Elongation Arrest Is Not a Prerequisite for Secretory Protein Translocation across the Microsomal Membrane

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ABSTRACT Signal recognition particle (SRP) is a ribonucleoprotein consisting of six distinct polypeptides and one molecule of small cytoplasmic 7SL RNA. It was previously shown to promote the co-translational translocation of secretory proteins across the endoplasmic reticulum by (a) arresting the elongation of the presecretory nascent chain at a specific point, and (b) interacting with the SRP receptor, an integral membrane protein of the endoplasmic reticulum which is active in releasing the elongation arrest. Recently a procedure was designed by which the particle could be disassembled into its protein and RNA components.

We have further separated the SRP proteins into four homogeneous fractions. When recombined with each other and with 7SL RNA, they formed fully active SRP. Particles missing specific proteins were assembled in the hope that some of these would retain some functional activity. SRP(-9/14), the particle lacking the 9-kD and 14-kD polypeptides, was fully active in promoting translocation, but was completely inactive in elongation arrest. This implied that elongation arrest is not a prerequisite for protein translocation. SRP receptor was required for SRP(-9/14)-mediated translocation to occur, and thus must play some role in the translocation process in addition to releasing the elongation arrest.

Signal recognition particle (SRP) has been shown to couple the cytoplasmic protein synthesis machinery with the membrane-bound protein translocation machinery of the endoplasmic reticulum. The function of SRP is well established and can be readily assayed in vitro. Experiments performed in a wheat germ in vitro translation system that was supplemented with purified SRP and SRP-depleted microsomal membranes, and programmed with total pituitary RNA, coding primarily for the secretory protein preprolactin, and/or total reticulocyte RNA, coding primarily for the cytoplasmic protein globin, have led to the following model for SRP activity (called the "SRP cycle," for review see reference 1).

First, SRP binds with high affinity to ribosomes that are synthesizing secretory proteins (2) and arrests their synthesis at a specific point in the nascent chain (3), corresponding to that point where the signal sequence is fully exposed on the surface of the ribosome. Second, SRP interacts with its own receptor in the endoplasmic reticulum, called SRP receptor (4, 5) or docking protein (6, 7). This interaction results in a release of the translation arrest (5, 6), and a weakening of the high affinity binding between SRP and the ribosome (8). The nascent chain then traverses the membrane co-translationally (9, 10); several models have been proposed for the mechanism of the passage (11-13), but the details remain unclear. After the formation of the functional ribosome-membrane junction, SRP and SRP receptor are free to re-enter the cycle.

SRP is an 11S particle composed of four proteins (two monomers composed of a 19-kD polypeptide and a 54-kD polypeptide, and two heterodimers, one composed of a 9-kD and a 14-kD polypeptide, and the other composed of a 68-kD and a 72-kD polypeptide, respectively) (14, 15), and one 300-nucleotide molecule of 7SL RNA (16). The RNA is composed of both unique and repetitive genomic sequences (17-20). Approximately 100 nucleotides at the 5' end and 45 nucleotides at the 3' end are homologous to the human Alu right monomer sequence. The central "S segment" of 155 nucleotides shows no homology to Alu DNA and is unique to 7SL RNA. It has been possible to disassemble SRP into its protein and RNA components, and to reconstitute from them a functional SRP (15). Preliminary subfractionation of the

1 Abbreviations used in this paper: DTT, dithiothreitol; 7MeG, 7-methylguanosine-5'-monophosphate; NaP, sodium phosphate, pH 7.5; PMSF, phenylmethylsulfonylfluoride; SRP, signal recognition particle; TEA, triethanolamine/HOAc (pH 7.5, adjusted at room temperature).
SRP proteins has indicated that all the proteins except the 54-kD protein can bind to the RNA directly, and that the 54-kD protein probably joins the assembly through the 19-kD protein (15). In this study we have further fractionated the protein component into four homogeneous subfractions, and have used these subfractions to assemble partial SRPs. Our purpose in doing so was to attempt (a) to assign functional domains on the particle, and (b) to address questions concerning the dependency relationships of the various SRP activities that can be assayed in vitro, i.e., whether the completion of a given event in the SRP cycle is required in order for subsequent events to occur.

MATERIALS AND METHODS

Materials: [35S]Methionine (1,500 Ci/mmol), translation grade, was purchased from Amersham Corp., Arlington Heights, IL; [3H]-Bolton-Hunter reagent (1,900 Ci/mmol) from New England Nuclear, Boston, MA; Nikkol (octaethyleneglycol-mono-n-dodecyl ether), a nontonic detergent shown to stabilize SRP activity from Nikko Chemicals Co. Ltd., Tokyo, Japan; Trasylol (the protase inhibitor aprotinin, sold as a liquid at 10,000 U/ml) from FBA Pharmaceuticals (New York, NY); elastase from Boehringer Mannheim Biochemicals, Indianapolis, IN; and aminopentyl agarose from Sigma Chemical Co., St. Louis, MO). Most other reagents were from J. T. Baker Chemical Co., Phillipsburg, NJ. All preparative procedures were carried out at 4°C, unless otherwise noted. All glassware was siliconized.

