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Functional dissection of the mammalian signal recognition particle

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FUNCTIONAL DISSECTION OF THE MAMMALIAN SIGNAL RECOGNITION PARTICLE

by

Vivian Siegel

DISSERTATION

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Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

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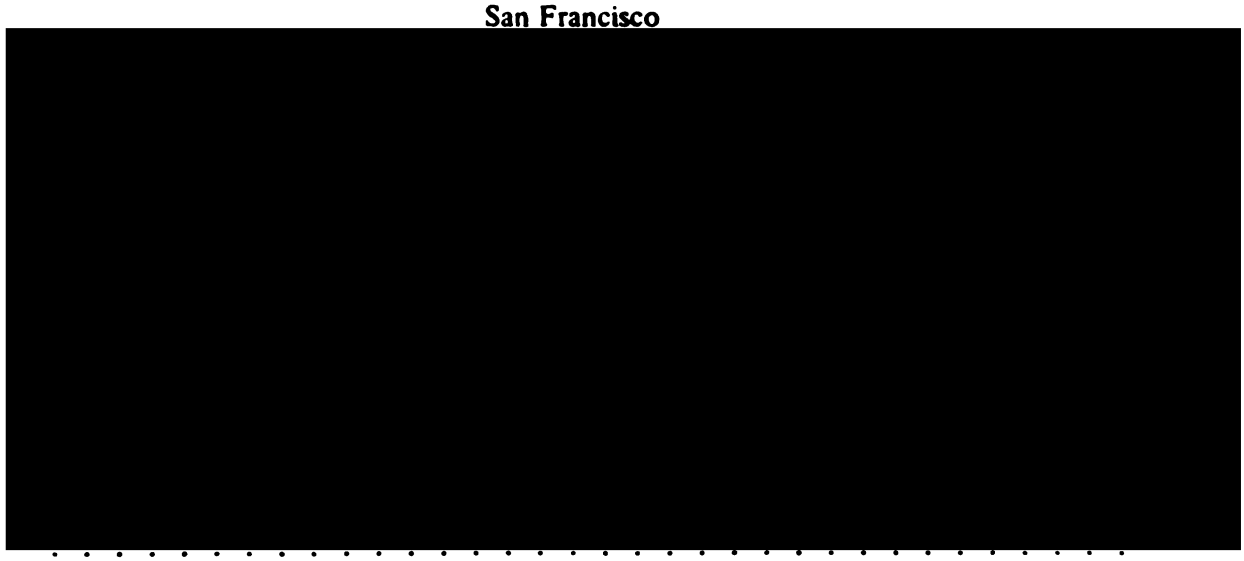

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Many people deserve thanks for their scientific and emotional support over the past years.

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FUNCTIONAL DISSECTION OF THE MAMMALIAN SIGNAL RECOGNITION PARTICLE

Vivian Siegel

Abstract

Signal recognition particle (SRP) has been shown to target nascent secretory, lysosomal, and membrane proteins to the endoplasmic reticulum (ER). It does this utilizing three activities which can be assayed in vitro: recognition of the signal peptide contained in these ER-bound proteins as it emerges from the ribosome, concomitant translational arrest of the nascent chain, and subsequent promotion of the translocation or insertion event itself.

SRP is a rod-shaped ribonucleoprotein composed of four proteins and a small RNA molecule. Exploiting the fact that SRP can be readily disassembled into protein and RNA components and then reconstituted, I have generated a series of "mutant" SRPs in which specific polypeptide or nucleic acid domains are either altered or removed. I have used these reconstitutes to assign each of the three activities to distinct domains on SRP and to map protein binding sites on the RNA molecule.

In our in vitro assay system, each activity requires a protein component. Thus, translational control is dependent on the 9/14 kd protein, signal recognition on the 54 kd protein, and protein translocation on the 68/72 kd protein. In contrast, the RNA seems to serve primarily as a template upon which the proteins are bound in specific geometry.

The structural map of SRP derived from footprint analysis places the signal recognition domain and the elongation arrest domain on opposite ends of the particle. This result is consistent with the model

that SRP concomitantly performs these two functions by physically bridging the distance between the nascent chain exit site and the aminoacyl tRNA site.

Using an assay for targeting to the ER membrane that is uncoupled from nascent chain elongation, I have re-examined the substrate requirements for the process. These studies show that the cotranslational nature of targeting reflects i) a requirement for the secretory chain to be ribosome-associated, and ii) a decrease in affinity of SRP for the nascent chain-ribosome complex as the chain becomes longer than 140 amino acids. These studies further demonstrate that SRP is required to initiate translocation of each nascent chain on a polysome.

Peter Walter 6/19/87

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 5

CHAPTER 6

CHAPTER 7

THE END

CHAPTER 1

INTRODUCTION:

SIGNAL RECOGNITION PARTICLE: A MEDIATOR IN THE RIBOSOME-COUPLED TARGETING OF PROTEINS TO THE ENDOPLASMIC RETICULUM MEMBRANE

Background

A characteristic feature of eukaryotic cells is the presence of intracellular membranes which define compartments differing in their morphology, contents, and the biochemical reactions that occur within them. It has been appreciated for some time that sorting mechanisms must exist that direct proteins to their proper compartments within the cell.

