Subcellular Distribution of Signal Recognition Particle and 7SL-RNA Determined with Polypeptide-Specific Antibodies and Complementary DNA Probe

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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. The particle was previously shown to function in protein translocation across and protein integration into the endoplasmic reticulum membrane.

Polypeptide specific antibodies were raised in rabbits against the 72,000-, 68,000-, and 54,000-mol-wt polypeptide of SRP. All three antibodies are shown to neutralize SRP activity in vitro. A solid phase radioimmune assay is described and used to follow SRP in various cell fractions. The partitioning of SRP is shown to be dependent on the ionic conditions of the fractionation. Under conditions approximating physiological ionic strength, SRP is found to be about equally distributed between a membrane associated (38%) and a free (15%) or ribosome associated (47%) state. Furthermore, it is shown that >75% of the total cellular 7SL-RNA is associated with SRP polypeptide in these fractions. Thus it is likely that the major—if not the only—cellular function of 7SL-RNA is as a part of SRP.

SRP consists of six distinct polypeptide chains (12) and one molecule of small cytoplasmic 7SL-RNA (13). Here we describe the preparation of specific antibody against three of the polypeptide components of SRP and their use to study its function and subcellular distribution.

MATERIALS AND METHODS

Preparation of Antibodies against Purified SRP Polypeptides: SRP was denatured by boiling in SDS and SRP polypeptides were fractionated by chromatography on hydroxyapatite (HTP; Biorad Laboratories, Richmond, CA) (14). The column was developed with a linear gradient of 10 column volumes of 50–400 mM Na phosphate (pH 6.5), 0.1% SDS, 1 mM dithiothreitol. The elution position of SRP polypeptides was determined by subjecting aliquots of the column fractions to PAGE in SDS. The 68,000-mol-wt SRP polypeptide could be well separated from the 72,000- and 54,000-mol-wt polypeptides. The peak fractions were trichloroacetic acid–precipitated, subjected to preparative PAGE in SDS and eluted from the gel as described (15). The eluates were concentrated on a small hydroxyapatite column and checked for purity on PAGE (see Fig. 1, lanes b–e). Antibodies were raised in white New Zealand rabbits against the 72,000-mol-wt, the 68,000-, and the 54,000-mol-wt SRP polypeptide. About 50 μg of each polypeptide were injected as emulsion in complete Freund’s adjuvant directly into lymph nodes. Rabbits were boosted twice at 10-d intervals with 50–100 μg antigen each, after which time a stable titer was reached (as assayed by dot blots [16]).

Polypeptide specific IgGs were prepared by immunoselection of the sera on
Sepharose columns to which the corresponding purified SRP polypeptide were covalently linked. To a 2-ml aliquot of serum a solution of 4 M NaCl and a solution of 10% Triton X-100, 2% SDS was added to yield final concentrations of 500 mM NaCl (taking into consideration that the serum contained ~150 mM NaCl), 0.1% Triton X-100, 0.02% SDS. The serum was then recharged for 10 times over a 0.5-ml column of Sepharose CL-4B having ~50 μg of purified SRP polypeptide bound to it. The column was washed with 2.5 ml of solution A (20 mM Na phosphate, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.02% SDS) followed by 2.5 ml of solution A without detergents, followed by 0.5 ml of a solution of 3.0 M NH4SCN, 50 mM Na phosphate, pH 7.5, followed by 1 ml of solution A without detergents. The eluates were collected on ice into a siliconized Corex tube containing 100 μl of a solution of 0.5 M Na4HPO4 to neutralize the first elution buffer. IgG was precipitated by the addition of an equal volume of saturated (NH4)2SO4 (30 min on ice) and collected by centrifugation for 30 min at 10,000 g. The pellet was dissolved in a small volume of PBS; the solution was precipitated with (NH4)2SO4 and the precipitate was finally dissolved in PBS containing 50% glycerol, 0.02% NaN3 and stored at −20°C. The protein concentration was determined by the method of Schaffner and Weissmann (17). When IgG fractions were to be added to in vitro translation systems, the phosphate buffer was first exchanged by gel filtration to a buffer containing 50 mM tris(hydroxymethyl)aminomethane - HCl pH 7.5, (TEA), 150 mM KOAc, 10% glycerol.

