Nucleotide Dependent Binding of the GTPase Domain of the Signal Recognition Particle Receptor β-Subunit to the α-Subunit.

Kyle R. Legate, Domina Falcone and David W. Andrews*

Department of Biochemistry,
McMaster University
1200 Main St. W.
Hamilton, Ontario, L8N 3Z5

* Corresponding Author

tel: 905 525 9140 X 22075
fax: 905 522 9033
Email: Andrewsd@fhs.mcmaster.ca

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Running title: Role of SRβ GTPase
The signal recognition particle (SRP) receptor is a heterodimer of two polypeptides (SRα and SRβ) that each contain a GTP binding domain. The GTP binding domain in the peripheral membrane SRα subunit has a well defined role in regulating targeting of SRP-ribosome-nascent chain complexes to the translocon. The only well established function for the transmembrane SRβ subunit is anchoring SRα on the endoplasmic reticulum membrane. Deletion of the amino-terminal transmembrane domain of SRβ did not effect receptor dimerization but revealed a cryptic translocation signal that overlaps the GTPase domain. We demonstrate that the domain of SRα that binds SRβ does so by binding directly to the nucleotide bound form of the GTPase domain of SRβ. A mutant SRβ containing an amino acid substitution that allows the GTPase domain to bind XTP dimerizes with SRα most efficiently in the presence of XTP or XDP, but not ATP. Our results suggest an additional level of regulation of SRP receptor function based on regulated dissociation of the receptor subunits.

The sorting of newly synthesized proteins to their correct subcellular destination is a major undertaking for all eukaryotic cells. Targeting of proteins and nascent polypeptides is facilitated by localization peptides within the amino acid sequence of the protein (reviewed in (1-3)). The majority of polypeptides destined for the endoplasmic reticulum (ER) contain an amino terminal signal sequence which is eventually cleaved from the protein during translocation across the endoplasmic reticulum membrane.
Nascent polypeptides are sampled by signal recognition particle (SRP)\(^1\) as they emerge from the ribosome in the cytoplasm\(^4\). When SRP encounters a signal peptide the 54 kDa subunit (SRP54) binds to it while two other SRP polypeptides, SRP9 and SRP14 in conjunction with SRP-RNA, pause translation via an interaction with the ribosome (reviewed in \(^4\)). The ribosome-nascent chain-SRP complex is then targeted to the ER where it interacts with SRP receptor (SR), a heterodimeric complex consisting of the subunits, SR\(\alpha\) and SR\(\beta\). During docking, SRP54 and SR\(\alpha\) interact and cooperatively bind two molecules of GTP resulting in the formation of a very stable complex \(^5\). The presence of a nascent chain acceptor, believed to be Sec61\(\alpha\), \(^6\) allows transfer of the nascent chain from SRP54 to the translocon that forms the aqueous pore that spans the ER membrane. Hydrolysis of GTP then dissociates the SRP-SR complex and SRP recycles back to the cytoplasm\(^5\). Thus, according to one model, GTP functions as a molecular switch to ensure that nascent chain transfer is unidirectional \(^7\).

In addition to the well characterized GTPases of SR\(\alpha\) and SRP54, a GTP binding domain has been identified in SR\(\beta\). The GTPase domain of SR\(\beta\) is more closely related to the ras superfamily of GTPases than to the characteristic SRP type GTPase domains found in SRP54 and SR\(\alpha\) \(^8\). Consistent with SR\(\beta\) belonging to the ras superfamily the affinity of SR\(\beta\) for GTP is 20 nM, a value more similar to the affinity of ras (8 nM) than of SR\(\alpha\) (14 \(\mu\)M) for GTP \(^9,10\). Unlike the SRP GTPases, little is known about the function of the SR\(\beta\) GTPase in translocation. Recently it was proposed that ribosomes bind to the GTP bound form of SR\(\beta\) and that the ribosome functions as the

\(^1\)The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; SR, SRP receptor; SRX2, amino terminal 151 amino acids of SR; XTP, Xanthosine triphosphate; XDP, Xanthosine diphosphate; HA, influenza haemagglutinin; RM, canine pancreatic microsome;
SRβ GTPase activating protein(10). However, SRβ also functions as a membrane anchor for SRα (8,11) via an interaction with an independently folded domain (SRX2) at the amino terminus of SRα (11). A translational pause encoded in the SRα mRNA ensures efficient targeting of SRα to the ER membrane by allowing the SRX2 domain to fold and interact with SRβ before translation of SRα has completed(12). Once formed, the dimer is stable in 1% non-ionic detergent and 500 mM KOAc. Therefore, it was surprising that in yeast the transmembrane domain of SRβ is not required for SR function or for dimerization with SRα(13). Moreover, SR binding to the ER membrane was dramatically decreased, but it was not abolished, in yeast expressing a mutant SRβ lacking the transmembrane region(13).

We have examined the role of SRβ in membrane assembly and heterodimerization of SR using a mammalian cell free system. Our data demonstrate that sequences amino terminal of the SRβ GTPase are required for membrane binding and for orienting SRβ with respect to the membrane. Moreover, this region of the molecule partially suppresses a cryptic ER translocation signal that overlaps the SRβ GTPase domain. Surprisingly, the SRβ GTPase domain is both necessary and sufficient for interaction with SRα via the SRX2 domain. Mutation of the SRβ GTPase to favour XTP over GTP allowed us to demonstrate that in the nucleotide free state, SRβ is unable to bind SRα. Along with the recent observation that the ribosome increases the GTPase activity of SRβ(10), these data suggest that binding of SRα to the ER membrane may be regulated during targeting and transfer of nascent polypeptides to the translocon.

