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GTP Hydrolysis by Complexes of the Signal Recognition Particle and the Signal Recognition Particle Receptor

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Abstract. Translocation of proteins across the endoplasmic reticulum membrane is a GTP-dependent process. The signal recognition particle (SRP) and the SRP receptor both contain subunits with GTP binding domains. One GTP-dependent reaction during protein translocation is the SRP receptor-mediated dissociation of SRP from the signal sequence of a nascent polypeptide. Here, we have assayed the SRP and the SRP receptor for GTP binding and hydrolysis activities. GTP hydrolysis by SRP was not detected, so the maximal GTP hydrolysis rate for SRP was estimated to be <0.002 mol GTP hydrolyzed \times mol of SRP⁻¹ \times min⁻¹. The intrinsic GTP hydrolysis activity of the SRP receptor ranged between 0.02 and 0.04 mol GTP hydrolyzed \times mol of SRP receptor⁻¹ \times min⁻¹. A 40-

fold enhancement of GTP hydrolysis activity relative to that observed for the SRP receptor alone was obtained when complexes were formed between SRP and the SRP receptor. GTP hydrolysis activity was inhibited by GDP, but not by ATP. Extended incubation of the SRP or the SRP receptor with GTP resulted in substoichiometric quantities of protein-bound ribonucleotide. SRP-SRP receptor complexes engaged in GTP hydrolysis were found to contain a minimum of one bound guanine ribonucleotide per SRP-SRP receptor complex. We conclude that the GTP hydrolysis activity described here is indicative of one of the GTPase cycles that occur during protein translocation across the endoplasmic reticulum.

THE process of protein translocation can be divided into a series of reaction steps that together implement the selective transport of a polypeptide across the RER membrane. Proteins that contain RER-specific signal sequences are selected in an initial sorting reaction when the signal recognition particle (SRP)¹ binds to the signal sequence as it emerges from the large ribosomal subunit (Walter and Blobel, 1981; Walter et al., 1981). The specificity of the sorting reaction in protein translocation is explained by the ability of SRP to discriminate between nascent chains that either contain or lack a RER signal sequence (Walter et al., 1981). The six protein subunits of SRP (72, 68, 54, 19, 14, and 9 kD) are organized into three functional domains by assembly onto the SRP (7SL) RNA (Siegel and Walter, 1985; Siegel and Walter, 1988*b*; Walter and Blobel, 1982; Walter and Blobel, 1983*a*). Nascent secretory chains

that contain photoactivatable amino acid analogues can be cross-linked to the 54-kD subunit of SRP (Krieg et al., 1986; Kurzchalia et al., 1986). Based upon this finding, as well as upon experiments using subparticles derived from SRP, the signal sequence recognition domain of SRP was shown to consist of SRP54 and SRP19 (Siegel and Walter, 1988*a,b*). Targeting of the SRP-ribosome-nascent chain complex to the membrane is accomplished by interaction of SRP with the SRP receptor (Gilmore et al., 1982) or docking protein (Meyer et al., 1982), a heterodimeric protein consisting of 68-kD (SR α) and 30-kD (SR β) subunits (Tajima et al., 1986). The signal sequence dissociates from SRP54 when the SRP-ribosome complex binds to the membrane bound SRP receptor (Gilmore and Blobel, 1983). Upon dissociation of the signal sequence from SRP, the nascent secretory polypeptide is inserted into a protein conducting channel in the membrane (Simon and Blobel, 1991).

The protein sequence of SR α , determined from canine and human cDNA clones (Hortsch et al., 1988; Lauffer et al., 1985), contains elements that are similar to the consensus motifs that comprise a GTP binding site (Connolly and Gilmore, 1989; Dever et al., 1987). Site-directed mutagenesis of SR α revealed that a functional GTP binding site in this subunit is essential for protein translocation across the endoplasmic reticulum (Rapiejko and Gilmore, 1992). SRP54 contains a GTP binding domain that is related to the

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1. *Abbreviations used in this paper:* PIC, protease inhibitor cocktail; PEI, polyethyleneimine; SRP, signal recognition particle.

G-domain of SR α (Bernstein et al., 1989; Römisch et al., 1989). Photolabeling with [α - 32 P] GTP suggested that both SR α and SR β bind GTP in a specific manner. SR α , but not SR β , binds GTP when affixed to a nitrocellulose membrane after denaturing gel electrophoresis (Connolly and Gilmore, 1989). Recently, sequence analysis of SR β disclosed the presence of a GTP binding site (Ogg et al., 1992). Thus, the SRP and the SRP receptor contains three protein subunits (SR α , SRP54, and SR β) that belong to the GTPase superfamily. Several lines of experimental evidence suggest that the GTP binding and hydrolysis cycles of SRP54 and SR α regulate the affinity between the signal sequence, SRP, and the SRP receptor. The SRP receptor-initiated dissociation of the signal sequence from SRP is a GTP-dependent reaction (Connolly and Gilmore, 1989). The nonhydrolyzable GTP analogue guanylyl-5'-imidodiphosphate (GMPPNP) can replace GTP in the SRP-signal sequence displacement reaction (Connolly et al., 1991). However, SRP fails to dissociate from the membrane bound SRP receptor, demonstrating that dissociation of the SRP-SRP receptor complex requires hydrolysis of protein-bound GTP (Connolly et al., 1991). The latter observation suggests that a GTP hydrolysis cycle regulates the affinity between SRP and the SRP receptor.

The discovery of three GTP binding sites in SRP and the SRP receptor was unanticipated and raised questions concerning the functional role of each of these GTP binding sites. Speculation on this point has been hindered by a lack of sufficient data concerning the GTPase activity of SRP and the SRP receptor. To begin to address such questions, we have assayed purified preparations of SRP and SRP receptor for GTP binding and hydrolysis activities. These studies have established that the formation of a bimolecular complex of SRP and SRP receptor activates a GTP hydrolysis site. The GTP hydrolysis cycle that can be detected with SRP-SRP receptor complexes has a similar affinity for guanine ribonucleotides as the GTP binding site that was detected with *in vitro* protein translocation assays (Connolly and Gilmore, 1986; Rapijko and Gilmore, 1992).

