The β Subunit of the Signal Recognition Particle Receptor Is a Transmembrane GTPase that Anchors the α Subunit, a Peripheral Membrane GTPase, to the Endoplasmic Reticulum Membrane

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Abstract. The signal recognition particle receptor (SR) is required for the cotranslational targeting of both secretory and membrane proteins to the endoplasmic reticulum (ER) membrane. During targeting, the SR interacts with the signal recognition particle (SRP) which is bound to the signal sequence of the nascent protein chain. This interaction catalyzes the GTP-dependent transfer of the nascent chain from SRP to the protein translocation apparatus in the ER membrane. The SR is a heterodimeric protein comprised of a 69-kD subunit (SRα) and a 30-kD subunit (SRβ) which are associated with the ER membrane in an unknown manner. SRα and the 54-kD subunit of SRP (SRP54) each contain related GTPase domains which are required for SR and SRP function. Molecular cloning and sequencing of a cDNA encoding SRβ revealed that SRβ is a transmembrane protein and, like SRα and SRP54, is a member of the GTPase superfamily. Although SRβ defines its own GTPase subfamily, it is distantly related to ARF and Sar1. Using UV cross-linking, we confirm that SRβ binds GTP specifically. Proteolytic digestion experiments show that SRα is required for the interaction of SRP with SR. SRα appears to be peripherally associated with the ER membrane, and we suggest that SRβ, as an integral membrane protein, mediates the membrane association of SRα. The discovery of its guanine nucleotide-binding domain, however, makes it likely that its role is more complex than that of a passive anchor for SRα. These findings suggest that a cascade of three directly interacting GTPases functions during protein targeting to the ER membrane.

In eucaryotic cells the translocation of most secretory and the integration of most membrane proteins into the endoplasmic reticulum (ER) are cotranslational events. Targeting of ribosomes synthesizing such proteins from the cytoplasm to the ER is catalyzed by the signal recognition particle (SRP),[1] which binds to signal sequences on the polypeptide chains emerging from the ribosome (reviewed in Walter and Johnson, 1994). Subsequent to signal sequence recognition in the cytosol, the resulting complex is targeted to the cytoplasmic face of the ER membrane via the interaction of SRP with its membrane bound receptor (Gilmore et al., 1982*a*,*b*; Meyer et al., 1982). Upon binding to the SRP receptor (SR), SRP dissociates from both the signal sequence and the ribosome, allowing the engagement of the ribosome with the translocon, a protein apparatus in the membrane that forms a pore through which the nascent polypeptide moves across the lipid bilayer (Gilmore and Blobel, 1983; Simon and Blobel, 1992; Görlich and Rapoport, 1993; Crowley et al., 1994). Thus, SRP and SR are the "initiation factors" of protein translocation mediating both targeting and the formation of the ribosome/translocon junction.

Both SR and SRP are complex structures: SRP is a ribonucleoprotein consisting of six distinct protein subunits and one RNA subunit (Walter and Blobel, 1980; Walter and Blobel, 1982). The most phylogenetically conserved SRP protein subunit, SRP54, contains the signal sequence-binding site of SRP and mediates SRP binding to SR (Krieg et al., 1986; Kurzchalia et al., 1986; Miller et al., 1993; Brown et al., 1994). The SR is a heterodimeric membrane protein consisting of a 69-kD (SRα) and a 30-kD subunit (SRβ) (Tajima et al., 1986). It is now known how SR interacts with either SRP54 or the membrane of the ER.

GTP is required for multiple steps of the targeting reaction, and both SRα and SRP54 contain GTPase domains (Connolly and Gilmore, 1986, 1989; Bernstein et al., 1989; Römisch et al., 1989). The GTPase domains of SRα and SRP54 are related and define a new subfamily in the GTPase

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1. *Abbreviations used in this paper*: DIFP, diisopropylfluorophosphate; DTT, dithiothrietol; KOAc, potassium acetate; $Mg(OAc)_2$, magnesium acetate; SRP, signal recognition particle; SR, SRP receptor; TEA, triethanolamine.

superfamily (Bernstein et al., 1989; Römisch et al., 1989; Bourne et al., 1991). A model describing the cycle of GTP binding and hydrolysis by SRP54 during the protein targeting reaction has been proposed (Miller et al., 1993). According to this model, SRP54 becomes stabilized in a nucleotide-free state when SRP binds to a signal sequence exposed on the ribosome. Interaction of SRP with the SR on the ER membrane in the presence of translocon components stimulates GTP binding to SRP54 with the concomitant release of SRP from the signal sequence and ribosome, which then engage with the translocon to translocate the protein across the membrane. After release from the ribosome and signal sequence, the SRP, in its GTP-bound state, remains tightly bound to the SR. The SR then functions as a GTPase activating protein for SRP54 and, upon hydrolysis of the bound GTP, SRP is released from SR into the cytosol, free to enter into another round of targeting. SR and SRP work catalytically to promote the interaction of the nascent chain-ribosome complex with the translocon and do not remain associated after the ribosome/translocon junction is formed. Thus, during protein targeting the assembly and disassembly of complexes is regulated by GTP binding proteins, as are other protein–protein interactions that need to be formed and broken in cells in a coordinated and regulated manner (Bourne et al., 1990).

The individual contribution that the two subunits make to SR function is largely unknown. There is evidence that GTP binding to SRα is required for translocation, but it is not known what role this serves (Rapiejko and Gilmore, 1992). SRβ has no known function. Either one or both subunits could conceivably be required for any of the known SR functions of SRP binding, membrane binding, regulation of the SRP54 GTPase cycle, and promoting translocation. Here, we examine the role of the two subunits in the association of SR with SRP and with the ER membrane. We also show that, like SRα and SRP54, SRβ is a member of the GTPase superfamily. This brings to three the number of directly interacting GTPases, suggesting that a GTPase cascade of unprecedented complexity functions during protein targeting.

Materials and Methods

Materials

α-[^{32}P]GTP (3,000 Ci/mmole) was purchased from Amersham Corp. (Arlington Heights, IL); Na[^{125}I] (100 mCi/ml) from New England Nuclear (Boston, MA); Nikkol (octa-ethylene-mono-*n*-dodecyl ether) from Nikko Chemicals Co., Ltd. (Tokyo, Japan); nitrocellulose filters from Schleicher & Schuell, Inc. (Keene, NH); Trasylol (10,000 kallikrein inhibition units per ml) from FBA Pharmaceuticals (New York, NY); TPCK-trypsin from Worthington Biochemical Corp. (Freehold, NJ); aminopentyl agarose, cyanogen bromide (CNBr) and protease inhibitors from Sigma Chemical Co. (St. Louis, MO); Freund's complete and incomplete adjuvant, anti-mouse Ig and anti-rabbit Ig antibodies from Cappel Laboratories, Malvern, PA; CNBr-activated Sepharose CL-4B, CM-Sepharose, and protein A-Sepharose from Pharmacia Fine Chemicals (Uppsala, Sweden); DEAE Affigel Blue and hydroxylapatite from Bio-Rad Laboratories (Richmond, CA).

