Translocation of Proteins Across the Endoplasmic Reticulum III. Signal Recognition Protein (SRP) Causes Signal Sequence-dependent and Site-specific Arrest of Chain Elongation that is Released by Microsomal Membranes

PETER WALTER and GÜNTER BLOBEL
Laboratory of Cell Biology, The Rockefeller University, New York 10021

ABSTRACT The previously observed (Walter, et al. 1981 *J. Cell Biol.* 91:545–550) inhibitory effect of SRP selectively on the cell-free translation of mRNA for secretory protein (preprolactin) was shown here to be caused by a signal sequence-induced and site-specific arrest in polypeptide chain elongation. The M_r of the SRP-arrested nascent preprolactin chain was estimated to be 8,000 corresponding to ~70 amino acid residues. Because the signal sequence of preprolactin comprises 30 residues and because ~40 residues of the nascent chain are buried (protected from protease) in the large ribosomal subunit, we conclude that it is the interaction of SRP with the amino-terminal signal peptide of the nascent chain (emerged from the large ribosomal subunit) that modulates translation and thereby causes an arrest in chain elongation. This arrest is released upon SRP-mediated binding of the elongation-arrested ribosomes to the microsomal membrane, resulting in chain completion and translocation into the microsomal vesicle.

In the previous two papers we have described several functional properties of SRP. We have shown (*a*) that SRP binds, presumably via the signal sequence of the nascent chain, to in vitro assembled polysomes synthesizing secretory protein but not to those synthesizing cytoplasmic protein (1); (*b*) that SRP inhibits translation of mRNA coding for secretory protein but not of that coding for cytoplasmic protein (1); and (*c*) that SRP mediates binding of in vitro assembled polysomes synthesizing secretory protein (but not of those synthesizing cytoplasmic protein) to microsomal membranes (2). Polysome binding and translation-inhibitory effect were observed to be correlated (1) and the presence of microsomal membranes appeared to reduce the translation-inhibitory effect of SRP (2).

In this paper we describe studies on the translation-inhibitory effect of SRP and on the reversal of this effect by microsomal membranes.

MATERIALS AND METHODS

The preparation of various microsomal membrane fractions (RM, K-RM), the extraction and purification of SRP, the wheat germ translation system, and the quantitation of in vitro synthesized protein were described in the first paper of this series (1). The SRP preparation used was the eluate of the aminopentyl-agarose column.

RESULTS

To address questions concerning the nature of the SRP-induced inhibition of secretory protein synthesis (1) and the observed release of this inhibition by microsomal membranes (2), we decided to use a translation system that was synchronized with respect to polypeptide chain elongation (3, 4). Synchronization can be achieved by the addition of inhibitors of initiation a short time after the start of the incubation. Such a system allows the subsequent addition of membranes to be precisely timed with respect to chain length. Furthermore, protein synthesis stops after the initiated chains are completed. This allows us to detect any putative SRP-arrested states of preprolactin chain elongation and to ascertain that (upon release of the arrested state by microsomal membranes) the appearance of completed chains is due to chain completion of a previously elongation-arrested nascent chain, but not of a newly initiated chain.

To characterize our synchronized translation system, the following experiments were performed. Translation of prolactin mRNA was allowed to initiate and to proceed for 2 min after which time 7-methylguanosine-5′-monophosphate (^{7}mG) was added to the translation system. This compound completely blocks initiation of protein synthesis without effecting the rate of elongation (4). From the time-course of incorporation of [^{35}S]Methionine into polypeptide (Fig. 1, closed circles), it was apparent that translation plateaued after 15 min. It took 10 min for the first preprolactin chains to be completed (2) and ~15 min for all preprolactin chains to be completed (Fig. 1 and Fig. 2, left panel, no RM added). Protein synthesis in the

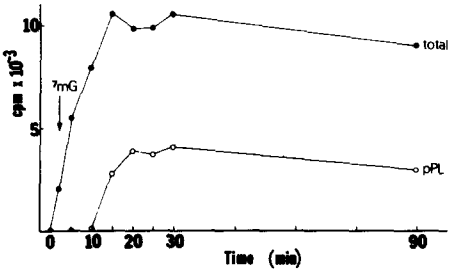

FIGURE 1 Time-course of incorporation of [^{35}S]Met into polypeptide in the synchronized translation system. Bovine pituitary RNA was translated in the wheat germ system in the absence of microsomal membranes. The system was incubated at 26°C. After 2 min, 7-methylguanosine-5′-monophosphate (^{7}mG) was added to 2 mM to inhibit initiation. At different time points, 10-μl aliquots were (*a*) spotted on filter paper and TCA-precipitated, and (*b*) TCA-precipitated and prepared for analysis by SDS-PAGE. The filter disks were boiled in 5% TCA as described and [^{35}S]Met incorporated into polypeptide was determined by scintillation counting (●). Bands corresponding to preprolactin (○) were located by autoradiography of the polyacrylamide gel, sliced from the gel, and their radioactivity was determined as described (11). The counting efficiency in both cases was 70%.

