GTPase Domain of the 54-kD Subunit of the Mammalian Signal Recognition Particle Is Required for Protein Translocation But Not for Signal Sequence Binding

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Abstract. The 54-kD subunit of the signal recognition particle (SRP54) binds to signal sequences of nascent secretory and transmembrane proteins. SRP54 consists of two separable domains, a 33-kD amino-terminal domain that contains a GTP-binding site (SRP54G) and a 22-kD carboxy-terminal domain (SRP54M) containing binding sites for both the signal sequence and SRP RNA. To examine the function of the two domains in more detail, we have purified SRP54M and used it to assemble a partial SRP that lacks the amino-terminal domain of SRP54 [SRP(−54G)]. This particle recognized signal sequences in two independent assays, albeit less efficiently than intact SRP. Analysis of the signal sequence binding activity of free SRP54 and SRP54M supports the conclusion that SRP54M binds signal sequences with lower affinity than the intact protein. In contrast, when SRP(−54G) was assayed for its ability to promote the translocation of preprolactin across microsomal membranes, it was completely inactive, apparently because it was unable to interact normally with the SRP receptor. These results imply that SRP54G plays an essential role in SRP-mediated targeting of nascent chain–ribosome complexes to the ER membrane and also influences signal sequence recognition, possibly by promoting a tighter association between signal sequences and SRP54M.

THE mammalian signal recognition particle (SRP),[1] a cytoplasmic RNP composed of six proteins and a single RNA molecule (SRP RNA) (Walter and Blobel, 1982), catalyzes the transfer of proteins across the membrane of the ER (reviewed in Walter and Lingappa, 1986). SRP binds to the signal sequences of secretory and transmembrane proteins as they emerge from translating ribosomes and inhibits further elongation of the nascent chain (elongation arrest) (Walter and Blobel, 1981). SRP then targets nascent chain–ribosome complexes to the ER membrane by binding to the heterodimeric SRP receptor (docking protein) (Gilmore et al., 1982*b*; Meyer et al., 1982; Tajima et al., 1986). This interaction is dependent on the binding of GTP (Connolly and Gilmore, 1989) and results in the release of the signal sequence from SRP and a relief of the elongation block (Walter and Blobel, 1981; Gilmore et al., 1982*a*; Meyer et al., 1982). Concomitant with the resumption of protein synthesis, nascent chain–ribosome complexes associate with a translocation machinery in the membrane ("translocon") which transports the growing nascent chain into the lumen of the ER.

Photocrosslinking experiments have shown that signal sequences are recognized by the 54-kD subunit of SRP (SRP54) as they emerge from the ribosome (Krieg et al., 1986; Kurzchalia et al., 1986). Primary sequence analysis of SRP54 revealed that the protein consists of an amino-terminal segment homologous to the carboxy-terminal region of the α-subunit of the SRP receptor (Bernstein et al., 1989; Römisch et al., 1989) and a unique carboxy-terminal segment. Limited proteolysis of SRP54 showed that these two segments comprise compactly folded structural domains (Römisch et al., 1990; Zopf et al., 1990). The amino-terminal domain (SRP54G) contains a consensus motif for GTP binding and has been shown experimentally to bind GTP (Miller, J., and P. Walter, unpublished observations). The carboxy-terminal domain (SRP54M) has a high content of methionine residues which have been proposed to play a key role in the formation of the signal sequence binding pocket on the basis of their evolutionary conservation and their physical properties (Bernstein et al., 1989). SRP54M has been shown to mediate the attachment of the protein to SRP RNA and to contain the site to which signal sequences are crosslinked (Römisch et al., 1990; Zopf et al., 1990; High and Dobberstein, 1991). On the basis of the latter result

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1. *Abbreviations used in this paper*: AF, arrested fragment; EKRM, EDTA- and salt-washed rough microsomes; Gpp(NH)p, guanylyl-5'-imidodiphosphate; PPL, preprolactin; PPL86, 86-mer of preprolactin; SRP, signal recognition particle; SRP54, 54-kD subunit of SRP; SRP54G, 33-kD amino-terminal GTPase domain of SRP54; SRP54M, 22-kD methionine-rich, carboxy-terminal domain of SRP54; SRP(−54G), SRP lacking SRP54G.

it was concluded that SRP54M contains the signal sequence binding pocket. Surprisingly, alkylation of cysteine residues in SRP54G inhibits signal sequence binding (Siegel and Walter, 1988*a*; Lütcke et al., 1992). This inhibition can be relieved upon proteolytic removal of the alkylated SRP54G domain by digestion with V8 protease, suggesting that SRP54G can influence the activity of the signal sequence binding site contained in SRP54M (Lütcke et al., 1992).

In two previous studies, the function of SRP54 has been analyzed using either intact or partially proteolyzed SRP (Zopf et al., 1990; High and Dobberstein, 1991). In neither case could the exact functional contribution of each domain be assessed because both were present during the reaction, even if they were not covalently linked. To overcome this problem, we took advantage of the observation that SRP can be disassembled under mild nondenaturing conditions and reconstituted into a fully functional particle by recombining the individual components (Walter and Blobel, 1983*a*). Using this approach, we generated a particle in which SRP54 has been replaced by purified SRP54M. Experiments with this particle have allowed us to infer the role of SRP54G in both signal sequence recognition and protein translocation. We have also taken advantage of the observation that free SRP54 can interact with signal sequences (Lütcke et al., 1992) to analyze the role of each domain in signal recognition under conditions which rule out possible influences of other SRP subunits.

Materials and Methods

Reagents

V8 protease (from *Staphylococcus aureus*), its inhibitor 3,4-dichloroisocoumarin (DCI) and guanylyl-5'-imidodiphosphate (Gpp(NH)p) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). The protease inhibitors Trasylol (10,000 U/ml) and diisopropylfluoro-phosphate (DFP) were purchased from FBA Pharmaceuticals (New York, NY) and Aldrich Chemical Co. (Milwaukee, WI), respectively. The nonionic detergent Nikkol (octa-ethyleneglycol-mono-*n*-dodecyl ether) was from Nikko Chemicals Co., Ltd. (Tokyo, Japan). The plasmid pBP4, encoding bovine preprolactin, has been described previously (Hansen et al., 1986). The plasmid pCYCΔ90 was a kind gift of Dr. Andrew Murray (University of California).

