protein[69]; or (*d*) for diversifying the organellar distribution of proteins (e.g., some proteins that may occur both within peroxisomes and the mitochondrial matrix); or (*e*) for anchoring polymeric structures in the membrane (e.g., free and membrane-bound forms of cytoskeletal proteins).

[69] D. Perlman, H. O. Halvorson, and L. E. Cannon, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 781 (1982).

[53] Signal Recognition Particle: A Ribonucleoprotein Required for Cotranslational Translocation of Proteins, Isolation and Properties

By PETER WALTER and GÜNTER BLOBEL

Microsomal membranes (RM)[1] lose their ability to translocate nascent secretory,[2,3] lysosomal,[4] and membrane[5] proteins upon salt extraction if they are assayed in the wheat germ cell-free translation system. From the salt extract, a ribonucleoprotein [termed signal recognition particle (SRP)] was purified to homogeneity and shown to be composed of six different polypeptide chains[3] and one 7 S RNA molecule.[6] Addition of the crude salt extract or purified SRP to salt-extracted RM (K-RM) restored their ability not only to catalyze translocation of secretory[2,3] and lysosomal[4] proteins across the microsomal membrane, but also to catalyze the asymmetric integration of transmembrane proteins[5] into the membrane. The function of SRP in the translocation process was shown to involve the recognition of the signal sequence of these proteins in their nascent state,[7] the specific binding of polysomes synthesizing these proteins to RM vesicles,[8] and the initiation of the translocation event. In the absence

[1] P. Walter and G. Blobel, this volume [6].
[2] G. Warren and B. Dobberstein, *Nature (London)* **273,** 569 (1978).
[3] P. Walter and G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 7112 (1980).
[4] A. H. Erickson, P. Walter, and G. Blobel, in preparation.
[5] D. J. Anderson, P. Walter, and G. Blobel. *J. Cell Biol.* **93,** 501 (1982).
[6] P. Walter and G. Blobel, *Nature (London)* **299,** 691 (1982).
[7] P. Walter, I. Ibrahimi, and G. Blobel, *J. Cell Biol.* **91,** 545 (1981).
[8] P. Walter and G. Blobel, *J. Cell Biol.* **91,** 551 (1981).

ISBN 0-12-181996-5

of RM vesicles, SRP modulates translation by reversibly arresting the elongation of nascent secretory proteins after their signal peptide and about 40 additional amino acids have been polymerized.[9] This translation arrest can be released if K-RM vesicles are added, resulting in completed and efficiently translocated secretory protein. The arrest-releasing activity of K-RM vesicles has been localized to a 72,000-dalton integral membrane protein, termed SRP-receptor,[10,11] or docking protein.[12]

Here we describe the purification of SRP which, as a component of the translocation machinery, has proved to be a valuable tool for studying the mechanism of protein translocation across the membrane of the endoplasmic reticulum.[7-9] Purified SRP has also been used as an affinity probe to purify SRP-receptor.[11] Finally, it has been helpful as an additional supplement in *in vitro* translation systems containing RM, where it can boost the efficiency of *in vitro* protein translocation to virtually 100%.

Properties of SRP

The SRP is composed of six different polypeptide chains (molecular weights: 72,000, 68,000, 54,000, 19,000, 14,000, and 9000) and one molecule of RNA (identified as 7 SL RNA, which is about 300 nucleotides long, has been sequenced,[13,14] and has a sedimentation coefficient of 11 S). The particle is negatively charged and interacts tightly with DEAE-ion exchange resins.[3] It also interacts (quite selectively) with hydrophobic-ion exchange resins (see footnote 3 and below). Taken together with the fact that SRP's activity is stabilized by very low concentrations of nonionic detergents,[3] this suggests that SRP contains exposed hydrophobic domains. The activity of SRP is dependent on free sulfhydryl group(s).[3,15] To stabilize SRP, we therefore include small amounts of nonionic detergent (Nikkol) and reducing agent (DTT) in most buffers.

