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The role of GTP binding proteins in the initiation of protein translocation

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The Role of GTP Binding Proteins in the Initiation of Protein Translocation

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ABSTRACT

The Role of GTP Binding Proteins in the Initiation of Protein Translocation

Joshua D. Miller

In higher eukaryotes, proteins bearing a signal sequence are translocated across the membrane of the endoplasmic reticulum (ER). The initial events of protein translocation are the binding of the signal sequence by the 54 kD subunit (SRP54) of the signal recognition particle (SRP) and the targeting of the ribosome nascent chain complex to the ER. Guanine nucleotide is required for these events to take place. Targeting is mediated by the binding of SRP to the SRP receptor, a membrane protein consisting of two different subunits, SRα and SRβ. Interaction of SRP and SRα/SRβ causes release of the signal by SRP54 in a GTP dependent manner and the engagement of the nascent chain with the membrane bound translocation apparatus. Both SRP54 and SRα contain homologous domains which include a predicted GTPase fold. The body of work presented here characterizes the targeting reaction structurally, by examining the interaction between SRP and SRα/SRβ, and functionally, by following GTP binding to and hydrolysis by these components. The results of biochemical extraction of ER membranes with high pH buffers or the detergent Triton X-114 suggest that SRβ is an integral membrane protein of the ER that anchors SRα to the membrane. SRα, in turn, is shown to be required for binding of SR to SRP. A functional dissection of SRP suggests that it is SRP54 that SRα interacts with. A monoclonal

antibody recognizing SRβ was used to isolate a cDNA clone encoding this protein. The amino acid sequence deduced from this clone contains a putative transmembrane domain, supporting the biochemical data that SRβ is an integral membrane anchor for SRα. It is unlikely that SRβ simply functions as a membrane anchor, however, because, in addition to the transmembrane domain, it also contains a GTP binding consensus sequence. This tripartite motif is similar to the one in SRα and SRP54 and is typified by the *ras* family of GTPases. SRβ shares no marked homology to any other proteins apart from the consensus sequence and is the first transmembrane protein found to contain this GTP binding motif. A UV crosslinking assay was used to demonstrate that all three proteins bind GTP specifically, and the SRα/SRβ complex functions to stimulate both GTP binding to and GTP hydrolysis by SRP54. A model is presented for the initiation of protein translocation across the ER in which SRα/β catalyzes a cycle of GTP binding, hydrolysis and release by SRP54 that regulates its dissociation from the signal sequence.

Peter Walter

Chair, Thesis Committee

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Chapter 1

Introduction

Background

A cell is defined by a lipid membrane bilayer which segregates its cytoplasm from the extracellular space. The membrane performs this function by forming a barrier to the passage of many small molecules, as well as macromolecules such as proteins. All cells have specialized functions that require the secretion of intracellularly synthesized protein into the extracellular space. This necessitates the proteins crossing the membrane. To accomplish this task, cells have a specific group of proteins that function to identify secretory proteins and to transport them across the membrane. In bacteria, proteins cross or "translocate" directly through the plasma membrane. In higher eukaryotic cells the endoplasmic reticulum (ER) has evolved to carry out protein translocation. Proteins in the cytoplasm that are destined to be secreted are translocated across the ER membrane and into the lumen of the ER. They then proceed by vesicular transport through a series of membranous structures to the plasma membrane. There, vesicles fuse with the plasma membrane and release their contents into the extracellular space.

Our current understanding of the translocation process in mammalian cells comes primarily from *in vitro* studies using canine pancreatic membranes as a model system (Nunnari and Walter, 1992; Rapoport, 1992). The primary requirement for a protein to enter the secretory pathway is that it contain a short stretch of amino acids known as a "signal sequence." Signal sequences are usually 20 - 30 amino acid, α-helical peptides containing a hydrophobic core (Gierasch, 1989). Most signal sequences are at the extreme N-terminus of secretory proteins and are cleaved off of the protein by signal peptidase upon entering the lumen of the ER.

The synthesis of signal sequence-bearing proteins is initiated on cytoplasmic ribosomes. The nascent chains must then be directed or "targeted" to the ER where translocation takes place. The selection and targeting of secretory nascent chains is mediated by a cytosolic factor known as the signal recognition particle (SRP). SRP is a ribonucleoprotein consisting of six polypeptides bound to a 300 nucleotide long RNA (referred to here as SRP RNA). SRP recognizes signal sequences as they emerge from the ribosome and then binds tightly to both the signal sequence and the ribosome. Signal sequence binding activity is mediated by the 54kDa subunit of SRP (SRP54) (Krieg et al., 1986; Kurzchalia et al., 1986). In this initial step of protein translocation, secretory proteins are segregated from the rest of the elongating polypeptides.

The result of the SRP/signal sequence interaction is that a complex of ribosome/nascent chain/SRP is formed. This entity is defined as a "targeting complex." SRP plays two roles in this targeting complex. First, it arrests the elongation of the nascent chain (Walter et al., 1981). The elongation arrest activity of SRP is thought to be important because once a signal sequence-bearing protein is fully synthesized and released from the ribosome, it adopts a conformation that can no longer interact productively with the translocation machinery. By arresting translation, SRP prevents ribosomal release and ensures that a targeting complex arrives at the membrane with a nascent chain that is competent to enter the translocation process.

The second function of SRP is to direct the ribosome/nascent chain to the ER membrane (Walter and Blobel, 1981). This targeting event requires the interaction of SRP with its membrane-bound receptor. The SRP receptor (SR) is a heterodimer consisting of a 72kD α subunit (SRα) and a 30kD β subunit (SRβ) (Tajima et al., 1986). While it was clear at the time this work was begun that SR resided in the ER, the nature of that association was unknown. Likewise, while

SR was known to bind to SRP, the specific subunits of each of these complexes that mediate their interaction had not been identified.

Binding of SRP to SR causes SRP to dissociate from the signal sequence and the ribosome (Gilmore and Blobel, 1983). The signal sequence is thus free to engage with the membrane-bound components of the translocation apparatus. The processes of signal sequence recognition, targeting and release of the signal sequence at the membrane are defined as the "initiation of protein translocation" to distinguish it from the actual passage, or translocation, of the protein across the membrane. Translocation initiation is mediated by SRP and SR, while the translocation step itself is mediated by membrane bound factors referred to collectively as the "translocon."

The translocon is thought to assemble from its individual components in response to the arrival of a targeting complex at the membrane. This assembly probably results in the formation of a pore that spans the membrane and allows for the passage of the elongating protein into the lumen of the ER. Upon completion of translocation the pore disassembles and awaits the arrival of another targeting complex. The ER contains large, ion conducting channels that behave in a manner that is consistent with their putative function as protein conducting pores (Simon and Blobel, 1991). During steady state translocation, the pore may contain or interact with, among other things, a signal sequence receptor, a factor that anchors ribosomes to the membrane, factors that modify the nascent chain and/or factors that regulate translocon assembly/disassembly. In contrast, SRP and SR function catalytically and cycle out of the translocation complex after targeting has been completed (Gilmore and Blobel, 1983). Ultimately SRP is released from SR and cycles back into the cytosol to be reused in another round of targeting.

The initiation of protein translocation is a regulated process

The translocation of proteins results from the combined action of several complex processes that must proceed with efficiency and fidelity. This is especially true at the level of translocation initiation, the point at which nascent proteins are committed to enter the secretory pathway. Initiation involves the formation and subsequent disassembly of a series of very specific protein complexes. SRP interacts with both the ribosome and the signal sequence. It then disengages from these components to assemble with SR while the ribosome and nascent chain, in turn, go on to interact with the translocon. In order to carry out these events in the proper order, the protein-protein interactions under consideration must be regulated in both a spatial and temporal manner. Highly ordered assembly and disassembly steps are often regulated by GTPases.

GTPases are able to regulate complex protein-protein interactions because they can adopt two distinct conformations depending on whether they are liganded to GTP or GDP (Bourne et al., 1990). For example, the α subunit of trimeric G-proteins ($G_{s\alpha}$) forms a complex with its β/γ subunits when it is liganded to GDP (Gilman, 1987). Stimulated hormone receptor interacts with this complex and causes $G_{s\alpha}$ to switch to the GTP-bound conformation. GTP binding to $G_{s\alpha}$, in turn, leads to the disassembly of the $\alpha/\beta/\gamma$ complex and stimulates the interaction of $G_{s\alpha}$ with its downstream effector, adenyl cyclase. Similarly, the translational elongation factor EF-Tu binds to its exchange factor, EF-Ts when liganded to GDP (Kaziro, 1978). This switches EF-Tu to the GTP-bound state which disassembles from EF-Ts and binds tightly to aminoacyl tRNA. Thus, in the GDP-bound conformation a GTPase can interact with one ligand, while the GTP-bound conformer recognizes another. By modulating its nucleotide bound state, a GTPase can control the way in which other proteins organize into functional groups. Given the parallels between protein

translocation and these GTP - regulated systems, it is, perhaps, not surprising to find that translocation is GTP-dependent and that three of the key proteins in initiation of protein translocation are GTPases.

SRP54, SRα, and SRβ contain GTP binding motifs

Molecular cloning of the genes that encode SRP54 (Bernstein et al., 1989; Römisch et al., 1989) and SRα (Lauffer et al., 1985; Connolly and Gilmore, 1989) revealed that they each contain a GTP binding consensus sequence: short stretches of amino acids that are conserved among most GTPases (Bourne, et al., 1990). From the crystal structure of the GTPases *ras* and EF-Tu, these sequence stretches are known to form part of the GTP binding site and to contact the bound nucleotide directly (Jurnak, 1985; Pai et al., 1990). The GTPase domains of SRP54 and SRα share significant additional amino acid sequence similarity with one another outside of the GTPase consensus sequences, which is not shared with other known GTPases. Thus, together SRP54 and SRα seem to comprise a new subgroup in the superfamily of GTPases. The extent of this sequence similarity continues N-terminal to the GTPase domain to define an additional region, called the N-domain. In addition, SRP54 possesses an extra C-terminal domain, called the M-domain, which is very rich in methionine residues (Bernstein, et al., 1989). The M-domain can be severed by proteases from the rest of SRP54. It contains the signal sequence binding site and interacts with SRP RNA to anchor SRP54 to the SRP complex (Zopf et al., 1990; Römisch et al., 1990; High and Dobberstein, 1991). Interestingly, alkylation of cysteine residues in the GTPase domain prevents signal sequence binding to the M-domain (Lütcke et al., 1992), suggesting that these two domains communicate to influence binding to their respective ligands.

The work presented in Chapter 2 demonstrates that, like SRα and SRP54, SRβ also contains a GTP binding motif. Except for the GTPase consensus sequences, the spacing of which is very close to that found in the *ras* protein, SRβ bears no primary structure similarity to any other known protein. In addition to the GTP binding motif, SRβ also contains a membrane-spanning region. SRβ is the first example of a protein with such a consensus sequence that contains a *bona fide* transmembrane domain.

GTP is required for the initiation of translocation

The finding that three of the key proteins in translocation initiation are putative GTPases is consistent with the observation that GTP is required for this process. Indeed, these targeting complexes will bind stably to membranes only if GTP or a non-hydrolyzable analog of GTP is present (Connolly and Gilmore, 1986). Because GTP but not GTP hydrolysis is required for this step, GTP is likely to play a regulatory role, rather than to provide energy from hydrolysis to the system. The stable association of targeted nascent chains with the membrane is thought to represent an interaction with the translocon, and therefore is dependent on the prior release of the signal sequence from SRP. The SR-catalyzed release of SRP from the signal sequence and ribosome requires GTP or a non-hydrolyzable analog (Connolly and Gilmore, 1989), suggesting that GTP binding to one or more of the identified GTPase domains in SRP and SR is required for this step. When this experiment is carried out with a non-hydrolyzable analog, SR and SRP are found in a tight complex, providing further evidence that the interaction of targeting components is modulated by GTP.

To dissect out the individual contributions of the putative GTPase domains, a more direct analysis of the role of GTP in translocation initiation was needed. One approach to this problem is to make site directed mutations that

affect GTP binding to each of the proteins. The GTPase *ras* has been very well studied in terms of its structure as well as its GTP binding and hydrolysis properties. A great number of mutations in this protein exist which have well defined biochemical phenotypes such as the inability to bind to GTP, the inability to bind to GDP and the inability to hydrolyze GTP (Barbacid, 1987). Working from the *ras* model, mutations predicted to disrupt GTP binding were made in SRα and these mutants were used to repopulate SRα-deficient membranes (Rapiejko and Gilmore, 1992). These mutant proteins were unable to promote protein translocation into the ER and failed to form stable complexes with SRP in the presence of non-hydrolyzable GTP. Although they were not tested directly for GTP binding, function could be restored to one of the mutant forms of SRα by a 50-fold increase in GTP concentration. This indicates that this mutant SRα has a decreased affinity for GTP and that the mutation does not simply interfere with proper protein folding. Therefore, it appears that GTP binding to SRα is a requirement for an early step leading to protein translocation.

Mutations predicted to disrupt GTP binding have also been made in SRP54. These mutations render SRP containing the mutant protein unable to promote protein translocation, although the mutant SRP can still bind to signal sequences and elicit an elongation arrest (H. Bernstein and P. Walter, unpublished results). A mutant SRP containing only the M-domain of SRP54, i.e. lacking the N- and GTPase domains, is also functional for signal sequence binding and translational arrest but fails to promote translocation (Zopf et al., 1993). Thus, GTP binding to SRP54 does not appear to be required for signal sequence recognition but is crucial for translocation. These experiments demonstrate the importance of GTP binding to both SRα and SRP54 during translocation initiation, yet they do not define the role that this binding serves. One way to examine this is to monitor GTP binding to the individual GTPase

domains at different stages of the SRP cycle. Chapter 2 describes the initial stages of this work in which an UV crosslinking assay is used to characterize the GTP binding properties of each of the three proteins. A biochemical dissection of the interaction of SR with the ER membrane and with SRP is also presented. The functional consequences of the SRP·SR interaction, focusing on GTP binding and hydrolysis by these components, is explored in Chapter 3. This chapter concludes with a model which postulates that a SR regulated cycle of GTP binding and hydrolysis by SRP54 is coupled to the properly timed release of the signal sequence.

All of the work presented in Chapters 2 and 3 make use of mammalian components purified from canine pancreas microsomal vesicles. Homologues for SRα, SRP54 and SRP RNA have also been identified both in the yeast *S. cerevisiae* (Hann et al., 1989; Hann and Walter, 1991; Ogg et al., 1992a)and in the bacterium *E. coli* (Bernstein, et al., 1989; Poritz et al., 1988; Ogg, et al., 1992a). While it is unknown if their functions are analogous to their mammalian counterparts, they appear to be involved in protein translocation into the ER (Hann, et al., 1989; Ogg et al., 1992b; Ribes et al., 1990; Phillips and Silhavy, 1992) and some of the same physical interactions have been demonstrated (Hann and Walter, 1991; Hann et al., 1992; Poritz et al., 1990; Luirink et al., 1992). Chapter 4 consists of a characterization of the GTP binding and hydrolysis properties of the bacterial components and demonstrates that these properties are remarkably similar to the mammalian components with respect to their requirements and their regulation.

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Chapter 2

The beta subunit of the SRP receptor is a novel, transmembrane, *ras*-like GTP binding protein that anchors a chain of GTP binding proteins to the endoplasmic reticulum membrane

Introduction

Secretory proteins, whose ultimate fate is to be released into the extracellular space, must cross a membrane barrier which is normally impermeable to such traffic. In higher eukaryotes there is a specialized mechanism which allows proteins tagged with a "signal sequence" to translocate across the membrane of the endoplasmic reticulum (reviewed in Nunnari and Walter, 1992). This process can be divided into two stages; targeting of nascent secretory proteins from the cytoplasm to the membrane of the ER, followed by the physical translocation of the protein across the membrane. For a translating ribosome to reach the ER in a functional manner, it must interact with the cytoplasmic signal recognition particle (SRP). SRP is a ribonucleoprotein consisting of six polypeptides and one RNA. SRP binds to both the signal sequence and the ribosome to establish a functional ribosome-signal sequence-SRP tertiary complex, or targeting complex. Signal sequence binding is mediated by the 54-kD protein subunit of SRP (SRP54), and it results in the arrest of translational elongation so as to maintain the nascent chain in a translocation competent state. In the absence of signal sequence binding and translational arrest by SRP, the nascent chain elongates to a length that is incompatible with its subsequent translocation.

Once the targeting complex is formed, it is targeted to the cytoplasmic face of the ER membrane via the interaction of SRP with its membrane bound receptor. The SRP receptor (SR) is a heterodimer consisting of a 69-kD subunit (SRα) and a 30-kD subunit (SRβ) (Tajima et al., 1986). Upon binding to SR, SRP dissociates from both the signal sequence and the ribosome (Gilmore and Blobel, 1983), allowing the formation of the ribosome-membrane junction and

translocation of the elongating nascent chain into the lumen of the ER. Detergent solubilized and purified SR can bind to SRP and release it from the targeting complex (Gilmore and Blobel, 1983) thereby restoring translation (Gilmore et al., 1982a). The individual contribution that the SRα and SRβ subunits make to receptor function is still unknown.

The events that transpire from the assembly of a targeting complex to the final formation of the ribosome-membrane junction require that many protein-protein interactions be made and disassembled in a coordinated and regulated manner. Often, complex assembly and disassembly is regulated by GTP binding proteins (Bourne et al., 1990). GTP is, in fact, required for the establishment of a stable ribosome-membrane junction (Connolly and Gilmore, 1986) and, more specifically, for SR stimulated release of signal sequence by SRP54 (Connolly and Gilmore, 1989). Intriguingly, both SRα and SRP54 contain *ras*-like GTP binding motifs in their primary structure (Bourne et al., 1991; Bernstein et al., 1989; Römisch et al., 1989; Lauffer et al., 1985; Connolly and Gilmore, 1989). We demonstrate here that SRβ is a transmembrane protein that contains a third *ras*-like GTP binding motif and is required to anchor SRα and SRP54 to the ER.

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, 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 (Walter and Blobel, 1983a; Walter and Blobel, 1983b; Gilmore and Blobel, 1983; 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 was identified as an IgM using a kit purchased from Boehringer Manheim Biochemicals, Indianapolis, IN. IgM secreted into ascites fluid 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: 1) 100 mM sodium carbonate, pH unadjusted (pH 11.2); 2) 100 mM sodium carbonate, adjusted to pH 12.0 by the addition of sodium hydroxide; and 3) 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, 1983a for definition of equivalent). After 30 minutes at 25 °C, the reactions were spun for 30 minutes 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 minutes, the reactions were transferred to a 37 °C water bath for 3 minutes to induce phase separation. Detergent-poor and detergent-

rich phases were separated by a 5 minute 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 membranes were diluted to 2 eq/μl in high-salt buffer: 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 one hour. Digestion was stopped by addition of 2 mM diisopropylfluorophosphate (DIFP), 1 mM phenylmethylsulfonylfluoride (PMSF) and 100 U/ml Trasylol. After 15 minutes on ice the membranes were either assayed as in Fig. 1B, or pelleted by centrifugation 50,000 rpm in a Beckman Ti 70.1 rotor for 30 minutes through a cushion of 250 mM sucrose in high-salt buffer containing 0.1 mM PMSF. The pellet was resuspended in high-salt buffer and the centrifugation was repeated. After this washing step, the pellet was dissolved in 50 mM triethanolamine, 250 mM sucrose and 1 mM DTT. Membranes were frozen in liquid nitrogen and stored at -80 °C for further use.