Materials and Methods

Preparation of SRP and the SRP Receptor

The triethanolamine buffer used for all preparative and analytical procedures was made as a 1 M stock solution, adjusted to pH 7.5 at 25°C with acetic acid and is abbreviated TEA. SRP and salt-extracted microsomal membranes (K-RM) were isolated from canine pancreas rough microsomal membranes using previously described methods (Walter et al., 1981). The concentration of the SRP was determined from the absorbance at 260 nm (Walter and Blobel, 1983a). SRP receptor was isolated from K-RM using a modification of a previous procedure (Gilmore and Blobel, 1983). Briefly, K-RM were adjusted to 0.25 M sucrose, 50 mM TEA, 100 mM KOAc, 1 mM DTT, and supplemented with a protease inhibitor cocktail (2 \times PIC). The final concentration of 2 \times PIC was 0.2 μ g/ml each of chymostatin, leupeptin, antipain, and pepstatin and 2 μ g/ml of aprotinin. The K-RM were then permeabilized by adjustment to 0.05% Nikkol (octaethyleneglycolmono-N-dodecyl ether; Nikko Chemicals Co., Ltd., Tokyo, Japan), and separated from luminal proteins by centrifugation for 1 h at 146,000 g_{av} in a Beckman Ti 50.2 rotor. The membrane pellet was resuspended in 50 mM TEA, 100 mM sucrose, 500 mM KOAc, 1 mM DTT, 2 \times PIC and then solubilized by adjustment to 1% Nikkol. Detergent high-salt insoluble material were removed by a 2 h centrifugation at 146,000 g_{av} in a Beckman Ti 50.2 rotor (Beckman Instruments, Inc., Fullerton, CA). The detergent extract was adjusted to 275 mM KOAc by dilution with 50 mM TEA and applied to a 10-ml column of Fractogel TSK DE-650 M that had been equilibrated with 50 mM TEA, 275 mM KOAc, 0.5% Nikkol, 1 mM DTT.

The protein fraction that did not bind to the anion exchange column was adjusted to 1 M KOAc and 10 mM NaPO $_4$, pH 6.5, and applied to a 10-ml hydroxylapatite column (BioGel HTP; Bio Rad, Richmond, CA) equilibrated with 10 mM NaPO $_4$, 1 M KOAc, 0.5% Nikkol. The hydroxylapatite column was washed with 20 ml of equilibration buffer, and the SRP receptor was eluted with 100 mM NaPO $_4$, 1 M KOAc, 0.5% Nikkol, 1 \times PIC. The hydroxylapatite eluate was diluted with 9 vol of 25 mM Hepes, pH 7.5, 0.25% Nikkol and applied to a 2-ml column of CM-Sepharose equilibrated with 25 mM Hepes, pH 7.5, 0.25% Nikkol, 1 mM DTT (Buffer A) adjusted to 100 mM KOAc. The CM-Sepharose column was washed with buffer A containing 200 mM KOAc, and eluted with buffer A containing 350 mM KOAc. The eluate of the CM-Sepharose column was concentrated by chromatography on a 100 μ l hydroxylapatite column as described above and further purified by sucrose density gradient centrifugation as described previously (Gilmore and Blobel, 1983). The concentration of the SRP receptor preparation was estimated from the Coomassie blue staining intensity of the 68-kD α subunit on polyacrylamide gels relative to the staining intensity of protein molecular weight markers of known concentration. The purity of the SRP receptor preparation obtained by this method is comparable to that of the previous method (Gilmore and Blobel, 1983).

GTP Hydrolysis Assay

GTP hydrolysis assays were conducted at 25°C in a total volume of 10 μ l of 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc) $_2$, 0.1% Nikkol, 1 mM DTT (GTP hydrolysis buffer) unless otherwise noted. Typical assays contained 1 μ Ci of [α - 32 P] GTP (410 Ci/mMol). The concentration of SRP and SRP receptor in individual assays is specified in the figure legends. Aliquots (0.2–0.5 μ l) were removed at 5-min intervals and spotted onto polyethyleneimine (PEI) cellulose thin layer plates (J. T. Baker, Inc., Phillipsburg, NJ). GDP was resolved from GTP using 0.75 M KH $_2$ PO $_4$, pH 3.3 as the solvent for the thin-layer chromatography (Der et al., 1986). The radioactivity in spots corresponding to GTP and GDP was quantitated using a Betagen Bioscope 630 Blot analyzer. The quantity of GTP hydrolyzed in a control assay lacking both SRP and SRP receptor was subtracted as background to determine the rate of protein-specific GTP hydrolysis. GTP hydrolysis rates were calculated from the linear phase of a GTP hydrolysis reaction when the percentage of GTP that had been hydrolyzed did not exceed 10%.

Dissociation constants for SRP-SRP receptor complexes were calculated from data obtained in GTP hydrolysis assays using a Scatchard analysis (Scatchard, 1949). The concentration of bound SRP was calculated for each concentration of added SRP using the following equation: (Bound SRP) = $[(V_o - V_{SR}) \times (SR_T)] / (V_{SR-SRP})$, where V_o is the observed GTP hydrolysis activity, V_{SR} is the basal GTP hydrolysis activity of the SRP receptor, SR_T is the SRP receptor concentration, and V_{SR-SRP} is the maximal GTP hydrolysis activity of the SRP-SRP receptor complex. V_{SR-SRP} was estimated to be 0.9 mol GTP hydrolyzed \times min $^{-1}$ \times (SR - SRP) $^{-1}$. The fractional saturation (ϕ) of the SRP receptor with SRP is defined as $\phi = (SR - SRP) / (SR_T)$, where (SR - SRP) is the concentration of the SRP-SRP receptor complex.