General Methods

Preparation of rough microsomal membranes, their salt extraction and purification of SRP and SRP receptor were performed as described previously (Gilmore and Blobel, 1983; Walter and Blobel, 1983*a*,*b*; Tajima et al., 1986). Immunoblotting was performed using ^{125}I-labeled secondary antibodies as previously described (Tajima et al., 1986). SRα was detected with the mouse monoclonal IgG antibody directed against epitope A (Tajima et al., 1986), mp30 with a rabbit polyclonal serum (Tajima et al., 1986), and SRβ with a mouse monoclonal antibody described here.

Preparation of Monoclonal Antibody to SRβ

The anti-SRβ antibody is an IgM made by injecting Freund's adjuvant emulsified SRβ (purified by preparative SDS-PAGE) into the foot pad of a mouse followed by dissection of the popliteal lymph node and fusion to myeloma cells to create a hybridoma cell line. Hybridoma cells were propagated as ascites tumors. The monoclonal antibody was identified as an IgM using a kit purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). IgM was purified from mouse ascites fluid. To this end, the IgM was bound to anti-mouse IgM–Sepharose, washed with 0.5 M sodium chloride/10 mM phosphate buffer, pH 7.5, 0.1% Triton X-100 and eluted with 3.5 M magnesium chloride.

Alkaline Extraction of Microsomal Membranes

Three different solutions were used for alkaline extraction: (*a*) 100 mM sodium carbonate, pH unadjusted (pH 11.2); (*b*) 100 mM sodium carbonate, adjusted to pH 12.0 by the addition of sodium hydroxide; and (*c*) 100 mM sodium hydroxide, pH 13.0. Membranes were diluted 1:100 into alkaline solution to obtain a final membrane concentration of 0.04 equivalents (eq)/ml (see Walter and Blobel, 1983*a*) for definition of equivalent). After 30 min at 25°C, the reactions were centrifuged for 30 min at 100,000 rpm in a Beckman TL 100.1 rotor. Supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblotting.

Triton X-114 Extraction of Microsomal Membranes

Membranes were solubilized at 0.3 eq/μl in 1% Triton X-114, 10 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, and 1 mM dithiothreitol (DTT). After incubation on ice for 15 min, the reactions were transferred to a 37°C water bath for 3 min to induce phase separation. Detergent-poor and detergent-rich phases were separated by a 5-min centrifugation in a microfuge through a cushion of 175 mM sucrose in the above buffer containing 0.06% Triton X-114.

Trypsin Treatment of Microsomal Membranes

Salt-extracted microsomal membranes were diluted to 2 eq/μl in high-salt buffer containing 50 mM triethanolamine (TEA), pH 7.5, 500 mM potassium acetate (KOAc), 5.5 mM magnesium acetate ($Mg(OAc)_2$), 0.5 mM (ethylenedinitrilo)tetraacetic acid (EDTA), 1 mM DTT. Trypsin-TPCK was added and the reaction was incubated on ice for 1 h. Digestion was stopped by addition of 2 mM diisopropylfluorophosphate (DIFP), 1 mM PMSF and 100 U/ml Trasylol. After 15 min on ice the membranes were either assayed as in Fig. 1 *B*, or pelleted by centrifugation at 50,000 rpm in a Beckman Ti 70.1 rotor for 30 min through a cushion of 250 mM sucrose in high-salt buffer containing 0.1 mM PMSF. The membrane pellet was resuspended in high-salt buffer and the centrifugation was repeated. After this washing step, the pellet was dissolved in 50 mM TEA, 250 mM sucrose, and 1 mM DTT. The membrane suspension was frozen in liquid nitrogen and stored at −80°C until further use.

SRP–Sepharose Chromatography

Trypsinized membranes were diluted to 1 eq/μl in 1% Nikkol, 50 mM TEA, pH 7.5, 375 mM KOAc, 250 mM sucrose, 1 mM DTT, 10 U/ml Trasylol, 0.5 mM PMSF, and 0.1 mM DIFP and were extracted for 15 min on ice. The soluble fraction was obtained as the supernatant after a 30-min centrifugation at 100,000 rpm in the Beckman TL 100.1 rotor. The solubilized membranes were adjusted to 0.13 eq/μl in equilibration buffer (50 mM TEA, pH 7.5, 50 mM KOAc, 5 mM $Mg(OAc)_2$, 250 mM sucrose, 1 mM DTT and 0.5% Nikkol) and 650 μl was applied to a 0.15 ml SRP–Sepharose column containing 0.15 mg of covalently coupled SRP. After washing with 0.6 ml of equilibration buffer, the column was eluted with 0.8 ml elution buffer (50 mM TEA, pH 7.5, 10 mM KOAc, 25 mM $Mg(OAc)_2$, 250 mM sucrose, 1 mM DTT, and 0.5% Nikkol). Coupling of SRP to CNBr–Sepharose was as described previously (Tajima et al., 1986).

Protein Sequencing

Immunopurified SR (Tajima et al., 1986) was adjusted to 5% twice recrystalized SDS and heated to 80°C for 10 min to dissociate the two subunits and then fractionated by reverse phase chromatography on an Alltech C4 column using an IBM HPLC to yield purified SRα and SRβ.

Peptide sequence of SRβ was determined by Edman degradation, using an ABI automated sequenator. Sequencing the intact protein yielded two identical, overlapping sequences that were staggered by two amino acids: XXMGDGGGVGGAFQPYLDSLR and XXXXMGDGGGVGGAFQPYLDSLR. The yield of PTH amino acid released/μg per cycle of Edman degradation was considerably lower than that obtained from proteolytic fragments. Therefore, it is likely that the true amino terminus is blocked and that we obtained sequence from a small amount of proteolytic breakdown product. We also sequenced the amino-terminus of a proteolytic fragment that was generated by lysyl-endopeptidase digestion of SRβ and purified on a microbore C18 reverse phase column from Vydac using a Rainin HPLC. The sequence read KWLAK.

A third peptide sequence was obtained by performing five rounds of Edman degradation on total CNBr cleaved SRβ to expose a proline residue at the amino-terminus of one of the CNBr fragments. The amino-termini of all the other CNBr fragments were then blocked with *ortho*-phthalaldehyde (OPA; Brauer et al., 1984). Because proline does not react with OPA, it remains unblocked and, therefore, susceptible to Edman degradation. Sequencing was then resumed yielding a single sequence from the CNBr fragment. The sequence read PLIACNKQD.

cDNA Cloning

To obtain a cDNA clone of SRβ, a Madin-Darby Canine Kidney (MDCK) cell cDNA library constructed in the plasmid vector pEX (Stanley and Luzio, 1984) was screened using the anti-SRβ monoclonal described here. A total of 3×10^5 bacterial colonies were screened by a modification of the colony blotting procedure of Stanley (Stanley, 1983). Expression of the cDNAs was induced by incubating the filters at 42°C, lysing the cells at 90°C in 5% SDS, probing with the monoclonal antibody and using an alkaline phosphatase-conjugated secondary antibody to detect positive colonies. Four positive colonies passed secondary and tertiary screening; these cDNA clones were partially sequenced using the double-stranded Sequenase (USB) protocol and identified as encoding SRβ sequences because their predicted translation products contained the amino acid sequence obtained from direct protein sequencing of the amino-terminus. They were then subcloned into a Bluescript-II vector (Stratagene Inc., La Jolla, CA), single-stranded DNA was synthesized and the entire cDNAs were sequenced on both strands using Sequenase. The predicted translation products of both clones contained both additional peptide sequences derived from sequencing SRβ, thus confirming the identity of the clones.