SRP-Sepharose chromatography

Trypsinized membranes were diluted to 1 eq/μl in 1% Nikkol, 50 mM triethanolamine, pH-7.5, 375 mM potassium acetate, 250 mM sucrose, 1 mM DTT, 10 U/ml Trasylol, 0.5 mM PMSF and 0,1 mM DIFP and were extracted for 15 minutes on ice. The soluble fraction was obtained as the supernatant after a 30 minute 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 triethanolamine, pH-7.5, 50 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 1 mM DTT and 0.5% Nikkol) and 650

microliters 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 triethanolamine, pH-7.5, 10 mM potassium acetate, 25 mM magnesium acetate, 250 mM sucrose, 1 mM DTT and 0.5% Nikkol).

Protein sequencing

Immunopurified SR (Tajima, et al., 1986) was treated with SDS to dissociate the two subunits and then fractionated by reverse phase chromatography on an Alltech C4 column run on an IBM HPLC to yield purified SRα and SRβ. Peptide sequence was determined by Edman degradation, using an automated sequencer from ABI, of: 1) the amino-terminus and 2) a proteolytic fragment generated by lysyl-endopeptidase digestion of SRβ and purified on a microbore C18 reverse phase column from Vydac using a Rainin HPLC. A third peptide sequence was obtained by performing five rounds of Edman degradation on total CNBr digested 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 the drug *ortho*-phthalaldehyde or OPA (Brauer et al., 1984). Because proline does not react with OPA, it remains unblocked and susceptible to Edman degradation. Sequencing was then resumed yielding a single sequence from the CNBr fragment beginning with the unblocked proline.

cDNA cloning

To obtain a cDNA clone of SRβ, a MDCK 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

making nitrocellulose filter lifts, inducing expression of the cDNAs by incubating the lifts 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 positives were found that passed secondary and tertiary screening. These clones were then sequenced using the double stranded Sequenase (USB) protocol and identified as correct clones by the presence of the proper amino-terminal amino acid sequence obtained from direct protein sequencing. Two sets of genes were isolated that differed only by the spacing between element I and element II of the GTP binding consensus sequence and by the length of the 3' poly A tail. The larger clone, which contained the longer poly A tail (~60 residues), was presumed to be the correct clone because its spacing between element I and II conformed to the consensus spacing. In the smaller clone, which contained a poly A tail of six residues followed by 500 bp of noncoding sequence, there is an in frame deletion of twenty six amino acids between elements I and II. Consequently, this spacing is probably too short to form a functional GTP binding site. The cDNA for the longer clone was subcloned into a Bluescript-II vector (Stratagene), single stranded DNA was synthesized and the entire cDNA was sequenced on both strands using the sequenase system.

The amino acid sequence deduced from the canine cDNA did not begin with a methionine. Therefore, a full length SRβ cDNA was isolated by screening a murine teratocarcinoma cDNA library constructed in λZAP (Stratagene) using the canine cDNA as a hybridization probe (Maniatis et al., 1982). Eight independent clones were obtained from the 1.2×10^6 plaques screened and were verified by DNA sequencing. All of the murine clones corresponded to the larger of the canine clones depicted in Fig. 3A.

GTP crosslinking assay

SRP-Sepharose purified SR (Gilmore and Blobel, 1983; Tajima, et al., 1986) and SRP were incubated with 0.3 μM α-^{32}P-labeled GTP or ATP at 25 °C in 50 mM triethanolamine, pH-7.5, 150 mM potassium acetate, 5 mM magnesium acetate, 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 minute incubation the reactions were placed in plastic weigh boats on ice and UV irradiated (6 cm from a 6000 W/cm^2 UV source) for 5 minutes to covalently crosslink the bound radiolabeled nucleotide to the protein (Nath et al., 1985). The reactions were then precipitated with trichloroacetic acid to remove uncrosslinked label and analyzed by SDS-PAGE and 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.

Results

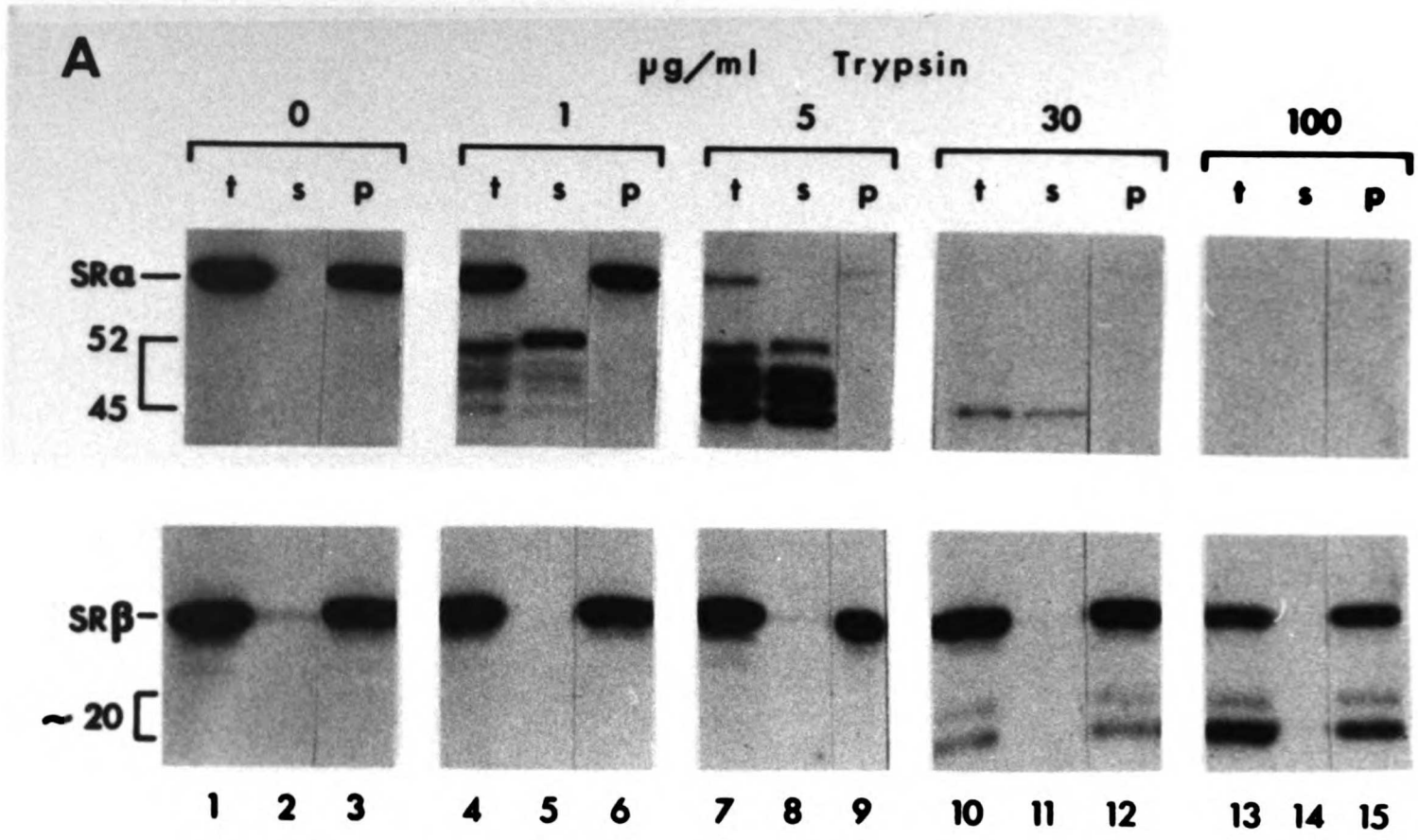
SRα mediates SR binding to SRP

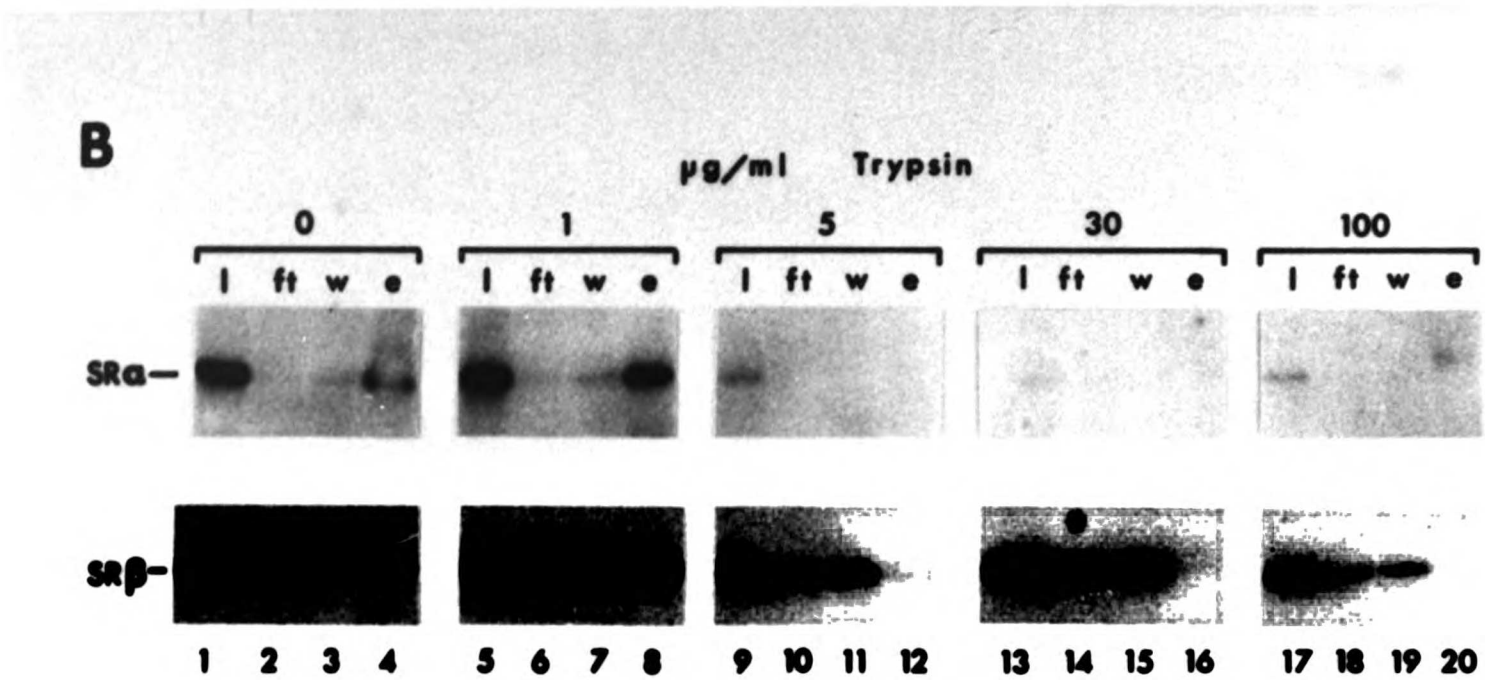
One of the most important functions of SR is its ability to bind SRP at the cytoplasmic surface of the ER. It is not known, however, what role each of the two SR subunits plays in this binding reaction. To address this question, we took advantage of the differential sensitivity of SRα and SRβ to the protease trypsin. As shown in Fig. 1A, concentrations as low as 1 mg/ml trypsin (lanes 4-6) will begin to degrade SRα while a minimal concentration of 30 mg/ml is required to detect breakdown products of SRβ (lanes 10-12). It has been demonstrated that solubilized SR will bind to SRP immobilized on a Sepharose resin (Gilmore et al., 1982b; Gilmore and Blobel, 1983; Tajima, et al., 1986). By digesting membranes with variable amounts of trypsin, then washing them free of digestion products prior to solubilization of the membrane-bound SR with the detergent Nikkol, we generated extracts containing different relative amounts of SRα and SRβ. Passing undigested extract over an SRP-Sepharose affinity column resulted in the binding of the SRα/SRβ complex to the resin (Fig. 1A, lanes 1-3), permitting recovery of the bound receptor by elution (Fig. 1B, lane 4). If, however, the integrity of some of the SRα polypeptide chains was compromised by slight tryptic digestion (1 μg/ml), intact SRβ began to appear in the column flow through and wash fractions (Fig. 1B, lanes 5-8). Increasing the amount of digestion by raising the trypsin concentration leads to greater amounts of SRβ in the flow through and wash (Fig. 1B, lanes 9-12, 13-16, and 17-20). We, therefore, conclude that SRα is required for binding of the SR complex to SRP. This is most likely through a direct interaction of SRα with SRP, but we can not eliminate the

Figure 1. Intact SRα is required for SR binding to SRP

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, SRβ and mp30 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 Fig. 1A were washed with high salt, solubilized with the nonionic detergent Nikkol and applied to an SRP-Sepharose affinity column. The column was washed and eluted as 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.


A
μg/ml Trypsin
0
1
5
30
100
t s p
SRα
52
45
SRβ
~ 20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
μg/ml Trypsin
0
1
5
30
100
l ft w e
SRα—
SRβ—
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

possibility that SRα is an allosteric regulator of SRβ that is required for SRβ to bind SRP (we, thus far, have no way of inactivating SRβ while leaving SRα intact).

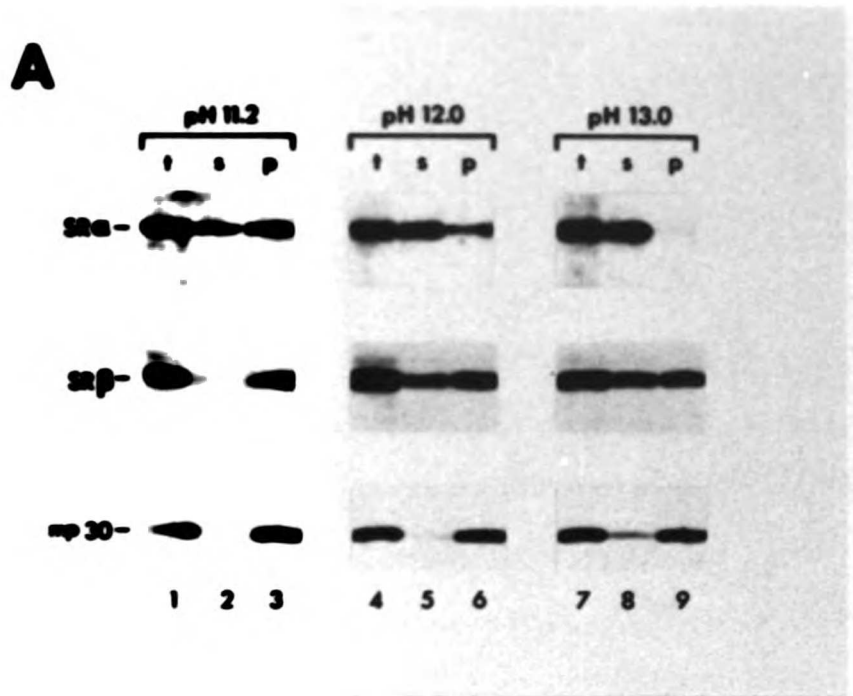
Membrane Association of SR

If SRα does in fact mediate SRP binding, perhaps SRβ functions to mediate the association of the SR complex to the ER membrane. The amino-terminus of SRα contains two hydrophobic stretches of amino acids that are of insufficient length to function as transmembrane domains but that are presumed to be involved in the association of the protein with the membrane (Lauffer, et al., 1985; Andrews et al., 1989). The demonstration that SRα can bind to the ER posttranslationally (Andrews, et al., 1989) suggests that it is a peripheral membrane protein that is anchored by either a specific lipid interaction (Sato and Ohnishi, 1983) or through another protein. If the later were correct, SRβ would be a likely candidate to tether SRα to the ER. To address these questions, we attempted to determine the disposition of the subunits in the membrane of the ER. Several criteria have been used to distinguish between peripheral and integral membrane proteins. One such method employs the alkaline extraction of membranes with carbonate buffer at pH 11.2 (Fujiki et al., 1982; Davis and Model, 1985). High pH is a nonspecific protein denaturant which disrupts the protein-protein interactions that bind peripheral proteins to the membrane without releasing integral membrane proteins from the lipid bilayer. After such an extraction, membranes can be pelleted along with their integral membrane proteins leaving peripheral membrane proteins in the supernatant. Fig. 2A shows the results of carbonate extraction on canine microsomal vesicles. As a control for these experiments we also followed the behavior of mp30, an ER

Figure 2. Membrane association of the SRP receptor

A) Alkaline extraction of canine microsomal membranes. Microsomes were extracted at either pH 11.2 (lanes 1-3), pH 12.0 (lanes 4-6) or pH 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 fractions (p) 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.


A
pH 11.2
pH 12.0
pH 13.0
t s p
t s p
t s p
SRα-
SRβ-
mp 30-
1 2 3 4 5 6 7 8 9


B
ug/ml Trypsin
0
25
t s p
t s p
SRα-
SRβ-
mp 30-
1 2 3 4 5 6

membrane protein of unknown function (Tajima, et al., 1986). Lanes 1-3 demonstrate that, at the standard pH of 11.2, mp30 behaved as a classic integral membrane protein in that it pelleted with the membranes. SRα and SRβ, on the other hand, behaved anomalously as they did not fractionate cleanly into either the supernatant or the pellet. Instead, they were partially extracted, with much more SRα being released from the membrane than SRβ. Upon raising the pH to 12.0 (lanes 4-6) most of SRα and approximately half of SRβ was extracted while all of the mp30 still pelleted with the membrane. At pH 13.0, SRα was fully extracted while half of SRβ remained membrane associated. Some mp30, however, began to be extracted from the membrane under these conditions implying that there may be some perturbation of the phospholipid bilayer. These results suggest that SRβ has a more hydrophobic character than SRα but they do not allow SRβ to be clearly assigned as an integral membrane protein.

Because of this anomalous behavior, we tested SR by another, independent, criteria for membrane association of proteins. Extraction of membranes with the detergent Triton X-114 has been shown to fractionate peripheral membrane components into an hydrophilic (detergent poor) "supernatant" phase and integral membrane proteins into an hydrophobic (detergent rich) "pellet" phase (Bordier, 1981). The results of such an experiment on canine microsomes are shown in Fig. 2B. Lanes 1-3 demonstrate that while mp30 behaved as a true integral membrane protein, partitioning solely into the detergent pellet, the SRP receptor subunits were again distributed into both supernatant and pellet. Comparison with the carbonate extraction results (Fig. 2A) shows that although SRα was again extracted to a greater extent than SRβ, the difference was much smaller, as much more SRβ was extracted with Triton X-114 than with the alkaline treatment.

