

Translocation of Proteins Across the Endoplasmic Reticulum I. Signal Recognition Protein (SRP) Binds to In-Vitro-Assembled Polysomes Synthesizing Secretory Protein

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ABSTRACT An 11S protein composed of six polypeptide chains was previously purified from a salt extract of dog pancreas microsomal membranes and shown to be required for translocation of nascent secretory protein across the microsomal membrane (Walter and Blobel 1980 *Proc. Natl. Acad. Sci. U. S. A.* 77:7112-7116). This 11S protein, termed signal recognition protein (SRP), has been shown here (a) to inhibit translation in the wheat germ cell-free system selectivity of mRNA for secretory protein (bovine preprolactin) but not of mRNA for cytoplasmic protein (alpha and beta chain of rabbit globin); (b) to bind with relatively low affinity (apparent $K_D < 5 \times 10^{-5}$) to monomeric wheat germ ribosomes; and (c) to bind selectively and with 6,000-fold higher affinity (apparent $K_D < 8 \times 10^{-9}$) to wheat germ ribosomes engaged in the synthesis of secretory protein but not to those engaged in the synthesis of cytoplasmic protein. Low- and high-affinity binding as well as the selective translation-inhibitory effect were abolished after modification of SRP by *N*-ethyl maleimide. High-affinity binding and the selective translation-inhibitory effect of SRP were largely abolished when the leucine (Leu) analogue β -hydroxy leucine was incorporated into the nascent secretory polypeptide.

We have previously reported the purification from dog pancreas rough microsomes of an 11S protein composed of six polypeptides that is required for translocation of secretory proteins (1). In this series of papers, we describe our elucidation of some of the specific molecular events involved in the 11S protein-mediated translocation of secretory proteins across the endoplasmic reticulum membrane. Our data show that the purified 11S protein functions in the recognition of the signal sequence of nascent secretory protein, and therefore it has been termed "signal recognition protein" (SRP).

In this first paper of the series, we show that SRP binds to nascent, in vitro assembled polysomes synthesizing secretory protein (bovine pituitary preprolactin) and not to those synthesizing cytoplasmic proteins (alpha and beta chain of rabbit globin). Moreover, SRP specifically inhibits translation of secretory protein (in the absence of microsomal vesicles) but not of cytoplasmic protein. This translation-inhibitory effect has been found to correlate with the polysome binding capacity of SRP.

MATERIALS AND METHODS

Materials

[³⁵S]Met (1,000 Ci/mmol) and [¹²⁵I]Bolton-Hunter reagent (2,000 Ci/mmol) were from New England Nuclear, Boston, Mass. Nikkol (octaethyleneglycol-mono-*N*-dodecyl ether) was from Nikko Chemicals Co., LTD, Tokyo, Japan.

Aminopentyl agarose was from Sigma Chemical Co., St. Louis, Mo. β -hydroxy-DL-leucine was from U. S. Biochemical Corp., Cleveland, Ohio. Trasylol (10,000 U/ml) was from FBA Pharmaceuticals, New York, N. Y. NCS tissue solubilizer was from Amersham Corp., Arlington Heights, Ill. The various protease inhibitors were from Sigma Chemical Co., St. Louis, Mo. and Boehringer, Mannheim, Germany.

All preparative procedures were done at 4°C. Optical absorbance determinations were performed in 1% SDS. The 1 M triethanolamine stock buffer was adjusted to pH 7.5 at room temperature with acetic acid and, as such, is referred to as TEA. The 4 M KOAc stock solution was adjusted with HOAc to pH 7.5 at room temperature. All percentages are weight per volume, except where indicated otherwise.

Buffers

(A): 250 mM sucrose, 50 mM TEA, 50 mM KOAc, 6 mM Mg (OAc)₂, 1 mM EDTA, 1 mM DTT (dithiothreitol), 0.5 mM PMSF (phenylmethylsulfonyl fluoride). (B): 250 mM sucrose, 50 mM TEA, 1 mM DTT. (C): 50 mM TEA, 500 mM KOAc, 5 mM Mg (OAc)₂, 1 mM DTT. (D): 50 mM TEA, 1 M KOAc, 10 mM Mg (OAc)₂, 1 mM DTT, 0.05% Nikkol.

