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# Disassembly and Reconstitution of Signal Recognition Particle

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## Summary

**Signal recognition particle (SRP) is a ribonucleoprotein consisting of six distinct polypeptides and one molecule of small cytoplasmic 7SL-RNA. The particle was previously shown to function in protein translocation across, and protein integration into, the endoplasmic reticulum membrane. A rapid procedure was developed to disassemble SRP into native protein and RNA components. The method utilizes unfolding of SRP with EDTA and dissociation on polycationic matrixes. SRP proteins prepared this way sediment below 7S and are inactive in activity assays. When recombined with 7SL-RNA in the presence of magnesium, the proteins are shown to reassociate stoichiometrically with 7SL-RNA to form fully active 11S SRP.**

## Introduction

The signal recognition particle (SRP) functions as an adapter between the cytoplasmic protein translation apparatus and the protein translocation machinery in the membrane of the endoplasmic reticulum. In the cytoplasm, SRP recognizes signal sequences on nascent polypeptides chains of secretory (Walter et al., 1981; Stoffel et al., 1981; Meyer et al., 1982a), lysosomal (Erickson et al., submitted), and certain classes of integral membrane proteins (Anderson et al., 1982). By virtue of a tight binding of SRP to the ribosomes that are in the process of synthesizing these proteins (Walter et al., 1981), SRP causes a site-specific arrest of the biosynthesis of the nascent protein (Walter and Blobel, 1981b). When these elongation-arrested polysomes interact with specific components of the membrane of the endoplasmic reticulum, their binding and the formation of a functional ribosome–membrane junction are observed (Walter and Blobel, 1981a). The direct interaction of SRP with the SRP receptor (Gilmore et al., 1982a, 1982b) (also termed docking protein; Meyer et al., 1982a), an integral membrane protein of the endoplasmic reticulum (Meyer et al., 1982b), was shown to be responsible for the release of elongation arrest. As translation resumes, the nascent polypeptide chain is now vectorially translocated into the lumen of the endoplasmic reticulum (Blobel and Dobberstein, 1975a, 1975b).

Structurally, SRP can be defined as a ribonucleoprotein (RNP) with a sedimentation coefficient of 11S, consisting of six different polypeptide chains (Walter and Blobel, 1980) and one molecule of the small cytoplasmic 7SL-RNA (Walter and Blobel, 1982) of known sequence (Ullu et al., 1982; Li et al., 1982). Both RNA and protein are required for SRP's activity, since it could be shown that nuclease treatment as well as alkylation with *N*-ethylmaleimide (Walter and Blobel, 1980) inactivates the particle. At present, little is known about the function of the individual building blocks of SRP.

We describe a procedure for a rapid and nondestructive disassembly of SRP into individual components, which can be reversed to reconstitute an active particle. These studies provide insight into the structure of SRP and open the way for detailed structure–function analyses.

## Results

### EDTA Causes Partial Unfolding of SRP

The integrity of many RNPs is dependent on the presence of divalent cations. Thus chelating agents could be successfully employed to unfold partially or to disassemble ribosomal subunits (Spirin, 1974; Newton et al., 1975; Blobel, 1971), RNAase P (Guthrie and Atchison, 1980) and small cytoplasmic RNPs (Mukherjee and Sarkar, 1981).

The data in Figure 1 demonstrate that SRP undergoes structural changes in the absence of $Mg^{2+}$ as well. We observed a time course of the hyperchromicity effect during nuclease digestion as a measure for the nuclease sensitivity of the particle. As the $Mg^{2+}$ concentration was decreased from 10 mM to 1 mM and finally to 0 mM by the addition of EDTA, SRP was rendered increasingly susceptible to nucleolytic attack. Interestingly, the kinetics of nuclease digestion of SRP in 10 mM $Mg^{2+}$ appeared to have a biphasic character: an initial rapid digestion of apparently exposed and accessible regions of 7SL-RNA, followed by a rather slow digestion of RNA regions that were probably protected by protein. The latter regions were rendered more nuclease sensitive as the $Mg^{2+}$ concentration was decreased. The nuclease digestion of naked 7SL-RNA (Figure 2, lanes d) was rapid and independent of the presence or absence of $Mg^{2+}$ (data not shown).

When a $Mg^{2+}$-depleted SRP preparation was analyzed for its integrity by sedimentation in sucrose gradients containing EDTA and 500 mM KOAc, SRP still sedimented at 11S and no polypeptide chains were dissociated (data not shown). Thus, although EDTA appears to unfold SRP, it is not sufficient to dissociate the particle under these conditions.

### Unfolded SRP Can Be Dissociated with Polycationic Substances

SRP binds to DEAE-ion exchange resins and can be eluted as an intact particle if all buffers contain $Mg^{2+}$. However, if SRP was incubated with DEAE-cellulose in the presence of EDTA, we observed differential elution only of SRP polypeptides—with the 7SL-RNA remaining bound to the column. Under the premise that this result was due to a dissociation of SRP, we optimized the conditions to obtain a maximum yield of dissociated SRP proteins. The introduction of a brief incubation at elevated temperature of SRP in the presence of both EDTA and DEAE-cellulose

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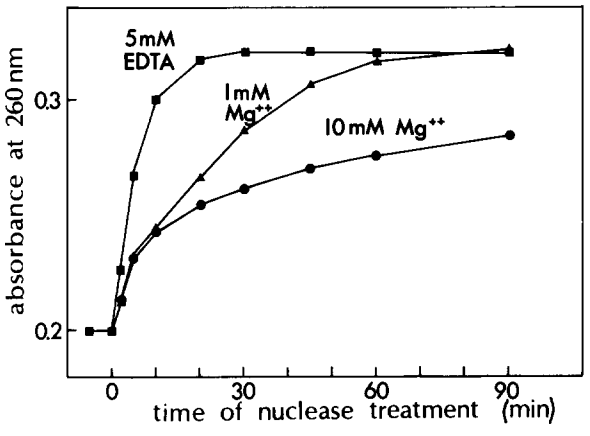


Figure 1. Nuclease Sensitivity of SRP

Gradient-purified SRP (see Experimental Procedures) was diluted to 0.2 $A_{260}$ units/ml, and the ionic concentration was adjusted to yield a final concentration of 50 mM TEA, 100 mM KOAc, 1 mM DTT, 0.01% Nikkol, containing 10 mM $Mg(OAc)_2$ (●), 1 mM $Mg(OAc)_2$ (▲), or 5 mM EDTA (■), respectively. The solutions were warmed to 30°C in a heated cuvette holder (Beckman DU8 spectrophotometer), and at time 0 min a solution of RNAase A (Sigma, 500 μg/ml) was added to yield a final concentration of 1 μg/ml. The absorbance at 260 nm was recorded as a function of time (Sarkar et al., 1981).