**EXPERIMENTAL PROCEDURES**

*Materials and General Methods*—General chemical reagents were obtained from either
Fisher, Sigma or Life Technologies, Inc. SURE™ *Escherichia coli* cells used for plasmid construction were purchased from Stratagene. Except where specified, restriction enzymes and other molecular biology enzymes were from New England Biolabs or MBI Fermentas. 35S-labeled methionine was from DuPont NEN. SP6 polymerase, *Taq* polymerase and RNAse inhibitor were from MBI Fermentas. Creatine kinase was from Boehringer Mannheim. A monoclonal antibody directed against a HA epitope was purchased from Berkeley Antibody Company.

Transcription reactions with SP6 polymerase were performed as described previously(14). Cell-free translation reactions were performed in rabbit reticulocyte lysate (RRL) and labeled with 35S-methionine as described previously (15); translation products were analyzed by SDS-PAGE and radiography. Canine pancreatic rough microsomes were prepared and either washed with 0.5 M KOAc (KRMs) or further purified by Sepharose CL-2B size exclusion chromatography (CRMs) as described (16).

Polyclonal antiserum directed against SRα was obtained as described (Young et al., 1995). Polyclonal antiserum against a synthetic peptide corresponding to the carboxyl terminal 20 amino acids of SRβ was obtained from ExAlpha Biologicals.

**Plasmids**—Construction of plasmids, sequencing and site directed mutagenesis were performed using standard techniques. Unless otherwise stated, all constructs were inserted following the SP6 RNA polymerase promoter in pMAC334, a version of pGEM3 containing the 5'-untranslated region of pSPUTK (17) and the 3'-untranslated region of bovine preprolactin. pMAC191, containing a modified full-length cDNA sequence of canine SRα; pMAC455, encoding SRβmad; and pMAC690, encoding SRβmad with an amino-terminal HA epitope-tag were previously reported(11).
Plasmid pMAC853 encodes SRβ-ΔTM, a fusion of the carboxyl terminal 206 amino acids of canine SRβ with an amino terminal HA epitope tag (MYPYDVPDYAA)_2. To assemble this plasmid the coding sequence for SRβ_md in pMAC690 was replaced by a PCR product generated by amplifying the appropriate region of the coding sequence from pMAC455 using an amino terminal primer CATGCCATGGCTAAGTTCATCCGGAGCAGA and a carboxyl terminal primer complementary to the T7 promoter. The PCR product was digested with *NcoI* and *EcoRI* and subcloned into the vector cut with the same enzymes.

pMAC747 encodes SRβ_C1, a version of SRβ_md lacking the carboxyl terminal six amino acids. The coding region for SRβ_C1 was amplified by PCR from pMAC455 using an amino terminal primer complementary to the SP6 promoter, and a carboxyl terminal primer GCTCTAGACCTACTTCTCCAGGTCCTGGATG. The PCR product was cut with *NcoI* and *XbaI* and subcloned into pMAC690 in place of the coding sequence for SRβ_md.

pMAC1300 encodes SRβ_C1-ΔTM, and was created by digesting pMAC747 (encoding SRβ_C1) with *BspEI* and *BamHI*, and subcloning cloning it into the corresponding region of pMAC853 (encoding SRβ-ΔTM).

pMAC1056 encodes SRβ_D4, the carboxyl terminal 195 amino acids of canine SRβ. The coding region for SRβ was amplified by PCR from pMAC853 using an amino terminal primer CATGCCATGGCTGTTCTTCTCGTGC and a carboxyl terminal T7 promoter primer. The PCR product was cut with *NcoI* and *BamHI* and used to replace the SRβ_md coding region in pMAC690 digested with the same enzymes.

pMAC1057 encodes SRβ_D5, the carboxyl terminal 182 amino acids of canine SRβ. The appropriate coding region was amplified from pMAC853 using an amino terminal primer.
CATGCCATGGGATTACTGTTTGTCAGGTTGTTAAC and a carboxyl terminal T7 promoter primer. This fragment was cloned into pMAC690 using NcoI and BamHI as above.

pMAC1082 encodes SRβ-Δloop, a version of SRβ-ΔTM containing a deletion of amino acids 185 to 219. To prepare the plasmid for PCR, an endogenous HindIII site was removed from a non-coding region of the plasmid by cutting pMAC853 with HindIII, treating with Klenow fragment and ligating. The resulting plasmid (pMAC853-HindIII) was amplified with a 5' primer AATTAAGCTTGGAAAGAAAGGCAAAGAATTTGAGT and a 3' primer AATTAAGCTTGGGGATTTTATG. The PCR product (the entire plasmid except for the deleted region) was digested with HindIII and ligated to yield pMAC1082.

pMAC1363 encodes SRβ-loop2, a version of SRβΔTM with amino acids 185-219 replaced with the pseudo-random sequence RSTISLQQASPLTGTPDKSGRSATLVAQQLALNL. This sequence was created as a pair of oligonucleotides containing a 5' BglII and a 3' HindIII site. The oligos were ligated into pSPUTK to give pSPUTK-loop2. The carboxyl terminal region of SRβ and T7 RNA polymerase promoter were amplified from pMAC853-HindIII using a 5' primer containing a HindIII site and a 3' primer containing a NheI site, and ligated into pSPUTK, giving pSPSRβ3'. pSPUTK-loop2 was digested with BglII and HindIII, and the fragment was ligated into pSRSRβ3' to give pSPSRβ3'loop2. The amino terminal region of pMAC853 including the SP6 RNA polymerase promoter and coding regions for the HA tag and the amino terminal part of SRβ were amplified using a 5' primer containing an AflIII site and a 3' primer containing a BglII site, and ligated into pSPSRβ3'loop2 to assemble the coding region for SRβloop2.

