

Protein Translocation Across the ER Requires a Functional GTP Binding Site in the α Subunit of the Signal Recognition Particle Receptor

Peter J. Rapiejko and Reid Gilmore

Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Abstract. The signal recognition particle (SRP)-mediated translocation of proteins across the RER is a GTP dependent process. Analysis of the primary amino acid sequence of one protein subunit of SRP (SRP54), as well as the α subunit of the SRP receptor (SR α), has indicated that these proteins contain predicted GTP binding sites. Several point mutations confined to the GTP binding consensus elements of SR α were constructed by site specific mutagenesis to define a role for the GTP binding site in SR α during protein translocation. The SR α mutants were analyzed using an in vitro system wherein SR α -deficient microsomal membranes were repopulated with SR α by in vitro translation of wild-type or mutant mRNA transcripts. SRP

receptors containing SR α point mutants were analyzed for their ability to function in protein translocation and to form guanylyl-5'-imidodiphosphate (Gpp[NH]p) stabilized complexes with the SRP. Mutations in SR α produced SRP receptors that were either impaired or inactive in protein translocation. These SRP receptors were likewise unable to form Gpp(NH)p stabilized complexes with the SRP. One SR α point mutant, Thr 588 to Asn 588, required 50- to 100-fold higher concentrations of GTP relative to the wild-type SR α to function in protein translocation. This mutant has provided information on the reaction step in protein translocation that involves the GTP binding site in the α subunit of the SRP receptor.

RIBOSOMES synthesizing proteins with signal sequences that specify translocation across the RER are selectively delivered to the membrane by the combined action of the signal recognition particle (SRP)¹ and the SRP receptor. SRP, a ribonucleoprotein particle composed of the SRP RNA and six polypeptide subunits (Walter and Blobel, 1982), binds to the signal sequence shortly after it emerges from the large ribosomal subunit (Walter and Blobel, 1981; Walter et al., 1981). Nascent polypeptides containing photoactivatable amino acid analogues can be cross-linked to the 54-kD subunit of the SRP (SRP54) (Krieg et al., 1986; Kurzchalia et al., 1986). The signal sequence binding site was shown to reside within a carboxyl-terminal methionine-rich domain of SRP54 (Zopf et al., 1990; High and Dobberstein, 1991) as initially postulated by Bernstein et al. (1989). The SRP-ribosome-nascent polypeptide complex is targeted to the membrane through an interaction with the SRP receptor, or docking protein (Walter and Blobel, 1981; Gilmore et al., 1982b; Meyer et al., 1982). The SRP receptor is a heterodimeric protein with an α subunit of 68 kD (SR α) and a β subunit of 30 kD (SR β) (Gilmore et al., 1982b; Tajima et al., 1986). After the SRP receptor mediated dissociation of the

SRP from the signal sequence (Gilmore and Blobel, 1983), translocation of the polypeptide across the membrane is proposed to occur through a proteinaceous transport site that is in proximity to at least three different integral membrane proteins that have been identified by cross-linking to nascent polypeptides (Wiedmann et al., 1987; Krieg et al., 1989; High et al., 1991; Kellaris et al., 1991).

Translocation of secretory proteins across and integration of membrane proteins into the RER requires GTP in a process that is distinct from elongation of the nascent polypeptide (Connolly and Gilmore, 1986; Hoffman and Gilmore, 1988; Wilson et al., 1988). Analysis of several sequential reaction steps in protein translocation disclosed that the SRP receptor mediated displacement of the SRP from the signal sequence is dependent upon GTP (Connolly and Gilmore, 1989). When GTP hydrolysis is blocked, subsequent rounds of protein translocation cannot occur because of the formation of a high affinity complex between the SRP and the SRP receptor (Connolly et al., 1991). Examination of the amino acid sequence of SR α revealed that it contains sequences similar to the GTP binding site consensus elements present in guanine nucleotide binding proteins (Connolly and Gilmore, 1989). The 54-kD subunit of the SRP was found to be homologous to SR α in a region that contains the GTP binding site consensus motifs (Bernstein et al., 1989; Romish et al., 1989). Neither SR α nor SRP54 contain an exact

1. *Abbreviations used in this paper:* K-RM, SRP-depleted RM; RM, rough microsomal membranes; SRP, signal recognition particle; T₅-K-RM, trypsin-digested K-RM.

match for the NKXD sequence motif that is present in more typical GTP binding proteins (Dever et al., 1987), but instead contain the sequence TKFD and TKLD, respectively (Bernstein et al., 1989; Connolly and Gilmore, 1989; Romish et al., 1989). More recently, analysis of the sequence of SR β has indicated that it, too, appears to be a GTP binding protein (J. Miller and P. Walter, personal communication).

The discovery of GTP binding sites in both the SRP and the SRP receptor raises questions concerning the reaction steps controlled by each of three possible GTP hydrolysis cycles. This complexity also presents several obstacles in devising experiments to study the role of the individual GTP-regulated proteins. One approach that has had success in other systems, including analysis of the GTP binding properties of the ras oncogene, is oligonucleotide-directed site-specific mutagenesis (Clanton et al., 1986; Sigal et al., 1986). By introducing specific point mutations into the putative GTP binding site of SR α , we were able to address fundamental questions regarding the role of GTP in SR α function. Several of the mutations described here are analogous to those constructed previously in H-ras p21 that were shown to either impair or abolish GTP binding (Clanton et al., 1986; Der et al., 1986; Sigal et al., 1986). Microsomal membranes containing these SR α mutants were defective in protein translocation. These defects in protein translocation could be ascribed to SR α subunits which lacked affinity for GTP. An SR α mutant that displayed a reduced affinity for GTP has proven to be a valuable tool for defining a role for guanine nucleotides in protein translocation.

Materials and Methods

Preparation of Microsomal Membranes, SRP, ¹²⁵I-SRP, SRP-depleted Rough Microsomal Membranes, and Trypsin-digested SRP-depleted Rough Microsomal Membranes

Rough microsomal membranes (RM) were isolated from canine pancreas as described (Walter and Blobel, 1983). SRP and SRP-depleted rough microsomal membranes (K-RM) were prepared from RM as described (Walter et al., 1981). Trypsin-digested K-RM (T₅-K-RM), which lack the cytoplasmically exposed domain of SR α , were prepared from K-RM by mild proteolytic digestion with trypsin (5.0 μ g/ml) for 1 h at 0°C as described previously (Gilmore et al., 1982a). SRP was radioiodinated using ¹²⁵I-Bolton-Hunter reagent essentially as described by Siegel and Walter (1988). Rabbit liver RM used in the protein immunoblotting studies were prepared using the same procedure as that used for preparing the canine pancreas RM (Walter and Blobel, 1983).

Cell-free Transcription and Translation

To permit transcription of the α subunit of the SRP receptor, preprolactin, and opsin using T7 RNA polymerase, the cDNAs of these proteins were cloned into pGEM vectors (Promega Biotech, Madison, WI). The full length cDNA of SR α (Lauffer et al., 1985) was excised from the plasmid pSR19P, a derivative of pSPSR9 (Andrews et al., 1989), by digestion with BamHI and HindIII. The resulting fragment (2,407 bp) was subcloned into pGEM-4 that had been digested with these same enzymes. The recombinant plasmid, pG4 α , contains 44 nucleotides from the 5' untranslated region of *Xenopus* β -globin, 13 nucleotides from pSP64T, 27 and 413 nucleotides from the 5' and 3' untranslated regions of SR α , respectively, and 1,910 nucleotides of coding sequence. The preprolactin containing plasmid, pG4BP4, was constructed by inserting a 900-bp fragment from pSPBP4 (Siegel and Walter, 1988) into pGEM-4 using the restriction endonucleases HindIII and EcoRI. Similarly, pG3OP was derived from pSPOPI (Hoffman and Gilmore, 1988) by subcloning a 1,427-bp HindIII-EcoRI fragment containing the full-length opsin cDNA into pGEM-3. Recombinant DNA tech-

niques were performed as described previously (Maniatis et al., 1982). RNAs encoding wild-type and mutant SR α , bovine preprolactin, and the truncated fragment of bovine opsin (op-156) were transcribed with T7 RNA polymerase after linearization of the plasmids as described previously (Connolly et al., 1989). In the case of op-156, the plasmid was linearized within the protein coding region with the restriction enzyme AhaII (Hoffman and Gilmore, 1988). The mRNA transcripts were isolated by extraction with phenol-chloroform and by successive precipitations with ethanol and with lithium chloride. In vitro translations of the mRNA transcripts utilized a rabbit reticulocyte lysate (Promega Biotech) translation system (Jackson and Hunt, 1983) supplemented with placental RNase inhibitor (RNasin; Promega Biotech).

