

Translocation of Proteins Across the Endoplasmic Reticulum

II. Signal Recognition Protein (SRP) Mediates the Selective Binding to Microsomal Membranes of In-Vitro-Assembled Polysomes Synthesizing Secretory Protein

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ABSTRACT Translocation-competent microsomal membrane vesicles of dog pancreas were shown to selectively bind nascent, in vitro assembled polysomes synthesizing secretory protein (bovine prolactin) but not those synthesizing cytoplasmic protein (alpha and beta chain of rabbit globin). This selective polysome binding capacity was abolished when the microsomal vesicles were salt-extracted but was restored by an 11S protein (SRP, Signal Recognition Protein) previously purified from the salt-extract of microsomal vesicles (Walter and Blobel, 1980. *Proc. Natl. Acad. Sci. U. S. A.* 77:7112-7116). SRP-dependent polysome recognition and binding to the microsomal membrane was shown to be a prerequisite for chain translocation. Modification of SRP by *N*-ethyl maleimide abolished its ability to mediate nascent polysome binding to the microsomal vesicles. Likewise, polysome binding to the microsomal membrane was largely abolished when β -hydroxy leucine, a Leu analogue, was incorporated into nascent secretory polypeptides.

The data in this and the preceding paper provide conclusive experimental evidence that chain translocation across the endoplasmic reticulum membrane is a receptor-mediated event and thus rule out proposals that chain translocation occurs spontaneously and without the mediation by proteins. Moreover, our data here demonstrate conclusively that the initial events that lead to translocation and provide for its specificity are protein-protein (signal sequence plus ribosome with SRP) and not protein-lipid (signal sequence with lipid bilayer) interactions.

In the preceding paper (1) we have described the effects of signal recognition protein (SRP) in a wheat germ cell-free translation system programmed with mRNA's for either rabbit globin (cytoplasmic protein) or bovine prolactin (secretory protein). We have demonstrated (1) that SRP binds specifically to monomeric ribosomes, albeit with relatively low affinity (apparent $k_D < 5 \times 10^{-5}$ M), and that it binds selectively and with 6,000-fold higher affinity (apparent $k_D < 8 \times 10^{-9}$ M) to in vitro assembled polysomes synthesizing secretory protein (but not to those synthesizing globin). This 6,000-fold enhancement most likely results from specific recognition by SRP of the signal sequence of the nascent secretory polypeptide.

In this paper we describe the specific effects of SRP in a wheat germ cell-free translation system that was supplemented with dog pancreas microsomal membrane vesicles. Our data show that SRP mediates the selective binding to microsomal membranes of nascent, in vitro assembled polysomes synthesizing secretory protein but not of those synthesizing cytoplasmic protein. Polysome binding is abolished if SRP is modified

by *N*-ethyl maleimide (NEM) or if the nascent secretory polypeptide is modified by incorporation of β -hydroxy leucine, an analogue of Leu.

MATERIALS AND METHODS

The preparation of various microsomal membrane fractions (RM, K-RM), the extraction and purification of SRP, the cell-free wheat germ translation system, and the quantitation of in vitro synthesized protein were described in the preceding paper (1). The SRP preparation used was the eluate of the aminopentyl-agarose resin, except when stated otherwise.

Assay for Binding of In Vitro Assembled Polysomes to Microsomal Membranes

The assay for nascent polysome binding to microsomal membranes was an indirect one. We measured the depletion of mRNA from the translation system resulting from recruitment into membrane-bound polysomes and removal of these in vitro assembled rough microsomes (RM) by differential centrifugation.

The initial incubation volume for each timepoint was 75 μ l. The wheat germ cell-free translation system plus additional components (specified in figure leg-

ends) were mixed on ice. The system was allowed to warm for 2 min in a 26°C water bath; mRNA was then added and preincubation (at 26°C) was started. The translations were arrested after various preincubation times by cooling the mixture to 0°C in ice-water. A 40- μ l aliquot was withdrawn (leaving behind a 35- μ l control aliquot), transferred to a centrifuge tube, and spun at 20 PSI in the A-100-30 rotor in a Beckman Airfuge kept at 4°C (Beckman Instruments, Inc., Fullerton, Calif.). To start the spin, the air pressure was slowly turned up to reach 20 PSI after 10 s. The sample was then centrifuged for an additional 90 s. The brake engaged after a 2-min coast. A 35- μ l portion was carefully taken from the top. Incubation of this sample and the 35- μ l control aliquot (not spun) at 26°C was continued until a total incubation time of 90 min for each sample was reached. A 25- μ l portion of each was then TCA-precipitated and prepared for SDS-PAGE.

