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The affinity of signal recognition particle for presecretory proteins is dependent on nascent chain length

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We have developed an assay in which incomplete preprolactin chains of varying lengths are targeted to the endoplasmic reticulum (ER) membrane in an elongation independent manner. The reaction had the same molecular requirements as nascent chain translocation across the ER membrane, namely, it was signal recognition particle (SRP) dependent, and required the nascent chain to be present as peptidyl tRNA (i.e. most likely ribosome associated) and to have its signal sequence exposed outside the ribosome. We found that the efficiency of the targeting reaction dropped dramatically as the chains grew longer than 140 amino acids in length, which probably reflected a decrease in affinity of the nascent chain-ribosome complex for SRP. Thus at physiological SRP concentrations (10 nM) there appears a sharp cut-off point in the ability of these chains to be targeted, while at high SRP concentrations (270 nM) all chains could be targeted. In kinetic experiments, high concentrations of SRP were found to change the time in elongation after which translocation of the nascent polypeptide could no longer occur.

Key words: protein targeting/endoplasmic reticulum/signal recognition particle/signal sequence

Introduction

A detailed analysis of the effects of signal recognition particle (SRP) on secretory protein synthesis and translocation (Walter *et al.*, 1981; Walter and Blobel, 1981a,b) led to a model for its role in the targeting of secretory proteins to the endoplasmic reticulum (ER) membrane (for review see Walter *et al.*, 1984). According to this model SRP serves as an adaptor betwen the cytoplasmic translation machinery and the membrane bound translocation machinery. In particular, SRP was found to bind with high affinity to ribosomes synthesizing the nascent secretory protein preprolactin (Walter *et al.*, 1981) and to arrest the synthesis of this protein at a specific point, leading to the synthesis of an arrested fragment (AF) (Walter and Blobel, 1981b). This elongation arrest was found to be released upon the interaction of SRP with a component of the ER membrane (Walter and Blobel, 1981b) termed SRP receptor (Gilmore *et al.*, 1982) or docking protein (Meyer *et al.* 1982). The interaction of SRP with its receptor was found to lead to the release of SRP from the nascent preprolactin-ribosome complex (Gilmore and Blobel, 1983), and to the translocation of preprolactin across the ER membrane by a mechanism that is unknown.

In this paper, we describe a targeting assay which does not depend on the continued synthesis of the preprolactin chain. A similar assay was first developed by Connolly and Gilmore (1986), who measured the molecular requirements of targeting of short preprolactin chains created by either SRP mediated elongation arrest or truncation of the mRNA. We have extended their assay to use as a targeting substrate a steady state distribution of nascent preprolactin chains that have been stabilized with the elongation inhibitor cycloheximide. This has allowed us to determine directly the interdependence between the length of the preprolactin nascent chain and its affinity for SRP.

Results

The ribosome stalls at several distinct points during the synthesis of preprolactin

In order to address questions regarding the requirements on the nascent chain for SRP recognition and targeting to the ER membrane, we took advantage of the fact that protein elongation is not linear with time, but rather pauses at discrete positions. We programmed a wheat germ cell free translation system with full length synthetic preprolactin mRNA, and after allowing synthesis to attain steady state, we enriched for nascent chains with the positively charged detergent cetyltrimethylammonium bromide (CTABr), which precipitates these chains by virtue of their covalent linkage to tRNA (Hobden and Cundliffe, 1978).

As can be seen in Figure 1, lane e, there appears during steady state a set of discrete bands comprising a spectrum of nascent chain lengths. We have determined the approximate lengths of these chains by comparing them with chains of identical sequence and known length generated by translation of truncated synthetic preprolactin mRNAs (data not shown). We depict the migration of a series of such chains alongside each of the figures; the length of each chain in number of amino acids is indicated in Figure 1. We found that the nascent chains attained by this procedure range from ~55 amino acids in length to what appears to be full length but non-terminated preprolactin. This same set of chains appears when the translation system is programmed with the natural mRNA coding for preprolactin (compare Figure 1, lanes e and c) obtained from bovine pituitary glands.

When the translation system is synchronized by the addition of initiation inhibitors after a short period of time, this spectrum of bands disappears and is replaced primarily by full length preprolactin (Figure 1, lane a). At this stage in translation, CTABr fails to precipitate any of these chains (data not shown). Thus the nascent chains shown in lanes c and f represent transient intermediates in the synthesis of preprolactin.