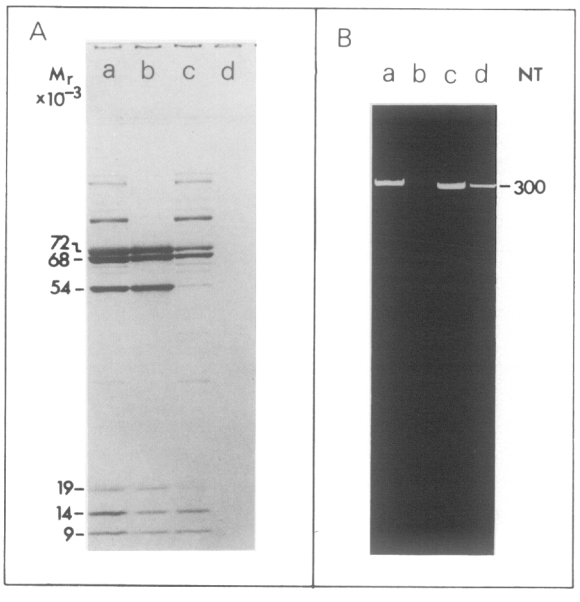


Figure 2. Dissociation of SRP

SRP was unfolded with EDTA and dissociated on DEAE-cellulose as described in Experimental Procedures. Equivalent aliquots of the various fractions (corresponding to 20 μl of the starting SRP preparation) were TCA-precipitated, and the polypeptides analyzed by PAGE in SDS on 10%–15% gradient gels followed by staining in Coomassie blue (A). Separate aliquots were treated with proteinase K (see Experimental Procedures), RNA was isolated by the perchlorate procedure (Lizardi and Engelberg, 1979) and analyzed by PAGE in 7 M urea on 7% gels followed by staining in ethidium bromide (B). The molecular weights ($M_r$) of the SRP polypeptides and the size of 7SL-RNA (number of nucleotides, NT) are indicated.

Lanes a show the SRP preparation used as starting material in this study. It was dissociated on DE53 in EDTA as described in Experimental Procedures to yield the SPR protein fraction (lanes b). 7SL-RNA was eluted from the DE53 resin by raising the salt to 1 M KOAc (lanes c) and freed of residual SRP polypeptides by proteinase K treatment and repeated phenol extractions (lanes d).

The wet Coomassie-blue-stained polyacrylamide gel (A; lanes a, b, and c) was scanned at 630 nm in the Beckman DU8 spectrophotometer. From the integrated absorbances the yields of the individual dissociated SRP polypeptides (lane b) were calculated to be 80% (72 kd), 75% (68 kd), 100% (54 kd), 100% (19 kd), 45% (14 kd), 55% (9 kd) with respect to the starting SRP preparation (lane a).

Note that some contaminating polypeptides in the original SRP preparation (A, lane a) bind to the DEAE-cellulose under dissociation conditions. They are thus absent in the SRP protein fraction (lane b), and all subsequent analyses in which this fraction was employed (Figures 3 and 5).

and the use of a DEAE-cellulose resin with a high surface density of charged groups (Whatman DE53) led to an almost quantitative disassembly of SRP (Figure 2, compare lanes b with lanes a). 7SL-RNA could subsequently be eluted with an increase in ionic strength from the DEAE-cellulose in intact form (Figure 2, lanes c), demonstrating that in the incubation steps no nucleolytic breakdown of the EDTA-unfolded SRP occurred. This 7SL-RNA fraction was subsequently treated with proteases and extracted with phenol to provide a 7SL-RNA fraction (Figure 2, lanes d) that was free of residual SRP proteins, to be used in the reconstitution studies. The dissociation reaction was independent of the time for which SRP was incubated with EDTA—i.e., the unfolding appeared to occur instantaneously. Therefore no long incubation steps, as described for a similar disassembly of RNAase P (Guthrie and Atchison, 1980), were necessary. Furthermore, prolonged incubation with DEAE-cellulose did not further increase the yield of SRP proteins.

Among the polycationic substances tested to dissociate SRP were polyetheleneimine (PEI) and various other preparations of DEAE–ion exchange resins (Whatman DE52; Pharmacia DEAE-Sephadex, DEAE-Sepharose, and DEAE-Sephacel). Although all of these substances caused SRP to dissociate, they were unpractical for the following reasons. PEI had to be precisely titrated into the dissociation reaction, as an excess of PEI prevented 7SL-RNA from precipitating. It only dissociated the 54, 68, and 72 kd SRP polypeptides, whereas the smaller polypeptides would precipitate together with 7SL-RNA and PEI. Furthermore, we were unsuccessful in attempts to recover protein or RNA in a form free of PEI from these pellet fractions. The other DEAE–ion exchange resins (listed above) produced SRP proteins only at a reduced yield, and in each case these SRP protein fractions were contaminated with residual 7SL-RNA. Thus it appears that the charge density on the surface of the ion-exchange resins is an important factor to obtain clear-cut fractionation of SRP in the dissociation reaction.

RNAase P has been successfully reconstituted after dissociation in urea (Kole and Altman, 1979). SRP could also be completely dissociated in 7 M urea in the presence of $Mg^{2+}$ or in 4 M urea in the presence of EDTA (data not shown). We have, however, not been able to reconstitute active SRP after removal of the urea (by gel filtration) and thus have not pursued this experimental approach.

