The Identification of Proteins in the Proximity of Signal-Anchor Sequences during Their Targeting to and Insertion into the Membrane of the ER

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Abstract. Using a photocross-linking approach we have investigated the cytosolic and membrane components involved in the targeting and insertion of signal-anchor proteins into the membrane of the ER. The nascent chains of both type I and type II signal-anchor proteins can be cross-linked to the 54-kD subunit of the signal recognition particle. Upon addition of rough microsomes the type I and type II signal-anchor proteins interact with a number of components. Both types of protein interact with an integral membrane protein, the signal sequence receptor, previously identified by its proximity to preprolactin during its translocation (Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. *Nature [Lond.]* 328:830–833). Three proteins, previously unidentified, were found to be cross-linked to the nascent chains of the signal-anchor proteins. Among them was a 37-kD protein that was found to be the main component interacting with the type I SA protein used. These proteins were not seen in the absence of membranes suggesting they are components of the ER. The ability of the nascent chains to be cross-linked to these identified proteins was shown to be abolished by prior treatment with agents known to disrupt translocation intermediates or ribosomes. We propose that the newly identified proteins function either in the membrane insertion of only a subset of proteins or only at a specific stage of insertion.

Two kinds of signal sequences have been identified which can direct nascent single-spanning membrane proteins to the ER (Wickner and Lodish, 1985). An NH_2-terminal signal sequence is present on many secreted and membrane proteins. This hydrophobic sequence is subsequently cleaved from the protein by signal peptidase during its translocation across the ER membrane. In the case of single-spanning membrane proteins with a cleaved NH_2-terminal signal sequence a stop transfer sequence is also present. The stop transfer sequence aborts translocation after the NH_2-terminus has crossed the membrane and stably anchors the protein into the membrane (Rapoport and Wiedmann, 1985).

A second class of proteins which possess an uncleaved signal sequence has been identified. The majority of such proteins are membrane proteins and a single sequence serves to both target the protein to the ER and to stably anchor the protein into the membrane. These proteins are known as signal-anchor (SA)[1] protein (Lipp and Dobberstein, 1988) to distinguish them from proteins with a cleaved signal sequence.

Single-spanning membrane proteins of the SA type can assume either a type I (NH_2-terminus extracytoplasmic) or type II (NH_2 terminus cytoplasmic) orientation. The distribution of charged residues flanking the hydrophobic core of the signal sequences seems to be important in determining the final orientation of SA proteins in the membrane (von Heijne, 1988; Haeuptle et al., 1989; Hartmann et al., 1989*a*). The known single-spanning membrane proteins with a cleaved NH_2-terminal signal are all of a type I topology.

The functions of a signal sequence can be seen as twofold: (*A*) targeting to the membrane; and (*B*) membrane insertion and translocation (secreted proteins) or retention (SA proteins). The targeting step is well characterized and known to be dependent upon signal recognition particle (SRP) for type I SA proteins (Hull et al., 1988), type II SA proteins (Lipp and Dobberstein, 1986; Holland and Drickamer, 1986), and proteins with NH_2-terminal-cleaved signal sequences (Walter and Lingappa, 1986). For these three types of proteins the release of the nascent chain from a 54-kD subunit of signal recognition particle (SRP54) requires the presence of GTP (High et al., 1991) in a process thought to be mediated by docking protein (DP) (SRP receptor) (Connolly and Gilmore, 1989). Thus the targeting and initial stages of translo-

1. *Abbreviations used in this paper*: AF, arrested fragment; DP, docking protein; ε-ANB-Lys, N^{ε}-5-Azido-2-nitrobenzoyl-Lys; ε-TDBA-Lys, N^{ε}-4-(3-trifluoromethyldiazarino)benzoyl-Lys; PPL, preprolactin; SA, signal-anchor; SRP, signal recognition particle; SRP54, 54 kD subunit of signal recognition particle; SSR, signal sequence receptor; TR, transferrin receptor.

cation (i.e., SRP release) are identical for type I and II SA proteins and for proteins with an NH_2-terminal–cleaved signal sequence.

The subsequent stages of translocation, involving the crossing of the membrane, are only well studied for the secreted protein preprolactin (PPL) which has a cleaved NH_2-terminal signal sequence. A photocross-linking approach has been used to analyze the next neighbors of PPL after its partial translocation across the membrane of the ER. The essence of the approach is the introduction at specific places within the nascent chain of a reagent which forms a highly reactive radical upon UV irradiation. After photolysis all the next neighbors of the nascent chain can be crosslinked to it allowing the nature of these proteins to be identified at different stages during the translocation process. Using this approach a membrane protein was identified which interacts with the PPL signal sequence (Wiedmann et al., 1987*b*) and denoted the signal sequence receptor (SSR). It has now been shown that the SSR also interacts with portions of the mature PPL chain (Kreig et al., 1989; Wiedmann et al., 1989) and that the protein mp39 identified by Krieg et al. (1989) is the same as SSR. These results suggest that SSR (mp39) may be part of a translocation complex. Very recently the SSR was found to be part of a complex in the ER which consists of at least two proteins, SSRα and SSRβ, though only SSRα can be crosslinked to the nascent chain of a secretory protein during translocation (Görlich et al., 1990).

The mechanism by which type I and type II SA proteins are inserted into the ER is not presently known. The insertion of either or both of these types of protein could occur in a number of ways: (*A*) using exactly the same machinery as secreted proteins; (*B*) using some of the same components as secreted proteins and in addition other components which interact exclusively with type I or type II SA proteins, (*C*) by a novel pathway unrelated to that used by secreted proteins. It is also possible that a sequential series of interactions mediates different stages of the insertion of membrane proteins. To clarify the mechanism of the membrane insertion of SA proteins we have used photocross-linking to study the cytosolic and membrane components which interact with type I and type II SA proteins. We find that SSR is crosslinked to both type I and type II SA membrane proteins used in this study. However, a 37-kD membrane protein (P37) is the major cross-link to the type I SA protein used.

Materials and Methods

Materials

T7 RNA polymerase and restriction enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany). ^{35}S-Methionine was from Amersham Buchler GmbH (Braunschweig, Germany). Cycloheximide, emetine, 7-methylguanosine 5′-monophosphate and puromycin were supplied by Sigma Chemical Co. (St. Louis, MO). N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB) was from Pierce Chemical Co. (Rockford, IL) and 4-(3-trifluoromethyldiazarino) benzoic acid (TDBA) was a gift from Dr. Josef Brunner, Swiss Federal Institute of Technology, Zürich, Switzerland.