In the presence of SRP, we found that the higher mol. wt nascent chains disappeared (Figure 1, lanes d and f). A

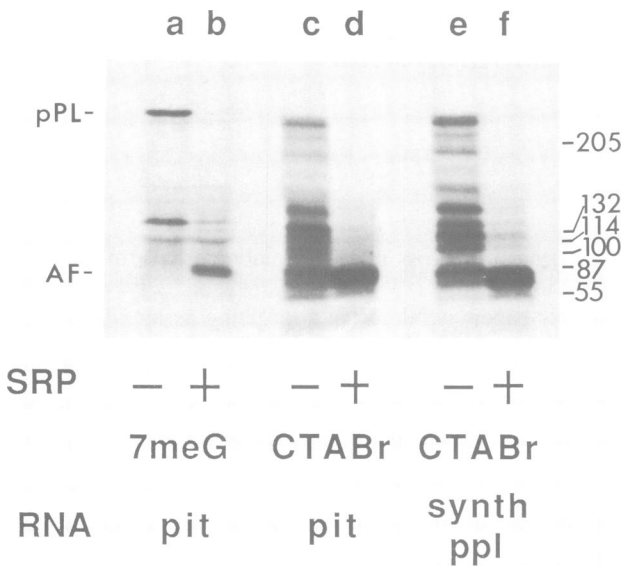

Fig. 1. There are several distinct pause sites in the synthesis of preprolactin. Pituitary RNA was used to program a wheat germ translation reaction (Erickson and Blobel, 1983) either in the absence (**lane a**) or presence (**lane b**) of 50 nM SRP. After 1 min, a mixture of 7MeG and edeine was added to a final concentration of 4 mM and 10 μM, respectively, to block further initiation. After 40 min, the translation was stopped by the addition of TCA to 10%. Pituitary RNA (**lanes c** and **d**) or full length synthetic preprolactin mRNA (transcribed from pSPBP4 with SP6 polymerase, **lanes e** and **f**) was used to program translation reactions either in the absence (lanes c and e) or presence (lanes d and f) of 50 nM SRP. After 40 min the translation was stopped by the addition of cycloheximide to 1 mM and precipitated with CTABr according to Gilmore and Blobel (1985). Plasmid pSPBP4 was cut with the following restriction endonucleases, transcribed with SP6 polymerase, and translated in a wheat germ extract to yield chains of the following sizes:

Enzyme	No. of amino acids	Mol. wt
*Fok*I	55	5818
*Pvu*II	87	9539
*Mbo*II	100	11964
*Hin*FI	114	12581
*Rsa*I	132	14689
*Fsp*I	205	22898

The migration of these translation products is indicated alongside this and other figures on the right hand side. The bands that correspond to preprolactin (pPL, 229 amino acids) and the arrested fragment (AF, 70 amino acids) are indicated along the left side.

predominant nascent chain remained, ~70 amino acids in length (referred to as the arrested fragment, or AF). We conclude that SRP has interacted with this chain and has blocked subsequent elongation. Interestingly, a nascent chain of similar mol. wt appears in the absence of SRP (lanes c and e), and as a novel translation product in a synchronized translation performed in the presence of SRP (lane b). Thus elongation arrest appears to occur at an SRP independent pause site in the synthesis of preprolactin.

Targeting of nascent chains to the microsomal membrane and translocation across the membrane have the same molecular requirements

We first asked whether such a steady state distribution of chains could be targeted to the ER membrane, and if so, whether this targeting reaction had the same requirements as had previously been determined for translocation across the ER membrane. If so, the targeting reaction should be SRP dependent, ribosome dependent, and signal sequence dependent.

Preprolactin mRNA was translated for a brief period of time to allow the accumulation of nascent chains. Further protein synthesis was inhibited by the addition of cycloheximide. An aliquot was immediately precipitated with TCA (Figure 2A, lane a), and another aliquot was precipitated with CTABr to determine the distribution of nascent chains (Figure 2A, lane b). Microsomal membranes were then added in the presence or absence of SRP. Sedimentation yielded a membrane bound (pellet) and an unbound (supernatant) fraction. The distribution of these chains was determined after TCA precipitation.

We found that SRP stimulated the binding of incomplete chains to the membrane (compare lanes d and f). This reaction appeared dependent on the continued association of these chains with the ribosome. Major bands that failed to be targeted and thus were recovered almost predominantly in the supernatant fraction corresponded to chains that were not (or were only very inefficiently) precipitated by CTABr [compare total (lane a) and CTABr precipitable (lane b) with lane e]. Thus the targeting reaction is both SRP dependent and, most likely, also depends on the continued association of the nascent chain with the ribosome, since only those chains still attached to tRNA were targeted.