### Characterization of the SRP Protein Fraction

When the SRP protein fraction was subjected to sedimentation analysis in sucrose gradients, all polypeptides sedimented below 7S (Figures 3A and 3B). This sedimentation behavior was independent of the presence or absence of $Mg^{2+}$ (data not shown). No material sedimented in the position of authentic SRP, and no distinct peak in the UV absorbance profile was observed—i.e., no detectable contamination with 7SL-RNA was present. SRP proteins were also fractionated by chromatography on CM-Sepharose columns (Figures 3C and 3D). The unusually high ionic strength required to elute five of the six SRP polypeptides from this resin is indicative of their very basic character and is consistent with their behavior on denaturing isoelectric focusing gels ($p_i$ >8.0, data not shown).

Interestingly, in both the sucrose gradient and ion exchange chromatography fractionation schemes two polypeptides behave as monomers (19 and 54 kd), whereas the 9 and the 14 kd polypeptides as well as the 68 and the 72 kd polypeptides appeared to be still bound to each other. These polypeptides sedimented consistently with the interpretation of being heterodimers (Figure 3B), and they also cochromatographed on CM-Sepharose columns (Figure 3D).

When subjected to activity assays, the SRP protein fraction (Figure 2, lanes b) and 7SL-RNA (Figure 2, lanes d) were completely inactive (Figure 4B, open symbols). SRP proteins did not compete with intact SRP in these assays, even when added in a fivefold molar excess (data not shown).

### Reconstitution of SRP

Having established that SRP proteins were no longer in a complex, but behaved as inactive monomeric and heterodimeric proteins, we attempted to reconstitute a functional particle with 7SL-RNA. As a starting point for these reconstitution experiments, we chose ionic conditions close to those reported by Traub and Nomura (1968, 1969)

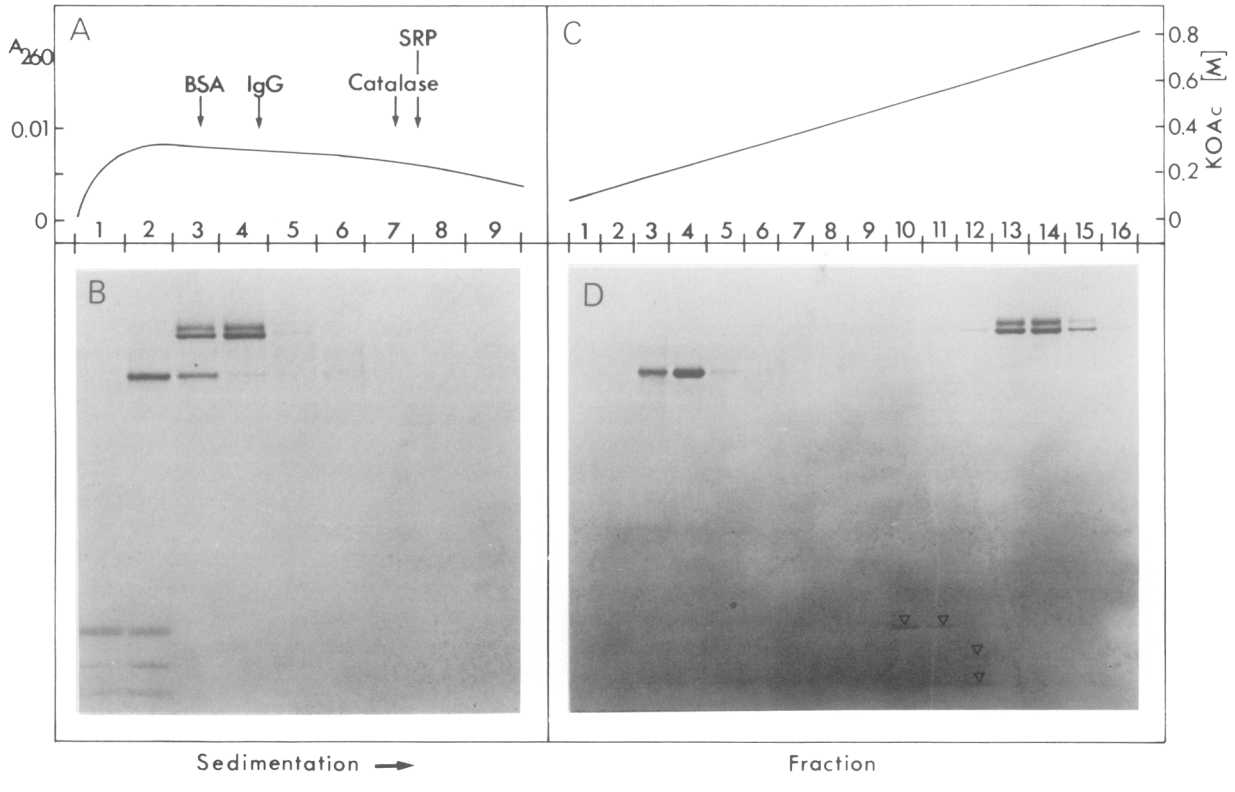


Figure 3. Fractionation of SRP Proteins

(A and B) Fractionation by sucrose gradient centrifugation. An aliquot (250 μl) of the SRP protein fraction (Figure 2, lanes b) was adjusted to 500 mM KOAc, 5 mM $Mg(OAc)_2$, layered on top of a 5%–20% sucrose gradient in 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% Nikkol, and centrifuged for 20 hr at 4°C at 40,000 rpm in a Beckman SW40 rotor. The gradient was fractionated using an ISCO gradient fractionator, and the absorbance at 260 nm was recorded (A). Fractions of 1.0 ml were collected. The top nine fractions were TCA-precipitated, and the polypeptides analyzed (B) by PAGE in SDS as described in Figure 2. The position of size markers (bovine serum albumin, BSA [4S], rabbit IgG [7S], bovine catalase [11S], and undissociated SRP) in parallel gradients was determined by recording the UV absorbance at 280 nm (A).

(C and D) Fractionation by chromatography on CM-Sepharose CI-6B. A 1.0 ml aliquot of SRP protein fraction (Figure 2, lanes b) was diluted with 4.0 ml of a solution of 50 mM TEA, 5 mM EDTA, 1 mM DTT, 0.01% Nikkol and passed over a 1.0 ml column of CM-Sepharose CI-6B at 4°C. The flowthrough fraction contained no detectable protein. The column was then developed with a linear 2 × 10 ml gradient (C) of increasing KOAc concentrations (50 mM to 1100 mM) in 50 mM TEA, 5 mM EDTA, 1 mM DTT, 0.01% Nikkol. Fractions of 1.0 ml were collected. An aliquot (250 μl) of each fraction was TCA-precipitated, and the polypeptides were analyzed by PAGE in SDS as described in Figure 2. The faint bands of the lower molecular weight SRP polypeptides are marked with an arrowhead.