The amino acid sequence of SRα predicts that it is a highly charged, very

hydrophilic molecule (Lauffer, et al., 1985). The fact that very stringent conditions, such as SDS denaturation are require to disrupt the SRα·SRβ interaction (not shown) suggests that these subunits bind tightly to one another. It is possible that the association of SRβ with the large, hydrophilic SRα is responsible for its anomalous extraction behavior. To test this hypothesis, we took advantage of the differential trypsin sensitivity of SRα and SRβ (Fig. 1A). At 25 μg/ml trypsin, SRα is virtually 100% degraded, while beta is essentially unaffected (Fig. 2B, compare lane 4 with lane 1). Repeating the Triton X-114 extraction on trypsinized membranes (lanes 4-6) reveals that SRβ now behaves similarly to the mp30 control, i.e.-like a true integral membrane protein. These results suggest that SRβ may be a true integral membrane protein that acts as a membrane anchor for SRα.

Cloning of SRβ

In order to further address the structure/function relationship of the SR subunits with each other and the ER membrane, the molecular cloning of SRβ was undertaken. Peptide sequence was obtained from the amino terminus as well as two internal sites. This sequence was used to confirm the identity of a canine cDNA clone obtained by screening an expression library with a monoclonal antibody directed against the SRβ protein (see Materials and Methods). The cDNA predicted a protein of the correct molecular weight (~30-kD) that contained all three of the SRβ peptide sequences , but had no amino-terminal methionine. To obtain a full length clone, the canine cDNA was used as a hybridization probe to isolate the full length murine cDNA clone from a teratocarcinoma λ-Zap library. The deduced amino acid sequences of both the canine and the murine SRβ protein are shown in Fig. 3A with the canine peptide sequences underlined. The two proteins are highly homologous except at the

Figure 3. Primary structure of SRβ

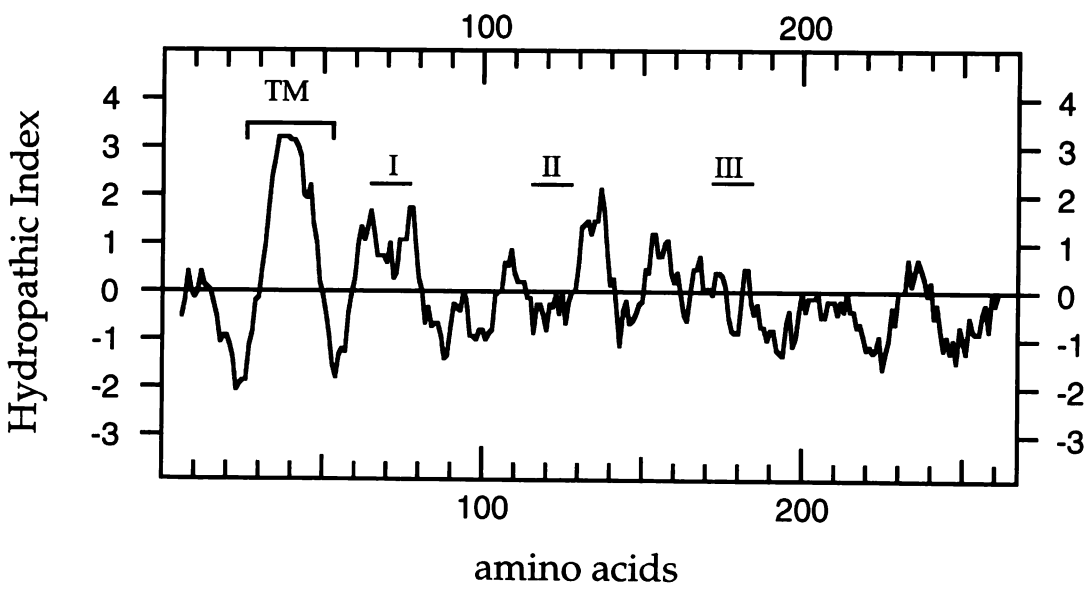
A) Amino acid sequence deduced from cDNA clone of SRβ. Full length sequence of the murine protein is given on the lower line. An incomplete clone of the canine gene that is truncated at the amino-terminus was also obtained. Differences from the murine protein are indicated on the upper line: - indicates no change from the murine protein; capital letters in the canine sequence indicate conservative amino acid changes; lower case letters in the canine sequence indicate non conservative amino acid changes; ◆ indicates an amino acid that is deleted in the murine protein with respect to the canine protein. Peptide sequences obtained from direct protein sequencing of canine SRβ are underlined. The predicted transmembrane domain is boxed and the tripartite GTP binding consensus sequence is labeled I, II, and III.

B) Hydropathy plot of SRβ. Hydrophobic sequences as predicted by Kyte and Doolittle (Kyte and Doolittle, 1982) fall above the line and hydrophilic sequences below the line. The predicted transmembrane domain is indicated (TM) as is the position of the tripartite GTP binding consensus sequence (I, II and III).

A

```
Dog SR β          p - M - - - G G V - - - - - - - - - - - - - - - -
Mouse SR β  M A S A N T R R V G D G ◆ ◆ A G G A F Q P Y L D S L R Q E L

- - - - - - - - - - - - - V - - V - - - - - - - - - - - - r - -
Q Q R D P T L L S V A V A L L A V L L T L V F W K F I W S R
                          I
R - - - - - - - - L - - - - N - - - - - - - - - - - - - - L - -
K S S Q R A V L F V G L C D S G K T L L F V R L L T G Q Y R
                                                      II
- - - - - - - - - - - - M - R - - - t - A t - - - - - - - - - -
D T Q T S I T D S S A I Y K V N N N R G N S L T L I D L P G

- - - - - L - F - E - - - A - - - - I - - - - - - - - - - -
H E S L R F Q L L D R F K S S A R A V V F V V D S A A F Q R

- - - - - - - - - - - - - - - - - - - - S - - - T - - F - - - - -
E Y K D V A E F L Y Q V L I D S M A L K N S P S L L I A C N
III
- - - - T - - - - - - - - - - - - - - - - - - - - - - - - - - - -
K Q D I A M A K S A K L I Q Q Q L E K E L N T L R V T R S A

- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
A P S T L D S S S T A P A Q L G K K G K E F E F S Q L P L K

- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Y E F L E C S A K G G R G D T G S A D I Q D L E K W L A K I
```

B



amino-terminus where there is significant divergence. A distinctive feature of this protein is that it contains a nineteen amino acid, putative transmembrane domain. According to Kyte and Doolittle hydropathy predictions (Kyte and Doolittle, 1982), a nineteen amino acid segment constitutes a membrane-spanning sequence if the average hydropathy is greater than +1.6. The average hydropathy of the SRβ segment is +2.7, falling easily into the transmembrane category. A Kyte and Doolittle hydropathy plot is shown in Fig. 3B with the predicted transmembrane region bracketed (TM). Thus, consistent with the extraction data shown in Fig. 2, SRβ appears to be a *bone fide* integral membrane protein. As shown in Fig. 3A, the transmembrane domain is flanked by a short (~30 amino acid) amino-terminal region with the majority of the protein being on the carboxy-terminal side. Trypsin digestion generates fragments of SRβ in the 20-kD range (Fig. 1A, lanes 10 and 13). These fragments have lost pieces much longer than 30 amino acids. Assuming that the membrane protects the luminal part of the protein from proteolysis, this result suggests that the larger, carboxy-terminal portion of SRβ resides on the cytoplasmic face of the ER where it is accessible to proteases, while the smaller, amino-terminal region is in the lumen of the ER.

The most striking feature of the cytoplasmic portion of SRβ is the presence of a consensus sequence for GTP binding (Dever et al., 1987; Bourne, et al., 1991) (elements marked I, II and III in Fig. 3A). This tripartite sequence is shared by a large number of GTP binding proteins as exemplified by p21-*ras* (Bourne, et al., 1991). Besides this consensus sequence, SRβ shares no homology to any other proteins in the data base. SRβ conforms very closely to *ras* not only in the consensus elements, but also with respect to the spacing between elements I, II and III. Thus, we expect that this domain will adopt the conserved structure which is known for *ras* (Pai et al., 1989; Pai et al., 1990) and another protein with

the tripartite motif, EF-Tu (Jurnak, 1985). SRβ is the first protein of this type found to contain a transmembrane domain, which may have important consequences for its function in the translocation process.

SRα, SRβ and SRP54 bind specifically to GTP

Given that SRα, SRβ and SRP54 all contain a GTP binding motif, there appears to be a cascade of at least three GTP binding proteins that function in initiation of protein translocation. To begin to address the ramifications of this finding we needed to be able to monitor GTP binding to each of these proteins independently. All of these proteins, however, exist in complexes with other proteins: SRα with SRβ, and SRP54 with the other SRP proteins. Standard GTP binding assays have insufficient resolution to allow the unambiguous quantification of GTP binding to an individual protein. Therefore, UV crosslinking was used to create a covalent nucleotide-protein crosslink and the reaction products were analyzed by SDS-PAGE and autoradiography, thus allowing the visualization of GTP binding to multiple proteins simultaneously.

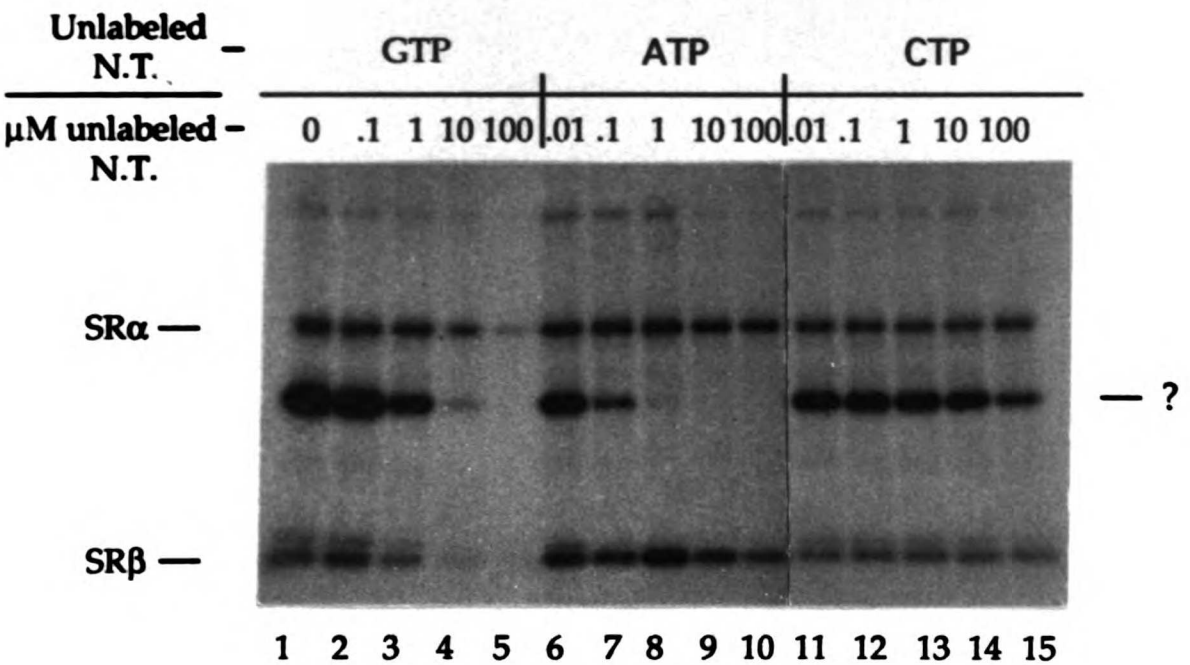
The results of crosslinking the purified SRα/SRβ complex to α-^{32}P-GTP is shown in Fig. 4A. Both SRα and SRβ were labeled with α-^{32}P-GTP as was an unidentified contaminant band (lane 1). The identity of the SRα and SRβ bands was confirmed by immunoprecipitation (not shown). Reactions using bovine serum albumin and lysozyme as negative controls showed no labeling of these proteins (not shown). Thus, the crosslinking reaction is specific for GTP binding proteins. To show that SRα and SRβ bind specifically to GTP, we titrated increasing amounts of unlabeled nucleotide into the reaction (lanes 2-15). The unlabeled GTP was able to compete away the labeling of SRα and SRβ (lanes 1-5), while neither unlabeled ATP (lanes 6-10) nor unlabeled CTP (lanes 11-15) had

Figure 4. SRα and SRβ bind specifically to GTP

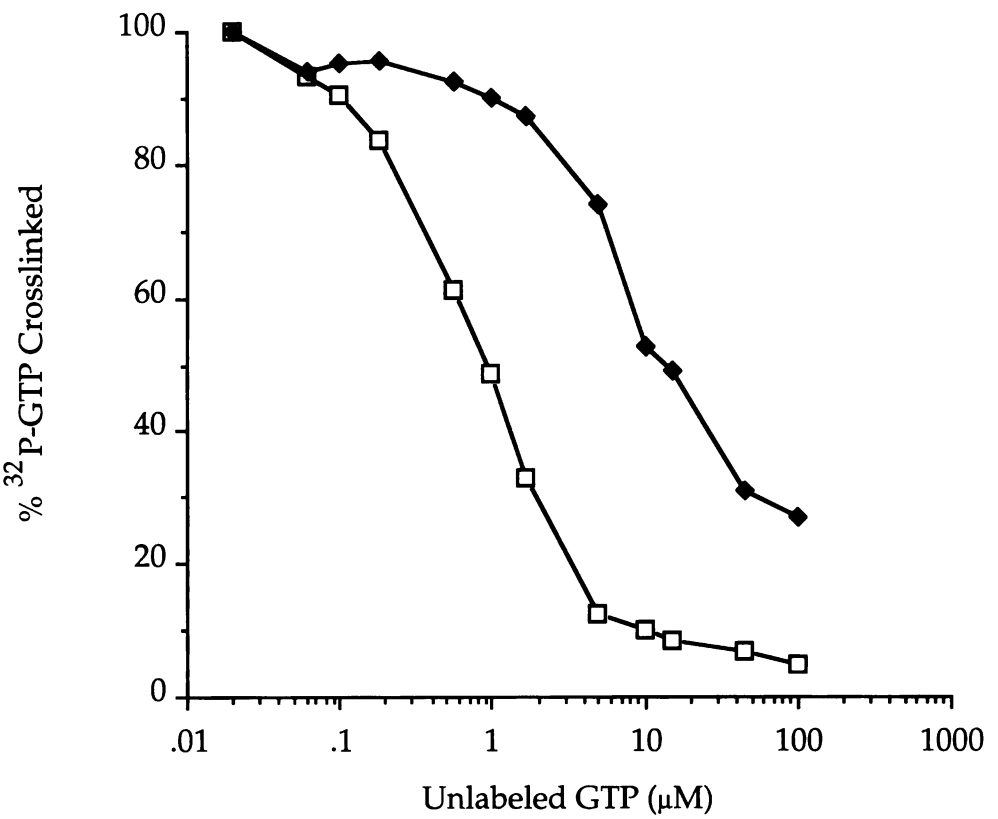
A) GTP crosslinking assay. Lane 1, purified SR was incubated with α-^{32}P-GTP and then UV irradiated to crosslink bound GTP to protein. In lanes 4-5, unlabeled GTP was included in the incubation at the indicated concentration. In lanes 6-10, unlabeled ATP was included in the incubation at the indicated 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.

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.

A


Unlabeled N.T.
GTP
ATP
CTP
μM unlabeled N.T.
0 .1 1 10 100 .01 .1 1 10 100 .01 .1 1 10 100
SRα
SRβ
— ?
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B


100
80
60
40
20
0
% 32P-GTP Crosslinked
.01
.1
1
10
100
1000
Unlabeled GTP (μM)

this effect. This was in contrast to the contaminant band which was more readily competed for by ATP than GTP suggesting that it is, in fact, an ATP binding protein. Thus, both SRα and SRβ bind specifically to GTP. This binding data is quantified in Fig. 4B. The amount of unlabeled nucleotide required to compete out 50% of the labeling of a given protein (EC_{50}) is related to the apparent affinity of the protein for that nucleotide. The EC_{50} for SRα is ~10 μM while for SRβ it is ~1 μM.

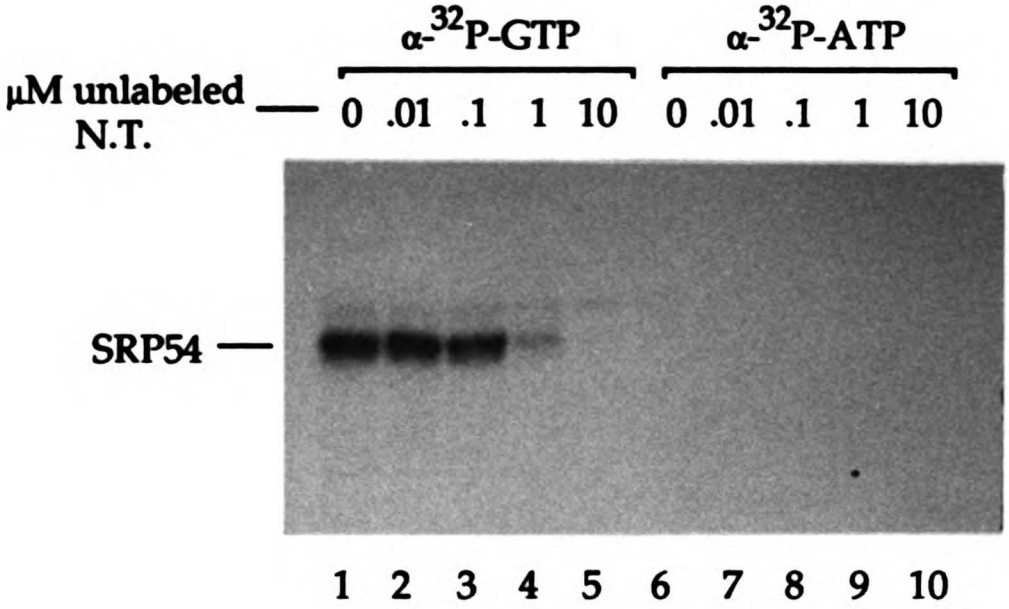
GTP binding to SRP was also examined using this method. When SRP was incubated with α-^{32}P-GTP and then irradiated, of the six SRP subunits, only SRP54 was labeled (Fig. 5A, lane 1). So, again, the crosslinking was specific for proteins with a GTP binding motif. Crosslinking to SRP54 was specific for GTP, because when α-^{32}P-ATP was substituted for the α-^{32}P-GTP, no labeling was detected (Fig. 5A, lane 6). As for SRα and SRβ, the labeling of SRP54 with α-^{32}P-GTP could be competed for by increasing concentrations of unlabeled GTP (Fig. 5A, lanes 2-5); the EC_{50} was ~0.5 μM (Fig. 5B).

