

# 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  $\alpha$ SSR, and the 22-kD polypeptide, the  $\beta$ SSR, represent a heterodimer. We report on the sequence of the  $\beta$ SSR, its membrane topology, and on the mechanism of its integration into the membrane. Cross-linking also produced dimers of the  $\alpha$ -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  $\beta$ -lactamase at different stages of their translocation through the membrane. The predominant cross-linked products obtained in high yields contained the  $\alpha$ SSR, 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.

**P**ROTEIN 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., 1987 *a, 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., 1987*b*). 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 (Hartmann 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  $F_{ab}$  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

AP	acceptor peptide
CMC	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-4-toluene sulfonate
DMS	dimethylsuberimidate
DSP	dithiobis(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 $MgCl_2$ , 80 mM KCl
RM	rough canine microsomes
SH	sulfhydryl
SRP	signal recognition particle
SSR	signal sequence receptor
sulfo-MBS	<i>m</i> -maleimidobenzoyl-sulfosuccinimidoester

## Materials and Methods

### Cross-Linking of the SSR in Microsomes

100  $\mu$ l 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 dithiobis (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., West-

bury, NY). 100  $\mu$ l of matrix-coupled affinity-purified  $\alpha$ SSR-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. They were then either directly subjected to SDS-gel electrophoresis or redissolved in 9 M urea, 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- $\mu$ l hydroxylapatite column. After washing, elution was carried out with 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. Diagonal electrophoresis was 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 ( $\beta$ SSR)

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 DTT, 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  $\alpha$ -methylmannoside, 50 mM Hepes/KOH pH 7.5, 0.5 M potassium acetate, 0.4% NP-40. The eluate was applied to a 2-ml hydroxylapatite 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 gradient (10–350 mM). SSR eluted at  $\sim$ 120 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) equilibrated 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 (100–900 mM). SSR is eluted at 400–500 mM. About 200  $\mu$ g of purified complex were obtained.

Immunoaffinity chromatography was carried out with the HSD-extract on a column to which affinity-purified  $\alpha$ SSR-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 hydroxylapatite 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 immunoaffinity chromatography with antibodies directed against the carboxy-terminal 15 amino acids of  $\beta$ SSR ( $\beta$ SSR-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 hydroxylapatite 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 M urea, 5 mM potassium phosphate pH 6.8, 300 mM potassium acetate, 50 mM Tris/HCl, and 0.2% NP-40 yielding the  $\beta$ SSR in the flow-through. The  $\alpha$ -subunit was eluted with 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, 0.1% SDS (analytical grade; Serva Fine Biochemicals Inc., Garden City Park, NY) after their separation. The extinction coefficients were calculated from the numbers of Trp and Tyr 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

by scanning of the Coomassie blue-stained gel. Similarly, defined aliquots of the recovered separated subunits were also subjected to electrophoresis and the ratio of the peaks determined by scanning. Final values of the molar ratio  $\alpha/\beta$  SSR of 1.08 (for 280 nm) and 1.15 (for 288 nm) were obtained.

### Amino Acid Sequencing of the $\beta$ SSR

Purified  $\beta$ SSR 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 $\beta$ SSR

Cloning was done with the same cDNA library that was used previously to clone cDNA for the  $\alpha$ SSR (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/T)AT(A/G)TT(A/G)TA(T/C)TG-3'; 5'-GT(A/G)AA(A/G)TT(A/G)AA(A/G)TANCCNGC-3' (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  $\beta$ SSR 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 Sma I 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-RM, SRP, and the glycosylation acceptor peptide (AP; benzoyl-Asn-Leu-Thr-*N*-methylamide) were added at final concentrations of 4 A<sub>280</sub> nm/ml, 0.03 A<sub>260</sub> nm/ml, and 30  $\mu$ M, respectively. Posttranslational proteolysis was carried out with 0.3 mg/ml proteinase K for 1 h at 0°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  $\alpha$ SSR ( $\alpha$ SSR-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 ( $\sim$ 50  $\mu$ g) to stoichiometric amounts of  $\alpha$ SSR-C antibodies before injection. This procedure yielded high titer antibodies that specifically recognized the SSR in solution. Antibodies to  $\beta$ SSR were raised in rabbits against two synthetic peptides comprising the first 15 ( $\beta$ SSR-N antibodies) and the last 15 amino acids ( $\beta$ SSR-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  $\alpha$ - or  $\beta$ SSR-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 DMS 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 pGEMBP1 (Connolly and Gilmore, 1986) was linearized with the restriction enzymes Pvu II and Rsa I and used in cell-free transcription reactions with T7 RNA polymerase to produce mRNA coding for the first 86 (86mer) or 132 (132mer) amino acids of preprolactin, respectively. Translation of the mRNAs in a wheat germ cell-free system (50  $\mu$ l) was carried out at 27°C for 10 min in the presence of 50 nM SRP as described

(Wiedmann et al., 1987a). Microsomal membranes, extracted with high salt (K-RM) (10 equivalents), were then added and the incubations continued for 3 min. The samples were placed on ice for 10 min and overlaid onto a 100- $\mu$ l cushion containing 250 mM sucrose, 50 mM Hepes/KOH pH 7.5, 5 mM MgCl<sub>2</sub>, 500 mM KCl in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) tube. After centrifugation for 5 min at 20 psi, the pellet was resuspended in 40  $\mu$ l MP buffer (10 mM Hepes/KOH pH 7.5, 250 mM sucrose, 3 mM MgCl<sub>2</sub>, 80 mM KCl) (membrane-targeted chains) (Gilmore and Blobel, 1985).