The first such sorting step distinguishes proteins that are synthesized in the cytoplasm from those that vectorially cross the membrane of the rough endoplasmic reticulum (and either remain there or progress to the Golgi apparatus, lysosomes, or plasma membrane, or are ultimately secreted). In cells synthesizing protein for secretion, the majority of ribosomes is attached to the membrane of the endoplasmic reticulum (Sabatini et al., 1966), yet no functional or structural difference between these and free ribosomes has been determined (Blobel & Sabatini, 1971). Experiments attempting to remove ribosomes from the ER membrane with EDTA, high salt, puromycin, or combinations thereof (Sabatini et al., 1966; Adelman et al., 1973) demonstrated that ribosomes were attached to microsomal membranes via two types of interactions: a direct one, presumably involving interaction of the large ribosomal subunit with the membrane proper, and an indirect one, in which the nascent polypeptide chain anchors the ribosome to the membrane. In the latter case, radioactively labelled nascent chains were found associated with the membrane, and the ribosome could only be released with a combination of puromycin and high salt. Together, these findings suggest that, *in vivo*, proteins cross the membrane of the endoplasmic reticulum cotranslationally.

The Signal Hypothesis

When mRNA for the secreted immunoglobulin light chain was translated *in vitro* in a heterologous system, the product differed in mobility from authentic light chain, having an apparent molecular weight of 1500 greater than the light chain (Milstein et al., 1972; Blobel & Dobberstein, 1975b). The existence of precursors to secretory proteins and the demonstrated association of nascent chains with the ER membrane led Blobel and Dobberstein in 1975 to propose the Signal Hypothesis (Blobel & Dobberstein, 1975). The essential feature of this hypothesis is that the initial event in translocation is the interaction of a discrete portion of the nascent chain, termed the signal sequence, with receptors in the membrane of the ER. As a result of this interaction, a functional ribosome-membrane junction is formed in continuity with a temporary proteinaceous pore through which the growing chain is passed. Upon completion of synthesis, the ribosomal subunits detach to enter the free cytoplasmic pool and the pore in the membrane disaggregates, with the newly synthesized protein localized exclusively within the cisternal space of the ER. Similarly, it has been proposed (Blobel, 1976-77) that transmembrane proteins, which span the membrane asymmetrically, are inserted into the membrane according to a modified version of the Signal Hypothesis, in which translocation does not proceed to completion but rather is arrested at a specific point by another sequence termed a stop-transfer sequence.

Studies in Cell-Free Systems Support the Signal Hypothesis and Show That Translocation is Co-Translational

The strongest evidence in favor of the Signal Hypothesis has come from studies in cell free systems in which the selective transfer of

proteins across membranes has been reconstituted from heterologous components. In 1975, Blobel and Dobberstein (1975b) demonstrated that IgG light chain, while synthesized as a precursor in a cell-free translation mix containing ribosomal subunits, was processed and vectorially translocated if canine pancreatic microsomal membranes were also present.

The processing/translocation reaction did not occur if membranes were heat inactivated, suggesting the involvement of a proteinaceous component in the sorting reaction, or were added after the precursor had been synthesized, demonstrating that the cotranslational nature of the reaction, which had been seen in vivo, was in fact obligatory in this system. Thus targeting to the endoplasmic reticulum differs from targeting to organelles such as mitochondria (Neupert & Schatz, 1981) and nuclei (Dingwall & Laskey, 1986), since in these cases proteins can be targeted after their synthesis is complete. It is not clear whether the cotranslational requirement reflects an incompatibility of folded domains to be translocated or simply to be recognized by the targeting machinery. It appears to be specific for higher eukaryotic systems, since microsomal membranes derived from the endoplasmic reticlulum of the yeast Saccharomyces cerevisiae can accept at least some proteins after precursor synthesis (Hansen et al., 1986, Rothblatt & Meyer, 1986, Waters & Blobel, 1986).

The existence of and requirement for N-terminal signal sequences has since been demonstrated for a large number of eukaryotic secretory proteins (Steiner et al., 1980) using in vitro translation/translocation assays. In bacteria, signal sequence mutations have been isolated in which the cell cannot translocate the mutant protein (Bassford &

Beckwith, 1979; Emr et al., 1980). Furthermore, it has been demonstrated in several systems that the signal sequence is sufficient to direct a marker protein out of the cell. Thus a signal sequence was sufficient to direct translocation of normally cytoplasmic globin, both in vitro (Lingappa et al., 1984) and in vivo (Simon et al., 1987).

It appears, then, that the signal sequence for targeting to the endoplasmic reticulum is the only signal absolutely required for secretion. Recent data from F. Weiland and J. Rothman support this: they show that an acyl-tripeptide N-acyl-Asn-[^{125}I]Tyr-Thr-NH_2, which becomes glycosylated in the RER, is efficiently and rapidly secreted from the cell. Because it is unlikely that such a small molecule could contain a signal, this result demonstrates that secretion can occur by bulk flow from the RER through the Golgi to the plasma membrane.

