The Signal Sequence Receptor Has a Second Subunit and Is Part of a Translocation Complex in the Endoplasmic Reticulum as Probed by Bifunctional Reagents

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Abstract. Bifunctional cross-linking reagents were used to probe the protein environment in the ER membrane of the signal sequence receptor (SSR), a 34-kD integral membrane glycoprotein (Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. Nature [Lond.]. 328:830–833). The proximity of several polypeptides was demonstrated. A 22-kD glycoprotein was identified tightly bound to the 34-kD SSR even after membrane solubilization. The 34-kD polypeptide, now termed αSSR, and the 22-kD polypeptide, the βSSR, represent a heterodimer. We report on the sequence of the βSSR, its membrane topology, and on the mechanism of its integration into the membrane. Cross-linking also produced dimers of the α-subunit of the SSR indicating that oligomers of the SSR exist in the ER membrane.

Various bifunctional cross-linking reagents were used to study the relation to ER membrane proteins of nascent chains of preprolactin and β-lactamase at different stages of their translocation through the membrane. The predominant cross-linked products obtained in high yields contained the αSSR, indicating in conjunction with previous results that it is a major membrane protein in the neighborhood of translocating nascent chains of secretory proteins.

The results support the existence of a translocon, a translocation complex involving the SSR, which constitutes the specific site of protein translocation across the ER membrane.

Protein transport across the ER membrane can be divided into two phases: (a) an initiation phase that generally involves the function of the signal recognition particle (SRP) (Walter and Blobel, 1981) and of the docking protein (also called SRP receptor) (Meyer et al., 1982; Gilmore et al., 1982), and (b) the actual membrane transfer of the polypeptide. Little is known about the second process. By photocross-linking a first attempt has been made to probe the molecular environment of a nascent polypeptide chain as it passes through the membrane (Kurzchalia et al., 1986; Krieg et al., 1986; Wiedmann et al., 1987a, b). Photoreactive chemical groups were incorporated into nascent preprolactin chains by the use of modified lysyl-tRNA, and upon irradiation, gave rise to cross-links with proteins located in close proximity. With the photoreactive groups present in the signal sequence, an integral glycoprotein of the ER membrane (with a molecular mass of 34 kD) was identified that has been provisionally termed signal sequence receptor (SSR) (Wiedmann et al., 1987b). Recent results indicate that various regions of the mature portion of a nascent polypeptide are also in close proximity to the SSR during the course of translocation (Krieg et al., 1989; Wiedmann et al., 1989). Thus, SSR may be part of a translocation complex through which or alongside which the nascent chain is guided across the membrane. However, the function of SSR in translocation requires further study since the cross-linking efficiency was rather low and since cross-linking in general can result in unspecific interactions. Furthermore, all previous experiments were performed with short preprolactin chains that did not undergo signal peptide cleavage and therefore presumably did not yet have a sizable portion exposed to the lumen of the ER. We have therefore used a different cross-linking strategy to further define the relation of translocating nascent secretory proteins and integral ER membrane proteins. Also, the studies were extended to another secretory protein and to a stage of translocation at which a truly transmembrane orientation of the translocating nascent chain can be expected.

We have previously purified a 34-kD protein that resembled the SSR in its molecular properties (Harmann et al., 1989). Antibodies directed against the purified protein partially precipitated the photocross-linked products of nascent...
preprolactin chains and the SSR (Wiedmann et al., 1989). Further evidence for the identity of the two proteins is provided in the present paper.

The SSR appears to be essential for the translocation process since antibodies directed against it and Fα fragments prepared from the antibodies inhibit the in vitro translocation of several secretory proteins (Hartmann et al., 1989). It is present in the ER membranes of various tissues in at least equivalent amounts compared with membrane-bound ribosomes and is in a more than 10-fold excess compared with SRP and its receptor (Hartmann et al., 1989). Such a relation is to be expected if SSR is indeed involved in the actual translocation rather than in the initiation phase since each membrane-bound ribosome is in the process of delivering a nascent chain through the membrane (Palade, 1975). Accordingly, the protein is segregated in the rough portion of the ER as shown by electronmicroscopic immunocytochemistry and cell fractionation (Vogel et al., 1990).

The sequence of the SSR shows that it contains only one hydrophobic membrane-spanning segment (Prehn et al., 1990). This fact and the small size of the protein make it unlikely that a single molecule of the protein forms a translocation site. It appears more probable that SSR is part of a larger protein complex in the ER membrane that may constitute the site at which translocation proceeds, named "translocon" by Walter and Lingappa (1986). To test this possibility and to identify components of the putative complex, we have probed the molecular environment of the SSR by means of bifunctional cross-linking reagents. We show here that SSR is indeed contained in a complex that includes a tightly bound second subunit.