Transfer of Proteins to Nitrocellulose and Detection with Antibody: Gel blots were set up and the antigens were detected as described by Fisher et al. (16) with the following modification. Immunoselected IgG fractions were used throughout this study. We usually employed 50 ng immunoselected IgG per lane of the SDS polyacrylamide gel to be analyzed. Detection of bound antibody was performed with iodinated second antibody (goat-anti-rabbit IgG, Cappel Laboratories, Cochranville, PA) labeled with 125I Bolton-Hunter reagent (18) to ~4 μCi/μg specific activity. After incubation with second antibody the nitrocellulose sheets were washed and dried as described and then autoradiographed using an intensifying screen. Radioactive bands were localized by aligning the film and the gel, they were excised from the gel and their radioactivity was determined by gamma counting.

Transfer of RNA to Nitrocellulose and Detection with Cloned cDNA: Samples up to 10 μg total nucleic acid were electrophoresed on 2 mm thick 1% agarose gels containing 2.2 M HCHO in 20 mM 3-N-morpholinolpropanesulfonic acid, 5 mM NaOAc, 0.1 mM EDTA at pH 7.0 (19). After electrophoresis the gels were soaked in 20 times 15 mM Na citrate, 150 mM NaCl, pH 7.0 (SSC) for 30 min at room temperature, blotted onto nitrocellulose overnight using 10 times SSC as transfer buffer. The nitrocellulose blots were exposed to 10 times over a 0.5-ml column of Sepharose Cl-4B having ~50 μg of purified SRP polypeptide bound to it. The column was washed with 2.5 ml of solution A (20 mM Na phosphate, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.02% SDS) followed by 2.5 ml of solution A without detergents, followed by 0.5 ml of a solution of 3.0 M NH4SCN, 50 mM Na phosphate, pH 7.5, followed by 1 ml of solution A without detergents. The eluates were collected on ice into a siliconized Corex tube containing 100 μl of a solution of 0.5 M Na4HPO4 to neutralize the first elution buffer. IgG was precipitated by the addition of an equal volume of saturated (NH4)2SO4 (30 min on ice) and collected by centrifugation for 30 min at 10,000 g. The pellet was dissolved in a small volume of PBS; the solution was precipitated with (NH4)2SO4 and the precipitate was finally dissolved in PBS containing 50% glycerol, 0.02% NaN3 and stored at −20°C. The protein concentration was determined by the method of Schaffner and Weissmann (17). When IgG fractions were to be added to in vitro translation systems, the phosphate buffer was first exchanged by gel filtration to a buffer containing 50 mM tris(hydroxymethyl)aminomethane - HCl pH 7.5, (TEA), 150 mM KOAc, 10% glycerol.