Preparation of SRP Subunits and Particle Reconstitution

Native SRP was prepared as described (Walter and Blobel, 1983*b*). SRP subunits were obtained using a modified version of a previously described protocol (Siegel and Walter, 1988*a*). A postribosomal supernatant from a high-salt extract of RM was diluted with an equal volume of buffer A (50 mM triethanolamine/HOAc, pH 7.5, (TEA), 1 mM DTT, 0.01% (w/v) Nikkol detergent, 0.5% PMSF, and 1% (v/v) Trasylol), and adsorbed onto DEAE cellulose (DE53, Whatman) in batch by incubation at 4°C for 30 min. Approximately 1 ml of packed resin, equilibrated in buffer A containing 250 mM KOAc and 2.5 mM $Mg(OAc)_2$, was used for each 25 ml of high-salt extract. The resin was washed three times with buffer A containing 250 mM KOAc and 2.5 mM $Mg(OAc)_2$. Bound SRP was then disassembled by the addition of buffer A containing 125 mM KOAc and 11 mM EDTA, and SRP proteins were eluted as described (Walter and Blobel, 1983*a*). One column volume of buffer A containing 125 mM KOAc and 11 mM EDTA was used in each elution step. The pooled eluate was diluted with 1.5 vol of buffer B [20 mM Hepes/KOH, pH 7.5, 1 mM DTT, 0.01% (w/v) Nikkol] containing 1% (v/v) Trasylol and loaded onto a 100-μl CM-Sepharose CL-6B column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). SRP54 was eluted with buffer B containing 10% glycerol, 0.1 mM EDTA, and 400 mM KOAc. All the other SRP proteins were eluted together with the same buffer containing 1 M KOAc. The peak fractions of each elution step were pooled and the protein concentration was determined by comparison to protein standards on a Coomassie blue–stained SDS–polyacrylamide gel. SRP RNA was eluted from the DE53 resin with 2 vol of 50 mM Tris·HCl, pH 8, 2 M NaCl, and 10 mM EDTA and purified as described (Siegel and Walter, 1985). SRP RNA was determined to be ~95% pure by electrophoresis on an acrylamide/8 M urea.

To isolate SRP54M, purified SRP54 was digested with V8 protease (Zopf et al., 1990). After digestion, the reaction was diluted eightfold with buffer B and rechromatographed on a 50-μl CM-Sepharose column. The column was washed with 5 vol of buffer B containing 50 mM KOAc and eluted with buffer B containing 600 mM KOAc. One 15-μl fraction and five 25-μl fractions were collected, and 2.5 μl of each fraction was analyzed by SDS-PAGE.

SRP particles were reconstituted as described (Walter and Blobel, 1983*a*) and sedimented at 4°C on 220-μl 5–20% (w/v) sucrose gradients in buffer B containing 500 mM KOAc and 5 mM $Mg(OAc)_2$ in a Beckman TLS 55 rotor at 55,000 rpm for 2.5 h (Bernstein, H. D., et al., submitted for publication).

Preparation of 4.5S RNA Resin

E. coli 4.5S RNA was prepared as described (Poritz et al., 1990). The RNA was then covalently coupled to hydrazide Avidgel Ax (BioProbe Int. Inc., Tustin, CA) after oxidation of the 3'-hydroxyl groups. The coupling reaction was performed following the manufacturer's instructions in 100 mM NaOAc, pH 5.0, at a concentration of 22 nmoles 4.5S RNA per ml of resin. Before coupling, the activated 4.5S RNA was ethanol precipitated to remove residual $NaIO_4$. The coupling efficiency was >95%. The resin was stored at 4°C in diethylpyrocarbonate-treated water and equilibrated shortly before use as indicated. The specific attachment of the 4.5S RNA through its 3' end allowed free access of SRP54 to its binding site on the RNA.

Photocrosslinking Reactions

Crosslinking reactions were performed as described previously (Zopf et al., 1990) except that a truncated form of preprolactin mRNA encoding the first 86 amino acids (PPL86) was used. The concentration of RNPs was 50 nM except in the crosslinking competition experiments, where it was 25 nM. Crosslinked products were released from the ribosomes after incubation with 1 mM puromycin in 500 mM KOAc for 15 min at 4°C and 15 min at 37°C (Blobel and Sabatini, 1971). To recover reaction products derived from free SRP54 or SRP54M, 40 μl of a 1:1 slurry of 4.5S RNA resin in buffer C [50 mM TEA, 1 M KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% (w/v) Nikkol] was added to unfractionated crosslinking reactions. After incubation for 30 min on ice, the beads were pelleted in a microcentrifuge, and the supernatant was removed. The beads were then washed three times with 100 μl of buffer C. Bound material was eluted by boiling the beads in SDS-PAGE sample buffer.

In crosslinking competition experiments, covalently linked nascent chain/free protein complexes were separated from nascent chain/SRP complexes by sucrose gradient sedimentation as described above. Gradients were fractionated into a 100-μl top fraction and a 150-μl bottom fraction that included the pellet. The bottom fraction was diluted with an equal volume of 50 mM TEA, 10 mM EDTA, 1 mM DTT, and 0.01% (w/v) Nikkol, and the SRP contained in this fraction was disassembled on DE53 cellulose as described (Walter and Blobel, 1983*a*). After disassembly, the magnesium ion concentration of the DE53 eluate was raised to 5 mM. SRP54 and SRP54M crosslinked products were then purified by 4.5S RNA chromatography. The top fraction recovered of the sucrose gradient was diluted with an equal volume of 50 mM TEA, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 0.01% (w/v) Nikkol before 4.5S RNA chromatography.

Activity Assays

In vitro translations were performed as described (Strub and Walter, 1990), except that 0.1 μl of each synthetic mRNA was used per 10-μl reaction. Elongation arrest assay reactions were incubated for 20 min at 26°C. To assay translocation activity, one equivalent (as defined in Walter and Blobel, 1983*b*) of EKRMs was added to each translation reaction. The reactions were incubated for 45 min. Percent elongation arrest and percent translocation were calculated as defined previously (Siegel and Walter, 1985). The targeting of nascent chain/ribosome complexes to the ER membrane was measured as described (Siegel and Walter, 1988*b*). RNPs were added to 15-μl translation reactions programmed with ~0.2 ng of synthetic PPL86 mRNA. After incubation at 22°C for 10 min further elongation was inhibited by the addition of cycloheximide to a final concentration of 1 mM.

Four equivalents of EKRMs were added and reactions were incubated for an additional 5 min at 22°C. EKRMs were pelleted and both the pellet fraction and the TCA-precipitated material from the supernatant fractions were then analyzed by SDS-PAGE and autoradiography.

Analysis of SRP/SRP-Receptor Interaction

SRP receptor was purified on an immunoaffinity column as described by Migliaccio et al. (1992). The Gpp(NH)p-dependent SRP/SRP-receptor interaction was assayed as described by Connolly et al. (1991). In brief, SRP receptor was incubated for 10 min at 25°C with a twofold molar excess of reconstituted SRP or SRP(−54G) in 20 μl of buffer D [50 mM TEA, 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.1% (w/v) Nikkol] containing no or 100 μM Gpp(NH)p. After adjusting the KOAc concentration to 500 mM, the reactions were placed on ice for 10 min and then analyzed on small 5–20% (w/v) sucrose gradients in buffer D containing 500 mM KOAc as described above.

Results

Assembly of a Partial SRP that Lacks SRP54G

To investigate the role of the individual domains of SRP54 in signal recognition and protein translocation, we took advantage of the observation that the M-domain can be isolated after partial proteolysis of SRP54 with V8 protease as a 22-kD COOH-terminal fragment (SRP54M) (Fig. 1, lane *1*) that can still bind efficiently to SRP RNA (Zopf et al., 1990). SRP54M was purified by CM-Sepharose chromatography after proteolytic digestion of SRP54. It was eluted from the resin as a pure component at a salt concentration of 600 mM KOAc (Fig. 1, lanes *6–8*), whereas the G-domain (SRP54G) and the protease were recovered in the flow-through fraction (Fig. 1, lane *2*).