Transcription and Translation

The plasmid used for transcription of truncated mRNA coding for PPL was pSPBP4 (Siegel and Walter, 1988) and was a gift from Peter Walter. The pSPBP4 was linearized with PvuII. For transcription of transferrin receptor (TR) the EcoRI fragment described by Zerial et al. (1986) which contained the entire coding region, was recloned into pGEM 1 under the T7 promoter and the resulting plasmid linearized with NdeI. IMC-CAT consists of portions of invariant chain, multiple colony–stimulating factor and chloroamphenicol acetyltransferase and is derived from the LMC-CAT construct (Haeuptle et al., 1989). EcoRI fragments carrying the coding region of IMC-CAT or of mutant IMC-CAT (see Fig. 1 *a*) were subcloned from pDS5 into pGEM 3 under the T7 promoter and the plasmid linearized with HindIII. To obtain a COOH terminally shortened protein the IMC-CAT template was truncated by the inclusion of the complementary oligonucleotide 5′CCCATATCACCAGCTCACCG3′ at 40 μg ml^{-1} in the wheat germ cell-free translation system (Haueptle et al., 1986). The predicted amino acid sequence of IMC-CAT$_{103}$ is as follows: MDDQRDLISNNEQLPML-GRRPGAPESKCSHQNETMVLASSTTSIHTMLLLLLMLFHLGLQALDI-TAFLKTVKKNKHKFYPAFIHILARLMNAHPEFRMAMKDG. The hydrophobic SA sequence is underlined.

Truncations were chosen to be as close to the point of SRP arrest as possible and allowed the production of essentially a single length polypeptide by SRP arrest rather than a series of chain lengths as is sometimes seen (Walter and Lingappa, 1986). Transcription was performed as described by the manufacturer (Promega Biotech, Madison, WI). The N$^{\epsilon}$-4-(3-trifluoromethyldiazarino) benzoyl-Lys (ϵ-TDBA-Lys)-tRNA was prepared as described by Wiedmann et al. (1987*a*) except that the modified Lys-tRNA was not submitted to BD-cellulose chromatography. The preparation of N$^{\epsilon}$-5-Azido-2-nitrobenzoyl-Lys (ϵ-ANB-Lys)-tRNA was done as described by Krieg et al. (1986) with the BD-cellulose chromatography also omitted. Translation in the wheat germ cell-free translation system was performed as described by Stueber et al. (1984). Cell-free translations were pulsed by addition of 7-methylguanosine 5′-monophosphate to a final concentration of 2 mM after 10 min. Translations were continued for a further 5 min and then cycloheximide added to 2 mM to prevent further chain elongation. For the analysis of nascent chain interactions with SRP components the mixture was then chilled on ice and subsequently irradiated. When used, salt washed rough microsomes were added to 0.1 OD_{280} U per 25 μl of translation and the mixture incubated at 25°C for a further 5 min before being chilled on ice and irradiated.

Disruption of Translocation Intermediates

After incubation with rough microsomes, and cooling on ice, an equal volume of ice cold 8 M urea or 0.05 vol of ice-cold 500 mM EDTA, pH 8, were added and samples maintained on ice for a further 5 min before irradiation.

Proteolysis

After irradiation, samples were incubated with 100 μg ml^{-1} Trypsin-TPCK at 0°C for 1 h. PMSF was added to 10 mM and samples were further processed by extraction with sodium carbonate solution as described below using a solution supplemented with 0.1 mM PMSF.

Irradiation

The optimal conditions for UV irradiation were determined empirically. Irradiation was done with a black light lamp equipped with a 100 W mercury bulb and a 365-nm filter (Spectroline model B-100/F; Spectronics Corporation, Westbury, New York). Samples in open polypropylene microfuge tubes were maintained at 0°C during irradiation which was for 10 min at a distance of 15 cm from the light source. After irradiation samples were further processed as described below.

Sample Analysis

Total proteins in the assay were recovered by precipitation with an equal volume of 20% TCA/50% Acetone. For immunoprecipitation the cell-free translation mixture was diluted with four volumes of IP buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, and 1% Triton X-100) and incubated with 1 μl of the respective serum at 4°C overnight. The anti-SRP54 peptide antibody, 981, has been previously described (Römisch et al., 1990). Subsequent stages of the procedure were performed as described by Haeuptle et al. (1989).

To analyze membrane components, the samples were subjected to extraction with sodium carbonate. Samples were made 0.1 M with respect to sodium carbonate, pH 11.5, and incubated on ice for 15 min. The membranes were recovered by centrifugation in an airfuge (Beckman Instruments Inc.,

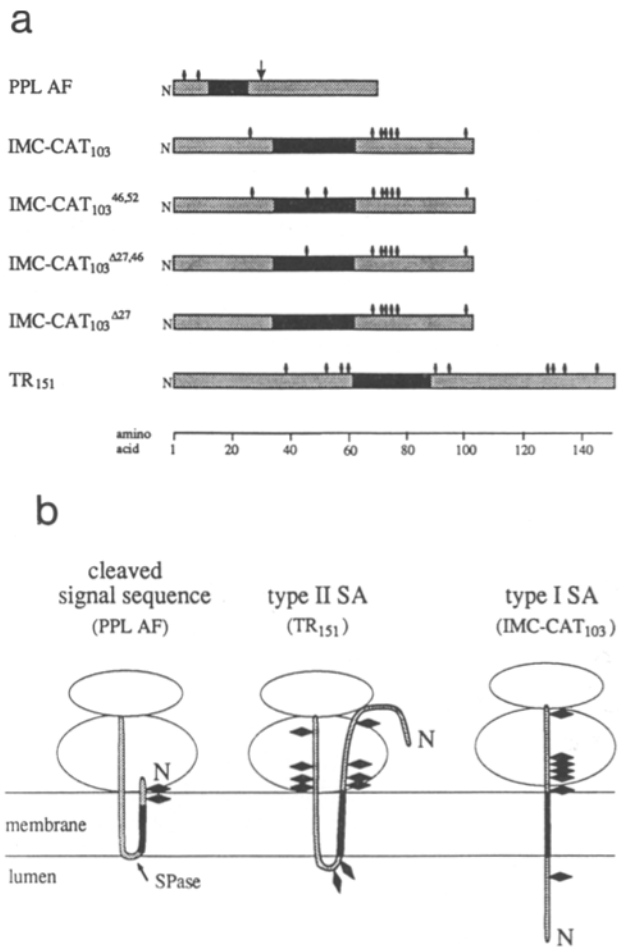

Figure 1. (*a*) Shows the position of the lysine residues (♦) present in the nascent chains of the SRP-arrested fragment of PPL, COOH-terminally truncated IMC-CAT ($IMC\text{-}CAT_{103}$) and variants of IMC-CAT, and COOH-terminally truncated transferrin receptor (TR_{151}). Black boxes indicate the hydrophobic core of signal or SA sequences and the arrow indicates the position of signal peptidase cleavage of PPL. The position of amino acid residues is indicated below the protein outlines. Lysine residues are present at positions 4 and 9 of the PPL AF nascent chain and at positions 39, 53, 58, 60, 90, 95, 128, 130, 134, and 145 of TR_{151}. Lysine residues were present at positions 27, 69, 72, 73, 75, 77, and 101 of the basic IMC-CAT construct. The mutant $IMC\text{-}CAT_{103}^{46,52}$ has additional lysine residues at positions 46 and 52. The mutant $IMC\text{-}CAT_{103}^{\Delta 27,46}$ has the lysine at position 27 replaced by a glutamine residue and a lysine inserted at position 46. The mutant $IMC\text{-}CAT_{103}^{\Delta 27}$ has the lysine residue at position 27 replaced by a glutamic acid residue. (*b*) Shows putative translocation intermediates of a secreted protein (PPL) (after Krieg et al., 1989), a type II SA protein (TR), and a type I SA protein (IMC-CAT) generated by using SRP-arrested complexes and truncated mRNAs as outlined in (*a*). For PPL the SRP arrest after the synthesis of roughly 70 amino acids generates the arrested fragment of PPL (PPL AF). For PPL AF and TR_{151} portions of the nascent chain which would be translocated in the full-length protein are retained by the continued association with the ribosome. In $IMC\text{-}CAT_{103}$ the NH_2 terminus is translocated across the membrane (High, unpublished data) in exactly the same way as for SRP-arrested IMC-CAT (High et al., 1991) and therefore the nascent chain may be at a late stage of membrane insertion. The hydrophobic regions of the cleaved signal sequence of PPL and the SA sequences of IMC-CAT and TR are indicated by black boxes and the approximate positions of the lysine residues are indicated by diamonds. The arrow indicates the cleavage site for signal peptidase (*SPase*).