For reaction with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-4-toluene sulfonate (CMC; Serva Fine Biochemicals Inc.), 200  $\mu$ l of a 2 mg/ml solution in MP buffer was added (4 mM final concentration). After incubation for 30 min on ice, free carbodiimide was removed by centrifugation of the membranes through a cushion containing 500 mM sucrose, 50 mM Hepes/KOH pH 7.5, 5 mM MgCl<sub>2</sub>, 500 mM KCl for 5 min at 20 psi in an airfuge. The pellets were resuspended in 40  $\mu$ l MP buffer and incubated for 30 min on ice. Quenching of the cross-linking reagent was carried out with 50 mM glycine/HCl pH 7.5.

For reaction with *m*-maleimidobenzoyl-sulfosuccinimidoester (sulfo-MBS; Pierce Chemical Co.), the reagent was added to a final concentration of 0.12 mM and the mixture was incubated at 0°C for 10 min. The reaction was stopped by addition of 5 mM  $\beta$ -mercaptoethanol, 50 mM glycine/HCl pH 7.5.

For binding to Con A-Sepharose, the proteins were precipitated with TCA, redissolved in SDS buffer (0.1 M Tris/HCl pH 7.5, 1% SDS) and diluted 1:20 in Con A-binding buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1% NP-40). After incubation with 20  $\mu$ l Con A-Sepharose overnight, the resin was washed thoroughly and the bound material was eluted by boiling with SDS sample buffer (Laemmli, 1970).

Alkali treatment of microsomal membranes was carried out with 0.1 M Na<sub>2</sub>CO<sub>3</sub> as described except that 5 mM Tris-base was added (Wiedmann et al., 1987b). A high salt detergent extract was obtained by resuspending the alkali-extracted membranes in a buffer containing 50 mM Tris/HCl pH 7.5, 500 mM potassium acetate, 5 mM magnesium acetate, 1% NP-40, and centrifugation for 10 min at 20 psi in an airfuge.

Protease digestion was carried out directly after quenching of the cross-linking reagent by addition of proteinase K or trypsin to a final concentration of 0.1 mg/ml. After incubation for 1 h on ice, the reaction was stopped with 2 mM PMSF and the proteins were precipitated with TCA.

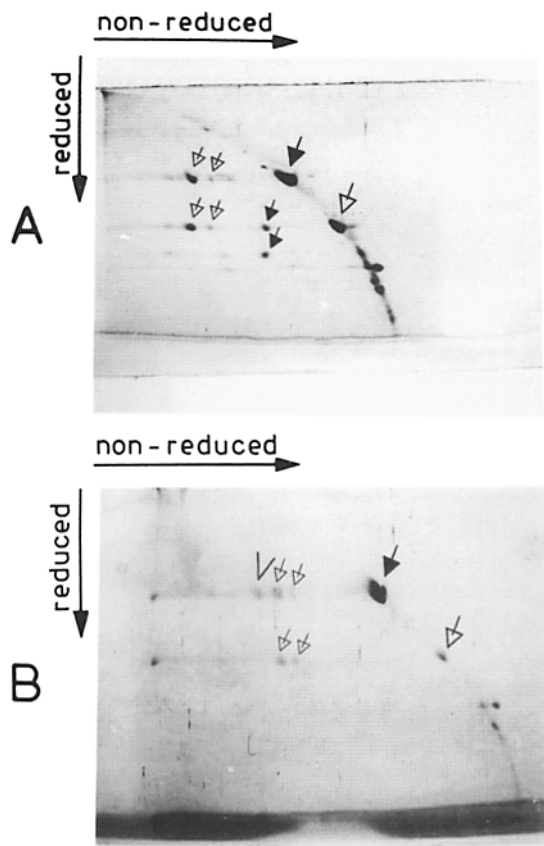
Puromycin (2 mM final concentration) was added to membrane-targeted chains for 5 min at 27°C, then KCl was added to a final concentration of 500 mM, and the incubation was continued for 5 min. The samples were placed on ice and 5 vol of 2 mg/ml CMC were added.

Immunoprecipitations with prolactin antibodies were performed as described (Wiedmann et al., 1987a). For immunoprecipitations with SSR antibodies after CMC-cross-linking, the samples were treated with 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH  $\sim$ 9, and 1% SDS at 95°C for 5 min. They were then diluted 1:20 in 0.01 M Tris/HCl pH 7.4, 0.5 M NaCl, 1% NP-40, 0.1% BSA, and centrifuged before addition of 15  $\mu$ l  $\alpha$ SSR-C agarose containing  $\sim$ 20  $\mu$ g coupled affinity-purified immunoglobulin. Presaturation of the antibodies was carried out for 1 h with  $\sim$ 30  $\mu$ g purified  $\alpha$ SSR (electrophoretically homogeneous). After incubation for 8 h at 4°C, the resin was thoroughly washed and the bound material was released with SDS sample buffer.

SDS-gel electrophoresis was carried out according to Laemmli (1970) using 10–15% linear acrylamide gels.

### Cross-Linking of Nascent $\beta$ -Lactamase

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  $\mu$ l 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  $\mu$ l. 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 protein A-Affigel ( $\sim$ 10  $\mu$ g bound immunoglobulin). After incubation for 2 h at room temperature, the resin was washed, and the bound material was eluted with SDS sample buffer.