Glossary

| Acronym | Definition |
|---------|------------|
| AP      | acceptor peptide |
| CMC     | 1-cyclohexyl-3-(2-morpholinomethyl) carbodiimide-metho-4-toluene sulfonate |
| DMS     | dimethylsuberimidate |
| DSP     | diethobis(succinimidyl propionate) |
| DTSSP   | 3,3'-dithiobis(sulfosuccinimidyl propionate) |
| EK-RM   | microsomal membranes washed with salt and EDTA |
| HSD     | high salt detergent |
| K-RM    | microsomal membranes washed with salt |
| MP buffer | 10 mM Hepes/KOH pH 7.5, 250 mM sucrose, 3 mM MgCl₂, 80 mM KCl |
| RM      | rough canine microsomes |
| SH      | sulfhydroxyl |
| SRP     | signal recognition particle |
| SSR     | signal sequence receptor |
| sulfo-MBS | m-maleimidobenzoyl-sulfosuccinimidoester |

Materials and Methods

Cross-Linking of the SSR in Microsomes

100 μl of a suspension of canine microsomal membranes containing 200 equivalents (see Walter et al., 1981) were diluted to 1 ml with 50 mM Hepes/KOH pH 7.5, 250 mM sucrose, and diethobis (succinimidyl propionate) (DSP; Pierce Chemical Co., Rockford, IL) was added to a final concentration of 0.2 mg/ml from a solution in DMSO (50 mg/ml). After incubation for 10 min on ice, the reaction was stopped with 50 mM Tris/HCl pH 7.5. The membranes were solubilized by addition of 1% NP-40 and 500 mM potassium acetate (final concentrations) and aggregates were removed by a 15-min spin in an Eppendorf centrifuge (Brinkmann Instruments Co., Westbury, NY). 100 μl of matrix-coupled affinity-purified αSSR-C antibodies (see below) were added. After incubation at 4°C overnight, the resin was washed with 50 ml of Tris/HCl pH 7.5, 500 mM potassium acetate, 0.2% NP-40, and the proteins were eluted with 1.5 ml of 0.1 M glycine/HCl pH 2.2, 0.1% NP-40, and precipitated with TCA. The proteins were then directly subjected to SDS-gel electrophoresis or redisolved in 9 μl of 50 mM Tris/HCl pH 7.5, 300 mM potassium acetate, 5 mM potassium phosphate pH 6.8, and 0.2% NP-40 by incubation for 5 min at 80°C and applied to a 100-μl hydroxyapatite column. After washing, elution was carried out with 9 μl of 9 M urea, 600 mM potassium acetate, 300 mM potassium phosphate pH 6.8, 0.2% NP-40. The proteins were precipitated with TCA and submitted to SDS-gel electrophoresis. For electrophoresis, the samples were carried out in the first dimension under nonreducing conditions (7.5-12.5% acrylamide gradient gel) (Sommer and Traut, 1974). For electrophoresis under reducing conditions in the second dimension, the strip was cut out, soaked for 15 min in 100 mM DTT, 75 mM Tris/HCl pH 6.8, 0.1% SDS, 20% glycerol and placed onto a 12.5% gel. After electrophoresis, the proteins were visualized by a modified Coomassie blue-staining procedure according to Harlow and Lane (1988).

Purification of the 22-KD Polypeptide (βSSR)

Purification by conventional chromatographic procedures was carried out as follows. 20 ml of a suspension of rough canine microsomes (RM) containing 20,000 equivalents were washed with high salt (Walter and Blobel, 1983). The resulting K-RM were preextracted with 50 mM Hepes/KOH pH 7.5, 50 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1% NP-40 (LSD-K-RM) and thereafter solubilized with the same buffer except that the potassium acetate concentration was raised to 500 mM. After sedimentation of undissolved material, the high salt detergent extract (HSD-extract) was incubated with Con A-Sepharose for 3 h at 4°C. The resin was filled into a column and washed with 50 ml of Con A buffer (see above). The bound material was eluted overnight at 25°C with 10-column vol of 0.7 M α-mannosides, 50 mM Hepes/KOH pH 7.5, 0.5 M potassium acetate, 0.4% NP-40. The eluate was applied to a 2-md hydroxyapatite column equilibrated with 10 mM potassium phosphate pH 6.8, 0.5 M potassium acetate, 0.4% NP-40. Elution was carried out with a linear potassium phosphate/EDTA gradient (10-350 mM). SSR eluted at ~130 mM potassium phosphate. The peak fractions were diluted 1:6 with 20 mM Tris/HCl pH 7.5, 0.4% NP-40 and applied to a QAE-Sephadex column (2 ml) eluted in 20 mM Tris/HCl pH 7.5, 50 mM potassium acetate, 0.4% NP-40. After washing with this buffer, elution was carried out with a linear gradient of potassium acetate (100-500 mM). SSR is eluted at 400-500 mM. About 200 μg of purified complex was obtained.

Immunofluorescence chromatography was carried out using the HSD-extract on a column to which affinity-purified αSSR-C antibodies (see below) had been coupled. 20 ml of the extract were applied to a 1 ml column. After washing with 200-column vol of 50 mM Hepes/KOH pH 7.5, 500 mM potassium acetate, and 0.2% NP-40, elution of the SSR was performed either with 5 ml of the synthetic peptide used for raising the antibodies, dissolved in the washing buffer (1 mg/ml), or with 5 ml glycine buffer as described above. In the former case, excess of the peptide was removed by passing the eluate over a hydroxyapatite column and step-eluting the SSR with 0.35 M potassium phosphate pH 6.8, 0.5 M potassium acetate, and 0.2% NP-40. About 1 mg of purified complex was obtained with either elution method. Identical results were obtained after immunofluorescence chromatography with antibodies directed against the carboxyl-terminal 15 amino acids of βSSR (βSSR-C antibodies), which were coupled to an agarose column.