Figure 5. SRP54 binds specifically to GTP

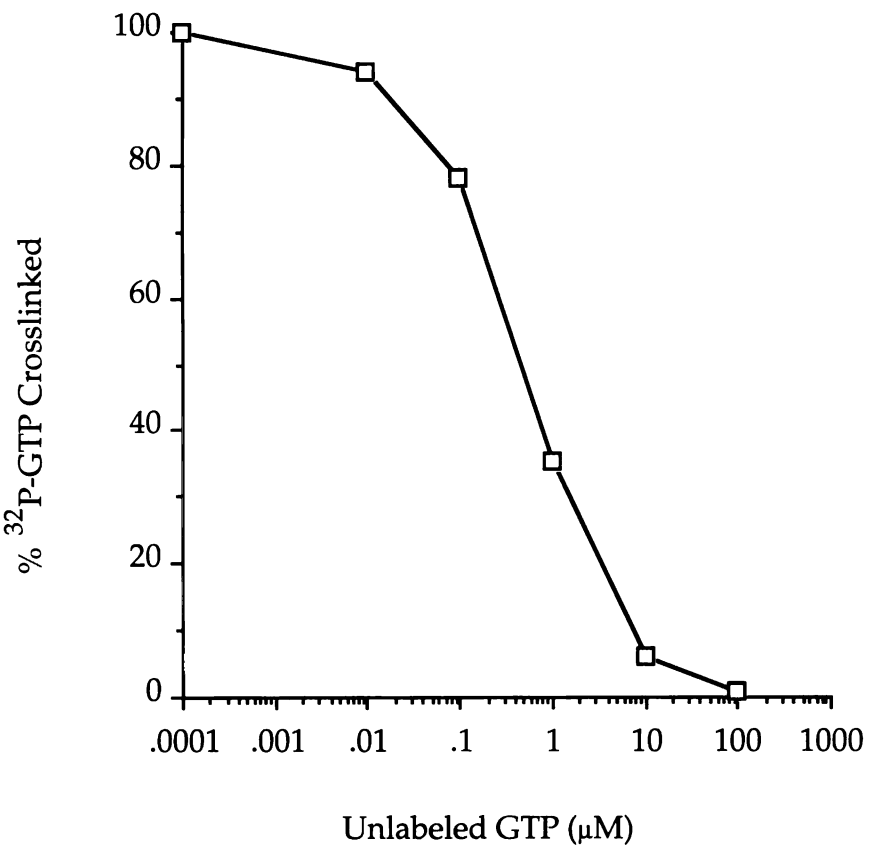
A) Nucleotide crosslinking assay. SRP was incubated with either α-^{32}P labeled GTP (lanes 1-5) or ATP (lanes 6-10) and then UV irradiated to crosslink bound nucleotide to protein. Unlabeled competitor GTP (lanes 4-5) or ATP (lanes 7-10) was added to the concentration indicated.

B) Quantitation of GTP crosslinking to SRP54. The amount of labeling of SRP 54 (□) at a given concentration of unlabeled competitor GTP was determined by densitometry and plotted against the log of the concentration of the competitor GTP.

A


α-32P-GTP
α-32P-ATP
μM unlabeled N.T.
0 .01 .1 1 10 0 .01 .1 1 10
SRP54
1 2 3 4 5 6 7 8 9 10

B


100
80
60
40
20
0
% 32P-GTP Crosslinked
.0001
.001
.01
.1
1
10
100
1000
Unlabeled GTP (μM)

Discussion

SR has a number of functions thought to be important for translocation. The relative contribution of SRα and SRβ to these functions has been difficult to address because the two subunits have not been purified away from one another under nondenaturing conditions. Here we demonstrate that SRα is required for SRP binding and that SRβ may mediate the association of SR with the ER membrane. The proteolysis approach used here is limited in its utility because it only inactivates SRα. The isolation of the cDNA clone for SRβ, along with the previously cloned cDNA for SRα (Lauffer, et al., 1985), should make it possible to express the two subunits independently of one another. By assaying the two components separately and in conjunction, the requirement for each subunit in receptor function could be directly assessed.

The proteolysis technique has been used previously to demonstrate a requirement for SRα in protein translocation (Gilmore, et al., 1982a). Whether SRβ is also required for translocation is unknown as of yet. This work suggests that, at the very least, SRβ is required to anchor SRα to the membrane. The fact that SRβ binds GTP, however, makes it likely that its role is greater than that of a simple anchor. SRβ is an unusual GTP binding protein in that it contains a membrane-spanning region. Although one other transmembrane protein, GP85, has been demonstrated to bind GTP (Lokeshwar and Bourguignon, 1992), this is a radically different type of GTP binding protein that does not contain the tripartite consensus sequence that is common to the GTPase superfamily typified by *ras* (Bourne, et al., 1991). A transmembrane GTP binding protein could be a key regulator of the events that govern the assembly of other transmembrane proteins to form the translocation apparatus. If SRβ has such a function, it could

provide the link between the targeting machinery and the more downstream components of the translocon.

The existence of this chain of GTP binding components anchored to the membrane by SRβ represents an interaction of unprecedented complexity in the GTPase field. By developing a binding assay, we have laid the groundwork for an examination of the effect of GTP on the interaction between these components and on their mode of action during translocation. This work is the subject of the next chapter.

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Chapter 3

A novel GTP cycle during initiation of protein translocation

Introduction

Targeting of ribosomes synthesizing secretory and membrane proteins from the cytosol to the endoplasmic reticulum (ER) membrane is a GTP-dependent process (Connolly and Gilmore, 1986; Nunnari and Walter, 1992). It is mediated by the signal recognition particle (SRP), a ribonucleoprotein consisting of one RNA (SRP RNA) and six protein subunits. The 54 kDa subunit of SRP (SRP54) binds to the signal sequence of the nascent polypeptide chain as it emerges from the ribosome (Kurzchalia et al., 1986; Krieg et al., 1986). SRP54 contains two distinct domains: an N-terminal domain that contains a putative GTP binding site and a C-terminal domain that binds signal sequences and SRP RNA (Bernstein et al., 1989; Römisch et al., 1989; Zopf et al., 1990; Römisch et al., 1990; High and Dobberstein, 1991). Binding of the signal sequence to SRP creates a targeting complex that contains the nascent polypeptide chain, the translating ribosome, and SRP. This complex is directed to the ER membrane via the interaction of SRP with the membrane-bound SRP receptor (SR). SR is composed of two subunits, SRα and SRβ, each of which also contains a GTP binding domain (Miller & Walter, unpublished; Tajima et al., 1986; Connolly and Gilmore, 1989). In the presence of GTP, SR binding to SRP causes dissociation of SRP from both the signal sequence and the ribosome (Gilmore and Blobel, 1983; Connolly and Gilmore, 1989). GTP hydrolysis is then required for SRP to be released from the SR and returned to the cytosol: if GTP is replaced by the non-hydrolyzable GTP analog guanylyl-imidodiphosphate (GMP-PNP), SRP and SR form a stable complex (Connolly et al., 1991). Thus, the three predicted GTP binding proteins in SRP and SR mediate the initiation of protein translocation and organize formation of the junction between the ribosome and the membrane components

comprising the protein translocation apparatus or translocon. Here we describe experiments that elucidate the function of GTP binding to SRP54 in this process.

GTP hydrolysis results from interaction of SR with SRP54 bound to SRP RNA

To begin to decipher the role of GTP in protein targeting to the ER, we pursued an observation made by Connolly and Gilmore (unpublished) that GTP hydrolysis results when SRP and SR interact. We found that purified SRP showed no detectable GTPase activity and that purified SR hydrolyzed GTP only poorly (Fig. 1, reactions 1 and 2). In contrast, when SRP and SR were combined, the rate of GTP hydrolysis increased approximately ten-fold over that catalyzed by SR alone (Fig. 1, reaction 3). These results suggest that an SRP-SR complex forms (Tajima, et al., 1986; Gilmore et al., 1982) that catalyzes GTP hydrolysis at the increased rate.

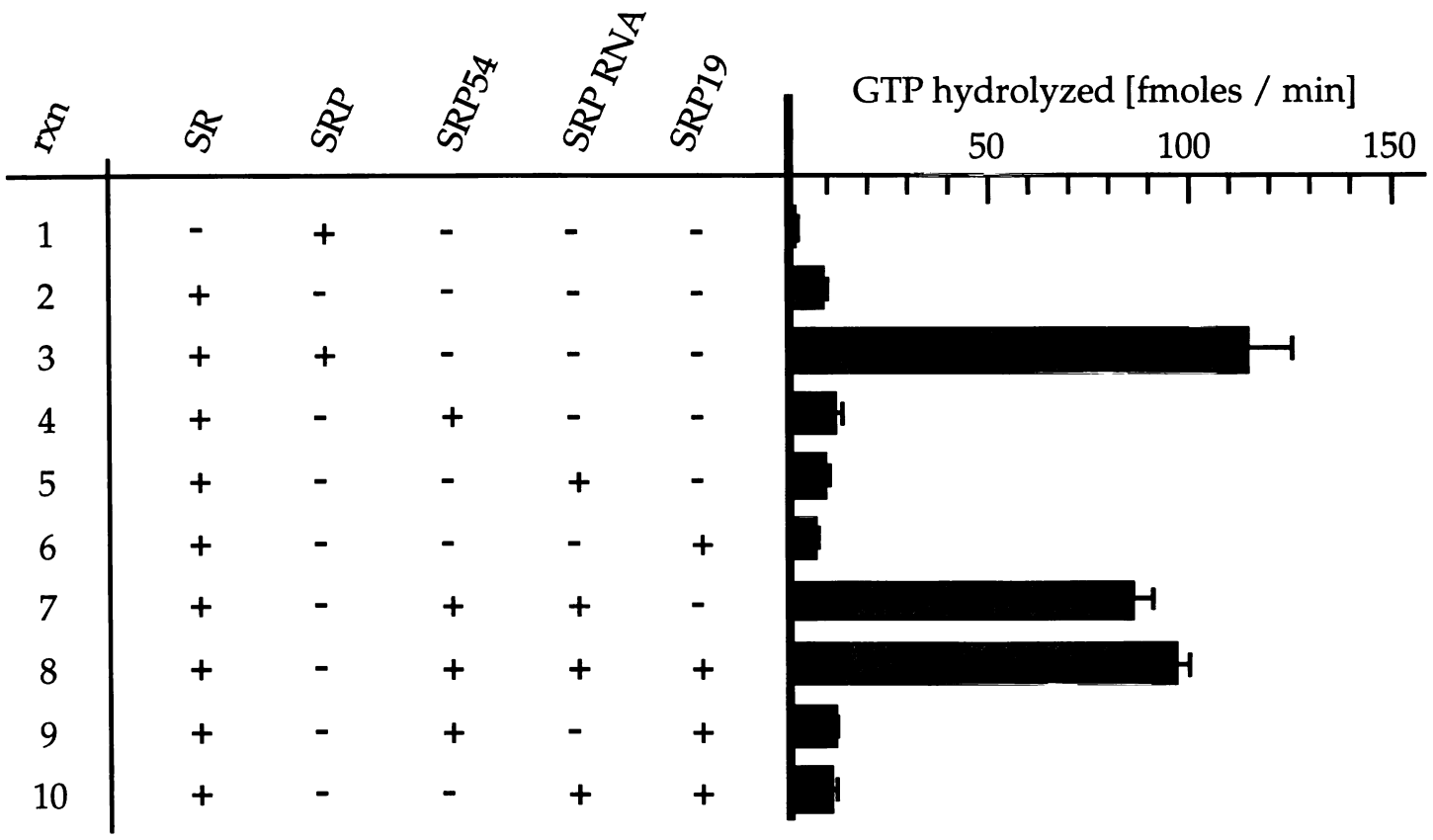
To determine which of the SRP subunits mediate the interaction with SR and elicit GTP hydrolysis, SRP was dissociated into its protein and RNA subunits under non-denaturing conditions (Walter and Blobel, 1983a). The dissociated SRP proteins can be fractionated, purified and reconstituted with SRP RNA to regenerate fully functional SRP.

Partially reconstituted SRP complexes were used to determine the contribution of individual SRP subunits to the SR-dependent GTP hydrolysis. Surprisingly, a partial SRP, comprised of SRP19, SRP54 and SRP RNA, was almost as active as native SRP in the presence of SR (Fig. 1, compare reactions 8 and 3), but was inactive in the absence of SR (not shown). Moreover, SRP19, which is known to stabilize the binding of SRP54 to SRP RNA (Siegel and Walter, 1985), could be omitted with only a minimal loss of activity (Fig. 1, reaction 7). SRP RNA and SRP54, however, were both essential for GTP hydrolysis to occur (Fig. 1). We conclude that both SRP54 and SRP RNA are necessary and sufficient

Figure 1. Stimulated GTPase activity of SRP and partially reconstituted SRPs

GTP hydrolysis rates are the average of three independent experiments; the standard deviation of the measurements is indicated. The reaction was linear with time over the period analyzed. tRNA could not replace SRP RNA in this reaction. In the presence of the SR, all partially reconstituted SRPs which contained both the SRP RNA and SRP54 were about equally active, i.e. the additional presence of SRP68/72 and/or SRP9/14 had no effect on the reaction. In the absence of SR, purified SRP proteins, SRP RNA and all partially reconstituted SRPs were inactive.

Methods. SRP and SRP receptor were purified as described previously (Walter and Blobel, 1983b; Gilmore and Blobel, 1983) as were the individual SRP components (Siegel and Walter, 1985) . Partially reconstituted SRPs were formed by mixing the various components at a concentration of 500 nM each in 300 mM KOAc, 5 mM $Mg(OAc)_2$, 25 mM Hepes (pH 7.5), 0.01% Nikkol detergent, 1 mM DTT. After mixing, the reactions were incubated for ten minutes on ice, ten minutes at 37 °C and then stored on ice until use in the GTPase reaction. Twenty µl GTPase reactions contained 20 nM SR and/or either 20 nM SRP or 20 nM partially reconstituted SRPs in GTP hydrolysis buffer containing 50 mM KOAc, 50 mM triethanolamine (pH 7.5), 2.5 mM $Mg(OAc)_2$, 0.5% Nikkol detergent (octaethyleneglycol mono n-dodecyl ether, Nikko Chemical Corp., Tokyo), 1 mM dithiothreitol. GTP was at 1 µM including 0.5 mCi/ml of γ-^{32}P-GTP (ICN). Reactions were incubated at 25 °C for 20 min and assayed by charcoal adsorption followed by Cerenkov counting.


rxn
SR
SRP
SRP54
SRP RNA
SRP19
GTP hydrolyzed [fmoles / min]
50
100
150
1 - + - - -
2 + - - - -
3 + + - - -
4 + - + - -
5 + - - + -
6 + - - - +
7 + - + + -
8 + - + + +
9 + - + - +
10 + - - + +

to interact functionally with the SR, and that this interaction elicits the increased rate of GTP hydrolysis. All subsequent analyses were carried out with this "minimal SRP" [SRP(54/RNA)] lacking five of the six SRP protein subunits.

The SRP Receptor Stimulates GTP Binding to SRP54

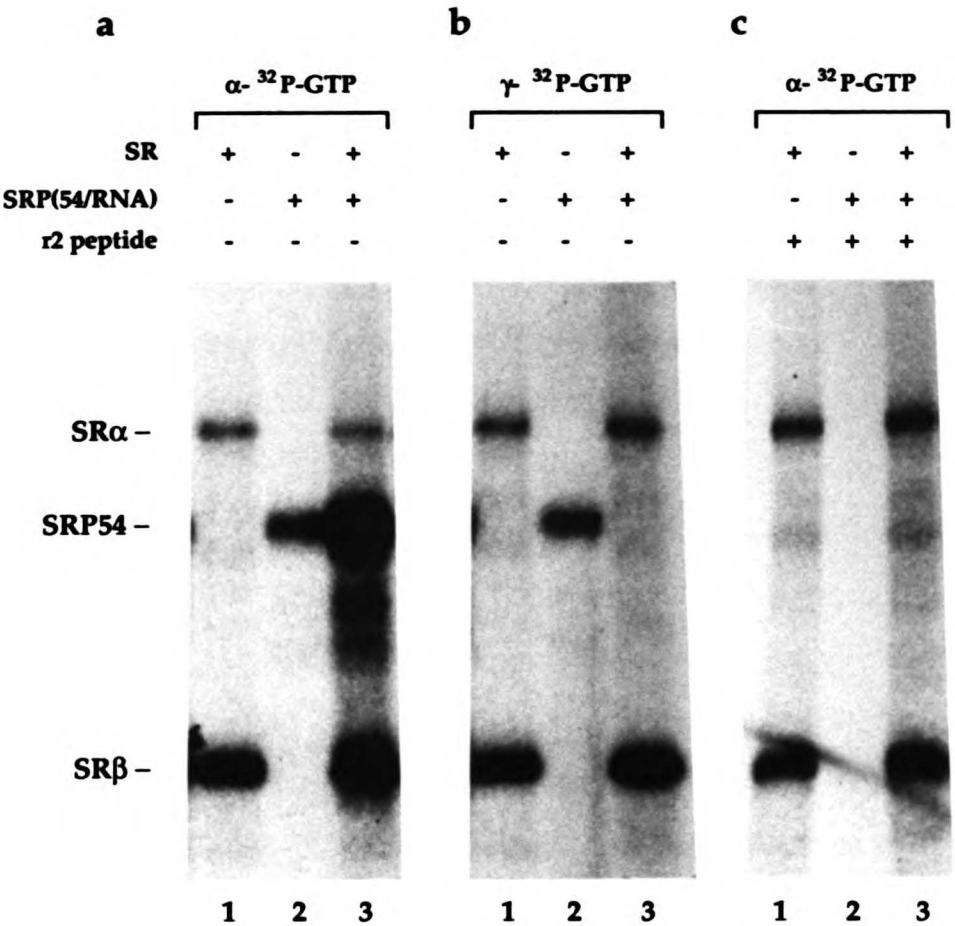
To determine which of the three GTP binding proteins, SRP54, SRα or SRβ, catalyzes the observed GTP hydrolysis, we used a UV crosslinking assay (Nath et al., 1985; Pashev et al., 1991) to monitor nucleotide binding to the proteins. The crosslinking approach allowed us to detect relatively low affinity GTP binding to these proteins, which have K_D's in the micromolar range (see Chapter 2), and made it possible to observe GTP binding to specific proteins in the presence of other GTP binding proteins.

To measure GTP binding by UV crosslinking, reactions were incubated with radiolabeled GTP, placed on ice and irradiated (Fig. 2). Proteins covalently crosslinked to radiolabeled GTP were separated by SDS-PAGE and then visualized by autoradiography. When SR was incubated with α-^{32}P-GTP and UV crosslinked, both SRα and SRβ were labeled (Fig. 2a, lane 1). Similarly, when SRP(54/RNA) was used, SRP54 was labeled (Fig. 2a, lane 2). In reactions containing the entire SRP complex, only SRP54 was labeled, while the remaining five SRP protein subunits which lack a GTP binding consensus sequence were not (not shown). This indicates that the labeling reaction was specific for GTP binding proteins. Thus, we conclude that SRP54, SRα and SRβ are indeed GTP binding proteins as predicted from their amino acid sequences, and that the UV crosslinking assay can be used to monitor nucleotide binding to these proteins.

