ASSEMBLY OF SRP RECEPTOR

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ABSTRACT

Co-translational targeting of secretory and integral membrane proteins to the endoplasmic reticulum (ER) requires two key mediators, the signal recognition particle (SRP) and its receptor. The SRP receptor is composed of two tightly associated subunits termed SRα and SRβ. Very little is known about the mechanism of membrane assembly of these two subunits of the SRP receptor. Therefore, it is the aim of this thesis to study the interactions between SRα and SRβ on the ER membrane as well as the role of SRα and SRβ in membrane assembly of functional SRP receptor.

Unlike typical endoplasmic reticulum (ER) integral membrane proteins, both subunits of the SRP receptor were extracted from the ER membrane with 0.08% deoxycholate; 0.2M Tris pH 9.0. Nevertheless, SRβ could be targeted to the ER only when the SRP dependent pathway of translocation was functional, similar to other integral membrane proteins of the ER. Urea resistant anchoring of SRα on the ER membrane was sensitive to limited digestion of the membranes with trypsin (Andrews *et al.*, 1989). However, anchoring of SRα was restored by incorporating exogenous SRβ into trypsin treated membranes, confirming that one function of SRβ is anchoring of SRα. Consistent with this is the observation that, SRβ could be immunoprecipitated in a complex with SRα but not with SRα mutants containing deletions in the anchoring domain. Finally, an antiserum to the GTP binding domain of SRβ inhibited translocation of the secretory protein preprolactin suggesting that SRβ also has a direct role in translocation.

DEDICATIONS

This thesis is dedicated to my parents Gino and Maria Ursini, for it was made possible by the endless love and support they have always given me.

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CMC	critical micellar concentration
CRM	column washed rough dog pancreatic microsomes
DTT	dithiothreitol
EMC	encephalomyocarditis virus
ER	endoplasmic reticulum
eq	equivalent to 1 μl of CRM at 50 A_{280} U/ml
GMP PNP	guanylyl-imido diphospate guanosine monophosphate
GST	glutathione-S-transferase
HA	hemagluttinin
IPTG	iso-propyl-thiogalactoside
kDa	kilodaltons
KRM	CRM extracted with 0.5M KOAc
NEM	N-ethylmaleimide
OST48	48 KDa subunit of oligosaccharyltransferase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SRP	signal recognition particle
SRα	alpha subunit of SRP receptor
SRβ	beta subunit of SRP receptor
SSRα	phosphoglycprotein 35
SSRβ	glycoprotein 25H
T_5KRM	KRM digested with 5 μg/ml of trypsin

TAC	trp/lac hybrid promoter
TCA	trichloroacetic acid
TRIS	2-(hydroxymethyl)-2-nitro-1,3-propendiol

CHAPTER ONE

INTRODUCTION

Most protein synthesis begins in the cytoplasm of the cell irrespective of the molecule's final destination. Protein targeting is the process by which the cell directs a polypeptide to its correct location, based on information encoded in the primary sequence of the molecule. Sorting of secretory proteins, as well as integral membrane proteins of the endoplasmic reticulum, Golgi, lysosome and plasma membrane begins with co-translational targeting to the endoplasmic reticulum membrane. This initial targeting event utilizes at least two components, signal recognition particle (SRP) and SRP receptor, along with the signal sequence of the secretory protein (Walter and Blobel, 1981; Gilmore *et al.*, 1982). SRP is a cytoplasmic complex which recognizes and binds amino terminal signal sequences of secretory proteins as they emerge from the ribosome (Walter *et al.*, 1981). This interaction results in a transient arrest in elongation, allowing the nascent chain to remain in a translocation competent state (Walter and Blobel, 1981b; Wolin and Walter, 1989). SRP then targets the nascent chain/ribosome complex to another complex on the endoplasmic reticulum membrane termed SRP receptor (Walter and Blobel, 1981b; Gilmore *et al.*, 1982). The interaction of SRP with SRP receptor results in the hydrolysis of a bound GTP molecule coincident with the release of SRP into the cytoplasm, the release of elongation arrest and the transfer of the nascent chain to the translocation machinery of the ER (Connolly and Gilmore, 1986; Connolly and Gilmore, 1989; Connolly *et al.*, 1991; Connolly and Gilmore, 1993).

SRP STRUCTURE AND FUNCTION

Co-translational translocation of secretory proteins across microsomal membranes in a cell free wheat germ system is abolished by prior extraction of the membranes with high ionic strength buffers (Walter and Blobel, 1980). This function can be reconstituted by addition of the salt extract to the cell free translation reaction, suggesting that a component was removed from the microsomes which is necessary for translocation to proceed (Walter and Blobel, 1980). Using both hydrophobic and ion exchange chromatography, a complex was purified from dog pancreatic microsomes which was sufficient to restore translocation function to the depleted membranes (Walter and Blobel, 1980). This complex bound with increased affinity to ribosomes translating secretory proteins, resulting in arrest in elongation of the nascent chain (Walter and Blobel, 1981; Walter and Blobel, 1981b). For these reasons, the complex was termed signal recognition protein (SRP) (Walter and Blobel, 1981). SRP is composed of six different polypeptides of various molecular weights ranging from 72 to 9 kilodaltons. Four of these were purified as heterodimers (68/72kDa, 9/14kDa) and two exist as monomers (19kDa, 54kDa) (Walter and Blobel, 1980; Scoulica *et al.*, 1987). SRP also contains a 7S RNA species with high homology to Alu consensus sequences. Therefore, SRP was renamed signal recognition particle since the 7S RNA is required for both the structural and functional properties of the complex (Walter and Blobel, 1982). The 7S RNA of has been shown to span the entire length of the molecule (Andrews *et al.*, 1987) and is required for the ribonucleoprotein to promote both translational arrest and translocation of the nascent polypeptide (Walter and Blobel, 1982).

SRP complexes which were reassembled without various subunits or modified by alkylation with N-ethylmaleimide (NEM), provided a great deal of

evidence concerning the functions of each the subunits in the translocation process (Siegel and Walter, 1985; Siegel and Walter, 1988). Partial SRP complexes reassembled without the 9/14kDa heterodimer or modification of the 9/14kDa heterodimer with NEM resulted in the loss of translational arrest, although the partial SRP complexes were still active in promoting translocation.

In contrast, SRP containing an alkylated 68/72kDa heterodimer was able to arrest translation but could not promote translocation. This resulted from the inability of the modified SRP to target the nascent chain to the ER membrane (Siegel and Walter, 1988). SRP containing modified 68/72kDa heterodimer bound to an SRP receptor affinity column with reduced affinity compared to SRP complexes with non-alkyated 68/72kDa heterodimers (Siegel and Walter, 1988).

Finally, alkylation of the 54kDa subunit from SRP or reassembly of partial SRP complexes missing the 54kDa species, resulted in a particle without elongation arrest or translocation functions (Siegel and Walter, 1985; Siegel and Walter, 1988). This particle did not bind ribosomes synthesizing secretory proteins, suggesting that it no longer interacted with the signal sequence of the nascent chain (Siegel and Walter, 1988). Several groups used crosslinking techniques to verify that SRP54 is the component of SRP which binds to signal sequences. Nascent chains were synthesized in a cell free system in the presence of a chemical crosslinker (εANB-Lys-tRNA) which is a functional analogue of the amino-acyl Lysyl tRNA. A photoreactive group was covalently attached to the amino acid so that the probe could be metabolically incorporated into the nascent chain (Krieg *et al.*, 1986). Upon photolysis, the nascent chain formed crosslinks with a species of 54kDa. Immunoprecipitation of the crosslinked species with SRP54 antisera confirmed that the signal

sequence interacts with the 54kDa subunit of SRP (Krieg *et al.*, 1986, Kurzchalia *et al.*, 1986).

SRP54 is composed of two domains, an N-terminal domain containing a GTP binding site (Zopf *et al.*, 1990; Miller *et al.*, 1993) and a C-terminal domain rich in methionine residues (Zopf *et al.*, 1990). Limited proteolysis of SRP can selectively remove the G-domain but leave the M-domain attached to the core of the particle (Zopf *et al.*, 1990). The nascent chain was also crosslinked to SRP particles containing only the M-domain of the 54kDa subunit. The crosslinked species were immunoprecipitatable with anitsera to the C-terminus of SRP54 (Zopf *et al.*, 1990) suggesting that the M domain of SRP54 binds the signal sequence of the nascent chain.

Although SRP particles lacking the G-domain of SRP54 can interact with signal sequences and induce elongation arrest, they cannot promote translocation across the ER membrane. SRP particles lacking the G domain are unable to target the nascent chain to the ER membrane. Indeed, complexes could not be formed between partial SRP particles lacking the G-domain of SRP54 and purified SRP receptor (Zopf *et al.*, 1993). This is consistent with the result that the hydrolysis of GTP by SRP54 is necessary for the release of the bound signal sequence from SRP (Miller *et al.*, 1993).

Although SRP and SRP receptor can bind GTP, neither complex can promote hydrolysis of the bound GTP molecule (Miller *et al.*, 1993; Miller *et al.*, manuscript in preparation; Connolly and Gilmore., 1993). However, SRP/SRP receptor complexes can hydrolyze the bound GTP molecule (Connolly and Gilmore, 1993; Miller *et al.*, 1993). GTP hydrolysis is required to release SRP from the ribosome since a non-hydrolyzable analogue (GMP PNP) maintains stable SRP/SRP receptor complexes which are resistant to extraction to high ionic strength conditions (Connolly *et al.*, 1991). Consistent with the fact that

SRP54 is the only SRP protein with a GTP binding site, SRP54/7S RNA complexes are both necessary and sufficient to hydrolyze the bound GTP molecule in the presence of SRP receptor (Miller *et al.*, 1993). Upon complex formation, SRP receptor acts as a GTPase activating protein to stimulate GTP hydrolysis by SRP54 (Miller *et al.*, 1993). In addition, SRP receptor is a guanine nucleotide loading protein which increases the affinity of GTP binding to the empty site on SRP54 (Miller *et al.*, 1993).

SRP RECEPTOR STRUCTURE AND FUNCTION

SRP receptor was originally identified as the ER membrane protein which relieved the SRP induced translational arrest of secretory proteins. Therefore, SRP receptor couples translation with translocation of the nascent chain across the ER (Meyer *et al.*, 1982; Gilmore *et al.*, 1982). Solubilization of microsomes with 1% Nikkol and 250mM KOAc released a 72kDa species which co-migrated on sucrose gradients with the activity to release elongation arrest. A 30kDa polypeptide was also present but initially was not considered to be a subunit of SRP receptor due to slight variations in migration in the sucrose gradients (Gilmore *et al.*, 1982b).

In an attempt to purify SRP receptor from solubilized microsomes, affinity chromatography was performed after solubilization using an SRP Sepharose column (Tajima *et al.*, 1986). Elution of bound protein from the column resulted in two polypeptides of apparent molecular weight 72 and 30kDa in SDS-PAGE. These polypeptides were referred to as SRα and SRβ respectively (Tajima *et al.*, 1986). Endogenous SRβ is present in equimolar or in slight excess over SRα on microsomal membranes. In addition, the complex can be immunoprecipitated with antisera to either SRα or SRβ, suggesting that the two

proteins form a very tight complex stable after detergent solubilization in 250mM KOAc (Tajima *et al.*, 1986).

Digestion of endogenous SRP receptor on microsomal membranes using limited proteolysis with either trypsin or elastase has revealed that SRα is cleaved to produce two separate domains while SRβ remains apparently intact on one dimensional SDS-PAGE (Gilmore *et al.*, 1982; Hortsch *et al.*, 1985; Andrews *et al.*, 1989). At low concentrations, both proteases cleave SRα at distinct sites resulting in a cytoplasmic soluble fragment as well as a membrane anchored fragment (Hortsch *et al.*, 1985). Microsomes containing only the membrane anchored portion, produced with either trypsin or elastase digestion, are abolished for both translocation and arrest-releasing activities (Hortsch *et al.*, 1985). However, reconstitution of these trypsin or elastase treated membranes with the 59kDa soluble elastase fragment of SRα is both necessary and sufficient to restore both of these processes. The 46kDa trypsin soluble fragment of SRα does not restore either of these functions since it does not bind to proteolyzed membranes (Hortsch *et al.*, 1985).

SRα is a 638 residue polypeptide with no cleavable signal sequence (Lauffer *et al.*, 1985). Furthermore, protein sequencing of the 60kDa soluble elastase fragment reveals that its amino terminus begins at residue 151 of the full length molecule. This suggests that the amino terminal 150 residues comprise the amino terminal anchoring fragment of SRα (Lauffer *et al.*, 1985). The amino terminal anchoring domain of the molecule contains two stretches of hydrophobic residues followed by three stretches of positive charges, contributing to the overall basic character of SRα (Lauffer *et al.*, 1985). The carboxyl terminal region of the molecule contains a GTP binding consensus sequence which has been shown to bind GTP (Lauffer *et al.*, 1985; Rapiejko and Gilmore, 1992; Miller *et al.*, 1993).

The primary structure of the complete murine and partial canine SRβ cDNAs reveals a molecule with a single putative transmembrane domain near the amino terminus and a GTP binding site near the carboxyl terminus of the molecule (Miller *et al.*, manuscript in preparation). The 19 residue hydrophobic domain near the amino terminus of SRβ is flanked on the carboxyl end by a cluster of positive charges, consistent with a type I membrane topology (amino terminal lumenal; carboxyl terminal cytoplasmic) according to the positive inside rule (Von Heijne, 1989).

ASSEMBLY OF SRP RECEPTOR ON THE ER MEMBRANE

SRα has been shown to post-translationally assemble onto microsomes that have been inactivated for translocation by digestion with trypsin or alkylation by NEM. This suggests that SRα is assembled onto membranes by a novel mechanism involving neither SRP nor its receptor (Andrews *et al.*, 1989). In contrast to cytochrome b5 (Anderson *et al.*, 1983), SRα does not use an insertion sequence motif to associate with the membrane since urea resistant anchoring of SRα is abolished by treatment of the membranes with low concentrations of trypsin (Andrews *et al.*, 1989). In addition, assembly of SRα is specific for the ER membrane since the molecule did not associate with artificial phospholipid vesicles or mitochondria (Andrews *et al.*, 1989). Therefore, a novel mechanism exists for assembly of SRα onto microsomal membranes requiring additional, as of yet, undefined ER membrane proteins.

Assembly of SRα onto microsomal vesicles has been shown to occur by a two-step mechanism. The initial step involves targeting of the molecule to microsomes. Membrane targeting is followed by the stable anchoring of SRα onto the membrane as judged by the ability of the molecule to remain associated with the membrane in the presence of aqueous perturbants such as

2M urea (Andrews *et al.*, 1989). Targeting and anchoring are separate and distinct processes since SRα can target onto microsomes treated with either trypsin or alkylating agents such as NEM, however it cannot stably anchor onto membranes which have been previously digested with trypsin (Andrews *et al.*, 1989). The identity of the component on the ER membrane which is responsible for anchoring SRα is unknown, but a possible candidate is the β subunit of SRP receptor.

AIM OF STUDY

I examined the interaction between the α and β subunits of the SRP receptor and the ER membrane. In particular, the mechanisms that SRα and SRβ use to assemble onto microsomes were addressed. Because concentrations of at least one percent Nikkol are required to solubilize SRP receptor from the membrane (Gilmore *et al.*, 1982b) and based on the amino acid sequences deduced from the cDNA clones of the molecules (Lauffer *et al.*, 1985; Miller *et al.*, manuscript in preparation), SRP receptor is thought to be an integral membrane protein. However, recent evidence suggests that SRα is a peripheral membrane protein (Young *et al.*, submitted). Evidence is presented here that SRα is anchored to the ER membrane via an interaction with the transmembrane β subunit.

In contrast to SRα, very little is known about the membrane assembly of SRβ. Using adaptations of two standard methods to assay membrane integration, the assembly of the SRα/SRβ complex on the ER membrane was found to be atypical of integral membrane proteins. We have shown that assembly of SRβ, as a putative transmembrane protein, requires a functional SRP dependent pathway. It has been proposed that SRβ may function in the

initial targeting or stable anchoring of SRα to the ER membrane (Andrews *et al.*, 1989). It was possible to assess whether SRβ is the molecule responsible for anchoring SRα by incorporating exogenous SRβ into trypsin digested membranes, which on their own cannot anchor SRα. Urea resistant anchoring of SRα to these membranes was then assayed. Evidence is provided in this thesis which suggests that SRβ is the anchor for SRα. Consistent with this result, SRβ can form stable complexes with SRα but not with SRα mutants with deletions in the membrane assembly domain. Finally, evidence is provided which suggests that SRβ also contributes to the translocation function of SRP receptor.

CHAPTER TWO

MATERIALS AND METHODS

2.0-MATERIALS

Restriction endonucleases were purchased from New England Biolabs and were used according to maunufacturer's instructions. *E. Coli* SURE cells were purchased from Stratagene. SP6 RNA Polymerase was obtained from Cedarlane Biolabs. RNAguard was purchased from Pharmacia, creatine kinase was obtained from Boehringer Mannheim and ^{35}S Met was from NEN Dupont. Ultra pure urea, Tris and sucrose were purchased from ICN Biomedicals. Deoxycholate and sodium carbonate were purchased from Sigma and BDH respectively.

Rabbit reticulocyte lysate was prepared as previously described (Jackson and Hunt, 1983). Cell free transcription linked translation reactions were performed as published (Gurevich *et al.*, 1991; Andrews, 1992). Post translational assembly of SRα onto microsomes was assayed as described (Andrews *et al.*, 1989) Canine pancreatic microsomes were prepared according to published procedures and were either column washed (CRM) or extracted with 500 mM KOAc (KRM) (Walter and Blobel, 1983). Trypsin treated membranes (T_5KRM) were prepared by incubating KRMs with 5μg/ml of trypsin for one hour at 0°C, as described (Andrews *et al.*, 1989). The soluble trypsin fragment of SRα was removed from the T_5KRM by washing with buffer containing 500mM KOAc.

Antibodies to calnexin, the 48KDa subunit of oligosaccharyl transferase (OST48), SSRα and SSRβ were the generous gifts of J.J.M Bergeron, R.

Gilmore and T. Rapoport respectively. Antisera to SRP54 was produced using a GST fusion protein by Fabiola Janiak using conventional methods.

2.1.0-GENERAL METHODS

A polyclonal antiserum to SRβ was generated using a construct in which the 40 amino acids from the carboxyl end of SRβ were fused to to glutathione-S-transferase (GST) (section 2.1.2). The open reading frame was placed under the control of a TAC promoter and synthesis of the fusion protein was induced in the presence of IPTG. Total bacterial lysate was prepared according to published procedures and the fusion protein was purified using a 1ml glutathione Sepharose column (Pharmacia). Approximately 1mg of fusion protein was emulsified in an equal volume of Freund's complete adjuvant (Sigma) and was injected subcutaneously into a New Zealand white rabbit. The animal was given further monthly injections of fusion protein (0.5mg) in Freund's incomplete adjuvant (Sigma). Small scale bleeds were performed every 2-3 weeks using the marginal ear vein. The final bleed was performed by cannulation. Blood was clotted using several units of thrombin and incubated at 37°C until the clot formed. The clot was then separated from the serum by centrifugation in a refrigerated Sorvall RT6000B for 15 minutes at 10 000 xg. 0.01% sodium azide was added as a preservative and serum was stored at -80°C. Bleeds and processing of the serum was performed by Brian Leber.

Proteins were separated by SDS PAGE electrophoresis using either 16% Laemmli (Laemmli, 1970) or 10% Tricine gels (Shagger and Von Jagow, 1987). *In vitro* synthesized proteins labelled with ^{35}S methionine were visualized by fluorography followed by autoradiography.

2.1.1-RECOMBINANT DNA TECHNIQUES

Recombinant DNA techniques were carried out as described by (Sambrook *et al.*, 1989) where small scale isolation of DNA was performed using the alkaline lysis method. DNA was purified from agarose using the "gene clean" procedure (Biorad) following manufacturers instructions.

Large scale plasmid isolation was performed as described in Ausubel and Frederick, 1988. Briefly, cells were saturated overnight in 100 mls superbroth (3.2% bactotryptone; 2% yeast extract; 85mM NaCl; 5mM NaOH) plus 100 µg/ml ampicillin and lysed using the large scale alkaline lysis method. DNA was precipitated with 0.6 volumes of isopropanol and pelleted by centrifugation in the Hermle Z380 at 7000 xg for 10 minutes. The pellet was resusupended in 3 ml TE (10mM Tris pH 8; 1.0mM EDTA) and an equal volume of ice cold 5M LiCl was added to precipitate the RNA. The RNA was pelleted by centrifugation in the Hermle Z380 for 10 minutes at 4000 xg. The DNA was once again precipitated with 0.6 volumes of isopropanol, the pellet was resuspended in 300 µl TE + 20 µg/ml RNase A and incubated at room temperature for 30 minutes. The DNA was precipitated in an equal volume of 26% PEG in 1.6M NaCl and again pelleted by centrifugation in a microcentrifuge for 15 minutes at 4°C. The pellet was dissolved in 400 µl TE, extracted once with an equal volume of chloroform, twice with an equal volume of TE saturated phenol and once again in an equal volume of chloroform. The DNA was ethanol precipitated by adding 0.1 volumes of 5M ammonium acetate and 2.5 volumes of 100% ethanol. The plasmid DNA was pelleted by centrifugation, washed with 70% ethanol and quantitated by measuring the OD_{260}. All DNA solutions were adjusted to a final concentration of 1 mg/ml with TE.