The separation of the two subunits of the SSR was achieved as follows. The eluate of the immunocolumn was applied to a hydroxyapatite column (0.5 ml) equilibrated with 5 mM potassium phosphate, pH 6.8, 50 mM Tris/HCl, 500 mM potassium acetate, 0.2% NP-40. The column was washed with 9 ml of 9 M urea, 5 mM potassium phosphate pH 6.8, 300 mM potassium acetate, 50 mM Tris/HCl, and 0.2% NP-40 yielding the βSSR in the flowing-through. The α-subunit was eluted with 9 ml of 9 M urea, 300 mM potassium phosphate pH 6.8, 600 mM potassium acetate, and 0.2% NP-40.

The stoichiometric ratio of the two subunits of the SSR was determined from the absorption at 280 and 288 nm in 50 mM sodium phosphate pH 7.5 containing 2% SDS (analytical grade; Serva Fine Chemicals Inc., Garden City Park, NY) after their separation. The extinction coefficients were calculated from the numbers of Tyr and Trp residues as described by Edelhoch (1967). The ratio was corrected for the relative yields of the subunits during their purification. To this end, the SSR complex was subjected to SDS-gel electrophoresis and the ratio of the peaks of the two subunits was determined.
Amino Acid Sequencing of the βSSR

Purified βSSR was either subjected directly to automated sequencing or cyanogen bromide peptides were produced and separated by HPLC. One of the peptides was further cleaved with trypsin and the fragments were again separated by HPLC. Two of them were subjected to automated sequencing (see Fig. 3).

cDNA Cloning of βSSR

Cloning was done with the same cDNA library that was used previously to clone cDNA for the αSSR (produced from poly(A)-RNA of MDCK cells) (Prehn et al., 1990). Hybridization screening of colonies was carried out with the following two oligonucleotides: 5'-AC(A/G)TT(A/G)TA (A/G)TT(A/G)TA(T/C)TGG-3'; 5'-GTA(A/G)AA(A/G)TT(A/G) AA(A/G)TANCCNGC-Y (N indicates that all four bases were inserted at that position), corresponding to the peptides QYNIYNV and AGYFNFT, respectively. Labeling of the oligonucleotides and screening were performed as described (Prehn et al., 1990). Two positive clones were analyzed by nucleotide-sequencing of both strands. The inserts were either cut out with Bam HI and sequenced with the Sanger method after recloning in M13 phage, or analyzed directly by plasmid sequencing using primer oligonucleotides corresponding to the vector and to the insert.

In Vitro Transcription Translation

The insert of a βSSR clone was cut out with the restriction enzyme Not I, the ends were filled in with Klenow enzyme, and the DNA fragment was cloned into the SmalI site of the vector pGEM2. In vitro transcription with T7 RNA polymerase and translation in the wheat germ system were carried out as described (Prehn et al., 1990). Where indicated, K-RR, SRP, and the glycosylation acceptor peptide (AP: benzoyl-Asn-Leu-Thr-N-methylamide) were added at final concentrations of 4 μg/ml, 0.03 μg/ml, and 30 μM, respectively. Posttranslational proteolysis was carried out with 0.3 mg/ml protease K for 1 h at 37°C in the absence or presence of 0.3% NP-40.

Antibodies: Their Affinity Purification and Matrix Coupling

Antibodies to a peptide comprising the COOH-terminal 15 amino acids of αSSR (αSSR-C antibodies) have been characterized before (Prehn et al., 1990). Antibodies against the native SSR complex were raised in rabbits by binding the purified complex (∼50 μg) to stoichiometric amounts of αSSR-C antibodies before injection. This procedure yielded high titer antibodies that specifically recognized the SSR in solution. Antibodies to βSSR were raised in rabbits against two synthetic peptides comprising the first 15 (SSR-N antibodies) and the last 15 amino acids (βSSR-C antibodies) (indicated in bold face in Fig. 3).

Peptide antibodies were affinity-purified on a column to which the synthetic peptide had been coupled. The peptide was dissolved in isopropanol and coupled to Affigel 10 by incubation overnight at room temperature (20 mg per ml resin). Antibodies against the native SSR were affinity-purified on a column containing purified SSR complex coupled to Affigel 15. Elution of the antibodies was carried out in series at pH 12 and 2.2.

The immunocolumns with α- or βSSR-C antibodies were prepared by incubating affinity-purified peptide antibodies with 1 ml protein A-agarose for 1 h at room temperature. The resin was washed with 10 mM Hepes/KOH pH 7.5, 100 mM NaCl, and 0.2 M borate/NaOH pH 9.0, and then treated with 20 mM DMSO for 30 min at room temperature to cross-link the antibodies to protein A. The reaction was quenched with Tris/HCl pH 7.5.

