Signal Recognition Particle Causes a Transient Arrest in the Biosynthesis of Prepromelittin and Mediates its Translocation across Mammalian Endoplasmic Reticulum

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Abstract. The translocation of prepromelittin (pPM) across mammalian endoplasmic reticulum was studied in both wheat germ and reticulocyte lysate. In the wheat germ system, signal recognition particle (SRP) caused a transient arrest in the synthesis of pPM. This was indicated by a slowdown in the rate of synthesis of pPM in the presence of SRP. The arrest was specific, dependent on the concentration of SRP, and more effective at early incubation time. In a tightly synchronized translation system, SRP had no apparent effect on the elongation of pPM, indicating that the effect of SRP on pPM chain synthesis might be at the final stages of chain elongation and release from the ribosome. This was reflected in a transient accumulation of pPM as peptidyl tRNA. Because pPM is composed of only 70 amino acids, arrest by SRP may be very close to chain termination. Arrest at this stage of chain synthesis seems to be unstable and the nascent chain gets terminated and released from the ribosome after a transient delay.

The translocation of pPM was shown to be dependent on both SRP and docking protein. The difference in the translocation efficiency of pPM in reticulocyte and wheat germ lysates may reflect a difference in the targeting process in the two systems.

SECRETORY and membrane proteins are synthesized with signal sequences that enable them to cross the membrane or integrate into it (for reviews see Hortsch and Meyer, 1986; Wickner and Lodish, 1985; Walter et al., 1984). This process was found to be receptor-mediated as initially proposed in the signal hypothesis (Blobel and Dobberstein, 1975a, b). Two components were isolated from mammalian endoplasmic reticulum (ER) and found to mediate the translocation of proteins across the ER membrane. These are signal recognition particle (SRP), a ribonucleoprotein particle (Walter and Blobel, 1980) and docking protein (DP) or SRP-receptor, an integral membrane component (Meyer et al., 1982; Gilmore et al., 1982a, b). Furthermore, SRP was found to cause arrest in the elongation of nascent secretory polypeptides. It recognizes the signal sequence when it emerges from the large ribosomal subunit, and arrests further chain elongation until the nascent chain is targeted to the membrane (Meyer et al., 1982; Walter and Blobel, 1981). Interaction of the arrested chain with the membrane results in the release of the elongation arrest, presumably by the displacement of SRP from the ribosome (Gilmore and Blobel, 1983). The nascent chain then integrates in the membrane in an unknown manner. Interaction between the signal sequence and a putative signal sequence receptor was thought to play a role in this integration step and the subsequent step of chain segregation across the membrane (Friedlander and Blobel, 1985). Some of the subunits of the signal peptidase complex were suggested as candidates for such a receptor (Evans et al., 1986).

The arrest of nascent chain synthesis by SRP has been an intriguing phenomenon. It was proposed to target the chain to the membrane by an SRP-DP recognition process (Meyer et al., 1982; Gilmore et al., 1982a, b). However, in homologous mammalian lysates, SRP does not cause arrest in nascent chain synthesis, yet the chain is very efficiently targeted to the membrane (Meyer, 1985). Furthermore, SRP subparticles that lack the arrest function were isolated and found to target the nascent chain to the membrane, though through a narrower time window than did complete particles (Siegel and Walter, 1985; Siegel and Walter, 1986).

In the initial observation of nascent chain arrest by SRP, chain elongation was arrested at an early specific site when about 70 amino acids had been polymerized; these appeared as a distinct arrested peptide that could be chased to the mature protein by the addition of membranes (Walter and Blobel, 1981) or to the preprotein by the addition of purified docking protein (Meyer et al., 1982; Gilmore et al., 1982a, b; Meyer, 1985). This release of arrest could take place even after 90 min of incubation, indicating that the arrested com-

1. Abbreviations used in this paper: CTABr, cetyltrimethylammonium bromide; DP, docking protein; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; PM, promelittin; pPM, prepromelittin; RM, rough microsomes; RM-KN, salt-washed and nuclease-treated rough microsomes; SRP, signal recognition particle.
plex is rather stable. Subsequently, it was observed that arrest could take place late in the elongation process (Ainger and Meyer, 1986). Furthermore, for some proteins, multiple arrested peptides ranging in size from 70 to 140 amino acids were detected (Ibrahimi and Fuchs, 1986). These observations indicated that arrest of chain elongation by SRP could take place at more than one specific site. It is thought that the polymerization of 70 amino acids is needed to span the large ribosomal subunit and to expose the signal sequence for recognition by SRP, thus representing the earliest arrested stage.