With increasing preincubation time, increasing amounts of prolactin mRNA would be expected to be assembled into membrane-bound polysomes and, therefore, to be removed from the translocation mix by sedimentation. Incubation of the supernatant fluid fraction would be expected to result in the translation of all free mRNA and mRNA engaged in free polysomes. Under the conditions used, the amount of preprolactin made in our translation system is proportional to the amount of mRNA added to the translation. The final amount of preprolactin therefore is a function of the amount of translatable mRNA left in the supernatant fluid after the centrifugation step, and thereby a measure of the amount of mRNA not attached to the microsomal membrane at any given time of preincubation.

RESULTS

SRP was purified (2) from a salt extract of microsomal vesicles (3) on the basis of its ability to restore the extracted membrane's capacity for cotranslational translocation of secretory protein. Translocation of the nascent secretory protein preprolactin into the lumen of the microsomal vesicles is accompanied by cleavage of the signal sequence yielding "processed" prolactin molecules (4-6). The ratio of prolactin to preprolactin molecules is therefore a measure of the membrane's translocation activity (2, 7). The data in Fig. 1 demonstrate that SRP, in a concentration-dependent manner, restored translocation activity to salt-extracted microsomal vesicles (K-RM). Translation of prolactin mRNA in the wheat germ cell-free system supplemented with K-RM yielded synthesis only of preprolactin molecules. However, in the presence of increasing amounts of SRP (upper panel) there was an increasing appearance of prolactin concomitant with a decreasing appearance of preprolactin. At the highest concentrations of SRP (Fig. 1, upper and middle panel), virtually all preprolactin molecules were processed and therefore translocated. As previously observed for SRP alone (1), the combined presence of K-RM and increasing concentrations of SRP did not affect globin synthesis (Fig. 1, upper panel).

The specific translation-inhibitory effect of SRP on preprolactin synthesis (1) that was previously observed in the wheat germ system in the absence of membranes (Fig. 1, lower panel, open circles) was considerably reduced when K-RM were present (Fig. 1, lower panel, closed circles). This striking release of SRP-induced, secretory protein-specific inhibition of translation will be analyzed in the following paper of this series (8).

The specific binding of SRP to polysomes synthesizing secretory protein (1) suggested that SRP functions in the early steps of the overall translocation event, i.e. SRP would be required to recognize nascent polysomes and to mediate their specific binding to membranes. This binding has been postulated (9) to occur early in the secretory proteins' synthesis, presumably shortly after the signal sequence is synthesized (10, 11). To relate chain length to polysome binding it was necessary to determine the time required to synthesize a complete polypeptide chain in our system. Whereas the incorporation of [³⁵S]Methionine into polypeptide appeared to start without any delay at time zero (Fig. 2, filled squares), it took ~10 min for a complete preprolactin (Fig. 2, filled circles) or prolactin (Fig.