In Figure 2B, we determined the targeting efficiency of preprolactin chains of defined lengths synthesized by programming the translation with incomplete synthetic preprolactin mRNAs (see legend to Figure 1). We found that chains generated from a *Pvu*II cut plasmid (87 amino acids in length and shorter) (lanes c and d) and chains generated from an *Rsa*I cut plasmid (132 amino acids in length and shorter) (lanes e and f) were very efficiently targeted to the membrane in the presence of SRP. In contrast, chains generated from a *Fok*I cut plasmid (55 amino acids in length and shorter) (lanes a and b) were not targeted by SRP (the percentage of chains pelleting with the membranes is comparable to the percentage that pellet with membranes in the absence of SRP; see for example Figure 2C, lanes b and c). The lengths of these chains are too short for the signal sequence to be fully exposed on the surface of the ribosome. Thus the targeting reaction is signal sequence dependent.

We compared the efficiency of targeting when SRP was present during elongation [as in the Connolly and Gilmore assay (1986)] to that when SRP was added subsequent to elongation and the addition of cycloheximide. We found the percentage of chains associated with the membrane to be approximately equal (compare Figure 2C, lanes e and j). Thus the assay qualitatively and quantitatively resembles previously developed targeting (Connolly and Gilmore, 1986) and translocation assays (Ainger and Meyer, 1986).

Gilmore and Blobel (1985) found that puromycin can release SRP-arrested chains from the ribosome, indicating that in the presence of SRP the A site is at least occasionally empty. This finding led to the suggestion that SRP blocks elongation by sitting partially in the A site. However, since in the presence of cycloheximide the peptidyl tRNA remains in the A site (Vasquez, 1979), the finding that cycloheximide stabilized chains can be efficiently targeted indicates that the A site need not be empty for SRP to promote targeting.