Preparation of Microsomal Membranes

Rough microsomal membranes were prepared from freshly excised dog pancreas (2) by a modification of the procedure described (3). The tissue was extensively minced with a razor blade, passed through a tissue press, and homogenized in 4 vol of Buffer A with 5 strokes in a motor driven Potter-Elvehjem homogenizer (Kontes Co., Vineland, N. J.). The homogenate was centrifuged for 10 min at 1,000 *g*_{av} and for 10 min at 10,000 *g*_{av}. Crude rough microsomes (RM) were collected by centrifugation of the 10,000 *g*_{av}-supernate

for 2.5 h at 140,000 g_{av} through 10–15-ml cushions of 1.3 M sucrose in Buffer A. The resulting pellets were resuspended by manual homogenization in a Dounce homogenizer (A-pestle) in Buffer B to a concentration of 50 A_{280} U/ml.

Adsorbed ribosomes and proteins were removed by passing the resuspended crude rough microsomes through a Sepharose C1-2B column (4) in a low-salt buffer [50 mM TEA / 0.5 mM Mg (OAc)₂ / 1 mM DTT]. A 20-ml portion was loaded on a 200-ml column (upward flow, 15 ml/h). The turbid fractions were pooled, and the membranes were collected by centrifugation (15 min at 50,000 g_{av}). The resulting washed RM were resuspended in 20 ml of Buffer B, aliquoted, and rapidly frozen in liquid nitrogen and stored at -80°C .

Salt-extraction of Washed Rough Microsomes

10 ml of an ice-cold salt solution (1.5 M KOAc / 15 mM Mg (OAc)₂) was slowly added to 20 ml of RM. The mixture was incubated for 15 min on ice. The membranes were sedimented for 1 h at 120,000 g_{av} through a cushion of 0.5 M sucrose in Buffer C, and the resulting pellet of salt-extracted RM (K-RM) was resuspended in 20 ml of Buffer C containing 250 mM sucrose. The supernatant fluid fraction, not including the cushion, was recentrifuged (3.5 h at 200,000 g_{av}) to deplete it of ribosomes (post-ribosomal salt extract).

Fractionation of the Salt Extract by Hydrophobic Chromatography

A 2-ml column of aminopentyl-agarose (5.7 $\mu\text{mol/ml}$ of 1,5-diaminopentane) was prewashed with 10 ml of 2 M KOAc and then equilibrated with 20 ml of Buffer C. The post-ribosomal salt extract (24 ml) was passed over the column (6 ml/h). The column was then washed with 10 ml of Buffer C and eluted with Buffer D. As soon as Buffer D appeared in the eluent (detected by an abrupt change in drop size due to the presence of detergent), a 2-ml fraction was collected. This fraction is referred to as SRP and is essentially homogeneous (1).

Further Purification of SRP

For some experiments (as indicated in figure legends) as well as after the radioiodination procedure, SRP was further purified by sucrose gradient centrifugation. A 100- μl portion of column-purified SRP solution (5 U/ μl) was layered on top of a 5–20% sucrose gradient (5 ml) in Buffer C containing 0.01% Nikkol and centrifuged for 6 h at 50,000 rpm in the Beckman SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The gradients were fractionated using an Instrument Specialities Co. (ISCO, Lincoln, Neb.) gradient fractionator with a continuous absorbance monitor and the 11S peak was collected. When iodinated material was repurified, the gradients contained 100 $\mu\text{g/ml}$ autoclaved gelatin as carrier protein.

Labeling of SRP with Bolton-Hunter Reagent

A 100- μl portion of column-purified SRP solution (5 U/ μl , protein concentration 40 $\mu\text{g/ml}$) was incubated with 400 μCi ^{125}I -Bolton-Hunter reagent (2,000 Ci/mmol) for 150 min on ice. Tris-HOAc pH 7.5 was added to 50 mM to terminate the reaction. The mix was then passed over a 1-ml Sephadex G-25 superfine column in buffer C (containing 100 $\mu\text{g/ml}$ autoclaved gelatin and 0.01% Nikkol) and the excluded volume was collected (120 μl). The sample was then repurified on a sucrose gradient as described above. The 11S peak was collected in 550 μl . The radioactivity was determined by gamma counting to be 7,550 cpm/ μl . From these data we calculated that about one in ten SRP molecules was modified.

Cell-free Protein Synthesis

Bovine pituitary RNA and/or rabbit reticulocyte RNA (0.2 A_{260} U per 25 μl of translation mix) were translated in a staphylococcal nuclease-treated (5) wheat germ system (6 μl of wheat germ S23 and 25 μCi [^{35}S]Met (methionine)/25 μl translation mix). All translations were supplemented with human placental RNase inhibitor (6) at a final concentration of 0.01 A_{260} U/ml and a cocktail of selected protease inhibitors (which do not interfere with protein synthesis) at the following final concentrations: pepstatin A 0.1 $\mu\text{g/ml}$, chymostatin 0.1 $\mu\text{g/ml}$, antipain 0.1 $\mu\text{g/ml}$, leupeptin 0.1 $\mu\text{g/ml}$, and trasylol 10 U/ml. The ions that were added with various fractions were taken into account and compensated for to yield final ion concentrations of 150 mM KOAc and 2.0 mM Mg (OAc)₂ in all translations. The nonionic detergent Nikkol was present in all translations at a final concentration of 0.002% to stabilize SRP activity (1). Membranous fractions were always added last, after all of the other components were mixed and the detergent uniformly diluted.