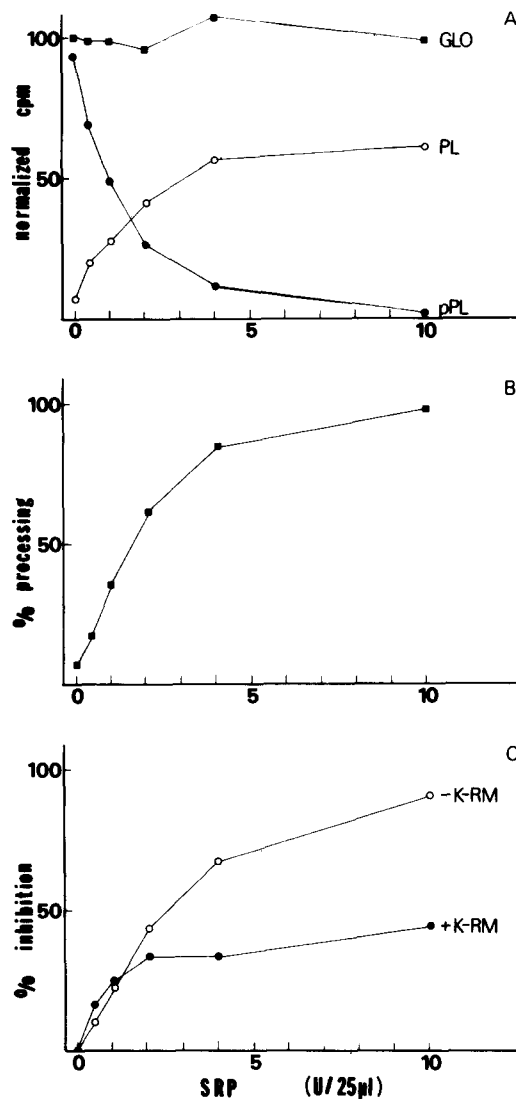


FIGURE 1 Titration of purified SRP in the translocation assay. Bovine pituitary and rabbit reticulocyte RNA were translated in a 25- μ l wheat germ system in the presence of 1 eq of salt-extracted RM with increasing amounts of SRP added. The translation products were separated by PAGE in SDS. Bands corresponding to preprolactin (*pPL*), prolactin (*PL*), and globin (*GLO*) were located by autoradiography, sliced from the dried gel, and the radioactivity was determined (7). The cpm values obtained in the absence of SRP were: for globin 389,000 cpm, for preprolactin 105,000 cpm, and for prolactin 10,900 cpm. These cpm values were normalized to be 100 for globin and for the sum of preprolactin plus prolactin. SRP was gradient-purified and its activity was determined in units (U) (see preceding paper [1]). Panel A: normalized cpm in globin (■), in preprolactin (●), and in prolactin (○). Panel B: the ability of SRP to restore translocation activity to salt-extracted RM was expressed as percentage processing = (cpm PL \times 100)/(cpm pPL + cpm PL) (■). Panel C: the quantitation of SRP's ability to inhibit synthesis of (1) preprolactin in the absence (●) of salt-extracted microsomal membrane (K-RM) and of (2) preprolactin plus prolactin in the presence (○) of K-RM.

2, open circles) molecule to be synthesized in our RM-containing translation system. After 10 min the radioactivity in preprolactin and prolactin increased linearly with time for ~20 to 40 min. Translation leveled off after 90 min of incubation.

Next, we designed an assay that would permit us to examine the binding of nascent polysomes to microsomal vesicles. For

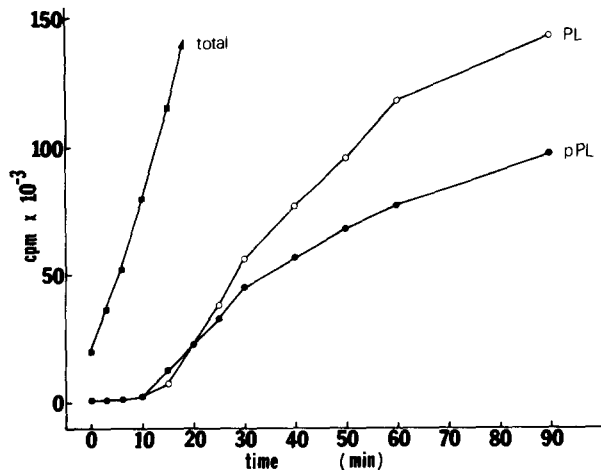


FIGURE 2 Time-course of incorporation of [^{35}S]Met into total protein, preprolactin, and prolactin. Bovine pituitary RNA was translated in the wheat germ system in the presence of 50 eq/ml of RM (1). This concentration of membranes allowed ~60% of the chains to be translocated (60% processing). The system was incubated at 26°C. At different time points, 10- μl aliquots were (a) spotted on filter paper and TCA-precipitated, or (b) TCA-precipitated and prepared for analysis by PAGE in SDS. The filter disks were boiled in 5% TCA as described (1) and [^{35}S]Met incorporated into polypeptide was determined by scintillation counting (■). Bands corresponding to preprolactin (●) or prolactin (○) were located by autoradiography of the polyacrylamide gel, sliced from the gel, and their radioactivity was determined as described (7).