Oligonucleotide-directed Site-specific Mutagenesis of SR α

A 1,045-bp HpaI-BamHI fragment of pG4 α was subcloned into M13 mpl8 to create mR α -1045, the template for site-directed mutagenesis. To generate mutations within the putative GTP binding consensus elements of SR α , the following oligonucleotides were synthesized: 3-1, 5'-CCTTACCGAATTGTA-3'; 3-2, 5'-GTCCTTAACAAATTTG-3'; 3-3, 5'-GTCCTTAAGAAATTTG-3'; 3-5, 5'-CAAATTTGCTACCAT-3'; 3A-4, 5'-CTGTCAAACACCCG-3'; 2-6, 5'-GCTGGCCAGATGCAAG-3'; 1-7, 5'-TGGGGAACTCTACTA-3'. The underlined base(s) differ from the wild-type sequence. Oligonucleotide directed site specific mutagenesis was performed essentially as described by Nakamaye and Eckstein (1986) using a site-directed mutagenesis kit and the experimental protocols provided by the manufacturer (Oligonucleotide-Directed in vitro Mutagenesis System Version 2; Amersham Corp., Arlington Heights, IL). The generation of SR α mutants was verified by DNA sequencing of the recombinants (Sanger et al., 1977; Chen and Seeburg, 1985). Full-length SR α mutants were regenerated by subcloning an 818-bp NcoI-BamHI fragment (or a 290 HincII-SphI fragment in the case of mutant SR α 1-7) back into pG4 α that had been cut at those same restriction sites.

Repopulation of Microsomal Membranes with SR α

The T₅-K-RM were repopulated with the various SR α mutants according to the procedure of Andrews et al. (1989). Briefly, rabbit reticulocyte translation reactions (Jackson and Hunt, 1983) programmed with a SR α mRNA transcript (20 ng/ μ l of final volume) were incubated for 40 min at 25°C. After supplementation with T₅-K-RM (3–5 equivalents [eq]/20 μ l of translation; eq, as defined previously) (Walter et al., 1981), the samples were then incubated for an additional 15 min at 25°C to generate membranes that were repopulated with wild-type or mutant SR α . The repopulated membranes typically contain 2–3 fmol of SR α per eq of microsomal membranes.

Sucrose Gradient Analysis of SRP-SRP Receptor Complexes

Membranes were repopulated with wild-type or mutant SR α by in vitro translation in the presence of [³⁵S]methionine. The 100- μ l translation reaction containing the repopulated membranes was adjusted to 250 μ M cycloheximide and applied to a 1.0 ml Sepharose CL-2B (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated in 50 mM KOAc, 5 mM Mg(OAc)₂, 50 mM triethanolamine-OAc, (TEA) pH 7.5, 0.002% Nikkol (octaethyleneglycol-mono-N-dodecyl ether; Nikko Chemical Co., Ltd., Tokyo, Japan), and 1 mM DTT (Buffer A) to separate ribonucleotides from the repopulated membranes. The membranes eluting in a 150- μ l void volume fraction were collected. From this, 60- μ l aliquots containing \sim 10 eq of repopulated membranes were incubated for 30 min at 25°C with 3.6 pmol of SRP in Buffer A supplemented with gelatin (0.1 mg/ml) in the presence of 0–5 mM Gpp(NH)p. The KOAc concentration was raised to 300 mM, and the samples were chilled on ice for 5 min. The KOAc concentration was then reduced to 200 mM and the membranes solubilized by adjustment to 1% Nikkol. The SRP receptor was separated from SRP-SRP receptor complexes by 5–20% sucrose density gradient centrifugation in Buffer A containing gelatin (0.1 mg/ml), 200 mM KOAc, and 0.1% Nikkol. Centrifugation was for 6 h at 50,000 rpm in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Fullerton, CA) at 4°C. The 5.0-ml gradients were fractionated with a gradient fractionator (Isco, Lincoln, NE) into 300- μ l fractions. The radiolabeled SR α was collected by TCA precipitation onto 0.45- μ m nitrocellulose filters (Schleicher and Schuell, Keene, NH) using a modification of the procedure of Schaffner and Weissman (1973). Briefly, the sucrose gradient fractions were collected into tubes containing 33.3 μ l

of a 1% SDS, 1.0 M Tris-OAc solution, pH 7.4, and the samples were incubated for 2 min at room temperature after adjustment to 10% TCA. The dried filters were subjected to scintillation counting.

GTP-dependent Membrane Integration of Bovine Opsin

The truncated op-156 transcript was translated for 15 min at 30°C in a rabbit reticulocyte lysate translation system in the presence of [³⁵S]methionine. After the addition of cycloheximide to 250 μM, ribosomes bearing the nascent op-156 polypeptide were separated from ribonucleotides by chromatography on a 1.0-ml Sephacryl S-200 column equilibrated with 50 mM triethanolamine-OAc, pH 7.5, 150 mM KOAc, 2.5 mM Mg(OAc)₂, 0.002% Nikkol, and 3 mM DTT (Buffer B) as described previously (Connolly and Gilmore, 1986). Simultaneously T₅-K-RM that were repopulated with either the wild-type or a mutant SRα by translation in the absence of [³⁵S]-methionine were rendered ribonucleotide free by Sepharose CL-2B gel filtration chromatography in Buffer B. The gel-filtered membranes were then supplemented with a nucleotide regenerating system (10 mM creatine phosphate, 0.3 mM ATP, 2.0 mg/ml creatine phosphokinase). Aliquots (25 μl) of the repopulated membranes were incubated with 8 μl of the ribonucleotide depleted op-156 polysomes in a total volume of 35 μl for 30 min at 25°C in the presence of increasing concentrations of GTP.

The integration of the op-156 polypeptides into microsomal membranes was assayed as described previously (Hoffman and Gilmore, 1988) using an alkaline sodium carbonate extraction procedure. Briefly, the samples were adjusted to 90 mM Na₂CO₃, pH 11.5, and incubated on ice for 10 min. The 175-μl sample was layered onto a 50-μl cushion of 200 mM sucrose, 100 mM Na₂CO₃, pH 11.5, 100 mM KOAc, 2.5 mM Mg(OAc)₂, and separated into supernatant and pellet fractions by an 8 min centrifugation at 30 psi in an airfuge using the A-100/30 rotor (Beckman Instruments, Inc.). After centrifugation, the supernatant and pellet fractions were prepared for SDS-PAGE. Alternatively, the pellets were resuspended in 150 μl of 100 mM Tris-OAc, pH 7.4, 0.1% SDS and the radioactive peptides collected by filtration through 0.45-μm nitrocellulose filters after TCA precipitation. The supernatant was similarly TCA precipitated to monitor the total recovery of op-156 peptides.

Miscellaneous Procedures

Preprolactin and prolactin were immunoprecipitated from in vitro translation reactions as described previously (Connolly and Gilmore, 1986). For immunoblotting, proteins were resolved by PAGE in SDS, and transferred to nitrocellulose sheets (0.45 μm) (Schleicher and Schuell) as described by Connolly and Gilmore (1989). The nitrocellulose blots were probed with mAbs that recognize the α or β subunit of the SRP receptor (Tajima et al., 1986). After washing to remove unbound primary antibodies, the nitrocellulose sheets were probed with HRP-coupled second antibodies specific for mouse immunoglobulins. Bound second antibodies were visualized using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Corp.) following the manufacturer's recommendations.

Results

GTP Binding Site Mutants Are Defective in Protein Translocation

When the protein sequence of the α subunit of the SRP receptor was first compared with the GTP binding consensus elements, no precise match for the NKXD motif that is highly conserved in other GTP binding proteins (Dever et al., 1987) was found in SRα (Connolly and Gilmore, 1989). Instead, SRα has two potential guanine recognition (GTP-3) motifs, NTPD, designated here as GTP-3A, and TKFD, designated as GTP-3B (Fig. 1 A). Several other GTP binding proteins have recently been identified that contain threonine instead of asparagine as the initial residue within the third consensus element (Didsbury et al., 1989; Rothman et al., 1990). Sequence alignment of SRα with the closely related GTP binding protein SRP54 suggested that the consensus element most likely used by SRα was GTP-3B rather than

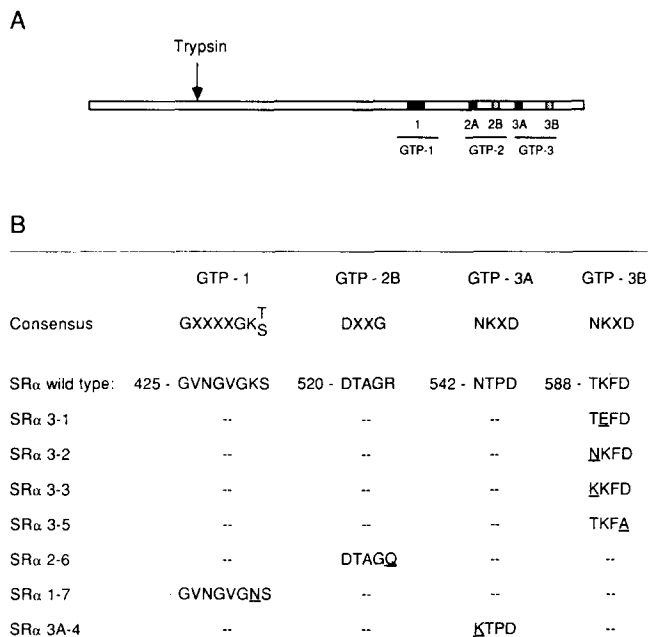


Figure 1. Point mutations in the GTP binding consensus elements of SRα. Seven point mutations within the GTP binding consensus elements of SRα were constructed as described in Materials and Methods. (A) The locations of the GTP binding consensus motifs (GTP-1, GTP-2, and GTP-3) are indicated in the linear representation of the polypeptide sequence of SRα. SRα has two potential GTP-2 sequence elements, DAAG (GTP-2A) and DTAG (GTP-2B) and two potential GTP-3 consensus elements, NTPD (GTP-3A) and NKFD (GTP-3B). Digestion of K-RM with 5.0 μg/ml of trypsin results in the cleavage of SRα in the vicinity of the elastase cleavage site at Met 152 (arrow) (Gilmore et al., 1982b). (B) The sequence and nomenclature of the seven point mutants. The amino acid sequence of the guanine nucleotide binding elements in SRα is shown below the consensus sequences. The number preceding each sequence is the position of the first amino acid residue of the element in the SRα sequence. The altered amino acid in each mutant relative to the wild-type SRα sequence is underlined.