The predicted translation productions of both clones contained consensus sequences for GTP-binding proteins (Bourne et al., 1991). Two sets of clones (1.0 and 1.2 kb in length) were isolated; they differed from each other by the spacing between region G-1 and region G-3 of the GTP-binding consensus sequence, by the length of the 3′ poly(A) tail, and by the overall length of the cDNA insert. The 1.0 kb clone presumably encodes SRβ. It contains an ~60 nucleotide-long poly(A) tail, and the spacing between regions G-1 and G-3 of the predicted protein product conforms to the spacing found in other GTPases. In contrast, the 1.2 kb clone contains only six A residues at the polyadenylation site followed another 500 bp of noncoding sequence. This clone also contains an in frame deletion of 26 amino acids between regions G-1 and G-3 (residues 82-107 from the SRβ sequence shown in Figs. 3 and 4 would be deleted). We consider it likely that this cDNA clone was derived from an alternately or erroneously spliced form of the mRNA.

The amino acid sequence deduced from the canine cDNA does not begin with a methionine and extends just past the amino acid sequence derived from amino-terminal sequencing. Therefore, a full-length SRβ cDNA was isolated by screening a murine teratocarcinoma cDNA library constructed in λ-ZAP (Stratagene) using the canine cDNA as a hybridization probe (Maniatis et al., 1982). Eight independent clones were obtained from 1.2×10^6 plaques screened and were verified by DNA sequencing. All of the murine clones corresponded to the 1 kb canine clones described above. The differences between the predicted canine and murine proteins are listed in Table I.

Table I. Comparison of Canine and Murine Proteins

Amino acid no.	7	9	12′	12″	13	39	42	56	59	67	72	86	100
Canine SRβ	P	M	G	G	V	V	V	R	R	L	N	L	M
Murine SRβ	R	V			A	A	L	W	K	F	D	Q	I

Amino acid no.	102	106	108	109	124	126	128	132	137	166	170	173	183
Canine SRβ	R	T	A	T	L	F	E	A	I	S	T	F	T
Murine SRβ	K	N	G	N	F	L	D	S	V	A	S	L	A

The positions where the two sequences differ are indicated. The amino acid numbers refer to the murine protein as shown in Fig. 3 *A*. In the canine sequence there is an insertion of GG, labeled 12′ and 12″, between amino acids 12 and 13 of the murine protein.

Sequence Analyses

Homology searches were conducted using the BLAST network at the NCBI (Altschul et al., 1990). The *Saccharomyces cerevisiae* SRβ homologue was found as a predicted open reading frame in genomic sequence from chromosome 11 (accession no. Z66877). Limited sequence similarity was also found with the *S. cerevisiae IRA2* protein (accession no. RGBYI2; residues 191-218 of murine SRβ are 59% identical to residues 439–466 of *S. cerevisiae* Ira2p) and the *S. cerevisiae SWI4* protein (accession no. S07106; residues 164–228 of murine SRβ are 33% identical to residues 800–864 of *S. cerevisiae* Swi4p); however, in both cases the similarities were not phylogenetically conserved between mammalian and yeast SRβ sequences and are therefore unlikely to be functionally significant. Secondary structure predictions based on amino acid sequence were done using Chou-Fasman parameters (Rawlings et al., 1983).

GTP Cross-linking Assay

SRP-Sepharose purified SR (Gilmore and Blobel, 1983; Tajima et al., 1986) was mixed at 20 nM with 0.3 μM α-[^{32}P]-labeled GTP at 25°C in 50 mM TEA, pH 7.5, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 0.5% Nikkol. Some reactions were supplemented with unlabeled nucleotide to compete for binding with the radiolabeled substrate. After a 20-min incubation at 25°C the 20-μl reactions were placed in plastic weigh boats on ice and UV irradiated (6 cm from a 6000 W/cm^2 UV source) for 5 min to form covalent cross-links of the bound radiolabeled nucleotide to the protein (Nath et al., 1985; Miller et al., 1993). The reactions were then precipitated with an equal volume of 30% trichloroacetic acid to remove uncrosslinked radiolabeled nucleotide and analyzed by SDS-PAGE followed by autoradiography. Quantitation was done using a Bio-Rad densitometer to scan autoradiograms that were determined to be in the linear range of both the film and the machine. Data points are experimental and the line is generated as a best fit to the equation: $B = B_{max}(1 - [I]/\{[I] + K_i[1 + ([S]/K_D)]\})$ (a modification of equation III-5 described by Segel [1975]) using the program Kaleidagraph (Abelbeck Software, 1989) on a Macintosh II computer. B, amount of α-[^{32}P]GTP cross-linked to SRP54; B_{max}, amount of a α-[^{32}P] GTP crosslinked to SRP54 in the absence of competitor; [I], concentration of competitor; K_i, dissociation constant of competitor; K_D, dissociation constant of α-[^{32}P] GTP; [S], concentration of α-[^{32}P]GTP. GTP hydrolysis was measured in these reactions as described previously (Miller et al., 1993) and found to be negligible (not shown).

Results

SRα Is Required for SR Binding to SRP

To investigate the individual roles of the two SR subunits in the interaction with SRP, we took advantage of the different sensitivities of SRα and SRβ to proteolytic digestion. As shown in Fig. 1 *A*, trypsin at a concentration as low as 1 μg/ml begins to degrade SRα (lanes 4–6), while a minimal concentration of 30 μg/ml is required to begin to degrade SRβ (lanes *10–12*). To generate extracts containing different relative amounts of SRα and SRβ, we first incubated rough microsomal membrane vesicles with variable amounts of trypsin, extracted peripherally attached digestion products with high salt and then solubilized the remaining membrane-associated SR fragments with detergent. Passing a detergent extract prepared from undigested membranes over an SRP–Sepharose affinity column resulted in the quantitative binding of the SRα/SRβ complex to the resin (Fig. 1 *B*, lanes *1–3*) and allowed the recovery of the bound receptor by elution (Fig. 1 *B*, lane *4*). This result was expected, as SR was originally purified by a similar procedure using affinity chro-

