Evidence for a Two-Step Mechanism Involved in Assembly of Functional Signal Recognition Particle Receptor

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Abstract. The signal recognition particle (SRP) and SRP receptor act sequentially to target nascent secretory proteins to the membrane of the ER. The SRP receptor consists of two subunits, SRα and SRβ, both tightly associated with the ER membrane. To examine the biogenesis of the SRP receptor we have developed a cell-free assay system that reconstitutes SRP membrane assembly and permits both anchoring and functional properties to be assayed independently. Our experiments reveal a mechanism involving at least two distinct steps, targeting to the ER and anchoring of the targeted molecule on the cytoplasmic face of the membrane. Both steps can be reconstituted in vitro to restore translocation activity to ER microsomes inactivated by alkylation with N-ethyl-maleimide. The characteristics elucidated for this pathway distinguish it from SRP-dependent targeting of secretory proteins, SRP-independent ER translocation of proteins such as prepromelentin, and direct insertion mechanisms of the type exemplified by cytochrome b5.

The initial step in sorting proteins to a number of intracellular organelles as well as to the exterior of the cell involves targeting the nascent polypeptide to the ER membrane. Cell-free systems in which this targeting event is reconstituted have allowed the identification of three distinct pathways used to direct proteins to the ER membrane of higher eukaryotes (reviewed in Perara and Lingappa, 1988; Sabatini et al., 1982). Two of these pathways involve recognition of a signal sequence encoded in the nascent protein by specific receptors in the cytoplasm and on the ER membrane. These two pathways differ in that one pathway is dependent on a cytoplasmic adaptor called signal recognition particle (SRP). This SRP-dependent targeting pathway (reviewed in Walter et al., 1984) appears to be the primary route of targeting of both secreted and integral membrane proteins to the ER. The other pathway is independent of SRP, appears to be receptor mediated, and is responsible for targeting a few highly specialized small proteins such as prepromelitin (Muller and Zimmermann, 1987). The third pathway, typified by cytochrome b5, uses a receptor-independent hydrophobic insertion sequence instead of a receptor-mediated signal sequence. Such sequences have been identified for only a small number of molecules and tend to be located at the extreme carboxyl terminus of the protein anchoring them to the cytoplasmic face of the membrane (Sabatini et al., 1982).

Two essential components of the SRP-mediated ER targeting system, SRP and SRP receptor (also called docking protein) have been isolated and characterized in some detail (Siegel and Walter, 1988; Tajima et al., 1986; Meyer et al., 1982). These two macromolecular complexes act sequentially to direct ribosomes synthesizing secreted and transmembrane proteins to the ER membrane. The process begins with the emergence of a signal sequence from the ribosome. SRP binds both the signal sequence and the ribosome directly (Krieg et al., 1986; Kurzchalia et al., 1986). This interaction slows elongation of the nascent polypeptide and maintains the nascent-chain ribosome complex in a translocation competent state. This complex is presumed to target to the ER membrane due to the affinity of SRP for its receptor on the cytoplasmic face of the ER (Gilmore et al., 1982b). After interaction with the SRP receptor, SRP loses its affinity for the signal-bearing translation complex and releases the now targeted nascent protein to the translocation machinery in the ER membrane (Gilmore and Blobel, 1983).

The SRP receptor is composed of two subunits, termed SRα and SRβ, which cofractionate in sucrose gradients and can be coprecipitated using antisera directed against either molecule (Tajima et al., 1986). The cDNA of the larger of the two subunits SRα, has been cloned from both canine (Lauffer et al., 1985) and human (Hortsch et al., 1988) cells. SRα has a deduced molecular mass of ~69,700 D and migrates in SDS-PAGE with an apparent molecular mass of ~72,000 D. It is this subunit that is believed to interact with SRP to release translation arrest and initiate translocation (Gilmore et al., 1982a).

1. Abbreviations used in this paper: EF, elastase fragment; NEM, N-ethylmaleimide; SRα, signal recognition particle receptor alpha subunit; SRβ, signal recognition particle receptor beta subunit. SRP, signal recognition particle.
SRα has a large cytoplasmically disposed domain that can be cleaved from the membrane by low concentrations of either trypsin or elastase (Gilmore et al., 1982a; Meyer and Dobberstein, 1980). Amino terminal sequencing of this domain was used to confirm that it corresponds to a fragment of the molecule beginning at amino acid 152 and continuing to the end of the molecule, amino acid 638 (Lauffer et al., 1985). This cytoplasmic fragment has been shown to have a greatly reduced affinity for SRP suggesting that part of the molecule remains membrane associated contributes to a functionally important domain of the receptor (Lauffer et al., 1985).

Microsomes treated with either trypsin or elastase to remove SRα are not functional in promoting nascent preprotein translocation or in releasing the SRP-induced arrest of translation observed in wheat germ translation reactions (Gilmore et al., 1982a). However both functions can be restored by adding back purified cytoplasmic fragment of SRα to the digested microsomes (Gilmore et al., 1982b).

Examination of the deduced amino acid sequence for SRα from a canine cDNA clone revealed several remarkable features (Lauffer et al., 1985) also found in the human sequence (Hortsch et al., 1988). At the amino terminus of the molecule are two hydrophobic sequences, amino acids 1-22 and 64-79, believed to anchor the molecule on the ER membrane. After the hydrophobic sequences are three highly charged stretches of amino acids, including residues 84-97, 129-175, and 205-243. The primary elastase cleavage site, between residues 151 and 152, is approximately midway through the second highly charged region of SRα.

SRα molecules synthesized in wheat germ cell-free translation reactions and then added to microsomes were observed to cofractionate with the membranes even after disruption of the microsomes with sodium carbonate. These experiments have been used to suggest the molecule may associate with microsomes posttranslationally (Hortsch and Meyer, 1988). However, the lack of a functional assay combined with the difficulties involved in controlling for non-specific association in such an assay has made interpretation of the data difficult.