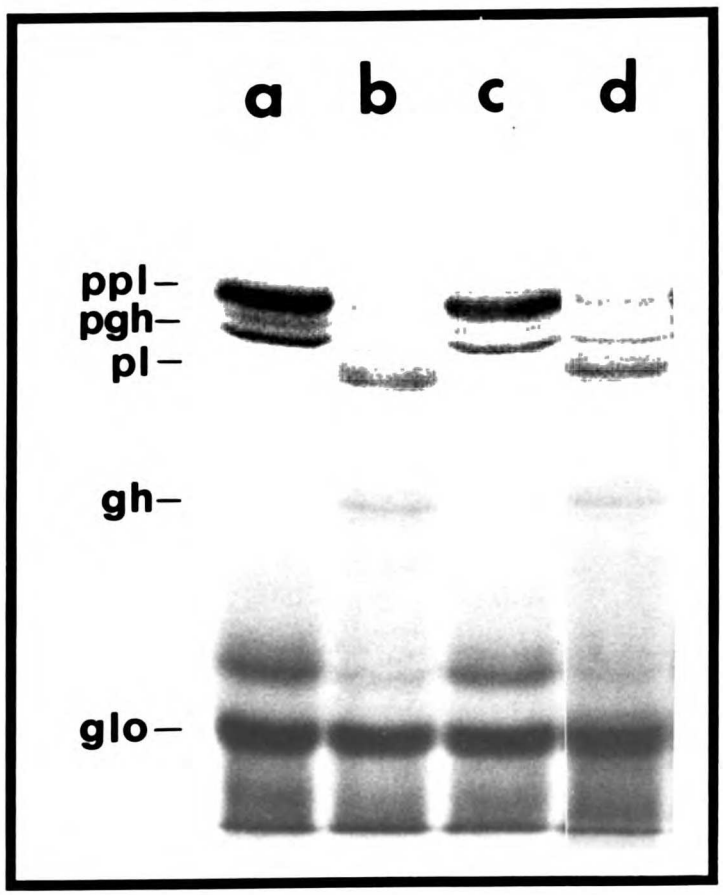
Purification of SRP

Prolactin is synthesized as a precursor in a cell free translation system derived from wheat germ (Fig. 1, lane a), and the addition of microsomal membranes results in translocation of the nascent chain and processing to mature prolactin (Fig. 1, lane b). If the microsomal membranes are salt-extracted prior to their addition to the wheat germ system, translocation no longer occurs (Fig. 1, lane c), i.e., salt extraction renders these membranes translocation "incompetent". Furthermore, the translocation competence of the membranes in wheat germ can be restored by adding back the microsomal salt wash to the assay. These observations led to a functional assay for the soluble (salt extractable) factor required for translocation in the wheat germ system, and hence ultimately to its purification (Walter & Blobel, 1980).

It would be useful at this point to reflect on what kind of compo-

Figure 1. Translation of pituitary and reticulocyte RNAs in the wheat germ cell free translation system.

A mixture of reticulocyte RNA, coding primarily for the cytoplasmic protein globin, and pituitary RNA, coding primarily for the secretory proteins prolactin and growth hormone, were used to program a cell free translation system derived from wheat germ, either in the absence or presence of microsomal membranes. Translation was for 60 min at 26°C. Lane a, no membranes; lane b, rough microsomal membranes; lane c, salt-extracted microsomal membranes; lane d, salt extracted microsomal membranes + SRP.


a
b
c
d
ppl–
pgh–
pl–
gh–
glo–

nent was expected to fall out of this purification scheme. The Signal Hypothesis proposed the existence of a signal receptor in the membrane of the endoplasmic reticulum, and the ability of microsomal membranes to promote translocation in vitro supported this view. Because the factor was extracted from the membrane with high salt, it was presumed to be a peripheral membrane component. It was probably a complete surprise, then, to find that this component was a cytoplasmic particle that interacted not only with the signal sequence and with the ER membrane, but also with the translational machinery of the cell, thus allowing and perhaps ensuring the cotranslational nature of the translocation event.

The key step in the purification of this factor resulted from the discovery that the activity of the microsomal salt wash was maintained and even restored if low concentrations of nonionic detergent were added. On the premise that detergent stabilization of the active component might be caused by amphiphilic interaction, hydrophobic chromatography was chosen as a means of exploiting this particular property of the activity. The activity bound to both ω-aminopentyl- and ω-aminohexyl-agarose columns. Elution of the activity by raising the salt and including detergent in the elution buffer allowed 100% recovery from the aminopentyl-agarose column. Six polypeptides (72 kd, 68 kd, 54 kd, 19 kd, 14kd, and 9 kd) were reproducibly obtained in this eluate.

Later steps in the purification included DEAE-Sepharose chromatography as a concentration step, and sucrose gradient sedimentation. It was eventually found that in addition to the six polypeptides that consistently co-purified with the activity (other attempts to fractionate these polypeptides from each other and the activity included gel filtration and hydroxylapatite chromatography), the factor, now called

Signal Recognition Particle, contained a molecule of RNA (see below).

Hydrophobic chromatography may be generally useful for the purification of ribonucleoproteins. When SRP RNA was the only RNA present in the material loaded onto the aminopentyl agarose column, SRP could be purified essentially to homogeneity using only this step. However, when the original procedure for extracting SRP was modified, so that 5S RNA was also present in the material loaded onto this column, it and its associated protein (ribosomal protein L5) copurified with SRP (Walter & Blobel, 1983c).