RESULTS

Antibodies against SRP Polypeptides: To raise antibodies specific to individual SRP polypeptides, we first denatured purified SRP in SDS and then applied separation techniques in SDS to fractionate the polypeptides. We found it sufficient to chromatograph the polypeptide mixture on hydroxylapatite columns (14) and then to subject the peak fractions to preparative PAGE in SDS. As shown in Fig. 1, lanes h–d, we were able to obtain fractions of the larger SRP polypeptides that showed no detectable cross-contamination. These fractions were used as antigen in immunization and in immunoselection procedures (see Materials and Methods) to obtain high titer, monospecific IgG fractions. When a total pancreatic protein profile was blotted onto nitrocellulose (Fig. 1, lane f) and probed with these IgG fractions, only one band of the same molecular weight as the corresponding SRP polypeptide was detected (Fig. 1, lanes h–j). It is apparent that these three polypeptides were not cross-reactive, nor did the antibodies detect any of the three small molecular weight SRP polypeptides. Antigens of the same molecular weight could also be detected in different tissues of various mammalian species, namely dog liver (Fig. 2, lanes a–c), bovine pituitary (Fig. 2, lanes d–f), human placent (Fig. 2, lanes g–i) and rabbit reticulocytes (E. Evans and P. Walter, unpublished observations). In bovine pituitary as well as human placenta the anti-54,000-mol-wt antibody also detected some minor bands, in addition to the 54,000-mol-wt polypeptide (Fig. 2, lanes i/ and j). At present, we cannot distinguish whether these bands arise from breakdown of 54,000-mol-wt SRP polypeptide and/or additional cross-reactive proteins in these tissues. No SRP cross-reactive polypeptides were detected in amphibian, insect, plant, yeast, or procaryotic total cell lysates (data not shown).

Antibodies against SRP Polypeptides Neutralize SRP Activity In Vitro: When antibodies against the individual SRP polypeptides were titrated into an in vitro protein translation assay (20)
the following data were obtained (see Fig. 3). In each of the three cases an inhibition of the protein translocation process was observed as the IgG concentration was raised. These results indicate that all three IgG fractions were able to recognize native SRP (even though they were raised against SDS denatured polypeptides) and to block the function of the particle. As a result, the SRP-dependent process of protein translocation could no longer take place. All three of the specific IgG fractions caused <30% inhibition on protein synthesis in the absence of RM (data not shown).

Apparently only a fraction of the IgG molecules (raised and immunoselected against SDS-denatured polypeptides) was capable of binding to native SRP. This explains the excess of IgG over SRP required to block translocation (at 100 μg/ml IgG is in about 20-fold molar excess over SRP in these reactions). In addition, some of the IgG might have been irreversibly denatured in the immunoselection step (see below).

It is interesting to note that while increasing concentrations of IgG, in all three cases, led to a decrease in the synthesis of translocated prolactin (PL) only the presence of anti-54,000-mol-wt IgG did not cause a concomitant increase in proprolactin (pPL) synthesis. This result might imply that while anti-72,000-mol-wt IgG and anti-68,000-mol-wt IgG blocked some of the early functions of SRP, the anti-54,000-mol-wt IgG apparently still allowed SRP-dependent elongation arrest of pPL synthesis to take place, but interfered with the subsequent release of this arrest by SRP receptor. We have attempted to reconfirm these results using Fab fragments of IgG fractions, to avoid possible artifacts due to cross-linking of individual SRP molecules with divalent IgG. However, so far we have failed to produce Fab fragments with detectable activity. In our hands, the immunoselected IgG fractions tended to precipitate irreversibly in the papain digestion step. The effect might have been caused by the considerable dilution and/or partial denaturation in the immunoselection step. This step was, however, required to produce IgG of sufficient specific activity to show an effect in the in vitro assay. Thus, although we could demonstrate that all three IgG fractions inhibit SRP activity, the above mentioned molecular details of this inhibition have to be interpreted with caution, due to the lack of monovalent Fab fragments as probes.

Distribution of SRP during Cell Fractionation

The insert in Fig. 4 shows that the blot assay as used in Figs. 1 and 2 can be used in a quantitative way, such that the signal of second antibody bound (measured in counts per minute in a specific band on the nitrocellulose blot) is directly proportional to the amount of antigen present in the original sample. We have used this assay to follow the 68,000-mol-wt SRP polypeptide as a representative for SRP through the cell...
fractionation scheme that is generally used by us to prepare
dog pancreatic microsomal membranes (Fig. 4). We estimated
from the data shown in Fig. 4 that we recovered ~35% of
the SRP present in the homogenate (Fig. 4A) in the rough
microsomal fraction (Fig. 4G), whereas 27% were lost in the
nuclear pellet (Fig. 4B), 7% in the mitochondrial pellet (Fig.
4D) and only 2% were left in the final cytoplasmic supernatant
(Fig. 4F). Another 30% were unaccounted for, repre-
senting the additive losses in the three subsequent fractiona-
tion steps (Fig. 4, compare A' to A, C' to C, and E' to E).

