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 required. 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) 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...
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). Cytochrome c, 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 Brunsw, 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; Haueuptle 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 (Haueuptle et al., 1989). An EcoRI fragment carried by IMC-CAT was subcloned from pDS3 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 Ndel (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 at 25°C. Cycloheximide was added to a final concentration of 0.25 mM, emetine to 1 mM when used. The mixture was then added to rough microsomes and assayed for membrane insertion directly or subjected to centrifugation through a high salt/sucrose cushion (see below).

**Apyrase Treatment**

The products of a 25 μl 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 OD260 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 translation buffer using 9.5 μl per 25 μl of translation mixture loaded onto the cushion. 20 μl of this resuspended mixture was incubated in the presence of 0.06 OD260 U of rough microsomes and in the absence of nucleotides or the presence of 500 μM 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 PvulI (P) or NdeI (N) as discussed in the Materials and Methods section.

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 2 and 3 with lanes 1 and 4). Pretreatment of the microsomes with apyrase had little effect (compare Fig. 3, lanes 6...
brane insertion (see Fig. 3) both ATP and GTP were in-
germinated lysate was added back the pattern of protease protected
place of wheat germ lysate (data not shown). When wheat
an antibody specific for the NH2-terminal invariant chain-
clude the NH2-terminal region. Both the 6.5- and 10-kD
respectively (see Fig. 4 b, lanes 2 and 4). Since the only
fragment as well as the 10-kD fragment (Fig. 4 a, lane 1).
This pattern was also seen when HeLa cytosol was added in
insertion using the isolated complex. Two major protease
protected fragments were observed, with a predominant 6.5-kD
lane 3).  

The results of a time course experiment are shown in Fig.
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 pro-
tease protection. The limit digest product is indicated (X).

Apyrase WG + + - -
Apyrase RM - - - +

Figure 3. Apyrase treatment abolishes membrane insertion. After
addition of cycloheximide to the translation reactions samples were
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Membranes which had been apyrase treated (Apyrase RM) or mock
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tease protection. The limit digest product is indicated (X).

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 ef-
ciently than GTP but over the long incubation time used
both nucleotides allowed membrane insertion to reach com-
pletion. 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; Wil-
son et al., 1988) GTPγS was known to inhibit other GTP-
dependent processes (reviewed by Bourne, 1988). We decided
to make use of the GTP analogues: GMPPNP, GMPPCP, and
GTPγS and determine their efficiency in promoting
membrane insertion.

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 translo-
cation.

Analysis of Nucleotide Requirement Using
the Purified System

By using apyrase treatment we had shown a nucleotide de-
pendence for the membrane insertion of IMC-CAT. To deter-
mine the type of nucleotide required we used the isolated
SRP-arrested complex. We found that the membrane inser-
tion of IMC-CAT, as judged by the appearance of the 6.5-kD
protease-protected fragment, showed an absolute require-
ment 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). Com-
binations of GTP and ATP or GMPPNP and AMPPNP were
no more efficient than GTP or GMPPNP alone.

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The results of a time course experiment are shown in Fig.
6. The relative efficiency of membrane insertion in the pres-
ence 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 GTPγS showed no stimulation above back-
ground with membrane insertion being less than 1% of the
control value after 15 min (data not shown). Commercial
preparations of GTPγS 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 (Conn-
olly and Gilmore, 1986). We found that the inclusion of low
concentrations of GDP strongly inhibited the membrane in-
sertion of IMC-CAT (data not shown) and conclude that the
failure of GTPγS to stimulate membrane insertion in our as-
say 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 NH$_2$-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 nM ATP; lane 3, 500 nM GTP; lane 4, 500 nM ATP; and 500 nM GTP; lane 5, 2 mM AMPPNP; lane 6, 2 mM AMPPNP; lane 7, 2 mM GMPPNP; lane 8, 1 mM AMPPNP; 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 preprolactin 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 nM. 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 nM 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 (Y) 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).

We used PPL as a model-secreted protein with a cleaved NH₂-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 proteinase 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 NH₂-terminus of IMC-CAT was clearly glycosylated (Fig. 7, lane 3). Since the NH₂-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 NH₂-terminal domain of TR have not been shown to be functional either in vitro or in vivo. The nascent
Membrane insertion of TR requires GTP. The TR<sub>151</sub>/ribosome/SRP complex was incubated in WG compensation buffer, with rough microsomes, in the absence of nucleotides (lane 1), or the presence of 500 μM GTP (lanes 2 and 4) or 500 μM GMPPNP (lane 3). Samples were subjected to proteolysis in the presence or absence of NP40 and analyzed by gel electrophoresis.

Concomitant with the reduction of crosslinking of the nascent chains to SRP54 was the appearance of weak crosslinks between PPL and IMC-CAT<sub>103</sub> 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 TR<sub>151</sub> 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-CAT<sub>103</sub> a protein of 37 kD was crosslinked (Fig. 9, lanes 10 and 11; closed circle). The further analysis of the component crosslinked to IMC-CAT<sub>103</sub> 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 (Haeuptle et al., 1989; Hartmann et al., 1989). Nothing is known about the components which mediate the NH<sub>2</sub> 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 NH<sub>2</sub> 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 NH<sub>2</sub> 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 8. Membrane insertion of TR requires GTP. The TR<sub>151</sub>/ribosome/SRP complex was incubated in WG compensation buffer, with rough microsomes, in the absence of nucleotides (lane 1), or the presence of 500 μM GTP (lanes 2 and 4) or 500 μM GMPPNP (lane 3). Samples were subjected to proteolysis in the presence or absence of NP40 and analyzed by gel electrophoresis.
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 μM GTP, 500 μM GMPPNP, and 500 μM 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 μM 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 isolated this region 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 (Deshayes 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-
release 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 NH$_2$-terminal signal sequence and found the process to be GTP dependent. Since both DP$_\alpha$ and SRP$_{54}$ 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 SRP$_{54}$ from the nascent chain can occur. Whether nascent chain release from SRP$_{54}$ 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 SRP$_{54}$ consistent with its ability to efficiently promote membrane insertion. GDP did not promote the release of nascent chains from SRP$_{54}$, consistent with its inhibitory effect on the translocation of PPL (Connolly and Gilmore, 1986). For both PPL and IMC-CAT$_{103}$, the reduction in efficiency of crosslinking to SRP$_{54}$ 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 SRP$_{54}$. Since GTP is a prerequisite for the release of the signal sequence from SRP$_{54}$, 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-CAT$_{103}$ 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-CAT$_{103}$ 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 SRP$_{54}$ 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 SRP$_{54}$ is only required after the release of SRP$_{54}$ 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 SRP$_{54}$ 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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