Signal Sequence Recognition and Targeting of Ribosomes to the Endoplasmic Reticulum by the Signal Recognition Particle Do Not Require GTP

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The identification of GTP-binding sites in the 54-kDa subunit of the signal recognition particle (SRP) and in both the α and β subunits of the SRP receptor has complicated the task of defining the step in the protein translocation reaction that is controlled by the GTP-binding site in the SRP. Ribonucleotide binding assays show that the purified SRP can bind GDP or GTP. However, crosslinking experiments show that SRP54 can recognize the signal sequence of a nascent polypeptide in the absence of GTP. Targeting of SRP-ribosome-nascent polypeptide complexes, formed in the absence of GTP, to microsomal membranes likewise proceeds normally. To separate the GTPase cycles of SRP54 and the α subunit of the SRP receptor (SR α), we employed an SR α mutant that displays a markedly reduced affinity for GTP. We observed that the dissociation of SRP54 from the signal sequence and the insertion of the nascent polypeptide into the translocation site could only occur when GTP binding to SR α was permitted. These data suggest that the GTP binding and hydrolysis cycles of both SRP54 and SR α are initiated upon formation of the SRP-SRP receptor complex.

INTRODUCTION

Ribosomes engaged in the synthesis of proteins bearing an amino-terminal endoplasmic reticulum (ER)¹-specific signal sequence are cotranslationally recognized by the signal recognition particle (SRP). The SRP is a ribonucleoprotein particle consisting of six polypeptide subunits and the 7SL, or SRP RNA (Walter and Blobel, 1982). The SRP specifically binds to the signal sequence of the nascent polypeptide as it emerges from the large ribosomal subunit (Walter and Blobel, 1981a; Walter *et al.*, 1981). Crosslinking studies have shown that the 54-kDa subunit of the SRP (SRP54) corresponds to the signal sequence recognition site of the SRP (Kellaris *et al.*, 1991; Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). A carboxy-terminal methionine-rich domain (M-domain) of SRP54 contains the binding sites for both the 7SL RNA and the signal sequence (High and Dobberstein,

1991; Zopf *et al.*, 1990). Targeting of the SRP-ribosome-nascent polypeptide complex to the ER is mediated via the binding of the SRP to the SRP receptor, or docking protein. The SRP receptor is a heterodimeric integral membrane protein consisting of a 68-kDa α subunit and a 30-kDa β subunit (for review see Rapoport, 1992).

Targeting of the ribosome to the ER results in the subsequent insertion of the nascent polypeptide into a proteinaceous transport channel, or translocon, through which the nascent polypeptide traverses the membrane (Gilmore and Blobel, 1985; Simon and Blobel, 1991; Crowley *et al.*, 1993). Electrophysiological experiments have revealed protein transport channels in the ER that are proposed to open in response to signal sequences (Simon and Blobel, 1992) and have been shown to close upon ribosome detachment from the membrane (Simon and Blobel, 1991). Several ER integral membrane proteins including mp39 (Krieg *et al.*, 1989), translocating chain-associated membrane protein (TRAM) (Görllich *et al.*, 1992a), P37 (High *et al.*, 1991b), imp34 (Kellaris *et al.*, 1991), and the yeast Sec61 protein (Müsch *et al.*, 1992; Sanders *et al.*, 1992)

¹ Abbreviations used: DSS, disuccinimidyl suberate; ER, endoplasmic reticulum; GMPPNP, guanylyl-5'-imidodiphosphate; K-RM, SRP-depleted RM; RM, rough microsomal membranes; SRP, signal recognition particle; T₁-K-RM, trypsin-digested K-RM; TEA, triethanolamine-OAc.

have been identified as components of the translocation channel by chemical or photochemical crosslinking to translocating nascent polypeptides. The P37 protein, which may be identical to imp34, was shown to be the mammalian homologue of the yeast Sec61 protein (Görlich *et al.*, 1992b). Likewise, TRAM and mp39 are probably alternative designations for the same polypeptide (Görlich *et al.*, 1992a). The recent reconstitution of translocation-competent proteoliposomes from purified components has shown that the SRP receptor and the Sec61 complex are both essential for translocation of secretory polypeptides, whereas TRAM was found to stimulate translocation of some secretory proteins (Görlich *et al.*, 1993).

An analysis of the nucleotide dependence of the protein translocation reaction established that a reaction step that occurs before membrane insertion of the nascent polypeptide is dependent upon GTP (Connolly and Gilmore, 1986). This GTP-dependent reaction was subsequently localized to the SRP receptor-mediated dissociation of the SRP from the signal sequence (Connolly and Gilmore, 1989). When GTP hydrolysis is blocked by the substitution of guanylyl-5'-imidodiphosphate (GMPPNP) for GTP, the SRP fails to dissociate from the SRP receptor, thereby inhibiting subsequent rounds of nascent polypeptide chain targeting (Connolly *et al.*, 1991). Examination of the amino acid sequences of the α subunit of the SRP receptor (Connolly and Gilmore, 1989) and SRP54 (Bernstein *et al.*, 1989; Römisch *et al.*, 1989) revealed that these proteins contain the consensus protein sequences common to GTP-binding proteins (Dever *et al.*, 1987). The β subunit of the SRP receptor also contains a GTP-binding site (Ogg *et al.*, 1992). Several sequence motifs present in the GTP-binding domains of SR α , SRP54, and their yeast and bacterial homologues (Bernstein *et al.*, 1989; Hann *et al.*, 1989; Römisch *et al.*, 1989; Amaya *et al.*, 1990) suggest that these proteins belong to a distinct subfamily of GTP-binding proteins (Valencia *et al.*, 1991).