Structure of SRP

SRP is an 11S ribonucleoprotein consisting of six nonidentical polypeptide chains (72 kd, 68 kd, 54 kd, 19 kd, 14kd and 9 kd) and one molecule of RNA of about 300 nucleotides (Walter & Blobel, 1980,1982), all in approximately stoichiometric proportions. A variety of electron microscopic techniques have been applied to directly visualize the particle (Andrews et al., 1985). Images were obtained either after negative staining with or without fixation or after platinum shadowing. Also, unstained specimens were examined by darkfield EM. All images are internally consistent and show SRP as a rod-like structure of about 24 nm length and 5 nm width. From these dimensions we calculate a volume of 525 nm^3 assuming a perfect cylindrical shape, which is in good agreement with the volume calculated based on SRP's molecular composition (the combined molecular weight of RNA and protein components is 325 kd, resulting in an expected volume of 380 nm^3) if one takes into account that various crevices and indentations are reproducibly seen in the images and SRP therefore deviates significantly from perfect rod shape. More recently, electron spectroscopic imaging has been used to

localize the RNA within the particle. The RNA was found to be concentrated at the two ends of the particle, suggesting that the RNA spans the length of SRP, forming an extended stem structure which serves as a backbone for SRP assembly (Andrews et al., submitted).

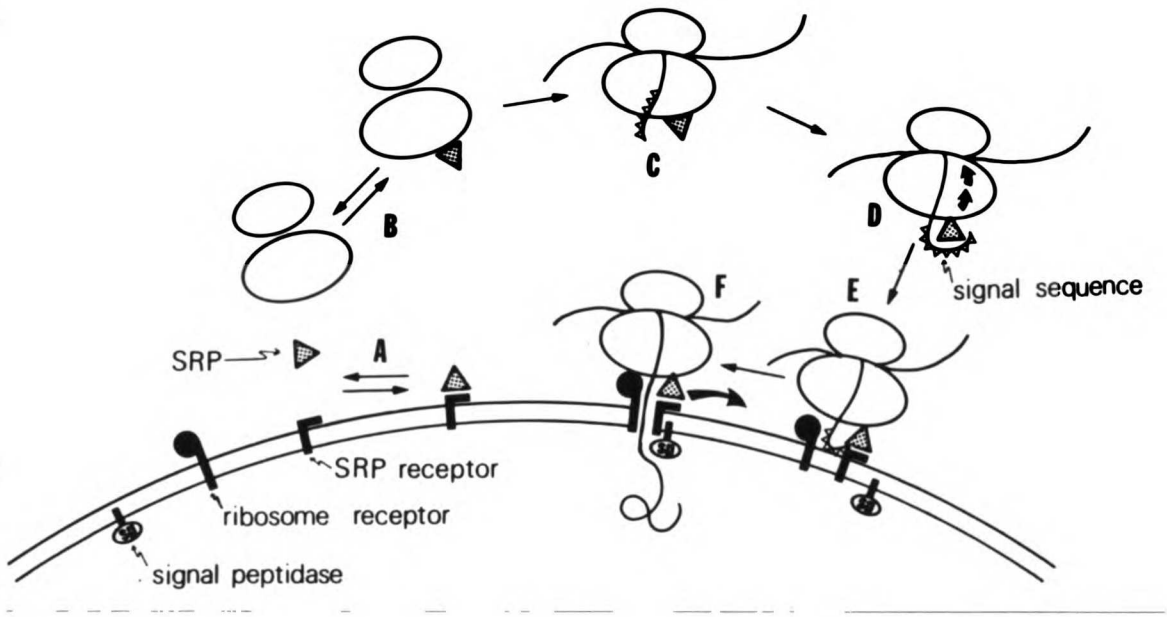
When the SRP RNA was sequenced, it was found to be identical to the abundant small cytoplasmic RNA known as 7SL RNA (Walter & Blobel, 1982). The sequence of this RNA is quite interesting since it is comprised of both unique and repetitive genomic sequences (Weiner, 1980; Li et al., 1982; Ullu et al., 1982; Ullu & Tschudi, 1984). Approximately 100 nucleotides at its 5' end and 50 nucleotides at its 3' end are about 80% homologous to the consensus sequence for the highly repetitive Alu-like sequences found widely dispersed in the higher mammalian genome; there is evidence based on evolutionary grounds (Ullu & Tschudi, 1984) that 7SL RNA may in fact be the parent RNA from which the Alu-like sequences are derived. The core portion (termed the S segment, about 150 nucleotides long) shows no homology to Alu DNA. In proposed secondary structures for the RNA (Gundelfinger et al., 1984; Zwieb, 1985; E. Ullu, unpublished, and P. Walter, unpublished), the 5' and 3' Alu segments base pair with each other to form one "end" in the folded structure.

Activity of SRP

With SRP purified to homogeneity, it became possible to study its activity in greater detail. Results of experiments testing both the effect of SRP on the preprolactin nascent chain and its binding properties to various components in the wheat germ translation/translocation system (Walter et al., 1981; Walter & Blobel, 1981a,b) have led to the model for the "SRP cycle" shown in Figure 2.

SRP is thought to bind with loose affinity to monosomes in a signal

Figure 2. Model for the function of SRP in the targeting of proteins to the endoplasmic reticulum membrane. Reprinted with permission from Walter et al., 1984.