2.1.2-PLASMIDS

Unless otherwise stated, constructs were cloned into the vector pSPUTK behind the SP6 RNA polymerase promoter (Falcone and Andrews., 1991). Plasmids encoding SRα, SREF, SRD3, SRD4, SR18 and bovine preprolactin have been published (Young *et al.*, in preparation; Falcone and Andrews, 1991). Plasmids pMP205 (SRX2) and pMP55 (SRD4) were made by David Andrews. Plasmids pMP3 (SR18) and pMP42 (SREF) were constructed by Leander Lauffer. Plasmids pMP456 (SRD3) and pMP359 (pSPGEXSRβ1) were made by Kathy Vassilakos. Plasmid pMP433 (pSPSRβM1) was constructed by Jason Young. Plasmid pMP670 (HA-SRβ$_{MD}$) was constructed by Kyle Leggat. Plasmid pMP10 (cyt b5) was obtained from Dr. David Meyer. pSPUTK was generated by Mina Falcone.

pSPGEXSRβ1: A partial cDNA encoding nt 75-799 of canine SRβ was cloned into the Pst I site in pSPUTK (plasmid pMP295). The partial cDNA sequence was then inserted behind the GST coding sequence in pSPGEX (a derivative of the plasmid pGEX-2T, Pharmacia). This was accomplished using the *Nco* I and *Sal* I sites in pMP295 and in the multiple cloning region of pSPGEX. In order to adjust the reading frame of SRβ to that of the GST sequence, the plasmid was digested with *Nco* I and *Bst* XI. The overhanging ends of the plasmid were filled in using the Klenow fragment of DNA polymerase and the plasmid was religated. The resulting plasmid (pSPGEXSRβ1) encodes the GST domain fused to amino acids 208-265 of canine SRβ.

SRβ$_{MD}$: To examine the interaction of SRβ with canine SRα, ideally canine SRβ should be used. However, the clone for canine SRβ is incomplete at the amino terminal end. Therefore, a hybrid clone was constructed containing the

amino terminal putative lumenal domain of murine SRβ an the rest of canine SRβ. The canine SRβ cDNA coding sequences, initially obtained as an insert in pBluescript II (Miller *et al.*, manuscript in preparation), were subcloned into pSPUTK using *Xba* I and *Eco* RI sites. The murine SRβ cDNA, obtained as a PCR fragment, was also cloned into the multiple cloning site of pSPUTK using the *Nco* I and *Sal* I sites included in the PCR primers. The hybrid clone was then constructed using the *Pst* I site in both the murine (nt 95) and canine (nt 74) cDNAs and the *Sma* I site in the polylinkers. The resulting plasmid encodes a hybrid molecule ($SR\beta_{MD}$) composed of the first 29 residues of murine SRβ followed by the putative transmembrane and cytoplasmic domains of canine SRβ (amino acids 25-265).

HA-$SR\beta_{MD}$: DNA encoding an 11 amino acid hemagluttinin tag was placed amino terminal ot the $SR\beta_{MD}$ cDNA to provide an epitope at the amino terminus of $SR\beta_{MD}$ for use in co-immunoprecipitation reactions with full length SRα or various SRα deletion mutants. The coding sequence for $SR\beta_{MD}$ was excised from the vector pSPUTK as an *Nco* I/*Sph* I fragment. The plasmid pG7SCTHA2 (containing the HA tag) was digested with the same restriction enzymes. The resulting plasmid contains the $SR\beta_{MD}$ sequence immediately following the hemagluttinin epitope. The plasmid pG7SCTHA2 and the HA-antisera were gifts from John Glover.

2.1.3-CARBONATE AND DEOXYCHOLATE EXTRACTION OF ER MEMBRANES

Two mls of column washed microsomes (50 A_{280} U/ml) were applied to a 100 ml Sepharose CL2B gel exclusion column equilibrated in either 0.2M Na_2CO_3; 1M NaSCN; 10mM DTT (4°C) or 0.08% deoxycholate; 0.2M Tris

pH9.O (22°C). Fractions were collected beginning just prior to the pre-determined excluded volume. Fifty 1.5ml fractions were collected from the column where fractions 2-8 contain the peak of the excluded volume and fractions 20-40 contain the included volume of the column. Representative fractions were concentrated by TCA precipitation and the location of marker proteins was analyzed by immunoblotting after separation by SDS-PAGE and electrophoretic transfer of the polypeptides onto nitrocellulose membranes. The blots were probed with antisera to the indicated proteins and were developed colorimetrically using an alkaline phosphatase conjugated secondary antibody. The carbonate and deoxycholate extractions of CRMs was performed by David Andrews.

The analysis of trypsin treated membranes was performed similarily except that 20eq of membranes (1eq/ul) were incubated in the appropriate extraction buffer for 10 minutes prior to passage chromatography in a 0.8ml CL2B column. Six fractions were collected where fraction 2 contains the void volume and fractions 4-6 contain the included volume of the column.

2.1.4-MEMBRANE TARGETING AND ANCHORING OF SRβ_{MD} AND SRα TRANSLATION PRODUCTS

Reticulocyte lysate translation reactions for SRβ_{MD} (20µl) contained one equivalent of mock treated KRM or T_5KRM. As SRα has been shown to target to microsomes in a post-translational manner, the ribosomes were removed from the translation reaction (20µl) by centrifugation (Andrews *et al.*, 1989) prior to the addition of 5 equivalents mock treated KRM or T_5KRM. The microsomes were incubated with post-ribosomal supernatants of SRα for 15 minutes at 24°C. The reaction containing 10mM Tris Ac pH7.5/50mM KCl/2.5mM $MgCl_2$/10mM DTT was adjusted to 2M urea/25mM EDTA, incubated on ice for

10 minutes and loaded onto a 100µl 0.5M sucrose cushion. Microsomes and microsome associated proteins were pelleted by centrifugation at 4°C in a Beckman airfuge at 160 000 xg for 15 minutes. The top 75µl, containing proteins stripped from the membranes as well as cytosolic proteins, were collected. The bottom half of the cushion was discarded and the microsomal pellet was resuspended in 75µl 1% SDS/0.1M Tris pH9 and incubated at 65°C for 10 minutes to ensure complete solubilization. Equal volumes of the supernatant and pellet fractions were analyzed by SDS-PAGE followed by fluorography.

2.1.5-RECONSTITUTION OF TRANSLOCATION ACTIVITY OF T_5KRM

Post ribosomal supernatants of SRα or SRD3 (10µl) were incubated with 1µl T_5KRM for 15 minutes at 24°C. New translation reactions encoding preprolactin (10µl) or $SR\beta_{MD}$ (20µl) were added to the post-ribosomal supernatants and incubated for an hour at 24°C. Total translation products were used to assay reconstitution of preprolactin translocation by SDS-PAGE on 16% Laemmli gels.

To analyse the membranes for reconstitution of $SR\beta_{MD}$ assembly, the translation products were applied to a 0.8ml Sepharose CL2B column equilibrated in Buffer R (10mM Tris Ac pH 7.5; 100mM KOAc; 2.5mM $MgCl_2$) and 1M NaCl. Fractions 4-13 were collected and analyzed on 10% Tricine gels. Fraction 5 (the void volume) contains microsome and microsome associated proteins.

2.1.6-RECONSTITUTION OF ANCHORING OF SRα ONTO TRYPSIN TREATED MICROSOMES

A post ribosomal supernatant of SRα was incubated with one equivalent of T_5KRM for 15 minutes at 24°C. Either a newly assembled translation reaction or a post-ribosomal supernatant for $SR\beta_{MD}$ was added to the reaction and incubation continued for an hour at 24°C. The translation products were adjusted to 2M urea and 25mM EDTA, incubated on ice for 10 minutes, loaded onto a 0.5M sucrose cushion and the membranes were pelleted by centrifugation as above. The samples were fractionated into two 70µl aliquots, referred to as the top and middle fractions, the pellet was was resusupended in 70µl 1%SDS/0.1M Tris pH9 and the samples were analyzed by SDS-PAGE as above (section 2.1.4).

The amount of SRα recovered in the pellet fraction was determined by densitometry. The fraction of SRα molecules anchored on T_5KRM was obtained by subtracting the amount of SRα in the pellet when $SR\beta_{MD}$ was added post-translationally (non-specific binding) from the SRα in the pellet of a parallel reaction in which $SR\beta_{MD}$ was added co-translationally.

2.1.7-IMMUNOBLOTTING

Protein samples were separated electrophoretically by SDS-PAGE and transferred onto nitrocellulose using a Hoefer semi-phor transblotter (model # TE77) for one hour at 100 mA. The gel, nitrocellulose and Whatman 3MM chromatography paper were all soaked in transfer buffer (30mM Tris base; 240mM glycerol; 20% methanol) prior to assembly of the transfer apparatus. The nitrocellulose was blocked for one hour in 140mM NaCl; 10mM KPO_4; 0.02% NaN_3; 0.5% skim milk at room temperature. The primary antibody was added (1:1000 dilution) to the nitrocellulose and incubated at room temperature for 4 hours or overnight at 4°C. Blots were incubated in 0.1% Triton X100; 560mM NaCl; 0.02 %SDS; 10 mMKPO_4; 0.01% NaN_3; 1% BSA with polyclonal

antiserum. Blots probed with monoclonal antibodies were incubated in a similar buffer without SDS. The blots were washed several times in 140mM NaCl; 10mM KPO_4; 0.02% NaN_3; 0.1% Triton X-100. The blots were then incubated for several hours at room temperature in the monoclonal buffer containing the secondary antibody coupled to either (AP) alkaline phosphatase or (HRP) horseradish peroxidase at a 1:2000 dilution (Jackson Labs). Blots probed with AP linked secondary antibodies were developed colorimetrically in 10 mls of AP buffer (100mM Tris pH 9.5; 100mM NaCl; 5mM $MgCl_2$) using the substrates NBT and BCIP according to the manufacturers instructions (Gibco BRL). Blots probed with HRP linked secondary antibodies were developed colorimetrically in 10 mls of HRP buffer (140mM NaCl; 10mM KPO_4) with 0.1% hydrogen peroxide and 10 mg of diaminobenzidine per blot dissolved in 200 µl of dimethylformamide.

2.1.8-IMMUNOPRECIPITATIONS

Cell free synthesized translation products or solubilized membrane proteins were incubated in at least 10 fold excess of immunoprecipitation buffer (100mM Tris pH8; 100mM NaCl; 10mM EDTA; 1% Triton). To denature proteins prior to immunoprecipitation, the translation reaction was boiled in 10 volumes of 1% SDS/0.1M Tris pH 9.0. The SDS was then diluted with ten fold excess of immunoprecipitation buffer. Monoclonal antibodies to SRα (S1) coupled to Sepharose, polyclonal antisera to SRβ or SRα or monoclonal antibodies to HA were added and incubated as described below. Competition assays were also performed with 10 µl of the polyclonal antisera to SRβ and 25, 50 or 100 µg of GST-SRβ fusion protein (section 2.1.2).

Samples were incubated with the primary antisera for four hours at 4°C on an end over end rotator. Ten µl of diluted Protein A beads (3 µl Protein A

and 7 µl CL4B Sepharose) was added to the immunoprecipitations with the various antisera and incubated for an additional two hours at 4°C on the rotator. Immunoprecipitates were then washed three times with 1 ml of immunoprecipitation buffer and once with 1.25 ml of 0.1M Tris pH8/0.1M NaCl. The beads were heated up to 80°C in SDS-PAGE sample buffer to elute the bound protein.

2.1.9 - CO-IMMUNOPRECIPITATIONS OF SRα AND SRβ

SRα, SRD3, SR30, SRX2, SRD4, SR18 and SRβ were transcribed under the control of an SP6 promoter and translated in a rabbit reticulocyte lysate in the absence of membranes. An equal amount of SRα (or SRα deletion mutants) and SRβ translation products were incubated for 30 minutes at 24°C to allow complex formation. A 10 fold excess of immunoprecipitation buffer was added and the samples were immunoprecipitated with antisera to SRα, SRβ or HA as described above (section 2.1.8). Densitometry was used to determine the ratio of SRα and SRβ co-immunoprecipitated. The values obtained for each molecule were normalized for the number of methionine residues

2.1.10-INHIBITION OF PREPROLACTIN TRANSLOCATION WITH POLYCLONAL ANTISERA TO SRβ

Ten microlitres of column washed and salt extracted microsomes (4 eq/µl) were incubated with SRβ or SRα antisera at various dilutions ranging from 0-5µl antibody/eq of membranes and allowed to interact for one hour on ice. To remove unbound antisera, the membranes were layered onto a sucrose cushion containing 50mM TEA; 0.5M sucrose and pelleted by centrifugation at 100 000xg for 10 minutes in a Beckman airfuge. The pellets were then resuspended in 20 µl of Buffer C (50mM TEA; 0.25M sucrose) and 5µl were

used in an immunoblot. Membrane bound antibodies were visualized using a colorimetric reaction after probing the nitrocellulose membrane with goat anti-rabbit antisera linked to alkaline phosphatase (section 2.1.7). The resuspended membranes were assayed for translocation activity by adding 1μl to a 10μl translation reaction for preprolactin. Conversion of preprolactin to the mature form by signal peptide cleavage was used as a measure of translocation activity. The relative amounts of preprolactin and prolactin were determined by densitometry of the fluorograms and normalizing for the number of methionines in preprolactin and prolactin. These membranes were also tested for anchoring of endogenous SRα molecules or stable assembly of in vitro translated SRα as described above.

CHAPTER THREE

RESULTS

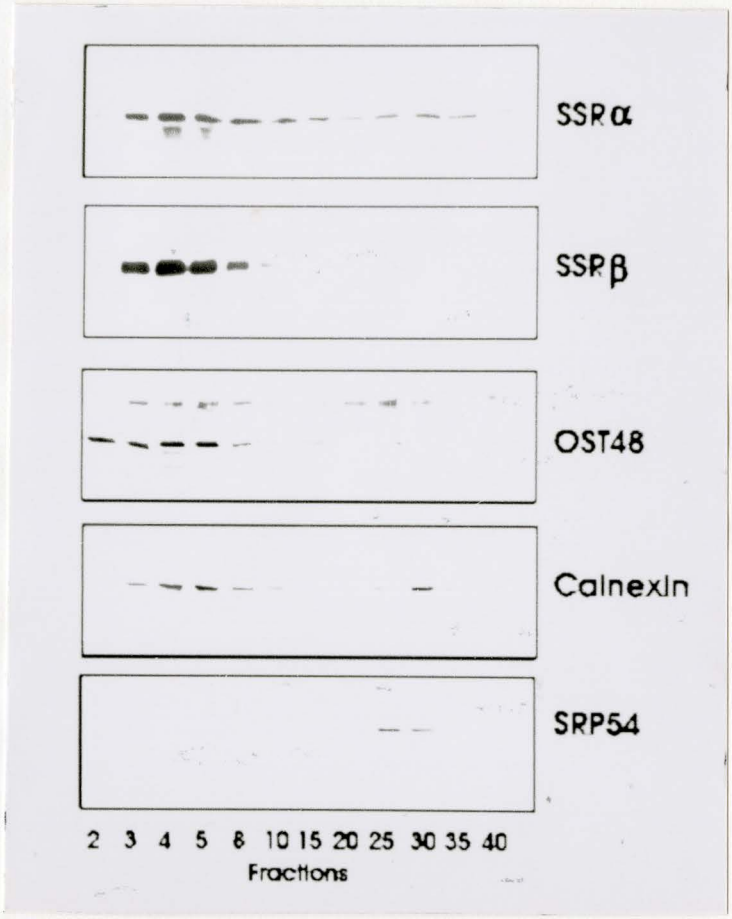
INTERACTION OF SRβ WITH ER MEMBRANES

To characterize the interaction of the SRP receptor with the ER membrane two traditional approaches were used to examine canine pancreatic rough microsomes. First, incubation in deoxycholate at a concentration just below the CMC of the detergent (0.08%) was used to isolate integral membrane proteins by permeabilizing the membrane such that lumenal and peripheral membrane proteins are released, but the lipid bilayer remains largely intact (Kriebach and Sabatini, 1974) (Figure 1). Second, alkali extraction with sodium carbonate pH 11.5 converts closed vesicles to open membrane sheets thereby releasing the lumenal and peripheral membrane proteins (Fujiki *et al.*, 1982). To increase the stringency of the alkali extraction, 1M NaSCN and 10mM DTT were also included in the incubation.

To clearly distinguish membranes from large protein complexes the extracted microsomes were analyzed by gel filtration chromatography using Sepharose CL2B equilibrated and eluted in the appropriate extraction buffer. Conventionally, deoxycholate extraction is performed at pH 7.5 (Kriebach and Sabatini, 1974) however, more efficient release of both peripheral and lumenal proteins has been observed at pH 9.0 (Andrews *et al.*, 1992). The observation that incubation at pH 9.5 alone is sufficient to release ER lumenal contents is consistent with this observation (Nicchitta and Blobel, 1993).

As expected, extraction with 0.08% deoxycholate/Tris pH9.0 did not release significant quantities of the ER integral membrane proteins SSRα, SSRβ, the 48KDa protein of oligosaccharyl transferase (OST48) and calnexin

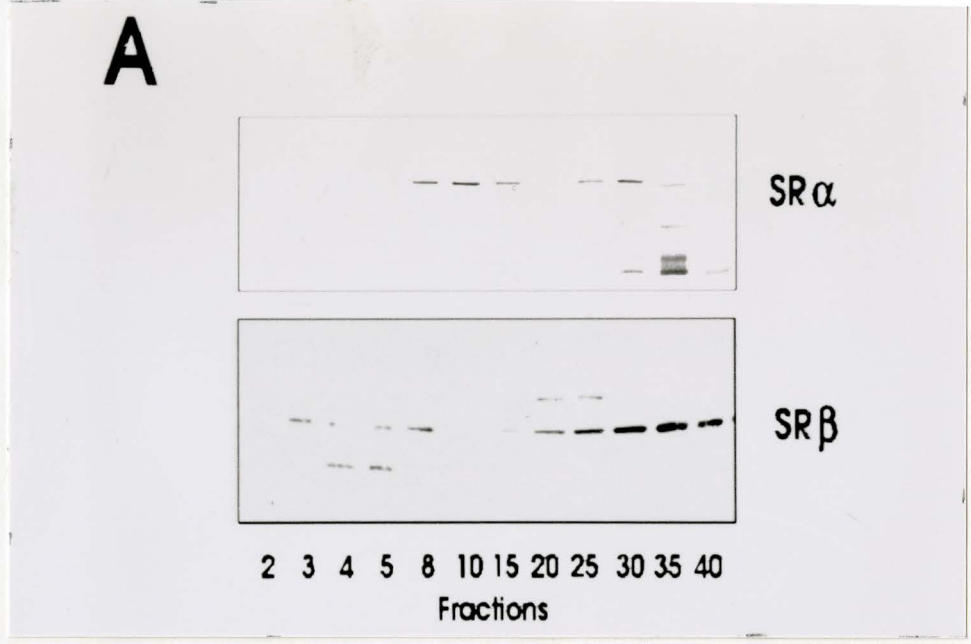
FIGURE 1: Gel filtration in 0.08% Deoxycholate/0.2M Tris pH 9.0 distinguishes integral and peripheral membrane proteins. Column washed microsomes (CRM) were applied to a Sepharose CL2B gel exclusion column equilibrated and eluted in 0.08% deoxycholate/0.2M Tris pH 9.0. Fifty fractions were collected where fractions 2-8 contain the peak of the excluded volume and factions 20-40 contain the included volume of the column. Representative fractions were concentrated by TCA precipitation. The location of several marker proteins including, SSRα, SSRβ, OST48, Calnexin and SRP54, were determined by immunoblots. The nitrocellulose membranes were probed with antisera to the indicated proteins at a 1:1000 dilution and developed colorimetrically using an alkaline phosphatase conjugated donkey anti-rabbit secondary antibody at a 1:2000 dilution. The migration positions of SSRα, SSRβ, OST48, Calnexin and SRP54 are indicated to the right of each panel.



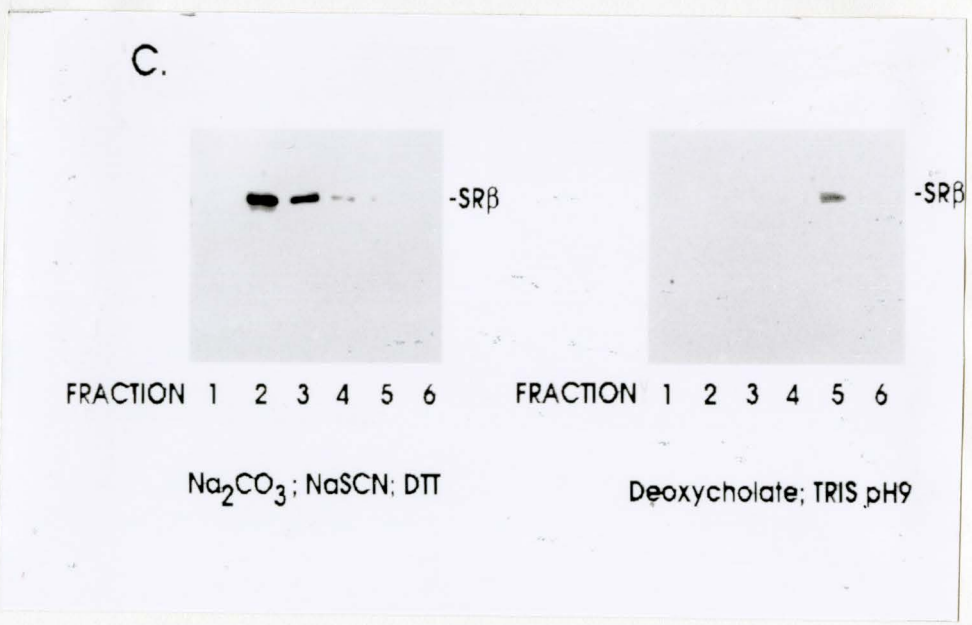
(Figure 1, fractions 2-8). However, a subset of the SSRα and calnexin was extracted into the included volume of the column (Figure 1, fractions 25-35). Calnexin, SSRα and SSRβ each have single transmembrane domains (Wada *et al.*, 1991; Hartmann *et al.,* 1989; Gorlich *et al.,* 1990) while OST48 spans the membrane seven times (Silberstein *et al.*, 1992). In contrast, the peripheral membrane control SRP54 (the 54KDa subunit of SRP) was recovered exclusively in the included fractions from the gel filtration column (Figure 1, fractions 25-30).