Palo Alto, CA) at 20 psi for 10 min. The supernatant was removed and the membrane pellet resuspended in 0.1 M sodium carbonate and reprocessed as above. The pooled supernatants of the two extractions were TCA precipitated by addition of 0.1 vol of 100% TCA solution. For direct analysis the samples were solubilized in sample buffer at 95°C for 5 min before gel electrophoresis.

Before immunoprecipitation the membrane pellets obtained after carbonate extraction were solubilized in 100 mM Tris.HCl, pH 7.5, 1% SDS by heating at 37°C for 30 min followed by 95°C for 5 min. The solubilized membrane pellets were then diluted 1 in 20 with IP buffer. For immunoprecipitation of the SSR we used a mixture of affinity-purified antibodies directed against the purified SSRα subunit (Hartmann et al., 1989*b*) a COOH-terminal peptide of SSRα (Prehn et al., 1990) and the native SSR complex (Görlich et al., 1990). The combined anti-SSR antibodies or control antiserum were preincubated with protein A Sepharose for 2 h, washed with IP buffer four times, and then added to the solubilized membrane fraction diluted with IP buffer. The samples were incubated at 4°C overnight and then further processed as described above. All samples were analyzed on 10–15% SDS-polyacrylamide gels and were subjected to fluorography with Entensify (New England Nuclear, Boston, MA) as directed by the manufacturer.

Results

SA Sequences Bind to SRP54

The incorporation of modified lysine residues into a nascent chain by cell-free translation and subsequent UV irradiation was first used to identify SRP54 as the component of SRP which interacts directly with the signal sequence of PPL (Kurzchalia et al., 1986; Krieg et al., 1986; Wiedmann et al., 1987*a*). We have used this approach to study the interac-

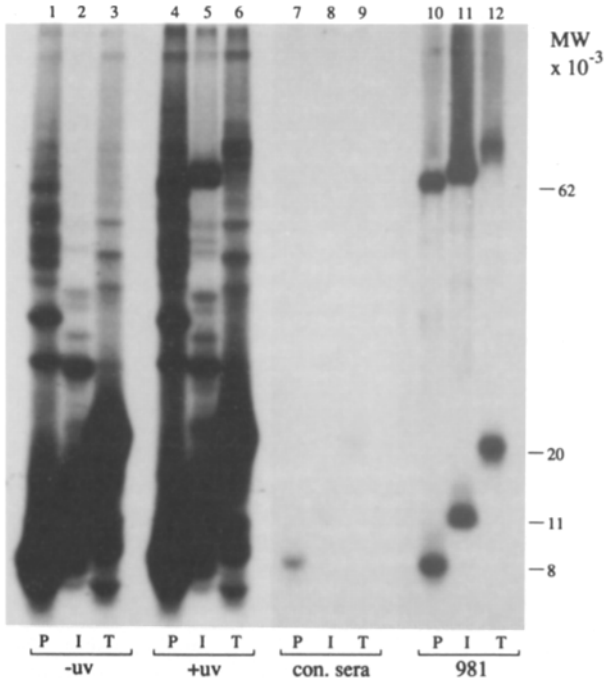

Figure 2. Photocross-linking of nascent chains to SRP54. The UV-activated cross-linking reagent ε-TDBA, present as ε-TDBA modified Lys, and ^{35}S-methionine were incorporated into SRP-arrested nascent chains of PPL AF (*P*), $IMC\text{-}CAT_{103}$ (*I*), and TR_{151} (*T*). Products after irradiation (lanes *4–6*) or controls which received no irradiation (lanes *1–3*) were characterized by SDS-PAGE and autoradiography. A portion of the irradiated mixture was immunoprecipitated with control serum (lanes *7–9*) or the antiserum 981 raised against a peptide of SRP54 (lanes *10–12*).

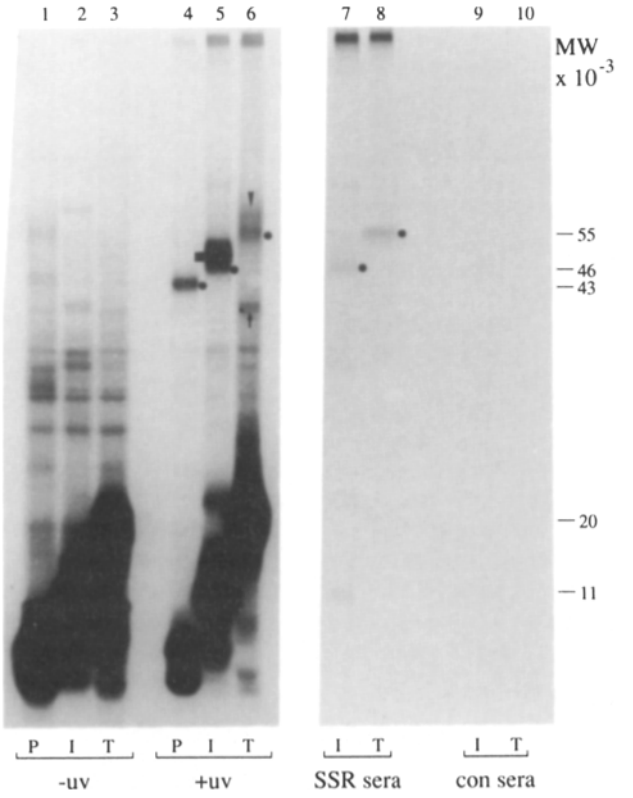

Figure 3. Photocross-linking of membrane components to nascent chains containing ε-TDBA-Lys. Nascent chain/ribosome complexes were allowed to interact with microsomal membranes (RMs) and then UV irradiated. Proteins present in the membrane pellet after sodium carbonate extraction before (lanes *1–3*) and after (lanes *4–6*) UV irradiation are shown. The nascent chains used were PPL AF (*P*), IMC-CAT_{103} (*I*), and TR_{151} (*T*). Proteins were immunoprecipitated with a mixture of antibodies specific for SSRα and SSRβ (lanes *7* and *8*) or control serum (lanes *9* and *10*). The estimated molecular weights of IMC-CAT_{103} (11) and TR_{151} (20) are indicated to the right of the figure together with the molecular weight of a 35-kD component cross-linked to PPL AF (43), IMC-CAT_{103} (46), and TR_{151} (55). The 35-kD protein SSRα (*) was found cross-linked to all the nascent chains. A 37-kD component, P37, (■) was the major crosslink to IMC-CAT_{103} (lane *5*) while additional components of 19 (*arrow*) and 42 kD (*arrowhead*) were crosslinked to TR_{151} (lane *6*). The samples were all run on one gel, lanes *1* to *6* are from a 3-d exposure while lanes *7* to *10* are from a 20-d exposure.

