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An Amino-terminal Domain Containing Hydrophobic and Hydrophilic Sequences Binds the Signal Recognition Particle Receptor α Subunit to the β Subunit on the Endoplasmic Reticulum Membrane*

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The signal recognition particle receptor consists of two subunits of 72 kDa (SRα) and 30 kDa (SRβ). Assembly of SRα on the endoplasmic reticulum membrane can occur independent of the signal recognition particle-mediated translocation pathway. To identify the sequences within SRα necessary for membrane binding, a series of amino-terminal and internal deletion mutants was constructed and translated in a cell-free system. In addition, nascent SRα polypeptides of varying lengths were generated by cycloheximide treatment of translation reactions. Microsome binding assays performed on these polypeptides revealed a membrane binding domain consisting of the amino-terminal 140 residues of SRα. This domain includes the two hydrophobic sequences originally proposed to bind to membranes and a highly charged region not previously implicated in membrane assembly. Furthermore, the domain forms a protease-resistant folding unit that after proteolysis can target and anchor onto microsomes. Extraction of microsomal SRα at high pH supplemented with 1 M NaSCN suggests that SRα and the membrane binding domain are not integrated in the endoplasmic reticulum membrane. The membrane binding domain is also the major site of tight binding with SRβ, suggesting that SRβ plays a role in the membrane assembly of SRα.

In mammalian cells, secretory signal sequences of nascent polypeptide chains are bound by the ribonucleoprotein signal recognition particle (SRP)[1] as they emerge from the ribosome. Targeting to polypeptide translocation sites on the endoplasmic reticulum (ER) membrane then occurs via the interaction of SRP with the SRP receptor on the cytoplasmic face of the ER membrane (1, 2). The major components of this targeting pathway are conserved in eukaryotes and possibly in prokaryotes (for review, see Ref. 3).

The SRP receptor has been isolated as a heterodimer of two polypeptides that migrate in SDS-PAGE as 72-kDa (SRα) and 30-kDa (SRβ) species (4). Both subunits are resistant to extraction from the membrane with urea or high salt and have been characterized as integral membrane proteins by resistance to extraction at high pH (2, 4, 5). Protease dissection of SRα on microsomes or purified by affinity chromatography revealed a translocation active cytoplasmic fragment of about 58 kDa and a fragment of about 14 kDa containing a putative membrane anchor (5–7). The cDNA for SRα encodes a 638-residue polypeptide containing two stretches of hydrophobic amino acids (residues 1–22 and 64–79) near the amino terminus that were proposed to serve as membrane anchors, as well as three clusters of charged (mostly basic) residues between residues 84 and 243 (8). The cytoplasmic elastase fragment of SRα was shown to consist of the sequence from residue 152 to the carboxyl terminus and contains a GTP binding site (8, 9). The cytoplasmic elastase fragment can assemble on trypsin-digested membranes to restore translocation activity, suggesting that it may bind SRβ directly (10). SRβ is predicted from the primary amino acid sequence to have a single transmembrane domain near the amino terminus and a GTP binding site near the cytoplasmic carboxyl terminus (11).

SRα has previously been shown to target and anchor onto the ER membrane *in vitro* by a mechanism independent of the SRP-mediated pathway (10). Membrane assembly and functional reconstitution of SRα can occur post-translationally and in the absence of GTP or ATP. Cell-free synthesized SRα can also restore SRP-mediated translocation activity to microsomes in which the endogenous SRα has been inactivated by digestion with trypsin or by alkylation of free sulfhydryls. The binding of SRα onto trypsin-digested microsomes is labile to urea, suggesting that the subunit is not assembled on the membrane by spontaneous insertion into the lipid bilayer (10).

The exact mechanism by which SRα assembles on the membrane is unknown. Furthermore, the sequences within SRα required for interaction with SRβ have not been identified. To investigate these issues, we have assayed deletion mutants of SRα translated in a cell-free system for salt-resistant binding onto ER microsomes. An amino-terminal domain of SRα including amino acids 1–140 was found to be necessary for membrane binding. Immunoprecipitation experiments indicate that the domain is also responsible for binding to SRβ. The SRα membrane binding domain appears to be an independent folding unit that is tightly bound to SRβ but not integrated into the ER membrane. A new model of SRα membrane assembly is proposed in which both hydrophobic and hydrophilic regions of

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[1] The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; RRL, rabbit reticulocyte lysate; SRα and SRβ, SRP receptor α and β subunits; KRMs, canine pancreatic rough microsomes extracted with 0.5 M KOAc; CRMs, CL-2B column washed canine pancreatic rough microsomes; CTAB, cetyltrimethylammonium bromide; OST48, the 48 kD subunit of oligosaccharyl transferase.

SRα anchor the protein to the membrane primarily by interacting with the transmembrane SRβ.

EXPERIMENTAL PROCEDURES

Materials and General Methods—General chemical reagents were obtained from either Fisher, Sigma, or Life Technologies, Inc. SURE™ *Escherichia coli* cells used for plasmid construction were purchased from Stratagene. Except where specified, restriction enzymes, other molecular biology enzymes, and reagents were from New England Biolabs. ^{35}S-Labeled methionine was from DuPont NEN. SP6 polymerase was purchased from Epicentre Technologies. Creatine kinase, staphylococcal nuclease, and various proteases were from Boehringer Mannheim, and RNAguard (an RNase inhibitor) was from Pharmacia Biotech Inc.

Transcription reactions with SP6 polymerase were performed as described previously (14). Cell-free translation reactions were performed in rabbit reticulocyte lysate (RRL) and labeled with [^{35}S]methionine as described previously (12); translation products were analyzed by SDS-PAGE (15, 16) followed by fluorography. Canine pancreatic rough microsomes were obtained as described, and either extracted with 0.5 M KOAc (KRMs) or washed by Sepharose CL-2B gel exclusion chromatography (CRMs) (13).

Polyclonal antisera against SRα and SRβ were raised in rabbits injected with purified bacterially overexpressed fusion proteins. Plasmid pMAC142 contained the sequence encoding amino acids 39–295 of SRα inserted into pRIT-2T (Pharmacia) resulting in a fusion protein with *S. aureus* Protein A. Plasmid pMAC359 encoded amino acids 208–265 of canine SRβ fused to glutathione *S*-transferase in the vector pMAC241, a modification of pGEX-2T (Pharmacia) with an enhanced polylinker. The SRα and SRβ fusion proteins were purified using IgG-Sepharose and glutathione-Sepharose columns, respectively. Other antisera were kind gifts of J. J. M. Bergeron, R. Gilmore, and T. Rapoport.

Plasmids—Construction of plasmids, sequencing and site-directed mutagenesis were performed using standard techniques (36). Unless otherwise stated, all constructs were inserted following the SP6 RNA polymerase promoter in pSPUTK (37). The deletion mutants of SRα and the relevant restriction sites are outlined in Fig. 1 and briefly described below. Full construction details for each of the plasmids are available from the authors on request.