Under these conditions mammalian polysomes still attached to the RM preparation do not read out in the plant translation system, and therefore, no

“stripping” or nuclease treatment of the microsomes to deplete them of their endogenous mRNA activity is necessary.

Quantitation of *in Vitro* Synthesized Proteins

An isotope dilution experiment was carried out to determine the amount of free, nonradioactive Met in our translation system. Nonradioactive Met at $8.5 \pm 1.5 \mu\text{M}$ was found to decrease the amount of radioactive Met incorporated into polypeptide chains by 50%. Therefore, calculations to determine the concentrations of *in vitro* synthesized protein assume an endogenous Met concentration of 8.5 μM .

Polyacrylamide Gel Electrophoresis (PAGE) in Sodium Dodecyl Sulfate (SDS)

Procedures for SDS-PAGE and subsequent autoradiography of dried slab gels were done as described (7, 8). The samples for electrophoresis were precipitated with TCA, reduced with DTT (100 mM) at 100°C for 5 min, and alkylated with iodoacetamide (0.5 M) at 37°C for 1 h. Polyacrylamide gradient (10–15%) slab gels were used throughout. To quantitate the radioactivity in specific polypeptide species, bands were located by autoradiography, excised, and rehydrated for 1 h in 100 μl of H₂O. The rehydrated slices were then incubated in 1 ml of NCS tissue solubilizer / H₂O, 9:1 (vol/vol) for 6 h at 50°C . After cooling, 50 μl of glacial acetic acid were added and radioactivity was determined by liquid scintillation counting in 10 ml of Aquasol. The counting efficiency was determined to be 70%.

Definitions

% Processing is defined as [cpm in prolactin / (cpm in prolactin + cpm in preprolactin)]. It is used as a measure of the translocation activity of membranes added in subsaturating amounts (1).

1 equivalent (eq) is the amount of a fraction (supernatant fluid or membrane) that is derived from 1 μl of a RM suspension at a concentration of 50 A_{280} U/ml. 1 eq is derived from ~ 1 mg of tissue.

1 Unit of translocation activity (U) (a) for a membrane fraction is the amount of membranes that gives the same amount of processing (i.e., translocation) as 1 eq of RM, (b) for SRP is the amount that has to be added back to 1 eq of K-RM to restore activity to that of 1 eq of RM.

RESULTS

During purification of SRP, we noted that SRP inhibited protein synthesis in the wheat germ cell-free translation system if added in the absence of salt-extracted membrane. We decided to investigate the specificity of this phenomenon by comparing the effect of SRP on the translocation of secretory versus cytoplasmic proteins. Rabbit reticulocyte RNA was chosen as a readily available mRNA pool containing species coding almost exclusively for the cytoplasmic proteins alpha and beta globin. When SRP was added in increasing amounts to a translation containing both globin and prolactin mRNA's (but no microsomal vesicles), a concentration-dependent inhibition of preprolactin synthesis was observed (Fig. 1). However, no significant effect on the amount of globin synthesized could be detected. At the highest concentration of SRP used, preprolactin synthesis was essentially completely inhibited.

These findings indicated that SRP was able to affect the translation of specific mRNA's (i.e. inhibition of preprolactin synthesis and not of globin synthesis). On the premise that this effect of SRP on preprolactin synthesis was exerted via a direct interaction of SRP with polysomes synthesizing preprolactin, we attempted to demonstrate an affinity of SRP for these polysomes. It was therefore necessary to radioactively label SRP. Because its metabolic labeling was not feasible, we tested several protein iodination procedures to obtain radioiodinated SRP which, in spite of the perturbation caused by the covalent modification of the protein, would retain most of its properties.