GTP-3A (Bernstein et al., 1989; Romish et al., 1989). We sought to determine whether one or both of these putative GTP binding elements is essential for the function of the SRP receptor by making a comparable mutation in both GTP-3A and GTP-3B. Conversion of Asn 116 to Lys 116 in the guanine recognition element of H-ras p21 yields a protein that lacks detectable affinity for GTP (Clanton et al., 1986). By analogy, the first amino acid of the GTP-3A and GTP-3B motifs was converted to lysine in the SRα mutants 3A-4 and 3-3, respectively (Fig. 1 B). Several other point mutants were made in segments of SRα that, by analogy with the x-ray crystal structure data for EF-Tu (la Cour et al., 1985) and H-ras p21 (Pai et al., 1989, 1990), should be in the vicinity of the triphosphate (SRα 1-7), magnesium (SRα 2-6), and guanine ring (SRα 3-1 and 3-5) binding domains of SRα. Since the GTP-3B sequence motif of SRα does not match precisely the typical consensus sequence, we constructed SRα 3-2 in which the atypical threonine was replaced by the more common asparagine (Fig. 1 B). The SRα mutants were constructed by site-directed mutagenesis as described in Materials and Methods.

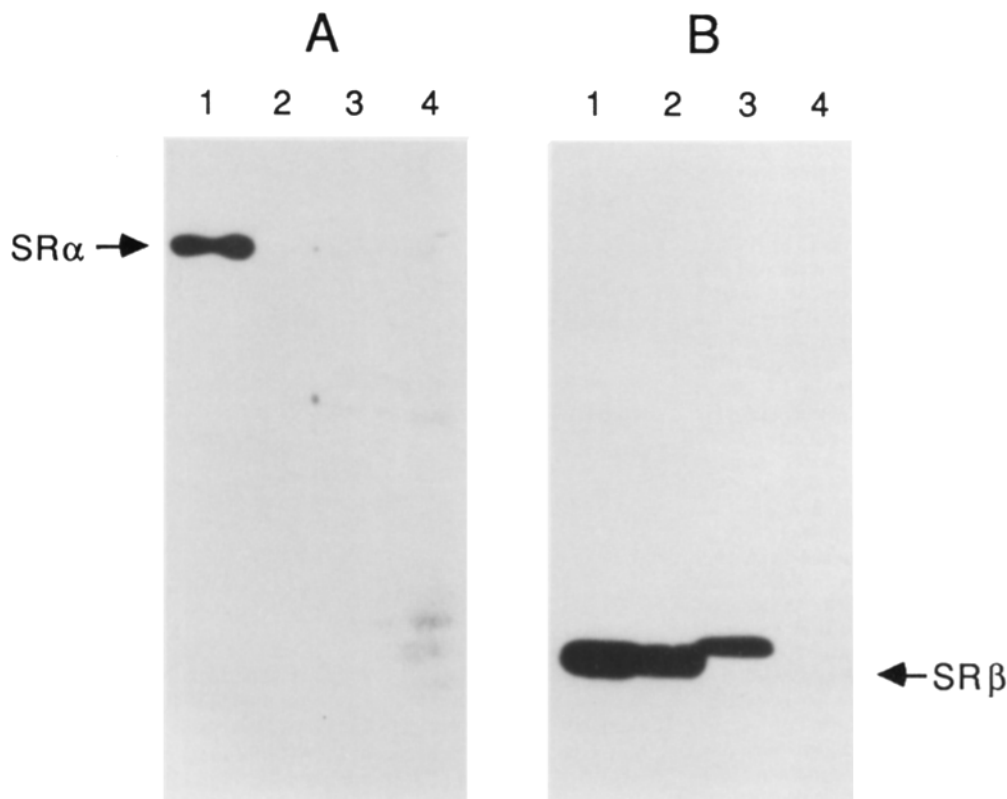


Figure 2. Immunodetection of SRP receptor subunits. Protein immunoblots of 10% polyacrylamide gels were probed with mAbs that recognize SR α (A) or SR β (B). The individual gel lanes for both blots correspond to 6 eq of: (lane 1) SRP depleted microsomal membranes from canine pancreas (K-RM), (lane 2) trypsinized K-RM (T₅-K-RM), (lane 3) rabbit liver microsomal membranes, or (lane 4) 16 μ l of rabbit reticulocyte lysate precipitated with 67% (NH₄)₂SO₄. Peroxidase-labeled second antibodies were detected by enhanced chemiluminescence.

The SR α mutants were assayed for SRP receptor function using an *in vitro* assay system for protein translocation. To obtain reliable assays of SRP receptors that contain altered α subunits, we used a membrane repopulation procedure developed by Andrews et al. (1989). Microsomal membranes that have been rendered translocation incompetent by trypsin digestion of the endogenous SRP receptor α subunit can be functionally reconstituted by the addition of the soluble cytoplasmic fragment of SR α (Meyer and Dobberstein, 1980; Gilmore et al., 1982a). More recently, it has been shown that the trypsinized microsomes can be repopulated with a functional SRP receptor by *in vitro* translation of an SR α mRNA transcript (Andrews et al., 1989). This approach provides the means to assay the biochemical and functional consequences of point mutations in SR α in a context resembling the native membrane without interference from the endogenous wild-type SR α subunit.

The biological reagents used for the repopulation procedure were characterized by protein immunoblot analysis using mAbs raised against SR α and SR β (Tajima et al., 1986). Trypsin digestion of the microsomal membranes results in the virtual removal of SR α (Fig. 2 A, lanes 1 and 2) without reducing significantly the SR β content of the membrane (Fig. 2 B, lanes 1 and 2). Long exposures of the immunoblot disclosed that the trypsinized membranes (T₅-K-RM) contained <2% of the initial quantity of full-length SR α as judged by densitometric scans of the immunoblots (data not shown). Optimal repopulation of T₅-K-RM is obtained when the rabbit reticulocyte lysate translation system is used to translate the SR α mRNA transcript (Andrews et al., 1989). Protein blots of the rabbit reticulocyte lysate and rabbit liver rough microsomes were probed with antibodies

specific for SR α and SR β to determine whether the translation extract contained detectable quantities of either subunit of the SRP receptor. Although we did not detect SR α in the reticulocyte lysate (Fig. 2 A, lane 4), we noted that the antibody raised against the canine protein did not recognize SR α in the rabbit liver rough microsomes (Fig. 2 A, lane 3). A second blot, probed with an antibody specific for SR β , revealed the presence of a 31-kD β subunit in rabbit liver microsomal membranes (Fig. 2 B, lane 3) indicating cross-reactivity of this antibody with rabbit SR β . The β subunit of the SRP receptor was not detected by this antibody in the reticulocyte lysate (lane 4) even upon extended exposure of the film to the immunoblot. Therefore, we conclude that any background translocation activity that might be detected in the repopulation experiments is most likely because of the low, yet detectable amounts of SR α in the T₅-K-RM preparation, and not because of contamination of the rabbit reticulocyte lysate with SRP receptor or microsomal membranes.