Guanine Nucleotide Binding Assays

The nitrocellulose filter assay for protein bound GTP was based upon methods used to measure [α - 32 P] GTP or [35 S] GTP γ S binding to G-proteins (Brandt and Ross, 1986). SRP, SRP receptor or both proteins together were incubated in a total volume of 100 μ l of 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc) $_2$, 0.1% Nikkol, 1 mM DTT, and 2 μ M GTP (including 20 μ Ci of [α - 32 P] GTP (410 Ci/mMol). Binding of GTP was initiated by addition of SRP or SRP receptor. Aliquots (8 μ l) were withdrawn at frequent time intervals and diluted 25-fold into ice-cold 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc) $_2$, 20% PEG-6000. The samples were vacuum filtered through 25 mm BA85 nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH), and the filters were rapidly washed three times with 1.5 ml of 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc) $_2$. Radioactivity bound to nitrocellulose filters was determined by scintillation counting in Optifluor (Packard Instruments, Meriden, CT). Control experiments demonstrated that the nitrocellulose filters retained \sim 80% of a sample of detergent solubilized K-RM protein after precipitation with the wash buffer containing 20% PEG-6000.

Protein bound GTP was also detected after gel filtration chromatography. SRP and the SRP receptor were incubated alone or in combination at 25°C in a total volume of 10–30 μ l of 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc) $_2$, 0.1% Nikkol, 1 mM DTT, and 1 μ M [α - 32 P] GTP (410

Ci/mMol). After a 10–30-min incubation, the samples were chilled on ice and applied to 1.0 ml Sephadex G-50 columns equilibrated in 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc)₂, 0.1% Nikkol, 1 mM DTT, 1 μg/ml of BSA. Fractions of 100 μl were collected into scintillation vials, and the amount of [α -³²P] GTP in each fraction was quantitated by scintillation counting in Optifluor. Protein-bound ribonucleotides eluted in fractions 4–6, while the peak of unbound GTP eluted in fractions 7–11.

Results

SRP–SRP Receptor Complexes Hydrolyze GTP

SRP and the SRP receptor were incubated with GTP to determine whether either protein complex has an intrinsic GTPase activity. The GTP hydrolysis assays contained 1 μM [α -³²P] GTP, based upon the previous observation that micromolar concentrations of GTP or Gpp(NH)p were sufficient for the guanine ribonucleotide-dependent dissociation of SRP from the signal sequence in a protein translocation reaction (Connolly and Gilmore, 1986; Rapiejko and Gilmore, 1992). As the intrinsic GTP hydrolysis rates of purified GTP binding proteins are often as low as 0.01 mol of GTP hydrolyzed \times mol of protein⁻¹ \times min⁻¹ (Bourne et al., 1990), the GTPase assay was designed to detect low hydrolysis rates when using 1 μM [α -³²P] GTP as the substrate. Nikkol (0.05%) is present in the SRP storage buffer to stabilize SRP activity (Walter and Blobel, 1980), and is included here to stabilize SRP and to maintain the solubility of the SRP receptor. The signal recognition particle did not hydrolyze GTP at a rate that significantly exceeded the rate obtained with the assay buffer alone (Fig. 1). Several different SRP preparations were assayed for GTP hydrolysis activity and comparable results were obtained. The maximal GTP hydrolysis rate calculated from the data shown here is 0.002 mol GTP hydrolyzed \times mol of SRP⁻¹ \times min⁻¹. This value should be considered as a maximal estimate for the intrinsic GTP hydrolysis rate for SRP. Hydrolysis of GTP by the SRP receptor was quite low, yet readily detectable (Fig. 1). Several different SRP receptor preparations were assayed, and the intrinsic GTP hydrolysis rate ranged between 0.02 and 0.04 mol of GTP hydrolyzed \times mol of SRP receptor⁻¹ \times min⁻¹. The low intrinsic GTPase activity of the SRP and the SRP receptor suggest that the hydrolysis reactions catalyzed by these two GTPases are controlled by accessory factors.

High affinity SRP–SRP receptor complexes are formed by coincubation of the two proteins with Gpp(NH)p, but not GTP or GDP, suggesting that a GTPase cycle is initiated upon contact between SRP and the SRP receptor (Connolly et al., 1991). A significant enhancement of GTP hydrolysis activity was observed in an assay that contained 60 nM SRP and 15 nM SRP receptor (Fig. 1). Because SRP was present in a fourfold excess relative to the receptor in this experiment, the GTP hydrolysis activity was arbitrarily expressed with respect to the SRP receptor concentration.

GTP hydrolysis, but not GTP γ S binding, by the heterotrimeric G protein G_s is inhibited when the nonionic detergent Lubrol is present in assays at concentrations above the critical micelle concentration (Brandt et al., 1983; Brandt and Ross, 1985). In the case of G_s, the detergent inhibition of the GTPase activity was alleviated when the detergent concentration was reduced (Brandt and Ross, 1985). To determine whether the GTP hydrolysis rates determined here for

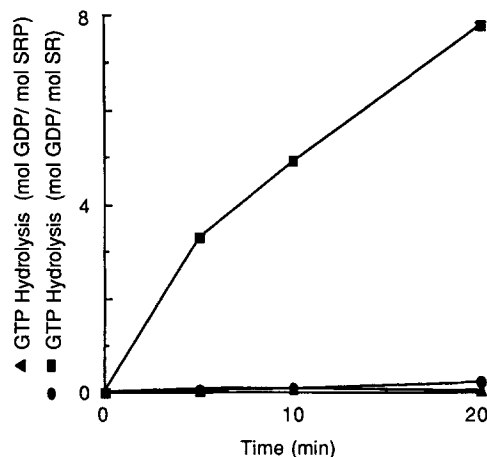


Figure 1. GTP hydrolysis by SRP and the SRP receptor. GTP hydrolysis was assayed in a total volume of 10 μl of GTP hydrolysis buffer (50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc)₂, 0.1% Nikkol, 1 mM DTT) containing 1 μM GTP (including 2 μCi of α -³²P GTP). Individual assays contained (▲) 60 nM SRP, (●) 15 nM SRP receptor, or (■) 60 nM SRP plus 15 nM SRP receptor. Ribonucleotides were resolved on polyethyleneimine (PEI) cellulose thin-layer plates as described in Materials and Methods. The fraction of GTP hydrolyzed at each time point was quantitated with a Betagen Bioscope 630 Blot Analyzer (Betagen, Waltham, MA). The GTP hydrolysis rate is expressed as mole GDP formed/mole of protein.