tion of SRP with SA sequences using IMC-CAT (High et al., 1991) and the TR (Schneider et al., 1984) as models for type I and type II SA proteins respectively (Fig. 1 *b*). Both proteins show an SRP-mediated arrest of translation in the wheat germ cell-free translation system (High et al., 1991; Zerial et al., 1986). After translation in the presence of SRP and ε-TDBA-Lys-tRNA samples were divided and a portion subjected to UV irradiation while the control received no irradiation. High molecular weight products, dependent upon prior UV irradiation, were seen for all the nascent chains used (Fig. 2, lanes *4–6*). These products were immunoprecipitated by antibody 981, an antipeptide antibody specific for SRP54 (Römisch et al., 1990) (Fig. 2, lanes *10–12*) but not by a control serum (Fig. 2, lanes *7–9*). In all cases the nascent chain is therefore cross-linked to SRP54. Antiserum 981 also immunoprecipitated significant amounts of all three non-crosslinked nascent chains (Fig. 2, lanes *10–12*, bands of 8, 11, and 20 kD). This precipitation is specific since almost no labeled nascent chains were immunoprecipitated with the control serum (Fig. 2, lanes *7–9*). The interaction between SRP54 and a ribosome/nascent chain complex has previously been found to be resistant to both high salt and 1% NP40 (S. High, unpublished data). It is therefore most likely that the uncross-linked nascent chains are coprecipitated with antiserum 981 because of their interaction with SRP54. In the case of PPL and IMC-CAT the cross-linking product was also immunoprecipitated by antibodies specific for the nascent chain (data not shown). No antibody was available to the small NH_2-terminal portion of the TR synthesized in these experiments.

Nascent SA Proteins Can Be Cross-linked to a Small Number of ER Membrane Proteins Including SSRα

Having shown that SA proteins and secreted proteins interact with the same component of SRP we investigated the interaction of SA proteins with components of the ER which might be involved in their translocation. The procedure used was that already established to identify SSRα (mp39) (Wiedmann et al., 1987*b*; Kreig et al., 1989; Wiedmann et al., 1989). It involved generating an elongation arrested SRP/nascent chain/ribosome complex which was allowed to interact with RMs. Upon UV irradiation the interaction of the signal sequence with membrane components later in the translocation pathway could be detected. We have previously shown that the addition of rough microsomes to the SRP-arrested complexes caused a large reduction in the amount of SRP54-nascent chain photo-crosslinking product in all cases, verifying that the transfer of the signal sequence was efficient (High et al., 1991). Under those conditions both IMC-CAT and TR were stably integrated into the membrane by a GTP-dependent mechanism which requires SRP and ribosome associated nascent chains (High et al., 1991). We now wished to identify the membrane components with which the nascent chains interact.

After the addition of membranes, samples were irradiated and then subjected to extraction with sodium carbonate to select for integral membrane proteins (Fujiki et al., 1982). For all of the nascent chains used specific UV-dependent cross-linked components were seen in the membrane pellet remaining after extraction with sodium carbonate solution (compare Fig. 3, lanes *1–3* and *4–6*). These cross-linked components were not seen in the absence of added membranes (compare Figs. 2 and 3) nor when the truncated mRNAs were translated in the absence of SRP and RMs (data not shown). While these results make it unlikely that any of the observed cross-links are to ribosomal proteins we cannot rule out this possibility. It is conceivable that the nascent chain of a membrane bound ribosome contacts a ribosomal protein which is not contacted in the free ribosome/nascent chain complex. In the case of PPL AF (Fig. 3, lane *4*) a 43-kD cross-linking product (*) is seen which represents a 35-kD protein cross-linked to the 8-kD nascent chain. This protein has previously been defined as the signal sequence receptor, SSRα, (Wiedmann et al., 1987*b*; Wiedmann et al., 1989), or mp39 (Krieg et al., 1989) and, as expected, was