Repopulated membranes bearing each of the SRP receptor α subunit mutants were generated by *in vitro* translation. The translation reactions containing the repopulated membranes were then supplemented with an aliquot of a newly assembled translation reaction programmed with a preprolactin mRNA transcript, the reporter for translocation activity. After incubation at 25°C, the ³⁵S-labeled preprolactin (pPL) and the translocated, signal peptidase-processed, mature prolactin (PL) were recovered by immunoprecipitation with an antibody raised against prolactin. Microsomal membranes, but not trypsinized microsomal membranes, translocate and process preprolactin in translation reactions lacking the SR α mRNA transcript (Fig. 3). As shown previously (Andrews et al., 1989), trypsinized membranes repopulated

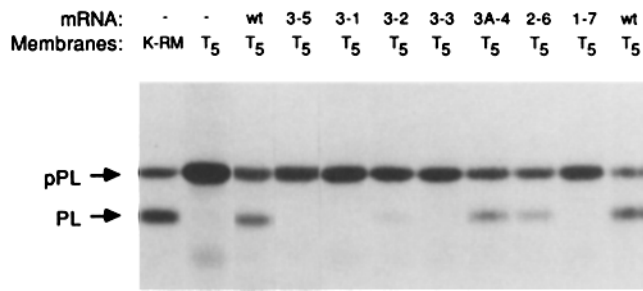


Figure 3. Translocation activity of SR α mutants. T₅-K-RM (3 eq) were repopulated with the wild-type or mutant SRP receptor α subunit by translation of 400 ng of the various mRNA transcripts in 20 μ l rabbit reticulocyte lysate translation reactions that included [³⁵S]methionine as described in Materials and Methods. Control translations containing no mRNA were used for mock repopulation of K-RM or T₅-K-RM (T₅). After repopulation, the translation reactions were incubated for 40 min after supplementation with 3 μ l of a newly assembled translation reaction containing 420 ng of preprolactin mRNA transcript. Preprolactin (pPL) and prolactin (PL) were recovered by immunoprecipitation with an antibody directed against prolactin and were resolved by 12% PAGE in SDS. Shown is an autoradiogram of the fluorographed, dried gel. The assays of the mutant SR α 3-5 and the adjacent SR α wild-type control were taken from a separate experiment.

with wild-type SR α are able to mediate protein translocation as shown by the appearance of the processed form of prolactin. Examination of the SR α point mutants revealed that they could be divided into three classes based upon the translocation activity of the repopulated membranes (Fig. 3). Membranes repopulated with SR α 2-6 and SR α 3A-4 were competent for protein translocation and typically were 50-70% as active as membranes repopulated with the wild-type SR α . Repopulation of membranes with SR α 3-1, SR α 3-3, SR α 3-5, and SR α 1-7 did not reconstitute translocation activity above the background level present in the trypsinized membrane. One mutant (SR α 3-2) displayed a markedly reduced, yet detectable ability to reconstitute the translocation activity of the proteolyzed membranes. These results indicate that alterations within the GTP-3B element are deleterious, while the one mutation we have tested within the GTP-3A element was without significant effect.

Differences in the translation efficiency of the SR α mRNA transcripts will affect the extent of prolactin processing, since the reconstituted SRP receptor is the limiting component in the repopulated membranes (Andrews et al., 1989). The amount of each SR α mutant translated in a repopulation reaction was quantified by gel electrophoresis of nonimmunoprecipitated translation products, and was found to vary by \pm 30%. These changes in SR α translation varied between mRNA transcript preparations, but were not of sufficient magnitude to account for the lack of translocation activity of the membranes repopulated with SR α 3-1, SR α 3-3, SR α 3-5, and SR α 1-7. The reduced activity of SR α 2-6 relative to the wild-type SR α in the experiment shown here, however, can be partially ascribed to a reduced level of translation of the SR α 2-6 mRNA transcript. Although our analysis was limited to single amino acid changes confined to the putative GTP binding consensus elements of SR α , these subtle alterations could conceivably affect the ability of a mutant

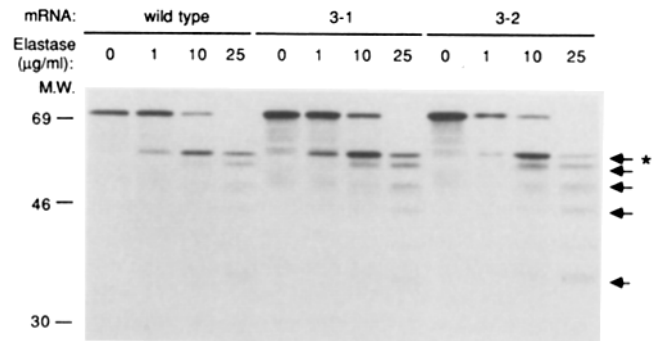


Figure 4. Proteolytic sensitivity of SR α mutants. mRNA transcripts of SR α wild type, SR α 3-1, and SR α 3-2 were translated in a rabbit reticulocyte lysate system that contained [³⁵S]methionine. Aliquots of the translation reactions were first adjusted to 250 μ M cycloheximide, and were then incubated for 30 min on ice with 0, 1, 5, or 25 μ g/ml of elastase in the presence of aprotinin (1 mg/ml). After the addition of PMSF to 10 mM, the samples were solubilized and subjected to 12% PAGE in SDS followed by autoradiography. The arrows at the right designate a series of proteolytic fragments derived from the SR α wild type and all SR α mutants when digested with 25 μ g/ml elastase. The asterisk indicates the 58-kD fragment of SR α that is derived by cleavage of SR α at residue 152 (Lauffer et al., 1985).

SR α to be efficiently incorporated into microsomal membranes. Incorporation of the SR α mutants into microsomes was assayed by centrifugation of the repopulated membranes through sucrose cushions as described by Andrews et al. (1989). All of the SR α mutants were incorporated into microsomal membranes with an efficiency that was comparable to that of the wild-type SR α (data not shown). Whereas cosedimentation with the membrane vesicles indicates that the receptor mutants were incorporated into the membrane, these cosedimentation experiments do not preclude the possibility that the membranes were repopulated with a malformed protein. To address this possibility, the sensitivity of in vitro translated SR α mutants to proteolytic digestion was examined since it is likely that grossly misfolded proteins would display an altered sensitivity to proteolysis. The SR α mutants were translated in vitro in the presence of [³⁵S]methionine, and subjected to proteolytic digestion with elastase as described in the legend to Fig. 4. SR α wild type, SR α 3-1, and SR α 3-2 displayed similar sensitivities to proteolytic digestion (Fig. 4). Proteolysis with low concentrations of elastase resulted in the production of the well-characterized 58-kD fragment of SR α designated by the asterisk (Meyer and Dobberstein, 1980; Hortsch et al., 1985; Lauffer et al., 1985). Additionally, digestion of the various SR α mutants with higher concentrations of elastase resulted in a similar pattern of proteolytic fragments (Fig. 4, arrows). All of the SR α mutants were subjected to this same analysis with identical results (data not shown). As previous research has shown that elastase digestion of the immunopurified SRP receptor yields the 58-kD cytoplasmic fragment (Hortsch et al., 1985), the use of total SR α translation products rather than the membrane incorporated SR α in this experiment is valid. Furthermore, trypsin digestion of SR α that was repopulated into membranes did not disclose significant differences in the protease sensitivity of the wild type and

SR α mutants (data not shown). From these experiments, we conclude that protein misfolding is unlikely to be responsible for the lack of translocation activity shown by the SR α point mutants.

GTP Binding Affinity of the SR α Mutants

SR α point mutants that could effectively replace the wild-type protein had alterations that were either immediately adjacent to (SR α 2–6) or far from (SR α 3A–4) the conserved residues within the GTP binding sites (Fig. 1). The SR α point mutants that were inactive in translocation (SR α 3–1, 3–3, 3–5, and 1–7) correspond to alterations in residues that should be directly involved in ribonucleotide binding by analogy to other GTP binding proteins. Conceivably, the reduced translocation activity of SR α 3–2 might be because of a reduced affinity for GTP. The GTP content of the translocation reactions shown in Fig. 3 is not known with certainty. To assess the GTP binding affinity of the SR α mutants directly, the repopulation assay was modified so that the protein translocation reaction was dependent upon the addition of exogenous GTP. A truncated mRNA transcript encoding the amino-terminal 156 residues of bovine opsin was translated to assemble op-156-ribosome complexes as the source of a translocation substrate. Previous research from this laboratory has shown that op-156 is integrated into microsomal membranes in a GTP-dependent reaction that can be monitored either by acquisition of N-linked oligosaccharide or by resistance to alkaline extraction (Hoffman and Gilmore, 1988). As protein synthesis also requires GTP, the repopulated membranes and the preassembled op-156 polysomes were generated in separate rabbit reticulocyte lysate translocation reactions that contained endogenous GTP before the removal of ribonucleotides by gel filtration chromatography (Connolly and Gilmore, 1986). The ribonucleotide depleted membranes and the op-156 polysomes were then incubated together in the presence of cycloheximide (250 μ M), a ribonucleotide triphosphate regenerating system, and increasing concentrations of GTP. Sodium carbonate extraction of the microsomal membranes followed by airfuge centrifugation was used to separate the samples into supernatant and membrane pellet fractions that contain unincorporated and membrane-integrated op-156 chains, respectively (Fig. 5 A). The GTP-dependent membrane integration of op-156 was further confirmed by the appearance of glycosylated op-156 (g-op-156) in the pellet fraction when the trypsinized membranes were repopulated with the wild-type SR α (Fig. 5 A). Furthermore, the ability of GTP to mediate nascent chain insertion was concentration dependent as shown by quantification of the membrane-integrated op-156 polypeptide (Fig. 5 B). In membranes repopulated with wild-type SR α , half-maximal integration of the nascent opsin polypeptide occurred at \sim 1.0 μ M GTP. A representative of those mutants unable to mediate protein translocation, SR α 3–5, was refractory to 1 mM GTP. The small increase in membrane-integrated op-156 observed with SR α 3–5 between 0.1 and 10.0 μ M GTP can be attributed to residual undigested wild-type SR α present in the T₅-K-RM as determined in control integration experiments using mock-repopulated T₅-K-RM membranes (data not shown). Other SR α mutants that were defective in prolactin translocation (SR α 1–7, 3–1, and 3–3) were also defective in op-156 glycosylation when assayed at