All of the GTPase mutants were created by oligonucleotide-directed point mutation and PCR using a method described previously (18) using an Apal site. All plasmids were sequenced to
confirm the presence of the desired mutations.

Quantification of in vitro translation products—1 µL of translation reaction products were added to 50 µL 200 mM NaOH and 2.5 µL H₂O₂, and spotted onto glass microfibre filters (Whatman). The filters were soaked in 10% w/v trichloroacetic acid (TCA) for 10 minutes, washed 3 times in 5% TCA, once in 95% ethanol, and allowed to dry. The dry filters was added to scintillation fluid, and the measured radioactivity was converted to fmoles of protein by the following equations:

\[
Ci = \frac{\left( \frac{CPM \times \%P}{M} \right) \times EF}{2.2 \times 10^{12}},
\]

where Ci is the number of curies per sample, \%P is the percentage of counts from the translated protein, M is the number of methionines in the protein, and EF is the efficiency factor of the scintillation counter.

\[
fmoles_{protein} = \left\{ \frac{Ci}{f} \right\} \times 10^{12},
\]

\[
\frac{1}{SA_0} - \frac{(1 - f)}{1494}
\]
where $f$ is the radioactive decay factor and $SA_0$ is the specific activity of the isotope. Periodically, 0.5 µL of reaction products were run on SDS-PAGE gels and subjected to Phosphorimager analysis to determine the percentage of radioactivity arising from the product of interest ($\%P$), as compared to non-specific translation products. The determination of fmoles of protein rather than simply measuring radioactivity and correcting for the number of methionines was used to permit repeat experiments to be performed with identical quantities of *in vitro* translation products irrespective of the specific activity of the $^{35}$S methionine.

**Immunoprecipitations and Proteolysis**—For immunoprecipitation of dimers consisting of SRα and the specified SRβ mutant, equimolar amounts of the two molecules were incubated for 15 minutes at 24°C. The reactions were then diluted in TXSWB (100 mM Tris-Cl, pH 8.0, 500 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5% glycerol) at 4°C and incubated rotating end over end for 8-12 hours with either a polyclonal antibody to SRα conjugated to CNBr-activated Sepharose (Pharmacia), or a polyclonal antibody to the HA epitope bound to Protein G Affi-Gel (Bio-Rad). Immunoprecipitates were washed three times in TXSWB, and washed two times with buffer containing 0.1 M Tris-Cl, pH 8.0, 0.1 M NaCl, prior to addition of SDS-PAGE loading buffer.

For immunoprecipitation with anti-SRβ<sub>COOH</sub>, SRβ and SRα translations were mixed such that the proteins were present in a 2:1 ratio, respectively. Reactions were added to 500 µL of TXSWB with anti-SRβ<sub>COOH</sub> and Protein G Sepharose and incubated with end over end rotation for 12 hours at 4°C. The beads were pelleted and the supernatant was recovered and used for immunoprecipitation with anti-HA antibodies to isolate the remaining SRβ molecules.

To determine the membrane topology and protease sensitivity of the mutants bound to
membranes, microsomes were added to 20 microliter translation reactions. After translation for 60 minutes the membranes and membrane bound proteins were isolated by gel-filtration chromatography and analyzed by proteolysis as described previously (19).

GTP depletion- Depletion of small molecules from translation reactions was performed essentially as described (20). Briefly, translation reactions were loaded onto a 20X bed volume of Sephadex G25 resin equilibrated in 250 mM sucrose, 25 mM HEPES-KOH, pH 7.5, 10 mM KOAc, 1 mM DTT, 5% glycerol and centrifuged at low speed at 4°C. The flow through was collected and the procedure was repeated. To determine how effectively this procedure removed nucleotides a control reaction containing $^{35}$S labeled ATP was analyzed. For nucleotide titration experiments, the appropriate amount of nucleotide was added to 10 µL of depleted SRβ reaction products and incubated for 20 minutes on ice prior to mixing with translation reactions for SRα for 15 minutes at 24°C.

RESULTS

To examine membrane assembly and identify the SRα-binding domain within SRβ, a series of deletion mutants were created (Figure 1). These mutants were synthesized in a reticulocyte lysate cell free system and assayed for both correct assembly into ER membranes and for heterodimerization with SRα. As a starting point for this analysis we used the SRβ<sub>md</sub> chimera consisting of the first ten amino acids of murine SRβ followed by residues 10 to the carboxyl-terminus (residue 265) of canine SRβ(11). This chimera, referred to here as SRβ, was used because it has been previously demonstrated to form heterodimers with canine SRα that are indistinguishable from the canine proteins found on microsomes(11).
The chimeric amino-terminus and the transmembrane domain were deleted in SRβ-ΔTM which therefore, comprises the cytoplasmic domain of canine SRβ. The additional sequences deleted in SRβD4 and SRβD5 include the positively charged region or the positively charged region and the G1 GTPase consensus sequence, respectively. SRβC1 is a mutant of SRβmd lacking only the carboxyl-terminal six amino acids, while in SRβC1-ΔTM the amino terminus and transmembrane domain are also deleted. Alignment of SRβ with members of the ras family of GTPases identifies a putative insertion of 34 amino acids between the G4 and G5 consensus sequences(8). Deletion of this sequence from SRβ-ΔTM yielded SRβ-Δloop, and replacement of the loop with a scrambled version of the sequence resulted in SRβ-loop2. Finally, deletion of the SRβ cytoplasmic region adjacent to the putative transmembrane domain that is enriched in lysine and arginine residues yielded SRβ-Δch. Preliminary immunoprecipitation studies (see below) determined that antibodies raised to a carboxyl-terminal peptide of SRβ precipitated only monomeric SRβ. Therefore to facilitate immunoprecipitation, the deletion mutants were tagged at the amino terminus by adding the well characterized HA epitope, unless specified otherwise.