The question arises, then, of how secretory proteins whose full size is about 70 amino acids are targeted to the membrane. This question was previously investigated by studying the translocation of shortened polypeptides across the ER membrane (Ibrahimi et al., 1986). The main drawback of this approach was the fact that, because of the absence of a termination codon, the shortened polypeptides remained attached to tRNA (Haeuptle et al., 1986), which complicated the analysis of the targeting process.

Prepromelittin (pPM) is a secretory protein of the venom gland of queen honey bee. Its full size is about 70 amino acids (Suchanek et al., 1978). It serves as a suitable model system for the analysis of the translocation of short secretory proteins. I report here that the synthesis of prepromelittin is only transiently arrested by SRP, in contrast to prelight chain, whose synthesis is arrested by SRP permanently. This transient arrest may provide only a narrow time window for pPM targeting to the membrane, resulting in only inefficient translocation. Otherwise, pPM is translocated across the ER membrane in a co-translational and SRP-DP-dependent manner as a typical secretory protein.

Materials and Methods

[35S]Methionine (1,000 Ci/mmol), [3H]Proline (100 Ci/mmol), and reticulocyte lysate (N150) were obtained from Amersham International, Amsersham, England. Protease K was obtained from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany; phenylmethylsulfonyl fluoride was from Sigma Chemical Co., St. Louis, MO; and 7-methylguanosine phosphate was from P-L Biochemicals, Inc., Milwaukee, WI. Tritium-labeled pPM standard and mRNA from queen honey bee venom gland containing mainly pPM mRNA (Kinds-Mügge et al., 1974) were a generous gift from G. Kreil, Institut für Molekularbiologie, Österreichische Akademie der Wissenschaften, Salzburg, Austria. IgG against light chain mRNA was isolated from MOPC-41 mouse myeloma. Cell-free lysates from wheat germ, rough microsomes (RM), salt-extracted and nuclease-treated microsomes (RM-KN), N-ethylmaleimide (NEM)-treated microsomes, and SRP were prepared and used as previously described (Walter et al., 1981; Gilmore et al., 1982).

Protein Synthesis and Translocation Assays

mRNA was translated in either the wheat germ or reticulocyte lysate. Translations in the wheat germ system were done as previously described (Walter et al., 1981) and those in reticulocyte lysate were done according to the manufacturer's protocol. [3H]Proline and [35S]Methionine were used at 10 and 15 μCi per 10 μl assay, respectively. Incubations were done at 27°C for wheat germ and at 30°C for reticulocyte lysate for 60 min, unless otherwise indicated. Microsomes and SRP were added to the translation assays as indicated in the figure legends at a concentration of 0.8A280/ml for the microsomes and 0.08A280/ml for SRP, unless otherwise indicated. This concentration was considered to be 1 U in each case.

Continuous and Synchronized Translation Assays

Total mRNA from queen bee venom gland was titrated in the wheat germ translation system for synthesis of pPM. From a stock solution with 50A280/ml, 1 μl was chosen as a sublimiting amount of mRNA in a 25-μl translation assay.

For studying the time course of the effect of SRP on the synthesis of pPM, the above amount of mRNA together with rabbit globin mRNA were translated in the wheat germ lysate in the absence and presence of SRP. At the indicated time intervals (see Results), 10 μl samples were taken from each incubation mixture into 10 μl of 10% TCA.

The above concentration of mRNA was used threefold for synchronized translations. All the components of the translation assay except mRNA were mixed and SRP was added. The mixture and mRNA were prewarmed at 27° and then mixed together. After 1 min of further incubation, 7-methylguanosine phosphate was added to a final concentration of 8 mM. Samples were withdrawn at time intervals and precipitated in 10% TCA.

Posttranslational Treatments

Cycloheximide was added at the end of the incubation period to a final concentration of 200 μM for posttranslational processing. This was followed by the addition of microsomes and the incubation was continued for another 30 min. After incubation, samples were digested with proteinase K at a final concentration of 200 μg/ml in the presence of 10% sucrose where indicated. Digestion was allowed to proceed for 15 min on ice, after which it was stopped by phenylmethylsulfonyl fluoride at a concentration of 2 mM. Samples were precipitated with TCA and quickly prepared for gel electrophoresis.