Cross Linking of Nascent Preprolactin

The plasmid pLAC81M1 (a generous gift of Drs. D. Andrews and V. Lingappa, University of California, San Francisco) was linearized with Ava II and transcribed in vitro with SP6 RNA polymerase. The RNA was translated in 25 μl of a reticulocyte lysate for 15 min at 30°C as described (Pelham and Jackson, 1976) in the presence of nuclease-treated canine microsomes prewashed with EDTA and high salt (EK-RM) (Walter and Blobel, 1983). The membrane-targeted chains were centrifuged through a cushion containing high salt and taken up in MP buffer at a membrane concentration of 1 equivalent/50 μl. The reaction with sulfo-MBS was carried out as described above. For binding to Con A-Sepharose and for immunoprecipitation, the samples were first adjusted to 0.5 M potassium acetate, 1% NP-40. After dilution to 150 mM potassium acetate, immunoprecipitation was carried out with affinity-purified antibodies directed against the native SSR complex that were prebound to Affigel (100 μg bound immunoglobulin). After incubation for 2 h at room temperature, the reaction was washed, and the bound material was eluted with SDS sample buffer.
Results

Identification of Proteins in Proximity to the SSR in the ER Membrane

To identify membrane proteins that are in close proximity to the SSR in the ER membrane and would be candidates for constituents of a translocation complex, rough canine microsomes were treated with the bifunctional cross-linking reagent DSP and solubilized with nonionic detergent at high salt. The extract was passed over an immunocolumn to which SSR antibodies (αSSR-C antibodies) had been coupled. The bound material was analyzed directly by diagonal electrophoresis (first dimension nonreducing, second dimension after reduction with DTT). The bound material was treated with 9 M urea and applied to a hydroxylapatite column. The material eluted at high salt concentration was subjected to diagonal SDS-gel electrophoresis. In the diagonal, representing noncross-linked species, there were found two main spots, one corresponding to the 34-kD SSR (Fig. 1A, large solid arrow) the other with a size of ~22 kD (large open arrow). The latter spot was seen regardless of whether or not the cross-linking reagent had been present. Thus, the noncross-linked 22-kD polypeptide appears to be associated with the 34-kD SSR after membrane solubilization. Several other small polypeptides, some of which apparently contain disulfide bridge(s) (note that some migrate slightly off the diagonal), were also observed in variable amounts among the noncross-linked species copurified with the SSR, indicating that they are loosely bound under the conditions used for solubilization.

The main cross-linked product (to the left of the diagonal) corresponds to the heterodimer of the 34-kD SSR and the 22-kD polypeptide (small open arrows on the left). Several other cross-linked products were observed, including a prominent one between the 22- and a 20-kD polypeptide (small solid arrows). This protein also gave rise to cross-links with the 34-kD SSR and to a ternary product containing all three components. Minor cross-linked products were also observed with a 18-kD protein.

For further enrichment of the cross-linked products containing the 34-kD SSR, we have used the property of urea-denatured SSR to bind tightly to a hydroxylapatite column (see below). As shown in Fig. 1B, most of the noncross-linked 22-kD polypeptide could be removed (see large open arrow) and only a few cross-linked species remained. One is the heterodimer of the 34- and 22-kD polypeptides mentioned before (small open arrows). A second, faster migrating cross-linked product that also contained the 34- and 22-kD polypeptides was reproducibly observed (small open arrows on the right, see also Fig. 1A). It is possible that different populations of cross-linked dimers have different mobilities in SDS gels. In addition to the heterodimers, one major cross-linked product must be a homodimer of the 34-kD polypeptide based on its size and on the fact that no other constituent could be found (even after silver staining) (see large arrowhead in Fig. 1B).

Experiments using other cross linking reagents reacting with amino groups, such as 3,3'-dithiobis(sulfosuccinimidypropionate) (DTSSP) and DMS, also gave rise to a similar pattern of products (data not shown).

These data show that the 22-kD polypeptide is the most prominent neighbor of the 34-kD SSR that remains bound even after solubilization of the membranes. However, it is clear that other polypeptides, including a 20-, an 18- and a 20-kD polypeptide, and a second copy of the 34-kD SSR are also in its spatial proximity.

Purification of the SSR-associated 22-kD Polypeptide

The purification of the 22-kD polypeptide was based on its tight association with the 34-kD SSR after solubilization of...
canine microsomal membranes with nonionic detergents. Although it was evident that immunoaffinity chromatography with SSR antibodies could be used for purification, we have initially used conventional separation techniques to avoid the purification of immunologically cross-reacting proteins and to test the stability of the complex under various conditions (Fig. 2 A).

A remarkable enrichment of the SSR could be achieved by preextracting microsomal membranes with detergent at low salt concentrations. SSR remained in the sedimentable membrane remnants (Fig. 2 A, lane 3, LSD-KRM) whereas many other proteins, including those located in the lumen of the ER, were removed. Subsequent solubilization of the SSR was achieved by treatment with detergent at high salt concentration (Fig. 2 A, lane 4, HSD-extract).