We chose to label SRP with ^{125}I -Bolton-Hunter reagent which allowed labeling under mild conditions. No decrease in

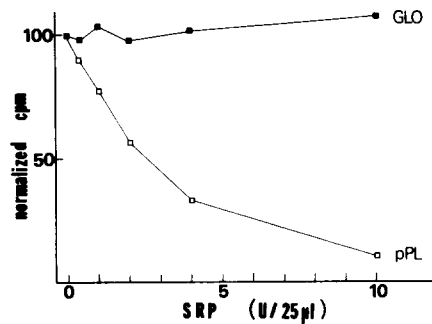


FIGURE 1 SRP inhibits synthesis of preprolactin but not of globin. Bovine pituitary and rabbit reticulocyte RNA were translated together in a wheat germ system (25 μ l) either in the absence or in the presence of various amounts of gradient-purified SRP. The translation products were separated by PAGE in SDS. Bands corresponding to preprolactin (pPL) and globin (GLO) were located by autoradiography, sliced from the dried gel, and the radioactivity was determined (16). The hot acid-insoluble radioactivity in the absence of SRP was: for globin, 416,000 cpm and for preprolactin, 129,000 cpm. These cpm values were normalized to be 100 for globin and preprolactin, respectively. Normalized cpm in globin (■) and preprolactin (□). SRP was gradient purified (see Materials and Methods) and its activity determined in units (U) as defined in Materials and Methods (1).

activity of SRP was detected after the labeling reaction was completed. We estimated that we modified about one in ten SRP molecules. After labeling, SRP was repurified by sucrose gradient centrifugation. Labeled SRP had a sedimentation coefficient of 11S, indistinguishable from unlabeled material (Fig. 2). SDS-PAGE showed the distribution of label among the six polypeptide chains of SRP. Most label was contained in the 54,000 dalton chain; the 70,000 dalton and 68,000 dalton chains were labeled to a lesser extent, and the smaller polypeptide chains were not visualized on the autoradiogram (Fig. 2). We also used oxidative radioiodination procedures (Chloramine T, Lactoperoxidase, Iodogen) to obtain SRP of even higher specific activity, but SRP was inactive and dissociated on sucrose gradients after these procedures.

Because ^{125}I -labeled SRP was indistinguishable from unlabeled SRP with respect to its sedimentation behavior and because it could be rebound to salt-extracted membranes under conditions of low ionic strength (data not shown) we concluded that the perturbation caused by the labeling procedure itself did not severely alter the structure of SRP. ^{125}I -labeled SRP could therefore be used as a probe to study direct physical interactions with preprolactin-synthesizing polysomes.

To assemble preprolactin synthesizing polysomes, a staphylococcal nuclease-treated wheat germ cell-free translation system was used. If exogenous globin or prolactin mRNA was translated, polysomes were formed but in insufficient amounts to be visualized in the UV absorption profile of sucrose gradients (Fig. 3, upper panels).

In vitro assembled polysomes could, however, be readily detected if they were labeled by incorporation of [^{35}S]Met into their nascent polypeptide chains (Fig. 3, middle panels). For these analyses, the translations were allowed to proceed for 20 min, at which point incorporation of label into protein was linear with time (9). Therefore, the translation reaction could be considered to be in a steady-state situation (new ribosomes initiate while others terminate protein synthesis). Because the specific activity of the labeled Met in the translation system was known (see Materials and Methods), we were able to

compute the concentration of nascent chains (or protein-synthesizing ribosomes) in the different gradient fractions (Table I). For these calculations we assumed (based on the steady state argument above) the average Met content of the nascent chains to be one-half of the Met content of the finished molecule. (The completed proteins contain one Met each in rabbit alpha and beta globin (10) and eight Met (11, 12) in a bovine preprolactin molecule.)

If instead of labeled Met we added ^{125}I -labeled SRP and analyzed the polysome profile on sucrose gradients after 20 min of translation, an association of SRP with polysomes synthesizing preprolactin was observed (Fig. 3, lower panels). In this case, the amount of ^{125}I -labeled SRP at the top of the gradient was considerably reduced (relative to that present when no mRNA was translated) and labeled SRP appeared in the position of polysomes (see Table II). In contrast, little or no labeled SRP cosedimented with globin-synthesizing poly-