Preparation of Salt-extracted Microsomal Membranes and SRP: Microsomal membranes were prepared according to the method of Walter and Blobel (21), with the following modifications. The column-washing step was replaced by two consecutive washes of the membranes by pelleting (90,000 g [av], 45 min) and resuspending the membranes in twice the original volume of 50 mM triethanolamine/NaOAc (pH 7.5) (TEA), 1 mM MgOAc2, 0.5 mM EDTA (pH 7.5), 1 mM diithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Trasylol.

SRP was prepared from these membranes as described (22). We found that the purification of SRP from the microsomal salt extract was dependent on the particular batch of the aminopentyl agarose used, being optimal, under the conditions described (22), at a density of 5.7 g/ml diaminopentane coupled per milliliter of resin.

Disassembly of SRP and Separation of SRP Polypeptides: SRP was disassembled into protein and RNA components essentially as described previously (15). A solution of 175 µCi [3H]-Bolton-Hunter protein (lane c) at 2.5 µM in Buffer A containing 0.4 M NaOAc (25% of theoretical yield); and (d) 50 µl of 9/14-kD protein (lane e) at 2.5 µM in Buffer A containing 1 M KOAc (25% of theoretical yield). The concentration of the SRP proteins was estimated by comparing the activity of a standard of known concentration. A typical preparation (as in Fig. 1, Panel A) was equilibrated DEAE cellulose (DE53, Whatman Inc., Clifton, NJ). The slurry was incubated 10 min on ice and 10 min at 37°C, with mixing by inversion once each minute. The resin was then pelleted in a microfuge for 2 min and the supernatant removed. The pellet was resuspended twice more in 50 µl 300 mM NaOAc, 1 mM EDTA, 1 mM DTT, 0.01% Nikkol. RNA was added last, also in stoichiometric amounts with respect to the proteins. The concentration of the SRP proteins was estimated by comparing the activity of a standard of known concentration. A typical preparation (as in Fig. 1, Panel A). The concentration of the SRP proteins was estimated by comparing the activity of a standard of known concentration. A typical preparation (as in Fig. 1, Panel A).

Concentration of 50 mM NaPi and 1 M KOAc, respectively: (a) 50 mM NaPi, (b) 150 mM NaPi, (c) 250 mM NaPi, (d) 300 mM NaPi, and loaded it onto a 0.5-ml hydroxyl apatite column (HTP, Bio-Rad Laboratories) poured in nylon mesh-sealed 200-µl glass capillary tubes, and equilibrated in Buffer A containing 50 mM KOAc. The columns were eluted with two steps of Buffer A containing 0.4 M and 1 M KOAc, respectively. One 30-µl fraction and six 50-µl fractions were collected at each step. Aliquots of 5 µl of each fraction were TCA-precipitated, and the polypeptides separated and displayed by SDS PAGE as described above. The 54-kD protein eluted at 0.4 M KOAc in Buffer A, and the other proteins eluted at 1 M KOAc in Buffer A.

The described fractionation scheme resulted in four fractions containing essentially homogenous SRP proteins. The proteins were concentrated enough for subsequent reconstitution of SRP and were in a buffer that was compatible with both reconstitution and subsequent activity assays. Aliquots of 5 µl of each protein fraction were frozen in liquid N2 and stored at −80°C. They could be thawed and rapidly refrozen at least twice with no measurable loss of activity (as measured by the reconstitution of a functional SRP).

The concentration of the SRP proteins was estimated by comparing the band intensity after SDS PAGE and Coomassie Blue staining to that of an SRP reference standard. The purity of the SRP protein preparations was determined by SDS PAGE and Coomassie Blue staining, and was found to be >85% pure.

Purification of 7SL RNA: Gradient-purified SRP (150 µg in 1 ml) was precipitated with 2.5 vol of ethanol by freezing the samples in liquid N2 for at least 30 min, and then spinning them at top speed in a microfuge for 15 min at 4°C. The pellet was resuspended in a solution containing 25 mM TEA, 2.4% SDS, 100 mM NaCl, 15 mM EDTA, 200 µg/ml protease K (Boehringer Mannheim Biochemicals), and incubated for 30 min at 37°C. This solution was then extracted with an equal volume of phenol, followed by an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and finally with an equal volume of chloroform/isoamyl alcohol (24:1). NaOAc was added to a final concentration of 300 mM and the solution was ethanol-precipitated as above. The resulting pellet was washed twice more in 50 µl 300 mM NaOAc, 1 M NaCl, and redissolved in 125 µl of water. The resulting solution was added and the mixture was incubated 2-3 h on ice, with gentle vortexing every 30 min. The reaction was stopped by adding Tris/NaOAc (pH 8.0) to a 5% final concentration of 50 mM. No attempt was made to separate the unincorporated Bolton-Hunter reagent, as previous attempts to remove it by gel filtration on a variety of resins resulted in a substantial loss of labeled protein, presumably caused by irreversible binding to the gel filtration column.