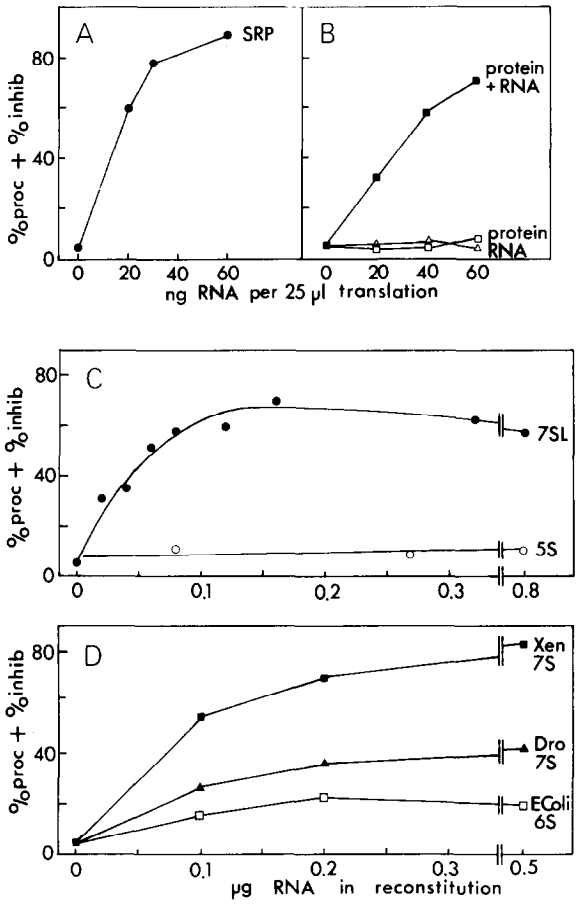


Figure 4. Activity Assay of Reconstituted SRP

SRP activity (expressed as % processing + % inhibition) was determined in a wheat germ cell-free translation system, programmed with bovine pituitary RNA, and supplemented with SRP-depleted microsomal vesicles as described in Experimental Procedures. (A) shows a titration of undissociated SRP (●) (Figure 2, lanes a) into the assay system. (B) shows titrations of either SRP proteins (□) (Figure 2, lanes b), 7SL-RNA (Δ), (Figure 2, lanes d), or SRP proteins plus 7SL-RNA (■) into the assay system after incubation under reconstitution conditions (see Experimental Procedures for concentration of components in the reconstitution reaction). The amount of SRP (●), reconstituted SRP (■), or 7SL-RNA (Δ) is expressed as the amount of RNA contained in these samples; the amount of SRP proteins (□) is the same as the amount of SRP protein present in the reconstituted SRP sample (■) at each given data point.

(C and D) shows activity assays of reconstituted SRP preparations where the amount of RNA in the reconstitution reaction (total volume 6 µl, see Experimental Procedures) was varied, while the amount of SRP proteins in the reconstitution reaction was kept constant as given in Experimental Procedures. A 4 µl aliquot of each reconstituted SRP preparation was assayed in a 25 µl in vitro translation system. The following RNA preparations were used in these experiments. (C) canine 7SL-RNA (●), canine ribosomal 5S RNA (○); (D) D. melanogaster 7S RNA (▲), X. laevis 7S RNA (■), E. coli 6S RNA (□).

for the reconstitution of functional 30S ribosomal subunits (370 mM $K^+$, 20 mM $Mg^{2+}$). SRP activity could be reconstituted (Figure 4B) by simply recombining SRP proteins with a stoichiometric amount of 7SL-RNA in the presence of $Mg^{2+}$. A short incubation step at elevated temperature (10 min at 37°C) was required to drive the reconstitution to completion. If this incubation step was omitted, functional SRP was obtained nevertheless, albeit at a reduced yield (about 30% of the activity shown in Figure 4B). In contrast to the reconstitutions of ribosomal subunits, we observed that the ionic conditions could be varied over a wide range (150–500 mM $K^+$, 4–20 mM $Mg^{2+}$) without affecting the efficiency of the reaction.

When reconstituted SRP was analyzed by sucrose gradient centrifugation, we observed that all six SRP polypeptides had reassembled in about stoichiometric amounts with the 7SL-RNA and formed an active 11S particle (Figures 5A, 5B, and 5C). 7SL-RNA was added in stoichiometric amounts with respect to the 9/14 kd SRP protein, which is the limiting component in the SRP protein fraction (see Figure 2). Thus all other SRP proteins were in molar excess and remained in part on top of the gradient (Figure 5B). A second, more slowly sedimenting peak, comprising about half of the absorbance of the reconstitution reaction, was observed (Figure 5A), which consisted of 7SL-RNA together with the 9/14 kd SRP protein (Figures 5A, 5B, fraction 6). 7SL-RNA in both peaks was not degraded (Figure 5A, inset), and only the 11S peak (Figures 5A, 5B, fraction 7) was active in the translocation assay (Figure 5C). We cannot distinguish whether the slower sedimenting peak constitutes an assembly intermediate of SRP or whether it is an incorrectly assembled form acting as a dead-end in the assembly process.

From the activity assay shown in Figure 4 it is apparent that the reconstituted SRP fraction was about half as active (Figure 4B) as the control SRP preparation (Figure 4A). However, because the amount of SRP and reconstituted SRP is given in Figure 4 as the amount of RNA contained in the fraction, and because from the data in Figures 5A, 5B, and 5C it follows that only half of the 7SL-RNA in the reconstitution mixture assembled into active 11S SRP, we conclude that the fully reconstituted SRP is as active as the undissociated control preparation. Thus the assembly process occurred with about 50% yield with respect to the limiting components. To test for cooperativity in the reconstitution process, we varied the concentration of 7SL-RNA in the standard reconstitution reaction (Figure 4C). We calculated that 70 ng 7SL-RNA would be approximately equimolar with respect to the limiting amount of the 9/14 kd SRP protein in the reconstitution mixture. As shown by the data in Figure 4C (filled circles), we indeed saturated the reconstitution reaction around 50–100 ng 7SL-RNA. At higher RNA conconcentrations the activity of reconstituted SRP remained on a plateau—i.e., in contrast to the reconstitution of ribosomal subunits, a molar excess of 7SL-RNA did not compete in the in vitro assembly process of SRP.