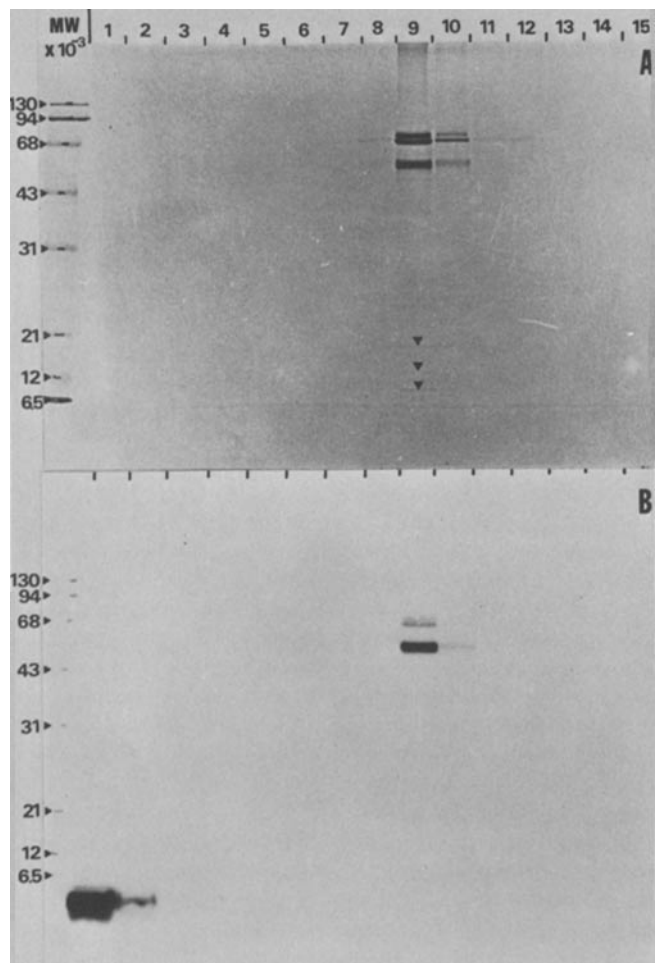


FIGURE 2 Sucrose gradient centrifugation of ^{125}I -labeled SRP. Column-purified SRP was labeled with Bolton-Hunter reagent as described in Materials and Methods. Labeled SRP (50,000 cpm) was mixed with 2,500 eq of column-purified, unlabeled SRP in 500 μ l of Buffer C. The sample was layered on top of a 5–20% sucrose gradient (13 ml) in Buffer C and was centrifuged for 20 h at 4°C at 40,000 rpm in the Beckman SW 40 rotor (Beckman Instruments, Inc.). Fifteen fractions were collected using an ISCO (Instrument Specialties Co.) gradient fractionator. Each fraction was TCA precipitated and the proteins were visualized by Coomassie blue staining after PAGE in SDS (panel A). Autoradiography using an intensifying screen was used to visualize the radioactively labeled polypeptide chains (panel B).

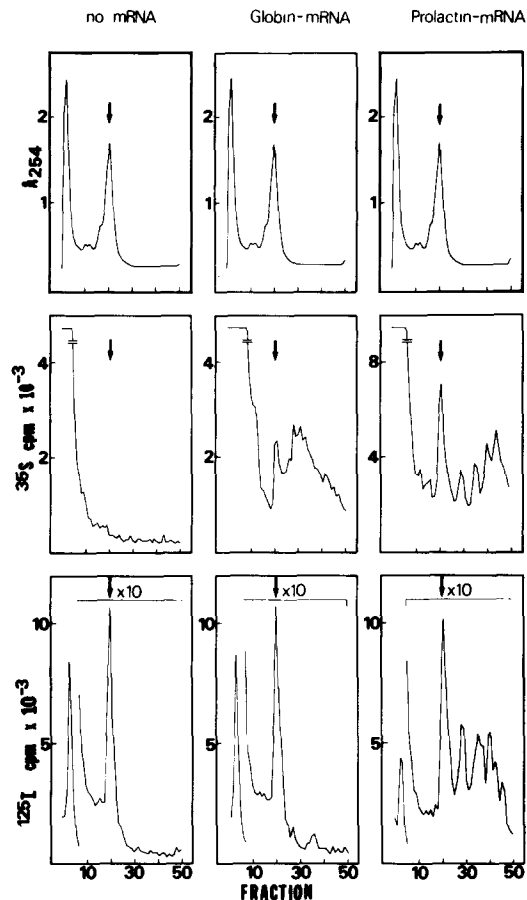


FIGURE 3 Specific binding of SRP to in vitro assembled polysomes. Prolactin or globin mRNA were translated in the wheat germ system in the presence of [^{35}S]Met and SRP (5U/100 μl). After 20 min, the translations were arrested by cooling on ice. A 100- μl portion of translation mix was then layered on top of a 10–30% sucrose gradient (13 ml in 50 mM TEA, 100 mM KOAc, 5 mM Mg (OAc) $_2$ and centrifuged at 4°C at 39,000 rpm for 120 min in the Beckman SW 40 rotor (Beckman Instruments, Inc.). Fifty-two fractions were collected using an ISCO gradient fractionator, and the UV absorbance was recorded with a continuous flow cell (upper panels). 100 μl of each fraction was spotted onto Whatman 3MM filter disks (Whatman, Inc.) and TCA-precipitated for 30 min in 10% TCA at 0°C. The filters were then boiled for 10 min in 5% TCA, dried using subsequent washes with ethanol, ethanol-ether (1:1) and ether, and their radioactivity was determined by scintillation counting in Liquifluor (New England Nuclear, Boston, Mass.) (middle panels). The samples loaded on the sucrose gradient had the following hot acid-insoluble radioactivity in 5 μl : 17,800 cpm (no mRNA added), 143,000 cpm (globin mRNA), 103,000 cpm (prolactin mRNA).