technical reasons (see below) this assay was an indirect one: instead of measuring directly the fraction of nascent polysomes that bound to microsomal membranes, we determined the fraction of nascent polysomes that did not bind under given conditions. This was done by measuring the amount of mRNA that remained in the translation system after removal (by differential centrifugation) of mRNA that was assembled into membrane-bound polysomes. We also attempted to directly measure the fraction of mRNA assembled into membrane-bound polysomes by resuspending the pelleted material and incubating it in the translation system. However, we have found that, due to difficulties in quantitatively resuspending the pellets, the obtained measurements were unreliable.

To define the parameters of this assay we first determined the optimum centrifugation conditions that would allow efficient pelleting of microsomal vesicles from the translation mix while minimizing the loss of ribosomes or nascent, free polysomes. We chose an arbitrary preincubation time of 5 min (preincubation time is the period of incubation before centrifugation (see Materials and Methods)), at which time approximately half of the preprolactin molecule has been synthesized (Fig. 2). RM were present in the translation system in subsaturating amounts sufficient to allow ~70% of the preprolactin chains to be processed (translocated) under appropriate conditions (Fig. 3, spin time = 0). If the translation mixture was spun in a Beckman Airfuge (Beckman Instruments, Inc.) for increasing periods of time, there was a rapid depletion of membrane-bound polysomes (evident from a decrease in the amount of prolactin formed [Fig. 3, open circles]) to background level (Fig. 3, filled square) after a 90-s spin. The amount of preprolactin formed (by polysomes not functionally attached to membranes) increased slightly from a 0 to a 60-s spin (presumably because some protein synthesis inhibiting components [possibly SRP, see following paper] were removed with

the membranes) and then decreased rapidly if the spin was continued for longer than 90 s (due to the loss of free polysomes synthesizing preprolactin). We therefore chose a spin time of 90 s for all subsequent experiments.

To demonstrate that this assay could be used in a semiquantitative manner we added a limiting amount of RM to a translation of prolactin mRNA, so that only 50% of the newly synthesized preprolactin molecules would be translocated (if the membranes were not removed by centrifugation). Consequently, only half of the preprolactin-synthesizing polysomes would be expected to be functionally engaged with the membranes, whereas the other half would exist as free polysomes in the translation mix. We then preincubated this translation mix for various time periods, after which the translation mixture was divided into two portions. From one aliquot the membranes and their attached polysomes were removed by centrifugation, whereas the other aliquot was not subjected to centrifugation. Translation was then continued for 90 min and the translation products were displayed by SDS-PAGE (Fig. 4). In the case where centrifugation was omitted, no change in the ratio of translation products was observed; preprolactin and prolactin were synthesized, as expected, in approximately equal and constant amounts, independent of the length of the preincubation time. If the membranes were removed after 0 min preincubation (before mRNA had been engaged in polysomes), preprolactin was made from all the mRNA available (Fig. 4B, open triangle at 0 min). Approximately the same amount of product (but now represented in preprolactin plus prolactin) (Fig. 4B, filled squares) was obtained if translation was allowed to continue in the presence of membranes (i.e. if the spin was omitted). If preincubation was allowed to proceed, with time, increasing amounts of mRNA engaged with the membranes (as polysomes formed) and were removed in the centrifugation step. Therefore, the final amount of preprolactin formed (Fig. 4B, open triangles) decreased with increasing preincubation time. After 4 min of preincubation time, ~50% of the translatable mRNA was engaged with the microsomes, thereby satu-