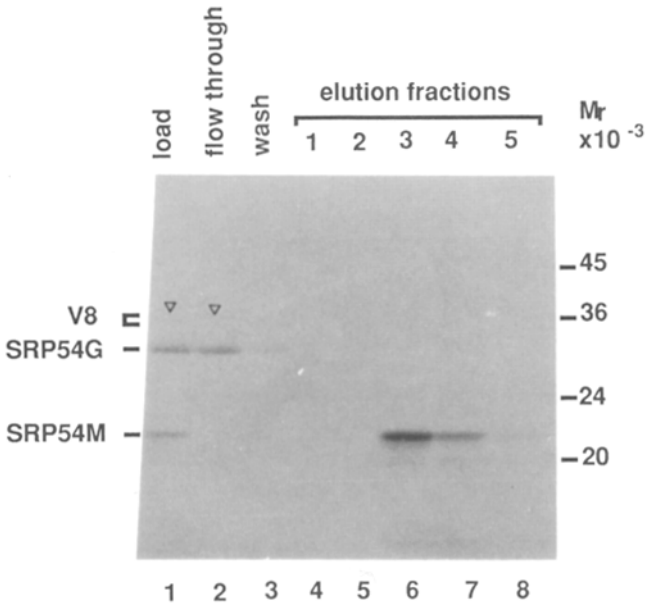


Figure 1. Purification of SRP54M. 40 μg of purified SRP54 was digested with V8-protease, and the products were separated by CM-Sepharose chromatography as described in Materials and Methods. One-fiftieth of the digestion reaction (lane *1*, *load*) and the flow-through fraction (lane *2*), and one-tenth of the wash fraction (lane *3*) and of each of the elution fractions (lanes *4–8*) was precipitated with TCA and loaded onto a 10–15% SDS-polyacrylamide gradient gel. Proteins were visualized by Coomassie blue staining. Migration positions of the proteolytic products (*SRP54G* and *SRP54M*) and the protease (*V8*) are indicated. The protein eluted in fraction 3 was used in subsequent experiments.

Purified SRP54M was mixed with approximately equimolar amounts of all the other purified SRP components and incubated under conditions which promote particle assembly (Siegel and Walter, 1985) to generate a particle that lacks the G-domain of SRP54 [SRP(−54G)]. Reconstitution reactions were subjected to sucrose gradient sedimentation to monitor the extent of particle assembly and to separate particles from free components. As shown in Fig. 2 *a*, SRP54M sedimented at 11S (Fig. 2 *a*, lanes *8* and *9*), indicating that it had bound to the core of SRP. SRP54M bound approximately as efficiently as control SRP54 (Fig. 2 *c*, lanes *8* and *9*), indicating that protease treatment and purification did not impair the RNA binding property of SRP54M. The appearance of unassembled SRP components at the top of the sucrose gradients may be due to incomplete reconstitution and to some inaccuracies in the determination of the protein concentrations.

Signal Sequence Recognition Activity of SRP(−54G)

To determine which step of SRP function was dependent on the presence of SRP54G, we first analyzed the ability of SRP(−54G) to recognize a signal sequence using a crosslinking approach. Sucrose gradient-purified reconstituted SRP or SRP(−54G) was added to wheat germ translation reactions supplemented with ^{35}S-methionine to radiolabel the nascent chains and N^{ϵ}-(5-azido-2-nitrobenzoyl)-lysine tRNA to incorporate photoreactive lysine residues into the signal sequence of preprolactin at positions −27 and −22. A truncated synthetic mRNA encoding the first 86 amino acids of preprolactin (PPL86) was translated in each reaction. The binding of SRP to the PPL86 signal sequence produces an elongation-arrested fragment (AF) of ~70 amino acids that can be crosslinked only via the two lysines in the signal sequence. After a brief incubation at 26°C, samples were irradiated with UV light and nascent polypeptide chains were released from ribosomes with puromycin and high salt (Krieg et al., 1986; Zopf et al., 1990). The products of the crosslinking reactions were then analyzed by sucrose gradient sedimentation (Fig. 2, *b* and *d*). A radiolabeled product of ~27 kD that sedimented at 11S was detected in the translation reaction containing SRP(−54G) (Fig. 2 *b*, lanes *7–10*, *SRP54M*AF*). This band corresponds to SRP54M crosslinked to the AF. A crosslinked product of a similar size was identified in previous experiments in which SRP54 was subjected to V8 proteolysis after it had been crosslinked to the signal sequence of PPL86 (Zopf et al., 1990). As expected from previous experiments, a 62-kD radiolabeled product sedimenting at 11S was observed in the control reaction containing reconstituted SRP (Zopf et al., 1990). This band corresponds to SRP54 crosslinked to the AF (Fig. 2 *d*, lanes *7–10*, *SRP54*AF*).

We next monitored the activity of SRP(−54G) in an elongation arrest assay. This assay takes advantage of the observation that SRP-mediated inhibition of nascent chain elongation is strictly dependent upon the binding of SRP to the signal sequence (Walter and Blobel, 1981; Wolin and Walter, 1989), and thus provides a quantitative measure of signal sequence recognition. Varying amounts of purified reconstituted SRP or SRP(−54G) were added to wheat germ translation reactions programmed with synthetic full-length