Based on the fact that SSR is glycosylated, Con A–Sepharose chromatography was used as a first step in the purification procedure after membrane solubilization (Fig. 2 A, lane 5). The glycoproteins eluted from the column with α-methylmannoside were further separated on a hydroxylapatite column using a phosphate concentration gradient. A 22-kD polypeptide exactly coeluted with the 34-kD SSR from the column (data not shown). The material from the peak fractions (Fig. 2 A, lane 6) was further purified on a QAE-Sephadex ion exchange column using a salt gradient for elution. Again, the 34- and 22-kD polypeptides exactly coeluted (not shown). After this step, the two proteins made up >98% of the total Coomassie blue-staining material (Fig. 2 A, lane 7). The 22-kD polypeptide is a glycoprotein as demonstrated by Con A blotting (Fig. 2 A, lane 8).

Copurification of the two polypeptides was also observed with other separation media under varying conditions (data not shown). As anticipated, the complex could be isolated by a one-step procedure using immobilized αSSR–C antibodies. Lanes 10 and 11, Coomassie blue-staining material of RM and of the purified complex, respectively. (C) Separation of αSSR and βSSR by dissociation of the complex with urea and subsequent hydroxylapatite chromatography. Lane 12, complex before dissociation; lane 13, αSSR, eluted by high salt from the column after dissociation of the complex; lane 14, βSSR found in the flow-through of the column. The two arrows indicate the positions of αSSR and βSSR.
The nucleotide sequence around the initiator methionine (Fig. 3, highlighted with a broken line) is similar to the consensus sequence determined by Kozak (1989) to be present at most sites of translation initiation in eukaryotic mRNA. The protein sequence determined from the NH2 terminus of the pSSR starts at residue 18 of the sequence deduced from cloned cDNA. The amino acid sequence derived from it is shown above and that determined from cloned eDNA. The amino acid sequence derived for the expected amino acid sequence and coded for the expected amino acid sequence (Fig. 3).

The nucleotide sequence around the initiator methionine (Fig. 3, highlighted with a broken line) is similar to the consensus sequence determined by Kozak (1989) to be present at most sites of translation initiation in eukaryotic mRNA. The protein sequence determined from the NH2 terminus of authentic pSSR starts at residue 18 of the sequence deduced from the cDNA. The NH2 terminal extension (Fig. 3, triple-underlined area) has the typical characteristics of a cleavable signal sequence: it contains a stretch of hydrophobic amino acid residues and a cleavage site conforming to the -1,-3 rule of von Heijne (1983). The protein contains a sec-

**Amino Acid Sequence and Membrane Topology of the pSSR**

Partial amino acid sequences were determined both from the NH2 terminus of the intact polypeptide and of two tryptic peptides (Fig. 3, amino acid sequences shown in parenthesis in the one-letter code). To isolate cDNAs coding for pSSR, degenerate oligonucleotide probes corresponding to selected regions of these peptides were synthesized and used to screen a cDNA library prepared from mRNA of MDCK cells. Two positive clones were found that had identical nucleotide se-

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Figure 4. In vitro insertion of βSSR into microsomal membranes. RNA coding for βSSR was synthesized in vitro and translated in a wheat germ cell-free system with K-RM, SRP, and a glycosylation acceptor peptide (AP) present during the incubation, as indicated. Post-translational proteolysis with proteinase K (Prot.K) in the absence or presence of detergent (Det.) was carried out as indicated. glyc. βSSR, glycosylated βSSR; p-βSSR, precursor polypeptide; unglyc. βSSR, unglycosylated βSSR, lacking the signal peptide.

~19 kD in the wheat germ system (Fig. 4, lane 1). Microsomal membranes depleted of SRP yielded the same product (Fig. 4, lane 2). If SRP was also present during translation and if glycosylation was prevented by the addition of a competing acceptor peptide (AP), a smaller product was obtained (lane 3), indicating that translocation had occurred and that a signal peptide had been cleaved off. The processed protein was not completely protected against proteolytic attack. Proteinase K, added after translation, removed a ~0.5-kD segment (barely seen by comparing Fig. 4, lanes 3 and 4), indicating that a small portion of the βSSR is exposed on the cytoplasmic side. After disruption of the membrane barrier by detergent, the product was completely degraded by proteases (Fig. 4, lane 5). The existence of a small cytoplasmic tail of βSSR could be confirmed by immunoblot analysis after treatment of intact microsomal membranes with trypsin (data not shown). If glycosylation was not prevented in the cell-free system, a product corresponding in size to the authentic protein was obtained (Fig. 4, lane 6). The size difference of ~4 kD to the unglycosylated product indicates that two carbohydrate chains have been attached. The glycosylated protein was also only slightly shortened by proteolysis (Fig. 4, lane 7) and was completely degraded if detergent was present (lane 8).

These results show that the βSSR is synthesized with a cleavable signal peptide and inserted into the ER membrane by a SRP-dependent pathway. The mature protein has its NH₂ terminus exposed on the luminal side and a short segment (12 amino acids according to the sequence) on the cytoplasmic side. Two carbohydrate chains are attached to the luminal portion.

Proximity of Translocating Polypeptide Chains to the αSSR

We have used the bifunctional cross-linking reagents to extend the previous experiments on the environment of nascent polypeptide chains that are in the process of translocation across the ER membrane. Two secretory proteins were used, preprolactin and pre-β-lactamase.