absence of ^{7}mG was linear with time for 30–50 min (2). When RM were added to the synchronized translation system at different times after the initiation block by ^{7}mG, segregated prolactin was formed only when the membranes were added early enough (Fig. 2, left panels), i.e. before the growing chain lost its capacity for a functional interaction with the membranes (5). When the membranes were added late (Fig. 2, left panels, RM added at 14 min) no prolactin was formed, indicating that at this time-point no more nascent preprolactin molecules capable of being translocated were present.

Using the synchronized translation system we then proceeded to investigate the translation-inhibitory effect of SRP. When SRP was present from the beginning of translation, a decreased overall rate of incorporation of [^{35}S]Met into polypeptide is observed (Fig. 3, minus K-RM). Addition of salt-extracted microsomal membranes (K-RM) at each time-point resulted in the onset of significant further [^{35}S]Met incorporation (Fig. 3, plus K-RM). This new burst of protein synthesis plateaued after ~5–10 min, at a level comparable to the total [^{35}S]Met incorporation obtained in the absence of SRP (compare Figs. 1 and 3). This release of SRP-induced inhibition was observed even when K-RM were added after 20 min, i.e. at a time at which protein synthesis in the presence of SRP was arrested and in the absence of SRP was already completed (compare Figs. 1 and 3).

From the SDS-PAGE analysis of the translation products, it was apparent that in the presence of SRP and in the absence of K-RM essentially no preprolactin or prolactin was formed (Fig. 2, right panels, minus K-RM). Instead, the appearance of a distinct low-M_r band was observed (Fig. 2, right panels, arrow head) that could not be detected when SRP was omitted (Fig. 2, left panels, minus RM). When K-RM were added at early time-points, prolactin was formed (Fig. 2, right panels, plus K-RM at 3 min, 6 min, and 9 min). When the system was incubated for longer times in the presence of SRP and in the absence of K-RM the low-M_r band persisted (Fig. 2, right panels, minus K-RM). Upon K-RM addition however, the low-M_r band disappeared concomitantly with the appearance of primarily processed (translocated) prolactin. Even as late as 20 min after the initiation block when, in the absence of SRP (Fig. 1 and Fig. 2, left panels), no more translation was observed, added K-RM were able to release the SRP-induced elongation arrest (Fig. 2, right panels, plus K-RM at 20 min). Note however, that not all elongation-arrested preprolactin chains were converted into processed prolactin after the addition of K-RM; some preprolactin molecules were also formed (Fig. 2, right panels plus K-RM at 14 min); consequently release of translation arrest may not in all cases be followed by chain translocation in our assay system. Because reinitiation was prevented by ^{7}mG and no other major products besides prolactin were observed after the membrane addition, we are most likely dealing with a precursor-product relationship between the low-M_r band and processed (translocated) prolactin or preprolactin. However, definitive identification of the low-M_r band as a translation-arrested preprolactin fragment has to await characterization by sequencing. The molecular weight of the low-M_r polypeptide was determined and estimated to be 8,000 based on its relative mobility in a SDS-urea polyacrylamide gel system (Fig. 4).

DISCUSSION

We have demonstrated here that prolactin mRNA translation can be arrested by SRP. This arrest occurs at a distinct point in preprolactin synthesis, resulting in the formation of a low molecular weight polypeptide that comprises the amino-terminal portion of preprolactin. Its size of ~8,000 daltons corresponds to ~70 amino acids polymerized. This translation arrest explains the previously observed (1) SRP-induced inhibition of preprolactin synthesis. Preprolactin synthesis continues only if the translation arrest is released by the addition of salt-extracted microsomal membranes (K-RM), leading to the formation of completed and efficiently translocated prolactin molecules.

To our knowledge, this is the first example of a site-specific modulation in the synthesis of specific proteins at the translational level. SRP was previously shown (1, 2) to recognize polysomes synthesizing secretory protein by information contained in the nascent secretory protein. Because ~40 amino acids are required to span the large ribosomal subunit (6, 7) and because preprolactin chain elongation was arrested after ~70 amino acids were polymerized, an NH_2-terminal segment comprising 30 residues would be exposed on the "outside" of the ribosome. This exposed segment corresponds approximately to the size of the cleaved signal peptide of preprolactin (8). We have therefore demonstrated, that the information required for recognition by SRP of a polysome synthesizing secretory protein is contained in the amino-terminal signal peptide or closely adjacent residues. The previously reported observation (3, 4), namely that nascent chains of >80 residues could no longer be translocated, might be due to the inability of the longer chains to interact with SRP.