Reconstitution of Complete and Partial SRPs: Reconstitutions were performed under standard conditions as previously described (15). Protein was added in approximately stoichiometric amounts on ice. For the partial reconstitutions, a buffer equivalent in composition to that containing the missing protein was added so that the concentration of components in the different reconstitutions would be identical. The ionic conditions were adjusted by the addition of another buffer solution so that the final concentrations in the reconstitution mixture were 20 mM HEPES, 500 mM KOAc, 5 mM MgOAc2, 1 mM EDTA, and 0.01% Nikkol. RNA was added last, also in stoichiometric amounts with respect to the proteins. The concentration of proteins and RNA was 400 mM. The mixture was incubated for 10-15 min on ice and 10-15 min at 37°C. The reconstitutes were then returned to ice if they were to be used immediately in an activity assay, or frozen in liquid N2 and stored at −80°C. A single freeze-thaw cycle resulted in no detectable change in activity.

Activity Assays: Total bovine pituitary RNA, coding primarily for the secretory protein preprolactin, and total rabbit reticulocyte RNA, coding primarily for the cytoplasmic protein globin, were translated together in a wheat germ, cell-free system (25 µl final volume) in the presence or absence of salt-
extracted microsomal membranes (24). The ionic conditions of this assay were kept constant at 144 mM KOAc. 2.4 mM Mg(OAc) 2 in all cases. Temperature was kept constant at 26°C. The translation products were displayed by SDS PAGE, and bands were localized by autoradiography and quantitated by densitometer scanning of preflashed film, using a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments Inc, Fullerton, CA).

Two activities of SRP (or partial SRPs) were monitored: (a) promotion of the translocation of presecretory protein (preprolactin) across the microsomal membrane (thus allowing the processing of preprolactin to prolactin by signal peptidase), and (b) site-specific elongation arrest of preprolactin synthesis in the absence of microsomal membranes. Varying amounts of SRP or partial SRPs were added to in vitro translation reactions either containing SRP-depleted membranes (at 2 eq/25 μl [2]), to measure percent processing of preprolactin to prolactin, or not containing membranes, to measure percent inhibition of preprolactin synthesis. Translations were allowed to proceed for 1 h and were stopped by chilling on ice. The reactions were TCA-precipitated, resuspended in DTT sample buffer, denatured, and alkylated with iodoacetamide. Samples were then submitted to SDS PAGE on 12% gels, and visualized by autoradiography on preflashed film. The intensities of the preprolactin, prolactin, and globin bands were quantitated by densitometer scanning.

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\text{Percent processing} = \frac{(8/7)\text{prolactin}}{(8/7)\text{prolactin} + \text{preprolactin}} \times 100, \text{and}
\]

\[
\text{Percent inhibition at concentration a} = 1 - \frac{[\text{preprolactin(a)} \times \text{globin(0)}]}{[\text{preprolactin(0)} \times \text{globin(a)}]} \times 100.
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Synchronized Translations: Protein synthesis was initiated by the addition of RNA to 100 μl of a prewarmed extract (containing all components necessary for protein synthesis except mRNA). After 30 s, further initiation was inhibited by the addition of the cap analog 7-methylguanosine-5'-monophosphate (7MeG) (3) to a final concentration of 4 mM. To test translocation capability of the nascent chain as a function of elongation time, we added 10-μl aliquots of these extracts at various time points to tubes containing 1 μl of microsomal membranes, and continued incubation for a total of 30 min. To measure the time of completion of the preprolactin nascent chain, we TCA-precipitated 10-μl aliquots of these extracts at various time points after the initiation of translation to halt further protein synthesis. The products of the in vitro protein synthesis reactions were submitted to SDS PAGE, the gels fluorographed (3), and the bands visualized by autoradiography and quantitated as described above.

Purification of a 60-kD Elastase Fragment of SRP Receptor: The 60-kD elastase fragment of SRP receptor was purified by a modification of the procedure described by Meyer et al. (7); however, the pre-proteolytic detergent extraction was omitted. Purification was monitored by following the ability of the fragment to restore the translocation activity of trypsinized microsomal membranes as described (5), as well as by Western blotting (25) after SDS PAGE.

Salt-extracted microsomes (750 ml at 1 eq/ml [as defined in reference 2]) were further extracted with EDTA (13) and resuspended in half the original volume in a buffer containing 50 mM TEA, 500 mM KOAc, 5 mM Mg(OAc) 2, 1 mM DTT, and 1% Trasylol (to inhibit hydrolases other than elastase). Elastase was added to 1 μg/ml and the mixture was incubated for 1 h at 0°C. PMSF was added to 10 μM final concentration and the proteolytic microsomes were pelleted for 1 h at 140,000 g (av). The pelleted microsomes were resuspended in the same buffer as above and were digested a second time with 1 μg/ml elastase. The supernatants obtained from both digestions were combined, diluted with 2.5 vol of a solution containing 50 mM TEA, 1 mM DTT, and loaded onto a 20-ml CM-Sephadex A50 column. The column was developed with a step of 300 mM NaPi, 250 mM KOAc, 0.1 mM EDTA, 1 mM DTT. The eluted material was chromatographed on Biogel P200 (1.6 × 80 cm) to separate the 60-kD fragment from high molecular weight contaminants, concentrated on a small (0.3 ml) hydroxyapatite column as described above, and finally fractionated on a 5-20% sucrose gradient containing 50 mM TEA, 500 mM KOAc, 0.1 mM EDTA, 1 mM DTT (15 h at 60,000 rpm in the Beckman SW60 rotor). At this stage the fragment was essentially homogeneous. It was active in restoring translocation activity to trypsinized membranes and showed no contamination with intact SRP receptor as judged by Western blotting. Starting with 750 ml of a rough microsome preparation at 50 A 260 U/ml, we obtained ~200 μg of 60-kD SRP receptor fragment.