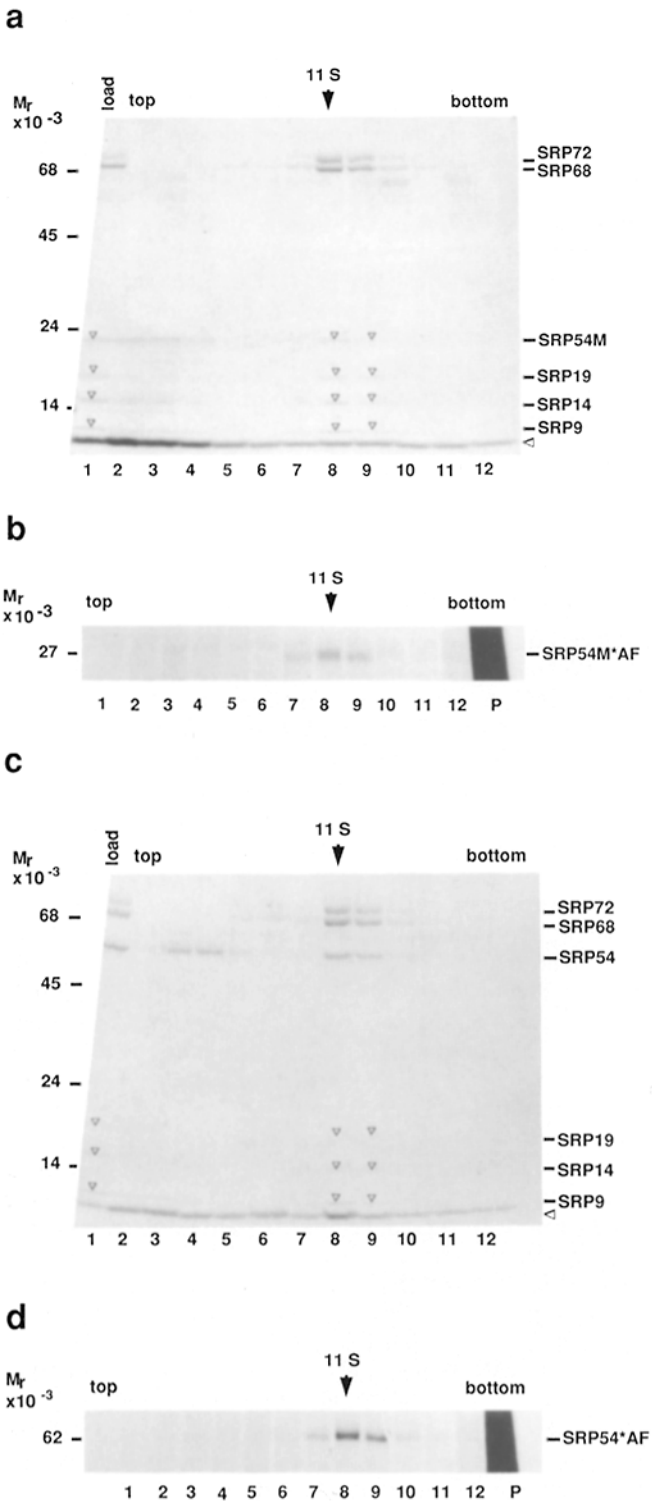


Figure 2. Sucrose gradient sedimentation of RNPs after reconstitution and photocrosslinking reactions. Purified SRP54M (*a*) or purified SRP54 (*c*) was mixed with SRP RNA and the five other SRP proteins at a final concentration of 2 μM and incubated under reconstitution conditions (Walter and Blobel, 1983*a*). One-fifth of each 25-μl reconstitution reaction was saved (lane *1*, *load*), and the remainder was sedimented on 5–20% (w/v) sucrose gradients (see Materials and Methods). Eleven fractions were collected from each gradient (lanes *2–12*). Proteins were precipitated with TCA in the presence of aprotinin as a carrier (*arrow head on right*), separated by SDS-PAGE, and visualized by Coomassie blue staining. RNPs in the 11S fractions (lanes *8* and *9*) of preparative sucrose gradients were pooled and used in subsequent experiments. The signal sequence of PPL was crosslinked to reconstituted SRP(−54G) (*b*) or reconstituted SRP (*d*) as described in Materials and Methods. Crosslinked RNPs were released from ribosomes after UV irradiation and sedimented on sucrose gradients as described above. Twelve fractions (lanes *1–12*) and a pellet fraction (*P*) were collected from each gradient. Proteins were TCA-precipitated and subjected to SDS-PAGE. Radiolabeled products were visualized by fluorography using En-Hance (New England Nuclear, Boston, MA). The radiolabeled products recovered in the pellet fraction are nonspecific and independent of ongoing protein synthesis and UV-irradiation.

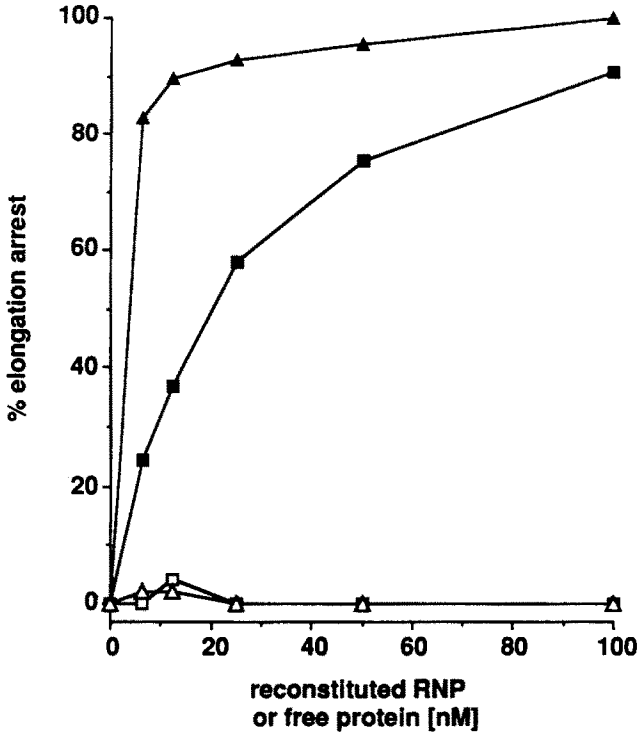


Figure 3. Elongation arrest activity of SRP(−54G). Increasing amounts of reconstituted SRP(−54G) (■), reconstituted SRP (▲), free SRP54 (□), or free SRP54M (△) were added to wheat germ translation reactions programmed with preprolactin and cyclin B Δ90 mRNA. ^{35}S-labeled translation products were separated by SDS-PAGE, and their level of synthesis was quantitated using a Phosphorimager (Molecular Dynamics, Inc.). Percent elongation arrest was defined previously (Siegel and Walter, 1985).

preprolactin mRNA (PPL). In addition, we added a truncated form of B cyclin mRNA to yield a control nonsecretory protein. The percent inhibition of PPL synthesis was measured as previously described (Siegel and Walter, 1985). SRP(−54G) inhibited the synthesis of PPL in a specific and concentration-dependent manner (Fig. 3), but approximately five times more SRP(−54G) than SRP was required to achieve 50% inhibition. At higher concentrations, the activity of both wild-type and mutant particles approached 100%. Consistent with this observation, the similar crosslinking efficiencies of SRP(−54G) and SRP in the experiments described above may be explained by the high RNP concentration (50 nM). As predicted from previous results (Siegel and Walter, 1985; Siegel and Walter, 1986), the elongation arrest observed here required the participa-