To our surprise both SRα and SRβ were efficiently released from CRMs by incubation in 0.08% deoxycholate; 0.2M Tris pH 9 (Figure 2A, fractions 25-40). A small fraction of the SRβ molecules remained associated with the membranes in 0.08% deoxycholate; 0.2M Tris pH 9.0 (Figure 2A, fractions 3-5). In contrast, SRα was completely extracted from the membranes (Figure 2A, fractions 3-5) but may exist in a large molecular weight complex as well as the monomer form (Figure 2A, fractions 8-15). Although SRα has previously been shown to interact with membranes less tightly than conventional transmembrane proteins (Young *et al.,* submitted; Miller *et al.,* manuscript in preparation), SRβ is believed to be a type I transmembrane protein (Miller *et al.,* manuscript in preparation). Therefore, the interaction of both subunits with ER membranes was examined by alkali extraction (Fujiki *et al.,* 1982) in a buffer containing 0.2M Na_2CO_3/1M NaSCN/10mM DTT. As expected from previous results using pH 12 or mixtures of urea and carbonate (Miller *et al.*, manuscript in preparation; Young *et al.*, submitted), most of the SRα was released (Figure 2B, 24-32). In contrast, SRβ remained membrane associated suggesting a transmembrane topology (Figure 2B, fractions 4-6). Taken together these results indicate that SRβ traverses the membrane but in a manner sensitive to extraction with deoxycholate.

FIGURE 2: The SRP receptor is atypical of ER membrane proteins. (A) SRβ and SRα were extracted by gel filtration chromatography in 0.08% deoxycholate/0.2M Tris pH 9.0. CRM were applied to a Sepharose CL2B column equilibrated in 0.08% deoxycholate/0.2M Tris pH 9.0. Fifty fractions were collected where fractions 2-8 contain the excluded volume and fractions 20-40 contain the included volume of the column. Representative fractions were concentrated by TCA precipitation and analyzed by for the location of SRα and SRβ by immunoblotting with SRα or SRβ antisera at a 1:1000 dilution. The blots were developed colorimetrically with an alkaline phosphatase conjugated secondary antibody at a (1:2000) dilution. (B) SRβ but not SRα resists extraction with alkali. Column washed membranes were applied to a Sepharose CL2B column equilibrated in 0.2M Na_2CO_3/1M NaSCN/10mM DTT and fractions were collected, processed and SRα and SRβ detected as in A. (C) SRβ is released from T_5KRM by 0.08% deoxycholate/0.2M Tris pH 9.0. Twenty equivalents of T_5KRM were incubated 10 minutes in 20μl of either deoxycholate (Panel A) or Na_2CO_3 (Panel B) extraction buffers prior to gel filtration chromatography as above. The column volume was 0.8ml and six fractions were collected where fraction 2 contains the peak of the excluded volume and fractions 4-6 contain the included volume of the column. The fractions were concentrated by TCA precipitation and analyzed for the location of SRβ by an immunoblot as in Panel A. The migration positions of molecular weight standards (in kilodaltions), SRα and SRβ are indicated on the left and right sides of the panels.


A
SRα
SRβ
2 3 4 5 8 10 15 20 25 30 35 40
Fractions


B
SRα
SRβ
4 5 6 8 12 16 20 24 28 32 36 40
Fractions


C.
-SRβ
-SRβ
FRACTION 1 2 3 4 5 6
FRACTION 1 2 3 4 5 6
Na2CO3; NaSCN; DTT
Deoxycholate; TRIS pH9

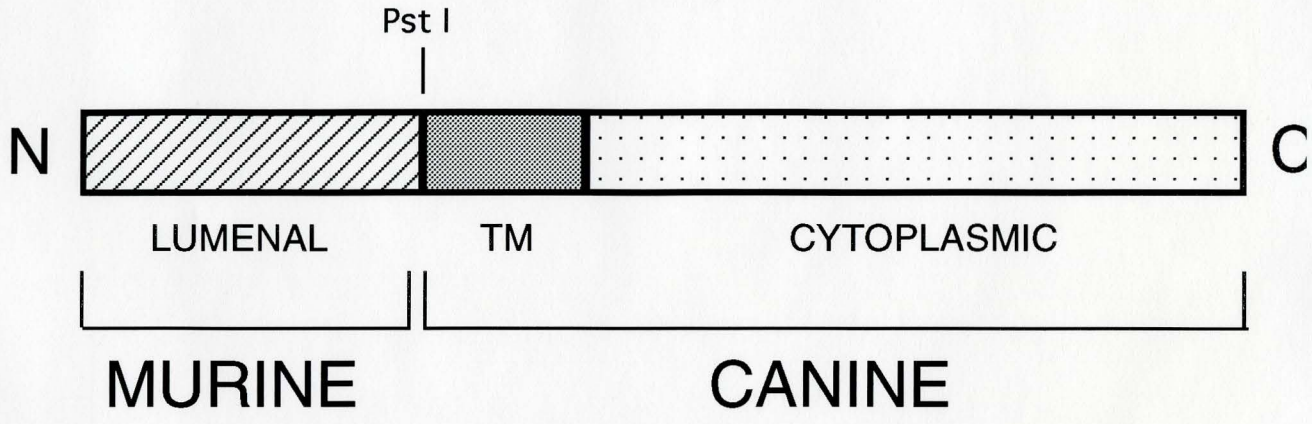
It has been shown that the interaction between SRα and SRβ is sufficient to redistribute SRβ molecules to the aqueous phase in TX-114 phase partitioning experiments (Young *et al.*, submitted; Miller *et al.,* manuscript in preparation). Therefore, it is possible that the association of SRα and SRβ results in the extraction by 0.08% deoxycholate/Tris pH9.0 of SRβ from CRMs (Figure 2A). Therefore, to examine the detergent solubility of SRβ independent of the bulk of SRα, the membranes were digested with 5μg/ml of trypsin. Limited trypsin digestion releases most of the SRα from the membrane without detectably altering SRβ (Andrews *et al.,* 1989). When T_5KRM were analyzed by extraction with either 0.2M Na_2CO_3/1M NaSCN/10mM DTT or 0.08% deoxycholate; Tris pH 9.0, the SRβ molecules remained resistant to alkali extraction (Figure 2C, fraction 2) but sensitive to extraction with 0.08% deoxycholate/Tris pH9.0 (Figure 2C, fraction 5). Taken together, these results suggest that SRβ interacts with the ER membrane in an unusual manner.

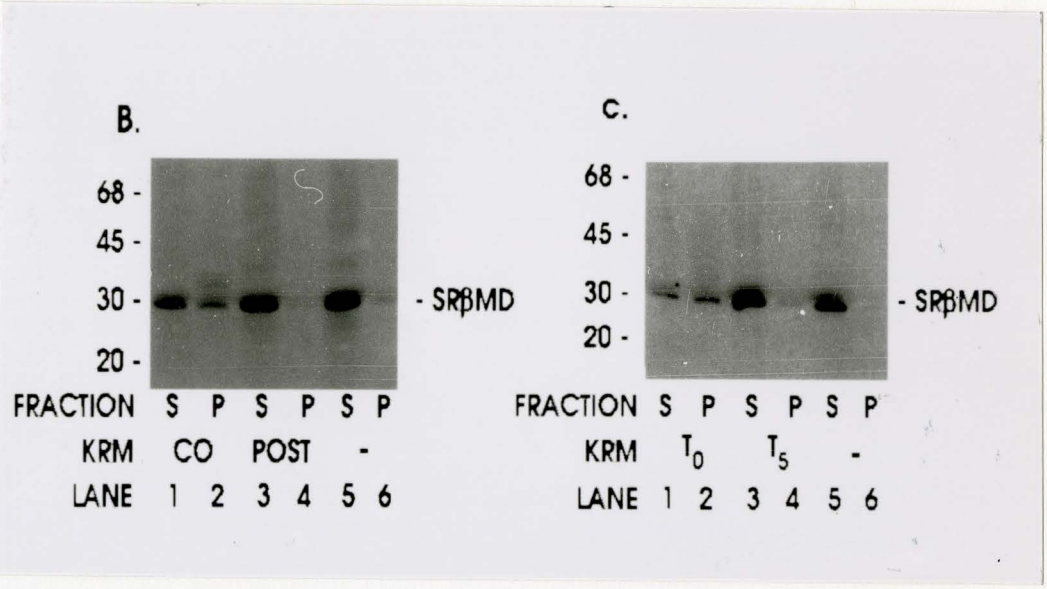
SRβ REQUIRES SRP RECEPTOR FOR MEMBRANE ASSEMBLY

To examine membrane targeting and assembly of SRβ, a reticulocyte lysate cell free system containing canine pancreatic microsomes was used. Ideally, to address the interaction of SRβ with canine SRα in this system a cDNA for canine SRβ would be used. However, the canine SRβ clone was not complete at the amino terminus. Therefore, DNA encoding the amino terminus of the murine SRβ was fused to the canine clone to generate a plasmid encoding $SR\beta_{MD}$. This hybrid contains the first 28 residues of murine SRβ followed by the transmembrane and cytosolic domains of canine SRβ (Figure 3A). The canine and murine SRβ molecules are very similar (88% amino acid identity) (Miller *et al.*, manuscript in preparation). Moreover, once targeted to the ER membrane, the murine portion preceding the transmembrane domain is

FIGURE 3: $SR\beta_{MD}$ membrane assembly is co-translational and trypsin sensitive. (A) Construction of the $SR\beta_{MD}$ coding region. The predicted lumenal domain of murine SRβ (residues 1-29) was fused to the transmembrane and cytoplasmic domains of canine SRβ (residues 25-265) ($SR\beta_{MD}$). The fusion protein was generated using the indicated Pst I site present in both cDNAs. (B) $SR\beta_{MD}$ assembles co-translationally onto membranes. Reticulocyte lysate translation products of $SR\beta_{MD}$ (20 μl) were incubated with 1 equivalent of KRM either co-translationally (lanes 1,2) or post-translationally for 15 minutes (lanes 3,4). The control reaction (lanes 5,6) did not contain KRM. The reactions were adjusted to 2M urea and 25mM EDTA, incubated on ice for 10 minutes and the membranes were subsequently isolated by centrifugation through a sucrose cushion at 160 000 xg for 15 minutes. The samples were divided into supernatant (lanes 1, 3, 5) and pellet (lanes 2, 4, 6) fractions. (C) $SR\beta_{MD}$ assembly requires a trypsin sensitive component on the ER membrane. $SR\beta_{MD}$ translation reactions (20μl) contained 1eq of KRM (lanes 1, 2), T_5KRM (lanes 3, 4) or no added membranes (lanes 5,6). The completed translation reactions were adjusted to 2M urea; 25mM EDTA and the membranes were isolated by centrifugation above. Samples were divided into supernatant (lanes 1, 3, 5) or membrane pellet (lane 2, 4, 6) fractions. The migration positions of molecular weight markers (in kilodaltons) and $SR\beta_{MD}$ are indicated on the sides.

A.


SRβMD
Pst I
N
C
LUMENAL
TM
CYTOPLASMIC
MURINE
CANINE


B.
68 -
45 -
30 -
20 -
- SRβMD
FRACTION S P S P S P
KRM CO POST -
LANE 1 2 3 4 5 6
C.
68 -
45 -
30 -
20 -
- SRβMD
FRACTION S P S P S P
KRM T_0 T_5 -
LANE 1 2 3 4 5 6

predicted to be in the lumen of the ER (Miller *et al.*, manuscript in preparation). As the interaction of SRβ with SRα is believed to take place on the cytosolic side of the ER (Tajima *et al.*, 1986; Andrews *et al.*, 1989), the predicted interacting domains are both canine.

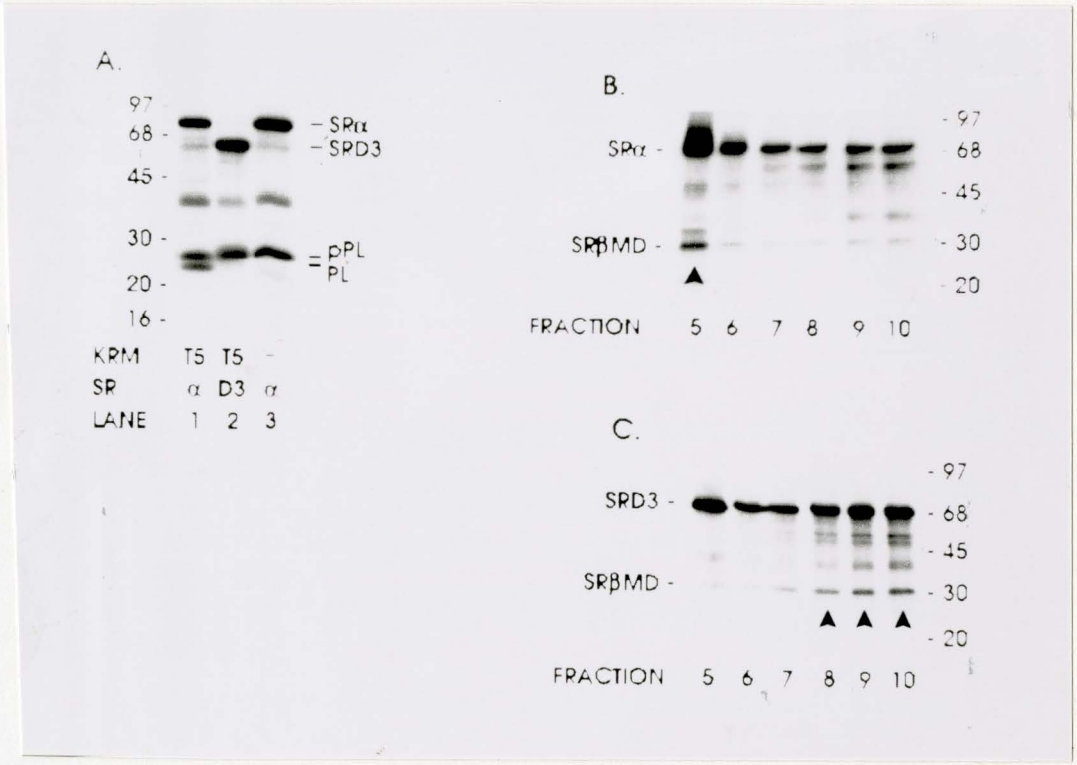
It has been shown that targeting of SRα to the ER membrane can occur post-translationally by a mechanism that does not require SRP (Andrews *et al.*, 1989). Therefore, to determine the sequence of events for $SR\beta_{MD}$ assembly, reticulocyte lysate translation products were incubated with KRM during or after translation was terminated. Anchoring of the protein was assayed by centrifugation of the microsomes through a sucrose step gradient in the presence of 2M urea. $SR\beta_{MD}$ molecules anchored on the membranes were recovered in the membrane pellet (P) while unassociated proteins remained in the supernatant (S). Urea resistant anchoring of $SR\beta_{MD}$ onto membranes occurred only when the membranes were present throughout the translation reaction (Figure 3B, compare lanes 1,2 and 3,4). Thirty eight percent of the $SR\beta_{MD}$ molecules anchored onto KRM when the protein was synthesized co-translationally in the presence of membranes. Conversely, when post ribosomal supernatants of $SR\beta_{MD}$ were incubated with the membranes, only 1.8% of the $SR\beta_{MD}$ molecules anchored onto the membranes in a urea resistant manner. Finally, incubation of KRM with $SR\beta_{MD}$ in a co-translational manner appears to result in poorer synthesis of $SR\beta_{MD}$ as opposed to incubation of the post-ribosomal supernatant with KRM (Figure 3B, compare lanes 1,2 and 3,4). However, densitometry of the total translation products both co-and post- translationally revealed that the similar levels of $SR\beta_{MD}$ were synthesized. Co-translational assembly of $SR\beta_{MD}$ onto KRM results in an uncharacterized post-translational modification to a fraction of the $SR\beta_{MD}$ molecules (Figure 3B, lane 2). Therefore, the modified $SR\beta_{MD}$ molecules were

also included in calculating the total amount of cell free synthesized $SR\beta_{MD}$ in the co-translational reaction.

Co-translational targeting of ER membrane proteins is generally mediated by SRP. Therefore, to assay the role of SRP in targeting, $SR\beta_{MD}$ was expressed in a wheat germ extract (which lacks endogenous SRP) in the presence of CRM. Unfortunately, the $SR\beta_{MD}$ molecules synthesized in a wheat germ extract aggregated, as determined by gel exclusion chromatography, thereby preventing meaningful analysis of membrane assembly (data not shown). $SR\beta_{MD}$ does not aggregate when synthesized in reticulocyte lysate, however this cell free system contains SRP. Therefore, the SRP pathway was inactivated by removing the GTP binding domain of SRα from the ER membrane by digestion with 5μg/ml of trypsin at 0°C for one hour (Andrews *et al.*, 1989). Trypsin digested membranes (T_5KRM) were unable to anchor $SR\beta_{MD}$ (Figure 3C, compare lanes 1,2 and 3,4) suggesting that a trypsin sensitive component on the ER membrane (possibly SRα) is required for $SR\beta_{MD}$ assembly. Co-translational synthesis of $SR\beta_{MD}$ in the presence of KRM (Figure 3C, lanes 1,2) was reduced two fold compared to the levels of translation product obtained with T_5KRM (Figure 3C, lanes 3, 4). It is known that elevated levels of microsomes are inhibitory to translation of exogenous mRNA in a rabbit reticulocyte lysate. Therefore, it is possible that the KRM were more concentrated than the T_5KRM resulting in the two fold inhibition of synthesis of $SR\beta_{MD}$.

Trypsin digested membranes (T_5KRM) support SRP dependent translocation of preprolactin when the digested SRα molecules are replaced with wild type SRα synthesized in reticulocyte lysate (Figure 4A, lane 1) (Andrews *et al.*, 1989). As expected, reconstitution of the trypsin digested membranes with SRD3 (an SRα mutant defective for translocation activity)

FIGURE 4: $SR\beta_{MD}$ requires functional SRP receptor for assembly on T_5KRM. (A) Addition of SRα reconstitutes translocation of preprolactin across T_5KRM. One equivalent of T_5KRM was incubated with post-ribosomal supernatants (10μl) of cell free synthesized SRα (lane 1) or SRD3 (lane 2) for 15 minutes at 24°C. Translation reactions encoding preprolactin (20μl) were added to the reconstituted microsomes and incubated for one hour at 24°C. The control reaction did not contain membranes (lane 3). The conversion of preprolactin (pPL) to prolactin (PL) indicates restored translocation function. (B) $SR\beta_{MD}$ assembles on T_5KRM reconstituted with SRα. T_5KRM (1eq) were incubated with a post-ribosomal supernatant of SRα (10μl) as above, a new translation reaction for $SR\beta_{MD}$ (20μl) was added and incubation continued for one hour at 24°C. The sample was analyzed for $SR\beta_{MD}$ membrane assembly by gel filtration chromatography using a Sepharose CL2B column equilibrated in Buffer R and 1M NaCl. Fraction 5 contains the peak of the excluded volume and fractions 8-10 contain the included volume (C) SRD3 does not reconstitute membrane assembly of $SR\beta_{MD}$. A post-ribosomal supernatant of SRD3 (10μl) was incubated with 1equivalent of T_5KRM as above and a translation reaction for $SR\beta_{MD}$ (20μl) was added to the reconstituted T_5KRM. Membrane assembly was analyzed as above. The migration of molecular weight markers in kilodaltons, SRα, SRD3, $SR\beta_{MD}$, pPL and PL are indicated to the side. Upward arrowheads indicate $SR\beta_{MD}$ in fractions containing most of the molecule.

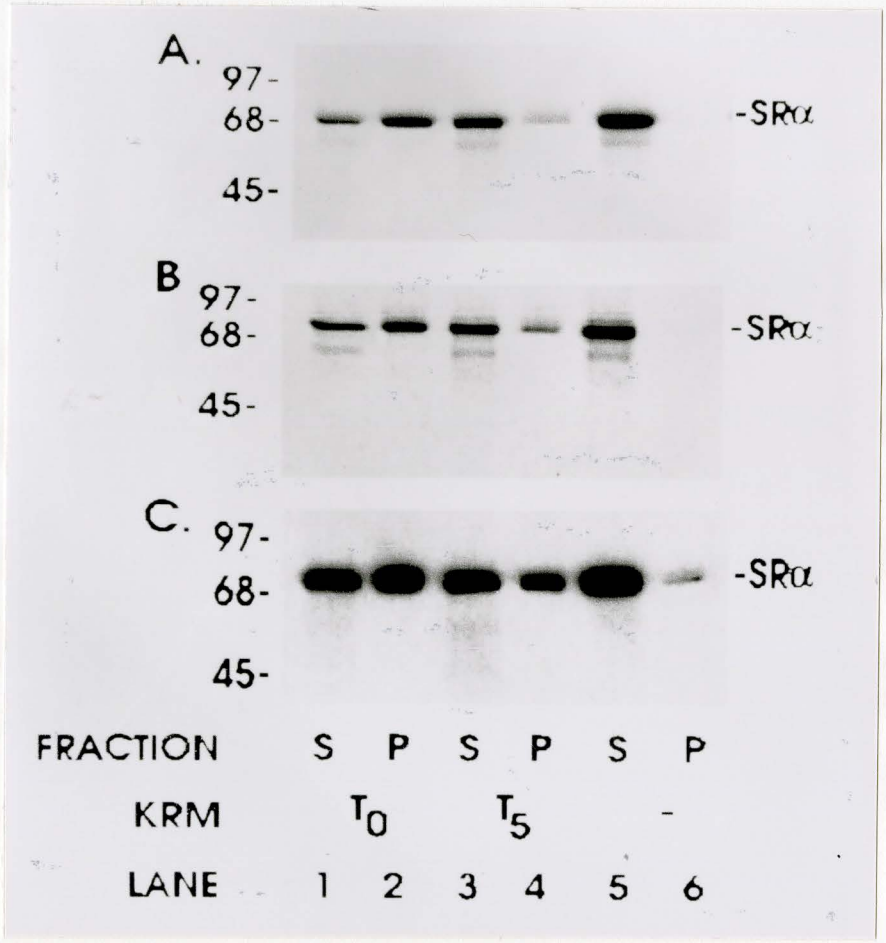

A.
97
68
45
30
20
16
SRα
SRD3
pPL
PL
KRM T5 T5 -
SR α D3 α
LANE 1 2 3
B.
SRα
SRβMD
97
68
45
30
20
FRACTION 5 6 7 8 9 10
C.
SRD3
SRβMD
97
68
45
30
20
FRACTION 5 6 7 8 9 10

0.1M Na_2CO_3, pH 11.5; or 1M NaCl) was added to the reaction and the membranes were collected by centrifugation. As reported previously, 2M urea removed SRα from T_5KRM but not KRM (Figure 5A, compare lanes 1,2 and 3,4). Furthermore, elevated pH also stripped SRα from T_5KRM but not KRM (Figure 5B, compare lanes 1,2 and 3,4). Finally, 1M NaCl did not release all of the SRα molecules from either membrane (Figure 5C, lanes 2 and 4) as expected from Figure 4. Although a small amount of SRα is found in the pellet fraction minus membranes with 1M NaCl (Figure 5C, lane 6), but not with urea or Na_2CO_3 (Figure 5A and B, lane 6), the level of synthesis of SRα in panel C was appreciably higher. However, the amount of SRα on T_5KRM in the presence of 1M NaCl is significantly more than in the absence of membranes (Figure 5C, compare lanes 4 and 6).