**Figure 1.** Cross-linking of the SSR in intact microsomes. Canine microsomal membranes were treated with the cleavable bifunctional cross-linking reagent DSP and solubilized with nonionic detergent at high salt. The extract was passed over an immunocolumn to which SSR antibodies ( $\alpha$ SSR-C antibodies) had been coupled. (A) The bound material was analyzed directly by diagonal electrophoresis (first dimension nonreducing, second dimension after reduction with DTT). (B) 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. The solid and open large arrows indicate the positions of noncross-linked 34- and 22-kD polypeptides ( $\alpha$ - and  $\beta$ SSR), respectively. The small open arrows indicate cross-linked products of these polypeptides. The solid small arrows indicate a cross-linked product of the 22-kD polypeptide with a  $\sim$ 20-kD polypeptide. The large arrowhead in B indicates the occurrence of a cross-linked  $\alpha$ SSR-dimer. The prominent spots on the left hand side of part B correspond to the origin of the first dimension.

## 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 that reacts with amino groups and contains a disulfide bridge cleavable by reduction. After incubation with the cross-linker, the membranes were solubilized with nonionic detergent and products containing the SSR were isolated by immunoaffinity chromatography, using antibod-

ies directed against a peptide comprising the COOH-terminal 15 amino acids of the SSR ( $\alpha$ SSR-C antibodies) (Prehn et al., 1990). The products were then analyzed by diagonal SDS-gel electrophoresis (first dimension without reduction, second dimension after cleavage of the cross-link bond with dithiothreitol) (Sommer and Traut, 1974) (see Fig. 1 A). It should be noted that the extent of cross-linking was kept low to avoid a perturbation of the system.

In the diagonal, representing noncross-linked species, there were found two main spots, one corresponding to the 34-kD SSR (Fig. 1 A, large solid arrow) the other with a size of  $\sim$ 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.

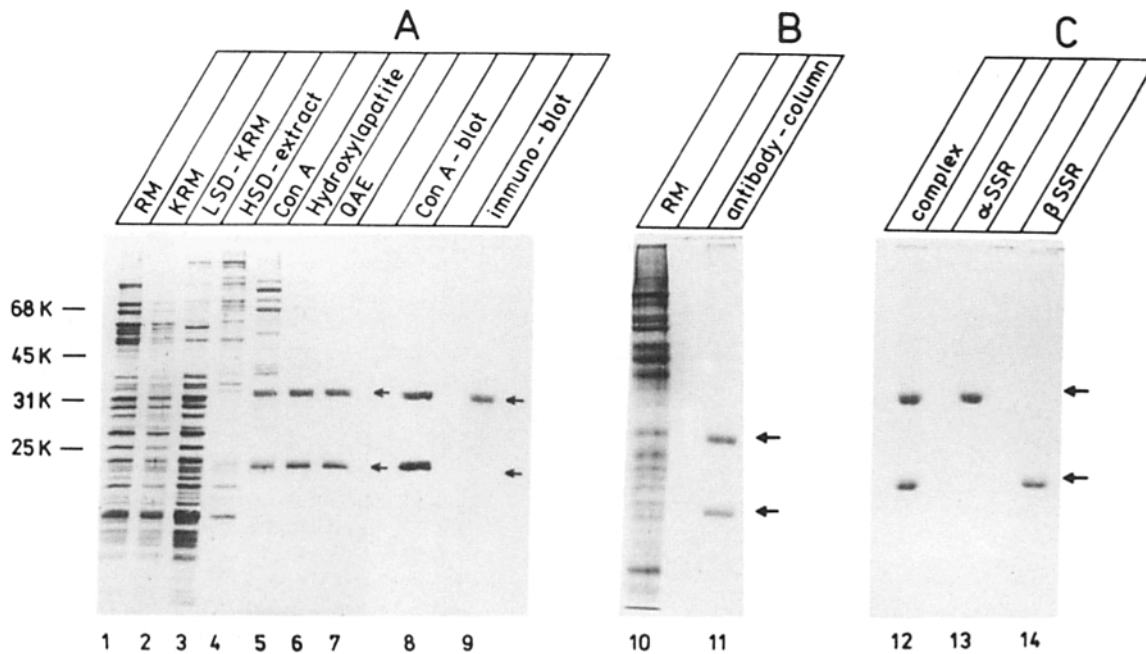
For further enrichment of the cross-linked products containing the 34-kD SSR, we have used the property of urea-denatured SSR to bind tightly to a hydroxylapatite column (see below). As shown in Fig. 1 B, most of the noncross-linked 22-kD polypeptide could be removed (see large open arrow) and only a 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. 1 A). 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. 1 B).

Experiments using other cross linking reagents reacting with amino groups, such as 3,3'-dithiobis(sulfosuccinimidyl propionate) (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-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