RESULTS

Separation of SRP Proteins

In the absence of divalent cations, SRP can be dissociated into RNA and protein components by a brief incubation with polycationic substances. This disassembly reaction is mild and non-denaturing as judged by the fact that fully active SRP can easily be reconstituted from these, by themselves inactive, subfractions (15). Having such a disassembly/reconstitution assay in hand, we decided to further fractionate the SRP protein fraction into its individual components.

We designed a fractionation scheme using a combination of hydroxylapatite and CM-Sepharose chromatography that allowed us to separate the SRP proteins into four homogeneous fractions (Fig. 1A). During this fractionation we noted that two SRP polypeptides (the 19-kD and 54-kD proteins) appeared to act as monomeric proteins (Fig. 1A, lanes c and d), whereas two pairs of SRP polypeptides under all of our conditions precisely co-chromatographed in approximately stoichiometric proportions and appeared to constitute dimeric proteins (the 68/72-kD protein and the 9/14-kD protein) (Fig. 1A, lanes b and e) as previously described (15). The described fractionation procedure is relatively fast and the resulting SRP proteins are obtained in good yields. They can readily be reconstituted into active SRP when they are recombined and mixed with stoichiometric amounts of 7SL RNA under reconstitution conditions (Fig. 2, A and B [3]). Note that the reconstituted SRP was active in both of our activity assays, namely (a) promotion of the translocation of nascent secretory proteins across the microsomal membrane (Fig. 2A), and

FIGURE 1 Separation of SRP proteins. SRP (180 μg/ml) was disassembled into protein and RNA, and the proteins were further fractionated by hydroxylapatite and CM-Sepharose chromatography as described in Materials and Methods. (A) This panel shows a 10-15% SDS polyacrylamide gel stained in Coomassie Blue of 50 μl SRP (5% of total, lane a) and 5 μl of peak fractions from CM-Sepharose columns (10% of total, lanes b-e). Molecular masses (×10⁻⁵) of the SRP polypeptides are indicated. Higher molecular weight bands are contaminants in the starting SRP preparation. (B) 10 μl SRP or 2 μl (diluted to 10 μl) peak fractions shown in A were labeled with 125I-Bolton-Hunter reagent. 1% of each sample was directly denatured in sample buffer and submitted to SDS PAGE. The bands were visualized by autoradiography. Exposure was for 5 h with an intensifying screen. Lanes correspond to those in A. The asterisk indicates unincorporated Bolton-Hunter reagent.
To assess the purity of the SRP protein fractions, we radiolabeled the proteins and subjected them to analysis by SDS PAGE followed by autoradiography. Even upon prolonged exposure, we did not detect cross-contamination of SRP polypeptides between the fractions (Fig. 1 B, lanes b–e), except a trace amount (<5%) of the 54-kD protein in the 9/14-kD fraction (not visible at exposure shown in Fig. 1 B, lane e). Also note that, during the disassembly and fractionation, all of the polypeptides that were contaminating our starting SRP preparation (mostly the high molecular weight bands in lanes a in Fig. 1, A and B) were completely removed and are absent in the four SRP protein fractions.

**SRP(-9/14) Is Active in Translocation, but at a Reduced Efficiency When Compared with Complete SRP**

After we had convinced ourselves of the purity of the separated SRP proteins and of their ability to reassemble into active particles, we proceeded to reconstitute partial SRPs by omitting specific SRP proteins from the reconstitution reactions. We reasoned that SRPs lacking certain proteins might exhibit partial functions and thereby allow us to map functional properties to specific SRP polypeptides.

We assembled the following partial SRPs: SRP(-9/14), SRP(-54), and SRP(-68/72) (or, the reconstitutes lacking the 9/14-, 54-, and 68/72-kD proteins, respectively). In our activity assays, we found that SRP(-54) and SRP(-68/72) were completely inactive in both translocation and elongation arrest (data not shown). Thus far, we have not assembled an SRP(-19). However, it was previously shown (15) that the binding of the 54-kD protein was dependent on the presence of the 19-kD protein. Therefore, SRP(-19) would essentially be an SRP(-19,-54), and thus is likely to be inactive as well.