In a separate experiment, prolactin and globin mRNA were translated in the wheat germ system (100 μl) in the presence of ^{125}I -labeled SRP (5 U, 40,000 cpm). After 20 min, the translations were arrested by cooling the mixture on ice and fractionated on sucrose gradients as described above. The distribution of radioactivity was then determined by gamma counting of the collected fractions (lower panels). Note that the scale is expanded by a factor of 10, starting with fraction number 7. Sedimentation is from left to right. The position of the 80S monosome peak is marked with an arrow.

somes (Fig. 3, lower panels; Table II), even though these polysomes were present in approximately fourfold higher amounts than preprolactin-synthesizing polysomes (Table I). It should also be noted that ^{125}I -labeled SRP was found in the position of the monosomes whether or not mRNA was being translated.

Having established that SRP does specifically bind to polysomes synthesizing a secretory protein, i.e. preprolactin, and not to globin-synthesizing polysomes, we designed experiments to localize the information for this specific interaction of SRP with polysomes (whether it is expressed via the nascent chain or other components [e.g. mRNA]). We took advantage of a recent demonstration (13) that incorporation of the Leu analogue, β -hydroxy leucine, into the nascent chain of preprolactin completely abolishes in vitro translocation of this molecule. When we translated prolactin mRNA in the presence of β -hydroxy leucine and in the absence of any exogenously added Leu, we observed a significant decrease in the concentration-dependent inhibition of SRP on preprolactin synthesis (Fig. 4). If, in addition to β -hydroxy leucine, L-Leu was added, β -hydroxy leucine was efficiently competed out (13), and SRP-dependent inhibition (Fig. 4) was fully restored. From the polysome profile of β -hydroxy leucine-incorporating polysomes (Fig. 5, middle panels), it was apparent that polysome formation was only inhibited by 35% (Table I), whereas the binding of ^{125}I -labeled SRP to these polysomes (Fig. 5, lower panels) was decreased by 70% (Table II). The binding of SRP to polysomes synthesizing preprolactin could be restored if β -hydroxy leucine was competed out with Leu (Fig. 5, lower panels, Table II).

We have previously demonstrated that the translocation activity of microsomal membranes is sensitive to the sulfhydryl modifying reagent *N*-ethyl maleimide (NEM) and that the NEM-sensitive site of the translocation machinery is located on SRP (1, 14). The question arose whether NEM modification of SRP would inhibit its binding to preprolactin-synthesizing polysomes or whether it would perhaps affects the translocation process at a later step. From the data in Fig. 6, it is apparent

TABLE I
Quantitation of Newly Synthesized or Nascent Polypeptide in Sucrose Gradients

Fraction	Newly synthesized or nascent polypeptide*			
	Globin	Preprolactin	Preprolactin (OH-Leu)	Preprolactin (OH-Leu + Leu)
Top	180	13	8.8	12
Monosomes	9.8	5.3	4.3	5.4
Polysomes	52	14	9.3	13

The cumulative radioactivity of the following gradient fractions (displayed in Fig. 3 and Fig. 5) was determined and converted to absolute concentrations (see Methods and Results): Top: fractions 1–18; Monosomes: fractions 19–26; Polysomes: fractions 27–50.

* [moles $\times 10^{14}$ /gradient]

TABLE II
Quantitation of SRP in Sucrose gradients

Fraction	SRP [moles $\times 10^{14}$ /gradient]				
	Globin	Preprolactin	Preprolactin (OH-Leu)	Preprolactin (OH-Leu + Leu)	–mRNA
Top	8.1	5.1	7.7	5.3	7.4
Monosomes	1.2	1.1	1.1	1.2	1.0
Polysomes	0.23*	2.4*	0.8*	2.6*	0.4

* The value for “–mRNA” was subtracted as background. The fractions are defined in Table I.

