The β-subunit of the signal recognition particle receptor (SRβ), a member of the Ras family of small molecular weight GTPases, is involved in the targeting of nascent polypeptide chains to the protein translocation machinery in the endoplasmic reticulum membrane. We purified SRβ from an expressing strain of *Escherichia coli* and investigated the properties of the isolated GTPase. We find that, unlike other Ras family GTPases, most SRβ purifies bound to GTP, and SRβ-bound GTP is not easily exchanged with solution GTP. SRβ possesses no detectable GTPase activity. Although a stable interaction between SRβ and ribosomes is observed, SRβ is not stimulated to hydrolyze GTP when incubated with ribosomes or ribosome-nascent chains. A GTPase mutant harboring a mutation in a region predicted to be functionally important, based on observations made in related GTPases, binds GTP with faster kinetics and appears to be a less stable protein but otherwise displays similar properties to the wild-type SRβ GTPase.

Our results demonstrate that as an isolated GTPase, SRβ functions differently from the Arf- and Ras-type GTPases that it is most closely related to by sequence.

Protein translocation across the mammalian endoplasmic reticulum (ER) membrane is a cotranslational process believed to be regulated by three GTPases. Nascent polypeptide chains being synthesized in the cytosol are sampled by the signal recognition particle (SRP) as they emerge from the ribosome (1). The GTPase in SRP, SRP54, binds to signal sequences in nascent polypeptides as they emerge from the ribosome, forming ribosome-nascent chain-SRP ternary complexes (2, 3). The ternary complexes are directed to the ER because of the affinity of SRP for its cognate receptor (SRP receptor; SR) on the surface of the ER membrane. Through a series of GTPase-controlled steps the ribosome-nascent chain is transferred to the protein-conducting channel, or translocon, which facilitates translation of the nascent chain across, or integration into, the ER membrane (reviewed in Ref. 4).

The concerted action of GTPases ensures that the targeting step and nascent chain transfer step are unidirectional processes (5). SRP54 and one of the subunits of the SRP receptor, SRα, bind GTP in a cooperative manner (6). Binding of GTP by SRP54 and SRα increases the affinity of these proteins for one another, thereby maintaining a direct physical link between the ribosome-nascent chain and the ER membrane (7, 8). Sequence comparisons revealed that the SRα and SRP54 GTPases define a specific subfamily of GTPases conserved in prokaryotes, yeast, and mammals that is now referred to as the Ras-family of GTPases (9, 10). The third GTPase that appears to be involved in regulating translocation is SRβ. It has been proposed that release of SRP from the nascent chain, and subsequent transfer of the nascent chain to the translocon, is controlled by SRβ (11).

Unlike SRP54 and SRα, SRβ shares significant homology within the GTP binding consensus sequences, or G boxes, of Ras-type GTPases (12). Structural analysis indicates that SRβ also bears significant structural homology to Ras-type GTPases (13). However, it differs from other Ras-type GTPases in two respects. First, although other Ras-type GTPases require prenylation at the carboxyl terminus to enable a reversible interaction with membranes (14), SRβ is permanently integrated into the ER membrane by an amino-terminal transmembrane domain. Second, SRβ contains a cysteine within the G1 GTPase consensus sequence where most Ras-type GTPases contain a glycine. The other exception is members of the Arf family of GTPases that all contain an aspartic acid at this position. The identity of this amino acid appears to be crucial to the activity of Arf and Ras GTPases (15–17), which raises the possibility that SRβ functions functionally from both Arf-like and other Ras-type GTPases.

The search for protein factors that influence the activity of SRβ has led to the observation that a factor associated with ribosomes possesses measurable GAP activity and may also function as a guanine nucleotide dissociation factor for SRβ. Incubation of ribosome-nascent chain complexes with either the SRα/SRβ dimer or a proteolysis product of the dimer lacking the SRα GTPase (SRΔα) both increases the GTPase activity of SRβ and decreases the affinity of SRβ for nucleotides (18). The influence of the ribosome on SRβ suggests a direct physical contact between the two. Supporting this prediction, crosslinking experiments have revealed an interaction between SRβ and a protein component of the ribosomal 60 S subunit (11). Whether this ribosomal protein is responsible for the observed GAP/guanine nucleotide dissociation factor activity is still unresolved.

In addition to proposed roles in nascent chain transfer and SRP release, SRβ also anchors SRα to the ER membrane via a tight physical interaction between the SRβ GTPase domain
and an amino-terminal domain of SRα (19, 20). This interaction is influenced by the nucleotide-bound status of SRβ. Generation of empty SRβ by gel filtration of an XTP binding mutant of SRβ, which has a decreased affinity for GTP, abolishes the SRα/SRβ interaction. Replenishing the reaction mixture with xanthosine 5'-diphosphate or XTP restores dimer formation, with XTP having a greater effect. Deletion of any part of the SRβ core GTPase also abolishes the interaction with SRα (21). All data gathered to date on the function of SRβ have been obtained in the context of a heterodimer. Attempts to isolate the SRβ GTPase in a study by indirect immunoprecipitation of SR with trypsin or elastase to specifically digest SRα. This method releases the GTP binding domain of SRα from SRβ but leaves an amino-terminal domain of SRα bound to SRβ (10, 22). Therefore, there are no data on the properties of SRα as an isolated GTPase. To address this issue directly we have expressed and purified from *Escherichia coli* a soluble version of SRβ, termed SRβATM.

Isolated SRβATM has no detectable GTPase activity, and most does not exchange GTP in vitro. The small fraction (3–6%) of SRβATM that does bind exogenous GTP undergoes a conformational change that can be detected by fluorescence spectroscopy. A direct interaction between SRβATM and the ribosome is confirmed, and the influence of the ribosome on the SRβ GTPase is examined. Our results suggest that SRβ GTPase function is unlike other Ras-type GTPases.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Construction of plasmids, sequencing, and site-directed mutagenesis were performed using standard techniques. All encoded products are under the control of a T7 promoter. The plasmids pMAC191 (containing a modified full-length cDNA sequence of canine SRα, pMAC455 (encoding SRβATM), pMAC1083 (encoding hemagglutinin-SRβC71D), and pMAC853 (encoding SRβATM, a fusion of the carboxyl-terminal 206 amino acids of canine SRβ with an amino-terminal hemagglutinin epitope tag) were reported previously (20, 21).