In contrast to these overall defective SRPs, we observed that an SRP(-9/14) reconstitute was active in promoting the translocation of preprolactin across microsomal membranes (Fig. 2A [1]), albeit at reduced (~50%) efficiency, under these assay conditions. We noted that SRP(-9/14) exhibits this reduced activity with respect to the complete particle over the entire concentration range, and that both curves plateau at about the same SRP concentrations. SRP(-9/14) is therefore qualitatively different in its behavior from a complete SRP in that it appears to allow a certain percentage of the nascent chains to escape the translocation process at any given SRP concentration, and thereby reduces the overall efficiency of the process. This behavior is not consistent with the alternative possibility that the absence of the 9/14-kD protein leads to a mixture of fully active and inactive particles (due for example to a defect in reconstitution), since if this were the case we would expect, as total RNA concentration was increased, that the activity would eventually reach the same level as that of the complete particle.

**SRP(-9/14) Does Not Inhibit the Elongation of Presecretory Proteins**

Very much to our surprise, we found that although SRP(-9/14) was active in promoting co-translational translocation of preprolactin across the endoplasmic reticulum, it did not arrest preprolactin synthesis. Fig. 2B [1] demonstrates that the amount of preprolactin synthesized in 60 min in the presence of SRP(-9/14) (or in the presence of any of the other

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**Figure 2** Activity assay of SRP and SRP(-9/14). (A and B) 7SL RNA and SRP proteins were mixed to ~400 nM final concentration of each component, and reconstituted under standard conditions. Varying amounts of SRP and SRP(-9/14) were added to elongation arrest and translocation assays, and the results quantitated as described (see Materials and Methods). (A) Percent processing as a function of SRP (•) and SRP(-9/14) (■) concentration. (B) Percent inhibition as a function of SRP (•) and SRP(-9/14) (■) concentration. (C) Titration of 9/14-kD protein back on SRP(-9/14). SRP(-9/14) was assembled at 600 nM final concentration of each component. Then, varying amounts of the 9/14-kD protein were added, and the mixture was diluted to 400 nM final concentration of the other components. The temperature was elevated to 37°C for 10 min and the samples were then returned to ice. Then, 6 μl of each sample was added to a 25-μl translation reaction to assay percent inhibition. Samples were processed and results quantitated as above.
partial particles) was the same as that synthesized in the absence of SRP. (Data is shown only for SRP(-9/14) in Fig. 2B.)

The elongation arresting activity of the particle could be restored by titrating back the purified 9/14-kD protein to an already assembled SRP(-9/14), as shown in Fig. 2C. SRP(-9/14) was first assembled at a slightly higher concentration (600 nM of each component) than was used for the experiments in Fig. 2, A and B. Then varying amounts of the 9/14-kD protein were added, the concentration of SRP(-9/14) was adjusted to 400 nM, and the mixtures were returned to elevated temperature. The samples were submitted to an elongation arrest assay, and the results are plotted in Fig. 2C. The elongation arresting activity increased as the concentration of the 9/14-kD protein increased, and, as expected, reached the same level as that of the complete reconstitute at stoichiometric proportions. We noted, however, that the elongation arresting activity at intermediate concentrations of the 9/14-kD protein is lower than it is at the same concentration of complete particle (cf. Fig. 2, B and C; see below).

We considered the possibility that although SRP(-9/14) did not exhibit a detectable elongation arrest when measured after a 60-min incubation, it might induce a transient one. We have tested this idea by measuring directly the amount of preprolactin synthesized in a synchronized translation system. Synthesis was started by addition of mRNA to a pre-warmed translation mix, and then synchronized after a 30-s initiation period by the addition of 7MeG (a cap analog preventing further initiation) (see reference 3).

We detected no measurable difference in the rate of preprolactin synthesis with (Fig. 3[] or without SRP(-9/14) (Fig. 3[[]); we detected the first completed preprolactin chains at 7 min, and ~70% of the total number of chains at 9 min. In the presence of complete SRP, essentially no preprolactin was formed (due to the SRP-mediated elongation arrest) (Fig. 3[●]). We concluded from these data that SRP(-9/14) did not measurably delay or arrest protein synthesis, and that therefore elongation arrest was not a prerequisite for the protein translocation demonstrated in Fig. 2A.

**SRP and SRP(-9/14) Recognize Nascent Secretory Proteins with Similar Affinity**

Although SRP and SRP(-9/14) are qualitatively different with respect to their ability to arrest presecretory protein synthesis, they must share the ability to recognize signal sequences and thus promote protein translocation across the microsomal membrane. We were therefore interested to know whether both particles would recognize signal sequences with equal efficiency. This could be tested by assaying the ability of SRP(-9/14) to compete with SRP and inhibit elongation arrest. Data addressing this question are contained in the experiment described in Fig. 2C. When we demonstrated that arresting activity could be restored by readdition of the 9/14-kD protein to SRP(-9/14), we noted that at intermediate concentrations of 9/14-kD protein, there actually exists a mixture of complete SRP and SRP(-9/14). In this titration, SRP(-9/14) was held constant at 96 nM. It follows that at the point where 24 nM 9/14-kD protein was added back, we were actually assaying arresting activity of a mixture consisting of 24 nM SRP and 72 nM SRP(-9/14). Note that at 24 nM of 9/14-kD protein (Fig. 2C), the arresting activity of this mixture of SRP and SRP(-9/14) is 24%, whereas completely reconstituted SRP exhibits 24% inhibition at only 14 nM (Fig. 2B). In other words, a threefold molar excess of SRP(-9/14) effectively halved the arresting activity of SRP. We conclude from these results that SRP(-9/14) does compete with complete SRP with similar (same order of magnitude) affinity.