We have also attempted to use unrelated RNA species in the reconstitution reaction to demonstrate specificity for 7SL-RNA. When ribosomal 5S RNA was used, no SRP activity could be detected (Figure 4C, open circles). When the products of such a reconstitution reaction were analyzed on sucrose gradients, none of the SRP proteins was found to associate with the RNA (data looked identical to Figure 3B). A tenfold molar excess of ribosomal 5S RNA

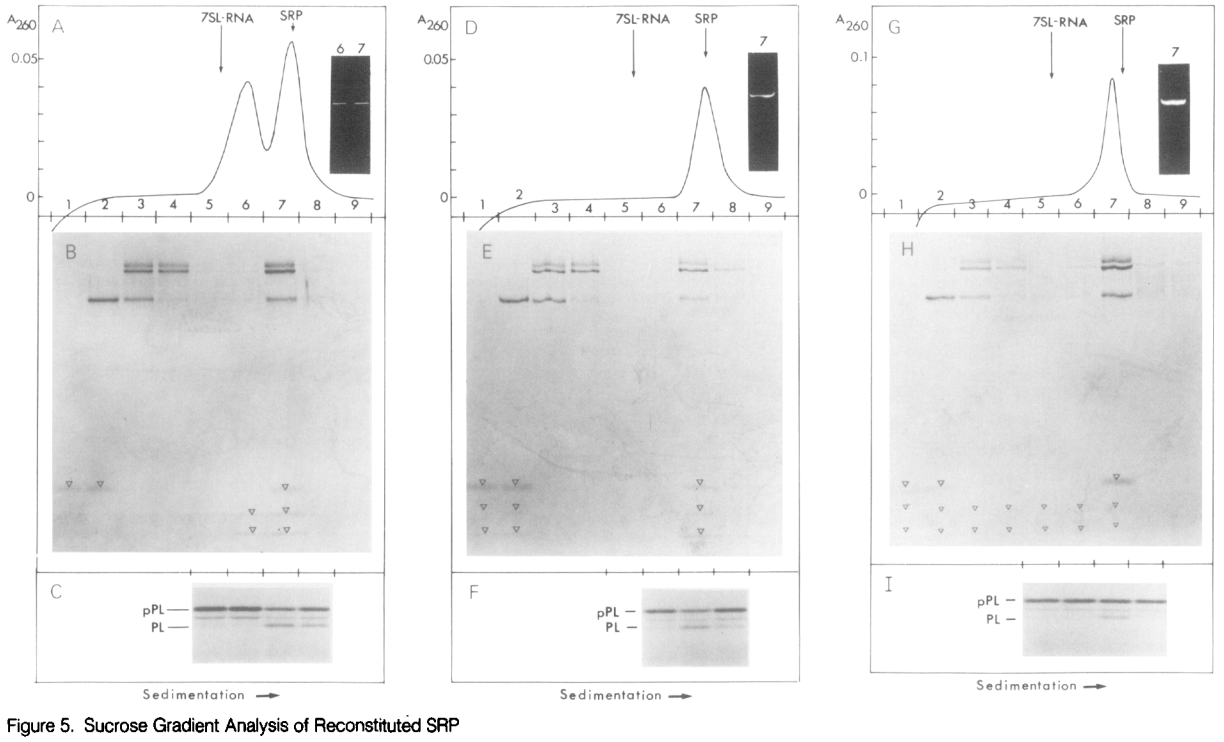


Figure 5. Sucrose Gradient Analysis of Reconstituted SRP

Aliquots (250 μl) of the SRP protein fraction (Figure 2, lanes b) were incubated under reconstitution conditions (see Experimental Procedures) with either canine 7SL-RNA (A, B, and C), X. laevis 7S RNA (D, E, and F), or D. melanogaster 7S RNA (G, H, and I). The reconstituted SRP preparations were then layered on 5%–20% sucrose gradients in 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% Nikkol and centrifuged for 20 hr at 40,000 rpm at 4°C in a Beckman SW40 rotor. Gradients were fractionated using an ISCO gradient fractionator, and the absorbance at 260 nm was recorded (A, D, and G). Fractions of 1.0 ml were collected. An aliquot (800 μl) of each of the nine top fractions was TCA-precipitated, and the polypeptides were displayed after PAGE in SDS (B, E, and H) as described in Figure 2. The faint bands of the lower molecular weight polypeptides are indicated with arrowheads. A 6 μl aliquot of the peak fractions was subjected to an activity assay as described in Experimental Procedures. The autoradiogram of the polyacrylamide gel displaying the in vitro translation products of the assay system is shown (C, F, and I). Note the SRP-dependent conversion of preprolactin (pPL) to prolactin (PL) as translocation of the secretory protein across the microsomal membrane takes place. A 150 μl aliquot of the peak fractions was ethanol-precipitated, and RNA was extracted as described in Figure 2. The RNA was subjected to PAGE in 7 M urea and stained with ethidium bromide (A, D, and G; insets). All lanes were cut from the same gel, and the RNA bands in A, D, and G had identical mobilities. The positions of authentic SRP and naked canine 7SL-RNA were determined in parallel gradients and are indicated in A, D, and G.

also did not compete with 7SL-RNA in the reconstitution reaction (data not shown).

## RNA-Binding Proteins of SRP

To address the question which of the individual SRP proteins would be capable of binding directly to 7SL-RNA in the absence of other proteins, we performed a crude fractionation of the SRP protein fraction by ion-exchange chromatography into three distinct fractions, comprising the 9/14 kd and the 19 kd protein; the 54 kd protein; the 68/72 kd protein. Each of these fractions and various combinations thereof were tested for their ability to bind to an excess of 7SL-RNA under stringent reconstitution conditions (500 mM $K^+$). The data summarized in Table 1 demonstrate that the 9/14 kd, 19 kd protein fraction, as well as the 68/72 kd protein can bind to 7SL-RNA independently of the other proteins, whereas the 54 kd protein requires the presence of the 9/14 kd and/or the 19 kd protein to stably interact with 7SL-RNA. These data are illustrated in a preliminary in vitro assembly map of SRP (Figure 6).