Plasmid pMAC191 contains the full-length cDNA sequence of canine SRα (8), with a C→G point mutation at nucleotide 4 of the open reading frame in the plasmid vector pSPUTK (37). The mutation introduces an *Nco*I site at the start codon of SRα. The overall translation efficiency of SRα in the cell-free system is increased by this mutation, but the resulting leucine to valine substitution does not affect the membrane targeting behavior or translocation activity of the polypeptide (data not shown). The mutant polypeptide is termed SRαN to distinguish it from polypeptides with the wild-type sequence and microsomal SRα. Plasmid pMAC42 encoding the polypeptide SR-EF, corresponding to the soluble elastase fragment of SRα, has been reported previously (10).

Plasmid pMAC3 encodes the polypeptide SRD1, containing amino acids 79 to the stop codon of SRα and therefore having the two hydrophobic regions deleted from the amino terminus of SRα. Plasmid pMAC456 encodes the polypeptide SRD3 in which residues 156–250 of SRαN are deleted, removing part of the second and all of the third charged regions of SRα. Plasmid pMAC55 encodes the polypeptide SRD4 containing an initial methionine followed by a glycine residue and residues 28 to the stop codon of SRα, deleting the first hydrophobic region of SRα.

Plasmid pMAC205 encodes the first 176 amino acids of SRαN followed by Ser-Asn-Tyr-Ser-Arg-stop codon. This polypeptide, SRX2, includes the two hydrophobic regions and the first two charged regions of SRα. Plasmid pMAC268 encodes SRX3, containing the polypeptide sequence Met-Gly-Ala-Pro followed by amino acids 28 to the stop codon of SRX2 and deleting the first hydrophobic region from SRX2. Plasmid pMAC135 encodes SRX6, containing residues 1–38, Asn-Ser and residues 79 to the end of SRX2, thereby deleting the second hydrophobic region of SRX2. Plasmid pMAC362 encodes SRX7, containing residues 1–79 and 103 to the stop codon of SRX2 and deleting the first charged region of SRX2.

Plasmid pMAC459 encodes SRD6, containing the sequence of SRαN with amino acids 39–79 replaced by Asn-Ser and thus deleting the second hydrophobic region from SRαN. Plasmid pMAC494 encodes the polypeptide SRD7, having the sequence of SRαN with amino acids 79–103, and therefore the first charged region, deleted.

Plasmid pMAC455 codes for a chimaeric SRβ polypeptide (SRβ-MD), containing the first 29 residues of mouse SRβ followed by the predicted transmembrane and cytoplasmic domains of canine SRβ. The chimaeric polypeptide was used because the cDNA sequence of canine SRβ was incomplete and the encoded protein was missing the initiation site and an unknown number of amino-terminal residues. However, the missing residues were predicted to be in the ER lumen (11) and less likely to interact with SRα. The lumenal domain of canine SRβ was therefore replaced with the complete amino-terminal lumenal domain of mouse SRβ, and the DNA sequence encoding this polypeptide was inserted into the vector pSPUTK. For immunoprecipitation experiments, plasmid pMAC690 was constructed encoding SRβ-MD with two copies of the influenza hemeagglutinin epitope tag at the amino terminus (HASRβ-MD). The sequence of the epitope tag was provided by inserting the DNA encoding SRβ-MD into the plasmid pG7SCTHA2 (35). The resulting coding sequence was inserted behind the SP6 promoter of plasmid pMAC334, a version of pGEM3 with the 5′-untranslated region of pSPUTK and the 3′-untranslated region of bovine preprolactin. Plasmid pMAC508 encoding the integral membrane protein S_LS_TgPA has been previously reported (38).

Cell-free Translations and Membrane Targeting—For post-translational targeting reactions, translation was terminated by chilling on ice, and then ribosomes were removed by centrifugation at 30 psi (180,000 × *g*) for 5 min in an Airfuge. A 20-μl aliquot of the supernatant was incubated with either 10 equivalents of CRMs or an equal amount of buffer for 5 min at 24 °C. The mixture was then loaded onto a 0.5-ml column of Sepharose CL-2B in a 1-ml syringe equilibrated with 500 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. The column was eluted with equilibration buffer, and fractions (single drops) from the column were collected. 1.5-μl samples of these fractions were analyzed by SDS-PAGE. The excluded volume of each column was calibrated by passing CRMs over the columns and identifying microsomal SRα by immunoblot analysis. The included volume was identified by the red color of the globin from the RRL.

Incomplete nascent SRαN polypeptides of different lengths were generated by terminating cell-free translation reactions at various times with 1 mM cycloheximide. To assay membrane targeting of these polypeptides, a 20-μl aliquot of each reaction was incubated with 5 equivalents of KRMs for 5 min at 24 °C. An equal volume of buffer containing 1 M NaCl, 50 mM EDTA, and 20 mM Tris-Cl, pH 8.0, was added at 4 °C. The mixture was layered over a 100-μl sucrose step gradient containing 500 mM sucrose, 500 mM NaCl, 25 mM EDTA, and 20 mM Tris, pH 8.0, and the membranes were pelleted by centrifugation in an Airfuge at 20 psi (100,000 × *g*) for 10 min. The top 75 μl (supernatant) was recovered, and peptidyl-tRNA was precipitated by adding 500 μl of 2% cetyltrimethylammonium bromide (CTAB) and 500 μl of 0.5 M NaOAc, pH 5.0 (17). Equivalent portions of the pellet and supernatant fractions were analyzed by SDS-PAGE.

Proteolytic Digestions—Controlled proteolysis of RRL translation products was performed by adding Proteinase K at a final concentration of 10 μg/ml to a completed 25-μl translation reaction and incubating on ice. Digestion was terminated after 30 min with 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin, and the reaction was incubated with 2.5 equivalents of KRMs for 5 min at 24 °C. The mixture was adjusted to 2 M urea, and the membranes were pelleted as described previously (10). The supernatant and pellet fractions were analyzed by SDS-PAGE.

Proteinase K digestions of KRMs at 1 equivalent/μl were performed for 1 h at 4 °C with either 0 or 10 μg/ml proteinase K. The reactions were terminated with 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin. The microsomes were adjusted to 500 mM NaCl and pelleted in an Airfuge at 20 psi (100,000 × *g*) for 10 min. Immunoblots were probed for SRα and visualized using an alkaline phosphatase color reaction.

Membrane Extractions and Immunoprecipitations—The Triton X-114 cloud point partitioning assay (18) was adapted to enhance solubilization of SRα by the addition of 5% glycerol to the solubilization buffer and 1% glycerol to the sucrose cushion (19). The immunoblot was probed with monoclonal antibodies against both SRα and SRβ and visualized using a two-color enzymatic system to permit unambiguous identification of the polypeptides (20). Immunoblots probed for other proteins were visualized with the alkaline phosphatase reaction.