SRα is a cytoplasmic, peripheral ER protein therefore, it can only bind to those regions of SRβ that are on the cytoplasmic side of the membrane. Since the detailed topology of SRβ has not been verified experimentally, it was examined by membrane binding and proteolysis. To this end, selected molecules were synthesized in vitro in the presence of canine microsomes (RMs) and then the membranes and membrane bound SRβ molecules were isolated by gel-filtration chromatography (Figure 2, lanes indicated RM). The topologies of membrane bound SRβ and the specified mutants were determined by digestion with proteinase K (Figure 2, lanes indicated PK). In the absence of
membranes, a single primary translation product was obtained for SRβ. However, to our surprise, translation of SRβ in the presence of membranes resulted in two bands, each representing membrane bound proteins (Figure 2, lane 2). The upper band is glycosylated as after treatment with endoglycosidase H it co-migrates with the lower band (data not shown). There is only one potential glycosylation site in SRβ and it is positioned between the G-2 and G-3 sequences in the GTP binding domain (amino acids 110-112). Therefore, the glycosylated band comes from molecules oriented with the GTPase domain inside the endoplasmic reticulum. The glycosylated band was protected from protease digestion by the membrane (Figure 2, lane 3), suggesting that the entire molecule was translocated across the ER membrane. Although in the experiment shown this species was only 93% protected from protease, control experiments (not shown) demonstrated that most of the glycosylated molecules were translocated into the lumen of the microsomes. At 0.005 mg/ml protease the glycosylated SRβ was quantitatively protected from protease while type I molecules were completely digested. However, to obtain a single band representing the protease resistant core of the protein 0.03 mg/ml of protease was required (data not shown). Under the latter conditions the glycosylated SRβ is no longer completely protected from the protease (compare lanes 2 and 3, upper bands).

Only SRβ that co-fractionated with membranes during gel-filtration chromatography are shown in lane 2 therefore, it is possible to calculate the fraction of molecules that adopt each topology. Greater than 60% of the membrane bound SRβ molecules are not glycosylated and co-migrate with SRβ synthesized in the absence of membranes, as expected for a membrane protein with type I transmembrane (amino-terminus lumenal) orientation. When protease was added to the membranes, the molecules that comigrated with SRβ were partially digested and therefore, migrated
with lower apparent molecular weight (Figure 2, lane 3, triangle). This digestion pattern is typical of a type I molecule with a protease resistant core structure. Some molecules were almost completely digested, resulting in the small band (Figure 2, *). This band results from protection by the membrane of the amino-terminus and transmembrane domain of SRβ. Upon solubilization of the membrane, the larger protease resistant fragment of SRβ was further digested by the protease (Figure 2, lane 4, dot) because the amino-terminus is no longer protected by the membrane. Treatment of these samples with endoglycosidase H revealed that none of the proteolytic fragments were glycosylated. Therefore the bands indicated by the triangle, asterisk and dot resulted from proteolytic digestion of the non-glycosylated type I transmembrane molecules (Figure 2, lane 2, lower band).

A glycosylation site introduced at the extreme amino terminus of SRβ was efficiently glycosylated when the protein was synthesized in the presence of RMs (data not shown), confirming the hypothesis that SRβ spans the membrane as a typical type I integral membrane protein. Therefore, the amino acids at the amino-terminus of SRβ (approximately residues 1-40) reside within or span the ER membrane, where they are unlikely to come into contact with SRα. Thus, the sequence containing a series of positive charges (amino acids 40-58) following the transmembrane domain, is the most amino-terminal region of SRβ that is a candidate for binding to SRα.

Deletion of these residues from full length SRβ (SRβ-Δch) inverts the orientation of some of the type I molecules in the ER membrane. As a result, the topology of some of the non-glycosylated molecules are inverted as they are almost completely protected from the protease (compare the lower band in lane 6 with the corresponding band in lane 7). After proteolysis the
inverted non-glycosylated molecules migrated slightly further due to proteolysis of the amino-terminal HA tag and the amino acids that precede the transmembrane domain. These residues are susceptible to digestion because they are now on the cytoplasmic side of the membrane, confirming an inverted topology for the protein. Comparison of the ratio of the upper and lower bands in lane 2 with those in lane 6 revealed that the fraction of the total molecules recovered with membranes that was glycosylated only increased by 7%, suggesting that most of the molecules with inverted topology are not good substrates for glycosylation. Neither these nor the non-glycosylated molecules fold into a protease resistant conformation as there is no protease resistant band in lane 8. As expected, the amino-terminus of that fraction of the molecules that still adopt the type I topology is protected from proteinase K by the membrane (Figure 2, * in lane 7). Thus, it appears that one function of the positively charged region is to prevent translocation of the GTPase domain of SRβ during translocation of the transmembrane domain.