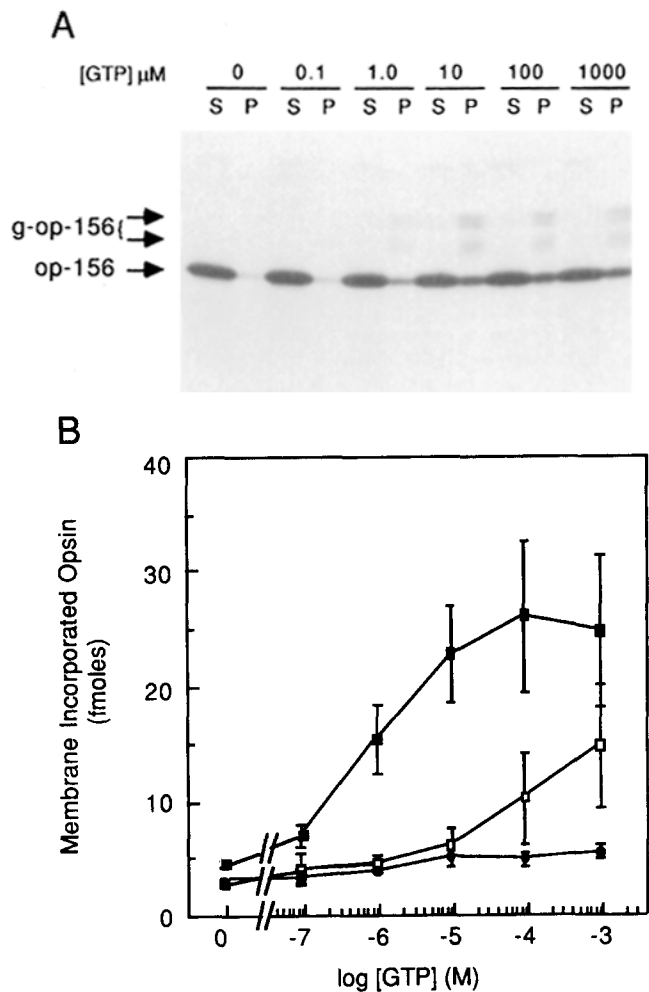


Figure 5. Opsin integration is dependent upon the GTP binding site in SR α . T₅-K-RM were repopulated with SR α wild type, SR α 3–2, or SR α 3–5 in the absence of [³⁵S]methionine as described in Fig. 3. The truncated mRNA encoding op-156 was translated in a separate reticulocyte lysate translation reaction that included [³⁵S]methionine to prepare op-156-polysomes. The repopulated membranes and the op-156 polysomes were separated from ribonucleotides by gel filtration chromatography (see Materials and Methods). The repopulated T₅-K-RM were then incubated with op-156 polysomes in the presence of an energy regenerating system and increasing concentrations of GTP. The samples were fractionated into supernatant (S) and membrane pellet (P) fractions by alkaline sucrose gradient centrifugation. (A) SDS 12–20% polyacrylamide gradient gel analysis of assays containing membranes repopulated with the wild-type SR α . Glycosylated op-156 (g-op-156) is generated upon membrane integration of op-156. (B) For quantitation, membrane pellet fractions were precipitated onto nitrocellulose filters with TCA and quantitated by scintillation counting. The curves shown are from membranes repopulated with wild-type SR α (■), SR α 3–2 (□), or SR α 3–5 (●). The data shown are the mean \pm SEM of three separate experiments with the exception of the data for SR α 3–5, which is the average of two experiments.

a GTP concentration of 1 mM (data not shown). Membranes repopulated with SR α 3–2, in contrast, were capable of integrating op-156 when the GTP concentration was sufficiently high. From this data, we estimate that SR α 3–2 has more than a 50-fold reduced affinity for GTP relative to the

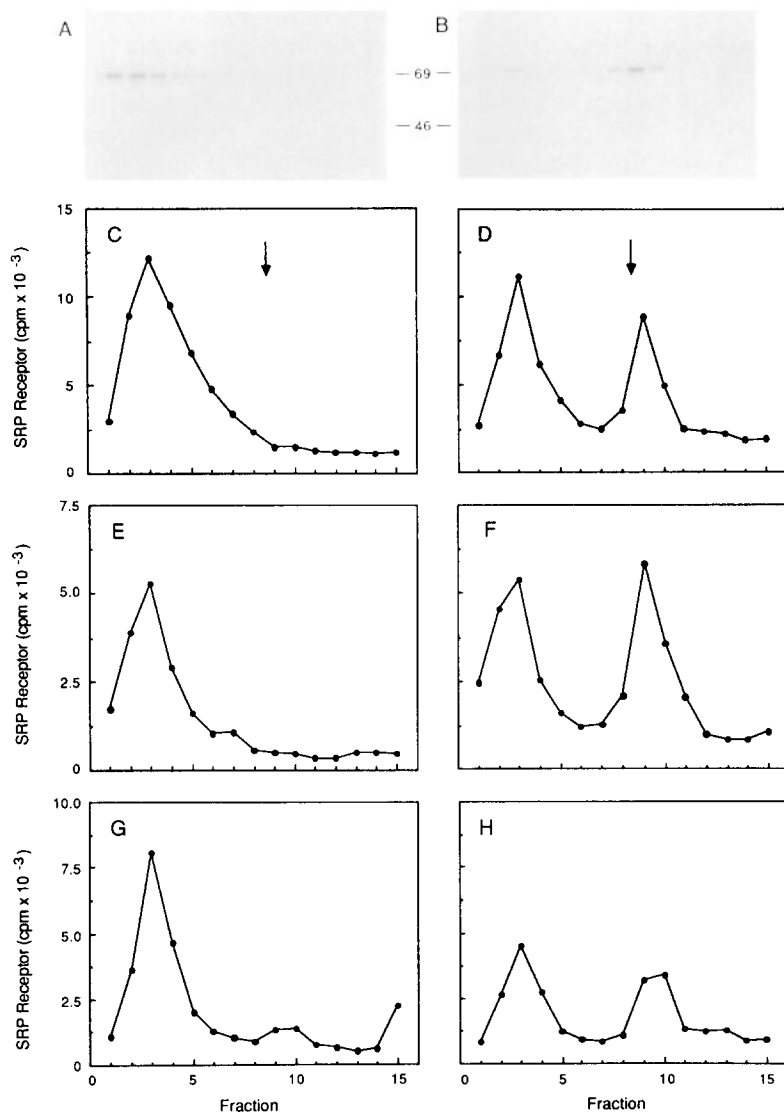


Figure 6. Complexes of the SRP and the SRP receptor are stabilized by Gpp(NH)p. T₅-K-RM were repopulated with the wild-type SR α as in Fig. 3. The repopulated membranes were separated from ribonucleotides by gel filtration chromatography in columns equilibrated with either 50 mM TEA, pH 7.5, 50 mM KOAc, 5 mM Mg(OAc)₂, 0.002% Nikkol, 1 mM DTT (A-F), or with the same buffer adjusted to 200 mM KOAc (G and H). The repopulated membranes were then supplemented with 3.6 pmol of SRP (A-E and H) and either no added ribonucleotides (A and C), 100 μ M Gpp(NH)p (B, D, and F-H), or 100 μ M GTP (E). After incubation under low ionic strength conditions, the membranes were solubilized by adjustment to 1% Nikkol and the SRP-SRP receptor complexes were resolved from free SRP receptor by sucrose density gradient centrifugation (see Materials and Methods). Fraction 1 represents the top of the gradient. The distribution of SR α was analyzed by autoradiography after PAGE in SDS (A and B) or by scintillation counting of TCA precipitated gradient fractions (C-H). The sedimentation position of an ¹²⁵I-labeled SRP standard run in parallel sucrose density gradients in the absence of repopulated membranes was typically in fractions 8 and 9 as indicated by the arrows in C and D.

wild-type protein. The impaired translocation of prolactin observed in Fig. 3 by SR α 3-2 can now be ascribed to a reduced affinity for GTP, since rabbit reticulocyte lysate extracts are reported to contain \sim 100 μ M GTP (Jackson and Hunt, 1983). Together these experiments show that the GTP binding site in SR α actively participates in the protein translocation reaction.