Plasmid pMAC1277 encodes SRβATM fused to an amino-terminal His tag and enterokinase (EK) cleavage site. This plasmid was assembled in two steps. First pMAC701, encoding SRβATM fused to an amino-terminal His tag and EK cleavage site, was generated by removing the SRβ coding sequence from pMAC455 by digestion with BglII and KpnI and inserting it into p EcoRI/BL (Invitrogen) digested with the same enzymes. The sequence encoding SRβATM was then excised from pMAC701 and EcoRI and inserted into pMAC853 (encoding SRβATM, a fusion of the carboxyl-terminal 206 amino acids of canine SRβ with a hemagglutinin epitope tag) previously reported (20, 21).

Plasmid pMAC1623 encoding SRβC71DATM fused to an amino-terminal His tag and EK cleavage site was generated from pMAC1277 by the method described in Ref. 23. Briefly, the entire plasmid was amplified by PCR using oligo958 (ATGGGGCCCTCGGCAAATCCTGGGAAAAC; desired mutation in bold) and oligo959 (ATGGGGCCCAAAACAGAAAGACGCTCT). The product was then digested with ApaI and the 3′ overhanging ends were blunted by incubation with the Klenow fragment of DNA polymerase, and the linear DNA was circularized by ligation with T4 DNA ligase.

Plasmid pMAC1624 encodes SRβC71DATM fused to an amino-terminal His tag and EK cleavage site was generated from pMAC1277 by the method described in Ref. 23. Briefly, the entire plasmid was amplified by PCR using oligo960 (ATGGGGCCCTCGGCAAATCCTGGGAAAAC; desired mutation in bold) and oligo959. The PCR product was digested with ApaI and end-repaired and ligated as above.

Plasmid pMAC1628 encodes SRβC71DATM fused to an amino-terminal His tag and EK cleavage site. SRβC71DATM was excised from pMAC853 using NcoI and EcoRI and inserted into pMAC853 digested with NcoI and EcoRI, replacing SRβATM with SRβC71DATM.

Plasmid pMAC1637 encodes SRβC71DATM fused to a carboxyl-terminal His tag. SRβC71DATM was amplified from pMAC1624 using oligo926 (AAGATTTCTAACGCAACATCCGGAAGC; desired mutation in bold) and oligo929 (CTAACGCAACATCCGGAAGC). The PCR product was digested with NcoI and EcoRI. The digested fragment was inserted into pET16b (Novagen) digested with NcoI and EcoRI.

**Protein Purification**—Plasmids encoding either His-SRβATM or His-SRβC71DATM were expressed in the salt-inducible BL21S strain by addition of NaCl to 300 mM final concentration for 2 h. All purification steps were carried out at 4 °C. Cell pellets were washed once in 50 mM Na2HPO4, pH 8.0, 1 mM phenylmethylsulfonyl fluoride and resuspended in lysis buffer (50 mM Na2HPO4, pH 8.0, 500 mM NaCl, 5 mM MgOAc2, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v)). Cells were lysed by a pressure cell, DNA was precipitated with 0.15% polyethyleneamine, and lysate was centrifuged at 18,000 *g* for 20 min in a Beckman JA-20 rotor. The lysate was further clarified by centrifuging at 110,000 *g* for 1 h in a Beckman T50.2 rotor prior to loading on Ni-NTA-agarose (Qiagen) equilibrated in 50 mM Na2HPO4, pH 8.0, 300 mM NaCl, 5 mM MgOAc2, 10% glycerol. The column was washed in 10 volumes of equilibration buffer, and His-SRβ was eluted with equilibration buffer + 50 mM imidazole. Protein containing fractions were dialyzed in BCA assay (Pierce) and pooled, and the sample was concentrated from 40 mM Tris-OAc, pH 7.8, 300 mM NaCl, 5 mM MgOAc2, 1 mM DTT, 25% glycerol. Dialyze was diluted with 6 volumes of 40 mM Tris-OAc, pH 7.8, 5 mM MgOAc2, 1 mM DTT, 25% glycerol to reduce the NaCl concentration and loaded immediately onto CM-Sephadex equilibrated in 40 mM Tris-OAc, pH 7.8, 50 mM NaCl, 5 mM MgOAc2, 1 mM DTT, 25% glycerol. The column was washed with 10 volumes of equilibration buffer, and His-SRβ was eluted in a single step in SRβ elution buffer (equilibration buffer + 100 mM NaCl). Protein containing fractions were detected by Bradford assay (Bio-Rad) and pooled. Protein concentration was determined by absorbance at 280 nm as described (24). Protein was frozen in small aliquots at −80 °C; material used for functional studies was thawed once and discarded.

**Immunoprecipitation**—Proteins were synthesized in vitro, quantified, and immunoprecipitated as described previously (21).

**HPLC Analysis of Bound Nucleotide**—10 nmols of SRβ were diluted to 250 μl in SRβ elution buffer. An equal volume of 8 μm urea, 20 mM Tris-OAc, pH 7.8, 100 mM NaCl was added, and the sample was incubated at 37 °C for 30 min. The sample was centrifuged through a 5-KDa cutoff filter (Millipore), and the filtrate was added to a Bakerbond QUAT 5-μm HPLC column (J. T. Baker Inc.) in 25 mM triethylamine borate, pH 7.2. Nucleotide was eluted from the column with a 5–100% gradient of triethylamine borate. Samples were analyzed with 32Karat, version 3.0 software (Beckman), and nucleotide was quantified by calculating the area under the curve and comparing to a standard curve of GTP or GDP. The recovery of nucleotides in these experiments (90% or desired) was determined by adding a known amount of GMP as an internal control.

**Fluorescence Experiments**—300 nm SRβ was incubated with 500 nm 2′-[3H]GTP (amino-ethylenamino)GTP (mant-GTP) in 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 5 mM MgOAc2, 2 mM DTT, and 10% glycerol in a 1-cm path length quartz cuvette. All measurements were performed with a fluorometer equipped with an 815 photomultiplier detection system (PTI, London, Ontario, Canada) with a 2-nm excitation slit width and a 2-nm emission slit width and compiled with Felix, version 1.4 software (PTI). Samples were excited at 280 or 285 nm, and emission spectra were recorded by scanning from 300 to 500 nm in 2-nm increments with an integration time of 0.2 s per data point. Emission spectra were corrected by subtracting a buffer blank, and peak values were manually selected for further calculation. All calculations and data plots were performed within MS Excel 2002.