**SRP(-9/14)-mediated Translocation Is Time Dependent**

The absence of an elongation arrest offered a possible explanation for the decreased translocation activity of SRP(-9/14)(Fig. 2A). We reasoned that now that protein synthesis was no longer arrested, the preprolactin-synthesizing ribosome had only a finite time window within which it must interact with the membrane of the endoplasmic reticulum in order for translocation to occur. If elongation were to proceed too far, then the nascent chain would assume a state that could no longer lead to the formation of a functional ribosome-membrane junction and no translocation would be observed. This hypothesis could be tested by measuring the percent translocation by SRP(-9/14) as a function either of membrane concentration or of the time of membrane addition in a synchronized system.

We titrated SRP-depleted microsomal membranes into a translocation assay (Fig. 4), keeping the concentration of SRP (●) or SRP(-9/14) (□) constant at 24 nM. We observed that the processing efficiency of SRP(-9/14) relative to complete SRP increased as the concentration of membranes was increased (Fig. 4). If translocation with SRP(-9/14) is dependent on forming a productive membrane junction within a finite period of time, then the translocation capacity of SRP(-9/14) should more closely approach that of SRP as the concentration of membranes is increased, since the probability of forming such a junction within a given time window would be higher. The data in Fig. 4 demonstrate that this is indeed the case. In fact, the translocation activity of SRP(-9/14) approached that of SRP at the highest concentration of membranes assayed. We conclude from this result that SRP(-9/14) is fully functional with respect to translocation.

In a second experiment, we tested directly the time dependence of the protein translocation process. In a synchronized translation experiment (see Materials and Methods), microsomal membranes were added at various times after the

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**Figure 3** Preprolactin synthesis in a synchronized translation assay. Translation reactions (50 µl) contain SRP (●) or SRP(-9/14) (□) at 96 nM, or no SRP (□). The amount of preprolactin synthesized at each time point was determined by densitometer scanning of fluorographed gels as described in Materials and Methods.
Varying amounts of SRP-depleted membranes (K-RM) were added to 25-µl translation reactions containing SRP (●), or SRP(-9/14) (□) at 24 nM. Translation was for 1 h at 26°C. Samples were processed and quantitated as described in Materials and Methods. The arrow indicates the "standard" microsome concentration used for the experiments shown in Fig. 2, which is saturating for the assay with SRP. For technical reasons we could not test any membrane concentration higher than that of the last point indicated.

As expected (Fig. 5A), if SRP was present throughout the translation, membranes could be added at any subsequent time point tested and productive translocation would still occur. On the other hand, if SRP(-9/14) was present (Fig. 5B), processing was strictly dependent on the time at which membranes were added; translocation (measured as percent processing) was decreased by 50% at ~3.6 min, and essentially abolished when microsomes were added after 4 min of elongation. A similar "cut-off time," after which translocation of the nascent chain could no longer take place, was also observed when complete SRP was not present throughout the translation, but rather was added together with microsomal membranes (as rough microsomes) at different time points (Fig. 5C). In this case, percent processing was halved at ~3.3 min, and further elongation beyond this point no longer allowed SRP to recognize and/or promote translocation of the nascent chain. Since preprolactin is fully translated with a t1/2 of ~8 min (see Fig. 3), this cut-off time corresponds to a stage in elongation where less than half of a preprolactin molecule has been polymerized, and agrees well with the size of the elongation-arrested fragment of preprolactin synthesized in the presence of SRP (3).

We therefore concluded that because SRP(-9/14) failed to arrest nascent preprolactin synthesis and thereby to hold the nascent chain in a translocation-competent state, the efficiency of protein translocation was now the result of a race between protein elongation and the time required to form a functional ribosome-membrane junction.

SRP Receptor Is Required for Translocation Mediated by SRP(-9/14)

The SRP receptor has been shown to be required for the translocation process in vitro (4-7), and to release the SRP-induced elongation arrest (5, 6). In this context, the interesting question arose as to whether, in a case where translocation occurred without any prior arrest in protein synthesis, mediated by SRP(-9/14), there would still be an absolute requirement for SRP receptor. The assay that allowed us to address this question directly was based on the observation that the cytoplasmic domain of the SRP receptor can be proteolytically severed from the membrane and then added back to it to reconstitute SRP receptor function (4).