SRP and SRP receptor were artificially low due to detergent inhibition, we reduced the concentration of Nikkol in the GTP hydrolysis assay. The critical micelle concentration for Nikkol is 7.1×10^{-5} M (0.0038%). The GTP hydrolysis rate for SRP was not enhanced when the Nikkol concentration was reduced to 0.002%, a value that is almost twofold below the critical micelle concentration. Likewise, the GTP hydrolysis rate for the SRP receptor was not enhanced when the Nikkol concentration was reduced to 0.005%. Based upon these results, we conclude that the low intrinsic rates of GTP hydrolysis for SRP and SRP receptor are not caused by detergent inhibition. However, we did observe a 5–10% increase in the hydrolysis rate when assays containing 60 nM SRP plus 10 nM SRP receptor contained 0.01% Nikkol instead of 0.1% Nikkol (data not shown). A further twofold reduction in the Nikkol concentration was accompanied by a reduction in GTP hydrolysis activity.

The validity of expressing the data with respect to the SRP receptor content was explored in more detail. The concentration dependence of SRP stimulation of the hydrolysis activity was determined in assays containing 15 nM SRP receptor (Fig. 2 A). The SRP concentration required for maximal stimulation of the GTP hydrolysis activity was dependent upon the SRP receptor concentration used for the experiment (data not shown). A saturation curve like that shown here is consistent with activation of a GTP hydrolysis site upon formation of a complex between SRP and the SRP receptor. The data in Fig. 2 A was analyzed as an equilibrium between uncomplexed SRP and SRP receptor and a hydrolytically active SRP–SRP receptor complex using the method of Scatchard (Scatchard, 1949). The experimental data could be adequately fit using a K_d of 15 nM for the forma-

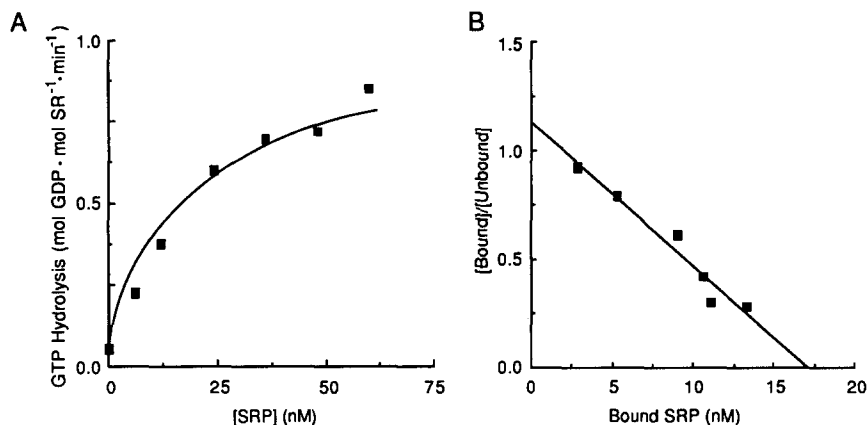


Figure 2. GTP hydrolysis by SRP-SRP receptor complexes. (A) GTP hydrolysis activity was assayed as in Fig. 1. Individual assays contained 15 nM SRP receptor plus the indicated concentration of SRP. Aliquots were removed from the hydrolysis assays at 5-min intervals during a 15-min time course to calculate hydrolysis rate as described in Fig. 1. (B) The data shown in A was analyzed using the method of Scatchard (Scatchard, 1949). The concentration of free and bound SRP was estimated as described in Materials and Methods. The K_d for SRP-SRP receptor complexes was calculated to be 15 nM.

tion of the SRP-SRP receptor complex and a hydrolysis rate of 0.90 mol of GTP hydrolyzed \times mol of SRP-SRP receptor complex⁻¹ \times min⁻¹ (Fig. 2 B). The total number of binding sites detected (17 nM) was in reasonable agreement with the SRP receptor concentration (15 nM) suggesting that a 1:1 complex of SRP and the SRP receptor is responsible for the observed GTP hydrolysis activity.

The GTP hydrolysis assays described in the preceding experiments were conducted under low ionic strength conditions (50 mM KOAc), to favor the formation of SRP-SRP receptor complexes (Connolly et al., 1991; Walter and Blobel, 1983b). The GTP hydrolysis activity decreased markedly as the KOAc concentration was raised (Fig. 3 A). A 2.5-fold lower GTP hydrolysis activity was obtained when the monovalent cation concentration was raised to a value that mimics physiological ionic strength (150 mM KOAc). Inhibition of the hydrolysis activity by increased ionic strength could be due to a direct effect upon the GTPase or it could be due to inhibition of SRP-SRP receptor complex formation. These alternative explanations were tested by determining whether the hydrolysis reaction was dependent upon the concentration of SRP when the ionic strength of the assay was increased (Fig. 3 B). The concentration of SRP required to achieve comparable levels of GTP hydrolysis was substantially higher when the assays were conducted at phys-

iological ionic strength. Analysis of these data using a Lineweaver-Burke plot or an Eadie-Hofstee plot yielded a hydrolysis rate of 0.85 mol GTP hydrolyzed \times mol of SRP-SRP receptor complex⁻¹ \times min⁻¹, and K_d of 126 nM for the SRP-SRP receptor complex. The calculated GTP hydrolysis rate for SRP-SRP receptor complexes at physiological ionic strength was in good agreement with the value calculated from the data presented in Fig. 2 B. A Hill plot of the data (insert in Fig. 3 B) indicated that a simple binding equilibrium between SRP and SRP receptor was operative. Likewise, the concentration of binding sites for SRP as detected by Scatchard analysis (14.7 nM) was in good agreement with the SRP receptor concentration (data not shown). These results demonstrate that the primary cause for the ionic strength dependence of the GTP hydrolysis activity was the altered affinity between SRP and the SRP receptor.