Microsomes were extracted with high pH following a modified procedure based on the published assay (21). 2 ml of KRMs at 1 equivalent/μl were loaded onto a 100-ml Sepharose CL-2B gel exclusion column equilibrated and eluted with 1 M NaSCN, 0.2 M Na_2CO_3, pH 11.5, and 10 mM dithiothreitol. 1.5-ml fractions were collected and concentrated by trichloroacetic acid precipitation for SDS-PAGE analysis. Immunoblots were visualized as above.

For immunoprecipitations of the SRα mutants with SRβ-MD, 10-μl

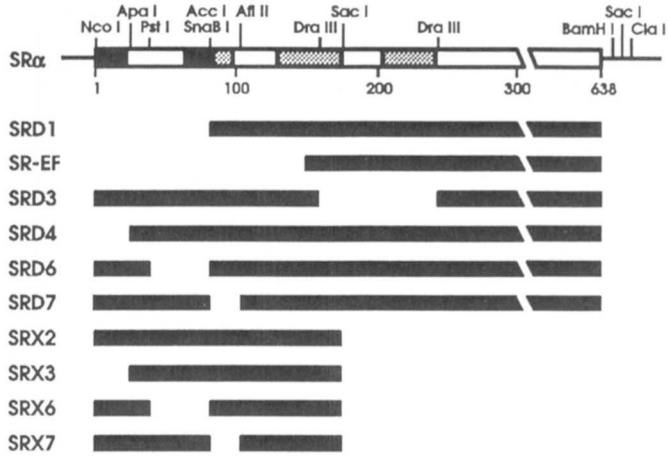

FIG. 1. **Mutants of SRα.** Diagram of the SRα coding region (*top bar*) with restriction enzyme sites in the DNA used to construct mutants. Amino acid residues are numbered *below bar*. Hydrophobic sequences are shown in *black*, and charged sequences are *shaded*. Deletion mutants are diagrammed *below* with *solid bars* indicating the region(s) expressed in each.

RRL translation reactions were mixed with 10-μl reactions of SRβ-MD after translation was complete and incubated at 24 °C for 30 min. The mixtures were then diluted in 500 μl of buffer (100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1% Triton X-100) at 4 °C, and the translation products were isolated using a monoclonal Sepharose affinity matrix. To prepare the affinity matrix, IgG against SRα was purified from ascites fluid (4) and coupled to CNBr-activated Sepharose. As controls, 10-μl translation reactions of SRαN, the deletion mutants and SRβ-MD were immunoprecipitated using the same monoclonal Sepharose.

To co-precipitate various SRα mutants with HASRβ-MD, RRL translation reactions synthesizing HASRβ-MD were carried out in the presence of KRMs. A 5-μl aliquot of the HASRβ-MD reaction was incubated with a 30-μl translation reaction of each SRα mutant at 24 °C for 30 min. The mixture was loaded onto a 0.8-ml Sepharose CL-2B column equilibrated and eluted with buffer containing 250 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. Fractions containing the excluded volume of the column were pooled, adjusted to 350 mM NaCl, 5% glycerol, and 1% Triton X-100, and HASRβ-MD was recovered using monoclonal antibodies against the hemeagglutinin epitope and Protein G Affi-Gel (Pharmacia).

RESULTS

Sequences within SRα Required for Membrane Binding—A plasmid was constructed encoding SRαN, a mutant of SRα (Leu replaced with Val) that has increased translational efficiency in our cell-free system but with the same functional and membrane targeting behavior as wild-type SRα (data not shown). Plasmid vectors encoding deletion mutants of SRαN (Fig. 1) were constructed to investigate the membrane binding of the receptor subunit. Previous experiments indicated that some portion of the amino-terminal region of the polypeptide, containing two relatively hydrophobic sequences, was involved in anchoring SRα to the ER membrane (5, 8, 10). Therefore, a series of plasmids was made containing deletions in the region encoding the two hydrophobic regions (SRD1, SRD4, and SRD6) and an adjacent region of charged amino acids (SRD7). A construct coding for the amino-terminal 176 amino acids of SRαN plus four additional residues (SRX2) was also made. Additional deletions were made within the SRX2 sequence (SRX3, SRX6, and SRX7). A broad deletion was also made in a central region of the SRαN sequence that was not expected to affect membrane binding (SRD3).

In a previous study of the membrane assembly of SRα, anchored and loosely bound molecules could be separated by a simple pelleting assay in the presence of 2 M urea (10). However, this assay could not clearly distinguish membrane-bound polypeptides from large insoluble aggregates. Therefore, to assay the deletion mutants for tight membrane binding, translation reactions containing microsomes were fractionated by Sepharose CL-2B gel exclusion chromatography at high ionic strength. Endogenous microsomal SRα was identified by immunoblotting and found to elute solely in the excluded volume of the columns (*fraction 4*, marked with *arrowhead* for all columns used in Fig. 2, data not shown). The included volume (*fractions 7–12* in all assays) was determined using the endogenous hemoglobin in the RRL lysate. Because the included volume eluted as a broad peak, *fractions 7*, *9*, and *11* are shown in Fig. 2 as representative fractions.

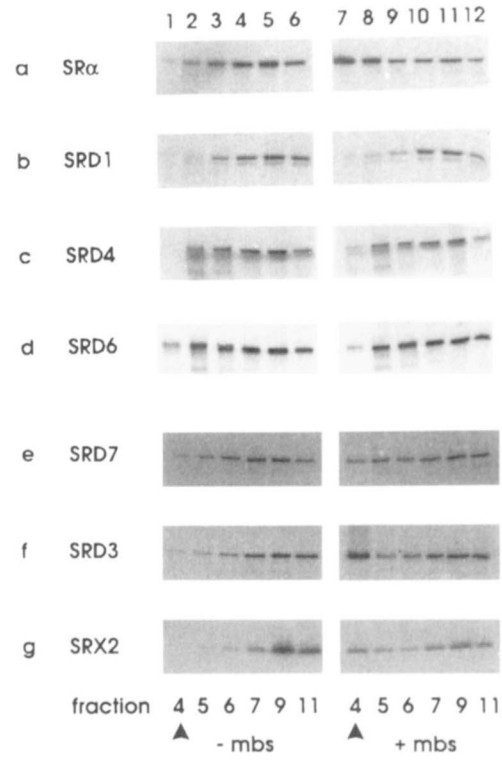

FIG. 2. **Membrane binding of SRα deletion mutants.** RRL translation reactions of SRαN and selected deletion mutants (*lanes 1–6*) or reactions incubated with microsomes (*lanes 7–12*) were loaded on 0.5-ml Sepharose CL-2B columns equilibrated and eluted in buffer containing 500 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. Membranes eluted in the excluded volume (*fraction 4*, *arrowheads*) while hemoglobin eluted as a broad peak in the included volume (*fractions 7–12*).