Urea is known to act by disrupting hydrophobic interactions between proteins (Kamoun, 1988), while Na_2CO_3 pH11.5 and NaCl disrupt electrostatic interactions (Creighton, 1984). Therefore, our results suggest that although both hydrophobic and electrostatic interactions are responsible for anchoring SRα to KRM the hydrophobic interaction was lost or reduced when membranes were digested with trypsin. Moreover, in the absence of the hydrophobic interaction the electrostatic component is sensitive to 0.1M Na_2CO_3 but not to 1M NaCl (Figure 5B and C, lane 4) suggesting that SRα is more tightly associated with the membrane than other peripherally associated proteins.

Resistance to 2M urea was chosen as the most appropriate criteria to define stable anchoring of SRα and $SR\beta_{MD}$ and to determine if $SR\beta_{MD}$ can restore anchoring of SRα to T_5KRM as outlined in figure 6A. Accordingly, T_5KRM were added to a post-ribosomal supernatant of SRα or SRD3 as described in figure 4. Translation reactions encoding either SRβ or preprolactin were then added to the reconstituted membranes co-translationally

FIGURE 5: Post-translational anchoring of SRα to ER membranes. Post ribosomal supernatants of SRα (20μl) were incubated with 5 equivalents of KRM (lanes 1, 2) or T_5KRM (lanes 3, 4) or without membranes for 15 minutes at 24°C (lanes 5, 6). (A) The samples were then adjusted to 2M urea and 25mM EDTA and left on ice for 10 minutes. Then the membranes were collected by centrifugation through a 0.5M sucrose cushion at 160 000 xg for 15 minutes. The samples were divided into supernatant (lanes 1, 3, 5) and pellet (lanes 2, 4, 6) fractions and analyzed by SDS PAGE and fluorography. Translation products extracted with 0.1M Na_2CO_3 and 25mM EDTA (B) or 1M NaCl and 25mM EDTA (C) were analyzed as above. Migration positions of molecular weight standards (in kilodaltons) and SRα are indicated to the sides of the panels.



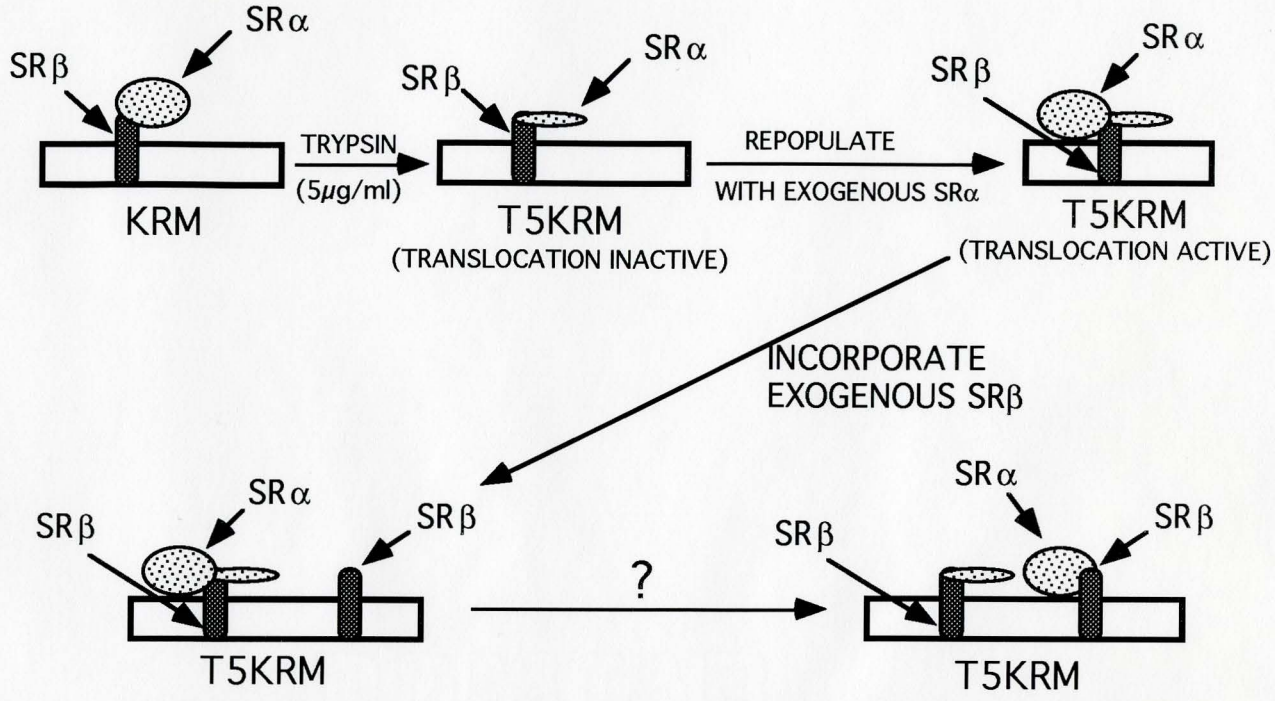
or to control reactions post-translationally. As expected from the data in figure 4, only T_5KRM reconstituted with SRα and not the translocation defective control molecule SRD3 restored translocation function to the T_5KRM (Figure 6B, lanes 1 and 2).

However, when SRβ_{MD} was assembled co-translationally onto T_5KRM reconstituted with SRα, on average 38 ± 16% std dev (n=4) of the SRα molecules became resistant to extraction with urea (Figure 6B, lanes 3-5; Figure 15). As expected, the interaction of SRβ_{MD} with reconstituted T_5KRM was urea resistant only when the SRβ_{MD} molecules were added co-translationally (Figure 6B, compare lanes 3-5 and 6-8). When the amount of nonspecific SRβ_{MD} in the pellet of the control reaction (Figure 6B, lane 8) was subtracted from the amount in the pellet of the experimental reaction (Figure 6B, lane 5), 10% of the SRβ_{MD} molecules were found to be anchored to the T_5KRM. This suggests that restoration of translocation activity to T_5KRM by added SRα was very poor. Consistent with this interpretation, translocation of preprolactin across these membranes was also inefficient in this experiment (Figure 6B, lane 1). When the amount of nonspecific SRα in the pellet of the control reaction was subtracted from the experimental reaction, 43% of the SRα molecules were anchored to the T_5KRM. Therefore, co-translational assembly of exogenous, SRβ_{MD} onto SRα reconstituted T_5KRM is sufficient to restore urea resistant anchoring of SRα. After normalizing for the number of methionine residues in SRα and SRβ_{MD}, the ratio of SRα:SRβ_{MD} present in the pellet of the experimental reaction (Figure 6B, lane 5) was 1.4:1. This suggests that one SRβ_{MD} anchors, on average, one SRα molecule onto T_5KRM.

To confirm and extend these results, stable complex formation between SRα (or SRα deletion mutants) with SRβ_{MD} was assayed by co-immunoprecipitation using antisera to each of the two subunits. The ability of

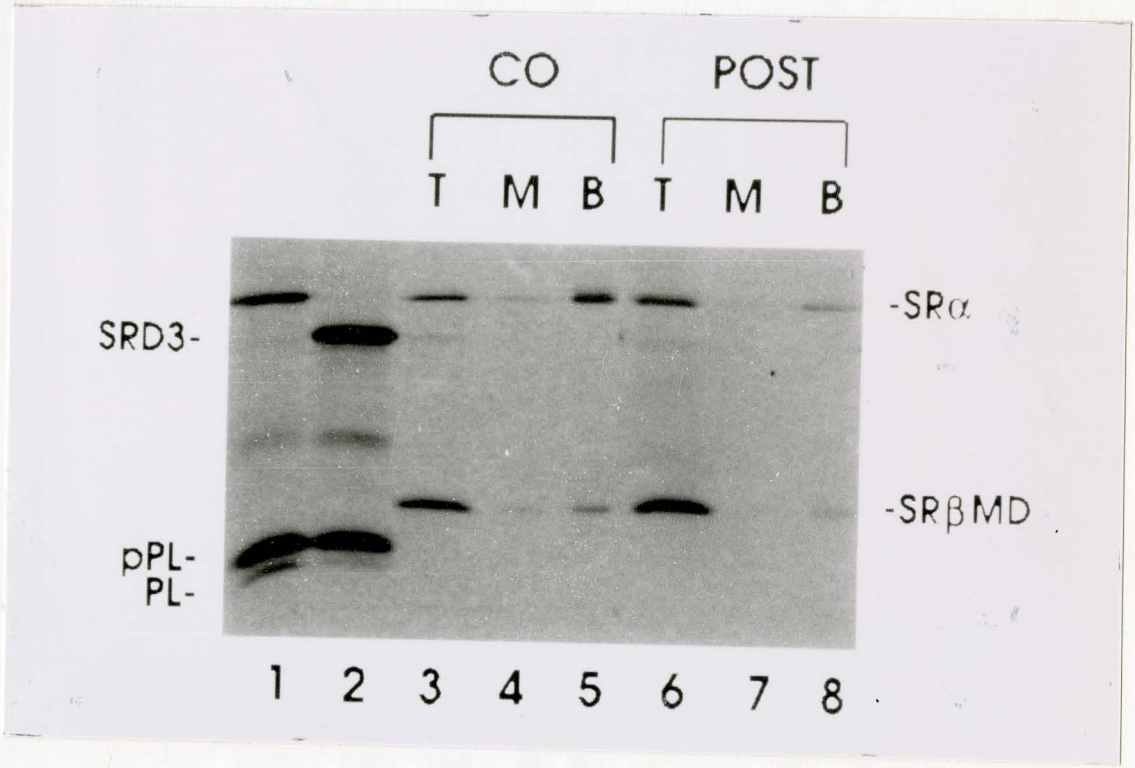
FIGURE 6: Newly synthesized SRβ anchors SRα to T_5KRM. (A) Schematic diagram illustrating the assay to determine whether SRβ is the molecule which anchors SRα to the ER membrane. (B) Post ribosomal supernatants of SRα or SRD3 (10μl) were incubated with 1 equivalent of T_5KRM for 15 minutes at 24°C. Translation reactions encoding preprolactin (pPL) (20μl) were added to the membranes reconstituted with SRα (lane 1) or SRD3 (lane 2) and incubated for one hour at 24°C. Conversion of preprolactin to prolactin indicates the extent of restoration of translocation activity to T_5KRM. Translation reactions encoding $SR\beta_{MD}$ (20μl) were added to T_5KRM reconstituted with SRα either before (lanes 3-5) or after (lanes 6-8) an additional one hour incubation at 24°C. The translation products were adjusted to 2M urea and 25mM EDTA and incubated for 10 minutes on ice. The membranes were then isolated by centrifugation over a 0.5M sucrose cushion at 160 000 xg for 15 minutes. Samples were divided into top (lanes 3, 6), middle (lanes 4, 7) and bottom (lanes 5, 8) fractions. Migration positions of SRα, $SR\beta_{MD}$, SRD3, pPL and PL are indicated to the sides of the panel.

A.



EXTRACT WITH 2M UREA: DO THE SR α MOLECULES REMAIN MEMBRANE ASSOCIATED?

B.



$SR\beta_{MD}$ to interact post-translationally with the various SRα deletion mutants was determined using reticulocyte lysate translation products.

The two functional domains of SRα are the amino terminal anchoring domain (SRX2) and the carboxyl terminal translocation active domain (SREF) (Figure 7; Young *et al.*, submitted). The epitope for the SRα antibody (S1) does not lie within SRX2 (data not shown). However, the deletion mutant, SRD3, contains the entire amino terminal anchoring domain of SRα but lacks the first 100 amino acids of the translocation active domain of the molecule (Figure 7; Young *et al.*, submitted). Furthermore, SRD3 is recognized by the S1 SRα antibody. Therefore, 10 µl cell free translation products of SRα, SRD3, or SREF were incubated with 10 µl $SR\beta_{MD}$ post-translationally in the absence of membranes for 30 minutes at 24°C to allow complex formation prior to immunoprecipitation with the S1 SRα antibodies. $SR\beta_{MD}$ formed stable complexes with SRα and SRD3, but not SREF (Figure 8A lanes 1-3). Moreover, both SRα and SRD3 co-immunoprecipitated $SR\beta_{MD}$ in a 3:1 ratio. Therefore, sequences within the amino terminal membrane anchoring domain are required for complex formation with $SR\beta_{MD}$. The region of SRX2 necessary for anchoring SRα consists of two hydrophobic regions followed by a positively charged region. Deletion of any of these regions impairs the ability of the molecule to anchor onto membranes (Young *et al.*, submitted). Deletion mutants of SRα missing either the first (SRD4) or both (SR18) hydrophobic regions (Figure 7) were assayed for their ability to interact with $SR\beta_{MD}$ as above. Cell free translation products of SRD4 or SR18 (10 µl) were mixed with 10 µl $SR\beta_{MD}$ post-translationally and complexes were detected by immunoprecipitation with the S1 SRα antibody. Neither SRD4 nor SR18 formed stable interactions with $SR\beta_{MD}$, although $SR\beta_{MD}$ was co-immunoprecipitated with full length SRα (Figure 8B, compare lanes 1-3).

FIGURE 7: Schematic diagram representing deletion mutants of SRα. The top bar represents the entire SRα coding region from residues 1-638. The positions of the two hydrophobic regions, H1-H2 (darker shaded boxes) and the three postively charged, +1-+3 (lighter shaded boxes) stretches in the anchoring domain are indicated on the diagram. Various deletion mutants of SRα are diagrammed below. The residues present in the deletion mutants are indicated below each box.

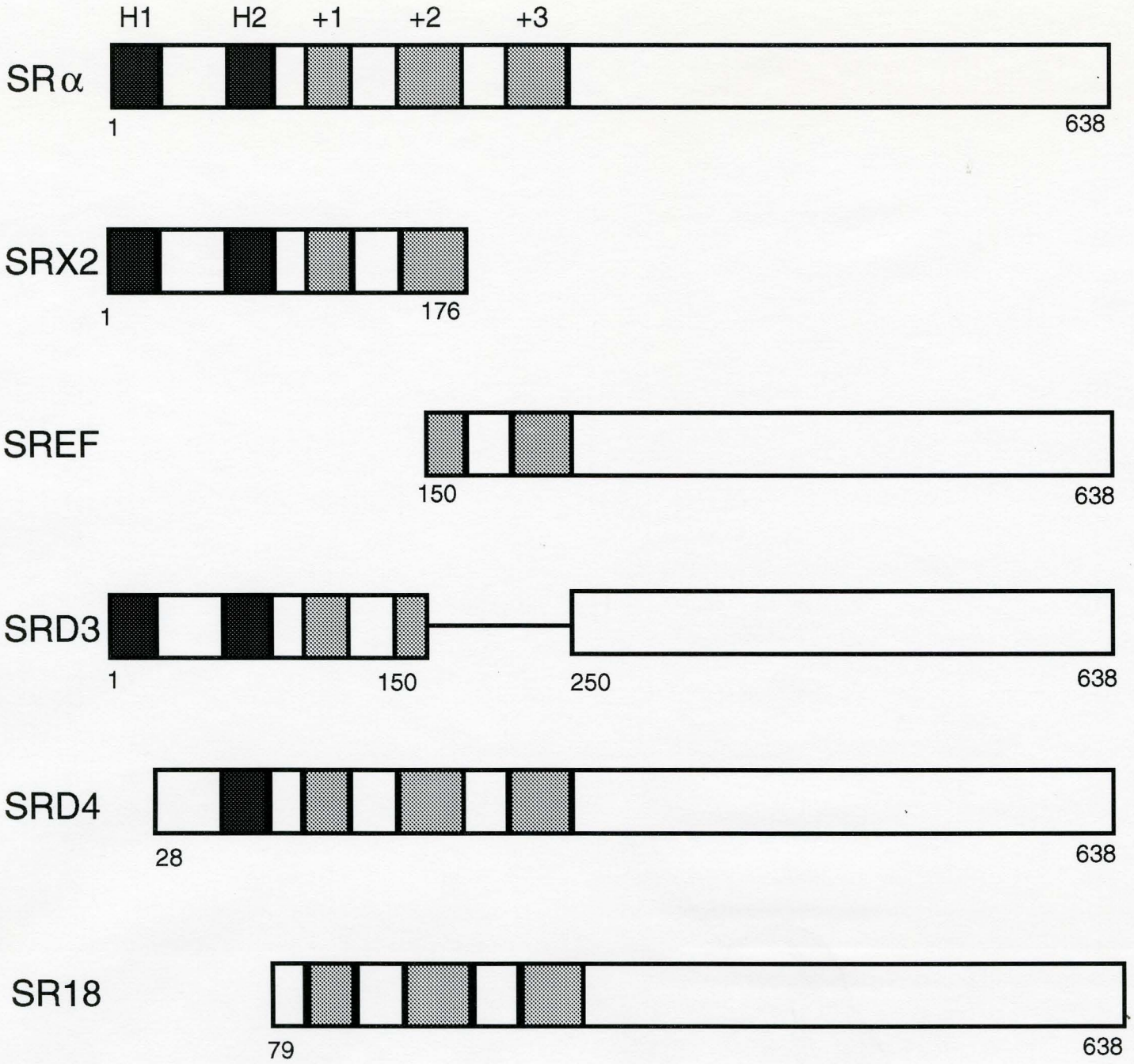
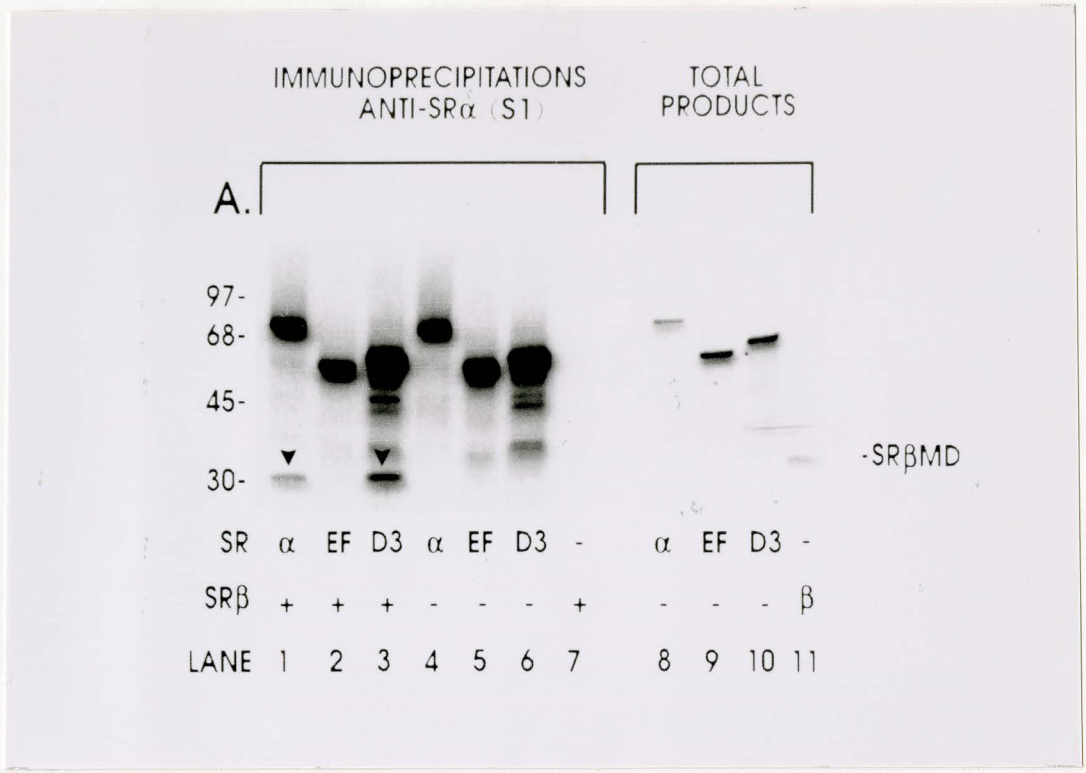
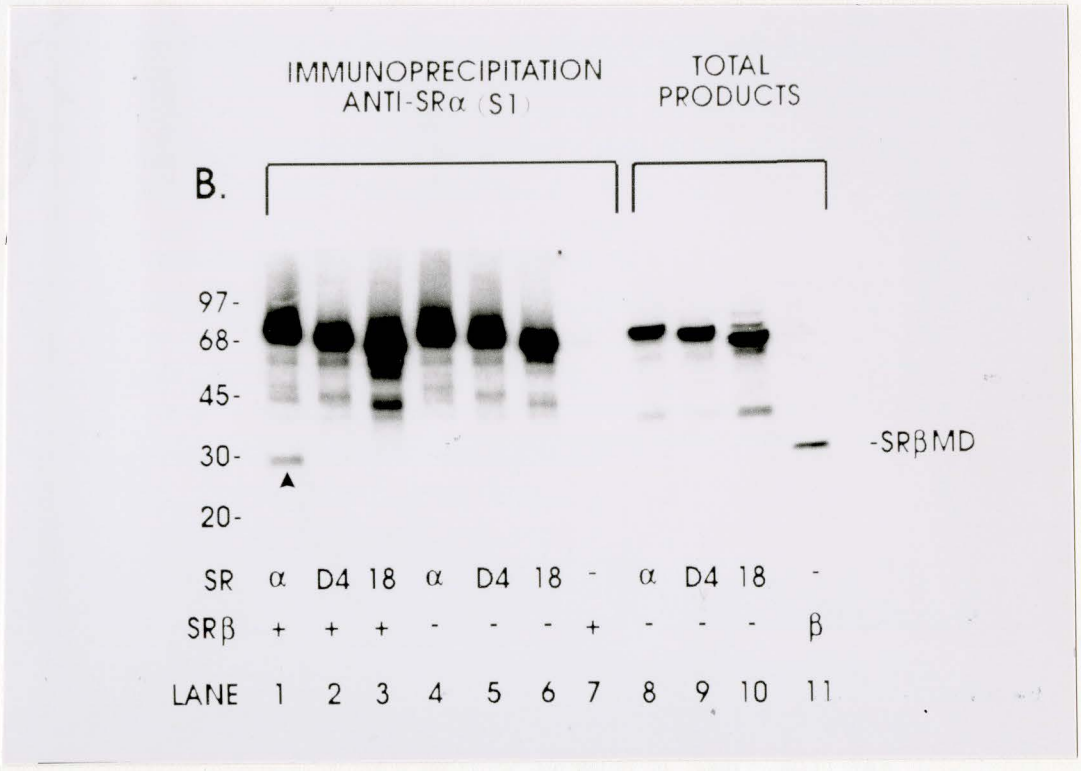

H1
H2
+1
+2
+3
SRα
1
638
SRX2
1
176
SREF
150
638
SRD3
1
150
250
638
SRD4
28
638
SR18
79
638

FIGURE 8: SRβ_{MD} interacts with the anchoring domain of SRα. (A) SRβ_{MD} reticulocyte lysate translation products were incubated post-translationally with an equal volume of cell free synthesized SRα (lane 1), SREF (lane 2) or SRD3 (lane 3) for 30 minutes at 24°C. The samples were immunoprecipitated with an SRα antibody (S1) coupled to cyanogen bromide activated (CNBr) Sepharose. In addition, control immunoprecipitations with SRα, SREF, SRD3 and SRβ_{MD} alone (lanes 4-7) were also performed. Total translation products for each of the molecules are in lanes 8-12. (B) SRβ_{MD} requires both hydrophobic regions in the anchoring domain of SRα for interaction. SRβ_{MD} reticulocyte lysate translation products were incubated post translationally with cell free synthesized SRα (lane 1), SRD4 (lane 2) and SR18 (lane 3) and the samples were immunoprecipitated with the monoclonal SRα antibody as above. Control immunoprecipitations for each of the molecules were performed (lanes 4-7) and total translation products (lanes 8-12) were also analyzed. The migration pattern of molecular weight markers (in kilodaltons) and SRβ_{MD} are indicated to the sides of the panels. Downward pointing arrowheads indicate the position of SRβ_{MD} in the co-immunoprecipitations with SRα and SRD3.


