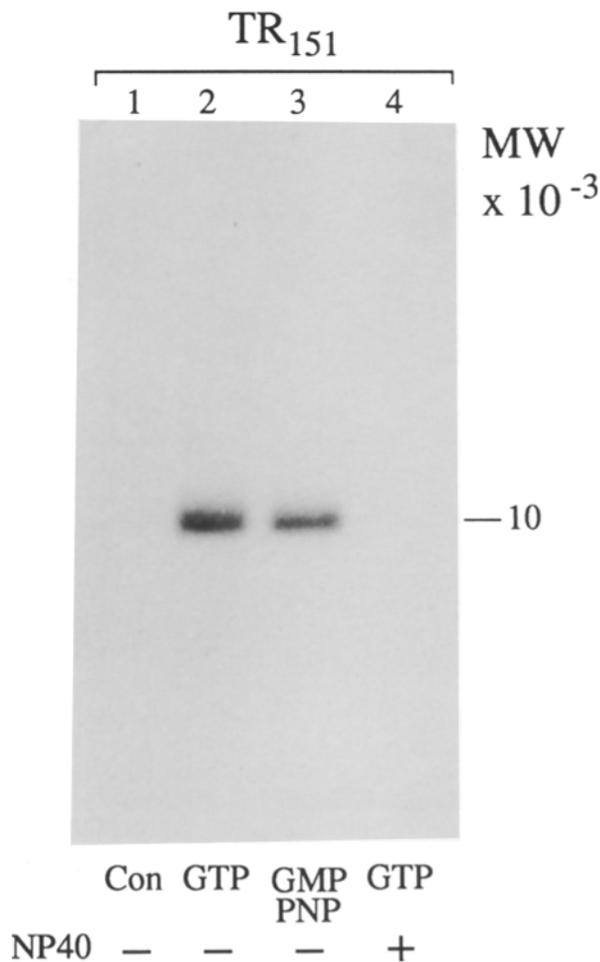


Figure 8. Membrane insertion of TR requires GTP. The TR₁₅₁/ribosome/SRP complex was incubated in WG compensation buffer, with rough microsomes, in the absence of nucleotides (lane 1), or the presence of 500 μ M GTP (lanes 2 and 4) or 500 μ M GMPPNP (lane 3). Samples were subjected to proteolysis in the presence or absence of NP40 and analyzed by gel electrophoresis.

chain/ribosome/SRP complex of TR₁₅₁ was isolated and tested in the reconstitution assay described above. It was found that GTP or GMPPNP were required for membrane insertion (Fig. 8, lanes 2 and 3) in agreement with previous data for other single spanning type II SA proteins (Wilson et al., 1988).

To identify components that interact with the nascent chains of PPL, IMC-CAT, and TR we synthesized their SRP-arrested nascent chains in the presence of ϵ -TDBA-Lys-tRNA. The lysine residues, of which several are close to the signal sequence of PPL (Kurzchalia et al., 1986; Krieg et al., 1986) and the SA sequences of IMC-CAT and TR (High et al., 1991), can then be crosslinked to proteins which are closely associated. After synthesis the nascent chain/ribosome/SRP complexes were isolated and incubated with RMs in the presence or absence of GTP and then irradiated with UV to initiate crosslinking. The resulting mixture was subjected to extraction with alkaline sodium carbonate, and then centrifugation to separate soluble components from integral membrane components. In the absence of added nucleotides,

or in the presence of 500 μ M GDP, the nascent chain remained bound to a cytosolic component of 54 kD (molecular weight estimated after subtraction of the contribution from the nascent chain; see Fig. 9, lanes 5, 8, 13, 16, 21, and 24, indicated by *arrowhead*). This cytosolic component has been shown to be SRP54 by immunoprecipitation (High et al., 1991). When either GTP or GMPPNP were added to 500 μ M the amount of nascent chain crosslinked to the SRP54 was significantly diminished in all cases (Fig. 9, lanes 6, 7, 14, 15, 22, and 23). These nucleotide conditions are identical to those already shown to promote the membrane insertion of IMC-CAT, TR (this study) and the translocation of PPL (Connolly and Gilmore, 1986).