Interestingly, GTP crosslinking to SRP54 was dramatically stimulated when SRP(54/RNA) and SR were mixed (Fig. 2a, lane 3) to elicit GTP hydrolysis (Fig. 1, reaction 7) . In contrast, there was no significant change in crosslinking to

Figure 2. GTP crosslinking during GTP hydrolysis

a SR (lane 1), SRP(54/RNA) (lane 2) or both complexes combined (lane 3) were UV crosslinked to α-^{32}P-GTP. **b** Reactions were identical to those in panel a except that γ-^{32}P-labeled GTP was used. **c** Reactions were identical to those in panel a except that the r2 signal peptide was added. UV crosslinking of labeled GTP to SRP54, SRα and SRβ was saturable and specific for GTP, as it could be inhibited by an excess of unlabeled GTP, but not ATP or CTP. **Methods.** Twenty μl reactions containing 20 nM SRP(54/RNA) and/or SR were incubated for 20 min in GTP hydrolysis buffer at 25 ^{o}C. The GTP concentration was 0.3 μM including 0.5 mCi/ml of α-^{32}P-GTP (Amersham) (panels a and b) or 0.5 mCi/ml of γ-^{32}P-GTP (panel c). The r2 signal peptide was added at 4 μM to the reactions in panel c. After 20 minutes, the reactions were pipeted into plastic weigh boats and UV irradiated at 6,000 W/cm^2 [using eight Model G15T8 15W Germicidal UV lamps (General Electric) at 6 cm distance from the sample] for 5 min on ice to covalently crosslink the bound radiolabeled nucleotide to the protein (Nath, et al., 1985). The reactions were then precipitated with TCA to remove uncrosslinked label and analyzed by SDS-PAGE followed by autoradiography.


a
b
c
α- 32P-GTP
γ- 32P-GTP
α- 32P-GTP
SR
SRP(54/RNA)
r2 peptide
SRα –
SRP54 –
SRβ –
1 2 3
1 2 3
1 2 3

either SRα or SRβ (Fig. 2a, lane 3). When SRP RNA was omitted, SRP54 crosslinked to GTP to the same extent as SRP(54/RNA), but the SRP RNA was required for stimulation of crosslinking by SR (not shown). Thus, stimulation of crosslinking of GTP to SRP54 and stimulated GTP hydrolysis (Fig. 1, reaction 4), are each dependent on a functional interaction between SRP54 and SR and both require SRP RNA.

Most GTP binding proteins bind GDP tightly and are stimulated to bind GTP by specific guanine nucleotide releasing proteins (GNRPs). GNRPs decrease the affinity of GTP binding proteins for GDP, thereby creating an empty nucleotide binding site into which GTP can enter (Bourne et al., 1991). By analogy, SR might stimulate GTP binding to SRP54 by acting as a GNRP. Alternatively, the GTP binding site of SRP54 may be unoccupied, and SR may trigger a conformational change in SRP54 to enhance its affinity for GTP directly.

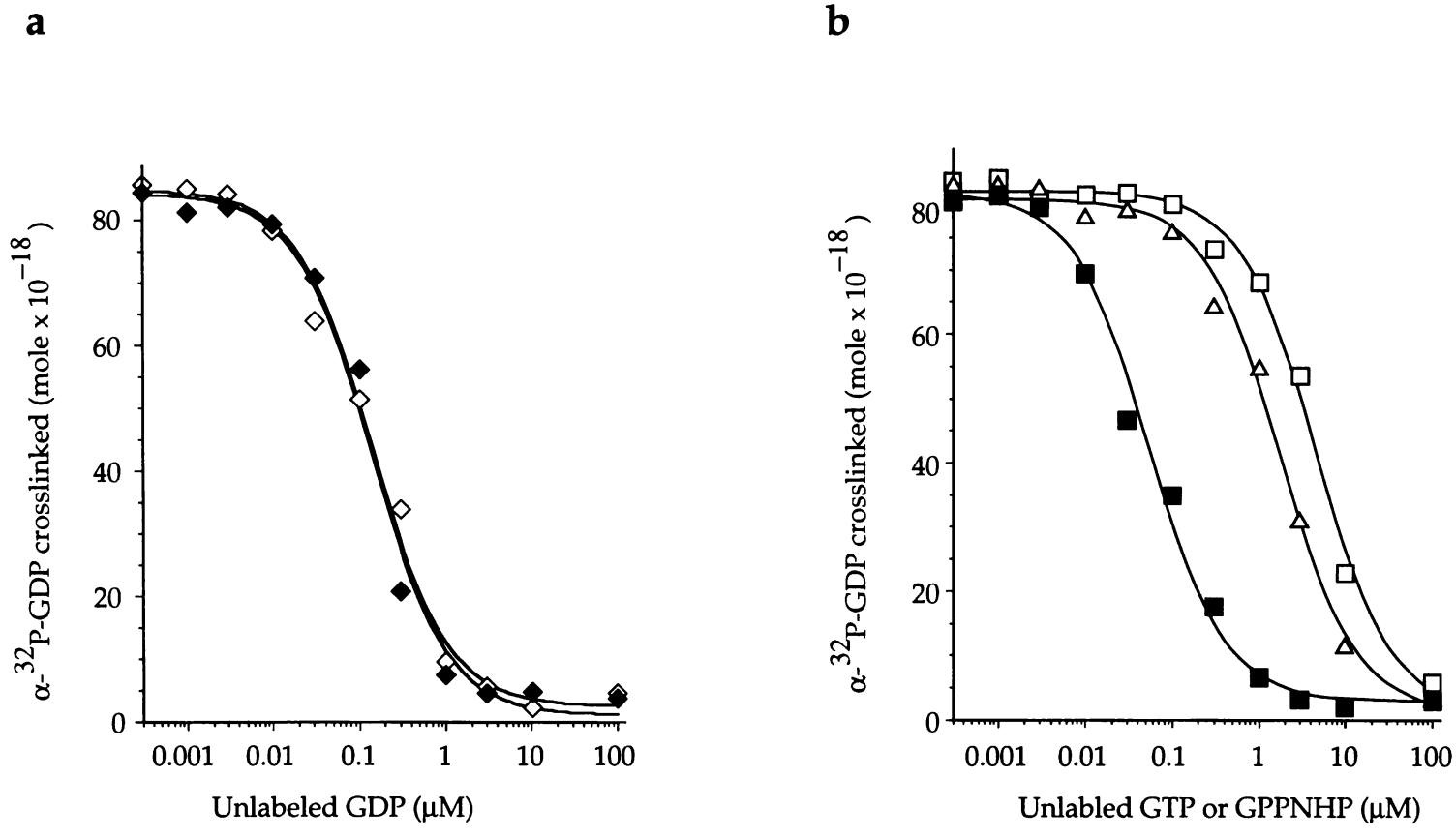
To distinguish between these two possibilities, we tested the effect of SR on the ability of SRP54 to bind GDP. To this end, SRP(54/RNA) was crosslinked to α-^{32}P-GDP in the presence of different concentrations of unlabeled competitor nucleotide. The concentration of nucleotide required to inhibit the crosslinking of SRP54 is a measure of its apparent affinity for the protein. As expected, crosslinking of SRP54 to α-^{32}P-GDP could be competed with unlabeled GDP (Fig. 3a, open diamonds). Surprisingly, the addition of SR to these reactions did not change the concentration of GDP required to inhibit the crosslinking (Fig. 3a, closed diamonds). Thus, we conclude that SR does not decrease the affinity of SRP54 for GDP.

When crosslinking of SRP(54/RNA) to α-^{32}P-GDP was competed with increasing concentrations of unlabeled GTP (Fig. 3b, open triangles), we found that approximately ten-fold more GTP than GDP was required for half-maximal inhibition (IC_{50}). This indicates that, in the absence of SR, the affinity of SRP54

Figure 3. SR increases the affinity of SRP54 for GTP

a Crosslinking of radiolabeled GDP is competed for by increasing amounts of unlabeled GDP in either the presence (◆) or absence (◇) of SR. The apparent K_i for GDP in the presence or absence of SR was 0.14 μM. **b** Crosslinking of radiolabeled GDP is competed for by increasing amounts of unlabeled GTP in the absence of SR (Δ) or GMP-PNP in the presence (■) or absence (□) of SR. The apparent K_i for GTP in the absence of SR was 1.7 μM. This is consistent with the apparent K_D of SRP54 for GTP (2 μM) as determined by crosslinking directly to labeled GTP. The apparent K_is for GMP-PNP in the absence and in the presence of SR were 4.5 μM and 0.05 μM, respectively. **Methods.** Twenty μl reactions contained 20 nM SRP(54/RNA) and/or SR in GTP hydrolysis buffer. All reactions also contained 0.1 μM GDP including 0.5 mCi/ml of α-^{32}P-GDP. Individual reactions were supplemented with unlabeled nucleotides to the concentrations indicated. Reactions were incubated for 4 h at 25 ^{o}C to reach equilibrium and then UV crosslinked. Under saturating conditions, the level of crosslinking of α-^{32}P-GDP to SRP54 was quantitatively identical to that of α-^{32}P-GTP (not shown), indicating that crosslinking efficiencies are invariant for different nucleotides. At saturation, $4x10^{-4}$ moles of nucleotide crosslinked to one mole SRP54. Products were analyzed as described in Figure 2. Quantitation of crosslinked product was

performed using a PhosphorImager (Molecular Dynamics). 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 (Segel, 1975)) using the program Kaleidagraph (Abelbeck Software, 1989) on a Macintosh II computer. B, amount of α-^{32}P-GDP crosslinked to SRP54; B_{max}, amount of α-^{32}P-GDP 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-GDP; [S], concentration of α-^{32}P-GDP.


a
b
α-32P-GDP crosslinked (mole x 10−18)
80
60
40
20
0
0.001
0.01
0.1
1
10
100
Unlabeled GDP (μM)
Unlabled GTP or GPPNHP (μM)

for GTP is approximately ten-fold lower than that for GDP. Because SR stimulates GTP hydrolysis, a similar competition study in the presence of SR would be difficult to interpret. To circumvent this difficulty, we used a non-hydrolyzable GTP analog, GMP-PNP for the experiment. Competition of α-^{32}P-GDP crosslinking with GMP-PNP in the absence of SR showed that the non-hydrolyzable analog binds to SRP54 with only a three-fold reduced affinity compared to that of GTP (Fig. 3b, open squares). This suggests that GMP-PNP binding to SRP54 closely mimics GTP binding. Interestingly, when SR was added to these reactions the IC_{50} for GMP-PNP decreased about 90-fold (Fig. 3b, closed squares), indicating that SR greatly increases the affinity of SRP54 for GTP. These results suggest that SR does not function like a GNRP to release GDP, but rather as a novel "guanine nucleotide loading protein" that promotes GTP binding to a presumably empty site by increasing the affinity of SRP54 for GTP.

The SRP Receptor Stimulates the Hydrolysis of GTP by SRP54

The finding that SR stimulates the binding of non-hydrolyzable GTP to SRP54 under conditions which lead to hydrolysis of GTP suggests that SRP54 is the GTPase that catalyzes nucleotide hydrolysis. If this were the case, then the labeled nucleotide crosslinked to SRP54 in Figure 2a, lane 3 might be hydrolyzed to GDP. To test this notion directly, the experiment shown in Figure 2a was repeated with GTP that is ^{32}P-labeled in the γ-position. γ-^{32}P-GTP would release the γ-phosphate upon hydrolysis resulting in the loss of the radiolabel from the crosslinked protein. SRα, SRβ and SRP54 were labeled with γ-^{32}P-GTP to the same extent as with α-^{32}P-GTP when reactions were carried out with either SR or SRP(54/RNA) alone (compare Fig. 2a and 2b, lanes 1 and 2), consistent with the observation that neither component alone hydrolyzes GTP significantly. In the GTP hydrolysis reaction containing both SR and the SRP(54/RNA), however, the

labeling of SRP54 was almost completely abolished (Fig. 2b, lane 3). In contrast, the labeling of SRα and SRβ remained unchanged. Because SRP54 was labeled when crosslinked after incubation with α-^{32}P-GTP under the same conditions (Fig. 2a, lane 3), we conclude that the crosslinked nucleotide must be GDP which has lost the γ-phosphate as the result of GTP hydrolysis. These results argue strongly that SRP54 hydrolyzes GTP upon interaction with SR. Thus, SR functions not only as a guanine nucleotide loading protein, but also as a GTPase activating protein.

Inhibition of the SRP Receptor-Dependent GTP Hydrolysis Reaction by Signal Peptides

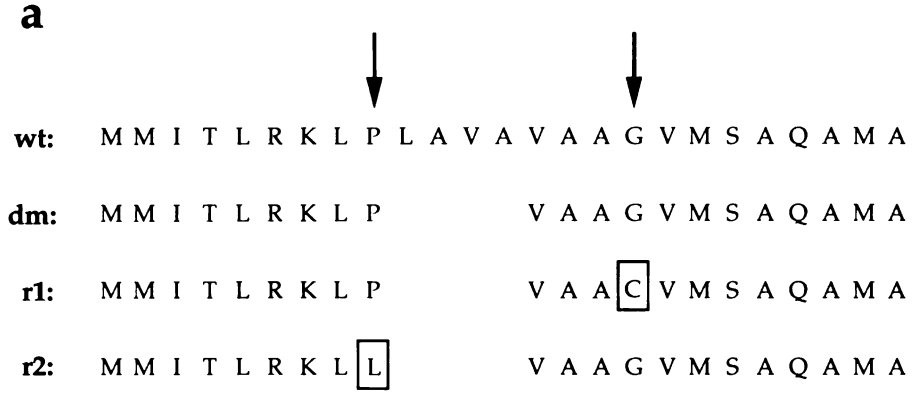
Because signal sequences are a physiological ligand of SRP54 during the targeting reaction, we tested the possibility that the binding of a signal sequence to SRP54 could influence the SR-dependent GTP hydrolysis reaction. We used a series of four closely related synthetic peptides (Fig. 4a) derived from the signal sequence of the bacterial outer membrane protein LamB. The first peptide corresponds to the wild type signal sequence (wt), the second peptide to a deletion mutant (dm) which renders the signal sequence inactive *in vivo*. The other two peptides (r1 and r2) have two different second site reversions which restore their signal sequence function *in vivo*. They differ from the deletion mutant only by single amino acid replacements (Emr and Silhavy, 1983; McKnight et al., 1989).

The three peptides corresponding to functional signal sequences (Fig. 4b, wt, open squares; r1, closed triangles and r2, open triangles) were all potent inhibitors of SR-dependent GTP hydrolysis by SRP54. Half-maximal inhibition was reached at about 2 μM for both the wild type and r1 peptide; the IC_{50} for the

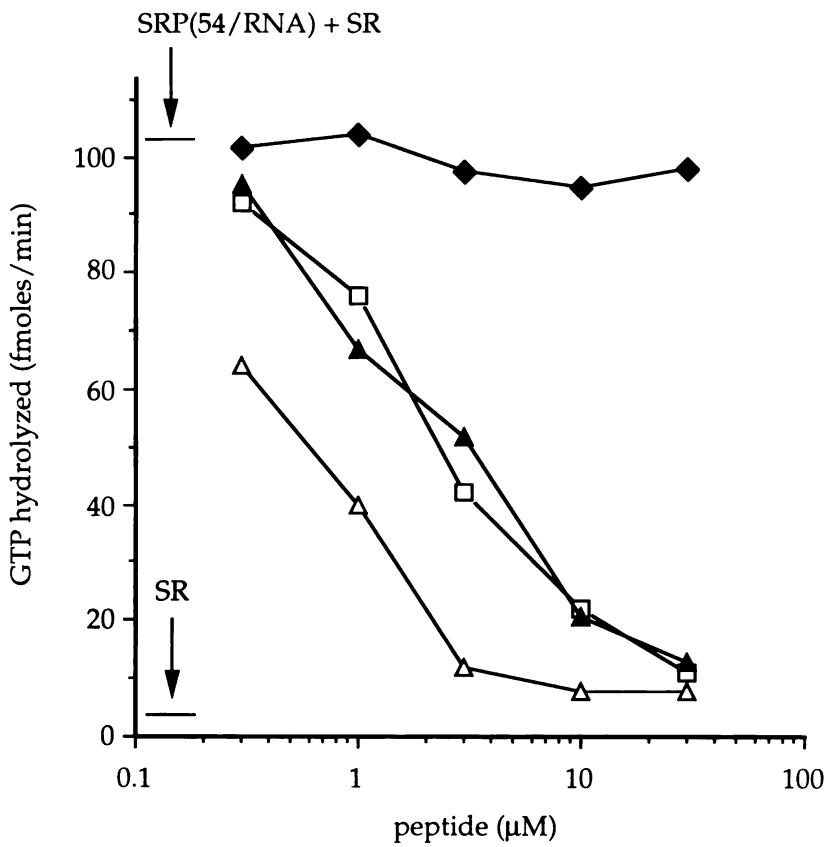
Figure 4. Functional signal peptides inhibit GTP hydrolysis

a Synthetic peptides used in the GTPase assay. The wt peptide corresponds to the signal sequence of the E. coli LamB protein. The LamB signal sequence functions efficiently in a mammalian *in vitro* translocation system (Watanabe et al., 1986), and the synthetic peptides used here inhibit in vitro protein translocation across the membrane of *E. coli* inverted vesicles (Chen et al., 1987). The synthetic wt peptide can readily adopt an α-helical conformation when analyzed by circular dichroism spectroscopy (McKnight, et al., 1989). The deletion mutant peptide (dm) removes four amino acids, thus bringing a proline and a glycine residue (arrows) closer together, such that these two residues function as helix breakers. The synthetic dm peptide does not form an α-helix (McKnight, et al., 1989), and the peptide does not function as a signal peptide in vivo (Emr and Silhavy, 1983). In the second site revertants r1 and r2 either the glycine or the proline residue are changed to a different amino acid, respectively. Both the r1 and r2 peptides regain the ability to form an α-helix and function as signal sequences in vivo (Emr and Silhavy, 1983; McKnight, et al., 1989). **b** GTP hydrolysis reactions (as in Figure 1) containing SRP(54/RNA) were supplemented with increasing concentrations of the synthetic wt (▲), dm (◆), r1 (□) or r2 (Δ) peptides. The level of GTP hydrolysis in the absence of signal peptide [SRP(54/RNA)+SR] and the basal level of hydrolysis by SR are indicated (SR). The peptides were added to the reactions from a 150 μM stock solution in water. The concentration of the stock solution was determined by amino acid analysis.

a


wt: M M I T L R K L P L A V A V A A G V M S A Q A M A
dm: M M I T L R K L P V A A G V M S A Q A M A
r1: M M I T L R K L P V A A C V M S A Q A M A
r2: M M I T L R K L L V A A G V M S A Q A M A

b


SRP(54/RNA) + SR
SR
GTP hydrolyzed (fmoles/min)
0
20
40
60
80
100
0.1
1
10
100
peptide (μM)