In the absence of microsomes, SRαN synthesized in RRL fractionated in the included volume (Fig. 2*a*, *lanes 3–6*). As expected, after incubation with membranes, much of the SRαN fractionated in the excluded volume together with the microsomes (Fig. 2*a*, compare *lane 1* with *lane 7*), indicating that these polypeptides were tightly bound on the membranes. SR-EF (which lacks the amino-terminal 151 residues of SRα) has been shown to behave as a peripheral membrane protein (2, 5, 8, 10). Consistent with this, SR-EF was found only in the included volume of the columns in either the absence or presence of membranes (data not shown).

The two hydrophobic stretches in SRα were deleted in SRD1 and, as expected, this polypeptide did not fractionate with microsomes as it was recovered only in the included volume (Fig. 2*b*, compare *lanes 1–6* with *lanes 7–12*). Constructs that removed only the first (SRD4) or second (SRD6) of the hydrophobic sequences were also assayed. Although a fraction of SRD6 aggregated in RRL and therefore is recovered in fractions 5 and 6, the aggregates were clearly resolved from fraction 4 containing membranes (Fig. 2*d*, compare *lanes 8* and *9* to

lane 7). Neither SRD4 nor SRD6 were able to bind efficiently onto microsomes (Fig. 2, *c* and *d*, compare *lanes 1* and *7*). Surprisingly, a construct (SRD7) that left both the hydrophobic regions intact but deleted an adjacent section of strongly charged residues (amino acids 79–103) was also unable to bind efficiently onto microsomes (Fig. 2*e*, compare *lanes 1* and *7*). Although analysis of this molecule was complicated by the presence of large aggregates (Fig. 2*e*, *lane 1*), there was still a large portion of unaggregated polypeptide (Fig. 2*e*, *lanes 10–12*) that was expected to be targetted to the membrane. As a control, a construct with a deletion in a region of the SRα sequence containing numerous basic amino acids (residues 156–250) but containing an intact amino terminus (SRD3) was found to fractionate with microsomes as expected (Fig. 2*f*, compare *lanes 1* and *7*).

These results suggested that sequences beyond the predicted membrane anchor of SRα (8) may be required for membrane assembly. The carboxyl-terminal domain of SRα (residues 152–638) has been shown to target to translocation sites on the ER but not anchor to the membrane in a manner resistant to high salt or urea concentrations (1, 10). To directly examine the membrane assembly of the amino-terminal region of SRα, a construct (SRX2) containing the first 176 amino acids of SRαN was assayed. Although the putative carboxyl-terminal targeting domain was deleted from SRX2, the polypeptide bound onto microsomes (Fig. 2 *g*, compare *lanes 1* and *7*). Therefore, there are at least two targeting sequences in SRα, but only the amino-terminal sequence mediates tight binding onto membranes. To analyze the sequence of SRX2 further, plasmids were constructed with deletions within the SRX2 coding region (SRX3, SRX6, and SRX7, Fig. 1). However, cell-free synthesized SRX3, SRX6, and SRX7 were unable to clearly bind onto membranes and formed very large aggregates in the presence or absence of microsomes (data not shown).

The positively charged amino acid sequence deleted in SRD7 may be specifically required for membrane binding. However, it is also possible that the deleted sequence is not itself involved in membrane assembly but that deletion adversely affects protein folding around an adjacent membrane anchor sequence. To address this issue, cell-free translation reactions of SRαN were terminated with cycloheximide at different times after initiation to generate a series of ribosome-bound peptidyl-tRNA translation intermediates with a range of lengths. Ribosome-bound nascent polypeptides prepared in this manner should be free of aggregates. The reactions were incubated with microsomes to allow targeting of the nascent chains and then adjusted to 500 mM NaCl and 25 mM EDTA. The membranes were separated by centrifugation and analyzed for the presence of bound polypeptides. Peptidyl-tRNA was precipitated from the supernatant with CTAB (17) to recover nascent chains not bound to the microsomes. It was expected that if the amino-terminal hydrophobic regions of SRαN (up to around residue 80) were sufficient for membrane binding while attached to ribosomes, then polypeptides of molecular weight greater than or equal to 13 kDa (corresponding to about residue 120, presuming 40 amino acids at most are sequestered within the ribosome (22, 23)) would be detected in the membrane fraction. On the other hand, if membrane binding required sequences beyond the hydrophobic regions, then only larger polypeptides (approximately 190 amino acids for a 150 residue membrane binding domain) would be recovered with the microsomes.

Nascent SRαN polypeptides of discrete sizes from 10 kDa upward (estimated by migration in SDS-PAGE) could be detected after precipitation with CTAB (Fig. 3, *lanes 7–12*). The CTAB-precipitated products reflected the polypeptides present in the total translation reaction (Fig. 3, *lane 13*). However, no

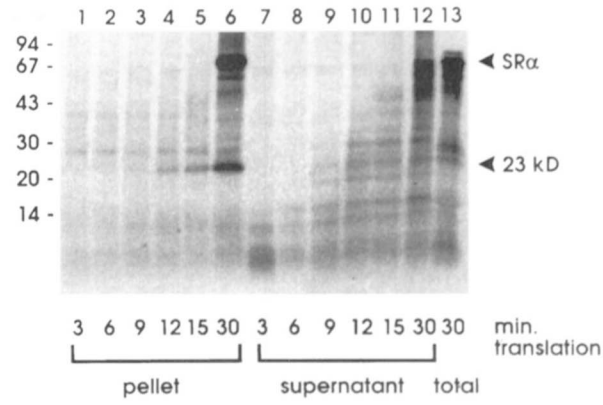

FIG. 3. **Membrane binding of SRα nascent chains.** RRL translation reactions of SRαN were terminated with 1 mM cycloheximide at various times after initiation. The terminated reactions were incubated with microsomes and then adjusted to 500 mM NaCl and 25 mM EDTA. Membranes were pelleted by centrifugation in an Airfuge (*lanes 1–6*), and peptidyl-tRNA was precipitated from the resulting supernatant with CTAB (*lanes 7–12*). A sample of the total translation reaction terminated after 30 min was also analyzed (*lane 13*). A 23-kDa translation intermediate that anchored into membranes is marked.

polypeptides smaller than a 23-kDa translation intermediate were recovered with microsomes (Fig. 3, *lanes 1–6*). This 23-kDa product was chased to full-length SRαN when the translation reactions were allowed to proceed for 1 h and is therefore a true translation intermediate (data not shown). The deletion mutant SRX2 containing 180 amino acids also migrates as a 23 kDa band, suggesting the 23-kDa nascent polypeptide contains a similar number of residues. This is too large to consist of the hydrophobic regions of SRαN alone, but it is consistent with a membrane binding domain of approximately 140 amino acids. These data therefore support the hypothesis that sequences carboxyl-terminal to the hydrophobic regions of SRα are necessary for membrane assembly.