GTP hydrolysis and guanine nucleotide exchange are required for multiple rounds of protein translocation. When assayed at 1 mM GTP, membranes repopulated with the wild-type SR α , or SR α 3-2, integrated 8 and 6 fmols of op-156 per fmol of SRP receptor α subunit present in the membranes, respectively (data not shown). Replacement of GTP with a nonhydrolyzable nucleotide analog such as guanylyl-5'-imidodiphosphate (Gpp[NH]p), results in an inhibition of SRP-SRP receptor cycling, effectively limiting each SRP receptor to one round of nascent polypeptide chain insertion (Connolly et al., 1991). In microsomal membranes repopulated with wild-type SR α , membrane integration of op-156 polypeptides was reduced by nearly 90% when GTP (1 mM) was replaced with 100 μ M Gpp(NH)p (data not shown).

Similarly, the SR α mutants capable of mediating protein translocation (SR α 3-2, 3A-4, and 2-6) also displayed this reduced ability to integrate op-156 in the presence of Gpp(NH)p (data not shown).

Stabilization of SRP-SRP Receptor Complexes by Gpp(NH)p

When purified SRP and SRP receptor are incubated together under low ionic strength conditions in the presence of the nonhydrolyzable nucleotide Gpp(NH)p, a complex is formed that is remarkably resistant to dissociation by high ionic strength buffers (Connolly et al., 1991). Unlike the assays for prolactin translocation or op-156 insertion, a single guanine nucleotide exchange reaction is monitored in the complex formation assay. The protein subunit occupied by Gpp(NH)p in the high affinity complex is not known, and could conceivably be SR α , SR β , or SRP54. In an effort to understand more precisely the role of the GTP binding site in SR α , we determined whether the SR α mutants could form Gpp(NH)p stabilized complexes with the SRP. In vitro translated ³⁵S-labeled SR α mutants were used to repopulate T₅-K-RM.

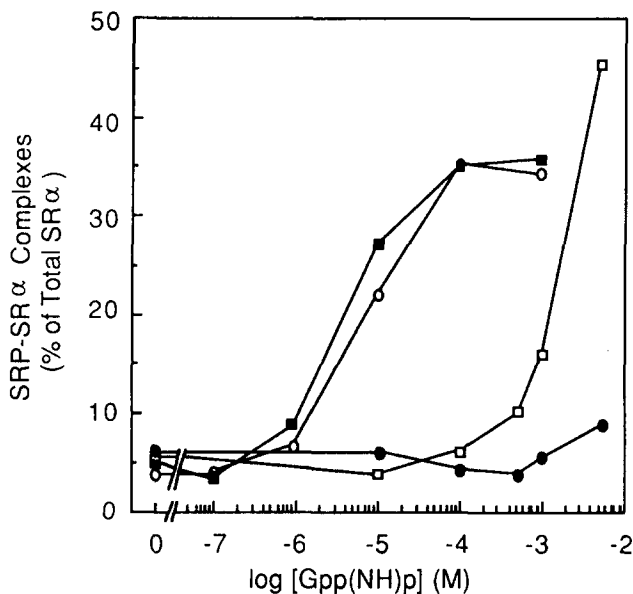


Figure 7. The effect of mutations within the GTP binding consensus elements of SR α on SRP-SRP receptor complex formation. T₅-K-RM were repopulated with the wild-type SR α (■), SR α 2-6 (○), SR α 3-2 (□), or SR α 3-5 (●) as in Fig. 3. The repopulated membranes were separated from ribonucleotides by gel filtration chromatography under low ionic strength conditions. Aliquots of the repopulated membranes were supplemented with 3.6 pmol of SRP and increasing concentrations of Gpp(NH)p and incubated as in Fig. 6. After solubilization of the membranes, SRP-SRP receptor complexes were resolved from free SRP receptor by sucrose density gradient centrifugation. The distribution of SR α in the sucrose gradients was determined by scintillation counting of TCA-precipitated gradient fractions. The cpm value for the fraction from each gradient that contained the lowest cpm (typically fraction 14, see Fig. 6) was subtracted as background from all other fractions to correct for artifactual sedimentation of some SR α in fractions 8-10 in the absence of Gpp(NH)p (Fig. 6, C or E). The percentage of SR α in complex with the SRP was then calculated using the following formula: [(cpm in fractions 8-10)/(cpm in fractions 8-10 + cpm in fractions 1-5)] \times 100.

Ribonucleotides as well as any nonmembrane-associated SR α were removed by gel filtration chromatography using a low ionic strength buffer. The repopulated membranes were then incubated with purified SRP in the presence or absence of 100 μ M Gpp(NH)p in a buffer containing 50 mM KOAc. After raising the KOAc concentration to 300 mM, the repopulated membranes were solubilized with the nonionic detergent Nikkol, and the proteins were subjected to sucrose density gradient centrifugation. Since SDS-PAGE analysis of the gradient fractions revealed that the only radiolabeled protein was SR α (Fig. 6, A and B), the sedimentation position of SR α was determined by scintillation counting of TCA-precipitated gradient fractions (Fig. 6, C-H). SR α remained at the top of the gradient when no ribonucleotides were added to the incubation (Fig. 6, A and C). The inclusion of Gpp(NH)p in the incubation buffer resulted in a shift of 40-60% of the wild-type SR α to a high S form (Fig. 6, B and D) that cosedimented with the ¹²⁵I-labeled SRP standard designated by the arrows in Fig. 6, C and D. Previous analysis of the high affinity complex used purified prepara-

tions of both the SRP and SRP receptor (Connolly et al., 1991). As the experiments reported here used a less well-defined system that included trypsinized membranes and the *in vitro* protein translation system, it was important to define the protein and nucleotide components that were required for high affinity complex formation. The rapidly sedimenting complex was not formed when Gpp(NH)p was replaced by GTP (Fig. 6 E), but was still detected when purified SRP was not included (Fig. 6 F). Reticulocyte lysate extracts contain endogenous SRP (Meyer et al., 1982), thus it seemed possible that the complex observed in Fig. 6 F was assembled by binding of rabbit SRP to the SRP receptor shortly after synthesis of SR α . To test this hypothesis, the repopulated membranes were resolved from the SRP as well as the nucleotides present in the reticulocyte extract by chromatography in a buffer containing 200 mM KOAc, a salt concentration known to disrupt SRP-SRP receptor complexes that do not contain Gpp(NH)p (Gilmore et al., 1982b; Connolly et al., 1991). Rapidly sedimenting complexes containing SR α were formed upon subsequent incubation of the repopulated membranes with Gpp(NH)p only in samples supplemented with purified SRP (compare Fig. 6, G and H).

Previous studies have shown that the Gpp(NH)p stabilized complex formed using purified SRP and SRP receptor contains both SR α and SR β (Connolly et al., 1991). The experiments described above (Fig. 6) were performed using T₅-K-RM repopulated with SR α . Because the mild trypsinization used in the preparation of T₅-K-RM leaves the SR β largely intact (Fig. 2 B), it is likely that the SRP-SRP receptor complexes formed here also contain SR β .

The guanine nucleotide-dependent interaction of the SRP and the SRP receptor was further examined by quantifying the amount of SRP-SRP receptor complexes formed as a function of the Gpp(NH)p concentration for each SR α mutant (Fig. 7). The amount of radiolabeled SR α migrating in the high S form is expressed as a percentage of the total SR α migrating in both peaks at each concentration of Gpp(NH)p. In this way, the SR α mutants could be assayed for their relative ability to form high affinity complexes with the SRP as a function of Gpp(NH)p concentration. Half-maximal complex formation for the wild-type SR α occurred at 4 μ M Gpp(NH)p (Fig. 7, ■), in good agreement with the affinity measured for GTP in the nascent chain integration assay (Fig. 5). When the translocation competent mutant SR α 2-6 was tested for complex formation, a nearly identical dose response curve for the nonhydrolyzable guanine nucleotide analog was observed (Fig. 7, ○). An SR α mutant defective in protein translocation and nascent chain integration, SR α 3-5, did not form high affinity complexes with the SRP at Gpp(NH)p concentrations below 5.0 mM (Fig. 7, ●). Similar results were obtained using mutant SR α 3-3 (data not shown). When mutant SR α 3-2 was examined in this assay, a decrease in affinity for Gpp(NH)p of nearly two orders of magnitude was observed (Fig. 7, □). This is the same SR α mutant that was marginally active in prolactin translocation (Fig. 3) and displayed a lower affinity for GTP in the nascent chain integration assay (Fig. 5). The remaining mutants, SR α 3-1, SR α 1-7, and SR α 3A-4 were each assayed for complex formation at 100 μ M Gpp(NH)p. Of these, only the SR α mutant capable of mediating protein translocation, SR α 3A-4, was capable of forming stable complexes with the SRP. As none of the mutants we constructed appeared capa-

ble of forming a ribonucleotide-independent complex with the SRP, it appears unlikely that any of the SR α mutants described here mimic the GTP rather than guanosine diphosphate (GDP) bound form of the protein.