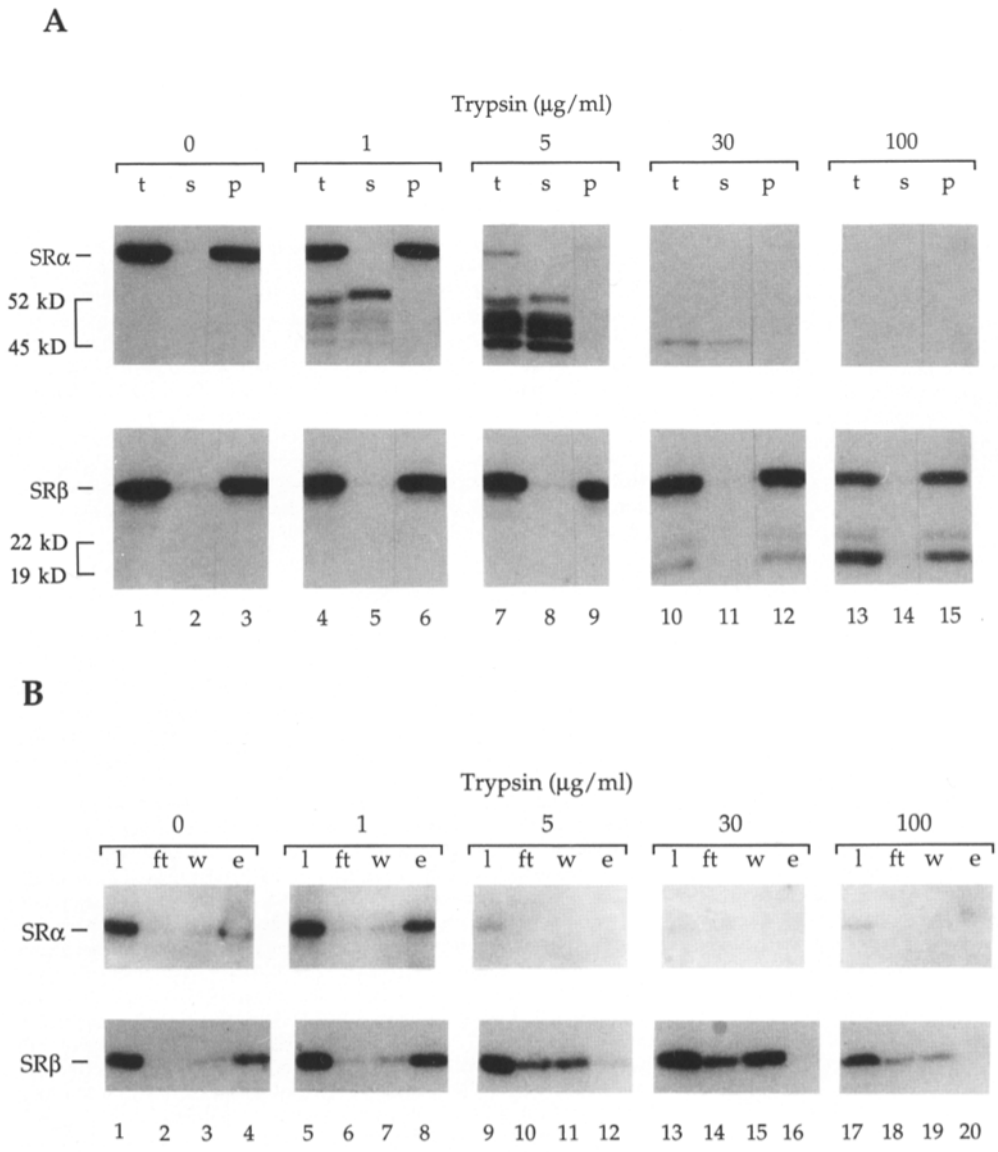


Figure 1. Requirement for intact SRα for SRP binding. (*A*) tryptic digestion pattern of SR. Canine microsomal membranes were digested in high salt (500 mM potassium) with the indicated concentration of trypsin-TPCK. Proteolysis was stopped by the addition of protease inhibitors and the membranes were pelleted by centrifugation. Equivalent amounts of the total reaction mixture (*t*), and of the supernatant (*s*) and pellet fractions (*p*) were separated by SDS-PAGE and immunoblotted for SRα and SRβ as indicated. The position of undigested protein is labeled and the position of proteolytic breakdown products are indicated by brackets. (*B*) Binding of proteolyzed SR to SRP-Sepharose. The trypsinized membranes from *A* were washed with high salt buffer, solubilized with the nonionic detergent Nikkol and applied to an SRP-Sepharose affinity column. The column was washed and eluted as described in Methods. Equivalent amounts of the load (*l*), flow through (*ft*), wash (*w*), and elution (*e*) fractions were separated by SDS-PAGE and immunoblotted for SRα and SRβ as indicated.

matography on SRP-Sepharose resins (Gilmore et al., 1982*b*; Gilmore and Blobel, 1983; Tajima et al., 1986). In contrast, when extracts were prepared from tryspin-digested membranes, intact SRβ was recovered in the flow through and wash fractions. The amount of SRβ recovered was roughly proportional to the amount of SRα that was degraded (Fig. 1 *B*, lanes *5–20*). Thus, we conclude that SRα is required for binding of the SRα/SRβ complex to SRP. In the simplest scenario this would occur through a direct interaction of SRα with SRP. However, more complicated possibilities, e.g., that SRα is an allosteric regulator of SRβ and, as such, is required for SRβ to bind SRP, cannot be ruled out from these data.

Membrane Association of SR

SRα was proposed to associate with the ER membrane through its amino-terminal region (Hortsch et al., 1985; Lauffer et al., 1985; Andrews et al., 1989). The two stretches of hydrophobic amino acids that are present in this region are, however, of insufficient length to function as conventional transmembrane helices. The SRα/ER membrane interaction may involve protein–lipid and/or protein–protein interactions, and one function of SRβ might be to tether SRα to the ER membrane. To determine whether the SR subunits behave as peripheral membrane proteins or as integral membrane proteins, we performed carbonate extraction of microsomal vesicles. Carbonate solutions at pH 11.2 are nonspecific protein denaturants that disrupt protein–protein interactions that bind peripheral membrane proteins to the membrane but do not disrupt protein–lipid interactions that retain integral membrane proteins in the lipid bilayer (Fujiki et al., 1982; Davis and Model, 1985). After extraction, lipid bilayers containing integral membrane proteins are collected by centrifugation leaving peripheral membrane proteins in the supernatant. When microsomal vesicles were subjected to carbonate extraction at pH of 11.2, neither SRα nor SRβ par-