Domain Structure of the SRα Membrane Binding Sequence—To determine whether the membrane binding sequence of SRα forms an independently folded protein domain, we examined the sensitivity of SRα and several deletion mutants to protease digestion. Elastase dissection of purified SRα previously revealed a 14-kDa amino-terminal fragment presumed, but not demonstrated, to bind onto membranes (5, 8), as the fragment could not be detected on microsomes digested with elastase using SRα antisera (5). Therefore, to identify folding units within SRα that are competent in membrane targeting, we assayed proteolysis fragments of cell-free synthesized SRα for membrane anchoring. Cell-free translation reactions of SRαN were digested on ice with 10 μg/ml proteinase K for 30 min and then incubated with KRMs to allow membrane assembly. The reactions were adjusted to 2 M urea, and microsomes were recovered by centrifugation. Both the supernatant (Fig. 4*a*, *lane 1*) and pellet (Fig. 4*a*, *lane 2*) were analyzed for the presence of proteolytic fragments. Proteolytic fragments with a range of sizes were detected in the membrane fraction (Fig. 4*a*, *lane 2*), and the smallest of these fragments had an apparent molecular size of 16 kDa as estimated by migration in SDS-PAGE (Fig. 4*a*, *lane 2*). Since the amino-terminal deletion mutants SRD1 and SRD4 were unable to bind onto microsomes (see Fig. 2), it is likely that the membrane-anchored proteolytic fragment contained an intact amino terminus.

Our polyclonal antisera recognizes the amino-terminal region of SRα on immunoblots. Therefore, to confirm that the 16-kDa membrane binding fragment includes the amino-terminal domain, microsomes were digested with 10 μg/ml Proteinase K for 1 h, adjusted to 500 mM NaCl, and recovered by centrifugation. Immunoblots of the digested microsomal pro-

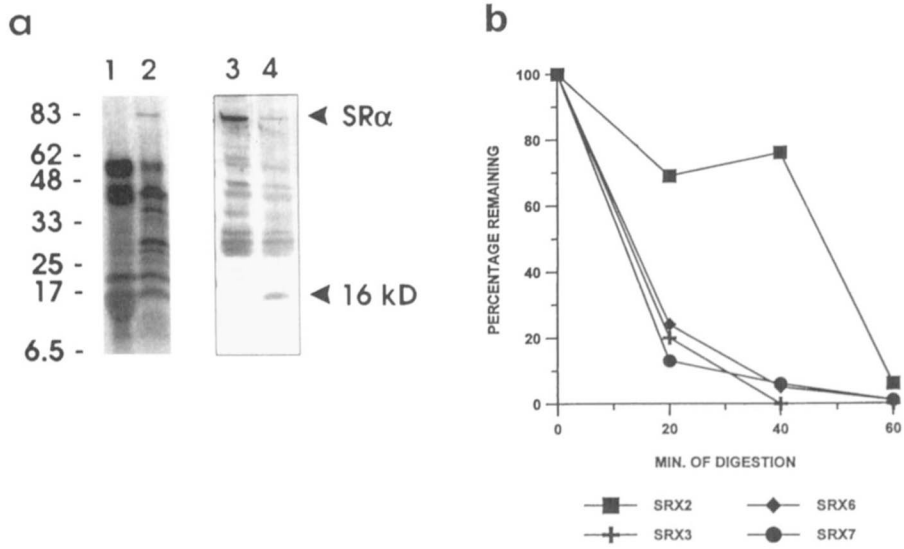

FIG. 4. **Protease dissection of SRα.** *a*, SRαN synthesized in RRL was digested with 10 μg/ml proteinase K for 30 min and incubated with microsomes after digestion was terminated. The reaction was adjusted to 2 M urea and separated into supernatant (*lane 1*) and pellet (*lane 2*) fractions by centrifugation in an Airfuge. The entire pellet and 25% of the supernatant were analyzed. A fragment with an apparent molecular size of 16 kDa (marked) produced by the proteolysis were observed to pellet with microsomes. Also, microsomes digested with 0 (*lane 3*) and 10 μg/ml proteinase K (*lane 4*) were adjusted to 500 mM NaCl, and the membranes were recovered for immunoblot analysis by centrifugation in an Airfuge. Immunoblots were probed with polyclonal antisera raised against an amino-terminal segment of SRα. A 16-kDa proteolysis product immunoreactive with SRα antisera (marked) remained on membranes. *b*, RRL translation reactions of SRX2, SRX3, SRX6, and SRX7 were digested with 10 μg/ml proteinase K, and samples were analyzed at 20-min intervals by SDS-PAGE and fluorography. The amount of translation product remaining was quantified for three independent experiments and plotted as a percentage of the amount present before digestion. The average standard deviation was ±5.2.

teins as well as proteins from mock-digested membranes were probed with the antisera against the amino terminus of SRα. As expected, full-length SRα was detected in the membrane pellet from mock digests (Fig. 4*a*, *lane 3*), and, as predicted, a 16-kDa fragment generated by proteolysis also pelleted with membranes (Fig. 4*a*, *lane 4*). This suggests that the 16-kDa membrane binding fragment in Fig. 4*a*, *lane 2*, consists of the amino-terminal membrane binding domain. The apparent molecular size of the 16-kDa fragments produced by proteolysis of both cell-free synthesized or endogenous microsomal SRα is consistent with the 140-residue amino-terminal domain suggested by Fig. 3.

The comparative resistance of the amino-terminal fragment of SRα to proteinase K digestion whether or not the protein is attached to membranes suggests that the anchoring domain forms a folded unit. To test this directly, the deletion mutants SRX2, SRX3, SRX6, and SRX7 (see Fig. 1) were assayed for resistance to Proteinase K. SRX2 contains the complete amino-terminal domain of SRαN, and the other polypeptides have deletions within the SRX2 sequence. Cell-free translation reactions of the polypeptides were digested with 10 μg/ml Proteinase K on ice for up to 1 h and analyzed at intermediate time points. The SRX3, SRX6, and SRX7 polypeptides were rapidly degraded under these conditions, with less than 30% of the initial populations remaining after 20 min (Fig. 4*b*). In contrast, more than 60% of the initial population of SRX2 polypeptide remained after 40 min of digestion (Fig. 4*b*). Interestingly, these data are reflected in the membrane binding behavior of SRX3, SRX6, and SRX7 reported above. In addition, the deletion mutants SRD4, SRD6, and SRD7 containing deletions in the SRX2 region of SRαN cannot bind onto membranes (Fig. 1). Taken together, these data suggest that the deletions within SRX2 lead to misfolding, and that SRX2 forms an independently folded protein domain.