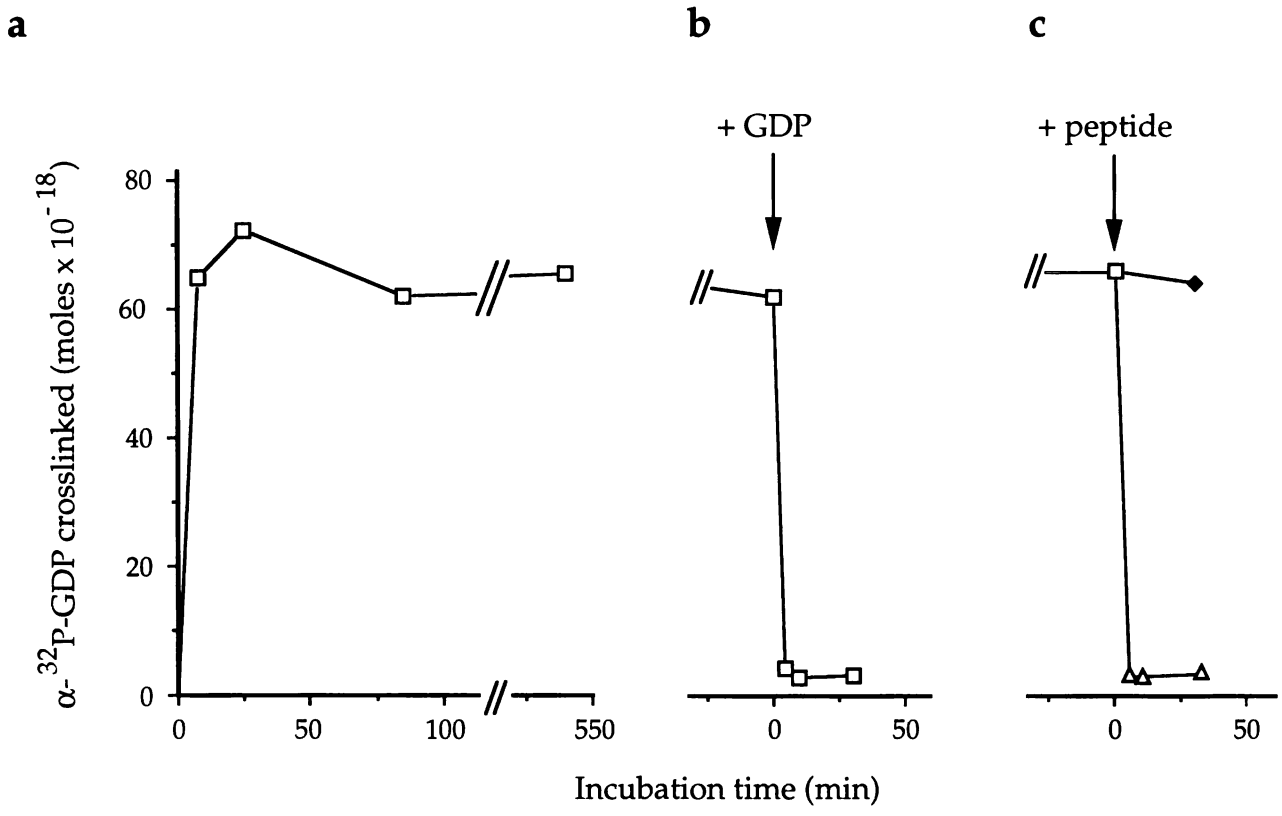
r2 peptide was about five-fold lower. In contrast, the dm control peptide (Fig. 4b, closed diamonds) did not affect the reaction even at 30 μM, the highest concentration tested.

The signal peptides could exert their inhibitory effects on GTP hydrolysis by either inhibiting GTP binding to SRP54 or by blocking hydrolysis. To distinguish between these two possibilities, a UV crosslinking experiment was performed in the presence of the peptides. The data in Figure 2c show that crosslinking of α-^{32}P-GTP to SRP54 was drastically reduced in the presence of a functional signal peptide, whether SRP(54/RNA) was incubated in the absence (compare Fig. 2a and 2c, lanes 2) or in the presence of SR (compare Fig. 2a and 2c, lanes 3). Note that the GTP crosslinking to SRα and SRβ was unaffected by the peptide (compare Fig. 2a, lanes 1 and 3 with Fig. 2c, lanes 1 and 3), indicating that the inhibition was not due to non-specific effects. As an additional control, we tested the dm peptide which, as expected, did not inhibit GTP crosslinking to SRP54, SRα or SRβ (not shown). We conclude that binding of a functional signal peptide to SRP54 prevents GTP binding and, as a consequence, inhibits GTP hydrolysis.

Two mechanisms might account for the observed signal peptide-mediated inhibition of GTP binding. SRP54 may co-purify with nucleotide and the signal peptide may stabilize the nucleotide-bound state, thereby preventing labeled GTP from entering the occupied binding site. Alternatively, the signal peptide may stabilize SRP54 in a nucleotide-free state that contains neither GTP nor GDP. To discriminate between these mechanisms, α-^{32}P-GDP was prebound to SRP54 (Fig. 5a), and then an excess of unlabeled GDP was added (Fig. 5b). Crosslinking of labeled GDP to SRP54 diminished completely even at the earliest time point after addition of unlabeled GDP, indicating that the radiolabeled GDP dissociated rapidly. Identical results were obtained when the r2 signal peptide

Figure 5. Time course of GDP crosslinking to the SRP(54/RNA) particle

a α-^{32}P-GDP was mixed with SRP(54/RNA) in GTP hydrolysis buffer. Aliquots were removed for UV crosslinking at the times indicated. The amount of GDP crosslinked to SRP54 was determined after SDS PAGE by quantitation with a PhosphorImager. **b** SRP(54/RNA) was incubated with GDP as in panel a. At time zero, unlabeled GDP is added to a final concentration of 10 μM. Aliquots were removed for UV crosslinking at the indicated times to monitor the dissociation of the prebound α-^{32}P-GDP. **c** SRP(54/RNA) was incubated with GDP as in panel a. At time zero, the r2 (Δ) or dm (◆) peptide were added to final concentrations of 4 μM. All reactions contained 20 nM SRP(54/RNA) in GTP hydrolysis buffer and 0.1 μM GDP including 0.5 mCi/ml α-^{32}P-GDP. UV crosslinking and analysis were performed as in Figure 3.


a
b
c
+ GDP
+ peptide
α-32P-GDP crosslinked (moles x 10-18)
80
60
40
20
0
0
50
100
550
0
50
0
50
Incubation time (min)

was added to SRP54 containing prebound α-^{32}P-GDP (Fig. 5c, open triangles), indicating that the signal peptide does not stabilize a GDP-bound state. Addition of the control dm peptide (Fig. 5c, closed diamonds) did not reduce GDP crosslinking, demonstrating that a functional signal sequence was required to exert this effect. Thus, we conclude that a functional signal sequence stabilizes an empty guanine nucleotide binding site in SRP54. While this is the most likely interpretation, we cannot formally exclude the possiblity that a signal sequence bound to SRP54 still allows nucleotide binding, but causes a drastic conformational change in the protein that completely disrupts the ability of a bound nucleotide to become UV crosslinked.

Model for GTP utilization by SRP54

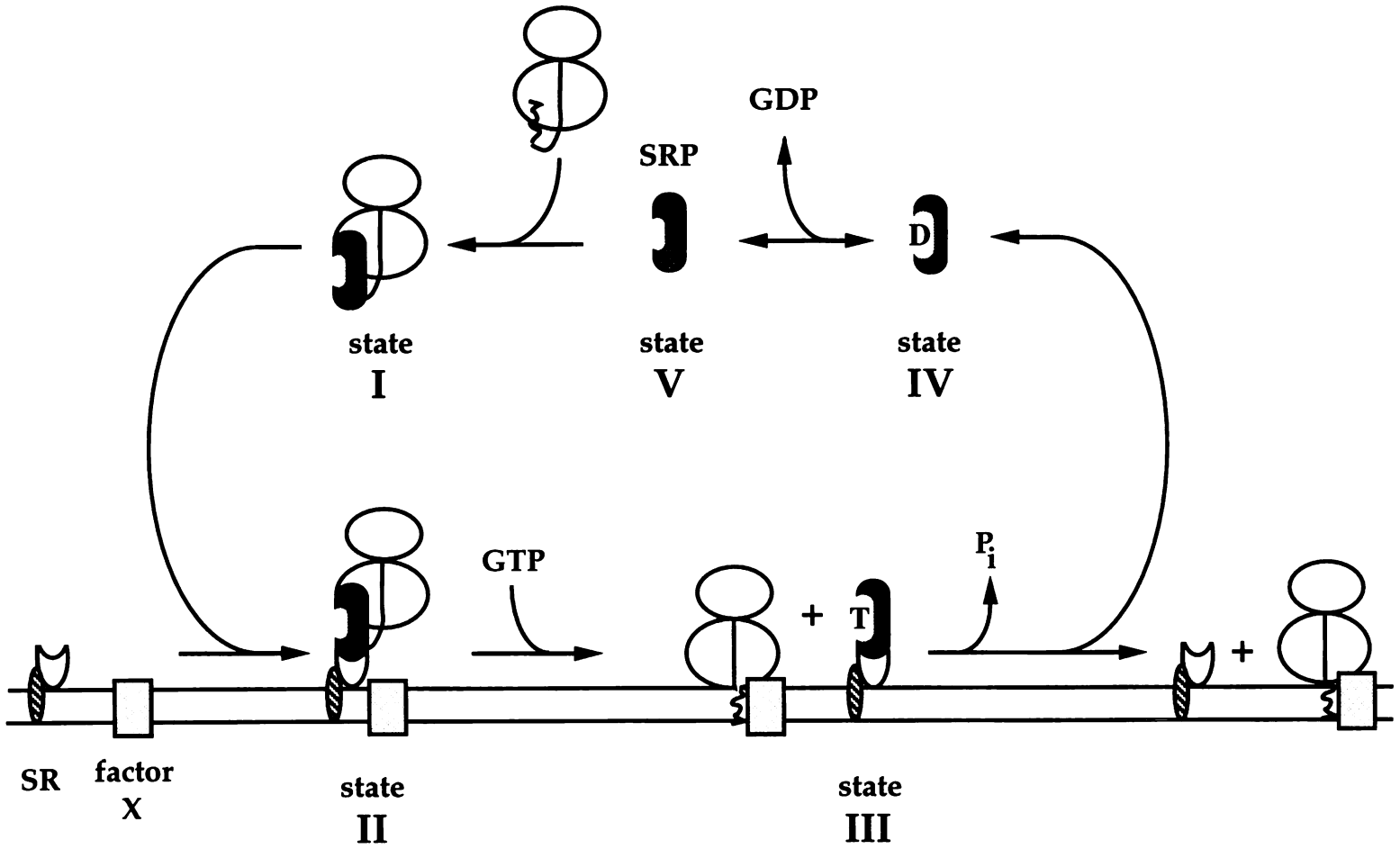
We have shown that the guanine nucleotide-bound state of SRP54 can be influenced by two ligands, the signal peptide and SR, indicating that the GTPase domain of SRP54 is used to integrate information received from both the nascent chain and the ER membrane. These data suggest a model (Fig. 6), in which the occupancy of the SRP54 guanine nucleotide binding site defines discrete steps in a cycle of GTP binding and hydrolysis that operates during protein targeting to the ER membrane.

Signal sequence and guanine nucleotide bind to structurally separate domains on SRP54 (Zopf, et al., 1990; Römisch, et al., 1990). Chemical modification of the GTP binding domain prevents signal sequence binding (Lütcke et al., 1992). Our data suggest that binding of a signal sequence to one domain stabilizes an empty nucleotide binding site in the other. Thus, taken together, these results indicate that the two domains can communicate in both directions and that the binding of a signal peptide and guanine nucleotide (GTP or GDP) to SRP54 may be mutually exclusive. Because SR stimulates GTP binding to SRP54, it is attractive to speculate that the targeting complex arrives at the ER membrane with SRP54 in a nucleotide free state (Fig. 6, I), and that an SR-catalyzed conformational change in SRP54 (Fig. 6, II) favors GTP binding and concomitantly leads to a reduction in the affinity for the bound signal sequence (Fig. 6, III).

An integral part of any model for targeting to the ER is that SR causes the release of the signal sequence from SRP. To this point, signal sequence release has only been studied in unfractionated translation systems and membrane extracts. Never before has it been examined in such a highly purified system as

Figure 6. Model depicting the role of GTP binding to and hydrolysis by SRP54

During the initiation of protein translocation, SRP54 is proposed to cycle between three forms with respect to bound nucleotide: a nucleotide-free or "empty" state, a GTP-liganded state (T), and GDP-liganded (D). The different states of SRP with respect to its multiple ligands are labeled with roman numerals (I through V). The putative factor X is indicated by the stippled box. The model is discussed in the text.


GDP
SRP
D
state
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state
IV
GTP
P_i
+ T
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SR
factor
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III

described here. That SR causes this release in less purified systems is well documented and lies at the very heart of SR function (Gilmore et al., 1982a; Gilmore and Blobel, 1983, Connolly and Gilmore, 1989). Our data indicate, however, that in the minimal system used here, SR alone is not sufficient to cause dissociation of the signal peptide from SRP. In the experiment shown in figure 2, GTP crosslinking to SRP54 is an indicator of whether a signal sequence is bound to SRP54 (compare Fig. 2a, lane 2 with Fig. 2c, lane 8) Note that the signal sequence inhibition of GTP binding to SRP54 (Fig. 2c, lane 8) is maintained in the presence of SR (Fig. 2 c, lane 9). If SR were to cause signal sequence release in this assay, GTP binding to SRP54 would have been restored. Therefore, something is missing from the purified system that,normally, allows for signal sequence release in other assays. Thus, an additional factor(s) (Fig. 6, depicted as "factor X") is required in conjunction with SR to stimulate GTP binding to SRP54 and to release the signal sequence. In principle, the proposed factor X could be a component of the targeting complex not included in the reconstituted assays, such as the ribosome, nascent chain, or another soluble protein. More interestingly, however, factor X could be an additional membrane component as depicted in Figure 6. This latter notion is appealing as this process could ensure that the signal sequence is not released from SRP unless essential components of the translocon are available and properly pre-assembled. Thus, the mechanism would introduce a molecular check point: unless proper translocation is ensured, the reaction is not allowed to proceed further.

Upon GTP binding to SRP54, SRP and SR leave the translocation site as a complex (Fig. 6, state III). We have demonstrated that SR activates the GTPase of SRP54 by stimulating both GTP binding (Fig. 2a), as well as hydrolysis (Fig. 2c). Moreover, since SRP and SR form a stable complex in the presence of a non-hydrolyzable GTP analog (Connolly, et al., 1991), we reason that the hydrolysis

of GTP by SRP54 is required for SRP to dissociate from SR and for SRP to return, liganded to GDP, to the cytosol (Fig. 6, state IV). The energy of GTP hydrolysis would therefore be used to provide unidirectionality to the SRP cycle. Given the rapid dissociation rate of GDP from SRP54 (Fig. 3a and 5b), nucleotide-bound SRP54 probably exists in equilibrium with empty SRP54 (Fig. 6, state IV→ state V), which can bind to a signal sequence to initiate another round of targeting (Fig. 6, state V→ state I).

According to our model, the function of SRP54 closely resembles that of other GTPases (e.g. the trimeric G-proteins or EF-Tu), in that interconversion between different nucleotide occupied states causes the GTPase to interact in temporal succession with its effectors (Bourne et al., 1990; Gilman, 1987). Thus, these GTPases function as molecular switches to monitor the accurate assembly of supramolecular complexes. Unlike the typical case where the triggering conversion is that between the GTP-bound state and the GDP-bound state, however, the crucial conformational switch in SRP54 is the interconversion between the empty state and the GTP-bound state.

Our data suggest that SRP54 is one of a limited number of GTP binding proteins, such as the Sec4 protein, that do not bind tightly to GDP (Sasaki et al., 1991). In the case of Sec4, a GDP dissociation inhibitor protein was discovered that stabilizes GDP binding. A similar, as yet unidentified, GDP dissociation inhibitor might also exist for SRP54 so as to allow GDP release only upon the interaction of SRP with the ribosome and/or signal sequence. The additional SRP subunits do not function in such a role since the nucleotide binding and hydrolysis properties that have been described here for SRP(54/RNA) are indistiguishable from those measured for native SRP (Fig. 1 and data not shown).

Although our model is consistent with all of the available data, it fails to provide a function for the additional GTPase domains in SRα and SRβ. It is likely

that additional check points in the assembly of a functional ribosome-membrane junction exist which are monitored by guanine nucleotide switches in SRα and SRβ. It has been shown that a functional GTP binding domain in SRα is required during the initiation of protein translocation and for the formation of a stable SRP-SR complex (Rapiejko and Gilmore, 1992). Thus, we surmise that, SRα also needs to be in a defined guanine nucleotide-bound state for these reaction steps to proceed and that progression of SRP54 through the cycle shown in Figure 6 (e.g. the SRα-catalyzed state II→ state III) is dependent on the progression of SRα through a similar a similar cycle. We can speculate further that, just as SRP recruits ribosomes with nascent chains from the cytosol to the translocation site, so may SR recruit essential translocon components from the plane of the membrane in to the complex, perhaps including the proposed factor X.

It is not yet known whether a functional GTPase domain in SRβ is also required for SR function, although the intimate association of the two SR subunits suggests that this is the case. If we were to extend our model, SRβ could perform a critical role in translocation by signaling the arrival of the signal sequence at the surface of the membrane. In such a way, SRβ could act to connect the targeting events with the actual translocation events in a temporal and, possibly, spatial manner. Thus, there may be a cascade involving three distinct, directly interacting GTPases that monitor the formation of the ribosome membrane junction during the initiation of protein translocation across the ER membrane.

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Chapter 4

The GTPase activity of the E. coli Ffh protein is regulated by the E. coli FtsY protein and by signal sequences

The ability to secrete proteins from the cytoplasm to the extracellular space is a conserved function of all cells from bacteria to higher eukaryotes. In general, secretory proteins are tagged with a signal sequence that targets them to a specialized membrane through which they are translocated. In E. coli, a number of distinct approaches have been taken to identify factors important for the translocation of signal sequence-bearing proteins, referred to as preproteins, across the inner membrane. Genetic manipulations have yielded several secretion, or sec, mutants that are impaired in either the targeting of preproteins to the membrane or in the translocation event itself (reviewed in Schatz and Beckwith, 1990). Other sec gene candidates have been identified based on their homology to components of the signal recognition particle (SRP) mediated targeting mechanism in mammalian cells (Poritz et al., 1988; Bernstein et al., 1989; Ogg et al., 1992).