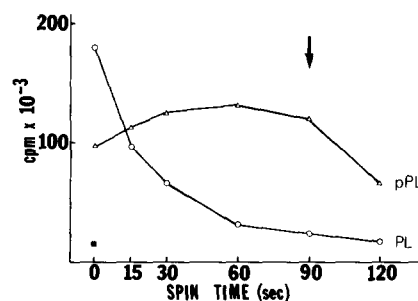


FIGURE 3 Removal of microsomal vesicles from the translation system as a function of centrifugation conditions. Pituitary RNA was translated in the wheat germ system in the presence of 4 eq of RM in a 50- μl translation mix for 5 min at 26°C. This concentration of membranes allowed ~70% of the chains to be translocated (70% processing). The samples were then chilled on ice and centrifuged for various periods of time as described in Materials and Methods. The spin time indicated on the abscissa does not include the 10-s acceleration time and the deceleration time as defined in Materials and Methods. The zero-time sample was not centrifuged at all but was kept at 4°C for an analogous period of time (5 min). After centrifugation, the supernatant fluid was incubated for an additional 85 min at 26°C, and the translation products were quantitated as in Fig. 2. Cpm in preprolactin: (Δ), cpm in prolactin: (\circ). The background radioactivity in an equally sized gel slice from a region with no visible band is indicated (■). The arrow indicates the spin time chosen to be optimal and used in all further assays.

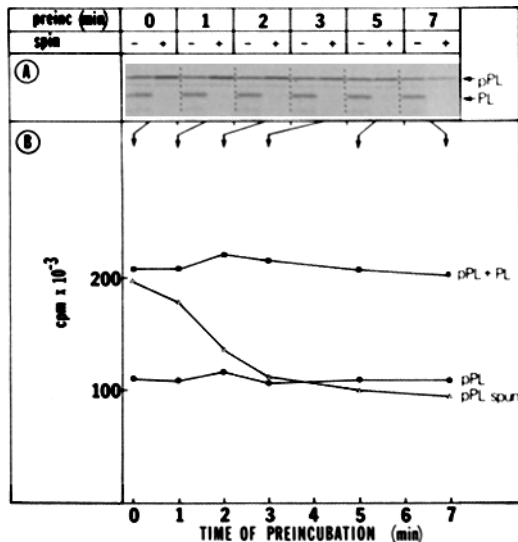


FIGURE 4 Binding of preprolactin synthesizing polysomes to microsomal membranes. Pituitary RNA was translated in a 75- μ l wheat germ system in the presence of 4.5 eq of RM (50% processing). The binding of nascent polysomes was assayed, as described in Materials and Methods, as a function of the preincubation time. For each time-point of preincubation, a control sample was analyzed that was not subjected to the centrifugation step (*spin* -). The translation products were analyzed by PAGE in SDS and bands corresponding to preprolactin (pPL) and prolactin (PL) localized by autoradiography (panel A). The radioactivity in the individual bands (panel B) was determined (see Fig. 2) for preprolactin spun (Δ) (i.e. centrifugation performed), for preprolactin (\bullet) in the control sample (i.e., centrifugation omitted), and for the sum of both [preprolactin and prolactin] (\blacksquare) in the control sample (i.e., centrifugation omitted). In all samples where no membranes have been added or in samples where the membranes have been removed by centrifugation ("spun"), no visible bands in the prolactin (PL) region of the gel were observed (i.e. PL = 0). The background radioactivity in this region of the gel was in all cases <10% that of the preprolactin value in the same lane.

rating the translocation activity of the membranes. Therefore, longer periods of preincubation did not lead to a further reduction in preprolactin synthesis but resulted in a constant level of preprolactin at the amount that was synthesized in the presence of membranes (Fig. 4, filled circles).

We have demonstrated in Fig. 4 that mRNA assembled *in vitro* into polysomes and functionally engaged with membranes (as judged by the amount of prolactin translocated) was physically bound to the membranes after 3–4 min of preincubation of the translation system and could be removed with the membranes by centrifugation. We therefore were able to use this assay to look at the polysome binding capacity of salt-extracted microsomal vesicles in the absence and presence of SRP. To examine the specificity of polysome binding we used globin mRNA as a control.