**Filter Binding**—100 pmols of SRβ (1 μM) were incubated at the specified temperatures with 10 μM GTP including 25% [3H]GTP (specific activity 31 Ci/mol) in 50 mM Tris-OAc, pH 7.8, 200 mM NaCl, 5 mM MgCl2, 10% glycerol, 2 mM DTT. At the appropriate time points samples were withdrawn and diluted to 2 ml in ice-cold filter binding buffer (20 mM Tris-OAc, pH 7.8, 200 mM NaCl, 5 mM MgCl2, 10 mM NH4Cl). Samples were applied to prewashed nitrocellulose discs (Whatman) and the discs were washed with 3 × 3 ml filter binding buffer in a Millipore 1225 filtration sampling manifold (Millipore). Discs were dried, and bound nucleotide was quantified in a scintillation counter.

**Nucleotide Exchange**—Nucleotide exchange reactions were performed as described previously (25, 26). Briefly, SRβATM (1 μM) was incubated with 20 μM GTP including 0.2 μM [γ-32P]GTP for 10 min at 30 °C in final buffer conditions containing 20 mM Tris-OAc, pH 7.8, 100 mM NaCl, 10 mM MgCl2, 10 mM EDTA, 1 mM DTT, 10% glycerol. After 10 min MgCl2 was added to a concentration of 20 mM. The extent of nucleotide exchange was quantified by filter binding. To prepare SRβATM for GTPase assay, free nucleotide was separated from bound nucleotide by preparative SDS-PAGE (10% gel) and stained with Coomassie blue. The gel was destained overnight in 2 volumes of 10 mM Tris-OAc, pH 8.0, 1% SDS. The gel was scanned by a Molecular Dynamics PhosphorImager, and the radioactive band corresponding to SRβATM was excised from the gel, and the radioactivity was determined from the dried gel.
with UV light at 5000 micswatts/cm² for 5 min. Samples were precipitated with trichloroacetic acid and washed in ethanol, ether (1:1) to remove free nucleotide, resolved by SDS-PAGE, and analyzed using a PhosphorImager.

**GTPase Assay**—40 nm nucleotide-bound SRβ or 5.0 A_{260} units/ml ribosomes was incubated with 83.5 nM [γ-32P]GTP in GTPase buffer (50 mM Tris-OAc, pH 7.8, 150 mM KOAc, 5 mM MgOAc, 2 mM DTT) at 24 °C. At the indicated time points samples were removed and quenched by adjusting the EDTA concentration to 50 mM on ice. Samples were spotted onto polyethyleneimine cellulose TLC plates and resolved in 0.375 M KH2PO4, pH 3.5, for 1 h. Plates were dried and exposed to a PhosphorImager screen for quantitative analysis. To generate the Lineweaver-Burke plot reactions were supplemented with cold GTP to concentrations up to 5 μM, and the reaction was monitored using hydrolysis of [γ-32P]GTP to estimate hydrolysis of all GTP.

To assess the effect of ribosomes on the SRβ GTPase, 10 nM [γ-32P]GTP-loaded SRβ{τ}TM and 20 nm 80 S ribosomes were incubated at 24 °C in GTPase buffer. Samples were removed at the indicated time points and quenched with 50 mM EDTA, and [γ-32P]GTP was resolved from 2-P by TLC.

**Ribosome Binding Experiments**—Canine pancreatic ribosomes and wheat germ RNAs were prepared as described elsewhere (11, 18) and stored at a concentration of 100 A_{260} units/ml in 25 mM HEPES-KOH, pH 7.6, 5 mM MgOAc, 150 mM KOAc, 1 mM DTT (+1 mM cycloheximide for RNAs). 5 μM SRβ was incubated with 20 A_{260} units/ml ribosomes or RNAs for 1 h at 24 °C in the above buffer and then added to the top of 30 ml of linear 0.3–1.2 M sucrose gradients. The gradients were centrifuged in an SW28 rotor for 16 h at 48,000 × g. 1-ml fractions were collected and precipitated with trichloroacetic acid, resolved by SDS-PAGE, and analyzed by Western blotting using an antibody directed against SRβ.

**Data Analysis**—All data analysis was performed using SigmaPlot 8.02.

### RESULTS

Although sequence comparisons clearly indicate that SRβ belongs to the Ras superfamily of small molecular weight GTPases it defines its own subfamily. One method of sorting GTPase family members is to define regions of homology within the residues lining the GTP binding pocket. Ras-type GTPases contain sequences of conserved residues called G boxes that are arranged at discrete intervals throughout the primary sequence (27). Arf family GTPases are distinguished from other Ras-type GTPases by the presence of an aspartic acid instead of a glycine residue in the G1 box (Table I). The identity of this residue within SRβ is not strictly conserved among lower eukaryotes, but higher eukaryotes contain a cysteine in this position (Table II). The G1 box of canine SRβ differs from Arf GTPases in only one other position in which an Ala not conserved in other Ras GTPases is replaced by a Ser.

In an attempt to identify the importance of the cysteine in the function of SRβ two point mutants at this site were generated. The first, SRβ{τ}C71M, converts the cysteine to an aspartic acid, converting SRβ into an Arf family GTPase. The second, SRβ{τ}C71T, converts the cysteine to a glycine to resemble other Ras-type GTPases. Binding of SRβ and SRβ mutants to SRαs was assayed by coprecipitation. SRαs, wild-type SRβ, both cysteine point mutants, and another GTPase point mutant, SRβ{τ}D72T, shown previously to switch the nucleotide binding preference from GTP to XTP (11, 21), were synthesized in vitro in a rabbit reticulocyte lysate system. Nucleotides were removed from some samples by gel filtration (–GTP) and separate reactions containing equimolar amounts of SRαs and each of the SRβ variants were incubated together to allow complex formation. Complexes were immunoprecipitated with an antibody against SRαs (Fig. 1). All of the SRβ molecules bound SRαs in the presence of nucleotides contributed by the translation mix. As reported previously (21), binding of SRαs to the XTP-prefering version of SRβ was greatly reduced in the absence of nucleotide, because the reduced affinity of SRβ{τ}D72T for GTP allows this SRβ variant to be emptied by gel filtration. Binding of either cysteine point mutant to SRαs was unaffected by nucleotide depletion demonstrating that, despite the mutation in the G1 box, these mutants retain SRαs binding activity under conditions that serve to empty SRβ{τ}D72T.