To our surprise, deletion of the first 59 amino acids of SRβ resulted in a molecule with reduced but not abolished interaction with membranes (Figure 2, compare lanes 1 and 2 with lanes 13 and 14). Both type I and type II membrane associated molecules were detected bound to ER membranes. The membrane protected amino-terminus of SRβ−∆TM was too small to detect by SDS-PAGE but the disappearance of the band that co-migrates with full length non-glycosylated SRβ−∆TM upon addition of protease (Figure 2, compare lanes 14 and 15) is diagnostic for type I transmembrane molecules. In contrast, the lumenally disposed glycosylated SRβ−∆TM molecules were protected from added protease unless non-ionic detergent was added to solubilize the membranes (Figure 2, compare lanes 13-16) confirming that there is a cryptic signal anchor sequence
within SRβ that translocates the GTPase domain of SRβ across the ER membrane. Extraction with sodium carbonate pH 11.5 revealed that both the glycosylated and the type I molecules were integrated into the bilayer (data not shown). Since type I membrane bound SRβ–ΔTM molecules were observed, a similar cryptic signal anchor sequence within SRβ probably explains membrane binding observed previously for a corresponding mutant of yeast SRβ(13). Membrane binding by SRβ–ΔTM may also explain why this yeast mutant complemented a yeast strain deleted for the endogenous SRβ(13).

Of the 16 residues between the transmembrane segment and G1 consensus sequence in SRβ, 5 of the first 11 are positively charged. Although the next 5 residues are hydrophobic, signal peptides require a longer contiguous stretch of uncharged amino acids. Thus, the cryptic signal sequence must at least overlap the GTPase domain of SRβ. Consistent with this interpretation the first 6 residues of the GTPase domain are uncharged. Control experiments using deletion mutants without the amino-terminal HA tag verified that the epitope sequence does not contribute significantly to the cryptic signal sequence (data not shown). As expected, membrane binding and translocation of SRβ–ΔTM occurred only co-translationally, demonstrating that membrane binding was specific (data not shown) and not due to aggregation on the surface of the ER. Finally, it is possible that deletion of residues 1-59 from SRβ impairs folding of the membrane bound molecules as less of the protease resistant core fragment was detected (Figure 2, lane 16).

Deletion of the last 6 amino-acids from the carboxyl-terminus of the SRβ GTPase domain resulted in a molecule (SRβC1) with primarily type I topology, similar to SRβ, (Figure 2, compare lanes 1-3 with lanes 9-11). However it appears that this molecule is somewhat impaired for folding
as the intensity of the band resulting from the protease resistant structure was substantially reduced (Figure 2, lane 11, triangle).

Since the positive charges following the transmembrane domain appear to dictate the membrane orientation of SRβ, this region is unlikely to be involved in binding to SRα. Therefore, the GTP-binding domain and the putative loop region are the main candidates for an SRα-binding motif. To identify the SRα binding site in SRβ we used coimmunoprecipitation to assay a series of SRβ deletion mutants for interaction with SRα (Figure 3). An antibody directed against the HA epitope at the amino terminus of all of the SRβ mutants was used to precipitate SRβ because antibody binding to an HA tag at the amino-terminus was not expected to effect the SRβ/SRα interaction. To precipitate SRα we used an antibody directed against the linker region between the SRX2 domain of SRα that binds it to SRβ and the SRα GTPase domain. Equimolar amounts of in vitro translation products for SRα and the specified SRβ mutants were mixed together (Figure 3, panel A) and then immunoprecipitated with anti-HA (Figure 3, panel B) or anti-SRα antibodies (Figure 3, panel C).

Consistent with the hypothesis that the amino terminus of SRβ is not required for binding to SRα, dimerization of either SRβ–ΔTM or SRβD4 with SRα was detected with both antibodies (Figure 3, Panels B-C, lanes 1 and 2, respectively). Deletion of the G-1 region, (SRβD5) which comprises part of the SRβ GTPase domain, abolished binding to SRα (Figure 3, Panels B-C, lane 3). Deletion of as few as six amino acids from the carboxyl-terminus of SRβ also abolished binding to SRα (Figure 3, Panels B-C, lane 4). The last 6 amino acids of SRβ are predicted to form part of
the α5 helix of the GTPase domain(8). Thus, our results demonstrate that deletion of either end of the GTPase domain prevents dimer formation. In addition, both of these SRβ mutants (SRβD5 and SRβC1-ΔTM) lack the protease resistant fragment observed for SRβ-ΔTM (Figure 2 and data not shown) suggesting that the protease resistant fragment probably results from correct folding of the SRβ GTPase domain. Taken together, these data imply that a properly folded SRβ GTPase domain is required for efficient binding of SRα. Furthermore, these SRβ mutants interact less efficiently with the anti-HA antibody (Figure 3b). Control experiments (not shown) demonstrated that inefficient precipitation of the SRβ mutants was not related to dimer formation or due to increased proteolysis of these molecules. Therefore we presume that misfolding of the protein renders the HA tag less accessible to the antibody.