Membrane Binding of SRα Correlates with Binding to SRβ—SRα has been previously described as an integral membrane protein since solubilization could be achieved only in the presence of detergent and high salt concentrations (2). In addition, SRα was detected in the membrane pellet after extractions of microsomes at pH 11 (5). Interestingly, SRα has recently been shown to become largely extracted at pH 13, along with roughly half of the SRβ population (11). Furthermore, SRα and SRβ were found in both the aqueous and hydrophobic phases after Triton X-114 cloud point extractions of membranes (11). To extend and clarify these results, the behavior of microsomal SRα in high pH and cloud point extractions was re-examined.

In studies of the translocation and membrane integration of proteins in cell-free systems, it has been observed that extraction with high pH alone was not always sufficient to distinguish peripherally bound proteins from integrated polypeptides (24). To increase the stringency of the high pH extractions, microsomes were extracted in buffer containing 1 M NaSCN, 0.2 M Na_2CO_3, pH 11.5, and 10 mM dithiothreitol, and membranes were separated from extracted material by Sepharose CL-2B gel exclusion chromatography. Fractions were analyzed for the presence of SRα and SRβ by immunoblot analysis. As controls, the immunoblots were also probed for the integral membrane proteins SSRα and the 48-kDa subunit of oligosaccharyl transferase (OST48), the cytosolic protein actin, and the 54-kDa subunit of the peripheral membrane SRP (SRP54) (25–30).

Under these conditions, microsomal SRβ was detected solely in the predetermined excluded volume of the column, corresponding to fractions 4–6 (Fig. 5*a*, *lanes 1–3*). While a fraction of microsomal SRα eluted in fractions 4 and 5 (Fig. 5*a*, *lanes 1* and *2*), the majority of the SRα polypeptides eluted in a broad peak between fractions 24 and 32 (represented by *fractions 24*, *28*, and *32*, Fig. 5 *a*, *lanes 8–10*). Visual inspection of this experiment and replicate trials indicated that approximately 20% or less of the SRα population remained on membranes in the excluded volume. As expected, the integral membrane control proteins SSRα and OST48 were observed almost exclusively in the membrane fractions (Fig. 5*a*, *lanes 1–3*), while actin and peripherally bound SRP54 eluted in a broad peak

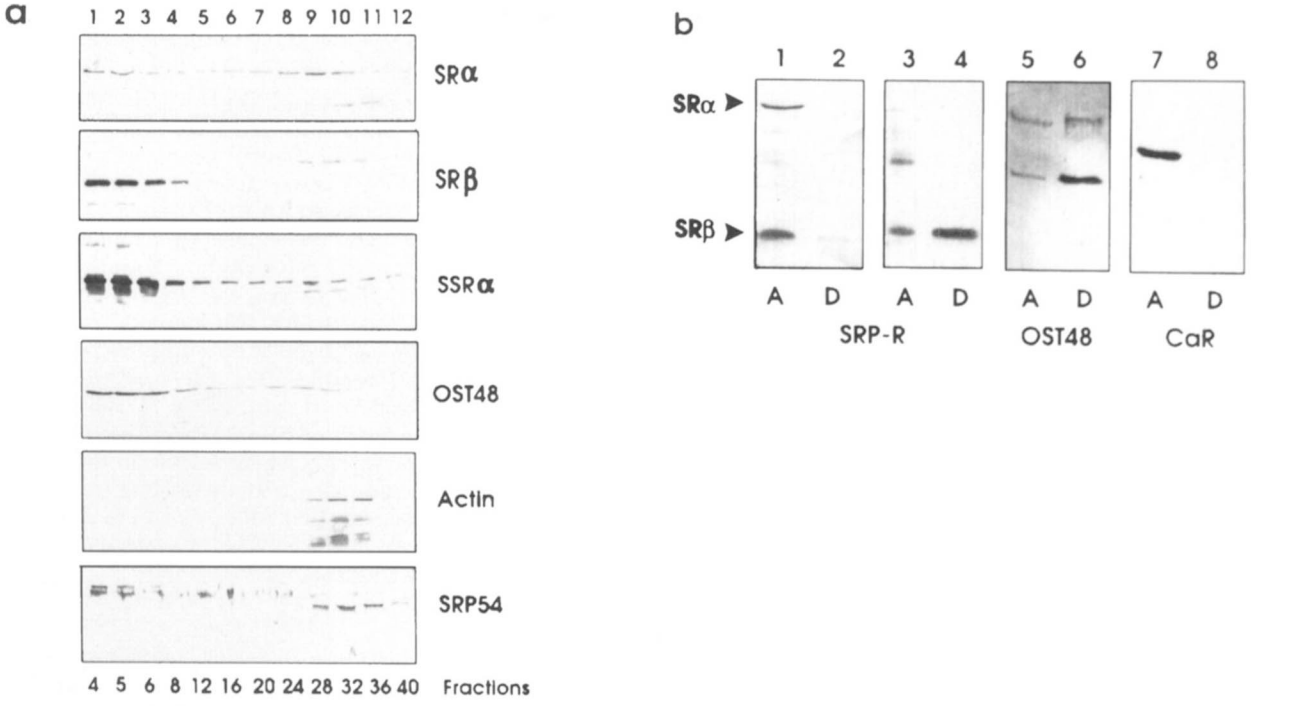

FIG. 5. **Membrane extraction of the SRP receptor.** *a*, microsomes were loaded onto a Sepharose CL-2B gel exclusion column equilibrated and eluted with 1 M NaSCN, 0.2 M Na_2CO_3, pH 11.5, and 10 mM dithiothreitol. Fractions were collected for SDS-PAGE and immunoblot analysis. Immunoblots of selected fractions were probed with monoclonal antibodies against SRα and polyclonal antibodies against SRβ, SSRα, OST48, actin, and SRP54. *b*, membranes either before (*lanes 1–2* and *5–8*) or after (*lanes 3* and *4*) digestion with 5 μg/ml trypsin were partitioned by cloud point extraction after solubilization with Triton X-114. The aqueous phases (*lanes 1, 3, 5,* and *7*, marked *A*) and detergent phases (*lanes 2, 4, 6,* and *8*, marked *D*) were resolved by SDS-PAGE followed by immunoblot identification of SRα and SRβ using specific antibodies and a two-color dye reaction (*lanes 1–4*) or antibodies against OST48 (*lanes 5–6*) and calreticulin (*lanes 7–8*).

centered around fraction 30 (Fig. 5*a*, *lanes 9–11*). It appears that while perturbation of the membrane at pH 11 was not sufficient to extract SRα (5), most SRα polypeptides can be clearly separated from integral membrane ER proteins under the conditions used here.

SRα and SRβ have been previously observed to partition into both phases following cloud point separation (11). However, SRα is known to be fully solubilized only in the presence of detergent and moderately high ionic strength (250 mM KOAc and above) (2), and the cloud point assay uses solubilization conditions at physiologic ionic strength (150 mM NaCl) (18). As expected, we discovered that a large portion of microsomal SRα and SRβ remained insoluble in the original cloud point solubilization buffer. However, both subunits became fully solubilized when the buffer was supplemented with 5% glycerol (data not shown). We therefore assayed microsomes solubilized in this manner by cloud point separation, to confirm and extend previous results. Immunoblots were probed for SRα and SRβ and as controls for the integral membrane protein OST48 and the lumenal protein calreticulin (39).