Defining the role of GTP in the translocation reaction is complicated by the presence of three GTP-binding sites (SR α , SR β , and SRP54) in these two interacting protein complexes. In a previous study we utilized site-directed mutagenesis of the GTP binding consensus motifs in SR α . This investigation revealed that nascent polypeptide chain insertion requires a functional GTP-binding site in SR α (Rapiejko and Gilmore, 1992) and suggested a model in which GTP binding to SR α represents the first committed step in nascent chain insertion (Rapiejko and Gilmore, 1993). Two studies that directly examined the GTP binding and hydrolysis activities of the SRP and the SRP receptor have determined that both protein complexes must be present for significant GTP hydrolysis to occur (Connolly and Gilmore, 1993; Miller *et al.*, 1993). To define a role for the putative GTP-binding site within SRP54, we have now focused our attention on the initial events in protein

translocation involving the binding of the SRP to the signal sequence. The experiments reported herein were designed as a systematic dissection of the SRP-mediated translocation scheme. At each point, SRP function was tested in the absence or presence of guanine ribonucleotides to determine whether occupancy of the GTP binding site within SRP54 was required before contact between the SRP and the SRP receptor.

MATERIALS AND METHODS

Preparation of Microsomal Membranes, SRP, K-RM, and T₅-K-RM

SRP and SRP-depleted rough microsomal membranes (K-RM) were prepared from canine pancreas rough microsomal membranes (RM) (Walter and Blobel, 1983) using procedures described previously (Walter *et al.*, 1981). K-RM were digested with trypsin (5 μ g/ml) as described previously (Gilmore *et al.*, 1982) to prepare microsomal membranes (T₅-K-RM) that lack the 52-kDa cytoplasmic domain of the α subunit of the SRP receptor.

Guanine Ribonucleotide Binding to SRP

The binding of [α ³²P]GDP and [α ³²P]GTP to the SRP was measured using a filter binding assay described previously (Connolly and Gilmore, 1993). Briefly, 1 μ M [α ³²P]GTP or [α ³²P]GDP (400 Ci/mmol) was incubated with purified SRP (650 fmol) in binding buffer (50 mM triethanolamine-OAc [TEA] pH 7.5, 50 mM KOAc, and 2.5 mM Mg(OAc)₂) for 20 min at 25°C. The amount of [³²P]-labeled GTP or GDP bound to SRP was determined by taking three aliquots (7 μ l) of each of the binding reactions, adjusting them to 250 μ l with ice-cold binding buffer that contained 20% PEG-6000, and subjecting them to vacuum filtration through nitrocellulose filters (Schleicher and Schuell, Keene, NH) after incubation on ice for 30 sec. The filters were washed four times with 1.5 ml ice-cold binding buffer, and the dried filters were subjected to scintillation counting. Background counts were determined in reactions that lacked SRP.

[α ³²P]GDP was prepared by enzymatic digestion of [α ³²P]GTP (Amersham, Arlington Heights, IL). Two micromolars [α ³²P]GTP (400 Ci/mmol) was incubated for 3 min at 25°C in 50 mM TEA (pH 7.5), 20 mM glucose, 1 mM Mg(OAc)₂, and 0.4 U/ μ l of hexokinase. The enzyme was inactivated by a 3-min incubation at 90°C. The conversion of [α ³²P]GTP to [α ³²P]GDP was monitored by chromatography on polyethyleneimine cellulose thin layer plates as described previously (Connolly and Gilmore, 1993).

Cell-free Transcription and Translation

The plasmid pDM9G (Connolly *et al.*, 1989), containing a cDNA encoding the G protein of vesicular stomatitis virus, was linearized within the coding region by digestion with the restriction endonuclease *Hin*FI. A truncated mRNA transcript (pG64) was prepared by *in vitro* transcription using SP6 RNA polymerase (Gurevich *et al.*, 1991) and was isolated by extraction with phenol-chloroform followed by successive precipitations with ethanol and with lithium chloride. The plasmids pG4 α and pG4 α 3-2 were transcribed with T7 RNA polymerase to obtain mRNA encoding the wild-type and mutant forms of the α subunit of the SRP receptor (Rapiejko and Gilmore, 1992). The point mutant, designated SR α 3-2, contains a single amino acid substitution at residue 588 of SR α (Thr to Asn).

Separation of Ribonucleotides from In Vitro-Assembled Polysomes

The pG64 mRNA transcript was translated in a wheat germ cell-free translation system using standard procedures (Connolly *et al.*, 1989).

A 100- μ l wheat germ translation reaction containing 1 μ g of pG64 mRNA transcript was allowed to proceed for 15–20 min at 25°C before adjustment to 250 μ M cycloheximide. Free ribonucleotides were separated from ribosome-bound nascent polypeptides by gel filtration chromatography as described previously (Connolly and Gilmore, 1986). The 1-ml Sephacryl S-200 gel filtration columns were equilibrated with 50 mM TEA (pH 7.5), 1 mM dithiothreitol (DTT), 100 mM KOAc, 5 mM Mg(OAc)₂, and 0.002% Nikkol (octaethyleneglycol-mono-N-dodecyl ether, Nikko Chemical, Tokyo, Japan) (buffer A).

The mRNA transcripts encoding SR α and SR α 3-2 were translated in a rabbit reticulocyte lysate (Promega Biotech, Madison, WI) translation system (Jackson and Hunt, 1983) at a concentration of 20 ng/ μ l. Trypsin digested K-RM (T₅-K-RM) were repopulated with wild-type (SR α) or mutant (SR α 3-2) SRP receptors as described previously (Andrews *et al.*, 1989; Rapiejko and Gilmore, 1992). Ribonucleotides were separated from repopulated membranes by gel filtration chromatography using a 1-ml Sepharose CL2B column (Rapiejko and Gilmore, 1992).

Crosslinking of Nascent Polypeptides to Translocation Components

The nascent pG64 polypeptide was crosslinked to translocation components by reaction with 1 mM disuccinimidyl suberate (DSS) for 20 min at 25°C as described previously (Kellaris *et al.*, 1991) unless otherwise indicated. The 50 mM DSS stock solution in dimethyl sulfoxide (DMSO) was freshly prepared before each experiment. Control samples that lacked DSS were also adjusted to 2% DMSO. The crosslinking reactions were quenched by addition of one-tenth volume of 1 M glycine in 0.1 M NaHCO₃ (pH 8.0) or 1 M ethanolamine (pH 8.0). The crosslinked products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12–20% polyacrylamide gradient gels.