**Figure 2.** Purification of the SSR complex and of its constituents. (A) Purification of the SSR complex with conventional separation techniques. Lanes 1-7 show the protein pattern at different stages of the purification (Coomassie blue-staining after SDS-gel electrophoresis, 10% acrylamide gel). *RM*, rough microsomes; *KRM*, RM washed with high salt; *LSD-KRM*, KRM preextracted with detergent at low salt; *HSD-extract*, detergent extract after solubilization with detergent at high salt; *Con A*, material bound to and eluted from Con A-Sepharose with  $\alpha$ -methylmannoside; *Hydroxylapatite*, pooled peak fractions containing SSR after chromatography on a hydroxylapatite column; *QAE*, pooled peak fractions containing SSR after chromatography on QAE-Sephadex. Lane 8, the purified material was separated in an SDS-gel, transferred onto nitrocellulose and probed with Con A coupled with peroxidase followed by reaction with a chromogenic substrate (*Con A-blot*). Lane 9, Immunoblot analysis of the purified material using antibodies to intact SSR and  $^{125}\text{I}$ -protein A (autoradiograph). (B) Purification of the SSR complex by immunoaffinity chromatography using a column with immobilized  $\alpha$ SSR-C antibodies. Lanes 10 and 11, Coomassie blue-staining material of RM and of the purified complex, respectively. (C) Separation of  $\alpha$ - and  $\beta$ SSR by dissociation of the complex with urea and subsequent hydroxylapatite chromatography. Lane 12, complex before dissociation; lane 13,  $\alpha$ SSR, eluted by high salt from the column after dissociation of the complex; lane 14,  $\beta$ SSR found in the flow-through of the column. The two arrows indicate the positions of  $\alpha$ - and  $\beta$ SSR.

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  $\alpha$ -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 affinity-purified antibodies directed against the COOH-terminus of either the 34-kD SSR (Fig. 2 B, lane 11) or against the 22-kD polypeptide (data not shown).

The 22-kD polypeptide could be coimmunoprecipitated with SSR antibodies even after pretreatment of the complex at high or low pH (pH 11.5 or 2.2) or at high salt concentration (up to 1 M potassium acetate tested). Thus, the complex is either very stable or is rapidly reformed from its dissociated constituents under physiological conditions.

To determine the stoichiometric ratio of the two polypeptides in the complex, the proteins were dissociated in 8 M urea and then quantitatively separated by hydroxylapatite chromatography (Fig. 2 C). Under low salt conditions, the 34-kD polypeptide remained bound to the column whereas

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-17      +
1  CGGCCCGCGTGGTCCCCCTGAAAGGACTGTGGGGTGC CGCGATGAGGCTTCTGGCTTCTGTGCTGTGGCC
      +
- 7  LeuPheAlaValSerHisAlaGluGlyAlaArgLeuLeuAlaSerLysSerLeuLeuAsnArgTyrAla
73  CTGTTTGTCTCAGTCATCGGAGGAAGGCCCGCTCTGGCCCTCCAAGTCACTGCTGAACAGATACGCC
      (E--E--G--A--R--L--L--A--S--K--S--L--L--N--R--Y--A--
      - - - - -
18  ValGluGlyArgAspLeuThrLeuGlnTyrAsnIleTyrAsnValGlySerSerAlaAlaLeuAspValGlu
145  CTGGAGGGCGGAGACCTGACCTTGCGTACCAACATGCTGCTCAAGTGTGCTATAGACGTGGAG
      -V--E--G--R--D--L--T--L--Q--Y--N--I--Y--N--V--G)
      - - - - -
42  LeuSerAspAspSerPheProProGluAspPheGlyIleValSerGlyMetLeuAsnValLysTrpAspArg
217  TTATCTGATGATTCCTTCCCCAGAGGACTTTGGCATGTGTCTGGAATGCTCAATGTCAAATGGGACCGG
      (CHO)
66  IleAlaProAlaSerAsnValSerHisThrValValLeuArgProLeuLysAlaGlyTyrPheAsnPheThr
289  ATTGCCCTGCTAGCAATGTCTCCACACCGTGGTCTGCGCCGCTCAAGGCTGGCTATTTCACACTTACT
      (I--A--P--A--S--?--V--S--H--T--V--V--L--R--P--L--K)(A--G--Y--F--?--F--T--
      - - - - -
90  SerAlaThrValThrTyrLeuAlaGlnGluAspGlyProValValIleGlyPheThrSerAlaProGlyGln
361  TCGGCTACTGTTACTTACCTGGCTCAGGAGGATGGGCTCTGCTGATTGGCTTTACCAGCGCCCTGGACG
      -S--A--T--V--T--Y--L--A--Q--E--D--G--P--V)
      - - - - -
114 GlyGlyIleLeuAlaGlnArgGluPheAspArgArgPheSerProHisPheLeuAspTrpAlaAlaPheGly
433  GGAGGGATCCTGGCCAGCGGGAGTTGTAGGAGATTCTCCCGCATTTTCTGGACTGGGCAGCCTTCGGA
      + + +
138 ValMetThrLeuProSerIleGlyIleProLeuLeuLeuTrpTyrSerSerLysArgLysTyrAspThrPro
505  GTCATGACCCCTGCCCTCCATCGGCATCCCCCTGCTGCTGTGTTACTCCAGCAAGAGGAAATATGACACTCC
      + + +
162 LysSerLysLysAsn---
577  AAGTCCAAGAAGAACTGAGGGGCTTCCGAGGCCGCTGCCTAGGAGATCCAGGTGCTTTCAGACTCCGGAGG
649  CCTCTGGAGTGGCTCTCTCATCCCCAGCCCTGCCACCTTCTCCAGGATCTGCCTGCTCTCAACCAGAGT
721  CGGGGACCAGGCCCTCGGTGTCTGTGAAAGCCTCCTCTCTCTCCCAAAGCCATATGAGCAAGAACGAGCAG
793  GAATGCCTCAGCAACAAGCCTCGGGACCTCATCTGTGTGCCTTGTGGTGTGCACTGTAAGTGAAGGGGCC
865  CAGGAGGGGGCACTAAGGAACTGCCTGACTCTACCCCATCCCTGCTCTCCCTCTCGGAAGAGGGTGGGA
937  AGATCAGATGAAGCCAAGTGAAGTCTCGGAGGCTACCCAATCTCTGTTCTGGGTGAGGGAGCTCTAACCAATG
1009 GAGTTTCTAATAAAAAATCGCAAACTGAAAAA.....