B
C
D
E
F
signal sequence
SRP
A
SRP receptor
ribosome receptor
signal peptidase

sequence independent manner (Fig. 2A,B). Upon emergence of the signal sequence (Fig. 2C), the affinity of SRP for the ribosome increases (Fig. 2D), in the case of preprolactin the affinity increases three to four orders of magnitude (Walter et al., 1981). Interestingly, concomitant with this increase in affinity, SRP specifically blocks further elongation of the preprolactin nascent chain (Walter & Blobel, 1981b). This "elongation arresting" activity of SRP, which can be demonstrated by adding SRP to a wheat germ translation system in the absence of microsomal membranes, is thought to hold the nascent chain in a "translocation competent" state. In other words, membranes can be added at any point in time to this SRP-arrested system and productive translocation, as evidenced by signal peptidase cleavage, will still occur. The membrane component that specifically targets the SRP-translation complex to the membrane (Fig. 2E) is SRP-receptor (Gilmore et al., 1982a,b), also known as docking protein (Meyer et al., 1982a,b), a heterodimeric protein consisting of a 70 kd (Lauffer et al., 1985) and a 30 kd subunit (Tajima et al., 1986), which has been localized by immune-electron microscopy and biochemical fractionation primarily to the rough ER membrane (Hortsch & Meyer, 1985; Tajima et al., 1986). In addition to its targeting function, SRP receptor has been shown to release the elongation arrest induced by SRP. This function requires the 70 kd subunit, while the 30 kd subunit appears to be required for anchoring the receptor in the ER membrane (Lauffer et al., in preparation). Subsequent to targeting, SRP and SRP receptor are freed from the translocation complex (and can re-enter the cycle (Gilmore & Blobel, 1983), Fig. 2F,A), and translocation proceeds (Fig. 2E,F) through an environment that is accessible to aqueous perturbants (Gilmore & Blobel, 1985)

by a mechanism which requires GTP for its initiation (Connolly & Gilmore, 1986), but is otherwise undefined.

The knowledge of the molecular dimensions of SRP has considerably influenced our view of how SRP could functionally interact with the protein translocation machinery. While the molecular details remain nebulous they raise the question of how SRP could recognize information in the nascent chain and simultaneously affect reactions involved in elongation, since the nascent chain exit site and the peptidyl transferase center are believed to be physically separated by about 16 nm (Bernabeau et al., 1983). While it is of course possible that such a mechanism involves allosteric changes across the ribosome itself, it is also conceivable that SRP physically bridges the distance between the two sites, thus recognizing signal sequences with one end and modulating the elongation reaction with the other (possibly by blocking the aminoacyl-tRNA binding site).

Assigning Functions to Specific SRP Domains: Polypeptide Specific Antibodies to SRP

In order to study the function of specific polypeptide components of SRP, polyclonal antibodies were raised against the three large polypeptides (Walter & Blobel, 1983d). Antibodies against the individual polypeptides were titrated into an *in vitro* protein translocation assay, with the following results: In each of the three cases an inhibition of the protein translocation process was observed as the IgG concentration was raised. In the case of antibodies raised to the 72 kd and to the 68 kd polypeptide, elongation arrest by the particle was also inhibited. When the antibody raised against the 54 kd polypeptide was tested, translocation was blocked but elongation arrest was unaffected.

This result might imply that while anti-72 kd IgG and anti-68 kd IgG blocked some of the early functions of SRP, the anti-54 kd IgG apparently still allowed elongation arrest of preprolactin synthesis to occur, but interfered with the subsequent release of the arrest by SRP receptor. However, since it has not been possible to reconfirm these results with monovalent Fab fragments of IgG fractions, the above mentioned molecular details of the inhibition have to be interpreted with caution, since it could result simply from cross-linking of SRP molecules with the divalent IgG.

Disassembly of SRP and Reconstitution

The integrity of many RNPs is dependent on the presence of divalent cations. Thus chelating agents have been successfully employed to partially unfold or to disassemble ribosomal subunits (Spirin, 1974; Newton et al., 1975; Blobel, 1971), RNAse P (Guthrie & Atchison, 1980), small cytoplasmic RNPs (Mukherjee & Sarkar, 1981), as well as SRP (Walter & Blobel, 1983a). In all cases which have been studied, EDTA seems to unfold the RNP but does not cause its disassembly into RNA and protein. In other words, when a sample of the RNP is treated with EDTA and sedimented on a sucrose gradient, the RNA and protein still cosediment.

It is important to understand what effect EDTA has on the structure of the RNP. Newton et al. (1975) showed that for the case of the ribosome, EDTA destroyed the specificity of the protein/RNA interaction, and that the proteins, although still bound, were now randomly distributed along the RNA. This was shown by the following experiment. 30S subparticles (labelled in both RNA and protein) were mixed with labelled 23S RNA, with or without the addition of EDTA to 5mM. The components

were then separated on a nondenaturing gel to examine the distribution of 30S proteins between the two RNAs. When the components were mixed together in the absence of EDTA, no significant transfer of proteins between the 30S particle to the 23S RNA occurred. However, when the same mixture was treated with EDTA, the proteins became almost equally distributed between the two RNAs. All proteins tested, irrespective of the initial strength of their binding to the RNA, were able to participate in the exchange. The result shows clearly that in the presence of EDTA the proteins are free to move from one RNA strand to another, which in turn implies that they can move freely within the confines of their own RNA strand.

Such a result suggests two things. First, since 5mM EDTA has no effect on the secondary structure of the RNA, the site specificity of the protein binding must rely on RNA tertiary structure. Second, since heterologous RNAs seem equally efficient at binding these proteins, the binding in the presence of EDTA must occur at sites all the RNA molecules have in common, i.e., the nonspecific interaction is most likely to be a charge interaction between the phosphate backbone of the RNA and the positively charged groups of the proteins.