Concomitant with the reduction of crosslinking of the nascent chains to SRP54 was the appearance of weak crosslinks between PPL and IMC-CAT₁₀₃ and new components which were found in the membrane pellet obtained after carbonate extraction (Fig. 9, lanes 2, 3, 10, and 11). The appearance of these crosslinks was dependent upon both UV irradiation and the presence of ϵ -TDBA-Lys-tRNA (High et al., 1991). For TR₁₅₁ no new crosslinks were visible in the postcarbonate membrane pellet when GTP or GMPPNP were present. For PPL, after subtraction of the contribution of the nascent chain, a protein of 35 kD was crosslinked (Fig. 9, lanes 2 and 3; *star*). In the case of IMC-CAT₁₀₃ a protein of 37 kD was crosslinked (Fig. 9, lanes 10 and 11; *closed circle*). The further analysis of the component crosslinked to IMC-CAT₁₀₃ is the subject of a separate study (High et al., 1991). In the case of PPL the 35-kD protein is expected to be the signal sequence receptor (SSR) identified by Wiedmann et al., 1987b) using a similar crosslinking approach. Unlike previous studies (Wiedmann et al., 1987b), the release of the nascent chain from SRP54 upon the addition of RMs which we observe is not complete. We attribute this lower efficiency to the fact that we are using purified nascent chain/ribosome/SRP complexes in our system.

Discussion

SA sequences can promote the membrane translocation of polypeptide segments either preceding or following the hydrophobic core of the signal sequence. The choice between which of the two domains is translocated appears to depend on a number of factors. The distribution of charged residues flanking the hydrophobic core of the SA sequence is important in this respect (Hauptle et al., 1989; Hartmann et al., 1989). Nothing is known about the components which mediate the NH₂ and COOH translocation of SA proteins. In this study we have investigated the requirement of membrane insertion of SA proteins for nucleotides and cytoplasmic factors. We found that the isolated nascent chain/ribosome/SRP complex was competent for membrane insertion and translocation of the NH₂ terminus of IMC-CAT across rough microsomes. The membrane insertion required only the addition of GTP and no other cytosolic components were found to be necessary. Further addition of SRP did not increase the efficiency of IMC-CAT NH₂ terminal translocation (data not shown) suggesting that the functionally bound SRP was tightly associated and resistant to the high-salt extraction procedure used during isolation. The finding that the nascent chain of IMC-CAT can be crosslinked to SRP54 in the iso-