When prolactin or globin mRNA was translated in the absence of membranes (Fig. 5, panels A and B), the amount of translation product (preprolactin or globin, respectively) did not change with an increase of the preincubation time, whether the sample was subjected to the centrifugation step (Fig. 5, open symbols) or not (Fig. 5, closed symbols). When the sample was subjected to centrifugation, the amount of product made was consistently reduced by 15–25%. This decrease was presumably due to nonspecific losses in the centrifugation step and did not perturb our analysis because it did not change as

a function of preincubation time, i.e., the losses were not due to sedimentation of newly assembled polysomes.

When RM were added to the translation system in saturating amounts (>90% processing) (Fig. 5, panels C and D), the amount of preprolactin made after the centrifugation step was sharply decreased with increasing preincubation time (Fig. 5 C, open triangles), whereas the amount of globin synthesized did not change. When RM were depleted of translocation activity by salt extraction (K-RM), and these translocation-inactive vesicles were assayed for their ability to bind nascent preprolactin polysomes, no binding was detected (Fig. 5 E, open triangles). However, when purified SRP was present in saturating amounts the binding capacity of K-RM for the secretory protein-synthesizing polysomes was fully restored (Fig. 5 G, open triangles). In all cases, the amount of globin synthesis was independent of preincubation time, i.e. nascent polysomes synthesizing globin did not interact with either RM or K-RM or K-RM plus SRP (Fig. 5 D, F, and H).

To demonstrate that the information for the specific attachment of polysomes synthesizing preprolactin to the microsomal vesicles was contained in the nascent chain, we perturbed its

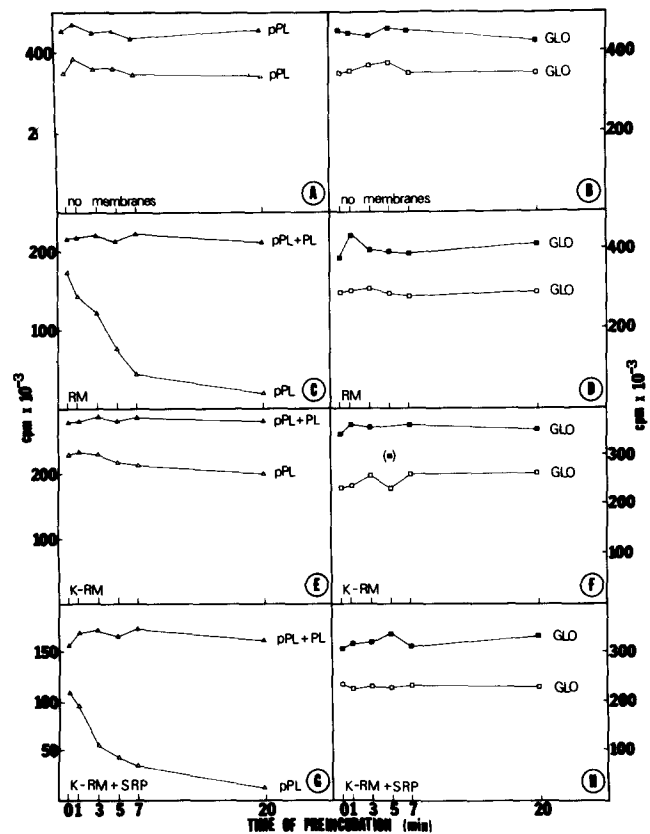


FIGURE 5 Nascent polysome binding to various microsomal membrane fractions. Pituitary RNA (panel A, C, E, G) or reticulocyte RNA (panel B, D, F, H) was translated and assayed for nascent polysome binding (see Materials and Methods) either in the absence (panel A, B) or in the presence of microsomal membranes (RM) at 6 eq/75 μ l translation (90% processing) (panel C, D); or in the presence of salt-extracted RM (K-RM) at 3 eq/75 μ l translation, either in the absence (<10% processing) (panel E, F) or in the presence of 20 U SRP/75 μ l (90% processing) (panel G, H). The radioactivity after PAGE in SDS was determined (see Fig. 2) for preprolactin (pPL) (Δ) and globin (GLO) (\square) of the samples subjected to centrifugation. For the control samples (centrifugation step omitted), the radioactivity was determined for the sum of [preprolactin plus prolactin] (\blacktriangle) and for globin (\blacksquare).