Instead of challenging the RNP with a heterologous RNA, or negatively charged molecule, one can add a positively charged matrix to the system. If the matrix has a high density of positive charges, it should compete with the RNA binding proteins for the RNA. Herein lies a method (Walter & Blobel, 1983a) that can probably be successfully employed to disassemble almost any RNP into RNA and protein components.

SRP was first unfolded by the addition of EDTA to a final concentration of 5mM. Then various polycationic substances were tested for

their ability to dissociate SRP; the material that was most successful for disocciation was Whatman DE53, a DEAE-cellulose resin with a very high surface density of charged groups. Under conditions of ionic strength (250mM KOAc) for which intact SRP has been shown to bind to DEAE when all buffers contain Mg^{++}, and with a brief incubation at elevated temperature in the presence of both EDTA and the resin, almost quantitative disassembly of SRP was obtained. The protein fraction was completely devoid of contaminating 7SL RNA, and the RNA could be subsequently eluted from the resin with an increase in ionic strength. The RNA was intact, indicating that no nucleolytic breakdown of the EDTA-unfolded SRP occurred, and could be subsequently treated with proteases and extracted with phenol to provide a 7SL RNA fraction that was free of residual SRP proteins. The dissociation was independent of the time for which SRP was incubated with EDTA, i.e., the unfolding appeared to occur instantaneously. Furthermore, prolonged incubation did not further increase the yield of SRP proteins.

Such a disassembly reaction is extremely powerful as a purification step because it uses a similar ion-exchange resin to one used earlier for SRP purification (DEAE-Sepharose was used to concentrate SRP after the aminopentyl agarose step). The only real difference is the presence or absence of divalent cation. Therefore, proteins that had co-eluted with SRP on the DEAE column in Mg^{++} containing buffers (e.g., some higher molecular weight contaminants that are sometimes found in SRP preparations) should still bind to DEAE in the presence of EDTA. Hence this method may be a reasonable way to identify proteins bound to a particular RNA even when the RNP has only been partially purified. This procedure has been used successfully in our laboratory (B. Bowerman and

P. Walter, unpublished) to identify Drosophila SRP polypeptides from a partially purified preparation.

SRP Requires Both Protein and RNA Components for Activity

When subjected to activity assays, both the SRP protein fraction alone and 7SL RNA alone were completely inactive. SRP proteins did not compete with intact SRP, even when added in five fold molar excess. Attempts were then made to reconstitute a functional particle with 7SL RNA. As a starting point for these experiments, ionic conditions were chosen that were close to those reported by Traub and Nomura (1968,1969) for the reconstitution of functional 30S ribosomal subunits. SRP activity could be reconstituted simply by recombining SRP proteins (either from the "crude" SRP protein fraction or from purified SRP proteins) with a stoichiometric amount of 7SL RNA in the presence of Mg^{++}. A short incubation step at elevated temperature was required to drive the reconstitution to completion. If this incubation step was omitted, functional SRP was obtained nevertheless, albeit at a reduced yield. Similar heat activation steps are required to reactivate EDTA-unfolded ribosomal subunits even without separation of protein and RNA components (Spirin, 1974), suggesting that simply by mixing the two components, we are returned to a similar state of nonspecific protein-RNA interaction as we were before the disassembly process. In contrast to the reconstitution conditions for ribosomes, the ionic conditions could be varied over a wide range (150-500mM K^{+}, 4-20mM Mg++) without affecting the efficiency of the reaction. Similarly, RNAse P can be reconstituted from protein and RNA components simply by mixing the two in the presence of Mg^{++} (Kole & Altman, 1979).

When reconstituted SRP was analyzed by sucrose gradient centrifu-

gation, it was found that all six proteins had reassembled in stoichiometric amounts with RNA and formed an active 11S particle. A second, more slowly sedimenting peak was also observed, which was comprised of the 7SL RNA and the 9/14 kd protein alone. We cannot distinguish whether the slower sedimenting peak constitutes an assembly intermediate of SRP or whether it is an incorrectly assembled form acting as a dead end in the assembly process.

RNA-Binding Proteins of SRP

To address the question which of the individual SRP proteins would be capable of binding directly to 7SL RNA, each of the SRP protein fractions and various combinations thereof were tested for their ability to bind to an excess of 7SL RNA under stringent reconstitution conditions (500mM K^+, 5mM Mg^{++}). It was found that the 9/14 kd, the 19 kd, and the 68/72 kd protein fractions can each bind to 7SL RNA independently of the other proteins, whereas the 54 kd protein requires the presence of the 19 kd protein to stably interact with 7SL RNA (Walter & Blobel, 1983a).

Since 7SL RNA is required for the reconstitution of SRP proteins into an 11S particle, we conclude that at least one of the functions of the RNA is structural: the RNA is providing a matrix for the coordinate assembly of the SRP proteins. This effect is clearly sequence specific, since unrelated RNA molecules are not able to replace 7SL RNA in this function (Walter & Blobel, 1983a), nor do any of the SRP proteins interact with measurable affinity to unrelated RNAs under these stringent reconstitution conditions. Much of the RNA seems to be involved in protein contact, since it is almost completely protected within the particle from nuclease digestion (Gundelfinger et al., 1983).

The data on the in vitro reassembly of SRP suggest that the process is ordered and cooperative. To test for cooperativity in the reconstitution process, the concentration of 7SL RNA in the reconstitution reaction was varied. The reaction was saturated by an approximately equimolar concentration of RNA to protein. As the concentration of RNA was increased beyond this concentration as much as five-fold, the activity of reconstituted SRP remained at a plateau, i.e., in contrast to the reconstitution of ribosomal subunits, an excess of 7SL RNA did not compete in the in vitro assembly process of SRP. This demonstrates the highly cooperative nature of the assembly process.