Cetytrimethylammonium Bromide (CTABr) Precipitation of Peptidyl tRNA

Peptidyl tRNA was precipitated from the translation assays essentially as described before (Gilmore and Blobel, 1985; Hobden and Cundliffe, 1978). The translation products (10 μl) were added to 250 μl of 2% wt/vol CTABr. The CTABr precipitates were induced and collected as described before (Gilmore and Blobel, 1985). The washing of the precipitates with acetone/HCl (19:1) to remove CTABr was found to result in extensive losses of pPM that may have been due to its extraction in acetone and/or hydrolysis by HCl. Therefore, 1 used n-butanol for washing the CTABr precipitates, which were then solubilized in sample buffer and subjected to PAGE and autoradiography. Preparation of CTABr precipitates for scintillation counting was done exactly as described before (Gilmore and Blobel, 1985).

Analysis of Translation Products

The translation products were separated by electrophoresis on Laemmli type (Laemmli, 1970) SDS polyacrylamide gels containing 6 M urea and 22% acrylamide (Ibrahimi et al., 1986). Where indicated, translation mixtures were extracted with n-butanol (Mollay et al., 1982) and the resulting fractions were subjected to SDS PAGE. Labeled bands were visualized by fluorography using EN3HANCE.

Results

 Biosynthesis and Characterization of pPM and PM

Promelittin (PM) is the predominant secretory protein of queen bee venom gland. In vitro translation of total mRNA from these glands results in a product that is larger than PM, and this has been shown to be pPM (Kaschnitz and Kreil, 1978). It contains a typical amino terminal signal sequence comprising 21 amino acid residues. Subcellular fractions from rat liver were shown to convert pPM to PM (Suchanek and Kreil, 1977). Since antibodies to melittin could not be produced, other criteria were used to establish the identity of the in vitro translation products. PM can be selectively separated from pPM by extraction with n-butanol (Mollay et al., 1982; Mollay et al., 1976). The identity of the separated products was previously established by sequence analysis (Kaschnitz and Kreil, 1978). The two components could also be separated by electrophoresis using SDS polyacrylamide gels containing 22% acrylamide and 6 M urea. A single major band that comigrated with standard pPM was obtained.
when total translation products were displayed after SDS PAGE (Fig. 1, lanes 1 and 6). Addition of RM from mammalian ER to the translation mixture in either wheat germ or reticulocyte lysate generated a lower molecular mass protein, probably due to the cleavage of the signal sequence from pPM (Fig. 1, lane 2). The two proteins could be separated from each other by extraction of the translation mixture with n-butanol. As expected, PM partitioned in the aqueous phase and pPM in the interphase (Fig. 1, lanes 3–5). The two products had the expected molecular size based on their mobility in SDS PAGE. Their identity was confirmed by amino acid sequence analysis (results not shown).

**SRP Causes a Transient Arrest in the Biosynthesis of pPM**

In a tightly synchronized translation assay that favored the formation of monosomes, the synthesis of pPM, in contrast to that of IgG κ prelight chain, was not affected by SRP (results not shown). Because the size of pPM is 70 amino acids, which is the expected size of the SRP-arrested peptide, the above results could be interpreted by assuming that chain termination takes place before SRP is able to bind to the nascent chain. An alternative possibility is that the two events coincide or fall close together, in which case the arrested peptide and the completed peptide would co-migrate as a result of being identical or different by only a few amino acids. To discriminate between these possibilities, I studied the effect of SRP on the rate of synthesis of pPM in a nonsynchronized translation system. Subliming concentrations of mRNA were used for translations in the wheat germ system in the presence and absence of SRP to enhance polysome formation and allow continuous chain synthesis. The logic of this experiment was that when mRNA is limiting, a transient arrest by SRP will delay the recycling of mRNA, and hence lower the overall rate of pPM synthesis. Samples were withdrawn at time intervals, precipitated with TCA, and subjected to SDS PAGE. A parallel experiment was done on light chain. Fig. 2 shows that SRP caused a permanent arrest in the synthesis of prelight chain. The arrest in the biosynthesis of pPM was only transient, as indicated by the slowdown in the rate of chain formation in the presence of SRP. Rabbit globin mRNA was included in the translations as a cytoplasmic protein marker whose synthesis is not affected by SRP. For each time point the amount of pPM or prelight chain formed in the presence of SRP was determined by densitometry and corrected by multiplying by the ratio of globin in the absence of SRP over globin in the presence of SRP. This ratio was always very close to 1.