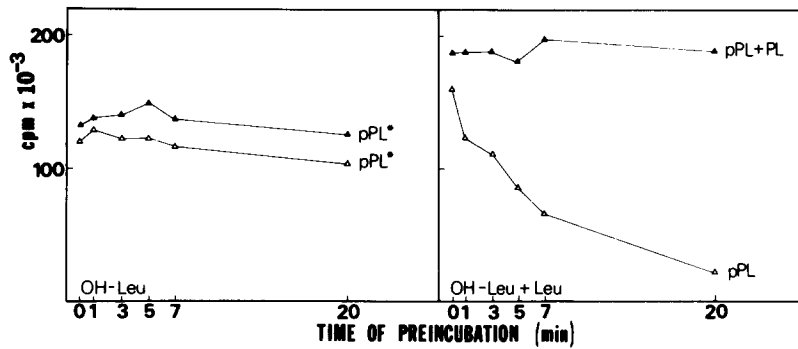


FIGURE 6 Inhibition of nascent preprolactin polysome binding to microsomal membranes by incorporation of beta-hydroxy leucine into nascent chains. Pituitary RNA was translated with 6 eq of RM (90% processing) per 75 μ l wheat germ system and assayed for nascent polysome binding (see Materials and Methods) in the presence of 10 mM beta-hydroxy-DL-leucine alone (left panel), or in the presence of 10 mM beta-hydroxy-DL-leucine plus 2 mM L-Leu (right panel). The radioactivity was quantitated after PAGE in SDS (see Fig. 2). Cpm in preprolactin (containing beta-hydroxy leucine residues) (*pPL**) when centrifugation was performed: (Δ), or when centrifugation was omitted: (\blacktriangle). Cpm in preprolactin (beta-hydroxy leucine replaced by leucine) when centrifugation was performed (Δ), or cpm in [preprolactin plus prolactin] when centrifugation was omitted (\blacktriangle).

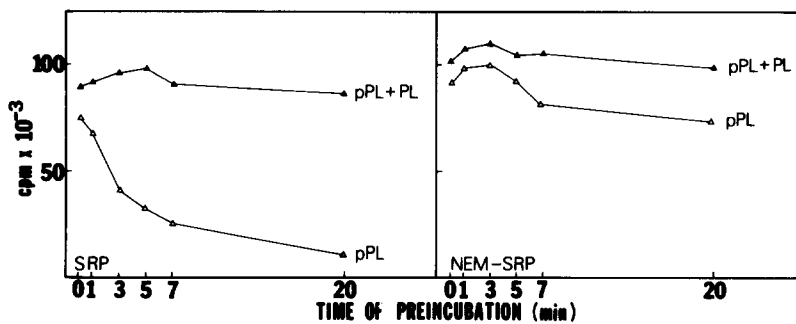


FIGURE 7 Inhibition of nascent polysome binding to salt-extracted microsomal membranes by modification of SRP with *N*-ethyl maleimide. Pituitary RNA was translated with 3 eq of K-RM and 20 U of SRP (left panel), or the equivalent amount of *N*-ethyl maleimide modified SRP (1) (right panel) present per 75 μ l wheat germ system and assayed for nascent polysome binding as described in Materials and Methods. The radioactivity was quantitated after PAGE in SDS (see Fig. 2). Cpm in preprolactin when centrifugation was performed (Δ); cpm in the sum of [preprolactin plus prolactin] when centrifugation was omitted (\blacktriangle).

structure (and thereby its information content) by incorporating β -hydroxy leucine (12), a Leu analogue, into nascent preprolactin (Fig. 6, left panel). A preincubation time-dependent attachment of the β -hydroxy leucine-incorporating polysomes to the microsomes was not observed. Attachment could be restored by competing out the β -hydroxy leucine with Leu (Fig. 6, right panel).

Finally, we demonstrated that NEM-treatment of SRP (which abolished its interaction with ribosomes or polysomes [1]), also inactivated its capacity to bind nascent polysomes synthesizing secretory proteins to salt-extracted RM (Fig. 7).