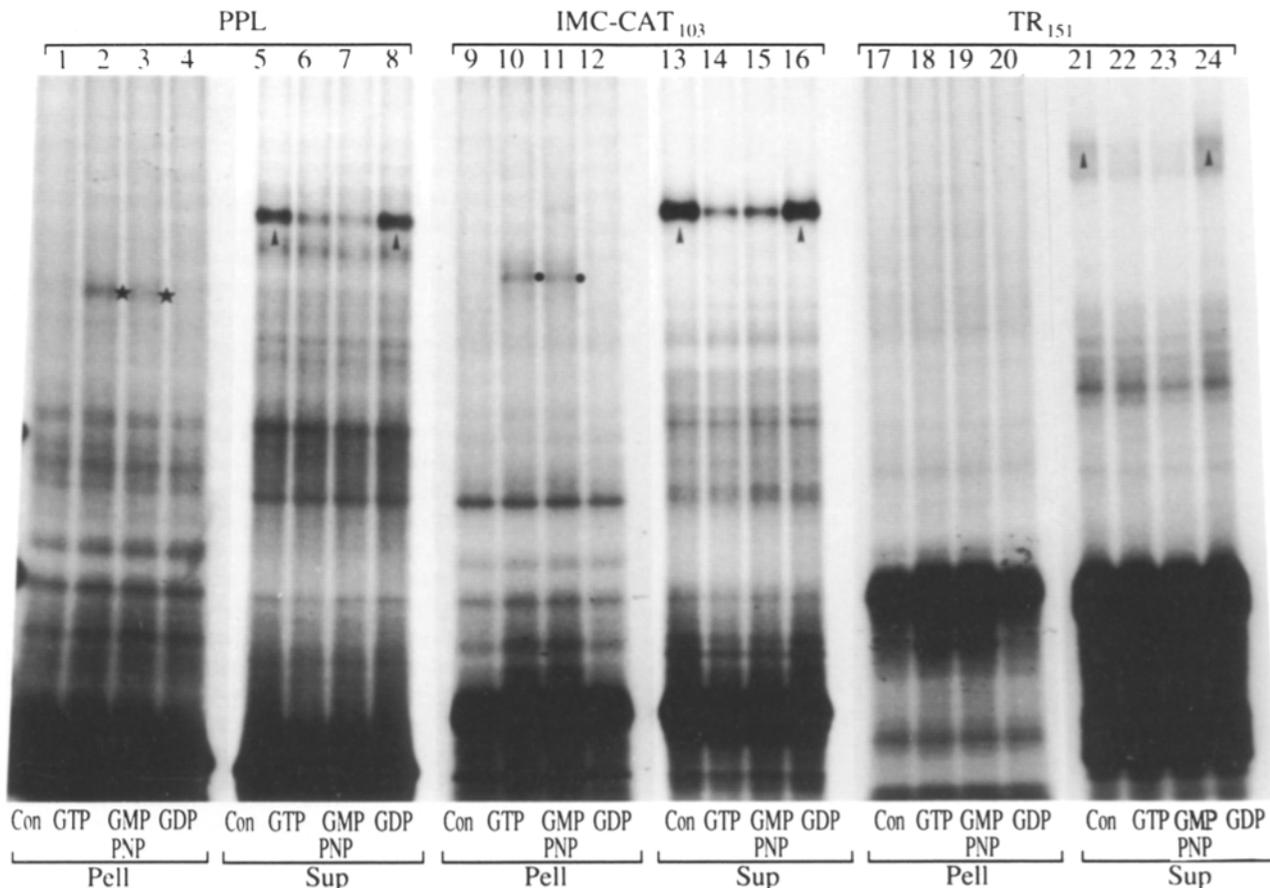


Figure 9. Nucleotide dependence of nascent chain release from SRP54. The indicated nascent chain/ribosome/SRP complexes synthesized in the presence of ϵ -TDBA-Lys-tRNA were incubated with rough microsomes in the absence of added nucleotides (Con) or with 500 μ M GTP, 500 μ M GMPPNP, and 500 μ M GDP, as indicated, for 5 min at 25°C. After UV irradiation on ice the samples were subjected to alkaline carbonate extraction, centrifuged, and the proteins present in the pellet (*Pell*) and supernatant (*Sup*) were analyzed. The cross-linked products of the various nascent chains and SRP54 in the absence of added nucleotide, or with 500 μ M GDP present, are indicated (arrowhead). Photocrosslinks to components of 35 kD (star) and 37 kD (●) which remain in the membrane pellet after carbonate extraction are also shown.

lated nascent chain/ribosome/SRP complex further supports this notion. The isolated nascent chain/ribosome/SRP complex of TR was also found to be competent for membrane insertion. The membrane insertion of TR required the presence of GTP. Both type I and type II SA proteins also showed membrane insertion in the presence of GMPPNP, a nonhydrolyzed analogue of GTP. GMPPNP had previously been shown to promote translocation of secreted proteins (Connolly and Gilmore, 1986) and type II SA proteins (Wilson et al., 1988).

Our results show that in a ribosome/SRP complex regions of the nascent chain both NH₂- and COOH-terminal of the hydrophobic core of an SA sequence remain translocation competent. We show this here for the NH₂ terminus of IMC-CAT and the COOH terminus of TR. Wilson et al., (1988) have previously shown comparable results for the COOH terminus of the hemagglutinin-neuraminidase glycoprotein. In yeast, where translocation of prepro- α -factor across the ER is SRP/ribosome independent, an association with heat-shock proteins is known to be important in maintaining the protein in a translocation competent state (Deshaies et al., 1988). Our results suggest that soluble chaperonins are not required for the membrane insertion of

the SA proteins tested. However, the relatively low efficiency of the posttranslational membrane insertion process means we cannot rule out a role for other cytosolic factors during cotranslational insertion *in vitro* or during the *in vivo* process.