We purified a 60-kD elastase fragment of SRP-receptor (Fig. 6) as described in Materials and Methods. Adding this pure protein fraction back to trypsinized microsomal membranes allowed us to restore their translocation activity in the presence of SRP (see Table I). Note that neither the purified SRP receptor fragment nor trypsinized membranes alone release the SRP-induced elongation arrest. However, arrest-
the figure shows a 10–15% SDS polyacrylamide gel stained in Coomassie Blue of the proteolytic extract (lane A), the fraction after CM-Sephadex chromatography (lane B), and the purified 60-kD fragment after sucrose gradient centrifugation (lane C).

**FIGURE 6** Characterization of the 60-kD elastase fragment of SRP-receptor. The cytoplasmic domain of SRP receptor was purified as described in Materials and Methods. The figure shows a 10–15% SDS polyacrylamide gel stained in Coomassie Blue of the proteolytic extract (lane A), the fraction after CM-Sephadex chromatography (lane B), and the purified 60-kD fragment after sucrose gradient centrifugation (lane C).

**TABLE I**

| Additions   | Activity |
|-------------|----------|
| SRP         | Membranes| SRP receptor | % Processing | % Inhibition |
| —           | —        | —            | 76           | <10         |
| —           | —        | K-RM*        | 9.6          | <10         |
| —           | —        | T5-K-RM*     | 1            | <10         |
| SRP         | —        | —            | ND           | 90          |
| SRP         | —        | 60 kD        | ND           | 90          |
| SRP         | K-RM     | —            | 94           | <10         |
| SRP         | T5-K-RM  | —            | 4            | 90          |
| SRP         | T5-K-RM  | 60 kD        | 75           | 35          |
| SRP(-9/14)  | K-RM     | —            | 47           | <10         |
| SRP(-9/14)  | T5-K-RM  | —            | 2            | <10         |
| SRP(-9/14)  | T5-K-RM  | 60 kD        | 48           | <10         |

Wheat germ translation reactions were programmed with pituitary RNA as described in Materials and Methods. SRP or SRP(-9/14) was added to 96 mM Membranes, where added, were at 2 eq/25 μl translation (eq is defined in reference 2). 60-kD receptor fragment was added to 27 mM (2 μl of a 20 μg/ml solution per 25 μl translation). In vitro translocations were allowed to proceed for 1 h at 26°C, and samples were processed and quantitated as described in Materials and Methods. In the reactions where membranes were omitted, percent processing was not determined (ND).

* K-RM, SRP-depleted membranes.
* T5-K-RM, trypsinized K-RM. The T5-K-RM fraction used corresponds precisely to that described by Gilmour et al. (see Fig. 5 in reference 4).

**DISCUSSION**

Our view of ribonucleoproteins has evolved over the years from that of rather static, multicomponent assemblies, to that of incredibly dynamic structures. In particular, studies on the ribosome have revealed that it exhibits many long range interactions. Compared to the ribosome, the signal recognition particle is a rather simple structure, consisting of six polypeptides (organized in four SRP proteins) and one 300 nucleotide RNA molecule. The particle has been purified to homogeneity and the RNA sequenced. Recent electron microscope studies have depicted SRP as a rod-like particle with a length of ~24 nm and a diameter of ~5 nm (26). In spite of this simple composition and the apparently rather extended physical structure of the particle, SRP is also likely to be governed by allosteric interactions between its constituents. This was most clearly demonstrated by the highly cooperative reconstitution of SRP from separated proteins and RNA (15).

"Single omission experiments" (the omission of one specific component in the reconstitution of an RNP) have been extensively used to study the function of ribosomes (27–31). To perform similar experiments on SRP, we have fully separated the SRP proteins into its four components and have shown these component fractions to be essentially free of cross-contamination. We then used these fractions to assemble partial and complete SRPs, and assayed them in in vitro elongation arrest and in vitro protein translocation assays. The complete particle was active in both assays, indicating that the separation process did not substantially inactivate any of the components.

Single omission experiments, when performed on the ribosome, were often difficult to interpret. Many different proteins, when omitted from the assembly, have been shown to affect the same function, but their omission tended to decrease rather than abolish activity (28, 29); conversely, a single omission has affected different functions (28–31). We were not surprised, therefore, to find that omission of an individual SRP protein in the in vitro reconstitution of the particle led in two of the three cases (see Results) to defective SRPs for which we were unable to demonstrate any activity in either of our relatively stringent assays. For both assays, we asked that a complex activity, rather than merely binding...
affinity, be retained. Possible explanations for those negative results are plentiful, and we cannot necessarily conclude that the omitted protein constitutes an essential functional domain, since its omission could simply result from an overall incorrect or severely altered assembly.

In marked contrast, we discovered that one of the partial particles, SRP(-9/14), was active in the translocation assay, although at a reduced level, under our standard assay conditions, relative to that of the complete particle. Surprisingly, this particle inhibited neither the rate nor the degree of preprolactin synthesis; the decreased translocation activity by SRP(-9/14) could be accounted for by the observation that without elongation arrest, the functional interaction of the polysome with the microsomal membrane had acquired a strict time dependence. In addition, we could more closely approach the translocation activity of complete SRP simply by increasing the membrane concentration. We concluded from this result that we had not in fact affected the translocation activity of the particle by removing these polypeptides. In other words, we have completely abolished one of the assayable activities of SRP (elongation arrest) without noticeably altering the other.