Ribonucleotide Dependence of the Hydrolysis Reaction

Several criteria must be met to establish that the GTP hydrolysis activity of the SRP-SRP receptor complex is of relevance to the GTP-dependent step in the protein translocation reaction. Previous studies have shown that GTP cannot be replaced by ATP to allow nascent chain insertion into the ER (Connolly and Gilmore, 1986; Hoffman and Gilmore,

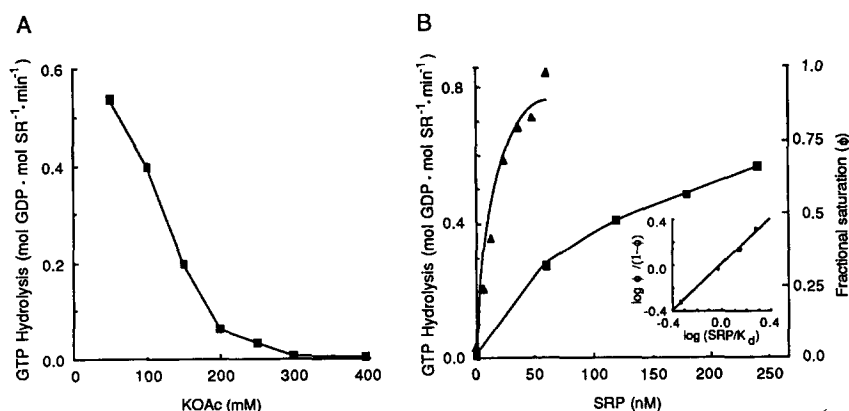


Figure 3. The ionic strength dependence of GTP hydrolysis. (A) GTP hydrolysis assays containing 60 nM SRP and 10 nM SRP receptor were conducted as in Fig. 1 except that the KOAc concentration of the GTP hydrolysis buffer was adjusted to 50–400 mM as indicated. (B) GTP hydrolysis assays containing 15 nM SRP receptor plus the indicated concentration of SRP were conducted as in Fig. 1 except that the GTP hydrolysis buffer was adjusted to 150 mM KOAc (■). Aliquots of the hydrolysis reactions were removed at 5-min intervals during a 15-min assay to determine GTP hydrolysis rates. The experimental data in Fig. 2 A has been replotted for comparison (▲). The inset in B is a Hill plot for

fractional saturation of SRP receptor with SRP, and this parameter was calculated as described in Materials and Methods.

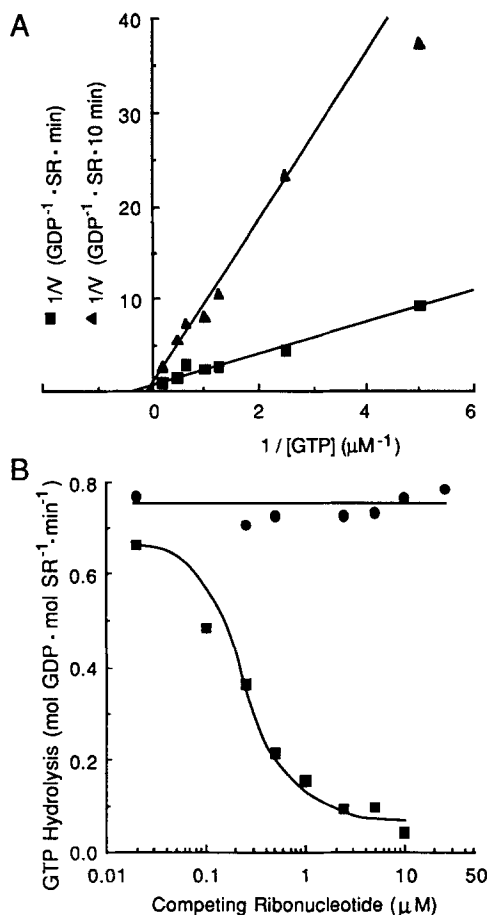


Figure 4. Nucleotide dependence of the GTP hydrolysis reaction. (A) GTP hydrolysis assays of 37.5 nM SRP receptor (\blacktriangle) or 15 nM SRP receptor plus 75 nM SRP (\blacksquare) were conducted as in Fig. 1 except that the GTP concentration was varied between 0.2 and 5 μM . The GTP hydrolysis activity for SRP-SRP receptor complexes was calculated from a 15-min time point and is expressed as mol GDP \times SR $^{-1}$ \times min $^{-1}$. GTP hydrolysis rates for the SRP receptor were calculated from samples removed at 10-min intervals during a 40-min time course and these hydrolysis rates are expressed as mol GDP \times mol SR $^{-1}$ \times 10 min $^{-1}$. (B) Standard GTP hydrolysis assays containing 60 nM SRP, 15 nM SRP receptor, and 1 μM GTP were further supplemented with 0–10 μM GDP (\blacksquare) or 0–25 μM ATP (\bullet). The GTP hydrolysis activity of assays that did not contain competing ribonucleotide have been plotted at 0.02 μM . This value accurately reflects the 2% contamination of a GTP stock with GDP, but is an overestimate of the ATP contamination of the GTP stock.

1988). SRP and the SRP receptor were inactive in ATP hydrolysis when assayed either alone or in combination (data not shown). Secondly, the K_m for GTP in the hydrolysis assay should be similar to the concentration of GTP that is required for half maximal stimulation of the GTP-dependent protein translocation reaction. The K_m for GTP hydrolysis was determined for the SRP receptor and for the SRP-SRP receptor complex (Fig. 4 A). The K_m for GTP in the hydrolysis reaction was 3 μM in the presence of SRP and ~ 15 μM in the absence of SRP. Half-maximal stimulation of the GTP-dependent translocation reaction was estimated to occur at 1–2 μM GTP (Rapiejko and Gilmore, 1992). The latter determination is subject to greater experimental error due to

the presence of other GTPases in the membrane preparation used for protein translocation assays. The similarity between these two values suggests that the GTP hydrolysis assay monitors a GTPase cycle that is of direct relevance to the protein translocation reaction. The turnover number (k_{cat}) for the SRP receptor was estimated to be 0.16 mol of GTP hydrolyzed \times mol of SRP receptor $^{-1}$ \times min $^{-1}$. The turnover number (k_{cat}) for the SRP-SRP receptor complex was 2.1 mol of GTP hydrolyzed \times mol of SRP-SRP receptor complex $^{-1}$ \times min $^{-1}$.