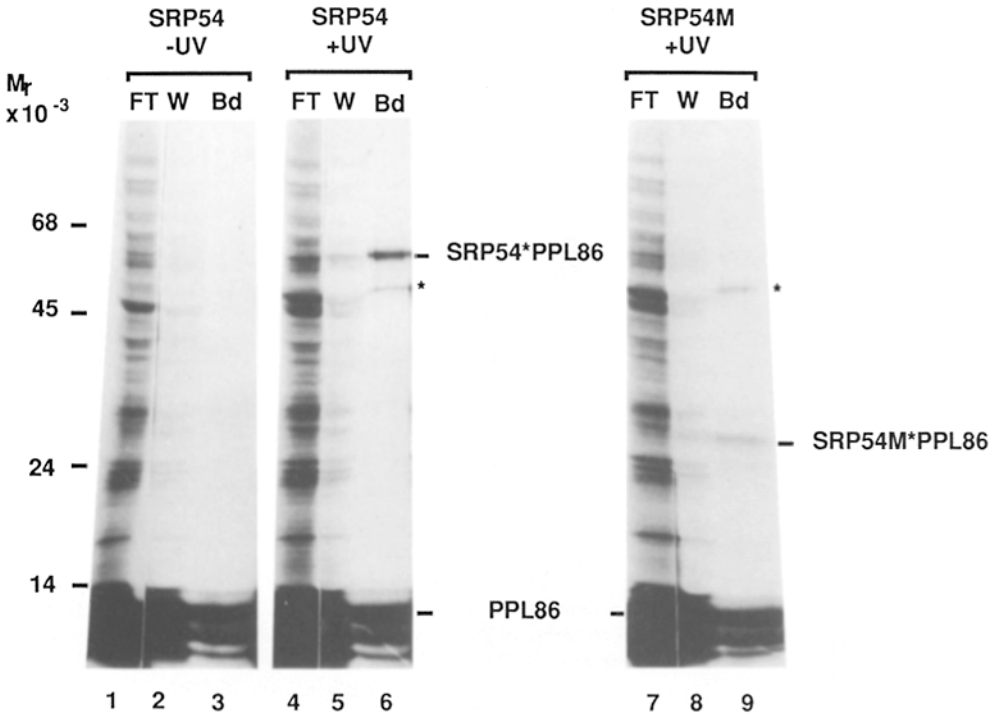


Figure 4. Binding of free SRP54 and free SRP54M to a signal sequence. Free SRP54 (lanes *1–6*) or free SRP54M (lanes *7–9*) were added at a concentration of 50 nM to 25-μl translation reactions programmed with PPL86 mRNA and supplemented with N$^{\epsilon}$-(5-azido-2-nitrobenzoyl)-lysine tRNA to incorporate photoactivatable crosslinking groups into the signal sequence. Crosslinked products were released from ribosomes before (lanes *1–3*) or after (lanes *4–9*) UV irradiation and purified from the crude translation mixtures using 4.5S RNA beads (see Materials and Methods). After TCA precipitation, the radiolabeled products in the flow-through fractions (lanes *1*, *4*, and *7*, *FT*) and wash fractions (lanes *2*, *5*, and *8*, *W*) were separated by SDS-PAGE and detected by fluorography. Material bound to the 4.5S RNA resin was eluted with SDS (lanes *3*, *6*, and *9*, *Bd*). Lanes *2*, *3*, *5*, *6*, *8*, and *9* were exposed approximately four times longer than lanes *1*, *4*, and *7*. An unidentified UV-dependent cross-linked product that bound to the affinity resin is labeled with an asterisk. A product of similar size has been observed previously and found to be associated with the large ribosomal subunit (Krieg et al., 1986).

tion of other subunits of SRP and neither free SRP54 nor SRP54M affected the synthesis of PPL (Fig. 3).

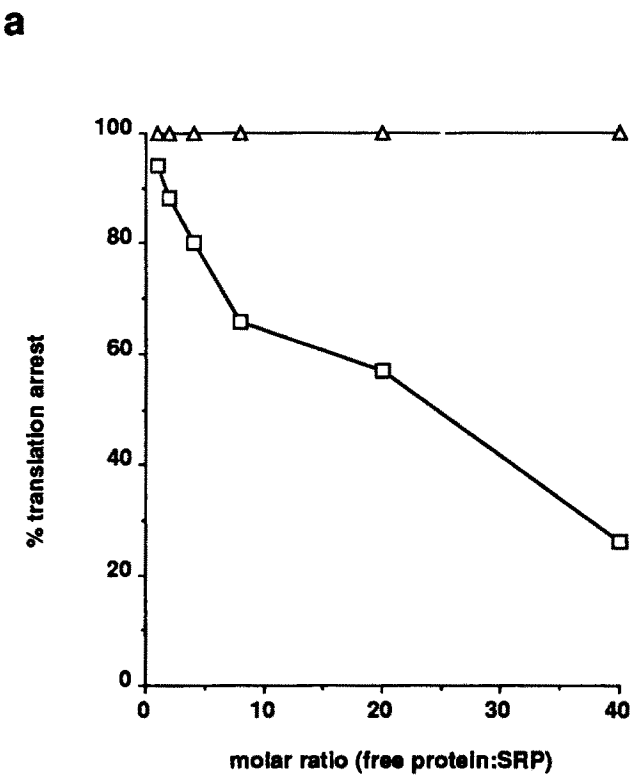
Taken together, these experiments show by two independent criteria that SRP(−54G) is active when assayed for signal sequence recognition. Thus, the G-domain of SRP54 is dispensable for this activity. The result that SRP(−54G) has a reduced specific activity in the elongation arrest assay suggests, however, that SRP54G may influence the affinity of the SRP/signal sequence interaction.

Free SRP54 Binds Signal Sequences More Tightly than SRP54M

In a simplified assay (similar to that described by Lütcke et al., 1992) that eliminates the influence of other SRP subunits, we measured the relative abilities of free SRP54 and SRP54M to interact with signal sequences. In these experiments, either purified SRP54 or SRP54M was added to UV crosslinking reactions containing photoreactive PPL86. Like the AF, PPL86 can be crosslinked only via its signal sequence. After crosslinking, nascent chains were released from ribosomes with puromycin in a high-salt buffer and specific crosslinked products were identified by passing the crude wheat germ translation mixtures over a resin to which *E. coli* 4.5S RNA was covalently attached. 4.5S RNA was used as an affinity ligand because both SRP54 and SRP54M specifically bind to it with high affinity (Römisch et al., 1990; Zopf et al., 1990). The 4.5S RNA affinity step allowed us to identify the crosslinked products containing SRP54 and SRP54M unambiguously over the high background of mostly UV-independent bands in these reactions (Fig. 4, lanes *1*, *4*, and *7*).

Radiolabeled products of ~62 kD (Fig. 4, lane *6*, *SRP54*PPL86*) and 27 kD (Fig. 4, lane *9*, *SRP54M*PPL86*) corresponding to the adducts of the crosslinking reaction between PPL86 and SRP54 or SRP54M, respectively, bound specifically to the 4.5S RNA resin. No radiolabeled product was bound to the 4.5S RNA resin if UV irradiation of the translation reactions containing SRP54 was omitted (Fig. 4, lane *3*). Coomassie blue staining of the gel shown in Fig. 4 indicated that, as expected, SRP54 was the only detectable protein in the fraction that bound to the 4.5S RNA resin (data not shown). One concern was that the free canine SRP54 used in these experiments might associate with wheat germ SRP components present in the translation extracts to form a heterologous particle. Because SRP54, which is bound to SRP RNA, cannot bind to 4.5S RNA (Zopf et al., 1990), it is clear that the observed crosslinked products were derived from free SRP54 and SRP54M. Quantitation of the products bound to the 4.5S RNA resin revealed that the reaction yielded five times more SRP54*PPL86 than SRP54M*PPL86. Consistent with the results of the elongation arrest assay described above, these data suggest that free SRP54 has a higher affinity for the PPL signal sequence than SRP54M.