To produce translocation intermediates, truncated nascent chains were synthesized in the presence of [³⁵S]methionine, SRP, and canine microsomes in a cell-free translation system using mRNAs prepared by in vitro transcription of plasmid DNA cut with restriction enzymes. The nascent chains remain bound to ribosomes that come to a halt at the end of mRNAs that lack a termination codon (Wiedmann et al., 1989; Krieg et al., 1989). The nascent chain–ribosome complexes targeted to the ER membrane were then isolated by centrifugation of the membranes through a sucrose cushion containing a high salt concentration (Gilmore and Blobel, 1985). The pellet fraction was treated at 0°C with various bifunctional cross-linking reagents and the products were analyzed.

If translocating nascent chains of preprolactin, containing 86 amino acids (86mer), were treated with a carbodiimide
Figure 5. Cross-linking of translocating preprolactin chains to the αSSR. (A) Translation of mRNA coding for the first 86 amino acids of preprolactin (86mer) was carried out in the presence of canine microsomal membranes and the membrane-targeted chains were pelleted as described in Materials and Methods. After reaction with CMC, the products were either directly analyzed (targeted chains, lanes 1-3 and 7-10) or subjected to various treatments: (lane 4) alkaline extraction of the membrane pellet (alk. pellet). (Lane 5) Solubilization of the alk. pellet with detergent at high salt (det. extract); (lane 6) binding to Con A-Sepharose (Con A bound). Lane 3 shows a control in which the nascent chains were released from the ribosomes by puromycin treatment before the addition of CMC. The products in lanes 9 and 10 were analyzed after treatment with trypsin or proteinase K, respectively. (B) After cross-linking of membrane-targeted 86mers with CMC, the products were solubilized either in SDS (lanes 1-3) or in NP-40 in the presence of high salt (lanes 4-7) and were subjected to immunoprecipitation with antibodies directed against SSR (lane 2), αSSR-C antibodies directed against the carboxy-terminal 15 amino acids of the SSR; lane 4, antibodies directed against the native SSR complex) or against prolactin (lanes 1 and 7). Controls were performed with antibodies presaturated with purified αSSR (lane 3), with purified SSR complex (lane 5), or with nonimmune immunoglobulin (lane 6). Equivalent amounts were used for the immunoprecipitations and the controls. (C) CMC cross-linking was performed with membrane-targeted preprolactin chains 86 or 132 amino acids long (86mer and 132mer, respectively) and the products in the alkali-extracted membrane pellet were analyzed. (D) Cross-linking with sulfo-MBS was performed with the membrane-targeted 86mer of preprolactin (cf. lanes 1 and 2). The products were further analyzed after solubilization of the membranes with NP-40 in the presence of high salt by binding to Con A–Sepharose (lane 3) or immunoprecipitation with αSSR-C antibodies (lane 4). (Lane 5) A control for the immunoprecipitation was performed with antibodies presaturated with purified SSR.
Figure 6. Cross-linking with sulfo-MBS of nascent β-lactamase chains to the αSSR. mRNA coding for the first 141 amino acids of β-lactamase (141mer) was translated in the reticulocyte lysate system in the absence (lane 1) or presence (lanes 2) of canine microsomal membranes pretreated with EDTA and high salt (EK-RM) (total products). The membrane-targeted nascent chains were isolated (lane 3) and subjected to reaction with sulfo-MBS (lane 4). The products were further analyzed by different methods (for these samples the assay was doubled): alkali extraction of the membrane pellet, lane 5; binding to Con A-Sepharose, lane 6 (there was probably some material lost during sample preparation); immunoprecipitation with antibodies directed against native SSR, lane 7; control with nonimmune immunoglobulin, lane 8. The arrowhead indicates the position of the cross-linked product containing the αSSR, the dot that containing a luminal soluble protein of about 53 kD.

(CMC), a reagent used to produce amide bonds between neighboring carboxyl and amino groups, one main labeled product of ~46 kD was obtained (Fig. 5 A, cf. lanes 2 and I, arrowhead). Such a product was only observed if the nascent chain had not completed translocation. If it was transferred across the membrane after its release from the ribosome by treatment with puromycin, the yield of the 46-kD cross-linked product was drastically diminished (Fig. 5 A, lane 3).

The 46-kD product was largely recovered in the membrane fraction after carbonate extraction (Fig. 5 A, lane 4), indicating that the nascent chain had been cross-linked to an integral membrane protein. The amount of the 86mer noncross-linked to that protein in the alkali-extracted membrane pellet was reproducibly increased by CMC, perhaps indicating that it had been cross-linked to phospholipids. The 46-kD cross-linked product could be solubilized by detergent at high salt concentrations (Fig. 5 A, lane 5). It was glycosylated, in contrast to the 86mer, as demonstrated by its binding to Con A-Sepharose (Fig. 5 A, lane 6). The cross-linked product was shortened by ~5 kD after treatment with high concentrations of trypsin (Fig. 5 A, cf. lanes 9 and 8) and slightly less so after treatment with proteinase K (lane 10), whereas the 86mer itself was completely resistant to proteolysis. Thus, cross-linking must have occurred to a portion of an integral glycoprotein of the ER membrane not accessible to proteases from the cytoplasmic side. The membrane protein has a size of ~36 kD (46–10 kD [86mer] = 36 kD) and a segment of ~5 kD exposed to the cytoplasm. These properties of the cross-linked membrane protein are similar to those deduced for the αSSR from photocross-linking experiments with the probes incorporated into the nascent chain (Wiedmann et al., 1987b; Krieg et al., 1989; Wiedmann et al., 1989).