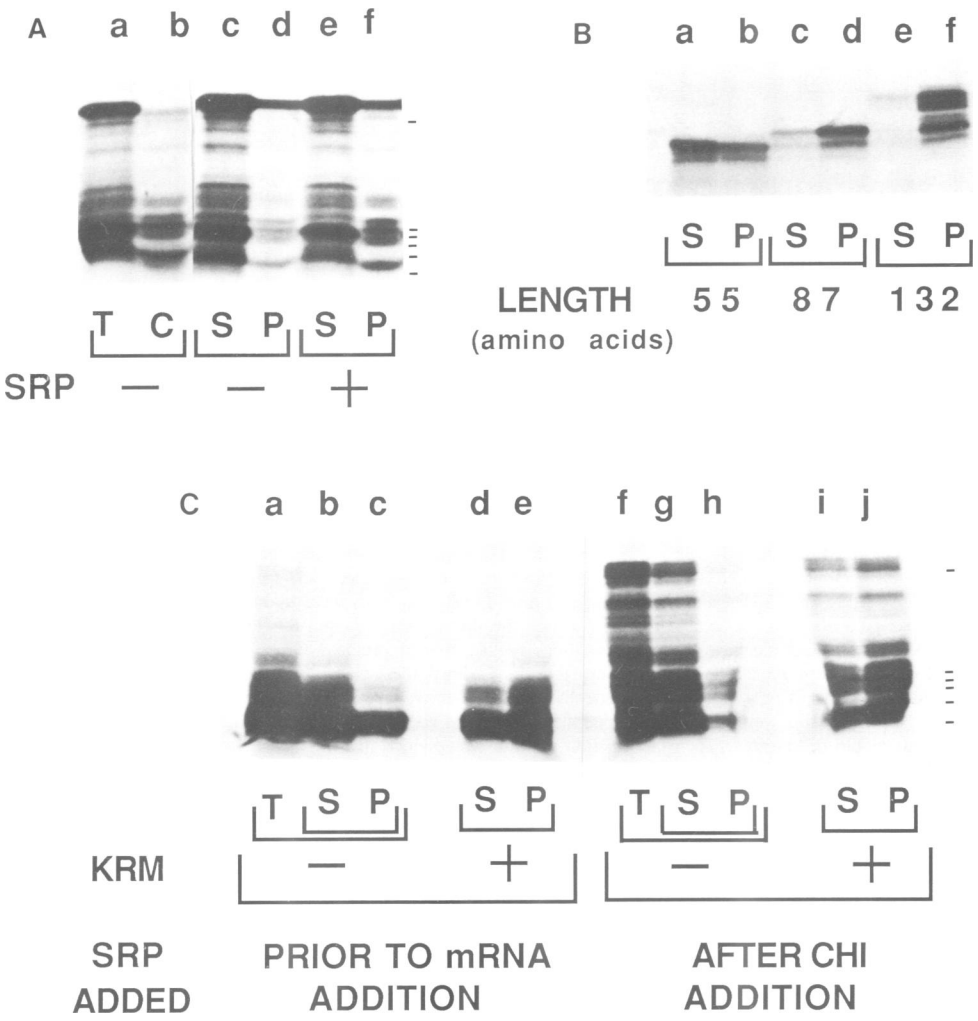

Fig. 2. Targeting of incomplete chains after inhibition of elongation by cycloheximide. (**A**) Synthetic preprolactin mRNA was used to program a 100 μl translation reaction. Translation was for 14 min at 22°C, after which cycloheximide was added to block further elongation. The reaction was then divided into 15 μl aliquots. One aliquot was TCA precipitated ('T', **lane a**) and one was CTABr precipitated ('C', **lane b**). One aliquot (**lanes e** and **f**) received SRP (10 nM final) and one (**lanes c** and **d**) the identical buffer without SRP. After a 10 min incubation at 22°C, four equivalents of SRP-depleted microsomal membranes were added to the latter two samples, which were then incubated for a further 5 min at 22°C. The samples were then layered onto 0.5 M sucrose cushions (see Materials and methods) in a polyallomer airfuge tube, and sedimented to obtain a supernatant ('S', unbound, lanes c and e) and a pellet ('P', membrane bound, lanes d and f) fraction. (**B**) Targeting of truncated preprolactin chains to microsomal membranes. *Fok*I (**lanes a** and **b**), *Pvu*II (**lanes c** and **d**) and *Rsa*I (**lanes e** and **f**) cut plasmids pSPBP4 (see legend to Figure 1) were transcribed and translated as described. Then SRP (10 nM) and membranes (four equivalents per 15 μl) were added and the samples divided into supernatant (lanes a, c and e) and pellet (lanes b, d and f) fractions as in (A) which were then CTABr precipitated. Because the translation reactions in this experiment are not synchronized, several ribosomes can traverse each message. Therefore, additional bands resulting from a 'piling up' of ribosomes at the end of the truncated message will appear, as well as pause sites such as those seen on the full length message. This results in the rather complex pattern of bands seen. (**C**) Comparison of targeting efficiency when SRP is present during synthesis and when it is added after cycloheximide addition. Synthetic preprolactin mRNA was used to program a translation reaction, which was incubated for 14 min at 22°C in the presence (**lanes a–e**) or absence (**lanes f–j**) of 10 nM SRP. After cycloheximide addition, SRP or SRP-compensating buffer was added so that both samples contained SRP at the same final concentration. After a 10 min incubation at 22°C, the samples were divided into 15 μl aliquots. One aliquot was CTABr precipitated, one aliquot received four equivalents of SRP-depleted membranes, and one received membrane compensation buffer. Targeting was assayed as described in (A), and supernatant and pellet fractions were CTABr precipitated. Lanes a and f: CTABr precipitate; lanes b and g: supernatant (−membranes); lanes c and h: pellet (−membranes); lanes d and i: supernatant (+membranes); lanes e and j: pellet (+membranes).

The ability of nascent chains to be targeted is dependent on the length of the nascent chain

We have previously shown that if elongation of preprolactin was allowed to proceed beyond a critical cut-off point, then translocation of preprolactin across the microsomal membrane was no longer observed (Siegel and Walter, 1985). We were therefore surprised to find (Figure 2C, lane j) that virtually full length preprolactin was efficiently targeted to the microsomal membrane.

One explanation for this result is that these longer chains are not themselves targeted to the membrane, but rather are brought to the membrane along polysomes also containing shorter chains. Figure 3 shows that this is indeed the case.