Surprisingly, both SRα and SRβ were detected only in the aqueous supernatant (Fig. 5*b*, compare *lanes 1* and *2*). The partitioning of SRα into the aqueous phase is consistent with its strongly hydrophilic primary sequence (8) and the apparently anomalous membrane interaction demonstrated in Fig. 5*a*. While SRβ appears to be integral membrane in high pH extractions supplemented with 1 M NaSCN (Fig. 5*a*), it is possible that the tight interaction between the receptor subunits (4) causes SRβ to partition in the aqueous phase with SRα. We therefore digested microsomes with 5 μg/ml trypsin for 1 h at 4 °C to proteolyze SRα while leaving SRβ unaffected (10) and then solubilized the membranes as above. After partitioning, tryptic fragments of SRα (Fig. 5*b*, *lane 3*), but no full-length protein, were detected in the aqueous phase, and SRβ was detected predominantly in the detergent phase (Fig. 5*b*, compare *lanes 3* and *4*). This agrees with recent results indicating the integral membrane nature of SRβ (11). As expected, in our solubilization conditions, OST48 was observed almost entirely in the detergent phase after cloud point separation (Fig. 5*b*, compare *lanes 5* and *6*), and calreticulin partitioned solely into the aqueous phase (Fig. 5*b*, compare *lanes 7* and *8*).

These results suggest that SRα is anchored largely by binding to the transmembrane SRβ polypeptide. To determine if this interaction is mediated by the membrane binding domain of SRα, we assayed the SRα deletion mutants used to map the SRα anchoring domain for the ability to bind SRβ in co-immunoprecipitations. A cDNA encoding canine SRβ was available but lacked a complete amino terminus (11). However, a complete cDNA of mouse SRβ was available (11), so a plasmid coding for a hybrid murine/canine SRβ (SRβ-MD) was constructed. The mouse and dog sequences are highly homologous (88% identity, 93% similarity), both having a single putative transmembrane domain and a carboxyl-terminal GTP-binding consensus sequence predicted to be on the cytoplasmic side of the ER (11). The SRβ-MD hybrid was constructed to contain the amino-terminal lumenal domain of mouse SRβ and the transmembrane and carboxyl-terminal cytoplasmic domains of canine SRβ. The junction between the sequences was selected because the binding site for SRα was expected to be in the transmembrane or cytoplasmic domain of SRβ.

RRL translation reactions of SRβ-MD were mixed with translation reactions of various SRα deletion mutants and immunoprecipitated using monoclonal antibodies against SRα (4). A fraction of SRβ-MD was observed to co-precipitate with SRαN (Fig. 6*a*, *lanes 1* and *4*) and SRD3 polypeptides (Fig. 6*a*, *lane 3*), but not with SR-EF (Fig. 6 *a*, lane 2). Furthermore, SRβ-MD did not co-precipitate with SRD4 or SRD1 (Fig. 6*a*, *lanes 5* and *6*) nor with SRD6 or SRD7 (data not shown). Control experiments (data not shown) suggest that the relatively poor co-precipitation of SRβ-MD with SRαN is likely due

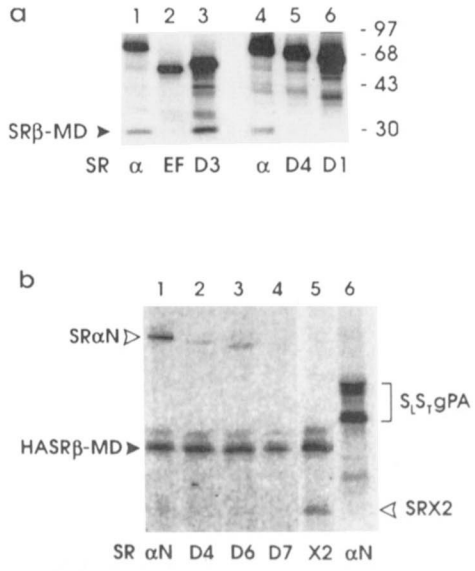

FIG. 6. **Co-precipitation of SRα mutants with SRβ.** *a*, cell-free translation reactions of SRαN (*lanes 1* and *4*), SR-EF (*lane 2*), SRD3 (*lane 3*), SRD4 (*lane 5*), and SRD1 (*lane 6*) were mixed with translation reactions of SRβ-MD and then immunoprecipitated with monoclonal antibodies against SRα. The SRβ-MD protein band is indicated with an *arrowhead*. *b*, translation reactions of SRαN (*lane 1*), SRD4 (*lane 2*), SRD6 (*lane 3*), SRD7 (*lane 4*), SRX2 (*lane 5*), and S_LS_TgPA (*lane 6*) were incubated with microsomes populated with HASRβ-MD. The mixtures were loaded onto Sepharose Cl-2B columns equilibrated and eluted with buffer containing 250 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. Fractions containing membranes were pooled, solubilized, and immunoprecipitated using antibodies against the hemeagglutinin epitope tag (*lanes 1–5*) or using IgG-Sepharose (*lane 6*). Because SRX2 contains three labeled methionine residues compared to 17 for SRαN, *lane 5* shows a 5-fold longer exposure. The SRαN and SRX2 protein bands are indicated by *open arrowheads*, and the HASRβ-MD band is indicated with a *solid arrowhead*. The glycosylated, unglycosylated, and signal-cleaved protein bands of S_LS_TgPA are indicated by the *bracket*.

to inefficient formation of dimers in the absence of membranes. As expected in control immunoprecipitations of translation reactions containing only SRαN, the deletion mutants or SRβ-MD, no protein bands corresponding to SRβ-MD were observed (data not shown). These results suggest that the polypeptide sequences involved in the membrane anchoring of SRα are sufficient for interaction with SRβ in the absence of membranes.