## Heterologous Reconstitution

Since 7SL-RNA is highly conserved through evolution (Ullu and Melli, 1982), attempts to create "chimeric SRPs" from mammalian SRP proteins and amphibian or insect 7SL-RNAs appeared promising. 7S RNA was prepared from either Xenopus laevis or Drosophila melanogaster. Following a fractionation scheme that would render all cellular SRP soluble by raising the salt in the homogenate to 500 mM (Warren and Dobberstein, 1978; Walter and Blobel, submitted), RNA was extracted from a postribosomal supernatant and fractionated by preparative polyacrylamide gel electrophoresis. From both species we obtained a major RNA band that comigrated with mammalian 7SL-RNA in denaturing polyacrylamide gels. Figure 4D shows the activity assays; Figures 5D–5F and Figures 5G–5I the analyses on sucrose gradients of heterologously reconstituted SRP using mammalian SRP protein (Figure 2, lanes b) and 7S RNA prepared from X. laevis or D. melanogaster, respectively.

X. laevis 7S RNA reconstitutes an apparently fully functional SRP (the activity curve, Figure 4D, filled squares, is

Table 1. Binding of Separated SRP Polypeptides to 7SL-RNA

| Polypeptides in Reconstitution | Polypeptides Bound to 7SL-RNA | Polypeptides Not Bound |
|---|---|---|
| 9/14 kd, 19 kd | 9/14 kd, 19 kd | |
| 54 kd | | 54 kd |
| 68/72 kd | 68/72 kd | |
| 9/14 kd, 19 kd, 54 kd | 9/14 kd, 19 kd, 54 kd | |
| 9/14 kd, 19 kd, 68/72 kd | 9/14 kd, 19 kd, 68/72 kd | |
| 54 kd, 68/72 kd | 68/72 kd | 54 kd |
| 9/14 kd, 19 kd, 54 kd, 68/72 kd | 9/14 kd, 19 kd, 54 kd, 68/72 kd | |

SRP proteins were fractionated by chromatography on CM-Sepharose CI-6B as described in Figures 3C and 3D. The column fractions of 750 μl were combined in three pools: fractions 3 and 4; fractions 10, 11, and 12; and fractions 13 and 14; comprising solutions of the 54 kd polypeptide; the 9/14 kd and 19 kd polypeptides; and the 68/72 kd polypeptide, respectively. Each pool was diluted with an equal volume of water, reapplied to a CM-Sepharose CL-6B column (50 μl bed volume), and eluted with 200 μl of a solution of 50 mM TEA, 800 mM KOAc, 5 mM EDTA, 1 mM DTT, 0.01% Nikkol.

Aliquots of 30 μl of the 54 kd protein solution and the 68/72 kd protein solution and aliquots of 65 μl of the 9/14 kd, 19 kd protein solution were mixed as indicated in the first column, and the ionic conditions were adjusted to reconstitution conditions (see Experimental Procedures). A solution containing (4 μg) of 7SL-RNA (this constitutes an about 5-fold molar excess of 7SL-RNA with respect to the proteins) was then added to each reaction, and the mixtures were incubated for 10 min at 37°C. The samples were layered on top of 5%–20% sucrose gradients, and fractionated after centrifugation as described in Figure 5. The nine top fractions were TCA-precipitated, and the polypeptides analyzed by PAGE in SDS. Polypeptides which cosedimented with 7SL-RNA are listed in the second column; polypeptides that remained on top of the gradients are listed in the third column. Two SRP proteins behave as heterodimers in the fractionations. This fact is indicated by listing them as the pair of their polypeptide chains (9/14 kd and 68/72 kd).

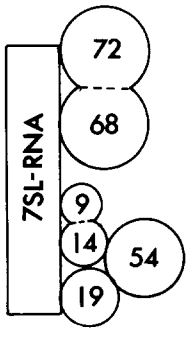


Figure 6. Preliminary Assembly Map of SRP
For discussion see Results.

almost superimposable on that of homologous mammalian SRP, Figure 4C, filled circles), with all six polypeptides assembled as an 11S particle. Preliminary sequence analyses show that X. laevis 7SL-RNA is about 80% homologous to mammalian 7SL-RNA (E. Ullu, personal communication). In contrast, SRP reconstituted with D. melanogaster 7S RNA was less active (Figure 4D, filled triangles) and had a slightly lower sedimentation coefficient than authentic SRP (Figure 5G). The 9/14 kd protein appeared to bind with a lower affinity to the reconstituted particle, such that upon sucrose gradient centrifugation in the high-salt buffer the protein was slowly released from the particle, and as a result was found streaking all across the gradient profile (Figure 5H, small arrowheads). In spite of these minor differences, both heterologous SRP preparations were active in restoring the translocation activity of salt-extracted microsomal membrane vesicles with fidelity (Figures 5F and 5I).

A third heterologous reconstitution was attempted with procaryotic 6S RNA. 6S RNA in Escherichia coli is a relatively abundant RNA of unknown function (Brownlee, 1971; Lee et al., 1978). It has, however, some sequence homology with mammalian 7SL-RNA (P. Walter, unpublished), and there is evidence that it can be immunoprecipitated by an antibody raised against the gene product of a genetically defined component (*secA*) of the procaryotic secretory machinery (Liebke et al., unpublished data). Thus 6S RNA is a likely candidate for the procaryotic equivalent of SRP RNA. However, our attempts to reconstitute a functional SRP from E. coli 6S RNA and mammalian SRP proteins failed. In activity assays we only observe an ambiguous elevated background (compare Figure 4D, open squares, to Figure 4C, open circles), and in sedimentation analyses we were not able to detect any binding of SRP proteins to 6S RNA (data not shown). 6S RNA also did not compete with 7SL-RNA in reconstitution reactions, even when added in a tenfold molar excess (data not shown).