To address questions of the in vivo distribution of SRP
between the different cellular structures with which SRP is
interacting functionally, we decided to investigate its associa-
tions in the postmitochondrial supernatant (Fig. 4E) more
closely. For this purpose, we fractionated the postmitochond-
ial supernatant further on sucrose gradients (Fig. 5). Since
it was known that the interaction of SRP with rough micro-
somal membranes is salt-dependent (12, 21), we performed
this gradient analysis at different salt concentrations. When
the UV profile of the sucrose gradients was analyzed (Fig.
5, A–E), three distinct peaks could be distinguished, one on
the top of the gradient corresponding to a cytoplasmic frac-
tion, a second corresponding to 80S monomeric ribosomes
possibly containing a tail of partially degraded free polysomes
and a third peak corresponding to the rough microsomal
membrane SRP. SRP was quantitated measuring the
amount of 68,000-mol-wt SRP polypeptide in fractions across
the gradients (Fig. 5, F–J). From these data (summarized in
Fig. 5 K) it was apparent that at low salt concentrations (50
mM KOAc) most of SRP was membrane-associated. At salt
concentrations approximating physiological conditions (150
mM KOAc) some SRP dissociated from the membrane such
that an about equal distribution between membrane-bound
SRP (38%) and ribosome-associated SRP (47%), as well as a

![Figure 4](image-url)

**Figure 4** Distribution of SRP during cell fractionation. Detection of the 68,000-mol-wt SRP polypeptide using immunoselected anti-68,000-mol-wt IgG on nitrocellulose blots (see Materials and Methods) was used to quantify the distribution of SRP during cell fractionation of dog pancreas. Cell fractionation was carried out as described (20), equivalent aliquots of the various fractions were precipitated by trichloroacetic acid, subjected to PAGE in SDS and transferred to nitrocellulose. The anti-68,000-mol-wt SRP polypeptide was detected by incubating the blot with 68,000-mol-wt IgG (50 μg/lane), followed by 121 rabbit anti goat IgG (50,000 cpm/lane) and subsequent autoradiography. The autoradiograph was used to localize the bands on the nitrocellulose sheet. The bands were excised and their radioactivity determined by gamma counting. The *inset* shows a titration of rough microsomes (%M) to demonstrate that the assay is linear. The fractions analyzed (see reference 20 for detailed conditions for cell fractionation) were homogenate (A), nuclear pellet (B), postnuclear supernatant (C) (after 10 min at 1,000 g), mitochondrial pellet (D), postmitochondrial supernatant (E) (after 10 min at 10,000 g), high-speed supernatant (F) and rough microsomes (G) (after 2.5 h at 140,000 g). The fractionation is diagrammatically displayed in the right hand panel. To show the recovery of SRP, the sums of the fractions resulting in each fractionation step are given (A', C', and E') and should be compared to the corresponding starting material (A' to A, C' to C and E' to E, respectively).

![Figure 5](image-url)