To examine the isolated SRβ GTPase in greater detail, a recombinant protein consisting of the cytoplasmic portion of SRβ fused to an amino-terminal hexahistidine tag (Hisβ) was expressed in E. coli. The protein (SRβ{τ}ATM) was purified to apparent homogeneity in two steps involving Ni-NTA-agarose and CM-Sepharose (Fig. 2a). Gel filtration analysis of the purified product confirmed that SRβ{τ}ATM is a monomer in solution (data not shown). One of the cysteine point mutants, SRβ{τ}C71T, was purified with a carboxyl-terminal Hisβ tag and purified using conditions identical to those used to purify SRβ{τ}ATM (Fig. 2b).

GTPases are generally purified in the GDP-bound form. This holds true for both tissue-derived proteins and recombinant proteins that lack GAP homologues in E. coli (28–30). Therefore, we expected that SRβ purified from E. coli would be GDP-bound. To identify and quantify the nucleotide that copurified with SRβ{τ}ATM, 10 nmol of SRβ{τ}ATM or SRβ{τ}C71T were denatured in 4 M urea to release the bound nucleotide into solution. The protein was removed by filtration, and the released nucleotide was analyzed by HPLC and compared with standards of GTP and GDP examined in parallel (Fig. 3). The retention times for GDP and GTP on the HPLC column were 7.9 and 9.6 min, respectively (Fig. 3a). Surprisingly, the supernatant from denatured SRβ contained a single major peak that eluted at 9.6 min, indicating the presence of GTP. A smaller peak was detected at 7.9 min, corresponding to a small amount of GDP (Fig. 3b). Calculating the area under the curves and comparing these values against values obtained from GTP and GDP standards and correcting for 10% loss (measured using GMP as an internal standard) revealed that 72% of SRβ{τ}ATM contains bound GTP whereas only 2.2% was bound to GDP. Similarly, 71% of purified SRβ{τ}C71T contains GTP, and 2.8% was bound to GDP. Therefore both wild-type SRβ and the GTPase point mutant remain bound to GTP throughout purification. The remaining 26% is not bound to nucleotide. This population does not arise from dissociation of nucleotide from SRβ{τ}ATM, as indicated by both the absence of XTP and the inability of SRβ{τ}C71T to bind to GTP in the timescale examined (Fig. 4).

Therefore we presume that this population consists of SRβ that has become structurally unstable in the absence of bound GTP, and the resulting loss of conformation prevented the uptake of exogenous GTP. A structurally unstable empty state is a common feature of Ras-type GTPases (31, 33, 34). Addition of 10 μM GTP to the buffers used during purification did not decrease the percentage of empty SRβ (data not shown), suggesting that this population does not arise from dissociation of nucleotide during purification.

Two methods were used to determine what fraction of recom-
binant SRβ is able to bind to or exchange bound GTP for exogenous GTP. The first method measured the ability of aromatic amino acids within SRβ to transfer energy to a fluorescent GTP analogue, mant-GTP (Fig. 4). Resonance energy transfer (RET) can be measured by monitoring the decrease in fluorescence output of an excited donor molecule (aromatic amino acids) and concomitant increase in acceptor molecule (mant) fluorescence (Fig. 4a). Mant fluorescence did not change in a control cuvette lacking protein, nor was there an increase in mant fluorescence attributable to binding to SRβ when the dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluores-
cence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-
due to transfer energy to a fluorescence dye was excited directly at 350 nm (data not shown). Therefore, the increase in mant fluorescence arises solely from RET be-

![Fig. 1. Immunoprecipitation of SRα and SRβ GTPase mutants.](image1)

![Fig. 2. Purification of His-tagged SRβ molecules.](image2)

![Fig. 3. Identification of nucleotide bound to SRβ.](image3)

![Fig. 4. Resonance energy transfer (RET) measurements.](image4)

**TABLE II**

Alignment of G1 box sequences of SRβ homologues

| Organism               | Gene/ORF name | G1 GTPase box sequence |
|------------------------|---------------|------------------------|
| *Saccharomyces cerevisiae* | Srp102p       | GPONSGKT               |
| *Schizosaccharomyces pombe* (putative) | O13950         | GPONSGKT               |
| *Arabidopsis* (putative) | AAD09846      | GLDSGKT                |
| *Caenorhabditis elegans* (putative) | NP_590245     | GLDCSGKT               |
| *Mus musculus*         | SRβ           | GLDCSGKT               |
| *Canis familiaris*     | SRβ           | GLDCSGKT               |
| *Homo sapiens*         | SRβ           | GLDCSGKT               |

**Note:** The limitations of RET require that mant-GTP is bound to SRβ rather than overestimate the rate of GTP binding. The distance between the mant fluorophore and aromatic side chains within SRβ molecules.

### Methods

**Immunoprecipitation of SRβ**

- Expression of His-tagged SRβ proteins in BL21SI strain of *E. coli*.
- Purification of SRβ by Ni-NTA chromatography followed by CM-Sepharose chromatography.
- Analysis of total protein by SDS-PAGE and visualization using Coomassie stain.

**Identification of nucleotide bound to SRβ**

- Analysis of nucleotide bound to SRβ by HPLC using quaternary amine column.
- Detection of nucleotide by absorbance at 260 nm.
- Identification of nucleotide bound to SRβ by autoradiography.

**Resonance energy transfer (RET) measurements**

- Monitoring of decrease in fluorescence output of an excited donor molecule (aromatic amino acids) and concomitant increase in acceptor molecule (mant) fluorescence.
- Assumption that the increase in mant fluorescence arises solely from RET due to transfer energy to a fluorescence dye.
- Validation of RET measurements by comparing the rate of GTP binding to SRβ with those of other GTPases.
and 500 nM mant-GTP were excited at 280 nm, and energy transfer was monitored by measuring the increase in mant-GTP fluorescence emission at 340 nm (Fig. 4b). Both SRβΔTM and SRβC71DΔTM bound mant-GTP, with SRβΔTM following biphasic binding kinetics. The first mode is complete after 45 min; the second mode is slower and takes an additional hour to complete. SRβC71DΔTM shows a single mode of GTP uptake that is complete after 45 min. The initial rate of increase in mant fluorescence is greater for SRβC71DΔTM than for SRβΔTM, indicating that the cysteine mutation permits mant-GTP more rapid access to the GTP binding site in SRβ.