Discussion

A role for guanine ribonucleotides in protein translocation across the RER was initially detected as a GTP requirement for the membrane insertion of nascent polypeptides (Connolly and Gilmore, 1986; Wilson et al., 1988; Hoffman and Gilmore, 1988). Subsequent experiments established that the SRP remains bound to the signal sequence of the nascent polypeptide in the absence of GTP (Connolly and Gilmore, 1989). When both the SRP and the SRP receptor were found to contain protein subunits with GTP binding sites (Connolly and Gilmore, 1989; Bernstein et al., 1989; Romish et al., 1989), it became apparent that further experimentation would be required to determine which of these nucleotide binding sites was functionally active during the SRP receptor-mediated dissociation of SRP54 from the nascent signal sequence. Since a separate GTP-dependent reaction may be mediated by each of these potential GTP binding proteins, it was necessary to devise a strategy that would enable the evaluation of each of the GTP binding sites independently. Here, we have used site-directed mutagenesis of SR α to determine which reaction steps in protein translocation are dependent upon the predicted GTP binding site in SR α . Mutagenesis of SR α was restricted to the GTP binding consensus elements to insure that alterations in SRP receptor function could be ascribed to alterations in the ability of SR α to bind or hydrolyze GTP. Our analysis of the various SR α mutants relied upon the ability of *in vitro* translated SR α to functionally repopulate microsomal membranes depleted of endogenous SR α by trypsin digestion (Andrews et al., 1989). By expressing the SR α mutants in this system, we were able to analyze microsomal membranes that had a homogeneous population of SRP receptors containing either wild-type or mutant SR α subunits.

The first consensus element in SR α (GVNGVGKS) is presumed to correspond to the triphosphate binding site based upon the function of the analogous sequences within *E. coli* elongation factor Tu (la Cour et al., 1985) and H-ras p21 (Pai et al., 1989, 1990). The ϵ -amino group of Lys 16 in H-ras p21 makes contact with the β and γ phosphates of the ribonucleotide (Pai et al., 1990). Substitution of asparagine for Lys 16 in H-ras p21 produces a protein with more than a 100-fold reduced affinity for both GDP and GTP (Sigal et al., 1986). The corresponding mutant in SR α (SR α 1-7) (Fig. 1 B) was unable to function in a protein translocation assay, demonstrating that Lys 431 in SR α is essential for function. The DXXG motif (GTP-2) contributes portions of the binding site for Mg²⁺ and the γ phosphate of the ribonucleotide (Pai et al., 1989, 1990). An adjacent, highly conserved glutamine residue (DXXGQ) is proposed to participate in GTP hydrolysis by abstracting a proton from a water molecule adjacent to the γ phosphate of GTP (Pai et al., 1990). This function is supported by mutagenesis studies showing that replacement of Gln 61 in H-ras p21 reduces the GTP hydrolysis rate (Der et al., 1986). Interestingly, SR α and SRP54, as well as two related proteins from *E. coli* (Bernstein et al., 1989; Romish et al., 1989) all contain argi-

nine at this site (DXXGR). Conversion of Arg 524 to a glutamine residue (SR α 2-6) caused no pronounced change in SRP receptor function. Since SR α is the limiting component in the translocation assays performed with repopulated membranes, we would anticipate reduced translocation activity from membranes repopulated with an SR α subunit that was grossly defective in GTP hydrolysis.

Mutations within the third GTP binding motif of H-ras p21 strongly influence that protein's affinity for guanine ribonucleotides (Clanton et al., 1986; Sigal et al., 1986; Walter et al., 1986), presumably because of a requirement for specific hydrogen bonds between these residues and the guanine base (deVos et al., 1988; Pai et al., 1989, 1990). Previous work demonstrating that the conversion of Asn 116 to Lys 116 abolished the affinity of H-ras p21 for GTP (Clanton et al., 1986) provided us with a means to discriminate between the two sequences that resemble a third GTP binding site motif in SR α . By constructing two analogous mutations within SR α (SR α 3-3 and SR α 3A-4), we were able to determine that mutation of Thr 588 (SR α 3-3) was not tolerated, whereas mutation of Asn 542 (SR α 3A-4) was relatively innocuous. This observation confirms the assignment of TKFD as the third consensus element in SR α that was made previously based upon an alignment of the SR α and SRP54 protein sequences (Bernstein et al., 1989; Romish et al., 1989). Furthermore, these data suggest that the assignment of DTAG as the second consensus element is reasonable based upon a comparable spacing of consensus elements in other GTP binding proteins (Dever et al., 1987).

Three additional mutants of the TKFD element were constructed, each targeting a different consensus amino acid. Whereas the mutant SR α 3-5 (TKFA) was based upon a ras mutant reported to display a 20-fold reduced affinity for GTP (Sigal et al., 1986), the rationale behind the construction of mutants SR α 3-1 and SR α 3-2 was to initiate a systematic investigation of this atypical GTP binding element. Since the first amino acid in the third motif is threonine instead of the more typical asparagine, SR α 3-1 was constructed to determine if an alteration at the second position was also permissible. Similarly, by returning the TKFD element back to the standard consensus sequence (SR α 3-2), we hoped to determine whether the substitution of threonine for asparagine in SR α corresponds to a neutral substitution with regard to guanine ribonucleotide binding affinity. The importance of the TKFD sequence was revealed by these studies, since alteration of either Lys 589 (SR α 3-1) or Asp 591 (SR α 3-5) was not tolerated in SR α . Detailed analysis of SR α 3-5 revealed that it was defective in the nascent op-156 insertion assay even at high concentrations of GTP. Moreover, SRP receptors containing the SR α 3-5 subunit were unable to form the Gpp(NH)p stabilized complexes with the SRP. We note that the loss of function produced by the SR α 3-5 mutation was considerably more severe than the 20-fold decrease in binding affinity shown by the corresponding mutation in the ras protein (Sigal et al., 1986). We conclude that both the aspartate and lysine residues in the third consensus element of SR α are required for GTP function consistent with the roles of the analogous residues in H-ras p21 (deVos et al., 1988; Pai et al., 1989, 1990).

Previous studies have established that deletion of GTP, or substitution with Gpp(NH)p, can cause defined blocks in the reaction steps that occur during the selective delivery of

SRP-ribosome nascent polypeptide complexes to the ER (Connolly and Gilmore, 1989; Connolly et al., 1991). Targeting of preassembled SRP-ribosome complexes to the membrane is a GTP-independent reaction (Connolly and Gilmore, 1986). The subsequent GTP and SRP receptor-dependent dissociation of the SRP from the signal sequence was initially ascribed to SR α based upon sequence analysis and GTP-photolabeling experiments (Connolly and Gilmore, 1989). If this assignment is correct, membranes repopulated with an SR α subunit that has a reduced affinity for GTP should be blocked at the same point in the translocation reaction as wild-type membranes depleted of GTP. Here, we found that membrane insertion of the opsin nascent chain was defective in membranes repopulated with SR α 3-2 unless the GTP concentration was 50-fold higher than that required by the wild-type receptor. Since op-156 was not integrated into microsomal membranes repopulated with SR α 3-2 at low GTP concentrations, we can conclude that the guanine nucleotide exchange reaction involving SR α is a prerequisite for the nascent chain insertion reaction.

In the presence of the nonhydrolyzable guanine nucleotide Gpp(NH)p, the dissociation of the SRP from the signal sequence proceeds normally even though the subsequent dissociation of the SRP from the SRP receptor is prohibited (Connolly et al., 1991). Gpp(NH)p stabilized SRP-SRP receptor complexes can be formed when the two purified proteins are incubated under conditions of low ionic strength (Connolly et al., 1991). Since incubation of the SRP with the SRP receptor under low ionic strength conditions activates a single GTP hydrolysis site (Connolly, T. and R. Gilmore, manuscript in preparation), it is likely that the Gpp(NH)p stabilized SRP-SRP receptor complex contains a single molecule of bound ribonucleotide. Results presented here establish that the formation of the Gpp(NH)p stabilized complex is dependent upon the presence of a functional GTP binding site in SR α . Based upon the results summarized above, we conclude that the GTP binding site in SR α is occupied by Gpp(NH)p in the high affinity complex. Our current data suggest that a guanine nucleotide exchange reaction in the GTP binding site of SR α is initiated by SRP-SRP receptor contact. Occupation of the SR α site by GTP is responsible for initiating the dissociation of the SRP from the signal sequence, perhaps as a direct consequence of the enhanced affinity between the SRP and the SRP receptor.

The roles for the GTP binding sites in SRP54 and SR β remain to be elucidated. As we have not conducted experiments where similar mutations were made in SR β and SRP54, we cannot exclude the possibility that alterations in these sites might cause translocation defects that are difficult to distinguish from those described here. However, if the GTP binding sites in SRP54, SR α , and SR β function in a sequential manner, discrete experimentally separable intermediates should accumulate after mutagenesis of each GTP binding site. If SRP54 binds GTP as a consequence of signal sequence recognition, this GTP requirement would not be detected by our current assays for GTP-dependent translocation reactions, because both the SRP and GTP are present during the in vitro translation reaction. As suggested previously, the ribonucleotide binding site in SRP54 could act to enhance the fidelity of signal sequence recognition (Bernstein et al., 1989). Alternatively, GTP binding to SRP54 may regulate the affinity between the SRP and the SRP

receptor in a manner that favors the productive targeting of SRP-ribosome nascent polypeptide complexes to the RER. In either case, GTP hydrolysis cycles involving the SRP and the SRP receptor would control the cyclic assembly and disassembly of the components of the membrane-bound translocation complex. Although extensive speculation concerning a role for the proposed binding site in SR β must await the development of assays that show a required function for SR β , it is tempting to postulate that a GTP hydrolysis cycle involving this subunit could regulate a cyclic association between the SRP receptor and the translocation components that are proposed to mediate transport of the nascent polypeptide across the membrane.