GDP was found to be a potent inhibitor of the GTP hydrolysis assay with an apparent K_i of 0.25 μM (Fig. 4 B). In contrast, ATP was not inhibitory when present in a 25-fold molar excess relative to GTP. Inhibition of the GTP hydrolysis assay by GDP is anticipated given that GTP binding proteins have affinity for both GDP and GTP (Bourne et al., 1990; Brandt and Ross, 1985). Previously, we had reported that GDP was a competitive inhibitor of the GTP-dependent insertion of nascent chains into the endoplasmic reticulum (Connolly and Gilmore, 1986). Half-maximal inhibition occurred at a GDP concentration of ~ 1 μM when the Gpp(NH)p concentration was 10 μM . Together these results indicate that the GTP binding site detected in the GTP hydrolysis assay has a similar affinity for guanine ribonucleotides as the GTP binding site that was detected in the protein translocation reaction.

GTP Binding Activity of the SRP and the SRP Receptor

The preceding experiments indicate that formation of an equimolar complex between SRP and the SRP receptor is a prerequisite for an active GTP hydrolysis cycle. Activation of a GTP hydrolysis cycle could occur either by increasing the hydrolysis rate of protein bound GTP or by increasing the rate of guanine nucleotide exchange (Bourne et al., 1990). We sought to determine whether either SRP or the SRP receptor binds GTP under the experimental conditions used for the GTP hydrolysis assay. Aliquots from a GTP hydrolysis assay containing [α - ^{32}P] GTP were collected on nitrocellulose filters and washed by vacuum filtration to remove unbound guanine ribonucleotide. When the SRP receptor was tested for GTP binding, we detected 5 fmol of bound guanine ribonucleotide per 150 fmol of SRP receptor after a 50-min incubation (Fig. 5). Substoichiometric quantities of bound GTP were also detected when SRP was assayed for GTP binding. After a 50-min incubation with GTP, 24 fmol of [α - ^{32}P] GTP were bound per 700 fmol of SRP. Two different explanations for substoichiometric binding of [α - ^{32}P] GTP to SRP and the SRP receptor were considered. The dissociation rate for GTP may be rapid enough to permit ribonucleotide dissociation during the time required to wash the nitrocellulose filters. Alternatively, SRP and the SRP receptor may not readily bind GTP in the absence of guanine nucleotide exchange proteins.

Hydrolysis reactions containing both SRP and SRP receptor were assayed to determine the rate and stoichiometry of nucleotide binding to SRP-SRP receptor complexes (Fig. 5). The quantity of bound guanine ribonucleotide increased rapidly during the first three min of a GTP hydrolysis reaction. A first order rate constant of 0.62 min $^{-1}$ for binding of gua-

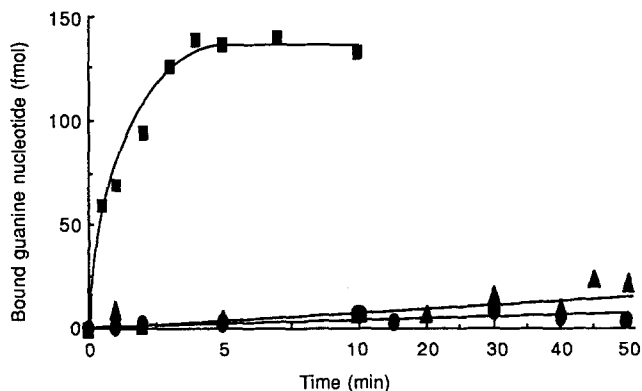


Figure 5. Quantitation of bound guanine ribonucleotide to SRP and the SRP receptor by filtration through nitrocellulose filters. Guanine ribonucleotide binding assays were conducted at 25°C in 50 mM TEA, 50 mM KOAc, 2.5 mM Mg(OAc)₂, 0.1% Nikkol, 1 mM DTT, 2 μM GTP (including 0.5 μM α-³²P GTP). The three time courses contained either 70 nM SRP (▲), 15 nM SRP receptor (●), or 70 nM SRP plus 15 nM SRP receptor (■). Aliquots were removed at frequent time intervals and the quantity of protein-bound guanine ribonucleotide in 10 μl of the reaction was determined as described in Materials and Methods. Note the compression of the abscissa after 10 min of the reaction.

nine ribonucleotide to the SRP-SRP receptor complex was calculated from the data shown in Fig. 5. We would expect to see occupation of all functional GTP hydrolysis sites during the first two minutes of incubation based upon a hydrolysis rate of 0.85 mol of GTP hydrolyzed × mol of SRP-SRP receptor complex⁻¹ × min⁻¹. The kinetics of ribonucleotide binding are in good agreement with the time course of GTP hydrolysis. Based upon the data presented in Figs. 2 and 3, SRP-SRP receptor complexes account for 90% of the 150 fmol of SRP receptor present in each sample. Hence, the plateau value of 135 fmol of bound ribonucleotide in each sample applied to the nitrocellulose filter is consistent with the detection of a single bound guanine ribonucleotide for each SRP-SRP receptor complex. Taken together these data suggest that the increase in GTP hydrolysis that occurs upon complex formation is due to an increase in the guanine ribonucleotide exchange reaction, rather than an increase in the rate at which protein bound GTP is hydrolyzed.

One possible explanation for the substoichiometric binding of GTP to SRP or the SRP receptor might be a lack of retention of one or both of these proteins by the nitrocellulose filter. As an alternative GTP binding assay, Sephadex G-50 gel filtration columns were used to separate protein bound ribonucleotide from free [α-³²P] GTP or GDP (Fig. 6). Protein bound ribonucleotide eluted in the void volume of the gel filtration column and was well resolved from unbound ribonucleotide (data not shown). Neither SRP nor the SRP receptor bound significant quantities of ribonucleotide when incubated with [α-³²P] GTP. In each case, the stoichiometry of bound ribonucleotide did not exceed the amount detected by the nitrocellulose filtration assay. Protein bound ribonucleotide was detected when GTP hydrolysis assays containing both SRP and the SRP receptor were applied to the G-50 gel filtration column. The elution of radiolabel with the protein was guanine nucleotide specific, as the addition of a 25-fold molar excess of unlabeled GTP, but not ATP,