To further test the role of GTP in membrane insertion we decided to analyze the interactions of nascent-secreted, type I SA, and type II SA proteins under different nucleotide conditions. To achieve this we used photocrosslinking of the nascent chain to determine closely associated components. Photocrosslinking had been used to identify SRP54 and SSR as proteins which interact with the signal sequence of PPL (Kurzchalia et al., 1986; Krieg et al., 1986; Wiedmann et al., 1987b). We synthesized nascent chains which contained lysine residues modified with ϵ -TDBA (Wiedmann et al., 1987a) and incubated them with RMs under different nucleotide conditions before crosslinking them to interacting proteins by UV irradiation. Our results show that for PPL, IMC-CAT, and TR, the absence of GTP caused the nascent chain to remain bound to SRP54. This strongly suggests that GTP is required for the release of the signal sequence from SRP54 and that the GTP-dependent step of translocation and membrane insertion is probably the same in each case. The re-

lease of SRP from the nascent chain of secreted proteins is known to require the DP (or SRP receptor) (Meyer et al., 1982; Gilmore et al., 1982). Connolly and Gilmore (1989) have studied the DP-mediated release of SRP from ribosomes which carry a nascent chain bearing a cleavable NH₂-terminal signal sequence and found the process to be GTP dependent. Since both DP α and SRP54 have potential GTP-binding sites our results do not rule out the possibility that binding of GTP to both of these proteins is required before release of SRP54 from the nascent chain can occur. Whether nascent chain release from SRP54 is the sole GTP requiring step of membrane insertion, or only one of several, remains to be determined.

The nonhydrolyzable GTP analogue GMPPNP was equally efficient in catalyzing the release of the nascent chain from SRP54 consistent with its ability to efficiently promote membrane insertion. GDP did not promote the release of nascent chains from SRP54, consistent with its inhibitory effect on the translocation of PPL (Connolly and Gilmore, 1986). For both PPL and IMC-CAT₁₀₃, the reduction in efficiency of crosslinking to SRP54 in the presence of GTP was accompanied by the appearance of new photocrosslinked products which remained in the membrane pellet after extraction with sodium carbonate solution. In the case of PPL the 35-kD membrane protein has the same molecular weight and Con A sepharose-binding properties (High et al., 1991) as the previously identified SSR (Wiedmann et al., 1987b). Wiedmann et al. (1987b) have shown that photocrosslinking of the signal sequence of PPL to SSR is accompanied by loss of photocrosslinking to SRP54. Since GTP is a prerequisite for the release of the signal sequence from SRP54, it would be expected that crosslinking of the SSR would require the presence of both membranes and GTP. This is exactly the situation which we observe. Since IMC-CAT₁₀₃ is an integral membrane protein the results of the carbonate extraction may be ambiguous and resistance of the photocrosslinked product to the procedure may reflect the properties of the nascent chain rather than the crosslinked partner. Thus, the component to which the IMC-CAT₁₀₃ nascent chain is transferred in the presence of GTP and rough microsomes could be a peripheral or integral protein of the endoplasmic reticulum.

The finding that GMPPNP can efficiently promote SRP54 release and membrane insertion *in vitro* should not be interpreted to suggest that GTP hydrolysis is unnecessary *in vivo*. It is possible that the hydrolysis of GTP bound to DP or SRP54 is only required after the release of SRP54 and membrane insertion have occurred. Under such circumstances differences between GTP and GMPPNP may only be observed when a "recycling" of this GTP-binding protein between two states is required for membrane insertion (see also Connolly and Gilmore, 1989). An assay where DP or SRP54 are present in only "catalytic" amounts may be required to show any dependence on GTP hydrolysis, a situation well documented for the role of GTP hydrolysis in the catalysis of polypeptide chain elongation by EF-Tu (Kaziro et al., 1978).

The data presented here support the view that the mechanism of SRP-mediated targeting to the ER membrane is identical for secreted, type I SA, and type II SA proteins. We find that there are no differences in the nucleotide requirements for translocation or membrane insertion of the three classes

of proteins studied. While the SSR is implicated in the translocation of PPL across the membrane (Wiedmann et al., 1987b; Krieg et al., 1989; Wiedmann et al., 1989) the nature of the subsequent stages in the translocation and membrane insertion of SA proteins is unknown. A common machinery may be responsible for the complete translocation of secreted proteins and the membrane insertion of SA proteins. Such a machinery would have to be able to accommodate proteins which will ultimately span the membrane once, in either of two possible orientations, as well as completely translocated proteins. We observe that the type I SA protein, IMC-CAT, is crosslinked to a 37-kD component of rough microsomes only when GTP is present. This result suggests that the 37-kD component may be involved at some stage in the integration of type I SA proteins.

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