Because we have also been able to demonstrate an SH-group dependent interaction of SRP with ribosomes regardless of their state of biosynthetic activity (1) it seems likely, that upon recognition of a signal sequence and in the absence of microsomal membranes, SRP transmits the information to arrest protein synthesis through ribosomal components causing a site-specific elongation arrest.

SRP arrests the elongation of secretory proteins once their signal sequence (marking them as secretory proteins) is expressed outside the ribosome. This arrest can be fully released when microsomal membranes are added, thereby allowing the secretory protein to be completed and concomitantly translo-

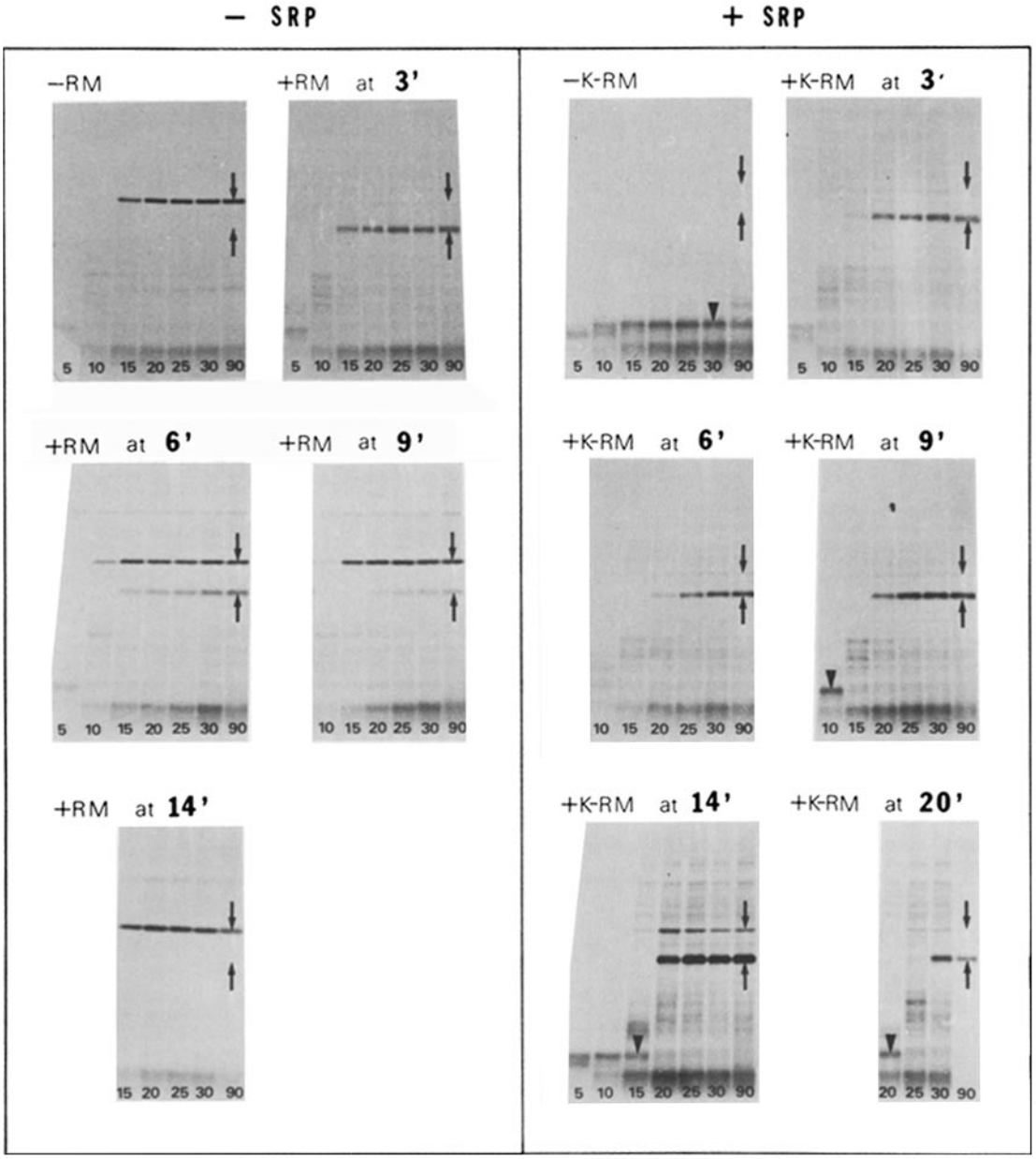

FIGURE 2 Function of SRP in a synchronized translation system. Bovine pituitary RNA was translated in the wheat germ system in the absence (left panels, *–SRP*) or presence (right panels, *+SRP*) of 40 U *SRP* per 100 μl translation. After 2 min, ^{7}mG was added to 2 mM to inhibit initiation. After 3, 6, 9, and 14 min, *RM* (8 eq) were added to the translation system (100 μl) not containing *SRP* (left panels). After 3, 6, 9, 14, and 20 min *K-RM* (4 eq) were added to the translation system (100 μl) containing *SRP* (right panels). At different time points (indicated on the bottom of each lane in min) 10-μl aliquots were TCA precipitated and prepared for SDS-PAGE. Fluorography of the PPO-impregnated polyacrylamide gel was performed. The positions of preprolactin and prolactin bands are indicated by downwards or upwards pointing arrows, respectively. The position of the "low-M_r band" is indicated with an arrow head. The reduced intensity of bands in the last lane of the lower right panel was due to loss of material during sample preparation.