Removal of the sequence from between G4 and G5 in SRβ that does not align with other ras type GTPases (a putative loop) results in reduced but detectable binding of SRβ−Δloop to SRα, as does replacement of this sequence with a pseudo-random sequence (SRβ−loop2). Precipitation of SRα via binding to these mutants was not very efficient (Figure 3, lanes 5-6, arrowheads in Panel B), therefore a darker exposure of these lanes of the autoradiogram is also shown (Figure 3, Panel D, lanes 1-2). When the precipitation is performed using antibodies to SRα, binding of SRβ−Δloop is on average 30% that of binding to SRβ−ΔTM while binding to SRβ-loop2 is about 15% that of binding to SRβ−ΔTM (Figure 3, Panel C, arrowheads in lanes 5 and 6, respectively). When synthesized in vitro, both SRβ−Δloop and SRβ−loop2 are less resistant than SRβ is to added proteases (data not shown) suggesting that the loop sequence contributes to the correct folding of the
SRβ-GTPase. Therefore, this sequence may contribute to, but is obviously not essential for, binding of SRβ to SRα.

SRX2, the previously identified minimum SRβ binding domain of SRα (11), binds to the GTPase domain of SRβ. In addition, the GTPase domain with the scrambled loop region (SRβ-loop2) also bound to SRX2 (Figure 3, Panel D, lanes 3-4). We were unable to identify any sequence similarities between SRX2 and other GTPase binding proteins. Moreover, the overall structural features found in SRX2, two extended hydrophobic regions and a positively charged sequence are not found in other GTPase binding proteins. Nevertheless, no other regions of SRα were observed to bind to SRβ (data not shown). Thus, we conclude that the SRX2 domain of SRα is a novel GTPase binding domain.

To assay co-precipitation of SRβ and SRα with an antibody directed against another region of SRβ, an antibody (anti-SRβCOOH) was raised against the carboxyl-terminal twenty amino acids of SRβ. However, consistent with the observation that the carboxyl-terminus of SRβ is required for SRα-SRβ dimerization, dimers were not precipitated with this antibody (Figure 3, Panel D, lanes 5-6). Instead, only SRβ-ΔTM was precipitated from mixtures of SRβ-ΔTM and SRα with anti-SRβCOOH (Figure 3, Panel D, lane 5). However, immunoprecipitation of the supernatant of this reaction with anti-HA antibodies precipitated SR dimers that were not recognized by anti-SRβCOOH (Figure 3, Panel D, lane 6). Therefore, we conclude that dimerization buries the carboxyl-terminus of SRβ within the dimer, where it cannot be bound by the antibody.

Since the GTPase domain of SRβ appears to be both necessary and sufficient for binding to
SRα, a series of GTPase point mutations (Table I) were created in SRβ−∆TM to determine whether a functional GTPase domain is required for SR dimerization. Similar GTPase mutations have also been examined for functional complementation of SRβ deletion in yeast(13). Coimmunoprecipitation experiments performed with these GTPase mutants illustrate that only a subset were competent for SR dimerization (Figure 4 and Table I). Identical results were obtained when binding to SRX2 was assayed (Figure 4). The mutants that bind to SRα include the G118L and H119L mutations believed to reduce the GTPase activity of GTP binding domains (21-24) and D181N that alters the nucleotide binding preference from GTP to XTP (25,26). Although the D181N mutation alters binding preference it does not eliminate GTP binding. Therefore, since our in vitro translation reactions contain at least 1 mM GTP it is likely that when synthesized in vitro, the D181N mutant is still primarily in the GTP bound form. Of the mutants that do not bind SRα K75I is predicted to have much reduced nucleotide affinity (27) while N178K is believed to be structurally unstable(24). Thus the common feature of all of the mutants that bind SRα is that they are all expected to bind nucleotide.

To determine whether the GTP-bound, GDP-bound form or the empty form of SRβ binds to SRα, small molecules, including GTP, were removed from in vitro translation reactions by gel filtration chromatography. Since the in vitro translation reactions contain very small amounts of SRβ, (approximately 100 fMoles) there was a practical limit on the size of gel filtration column that could be used, before there was unacceptable loss of SRβ due to non-specific binding. As a result, we were unable to reduce the amount of GTP in the reaction mixture to significantly less than 100 nM. Since SRβ has a $K_d$ for GTP of approximately 20 nM (10) sufficient GTP/GDP remains tightly
bound to SRβ that gel-filtration chromatography prior to incubation with SRα did not reduce binding of SRβ–ATM to SRα (Figure 5). However, after gel filtration chromatography the D181N mutant no longer dimerized with SRα. The D181N mutant is expected to possess a lower than wild-type affinity for GTP therefore, we presume that gel filtration chromatography reduced the concentration of GTP sufficiently to unload the D181N mutant, which was then unable to bind SRα (Figure 5). These data suggest that the empty form of the SRβ GTPase domain is unable dimerize with SRα. To confirm this result and examine the nucleotide specificity for binding, this experiment was repeated and dimerization was assayed after adding back specific nucleotides.

When dimer formation was assayed with added ATP (up to 1 mM) co-precipitation of D181N and SRα was not observed. In contrast, adding GTP, GDP, XTP or XDP restored some SRα-binding by D181N (Figure 6). The effect of the xanthosine nucleotides is more pronounced than the guanosine nucleotides, consistent with the predicted preference of D181N for XTP compared to GTP. Surprisingly, there seems to be little preference for the di- or tri-phosphate forms of either nucleotide. Addition of GTP or XTP to 10 mM did not increase dimer formation further while 10 mM GDP or XDP resulted in only a marginal increase in dimer formation. We were unable to restore dimer formation to the levels observed prior to nucleotide depletion, suggesting that similar to other small molecular weight GTPases, D181N may be destabilized in the absence of bound nucleotide (28). Taken together, these data reveal that an intact and nucleotide bound SRβ GTP binding domain is both necessary and sufficient for binding to the SRX2 region of SRα.