The observation that free SRP54 and SRP54M can bind to signal sequences led to the prediction that the free polypeptides can act competitively to inhibit the activity of complete SRP in vitro. Competition assays provided us with an additional method to quantitate signal sequence binding activity. Varying amounts of SRP54 or SRP54M were titrated into in vitro translation reactions containing a constant subsaturating amount of SRP. Free SRP54 interfered in a concentration-dependent manner with both the ability of



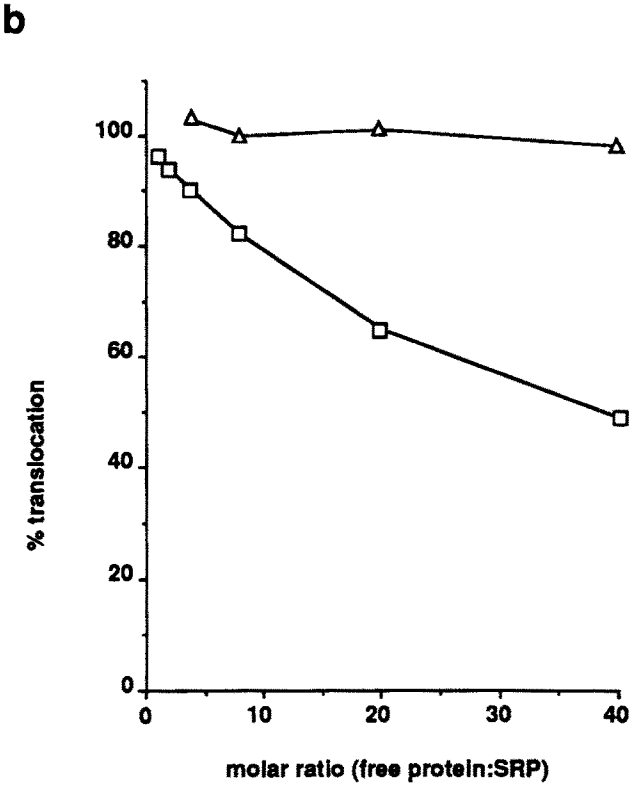


Figure 5. Effect of free SRP54 and SRP54M on SRP activities. Increasing amounts of free SRP54 (□) or free SRP54M (△) were added to 10 μl wheat germ translation reactions containing 6.25 nM SRP in the absence (*A*) or presence (*B*) of high-salt and EDTA-extracted rough microsomes. Translation reactions were performed and processed as described in the legend to Fig. 3. The addition of free proteins did not measurably affect the synthesis of cyclin B Δ90, a control nonsecretory protein.

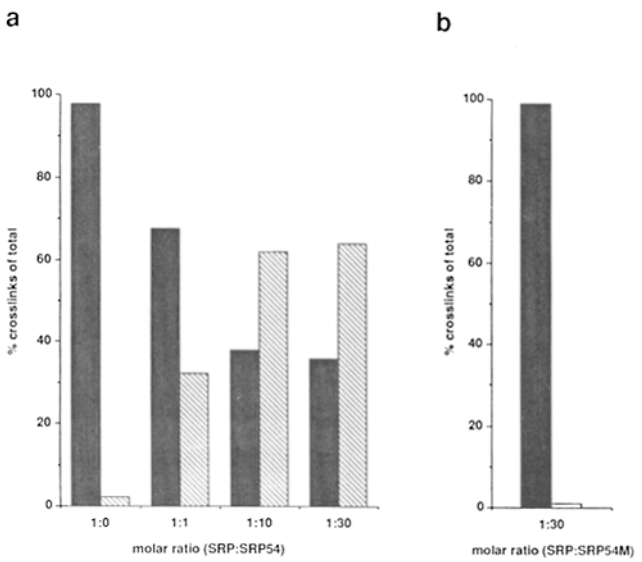


Figure 6. Effect of free SRP54 or SRP54M on the binding of SRP to a signal sequence. Crosslinking of SRP to the photoreactive signal sequence of PPL86 was performed in the presence of a constant amount of SRP (25 nM) and increasing amounts of free SRP54 or free SRP54M. SRP54 (*hatched bars*) and SRP54M (*open bars*) derivatives were separated from SRP (*stippled bars*) derivatives by sucrose gradient sedimentation as described in the legend to Fig. 2. The SRP54 derivative bound to SRP was dissociated by performing a disassembly reaction, and crosslinked products were isolated from each gradient fraction by 4.5S RNA chromatography. Crosslinked products eluted from the affinity beads were subjected to SDS-PAGE, and the radioactivity associated with the crosslinked products between SRP54 (62 kD) and SRP54M (27 kD) and the signal sequence of PPL were quantitated with a Phosphorimager (Molecular Dynamics, Inc.).

SRP to arrest the elongation of PPL (Fig. 5 *a*) and to promote its translocation across the membrane of microsomal vesicles (Fig. 5 *b*). A 40-M excess of free SRP54G blocked elongation arrest by more than 70% and translocation by ~50%. These results suggest that in both assays the free protein competed by binding nonproductively to the PPL signal sequence. Consistent with this notion, no effect was observed unless free protein was present at the outset of the reactions; free SRP54 could not displace SRP already bound to the signal sequence (data not shown). The requirement for a large excess of free protein to observe significant competition supports the view that free SRP54 has a lower affinity for the PPL signal sequence than SRP54 that is part of intact SRP. In contrast to SRP54, neither elongation arrest nor translocation was affected by the addition of any concentration of free SRP54M (Fig. 5).

The result that SRP54 but not SRP54M can compete with SRP was confirmed by UV crosslinking analysis, which measures signal sequence binding directly. A constant amount of SRP and varying amounts of SRP54 or SRP54M were added simultaneously to translation reactions synthesizing photoreactive PPL86 as described above. Nascent chains were released from ribosomes with puromycin after UV treatment. The relative crosslinking of PPL86 to SRP and the free polypeptides could be assessed by comparing the amounts of crosslinked product that sedimented at 11S and at the top of a sucrose gradient. The material recovered from each gradient fraction was incubated with 4.5S RNA