IMMUNOPRECIPITATIONS
ANTI-SRα (S1)
TOTAL
PRODUCTS
A.
97-
68-
45-
30-
-SRβMD
SR α EF D3 α EF D3 - α EF D3 -
SRβ + + + - - - + - - - β
LANE 1 2 3 4 5 6 7 8 9 10 11


IMMUNOPRECIPITATION
ANTI-SRα (S1)
TOTAL
PRODUCTS
B.
97-
68-
45-
30-
20-
-SRβMD
SR α D4 18 α D4 18 - α D4 18 -
SRβ + + + - - - + - - - β
LANE 1 2 3 4 5 6 7 8 9 10 11

This suggests that at least the first hydrophobic region of the anchoring domain of SRα is required to interact with SRβ$_{MD}$.

Coimmunoprecipitates between SRX2 and SRβ$_{MD}$ would directly demonstrate that SRβ$_{MD}$ interacts with the membrane anchoring domain of SRα. Therefore, a polyclonal antiserum was raised to the carboxyl terminal 40 residues of SRβ$_{MD}$ to test for complex formation between SRβ$_{MD}$ and SRX2. The antisera recognized only native and not denatured cell free synthesized SRβ$_{MD}$ in an immunoprecipitation reaction (Figure 9A, compare lanes 1-4 and 5). Moreover, the antisera is specific for SRβ$_{MD}$ since immunoprecipitation of the cell free synthesized product can be competed away with increasing amounts of GST-SRβ$_{MD}$, but not GST (Figure 9B, compare lanes 2,4,6 and 3,5,7). When, the SRβ antisera was used in a co-immunoprecipitation assay between either SRα, SREF, SRX2 or SRD3 and SRβ$_{MD}$, complexes were not observed (Figure 10A, lanes 1-4). Trace amounts of SRα, SRX2 and SRD3 were visible on long exposures, but were not above background levels in the absence of SRβ$_{MD}$ (data not shown).

Because the SRβ antisera could not be used to demonstrate complex formation between SRX2 and SRβ$_{MD}$, an SRα antisera (N1), raised to the anchoring domain of the molecule, was assayed in co-immunoprecipitations to test for complex formation between SRβ$_{MD}$ and SRX2. Unfortunately, the N1 SRα antisera was also unable to coimmunoprecipitate SRβ$_{MD}$ with SRα, SRX2 or SRD3 (Figure 11, lanes 1-3). Similar results were also obtained in other experiments when the amount of SRα immunoprecipitated was increased (data not shown).

In a final attempt to visualize SRX2/SRβ$_{MD}$ complex formation, a hemagluttinin (HA) epitope was fused to the amino terminus of SRβ$_{MD}$ (HA-SRβ$_{MD}$). Since SRβ$_{MD}$ is predicted to be a type I signal anchor protein, the

FIGURE 9: Characterization of polyclonal SRβ antisera. (A) The SRβ antisera recognizes only the native protein. Native reticulocyte lysate translation products of $SR\beta_{MD}$ were immunoprecipitated with either 10μl preimmune serum (lane 1) or 1-10μl of the SRβ antisera (lanes 2-5). Translation products were also boiled in 1% SDS/0..1M Tris pH9 to denature the protein prior to immunoprecipitation with 10μl of the SRβ antisera (lane 6). Total synthesis of $SR\beta_{MD}$ prior to immunoprecipitation is also indicated (lane 7). (B) GST-SRβ fusion protein can compete for the SRβ antisera. $SR\beta_{MD}$ reticulocyte lysate translation products were immunoprecipitated with 10μl of the SRβ antisera under conditions of antibody excess. Immunoprecipitation of $SR\beta_{MD}$ was performed in the absence (lane1) or presence of various amounts of GST (lanes 2, 4, 6) or GST-SRβ (lanes 3, 5, 7) fusion protein. Migration positions of molecular weight markers (in kilodaltons) and $SR\beta_{MD}$ are indicated to the sides of the panels.

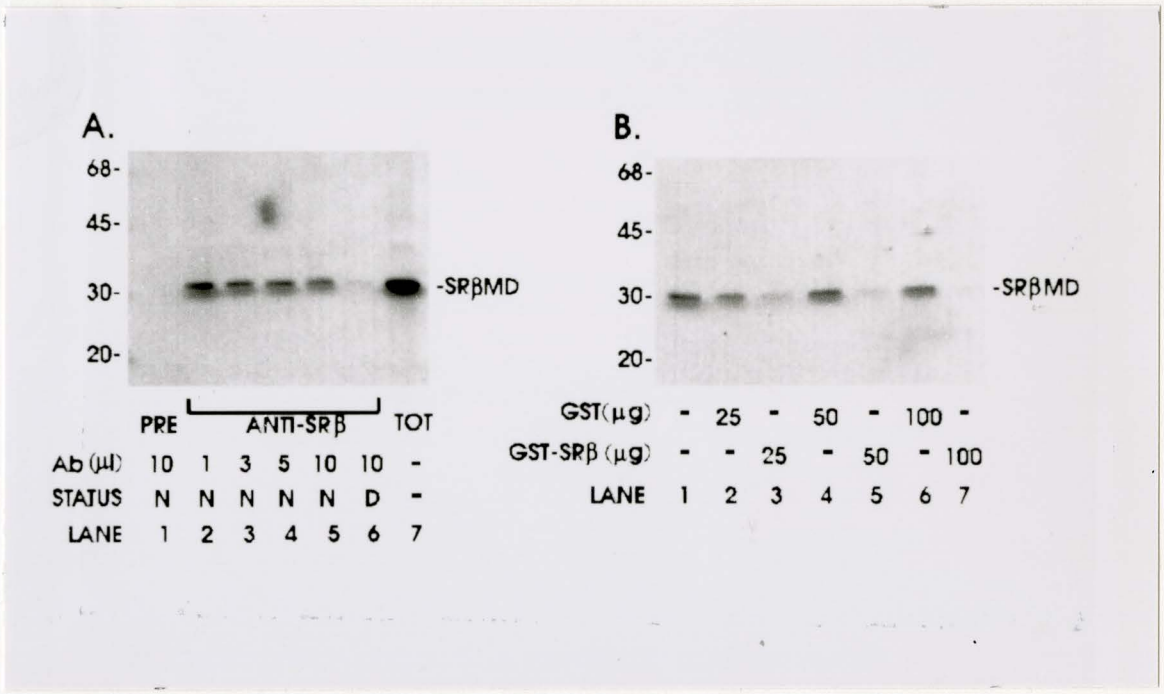


FIGURE 10: The polyclonal SRβ antisera cannot be used to detect complex formation between $SR\beta_{MD}$ and SRα. (A) $SR\beta_{MD}$ reticulocyte lysate translation products were incubated post-translationally with an equal volume of cell free synthesized SRα (lane 1), SREF (lane 2), SRX2 (lane 3) or SRD3 (lane 4) for 30 minutes at 24°C. The samples were immunoprecipitated with a purified IgG fraction of the polyclonal SRβ antisera. In addition, control immunoprecipitations for SRα, SREF, SRX2, SRD3 and $SR\beta_{MD}$ alone (lanes 5-9) were also performed. Total translation products for each of the molecules are in lanes 10-14. The migration position of molecular weight markers (in kilodaltons) are indicated on the left hand side of the panel.

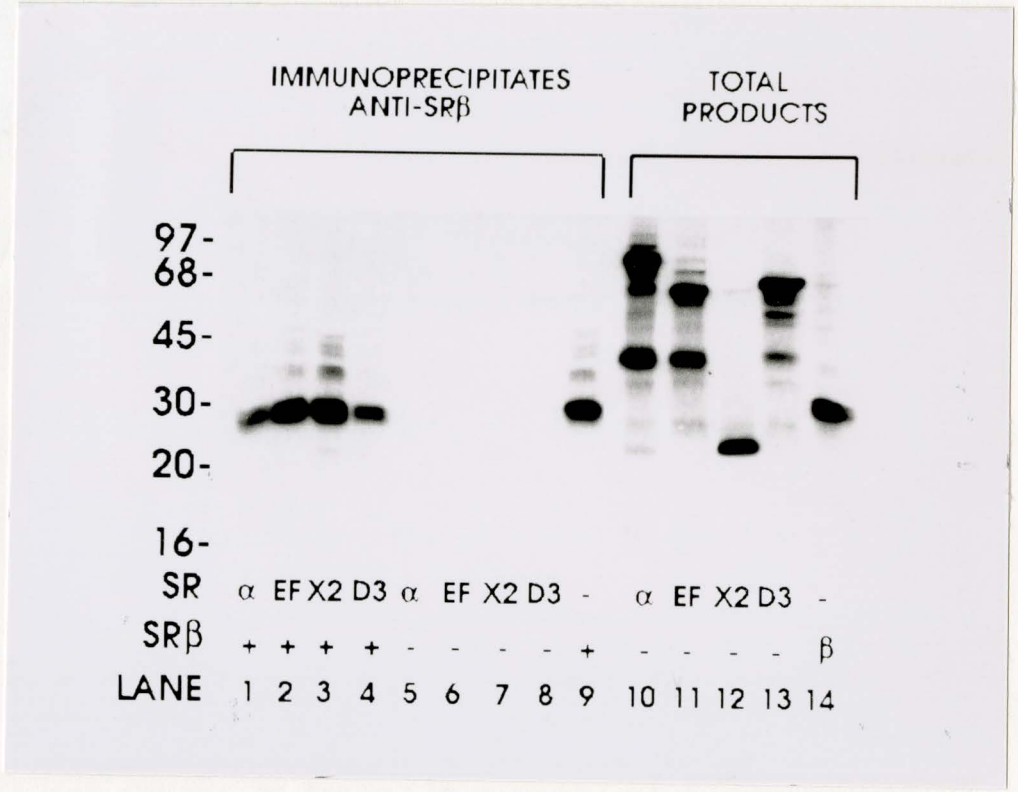
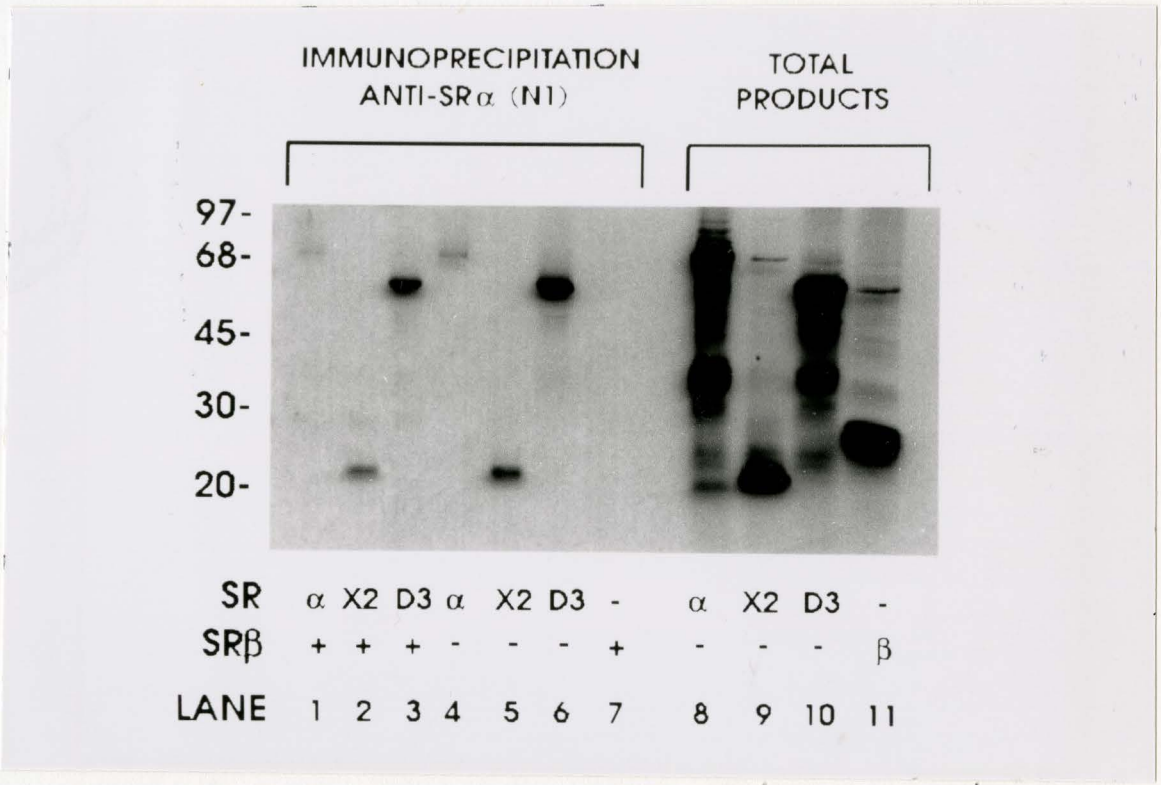


FIGURE 11: The polyclonal SRα antisera (N1) cannot be used to detect complex formation between SRβMD and SRα. SRβMD reticulocyte lysate translation products were incubated post-translationally with an equal volume of cell free synthesized SRα (lane 1), SRX2 (lane 2) or SRD3 (lane 3) for 30 minutes at 24°C. The samples were immunoprecipitated with a purified IgG fraction of the N1 SRα antisera. In addition, control immunoprecipitations for SRα, SRX2, SRD3 and SRβMD alone (lanes 4-7) were also performed. Total translation products for each of the molecules are in lanes 8-11. The migration pattern of molecular weight standards (in kilodaltons) are indicated on the left hand side of the panel.



HA residues from the HA-SRβ$_{MD}$ molecule would be lumenal (Miller *et al.*, manuscript in preparation). Moreover, since SRα is predicted to interact with the cytoplasmic domain of SRβ (Tajima *et al.*, 1986; Young *et al.*, submitted), the HA residues should not interfere with complex formation. Therefore, 10μl of SRα, SREF, SRX2 and SRD3 translation products were incubated post-translationally with 10 μl HA-SRβ$_{MD}$ and stable complexes were immunoprecipitated with anti-HA antibodies. The HA antibodies were able to co-immunoprecipitate both SRα and SRD3 (Figure 12, lanes 1 and 4), but not SREF (Figure 12, lane 2). However, due to the inefficiency of complex formation between SRα and SRβ$_{MD}$ in the absence of membranes, an SRX2/SRβ$_{MD}$ complex was likely obscured by the background bands (Figure 12, lane 3). The low levels of HA-SRβ$_{MD}$/SRα co-immunoprecipitated is unlikely a result of dissociation in the immunoprecipitation buffer since similar conditions were used to solubilize endogenous SRP receptor from microsomes and the solubilized SRα/SRβ complex remained intact (Tajima *et al.*, 1986). Therefore, it is likely that the efficiency of complex formation between SRβ$_{MD}$ and the anchoring domain of SRα is the limiting factor.

Stable complexes between SRβ$_{MD}$ and SRα were detected with the S1 SRα antibody (Figure 8). To determine optimal coimmunoprecipitation conditions, reticulocyte lysate translation products of SRα (10 μl) and SRβ$_{MD}$ (10μl) were mixed together post-translationally in the absence of membranes. The co-immunoprecipitations were performed with the S1 SRα antibodies in the presence of urea, NaSCN, Triton and various concentractions of salt (Figure 13A). Control immunoprecipitations with SRβ$_{MD}$ alone, were also performed to ensure that the co-immunoprecipitation of SRβ$_{MD}$ with the S1 SRα antibodies required the presence of SRα translation products (Figure 13B). High salt concentrations (2M NaCl) are insufficient to prevent non-specific aggregration

FIGURE 12: The HA antisera can be used to detect complex formation between HASRβ_{MD} and SRα. HASRβ_{MD} reticulocyte lysate translation products were incubated post-translationally with an equal volume of cell free synthesized SRα (lane 1), SREF (lane 2), SRX2 (lane 3) or SRD3 (lane 4) for 30 minutes at 24°C. The samples were immunoprecipitated with the HA antisera. In addition, control immunoprecipitations for SRα, SREF, SRX2, SRD3 and SRβ_{MD} alone (lanes 5-9) were also performed. Total translation products for SRα, SREF, SRD3 and SRX2 are in lanes 10-13. The migration pattern of molecular weight standards (in kilodaltons) is indicated on the left hand side of the panel. Downward pointing arrows indicate the migration postions of SRα and SRD3 in co-immunoprecipitations with HASRβ_{MD}.