SRP is relatively stable to nucleolytic breakdown. We normally observe little (<20%) or no degradation of SRP–RNA, even when taking no special precautions to keep solutions free of nuclease contamination. Preliminary experiments indicate that Mg^{2+} ions are important for SRP's structural integrity (see also below).

[9] P. Walter and G. Blobel, *J. Cell Biol.* **91,** 557 (1981).
[10] R. Gilmore, G. Blobel, and P. Walter, *J. Cell Biol.* **95,** 463 (1982).
[11] R. Gilmore, P. Walter, and G. Blobel, *J. Cell Biol.* **95,** 470 (1982).
[12] D. I. Meyer, E. Krause, and B. Dobberstein, *Nature (London)* **297,** 647 (1982).
[13] E. Ullu, S. Murphy, and M. Melli, *Cell* **29,** 195 (1982).
[14] W. Y. Li, R. Reddy, D. Henning, P. Epstein, and H. Busch, *J. Biol. Chem.* **257,** 5136 (1982).
[15] R. C. Jackson, P. Walter, and G. Blobel, *Nature (London)* **284,** 174 (1980).

We routinely freeze SRP preparations in small aliquots in liquid nitrogen. It can be stored at −80° for at least 1 year, and there is no apparent loss of activity upon three cycles of freezing and thawing.

Solutions

A stock solution of 1.0 *M* triethanolamine 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. Stock solutions of 2.5 *M* sucrose and 1.0 *M* $Mg(OAc)_2$ were not further adjusted. All stock solutions mentioned above were filtered through a 0.45 μm Millipore filter, except the sucrose solution which was filtered through a 1.2 μm Millipore filter. Dithiothreitol (DTT) was kept in small aliquots as a 1.0 *M* stock solution at −20° and diluted into the buffers immediately before use. The nonionic detergent Nikkol (octaethylene glycol dodecyl ether) was purchased directly from Nikko Chemical Corp. in Tokyo. It was chosen because it is available in a chemically pure form and does not absorb UV light at 260 nm or 280 nm (thereby allowing RNA and protein to be followed by optical absorbance). We keep the detergent as a 20% (w/v) stock solution at 4°. In its properties, Nikkol resembles Triton X-100, which can also be used to stabilize SRP activity (however, we have not yet tested to use Triton in the hydrophobic chromatography steps).

Buffer I: 50 m*M* TEA, 500 m*M* KOAc, 5 m*M* $Mg(OAc)_2$, 1 m*M* DTT

Buffer II: 250 m*M* sucrose, 50 m*M* TEA, 1 m*M* DTT

Buffer III: 50 m*M* TEA, 1.0 *M* KOAc, 10 m*M* $Mg(OAc)_2$, 1 m*M* DTT, 0.05% Nikkol

Buffer IV: 12 m*M* TEA, 250 m*M* KOAc, 2.5 m*M* $Mg(OAc)_2$, 1 m*M* DTT, 0.01% Nikkol

Buffer V: 50 m*M* TEA, 350 m*M* KOAc, 3.5 m*M* $Mg(OAc)_2$, 1 m*M* DTT, 0.01% Nikkol

Buffer VI: 50 m*M* TEA, 600 m*M* KOAc, 6.0 m*M* $Mg(OAc)_2$, 1 m*M* DTT, 0.01% Nikkol

Salt Extraction of RM

We have prepared salt extracts from either column-washed RM or EDTA-stripped RM preparations.[1] SRP can be purified from either preparation, but column-washed RM yield consistently cleaner preparations.