Three of the four SRP protein fractions can bind specifically in the absence of the other SRP proteins. There are therefore two explanations for the cooperative nature of the assembly reaction. The first is that any of the SRP proteins may bind initially to the RNA, but that the binding of the first protein increases the binding affinity of the other proteins, either by protein-protein interactions or by a conformational effect on the RNA. The other possibility is that only one of these three proteins can bind initially to the RNA to nucleate the in vitro SRP assembly. Such an "assembly initiator protein" for SRP has not yet been identified, but Nowotny and Nierhaus (1982) described two different techniques that were successfully used to identify such proteins for the large ribosomal subunit, and would probably be applicable here. The interested reader is encouraged to refer to their elegant work for further discussion.

Reconstitution of Canine SRP Proteins With 7SL RNAs From Other Species

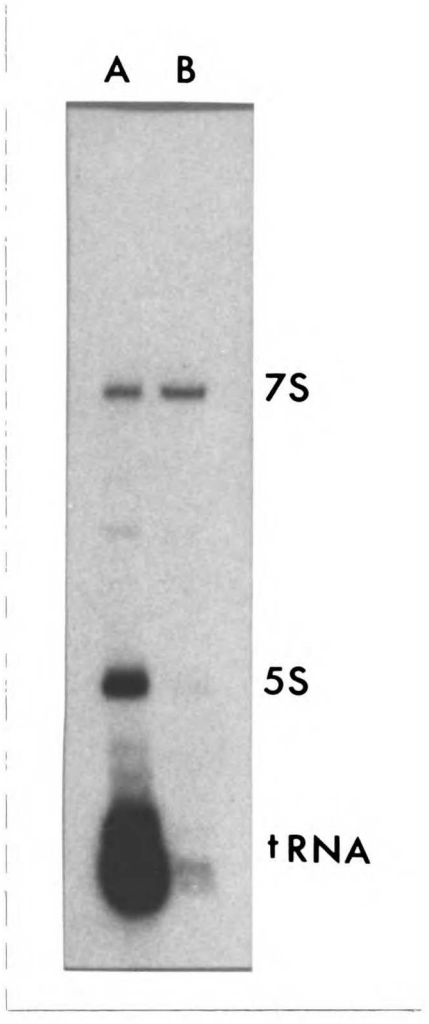
7SL RNA is highly conserved through evolution, and similar secondary structures can be drawn for human and drosophila 7SL RNAs (Ullu &

Tschudi, 1984; Gundelfinger et al., 1984; Zwieb, 1985). Because of this, attempts to create "chimeric SRPs" from mammalian SRP proteins and amphibian or insect 7SL RNAs appeared promising. Following a fractionation scheme that would render all cellular SRP soluble by raising the salt in the homogenate to 500mM, RNA from either Xenopus laevis or Drosophila melanogaster was extracted from a postribosomal supernatant and fractionated by polyacrylamide gel electrophoresis (Walter & Blobel, 1983a). The 7S RNA band was selected simply by the criterion of comigration with canine 7SL RNA on denaturing polyacrylamide gels. The reconstitute formed from Xenopus laevis RNA and canine SRP proteins was fully functional, with all six polypeptides assembled onto an 11S particle. In contrast SRP reconstituted with D. melanogaster 7S RNA was less active and had a slightly slower sedimentation coefficient than authentic SRP. The 9/14 kd protein appeared to bind with a lower affinity to the reconstituted particle, such that upon sucrose gradient sedimentation in the high salt buffer, the protein was slowly released from the particle, and as a result was found streaking all across the gradient profile. The ability to generate functional SRPs from a mixture of heterologous components demonstrates a) that the particle is highly conserved across evolution, and b) that the 7S RNAs that had been purified simply on the basis of size can in fact function as SRP RNAs.

Another method to assay the binding of heterologous RNA to canine SRP proteins involves nitrocellulose binding. At 500mM K+, 7SL RNA does not by itself bind to nitrocellulose, but if SRP proteins are included and the mixture is subjected to standard reconstitution conditions, the RNA now is retained by the filter, presumably through interaction with the protein. This assumption can be confirmed by eluting the RNA from

Figure 3. Nitrocellulose binding assay for the interaction of SRP RNA with its proteins.

Chinese hamster ovary cells were labelled overnight with ^{32}P phosphate. Cells were lysed by homogenization and extracted with 500mM KOAc and 5mM $Mg(OAc)_2$ for 15 min. The lysate was spun at 100,000 g for 3.5 hr to yield a postribosomal supernatant, shown in lane a. An aliquot of this supernatant was mixed with 2.5 pmol canine SRP proteins and incubated under standard reconstitution conditions (Walter & Blobel, 1983a). The mixture was passed over a nitrocellulose filter and washed 3 times with 500mM KOAc, 5mM $Mg(OAc)_2$, one time with 1M KOAc, 10mM $Mg(OAc)_2$, and one time with 100mM KOAc, 1mM $Mg(OAc)_2$. The filter was eluted with the proteinase K/ SDS buffer described by Walter and Blobel (1982). The eluted RNA was precipitated with ethanol and then resolved on a 6% acrylamide, 7M urea gel. The bands were visualized by autoradiography. Lane a, total RNA used in reconstitution; lane b, RNA bound to filter.