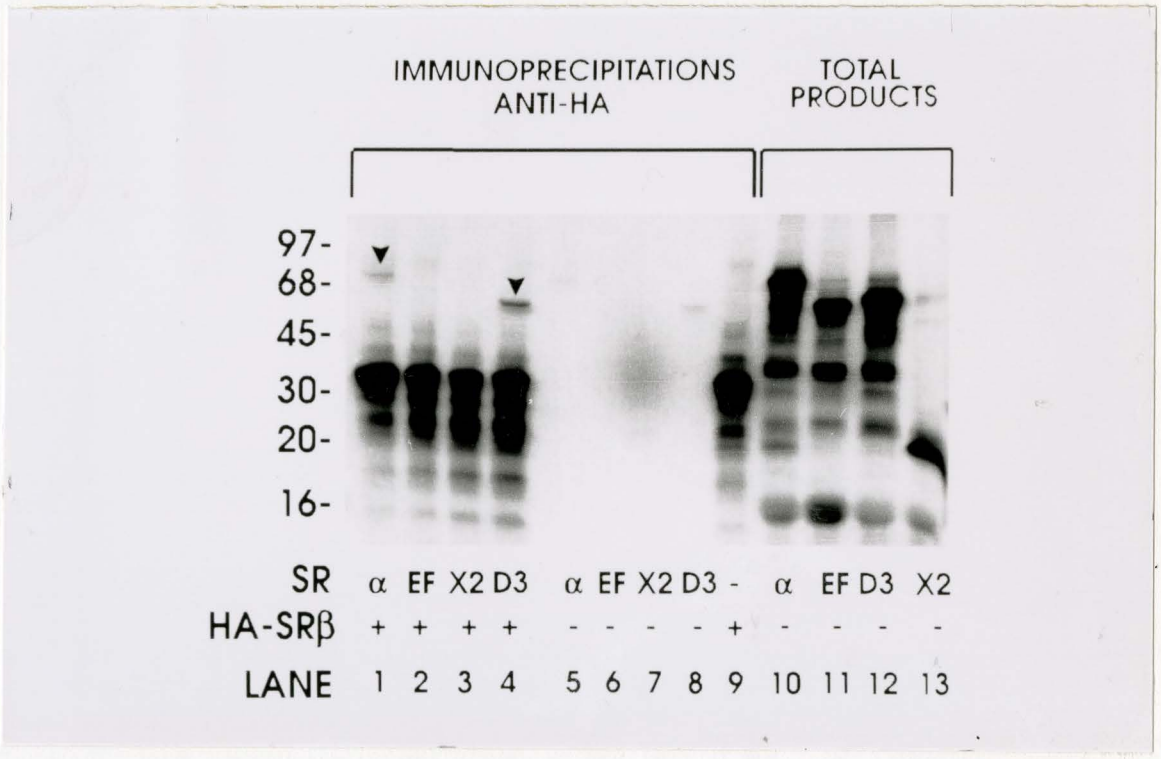
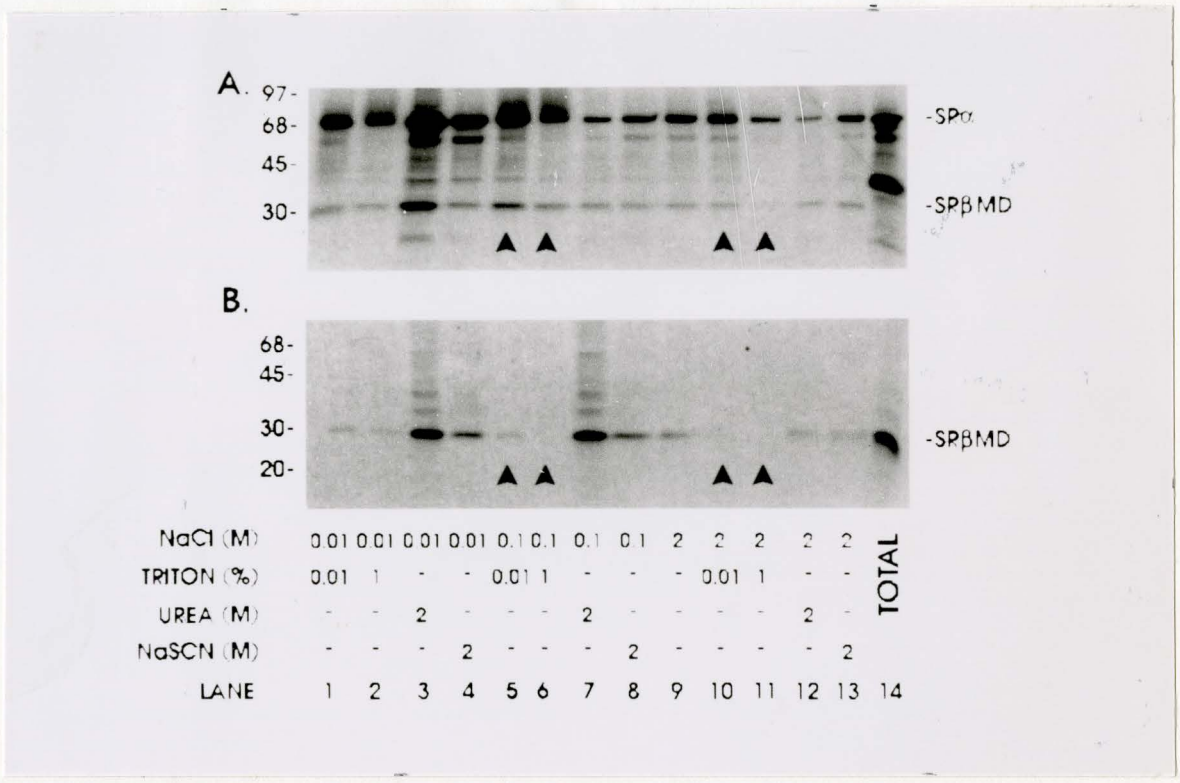


FIGURE 13: SRα/SRβ$_{MD}$ complexes are stable to high salt and detergent concentrations. (A) Reticulocyte lysate translation products of SRα and SRβ$_{MD}$ were incubated post-translationally for 30 minutes at 24°C to allow complex formation. The samples were then immunoprecipitated under various conditions with the S1 SRα antibody. The samples were immunoprecipitated with either 10mM (lanes 1-4), 100mM (lanes 5-8) or 2M NaCl (lanes 9-13). Triton at 0.01% (lanes 1, 5, 10) or 1% (lanes 2, 6, 11), 2M urea (lanes 3, 7, 12) and 2M NaSCN (lanes 4, 8, 13) were also used in the immunoprecipitations. Total translation products of SRα are indicated in lane 14. (B) Control immunoprecipitations with the monclonal SRα antibody using the various conditions described above were also performed on SRβ$_{MD}$ alone. Total translation products for SRβ$_{MD}$ are indicated in lane 14. The migration of patterns of molecular weight standards (in kilodaltons), SRα and SRβ$_{MD}$ are indicated to the sides of the panels. Upperward pointing arrows indicate conditions which resulted in specific co-immunoprecipitation of SRα and SRβ$_{MD}$.


A.
97-
68-
45-
30-
-SPα
-SPβMD
B.
68-
45-
30-
20-
-SPβMD
NaCl (M) 0.01 0.01 0.01 0.01 0.1 0.1 0.1 0.1 2 2 2 2 2
TRITON (%) 0.01 1 - - 0.01 1 - - - 0.01 1 - -
UREA (M) - - 2 - - - 2 - - - - 2 -
NaSCN (M) - - - 2 - - - 2 - - - - 2
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TOTAL

of SRβ$_{MD}$ with the antibody and/or Protein A Sepharose beads (compare Figure 13A and B, lane 9) Unfortunately, 2M urea or 2M NaSCN also resulted in the non-specific interaction of SRβ$_{MD}$ with the antibody and/or the Protein A Sepharose beads even in the presence of 2M NaCl (compare figure 13A and B, lanes 3, 4, 7, 8, 12 and 13). However, Triton either below (0.01%) or above (1%) the CMC in combination with 100mM NaCl resulted in the specific co-immunoprecipitation of one SRβ$_{MD}$ molecule per every four SRα molecules using the S1 SRα antibodies (compare figure 13A and B, lanes 5 and 6) When the ionic strength was increased to 2M NaCl, stable SRα/SRβ$_{MD}$ coimmunoprecipitates were still detected in the presence of 0.01% at a ratio of 5 SRα molecules/SRβ$_{MD}$ molecule or 1% triton at a ratio of 6 SRα molecules/SRβ$_{MD}$ molecule (compare figure 13A and B, lanes 10 and 11) Therefore, high detergent and salt concentrations do not completely abolish the interaction between SRα and SRβ$_{MD}$ but do disrupt the interaction between a population of the SRα and SRβ$_{MD}$ molecules.

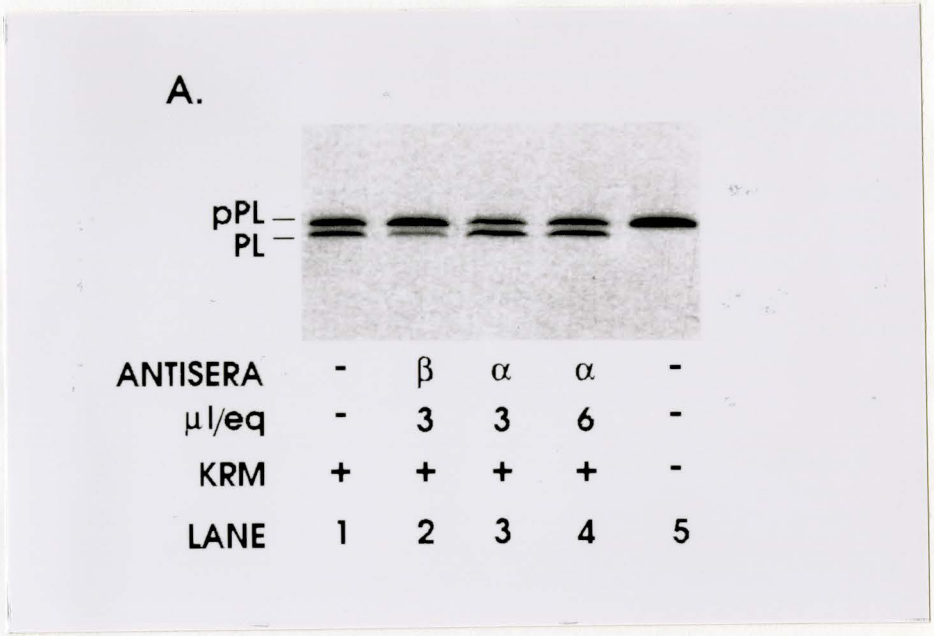
SRβ HAS A FUNCTION IN TRANSLOCATION

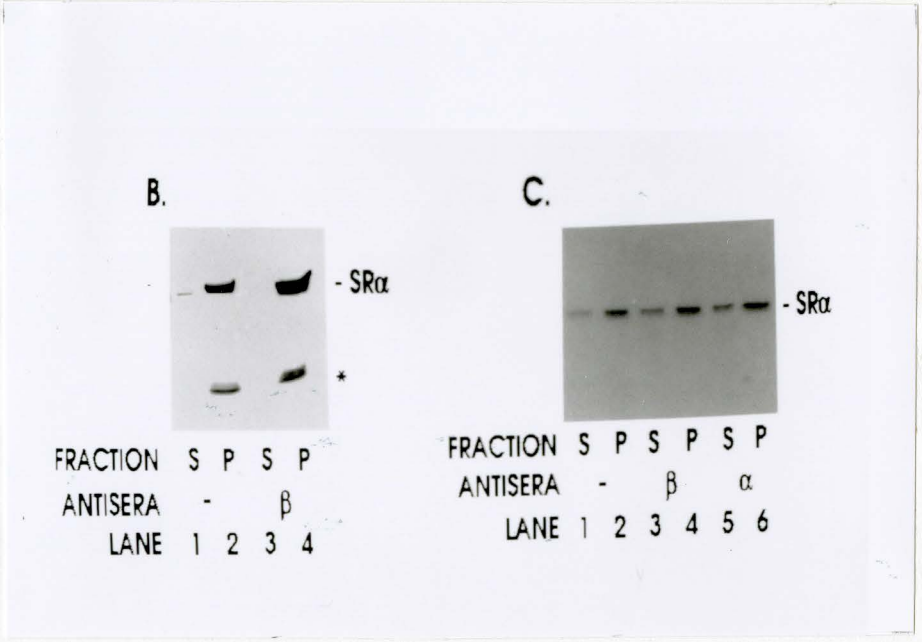
Although our data suggest that one role for SRβ is anchoring SRα on the ER membrane, SRβ has also been shown to bind GTP suggesting that it has an additional role in translocation (Miller *et al.*, 1993; Miller *et al.*, manuscript in preparation). A method used previously to demonstrate that a molecule is involved in or adjacent to the translocation machinery is inhibition of translocation by specific antisera (Hartmann *et al.,* 1989; Watanabe and Blobel, 1989). Therefore, column washed, KRM were incubated for one hour with antisera either to the last 40 amino acids of SRβ or to the membrane targeting domain of SRα. The membranes were then isolated by centrifugation through a sucrose cushion, resuspended and assayed for translocation of preprolactin as

above. Binding of equivalent amounts of the antibodies to the membranes was confirmed by immunoblotting (data not shown). When one equivalent of control membranes incubated without serum were added to a preprolactin translation reaction, 40% of the preprolactin molecules were translocated and cleaved to prolactin (Figure 14A, lane 1). When membranes were added that had been incubated with 3µl/eq of SRβ antiserum translocation of preprolactin was reduced to 19% (Figure 14A, lane 2). However, incubation of KRM with 3µl of SRα antisera/eq of membranes did not affect preprolactin translocation as 55% of the preprolactin molecules were processed to the mature form (Figure 14A, lane 3). When the amount of SRα antiserum added to the membranes was doubled, 47% of the preprolactin molecules were still translocated (Figure 14A, lane 4). Therefore, preincubation with the SRα antiserum has a negligible effect on the translocation activity of membranes in reticulocyte lysate. Moreover, it is unlikely that the inhibition of translocation observed when membranes were incubated with the SRβ antisera was due to a non-specific effect of the serum. In addition, since SRα and SRβ are part of the same complex on the ER membrane, the inhibition of translocation was unlikely to be due to crosslinking of the receptors.

Previous results suggest that SRβ is involved in anchoring SRα to the ER membrane (Figure 6). It is possible therefore, that the inhibition of translocation activity was due to displacement of SRα from either the ER membrane or SRβ. To examine this possiblity, microsomes incubated with 5µl antisera/eq of membranes and isolated by centrifugation were used to assay urea resistant anchoring of endogenous SRα. After incubation with antisera to SRβ the endogenous SRα molecules remained stably anchored to membranes extracted with 2M urea suggesting that the antisera does not dissociate the SRP receptors on the ER membrane (Figure 14B, compare lanes 2 & 4).

FIGURE 14: SRβ antisera inhibits translocation of preprolactin but not assembly of SRα. (A) SRβ but not SRα antisera inhibits translocation of preprolactin. Microsomes (KRM) incubated without the antisera or the relative amount (μl/eq KRM) indicated. The membranes were layered onto a 0.5M sucrose cushion and isolated by centrifugation at 100 000xg for 10 minutes. Reticulocyte lysate translation reactions encoding preprolactin (10μl) were incubated with 1μl KRM as specified (lanes 1-4). Conversion of preprolactin (pPL) to prolactin (PL) indicates translocation activity. (B) SRβ antisera does not dissociate SRα and SRβ on the ER membrane. Microsomes (KRM) were incubated without (lanes 1, 2) or with 5μl/eq of SRβ antisera (lanes 3, 4). The membranes were isolated as above, resuspended, adjusted to 2M urea, layered onto a sucrose cushion and centrifuged for 15 minutes at 160 000 xg. Samples were divided into supernatant (lanes 1, 3) and pellet (2, 4) fractions. The supernatant fractions were concentrated by TCA precipitation. The supernatant and pellet fractions were analyzed by an immunoblot using monoclonal SRα antibodies. The lower band (*) represents a degradation product of SRα. (C) SRβ and SRα antisera do not prevent SRα anchoring to the membrane. Microsomes were incubated with 5μl/eq of antisera as indicated and isolated by centrifugation as above. Post-ribosomal supernatants of SRα (20μl) were incubated with 5μl of the resuspended membranes for 15 minutes at 24°C. The samples were adjusted to 2M urea/25mM EDTA and fractionated by centrifugation as above. Samples were divided into supernatant (lanes 1, 3, 5) and pellet (lanes 2, 4, 6) fractions. Migration positions of SRα, pPL, and PL are indicated to the side of the panels.


A.
pPL
PL
ANTISERA - β α α -
µl/eq - 3 3 6 -
KRM + + + + -
LANE 1 2 3 4 5


B.
- SRα
*
FRACTION S P S P
ANTISERA - β
LANE 1 2 3 4
C.
- SRα
FRACTION S P S P S P
ANTISERA - β α
LANE 1 2 3 4 5 6

Furthermore, preincubation of the membranes with either SRα or SRβ antisera had no effect on the urea resistant anchoring of exogenously added SRα molecules (Figure 14C, compare lanes 2, 4 & 6). Therefore, the ability of the SRβ antisera to inhibit preprolactin translocation is unlikely to be due to physical displacement of the SRα from the ER membrane. Taken together these results suggest that SRβ is involved in another as yet undefined process in translocation.

CHAPTER FOUR

ANCHORING EFFICIENCY OF SRα ON T5KRM

The urea resistant anchoring of SRα on T5KRM required the presence of exogenous, full length $SR\beta_{MD}$, as presented in Figure 6. This suggests that SRβ is the ER membrane component responsible for anchoring SRα. Over four separate experiments, the average amount of SRα on T5KRM stable to extraction with 2M urea was found to be 38 ± 16%(n=4) (Table 1A). In addition, over three of the experiments, on average 1.4 SRα molecules are anchored onto T5KRM for every $SR\beta_{MD}$ molecule (Table 1B). This suggests that each membrane assembled $SR\beta_{MD}$ molecule can anchor one SRα molecule to the ER membrane.

Over the four separate experiments the limiting factor in the reconstitution of anchoring of SRα to T5KRM was efficient membrane assembly of $SR\beta_{MD}$. On average, 38 ± 16% of the SRα molecules and only 10.4 ± 6.2% of the $SR\beta_{MD}$ molecules were anchored to T5KRM. It seems that once an $SR\beta_{MD}$ molecule is assembled into T5KRM it can anchor a pre-existing SRα molecule quite efficiently. Therefore, to anchor greater than 38% of the SRα molecules to T5KRM, the efficiency of anchoring of $SR\beta_{MD}$ must be improved.

TRANSLOCATION ACTIVITY OF T5KRM

The major cause for the low efficiency of assembly of $SR\beta_{MD}$ onto T5KRM is the poor translocation activity of the membranes. The data presented in Figure 6 shows that the reconstitution of translocation of preprolactin across the T5KRM was 18%. Therefore, the assembly of only 10% of the $SR\beta_{MD}$ molecules onto T5KRM was not unexpected (Figure 6). On the other hand,

TABLE 1: Quantitative analysis of SRα anchoring onto T_5KRM reconstituted with $SR\beta_{MD}$. (A) To determine the average amount of anchored SRα molecules on T_5KRM over four experiments, the amount of SRα in the top, middle and bottom fractions for both the control and experimental conditions were quantitated by densitometry. The percentage of SRα in the pellet was calculated by dividing the amount present in the pellet by the total amount of protein produced (B/T+M+B). To determine the percentage of SRα which were anchored to the T_5KRM, the percent of the molecules in the pellet in the control reaction ($SR\beta_{MD}$ added post-translationally) were subtracted from the percent of the molecules in the pellet in the experimental reaction ($SR\beta_{MD}$ added co-translationally). The data for Figure 6 is represented in experiment #1. (B) To determine the average ratio of SRα :$SR\beta_{MD}$ which were anchored onto T_5KRM in the experimental reaction, the amount of the two molecules present in the pellet was determined by densitometry. The percent of SRα and $SR\beta_{MD}$ present in the pellet of the control reaction was subtracted from the total amount of SRα and $SR\beta_{MD}$ in the pellet of the experimental condition. These values were then normalized for the number of methionine residues in each of the proteins and the ratio of SRα/$SR\beta_{MD}$ on T_5KRM was then obtained. The data for Figure 6 is represented by experiment #1. Data for experiment #2 were not obtained.

A.

	SRα in pellet		
Expt. #	EXPERIMENTAL[1]	CONTROL[2]	Δ[3]
1	61%	18%	43%
2	80%	31%	49%
3	62%	18%	44%
4	56%	41%	15%
		Average:	38±16%

[1] $SR\beta_{MD}$ was assembled onto the reconstituted membranes co-translationally.

[2] $SR\beta_{MD}$ was assembled onto the reconstituted membranes post-translationally.

[3] Difference between percentages from 1 and 2.

B.

Expt. #	SRα in pellet[1]	$SR\beta_{MD}$ in pellet[2]	SRα/$SR\beta_{MD}$[3]
1	151	109	1.4/1
3	176	162	1.1/1
4	153	91	1.7/1
		Average:	1.4±0.3

[1] This value represents the total amount of SRα anchored to the T_5KRM after normalizing for the levels nonspecific pelleting and the number of methionines in the molecule. .

[2] This value represents the total amount of $SR\beta_{MD}$ anchored to the T_5KRM after normalizing for the levels nonspecific pelleting and the number of methionines in the molecule. .

[3] This value represents the ratio of the SRα/$SR\beta_{MD}$ molecules which are anchored to the T_5KRM..

when T_5KRM were able to translocate 50% of the preprolactin molecules, 61% of the SRβ_{MD} molecules were stably assembled onto the membranes (Figure 4). Therefore, the translocation activity of the membranes must be improved in order to optimize the ability of the membranes to anchor SRα. Several attempts were made at varying the amounts of preprolactin and SRβ_{MD} synthesized with respect to the SRα used to reconstitute translocation to the T_5KRM. By decreasing the amount of preprolactin translation products, the translocation activity of T_5KRM was marginally increased (data not shown). However, when the volume of the translation reaction for SRβ_{MD} was decreased accordingly, the synthesis of SRβ_{MD} was significantly reduced making the results uninterpretable (data not shown). Therefore, a new batch of T_5KRM must be prepared which can translocate at least 50% of the preprolactin molecules after reconstitution with SRα. Only under such conditions can one ensure that enough SRβ_{MD} molecules are properly assembled onto T_5KRM.

INHIBITION OF TRANSLATION OF SRβ_{MD}

Once T_5KRM are prepared which are competent for SRP receptor mediated translocation of SRβ_{MD}, a secondary problem exists which may also contribute to the low levels of membrane assembled SRβ_{MD} molecules. The total synthesis of SRβ_{MD} was reduced when the translation reactions were added co-translationally to membranes reconstituted with SRα. The reduction of SRβ_{MD} synthesis was calculated by determining the total translation products generated when SRβ_{MD} was added to T_5KRM either co- or post-translationally. The average synthesis of SRβ_{MD} over four separate experiments was 1.9 ± 1.2 fold less when the molecule was translated in the presence of T_5KRM reconstituted with SRα (Table 2).

TABLE 2: Quantitative analysis of the inhibition of translaton of $SR\beta_{MD}$ (B) Densitometry was used to determine the average inhibition of $SR\beta_{MD}$ synthesis when translation reactions were added to post ribosomal supernatants of SRα co-translationally. The total amount of $SR\beta_{MD}$ produced when added either co- or post-translationally was determined by adding the amount of the protein in the top, middle and bottom fractions. The total amount of $SR\beta_{MD}$ produced from the post-translational control was divided by the amount of $SR\beta_{MD}$ produced when added co-translationally to the membranes. The data in Figure 6 is represented in experiment #1 from the table..

Experiment #	SRβ (POST)[1]	SRβ (CO)[2]	POST/CO
1	4448	3372	1.3
2	18421	13638	1.4
3	11311	9744	1.2
4	6541	1795	3.6
		Average:	1.9± 1.2

[1] $SR\beta_{MD}$ was assembled co-tranlationally onto the reconstituted membranes.

[2] $SR\beta_{MD}$ was assembled post-translationally onto the reconstituted membranes.

$SR\beta_{MD}$ synthesis is not affected by the presence of T_5KRM during the translation reaction (Figure 3C), but is reduced when $SR\beta_{MD}$ is translated in the presence of T_5KRM reconstituted with a post-ribosomal supernatant of $SR\alpha$ (Figures 4 and 6). This suggests that the reticulocyte lysate changes over the course of the hour in the translation/incubation reaction. One possible way to improve the translation levels of $SR\beta_{MD}$ would be to isolate the T_5KRM reconstituted with $SR\alpha$ prior to addition of the translation reaction for $SR\beta_{MD}$ to remove the reticulocyte lysate. Therefore, trypsin treated membranes were incubated with a post-ribosomal supernatant of $SR\alpha$ and then isolated by centrifugation through a sucrose cushion. The membranes were resuspended in the new translation reaction encoding $SR\beta_{MD}$ and translation was allowed to proceed for one hour. Unfortunately, the translocation activity of the T_5KRM was almost completely abolished since only 6% of the $SR\beta_{MD}$ molecules were membrane assembled (Figure 15, lane 3). However, all of the $SR\alpha$ molecules remained associated with the membranes whether the $SR\beta_{MD}$ was assembled onto the membranes co- or post- translationally (Figure 15, compare lanes 3 and 6).