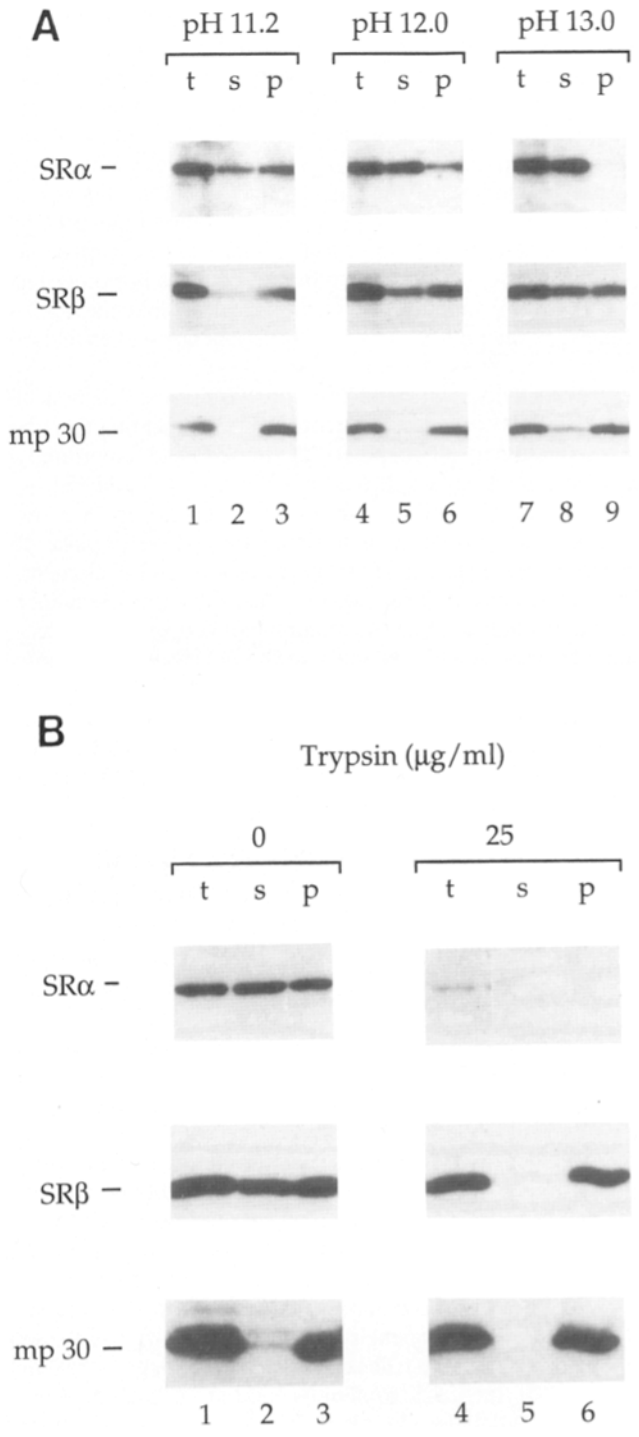


Figure 2. Membrane association of SR. (*A*) Alkaline extraction of canine microsomal membranes. Microsomes were extracted at either pH 11.2 (lanes *1–3*), 12.0 (lanes *4–6*), or 13.0 (lanes *7–9*) and then pelleted by centrifugation. Equivalent amounts of the total reaction mixture (*t*) and of the supernatant (*s*), and pellet (*p*) fractions were separated by SDS-PAGE and immunoblotted for SRα, SRβ, and mp30 as indicated. (*B*) Triton X-114 extraction of canine microsomal membranes. Membranes were either mock proteolyzed (lanes *1–3*) or treated with 25 μg/ml trypsin (lanes *4–6*) and then extracted with the detergent Triton X-114 as described in Methods. Equivalent amounts of the total reaction mixture (*t*), and of the "detergent-poor" supernatant (*s*), and "detergent-rich" pellet (*p*) fractions were separated by SDS-PAGE and immunoblotted for SRα, SRβ, and mp30 as indicated.

titioned cleanly into either the supernatant or the pellet fraction (Fig. 2 *A*, lanes *1–3*). Both subunits were partially extracted, with more SRα being released from the membrane than SRβ. In contrast, mp30, a control bona fide ER integral membrane protein of unknown function (Tajima et al., 1986) (J. Moskaug and P. Walter, unpublished observation), was exclusively recovered in the membrane pellet fraction. To obtain more stringent extraction conditions, we adjusted the pH to increasingly alkaline conditions. At pH 12.0 and 13.0 most of SRα and approximately half of SRβ was extracted, while most of the mp30 still pelleted with the membranes (Fig. 2 *A*, lanes *4–9*). Taken together, these results suggest that SRβ has a more hydrophobic character than SRα, but do not allow SRβ to be unambiguously characterized as an integral membrane protein.

Because of this anomalous behavior, we examined the membrane association of SR by another, independent, means. Solutions of the nonionic detergent Triton X-114 undergo a phase separation when warmed from 4°C to room temperature, and proteins dissolved in the detergent solution partition between the hydrophilic, detergent-poor supernatant phase and the hydrophobic, detergent-rich pellet phase according to their hydrophobicity (Bordier, 1981). Usually, integral membrane proteins are exclusively recovered in the detergent-rich phase. The analysis of a Triton X-114 extract of microsomal vesicles is shown in Fig. 2 *B*. Both SRα and SRβ again distributed ambiguously into both detergent-poor supernatant and detergent-rich pellet fractions (lanes *1–3*), while mp30 behaved as a true integral membrane protein, partitioning exclusively into the detergent-rich pellet fraction.

The ambiguous results obtained in these experiments must be caused by structural features of SR that distinguish it from "classical" integral and peripheral membrane proteins. SRα is a highly charged, hydrophilic molecule (Lauffer et al., 1985), and SRα and SRβ bind tightly to one another (Tajima et al., 1986). We therefore considered the possibility that, although SRβ may be an integral membrane protein, its hydrophobic character may be obscured during the fractionation procedures described above because of its association with the large, hydrophilic SRα moiety. To test this hypothesis, we again took advantage of the differential sensitivity to trypsin digestion of SRα and SRβ (Fig. 1 *A*). At 25 μg/ml trypsin, SRα was almost completely degraded, while SRβ was essentially unaffected (Fig. 2 *B*, compare lane *4* with lane *1*). Repeating the partitioning after Triton X-114 extraction of such trypsinized membranes (Fig. 2 *B*, lanes *4–6*), resulted in the almost quantitative partitioning of SRβ into the detergent-rich phase, similar to the mp30 control. These results suggest that SRβ is indeed a true integral membrane protein and, as such, could function to anchor SRα to the membrane.

Cloning of SRβ

To confirm this conclusion and to address the structure/function relationship of the SR subunits in further detail, we isolated and sequenced a cDNA clone encoding SRβ. A canine cDNA clone was obtained by screening an expression library with a monoclonal antibody directed against the SRβ protein (see Materials and Methods) and its sequence predicts a protein of the correct molecular mass (~30 kD). Peptide sequence data was obtained from the amino terminus and from

two internal sites (see Materials and Methods) of purified SRβ which confirmed the identity of the cDNA clone. Because different cDNA clones isolated from the canine library all lacked an amino-terminal methionine, we used the canine cDNA as a hybridization probe to isolate a clone from a murine cDNA library. Primer extension studies performed with poly(A)+ RNA (data not shown) and the presence of a putative initiator methionine indicated that the isolated clone was full-length. The mouse and canine cDNA clones predict highly homologous proteins; the few differences between their predicted amino acid sequences are listed in Table I. The predicted sequences were used to search the Genbank database. This search resulted in the identification of a highly similar protein predicted by an open reading frame of unknown function in *S. cerevisiae*. The deduced amino acid sequences of both the murine and the putative yeast SRβ proteins are aligned in Fig. 3. The two proteins are ~23% identical.

Consistent with the Triton X-114 partitioning data of the proteolyzed SR, the protein sequences predict a 19-amino acid, putative transmembrane domain (shaded box in Fig. 3) that is conserved between the mammalian and yeast proteins. This supports the notion that SRβ is a bona fide integral membrane protein. On its amino-terminal side, the transmembrane domain of mammalian SRβ is flanked by a short, ~30-amino acid-long region. The bulk of the protein is carboxyl-terminal to the transmembrane region. Extensive trypsin digestion of intact microsomal vesicles generates fragments of SRβ in the 20-kD range (Fig. 1 *A*, lanes *10* and *13*). Because these fragments have lost pieces much longer than the 30-amino acid amino-terminal tail and because the ER lumenal part of the protein is protected from proteolysis by the membrane of the microsomal vesicles, this result predicts that SRβ is a type I integral membrane protein whose carboxy-terminal portion resides on the cytoplasmic face of the ER. The transmembrane region is not preceded by a cleavable signal sequence, suggesting that the transmembrane region functions as a signal-anchor sequence during the biogenesis of SRβ.