cated across the membrane. SRP may act to prevent the synthesis of this class of proteins until their sequestration out of their biosynthetic compartment (i.e. cytoplasm) is guaranteed. Teleologically this mode of action appears plausible if one considers that many secretory (and lysosomal) proteins may express enzymatic activities that would be potentially harmful to the cell if fully synthesized in the cytoplasm. It should be noted, however, that the observed SRP-dependent elongation arrest might be an experimentally generated condition that may never or rarely arise in the intact cell, where endoplasmic reticulum (ER) membranes are always present.

Although we have only described experiments using prolactin as a model secretory protein and the two globin chains as a model cytoplasmic protein, we feel certain that the results obtained can be generalized to all proteins that use the translocation machinery of the ER. Although a systematic study with other proteins has not yet been done, several lines of evidence argue in favor of such a generalization: (*a*) the integration of a membrane protein, such as the delta-subunit of the acetylcholine receptor into membranes is SRP-dependent (Anderson et al. Manuscript in preparation); and (*b*) the synthesis of the above mentioned integral membrane protein,

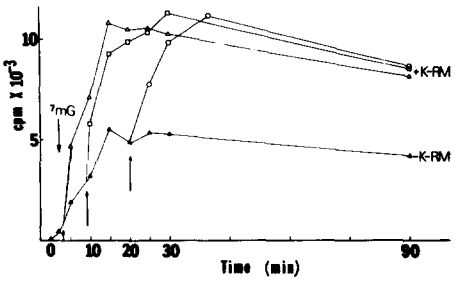

FIGURE 3 Release of SRP-induced inhibition on prolactin synthesis by *K-RM*. Bovine pituitary RNA was translated in the wheat germ system in the presence of SRP (40 U/100 μl). The system was incubated at 26°C. After 2 min, ^7mG was added to 2 mM to inhibit initiation. After 3 (△), 9 (□), and 20 (○) min *K-RM* were added to 4eq/100 μl. At different time points, 10-μl aliquots were spotted on filter paper, TCA-precipitated and the radioactivity in polypeptide determined (1). Control with no *K-RM* added: (▲).