## Discussion

The parallels in the properties of SRP with other well characterized RNPs were used to develop a rapid procedure to disassemble SRP into protein and RNA. The procedure utilizes an unfolding step of SRP with EDTA, followed by a displacement of the basic SRP polypeptides from the 7SL-RNA molecule with polycationic substances. The method is nondestructive, and both protein and RNA can be recovered in >50% yield. Four distinct SRP proteins are obtained in this manner. They are either monomeric (19 and 54 kd) or consist of two polypeptide chains as heterodimers (9/14 kd and 68/72 kd). The proteins in solution are well behaved and do not tend to aggregate over a wide range of ionic conditions (50 mM–1.0 M KOAc), a tendency that was observed when ribosomes were dissociated in chaotropic reagents (Gulik et al., 1978; Giri and Franz, 1978). Although by themselves inactive, the SRP proteins retained their ability to reassociate readily

and stoichiometrically with 7SL-RNA to form active 11S SRP. Because of the mild dissociation conditions, we feel confident that the structure of SRP proteins in solution retains features of the structure of the proteins in the particle. For example, it is likely that the SRP polypeptides that exist in heterodimeric form in solution also exist in a nearest neighbor relationship with each other in SRP.

Since 7SL-RNA is required for the reconstitution of SRP proteins into an 11S particle, we conclude that at least one function of the RNA molecule in SRP is structural. The RNA is providing a matrix for the coordinate assembly of the SRP proteins. This effect is clearly RNA sequence specific, since unrelated RNA molecules are not able to replace 7SL-RNA in this function, nor do any of the SRP proteins interact with measurable affinity to unrelated RNAs.

Our data on the in vitro reassembly of SRP suggest that this process is ordered and cooperative. When the separated SRP proteins were assayed for their ability to bind independently to 7SL-RNA (Table 1), we found that two of the three fractions that we had generated were able to bind specifically to 7SL-RNA in the absence of the other SRP proteins. Thus, whereas at least two SRP proteins (monomers or heterodimers), namely 68/72 kd and 19 and/or 9/14 kd, act as RNA-binding proteins in SRP, only one of them must bind initially to 7SL-RNA to nucleate the in vitro SRP assembly. If two or more SRP proteins would initially bind to 7SL-RNA independently, then an excess of 7SL-RNA would compete in the assembly process, because different SRP proteins would become tied-up on different RNA molecules, and the observed cooperativity could not be explained. This situation is similar to the ribosome, where in the small subunit 12 of 21 proteins (Mizushima and Nomura, 1970; Hochkeppel et al., 1976), and in the large subunit 17 of 32 proteins (Garrett et al., 1974; Marquardt et al., 1979), were shown to be RNA-binding proteins, but in each case only 2 or 3 proteins could be accounted for (Nomura et al., 1969) or identified (Nowotny and Nierhaus, 1982) to be such "assembly initiator proteins" in in vitro reconstitution reactions.

The fact that amphibian and insect 7SL-RNAs are capable of reconstituting active SRP with mammalian SRP proteins reinforces the notion that both 7SL-RNA and SRP—and thus the mechanism of cotranslational protein translocation across membranes—are highly conserved through evolution (Müller et al., 1982). It also demonstrates how interspecies reconstitutions of SRP can be taken advantage of to introduce perturbations into SRP structure. For example, when SRP was reconstituted with insect 7SL-RNA, the 9/14 kd SRP protein bound with a lower apparent affinity and readily dissociated on sucrose gradients, whereas the other SRP proteins remained bound. In a different experiment we demonstrated that the binding of the 54 kd protein to 7SL-RNA was dependent on the presence of the mixture of the 9/14 kd and the 19 kd SRP proteins, which we did not have available in separate fractions. We could therefore not distinguish whether the 9/14 kd, the 19 kd, or both proteins were required for the binding of the 54 kd protein. Together with the data discussed above, however, these data enable us to conclude that the binding of the 54 kd protein must be mediated through the 19 kd protein, since as the 9/14 kd protein was gradually displaced from the chimeric SRP on the sucrose gradient, the 54 kd protein (and the 19 kd protein) remained tightly bound.

As it was summarized in the Introduction, the function of SRP in the process of protein translocation across the membrane of the endoplasmic reticulum is well established and can be readily assayed in vitro. Thus we anticipate that the relatively simple molecular composition of SRP and the herein demonstrated ease with which the particle can be manipulated, fractionated, and specifically perturbed will allow us in future studies to relate structural features of SRP to its intriguing multiple functions in the cellular translation and translocation machinery.

## Experimental Procedures

### Materials

A stock solution of 1.0 M triethanolamine (Sigma) was adjusted to pH 7.5 at room temperature with acetic acid and, as such, is referred to as TEA. A stock solution of 4.0 M KOAc was adjusted to pH 7.5 at room temperature with acetic acid.

The nonionic detergent Nikkol (octaethyleneglycoldodecylether) was purchased directly from Nikko Chemical Corp., Tokyo, Japan. Nikkol was previously shown to stabilize SRP activity (Walter and Blobel, 1980). We therefore also included the detergent at low concentration in all buffers of the disassembly and reassembly reactions of SRP. So far, however, we have no evidence that this precaution is necessary.

### Preparation of Microsomal Membranes and SRP

Salt-extracted (SRP-depleted) microsomal membranes were prepared as described elsewhere (Walter and Blobel, 1983a), except that the column-washing step (chromatography on Sepharose CI-2B) was replaced by three consecutive washes of the membranes by pelleting (30 min, 100,000 × g) and resuspending of the membranes in 50 mM TEA, 1.5 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF.

SRP was prepared from these membranes as described (Walter and Blobel, 1983b). It was purified on an $\omega$-aminopentyl agarose column, concentrated on DEAE-Sepharose CI-6B, and subjected to sucrose gradient centrifugation.

### Activity Assay

Bovine pituitary and rabbit reticulocyte RNA were translated together in a wheat germ cell-free system (25 $\mu$l final volume) in the presence of 1 equivalent of salt-extracted (SRP-depleted) microsomal membranes as described elsewhere (Walter and Blobel, 1983a). Translation products were displayed by PAGE in SDS, and bands were localized by autoradiography and quantitated by scintillation counting of the excised gel pieces (Walter et al., 1979).