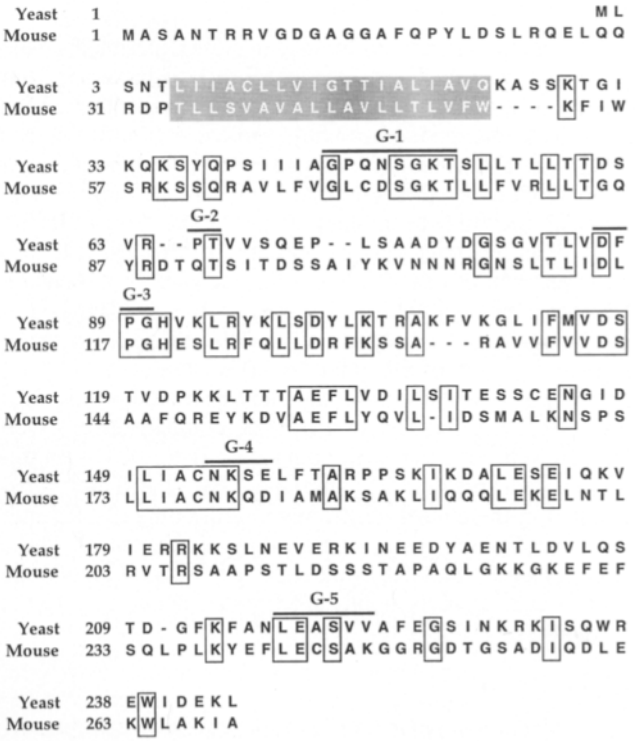


Figure 3. Primary structure of SRβ. Amino acid sequence deduced from cDNA clone of SRβ. The sequence of the murine protein is given on the lower line and the yeast sequence on the upper line. Identical residues are boxed and the predicted transmembrane domain is shaded. The GTP binding consensus motifs are labeled G-1 through G-5. Refer to the Results and to the legend to Fig. 4 for a description of the motifs and their placements.

Unexpectedly, we found that the cytoplasmic portion of SRβ contains consensus sequences for GTP binding that are characteristic to all members of the GTPase superfamily (elements marked G-1 through G-5 in Fig. 3 *A* and Fig. 4) (Bourne, et al., 1991). An unusual feature is that the *S. cerevisiae* SRβ contains a glutamate residue in place of an otherwise invariant aspartate residue the G-4 element. This change has not been seen in other GTPases. Sequence alignments indicate that the mammalian and yeast SRβ proteins form a discrete subfamily in the GTPase superfamily. Based on sequence alignments, the most closely related GTPases are those of the ARF and Sar1 subfamilies which function in membrane vesicle trafficking. The GTPase domains of mammalian SRβ are ~25% identical at the amino acid level to mammalian ARF and Sar1 (Fig. 4).

Structure Prediction of the SRβ GTPase Domain

The three-dimensional structures of several different GTPases are known: *ras* (Pai et al., 1989, 1990), EF-Tu (Jurnak, 1985), transducin (Noel et al., 1993), and $G_{\alpha s}$ (Lambright et al., 1994). The structures reveal the conserved structural core of the GTPase superfamily: a six-stranded β-sheet surrounded on both sides by five conserved α-helices. In the GTPase fold, the core β-sheet is largely buried beneath the hydrophobic faces of the enclosing α-helices. The signatures of this GTPase fold are the conserved motifs G-1–G-5 (which have been noted above) corresponding to connecting loops which interact with and respond to the bound nucleotide. The known structures also reveal the features that vary between different GTPases and confer the unique specificity and regulatory features of each protein (Bourne et al., 1991). As the G-1–G-5 motifs can be identified in the sequence of SRβ, and as the protein can be shown to interact with GTP (see below), it is reasonable to suggest that the structure of the SRβ protein is similar to that of other GTPases. We have exploited this idea in the alignment presented in Fig. 4.

The alignment of residues 62–187 of the amino acid sequence of SRβ with the sequences of ARF, Sar-1 and *ras* is relatively straightforward and can be accomplished without the introduction of substantial gaps or insertions. The alignment of motifs G-1, G-3, and G-4 is supported by the hydrophobic character of the sequences preceding each motif (e.g., AVLFV before G-1, LTLIDLP before G-3, and LLIAC before G-4), consistent with the presence of the β-strands which precede each motif. Strand β4 of the sheet, which is not followed by a conserved loop sequence, can also be located based on the hydrophobic character of the sequence VVFVV. The region between motifs G-1 and G-3 (amino