To prepare the salt extract from column-washed RM we aimed to keep ribosomes (and SRP) from unfolding and therefore included magnesium ions in our extraction buffers. This allowed us to obtain a ribosome-free

salt extract by removing intact, coextracted ribosomes in an additional spin after the initial extraction.[3]

All preparations were carried out at 0–4°. Ten milliliters of an ice-cold salt solution [1.5 *M* KOAc, 15 m*M* $Mg(OAc)_2$] were slowly added to 20 ml of column-washed RM (at a concentration of 1000 eq/ml). The mixture was incubated on ice for 15 min. The membranes were sedimented for 1 hr at 120,000 g_{av} through a cushion of 0.5 *M* sucrose in buffer I. The ratio of load to cushion was about 3 to 1. The supernatant fraction (including the upper half of the cushion) was carefully removed from the top with a Pasteur pipet. To remove ribosomes the supernatant was then recentrifuged for 3.5 hr at 200,000 g_{av} over a cushion of 0.5 *M* sucrose in buffer I. The ratio of load to cushion was again about 3 to 1. The supernatant was carefully removed from the top (again including the upper half of the cushion), yielding a postribosomal salt-extract fraction. Including the upper half of the cushions in the corresponding supernatant fractions allowed essentially complete recovery of SRP in spite of its relatively high sedimentation coefficient.

The membrane pellet from the first centrifugation step was resuspended in 20 ml of buffer II by manual homogenization in a Dounce homogenizer (pestle A, 2–3 strokes). The suspension was then sedimented (1 hr at 120,000 g_{av}) through a cushion of 0.5 *M* sucrose in buffer II and finally resuspended in 20 ml of buffer II to yield the salt-extracted rough microsome fraction (K-RM) at a concentration of 1000 eq/ml.

The extraction of EDTA-stripped RM was carried out identically to the extraction of column-washed RM, except that the membranes were removed from the salt extract by centrifugation for 1.5 hr at 200,000 g_{av} through a cushion of 0.35 *M* sucrose in buffer I. The second centrifugation step was omitted since ribosomes had been unfolded and extracted in the previous EDTA washes. A K-RM fraction prepared from EDTA-stripped RM was functionally indistinguishable from K-RM prepared from column-washed RM.

Fractionation of the Salt Extract and Hydrophobic-Ion Exchange Chromatography

The salt extracts prepared from column-washed RM or EDTA-stripped RM were treated identically. Chromatography of the salt extracts on ω-aminoalkylagarose resins[3,16,17] resulted in a 50-fold enrichment[3] of SRP.

[16] S. Shaltiel, this series, Vol. 34, p. 126.

[17] S. Shaltiel, S. P. Adler, D. Purich, C. Caban, P. Senior, and E. R. Stadtman, *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3397 (1975).