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**Figure 3.** Nucleotide and amino acid sequence of the  $\beta$ SSR. The nucleotide sequence was determined from cloned cDNA. The amino acid sequence derived from it is shown above and that determined directly below (given in parenthesis in the one-letter code). The sequences against which antibodies were raised are given in bold face. The cleaved-off signal sequence and the hydrophobic membrane-spanning region are singly and doubly underlined, respectively. Glycosylation sites are indicated by (CHO). The broken line indicates the nucleotide sequence characteristic for a translation initiation site. The sequence data are available from EMBL/GenBank/DBJ under accession number X53529.

the 22-kD polypeptide ran through (Fig. 2 C, lane 14). The 34-kD polypeptide was eluted at high salt concentrations (Fig. 2 C, lane 13). The relative amounts of the two polypeptides was then determined by UV spectroscopy and evaluated on the basis of their content of aromatic amino acids (see below). A molar ratio close to 1:1 was obtained (see Materials and Methods). It should be noted that the same 1:1 ratio was obtained if the complex was isolated with antibodies to the 22-kD polypeptide and that one polypeptide could be quantitatively removed from a detergent extract by antibodies to the other polypeptide (data not shown). This indicates that most, if not all, 34- and 22-kD molecules are contained in the complex.

On the basis of these results, the 22-kD polypeptide can be considered to be a second subunit of the SSR. Accordingly, the 34- and 22-kD polypeptides have been named  $\alpha$ - and  $\beta$ -subunits of the SSR, respectively.

**Amino Acid Sequence and Membrane Topology of the  $\beta$ SSR**

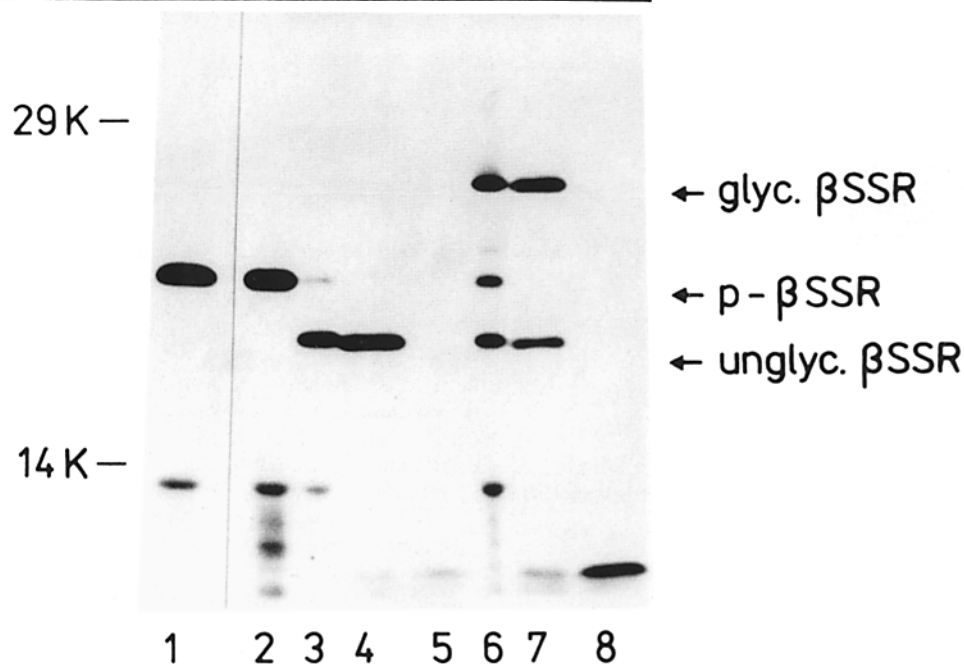
Partial amino acid sequences were determined both from the NH<sub>2</sub> terminus of the intact polypeptide and of two tryptic peptides (Fig. 3, amino sequences shown in parenthesis in the one-letter code). To isolate cDNAs coding for  $\beta$ SSR, degenerate oligonucleotide probes corresponding to 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-

quences 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 NH<sub>2</sub> terminus of authentic  $\beta$ SSR starts at residue 18 of the sequence deduced from the cDNA. The NH<sub>2</sub> terminal extension (Fig. 3, underlined sequence 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 second hydrophobic region close to the COOH terminus (Fig. 3, double-underlined area). As it is preceded by a cleaved signal sequence, it is likely to function as a classical membrane-spanning segment. Two N-glycosylation sites are found in the central region of the protein (indicated by (CHO) in Fig. 3). Since the sequencing of the tryptic peptides gave gaps at the positions of the asparagines, they indeed appear to carry carbohydrate chains. Further evidence for the conclusions drawn from the sequence is given below. The sequence of the  $\beta$ SSR bears no homology to any other protein so far registered.

The membrane topology of the  $\beta$ SSR and the mechanism of its biosynthetic membrane integration was studied by in vitro transcription-translation experiments (Fig. 4). mRNA obtained by in vitro transcription of plasmid DNA coding for the 22-kD polypeptide gave a single translation product of

K-RM	-	+	+	+	+	+	+	+
SRP	-	-	+	+	+	+	+	+
AP	-	-	+	+	+	-	-	-
Prot. K	-	-	-	+	+	-	+	+
Det.	-	-	-	-	+	-	-	+



**Figure 4.** In vitro insertion of  $\beta$ SSR into microsomal membranes. RNA coding for  $\beta$ SSR 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.  $\beta$ SSR*, glycosylated  $\beta$ SSR, *p- $\beta$ SSR*, precursor polypeptide; *unglyc.  $\beta$ SSR*, unglycosylated  $\beta$ SSR, lacking the signal peptide.