Subsequent to chain elongation, micrococcal nuclease was added (at levels 5-fold higher than those standardly used to destroy synthesis of endogenous wheat germ mRNAs) in order to digest the mRNA between the ribosomes and yield chains that should be targeted to the membrane independently

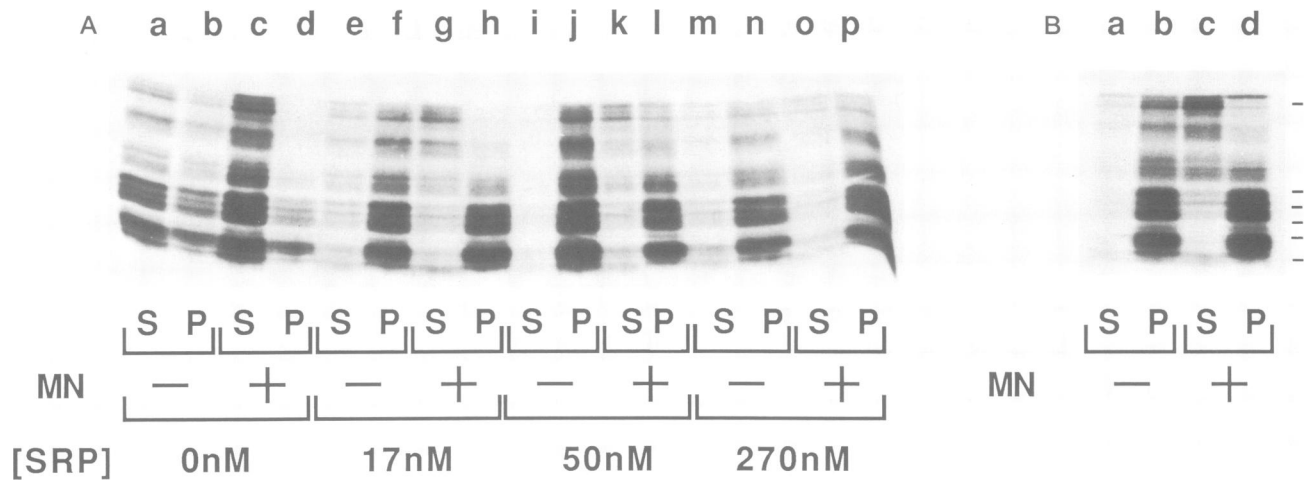

Fig. 3. (**A**) Effect of microsomal membranes on targeting of preprolactin nascent chains at various SRP concentrations. Preprolactin nascent chains were allowed to accumulate over a 14 min period. The sample was then divided into two aliquots, and one was digested with micrococcal nuclease. The samples were further divided into 15 μl aliquots. SRP was added to various concentrations and incubated for 10 min. Then four equivalents of SRP-depleted microsomal membranes were added and incubated for 5 min. Finally, samples were divided into supernatant and pellet fractions and CTABr precipitated. Note that even after micrococcal nuclease digestion the shortest chain is still efficiently targeted, in contrast to Figure 2B, lanes a and b. Mapping of micrococcal nuclease cleavage sites on polysomes synthesizing preprolactin have indicated that the amount of nuclease used in this experiment is insufficient to cut between the densely piled-up ribosomes with nascent chains 70 amino acids in length and shorter (S.Wolin and P.Walter, unpublished). Thus this chain is not targeted independently even after micrococcal nuclease digestion. (**B**) Effect of micrococcal nuclease on already targeted chains. Nascent chains were allowed to accumulate, SRP was added to 10 nM, and membranes (0.2 eq/μl) were added under conditions such that all chains would be targeted. Then the reaction mixture was divided in two, and micrococcal nuclease was added to one sample (lanes c and d) as in (A). After addition of EGTA, samples were divided into a supernatant (**lanes a** and **c**) and a pellet (**lanes b** and **d**) fraction and CTABr precipitated.

of one another. We then compared the distribution of chains targeted to the membrane with and without micrococcal nuclease digestion.

We found that at physiological concentrations of SRP (Figure 3A, lanes a−h), the addition of micrococcal nuclease had no effect on the efficiency of targeting of chains up to ~140 amino acids in length, but that as chains became longer than 140 amino acids, micrococcal nuclease digestion diminished the ability of SRP to promote targeting. We conclude that in the absence of micrococcal nuclease, these longer chains had been brought to the membrane along polysomes also containing shorter chains.

Furthermore, when micrococcal nuclease was added subsequent to incubation of the nascent chain complexes with microsomal membranes (Figure 3B), we found that the longer chains were released from the membrane and were recovered in the supernatant fraction (Figure 3, lane c). We conclude from this result that, even though these chains had been brought into close proximity with the microsomal membrane, that this proximity was not sufficient for the establishment of a stable ribosome−membrane junction.

The length dependence of nascent chain targeting results from a decrease in affinity of nascent chains for SRP

Most interestingly, we noted that as we increased the SRP concentration (Figure 3A, lanes i−l show targeting reactions containing 50 nM SRP, lanes m−p show reactions containing 270 nM SRP), the longer chains could also be targeted to the membrane after micrococcal nuclease digestion. The targeting efficiency was not noticeably affected by the length of time that the cycloheximide-arrested chains were incubated prior to SRP addition, or to freeze/thawing of the cycloheximide-arrested chains (data not shown). These results indicate that longer nascent chains fail to be targeted at 17 nM SRP because the affinity of SRP for the nascent chain−ribosome complex decreases as the nascent chain increases in length.