To examine the biogenesis of the SRP receptor we have developed a cell-free system that permits the introduction of recombinant molecules of SRα. In this assay both targeting and functional properties of the introduced molecules can be assayed independently. Here we show that SRα can be specifically recruited to functional sites on the ER membrane in vitro. Furthermore, targeting and anchoring are independent, separable steps. Targeting is saturable and can occur postranslationally. SRα molecules target to microsomes treated with either trypsin or the alkylation agent N-ethylmaleimide (NEM). Stable anchoring in the membrane is also NEM resistant but can be abolished by pretreatment of microsomes with trypsin. Furthermore we confirm that amino terminal sequences removed from the full length molecule by elastase are required to achieve a stable association with the ER membrane. Together these results suggest SRα molecules are targeted to the ER membrane by a novel mechanism involving an as yet unidentified protein intermediary.

Materials and Methods

General Methods

Restriction endonucleases were obtained from Boehringer Mannheim Diagnostics, Inc. (Houston, TX) or from New England BioLabs (Beverly, MA) and were used according to the manufacturer's instructions. Placental RNase inhibitor was from Promega Biotech (Madison, WI). Rabbit anti-ovine prolactin was from United States Biochemical Corp. (Cleveland, OH). Preparation and characterization of the monoclonal antibodies directed against SRα and SRβ have been described (Tajima et al., 1986). Proteinase K was from E. Merck (Darmstadt, FRG). [35S]methionine translable from ICN Biomedicals, Inc. (Costa Mesa, CA), and Triton X-100 from Boehringer Mannheim Biochemicals (Indianapolis, IN). A mitochondria-enriched vesicle fraction was prepared from canine pancreas as described (Greenwalt, 1974), except that tissue homogenization and the initial centrifugation step employed a buffer containing 50 mM TEA, pH 7.5, 0.25 M Sucrose, 50 mM KOAc, 6 mM MgAc, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF.

Canine pancreatic microsomal membranes were prepared as described (Walter and Blobel, 1983). All microsome fractions were washed twice with 0.5 M KOAc to remove endogenous SRP and then EDTA stripped as described (Walter and Blobel, 1983). Trypsinized and NEM-treated microsomes were prepared as described (Gilmore et al., 1982a). Unless indicated the concentrations of trypsin and NEM used were 5 µg/ml and 2 mM, respectively. Inactivated microsomes were prepared in 100-µl aliquots at a concentration of five equivalents per microliter and were pelleted by centrifugation at ~110,000 g (20 psi for 10 min in an air-fuge [rotor A-100/30; Beckman Instruments, Inc., Fullerton, CA]).

Recombinant DNA Constructs

The complete coding sequence of SRα was assembled from recombinant phage clones 1SR31 and 1SR50 using a unique Nco I site at nucleotide position 1,546 of the cDNA sequence (Lauffer et al., 1985). To facilitate further

![Figure 1. Cofractionation of SRα but not SRα-EF with microsome. Reticulocyte lysate translation products of SRα (lanes 1-6) and SRα-EF (lanes 7-12) were synthesized in the presence or absence of added microsomes as indicated by Mb. Reactions were adjusted to 2 M urea, layered on sucrose step gradients, and centrifuged for 15 min at 30 psi (178,000 g). Gradients were divided into top, middle, and bottom fractions, indicated above the lanes as t, m, and b, respectively. Migration positions of molecular mass markers, in kilodaltons, and of SRα and SRα-EF are indicated on the sides.](image-url)
manipulation the plasmid pSPSR9 was generated from nucleotides 1 to
2,555 of the SRct cDNA as well as the Eco RI adaptors used for cDNA cloning by
inserting them as an Eco RI fragment into the Eco RI site in the polylinker
region of pSP64. For expression of SRct in vitro the coding region of SRct was inserted into pSP64T (cut with Bgl II and Bam HI) as a Bam HI
fragment from pSPSR9 (one Bam HI site comes from the polylinker of pSP64 the other is located in the 3' untranslated region of SRct at
position 2364). In addition to the SRct coding sequence, the resulting plas- 
mid pSPSRSR contains the 5' untranslated region of Xenopus β globin, 22
nucleotides from the polylinker region of pSP64, 15 nucleotides from the
Eco RI adaptor used in cDNA cloning, 41 nucleotides of the original 5' un-
translated SRoL sequence, and 308 nucleotides of the 3' untranslated region
of SRct under transcriptional control of the SP6 promoter.

The elastase fragment (EF) of SRct starts with a methionine encoded by
an ATG at position 495 of the SRct cDNA. This codon was used for initia-
tion of translation of SRct-EF in vitro employing an Ava II site at position
490 of the SRct cDNA. Plasmid pSPSR10 was constructed by excising the
SRct coding sequence from pSPSR9 with Eco RI and Bam HI and inserting it
into pSP64T with Eco RI and Bam HI. A partial digest of this plasmid
with Ava II (there are three sites in the SRct cDNA) and Bam HI digest
the resulting Ava II-Bam HI fragment was blunt ended using Klenow frag-
ment of polymerase and ligated into the Hind III site in the polylinker
region of pGEM-1 to obtain plasmid pSPSR21. To enable transcription in plasmid
pSP64T, pSPSR21 was cut with Hind III and Eco RI, and the excised frag-
mant was cloned into pSP64T with Bgl II and Bam HI after filling in the
ends of both the fragment and vector sequences with Klenow fragment of
polymerase. The resulting plasmid, pSPSR-EF, contains the Xenopus β
globin 5' untranslated region, 17 nucleotides carried over from the pGEM-1
polylinker region, 4 nucleotides of SRct cDNA preceding the SRct-EF initi-
atng ATG, 1461 nucleotides encoding SRct-EF, and 308 nucleotides of the
SRct 3' untranslated region under transcriptional control of the SP6 pro-
moter.