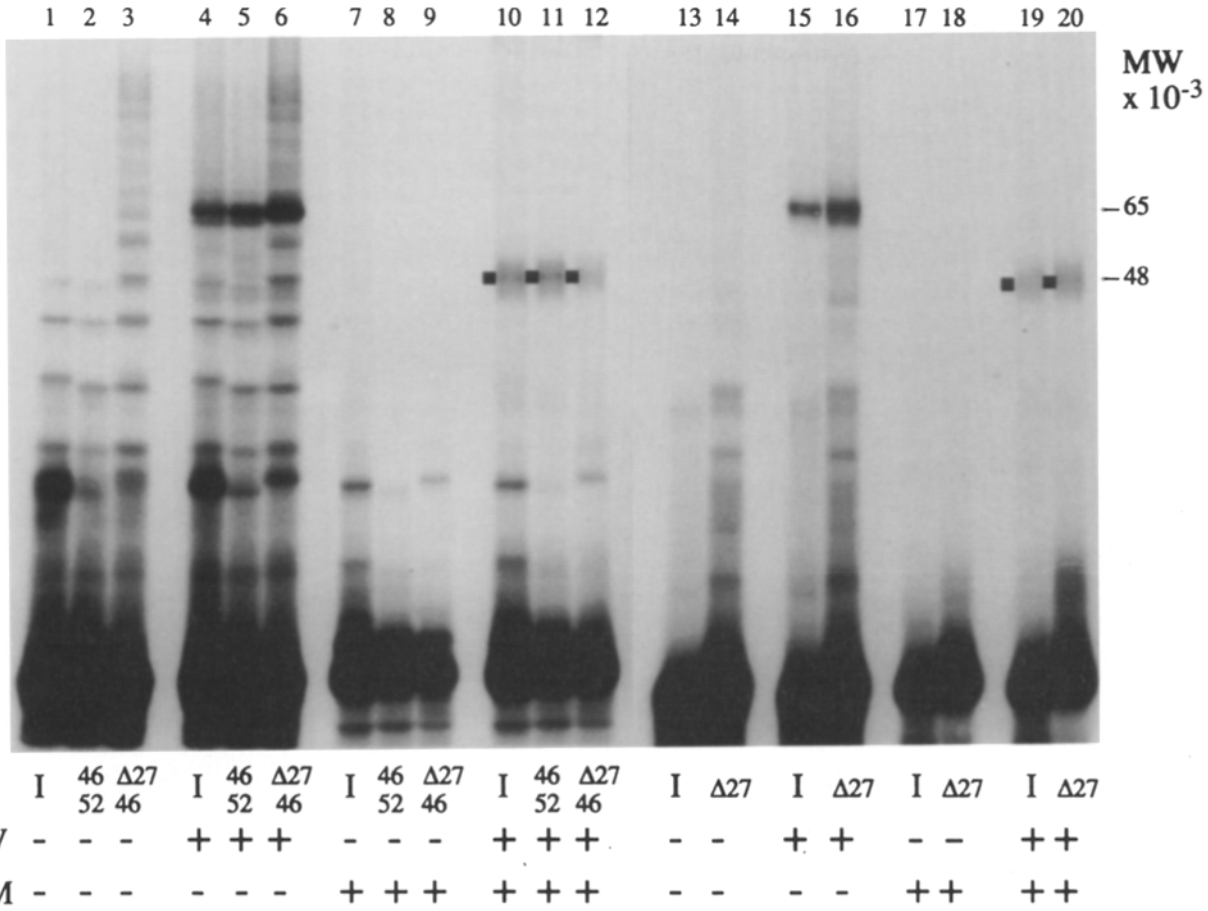

Figure 4. Photocross-linking of IMC-CAT lysine variants in the absence and presence of RMs. IMC-CAT$_{103}$ (*I*) and the variants IMC-CAT$_{103}^{46,52}$ (46,52) IMC-CAT$_{103}^{\Delta 27,46}$ (Δ27,46), and IMC-CAT$_{103}^{\Delta 27}$ (Δ27) were UV cross-linked, or processed without irradiation, in the absence or presence of rough microsomes (*RM*) as indicated. The TCA-precipitated material (*−RM*) or the membrane pellet after extraction with alkaline carbonate (*+RM*) were analyzed. Some slight alterations in mobility of the nascent chains, resulting from charge differences of the variants, are reflected by an altered mobility of the cross-linking products. Lanes *1* to *12* and *13* to *20* are results from two separate experiments. The calculated molecular weights for cross-links to SRP54 (65) and P37 (48) are indicated on the right. The cross-link to P37 is marked by a solid square.

bound by Con-A Sepharose (data not shown). The 43-kD cross-linking product was also immunoprecipitated by antibodies to prolactin (data not shown).

Using IMC-CAT$_{103}$ (Fig. 1 *b*) as the nascent chain probe a more complicated pattern of cross-linking products is seen (Fig. 3, lane *5*). To estimate the sizes of the proteins cross-linked to IMC-CAT$_{103}$ the contribution of the nascent chain (11 kD) was subtracted from the apparent molecular weights of the cross-linking products. The most prominent cross-link is to a set of proteins ranging from 33 to 42 kD with a 37-kD protein (P37) predominating (■) after a short exposure time of autoradiography (not shown). A crosslink to an 11-kD protein (visible as a 22-kD band) together with a weaker crosslink to a 60-kD protein (visible as a faint 71-kD band) were also seen. Immunoprecipitation from the solubilized pellet after a carbonate extraction, using an antibody specific for the nascent chain, brought down the cross-linking products of 33 to 42 kD (data not shown). Immunoprecipitation using a mixture of antisera specific for SSRα and SSRβ selectively precipitates IMC-CAT$_{103}$ cross-linked to a 35-kD protein (Fig. 3, lane *7*, *) and the photo–cross-linked protein also binds to Con-A Sepharose (data not shown). Thus, IMC-CAT$_{103}$ can be cross-linked with low efficiency to the integral membrane protein previously defined as the SSRα. Some of the 60-kD cross-linked protein is also immunoprecipitated by the SSR antibodies used (Fig. 3, lane *7*) but was not apparent in the Con-A Sepharose bound material (data not shown). Unlike the other cross-linked components, a UV-dependent cross-link to an 11-kD component was not observed in all experiments (see Fig. 4, lanes *10–12*, *19*, and *20*) and so the significance of this component is unclear at present.

Using TR$_{151}$ (Fig. 1 *b*) as the nascent chain for cross-linking in the presence of membranes a series of cross-linking products are observed (Fig. 3, lane *6*). The nascent chain of TR$_{151}$ has a molecular weight of 20 kD which was subtracted from the apparent molecular weights of the cross-linking products to estimate the size of the cross-linked partner as above. Three prominent cross-linked components of 42 kD (*arrowhead*), 35 kD (*) and 19 kD (*arrow*) are observed. The 35-kD partner is immunoprecipitated by antibodies specific for SSRα and SSRβ (Fig. 3, lane *8*, *), but not by control serum (Fig. 3, lane *10*), and also binds to Con-A Sepharose (data not shown). Thus the translocation

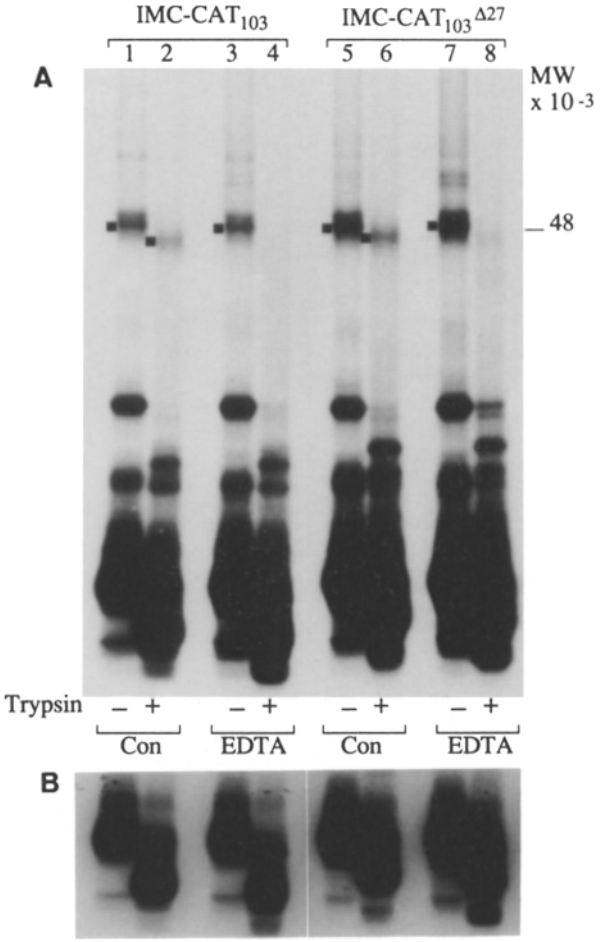

Figure 5. (*A*) Trypsin sensitivity of the IMC-CAT_{103} and IMC-$CAT_{103}^{\Delta 27}$ cross-links to P37 before and after dissociation of the ribosome with EDTA. After irradiation of IMC-CAT_{103} or IMC-$CAT_{103}^{\Delta 27}$ in the presence of rough microsomes the samples were treated with EDTA to disassemble the ribosomes (*EDTA*) or kept in Mg^{+2} containing buffer (*Con*) and subjected to trypsin treatment or mock treatment as indicated. After proteolysis the membrane pellet after carbonate extraction was analyzed. The cross-link to P37 is indicated by its molecular weight before proteolysis (48) and by a solid square. (*B*) Shows a shorter exposure of part of the autoradiograph shown in *A*. The reduction of 3 kD in the molecular weight of the IMC-CAT_{103} and IMC-$CAT_{103}^{\Delta 27}$ nascent chains after trypsin treatment can be seen.

intermediate of TR_{151} can be cross-linked to the SSRα. The cross-linking products of 42 and 19 kD are not immunoprecipitated by the SSRα/SSRβ specific antisera (Fig. 3, lane *8*) nor are they glycosylated as judged by Con-A Sepharose binding (data not shown). The pattern of cross-linked products recovered in the membrane pellet after extraction with sodium carbonate solution was identical when the cross-linking of IMC-CAT_{103} and TR_{151} was repeated using ϵ-ANB-lys tRNA in place of ϵ-TDBA-lys tRNA (data not shown).