$\sim 19$  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 (Fig. 4, 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  $\sim 0.5$ -kD segment (barely seen by comparing Fig. 4, lanes 3 and 4), indicating that a small portion of the  $\beta$ SSR 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  $\beta$ SSR 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  $\sim 4$  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  $\beta$ SSR is synthesized with a cleavable signal peptide and inserted into the ER membrane by a SRP-dependent pathway. The mature protein has its  $\text{NH}_2$  terminus exposed on the luminal side and a short seg-

ment (12 amino acids according to the sequence) on the cytoplasmic side. Two carbohydrate chains are attached to the luminal portion.

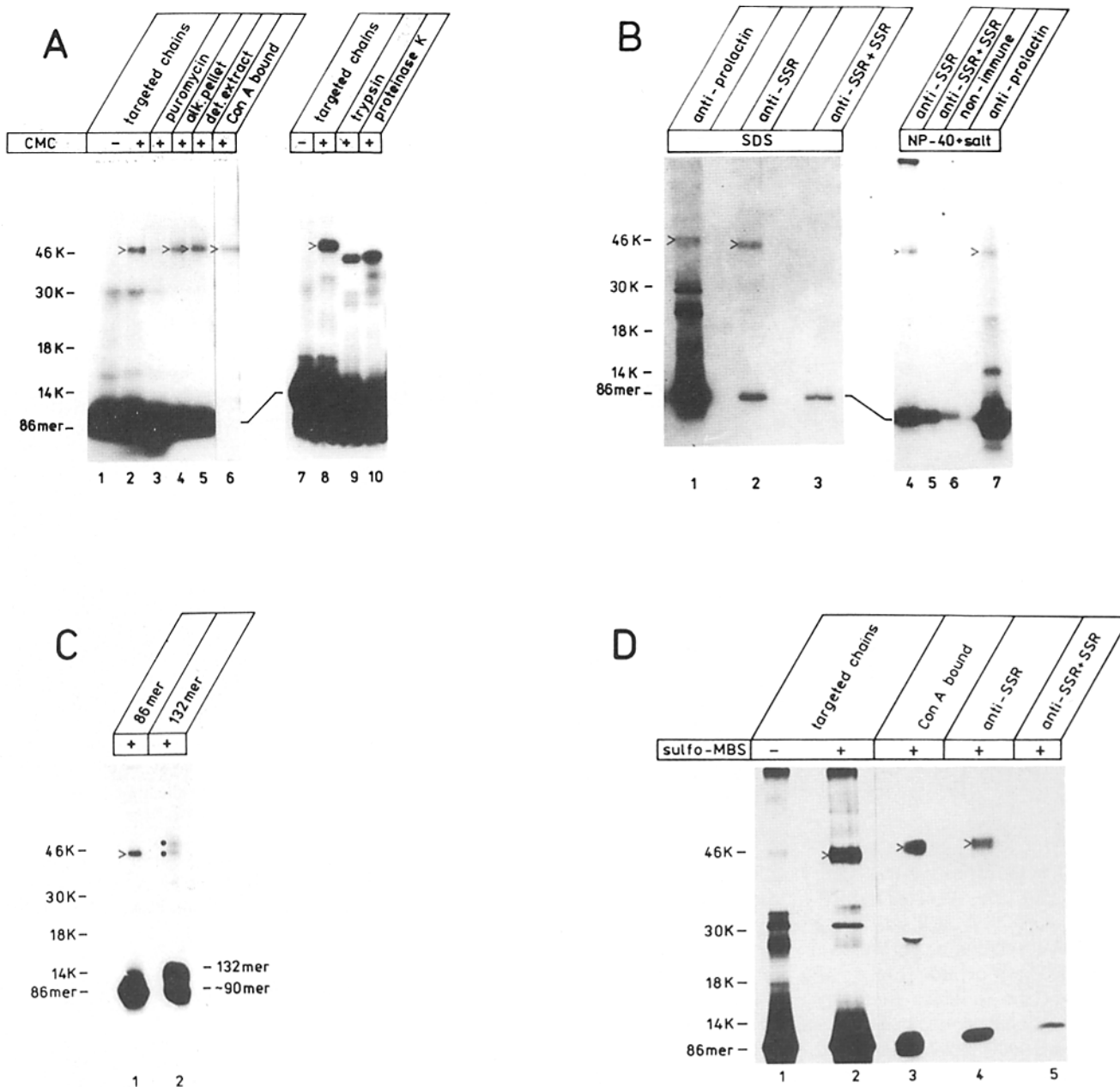
#### *Proximity of Translocating Polypeptide Chains to the $\alpha$ SSR*

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- $\beta$ -lactamase.

To produce translocation intermediates, truncated nascent chains were synthesized in the presence of [ $^{35}\text{S}$ ]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  $0^\circ\text{C}$  with various bifunctional cross-linking reagents and the products were analyzed.