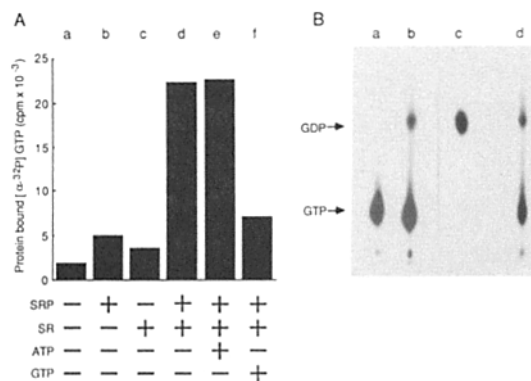


Figure 6. Quantitation of protein bound ribonucleotide by gel filtration chromatography. (A) GTP hydrolysis assays were conducted as in Fig. 1. The individual assays contained (a) no additions, (b) 60 nM SRP, (c) 7.5 nM SRP receptor, (d-f) 60 nM SRP and 7.5 nM SRP receptor. After 10 min at 25°C, samples e and f were adjusted to 25 μM ATP (e) or 25 μM GTP (f). After a total incubation of 20 min, the assays were chilled on ice and protein bound ribonucleotides were separated from unbound ribonucleotides by gel filtration chromatography at 4°C (see Materials and Methods). Protein bound ribonucleotide was quantitated by scintillation counting. (B) The load and selected eluate fractions from a gel filtration column were analyzed by thin layer chromatography on PEI cellulose plates. Aliquots were removed from GTP hydrolysis assays containing (lane a) no additions or (lane b) 60 nM SRP and 7.5 nM SRP receptor after 10 min at 25°C. The remainder of the assay in lane b was chilled on ice, and protein bound ribonucleotides (lane c) were separated from unbound ribonucleotides (lane d) by gel filtration chromatography.

significantly reduced the quantity of radiolabeled protein-bound guanine ribonucleotide. Thin layer chromatographic analysis of the protein bound radioactivity demonstrated that the bound ribonucleotide was primarily [α-³²P] GDP (Fig. 6B). Based upon results obtained in three separate experiments, the quantity of bound ribonucleotide detected by gel filtration chromatography was 0.5 mol of bound ribonucleotide per mol of SRP-SRP receptor complex. The lower binding stoichiometry obtained using this procedure may be due to dissociation of the ribonucleotide during chromatography, or incomplete recovery of the SRP-SRP receptor complex in the excluded volume fractions.

Discussion

Previous research from this laboratory demonstrated that GTP was required for the SRP receptor-mediated dissociation of SRP from the signal sequence of a nascent polypeptide (Connolly and Gilmore, 1989). Here, we have assayed SRP and the SRP receptor for GTP hydrolysis activity to gain insight into the functional significance of the GTP binding sites that have been detected in SRP54 and SRα by protein sequence comparisons (Bernstein et al., 1989; Connolly and Gilmore, 1989; Römisch et al., 1989). Several significant results were obtained from this analysis. Neither SRP nor the SRP receptor displayed robust GTP hydrolysis activities when assayed separately. Our maximal estimate for the intrinsic hydrolysis rate of the SRP (<0.002 min⁻¹) is comparable to the extremely low hydrolysis rates reported

for the Sec4 protein (Kabcenell et al., 1990) and the ras p21 protein (Gibbs et al., 1984). The intrinsic hydrolysis rate for the SRP receptor (0.02 min^{-1}) was lower than the value of 0.4 min^{-1} reported for G_i (Brandt and Ross, 1985), but comparable to rates reported for G_i (Sunyer et al., 1984) and G_p (Tamir et al., 1990). We were not able to detect a significant amount of protein bound ribonucleotide when the SRP receptor was incubated with GTP. For this reason, we cannot be certain that the GTPase activity displayed by our SRP receptor preparation was not due to a subpopulation of altered receptors or a protein contaminant. However, it should be noted that the low apparent K_m for GTP ($15 \mu\text{M}$) in the GTP hydrolysis assay may preclude detection of bound ribonucleotide to the SRP receptor using the nitrocellulose filtration method.

GTP binding proteins cycle between an active GTP-bound conformation and an inactive GDP-bound conformation (Milburn et al., 1990). The hydrolysis rate of GTP binding proteins is controlled by accessory proteins that catalyze guanine nucleotide exchange reactions or accelerate the hydrolysis of protein bound GTP (Bourne et al., 1990). Hence, the low hydrolytic activity of the SRP and the SRP receptor was not unexpected. Catalysis of the guanine ribonucleotide exchange reaction is the mechanism by which membrane bound hormone receptors accelerate the hydrolysis rate of signal transducing G-proteins (Gilman, 1987). The GTPase activating protein (GAP) preferentially interacts with the GTP-bound form of the *ras* p21 protein to stimulate hydrolysis of the protein bound GTP (Trahey and McCormick, 1987; Vogel et al., 1988). Accessory factors analogous to those identified for other GTP binding proteins are presumably required to initiate the GTP hydrolysis cycle for SRP and the SRP receptor. The observation that neither the SRP nor the SRP receptor bind stoichiometric quantities of GTP when tested separately suggests that the nucleotide binding sites in both proteins is either unoccupied, or is in the GDP-bound form in the absence of a guanine nucleotide exchange or release factor (GNRP). Based upon analogies to other GTP binding proteins, we propose that the GTP hydrolysis reactions catalyzed by SRP and SRP receptor are limited, at least in part, by the conditional binding of GTP to the guanine nucleotide binding sites. The initiation of a hydrolysis cycle upon combination of the two proteins strongly suggests that the conditional binding of GTP to at least one site is initiated upon formation of the SRP-SRP receptor complex. Hence, one of these proteins acts as a guanine nucleotide exchange factor for the other protein.

When SRP was added to hydrolysis assays containing SRP receptor, a saturable stimulation of GTP hydrolysis was observed. The hydrolysis activity was GTP specific as shown by the ability of GDP, but not ATP to act as a competitive inhibitor of the hydrolysis reaction. The results of the GTP hydrolysis assays were entirely consistent with the formation of a hydrolytically active bimolecular complex between SRP and SRP receptor. The ionic strength dependence of the hydrolysis assay matched the ionic strength dependence of the association between SRP and the membrane bound SRP receptor (Connolly et al., 1991; Walter and Blobel, 1983b). The reduced GTPase activity observed at physiological ionic strength should minimize nontranslocation associated GTP hydrolysis activity by SRP and SRP receptor within the cell.