Previously, a time point in elongation was defined beyond which translocation of preprolactin could no longer occur (Siegel and Walter, 1985). If this kinetically defined cut-off point results primarily from a decrease in affinity of the nascent chain−ribosome complex for SRP, then it should be possible to shift the point in time at which it occurs by changing the SRP concentration. To test this hypothesis, we performed the following experiment.

Synthesis of preprolactin was initiated by the addition of full length synthetic preprolactin mRNA. After 2 min, further initiation was blocked by the addition of the initiation inhibitors 7-methyl-guanosine-5′-monophosphate (7MeG) and edeine. At various time points, the translation reaction was added to mixtures of SRP and SRP-depleted microsomal membranes (KRMs), elongation was allowed to come to completion, and the amount of translocation assayed as previously described (Siegel and Walter, 1985). The amount of SRP was varied to give final concentrations of 10, 27 or 270 nM. To measure the rate of preprolactin synthesis, identical aliquots of the translation reaction were added directly to ice cold trichloroacetic acid (TCA). The results of this experiment are depicted in Figure 4.

When SRP was added together with microsomal membranes, the amount of translocation decreased with time, dropping to half-maximal at ~3.8 min when SRP was present at 10 or 27 nM. However, when SRP was added to 270 nM, translocation still occurred very efficiently at 4 min of synthesis, and dropped to half-maximal at 4.9 min. Thus the increased level of SRP indeed shifts the position of the kinetically defined cut-off point.

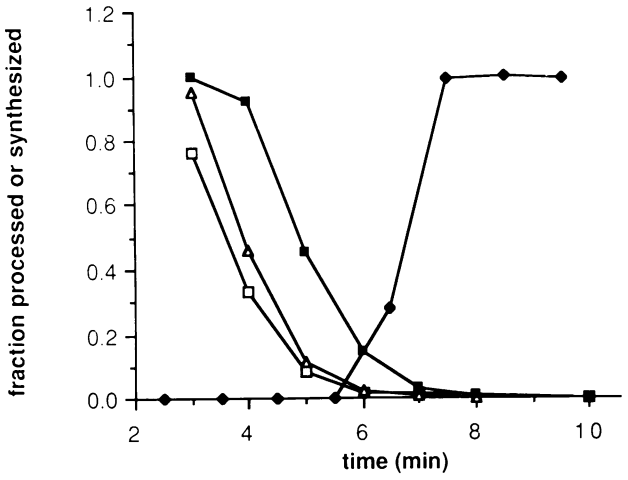

Fig. 4. Effect of SRP concentration on kinetic parameters of translocation. A wheat germ translation extract was prewarmed for 2 min at 26°C prior to the addition of full length synthetic preprolactin mRNA to initiate protein synthesis. After 2 min, initiation inhibitors were added. At the time points indicated, 5 μl of the translation was added to a tube containing a mixture of SRP and SRP-depleted membranes (KRMs) to give a final SRP concentration of 10 (□), 27 (△) or 270 nM (■), and a final membrane concentration of 1 eq per 10 μl. Synthesis was allowed to proceed for a total of 30 min. Alternatively, 5 μl of translation was added directly to ice-cold TCA (◆) to measure the rate of preprolactin synthesis. Samples were analyzed by SDS–PAGE. The amount of preprolactin synthesized was determined by densitometry of the film after autoradiography (◆); similarly, the fraction of processed preprolactin was determined from the amounts of preprolactin and translocated prolactin as previously described (Siegel and Walter, 1985).

Discussion

We have taken advantage of ribosome pausing during translation to create an assay for targeting of nascent presecretory proteins to microsomal membranes *in vitro* that does not depend on the continued elongation of the targeted substrate. With such an assay in hand, we have been able to assess directly the substrate requirements for this process.

In particular, we conclude that the signal sequence bearing chains needed to be associated with the ribosome for targeting to occur. Chains that were not precipitable with the positively charged detergent CTABr were found predominantly in the unbound fraction (Figure 2A). This finding is consistent with all published examples of SRP-dependent 'post-translational' translocation of proteins in systems using mammalian microsomal membranes (Mueckler and Lodish, 1986; Perara *et al.*, 1986; Caulfield *et al.*, 1986). In each case the translocation event seemed to require continued association of the secretory protein chain with the ribosome. Our results suggest that the ribosome is strictly required for the targeting step of the translocation reaction.