**SRP Causes a Specific and Concentration-dependent Arrest in the Biosynthesis of pPM**

Fig. 2 shows that after 15 min of incubation in the presence of SRP, the synthesis of pPM was 80% inhibited by SRP, while after 90 min the inhibition was only 18%. Fig. 3 shows that this inhibition is specific and dependent on the concentration of SRP. The incubation was carried out, as in Fig. 2, for 15 min in the presence of decreasing amounts of SRP. It is clear that the synthesis of globin was not essentially affected by SRP while that of pPM was arrested by SRP in a concentration-dependent manner.

**SRP Slows Down the Release of pPM from Peptidyl tRNA**

Because SRP slows down the rate of pPM synthesis, the possibility arose that pPM might stay in association with the ribosomes as a peptidyl tRNA for a longer period in the presence of SRP than in its absence. This possibility was tested by precipitating peptidyl tRNA from translation assays in the absence and presence of SRP. Translation assays were done in wheat germ lysates using excess mRNA. After 5 min of incubation, further initiation was blocked by adding 7-methylguanosine phosphate. Aliquots of 10 μl were withdrawn at the time intervals shown in Table I and put on ice. One-half of each aliquot was precipitated with CTABr and prepared...
Table I. Determination of Peptidyl tRNA in the Translation Assays in the Presence and Absence of SRP

| Time of incubation | -SRP cpm | +SRP cpm |
|-------------------|----------|----------|
| 10 min            | 39       | 100      |
| 20 min            | 11       | 72       |
| 40 min            | 11       | 12       |

Precipitation of peptidyl tRNA from samples of the translation assays and its preparation for scintillation counting were done as described in Materials and Methods. The cpm at 10 min in the presence of SRP (382047) were set equal to 100%. All other values were calculated relative to this value. All counts were corrected by subtracting the counts obtained from an incubation to which no mRNA was added.

The Translocation of pPM across ER Membranes Is Inefficient but Dependent on SRP

In a further series of experiments, I investigated the influence of SRP on the translocation of pPM. Protein synthesis was done in wheat germ lysate in the absence and presence of RM-KN and SRP. Fig. 4 A shows that the salt-washed and nuclease-treated microsomes caused no significant processing of pPM to PM. When SRP was added together with the microsomes, a low but significant degree of processing was observed. The processed PM was translocated inside the microsomes, as indicated by its protection from proteinase K digestion. Fig. 4 B shows that the translocation of pPM across ER membranes was more efficient in the reticulocyte lysate than in the wheat germ lysate. Exogenous SRP was not required for the processing or translocation process in these lysate.

The Translocation of pPM Does Not Take Place Posttranslationally or by NEM-treated Microsomes

Translocation of proteins across mammalian ER membranes is a co-translational and an SRP-DP-dependent process. I investigated the translocation of pPM in this respect. Fig. 5 shows that untreated RM were unable to translocate pPM when added after inhibiting protein synthesis by cyclohexi-
The NEM-treated microsomes were also inactive when included during the translation process.

**Discussion**

Protein translocation across mammalian ER membranes is a receptor-mediated and co-translational process. Three components, namely SRP (Walter and Blobel, 1980), DP (Meyer et al., 1982; Gilmore et al., 1982), and signal peptidase (Evans et al., 1986) were isolated from the membrane and found to be required for the translocation process. Furthermore, in the wheat germ lysate, in which the requirement for SRP in protein translocation can be tested, SRP induced an arrest in the elongation of nascent secretory chains (Walter et al., 1981). This was true for all secretory proteins that were tested in this manner (Hortsch and Meyer, 1986).

In this report, I studied the translocation of pPM across mammalian ER membranes. Several unique features of this secretory protein made the study of its translocation an interesting task. First, and most interesting, is the fact that it is composed of only 70 amino acids, which is equal to the size of the postulated arrested peptides for preprolactin (Walter and Blobel, 1981) and prelight chain (Meyer et al., 1982). Thus, I suspected that SRP might influence the synthesis of pPM in a novel manner. Second, pPM has, in addition to the signal sequence, a very hydrophobic core that might play a special role in the translocation of the protein across the ER membrane. Finally, melittin is toxic and capable of interacting with and disrupting natural and artificial membranes (Mollay et al., 1976; Levin et al., 1982; Posch et al., 1983). It is not clear whether PM and pPM might display such properties to any extent.