Targeting of Nascent Polypeptides to Microsomal Membranes

Ribonucleotide-depleted pG64 polysomes were incubated for 15 min at 25°C with K-RM in a buffer that contained 40 mM TEA, 110 mM KOAc, 1.7 mM Mg(OAc)₂, 0.0012% Nikkol, 0.6 mM DTT, and 50 mM sucrose. To assess targeting of pG64 polysomes to membranes, the 30- μ l reactions were layered over 50 μ l sucrose cushions (0.5 M sucrose, 50 mM TEA, 100 mM KOAc, 2.5 mM Mg(OAc)₂, 1 mM DTT) and centrifuged in a Beckman airfuge (Fullerton, CA) using the A-100/30 rotor for 3 min at 20 psi as described previously (Connolly and Gilmore, 1986). To fractionate the products of 200- μ l crosslinking reactions, four 50- μ l aliquots of each sample were layered over separate 50 μ l sucrose cushions, and the appropriate supernatant and pellet fractions were combined after centrifugation. The supernatants were precipitated with 10% trichloroacetic acid before solubilization for SDS-PAGE, whereas the pellets were solubilized directly for SDS-PAGE.

Quantification of Radioactive Species

Radioactive products that had been resolved by SDS-PAGE were quantified by densitometric scanning of autoradiograms using a soft laser scanning densitometer (Biomed Instruments, Fullerton, CA).

RESULTS

Guanine Ribonucleotide Binding to SRP

The previous assays used to detect a GTP-dependent step in the protein translocation reaction monitored the GTP-dependent delivery of the nascent polypeptide to membrane-bound components of the translocation complex (Connolly and Gilmore, 1986, 1989). Experi-

mentally, SRP-ribosome-nascent polypeptide complexes were assembled by *in vitro* translation, unbound ribonucleotides were removed, and the *in vitro*-assembled polysomes were incubated with microsomal membranes in the absence or presence of GTP or GTP analogues. Although this approach demonstrated a GTP-dependent step for nascent chain insertion, this analysis could not have detected a GTP requirement for SRP function if signal sequence recognition or targeting of SRP-ribosome complexes to the membranes were dependent upon GTP binding to SRP54 during the *in vitro* translation phase of the assay. Because the *in vitro* translation system contained both SRP and guanine ribonucleotides, the nucleotide bound to SRP54 could be either GTP or GDP.

Because purified GTP binding proteins frequently contain bound GDP (Ferguson *et al.*, 1986; Kahn and Gilman, 1986), the GTP-binding site in SRP54 could conceivably contain GDP when SRP is added to the *in vitro* system. Recent studies have shown that GTP hydrolysis occurs upon formation of complexes between the SRP and the SRP receptor (Connolly and Gilmore, 1993). Furthermore, experiments using purified preparations of SRP have shown that photolabeling of SRP54 by GDP or GTP is markedly enhanced upon inclusion of the SRP receptor (Miller *et al.*, 1993). To obtain additional information concerning the stoichiometry of GDP binding to SRP54, the ability of SRP to bind 1 μ M [α ³²P]GDP was measured using a nitrocellulose filtration assay (Connolly and Gilmore, 1993). Binding of [α ³²P]GDP to the SRP was readily detected after a 20-min incubation with the ribonucleotide (Figure 1A). Comparable results were obtained after briefer incubations of SRP with [α ³²P]GDP. Typically, the quantity of bound [α ³²P]GDP detected by this procedure was equivalent to 25% occupancy of the SRP54 site. In agreement with previous results (Connolly and Gilmore, 1993), the stoichiometry of GTP binding to the SRP detected using this assay was much lower, with ~4% of the SRP containing bound [α ³²P]GTP. The specificity of GDP binding to the SRP was examined in nucleotide competition experiments using 1 μ M [α ³²P]GDP and either 100 μ M or 1 mM GMPPNP, GDP, or GTP (Figure 1B). As expected, the protein bound [α ³²P]GDP was reduced to background levels when a 100- to 1000-fold excess of unlabeled GDP was included. Despite the fact that substoichiometric amounts of protein-bound GTP were detected by the filtration assay, the inclusion of GTP reduced the protein bound GDP by more than 10-fold (Figure 1B), indicating that SRP54 can bind either GTP or GDP in the absence of a guanine nucleotide exchange factor. The nonhydrolyzable GTP analogue GMPPNP was less effective as a competitive inhibitor of GDP binding when tested at either 100- or 1000-fold molar excess (Figure 1B). When 1 mM GMPPNP was included as the competing ribonucleotide, GDP binding was reduced fourfold. The competition exper-

Figure 1. Guanine ribonucleotide binding to SRP. Purified SRP was incubated in the presence of 1 μ M [α^{32} P]GTP or 1 μ M [α^{32} P]GDP in 50 mM TEA (pH 7.5), 50 mM KOAc, and 2.5 mM Mg(OAc)₂ for 20 min at 25°C. The amount of protein-bound guanine ribonucleotide was determined as described under MATERIALS AND METHODS. (A) The data presented represent the mean $\pm$ SEM for [α^{32} P]GDP and [α^{32} P]GTP binding ($n = 6$ and $n = 4$, respectively) to 180 fmol of purified SRP. (B) Competition of [α^{32} P]GDP binding to purified SRP by GDP, GTP, or GMPPNP. The 1 mM GMPPNP and 100 μ M GTP data points represent the average of triplicate determinations from a single experiment, whereas all other points are the averages of triplicate determinations taken from three separate experiments. (C) Time course for the dissociation of bound [α^{32} P]GDP from SRP. SRP that had been incubated in the presence of 1 μ M [α^{32} P]GDP for 20 min at 25°C was adjusted to 1 mM GDP, and the amount of [α^{32} P]GDP bound to the SRP was determined at time points ranging from 1 to 30 min.

iments suggest that the rate of GTP dissociation from SRP is rapid enough to prevent detection of stoichiometric amounts of bound GTP by the nitrocellulose filtration method.