The Position of the Crosslinking Group within the Sequence of IMC-CAT Has No Effect on the Pattern of Proteins Cross-linked

While for the PPL AF the lysine residues which are available as potential candidates to cross-link to other proteins are NH_2-terminal of the hydrophobic core of the cleaved signal sequence (Wiedmann et al., 1987*b*) this is not the case for IMC-CAT_{103} and TR_{151} where lysine residues are found both NH_2- and COOH-terminal of the hydrophobic core of the SA sequence (Fig. 1 *a*). For IMC-CAT_{103} the first lysine residue is at position 27, 7 residues NH_2-terminal of the hydrophobic stretch of amino acids. The next lysine is at residue 69, 6 residues COOH-terminal of this region. We have used three variants of IMC-CAT_{103} constructed by site-directed mutagenesis (Dobberstein, B., N. Flint, M.-T. Haeuptle, and J. Lippo, paper submitted for publication) where lysine residues are inserted into the hydrophobic core of the SA sequence (IMC-$CAT_{103}^{46,52}$ and IMC-$CAT_{103}^{\Delta 27,46}$) or where the sole lysine residue in the NH_2-terminal hydrophilic domain is replaced by a glutamic acid residue (IMC-$CAT_{103}^{\Delta 27}$) (Fig. 1 *a*). The presence of one or two lysines placed within the SA sequence does not disturb the arrest of translation upon addition of canine SRP (S. High, unpublished data) or the subsequent membrane insertion of the proteins (Dobberstein, B., N. Flint, M.-T. Haeuptle, and J. Lippo, paper submitted for publication).

IMC-CAT_{103}, IMC-$CAT_{103}^{46,52}$, IMC-$CAT_{103}^{\Delta 27,46}$, and IMC-$CAT_{103}^{\Delta 27}$ (Fig. 1 *a*) were cross-linked to SRP54 when the SRP-arrested nascent chain ribosome complex was irradiated (Fig. 4, lanes *4–6*, *15* and *16*). Since in IMC-$CAT_{103}^{\Delta 27}$ the first lysine is at residue 69 this region must be outside the ribosome and close to SRP54. The presence of additional lysines in the hydrophobic core of the SA sequence did not lead to any additional cross-links to other subunits of SRP suggesting that only SRP54 is close to or in contact with the SA sequence of the nascent chains. While up to four lysine residues will be retained within the ribosome of the IMC-CAT_{103}/ribosome/SRP complex generated (Fig. 1 *b*), no major UV-dependent cross-links other than to SRP54 were observed. Thus it seems that under these conditions no major cross-links to ribosomal proteins occur.

When membranes were added before UV irradiation, and the membrane pellet remaining after extraction with sodium carbonate was analyzed, the pattern of cross-linked proteins seen with IMC-$CAT_{103}^{46,52}$, IMC-$CAT_{103}^{\Delta 27,46}$, and IMC-$CAT_{103}^{\Delta 27}$ was identical to that previously observed with IMC-CAT_{103} (Fig. 4, lanes *10–12*, *19* and *20*). Similar to the results obtained with IMC-CAT_{103} the crosslinking is mainly to P37 with a small amount of SSRα probably also cross-linked. The results obtained using IMC-$CAT_{103}^{\Delta 27}$ where the first lysine is at residue 69, after the hydrophobic core of the SA sequence of IMC-CAT_{103}, show that it is the nontranslocated portion of IMC-CAT that is in close proximity to P37.

Proteolysis of Cross-linking Products

To further analyze the proteins cross-linked to the nascent chains of IMC-CAT_{103} and TR_{151} we used proteolysis after cross-linking in the presence of membranes. The size of the cross-linked product between PPL AF and SSRα is reduced by 4 kD after trypsin treatment (data not shown) as previously described by Krieg et al. (1989) and Wiedmann et al. (1989). This reduction is probably due to the removal of the 4-kD cytoplasmically exposed segment of the SSRα (Prehn et al., 1990).

For IMC-CAT_{103} a reduction of 3 kD in the size of the major cross-linking product is seen after trypsin treatment (Fig. 5 *A*, lane *2*). This is consistent with the loss of 3 kD

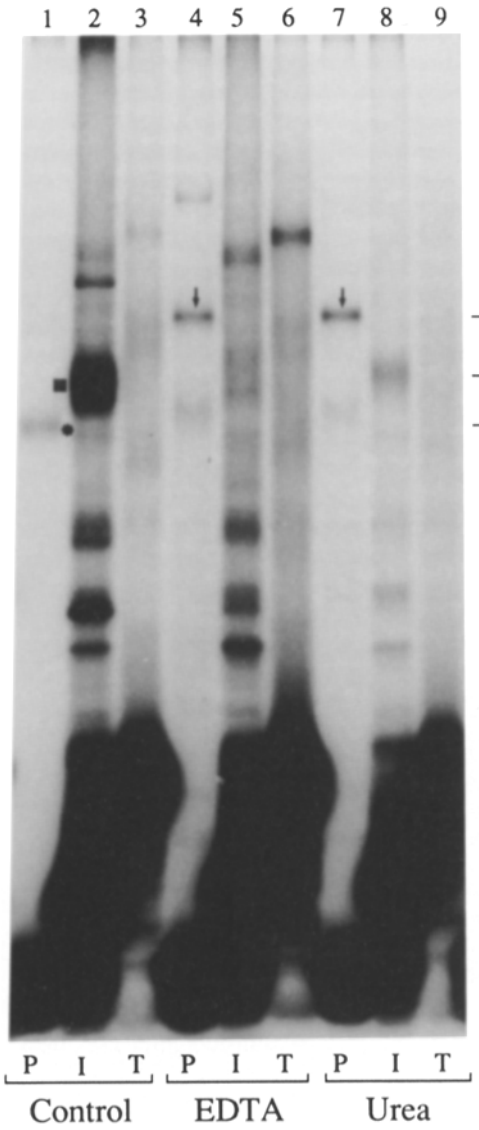

Figure 6. Disruption of translocation intermediates by EDTA and urea. After incubation of the ribosome/nascent chain complexes with rough microsomes the samples were treated with 25 mM EDTA or 4 M urea and then irradiated. The membranes were extracted with alkaline carbonate (pH 11.5), pelleted, and analyzed. The nascent chains used were PPL AF (*P*), IMC-CAT_{103} (*I*) and TR_{151} (*T*). Cross-linking products of PPL AF with SSRα (*) and a 56-kD component (*arrow*) and the cross-link product of IMC-CAT_{103} and P37 (■) are indicated by their respective molecular weights.

from the IMC-CAT_{103} nascent chain observed upon trypsin treatment (visible after short exposure time of autoradiography as shown in Fig. 5 *B*). Since the NH_2 terminus of IMC-CAT_{103} is translocated, and therefore protease protected (see Fig. 1 *b*), the 3 kD lost from IMC-CAT_{103} represents the COOH-terminus of the protein. This result shows that the major cross-linked protein, P37, is not accessible to protease digestion under these conditions. For TR_{151} trypsin treatment leads to proteolysis of the NH_2-terminus of the nascent chain and loss of most of the ^{35}S-Met label (data not shown). No further information could therefore be obtained for the interacting components.