SRP is a ribonucleoprotein that binds signal sequence containing nascent polypeptide chains in the cytoplasm and targets them to the endoplasmic reticulum (ER) (Nunnari and Walter, 1992). The *ffh* gene of E. coli encodes a protein (Ffh) that is highly homologous over its entire length to the 54-kD protein of SRP (SRP54) (Bernstein, et al., 1989). SRP54 can be proteolytically dissected into an amino-terminal domain which contains a GTPase consensus sequence (G-domain), and a carboxy-terminal, methionine rich, domain (M-domain) that both anchors the protein to the SRP RNA and also binds signal sequences (Zopf et al., 1990; Römisch et al., 1990; High and Dobberstein, 1991). The Ffh protein shares this structural organization, having both the GTPase consensus sequence and the methionine rich region (Hann et al., 1989) and yielding similar fragments on

proteolysis (Freymann and Walter, unpublished).

The product of the *ffs* gene of E. coli is the 4.5S RNA which shares conserved structural and sequence similarities with a domain of the 7S RNA of SRP (Poritz, et al., 1988). That 4.5S RNA is related to SRP RNA is indicated by the fact that they can substitute for each other in functional assays (Poritz et al., 1990; Ribes et al., 1990).

A third such gene, *ftsY*, codes for a protein (FtsY) that is homologous to the α-subunit of the SRP receptor (SRα) (Ogg, et al., 1992). The carboxy-terminal two-thirds of these proteins are over 50% similar and both contain a GTPase consensus sequence (Ogg, et al., 1992). The FtsY protein, however, is missing the first 130 amino acids of SRα and the rest of the amino-terminal third of the protein is much more divergent than the remainder of the molecule. Perhaps reflecting this truncation, the FtsY protein appears to be soluble (Bernstein, unpublished), as opposed to SRα which is a peripheral membrane protein of the ER (see Chapter 2).

Recently, genetic depletion of either Ffh or 4.5S RNA has been shown to create a secretion defect *in vivo* (Ribes, et al., 1990; Phillips and Silhavy, 1992). Therefore, Ffh and 4.5S RNA are similar to the sec proteins in that the loss of activity inhibits translocation *in vivo*. The role of FtsY has yet to be examined in this manner. The drawback of such genetic experiments is that they are unable to distinguish between a direct or indirect role of components in translocation. In the work described here, a biochemical approach is used to better understand how Ffh, 4.5S RNA and FtsY might function in translocation.

Ffh and 4.5S RNA have been shown biochemically to form an SRP like complex *in vivo* (Poritz, et al., 1990), and this complex can bind to signal sequences *in vitro* (Luirink et al., 1992). The mode of action of this complex,

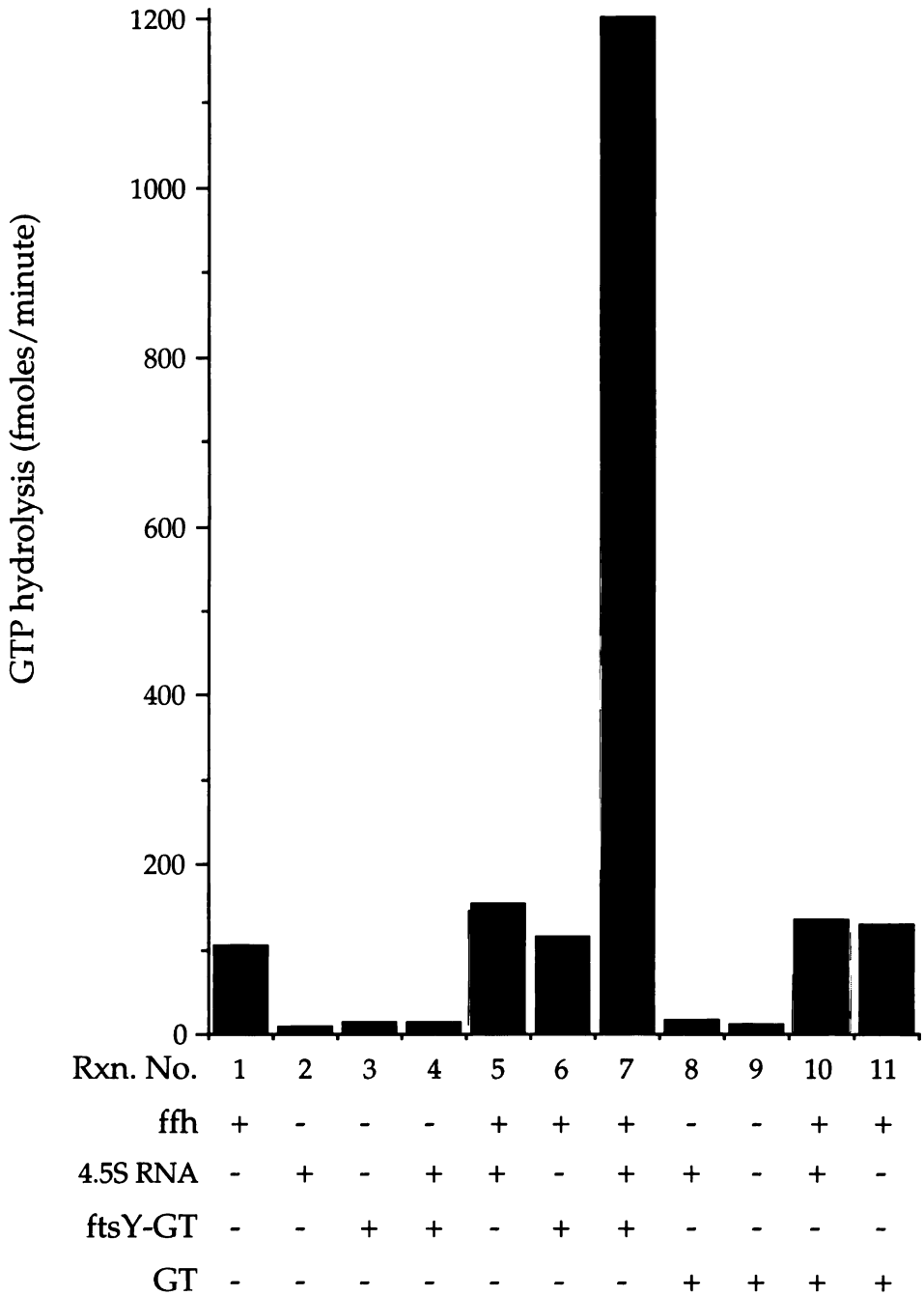
however, remains unknown. Even the ability of the complex to interact with its putative receptor FtsY has yet to be tested. The availability of purified Ffh, 4.5S and FtsY has made possible an *in vitro* analysis of the function of and interaction between these components. Since both Ffh and FtsY contain a GTPase consensus sequence, the first step in this analysis was to test them for the ability to hydrolyze GTP (Fig. 1). We found that the purified Ffh protein possessed GTP hydrolysis activity independent of any of the other components (Fig. 1, rxn 1). Ffh hydrolyzed GTP with Michaelis-Menten behavior (K_M = 0.4 μM) and was linear over the course of the assay (data not shown). Neither the 4.5S RNA (Fig. 1, rxn. 2) nor an FtsY-glutathione transferase fusion protein (FtsY-GT) (Fig. 1, rxn. 3) had significant hydrolysis activity separately, or in combination (Fig. 1, rxn. 4). Likewise, the addition of either 4.5S RNA (Fig. 1, rxn. 5) or FtsY-GT (Fig. 1, rxn. 6) to the Ffh GTPase reaction had little effect on the rate of hydrolysis as compared to Ffh alone. When both the FtsY-GT fusion protein and 4.5S RNA were combined with Ffh, however, a dramatic (~ 8-fold) stimulation of GTP hydrolysis took place (Fig. 1, rxn. 7). The glutathione transferase domain of the fusion protein (GT), which was purified in an identical manner to FtsY-GT, had no basal activity with or without 4.5S RNA (Fig. 1, rxns. 8 and 9 respectively) and possessed no stimulatory activity either in the presence or the absence of the RNA (Fig. 1, rxns. 10 and 11 respectively). This demonstrates that it is the FtsY-GT fusion and not a bacterial contaminant that is required for the stimulated GTP hydrolysis. This result is especially intriguing because it is the first demonstration of an interaction between the Ffh/4.5S RNA complex and its putative receptor, FtsY.

Given the similarity of Ffh to SRP54, it is likely to be a signal sequence binding protein as well as a GTPase. Signal sequence recognition by Ffh has been seen in a heterologous, *in vitro* system (Luirink, et al., 1992). To assay for a

Figure 1. GTP hydrolysis activity of Ffh

Purified Ffh and 4.5S RNA were assayed for the ability to hydrolyze GTP separately, together, or in conjunction with either an FtsY-glutathione transferase fusion protein (FtsY-GT) or the glutathione transferase (GT) itself. **Methods**: 100 microliter reactions were run for 20 minutes at 25 ^{o}C in 50 mM TEA, pH-7.5, 25 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM EDTA, 1 mM DTT, 0.1% Nikkol and 10% glycerol. Complexes containing both Ffh and 4.5S RNA were formed by reconstituting 10 μM Ffh/20 μM 4.5S RNA in 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.1% Nikkol and 10% glycerol for 10 minutes on ice followed by 10 minutes at 37 ^{o}C. In reactions containing Ffh but no RNA and reactions containing 4.5S RNA but no Ffh the SRP homologues were mock reconstituted. Ffh was used at 5 nM, 4.5S RNA at 10 nM, FtsY-GT and GT at 150 nM. GTP containing 5 microcuries of γ-^{32}P-GTP was added to a final concentration of 0.5 micromolar to start the reactions. GTP hydrolysis was measured by charcoal binding. In brief, after 20 minutes at 25 ^{o}C, 8 microliters of the reaction was mixed with 200 microliters of a 5% charcoal solution in 20 mM phosphoric acid, iced for 10 minutes and then spun for 10 minutes at maximum

speed in a microfuge. 100 microliters of the supernatant, containing the phosphate liberated in the reaction, were analyzed by Cerenkov counting in a liquid scintillation counter. Background was determined from a reaction run with buffer only and was subtracted from the data shown. Ffh protein was a generous gift of Dr. Doug Freymann. FtsY-GT and GT were the generous gifts of Dr. Harris Bernstein. γ-^{32}P-GTP, specific activity of 4,500 Ci/mmole, was obtained from ICN. The non-ionic detergent Nikkol was obtained from Nikko, Inc.


GTP hydrolysis (fmoles/minute)
1200
1000
800
600
400
200
0
Rxn. No. 1 2 3 4 5 6 7 8 9 10 11
ffh + - - - + + + - - + +
4.5S RNA - + - + + - + + - + -
ftsY-GT - - + + - + + - - - -
GT - - - - - - - + + + +

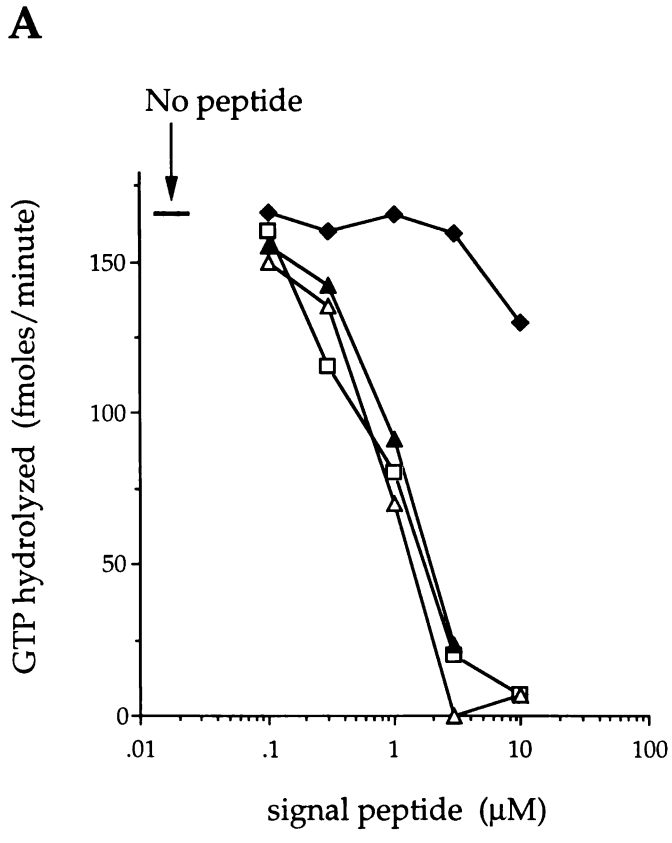
potential Ffh/signal sequence interaction in the GTPase reaction, synthetic signal peptides were added to the GTP hydrolysis reaction containing Ffh and 4.5S RNA. Four different synthetic peptides corresponding to the signal sequence of LamB, a bacterial outer membrane protein, were added to the assay: a peptide corresponding to the wild type signal sequence (wt), a deletion mutant (dm) that removes four amino acids from the hydrophobic core of the lam B signal rendering it inactive *in vivo*, and two different single amino acid, second site revertants (r1 and r2) of the lam B deletion mutant, both of which restore translocation promoting activity *in vivo*. The amino acid sequence of the peptides is given in the legend to Fig. 2. The data in Fig. 2A demonstrate that the wt signal peptide (open squares) effectively inhibited the reaction with an IC_{50} of ~ 1 mM. The dm peptide (closed diamonds), on the other hand, had little effect on GTP hydrolysis even at concentrations which were completely inhibitory for the wt peptide. Both reversion mutants r1 (closed triangles) and r2 (open triangles) restore inhibitory activity to the dm peptide which is similar to that of the wt peptide. Thus, only peptides corresponding to functional signal sequences (wt, r1 and r2) inhibit GTP hydrolysis. This is most likely through a specific interaction of the peptide with the M-domain that triggers a conformational change in the G-domain rendering it inactive.

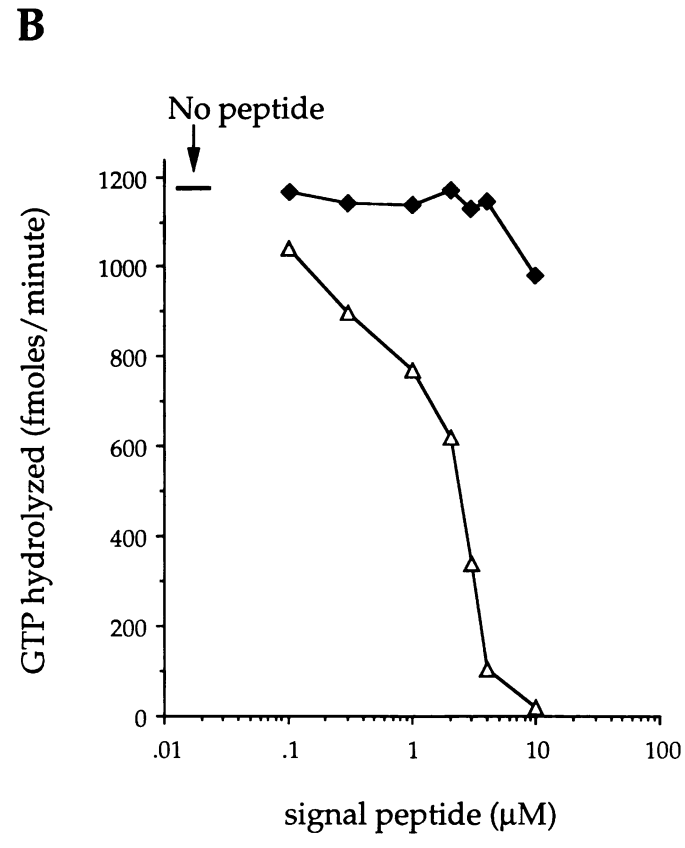
We next examined the effect of inhibitory peptide on the FtsY-GT stimulated reaction (Fig. 2B). The r2 peptide (open triangles) and the dm peptide (closed diamonds) were titrated into a GTP hydrolysis reaction containing both Ffh, 4.5S RNA and the FtsY-GT fusion protein. Again, the wt peptide inhibited the hydrolysis of GTP in a dose-dependent manner with an IC_{50} of ~2 μM and complete inhibition at 10 μM while the dm peptide had no effect. Therefore, even the FtsY stimulated GTPase is inhibited by functional signal peptide. The fact that high concentration of signal peptide completely inhibits both the

Figure 2. Effect of synthetic signal peptides on the GTPase reaction

A) Signal peptide was titrated into a GTP hydrolysis reaction containing Ffh and 4.5S RNA. Four different peptides were used: 1) wt peptide (□) corresponding to the signal sequence of lam B, a bacterial outer membrane protein; 2) dm peptide (◆) corresponding to a deletion mutant of the lam B signal sequence that renders it inactive *in vivo*; 3) r1 (▲) and r2 (Δ) peptides corresponding to second site, single amino acid revertants of the deletion mutant, both of which restore translocation promoting activity *in vivo*.

B) r2 peptide (Δ) or dm peptide (◆) was titrated into the FtsY-GT/Ffh/4.5S RNA GTP hydrolysis reaction. **Methods**: Reactions performed as described in Fig. 1 except that the reaction mixtures were pre-incubated for 30 minutes at 25 ^{o}C with synthetic signal peptide at the concentration indicated prior to the addition of GTP. Signal peptides were a generous gift of Dr. Lila Gierasch. The amino acid sequence of the peptides are: wt-MMITLRKLPLAVAVAAGVMSAQAMA, dm-deletion of residues L10-A13, r1-dm peptide with G17C change, r2-dm peptide with P9L change.


A
No peptide
GTP hydrolyzed (fmoles/minute)
150
100
50
0
.01
.1
1
10
100
signal peptide (μM)


B
No peptide
GTP hydrolyzed (fmoles/minute)
1200
1000
800
600
400
200
0
.01
.1
1
10
100
signal peptide (μM)

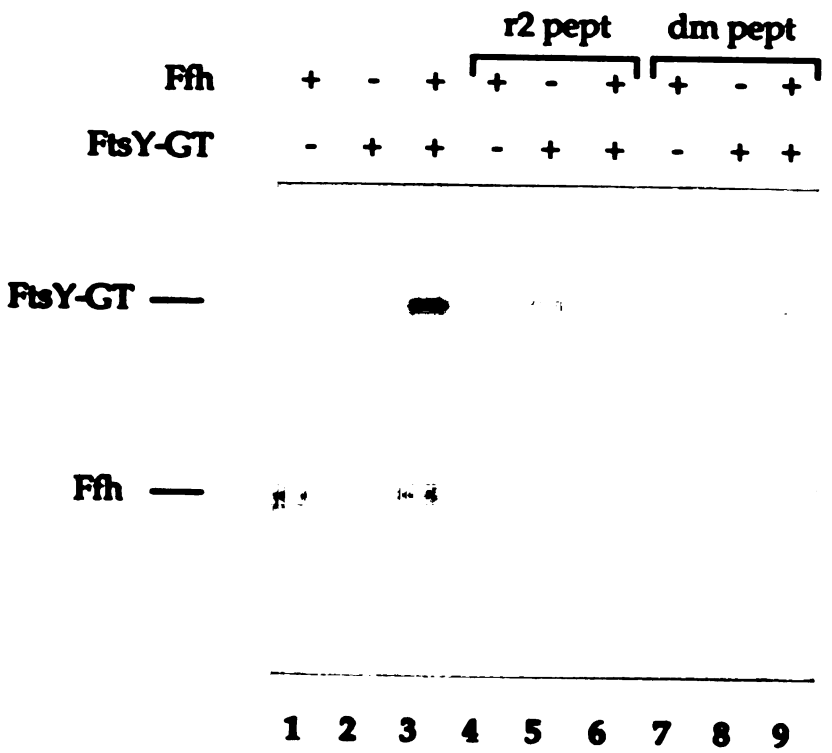
stimulated and unstimulated reactions suggests that Ffh is responsible for the hydrolysis in both these reactions.