We thank Dr. Peter Walter (University of California, San Francisco, CA) for the gift of the mAbs to the α and β subunits of the SRP receptor. We thank Dr. David W. Andrews (McMaster University, Hamilton, Ontario, Canada) for the plasmid pSR19P containing the cDNA of SR α and for helpful discussions concerning the membrane repopulation procedure. We thank Julie Johnson for her expert technical assistance and the construction of the plasmids pG4BP4 and pG3OP.

This work was supported by National Institutes of Health grant PHS GM 35687 and was conducted during the tenure of an Established Investigatorship of the American Heart Association (R. Gilmore).

Received for publication 12 December 1991 and in revised form 10 February 1992.

References

- Andrews, D. W., L. Lauffer, P. Walter, and V. R. Lingappa. 1989. Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. *J. Cell Biol.* 108:797-810.
- Bernstein, H. D., M. A. Poritz, K. Strub, P. J. Hoben, S. Brenner, and P. Walter. 1989. The amino acid sequence of the 54 kDa subunit of the signal recognition particle suggests a model for signal sequence recognition. *Nature (Lond.)*, 340:482-486.
- Chen, E. Y., and P. H. Seeburg. 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA (NY)*. 4:165-170.
- Clanton, D. J., S. Hattori, and T. Y. Shih. 1986. Mutations of the *ras* gene product p21 that abolish guanine nucleotide binding. *Proc. Natl. Acad. Sci. USA*. 83:5076-5080.
- Connolly, T., and R. Gilmore. 1986. Formation of a functional ribosome-membrane junction during protein translocation requires the participation of a GTP-binding protein. *J. Cell Biol.* 103:2253-2261.
- Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. *Cell*. 57:599-610.
- Connolly, T., P. Collins, and R. Gilmore. 1989. Access of proteinase K to partially translocated nascent polypeptides in intact and detergent-solubilized membranes. *J. Cell Biol.* 108:299-307.
- Connolly, T., P. J. Rapiejko, and R. Gilmore. 1991. Requirement for GTP hydrolysis for dissociation of the signal recognition particle from its receptor. *Science (Wash. DC)*. 252:1171-1173.
- Der, C., T. Finkel, and G. M. Cooper. 1986. Biological and biochemical properties of human *ras* genes mutated at codon 61. *Cell*. 44:167-176.
- Dever, T. E., M. J. Glynias, and W. C. Merrick. 1987. GTP-binding proteins: three consensus sequence elements with distinct spacing. *Proc. Natl. Acad. Sci. USA*. 84:1814-1818.
- deVos, A. M., L. Tong, M. V. Milburn, P. Matias, J. Jancarik, S. Noguchi, S. Nishimura, K. Miura, E. Ohtsuka, and S.-H. Kim. 1988. Three dimensional structure of an oncogene protein: catalytic domain of human c-H-ras p21. *Science (Wash. DC)*. 239:888-893.
- Didsbury, J., R. F. Weber, G. M. Bokoch, T. Evans, and R. Snyderman. 1989. *rac*, a novel *ras* related family of proteins that are botulinum toxin substrates. *J. Biol. Chem.* 264:16378-16382.
- Gilmore, R., and G. Blobel. 1983. Transient involvement of the signal recognition particle and its receptor in the microsomal membrane prior to protein translocation. *Cell*. 35:677-685.
- Gilmore, R., G. Blobel, and P. Walter. 1982a. Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. *J. Cell Biol.* 95:463-469.
- Gilmore, R., P. Walter, and G. Blobel. 1982b. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 95:470-477.
- High, S., and B. Dobberstein. 1991. The signal anchor sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition parti-

- cle. *J. Cell Biol.* 113:229-234.
- High, S., D. Gorlich, M. Wiedman, T. A. Rapoport, and B. Dobberstein. 1991. The identification of proteins in the proximity of signal-anchor sequences during their targeting to and insertion into the membrane of the RER. *J. Cell Biol.* 113:35-44.
- Hoffman, K., and R. Gilmore. 1988. Guanosine triphosphate promotes the post-translational integration of opsin into the endoplasmic reticulum. *J. Biol. Chem.* 263:4381-4385.
- Hortsch, M., D. Avossa, and D. I. Meyer. 1985. A structural and functional analysis of the docking protein. A characterization of active domains by proteolysis and specific antibodies. *J. Biol. Chem.* 260:9137-9145.
- Jackson, R. J., and T. Hunt. 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol.* 96:50-74.
- Kellaris, K. V., S. Bowen, and R. Gilmore. 1991. Endoplasmic reticulum translocation intermediates are adjacent to a nonglycosylated 34-kD integral membrane protein. *J. Cell Biol.* 114:21-33.
- Krieg, U. C., P. Walter, and A. E. Johnson. 1986. Photocrosslinking of the signal sequence of nascent preprolactin to the 54 kD polypeptide of the signal recognition particle. *Proc. Natl. Acad. Sci. USA.* 83:8604-8608.
- Krieg, U. C., A. E. Johnson, and P. Walter. 1989. Protein translocation across the endoplasmic reticulum: identification by photocross-linking of a 39-kD integral membrane glycoprotein as part of a putative translocation tunnel. *J. Cell Biol.* 109:2033-2043.
- Kurzchalia, T. V., M. Wiedmann, A. S. Girshovich, E. S. Bochkareva, H. Bielka, and T. A. Rapoport. 1986. The signal sequence of nascent preprolactin interacts with the 54 kD polypeptide of the signal recognition particle. *Nature (Lond.)* 320:634-636.
- la Cour, T. F. M., J. Nyborg, S. Thirup, and B. F. C. Clark. 1985. Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli*, as studied by X-ray crystallography. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2385-2388.
- Lauffer, L., P. D. Garcia, R. N. Harkins, L. Coussens, A. Ullrich, and P. Walter. 1985. Topology of signal recognition particle receptor in endoplasmic reticulum membrane. *Nature (Lond.)* 318:334-338.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
- Meyer, D. I., and B. Dobberstein. 1980. Identification and characterization of a membrane component essential for translocation of nascent secretory proteins across the endoplasmic reticulum. *J. Cell Biol.* 87:503-508.
- Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes—the role of the 'docking protein'. *Nature (Lond.)* 297:647-650.
- Nakamaye, K. L., and F. Eckstein. 1986. Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* 14:9679-9698.
- Pai, E. F., W. Kabsch, U. Krengel, K. C. Holmes, J. John, and A. Wittinghofer. 1989. Structure of the guanine-nucleotide binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. *Nature (Lond.)* 341:209-214.
- Pai, E. F., U. Krengel, G. A. Petsko, R. S. Goody, W. Kabsch, and A. Wittinghofer. 1990. Refined crystal structure of the triphosphate conformation of Ha-ras p21 art 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2351-2359.
- Romish, K., J. Webb, J. Herz, S. Prehn, R. Frank, M. Vingron, and B. Dobberstein. 1989. Homology of the 54K protein of signal recognition particle, docking protein and two *E. coli* proteins with putative GTP-binding domains. *Nature (Lond.)* 340:478-482.
- Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara, and T. H. Stevens. 1990. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. *Cell.* 61:1063-1074.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.
- Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56:502-514.
- Siegel, V., and P. Walter. 1988. Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: analysis of biochemical mutants of SRP. *Cell.* 52:39-49.
- Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, B. S. Wolanski, S. H. Socher, and E. M. Scolnick. 1986. Mutant ras-encoded proteins with altered nucleotide binding exert dominant biological effects. *Proc. Natl. Acad. Sci. USA.* 83:952-956.
- Tajima, S., L. Lauffer, V. L. Rath, and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. *J. Cell Biol.* 103:1167-1178.
- Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *J. Cell Biol.* 91:557-561.
- Walter, P., and G. Blobel. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. *Nature (Lond.)* 299:691-698.
- Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for co-translational protein translocation. *Methods Enzymol.* 96:84-93.
- Walter, P., I. Ibrahim, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* 91:545-550.
- Walter, M., S. G. Clark, and A. D. Levinson. 1986. The oncogenic activation of human p21^{ras} by a novel mechanism. *Science (Wash. DC)* 233:649-652.
- Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. A signal sequence receptor in the endoplasmic reticulum membrane. *Nature (Lond.)* 328:830-833.
- Wilson, C., T. Connolly, T. Morrison, and R. Gilmore. 1988. Integration of membrane proteins into the endoplasmic reticulum requires GTP. *J. Cell Biol.* 107:69-77.
- Zopf, D., H. D. Bernstein, A. E. Johnson, and P. Walter. 1990. The methionine-rich domain of the 54 kD protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4511-4517.