SRβ contains only one Trp located five amino acids from the carboxyl terminus. Excitation at 295 nm permits measurement of RET between this tryptophan and mant-GTP. No change in the apparent kinetics of GTP binding to SRβC71DΔTM was observed (Fig. 4c). However, SRβΔTM now showed a single mode of GTP binding, that resembles the second mode detected at 280-nm excitation in both slope and duration. Therefore, the first mode arises from RET between one or more of the Tyr (and Phe) residues scattered throughout SRβΔTM and mant-GTP, whereas the second mode arises from RET between the carboxyl-terminal Trp residue and mant-GTP.

Although fluorescence spectroscopy permits determination of binding kinetics it did not permit us to determine what fraction of SRβ can bind GTP. Therefore, a nitrocellulose filter binding assay was used to quantify the amount of GTP that could bind SRβ (Fig. 5). 100 pmols of SRβΔTM (diamonds) or SRβC71DΔTM (squares) was incubated with a 10-fold molar excess of GTP, including 25% [3H]GTP, at 24 °C for the indicated times. To analyze GTP binding the protein was bound to nitrocellulose filters and washed extensively to remove unbound nucleotide. The nucleotide remaining on the filter, representing the amount of solution GTP retained in a complex with SRβ, was quantified by scintillation counting. At 24 °C both proteins show that same t1/2 for nucleotide binding as calculated from fluorescence data. After 2 h SRβΔTM bound a maximum of 6.6 pmols of GTP, reflecting an occupancy of 6.6%. SRβC71DΔTM bound GTP at a faster rate than SRβΔTM, reaching a maximum of 3.5 pmols of GTP bound after 1 h followed by a steady decline throughout the rest of the experiment. A similar decline in binding was observed during RET experiments (Fig. 4) and may reflect structural instability of SRβC71DΔTM during extended incubation at 24 °C. These data reveal that ~10% of SRβ binds exogenous GTP (de novo) or by exchange). Therefore, 90% of SRβ is already tightly bound to nucleotide or in a conformation that is unable to bind nucleotide. Both RET and filter binding experiments indicate that SRβ binds added GTP slowly, consistent with observations made in other GTPases assayed in their nucleotide-bound states (30, 35, 36).

The Kd of SRβ for GTP has been reported to range from 1 μM for the purified, solubilized SR dimer (12) to 20 nM for the purified SR dimer reconstituted into liposomes (18). To determine the Kd of SRβ for GTP we cross-linked to [α-32P]GTP in the presence of an increasing concentration of cold competitor GTP (Fig. 6, ■).
Binding follows a characteristic sigmoidal curve with the inflection point occurring at 2 μM, demonstrating that recombinant SRβ and solubilized SR (12) have similar affinities for GTP. An identical Kᵰ was calculated for SRβ/C71D (Fig. 6, ▲). Therefore purified recombinant SRβ/C71D binds GTP with a similar affinity as native SRβ after solubilization of microsomes, and mutation of the cysteine in the G1 box does not affect the affinity of this protein for GTP. Because of the short incubation period prior to cross-linking this Kᵰ measurement reflects the loose binding conformation revealed by RET and not the majority of SRβ that is already bound to GTP.

Because the majority of SRβ remains bound to GTP throughout purification (Fig. 3) it is likely that the intrinsic GTPase activity of SRβ/C71D is negligible. To experimentally verify that the SRβ GTPase does not possess intrinsic catalytic activity, SRβ/C71D was incubated with [γ-32P]GTP, and hydrolysis was monitored by quantifying the liberation of the terminal phosphate by thin layer chromatography (Fig. 7). RNCs treated with N-ethylmaleimide (NEM) (identical to those used in previous attempts to assay the influence of the ribosome on the SRβ GTPase (18), demonstrated that NEM treatment is not sufficient to abolish GTPase activity associated with RNCs. Therefore, initial measurements were made in the absence of ribosomes. After 4 h of incubation at 24 °C no significant GTP hydrolysis was detected above a control reaction lacking SRβ (Fig. 7a, GTP). To ensure that the assay was sensitive enough to measure a low basal rate of GTP hydrolysis, the assay was repeated with varying concentrations of GTP, and a Lineweaver-Burke plot was generated to estimate a basal GTP hydrolysis rate (Fig. 7b). From the plot an estimated Kᵰ of 4.0 μM and kₗᵰ of 0.0005 min⁻¹ are derived, reflecting a negligible rate of GTP hydrolysis for SRβ/C71D. Consistent with the negligible rate of GTPase activity obtained using the thin layer chromatography assay, the efficiency of cross-linking [α-32P]GTP and [γ-32P]GTP to SRβ were identical (data not shown). Therefore SRβ is unable to hydrolyze GTP, suggesting the existence of a GAP.

A candidate GAP for SRβ has been proposed recently (18) to reside within the ribosome. Because ribosomes are a significant source of GTPase activity, this activity must be abolished to unambiguously assign GTPase activity arising from SRβ in reactions containing both SRβ and ribosomes. The use of alkylating reagents to modify ribosomal proteins has been shown to reduce, but not abolish, the activity of certain ribosome-associated GAPases (37) (see also Fig. 7a). Therefore, in addition to 80 S ribosomes, isolated ribosomal subunits and RNCs were treated with NEM in an attempt to decrease background ribosome-associated GTPase activity enough to detect GTP hydrolysis arising from SRβ. Treatment with NEM had no effect on the GTPase activity of 80 S ribosomes or RNCs (see Fig. 7a and Supplemental Fig. 1), but the GTPase activity of isolated 60 S subunits, already significantly decreased compared with intact ribosomes, was abolished following treatment with NEM (see Supplemental Fig. 1). Incubation of SRβ with NEM-treated 60 S subunits did not result in any additional GTP hydrolysis above background levels (data not shown).