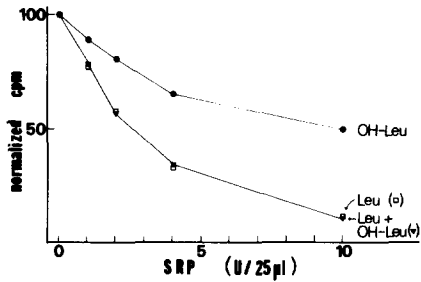


FIGURE 4 Translation-inhibitory effect of SRP is partially abolished after incorporation of beta-hydroxy leucine, a leu analogue. Bovine pituitary RNA was translated in the wheat germ system (25 μ l) either in the absence or in the presence of various amounts of gradient-purified SRP. In addition, the translation systems contained either 2 mM L-Leu (\square), or 10 mM beta-hydroxy-DL-leucine (\bullet), or 2 mM L-Leu plus 10 mM beta-hydroxy-DL-leucine (\blacktriangledown). Radioactivity was determined in the preprolactin band. The cpm values in the absence of SRP were 433,175 cpm (Leu), 347,880 cpm (OH-Leu), 404,380 cpm (OH-Leu plus Leu). These cpm values were normalized to be 100. In agreement with the report of Hortin and Boime (13), we found that the mobility of preprolactin in SDS-polyacrylamide gels was slightly decreased upon incorporation of beta-hydroxy leucine (data not shown).

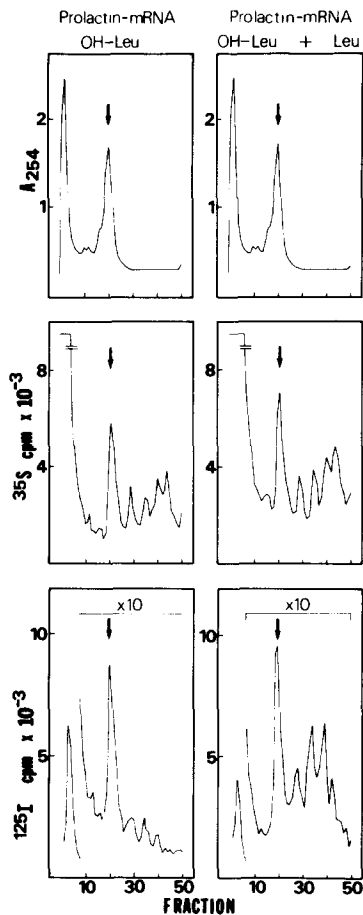


FIGURE 5 Beta-hydroxy leucine incorporation into nascent chains inhibits SRP binding to polysomes. Prolactin mRNA was translated and the mixture was analyzed as described in Fig. 3, except that the translation systems contained 10 mM beta-hydroxy-DL-leucine (left panels), or 10 mM beta-hydroxy-DL-leucine plus 2 mM L-Leu (right panels). Upper panels: UV absorption profile; middle panels: ^{35}S radioactivity insoluble in hot TCA; lower panels: radioactivity in ^{125}I -labeled SRP. Sedimentation is from left to right. The position of the 80S monosome peak is marked with an arrow.

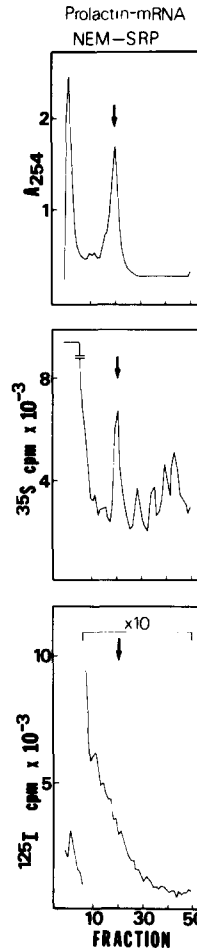


FIGURE 6 NEM-alkylation of SRP inhibits its binding to polysomes and monosomes. Prolactin mRNA was translated and the translation system analyzed as described in Fig. 3, except that ^{125}I -labeled SRP was alkylated with NEM as described in Fig. 7. Upper panel: UV absorption profile; middle panel: ^{35}S radioactivity insoluble in hot TCA; lower panel: radioactivity in NEM-treated ^{125}I -labeled SRP. Sedimentation is from left to right. The position of the 80S monosome peak is marked with an arrow.