To test the identity of the cross-linked membrane protein with the 34-kD putative αSSR (Hartmann et al., 1989), immunoprecipitation experiments were carried out (Fig. 5 B). Affinity-purified antibodies directed against the COOH-terminus of the αSSR (αSSR-C antibodies) were used (Prehn et al., 1990). The antibodies recognized the 46-kD cross-linked product after its solubilization in SDS (Fig. 5 B, lane 2). Antibodies presaturated with excess of purified αSSR gave a negative result (lane 3). The extent of immunoprecipitation was comparable to that with prolactin antibodies (Fig. 5 B lane 1). Thus, it may be concluded that the cross-linked membrane protein is identical with the previously purified αSSR.

Immunoprecipitation of the 46-kD cross-linked product could also be demonstrated after its solubilization with nonionic detergent at high salt concentrations using antibodies directed against the native complex of α- and βSSR (Fig. 5 B, lane 4). Controls carried out with antibodies presaturated with the purified complex (Fig. 5 B lane 5) or with nonimmune immunoglobulins (lane 6) were negative. Again, the extent of immunoprecipitation with SSR antibodies was similar to that with prolactin antibodies (Fig. 5 B, lane 7). It should be noted that a considerable amount of the noncross-linked 86mer was also found in the immunoprecipitate with SSR antibodies whereas much less was seen in the.
controls (Fig. 5 B, lane 4 vs. lanes 5 and 6). This may suggest that under these conditions even the noncross-linked chain is in tight association with the SSR.

If the cross-linking experiment with CMC was carried out with a fragment of preprolactin containing 132 amino acids (132mer), two cross-linked products of lower intensity were seen (Fig. 5 C, cf. arrowhead in lane 1 and dots in lane 2). In Fig. 5 C, lane 2, the upper band of ~48 kD corresponds in size to the cross-linked product of the 132mer and the αSSR, the lower one of 46 kD may originate from an interaction of αSSR with the shorter nascent chains of ribosomes that are stalled in front of those carrying the 132mer. Indeed, the shorter nascent chains of the stalled ribosomes are visible at the bottom of the gel (~90 amino acids, 90mer). Their contribution to the total membrane-targeted chains is, however, distinctly lower than those of the 132mer (not shown). Since the longer chain yields about the same amount of cross-linked product as the shorter one, it appears that the degree of juxtaposition of reactive groups of the nascent chain and the αSSR changes during the course of translocation.

The molecular environment of the 86mer translocation intermediate of preprolactin was also probed with sulfo-MBS, a hetero-bifunctional reagent reacting with NH2 as well as SH groups. A predominant cross-linked product of ~46 kD containing up to 5% of the radioactivity was again seen (Fig. 5 D, cf. lanes 1 and 2, arrowheads). It was bound to Con A-Sepharose (Fig. 5 D, lane 3) and could be immunoprecipitated with αSSR-C antibodies after its solubilization with nonionic detergent (lane 4). Controls with presaturated αSSR antibodies gave a negative result (Fig. 5 D, lane 5). Again, as in the experiment with CMC (Fig. 5 B, lanes 4–6), a significant amount of the noncross-linked 86mer was found in the immunoprecipitate with SSR antibodies and much less in the control.

Since the αSSR does not contain cysteines (Prehn et al., 1990), cross-linking by sulfo-MBS must have occurred with the amino group(s) of this protein and an SH-group(s) of the nascent preprolactin chain. The latter contains cysteines at positions 25, 34, and 41 (the signal peptide cleavage site is at position 30). Assuming that the COOH-terminal ~40 residues of the 86mer are buried in the ribosome (Malkin and Rich, 1967; Blobel and Sabatini, 1970), it is likely that these cysteines are located within the membrane, in particular if the signal sequence is inserted in a loop structure (Shaw et al., 1988).

The proximity of nascent preprolactin chains to the αSSR could also be demonstrated in experiments with DSS and DSP that produce cross-links between amino groups. Cross-linking of the 86mer yielded heterogeneous cross-linked products of ~46–50 kD but antibodies directed against αSSR specifically precipitated a single product of 46 kD (results not shown).

To test if the results with preprolactin hold for other secretory proteins, cross-linking experiments were carried out with β-lactamase. This protein also offered the opportunity to investigate a later stage of the translocation process at which the signal peptide of the nascent chain has been cleaved off. As may be seen from Fig. 6, lanes 1 and 2, the fragment of 141 amino acids of β-lactamase (141mer) was almost completely processed to a fragment of ~15 kD, corresponding to a 118mer lacking the signal peptide, if microsomal membranes were present during translation. Since signal peptide cleavage occurs at the luminal side of the ER membrane, the ribosome-bound processed chains should have had a truly transmembrane disposition. The molecular environment of such membrane-inserted chains was probed with low concentrations of sulfo-MBS. A major cross-linked product of 47 kD was obtained (Fig. 6, cf. lanes 4 and 3, arrowheads). Assuming that the 118mer gave rise to the product, the size of the interacting protein (47 kD - 15 kD = 32 kD) indicates that again the αSSR had been cross-linked. Indeed, the product was retained in the alkali-extracted membrane pellet (Fig. 6, lane 5) and was bound to Con A-Sepharose (Fig. 6, lane 6; for unknown reasons, the recovery was low in this experiment, but appeared to be nevertheless specific). The product was immunoprecipitated with antibodies directed against αSSR (Fig. 6, lane 7) but not with nonimmune immunoglobulin (lane 8). Thus, the processed β-lactamase chain appears to be in proximity of the αSSR during its transfer through the membrane.