Although isolated 60 S ribosomal subunits did not stimulate the SRβ GTPase, it is possible that ribosome-associated GAP activity requires an intact 80 S ribosome. The GTPase activity of 80 S ribosomes precluded analysis with exogenous nucleotide; therefore we chose to analyze hydrolysis of GTP bound by SRβ/C71D upon incubation with RNCs. If a protein within the ribosome acts as an SRβ GAP then incubation of SRβ with ribosomes in the absence of added GTP should result in the hydrolysis of SRβ-bound GTP to GDP. If the ribosome functions as a guanine nucleotide releasing factor then incubation of SRβ/C71D with ribosomes should lead to the release of SRβ/C71D-bound GTP or GDP. We assessed the effect of adding ribosomes to SRβ/C71D-bound GTP by removing the ribosomes by centrifugation at the end of the incubation and then assayed for...
nucleotide in the supernatant and bound to SRβΔTM using the HPLC method described above. Using this approach we were unable to detect any increase in hydrolysis of GTP because of the addition of ribosomes (data not shown). To increase the sensitivity of the assay and ensure that there were excess ribosomes present in the reaction we examined hydrolysis of 32P-labeled GTP using thin layer chromatography as described above. Because nucleotide exchange in SRβΔTM is very inefficient (see Fig. 5) we incubated SRβΔTM with [γ-32P]GTP and EDTA. In other low molecular weight GTPases this incubation step allows rapid nucleotide exchange. The exchange reaction was stopped by adding excess Mg2+ (25, 26). By monitoring the degree of exchange by nitrocellulose filter binding it was discovered that even in the presence of nucleotide and EDTA only 6% of SRβΔTM could exchange GTP for [γ-32P]GTP (data not shown), in agreement with the results obtained from time-dependent nucleotide exchange (Fig. 5). Unbound nucleotide was removed by repurifying SRβΔTM on CM-Sepharose. SRβΔTM was incubated alone or in the presence of >2-fold molar excess of 80 S ribosomes, and GTP hydrolysis over time was monitored by TLC. As expected, we detected no intrinsic GTPase activity in SRβΔTM alone. Moreover, the presence of ribosomes did not stimulate the GTPase activity of SRβΔTM (data not shown).

In addition to proposing that a ribosomal component acts as an SRβ GAP, Bacher et al. (18) measured a decreased affinity between SRβ and guanine nucleotides in the presence of ribosomes, suggesting that a ribosomal component also behaves as a guanine nucleotide releasing factor. However, if SRβ displays a lower affinity for GTP when incubated with ribosomes, it is likely that some GTP would dissociate from SRβ during the incubation and become available for hydrolysis by the ribosome. Because we did not observe any GTP hydrolysis we conclude that the GTP remains tightly bound to SRβ throughout the incubation with ribosomes.

Chemical cross-linking experiments have yielded a specific cross-link between SRβ and a protein within the 60 S ribosomal subunit, suggesting that a physical association does occur (11). We were unable to detect a cross-link between SRβ and a ribosomal protein using conditions that result in cross-links between SRβΔTM molecules and that in previous publications supported cross-linking between ribosomes and SRα/SRβ (see Supplemental Fig. 2). This raised the possibility that we do not detect an influence of the ribosome on SRβ, because the ribosome is unable to bind SRβ in the absence of SRα. To test this possibility SRβ binding to 80 S ribosomes and RNCs was assessed by sedimentation in sucrose density gradients. SRβΔTM or SRβC71DΔTM was incubated with purified 80 S ribosomes or RNCs, and ribosome-bound SRβ was separated from unbound SRβ by centrifugation on a 10–40% sucrose gradient. Fractions were collected and analyzed by Western blotting with an antibody against SRβ (Fig. 8). Both SRβΔTM and SRβC71DΔTM formed a stable complex with both untranslating ribosomes and RNCs, as revealed by their comigration in sucrose (Fig. 8, b–e). Prolactin, which is not expected to interact with ribosomes, remained at the top of the gradient (Fig. 8f). These data demonstrate that although the ribosome is unable to stimulate the SRβ GTPase, a stable interaction between the two can still occur. It should be noted that SRβ is present in excess over ribosomes in these experiments, so it is not possible to estimate the percentage of SRβ that is able to bind to ribosomes from this figure. By performing the experiment with equimolar amounts of SRβΔTM and ribosomes, we determined that 22% of SRβ is recovered in the ribosome-containing fractions (data not shown). Although it is not possible to estimate the amount of SRβ that can initially form a complex with ribosomes, the fact that 22% of SRβ remains bound to ribosomes throughout a

![Fig. 8. Binding of SRβΔTM and SRβC71DΔTM to ribosomes.](image-url)

16-h centrifugation step provides evidence that the interaction between ribosomes and SRβ is stable.

**DISCUSSION**

To examine the properties of the isolated SRβ GTPase, we expressed SRβΔTM and SRβC71DΔTM in *E. coli*. The transmembrane domain was deleted from both SRβ molecules to increase the solubility and yield in the expression system. The deleted region is not believed to contribute to the activity of SRβ, because SRβΔTM has already been shown to rescue translocation function in *vivo* in yeast containing two disrupted SRβ alleles (38).

Consistent with previous data, we have shown that recombinant SRβ binds GTP with a *Kd* of ~2 μM, similar to detergent-solubilized SR (12), but much higher than the *Kd* of SRα-SRβ dimers reconstituted into lipid vesicles (18). It must be noted that for all of these reports the *Kd* measurement is relevant only to the small fraction of SRβ that is able to bind solution GTP during the assay. In our experiments greater than 70% of the SRβΔTM was already bound to GTP, and less than 10% of the SRβΔTM added to the reaction binds GTP prior to cross-linking (Fig. 5). Furthermore, the short incubation time used in cross-linking studies favors the loose binding conformation (Fig. 4). The affinity for GTP of the larger population of SRβ that purifies bound to GTP is unknown, but it is presumably much higher than 2 μM, because there is no appreciable ex-
change with solution GTP during an 8-h incubation (Fig. 5), and bound GTP is removed during purification of the protein extremely slowly or not at all, despite the absence of solution GTP in the purification buffers.