The requirement for ribosome coupling seems to be intrinsic to the targeting machinery rather than to the substrate. In particular, the truly post-translational translocation of the yeast protein prepro-α-factor, which, in the homologous yeast system can occur after it is released from the ribosome and does not seem to require an SRP-like component (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986), did not occur in a wheat germ system supplemented with mammalian microsomes (Garcia and Walter, 1988). Instead, any translocation of full-length prepro-α-factor that did occur was dependent on SRP and required that the chain remain ribosome associated. Thus SRP, with its micromolar affinity for all ribosomes (Walter *et al.*, 1981), may have evolved to mediate exclusively a ribosome coupled process.

Most importantly, we found that the efficiency of targeting depends on the length of the nascent chain. Preprolactin chains of only 55 amino acids in length (Figure 2B) were not targeted, consistent with the idea that the signal sequence must be fully exposed on the surface of the ribosome for SRP to interact with it. Furthermore, while chains ranging from ~70 to ~140 amino acids in length were efficiently targeted at 10–17nM SRP (an approximately physiological concentration) (Figure 3), chains longer than this length were not targeted at this SRP concentration. However, longer chains could be targeted to the membrane efficiently when the SRP concentration was raised further to 270 nM.

At physiological SRP concentrations the longer nascent chains could also be brought to the membrane, but only as part of polysomes also synthesizing shorter chains. However, these chains did not become stably bound to the microsomal membrane. Thus when micrococcal nuclease was added to the translation extract after targeting, the ribosomes carrying these longer chains were released from the membrane (Figure 3B). We conclude that proximity to the membrane is not sufficient to mediate the formation of a stable ribosome–membrane junction, even though the concentration of these ribosomes in the vicinity of the membrane is vastly increased.

The dependence of the targeting reaction on the nascent chain length reflects a decrease in the apparent affinity of the longer nascent chain–ribosome complexes for SRP; as the SRP concentration was increased, the longer chains also became membrane bound (Figure 3A). Consistent with this finding, high concentrations of SRP can delay the kinetically defined cut-off point in elongation beyond which translocation is no longer observed (Figure 4). These results are consistent with the finding of Wiedmann *et al.* (1987) that chains longer than AF could still be cross-linked to the 54 kd polypeptide of SRP, but that the cross-linking efficiency dropped off as the chain length increased. We suggest that the decrease in apparent affinity reflects a sequestering of the signal sequence within the growing preprolactin chain. We envisage that the nascent chain is in equilibrium between having an exposed signal sequence and a sequestered one, and that the high concentration of SRP drives the equilibrium towards the exposed state.

Since the lengths at which nascent preprolactin chains can still be targeted depends on the SRP concentration, we can reconcile the apparent conflict between our earlier results (Siegel and Walter, 1985) and those of Ainger and Meyer (1986) which differ in the position of this cut-off point. Our work had been carried out at physiological levels of SRP (~15nM), while that of Ainger and Meyer (1986) had been carried out at ~10-fold higher concentrations. In the experiments shown in this paper, the $t_{1/2}$ for preprolactin synthesis was about 7 min. At physiological SRP concentrations, translocation was reduced to 50% at 3.8 min, corresponding to about 54% of the length of preprolactin polymerized, which agrees well with our earlier report of 48%. At 270 nM SRP, translocation was reduced 50% at 4.9 min, or 70%

of preprolactin polymerized, which agrees well with the Ainger and Meyer (1986) report of 66%.

While the targeting assay (Figure 3) and the translocation assay (Figure 4) agree well that increased SRP concentrations extend the window in nascent chain lengths in which targeting and translocation can occur, the two assays appear to differ in that almost full-length preprolactin chains could be targeted at 270 nM SRP, yet translocation was only observed until chains were ~70% polymerized. We attribute this difference to the experimental conditions under which the two respective assays are performed. In the targeting assay the nascent chain ribosome complexes are stabilized with cycloheximide and incubated with SRP, hence allowing considerable time for the signal sequence to become accessible for an interaction with SRP. In contrast, in the kinetic translocation assays the time window in which SRP can interact is comparatively short since protein synthesis is kept ongoing. In fact, the further nascent chains are polymerized, the shorter this time window becomes. For example, a 70% polymerized preprolactin chain becomes completed and terminated after only 2.3 min. Furthermore, because the relative accessibility of the signal sequence is expected to vary also depending on the nature of the protein being synthesized, the position of the cut-off point defining its maximal length of a translocation competent nascent chain at a given SRP concentration should also vary. In this regard it is interesting to note that Ainger and Meyer (1986) showed that nascent immunoglobulin light chains of virtually any length can be translocated.