The rate of GDP dissociation from the SRP at 25°C was examined by diluting SRP containing prebound [α^{32} P]GDP with an equal volume of binding buffer that contained 2 mM unlabeled GDP (Figure 1C). Nearly quantitative dissociation of bound [α^{32} P]GDP from the SRP occurred within the first few minutes. These data indicate that binding of GDP to SRP is readily reversible, consistent with a rapid GDP-dissociation rate that we estimate to have a half-time of <1 min. Photoaffinity labeling of SRP with GDP and GTP has shown that nucleotide binding to SRP54 is characterized by a rapid exchange of bound guanine ribonucleotide in the absence of the SRP receptor (Miller *et al.*, 1993). SRP bound [α^{32} P]GDP was also quantified by gel filtration chromatography at 4°C. Although protein bound GDP

was readily detected by gel filtration chromatography, the yield of bound nucleotide was lower than that measured by filter binding, suggesting that the GDP dissociation rate is relatively rapid even at 4°C. Thus, the ribonucleotide binding site in SRP54 is almost certainly in the empty-site conformation when the SRP is added to a translocation reaction because of the time required to purify the SRP relative to the rate of nucleotide dissociation. In the experiments described below, we have tested early steps in the translocation reaction for nucleotide-dependence based upon the finding that the nucleotide binding status of SRP can be controlled by the selective addition of individual ribonucleotides or ribonucleotide analogues. Because of the rapid dissociation of bound ribonucleotides from SRP54, attempts to preload SRP with GTP, GDP, or GMPPNP before addition to a translocation reaction were not effective.

Signal Sequence Recognition and Membrane Targeting of SRP-Ribosome Complexes

To determine if the early, SRP-mediated reaction steps of protein translocation were GTP dependent, we modified our previous assay to control the GTP content during incubation of SRP with ribosome-bound nascent polypeptides. A termination codon deficient mRNA transcript encoding the first 64 amino acids of the envelope glycoprotein (G protein) of the vesicular stomatitis virus (pG64) was translated in a wheat germ system to produce a ribosome-bound nascent polypeptide that would serve as a suitable substrate for recognition by the SRP. After translation of pG64 in the presence of [35 S] methionine and in the absence of SRP, unbound ribonucleotides were removed by gel filtration chromatography. Control experiments using [α^{32} P]GTP have shown that the total concentration of guanine ribonucleotides is reduced to <10 nM in the column eluate (Connolly and Gilmore, unpublished data). Aliquots of the pG64 polysomes were then incubated for 10 min with canine SRP in the presence or absence of 1 mM GTP, GDP, or GMPPNP. The length of this incubation was chosen based on previous results showing that the SRP-dependent steps in a protein translocation reaction occur within 5 min as determined in a synchronized translation experiment (Walter and Blobel, 1981b). Binding of SRP54 to the signal sequence of pG64 can be monitored by PAGE in SDS after treatment of SRP-ribosome-pG64 complexes with the crosslinking reagent DSS (Kellaris *et al.*, 1991). If GTP binding to SRP54 were a prerequisite for signal sequence recognition, we reasoned that the yield of the crosslinked product between SRP54 and pG64 would be enhanced in the presence of GTP or GMPPNP and reduced in the presence of GDP. Formation of the 61-kDa crosslinked product (designated by the asterisk) between pG64 and SRP54 required the presence of both DSS and canine SRP (Figure 2). Similar yields of the crosslinked product

were obtained in reactions that included GDP (Figure 2, lane 6), GTP (Figure 2, lane 9), GMPPNP (Figure 2, lane 12), or no added ribonucleotide (Figure 2, lane 3). The efficiency of crosslink formation between SRP54 and the nascent polypeptide was comparable to that observed with an unfractionated translation extract. These data suggest that the initial binding of the M-domain of SRP54 to the signal sequence of the nascent polypeptide does not depend upon GTP binding to the G-domain of SRP54.

Previous research has shown that targeting of polysomes to the ER requires SRP recognition of the signal sequence followed by a functional interaction between the SRP-ribosome complex and the SRP receptor (Walter and Blobel, 1981a; Gilmore and Blobel, 1985; Connolly and Gilmore, 1986). A potential requirement for GTP during delivery of SRP-ribosome complexes to the membrane was addressed by comparing the targeting efficiency of SRP-ribosome-pG64 complexes that were assembled in the presence or absence of GTP (Figure 3). The truncated pG64 mRNA transcript was translated either in the presence (Figure 3, A and B) or absence (Figure 3, C–F) of SRP to prepare ribosome-pG64 complexes. After removal of free GTP from both translation reactions, SRP was added to two aliquots from the sample that did not contain SRP during the *in vitro* translation reaction (Figure 3, samples C and D). Thus, Figure 3, samples A and B, differ from samples C and D with respect to the presence of GTP, provided by the *in vitro* translation system, at the point during the experiment when SRP was initially incubated with the ribosome-bound nascent polypeptide. Targeting of SRP-ribosome complexes to the membrane bound SRP receptor is assayed by cosedimentation of the radiolabeled nascent polypeptide with the membrane under physiological ionic strength conditions (Connolly and Gilmore, 1986). None of the samples were supplemented with GTP during the subsequent incubation of aliquots (Figure 3, B, D, and F) with microsomal membranes. As an additional control, ribosome-pG64 complexes prepared in the absence of SRP were assayed for targeting in the absence of SRP (Figure 3, E and F). In the absence of microsomal membranes, the pG64 nascent polypeptide was recovered in the supernatant (S) fraction (Figure 3, A, C, and E). A significant fraction (40%) of the pG64 polysomes was recovered in the pellet (P) fraction when microsomal membranes were included (Figure 3, B and D). Targeting of pG64 polysomes to the microsomal membranes was SRP dependent as shown by the recovery of >80% of the pG64 nascent polypeptide in the supernatant fraction when SRP was omitted (Figure 3F). The targeting efficiency of the SRP-ribosome-pG64 complex to the membrane was comparable in Figure 3, samples B and D, as determined by densitometric scanning of the autoradiogram. The most reasonable interpretation of these results is that binding of GTP to SRP54 is not a prerequisite for targeting of the SRP-