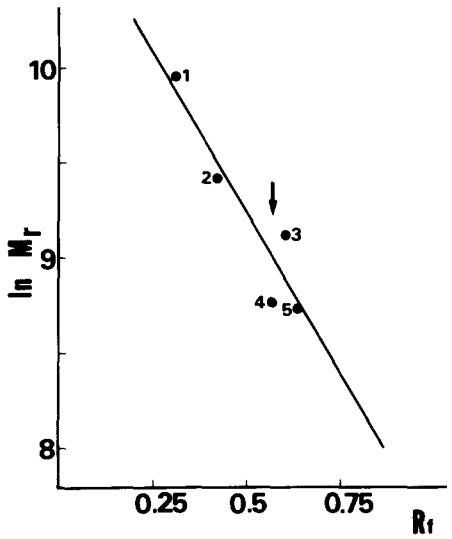

FIGURE 4 M_r-determination of the SRP-arrested translation product of prolactin mRNA. Bovine pituitary RNA was translated in a 25-μl wheat germ system containing 10 U SRP. After 2 min incubation at 26°C, ^{7}mG was added to 2 mM to inhibit initiation. Incubation was continued for 45 min. The translation was then TCA precipitated and prepared for SDS-PAGE. The sample was electrophorezed on a polyacrylamide slab gel (22.25% acrylamide/0.085% bisacrylamide) containing 6 M urea. The gel was fluorographed using PPO and the mobility (R_f) of the "low-M_r band" relative to the bromphenol blue dye front determined (arrow). The following M_r standards were run on the same gel: (*1*) soy bean trypsin inhibitor (M_r = 21,500); (*2*) cytochrome c (M_r = 12,500); (*3*) pre-f_1-bacteriophage coat protein (M_r = 8,900); (4) aprotinin (M_r = 6,500); (5) f_1-bacteriophage coat protein (M_r = 6,100 (17)). The connecting line represents the least-squares fit of the mol wt markers. The M_r of the SRP-arrested translation product of prolactin mRNA was determined to be ~8,000 daltons.

as well as of two additional secretory proteins, chicken lysozyme (unpublished observation) and rat apolipoprotein AI (Stoffel et al. Manuscript in preparation), was found to be arrested by SRP; in both cases arrest was released when microsomal vesicles were added and translocation occurred.

The relationship between SRP and protein fragment(s) generated by protease treatment of microsomal membranes and shown to be required for chain translocation (9–11) remains to be investigated. However, the finding (data not shown) that trypsin-treated K-RM (by themselves translocation inactive) could not be reactivated by SRP might suggest that the protein fragment(s) and SRP are unrelated.

Based on our findings, we would like to propose the scheme depicted in Fig. 5 as a model for the role of SRP in translocation of secretory (and lysosomal) proteins across and integration of membrane proteins into the membrane of the ER. SRP can be extracted from and rebound to microsomes. Although its precise in vivo interaction with the membrane remains to be elucidated, we would like to suggest an equilibrium (Fig. 5 *A*) between a membrane-bound and a free (soluble) form of SRP. Because of the in vitro observed low affinity binding of SRP to monomeric ribosomes we would also like to propose an equilibrium between the putative free form of SRP and monomeric ribosomes (Fig. 5 *B*). Upon translation of a mRNA coding for a secretory protein (Fig. 5 *C*) the expression of the nascent secretory protein's signal sequence causes a 6,000-fold enhancement in the apparent affinity of SRP for polysomes. Concomitantly, and presumably through the ribosome, SRP arrests the synthesis of the secretory protein (Fig. 5 *D*), preventing the completion of any secretory or presecretory protein in the cytoplasm. Only when membranes with "translocation competent sites" (phospholipid vesicles have no effect [unpublished data]) are offered to this arrested polysome, does it attach to the membranes (here by virtue of a putative SRP- and ribosome receptor) (Fig. 5 *E*) resulting in the assembly of the functional translocation machinery (here depicted as a pore like structure). Synthesis of the secretory protein then continues, concomitant with its translocation across the membrane (Fig. 5 *F*).

Our choice of the term signal recognition protein was made on the basis of the most striking property of this protein, namely its high affinity binding to nascent polysomes containing ER-targeted signal sequences in their nascent chains (12). Although we have not yet demonstrated a ribosome-independent and direct binding between such a signal peptide and SRP (and therefore have refrained from using the simpler term

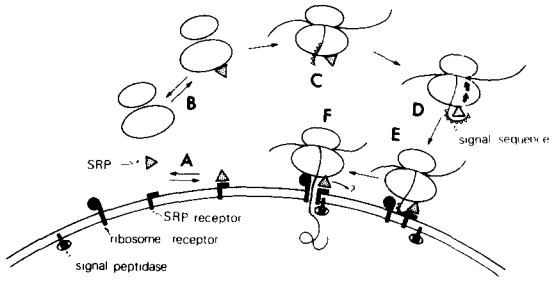

FIGURE 5 Model for the function of SRP in the translocation process.

"signal receptor") other lines of evidence suggest that such a direct interaction may indeed occur. For example, a signal peptide isolated from a tryptic digest of ovalbumin (5) as well as a chemically synthesized peptide containing the signal peptide region of preproparathyroid hormone (13) have been shown to compete when added to an in vitro translocation system containing translocation competent microsomal membranes. Likewise, preincubation of translocation competent microsomal membranes with in vitro synthesized preproinsulin was shown to reduce the vesicles' translocation activity (14). However, despite our demonstration (2) that the microsomal membranes' ability to selectively bind nascent polysomes synthesizing secretory protein can be localized to SRP, the results of the above mentioned competition experiments constitute only indirect evidence for a direct signal-signal receptor inter-

action. They do not rule out the possibility that the high affinity binding of SRP to nascent polysomes is due to a signal sequence-induced conformational change in the ribosome rather than the result of a direct binding of SRP to the signal sequence itself.

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