**Transcription-linked Translation**

Transcription of SP6 plasmids was as described previously (Perara and Lin-
gappa, 1985). Aliquots of the transcription reaction mixture were used
directly in the translation reactions at a final concentration of 20%. Transla-
tion reactions of this kind have been described for reticulocyte lysate (Perara
and Lingappa, 1985). Proteins synthesized in vitro were labeled by
incorporating ['S]methionine into the reaction mixture at a final concentration of 20%
just before translation. Translation reactions were mixed as described in the
methods.

Postribosomal supernatants were prepared by centrifugation at 28 psi
for 30 min in the air-fuge after translation was terminated by adding sodium
cacodylate to a final concentration of 100 mM. Unincorporated ['S]methio-
nine was removed by gel filtration as described above. These trans-
lation products were mixed with liposomes and/or microsomes and in-
cluded in the translation reaction mixture at a final concentration of 100
mM Tris-Ac, pH 7.5, 100 mM potassium acetate, 2 mM magnesium chloride, 1 mM DTT. The heavy su-
crose step was overlaid with 100 μl of a 0.25 M sucrose solution of 2 M sucrose. Final ion concentrations in both steps were the
same as for translation reactions: 10 mM Tris acetate, pH 7.5, 100 mM
potassium acetate, 2 mM magnesium chloride, 1 mM DTT. The heavy su-
crose step was overlaid with 100μl of a similar solution containing 0.25 M sucrose. Centrifugation in the air-fuge with the A-100/30 rotor was for 2 h
at 30 psi (~180,000 g). These gradients were fractionated into five fractions
50 μl each. The final fraction being the pellet solubilized after 5 min incu-
bation at 60°C in 50 μl of 1% SDS, 10 mM Tris-Ac, pH 9. Aliquots of these fractions were analyzed by SDS-PAGE (5 μl) and scintillation count-
ing (10 μl).

Liposomes were prepared as described previously (Doms et al., 1985)
except 0.75% [4C]cholesterol was included as a tracer. The ratio of lipids
used was 8:7:2:1 (phosphatidyl ethanolamine/phosphatidyl choline/choles-
terol/phosphatidyl serine). The final preparation was centrifuged for 10 s
in a microfuge to remove aggregates immediately before use.

**Results**

To examine the biogenesis of the SRP receptor we analyzed the mecha-
nism of targeting and membrane association for SRct. The strategy employed uses SRct molecules synthe-
sized in cell-free translation reactions to assay interaction with the ER membrane in vitro. Two plasmids were con-
structed to permit cell-free transcription using the SP6 pro-
moter. One plasmid called pSPSRSR contains the full length molecule SRct and the other, pSPSRcEF, encodes a deletion
mutant composed of amino acids 152–638. This deletion mut-
ant, termed SRct-EF, corresponds to the fragment of the molecule released from microsomes by digestion with elastase (Lauffer et al., 1985). After synthesis in reticulocyte
lysate a single major band of the expected molecular weight
is generated by each plasmid which, in control experiments, pro-
duced immunoprecipitable with a monoclonal antibody
(Tajima et al., 1986) directed against the authentic receptor (data not shown).

We used two criteria to establish an in vitro targeting assay. First SRct molecules had to become associated with micro-
somes in a manner indistinguishable from the endogenous
receptor. Second, association with microsomes must be
function; i.e., targeted SRct cell-free translation products
should interact with secretory protein nascent chain–ribo-
some complexes to permit translocation across the micro-
sonal membrane. To measure membrane association of
newly synthesized SRct we used a strategy similar to one
used previously to displace membrane-bound secretory pro-
tein translation complexes from microsomes (Gilmore and Blobel, 1985). In our use of this assay, translation reactions
synthesizing either SRct or SRct-EF were supplemented with micro-
somes incubated at 24°C, and then aqueous pertur-
bants were added and the microsomes were separated from
the reaction mixture by centrifugation over a 0.5 M sucrose
step gradient. A variety of aqueous perturbants were em-
ployed including salts (NaCl or KCl), EDTA, and urea.
Control experiments indicated that the most stringent condi-
tions compatible with maintaining the integrity of the

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endogenous receptor molecules on the cytoplasmic surface of vesicles by digestion with trypsin. Previously trypsinization of microsomal membranes it was necessary to first selectively inactivate the microsomes by proteolytic cleavage at the same location. SRαt molecules isolated from canine microsomes are very susceptible to proteolytic degradation at this site (unpublished observations). Regardless of the source of this band, in the pelleting assays in Fig. 1 it behaves the same as SRα-EE; these molecules restore 78 and 14% respectively, of the translocation activity observed with an equivalent amount of untrypsinized membranes. No processing of SRαt-EF did not cofractionate with added microsomes under the same conditions (lanes 4-6). SRαt-EF molecules synthesized in reticulocyte lysate translation reactions at the onset of synthesis to permit the test molecules SRαt, SRα-EE, or Pt to interact with the membranes cotranslationally. Translation was allowed to proceed for 40 min at 24°C and these microsomes (now referred to as preincubated) were assayed for regained ability to translocate preprolactin. Empirically it was found that sufficient preprolactin synthesis and the most consistent results were obtained if a single new translation reaction (30 μl) was assembled, programmed with RNA encoding preprolactin, and then subdivided into 3-μl aliquots which were added to each of the 20 μl reconstitution reactions. After an additional 40 min of protein synthesis, translation in the combined reactions was terminated by cooling to 0°C, and samples were prepared for electrophoresis. As expected when the original translation reaction contained intact microsomes, some of the preprolactin molecules synthesized were translocated as judged by signal processing to prolactin (compare lane 2 with 3). Moreover, translation reactions containing trypsinized membranes (lane 4) do not support preprolactin translocation when preincubated in translation reactions synthesizing the control molecule Pt. However, trypsinized membranes are functionally restored when preincubated with full length SRαt molecules and preprolactin is efficiently processed to prolactin (lanes 5 and 6). SRα-EE is also able to restore some function to trypsinized microsomes, as expected from previous work (Gilmor et al., 1982b) (lanes 7-9).