Signal peptides could inhibit the GTP hydrolysis reaction either through inhibition of GTP binding to the components of the GTP hydrolysis reaction or through inhibition of GTP turnover by these components. To examine the effects of signal peptide on GTP binding, we utilized a GTP crosslinking assay. In this assay purified components are mixed with α-^{32}P-labeled GTP and then irradiated with ultraviolet light. The UV irradiation causes the formation of radicals in the guanine ring of GTP which form covalent bonds with residues in the GTP binding pocket of the protein to which the GTP is bound (Nath et al., 1985; Pashev et al., 1991). The amount of GTP that is crosslinked to protein can be determined by SDS-PAGE and autoradiography.

The data shown in Fig. 3 demonstrate that Ffh could be crosslinked to GTP (Fig. 3, lane 1) as could FtsY-GT (Fig. 3, lane 2). The crosslinking was found to be UV dependent, to be specific for GTPase consensus containing proteins, and to be competed for by excess unlabeled GTP. Mixing of Ffh/4.5S RNA and FtsY-GT resulted in crosslinking of GTP to both proteins to approximately the same extent as when they were assayed separately (Fig. 3, lane 3). To test the effect of signal peptide on GTP binding, the crosslinking reactions were supplemented with r2 peptide (Fig. 3, lanes 4-6). The presence of peptide in the reaction containing Ffh completely inhibited GTP crosslinking (compare lane 4 to lane 1). The crosslinking to FtsY-GT, on the other hand, was unaffected by peptide (compare lane 5 to lane 2). When the FtsY-GT/Ffh/4.5S RNA/peptide combination was used, GTP crosslinking to Ffh was again completely inhibited while crosslinking to FtsY-GT was unaffected (compare lane 6 to lane 3). Conversely, the dm peptide had no effect on GTP crosslinking to either Ffh or FtsY-GT in any combination (Fig. 3, lanes 7-9). Thus, functional signal peptides appear to inhibit

Figure 3. Crosslinking of GTP to Ffh and FtsY-GT by ultraviolet irradiation

Lanes 1-3 show product of crosslinking α-^{32}P-GTP to either Ffh/4.5S RNA (lanes 1, 4 and 7), FtsY-GT (lanes 2, 5 and 8) or the mixture of Ffh/4.5S RNA and FtsY-GT (lanes 3, 6 and 9). Reactions shown in lanes 4-6 were supplemented with 4 micromolar r2 signal peptide. Reactions shown in lanes 7-9 were supplemented with 4 micromolar dm signal peptide. **Methods** : Reactions were performed as in Fig. 1 except that 10 microcuries of α-^{32}P-GTP is substituted for the γ-^{32}P-GTP used in the hydrolysis assays. After 20 minutes at 25 ^{o}C reactions were placed into plastic weigh boats on ice and UV irradiated for 5 minutes to covalently couple bound α-^{32}P-GTP to protein. Irradiation was performed at a distance of 6 cm from a 6,000 μW/cm^2 UV lamp. Labeled proteins were analyzed by SDS-PAGE and autoradiography.


r2 pept
dm pept
Ffh
+ - + + - + + - +
FtsY-GT
- + + - + + - + +
FtsY-GT
Ffh
1 2 3 4 5 6 7 8 9

both the stimulated and the unstimulated GTP hydrolysis reactions by altering GTP binding to Ffh. Again, this suggests that Ffh is responsible for GTP hydrolysis in the stimulated GTPase reaction. The alternative, however, that peptide could inhibit a GTP dependent interaction between Ffh and FtsY-GT that leads to GTP hydrolysis by FtsY-GT can not be ruled out.

The results presented here demonstrate that Ffh possesses a GTP hydrolysis activity that is regulated by signal sequences and FtsY. The fact that Ffh interacts with signal sequences suggests that it is likely to play a direct role in translocation of some substrates across the inner membrane. Additionally, as predicted by analogy to the mammalian system, Ffh complexed with 4.5S RNA interacts functionally with the FtsY protein, as though it acts as a receptor for an SRP like complex. The significance of this interaction is unclear as FtsY does not appear to be stably associated with the inner membrane (Bernstein and Walter, unpublished). In spite of this, FtsY may still play a role in bridging the gap between the cytoplasm and the membrane, perhaps through a more transient interaction of FtsY with the membrane.

Similar studies on the mammalian SRP54, SRP RNA and SRP receptor have demonstrated that they behave in a manner that is remarkably similar to the bacterial components (see Chapter 3). SRP receptor stimulates SRP54 to hydrolyze GTP in an SRP RNA dependent manner. Functional signal sequences inhibit this reaction by blocking GTP binding to SRP54. Given the strong similarity between these two systems, the fact that SRP54 hydrolyzes GTP in response to SRP receptor argues that Ffh hydrolyzes GTP in response to FtsY. These findings suggest that the bacterial components may play a direct role in the translocation process that is similar in function to that of SRP and SRP receptor.

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Chapter 5

Conclusions and future directions

Conclusions

The work described in this thesis represents the first attempt to understand the molecular mechanism by which GTP binding and hydrolysis regulates the initiation of protein translocation across the membrane of the ER. Analysis of the GTP requirement is complicated by the fact that at least three GTP binding proteins seem to be involved in this process. The ultimate goal is to determine their individual roles in targeting and/or more downstream events. The study of the structural and functional properties of these proteins has provided insight into the regulation of the targeting process and has also provided several novel examples of the different ways in which GTPases can function. Among these are the demonstration of three directly interacting GTPases, a transmembrane GTPase, a novel nucleotide binding promoting factor and the existence of a functional nucleotide free state.

Structurally, this system is unique because SRα, SRβ and SRP54 interact to form a cascade of GTP binding proteins that is anchored to the membrane of the ER by the integral membrane, GTP binding protein SRβ (see Chapter 1). The interaction of eIF-2 with GEF provides a precedent for GTP binding proteins that bind one another, but in this case, their are only two interacting proteins (Dholakia and Wahba, 1989). GTP binding to GEF regulates GTP binding to eIF-2. Likewise, we predict that the different guanine nucleotide bound states of any of the proteins described here will manifest an effect on the guanine nucleotide bound state of one or more of the others. In this manner, information may flow along this chain of GTP binding proteins and, potentially, be passed to the membrane through SRβ. This network of GTP binding proteins could terminate at the membrane with SRβ, or SRβ could tie into a larger group of, as yet

unidentified, GTP binding proteins. Alternatively, a completely distinct set of GTPases could function at stages of translocation other than initiation.

The GTPase dissection done in Chapter 3 also yielded important structural information about the targeting components. Previously, SR was thought to interact with SRP through the SRP68/72 complex (Siegel and Walter, 1988b). The identification of SRP(54/RNA) as the minimal particle required for SR stimulated GTPase coupled with the biochemical analysis of SR (Chapter 2) suggest a model for the SRP·SR interaction in which SRP54 or SRP RNA bind directly to SRα which is anchored to the membrane by SRβ. Direct binding studies will be required to determine if the SRP RNA requirement is for SR binding or for mediating an SR induced conformational change in SRP54 that triggers GTP hydrolysis. Another surprising result from this analysis is that SRP19 does not seem to be absolutely required for SRP54 to bind SRP RNA. Prior experiments suggested that SRP19 maintains the SRP RNA in a conformation that allows SRP54 to bind (Siegel and Walter, 1988a; Zwieb, 1991; Hann et al., 1992). Again, direct binding studies will be needed to resolve this question.

In terms of the function of GTP in translocation initiation, the most progress was made with SRP54 where a well defined cycle of GTP binding and hydrolysis is postulated here to regulate the release of the signal sequence at the membrane (see Chapter 3). This cycle has a number of interesting features centered around the fact that GDP dissociates rapidly from SRP54. In the SRP54 cycle, the GDP bound state does not seem to mediate assembly of a super molecular complex as it does with most GTPases (Bourne et al., 1990; Bourne et al., 1991; Gilman, 1987; Kaziro, 1978). Rather, it is the nucleotide free or "empty" state created by the dissociation of GDP that associates with signal sequences and ribosomes. The switch to the GTP bound state then causes the disassembly of this complex and stabilizes association with SR. Because GDP dissociates rapidly

from SRP54, the conformational switch due to GTP binding cannot be stimulated by a classic guanine nucleotide release protein (GNRP) that works by causing the dissociation of tightly bound nucleotide. Instead, SR seems to trigger GTP binding to SRP54 in a novel way, by increasing its affinity for GTP with respect to GDP. As a result of this stimulated binding, the signal sequence is released.

This model for signal sequence release by SRP54 is an extrapolation from the data presented in chapter 3. In point of fact, in our highly purified GTPase system, SR presumably could not cause signal sequence release. The interpretation of this is that the system is missing a factor (factor X) required for this activity. If this is true, then the GTPase assay is an excellent tool for its isolation. By inhibiting GTPase with signal peptide and then looking for a subcellular fraction that restores GTP hydrolysis, other important factors for targeting and/or translocation may be identified. Therefore, this GTPase reaction is likely to be a minimal, incomplete system that must now be developed to fully reconstitute the targeting reaction.

Proteins homologous to the mammalian targeting components have been identified in *E. coli*. While analysis of their function is not as far along as with the mammalian system, there are significant parallels in their GTP binding and hydrolysis properties. There are also some interesting differences between the two systems. With the mammalian components, SR as a detectable basal rate of hydrolysis while SRP54 does not. In the bacterial system, it is just the opposite, although the lack of a SRβ homologue could explain the absence of hydrolysis by FtsY. Also, it is very intriguing that FtsY is not membrane associated as would be expected for an SRα homologue. This raises the question of whether Ffh/4.5S RNA and FtsY are actually involved in membrane targeting and, if so, how is the cytoplasm-membrane gap bridged. While questions remain regarding the mode of action of the bacterial components, what is clear is that the same interactions

exist in both systems and, to a first approximation, produce the same results. The bacterial system may allow us to bring another tool, genetic manipulation, to bear on the problem. A combination of further biochemical analysis and, perhaps, genetic experiments should make it possible to define the cellular function of the bacterial components and, hopefully will contribute to a better understanding of the mammalian system.

Future directions

While much progress has been made in the course of this work in elucidating the function of GTP binding and hydrolysis by SRP54, the corresponding functions of SRα and SRβ remains a mystery. With respect to SRα, it is well documented that GTP binding is required for translocation to proceed (Rapiejko and Gilmore, 1992). It is still unknown, however, at which point during initiation does SRα bind to and presumably hydrolyze GTP, what stimulates these events, and what their functions are. In order to detect a cycle of binding to and hydrolysis by SRα, it may be possible to utilize the GTP crosslinking technique developed in chapter 3 to assay these events in crude systems such as membranes. Perhaps, for example, SRα only cycles while on the membrane and in response to the arrival of a targeting complex. Changes in GTP binding could be measured by crosslinking to α-^{32}P-GTP, followed by solubilization of the membrane and immunoprecipitation. The use of γ-^{32}P-GTP would allow for the monitoring of GTP hydrolysis as well. The *in vitro* translocation reaction is very sophisticated and can be broken into a number of partial reactions. The binding of GTP to SRα could be monitored as the reaction proceeds through its sequential steps and, hopefully, give an accurate description of the SRα GTPase cycle. The same type of experiments could be used to follow GTP binding to and hydrolysis by SRβ.

Unlike SRα, no direct role has been demonstrated for SRβ in translocation. The work presented in chapter 1 suggests that SRβ is needed to tether SRα to the membrane, but the fact that it binds GTP indicates another role. While unlikely, however, this role could be in another process, distinct from translocation. To really assess the individual functions of SRα and SRβ, the proteins must be expressed independently of one another. Both genes are now cloned (see chapter 2), making this an immediately attainable goal.

To test the anchor hypothesis, a number of interesting experiments could be done. All of them would involve immunodepletion of SRα and/or SRβ from solubilized membranes and then reconstitution of the membranes with mutant SR proteins. Obviously, the first experiment is to deplete SRβ and ask if SRα can bind to the ER and if translocation takes place. If SRβ is only required for membrane attachment of SRα, SRα could be anchored by giving it a PI linkage or a transmembrane domain. Both the SRβ transmembrane domain and a random transmembrane domain could be tested to see if there is a requirement for the SRβ domain. If SRβ is required for functional anchoring of SRα, such a domain swap could be performed on SRβ to see if its specific membrane spanning region is required.

With respect to the other SR functions, such as arrest release, SRP binding, SRP54 GTPase stimulation, etc., the contributions of the two subunits could be easily tested. Of particular interest is the effect of SRα and SRβ on the GTP binding properties of one another. It is possible that, for example, SRβ is an exchange factor or GNRP for SRα.

It is quite possible that SRβ will be required for membrane anchoring of SRα, but not for any of the known SR functions. In this case, the best way to ask if SRβ plays a role in translocation that is mediated by its ability to bind GTP is to make site directed mutations in the protein that would be predicted to affect GTP

binding. A large number of mutations in *ras* are known that affect its GTP binding and hydrolysis properties in a number of different ways. These mutations have been made in other related proteins such as *rab* with similar results. Because SRβ is so close to *ras* in its GTP binding domain (see chapter 2), there is a good chance that similar mutations would produce similar phenotypes with respect to nucleotide binding. The binding could then be characterized using the GTP crosslinking assay. Therefore, a mutant SRβ which is unable to bind GTP could be reconstituted into membranes and those membranes assayed for translocation ability. If GTP binding to SRβ is required for protein translocation, the reconstituted membranes would be nonfunctional.

As a transmembrane GTP binding protein, SRβ is ideally situated to regulate the membrane events of protein translocation. The translocating protein probably passes through a proteinaceous pore which assembles in the membrane to allow translocation and disassembles upon completion to reseal the membrane. This pore most likely consists of transmembrane proteins which assemble into a complex in response to some signal such as the arrival of the targeting complex. SRβ could pass such a signal from the other targeting components, SRα and SRP54, and pass it through its membrane-spanning domain to the pore components. In this way it could form the link between the targeting machinery and the translocation apparatus.

In this light, it would be very interesting to identify other components that interact with SRβ, especially those that do so in a nucleotide-dependent manner. Mutations in SRβ that alter its GTP binding properties could be powerful tools in this regard. GTP binding proteins generally assemble with different sets of proteins depending on their nucleotide-bound state. If this holds true, it might be possible to trap SRβ in interactions that are normally very transient by mutations that hold SRβ in a GTP-bound, GDP-bound or empty state. An

alternative approach to identifying SRβ interactions would be to biochemically isolate proteins that modulate its nucleotide binding and/or hydrolysis. If SRβ plays a critical role in protein translocation, it could provide a handle for walking though the interactions that comprise the translocation process.

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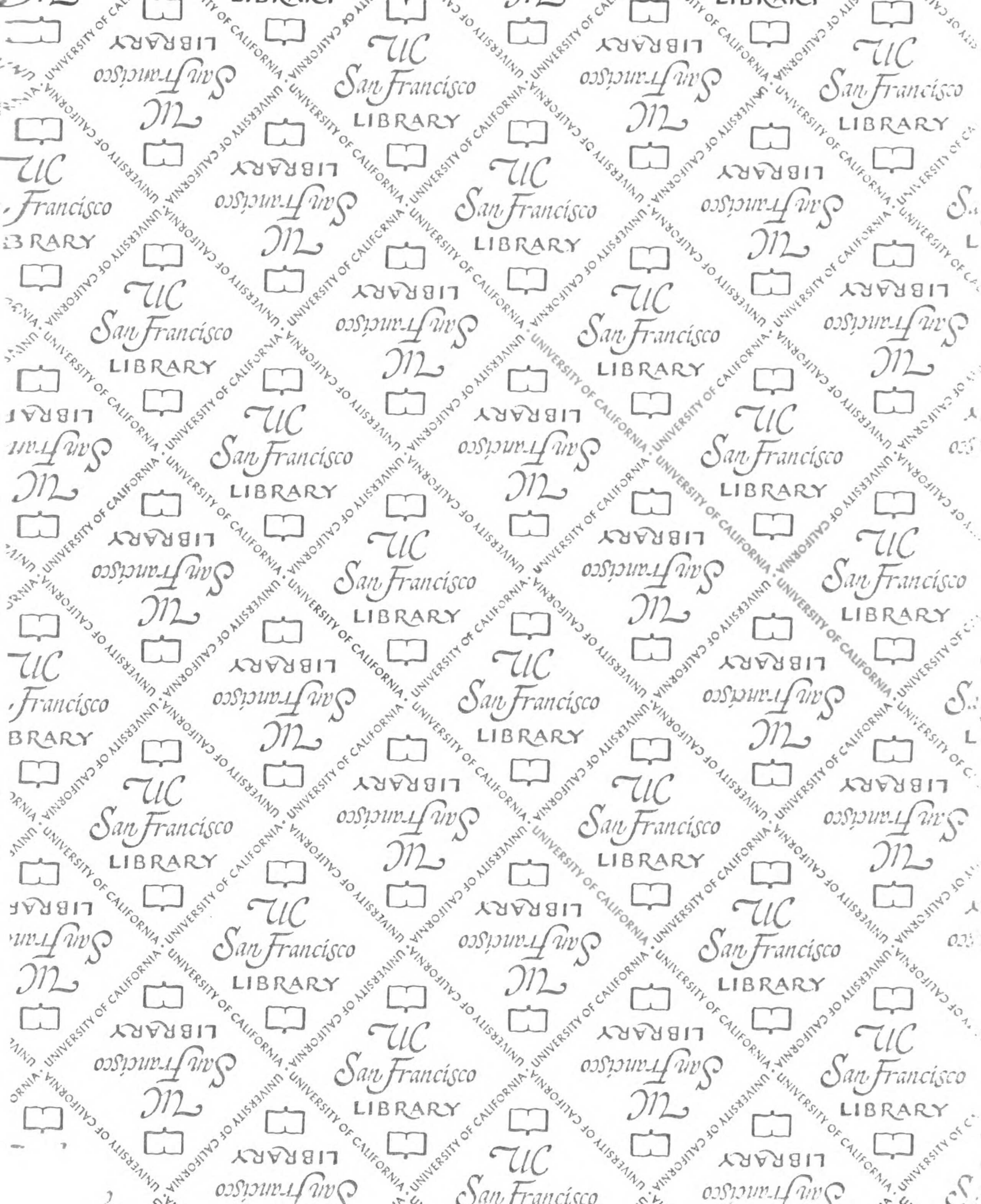
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