P37 Cross-linked to IMC-CAT_{103} becomes Trypsin Sensitive upon Ribosome Dissociation with EDTA

To further investigate the nature of the cross-link between IMC-CAT_{103} and P37 we used treatment with EDTA to release the ribosome before trypsin treatment. Results showed that the P37 cross-linking product was only protected from proteolysis when the ribosome was not dissociated (Fig. 5, lane *2*). Disruption of the ribosome by EDTA allowed the protease access to P37 as judged by the disappearance of the cross-linking product (Fig. 5, lane *4*). Similar results were obtained using IMC-$CAT_{103}^{\Delta 27}$ (Fig. 5, lanes *5–8*) where the cross-link must be at the cytoplasmic side of the membrane. This result suggests that the major cross-link is between the nontranslocated COOH-terminal region of IMC-CAT_{103} and a cytoplasmic domain of P37. Since the COOH-terminal 3 kD of IMC-CAT is removed by trypsin treatment in the absence of EDTA, but the cross-linking product is still visible, the lysine responsible for the cross-linking of IMC-CAT_{103} to P37 must be one of those just COOH-terminal of the hydrophobic core of the SA sequence. The trypsin sensitivity of the cross-linking product upon EDTA treatment suggests that either this region is shielded by the intact ribosome or that some other EDTA-sensitive interaction is responsible for the protection of the IMC-CAT_{103}-P37 cross-linked complex. Dissociation of the ribosome/nascent chain complex by treatment with puromycin and high salt yielded similar results to those obtained after EDTA treatment (data not shown). This is consistent with the view that an intact ribosome or ribosome–membrane interaction is essential for the protease protection of P37.

Disruption of the Ribosome or of Protein–Protein Interactions Greatly Reduces the Interaction of the Nascent Chains with SSRα and P37

If the interaction of the nascent chain with components of rough microsomes represents a true translocation intermediate then it should be sensitive to agents which disassemble the ribosome or are known to disrupt protein–protein interactions. Gilmore and Blobel (1985) showed that treatment with 25 mM EDTA, which dissociates the ribosome into its subunits, leaves the PPL AF associated with the membrane while treatment with 4 M urea largely disrupts the association. We subjected the translocation intermediates to treatment with EDTA or urea, before photo-activation of the cross-linker, and determined whether such pretreatment would disrupt any of the observed interactions. For PPL AF both treatments lead to the disappearance of the cross-link to the 35-kD SSRα and the appearance of a cross-link to a slightly larger 37-kD component. Unlike SSRα, the 37-kD component did not bind to Con A-Sepharose (data not shown). A strong cross-link to a 56-kD component (apparent molecular weight 64 kD including PPL AF) was also seen after these treatments (Fig. 6, lanes *4* and *7*). This band did not comigrate with residual SRP54-PPL AF–crosslinked product which remained in the supernatant after extraction with sodium carbonate solution (data not shown).

Treatment of the IMC-CAT_{103} translocation intermediate with 25 mM EDTA or 4 M urea caused almost complete loss of the major cross-link to the group of proteins in the region of P37, together with the disappearance of the cross-link to the 60-kD component (Fig. 6, lanes *5* and *8*). For TR_{151} the

treatment with 25 mM EDTA does not cause the loss of any of the identified cross-links (Fig. 6, lanes *3* and *6*) however treatment with 4 M urea causes a reduction in all the previously identified cross-links to TR_{151} (Fig. 6, lanes *3* and *9*). The treatment of both IMC-CAT_{103} and TR_{151} with EDTA also resulted in the appearance of new higher molecular weight photocross-linked products. In both cases the estimated molecular weight of the new cross-linked component was 65-kD. Treatment of the translocation intermediates with puromycin and high salt before photo-activation also lead to a reduction in the amount of SSRα or P37 which was cross-linked to the nascent chains (data not shown), although the efficiency was not as high as that seen upon EDTA or urea treatment. Neither treatment with EDTA nor urea led to any release of the nascent chains from the membrane. This can be deduced from the unchanged amounts of IMC-CAT_{103} and TR_{151} which are recovered with the membrane pellet after carbonate extraction (Fig. 6). Thus, although EDTA or urea treatment did not alter the amount of IMC-CAT_{103} or TR_{151} nascent chains which remained membrane associated they largely abolished the interaction of these nascent chains with P37 and SSRα.

Discussion

Two models can be proposed to account for the membrane targeting and insertion or translocation of proteins with cleaved signal sequences or SA sequences. Assuming that protein components are involved in this process then either the same components might mediate the process for all types of protein, or specific components might be required by subsets of the different proteins. To distinguish between these possibilities we have used an established, highly selective, cross-linking assay. This has allowed us to identify components interacting with nascent SA proteins during their targeting to the ER and their subsequent membrane integration.

SRP has been shown to be necessary for the correct targeting of both type I and type II SA proteins to the ER (Hull et al., 1988; Holland and Drickamer, 1986; Lipp and Dobberstein, 1986). We show here that the nascent chains of both IMC-CAT, a type I SA protein, and TR, a type II SA protein, can be cross-linked to SRP54. Thus SRP54 can recognize and interact with both cleaved signal sequences (Kurzchalia et al., 1986; Krieg et al., 1986) and SA sequences of type I and type II SA proteins. A hydrophobic core in these signal sequences is their only common component (von Heijne, 1988) and must therefore be the decisive feature for signal sequence recognition by SRP54. IMC-CAT mutants which had the possibility to cross-link from within the SA sequence did not yield cross-links to SRP proteins other than SRP54. Thus the hydrophobic core of the signal sequence, as well as the hydrophilic sequences both NH_2- and COOH-terminal, appear to be in contact or close to only SRP54. These results are consistent with only SRP54 being involved in direct signal binding as suggested by cross-linking studies with PPL (Kurzchalia et al., 1986; Krieg et al., 1986) and implied by biochemical studies of subunit function (Siegel and Walter, 1988). The characteristics of the interaction between SRP54 and SA sequences are identical to those observed between SRP54 and cleavable signal sequences since both GTP and RMs are required to obtain the release of the cleavable signals and SA sequences from SRP54 (High et al., 1991).

To identify the proteins in the vicinity of SA sequences during their membrane insertion and partial translocation we have added ribosomal complexes with SRP-arrested nascent chains to microsomal membranes. It has previously been shown that the interactions of the translocation intermediates formed under these conditions can be characterized by cross-linking (Wiedmann et al., 1987*b*; Krieg et al., 1989; Wiedmann et al., 1989). We found a protein of 35 kD crosslinked to the nascent chains of both IMC-CAT_{103} and TR_{151} which could not be removed from the membrane by extraction with sodium carbonate solution. The protein has the same size and glycosylation properties as the previously identified SSRα (mp39) (Wiedmann et al., 1987*b*; Krieg et al., 1989). The identity of this protein was confirmed by immunoprecipitation using antisera directed against the SSR. While SSRα was one of the major proteins cross-linked to PPL AF and TR_{151} it was only one of the minor proteins cross-linked to IMC-CAT_{103}. The cross-linking between the different nascent chains and SSRα was completely abolished upon prior treatment with EDTA, 4 M urea, or puromycin/0.5 M KCl, agents which either disassemble the ribosome or unfold proteins. This result suggests that the interaction with SSRα was specific and depended upon the context of the nascent chain/ribosome complex at the membrane rather than merely the presence of the nascent chain within the membrane. The observation that GTP is required before the signal sequence of membrane associated PPL AF can be transferred from SRP54 to SSRα (High et al., 1991) is further evidence that the interaction of the nascent chain with SSRα is of a specific nature.