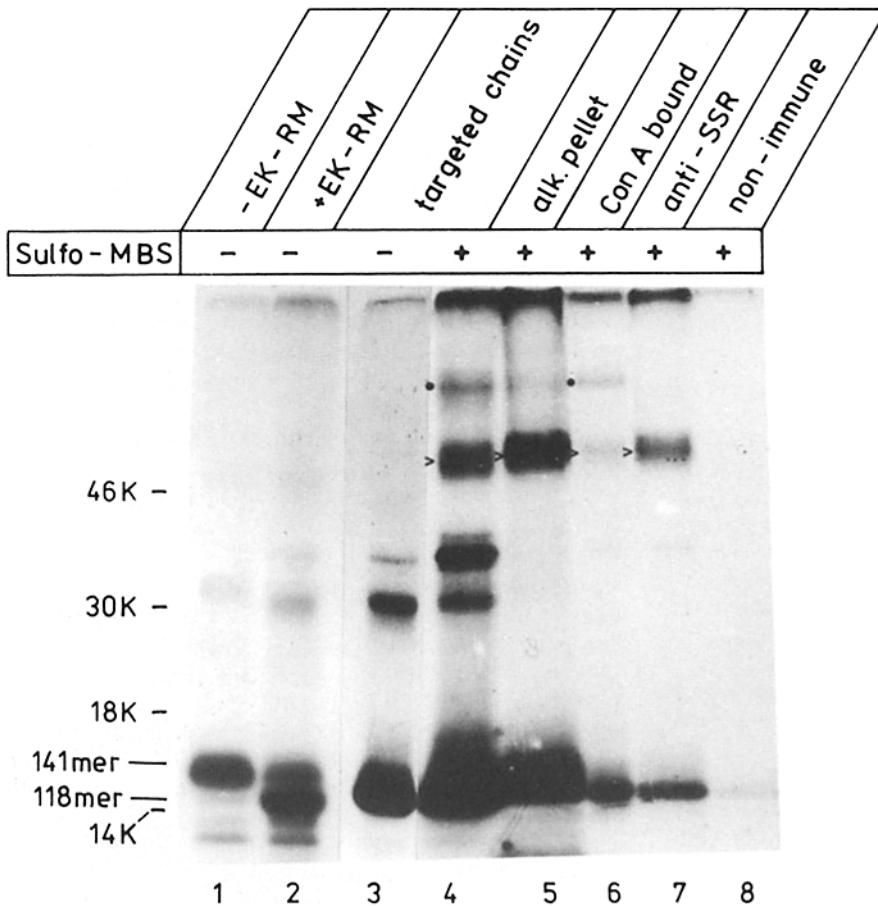
If translocating nascent chains of preprolactin, containing 86 amino acids (86mer), were treated with a carbodiimide





**Figure 5.** Cross-linking of translocating preprolactin chains to the  $\alpha$ SSR. (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),  $\alpha$ SSR-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  $\alpha$ SSR (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  $\alpha$ SSR-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  $\beta$ -lactamase chains to the  $\alpha$ SSR. mRNA coding for the first 141 amino acids of  $\beta$ -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  $\alpha$ SSR, the dot that containing a luminal soluble protein of about 53 kD.

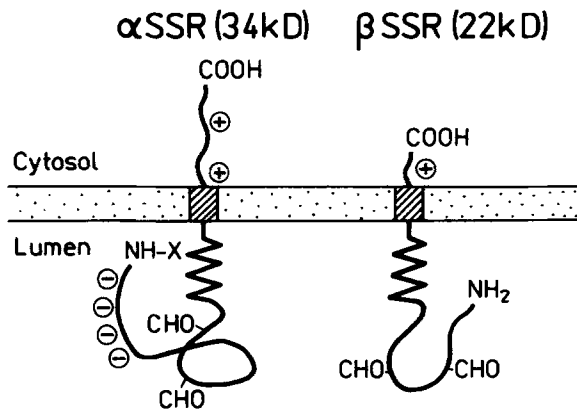
(CMC), a reagent used to produce amide bonds between neighboring carboxyl and amino groups, one main labeled product of  $\sim 46$  kD was obtained (Fig. 5 A, cf. lanes 2 and 1, arrowhead). Such a product was only observed if the nascent chain had not completed translocation. If it was transferred across 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  $\sim 5$  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  $\sim 36$  kD ( $46 - 10$  kD [86mer] = 36 kD) and a segment of  $\sim 5$  kD exposed to the cytoplasm. These properties of the cross-linked membrane protein are similar

to those deduced for the  $\alpha$ SSR 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  $\alpha$ SSR (Hartmann et al., 1989), immunoprecipitation experiments were carried out (Fig. 5 B). Affinity-purified antibodies directed against the COOH-terminus of the  $\alpha$ SSR ( $\alpha$ SSR-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  $\alpha$ SSR 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  $\alpha$ SSR.

Immunoprecipitation of the 46-kD cross-linked product could also be demonstrated after its solubilization with non-ionic detergent at high salt concentrations using antibodies directed against the native complex of  $\alpha$ - and  $\beta$ SSR (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



**Figure 7.** Schematic illustration of the putative membrane topology of the SSR subunits. Both subunits have one hydrophobic membrane-spanning region, two attached carbohydrate chains (indicated by *(CHO)*) and predicted  $\beta$ -pleated sheets at the luminal portion adjacent to the membrane anchor (*zig-zag lines*), which might be responsible for their tight interaction. Charge clusters are indicated. The *X* may simply represent a pyrroglutamate residue at the blocked  $\text{NH}_2$  terminus of  $\alpha$ SSR.