Previous attempts to measure the $K_d$ of SRβ (12) did not account for the possibility that much of the SRβ used in the assay may not be able to accept exogenous GTP. It is likely that these $K_d$ measurements reflect the affinity of the same small population of SRβ measured here that can bind to (or exchange with) exogenous GTP and do not reflect the affinity of the majority of SRβ in the assay. It is perhaps significant that 2–3% of SRβ purified from E. coli is bound to GDP. Our estimates of the fraction of SRβ that binds GTP, 3–6%, are similar enough to 2–3% that we speculate that the GDP bound form of SRβ is responsible for the binding activity that we measured. Bacher et al. (18), by incorporating purified SR into proteoliposomes, have measured a $K_d$ in close agreement with other Ras-type GTPases. It is possible that lipid binding by SRβ leads to a conformational change that permits exchange of bound GTP with exogenous GTP.

The GTP binding site in SRβΔTM differs from other low molecular weight GTPases in that it contains a cysteine at a position within the G1 GTPase consensus sequence that is highly conserved as either glycine or aspartic acid in other family members of Ras-type GTPases (Table 1). Structural analysis of Ras (39) and ARF-1 (40) does not provide insight into the functional role of the amino acid at this position, but mutation of this residue is invariably detrimental to the function of the protein (16, 17, 41, 42).

The identity of the amino acid at this position in SRβ is somewhat less conserved, suggesting that the side chain at this position is less important for the function of the protein than it is for other GTPases (Table II). In yeast, SRβ contains a glutamine at this position that simultaneously binds SRα and protrudes into the SRβ GTP binding pocket (13). SRβ shows greater sequence homology to Arf GTPases than to Ras (12); therefore we mutated the Cys to Asp in an attempt to convert SRβ into an Arf-type GTPase. Compared with wild type, SRβC71DΔTM appears to bind GTP with faster kinetics than SRβΔTM, suggesting that the conformation of the protein has changed such that GTP has easier access to the binding pocket. The cysteine normally at this position may contribute to the stability of the protein, because at 24 °C the mutant protein exhibits a gradual loss of nucleotide binding (see Figs. 4 and 5). Nucleotide preference was not affected by this mutation, because fluorescence assays failed to detect binding of mant-XTP to SRβC71DΔTM (data not shown). We were also unable to distinguish a difference in nucleotide affinity between SRβΔTM and SRβC71DΔTM for the small fraction of protein that binds exogenous nucleotide. Unlike the Asn in yeast SRβΔTM, the Cys in the canine protein is not likely to be involved in binding of SRβΔTM to SRα, because the C71D mutation does not appear to interfere with communoprecipitation of SRα with SRβC71DΔTM (Fig. 1). Finally, we were unable to detect GTPase activity arising from SRβC71DΔTM (Fig. 8) (data not shown), but because we could not detect GTPase activity from wild-type SRβΔTM the role of the cysteine in catalysis remains uncertain.

The use of fluorescence to study GTP binding to SRβΔTM revealed a two-step process (Fig. 4). The first step is rapid and was detected by monitoring energy transfer between Tyr (and Phe) residues within SRβ and a mant fluorophore incorporated into GTP. The second step is slower and was detected by monitoring energy transfer between a Trp residue located at the carboxyl terminus of SRβ and the mant fluorophore. This second step occurs on the same time scale as GTP binding monitored by a nitrocellulose filter binding assay, a technique that captures tightly bound protein-nucleotide complexes (Fig. 5). Taken together, these data suggest a two-step model for GTP binding to SRβ.

The first step involves GTP bound to SRβ in a loose conformation. The filter binding assay does not detect these complexes as loosely bound GTP is washed away. We detect these complexes by energy transfer between SRβ and mant-GTP. The time scale of this loose binding interaction is similar to GTP binding by other Ras-type GTPases assayed in their GDP-bound state (30, 35). The second step represents a tight binding conformation, detected by both filter binding and energy transfer between Trp and mant-GTP. Our data suggest that in SRβΔTM the carboxyl terminus of SRβ reorients such that energy transfer occurs between Trp and mant. We propose that this conformational change stabilizes the tight binding GTP-SRβΔTM complex. Comparison of the data in Fig. 4, b and c suggests that the increase in RET between the Trp and mant-GTP occurs subsequent to binding. The simplest explanation for the increase in RET with the Trp is that SRβΔTM undergoes a conformational change that moves the Trp closer to the GTP binding site. Consistent with a role for the carboxyl terminus of SRβ in stabilizing the structure of the protein we have shown previously (21) that the carboxyl-terminal six amino acids (including the one Trp in SRβ) are required for folding of the protease-resistant core of SRβ.

SRβC71DΔTM exhibits tight binding of GTP but does not undergo the conformational change that stabilizes the complex, because the rate of GTP binding is the same whether it is measured by RET or filter binding. It may be that access to the GTP binding site is altered in SRβC71DΔTM but the mechanism of GTP binding is not changed.

We have shown that the bulk (~70%) of both SRβΔTM and SRβC71DΔTM are tightly bound to GTP. In contrast other Ras-type GTPases are all purified in the GDP-bound state (28–30). Even ARF-1 purifies GDP-bound, yet it exhibits no measurable GTPase activity in vitro (29, 35). Ran purifies bound to both GTP and GDP, reflecting the equilibrium between the two populations within the cell (43). Thus, SRβ is the only Ras-type GTPase confirmed to purify predominantly in the GTP-bound state. This result is not specific for isolated SRβ, because SRβ-SRα complexes also purified in the GTP-bound state (13).

The finding that SRβ defaults to the GTP-bound state whereas other Ras-type GTPases default to the GDP-bound state makes some biological sense. GTP-bound SRβ binds to SRα more tightly than when SRβ is loaded with other nucleotides (21). Because SRβ is required to anchor SRα to the ER membrane, and SRα is found almost exclusively in the membrane fraction (38), unlike other Ras-like GTPases it makes sense to keep SRβ bound to GTP.

It has been reported previously (18) that ribosomes both stimulate the SRβ GTPase and decrease the affinity of SRβ for nucleotides. Furthermore, an interaction between SRβ and a 21-kDa ribosomal protein has been detected, which may provide the basis for the effect of the ribosome on nucleotide binding by SRβ (11). However, in the absence of SRα, we were unable to detect an influence of ribosomes or ribosomal sub-units on the SRβ GTPase. Attempts to detect a cross-link between a ribosomal protein and SRβ incubated with GTP or GDP were also unsuccessful (see Supplemental Fig. 2). Because SRβ can bind to ribosomes in the absence of SRα these results suggest that SRα changes the quality of the interaction between the ribosome and SRβ, either by regulating the structure of SRβ to facilitate GTP hydrolysis or by directly contributing residues that are required for GTP hydrolysis. This suggests an additional role for SRα in translocation as an effector of SRβ.
and is consistent with structural data that demonstrate that SRα binds SRβ predominantly through the SRβ switch I region (13).