Preincubation using SRα-EE molecules synthesized in vitro is much less efficient than with the full length molecule (compare lane 6 with 9; lane 9 is a longer exposure of lane 8). Densitometry of the autoradiogram in Fig. 2 permitted quantification of the relative translocation activity of trypsinized microsomes preincubated with SRαt and with SRα-EE. These molecules restored 78 and 14%, respectively, of the translocation activity observed with an equivalent amount of untrypsinized membranes. No processing of preprolactin was observed with the control membrane Pt after overexposure of lane 4 equivalent to lane 9. Thus both SRαt and SRα-EE molecules synthesized in reticulocyte lysate can associate functionally with microsomes in vitro.

We were surprised that the SRαt molecules synthesized in 20 μl reticulocyte lysate translation reactions could restore almost complete translocation activity to trypsinized membranes. Therefore, we measured the amount of SRαt synthesized in these reactions to compare with the number of endogenous receptors determined previously (Tajima et al., 1982a). Mild trypsinization does not inactivate other required components of the translocation machinery as these membranes can be restored by adding back the cytoplasmic portion of SRαt (Gilmor et al., 1982a).
Figure 3. SRα cofractionates with microsomes when added posttranslationally. (a) Reticulocyte lysate translation products of SRα (lanes 1–3) and SRα–EF (lanes 4–6), desalted by chromatography on Sephadex G25. (b) Translation products of SRα purified by affinity chromatography supplemented with microsomes as indicated by Mb. Reactions were incubated at 24°C for 20 min, adjusted to 2 M urea, layered on sucrose step gradients, and centrifuged for 15 min at 30 psi. Gradients were divided into top, middle, and bottom fractions, indicated above the lanes as t, m, and b, respectively. Migration positions of molecular mass markers, in kilodaltons, and of SRα and SRα–EF are indicated on the sides of the figure. The bands that migrate faster than SRα in b are presumed to result from degradation of intact molecules during purification.

1986). To measure SRα molecules cell-free translation products were labeled during synthesis with 3H-leucine and compared, by densitometry of autoradiograms of SDS-PAGE gels, with a prolactin standard labeled in exactly the same manner. This standard was assayed previously by two independent methods, radioimmuno assay and quantitative western blotting (data not shown). We estimate that the amount of SRα synthesized in a 20-μl reticulocyte lysate translation reaction varies between 1 and 20 fmol.

We routinely used 20-μl translation reactions to restore translocation activity to one equivalent of microsomes (defined in Walter and Blobel, 1983). The amount of endogenous SRα in canine microsomes is ~10-fold higher, ~90 fmol/equivalent (Tajima et al., 1986). Therefore 10% of wild-type levels of SRα synthesized in vitro can reconstitute 78% of translocation function. This suggests these molecules are assembled on the membrane in a conformation and at locations appropriate for efficient interaction with the translocation machinery. Moreover since SRα molecules are targeted to trypsinized microsomes, the targeting pathway must differ from the conventional, trypsin-sensitive, SRP-mediated pathway used by secretory and integral membrane proteins.

The targeting assays in Figs. 1 and 2 were performed with microsomes present during synthesis of SRα and SRα–EF. To determine whether or not membrane association was strictly a cotranslational event we used the cell-free targeting assay described above to assay posttranslational association of SRα and SRα–EF with untreated microsomes. To assay SRα targeting posttranslationally, cycloheximide was added to terminate translation and postribosomal supernates were prepared from these reactions by centrifugation. Microsomes were added at 1 eq/20 μl postribosomal supernate and then incubated at 24°C for 20 min, and membrane association was assayed by pelleting in 2 M urea step gradients. In initial experiments, SRα molecules were observed to cofractionate with microsomes (data not shown). Similar targeting was observed for SRα molecules from postribosomal supernates after gel filtration chromatography on G25 Sepharose (Fig. 3 a, lanes 1–3), suggesting small molecules in reticulocyte lysate translations are not required for membrane targeting. As expected, molecules of SRα–EF do not cofractionate with microsomes in 2 M urea step gradients (Fig. 3 b, lanes 4–6). This result is confirmed by our observation that immunopurified SRα also pellets in these gradients only when microsomes are included (Fig. 3 b). SRα molecules immunopurified without detergent migrate anomalously in SDS-PAGE (Fig. 3 b, lanes 1–3) and are found near the top of the resolving gel. However when microsomes are added to this same material the migration position returns to normal and SRα molecules cofractionate with microsomes (Fig. 3 b, lanes 4–6). Although other explanations are possible, without added microsomes the behavior of SRα molecules suggests these molecules aggregate after boiling in SDS. Nevertheless without added membranes SRα molecules remain at the top of 2 M urea step gradients (Fig. 3 b, lanes 1–3). When microsomes are added to purified SRα molecules, aggregation is markedly reduced, perhaps due to appropriate targeting of the molecules. We cannot conclude that targeting is independent of all reticulocyte lysate components because a small number of proteins were observed to copurify with SRα on the affinity column (data not shown).
Figure 4. SRα cofractionates with NEM-treated microsomes. Reticulocyte lysate translation reactions of SRα supplemented with microsomes indicated by Mb: M, untreated microsomes; N, microsomes alkylated with NEM; -, no membranes; and analyzed by sedimentation in sucrose step gradients. Gradient conditions (Gr) are indicated as: Ph, physiologic ions; HS, 0.5 M NaCl; HSE, 0.5 M NaCl and 25 mM EDTA; U, 2 M urea. After centrifugation (as in Fig. 3), gradients were divided into top, middle, and bottom fractions, indicated beneath the bars as t, m, and b, respectively. Positions of molecular mass markers, in kilodaltons, are indicated at the side of the figure. The band below SRα which remains at the top of the step gradients is presumed to result from initiation of translation at an internal methionine.