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  $\sim 48$  kD corresponds in size to the cross-linked product of the 132mer and the  $\alpha$ SSR, the lower one of 46 kD may originate from an interaction of  $\alpha$ SSR 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 ( $\sim 90$  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  $\alpha$ SSR 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  $\text{NH}_2$  as well as SH groups. A predominant cross-linked product of  $\sim 46$  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  $\alpha$ SSR-C antibodies after its solubilization with nonionic detergent (lane 4). Controls with presaturated  $\alpha$ SSR 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  $\alpha$ SSR 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  $\sim 40$  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  $\alpha$ SSR 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  $\sim 46$ –50 kD but antibodies directed against  $\alpha$ SSR 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  $\beta$ -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  $\beta$ -lactamase (141mer) was almost completely processed to a fragment of  $\sim 15$  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 ( $47 \text{ kD} - 15 \text{ kD} = 32 \text{ kD}$ ) indicates that again the  $\alpha$ SSR 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  $\alpha$ SSR (Fig. 6, lane 7) but not with nonimmune immunoglobulin (lane 8). Thus, the processed  $\beta$ -lactamase chain appears to be in proximity of the  $\alpha$ SSR during its transfer through the membrane.

Cross-linking must have occurred between the amino group(s) of the  $\alpha$ SSR and the cysteine at position 52 of the processed  $\beta$ -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  $\beta$ -lactamase fragment to a soluble, luminal protein of  $\sim 53$  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  $\beta$ -lactamase chain with the  $\alpha$ SSR, 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  $\alpha$ -(34 kD) and  $\beta$ -(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 coimmunoprecipitation with  $\alpha$ SSR antibodies of a 22-kD polypeptide after labeling of cells with [<sup>35</sup>S]methionine (Prehn et al., 1990). Furthermore, most if not all of the  $\alpha$ - and  $\beta$ SSR molecules in canine microsomes seem to be contained in the complex.

The sequence determined for the  $\beta$ SSR shares several features with that of the  $\alpha$ SSR: 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  $\beta$ -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  $\alpha$ SSR are within  $\sim 12$  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  $\alpha$ 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 data with bifunctional reagents support and extend previous results obtained with a photocross-linking technique (Wiedmann et al., 1987b, 1989; Krieg et al., 1989). With the new technique, the close proximity of  $\alpha$ SSR to nascent prolactin chains of 86 or 132 amino acids length was confirmed. The same intimate relation was found for  $\beta$ -lactamase. Recent results also indicate the close proximity of  $\alpha$ SSR to nascent chains of signal-anchor type membrane proteins at early stages of membrane insertion (High, S., D. Görlich, M. Wiedmann, T. A. Rapoport, and B. Dobberstein, unpublished results). With the  $\beta$ -lactamase we were able to demonstrate that the  $\alpha$ SSR is close to the translocating nascent chain even at a stage at which it had already a portion at the luminal side of the membrane, as indicated by signal peptide cleavage and by cross-linking of the chain to a soluble luminal protein. It appears, therefore, that translocating nascent chains in general are transferred through the membrane in close proximity of the  $\alpha$ SSR, even after the signal sequence has been cleaved off. This result supports the previous conclusion that  $\alpha$ SSR is not, or not only, a signal

sequence receptor (Krieg et al., 1989; Wiedmann et al., 1989). However, since there is not yet definitive proof against a receptor function, we have refrained from changing the name of the protein at this point.

The two cross-linking methods have both their merits and disadvantages. Photocross-linking with the probes incorporated into the nascent chain allows one to study interactions of selected regions of the chain since the photoreactive lysine derivatives can be precisely positioned. The photoreaction does not strongly depend on suitable chemical groups of the interacting neighbors. The high reactivity of both nitrenes and carbenes ensures specificity of cross-linking but also generally results in low yields due to the competing reaction with water. Cross-linking with reagents such as carbodiimides, *bis*-hydroxysuccinimide esters, or hetero-bifunctional reagents, of course, depends on suitable chemical groups of the neighboring proteins. Only rarely is it possible to precisely position the probes in a protein (see, however, our results with sulfo-MBS). Also, the reagents must have access to both partners that can be a problem for membrane proteins or for nascent chains still buried inside the ribosome. The yields with these reagents can be very high (up to 5% in our experiments) but it is possible that artifacts are produced by the extensive chemical modifications introduced. In view of these considerations, it is therefore remarkable that the  $\alpha$ SSR gave rise to a major cross-linked product with the nascent chains, regardless of the nature of the translocating protein, its chain length, and of the method of cross-linking or reagent used. It therefore appears that the  $\alpha$ SSR is a major membrane protein that is in close proximity of translocating nascent chains of secretory proteins. It cannot be excluded, however, that this only involves a portion of the total population of  $\alpha$ SSR molecules. In any case, the simple cross-linking pattern observed with the different methods argues against random encounters between the nascent chains and membrane proteins. It should be noted that we have no evidence as yet for proximity between the  $\beta$ SSR and translocating nascent chains.

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  $\alpha$ SSR (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  $\alpha$ SSR in intact microsomes (data not shown) and to the cleavage sites predicted from its sequence (Prehn et al., 1990).

Which region of the  $\alpha$ SSR is cross-linked to the nascent chains? Most if not all of the cytoplasmic portion of the  $\alpha$ SSR can be cleaved off by proteases without severing the cross-linked product into nascent chain and the bulk of the  $\alpha$ SSR (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  $\alpha$ SSR (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  $\alpha$ SSR 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  $\alpha$ SSR as a major protein in proximity of the translocating nascent chain and since  $\alpha$ SSR 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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