Figure 2. Binding of SRP to a signal sequence does not require GTP. Ribosomes bearing the pG64 nascent polypeptide were assembled by *in vitro* translation in the absence of SRP. After removal of unbound ribonucleotides by gel filtration chromatography in the presence of buffer A adjusted to 200 mM KOAc, aliquots of the ribosome-pG64 complexes (12.5 μ l) were incubated at 25°C in a final volume of 19 μ l with (lanes 1, 3, 4, 6, 7, 9, 10, and 12) or without (lanes 2, 5, 8, and 11) 32 nM SRP and in the absence (lanes 1–3) or presence of 1 mM GDP (lanes 4–6), 1 mM GTP (lanes 7–9), or 1 mM GMPPNP (lanes 10–12). After 10 min of incubation, DSS was added at a concentration of 1 mM to crosslink pG64 to adjacent proteins (see MATERIALS AND METHODS). The radiolabeled polypeptides corresponding to pG64 and the 61-kDa crosslinked product between SRP54 and pG64 (*) were resolved by PAGE in SDS.

ribosome complex to the microsomal membrane. Taken together with previous results (Connolly and Gilmore, 1986), we can conclude that targeting of the SRP-ribosome complex to the membrane bound SRP receptor is not dependent upon GTP binding to either SR α or SRP54.

GTP-dependent Insertion of Nascent Chains into Microsomal Membranes

Because one goal of the current work was to differentiate between the GTP binding and hydrolysis cycles of SRP54 and SR α , ribonucleotide-depleted ribosome-pG64 complexes were incubated with SRP and K-RM to determine whether the modified assay system could be used to analyze the subsequent steps in the protein translocation reaction (Figure 4). Sequential events in the translocation reaction were monitored by crosslinking of pG64 to previously identified translocation components with DSS. Ribonucleotide-depleted ribosome-pG64 complexes were preincubated with purified SRP before the addition of guanine ribonucleotides and microsomal membranes. This preincubation step, which results in binding of SRP to the signal sequence of pG64, prevents the sequestration of SRP in a GMPPNP-stabilized SRP-SRP receptor complex that can form when SRP is incubated with microsomal membranes in the

Figure 3. Targeting of SRP-ribosome complexes to the ER membrane is not dependent upon GTP binding to SRP54. The pG64 mRNA was translated in a wheat germ system in the presence (A and B) or absence (C, D, E, and F) of 60 nM SRP. The translations were adjusted to 250 μ M cycloheximide and subjected to gel filtration in the presence of buffer A adjusted to 150 mM KOAc and 2.5 mM Mg(OAc)₂. Aliquots (18 μ l) of the ribonucleotide-depleted pG64-ribosomes were supplemented with 48 nM SRP (C and D) and 3 eq of K-RM (B, D, and F). Targeting of the pG64 polypeptides to the membrane was assayed by airfuge centrifugation as described in MATERIALS AND METHODS. The radioactive pG64 polypeptide that was recovered in supernatant (S) and membrane pellet (P) fractions was resolved by PAGE in SDS.

presence of GMPPNP (Connolly *et al.*, 1991). In the absence of guanine ribonucleotides, incubation of the SRP-ribosome-pG64 complexes with K-RM did not result in dissociation of SRP from the signal sequence of pG64, as shown by the production of the 61-kDa crosslinked product between SRP54 and the nascent polypeptide (Figure 4, lane 3). The yield of the 61-kDa product decreased when GTP or GMPPNP was included during the incubation with K-RM (Figure 4, lanes 4 and 5). The disappearance of the 61-kDa crosslinked product was accompanied by the concomitant appearance of a more rapidly migrating crosslinked product of $\sim$ 43–45 kDa that is designated by the open triangle. The latter product corresponds to pG64 crosslinked to a nonglycosylated integral membrane protein with an apparent molecular weight of 34–37 kDa (imp34). The imp34 protein, which was identified in a previous analysis of membrane-bound translocation intermediates (Kellaris *et al.*, 1991), is a component of the translocation channel and is likely identical to P37 and Sec61 (High *et al.*, 1991b, 1993; Görlich *et al.*, 1992b). Therefore, the appearance of the 45-kDa crosslinked product is indicative of delivery of the nascent polypeptide to the translocation channel in the microsomal membrane. The efficiency of crosslink formation between imp34 and an adjacent nascent polypeptide was previously estimated to be 5–10% (Kellaris *et al.*, 1991). Although the efficiency of crosslink formation between pG64 and imp34 is not identical to that between pG64 and SRP54, the disappearance of the 61-kDa crosslinked product, along with the appearance of the 45-kDa crosslinked product, provides markers for both the release of the nascent polypeptide from the SRP and its subsequent delivery to the translocation channel in the microsomal membrane. As a control, trypsinized microsomal membranes (T₅-K-RM) that lack SRP receptor were added instead of intact K-RM (Figure 4, lanes 6–8). When the exper-

imental samples contained T₅-K-RM, the yield of the 61-kDa product was not significantly reduced by the inclusion of guanine ribonucleotides (Figure 4, lanes 6–8). The results of this experiment are consistent with previous observations concerning the role of the SRP receptor in the dissociation of the signal sequence from SRP (Gilmore and Blobel, 1983; Connolly and Gilmore, 1989). We conclude that the modified assay system contains all the protein components that are required for the delivery of the nascent polypeptide to the translocation channel. Thus, we need not be concerned that the experimental procedure used to deplete ribonucleotides has coincidentally removed a guanine nucleotide exchange factor that is required for the protein translocation reaction.