SSRα has been shown to be in close contact to the signal sequence of PPL (Wiedmann et al., 1987*b*) as well as other portions of the PPL nascent chain (Kreig et al., 1989; Wiedmann et al., 1989). SSRα can also be cross-linked to a translocation intermediate of β-lactamase (Görlich et al., 1990) and hybrid proteins containing the transmembrane region of IgM have been shown to cross-link to mp39 (SSRα) (Thrift et al., 1991). These results show that for a number of different secreted and membrane proteins one of the principal interactions is with SSRα. Antibodies raised against SSRα have been shown to inhibit the translocation of secreted proteins in a cell-free system (Hartmann et al., 1989*b*) and taken together these results suggest that SSRα (mp39) may be a ubiquitous component of the translocation machinery.

SSRα was not the only protein cross-linked to TR_{151}. Cross-links to two additional proteins of 19 and 42 kD were found. A 43-kD protein has been cross-linked to a synthetic signal sequence peptide and shown to have a large trypsin resistant fragment (Robinson et al., 1987). Since the bulk of label is lost from TR_{151} upon proteolysis we could not determine whether the 42-kD protein cross-linked to TR_{151} contains a large trypsin resistant fragment and therefore might be the same protein as that identified by Robinson et al. (1987).

The major component cross-linked to IMC-CAT_{103} is a nonglycosylated protein with an estimated molecular weight of 37 kD (P37). This protein is only seen upon the addition of rough microsomes consistent with it being a component of the ER. As already discussed above, we cannot at present completely rule out the possibility that the protein is ribosomal in origin. Derivatives of IMC-CAT with lysine residues placed within the hydrophobic core of the SA sequence showed an identical pattern of cross-linked proteins

to that obtained using IMC-CAT$_{103}$. Thus the only proteins in proximity to IMC-CAT$_{103}$ are P37 and, to a lesser extent, SSRα. Although in the construct IMC-CAT$_{103}^{\Delta 27}$ the only lysine residues present are in the nontranslocated COOH-terminal region of the polypeptide (Fig. 1, *a* and *b*) the efficiency of the crosslinking to P37 is the same as that obtained with IMC-CAT$_{103}$. This suggests that the principal interaction between IMC-CAT$_{103}$ and P37 is at the cytoplasmic face of the membrane.

The nature of the interaction between IMC-CAT$_{103}$ and P37 was further investigated by assessing the protease sensitivity of the cross-linking product. Trypsin treatment of the translocation intermediate showed that P37 is completely resistant to proteolysis when the nascent chain/ribosome complex is bound to the membrane. The observed loss of 3 kD from the cross-linking product is caused by the removal of the COOH-terminus of IMC-CAT$_{103}$. This cleavage could indicate that the COOH terminus of IMC-CAT$_{103}$ loops out of the ribosome and becomes protease accessible. Alternatively, the ribosome may detach from the membrane and expose a protease sensitive site of the nascent IMC-CAT$_{103}$. Different sensitivities to protease have been observed using nascent PPL and VSV G polypeptides of various lengths. From these studies it was suggested that the ribosome detaches from the membrane after about 100 amino acids of the mature protein have been synthesized, exposing a portion of polypeptide which is therefore protease accessible (Connolly et al., 1989). IMC-CAT$_{103}$ is 103 amino acids in length.

Upon EDTA treatment the IMC-CAT$_{103}$-P37 cross-linking product becomes completely accessible to proteolysis. As this is true for both IMC-CAT$_{103}$ and IMC-CAT$_{103}^{\Delta 27}$ we conclude that cross-linking has occurred on the cytosolic face of the membrane and that it is the COOH-terminal portion of IMC-CAT$_{103}$ that interacts with P37. When the translocation intermediate of IMC-CAT$_{103}$ (Fig. 1 *b*) is treated with EDTA, 4 M urea or puromycin and high salt before photoactivation, the efficiency with which IMC-CAT$_{103}$ is cross-linked to P37 is greatly reduced. The transfer of the nascent chain of IMC-CAT$_{103}$ from SRP54 to P37 has also been shown to occur only when both RMs and GTP are present (High et al., 1991). Taken together, these results provide further evidence that the interaction between IMC-CAT$_{103}$ and P37 is dependent not upon a random interaction between the nascent chain and membrane components, but upon an interaction of a highly specific nature.

Why does IMC-CAT$_{103}$ show a strong cross-link to P37 while there is no apparent cross-link to the two other proteins PPL AF and TR$_{151}$ which were used in this study? While it is possible that P37 represents a specific interaction with a type I SA protein a number of other explanations are possible. The NH_2 terminus of IMC-CAT$_{103}$ has been translocated across the membrane while the nascent chains of both PPL AF and TR$_{151}$ are probably still in the loop structure believed to be involved in membrane insertion (Inoyue and Halegoua, 1980; Shaw et al., 1988) as illustrated in Fig. 1 *b*. IMC-CAT$_{103}$ would have one or more lysines, just after the hydrophobic core of the SA sequence and very close to the cytoplasmic face of the membrane. Lysine residues in a similar position are not present in PPL AF and may still be within the ribosome in TR$_{151}$ (see Fig. 1 *b*). Thus P37 may represent a protein which is very close to the nascent chain where it enters the cytoplasmic face of the membrane. Strong cross-linking to a nonglycosylated protein which is slightly larger than mp39 (SSRα) has also been observed with other membrane proteins (Thrift et al., 1991).

Another possibility is that membrane insertion and translocation proceed in a stepwise manner and that the membrane-spanning IMC-CAT$_{103}$ represents a later stage of this process than the one in which PPL AF and TR$_{151}$ are cross-linked. These different stages of translocation could be reflected in different protein interactions. P37 could therefore be a component of the translocation apparatus which has a function later in the translocation process than that performed by SSRα. Krieg et al. (1989) have studied the pattern of RM proteins which are cross-linked to a number of PPL truncations of increasing chain lengths. They found that while mp39 (SSRα) was cross-linked to all the chain lengths used (86 to 131 amino acids), the longer nascent chains were also cross-linked to at least one additional protein which was non-glycosylated and larger than mp39. This maybe the same protein which we find as the major cross-link to IMC-CAT$_{103}$, that is, P37.

When membrane-inserted PPL AF was treated with EDTA or 4 M urea the nascent chain was then cross-linked to a protein which was larger than SSRα (37 kD) and also nonglycosylated. The protein therefore shows similar properties to P37 and a protein seen by Krieg et al. (1989) when using longer PPL nascent chains. It is possible that treatment with EDTA or 4 M urea causes PPL AF to move from SSRα into close proximity with P37.

Using different membrane inserted nascent chains as cross-linking probes we have identified several interacting components. Beside the SSRα, the most prominent cross-linked component was P37. It remains to be established whether this component is indeed a protein of the endoplasmic reticulum, and if so, what role it plays in the targeting or membrane insertion of proteins.

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