**Figure 5** Distribution of SRP as a function of ionic strength. Aliquots of postmitochondrial supernatant (fraction E in Fig. 4) were adjusted to various KOAc concentrations (see below) and layered on top of a 10–30% sucrose gradient in 50 mM TEA, 6 mM Mg(OAc)2, 1 mM EDTA, 1 mM dithiothreitol, containing KOAc at the following concentrations: 50 mM (A and J), 100 mM (B and G), 150 mM (C and H), 250 mM (D and I), and 500 mM (E and J). The gradient tubes also contained a 1.0-ml cushion of 70% sucrose in the same buffers at the bottom to prevent microsomes from pelleting. Gradients were centrifuged for 100 min at 4°C at 40,000 rpm in a Beckman SW40 rotor. They were fractionated using an ISCO gradient fractionator and the absorbance at 280 nm was recorded (A–E). Ten equally sized fractions were collected. Polypeptides were trichloroacetic acid-precipitated, subjected to PAGE in SDS and the amount of 68,000-mol-wt SRP polypeptide, was determined as described in Fig. 4. The cpm values were normalized, such that the sum of all fractions of each gradient equaled 1. The position of the 80S monomeric ribosome is indicated with arrows. In K the distribution of SRP is plotted as a function of the salt concentration. Three cellular fractions were defined as follows: free SRP (●) as the sum of the SRP contained in the two top fractions of the gradients; membrane-bound SRP (■) as the SRP contained in the two bottom fractions; and ribosome-bound SRP (○) as the SRP contained in the six middle fractions. Cpm values are given as percentage of SRP in one of these three fractions.
significant level of free SRP (15%) was observed. When the
salt concentration was raised further, SRP was found to
disassociate readily from both ribosomes and rough micro-
somes, and could be almost quantitatively recovered in the
cytoplasmic fraction.

These data confirm the rationale behind the cell fractiona-
tion scheme that generally precedes the purification of SRP.
A rough microsomal membrane fraction is prepared in under
low salt (50 mM KOAc) conditions and SRP is subsequently
extracted from these microsomes in a 500 mM salt-wash.

Correlation of 68,000-mol-wt SRP Polypeptide
with 7SL-RNA

Using the data described in Figs. 4 and 5 we designed a
fractionation scheme for canine pancreas that would render
the SRP doi soluble in the initial homogenate and would
yield a quantitative recovery (Fig. 6F). The rationale was to
dissociate SRP already at the stage of the homogenate from
all cellular structures and then to remove these organelles by
differential centrifugation. Nuclei were removed first in a low
speed spin to avoid breakage and the release of DNA. The
nuclear fraction was reextracted with 500 mM KOAc and the
extract was combined with the postnuclear supernatant. All
remaining material with a sedimentation coefficient larger
than 50S was removed from this supernatant during a high
speed centrifugation step.

The analysis of the resulting three fractions, the homogenate
H, the high-salt pellet fraction P, and the high-salt supernatant
S is shown in Fig. 6. The polypeptide profile (Fig. 6A) was
dominated by the massive amounts of secretory proteins
present in the secretory vesicles of the tissue. These major
bands were also dominant in fraction S, presumably due to
the breakage of some of the secretory granules. That a good
fractionation was obtained nevertheless was apparent from
the distribution of nucleic acid (Fig. 6B). Whereas all of the
DNA and ribosomal RNA was shown to pellet (present in
fractions H and P), the majority of 7S RNA and transfer
RNA was recovered in fraction S.

To correlate the cell fractionation behavior of SRP-poly-
peptides with that of 7SL-RNA, we used two specific probes to quantitate these components. The 68,000-mol-wt SRP polypeptide was almost quantitatively recovered (90%) from fraction S, as estimated by probing with anti-68,000-mol-wt IgG as described above (Fig. 6C). 7SL-RNA was quantitated on a nitrocellulose blot of a formaldehyde-agarose gel that was probed by hybridization (19) with a cloned restriction fragment of 7SL-RNA sequences. This clone (residues 105 to 230 of 7SL-RNA [22]) was kindly provided to us by Elizabetta Ullu and was devoid of the regions in 7SL-RNA which are homologous to the repetitive ALU-sequence family. It is thus a unique and specific probe for 7SL-RNA and does not crosshybridize with other ALU-like transcripts. Fig. 6E shows that this solid phase hybridization assay resulted in a linear response curve. We quantitated the amount of 7SL-RNA in the three cell fractions and, as shown in Fig. 6D, we were able to demonstrate that ~87% of all detectable cellular 7SL-RNA partitioned into fraction S.