In the preceding experiment, dissociation of the signal sequence from SRP54 occurred under conditions that should permit ribonucleotide binding to both SRP54 and SR α . Conceivably, signal sequence dissociation from SRP54 could be initiated by binding of GTP to SRP54, with a subsequent reaction step being dependent upon GTP binding to SR α . We next asked whether the GTP-dependent dissociation of SRP54 from the signal sequence could occur with GTP binding to SRP54 alone, independent of GTP binding to SR α . To accomplish this goal, we utilized microsomal membranes that contained SRP receptors bearing a point mutation in the

Figure 4. GTP dependence of crosslink formation between pG64 and imp34. The pG64 mRNA was translated in the absence of SRP, and ribonucleotides were removed by gel filtration chromatography in the presence of buffer A. Aliquots (7.5 μ l) of the ribonucleotide-depleted pG64-ribosomes were supplemented with 17 nM SRP (lanes 3–8) and preincubated for 10 min at 25°C before the addition of 5 eq of K-RM (lanes 3–5), or 5 eq T₅-K-RM (lanes 6–8) and ribonucleotides. The samples were adjusted to a final volume of 30 μ l and were incubated in the presence of buffer A containing 1 mM GTP (lanes 1, 2, 4, and 7), 1 mM GMPPNP (lanes 5 and 8), or no ribonucleotides (lanes 3 and 6) for 10 min at 25°C. The samples shown in lanes 2–8 were adjusted to 1 mM DSS and were incubated for 10 min at 25°C to crosslink pG64 to adjacent translocation components. The radio-labeled polypeptides corresponding to uncrosslinked pG64, and the 45- (Δ) and 61-kDa (*) crosslinked products were resolved by PAGE in SDS.

GTP binding site of SR α . Membranes with altered SRP receptors can be prepared by *in vitro* translation of mRNA encoding the mutant SR α in the presence of trypsin-digested microsomal membranes that lack the endogenous SR α (Rapiejko and Gilmore, 1992). One SRP receptor mutant (SR α 3-2) displays a 50- to 100-fold reduced affinity for GTP relative to the wild-type SR α as determined using a GTP-dependent translocation assay and a GMPPNP-dependent SRP-SRP receptor complex formation assay (Rapiejko and Gilmore, 1992).

Using membranes repopulated with SR α 3-2, the GTP binding and hydrolysis cycle of SR α can be selectively controlled by adjustment of the GTP concentration. Ribosome bound nascent polypeptides were assembled by translation of the pG64 mRNA in the absence of SRP. Crosslinking with DSS was used to evaluate the distribution of pG64 between SRP-bound and imp34-accessible forms. The crosslinked products were separated into soluble and membrane-bound fractions by differential centrifugation using physiological salt conditions (Figure 5). The supernatant fraction (S) should contain translocation intermediates that precede targeting of the SRP-ribosome complex to the membrane-bound SRP receptor, hence most of the pG64 nascent chains and essentially all of the 61-kDa crosslinked product was recovered in the supernatant fraction when pG64-ribosome complexes were incubated with mock repopulated T₅-K-RM and 1 mM GTP (Figure 5A). The corresponding pellet fraction (P) was devoid of both the 61- (*) and 45-kDa (Δ) crosslinked products, indicating that both the targeting and insertion phases of the translocation reaction were blocked. Approximately 12% of the uncrosslinked pG64 cosedimented with the mock repopulated T₅-K-RM in Figure 5, sample A. Additional control experiments, conducted to determine what factors were responsible for the recovery of this amount of pG64 in the pellet fraction, indicated that half of this background sedimentation occurs in the absence of both SRP and T₅-K-RM. The remainder is SRP independent and can be attributed to nonspecific sticking of pG64 to the microsomal membranes. The background contributed by these sources to the sedimentation of pG64 in the pellet fraction is negligible in comparison to the amount recovered under condition where bona fide insertion occurs.

Repopulation of T₅-K-RM with wild-type SR α reduced the amount of the 61-kDa product that was recovered in the supernatant fraction and caused the appearance of the 45-kDa crosslinked product in the membrane pellet fraction (Figure 5, B and C). Increasing the GTP concentration from 10 μ M (Figure 5, sample B) to 1 mM (Figure 5, sample C) caused a 25% increase in the amount of the 45-kDa crosslinked product in the pellet fraction. Membrane-targeted SRP-ribosome pG64 complexes were also observed when the trypsinized

Figure 5. Binding of GTP to SR α is a prerequisite for signal sequence dissociation from SRP54. Ribonucleotide-depleted ribosome-pG64 complexes, assembled by translation of the truncated pG64 transcript followed by gel filtration chromatography, were preincubated with 17 nM SRP in the absence of ribonucleotides for 5 min at 25°C. Aliquots (5 μ l) of these complexes were incubated with 150 μ l of a ribonucleotide-depleted repopulation reaction that contained T₅-K-RM that were either mock repopulated (A), repopulated with the wild-type SR α (B and C), or the mutant SR α 3-2 (D-F) for 20 min at 25°C in the presence of GTP at 10 μ M (B and D), 1 mM (A, C, and E), or 3 mM (F) in a total volume of 175 μ l before crosslinking in the presence of 200 μ M DSS for 2 min at 25°C. These samples were further processed into supernatant (S) and pellet (P) fractions by airfuge centrifugation over sucrose cushions adjusted to 5 mM Mg(OAc)₂ followed by SDS-PAGE as described in MATERIALS AND METHODS. One hundred fifty microliters of a ribonucleotide-depleted repopulation reaction contains ~25 eq of membranes. The radioactive species indicated by (#) represents a non-SRP dependent crosslinked product.

membranes were repopulated with the wild-type SR α as shown by the >10-fold increase in the quantity of the 61-kDa crosslinked product that was recovered in the membrane pellet fractions (Figure 5, B and C) as compared to the mock repopulated membranes (Figure 5A). Targeting and membrane insertion of pG64 was also confirmed by the enhanced recovery of the non-crosslinked nascent polypeptide in the membrane pellet fraction (Figure 5, B and C). The relative distribution of pG64 between the 45- and 61-kDa crosslinked products was similar when the experimental samples contained K-RM and either 10 μ M or 1 mM GTP. Thus, 10 μ M GTP is sufficient for all reaction steps that lead to membrane insertion of the nascent polypeptide.