Ribosomes are unlikely to interact with SRβ in the absence of SRα in vivo. Therefore, an SRα-SRβ-ribosome-nascent chain-translocon complex may be required for SRβ to hydrolyze GTP. Hydrolysis of GTP may then lead to dissociation of SRα from SRβ, contributing to transfer of the RNC from SR to the translocon (21).

We have shown previously that SRβ forms a tight physical association with SRβ, and this interaction is nucleotide-dependent and requires the intact GTPase domain of SRβ and is facilitated by the unique loop sequence located between the G4 and G5 boxes (20, 21). These characteristics are consistent with SRα assuming the role of an SRβ effector molecule. Our finding that the ribosome does not stimulate the GTPase activity of isolated SRβ, together with previous data demonstrating GTPase activity of SRβ only in the context of an SRα-SRβ-ribosome complex (18), leads us to speculate that SRα may regulate the GTPase activity of SRβ. A detailed structural analysis of SRβ alone and in a complex with SRα will be required to assess the extent that binding of SRα regulates SRβ function.

Acknowledgment—We thank Dr. A. E. Johnson for helpful discussions regarding fluorescence spectroscopy.

REFERENCES

1. Ogg, S. C., and Walter, P. (1995) Cell 81, 1075–1084
2. Krieg, U. C., Walter, P., and Johnson, A. E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8604–8608
3. Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., and Rapoport, T. A. (1986) Nature 320, 634–636
4. Legate, K. R., and Andrews, D. W. (2001) Biochim. Biophys. Acta 1489, 593–601
5. Millman, J. S., and Andrews, D. W. (1997) Cell 88, 673–676
6. Rapienko, P. J., and Gilmore, R. (1997) Cell 89, 703–713
7. Connolly, T., Rapienko, P. J., and Gilmore, R. (1991) Science 252, 1171–1173
8. Bacher, G., Luteke, H., Jungnickel, B., Rapoport, T. A., and Dobberstein, B. (1996) Nature 381, 248–251
9. Bernstein, H. D., Peritz, M. A., Strub, K., Hoben, P. J., Brenner, S., and Walter, P. (1989) Nature 340, 482–486
10. Romisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingrong, M., and Dobberstein, B. (1989) Nature 340, 478–482
11. Fulga, T. A., Sinning, I., Dobberstein, B., and Pool, M. R. (2001) EMBO J. 20, 2338–2347
12. Miller, J. D., Tajima, S., Lauffer, L., and Walter, P. (1995) J. Cell Biol. 128, 273–282
13. Schwartz, T., and Blobel, G. (2003) Cell 112, 795–803
14. Zhang, P. L., and Casey, F. J. (1996) Annu. Rev. Biochem. 65, 241–269
15. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
16. Trahey, M., and McCormick, F. (1987) Science 238, 542–545
17. Kahn, R. A., Clark, J., Rulka, C., Stearne, T., Zhang, C. J., Randazzo, P. A., Terui, T., and Cavenagh, M. (1993) J. Biol. Chem. 270, 143–150
18. Bacher, G., Pool, M., and Dobberstein, B. (1999) J. Cell Biol. 146, 723–730
19. Tajima, S., Lauffer, L., Rath, V. L., and Walter, P. (1986) J. Cell Biol. 103, 1167–1178
20. Young, J. C., Ursini, J., Legate, K. R., Miller, J. D., Walter, P., and Andrews, D. W. (1995) J. Biol. Chem. 270, 15650–15657
21. Legate, K. R., Falone, D., and Andrews, D. W. (2000) J. Biol. Chem. 275, 27439–27446
22. Lauffer, L., Garcia, P. D., Harkins, R. N., Coussens, L., Ulrich, A., and Walter, P. (1985) Nature 318, 334–338
23. Hughes, M. J., and Andrews, D. W. (1996) BioTechniques 20, 192–196
24. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) Anal. Biochem. 206, 74–80
25. Koyama, S., and Kikuchi, A. (2001) Methods Enzymol. 332, 127–138
26. Wang, Y., and Colielli, J. (2001) Methods Enzymol. 332, 139–151
27. Dever, T. E., Glyniás, M. J., and Merrick, W. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1814–1818
28. Poe, M., Scollnick, E. M., and Stein, R. B. (1985) J. Biol. Chem. 260, 3906–3909
29. Weiss, O., Holden, J., Rulka, C., and Kahn, R. A. (1989) J. Biol. Chem. 264, 21066–21072
30. Barlowe, C., d’Enfert, C., and Schekman, R. (1993) J. Biol. Chem. 268, 873–879
31. Feuerstein, J., Goody, R. S., and Wittinghofer, A. (1987) J. Biol. Chem. 262, 8455–8458
32. Shapiro, A. D., Riederer, M. A., and Pfeffer, S. R. (1993) J. Biol. Chem. 268, 6925–6931
33. John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., and Goody, R. S. (1990) Biochemistry 29, 6058–6065
34. Mistou, M. Y., Cool, R. H., and Parmeggiani, A. (1992) Eur. J. Biochem. 204, 179–185
35. Kahn, R. A., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7906–7911
36. Ferguson, K. M., Higashijima, T., Smigel, M. D., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7393–7399
37. Marsh, R. C., Chinnai, G., and Parmeggiani, A. (1975) J. Biol. Chem. 250, 8344–8352
38. Ogg, S. C., Barz, W. P., and Walter, P. (1998) Annu. Rev. Biochem. 67, 179–185
39. Amor, J. C., Harrison, D. H., Kahn, R. A., and Ringe, D. (1994) Science 252, 192–196
40. Terui, T., and Cavenagh, M. (1995) J. Cell Biol. 129, 143–150
41. Amer, J. C., Harrison, D. H., Kahn, R. A., and Ringe, D. (1994) Nature 372, 673–678
42. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V., and Levinson, A. D. (1984) Nature 312, 71–75
43. Jacquet, E., and Parmeggiani, A. (1988) EMBO J. 7, 2861–2867
44. Deleted in proof