Cross-linking must have occurred between the amino group(s) of the αSSR and the cysteine at position 52 of the processed β-lactamase fragment (118mer) since the other cysteine of the nascent chain should have been buried in the ribosome (position 98).

The experiments also provided evidence for cross-linking of the β-lactamase fragment to a soluble, luminal protein of ~53 kD (see dots in Fig. 6, lanes 4 and 6). This product was glycosylated (Fig. 6, lane 6), indicating its luminal location, but was not found in the alkali-extracted membrane pellet (Fig. 6, lane 5). The nature of the protein has yet to be clarified.

It should be noted that other reagents, such as CMC and DSS also gave rise to cross-linked products of the β-lactamase chain with the αSSR, but with lower yields (data not shown).

**Discussion**

We have used bifunctional cross-linking reagents to probe...
the protein environment of the SSR in the ER membrane. The 34-kD SSR was found in proximity to several integral membrane proteins, one of which is a 22-kD polypeptide that remains tightly bound to it even after solubilization of the membrane with detergent. The two proteins form a stable, stoichiometric complex and have therefore been termed α-(34 kD) and β-(22 kD) SSR. Both subunits have been found by immunological means in tissues of various mammals, in tissue culture cells and in the liver of birds (Hartmann et al., 1989; unpublished data). They appear to be in a similarly tight association in these cells as indicated by the coinmunoprecipitation with αSSR antibodies of a 22-kD polypeptide after labeling of cells with [35S]methionine (Prehn et al., 1990). Furthermore, most if not all of the α- and βSSR molecules in canine microsomes seem to be contained in the complex.

The sequence determined for the βSSR shares several features with that of the αSSR: both have amino-terminal cleavable signal peptides, one membrane-spanning segment, and two N-linked carbohydrate chains in the luminal portion (see scheme in Fig. 7). In addition, both are inserted into the ER membrane in a SRP-dependent manner. The reasons for the strong interaction of the two polypeptide chains are not entirely clear. Secondary structure predictions indicate that both subunits have β-pleated sheets adjacent to the membrane-anchor sequences at the luminal side that could be responsible for their interaction through hydrogen bonds (indicated by a zig-zag line in Fig. 7). Internal sequence regions of both subunits are indeed well protected against proteolysis of the solubilized SSR complex (unpublished data). Interestingly enough, the individual subunits synthesized in vitro appear to be more susceptible to proteolysis, perhaps because they are not assembled into the complex.

The cross-linking experiments with bifunctional reagents have shown that two molecules of the αSSR are within ~12 Å distance of each other in the ER membrane. Since we have tried to avoid a perturbation of the system by extensive cross-linking, we cannot exclude the possibility that more than two SSR molecules come close to each other in the ER membrane. In any case, it appears that SSR is present in a protein complex in the membrane that includes at least two copies of the heterodimer and several other polypeptides.

The present results strongly support the previous conclusion that the SSR found by cross-linking is identical with the purified 34-kD protein (Hartmann et al., 1989). Antibodies recognize the cross-linked products of nascent chains and the membrane protein, and size and glycosylation of the latter fit with the properties of the 34-kD protein. Also, the size of the cleaved-off cytoplasmic domain corresponds roughly to that estimated from the sequence of the αSSR (6 kD) (Hartmann et al., 1989; Prehn et al., 1990). Even the small size difference observed after treatment of the cross-linked products with trypsin or with proteinase K (Fig. 5 A, lanes 9 and 10) corresponds to the behavior of the noncross-linked αSSR in intact microsomes (data not shown) and to the cleavage sites predicted from its sequence (Prehn et al., 1990).

Which region of the αSSR is cross-linked to the nascent chains? Most if not all of the cytoplasmic portion of the αSSR can be cleaved off by proteases without severing the cross-linked product into nascent chain and the bulk of the αSSR (see Fig. 5 A). It follows that the participation of the cytoplasmic portion can at most involve residues very close to the membrane that may not be cleaved off by the proteases. An alternative site of cross-linking may be the luminal portion of the αSSR (the hydrophobic membrane anchor does not contain suitable chemical groups for the reaction with...
CMC, DSS, or sulfo-MBS. The experiments with sulfo-MBS and the 86mer of preprolactin raise the possibility that part of the presumably luminal portion of the αSSR that carries amino groups is actually located in the membrane.

The present experiments have provided evidence for the existence in the ER membrane of a translocation complex of several proteins, a constituent of which is the SSR. Since various cross-linking techniques have identified the αSSR as a major protein in proximity of the translocating nascent chain and since αSSR molecules are close to each other in the ER membrane, it appears that SSR forms the core of a translocation complex.

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