At 10 μ M GTP, membranes repopulated with the mutant SR α 3-2 were defective in membrane insertion of the nascent polypeptide as judged by the paucity of the 45-kDa crosslinked product in the pellet fraction (Figure 5D). The total yield of the 61-kDa product in the supernatant and pellet fractions was comparable to that obtained in assays containing the mock-repopulated T₅-K-RM (Figure 5A). Consistent with the accumulation of a membrane-targeted SRP-ribosome complex, the amount of the 61-kDa crosslinked product recovered in the pellet fraction (Figure 5D) was observed to be 13-

fold higher than that in the pellet fraction of mock repopulated membranes (Figure 5A). Increasing the concentration of GTP to 1 mM resulted in the formation of the 45-kDa crosslinked product, a reduction in the total yield of the 61-kDa product and an increase in the amount of pG64 recovered in the membrane pellet fraction to 23% (Figure 5E). Comparison of this sample (Figure 5E) to the mock repopulated control (Figure 5A) indicates that the alterations in the distribution of the crosslinked products can be attributed to the GTP-dependent activity of the SR α 3-2 mutant. The translocation efficiency of membranes repopulated with SR α 3-2 is not equivalent to that of membranes repopulated with the wild-type SR α even when 1 mM GTP is present (Rapiejko and Gilmore, 1992), therefore the reduced yield of the 45-kDa crosslinked product observed in the pellet fractions was not unexpected (compare Figure 5, E and C). Although a similar amount of 45-kDa crosslinked products were obtained after incubation in the presence of 3 mM GTP (Figure 5F), the amount of pG64 recovered in the pellet fraction was further increased to 36%. These data clearly demonstrate that membranes repopulated with the mutant SR α 3-2 respond to GTP in a concentration-dependent manner. Furthermore, the results presented here strongly suggest that SRP54 cannot dissociate from the signal sequence until the GTP binding site in SR α is occupied by GTP.

DISCUSSION

Whereas the participation of GTP-binding proteins in the protein translocation reaction has been established (Connolly and Gilmore, 1986), an elucidation of the precise role for each of the three GTP-binding sites that have now been identified in SRP and the SRP receptor has proven to be a problem of considerable complexity. Previous studies have shown that the SRP receptor-mediated dissociation of SRP from the signal sequence is a GTP-dependent reaction (Connolly and Gilmore, 1989; Connolly *et al.*, 1991; High *et al.*, 1991a). Thus, at least one of the three GTP-binding sites in the SRP and the SRP receptor functions at this point during the protein translocation reaction. Previous analysis of the GTP-binding site within SR α by site-directed mutagenesis demonstrated that a functional GTP-binding site in SR α was required for translocation of proteins across the ER (Rapiejko and Gilmore, 1992). More recent studies, which have directly measured the binding and hydrolysis of GTP by purified preparations of the SRP and the SRP receptor, indicate that GTP hydrolysis is observed upon formation of complexes between SRP and the SRP receptor (Connolly and Gilmore, 1993; Miller *et al.*, 1993). Moreover, GTP hydrolysis was observed when the SRP receptor was combined with a minimal ribonucleoprotein particle consisting of SRP54 and the SRP RNA (Miller *et al.*, 1993). Whereas significant binding of GTP to SRP is not readily detected using a

nitrocellulose filtration method (Connolly and Gilmore, 1993), we have observed significant, though substoichiometric, binding of GDP to the SRP. Furthermore, GDP rapidly dissociates from the SRP, suggesting that the SRP is likely in an empty-site conformation when isolated. The higher affinity for GDP relative to GTP suggests that SRP54 remains in the inactive conformation, requiring the presence of a guanine nucleotide exchange factor to initiate stable GTP binding.

Here we have examined several sequential events in the protein translocation reaction to determine whether either of two previously described functions of the SRP, nascent chain binding or ribosome-targeting to microsomal membranes, are dependent upon occupancy of the GTP binding site in SRP54. The analysis of the GTP requirements for SRP54 function was facilitated by the use of a nascent polypeptide, pG64, that remains bound to the ribosome as a peptidyl-tRNA. The pG64-ribosome complex is a suitable substrate for recognition by SRP in the absence of ongoing translation, presumably because of the lack of a folded structure that could occlude the signal sequence. Binding of SRP to the signal sequence of pG64 was detected by the formation of the well characterized crosslinked product between SRP54 and the signal sequence of a nascent polypeptide (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986; Kellaris *et al.*, 1991). Likewise, membrane insertion of the nascent polypeptide was monitored by formation of a 45-kDa crosslinked product between the nascent polypeptide and a component of the translocon (High *et al.*, 1991b; Kellaris *et al.*, 1991; Görlich *et al.*, 1992b).