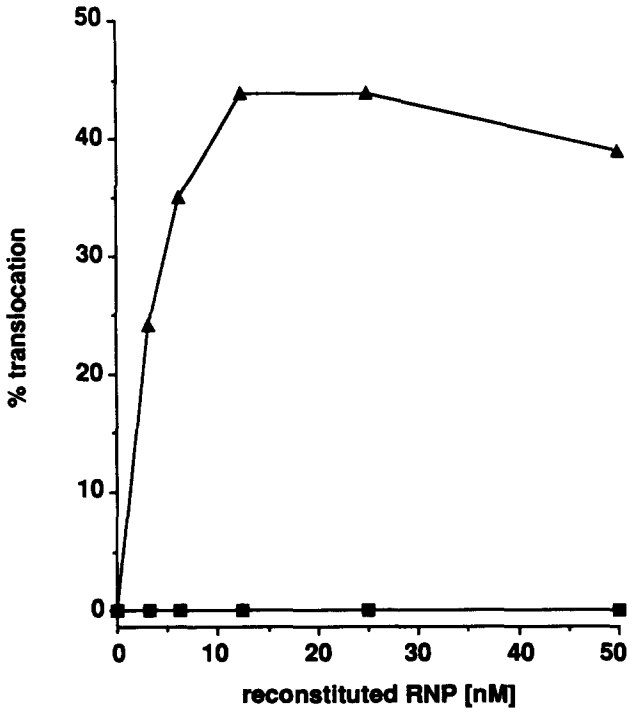


Figure 7. Translocation activity of SRP(−54G). Increasing amounts of reconstituted SRP(−54G) (■) or reconstituted SRP (▲) were added to wheat germ translation reactions supplemented with EKRMs and programmed with preprolactin and B cyclin Δ90 mRNA. Radiolabeled products were analyzed as described in the legend to Fig. 3. Percent translocation was defined previously (Siegel and Walter, 1985).

resin to recover the specific crosslinked products. Before this step, the SRP present in the fraction containing the 11S peak was first disassembled into its subunits (see Materials and Methods). The data shown in Fig. 6 *a* demonstrate that SRP54 competed effectively with SRP for binding to the signal sequence. At a 10-fold molar excess of SRP54, about two-thirds of the crosslinked products were recovered from the top fraction of the sucrose gradient. In the absence of SRP54, crosslinked products were observed only in the fraction containing the 11S peak, indicating that SRP remained intact throughout the experimental manipulations. When SRP54M was added instead of SRP54, no crosslinked products to SRP54M were observed even at a 30-fold molar excess of SRP54M (Fig. 6 *b*).

SRP(−54G) Cannot Target Nascent Chains to the ER Membrane

When tested in an in vitro activity assay, SRP(−54G) failed to promote the translocation of PPL across EDTA- and salt-washed microsomal membranes (EKRMs) to yield processed prolactin (Fig. 7). No activity was observed even at a concentration of 50 nM SRP(−54G), whereas reconstituted SRP promoted translocation efficiently at a concentration of 12.5 nM. The failure of SRP(−54G) to promote translocation could result from either an inability to target nascent chain–ribosome complexes to the ER membrane or, assuming targeting can occur, from an inability to release the signal sequence. To distinguish between these two possibilities, we tested whether SRP(−54G) could deliver nascent chain–ribosome complexes to the ER membrane (Connolly and Gilmore, 1986; Siegel and Walter, 1988*b*). Intact SRP efficiently mediated the insertion of nascent chains into EKRMs as indicated by the co-sedimentation of radiolabeled nascent chains with the membrane pellet (Fig. 8, compare lanes *6* or *8* with lanes *2* and *4*). Significant targeting was observed at an SRP concentration of 10 mM, and targeting vastly increased, if the concentration of SRP was increased to 50 nM (Fig. 8, compare lanes *6* and *8*). In contrast, no nascent chains above background were detected in the membrane pellet when SRP(−54G) was assayed, even if it was added to a concentration of 50 nM (Fig. 8, lanes *9–12*), indicating that SRP(−54G) could not promote nascent chain targeting.

The inactivity of SRP(−54G) in the targeting assay suggested that SRP54G may be required for a proper interaction of SRP with the SRP receptor. To test this notion directly, SRP and SRP(−54G) were incubated with purified SRP receptor in the presence of Gpp(NH)p, a nonhydrolyzable GTP-analog which has been shown previously to promote the formation of a stable SRP/SRP receptor complex (Connolly et al., 1991). Complex formation with SRP or SRP(−54G) was assessed by monitoring the shift of SRP receptor into the 11S fraction of sucrose gradients. The location

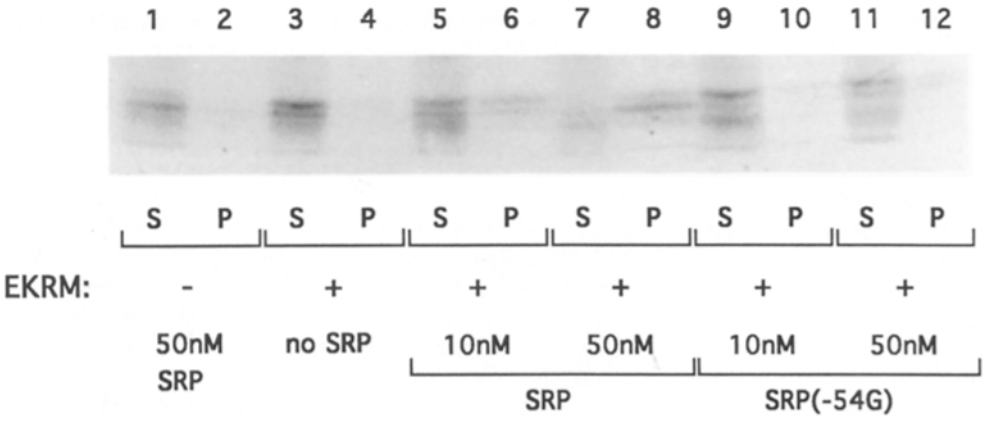


Figure 8. SRP(−54G) cannot target nascent chains to the ER membrane. SRP (lanes *1*, *2*, and *5–8*) or SRP(−54G) (lanes *9–12*) was added at the indicated concentrations to wheat germ translation reactions programmed with PPL86 mRNA. A control reaction contained only SRP buffer (lanes *3* and *4*). After a brief incubation, further nascent chain elongation was inhibited by the addition of cycloheximide, and incubation was continued either in the presence (lanes *3–12*) or absence (lanes *1* and *2*) of EKRMs. Reactions were then separated into an EKRM pellet (*P*) and a supernatant fraction (*S*). EKRMs and the TCA precipitated material of the supernatants were resolved by SDS-PAGE, and the nascent chains were visualized by autoradiography.

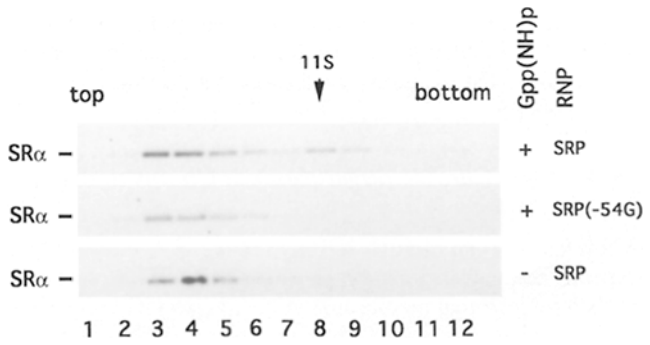


Figure 9. SRP(−54G) is unable to form a stable complex with SRP receptor in the presence of Gpp(NH)p. Reconstituted SRP (*upper* and *lower*) or SRP(−54G) (*middle*) were incubated with purified SRP receptor in the presence (*upper* and *middle*) or absence of Gpp(NH)p (*lower*). Protein complexes were separated on small 5–20% (w/v) sucrose gradients (see Materials and Methods), and 12 fractions were collected from each gradient (lanes *1–12*). Fractions were analyzed by Western blotting using a monoclonal antibody against the α subunit of SRP receptor (Taijma et al., 1986). Antigen was visualized by the chemiluminescent ECL method (Amersham Intl.).