To assess the possible role of free sulfhydryl groups on the microsome surface for targeting SRα molecules, microsomes were treated with 2 mM NEM and assayed both by pelleting in step gradients and by reconstitution of translocation activity. NEM has been shown to abolish SRP-dependent microsomal membrane translocation activity by alkylating the SRα molecules on the membrane surface thereby disrupting SRP recognition of the receptor (Gilmore et al., 1982a). NEM has also been suggested to block a later stage in SRP-mediated translocation (Hortsch et al., 1986). Moreover, modification with NEM also disables the SRP, SRP receptor–independent translocation process described for several polypeptides such as prepromelittin (Muller and Zimmerman, 1987).

In contrast to these processes targeting of SRα synthesized in vitro is not affected by pretreatment with NEM (Fig. 4) as assayed by pelleting in the presence of a variety of aqueous perturbants. The efficiency of SRα targeting to both mock NEM– (M) and NEM– (N) treated microsomes is identical in step gradients containing physiologic ions (Fig. 4 a, lanes I–9), 0.5 M NaCl (Fig. 4 a, lanes I0–I5), 0.5 M NaCl, 25 mM EDTA (Fig. 4 b, lanes I–9), and 2 M urea (Fig. 4 b, lanes I0–I5).

To determine whether or not this apparent targeting to NEM-treated microsomes results in a functional association with the membrane, translocation activity was measured for microsomes treated with NEM alone or in combination with trypsin. The rationale for digesting microsomes with trypsin before alkylation with NEM was to expose potential alkylation-sensitive sites on the ER membrane masked by trypsin-sensitive proteins including endogenous SRα. In contrast, trypsinization after NEM treatment might expose additional potentially functional sites on the ER membrane otherwise occupied by endogenous SRα.

Translocation activity is restored to microsomes alkylated with NEM by SRα molecules synthesized in reticulocyte lysate (Fig. 5). In this experiment cell-free translation products of plasmids encoding either SRα or, as a control, chimpanzee α globin were supplemented with microsomes after 40 min synthesis at 24°C. After an additional 20 min at this tem-
Incubation of microsomes, inactivated by trypsinization and/or alkylation, with $SR_{\alpha}$ restores translocation activity. Microsomes were incubated with $SR_{\alpha}$ (lanes 1–7) or Globin (lanes 9–16) and then supplemented with a newly assembled preprolactin translation reaction. The microsomes added were pretreated in two sequential steps, $Tr_1$ and $Tr_2$, respectively. Microsome treatments: $T$, trypsinized microsomes; $N$, NEM-alkylated microsomes; $-$, no membranes. The prefix $(m)$ indicates mock treatment. Molecular mass markers, lane 8, are albumin, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; and lactoglobulin, 18 kD. The migration positions of the translation products ($SR_{\alpha}$), preprolactin ($pP$), mature prolactin ($P$), and globin ($G$) are indicated at the side of the figure.

In almost all cases translocation activity is restored to close to wild-type levels. The one exception is membranes treated only with NEM (lane 6). In this experiment and others (Fig. 6 b and unpublished observations) translocation activity of microsomes treated only with NEM was restored to a lesser extent, discussed below.

If a step in the translocation pathway subsequent to signal cleavage was abolished by either trypsin or NEM, alone and in combination (compare lanes 3–6 with II–I4). In almost all cases translocation activity is restored to close to wild-type levels. The one exception is membranes treated only with NEM (lane 6). In this experiment and others (Fig. 6 b and unpublished observations) translocation activity of microsomes treated only with NEM was restored to a lesser extent, discussed below.

These experiments strongly suggest the $SR_{\alpha}$ molecules are not targeted to microsomal membranes by the conventional SRP-SRP receptor-mediated translocation pathway. However, they do not address the actual mechanism of targeting. One difference between $SR_{\alpha}$ and $SR_{\alpha}$-EF is the presence of two relatively hydrophobic stretches of amino
binding to liposomes by centrifugation in sucrose step gradients (Doms et al., 1985). In these experiments liposomes can be easily distinguished from microsomes because these vesicles rise to the top of sucrose step gradients while microsomes pellet during centrifugation. Liposome movement in step gradients was monitored by including a small amount, 0.75%, [14C]cholesterol during liposome preparation. As expected almost all of the radioactivity is recovered from the low density sucrose step (fractions 1 and 2) when liposomes are mixed with a mock SRα translation reaction, adjusted to 1.3 M sucrose, overlaid with a 0.25 M sucrose solution, and centrifuged at 30 psi for 2 h. The percent of total radioactivity in each fraction of one such gradient, measured by scintillation counting, is presented in Fig. 7 a. The bottom fraction, number 5, represents pelleted material.

To examine the specificity of SRα targeting we assayed

acids present in full length molecules. It has been postulated that these sequences act to anchor the receptor in the ER membrane. Therefore, the targeting event we are assaying could be due to simple partitioning of one or both of these sequences in the lipid bilayer of the microsome. Such a targeting event would be analogous to the insertion sequence-mediated membrane association of cytochrome b5 (Anderson et al., 1983). If a relatively nonspecific hydrophobic interaction is responsible for the cofractionation observed with microsomes then molecules of SRα would be expected to interact similarly with artificial phospholipid vesicles.