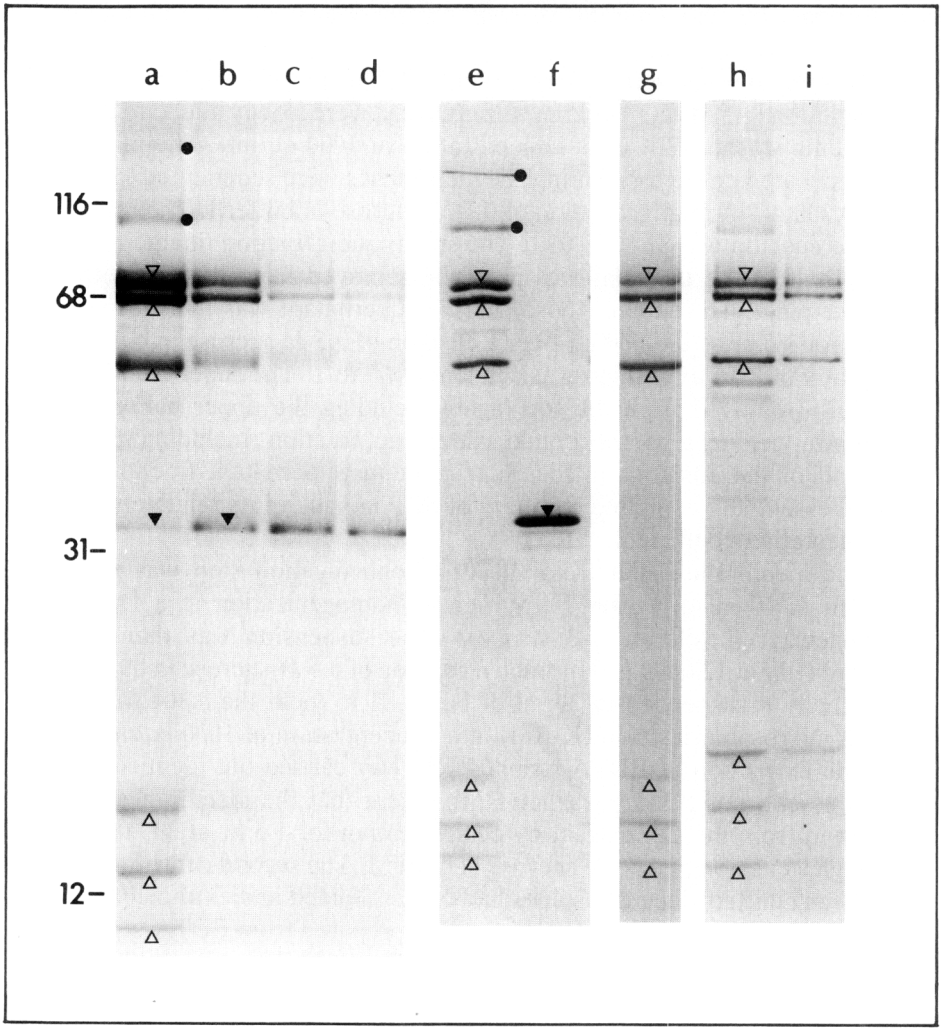

FIG. 1. Polypeptide profile in SDS–PAGE of different SRP preparations. Fractions obtained during the purification of SRP were TCA precipitated and their polypeptide composition visualized by Coomassie Blue staining after PAGE in SDS on 10–15% acrylamide gradient gels. The fractions were derived from either EDTA-stripped RM (lanes a–f) or column-washed RM (lanes g–i). Bands corresponding to polypeptides that have been designated as SRP polypeptides are marked with an open arrowhead. The low molecular weight polypeptides were weakly stained. The contaminating bands marked with dots or solid arrowhead were only obtained when EDTA-stripped RM was used as the starting material (lanes a–f, see text for discussion). The molecular weight standards were: cytochrome *c*, 12,000; DNase, 31,000; bovine serum albumin, 68,000; β-galactosidase, 116,000.

SRP was eluted from an ω-aminopentylagarose column and fractions of one column volume were collected. A 250-μl aliquot of fraction 1 (lane a), fraction 3 (lane b), fraction 5

All preparations were carried out at 0–4°. A 2-ml column of ω-aminopentylagarose (Sigma, 5.7 μmol of 1.5 diaminopentane coupled per milliliter of CNBr-activated agarose) was prewashed with 10 ml of 2 *M* KOAc (to exchange the counterion to acetate), washed with 10 ml of water, and equilibrated with 20 ml of buffer I. It is useful to layer a small amount of Sephadex G-15 on top of the ω-aminopentylagarose bed to prevent protein from binding to the very top surface, which, upon step elution, might become exposed to air. A 35–40 ml salt-extract fraction (obtained from 20 ml of a starting RM fraction) was passed over the column at a flow rate of 6 ml/hr. The column was then washed with 20 ml of buffer I and eluted with a step of buffer III. As soon as buffer III appeared in the eluent (detected by conductivity or the abrupt change in drop size due to the presence of detergent) 0.5-ml fractions were collected. Eluting protein was monitored by absorbance measurements at 260 nm or 280 nm and SRP activity was followed in the translocation assay. Typically, the bulk of the SRP eluted in 1–4 column volumes.