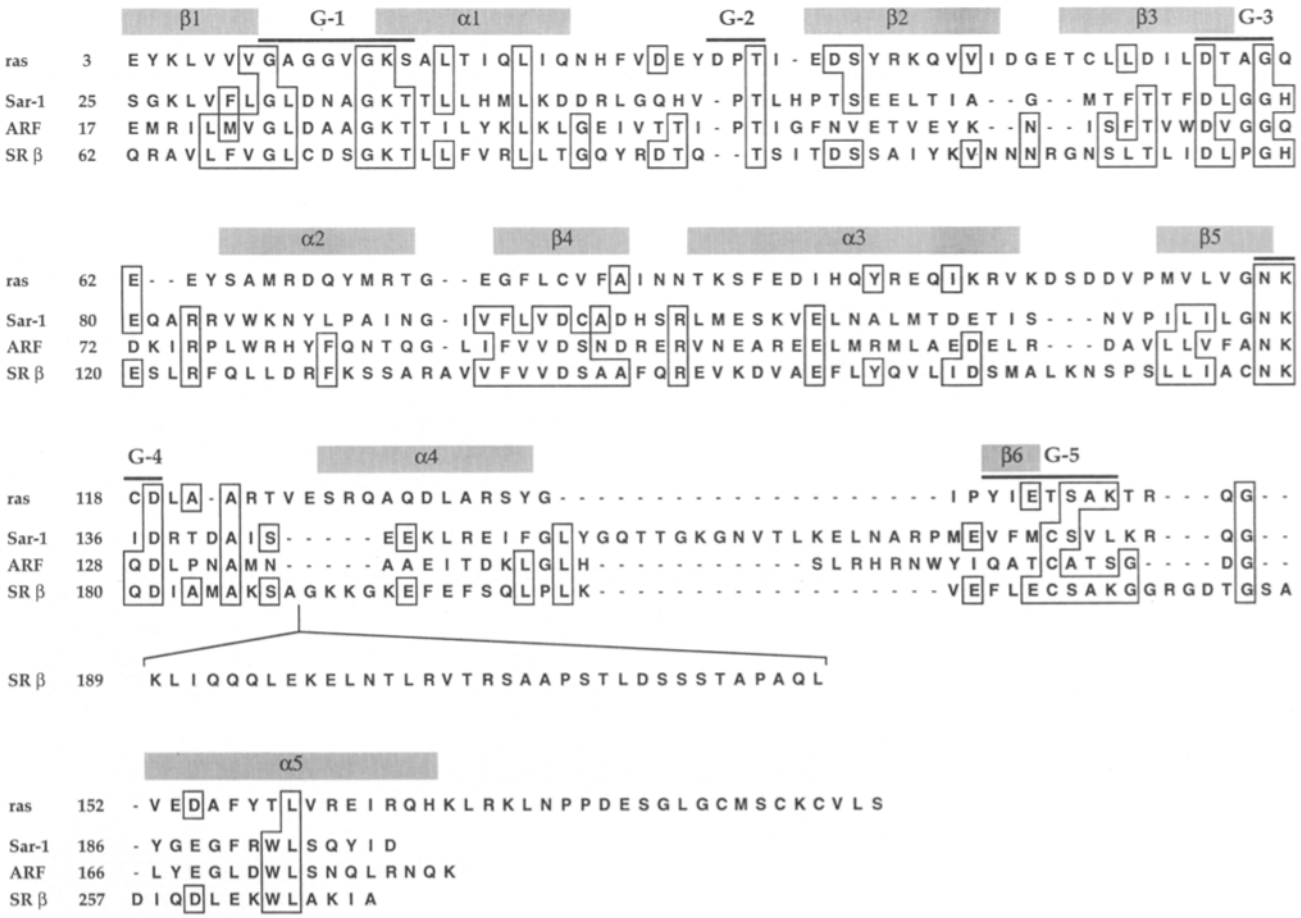


Figure 4. SRβ secondary structure prediction and homology to Sar-1, ARF and *ras*. Three criteria were used to generate this alignment: (*a*) placement of GTPase motifs G-1 through G-5 as defined by Bourne et al. (Bourne et al., 1991) and indicated by the solid lines over the sequence; (*b*) amino acid identity between SRβ and the other proteins (boxes); and (*c*) identification of potential secondary structures in SRβ consistent with those known for *ras* from x-ray crystallographic studies (gray boxes over sequence: α = alpha helix, β = beta sheet). G-1: GXXXXGK(T/S), X = any amino acid; G-2 varies between subfamilies of GTPases, but always contains a critical threonine; G-3: DXXG; G-4: NKXD; G-5, like G-2, varies between subfamilies, but is conserved within a family (see text for discussion of assignment of this motif. The bracketed sequence beginning with SRβ K189 is predicted to be an insertion that may form a loop on the surface of the core GTPase domain. All sequences displayed are mammalian: rat *ras* (Ruta et al., 1986), murine Sar-1 (Shen et al., 1993), and human ARF (Bobak et al., 1989).

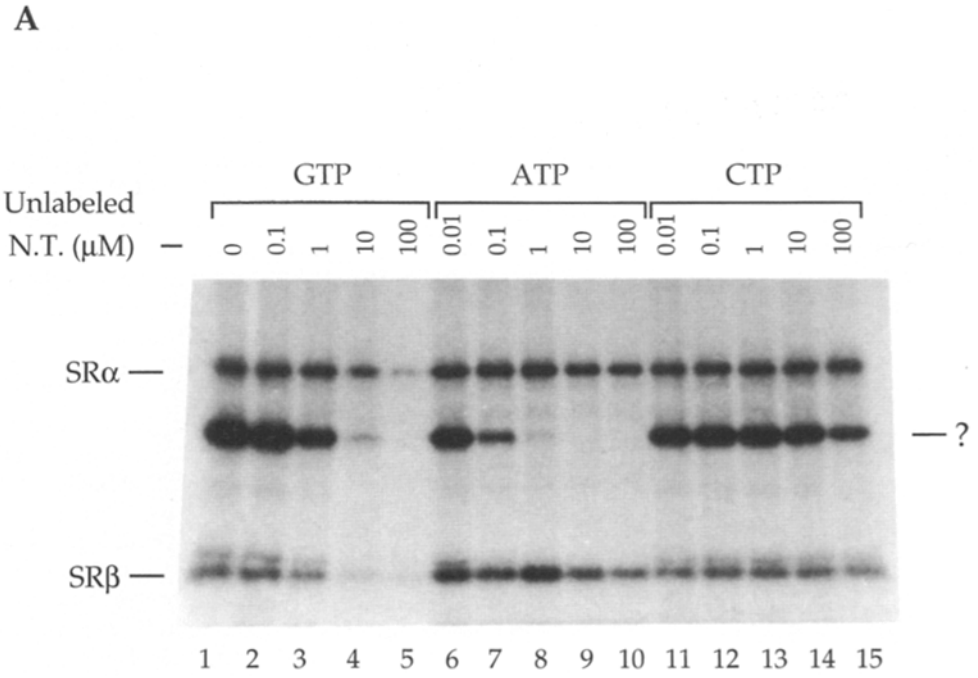
acids G85 to G108) is not as readily assigned, as this part of the protein differs among different members of the GTPase superfamily. It would be premature to assign secondary structure here. The G-2 motif contains a conserved threonine residue (T35 in ras) which in ras is essential for GTP hydrolysis and hydrogen bonds to the γ-phosphate of the bound GTP. T92 of SRβ may correspond to this residue.

Alignment of the carboxyl-terminal 80 residues of SRβ is more problematic, as the remainder of the GTPase domain of *ras* (i.e., helices α4 and α5 and strand β6) comprises only an additional 40 amino acids. We believe that two considerations resolve the difficulty here: (*a*) a carboxyl-terminal helix, α5, packs against the β-sheet, in effect closing the fold in each of the known GTPase structures; and (*b*) the loops between strand β5 and helix α4, and those between strand β6 and helix α5 have variable lengths in the different GTPase subfamilies. Secondary structure analysis and inspection of sequence conservation suggests that the carboxyl-terminal 13 amino acids of SRβ most likely form the carboxyl-terminal helix α5. This putative helix α5 in SRβ would include conserved residues (e.g., DxxxWL). Furthermore, it is preceded by a glycine-rich region consistent with the presence of a surface loop and this candidate loop region is preceded by a seven residue sequence (FLECSAK) which shows substantial similarity to the sequence of strand β6 and motif G-5 in *ras* (YIETSAK).

If these assignments hold, the amino-terminal end (to approximately residue 187) and the carboxyl-terminal end (beginning approximately with residue 241) of the GTPase fold are effectively "pinned." We, therefore, propose that the remaining residues form the missing helix α4 and a large loop, possibly between strand β5 and helix α4. This is indicated by the large insertion after motif G-4 in Fig. 4. The position of helix α4 remains speculative, however. Such a large surface loop could be a key element in the structure and function of this protein, possibly mediating binding to its effectors.