To examine the specificity of SRα targeting we assayed
Figure 7. SRα cofractionates with microsomes but not liposomes in sucrose step gradients. (a) Liposomes containing 0.75% [14C]cholesterol were adjusted to 1.3 M sucrose in a final volume of 100 μl and overlaid with 100 μl of a 0.25 M sucrose solution. After centrifugation at 30 psi for 2 h in an air-fuge (Beckman Instruments, Inc.), gradients were divided into five 50-μl fractions. The bottom fraction, number 5, represents any pelleted material solubilized in 50 μl of 1% SDS, 10 mM Tris, pH 9, after incubation at 60°C for 5 min. The radioactivity of each fraction was measured by scintillation counting and plotted as percent of the total. (b-d) Reticulocyte lysate translation products of SRα were desalted on Sephadex G25 and mixed with microsomes, liposomes, or microsomes plus liposomes, as indicated and subjected to centrifugation as in a. Aliquots of each fraction corresponding to 0.5 μl of SRα translation products were separated by SDS-PAGE and visualized by autoradiography to localize SRα molecules. The migration position of molecular mass standards is indicated at the side of the autoradiogram. Liposomes were localized by measuring total radioactivity in similar aliquots by scintillation counting.

at about four equivalents of microsomes in a 20-μl translation reaction. This pattern of binding suggested that 20 μl of translation products (~10 fmol SRα) could saturate the unused binding sites on approximately two to three equivalents of microsomes.

To demonstrate saturation of binding sites directly we held the concentration of microsomes constant and varied the amount of SRα molecules added to reactions. Post ribosomal supernatants of SRα translation reactions were prepared and salt exchanged as described above. The specified volume of this material was added to one equivalent of microsomes after the volume of the reaction was adjusted to 20 μl with buffer (10 mM Tris, pH 7.5, 100 mM potassium acetate, 2 mM magnesium chloride, 1 mM DTT) and then incubated at 24°C for 20 min. As expected when larger amounts of SRα translation products are added to a fixed concentration of microsomes the proportion of the total SRα recovered from the material pelleted in 2 M urea sucrose step gradients is reduced (Fig. 9 b). The shape of the curve is consistent with saturation of one equivalent of microsomes by ~10 μl of SRα translation products (~5 fmol of SRα).

These results suggest that there is a specific targeting site for SRα on the microsomal membrane. Furthermore, SRα binding experiments employing trypsinized microsomes suggest that resistance to extraction by 2 M urea is protein mediated (Fig. 10). Although molecules of SRα are able to restore translocation function to trypsinized microsomes (Figs. 2 and 5) this association is not as stable as with untreated or NEM-treated microsomes. As shown above, SRα binding to mock-treated microsomes and NEM-treated microsomes (Figs. 1, 3, and 4) results in an association with the microsome stable to 2 M urea. However, when SRα-reconstituted trypsinized microsomes are assayed as above using 2 M urea step gradients, SRα molecules are released (Fig. 10 a, compare lanes 1-3 with 4-6). Nevertheless, SRα molecules are interacting with microsomes because these molecules do cofractionate with microsomes in step gradients containing physiologic ions (lanes 7-12). Control experiments such as that shown above in Fig. 4 demonstrate that SRα molecules do not pellet in physiologic gradients without added microsomes.

Moreover, when trypsinized microsomes reconstituted with SRα are pelleted in sucrose step gradients under physiologic conditions and resuspended in a preprolactin translation reaction they are active in promoting translocation (Fig. 10 b). As a control mock trypsinized microsomes were resuspended in a prolactin translation reaction after a similar incubation. As expected resuspended pellets of intact microsomes are active in translocating preprolactin (lanes 1-3). Furthermore, the small number of SRα molecules that remain associated with trypsinized microsomes after pelleting also function to translocate prolactin (lanes 4-6). Trypsinized microsomes do not translocate prolactin without the reconstitution step as shown in lane 7. Together these results suggest that the targeting site for SRα is trypsin resistant while stable membrane anchoring is mediated by an additional trypsin-sensitive component. Also, stable membrane anchoring is not required for SRα to be active in promoting preprolactin translocation.

The most logical candidate protein to mediate one or both
of these steps in SRα biogenesis is SRβ. SRα and SRβ have been shown to form a complex stable to high salt (Tajima et al., 1986). Furthermore the two molecules have been shown to cofractionate with constant stoichiometry when rough and smooth liver microsomes are separated on sucrose gradients (Tajima et al., 1986). If SRβ plays a role in targeting SRα to the membrane we would expect it to be resistant to low concentrations of trypsin. However, if SRβ alone is responsible for mediating anchoring of SRα on the ER membrane it should be sensitive to digestion with trypsin at concentrations that prevent tight association between full length SRα molecules and microsomes in vitro, ~2 μg/ml.

The sensitivity of both SRα and SRβ molecules to digestion with trypsin was measured by incubating microsomes with different concentrations of the enzyme followed by immunologic identification of the digestion products after electrophoretic separation and blotting on nitrocellulose. As expected molecules of SRα are sensitive to low concentrations of trypsin (Fig. 11, top section). Degradation products of this molecule appear after incubation with as little as 1 μg/ml trypsin (lanes 4–6), and digestion is essentially complete by 5 μg/ml (lanes 7–9). In contrast, SRβ molecules are much less sensitive to the enzyme as degradation products first appear after incubation with 30 μg/ml trypsin (lanes 10–12). This pattern of trypsin sensitivity (Fig. 11, bottom section) is consistent with a role for SRβ in targeting rather than stable anchoring of SRα molecules on the ER membrane.