According to our model, recognition of nascent presecretory proteins will most effectively occur while the nascent chains are short. Since SRP is present at all times *in vivo*, this will naturally be the case. Once SRP is bound, protein synthesis may be slowed down due to the elongation arrest activity of SRP, but even if elongation continues, the nascent chain will remain an effective translocation substrate as long as SRP remains bound and keeps the signal sequence on the surface of the folding nascent chain. How long SRP can keep a nascent preprotein in this state will depend on the effective on and off rates of SRP (as determined by its affinity to the signal sequence-ribosome), and not necessarily on the elongation rate. However, as SRP dissociates from the signal sequence, the nascent chain may now fold such that the signal sequence becomes largely inaccessible (more likely so, if the nascent chain exposed outside the ribosome is long). Recognition of the signal sequence in this state requires unphysiologically high SRP concentrations or long incubation times to shift the equilibrium back to a translocation competent state and thus appears an unlikely pathway to be utilized *in vivo*.

It should be clear from the caviats discussed above that the measured parameters describe a particular experimental situation, yet are not easily extrapolated to physiological conditions. Since affinities, local concentrations of components, and the rates of the individual reactions *in vivo* are not known, and, in effect, may vary under different physiological conditions, our model can presently describe only qualitatively the constraints on the signal recognition and targeting reaction. However, the availability of assays for targeting that are independent of elongation, and in which the targeted complex is relatively stable, frees us of the strict ionic and complex factor requirements of a cotranslational assay. Thus it should now be possible to fully fractionate the system and determine if factors other than SRP promote or regulate the translocation event at this level.

Materials and methods

Materials

Micrococcal nuclease from *Staphylococcus aureus* was purchased from Boehringer Mannheim. SP6 polymerase was purchased from Promega. Restriction endonucleases were purchased from New England Biolabs. Other materials were purchased as described previously (Siegel and Walter, 1985).

Purification of SRP and microsomal membranes

Purification was as described previously (Siegel and Walter, 1985).

In vitro transcription

Plasmid pSPBP4, containing coding sequences for bovine preprolactin downstream of an SP6 promoter, was linearized with *Eco*RI and transcribed with SP6 polymerase as previously described (Siegel and Walter, 1988) to generate full length synthetic preprolactin mRNA. Other restriction endonucleases were used to linearize the plasmid at various positions within the coding sequence to generate incomplete (or 'truncated') synthetic preprolactin mRNAs.

In vitro translations

Pituitary RNA, containing predominantly the mRNAs encoding the secreted proteins prolactin and growth hormone, or full length synthetic preprolactin mRNA, was used to program a wheat germ cell free translation system (Erickson and Blobel, 1983). Generation of a distribution of preprolactin nascent chains for use in targeting assays was achieved by translating full length synthetic mRNA in the wheat germ cell free translation system for 14 min at 22°C. Truncated nascent chains were allowed to accumulate over a 30 min synthesis period at 26°C. After the chosen period of synthesis, cycloheximide was added to 1 mM to block further elongation and to encourage the nascent chains to remain bound to the ribosome.

Identification of nascent preprolactin chains

Translation reactions were solubilized in 250 μl 2% CTABr and nascent chains were precipitated using 50 μg calf liver tRNA according to Gilmore and Blobel (1985).

Targeting assays

All targeting assays were performed under physiological salt conditions. 15 μl of a wheat germ translation reaction (see above) was mixed with 4 equivalents (eq, as defined in Walter *et al.*, 1981) SRP-depleted microsomal membranes and incubated for 5 min at 22°C. This mixture was then layered on top of a 50 μl 0.5 M sucrose cushion containing 50 mM triethanolamine, pH 7.5, 140 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM dithiothreitol in a polyallomer airfuge tube. These tubes were spun for 3 min at 20 p.s.i. in a A-100/30 rotor at 4°C according to Connolly and Gilmore (1986). The supernatant, including the cushion, and the pellet were each solubilized in 2% CTABr and nascent chains were precipitated as above.

Micrococcal nuclease digestion of polysomes

After synthesis, calcium chloride was added to 1 mM and micrococcal nuclease was added to 10 U/ml and incubated for 10 min at 25°C. Digestions were stopped by the addition of EGTA to 2 mM.

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