GTP Binding of SRα and SRβ

To confirm experimentally that SRβ binds GTP, direct UV cross-linking was used to create covalent nucleotide-protein adducts (Nath et al., 1985; Pashev et al., 1991; Miller et al., 1993). When α-[^{32}P] GTP is included in the reaction, GTP binding to the individual SRα and SRβ chains can be analyzed by autoradiography after their separation by SDS-polyacrylamide gel electrophoresis (Fig. 5 *A*). Using



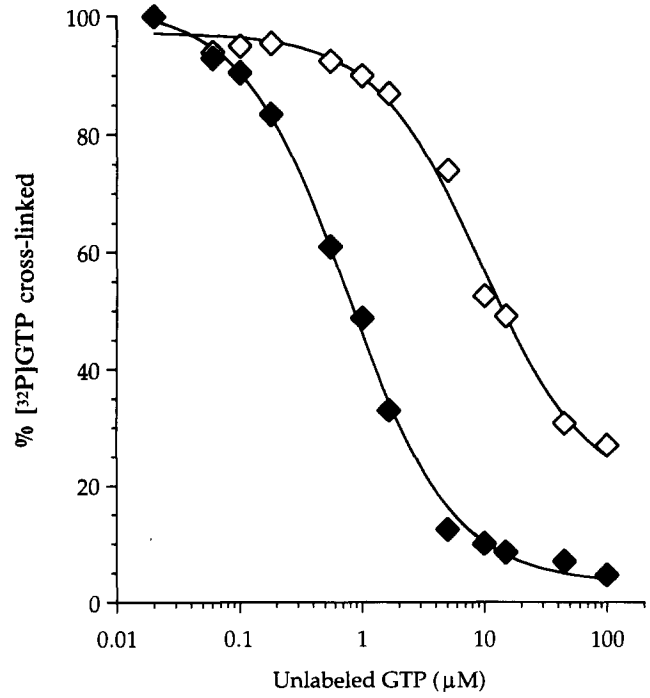


Figure 5. SRα and SRβ specifically crosslink to GTP. (*A*) GTP crosslinking assay. Lane 1, purified SR was incubated with α[^{32}P]GTP and then UV irradiated to crosslink bound GTP to protein. In lanes *4–5*, unlabeled GTP was included in the incubation at the indicated concentration. In lanes *6–10*, unlabeled ATP was included in the incubation at the indicated concentration. In lanes *11–15*, unlabeled CTP was included in the incubation at the indicated concentration. The reaction products were separated by SDS-PAGE and visualized by autoradiography. Bands corresponding to SRα and SRβ are indicated as is a contaminant band (labeled "?"). This contaminant is present in SRP–Sepharose-purified SR but not in immunopurified SR (not shown). (*B*) Quantitation of GTP crosslinking to SR. The amount of labeling of SRα (□) and SRβ (♦) at a given concentration of unlabeled competitor GTP was determined by densitometry and plotted against the log of the concentration of the competitor GTP.

this technique with purified SR, both SRα and SRβ were labeled with α-[^{32}P] GTP, as was an unidentified contaminant band (Fig. 5 *A*, lane *1*). The identity of the SRα and SRβ bands was confirmed by immunoprecipitation (not shown). Control reactions using bovine serum albumin and lysozyme showed no labeling of these proteins (not shown), indicating that, as previously established (Miller et al., 1993), the cross-linking reaction is specific for GTP-binding proteins. To further corroborate binding specificity, we added increasing amounts of unlabeled nucleotide to the reaction (lanes *2–15*). Unlabeled GTP inhibited the labeling of SRα and SRβ (lanes *1–5*), while neither unlabeled ATP (lanes *6–10*) nor unlabeled CTP (lanes *11–15*) showed this effect. In contrast, labeling of the unidentified contaminant band was readily competed by ATP, suggesting that nucleotide binding to this protein is not specific for GTP. The IC_{50} is the amount of unlabeled nucleotide required to inhibit the labeling of SRα and SRβ by 50% and it approximates the affinity of the protein for that nucleotide (Limbird, 1986). From the data shown in Fig. 5 *B*, the IC_{50} for SRα is ~10 μM and that for SRβ is ~1 μM. Thus, we conclude that both SRα and SRβ bind GTP specifically albeit with relatively low affinity when compared to other GTPases such as *ras*.

Discussion

We have shown that SRβ is a new member of the GTPase superfamily and have experimentally confirmed the ability of SRβ to bind GTP specifically. To our knowledge, it is the first such protein that is also a bona fide integral membrane protein. One other transmembrane protein, GP85, has been shown to bind GTP (Lokeshwar and Bourguignon, 1992). GP85, however, is a radically different type of GTP-binding protein that does not contain the consensus motifs common to the GTPase super family typified by *ras* (Bourne et al., 1991). We have identified a gene encoding a closely related yeast SRβ homologue that shares the features discussed for mammalian SRβ. Preliminary experiments from our laboratory indicate that disruption of the yeast SRβ gene leads to an identical phenotype to that of cells that have been deleted for the genes encoding SRα or any of the SRP components (S. Ogg and P. Walter, unpublished observation). This lends additional support to the assignment of the predicted yeast protein as an SRP receptor subunit, and provides in vivo evidence for the importance of SRβ in the SRP-dependent protein targeting reaction. Furthermore, we have demonstrated that SRα is a peripheral membrane protein that is required for the interaction of SRP with SR, while SRβ binds tightly to SRα and is predicted to span the ER membrane. These data suggest a model in which SRα mediates SRP binding (and thus, may regulate the GTPase cycle of SRP-54), while SRβ tethers SRα to the membrane. The unexpected discovery of the guanine nucleotide-binding domain in SRβ, however, makes it likely that its role is more complex than that of a passive membrane anchor.

GTP binding and hydrolysis by SRP54 is regulated by its interactions with different components of the targeting machinery (Miller et al., 1993). It is likely that the guanine nucleotide occupancy of SRα is also regulated, possibly by interaction with SRP, SRβ, or the translocon components. As SRP forms a functional targeting complex with the ribosome and nascent chains, so may SR form a complex with translocon components rendering them capable of accepting the targeting complex. Thus, the GTPases in SR and SRP would function as "molecular match makers" that establish the ribosome/translocon junction. Cycles of GTP binding and hydrolysis may guide the components through the sequential steps of a complex reaction.

The physiological importance of guanine nucleotide binding to SRβ still remains to be demonstrated. As the GTPase domain of SRβ is evolutionary highly conserved, however, we consider it very likely that nucleotide binding to SRβ is of functional significance. We can envision two different roles that a GTPase switch in SRβ could play. First, as for SRP54 and SRα, a cycle of GTP binding and hydrolysis on SRβ may be required for protein targeting and the initiation of translocation. This cycle might be regulated by specific effectors, such as SRα or translocon components, which could serve to enhance the fidelity of targeting and/or the assembly of the ribosome/translocon junction, to assure tight coupling of targeting to assembly of the translocon, or to regulate the activity of the translocon in response to the secretory needs of the cell.

According to a second, conceptually distinct hypothesis, the information flow would be reversed. Thus, the GTPase switch in SRβ may not be instrumental for targeting and translocation per se, but rather be set by these events. According to this scenario, effectors of SRβ would be used to adapt other cellular processes to the activity of the translocon. Such events could be downstream of protein translocation assuring that the secretory pathway has sufficient capacity to handle the load of proteins entering the ER. Alternatively, effectors could feed back on the synthesis of signal sequence-bearing proteins, thereby assuring that such proteins are only made if sufficient translocation sites are available to accommodate them.

The challenge now is to decipher the individual roles of the three directly interacting GTPases—SRP54, SRα and SRβ—that participate in protein targeting. Although still rather complex, the combination of the available biochemical and genetic tools should render this goal experimentally accessible.

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