Discussion

We have elucidated a previously uncharacterized mechanism for the biogenesis of SRα molecules that can be reconstituted in vitro. This pathway appears to involve at least two distinct steps: first, targeting to specific sites on the ER membrane and second, anchoring of the targeted molecule firmly to the cytoplasmic face of the membrane. The targeting step was assayed as the ability of SRα synthesized in vitro to substitute functionally for endogenous SRα on microsomal membranes inactivated by digestion with trypsin or by alkylation with NEM (Figs. 2, 5, and 6). The membrane-anchoring step was assayed by resistance to extraction with 2 M urea (Figs. 1, 3, and 4).

Targeting of SRα

The initial evidence for a highly selective targeting pathway comes from determination of the number of SRα molecules synthesized in vitro and the efficiency with which they act to restore translocation function to inactivated microsomes. We estimate that no more than 10 fmol of SRα molecules are synthesized in a 20-μl reticulocyte lysate translation reaction. Nevertheless, when these molecules are used to repopulate one equivalent of trypsinized microsomes, ~80% of the original translocation activity is restored (Fig. 2). In contrast, one equivalent of untreated microsomes has been shown to contain ~90 fmol of SRα (Tajima et al., 1986). Therefore, replacing 10% of the endogenous SRP receptor population restores almost 80% of the translocation activity. This result suggests SRα molecules synthesized in vitro are specifically targeted to sites on the ER membrane. Moreover it suggests that either SRP receptor concentration is not the limiting factor in the translocation of prolactin molecules across the ER membrane in vitro or that most of the SRP receptor on the surface of isolated microsomes is not capable of promoting translocation of an added substrate. Endogenous SRP receptor may appear nonfunctional for any of a variety of reasons including denaturation during microsome preparation and masking by other potential substrates or regulatory effectors.
Reconstitution of translocation function to microsomes inactivated by alkylation with NEM provides some clues as to the specific site the newly synthesized SRα molecules occupy on the ER membrane. Alkylation with NEM is unlikely to displace endogenous SRα from SRβ molecules. Therefore, newly synthesized SRα molecules are probably recruited to unoccupied sites on the membrane. The lower translocation activity of SRα reconstituted NEM-treated microsomes relative to those reconstituted similarly but inactivated with trypsin is consistent with there being a smaller number of potentially active sites on NEM-treated microsomes (Figs. 5 and 6). In contrast, NEM-treated microsomes treated with trypsin either before or after the alkylation step can be restored to the same level of translocation as microsomes treated only with trypsin. These results are consistent with the newly synthesized SRα molecules occupying additional sites exposed by the trypsinization step. Since the endogenous SRα molecules are trypsin sensitive it seems likely that the sites exposed by trypsin were previously occupied by endogenous SRα molecules. For this reason we refer to inactivated microsomes with reconstituted translocation activity due to added SRα molecules as repopulated microsomes.

Further evidence for specific targeting of SRα comes from estimates of the relative number of SRα molecules synthesized compared to the number of prolactin molecules translocated by SRα repopulated microsomes. The relative number of these molecules can be estimated from measured optical densities of bands on autoradiograms by correcting for the number of methionine residues in each molecule. Although the experiments presented in Figs. 2 and 5 can be used for this purpose the experiment presented in Fig. 10 b is the most appropriate. In this experiment microsomes were separated from unbound SRα molecules after the repopulation step by pelleting in step gradients. Therefore, when these microsomes were resuspended in a preprolactin translation reaction only those SRα molecules that cofractionated with microsomes could provide access to the translocation machinery. We calculate that the number of prolactin mole-
Figure II. SRβ is not sensitive to concentrations of trypsin which degrade SRα. Microsomes were incubated with trypsin at the concentrations indicated for 1 h at 0°C. The reaction products were divided into two equal aliquots and one was fractionated into a supernate and a microsome-associated pellet by centrifugation. The three fractions representing total products (t), products released from microsomes and found in the supernate (s), and those which remain bound to microsomes recovered in pellets (p) were then separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. SRα and SRβ molecules were identified by probing the nitrocellulose blots with monoclonal antibodies directed against each of the molecules and an anti-mouse IgG second antibody labeled with 125I, followed by autoradiography. Bands identified as SRα and SRβ and the migration positions of molecular mass markers, in kilodaltons, are indicated.

cules translocated in the experiment illustrated in Fig. 10 b was sixfold larger than the number of SRα molecules available to initiate translocation. This result strongly suggests a large fraction of the SRα molecules that became associated with microsomes during the repopulation step are active to translocate prolactin molecules.

Targeting of SRα molecules to the ER membrane differs from SRP-mediated targeting of secreted proteins in a number of ways. Targeting is independent of SRP receptor and can take place posttranslationally (Figs. 2, 3, and 4). In addition, there does not appear to be a requirement for small molecules such as nucleoside triphosphates, other than those possibly provided by the extensively washed microsome fraction. Although we have not measured the levels of such molecules directly, a gel filtration step that removes unincorporated [35S]methionine does not reduce targeting efficiency (Fig. 3 a). Moreover, targeting is independent of SRP, requiring at most a small subset of proteins in the translation reaction, as SRα molecules synthesized in vitro are still able to target to microsomes even after affinity purification (Fig. 3 b).

The fact that SRα is efficiently targeted to NEM-alkylated microsomes differentiates this pathway from the SRP-independent signal-mediated targeting pathway described for prepromelittin (Muller and Zimmermann, 1987). This pathway has been shown to be disabled when microsomes are alkylated with NEM (Zimmerman and Mollay, 1986). If NEM disables a step in this translocation pathway subsequent to targeting, it is possible that SRα targeting might overlap with the initial steps of this SRP-independent pathway. However, such a step cannot be shared with the SRP-dependent translocation pathway reconstituted to NEM-alkylated microsomes by SRα.