of SRP receptor was determined by Western blotting using a mAb against the α subunit of the SRP receptor (Tajima et al., 1986). As expected, a significant portion of the SRP receptor was detected in the 11S fraction of the sucrose gradient when it was incubated with intact SRP in the presence of Gpp(NH)p (Fig. 9, *upper*, lanes *8* and *9*). In contrast, SRP receptor was not shifted when SRP(−54G) was used in this assay instead of SRP (Fig. 9, *middle*, lanes *8* and *9*). Consistent with previous results, no stable complexes between SRP and its receptor were formed if Gpp(NH)p was absent (Fig. 9, *lower*). The presence of SRP in the 11S fraction of these gradients was confirmed by Western blotting using an antibody against SRP54 (data not shown). These results show that SRP54G is essential for a productive interaction between SRP and SRP receptor, and provide an explanation for the inability of SRP(−54G) to target nascent chains to the ER membrane.

Discussion

We have used a partially reconstituted SRP that lacks the NH_2-terminal domain of SRP54, SRP(−54G), to examine the functional contribution of its two separable domains. The in vitro assembly of "mutant" SRP particles in which individual SRP subunits were omitted or selectively alkylated has been used successfully in previous studies to analyze the functions of individual SRP proteins (Siegel and Walter, 1988*a*; Lütcke et al., 1992). Our studies presented here depended on the recovery of functional SRP54M after partial proteolysis and purification. SRP54M prepared by V8 protease digestion of SRP54 forms a compact and stable structure that is relatively resistant to further proteolysis and can be purified as a homogeneous product by conventional column chromatography (Fig. 1; Zopf et al., 1990). Purified SRP54M bound to the core of SRP with similar efficiency as intact SRP54 (Fig. 2), indicating that its RNA binding function was not impaired. Similar results were previously obtained when the binding of SRP54M to *E. coli* 4.5S RNA was tested (Römisch et al., 1990; Zopf et al., 1990). Translation of mRNA encoding SRP54M in vitro yields a polypeptide that folds properly and binds to RNA (Römisch et al., 1990). Small deletions from either end, however, abolish the ability of SRP54M to bind RNA (Lütcke et al., 1992). Thus, it is likely that SRP54M comprises one compact protein folding unit and that the recovery of full RNA binding activity from purified SRP54M is diagnostic for the native conformation of the entire domain.

As shown by two independent signal sequence binding assays, UV crosslinking and elongation arrest, SRP(−54G) can specifically bind to signal sequences. A reconstituted particle that contains SRP54M in place of SRP54, however, was about fivefold less active than complete SRP in the elongation arrest assay. The reduced activity suggests that SRP54G enhances the efficiency of signal sequence binding. This conclusion was supported by several experiments assessing the interaction of free SRP54 with signal sequences (Fig. 4; Lütcke et al., 1992). First, the signal sequence of PPL could be crosslinked to SRP54 several-fold more efficiently than to SRP54M (Fig. 4). Moreover, free SRP54 competed effectively with SRP in both elongation arrest and translocation assays, whereas SRP54M did not. The difference in the relative activity of SRP54 and SRP54M was greater in the competition assays than in other experiments. The simplest interpretation of this observation is that signal sequences bind less stably to SRP54M than to SRP54, and that in a competition they will eventually dissociate from SRP54M to bind with higher affinity to the full-length protein.

The notion that SRP54G modulates the signal sequence binding activity of SRP54M is further supported by recent results which show that alkylation of free sulfhydryl groups in SRP54 with *N*-ethylmaleimide inhibits the binding of signal sequences (Siegel and Walter, 1988*a*; Lütcke et al., 1992). The only cysteine residues in SRP54 are contained in SRP54G. Proteolytic removal of the modified SRP54G relieved the inhibition of signal sequence binding, suggesting that alkylation sterically hindered access to the binding pocket.

SRP54G could influence signal sequence binding by two general mechanisms. According to the first model, SRP54G directly stabilizes the binding of the signal sequence to SRP54M. Association between SRP54M and a signal sequence, for example, may change the positions of SRP54G relative to SRP54M such that it locks the signal sequence into its binding pocket. Alternatively, SRP54G may alter the conformation of SRP54M in such a way that it would bind signal sequences more tightly. Modulation of SRP54M activity via an intramolecular conformational switch would provide a means for regulating signal sequencing binding. One could envision that in the cytosol SRP54 has a high affinity for signal sequences. After association of SRP with the SRP receptor or appropriate components of the translocon, SRP54 would be switched to a low-affinity state which would favor the release of the signal sequence. According to the second, although not mutually exclusive model, SRP54G affects the efficiency of signal sequence recognition indirectly. SRP54G might mediate, for example, the attachment of SRP54G to the translating ribosome. This would allow the signal sequence binding pocket of SRP54M to be positioned in close proximity to the nascent chain exit site

on the large ribosomal subunit. Thus, SRP54G would not alter the affinity of SRP54M for signal sequences, but rather would increase the effective local concentration of signal sequences. Because a large molar excess of SRP54 over SRP is needed to achieve a 50% reduction in elongation arrest, it is likely that even if SRP54G can interact with ribosomes, other SRP subunits are required for optimal binding.

Previous observations have indicated that SRP54 mediates, at least in part, the crucial interaction between SRP and the SRP receptor. A chimeric particle composed of only SRP54 and *E. coli* 4.5S RNA, for example, functions like intact SRP to stimulate a GTPase activity when mixed with purified SRP receptor in vitro (Poritz et al., 1990). Recent experiments suggest that the SRP receptor promotes the binding of GTP to SRP54 and that GTP binding in turn promotes the release of the signal sequence and the initiation of translocation (Miller, J., H. Wilhelm, and P. Walter, manuscript in preparation). When we analyzed the SRP particle which lacks SRP54G, we observed that this particle although it could bind signal sequences, did not promote protein translocation. SRP(−54G) could neither target nascent chains to the ER nor form a salt stable complex with the SRP receptor in the presence of Gpp(NH)p. These results suggest that SRP54G plays an essential role in the binding of SRP to the SRP receptor. Interestingly, parallel results were obtained when mutations in the α subunit of the SRP receptor which impair nucleotide binding were analyzed (Rapiejko and Gilmore, 1992), implying that likewise, the G-domain of the α subunit of the SRP receptor is required for a functional interaction with SRP. Evidence that SRP54G is the domain that interacts with the SRP receptor has been provided by experiments which show that antibodies that recognize SRP54G (Zopf, D., and P. Walter, unpublished observations) block the elongation arrest release mediated by the SRP receptor (Walter and Blobel, 1983*c*). Taken together, these results support the previous suggestion that the binding of SRP to its receptor might involve an association between SRP54G and a homologous domain in the α subunit of the SRP receptor (Bernstein et al., 1989).

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