In Fig. 1 (lanes a–d) the elution profile of SRP from the ω-aminopentylagarose column is shown. Note that the salt extract was obtained from EDTA-stripped RM. Polypeptides of individual fractions were displayed by SDS–PAGE. SRP activity and SRP polypeptides (open arrowheads) eluted immediately as the elution buffer was applied to the column. Two contaminating polypeptides of high molecular weight (about 90,000 and 200,000, marked with dots) were also observed in these active fractions. Another distinct contaminant (about 34,000 daltons, marked with solid arrowhead) eluted delayed. If the pool of eluted material (Fig. 1, lanes a–c) was concentrated by DEAE-Sepharose chromatography and then further fractionated by sucrose-gradient centrifugation (see below), two distinct peaks, one at 7 S (Fig. 1, lane f) and one at 11 S (Fig. 1, lane e), were resolved. Only the 11 S peak showed SRP activity, but both peaks appeared to be ribonucleoproteins. A 5 S RNA could be extracted from the 7 S peak and 7 SL RNA (SRP-RNA) was extracted from the 11 S

(lane c), and fraction 7 (lane d) is displayed on the gel. Fractions 1–5 were pooled, concentrated on DEAE–Sepharose, and further fractionated by sucrose-gradient centrifugation. Two symmetrical peaks were obtained, one having a sedimentation coefficient of 7 S and one of 11 S (data not shown). The material displayed in lanes e (comprising the 11 S peak) and f (comprising the 7 S peak) corresponds to 250 μl of the pooled ω-aminopentylagarose eluate.

The eluate of ω-aminopentylagarose columns of two different SRP preparations starting from *column-washed RM* is shown in lanes g and h. Whereas the preparation shown in lane g appeared homogeneous, the material shown in lane h contained several contaminating bands and therefore required further purification by sucrose-gradient centrifugation. The 11 S peak of the gradient was collected and is shown in lane i.

peak. The 7 S RNP represents the known complex of ribosomal 5 S RNA and ribosomal protein L5 (35,000 daltons). It was previously shown[18] that this RNP could be specifically extracted from the large ribosomal subunit by EDTA treatment, consistent with our observation that this contaminant was only observed when the salt extract was prepared from EDTA-treated RM (rather than column-washed RM).

For comparison, the polypeptides of an ω-aminopentylagarose eluate prepared from column-washed RM are displayed in Fig. 1, lane g. Note that the 34,000-dalton polypeptide as well as the high molecular weight contaminants are absent. We feel that upon EDTA treatment of RM a "partial unfolding" of SRP takes place, thus allowing the high molecular weight contaminants to become unspecifically associated with the (highly charged) particle, although at present we have no experimental proof for this rationalization. In general, preparations obtained from column-washed RM are more homogeneous.

Chromatography on ω-aminoalkylagarose resins appears to involve both hydrophobic and ion-exchange effects.[16,17] Buffer III causes elution of SRP from the column by interfering with both types of interactions. The increase in ionic strength causes a weakening of ionic interactions, whereas the inclusion of small amounts of detergent interferes with hydrophobic interactions. Whereas an increase in ionic strength was required to elute SRP, omission of detergent led only to peak broadening.

The fact that the interaction of SRP with the ω-aminopentylagarose is indeed due to a combination of ionic and hydrophobic interactions is demonstrated by the data shown in Fig. 2. It can be seen that SRP under the ionic conditions of the salt extract (buffer I) does not interact with resins containing ω-aminopropyl- or ω-aminobutylagarose, but, as one increases the number of methylene groups in the alkyl chain (and thereby the hydrophobicity of the resin) to the length of ω-aminopentyl-, -hexyl-, or -octylagarose, binding does occur. If, however, the salt concentration in the salt extract is lowered by dilution to 200 m*M*, SRP also binds to ω-aminobutylagarose [indicating that the previously high salt (500 m*M*) prevented it from binding by interfering with presumably ionic interactions]. By analogy, SRP can be prevented from binding to ω-aminopentylagarose by increasing the salt concentration to 1.0 *M*. From these data we designed the elution conditions reflected in the composition of buffer III. Polypeptide profiles of eluted material bound at 200 m*M* salt to ω-aminobutylagarose were more heterogeneous than the material obtained from the ω-aminopentylagarose columns. Attempts to elute active SRP from ω-aminohexylagarose columns failed.