This uncoupling of elongation arrest and translocation by removal of a specific protein domain from SRP has allowed us to (a) assign a functional domain to the particle, and (b) ascertain the dependency relationships of partial reactions occurring in the SRP cycle.

SRP Contains an Elongation Arrest Domain

It seems reasonable that the portion of SRP comprised of the 9/14-kD protein and the RNA it binds to is contained in a structural domain separate from the rest of the particle. Limited micrococcal nuclease digestion of SRP (32) resulted in two separately sedimenting species in sucrose gradients, one containing the 9/14-kD protein and the Alu portion of 7SL RNA (with its 3' and 5' regions basepaired to each other), and the other containing the remaining proteins and the S fragment of 7SL RNA. Since it is the Alu-like portion of 7SL RNA that interacts with the 9/14-kD SRP protein which in turn is responsible for the elongation arrest activity of SRP, it seems likely that these separate structural domains represent separate functional domains as well. It remains to be tested whether a truncated SRP, lacking both the 9/14-kD protein and the Alu portion of 7SL RNA, like SRP(-9/14), is defective in arrest but not in signal recognition and translocation promoting activity. Furthermore, since the elongation arrest activity of SRP seems to reside in the portion of the molecule containing the Alu-like sequence of 7SL RNA, the provocative possibility arises that Alu transcripts in general may function in some aspect of translational control.

Elongation Arrest Is Not a Prerequisite for Protein Translocation

Since SRP(-9/14) is fully active in promoting secretory protein translocation but does not measurably arrest presecretory protein synthesis, it follows that elongation arrest is not itself a prerequisite for translocation, but rather—at least in our in vitro assays—that elongation arrest increases the efficiency of the translocation process, since it allows for an essentially infinite time window for the attachment of the polysome to the microsomal membrane. We can further conclude that SRP(-9/14) undergoes all biochemical and conformational changes that are necessary for translocation to occur. For example, if an increase in affinity of SRP to the ribosome is a prerequisite to translocation, then this particle must exhibit it; indeed, our data indicate that both SRP and SRP(-9/14) recognize signal peptides with approximately equal (same order of magnitude) affinities (see Fig. 2 and Results). This indicates that while the temporal sequence of events in the SRP cycle described earlier (see the introduction and reference 1) are confirmed, the dependency relationships need to be modified, and that, in particular, elongation arrest is not an obligatory step.

SRP Receptor Is Required for Protein Translocation, Even in the Absence of Elongation Arrest

SRP receptor releases the SRP-induced arrest of presecretory protein synthesis. It seemed possible that it was solely on the basis of the arrest-releasing activity that SRP receptor was required for translocation. Since SRP(-9/14) did not arrest protein synthesis, we were able to address the question of whether, in the absence of translation arrest, translocation could occur in the absence of SRP receptor.

Such translocation was not observed (see Table I), and we concluded therefore that SRP receptor was required for translocation, even in the absence of elongation arrest. This absolute requirement for SRP receptor may reflect merely the affinity between SRP and SRP receptor, i.e., that SRP receptor is required solely to correctly target the ribosome to the microsomal membrane. Alternatively, SRP receptor may be involved in the initiation of the translocation process itself, either directly or by organizing in its proximity whatever components are required for the translocation process.

In summary, the construction and assays of SRP(-9/14) have enabled us to gain considerable insight not only into structure/function relationships in SRP, but also into the role of the elongation arrest reaction. Given that elongation arrest is not absolutely required for protein translocation in vitro, the question about its function and importance in vivo becomes an even more interesting one. Elongation arrest clearly could serve a fidelity function to prevent synthesis of precursors in the cytoplasmic compartment, and, in addition, to improve the efficiency of the translocation reaction by retaining the nascent chain in a translocation-competent state. It may have been added as an evolutionary refinement to a more primitive SRP cycle, and in this regard it may be significant that arrest resides in a separate RNP domain. Elongation arrest could also be exploited as a regulatory step, providing the cell with a fast, possibly selective, on/off switch modulating specific secretory or membrane protein synthesis at the level of elongation.

There is some evidence that elongation arrest may be variable among different secretory or membrane proteins. For example, at a given SRP concentration, preprolactin synthesis is arrested better than preprolactin synthesis (Garcia, P., and P. Walter, unpublished results), which in turn is arrested better than ovalbumin, a secretory protein containing an uncleaved signal sequence. Also, a variety of membrane proteins containing uncleaved signal sequences failed to show demonstrable arrest in vitro (33). It is possible that the nascent chain itself plays a direct role in elongation arrest; alternatively, it may affect the affinity of SRP for the ribosome, and
thus indirectly affect both elongation arrest and translocation. Using SRP(-9/14) to compare the translocation efficiencies of these proteins in a system where elongation arrest is not a factor will enable us to determine at what level this variability is exerted.

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