The first 151 amino acids of SRα may function to promote efficient targeting as the deletion mutant SRα-EF appears to associate with microsomes with lower fidelity than the full length molecule as judged by reduced ability to restore translocation (Fig. 2). However, the experiments presented here do not permit us to differentiate a direct effect of the amino terminal region of SRα from improper folding of the deletion mutant. Efficient targeting is observed to microsomes digested with trypsin, suggesting the targeting receptor which may recognize the amino terminal region of SRα is protease resistant. Although we have no direct evidence, the resistance of SRβ to digestion with trypsin (Fig. 11) combined with previously reported colocalization experiments (Tajima et al., 1986), suggests this molecule may play a role in SRα targeting.

**SRα Membrane Anchoring**

Although SRα molecules are clearly targeted to trypsinized microsomes to restore function (Figs. 2, 5, and 10 b) these molecules are not anchored tightly to the membrane. Targeted SRα molecules cofractionate with trypsinized microsomes only in physiologic gradients and are largely released from the membrane by extraction with 2 M urea (Fig. 10 a). For this reason we suggest targeting and stable membrane association are distinct steps in SRα biogenesis, which can be dissociated by trypsinization of the membrane.

We examined the specificity of the membrane anchoring event to confirm that the acquisition of urea resistance does not result from simple hydrophobic interaction with the lipid.
bilayer. Even under physiologic conditions SRα molecules were not observed to interact with either artificial phospholipid vesicles or a mitochondria-enriched fraction used as nonspecific targets for SRα molecules in cell-free repopulation assays (Figs. 7 and 8). This lack of interaction with liposomes distinguishes the SRα anchoring mechanism from the insertion sequence mediated membrane anchoring of cytochrome b5 (Bendzko et al., 1982). Moreover, additional evidence for a specific microsomal protein required for SRα anchoring was provided by our demonstration that microsomal binding is saturable (Fig. 9). The results obtained by varying either the amount of microsomes or SRα were remarkably similar and suggest that the available sites on one equivalent of microsomes can be saturated by <10 fmol of SRα.

Previous studies have implicated the amino terminal 151 amino acids of SRα in membrane anchoring. Those experiments used proteolytic cleavage of endogenous SRα molecules with either elastase or trypsin to remove the amino terminus from the molecule. When this cytoplasmic fragment of SRα was added back to trypsinized microsomes, translocation activity was restored but SRα molecules did not become tightly associated with the membrane (Gilmore et al., 1982b; Meyer and Dobberstein, 1980; Hortsch et al., 1985). These observations led to the conclusion that the two hydrophobic stretches deleted from the molecule mediated SRα membrane association (Lauffer et al., 1985).

In view of the evidence discussed above for protein-mediated membrane anchoring and that showing full length SRα molecules do not become stably associated with trypsinized microsomes (Fig. 10), it is possible that release of the cytoplasmic portion of SRα by proteases is due to degradation of an anchoring protein rather than to cleavage of SRα. If this is true stable membrane association could result from interaction of this putative anchoring protein with any portion of SRα. However, our demonstration that SRα but not SRα-EF associates with intact microsomes in a urea-resistant manner confirms that the amino terminus of SRα is required for membrane anchoring (Figs. 1 and 3). Although it remains a formal possibility that the inability of SRα-EF to interact stably with the membrane is due to improper folding of the deletion mutant instead of loss of required sequences. We consider this possibility unlikely because SRα-EF can function to promote translocation, albeit with reduced efficiency (Fig. 2). Nevertheless, the fact that full-length SRα molecules do not associate tightly with trypsinized microsomes strongly suggests some trypsin-sensitive protein is, at least transiently, involved in SRα anchoring.

Alkylation of microsomes with NEM was used to probe the nature of this putative anchor protein. Alkylation does not prevent SRα binding to microsomes in a urea resistant manner (Fig. 4). Moreover, once anchored to microsomes SRα molecules are also unaffected by 0.5 M NaCl (Fig. 4 a, lanes 10-15), 0.05 M KClAc (not shown), 0.5 M NaCl, 25 mM EDTA (Fig. 4 b lanes 1-9), and 2 M urea, 0.5 M NaCl (not shown). These results, together with our demonstration that the amino terminus of SRα, which contains two hydrophobic stretches of amino acids, is required for tight membrane association, suggest the stabilizing interaction may be primarily hydrophobic. If so, this would explain why this putative membrane anchor is not seen in fractionation experiments in which the microsomal membrane is solubilized with detergents (Tajima et al., 1986).

Although such interaction may be mediated solely by SRβ we consider this unlikely because anchoring is abolished by pretreatment of microsomes with concentrations of trypsin which have no apparent effect on SRβ (compare Fig. 10 with Fig. 11). However, it is possible that the putative anchoring protein interacts with SRα only transiently to stabilize association with the microsome. In such a scenario, a structural or chemical modification catalyzed by the putative anchoring protein may attach SRα to either SRβ, some other microsomal component, or with the lipid bilayer. The results described here provide a logical framework and experimental system that will permit such questions to be addressed.

The authors thank Judy White and Jeffrey Glenn for help in preparing the phospholipid vesicles; and Alison Cowie for helpful discussions and critical reading of the manuscript.

This work was supported by National Institutes of Health grant GM-31626 to V. Lingappa and GM-32384 to P. Walter. D. W. Andrews is a recipient of a fellowship from the Medical Research Council of Canada. L. Lauffer is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

Received for publication 19 September 1988 and in revised form 7 November 1988.

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