DISCUSSION

Every step in the translocation of nascent polypeptides into and across the endoplasmic
reticulum membrane is highly regulated. Evidence of the extent of this regulation includes the identification of three GTPases, all with putative roles in the targeting of ribosomes to the translocon. Regulation begins when the signal peptide emerges from the ribosome where it is bound by SRP54, the GTPase in SRP. Transfer of the nascent polypeptide to the translocon occurs after the GTP-bound forms of SRP54 and SRα bind to each other. This binding interaction is unusually tight with a half-life for dissociation in vitro in excess of 6 hours (5). It has been proposed that transfer of the nascent polypeptide to the translocon is unidirectional because in the absence of GTP hydrolysis, binding of SRP54 to SRα is essentially permanent. Only after transfer of the nascent polypeptide to the translocon is GTP hydrolyzed by both SRP54 and SRα to release this interaction(5, 7, 29).

The role of the SRβ GTP binding domain has remained enigmatic, although the recent demonstration that the ribosome may be a GTPase activating protein for SRβ suggests a role in transfer of the ribosome to the Sec61 complex (10). Our results suggest an additional role for nucleotide binding in the regulation of dimerization of SRα and SRβ.

We have shown previously that SRα is anchored to the ER membrane through an interaction between the amino terminal SRX2 domain and SRβ(11). Our current data demonstrate that the minimal GTPase domain of SRβ is necessary and sufficient for binding to SRα via SRX2 (Figures 3 and 4). Since the sequences necessary for heterodimerization were localized to the GTP binding domain of SRβ, a number of point mutations were introduced to determine the effect of GTP binding on SR dimerization. Two mutants G118L, H119L that were expected to alter GTP hydrolysis without effecting GTP binding, retained the ability to coprecipitate SRα. In contrast, mutants that were unable to dimerize with SRα were either predicted to reduce the affinity of SRβ for both GTP and
GDP (K75I) or destabilize the GTPase fold (N178K) (Sigal et al., 1986; Pai et al., 1990).

However, the most convincing evidence that dimerization is regulated by nucleotide binding comes from the mutant D181N. This mutant is expected to have reduced affinity for GTP but a preference to bind XTP and XDP. In the absence of nucleotide this mutant was no longer able to bind to SRα. However, adding nucleotide back restored binding of SRβ to SRα. Surprisingly, GTP, GDP, XTP and XDP all supported dimerization suggesting that regulation is via the empty state. There is precedent for regulation of low molecular weight GTPases by guanine nucleotide exchange factors (30). However, a major difference between ras type and SRP type GTPases is that the empty state is transient for ras family GTPases while SRP GTPases are stable in the empty state (5, 31, 32). The similarity of the SRβ GTP binding domain to ras family GTPases suggests that nucleotide exchange by SRβ will be regulated by an as yet unidentified guanine nucleotide exchange factor. At present there are no candidates for such an exchange factor associated with the translocon.

Release of nucleotide from SRβ is predicted to dissociate the heterodimeric SR on the endoplasmic reticulum membrane. Is there any precedent for regulated binding of membranes by SR? In E. coli, the SR is composed of a single protein, FtsY, that is found in both the cytoplasm and on the plasma membrane (33). Membrane binding by FtsY is due in part to a direct interaction with lipids in the E. coli membrane (34). However, there is also evidence that FtsY membrane binding is regulated. First, since lipids are in a huge molar excess over FtsY, non-regulated binding to lipids is not compatible with the observed distribution of FtsY between membrane bound and soluble pools. Second, a large fraction of FtsY proteins are specifically cleaved after binding to the plasma membrane thereby limiting the total amount of FtsY bound to membrane (35). Finally, evidence has been presented for a membrane bound FtsY receptor (34).
What role could transient dissociation of SR play in protein targeting? The mechanism by which SRP-ribosome-nascent chain complexes are transferred from SR to the Sec61 complex appears to involve at least two steps, the details of which remain obscure (36). Furthermore, a direct interaction between SR and the Sec61 complex has not been demonstrated unambiguously. Membranes rendered translocation defective by proteolysis of Sec61α accumulate a post-targeting intermediate involving the ribosome-nascent chain (RNC) and SR (6). The next logical step is transfer of the RNC to the Sec61 complex. Taken together with the results presented here, a plausible albeit speculative role for regulated dissociation of SR would be to release the SRP-ribosome-nascent-chain complex from SRβ allowing it to be transferred to the Sec61 complex. Once transfer has occurred, the signal peptide would be released into the translocon triggering hydrolysis of GTP by SRP54 and SRα. Previously, this hydrolysis step was proposed to release SRP from SR for another round of targeting. According to our model this step releases SRP and SRα, allowing SRP to return to the cytoplasm and SRα to rebind to SRβ. Our future challenge is to devise a suitable system in which to test this model.
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Figure Captions

Figure 1

**SRβ deletion mutants.** Plasmids encoding deletion mutants of SRβ were created by PCR and cloned behind the SP6 promoter for translation *in vitro*. SRβ<sub>md</sub> is a chimera containing 10 amino acids of the murine and 255 amino acids of canine SRβ. The murine sequence is indicated by the bricked segment. The transmembrane domain is denoted by the hatched region and the scrambled sequence in SRβ-loop2 is denoted by pattern between residues 185 and 219. G1-G5 sequences represent the approximate locations of consensus GTPase sequences and are the same for all deletion mutants. (+), denotes a region enriched in arginine and lysine. Amino acid numbering is from SRβ<sub>md</sub>