[18] G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1881 (1971).

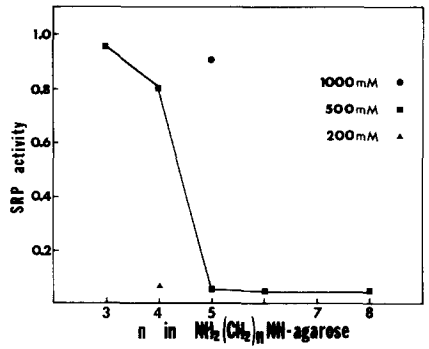

FIG. 2. Behavior of SRP on ω-aminoalkylagarose columns. Fractions of a postribosomal salt extract (500 μl) (ionic conditions of buffer I) were passed over 50-μl columns of ω-aminoalkylagarose of varying hydrophobicity ranging from ω-aminopropylagarose ($n = 3$) to ω-aminooctylagarose ($n = 8$). The material flowing through the column (i.e., not bound) was collected and assayed for SRP activity (■). Separate aliquots were made 1.0 *M* in KOAc prior to passage over the ω-aminopentylagarose column (●) or diluted to 200 m*M* KOAc prior to passage over the ω-aminobutylagarose column (▲). SRP activity is expressed as a fraction of the SRP activity of the starting material.

It is worth noting that *two* distinct RNPs were purified (Fig. 1, lanes e and f) by this chromatographic technique from the complex mixture of proteins in the salt extract prepared from EDTA-stripped RM. There are also reports from other laboratories[19] where chromatography on ω-aminoalkylagarose resins was used as a key step in the purification of RNPs. Thus, it is conceivable that RNPs share structural characteristics that can be recognized by these resins. An intriguing alternative interpretation would be that these interactions are not (or only to a small degree) of hydrophobic nature, but rather that RNPs bind to these resins on the basis of a sterically controlled ionic interaction. The increased affinity of the RNPs to resins with a higher number of methylene groups could therefore be due not (or not only) to an increased hydrophobicity of the matrix, but rather to an increased accessibility of the positively charged amino groups to the negatively charged RNA backbone in the particles. This interpretation is consistent with the fact that an *increase* in ionic strength caused weakening of the interactions and thus caused elution.

Further Purification of SRP

Signal recognition particles can be further purified by sucrose gradient centrifugation[3,8] under high salt conditions. This procedure removes all

[19] M. Hinterberger, I. Pettersson, and J. A. Steitz, *J. Biol. Chem.* **258,** 2604 (1983).

contaminating polypeptide chains that occasionally coelute in the hydrophobic-ion exchange chromatography (even if the salt extract was prepared from column-washed RM). An example is given in Fig. 1, lanes h–i. The relatively heterogeneous SRP preparation obtained after ω-aminopentylagarose chromatography (lane h) is purified to homogeneity (lane i). Prior to sucrose-gradient centrifugation it is helpful to concentrate SRP further by chromatography on DEAE–Sepharose Cl-6B.

For concentration by DEAE–chromatography, the eluate of the ω-aminopentylagarose column was diluted with three parts of ice-cold water to reduce the ionic strength. The solution (derived from 20 ml of RM) was passed over a 0.4-ml DEAE–Sepharose 6B-Cl column which was equilibrated in buffer IV. The column was washed with 2 ml of buffer V and eluted with a step of buffer VI. Signal recognition particle elutes immediately and can be completely recovered in a 0.4-ml fraction (the appearance of buffer VI in the eluate is monitored by conductivity, the appearance of SRP by optical absorbance at 260 nm and 280 nm).