Ribonucleotide binding assays detected a single bound

guanine nucleotide per SRP-SRP receptor complex during a GTP hydrolysis assay. However, the filter binding assays may underestimate the quantity of protein-bound ribonucleotide if dissociation of protein bound GTP or GDP occurs during washing of the nitrocellulose filter. These findings demonstrate that a minimum of one GTP binding site is hydrolytically active when the SRP-SRP receptor complex is formed. The experiments described in this manuscript do not allow us to determine which protein subunit (SR α , SR β , or SRP54) contains the GTP binding site that is active during the GTP hydrolysis reaction. However, the minimal proteins required for the GTP hydrolysis activity of the SRP-SRP receptor complex can be defined by combining partially reconstituted ribonucleoproteins derived from the SRP with the SRP receptor. This experimental strategy has revealed that SRP54 plus the 7S RNA comprise the minimal RNP that can form a hydrolytically active complex when combined with the SRP receptor (Poritz et al., 1990; Miller et al., 1993).

The binding affinity of the hydrolysis site for GTP is roughly 10–100-fold lower than that reported for other GTP binding proteins including *ras*, G_i (Brandt and Ross, 1985) and G_i (Sunyer et al., 1984). The lower affinity of the hydrolysis site for GTP is probably due to differences in the precise architecture of the GTP binding site in SR α or SRP54. The GTP binding sites in SR α and SRP 54 contain threonine instead of an asparagine in the third (NKXD) consensus element of the GTP binding site. In the H *ras* p21 protein, the primary role of the asparagine residue in the NKXD motif is to stabilize the nucleotide binding site by forming hydrogen bonds with several different elements of the nucleotide binding site (Pai et al., 1990). Clearly, the corresponding threonine residue within SR α or SRP54 may only make a subset of the hydrogen bonds ascribed to the asparagine residue present in more typical GTP binding proteins. Conversion of the atypical threonine residue in SR α to an asparagine residue by site directed mutagenesis led to the production of an SRP receptor with a 50–100-fold reduced affinity for guanine ribonucleotides (Rapiejko and Gilmore, 1992). The apparent affinity of the GTP hydrolysis site for GDP (apparent K_i of 250 nM) was more than 10-fold higher than the apparent affinity for GTP (K_m of 3.2 μM). More importantly, the K_m for GTP and K_i for GDP determined with the GTP hydrolysis assay were in good agreement with K_d and K_i values obtained for the GTP-dependent insertion of nascent polypeptides into the endoplasmic reticulum (Connolly and Gilmore, 1986; Rapiejko and Gilmore, 1992).

The ribonucleotide hydrolysis assay developed here presumably monitors a specific portion of the protein translocation reaction. Most likely, the reaction phase that is observed is initiated upon contact between the SRP-ribosome complex and the membrane bound SRP receptor. Recent experimental data indicate that SRP-dependent reactions that precede SRP receptor contact are not GTP-dependent (Zopf et al., 1993; Rapiejko, P., and R. Gilmore, manuscript in preparation). Hence, GTP hydrolysis cycles involving both SR α and SRP54 may initiate upon contact between the SRP-ribosome complex and the SRP receptor. As shown previously, contact between SRP and SRP receptor initiates a guanine nucleotide exchange reaction that ultimately results in dissociation of the signal sequence from SRP54 (Connolly and Gilmore,

1989; Connolly et al., 1991; High et al., 1991). Based upon the observation that point mutations in the GTP binding site of SR α block signal sequence dissociation from SRP54, we propose that binding of GTP to SR α is a prerequisite or a corequisite for GTP binding to SRP54 (Rapiejko and Gilmore, 1992; Rapiejko, P., and R. Gilmore, manuscript in preparation). Guanine ribonucleotide induced alterations in protein tertiary structure are believed to regulate the affinity between GTP binding proteins and downstream effector proteins (Bourne et al., 1990). Thus, binding of GTP to the G domain of SRP54 presumably reduces the affinity between the M-domain of SRP54 and the signal sequence. Experiments using nonhydrolyzable guanine nucleotides have shown that SRP-SRP receptor complexes are more stable when one or more of the GTP binding sites in the SRP-SRP receptor complex is occupied by the nonhydrolyzable GTP analogue Gpp(NH)p (Connolly et al., 1991). Formation of high affinity complexes between SRP and the SRP receptor is dependent upon a functional GTP binding site in SR α (Rapiejko and Gilmore, 1992) and requires the G-domain of SRP54 (Zopf et al., 1993). Taken together, these results suggest that the cyclic formation and dissociation of the SRP-SRP receptor complex probably involves two interlocking GTP hydrolysis cycles. In the context of a protein translocation reaction, GTP hydrolysis by the SRP-SRP receptor complex may be regulated by an auxiliary protein factor or GAP-like activity that monitors the successful insertion of the nascent polypeptide into the protein conducting channel in the ER (Simon and Blobel, 1991; Simon and Blobel, 1992). Recently, experimental data showing that a synthetic signal sequence can inhibit the GTP hydrolysis activity of SRP-SRP receptor complexes has been obtained (Miller et al., 1993).

To date, a function for the GTP binding site in the β subunit of the SRP receptor has not been proposed, due to a lack of data concerning the role of this subunit in the protein translocation reaction. Conceivably, the GTPase cycle of the β subunit might promote the cyclic assembly and disassembly of complexes between the SRP receptor and the SEC61 protein, as the latter polypeptide is believed to comprise the central core of the protein conducting channel (Görlich et al., 1992). Interestingly, the stoichiometry between membrane-bound ribosomes engaged in protein translocation and the SRP receptor is roughly 5:1 (Gilmore et al., 1982), suggesting that the SRP receptor may mediate ribosome targeting to a cluster of protein translocation channels. Further insight into the role of the three GTPases in SRP and the SRP receptor will be provided by the development of assay systems that incorporate additional components of the protein translocation machinery into reconstituted proteoliposomes.

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