The two effects of SRP in this assay system—namely, to catalyze the translocation of secretory protein (preprolactin) across the microsomal membrane (and concomitantly converting it from preprolactin to prolactin) and to cause some arrest of preprolactin synthesis (but not of globin synthesis, the control mRNA in this assay)—were quantitated as described previously (Gilmore et al., 1982a). Their sum expresses total SRP activity (% processing plus % inhibition) as the percentage of the total nascent preprolactin chains affected by SRP. The ionic conditions of the assay system were kept constant at 150 mM KOAc and 2 mM $Mg(OAc)_2$ in all cases.

### Dissociation of SRP

An aliquot (1 ml) of gradient-purified SRP (3.0 $A_{260}$ units/ml, 1.8 $\mu$M) in 350 mM sucrose, 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT,

0.01% Nikkol was diluted with an equal volume (1 ml) of a solution of 50 mM TEA, 10 mM EDTA, 1 mM DTT, 0.01% Nikkol on ice. DEAE-cellulose (DE 53, Whatman) was prepared by resuspending the resin in 4 M KOAc, followed by two washes in about 10 volumes of water, followed by two washes in about 10 volumes of dissociation buffer (50 mM TEA, 250 mM KOAc, 5 mM EDTA, 1 mM DTT, 0.01% Nikkol). The SRP solution prepared above was added to the pelleted DEAE-cellulose (1 ml volume of pellet) and incubated for 10 min on ice, followed by an incubation at 37°C for 10 min. The tube was mixed by inversion every minute. The supernatant was then removed after pelleting the DEAE-cellulose. The DEAE-cellulose was resuspended in 2 ml of dissociation buffer (see above) and incubated for 10 min at 37°C. The supernatant was removed, combined with the first supernatant, and is referred to as SRP protein fraction.

The remaining DEAE-cellulose pellet was eluted twice in batch with a total of 4 ml dissociation buffer containing 1 M KOAc at 37°C. The high-salt eluates were combined. Ice-cold ethanol, 10 ml, was added, and the mixture was incubated for 30 min at −80°C. It was then centrifuged for 10 min at 10,000 × g. The resulting pellet was resuspended in 0.5 ml of a solution of 1% SDS, 150 mM NaOAc, 20 mM Na phosphate, pH 6.5, containing proteinase K (Boehringer) at 200 μg/ml following incubation for 1 hr at 37°C. This suspension was then extracted three times with an equal volume of buffer-saturated phenol:chloroform:isoamyl alcohol (50:50:1), extracted once with an equal volume of diethyl ether, and reprecipitated with 2.5 volumes of ethanol after the salt was raised to 300 mM NaOAc. The ethanol precipitation was repeated twice more; the final pellet was washed with ice-cold 80% ethanol, dried in vacuum, and resuspended in sterile water. The concentration was estimated by absorbance determination (assuming 20 $A_{260}$ units equal 1 mg RNA) and adjusted to 2 mg/ml. From 500 μg SRP we obtained 60 μg RNA, which is about 60% of the theoretical yield. The RNA fraction, referred to as 7SL-RNA, was protein-free as determined by protein assay (Schaffner and Weissman, 1973) and PAGE in SDS and did not inhibit in vitro protein synthesis.

### Preparation of Other RNAs

Canine ribosomal 5S RNA was prepared by extractions of pancreatic microsomal membranes with EDTA and 0.5 M KOAc. The resulting salt extract was fractionated on ω-aminopentyl agarose columns, and the eluate was further fractionated on sucrose gradients as described (Walter and Blobel, 1983b). The 7S peak was collected, comprising the known complex of ribosomal protein L5 together with ribosomal 5S RNA. The RNA was extracted by proteinase K treatment followed by phenol extractions as described above.

Drosophila melanogaster 7S RNA and Xenopus laevis 7S RNA were prepared from 3 g of embryos (6–12 hr harvest) and 3 g of liver tissue, respectively. The material was homogenized in 12 ml of a solution of 250 mM sucrose, 50 mM TEA, 50 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM PMSF in a Potter-Elvehjem homogenizer. Ice-cold 4 M KOAc was added to the homogenate to a final concentration of 0.5 M KOAc to extract SRP from ribosomes and endoplasmic reticulum (Walter and Blobel, submitted). The homogenates were then centrifuged for 2.5 hr at 140,000 × g. RNA was extracted from the supernatant fractions after ethanol precipitation and proteinase K treatment as described above. The RNA was electrophoresed on a 7% preparative polyacrylamide slab gel in 7 M urea. Side strips were cut from the gel and stained in ethidium bromide. In the 7S region of the gel a major sharp band was detected in both cases which comigrated with canine 7SL-RNA. Stained side strips were used as markers, and this band was excised from the main unstained portions of the gel. The gel pieces were incubated for 1 hr in sterile water and minced with a sterile scalpel blade, and the RNA was eluted by shaking overnight into 12 ml of a solution of 0.5 M $NH_4OAc$, 10 mM $Mg(OAc)_2$, 1 mM EGTA. The eluates were diluted with an equal volume of water and passed over 100 μl DE53 columns. The RNAs were eluted with 250 μl 1 M NaOAc and ethanol precipitated as described above. The final yields were about 20 μg each.

Escherichia coli 6S RNA was prepared from a 1 M salt wash of E. coli ribosomes (which was a generous gift of Dr. Matthias Müller) by preparative PAGE and DEAE chromatography as described above.

### Reconstitution of SRP

SRP was routinely reconstituted at 500 mM KOAc and 5 mM $Mg(OAc)_2$, although different salt conditions (see Results) were equally successful. A 4 μl aliquot of SRP protein fraction was mixed with 1 μl of a solution of 7SL-RNA at 0.07 μg/μl on ice. This concentration made 7SL-RNA about stoichiometric with the limiting amounts of the 9/14 kd SRP protein (see Figure 2 and Results). A 1 μl aliquot of a solution of 100 mM TEA, 2M KOAc, 50 mM $Mg(OAc)_2$, 2 mM DTT, 0.02% Nikkol was then added to the mixture to yield the ionic conditions of the reconstitution reaction of 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% Nikkol. The mixture was incubated for 10 min on ice, followed by 10 min at 37°C. Reconstituted SRP could be rapidly frozen in liquid $N_2$ and stored at −80°C before analysis by activity assay or sucrose gradient centrifugation.

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