For sucrose-gradient centrifugation, the DEAE-concentrated SRP solution is layered directly on top of a 5–20% linear sucrose gradient in buffer I (containing 0.01% Nikkol). If the eluate of the ω-aminopentylagarose column was used without prior DEAE concentration, it was first diluted with one part of water to approximate the ionic conditions of the gradient. We either layered 250–500 μl on a 12.5-ml gradient (Beckman SW 40 rotor, 40,000 rpm, 20 hr) or 100–200 μl on a 5-ml gradient (Beckman SW 50.1 rotor, 50,000 rpm, 6 hr). The gradients were fractionated using an ISCO gradient fractionator with a continuous absorbance monitor, and the 11 S peak was collected.

Translocation Assay

To assay SRP for its activity, we used a wheat germ cell-free translation system.[20] It was programmed with bovine pituitary mRNA (coding mainly for the secretory protein prolactin) and supplemented with K-RM. Signal recognition particles reconstitute the translocation activity of K-RM which is monitored by the cotranslational conversion of preprolactin to prolactin.[3]

Bovine pituitary RNA (0.2 A_{260} units per 25 μl of translation mix) was translated in a staphylococcal nuclease-treated wheat germ system (6 μl of wheat germ S23 and 25 μCi [^{35}S]Met per 25 μl of translation mix). All translations were supplemented with human placental RNase inhibitor[21]

[20] A. H. Erickson and G. Blobel, this volume [3].
[21] P. Blackburn, *J. Biol. Chem.* **254,** 12484 (1979).

at a final concentration of 0.01 A_{280} units/ml from a 100-fold concentrated stock solution (kept at −80°) and a cocktail of selected protease inhibitors (which do not interfere with protein synthesis) from a 200-fold concentrated stock solution (kept at −20°) at the following final concentrations: pepstatin A 0.1 μg/ml, chymostatin 0.1 μg/ml, antipain 0.1 μg/ml, leupeptin 0.1 μg/ml, trasylol 10 units/ml. The ions that were added with the wheat germ extract and different amounts of SRP were taken into account and compensated for to yield final ion concentrations of 150 m*M* KOAc and 2.0 m*M* $Mg(OAc)_2$ in all translations. The nonionic detergent Nikkol was present in all translations at a final concentration of 0.002% to stabilize SRP activity. At this detergent concentration, the translocation activity of RM is unaffected. Furthermore, complete protection of the translocated polypeptide chain in posttranslational proteolysis assays[1,22] is maintained. To achieve breakdown of the lipid bilayer and expose signal peptidase, Nikkol concentrations in excess of 0.04% are required. Membranous fractions were always added last after all of the other components were mixed and the detergent uniformly diluted.

A typical assay for SRP activity contained K-RM at a concentration of 40 eq/ml (1 μl of K-RM/25 μl of translation mix). The eluate of the ω-aminopentylagarose column was diluted with one part of water and 0.5–3 μl of this solution was assayed in 25 μl of translation mix. After concentration by DEAE–chromatography or after sucrose-gradient centrifugation, the amount of SRP added to the translocation assay was adjusted according to the concentration or dilution the sample underwent. Usually, the highest amount of SRP that can be added to the translocation assay is limited by the salt and detergent added with the SRP solution. However, 3 μl of the ω-aminopentylagarose eluate assayed in a 25-μl translation mix constitutes a saturating amount, i.e., complete (>90%) translocation of nascent secretory proteins is usually obtained.

Instead of the K-RM fraction, the translation system can also be supplemented with RM or EDTA-stripped RM (at about 40 eq/ml) and SRP can be added to boost its translocation activity (try 1 μl of ω-aminopentylagarose eluate per 25 μl of translation mix as a starting point).

[22] G. Scheele, this volume [7].