DISCUSSION

We have described an assay for the functional attachment of *in vitro* assembled polysomes synthesizing secretory protein to microsomal vesicles. The assay is based on measuring the depletion of mRNA from the *in vitro* translation system after assembling the mRNA into membrane-bound polysomes, and removing these *in vitro* assembled rough microsomes by differential centrifugation.

In the past, attempts have been made to study the nature of the ribosome-membrane junction of the rough endoplasmic reticulum (ER) by performing binding experiments with isolated ribosomes and microsomal vesicles (13–16). However, the results of these studies could not correlate the observed binding event with the formation of a functional (with respect to translocation) ribosome-membrane junction.

Binding studies of *in vivo* assembled detached polysomes (polysomes isolated from rough microsomes after detergent solubilization) to microsomal membranes (17) pose an artificial situation as well. These polysomes, containing chains at various

stages of completion with the signal sequence already removed from many chains, do not exist in this form as free polysomes in the cell.

The assay system we describe here offers the advantage of being able to correlate the binding of nascent, *in vitro* assembled polysomes to membranes with the actual translocation of the newly synthesized secretory protein. The assay therefore simulates, being based on ongoing protein synthesis, the dynamic conditions occurring *in vivo*. However, it poses limitations in that it is restricted to conditions that are compatible with *in vitro* protein synthesis.

Using this assay we have demonstrated that microsomes that have been depleted of their translocation activity by salt extraction are unable to bind nascent preprolactin-synthesizing polysomes and that nascent polysome binding as well as preprolactin translocation can be restored only upon the readdition of SRP. The binding of nascent preprolactin polysomes to microsomes is therefore dependent on SRP.

The overall translocation event can be viewed as occurring in steps, where the recognition of nascent polysomes synthesizing secretory protein and their binding to the microsomal membrane precede the physical translocation of the nascent polypeptide chain across the membrane. From the data presented here and in the preceding paper (1), we can conclude that SRP is essential for recognition and binding to the microsomal membrane of nascent polysomes synthesizing secretory protein. Whenever we were able to interfere with the recognition by SRP of nascent *in vitro* assembled polysomes (1) we also observed an interference with SRP-mediated binding of these polysomes to the microsomal membrane. For example, NEM-modification of SRP, previously shown to abolish both

its low-affinity binding to the ribosome as well as its high-affinity binding to nascent polysomes synthesizing secretory protein (1), also abolished SRP's capacity to bind these polysomes to the microsomal membrane. Likewise, modification of the nascent chain via incorporation of β -hydroxy leucine, previously shown to abolish the high-affinity binding of SRP to nascent polysomes synthesizing secretory protein (1), also abolished binding of these polysomes to the microsomal membrane. Data in the following paper (8) will demonstrate that SRP's recognition of specific polysomes and its ability to bind these polysomes to the microsomal membrane may be separate events.

Considering the kinetics of the polysome binding, we have demonstrated that at least the binding step of the translocation process is a strictly cotranslational event. The binding of polysomes is essentially completed after only 3–4 min, i.e. after, at most, half of a prolactin chain is synthesized in our *in vitro* system. Therefore, each prolactin molecule translocated has been completed on bound polysomes. Taken together, our data here and in the preceding paper provide experimental support for the proposal (9, 18, 19) that protein translocation across the ER is a receptor-mediated process and conclusively rule out an alternative proposal originally advanced by Bretscher (20) and subsequently expanded upon by Wickner (21) as well as by Engelman and Steitz (22) that translocation across the lipid bilayer occurs spontaneously and does not require mediation by proteins. Our data also rule out translocation models (23–26) that, although relying on the participation of proteins, postulate a primary interaction of the signal sequence (due to its hydrophobic nature) with the lipid bilayer rather than with protein. If this were the case, binding of nascent polysomes synthesizing secretory protein to salt-extracted microsomal vesicles should have taken place in the absence of SRP. It is thus clear that the initial events that lead to translocation and provide its specificity are not protein-lipid interactions (signal sequence—lipid bilayer), but protein-protein interactions (signal sequence plus ribosome—SRP).

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