To examine the assembly of SRα-SRβ dimers on microsomes, selected deletion mutants of SRα were assayed for co-precipitation with a version of SRβ-MD tagged at the amino terminus with an influenza hemeagglutinin epitope (HASRβ-MD). As a control, SRαN was tested for co-precipitation with the integral membrane protein S_LS_TgPA (38). RRL translation reactions for SRαN, SRD4, SRD6, SRD7, and SRX2 were incubated with microsomes containing HASRβ-MD or S_LS_TgPA. To recover polypeptides associated with although not necessarily anchored to the membrane, the completed reactions were fractionated by Sepharose CL-2B chromatography at moderate ionic strength. After the microsomes were solubilized, immunoprecipitation using antibodies against the hemeagglutinin epitope recovered HASRβ-MD in each case (Fig. 6*b*, *lanes 1–5*). As predicted, SRαN was co-precipitated with HASRβ-MD (Fig. 6*b*, *lane 1*). Although significant amounts of SRD4, SRD6, and SRD7 fractionated with membranes after chromatography (data not shown), SRD4, SRD6, and SRD7 were co-precipitated very poorly with HASRβ-MD (Fig. 6*b*, *lanes 2–4*). Also, when membranes containing HASRβ-MD were added to translation reactions of SRD7 and the mixture was solubilized and immunoprecipitated with the same antibody, no SRD7 was co-precipitated even when the polypeptide was present in large excess (data not shown). In contrast, SRX2 was co-precipitated with HASRβ-MD at a level comparable with full-length SRαN (Fig. 6*b*, lane 5). As expected, no SRαN was detected in immunoprecipitations of the integral membrane protein S_LS_TgPA (Fig. 6*b*, *lane 6*), suggesting that the co-precipitation of SRαN and SRX2 with HASRβ-MD was not due to nonspecific aggregation of the hydrophobic sequences. PhosphorImager quantification revealed that after correction for the number of labeled methionine residues in the polypeptides, the ratio of SRαN to HASRβ-MD in the co-precipitation was 0.23:1, and the ratio of SRX2 to HASRβ-MD was 0.45:1. These ratios are reasonable given the expected low probability of contact between the SRα and HASRβ-MD polypeptides. The ratios of co-precipitated SRD4, SRD6, and SRD7 to HASRβ-MD were at least an order of magnitude lower than for SRαN. Therefore, these results indicate that the polypeptide sequences within the SRα membrane anchoring domain also mediate binding to SRβ. Taken together, our data suggest that SRα is bound to the ER membrane largely by interactions between the folded amino-terminal domain and the SRβ subunit.

DISCUSSION

We have shown here that a folded amino-terminal membrane binding domain of SRα containing hydrophobic and charged amino acids is required for tight binding to SRβ. The SRα membrane binding sequence contains approximately 140 residues and forms an independently folded protein domain (Fig. 4*b*). Membrane binding is observed when this region of SRα is generated by proteolysis of intact molecules either before or after targeting to microsomes (Fig. 4*a*) or by cell-free synthesis as an isolated polypeptide (Figs. 2*g* and 3). Furthermore, the SRα membrane binding domain binds directly to SRβ in the absence of other membrane proteins or lipids (Fig. 6*a*). Despite the presence of a membrane targeting signal within the carboxyl-terminal domain of SRα (10), deletion of either hydrophobic or charged sequences from the amino-terminal domain abolishes tight binding to the membrane (Fig. 2) and to SRβ (Fig. 6*b*). Our results therefore suggest that the membrane binding domain of SRα is not inserted into the membrane (Fig. 5), but the entire domain is involved in binding to SRβ. The remarkably strong interaction between the subunits is resistant to 1% nonionic detergent and high ionic strength (Fig. 6*b*), pH 11, and 2 M urea (2, 4, 5, 10) and most likely requires both hydrophobic and nonhydrophobic interactions. While the exact sequences within the SRα membrane binding domain that are in direct contact with SRβ remain to be determined, we expect they will include polar and nonpolar amino acids.

This model is not contradicted by the primary sequence of SRα, as the two hydrophobic regions within the anchoring domain are of comparatively low hydrophobicity and are both broken by lysine residues (8). Although the data cannot entirely discount the possibility of interactions between the membrane lipids and SRα, the relative extractibility of membrane bound SRα in Fig. 5*a* suggests that these interactions are not typical of a membrane protein with even a single transmembrane domain. Our results are more consistent with the hydrophobic regions of SRα contributing to intersubunit contacts.

Hydrophobic interactions alone are not sufficient for receptor dimer assembly, as the deletion mutant SRD7 that has the same hydrophobic sequences as full-length SRα was unable to bind SRβ (Fig. 6*b*). The importance of nonhydrophobic protein-protein interactions is further demonstrated by the cosegregation of both subunits as a complex in the aqueous phase after cloud point extraction (Fig. 5*b*). Moreover, SRα could be dissociated from membrane-bound SRβ by the combined disruption of polar and hydrophobic interactions with pH 11.5 and 1 M

NaSCN, without solubilizing the microsomal lipid bilayer (Fig. 5*a*).

A slight molar excess of SRβ over SRα on the ER membrane has been reported (1.1 mol of SRβ/mol of SRα) (4). In our model, novel SRα polypeptides targetted to the ER membrane would be anchored via these unpaired SRβ molecules. Anchoring of novel SRα is saturable at a concentration similar to that of excess SRβ on the membrane (10). The identity of the trypsin-sensitive membrane component required for anchoring of novel SRα (10) is still unresolved. However, despite the apparent resistance of SRβ to protease digestion (10, 11), our results suggest that SRβ is the required factor. The SRα membrane binding domain within SRX2 is necessary and sufficient for co-precipitation of SRα and SRβ (Fig. 6), and, similar to full-length SRα, the binding of SRX2 onto trypsin-treated microsomes is labile to urea (data not shown).

The domain of SRα required for membrane anchoring and tight binding to SRβ has been demonstrated to be unnecessary for functional assembly of the receptor on the ER membrane (10). Therefore, tight binding between the receptor subunits is not required for receptor activity. This suggests a dual role for SRβ, as a membrane anchor for SRα and as a part of the translocation machinery. A specific role for SRβ in translocation has not been directly demonstrated, but the GTPase activity of SRP54 requires binding to the SRP receptor (31), and SRβ has been shown to be labeled *in vitro* with GTP (11).

The two-domain structure of SRα is likely evolutionarily conserved. The sequence of a homologue of SRα has been obtained from yeast and contains a complete amino-terminal sequence (32). A yeast homologue of SRβ has now been identified (11), and we predict a similar pattern of interactions between these proteins. The *E. coli* homolog of SRα, FtsY, begins at residue 126 of the canine sequence (32, 33) and thus corresponds closely to the carboxyl-terminal domain of mammalian SRα. Interestingly, a bacterial homologue of SRβ has not been identified. Since the mammalian SRα anchoring domain that mediates binding to the β subunit is absent in FtsY, there may not be a homologue of SRβ in *E. coli*. However, FtsY has been reported to be resistant to high pH extraction despite the absence of hydrophobic domains (34), suggesting that it may also bind to an integral membrane protein.

As demonstrated in Fig. 3, nascent SRα polypeptides can assemble on microsomes while still attached to ribosomes. Since membrane binding appears to require a folded amino-terminal domain to interact with SRβ, targeting in this manner would still be essentially post-translational. However, this suggests that folding of SRα and receptor dimer assembly can occur cotranslationally, at least *in vitro*. While post-translational targeting of SRα molecules has been demonstrated *in vitro* (10), the subunit may assemble co-translationally *in vivo*. We have therefore begun to investigate the possibility that SRα assembles onto the membrane during a pause in translation.

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