To directly demonstrate that all 7SL-RNA contained in fraction S is indeed associated with SRP polypeptides (and thus most likely contained in SRP) we performed the immunoprecipitation experiments shown in Fig. 7. It is apparent that under the native immunoprecipitation conditions used, anti-68,000-mol-wt IgG was capable to bind and thus to precipitate 87% of 7SL-RNA (Fig. 7C) that were contained in fraction S (Fig. 6). This immunoprecipitation was shown to be specific, because nonimmune IgG did not precipitate 68,000-mol-wt SRP polypeptide (Fig. 7B) or 7SL-RNA (Fig. 7C). Furthermore, all stained transfer RNA (Fig. 7A), as well as all stained protein (which was not obscured by the IgG polypeptide chains) was not immunoprecipitated with anti-68,000-mol-wt IgG (data not shown).

Interestingly, all stainable 7SL RNA in fraction S was immunoprecipitable with anti-68,000-mol-wt IgG (Fig. 7A), indicating that all of it corresponds to 7SL-RNA. This finding allowed us to generalize the fractionation scheme shown in Fig. 6F and to prepare essentially pure 7SL-RNA from other sources, e.g., Xenopus laevis liver and Drosophila melanogaster embryos (23). In each case we obtained a major band in the corresponding fraction S which co-migrated with canine 7SL-RNA on polyacrylamide gels in 7 M urea. When eluted after preparative PAGE all of the RNA contained in these bands could be reconstituted with mammalian SRP polypeptides to form active SRP (23). Thus, all 7SL RNA in fraction S obtained from a variety of sources appeared to be functionally equivalent to 7SL-RNA.

**DISCUSSION**

Using polypeptide-specific antibodies against three of the six polypeptides of SRP, we have demonstrated here the existence of cross-reactive proteins of identical molecular weight in a variety of tissues and species. Although these analyses were limited to mammalian species due to the particular crossreactivity properties of the antibodies, many lines of evidence indicate that SRP or analogous particles exist in all eucaryotic and maybe even procaryotic cells. Most intriguing are experiments that demonstrate the wide interspecies interchangeability of the various functional components of the translation/translocation machineries in vitro assays. The most heterogeneous “in vitro chimeras” that have been shown to work with fidelity include (a) procaryotic secretory proteins synthesized on plant ribosomes, recognized by mammalian SRP and translocated across mammalian membranes (24) and (b) the demonstration that functional SRP themselves could be reconstituted from mammalian SRP protein and either amphibian or insect 7SL-RNA (23).

The intracellular distribution of SRP as determined by cell fractionation (Fig. 5) reflects its potential to interact with various cellular components. In particular, SRP was proposed to cycle between a cytoplasmic and a membrane-bound state (5). In the cytoplasm at least three different states can be distinguished (1): (a) a free form, (b) a form loosely bound to biosynthetically inactive ribosomes, and (c) a tight interaction with ribosomes involved in the synthesis of secretory proteins. At physiological ionic strength an about even distribution of SRP between the cytoplasmic and the membrane-bound state was observed. A significant amount of the cytoplasmic SRP appeared to be free. Depending on the ionic conditions, a different partitioning of SRP into subcellular fractions can be achieved. These results may explain some of the confusing data on the subcellular distribution of 7SL-RNA that have previously accumulated in the literature (25–28).

Using the SRP specific antibodies in a combination of radioimmunoassay and immunoprecipitation, we demonstrated here that >75% of the total cellular 7SL-RNA is associated with the 68,000-mol-wt SRP polypeptide that functions as the antigen. We can account only for 75% of the 68,000-mol-wt SRP polypeptide and thus the likely possibility remains that most of the unaccounted 7SL-RNA is also associated with SRP polypeptides. We therefore conclude.
that the main—if not the only—function of 7SL-RNA is as part of SRP. We can, of course, not exclude the possibility that SRP itself functions in more aspects of translation or protein translocation than have been described so far.

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