Therefore, it seems that the membranes did not survive the centrifugation process used to remove the reticulocyte lysate. However, it may be possible to assemble $SR\beta_{MD}$ on T_5KRM before the reticulocyte lysate becomes inhibitory to translation. Therefore, an $SR\alpha$ translation reaction was allowed to proceed co-translationally for 20 minutes in the presence of T_5KRM prior to the addition of a new translation reaction encoding either $SR\beta_{MD}$ or preprolactin. Presumably, this would provide sufficient time for the anchoring domain of $SR\alpha$ to emerge from the ribosome and target to the membrane. Trypsin treated membranes reconstituted in this manner translocated 30% of the preprolactin molecules and 16% of the $SR\beta_{MD}$ molecules (Figure 16A,

FIGURE 15: Isolation of the reconstituted membranes by centrifugation prior to SRβ_{MD} assembly causes SRα to pellet with the membranes. Post-ribosomal supernatants of SRα (10μl) were incubated with 1 equivalent of T_5KRM for 15 minutes at 24°C. The membranes were then isolated by centrifugation through a sucrose cushion at 100 000Xg for 10 minutes. The membranes were resuspended in translation reactions encoding SRβ_{MD} added either co-translationally (lanes 1-3) or post-translationally as a control (lanes 4-6). The translation products were extracted with 2M urea and 25mM EDTA for 10 minutes on ice. The membranes were then isolated by centrifugation over a 0.5M sucrose cushion at 160 000 xg for 15 minutes. Samples were divided into top (lanes 1, 4), middle (lanes 2, 5) and bottom (lanes 4, 6) fractions. Migration pattern of molecular weight markers (in kilodaltons), SRα and SRβ_{MD} are indicated on the sides of the panel.

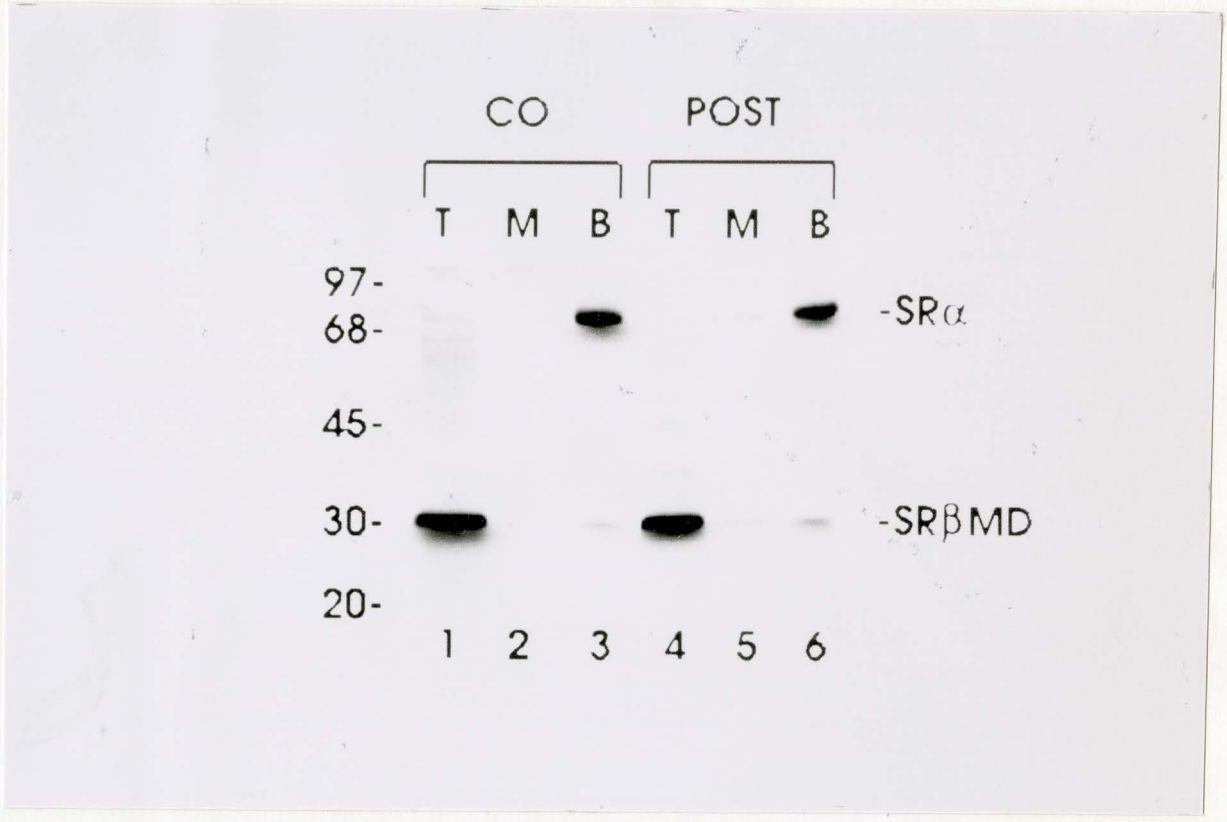
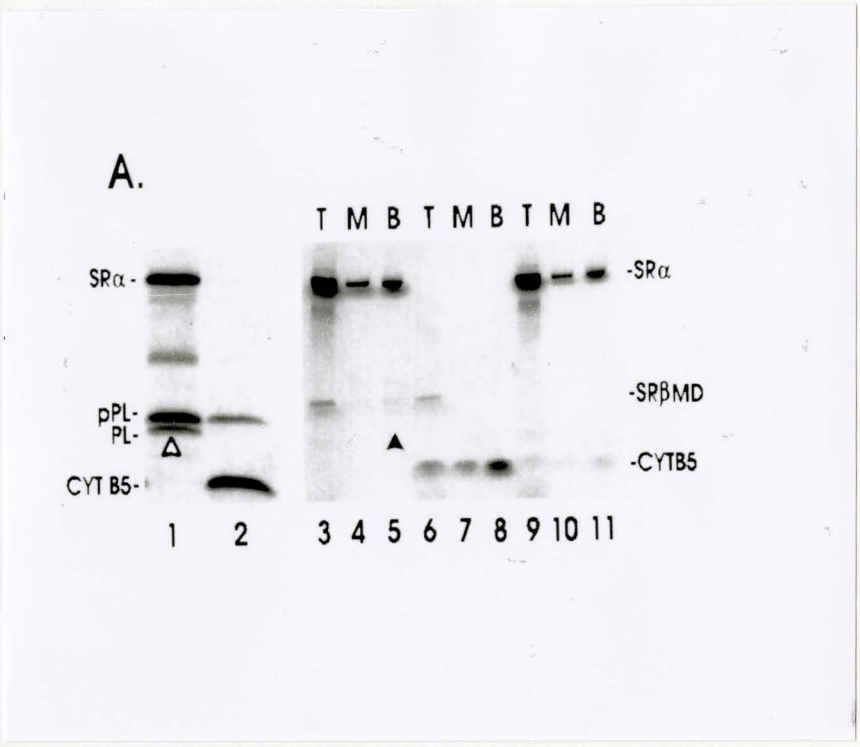
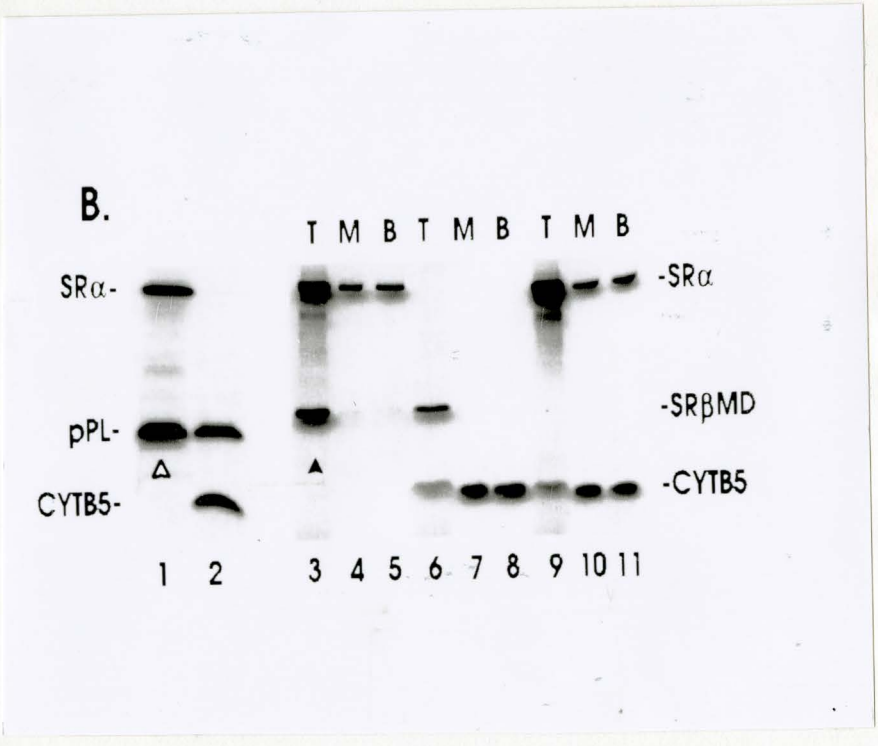

CO
POST
T M B T M B
97-
68-
45-
30-
20-
-SRα
-SRβMD
1 2 3 4 5 6

FIGURE 16: Co-translational reconstitution of T_5KRM with SRα prior to addition of $SR\beta_{MD}$. (A) Translation reactions encoding SRα or cytochrome b5 (10μl) were incubated co-translationally with 1 equivalent of T_5KRM for 20 minutes at 24°C. Translation reactions encoding preprolactin (pPL) (10μl) were added co-translationally to the membranes reconstituted with SRα (lane 1) or cytochrome b5 (lane 2). Translocation competence of the membranes was assayed by conversion of preprolactin to the mature form of the protein. Translation reactions encoding $SR\beta_{MD}$ were added co-translationally to T_5KRM reconstituted with SRα (lanes 3-5) or cytochrome b5 (lanes 6-8). As a control for SRα assembly, a translation reaction encoding cytochrome b5 was added instead to the membranes reconstituted with SRα (lanes 9-11). The translation products were extracted with 2M urea and 25mM EDTA for 10 minutes on ice. The membranes were then isolated by centrifugation over a 0.5M sucrose cushion at 160 000 xg for 15 minutes. Samples were divided into top (lanes 3, 6, 9), middle (lanes 4, 7, 10) and bottom (lanes 5, 8, 11) fractions. (B) Same as A except the translation reactions encoding SRα or cytochome b5 were only incubated co-translationally with one equivalent of T_5KRM for 10 minutes at 24°C. Migration patterns of SRα, $SR\beta_{MD}$, cytochrome b5, pPL and PL are indicated to the sides of the panels. Upperward pointing open arrowheads indicates the level of reconstitution of preprolactin translocation to the T_5KRM. Upperward pointing solid arrowheads indicates the level of reconstitution of assembly of $SR\beta_{MD}$ to T_5KRM.


A.
T M B T M B T M B
SRα-
pPL-
PL-
CYT B5-
-SRα
-SRβMD
-CYTB5
1 2 3 4 5 6 7 8 9 10 11


B.
T M B T M B T M B
SRα-
pPL-
CYTB5-
-SRα
-SRβMD
-CYTB5
1 2 3 4 5 6 7 8 9 10 11

lanes 1 and 5). However, pre-incubation of the SRα translation reaction for 20 minutes was sufficient to make the lysate inhibitory translation of $SR\beta_{MD}$. Therefore, the amount of $SR\beta_{MD}$ produced was low whether the translation reaction was added to T_5KRM reconstituted with SRα (Figure 16A, lanes 3-5) or mock reconstituted with cytochrome b5 (Figure 16A, lanes 6-8). The small population of membrane assembled $SR\beta_{MD}$ molecules anchored only 7% of the SRα molecules. Unfortunately, the ratio of SRα/$SR\beta_{MD}$ on the T_5KRM could not be determined since densitometry of SRα and $SR\beta_{MD}$ could not be performed from a single exposure.

In an attempt to improve translation of $SR\beta_{MD}$, the experiment was repeated but the translation of SRα was only allowed to proceed for 10 minutes in the presence of T_5KRM prior to the addition of the translation reaction for $SR\beta_{MD}$. In doing so, the levels of synthesis of $SR\beta_{MD}$ improved dramatically (Figure 16B, compare lanes 3 and 6). However, anchoring of both SRα and $SR\beta_{MD}$ onto the membranes was completely abolished (Figure 16B, compare lanes 5 and 8). In fact, T_5KRM repopulated with SRα in this manner only translocated 10% of the preprolactin molecules (Figure 16B, lane 1). A 10 minute translation is likely insufficient to allow enough time for synthesis and membrane assembly of SRα prior to the addition of $SR\beta_{MD}$ or preprolactin.

TARGETING OF SRα AND $SR\beta_{MD}$ TO SAME MICROSOME

Once the translocation function of T_5KRM and translation levels of $SR\beta_{MD}$ are optimized, one final factor exists which may affect the efficiency of assembly of $SR\beta_{MD}$ on T_5KRM. Since SRα and $SR\beta_{MD}$ are synthesized and targeted to T_5KRM in separate translation reactions, it is difficult to ensure that the SRα and $SR\beta_{MD}$ molecules would efficiently assemble in the vicinity of the same microsome. To improve this, the SRα and $SR\beta_{MD}$ molecules should be

targeted to the same site on the membrane during translation. Therefore, it would be useful to encode both SRα and SRβ$_{MD}$ in a bicistronic transcript so that both proteins can be synthesized in a single translation reaction in the vicinity of one another.

To generate this bicistronic transcript, one can make use of a particular sequence in the 5'UTR of the encephalomyocarditis (EMC) virus. The EMC leader allows the ribosome to initiate internally through secondary structural elements in the RNA (Jang and Wimmer, 1990). The ribosome then scans the message until it reaches a pyrimidine rich stretch which signals for efficient initiation of translation from the next AUG (Jang and Wimmer, 1990). In contrast, in most cellular protein synthesis the ribosome initiates at the beginning of a transcript and scans the message until it reach the start codon in the context of a Kozak consensus sequence (Kozak, 1989).

Therefore, the EMC leader sequence was used to generate two plasmids which would produce a bicistronic transcript encoding both SRα and SRβ$_{MD}$. First, the cDNA for SRα was cloned behind the EMC leader resulting in the plasmid EMC-SRα (Figure 17). The EMC-SRα DNA sequence was then placed downstream from the cDNA encoding SRβ$_{MD}$ in the vector pSPUTK (DV$_{\beta/\alpha}$ - Figure 17). The converse plasmid was also made in which the cDNA for SRβ$_{MD}$ was cloned behind the EMC leader resulting in the plasmid EMC-SRβ$_{MD}$. The EMC-SRβ$_{MD}$ DNA sequence was then cloned downstream of an SRα cDNA in the vector pSPUTK (DV$_{\beta/\alpha}$ - Figure 18).

The bicistronic transcripts produced from these plasmids should result in synthesis of SRα and SRβ$_{MD}$ in the vicinity of the same microsome and hopefully increase the efficiency of assembly of SRβ$_{MD}$ on T$_5$KRM. Theoretically, these transcripts may allow stable assembly of SRβ$_{MD}$ on T$_5$KRM from a single translation reaction without prior reconstitution of the

FIGURE 17: Construction of DVβ/α (pMP572): (A) The EMC leader was excised from the vector EMCSpPt (pMP266) as an *Nhe* I/*Nco* I fragment. An amino terminal deletion mutant of SRα, SRX1, in the vector pSPUTK was also cleaved with the enzymes *Nhe* I and *Nco* I. The EMC 5'UTR was inserted to the 5' end of the SRXI coding sequence resulting in the plasmid EMCSRX1. (B) The rest of the SRα coding region was added back onto the deletion mutant EMCSRX1 by cleaving both full length SRα (pMP191) and the N-terminal mutant with *Afl* II and *Cla* I resulting in the plasmid EMCSRα (pMP525). (C) To construct DVβ/α, the region encoding EMCSRα should be inserted behind the cDNA for SRβ_{MD} in the vector pSPUTK (pMP455). In order to accomplish this, the *Bam* HI site in the polylinker of pMP455 had to be removed from its original position and re-inserted after the *Eco* RI site. Therefore, SRβ_{MD} (pMP455) was cleaved with *Kpn* I, releasing the *Bam* HI site and subsequently religated. (D) The resulting plasmid was then linearized at a unique *Eco* RV site, following the *Eco* RI site, and a *Bam* HI linker was inserted. (E) EMCSRα and SRβ_{MD} (+*Bam* HI) were digested with *Bam* HI and *Eco* RI. In order to simplify the cloning procedure, EMCSRα was also digested with *Sca* I for better separation of the EMCSRα fragment which was then inserted behind SRβ_{MD} in the vector pSPUTK. (F) The resulting plasmid DVβ/α generates one bicistronic transcript from the SP6 promoter that contains the coding region for SRβ_{MD} behind the UTK leader followed by SRα behind the EMC leader.

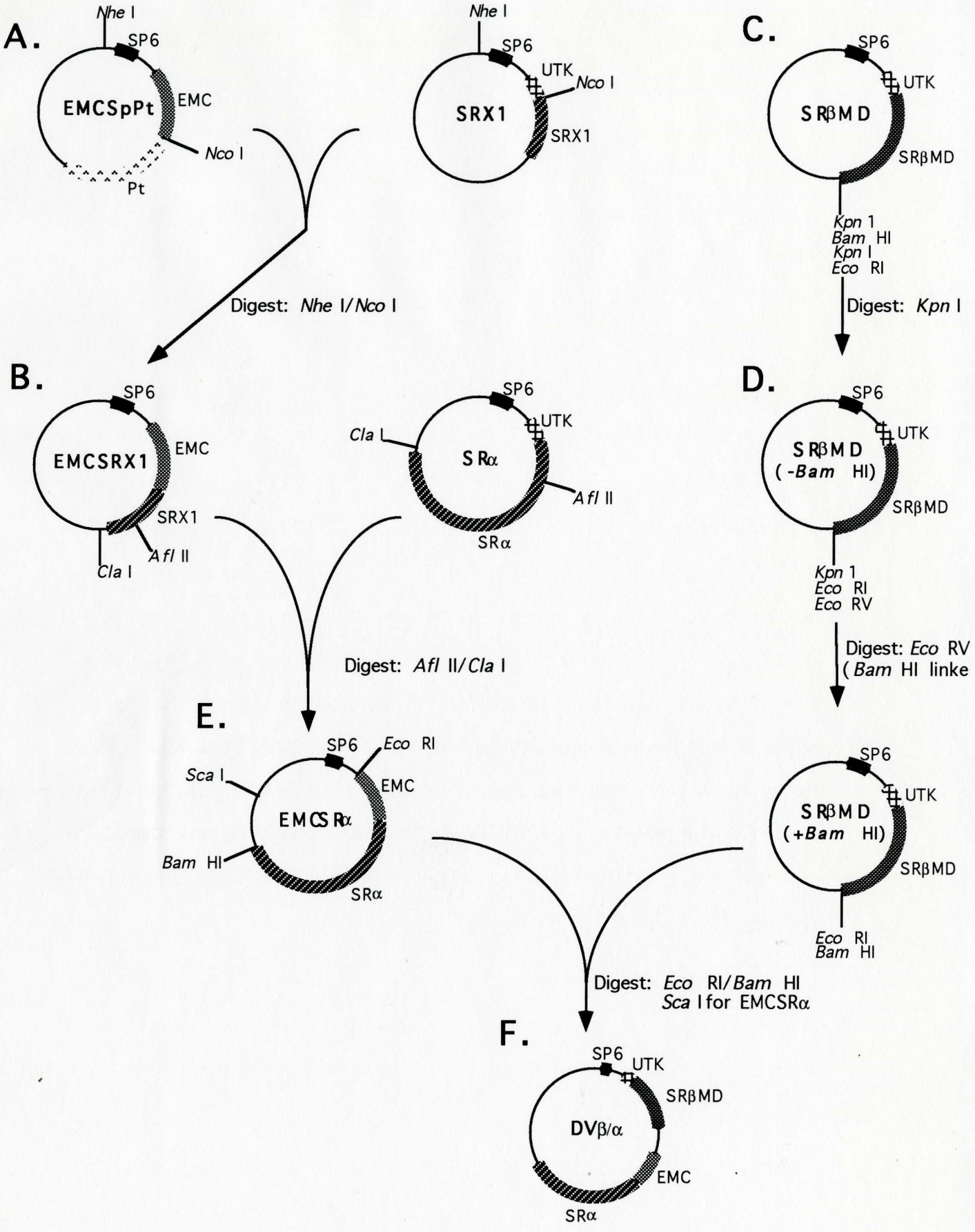
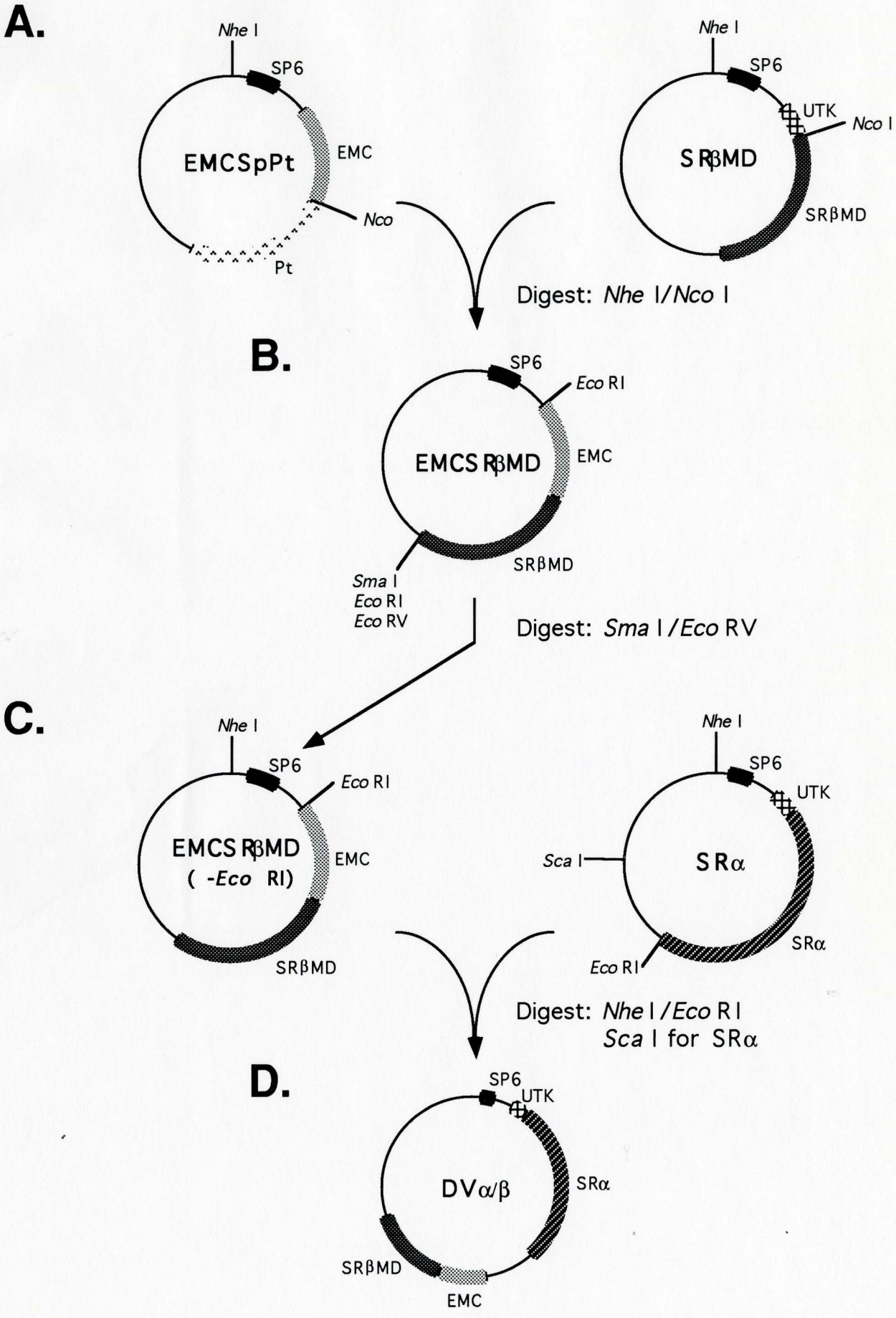