Our initial focus was upon SRP-dependent reaction steps that occur before the guanine nucleotide-dependent dissociation of SRP from the signal sequence. The experimental approach used here has allowed us to determine whether exogenous GTP must be added to allow progression between discrete intermediates in the protein translocation pathway. First, we tested the hypothesis that GTP binding to SRP54 enhances the affinity between the M-domain of SRP54 and the signal sequence, in analogy to the enhanced affinity between the GTP bound form of EF-Tu and an aminoacyl-tRNA (Cooper and Gorden, 1969; Kaziro, 1978.). High affinity binding of SRP to the signal sequence was detected by crosslinking of SRP54 to ribosome-bound nascent polypeptides. The results of these experiments indicate that the initial binding of SRP54 to the signal sequence of pG64 was not positively or negatively influenced by the presence of GTP, GDP, or a nonhydrolyzable GTP analogue. Based upon this finding, we conclude that binding of GTP to SRP54 is not a prerequisite for signal sequence recognition. Using an entirely different experimental approach, the G-domain of SRP54 was shown to be dispensable for the recognition of signal sequences by the SRP (Zopf *et al.*, 1993). However, removal of the G domain of SRP54 causes a reduction in the affinity of SRP54 for the signal sequence. More re-

cently, it has been proposed that binding of a signal sequence to SRP54 stabilizes the empty-site conformation of this GTP binding protein (Miller *et al.*, 1993).

As suggested by Bernstein *et al.* (1989), one conceivable function for the GTP hydrolysis cycle of SRP54 would be to discriminate between authentic signal sequences and other hydrophobic protein segments. Precedence for a GTP hydrolysis-mediated kinetic proofreading mechanism has been provided by the analysis of the mechanism by which EF-Tu enhances the fidelity of codon-anticodon recognition (Thompson *et al.*, 1986). Our finding that GMPPNP and GDP do not respectively enhance or inhibit signal sequence binding to SRP would appear to be inconsistent with a proofreading mechanism wherein GTP hydrolysis by SRP54 would be coupled to dissociation of SRP54 from the pG64-ribosome complex.

Alternatively, binding of SRP54 to the nascent polypeptide could occur in a GTP independent manner, followed by a guanine nucleotide exchange reaction that occurs if the nascent polypeptide chain contains an authentic signal sequence. Previously, we had suggested that GTP binding to SRP54 might be a prerequisite for efficient targeting of the SRP-ribosome-nascent polypeptide complex to the microsomal membrane (Connolly *et al.*, 1991). This hypothesis is supported by a recent study in which it was shown that a reconstituted SRP, which contained a truncated SRP54, retained the ability to bind to the signal sequence but was unable to target the nascent polypeptide to the microsomal membrane (Zopf *et al.*, 1993). Because the latter study employed a truncated form of SRP54 lacking the entire 33-kDa G-domain, it was not possible to deduce whether GTP occupancy of the nucleotide binding site in SRP54 was critical for the targeting reaction. We have extended these results by directly investigating the effect of GTP on targeting of the SRP-ribosome complex to the membrane. A comparison of the targeting efficiency of SRP-pG64-ribosome complexes formed in the presence and absence of GTP revealed, however, no significant difference in the binding of these complexes to the membrane-bound SRP receptor. Taken together, these two studies suggest that the G-domain of SRP54, rather than the bound ribonucleotide, is a critical structural element of SRP required for binding to the SRP receptor. Complex formation between SRP and the SRP receptor may be mediated by contact between the evolutionarily related G-domains in SRP54 and SR α as initially suggested by Bernstein *et al.* (1989).

Because the preceding analysis indicates that SRP does not require GTP for recognition of signal sequences or for efficient targeting of ribosomes to the ER, we would propose that SRP54 binds GTP after the SRP-ribosome complex binds to the SRP receptor. Unfortunately, simple experiments involving GTP addition or depletion cannot provide definitive information on the function of the GTP-binding site in SRP54 once

contact between SRP and the SRP receptor has occurred because of the presence of the additional GTP-binding sites in SR α and SR β . However, we were able to selectively block the function of the GTP-binding site in SR α , but not in SRP54, by using microsomal membranes that contained a mutant SR α subunit with a reduced affinity for GTP (Rapiejko and Gilmore, 1992). The activity of the mutant SR α could then be restored by simply raising the concentration of GTP to allow progression of the translocation reaction past the point that is dependent upon SR α . The results of these experiments indicate that binding of GTP to SR α is an obligatory reaction that precedes release of the signal sequence from SRP54. The binding of GTP to SR α may be a prerequisite or a corequisite for binding of GTP to SRP54. From the experiments described here, however, one cannot determine whether SR α and SRP54 bind ribonucleotides simultaneously as recently proposed (Ogg *et al.*, 1992) or whether binding to SR α precedes binding to SRP54.

The results described here suggest that the GTP binding and hydrolysis cycle of SR α , and presumably SRP54, are initiated upon binding of the SRP-ribosome complex to the SRP receptor. Based upon the finding that the GTP-binding site in SR α is essential for formation of the GMPPNP-stabilized high affinity complex between SRP and the SRP receptor (Rapiejko and Gilmore, 1992), we propose that GTP binding to SR α controls the cyclic assembly and disassembly of the SRP-SRP receptor complex by regulating the affinity between the two proteins. Conditional binding of GTP to SRP54, as initiated by GTP occupancy of the G-domain in SR α , could then be responsible for destabilizing the interaction between the M-domain of SRP54 and the signal sequence. Alkylation of cysteine residues within the G-domain of SRP54 inhibits signal sequence recognition (Siegel and Walter, 1988; Lütcke *et al.*, 1992), suggesting that conformational changes in the G-domain can regulate the affinity of the M-domain of SRP54 for the signal sequence (Lütcke *et al.*, 1992). Destabilization of the interaction between the M-domain of SRP54 and the signal sequence is consistent with the results presented in this manuscript provided that binding of GTP to SRP54 is initiated after contact with the SRP receptor. In this regard, we have obtained evidence that SRP does not hydrolyze GTP when assayed in the absence of the SRP receptor (Connolly and Gilmore, 1993; Miller *et al.*, 1993). As shown previously, GTP hydrolysis is required for the dissociation of the SRP-SRP receptor complex. In analogy to better characterized GTPase cycles, we suggest that the hydrolysis reactions of SRP54 and SR α are controlled by additional components of the translocation apparatus. One implication of the model presented above is that SRP receptor and SRP act as mutual guanine nucleotide exchange factors. A more precise elucidation of the mechanism by which GTP binding and hydrolysis of these two proteins are controlled remains an important goal.

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