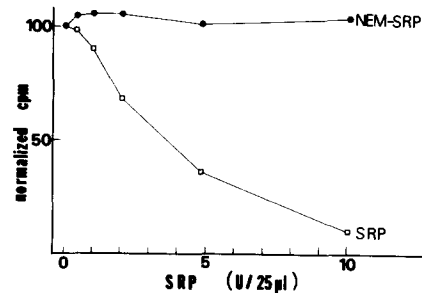


FIGURE 7 Translation-inhibitory effect of SRP is abolished after NEM-alkylation of SRP. Bovine pituitary RNA was translated in the wheat germ system (25 μ l) either in the absence or in the presence of gradient-purified SRP that was treated with NEM (\bullet) before assay. For NEM treatment, DTT was removed by passing SRP solutions through a Sephadex G-25 superfine column in Buffer C [without DTT]. The SRP solution was then made 2 mM in NEM and incubated for 30 min at 25°C. DTT was added to 5 mM to terminate the alkylation reaction. A control sample of SRP (\square) was incubated after DTT removal for 30 min at 25°C. DTT was added to 5 mM followed by NEM addition to 2 mM. The cpm values in the absence of SRP were 273,800 cpm (NEM-SRP) and 268,900 cpm (control SRP) and were normalized to be 100.

that NEM-modified SRP did not bind to preprolactin-synthesizing polysomes and, moreover, that even SRP's interaction with monosomes was completely abolished. If NEM-treated SRP does not interact with polysomes, it would also not be expected to exert the inhibitory effect on preprolactin synthesis. These expectations were borne out by the data in Fig. 7. NEM-modified SRP did not inhibit preprolactin synthesis even at the highest concentration assayed. The translation-inhibitory effect resulting from the binding of SRP will be analyzed in detail in the third paper of this series (15).

From the absolute concentrations of nascent polysomes and SRP as given in Tables I and II and an estimation of the monosome concentration from the UV absorption profile (8×10^{-10} mol/gradient), one can attempt to calculate maximum dissociation constants for the interactions of SRP with monosomes and of SRP with ribosome synthesizing preprolactin. The following values were obtained: (a) $K_D < 5 \times 10^{-5}$ (SRP-monomer) and (b) $K_D < 8 \times 10^{-9}$ (SRP-ribosome synthesizing preprolactin). Thus, due to the expression of the preprolactin nascent chain, the interaction of SRP with the ribosome is apparently at least 6,000-fold tighter than the SRP-monomer interaction. It should be noted, however, (based on our "slow" method of analysis and on the potential perturbations introduced by labeling SRP molecules) that the values obtained represent minimal estimates of apparent binding affinity.

DISCUSSION

We have demonstrated here a specific interaction between a purified 11S protein (1), termed "signal recognition protein (SRP), and secretory protein-synthesizing polysomes. This highly specific interaction was detected by two independent assays: (a) SRP specifically inhibited the translation (in the wheat germ cell-free system) of bovine preprolactin (secretory protein) mRNA, in a concentration-dependent manner, but had no effect on the translation of rabbit globin (cytoplasmic protein) mRNA; (b) [125 I]-labeled SRP bound selectively and with high affinity (apparent $k_D < 8 \times 10^{-9}$ M) to in vitro assembled polysomes synthesizing preprolactin and not to those synthesizing alpha and beta globin. The translation-inhibitory effect of SRP correlated with its selective polysome binding capacity. If the latter was impaired, the translation-inhibitory effect was also abolished.

Impairment of SRP's polysome binding capacity and, in parallel, impairment of its translation-inhibitory effect was observed after modification of SRP by NEM. The relatively low-affinity binding of SRP (apparent $k_D < 5 \times 10^{-5}$ M) to monosomes (not engaged with mRNA in protein synthesis) was also completely abolished after NEM modification of SRP, strongly suggesting that this low-affinity binding to ribosomes is also a specific interaction and not nonspecific adsorption.

Impairment of high affinity binding and abolition of the translation-inhibitory effect of SRP was also observed after modification of the preprolactin nascent chain. Incorporation of β -hydroxy leucine, an analogue of leucine, into nascent preprolactin chains of polysomes considerably reduced SRP's

high-affinity binding to these polysomes and, in parallel, reduced its translation-inhibitory effect. The effect of β -hydroxy leucine could be abolished when incorporation took place in the presence of β -hydroxy leucine and L-leucine, arguing against the possibility that the observed effect of β -hydroxy leucine was the result of free β -hydroxy leucine rather than of incorporated β -hydroxy leucine residues. These data strongly suggested that it is the nascent polypeptide chain – and not mRNA – which is mediating the high-affinity binding of SRP to polysomes. Taken together with the recent demonstration of Hortin and Boime (13) that replacement of Leu by β -hydroxy leucine abolishes in vitro translocation of secretory proteins that contain leucine-rich signal sequences (the signal peptide of bovine preprolactin contains eight Leu residues [11]) but not those that contain leucine-poor signal sequences, our data here suggest that it is the signal sequence of the nascent chain that mediates SRP's high affinity binding. Conclusive evidence for this conjecture will be presented in the third paper of this series (15).

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