A.
Nhe I
SP6
EMCSpPt
EMC
Nco I
Pt
Nhe I
SP6
UTK
Nco I
SRX1
SRX1
C.
SP6
UTK
SRβMD
SRβMD
Kpn 1
Bam HI
Kpn I
Eco RI
Digest: Nhe I/Nco I
Digest: Kpn I
B.
SP6
EMCSRX1
EMC
SRX1
Afl II
Cla I
SP6
UTK
Cla I
SRα
Afl II
SRα
D.
SP6
UTK
SRβMD
(-Bam HI)
SRβMD
Kpn 1
Eco RI
Eco RV
Digest: Afl II/Cla I
Digest: Eco RV
(Bam HI linke
E.
SP6
Eco RI
Sca I
EMC
EMCSRα
Bam HI
SRα
SP6
UTK
SRβMD
(+Bam HI)
SRβMD
Eco RI
Bam HI
Digest: Eco RI/Bam HI
Sca I for EMCSRα
F.
SP6
UTK
SRβMD
DVβ/α
EMC
SRα

FIGURE 18: Construction of $DV_{\alpha/\beta}$ (pMP642): (A) The EMC leader was excised from the vector EMCSpPT (pMP266) as an *Nhe* I/*Nco* I fragment. $SR\beta_{MD}$ in the vector pSPUTK was also cleaved with *Nhe* I and *Nco* I. The EMC 5'UTR was inserted amino terminal to the $SR\beta_{MD}$ coding sequence resulting in the plasmid $EMCSR\beta_{MD}$. (B) An *Eco* RI site in the polylinker of $EMCSR\beta_{MD}$ was removed by digestion with *Sma* I and *Eco* RV to release the restriction site and then religated, thereby leaving only the *Eco* RI site to the 5' end of the EMC leader. (C) Plasmids containing $SR\alpha$ (pMP191) and $EMCSR\beta_{MD}$ coding sequences were then cleaved with *Nhe* I and *Eco* RI. pMP191 was also digested with *Sca* I to allow for better separation of the desired fragment. The $SR\alpha$ cDNA was inserted in front of the $EMCSR\beta_{MD}$ sequence and the fragments were religated. (D) The resulting plasmid $DV_{\alpha/\beta}$ generates one bicistronic transcript from the SP6 promoter that contains the coding region for $SR\alpha$ behind the UTK leader followed by $SR\beta_{MD}$ behind the EMC leader.


A.
Nhe I
SP6
EMCSpPt
EMC
Nco
Pt
Nhe I
SP6
UTK
Nco I
SRβMD
SRβMD
Digest: Nhe I/Nco I
B.
SP6
Eco RI
EMCSRβMD
EMC
SRβMD
Sma I
Eco RI
Eco RV
Digest: Sma I/Eco RV
C.
Nhe I
SP6
Eco RI
EMCSRβMD
(-Eco RI)
EMC
SRβMD
Nhe I
SP6
UTK
Sca I
SRα
SRα
Eco RI
Digest: Nhe I/Eco RI
Sca I for SRα
D.
SP6
UTK
DVα/β
SRα
SRβMD
EMC

membranes with SRα. Both bicistronic transcripts were used in reticulocyte lysate translation reactions in the presence of T_5KRM or control membranes (KRM) and the urea resistant anchoring of either SRα or $SR\beta_{MD}$ was tested. SRα molecules produced from either transcript anchored on KRM in a urea resistant manner (Figure 19A and B, lanes 1-3). However, $SR\beta_{MD}$ molecules produced from $DV_{\alpha/\beta}$ anchored more efficiently to KRM than those molecules produced from $DV_{\beta/\alpha}$ (Figure 19A and B, lanes 1-3). In contrast, neither of these plasmids were able to anchor SRα or $SR\beta_{MD}$ on T_5KRM (Figure 19A and B, lanes 4-6). This result was unexpected since the SRα and $SR\beta_{MD}$ molecules should have been synthesized in the vicinity of the same microsome.

Prior to the knowledge that a one hour old reticulocyte lysate is inhibitory to translation, the trypsin treated membranes were reconstituted with SRα prior to the addition of translation reactions encoding the bicistronic transcripts. The $SR\beta_{MD}$ molecules produced from $DV_{\beta/\alpha}$ or DVα/β should assemble onto the reconstitued T_5KRM as in Figure 4. Reconstitution of anchoring of SRα may also become more efficient since newly synthesized SRα molecules should in the vicinity of the properly integrated $SR\beta_{MD}$ molecules. However, when the translation reaction encoding $DV_{\beta/\alpha}$ was added to T_5KRM reconstituted with either SRα or SRD3, synthesis of SRα and $SR\beta_{MD}$ from the bicistronic transcript was abolished (Figure 20, lanes 1-3 and 4-6). Therefore, a component exists in the reticulocyte lysate which also inhibits translation of SRα and $SR\beta_{MD}$ from the bicistronic transcript $DV_{\beta/\alpha}$. The experiment was performed with $DV_{\alpha/\beta}$ as well, and similar results were obtained (data not shown).

Therefore, in order to improve the efficiency of SRα anchoring on T_5KRM, the efficiency of assembly of $SR\beta_{MD}$ must be improved. Several strategies were attempted and to be proved unsuccessful. The most crucial

FIGURE 19: Membrane anchoring of SRα and SRβ_{MD} from DV$_{\beta/\alpha}$ and DV$_{\alpha/\beta}$. (A) Translation reactions encoding DV$_{\beta/\alpha}$ (20μl) were incubated co-translationally with 1 equivalent of KRM (lanes 1, 2) or T$_5$KRM (lanes 3, 4) for 1 hour at 24°C. In addition a minus membrane control was included (lanes 5, 6). The samples were then extracted with 2M urea and 25mM EDTA for 10 minutes on ice. Membranes were isolated by centrifugation through a 0.5M sucrose cushion at 160 000 xg for 15 minutes. The samples were divided into top (lanes 1, 4, 7), middle (lanes 2, 5, 8) and bottom (lanes 3, 6, 9) fractions. (B) Translation reactions encoding DV$_{\alpha/\beta}$ (20μl) were incubated co-translationally with 1 eq of KRM (lanes 1, 2), T$_5$KRM (lanes 3, 4) or in the absence of membranes (lanes 5, 6). The reactions were extracted with 2M urea and 25mM EDTA as described above. Membranes were isolated by centrifugation and the samples were fractionated as described in A. Migration of molecular weight standards (in kilodaltons), SRα and SRβ_{MD} are indicated on the sides. Upperward pointing open arrows indicates the migration position of SRα in the pellet fraction with T$_5$KRM. Upperward pointing solid arrows indicates the migration position of SRβ_{MD} in the pellet fraction with T$_5$KRM.

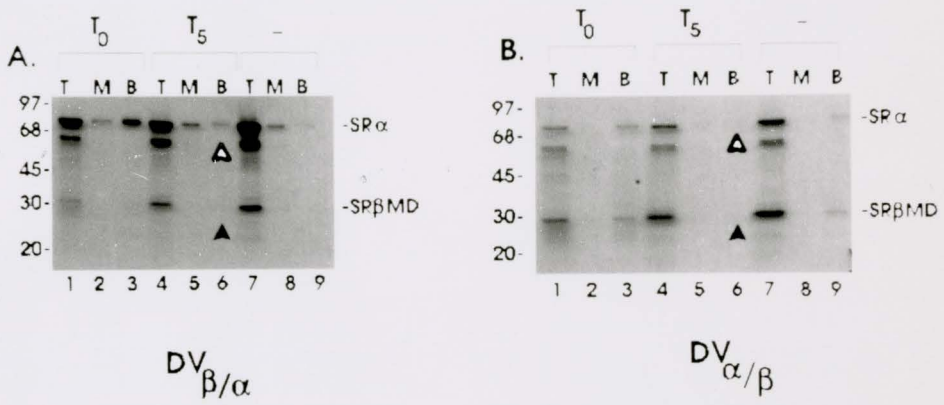
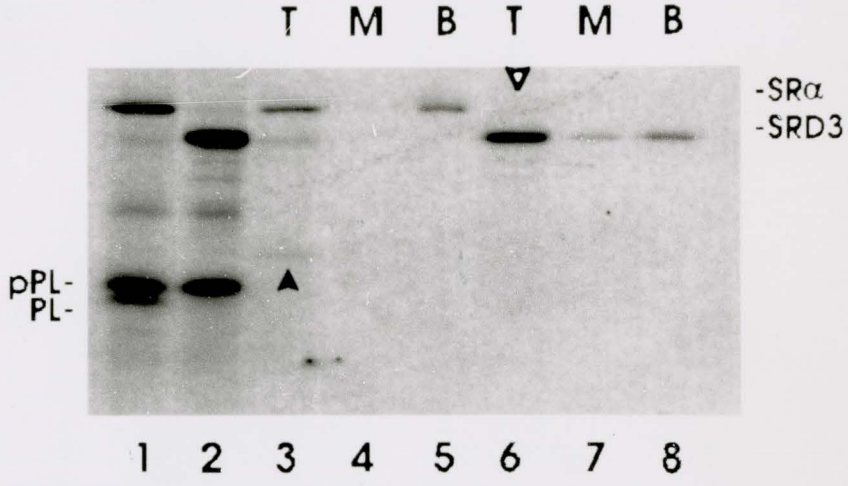

A.
T_0
T_5
–
T M B T M B T M B
97-
68-
45-
30-
20-
-SRα
-SRβMD
1 2 3 4 5 6 7 8 9
$DV_{\beta/\alpha}$
B.
T_0
T_5
–
T M B T M B T M B
97-
68-
45-
30-
20-
-SRα
-SRβMD
1 2 3 4 5 6 7 8 9
$DV_{\alpha/\beta}$

FIGURE 20: Synthesis of DVβ/α is inhibited when added to reconsituted T_5KRM. Post-ribosomal supernatants of SRα or SRD3 (10μl) were incubated with 1 equivalent of T_5KRM for 15 minutes at 24°C. Translation reactions encoding preprolactin were added to T_5KRM reconstituted with SRα (lane 1) or SRD3 (lane 2). Translocation activity was assayed by conversion to the mature form of the protein, prolactin Translation reactions encoding DVβ/α were also added co-translationally to T_5KRM reconstituted with SRα (lanes 3-5) or SRD3 (lanes 6-8). The translation products were extracted with 2M urea and 25mM EDTA for 10 minutes on ice. The membranes were then isolated by centrifugation over a 0.5M sucrose cushion at 160 000 xg for 15 minutes. Samples were divided into top (lanes 3, 6), middle (lanes 4, 7) and bottom (lanes 5, 8) fractions. Migration of SRα, SRD3, pPL and PL are indicated on the sides. The downward pointing open arrow indicates the migration po.sition of SRα and the upward pointing solid arrow indicates the migration position of SRβ_{MD}.


T M B T M B
-SRα
-SRD3
pPL-
PL-
1 2 3 4 5 6 7 8

factor to improve the assembly of $SR\beta_{MD}$ on T_5KRM is to obtain a set of membranes which can be easily reconstituted for SRP dependent translocation. process.

CHAPTER FIVE

DISCUSSION

The complete extraction of SRP receptor from microsomes in 0.08% deoxycholate; 0.2M Tris pH9.0 suggests that the interaction of the complex with the ER membrane is unlike other ER transmembrane proteins. Furthermore, the solubility of SRβ in 0.08% deoxycholate; 0.2M Tris pH9.0 does not require interaction with SRα, but is a property intrinsic to SRβ itself (Figure 2). SRβ is predicted to be a type I transmembrane protein (Von Heijne, 1989; Miller *et al.*, manuscript in preparation) with a lumenal domain of only 27 amino acids. It is unlikely that the behaviour of SRβ in deoxycholate is due to the size of the lumenal domain since other type I transmembrane proteins with small lumenal domains are resistant to extraction with 0.08%/deoxycholate/0.2M Tris pH9.0 (Andrews *et al.*, 1992).

In contrast, both endogenous SRβ and cell free synthesized $SR\beta_{MD}$ resist extraction with alkali pH in combination with 1M NaSCN/10mM DTT or 1M urea (Figure 2; see also Young *et al.,* submitted) similar to other ER transmembrane proteins. Therefore, SRβ may be a transmembrane protein since it resists extraction with alkali pH. However, SRβ may interact with other integral membrane proteins rather than the lipid bilayer, consistent with the solubility of SRβ in deoxycholate below the CMC of the detergent. Despite the unusual interaction of SRβ with the ER membrane, membrane assembly of SRβ is co-translational and requires functional SRP receptor, similar to most ER membrane proteins (Figure 4).

In contrast to SRβ, both endogenous and *in vitro* translated SRα molecules are efficiently extracted from membranes with either 0.08%

deoxycholate/0.2M Tris pH9.0 or a combination of alkali pH and various chaotropes (Figure 2; see also Young *et al.*, submitted; Miller *et al.*, manuscript in preparation). These results provide strong evidence that SRα is only peripherally associated with the ER membrane.

The interaction of SRα with the ER membrane is resistant to urea, salt and alkali pH, at concentrations which efficienty extract most peripheral membrane proteins, such as SRP (Figure 5; see also Gilmore *et al.*, 1982; Andrews *et al.*, 1989). In addition, anchoring of cell free synthesized SRα molecules to microsomes is abolished by prior treatment of the membranes with trypsin at 5μg/ml (Figure 5; see also Andrews *et al.*, 1989). This suggests that a trypsin sensitive component exists on the ER membrane which can tightly anchor SRα. Data presented in this thesis suggests that SRβ functions to anchor SRα to the ER membrane (Figure 6 and Table 1). Exogenous SRα molecules can assemble on T_5KRM but not in a manner resistant to extraction with 2M urea. If the SRα molecules are targeted to functional sites on the T_5KRM, SRβ can productively integrate into the ER membrane. A fraction of these SRβ molecules which assembled onto T_5KRM anchored SRα in a urea resistant fashion (Figure 6). Therefore, only the exogenous $SR\beta_{MD}$ molecules and not the pre-existing SRβ molecules on T_5KRM can anchor SRα.

After $SR\beta_{MD}$ was incorporated into T_5KRM, 38 ± 16% (n=4) of the SRα molecules were anchored in a urea resistant manner onto the membranes. However, given the poor translocation of $SR\beta_{MD}$ onto T_5KRM reconstituted with SRα, the inhibition of $SR\beta_{MD}$ synthesis observed when the $SR\beta_{MD}$ translation reaction was added co-translationally to a post-ribosomal supernatant of SRα and the probability of targeting SRα and SRβ to the same site on the microsome it was not expected that the reaction would be efficient. Therefore, the 38% anchoring of SRα on T_5KRM provides strong evidence that

the endogenous SRβ was somehow inactivated for anchoring SRα by prior treatment of the membranes with trypsin.

Data presented in this thesis provides strong evidence that exogenous SRβ is required to restore anchoring of SRα to T_5KRM. However, a shift in migration of endogenous SRβ is not observed by one-dimensional SDS-PAGE when microsomes are digested with trypsin at 5μg/ml (Andrews *et al.*, 1989). Two possibilities can explain this apparent contradiction. First of all, SRβ contains two potential cleavage sites within the last seven amino acids at the carboxy terminus of the molecule. Cleavage at either or both of these sites may impair urea resistant anchoring of SRα to the endogenous SRβ molecules, even though a shift in the migration of the protein is not detected. On the other hand, it is possible that SRα is the only component sensitive to trypsin treatment and that the membrane associated proteolytic fragment of SRα remains associated with endogenous SRβ thereby blocking the stable anchoring of newly synthesized SRα molecules on the membrane. In either case, addition of exogenous $SR\beta_{MD}$ to unoccupied sites on T_5KRM would result in the anchoring of newly added SRα molecules.

To determine if $SR\beta_{MD}$ is the trypsin sensitive component, an $SR\beta_{MD}$ deletion mutant missing the last seven amino acids can be used to assay for reconstitution of SRα anchoring to T_5KRM. At present, this experiment may be difficult, due to the low levels of assembly of $SR\beta_{MD}$ onto T_5KRM reconstituted with SRα. Since assembly of $SR\beta_{MD}$ onto reconstituted T_5KRM is only 10.4 ± 6.2% (n=4), it is not always possible to clearly visualize the increase of $SR\beta_{MD}$ molecules in the pellet fraction. Therefore, if it would be difficult to distinguish between the inability of the carboxyl terminal $SR\beta_{MD}$ mutant to anchor SRα and the inability of the T_5KRM to translocate the $SR\beta_{MD}$ deletion mutant. In addition, $SR\beta_{MD}$ deletion mutants may exist which are impaired but not

completely abolished in anchoring SRα. Therefore, assembly of $SR\beta_{MD}$ onto T_5KRM must be improved before deletion mutagenesis can be used to dissect the region of $SR\beta_{MD}$ involved in this process.

Consistent with our observations that SRβ anchors SRα to the ER membrane, $SR\beta_{MD}$ can form complexes with full length SRα or the membrane anchoring domain but not the cytoplasmic translocation competent domain of the molecule in co-immunoprecipitations with the S1 SRα antisera (Figure 8). This result is consistent with previous data suggesting that both hydrophobic stretches as well as the postively charged region of the SRα anchoring domain are required for membrane assembly on KRM (Young *et al.*, submitted).

In contrast to the S1 SRα antibody, the antiserum raised against the anchoring domain of SRα could not be used in an immunoprecipitation reaction to demonstrate stable SRα/$SR\beta_{MD}$ complexes. Since the antiserum is raised to the anchoring domain of SRα, the antibodies may be displacing the $SR\beta_{MD}$ molecules from the SRα/SRβ complex to expose the epitopes on SRα. In addition, antisera to the C-terminal 40 residues of SRβ could not be used to demonstrate stable complexes between SRα and $SR\beta_{MD}$. On the other hand, HA antisera could be used to demonstrate complex formation between SRα and HA$SR\beta_{MD}$ (in which the HA tag is fused to the amino terminus of $SR\beta_{MD}$). Therefore, the SRβ antisera may also dissociate the SRα/$SR\beta_{MD}$ complex, suggesting that the carboxyl terminal 40 residues of SRβ may interact with SRα.

Therefore, SRα and $SR\beta_{MD}$ are capable of forming complexes in the absence of membranes under high detergent and salt concentrations. However, the bulk of the SRα and $SR\beta_{MD}$ do not participate in complex formation. After normalizing for the number of methionine residues in SRα and $SR\beta_{MD}$, the ratio of SRα/SRβ molecules co-immunoprecipitated with the S1 SRα antibodies ranged from 3:1 - 10:1 while they were incubated post-

translationally in a 1:1 ratio prior to immunoprecipitation. It may be possible to improve the efficiency of complex formation by assembling the two subunits of the SRP receptor in the vicinity of the same microsome. To do so, one can translate one of the bicistronic transcripts (Figures 17, 18) which encode both SRα and $SR\beta_{MD}$ in the presence of KRM. This should allow synthesis of the two molecules in the vicinity of the same microsome. Preliminary experiments using the bicistronic message $DV_{\beta/\alpha}$ revealed that the SRα /$SR\beta_{MD}$ complex on KRM was resistant to extraction with alkali pH and could be co-immunoprecipitated in a 1:1 ratio with the S1 SRα antibody (data not shown).

In addition to a role in anchoring SRα to the ER membrane, SRβ is also predicted to be involved in the translocation process due to the identification of a GTP binding site in the molecule (Miller *et al.*, 1993; Miller *et al.*, manuscript in preparation). The inhibition of preprolactin translocation observed with the polyclonal antiserum to the GTP binding domain of SRβ also suggests a second function of the molecule in translocation of secretory proteins across the ER membrane.

In conclusion, data presented in this thesis supports the hypothesis that SRβ is an ER membrane transmembrane protein which interacts with the ER membrane in a manner atypical of other ER membrane proteins. However, SRβ utilizes the SRP dependent pathway of translocation to assemble onto microsomes. Finally, the data presented strongly suggests that endogenous SRβ is inactivated for anchoring of SRα to microsomes previously treated with trypsin as anchoring of SRα onto trypsin digested membranes can be restored by incorporating exogenous full length SRβ.

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