Figure 2

**Proteolysis of selected SRβ mutants.**

SRβ mutants were translated in the absence (lanes 1, 5, 9, 13) or presence (lanes 2-4, 6-8, 10-12, 14-16) of canine microsomes (RM). Membranes were isolated from the translation reactions by gel filtration chromatography and divided into three aliquots. Digestion with proteinase K (PK) was used to examine topology with respect to the membrane and to assay protein folding. Triton X-100, (TX100) was added to solubilize the membrane to permit digestion of protease sensitive fragments otherwise protected from the protease by the membrane. Triangles indicate type I transmembrane molecules including a protease resistant core (dots) and the transmembrane
amino-terminus protected by the membrane. Asterisks indicate the transmembrane amino-terminus protected by the membrane. One microliter of total translation products were analyzed in lanes 1, 5, 9, 13. All other lanes correspond to 7 microliters of the original translation reaction. The migration positions of molecular weight standards are indicated to the left of the panel.

Figure 3

An intact SRβ GTPase domain is sufficient to bind to SRα.

Panel A) Aliquots of translation reactions containing equimolar amounts of SRα and the specified SRβ deletion mutants were mixed together and incubated prior to immunoprecipitation. Panel B) Immunoprecipitation with anti-HA precipitates all of the SRβ mutants because they contain an amino-terminal epitope tag. Full length SRα precipitates efficiently with SRβ−∆TM and SRβD4, but precipitates poorly with SRβ−∆loop and SRβ-loop2 (upward arrowheads). Panel C) SRβ−∆TM, SRβD4, SRβ−∆loop and SRβ−loop2 precipitate with SRα. Precipitation of SRβ−∆loop and SRβ−loop2 (upward arrows) is 30% and 15% as efficient as precipitation of SRβ−∆TM, respectively. Panel D) 36 hours exposure (instead of 19 hours) illustrates binding of full length SRα with SRβ−∆loop and SRβ-loop2 (lanes 1-2). The amino terminal SRX2 domain of SRα (upward arrowheads) binds to both SRβD4 and SRβ−loop2 (lanes 3-4). Sequential precipitation of a translation reaction containing both SRα and SRβ with antibodies to the carboxyl-terminus of SRβ (lane 5) and then with antibodies to HA
(Lane 6) reveals that the carboxyl-terminus of SRβ is obscured by dimerization. The migration position of full length SRα is indicated by an arrow to the left of the panels. The dot indicates a SRα translation product that results from initiation of translation at an internal methionine. The bracket indicates the migration positions of the SRβ deletion mutants.

**Figure 4: Coimmunoprecipitation of SRα with SRβ GTPase mutants.** Translation reactions containing equimolar amounts of SRα and the SRβ GTPase mutants were mixed, incubated and then immunoprecipitated with anti-HA antibodies (panel A, precipitates the SRβ GTPase mutants) or anti SRα (panel B). SRX2 was also immunoprecipitated with selected GTPase mutants (inset, panel 1). Total products are unprocessed *in vitro* translation products (panel C). The migration position of full length SRα and SRβ are indicated by arrows to the left of the panels. The dot indicates a SRα translation product that results from initiation of translation at an internal methionine.

**Figure 5: D181N does not coimmunoprecipitate with SRα in the absence of GTP.** *In vitro* translation reactions were either depleted of nucleotides by gel filtration (-GTP) or left untreated (+GTP). Equimolar amounts of SRα and either SRβ−ΔTM or D181N molecules were mixed and then immunoprecipitated with anti-SRα antibodies. Total products are aliquots of the mixed *in vitro* translation products prior to immunoprecipitation. Migration positions of SRα and SRβ−
ΔTM are indicated by arrows. The dot indicates a SRα translation product that results from initiation of translation at an internal methionine.

**Figure 6: SRα binding to D181N can be restored by adding back nucleotide.** *In vitro* translation products were depleted of small molecules by gel filtration. Equimolar amounts of SRα and D181N were mixed and then the indicated nucleotide was added to the reaction. After incubation, immunoprecipitation with anti-SRα antibodies was used to measure the percentage of D181N that bound to SRα. The amount of SRβ–ΔTM that co-precipitated with SRα without depleting nucleotide was arbitrarily assigned a value of 100% (α/β) and is indicated for comparison. The results of one experiment (representative of three) are shown.
Table I: Effect of SRβ mutations on the ability to bind SRα

N/A = Not Applicable, nd = not determined, ts = temperature sensitive, wt = wild type

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| Mutant                  | Coimmunoprecipitate | GTPase box | Phenotype in yeast¹ |
|------------------------|---------------------|------------|---------------------|
| SRβ-ΔTM                | ++++                | N/A        | wt                  |
| SRβD4                  | ++++                | N/A        | nd                  |
| SRβD5                  | -                   | N/A        | nd                  |
| SRβ1C1-ΔTM             | -                   | N/A        | nd                  |
| SRβ-Δloop              | ++                  | N/A        | nd                  |
| SRβ-loop2              | +                   | N/A        | nd                  |
| K75I                   | -                   | G-1        | ts                  |
| G118L                  | ++++                | G-3        | ts                  |
| H119L                  | ++++                | G-3        | wt                  |
| N178K                  | +                   | G-4        | ts                  |
| D181N                  | ++++                | G-4        | wt                  |
| K75I/H119L             | -                   | G-1, G-3   | null                |
| G118L/D181N            | +                   | G-3, G-4   | nd                  |
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