The results presented here show that the translocation of pPM across ER membranes took place by a co-translational process and required both SRP and DP. Removal of SRP by salt extraction and/or inactivation of DP by treatment with NEM rendered the membranes translocation-incompetent. The small size of pPM resulted in two distinguishing features in its translocation across ER membranes in vitro, which are discussed below.

First, the nascent pPM chain seemed to be recognized by SRP at or very close to chain termination when the signal sequence had emerged from the large ribosomal subunit. In this unique situation, SRP might influence pPM chain finishing and release from the ribosome with no apparent effect on chain elongation. The results presented here indicate that this might indeed be the case. In a tightly synchronized translation assay designed to favor the formation of monosomes, SRP had no apparent influence on pPM chain elongation. However, in the presence of SRP, a transient accumulation of pPM as peptidyl tRNA was detected. Only under conditions where continued chain synthesis on polysomes was taking place was the influence of SRP on pPM chain synthesis detected as a slowdown in rate of chain synthesis. This effect was specific and dependent on the concentration of SRP. In contrast, the synthesis of prelight chain was permanently blocked by SRP and that of globin was not affected by SRP, as expected for typical secretory and cytoplasmic proteins, respectively. The fact that free SRP might be inactivated (Walter and Blobel, 1980) during incubation may explain why its arrest capacity diminishes with time in the case of pPM, but not prelight chain where SRP probably remains bound in the arrested complex.

Second, the translocation of pPM across ER membranes was more efficient in the reticulocyte lysate than the wheat germ lysate, in contrast to prelight chain, whose translocation was efficient in both translation systems. This difference might be due to the small size of pPM, which might reflect an intrinsic difference in the targeting processes of the two translation systems. One can envision that the inefficient translocation of pPM in the wheat germ translation system is due to the transient nature of its chain arrest by SRP, which might provide only a narrow time window for targeting the arrested nascent chain to the membrane. The permanent arrest of chain elongation by SRP provides a much broader time window for the targeting process in the case of prelight chain. In the reticulocyte lysate, exogenous SRP is not needed for translocation (Meyer et al., 1982) and neither exogenous or endogenous SRP produce arrest in nascent chain synthesis, leading to the conclusion that the targeting process might indeed be different from that in the wheat germ lysate. Since the translocation of pPM is expected to be efficient in vivo, it becomes very interesting to look for components of reticulocyte lysate that might play a role in the targeting process, resulting in a more efficient translocation. Short polypeptide hormones are invariably synthesized as part of longer precursors (Chretien et al., 1984), probably to guarantee efficient co-translational translocation across the ER membrane before the short polypeptide hormone is generated.

Results that led to different conclusions from those discussed here have recently been reported (Zimmermann and Mollay, 1986). The main discrepancy lies in the role SRP has in the synthesis and translocation of pPM. Zimmermann and Mollay suggested that the translocation of pPM is independent of SRP and proposed the requirement of an undefined component of the ER membrane for the translocation process. The discrepancy can be explained by the fact that the authors had not tested the influence of SRP on the synthesis and translocation of pPM directly. They relied on results obtained from membranes treated to deplete or inactivate SRP. However, such treated membranes may retain some processing activity, the extent of which varies depending on the particular secretory protein tested, and on the type of treatment, the batch of membranes, and the quantity used. In addition,
displaying only the translation products that are extracted in the aqueous phase, which contains PM and only residual amounts of pPM, does not allow one to get a true picture of the extent of processing. Indeed, in the experiment where the extent of processing by intact membranes was quantitated, it was found to be generally low and more efficient in reticulocyte lysate compared with wheat germ lysate, in agreement with the results presented here. Furthermore, when pPM was fused to a cytoplasmic protein, the translocation of the fusion protein across ER membranes was dependent on both SRP and DP (R. Zimmermann, personal communication). This observation supports the results presented here on the recognition of pPM by SRP; the transient nature of the arrest by SRP being a result of the small size of pPM.

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