A
B
7S
5S
tRNA

the nitrocellulose by incubation with proteinase K (Fig. 3). When a mixture of RNAs is included in the reaction, SRP RNA is specifically retained (compare Fig. 3, lanes a and b). The binding is not diminished even if a 20,000 fold molar excess of tRNA is added to the reconstitution reaction.

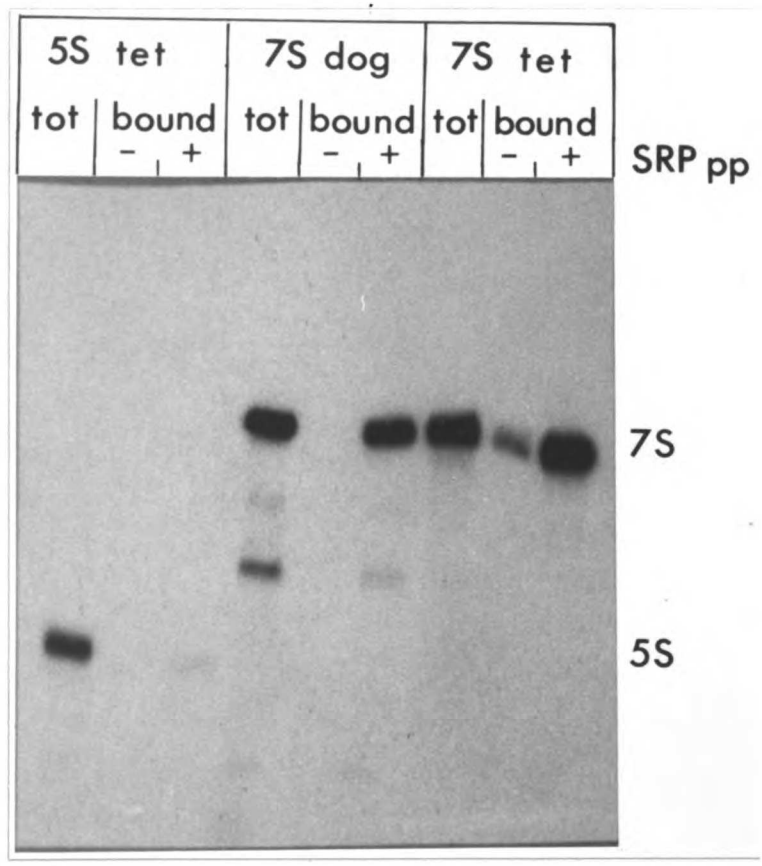
This assay has been successfully employed to test the specific binding of canine SRP proteins to 7S RNA from the widely divergent organism Tetrahymena (Fig. 4). The presence of canine SRP proteins significantly increased the retention of this RNA by nitrocellulose, suggesting that the RNA, which was isolated using its size as the only criterion, is the SRP RNA for this species. Nitrocellulose binding could also be used to select out an RNA that binds specifically to the SRP proteins from a mixture of labelled RNAs, allowing us to avoid the initial size criterion when searching for the SRP RNA in other organisms.

Assigning Function to Specific SRP Domains: The Goal of This Study

Signal recognition particle has been shown to mediate the targeting of nascent secretory proteins to the membrane of the endoplasmic reticulum. It is a complex of several components and exhibits several activities which can be assayed in vitro, among them signal recognition, elongation arrest, and protein translocation. I have sought to understand the relationship between the structure of SRP and its activities. My approach has been to exploit the ease with which the particle can be disassembled and reconstituted to remove or alter specific protein or RNA domains within the particle. I hoped to generate particles that were partially active, thus enabling me to map the missing functions to particluar domains. In Chapters 2-4 I present the results of these

Figure 4. Nitrocellulose binding of 5S RNA, SRP RNA, and Tetrahymena 7S RNA by canine SRP proteins.

The nitrocellulose binding assay descibed in Figure 3 was performed with purified RNA that had been labelled at the 3' end with ^{32}P-pCp and RNA ligase (see Chapter 5). Some reconstitution reactions contained a compensating buffer in place of SRP proteins. The first lane of each set shows total RNA used in the reaction, the second lane the amount bound to the filter in the absence of SRP proteins, and the third lane in each set shows the amount of RNA bound after reconstitution with canine SRP proteins.


5S tet
7S dog
7S tet
tot
bound
tot
bound
tot
bound
−
+
−
+
−
+
SRP pp
7S
5S

studies.

By nuclease protection analysis, I have positioned the protein components on the RNA (Chapter 5). Because the functions for these proteins have been defined by the analysis of the earlier chapters, such a structural map has functional significance, and influences our model of how SRP can simultaneously interact with the signal sequence at the nascent chain exit site and arrest elongation.

As has been stated earlier, the targeting of nascent chains to the microsomal membrane is unusual in that it is obligately cotranslational in nature. In other words, there is a window of time during elongation after which translocation no longer occurs. It was not clear whether this cut-off point reflects a loss in the ability of SRP to interact with these chains, or an incompatability of these chains with other components of the translocation machinery. I turn in Chapter 6 to an analysis of the substrate requirements for SRP-mediated targeting.

CHAPTER 2

ELONGATION ARREST IS NOT A PREREQUISITE FOR SECRETORY PROTEIN TRANSLOCATION ACROSS THE MICROSOMAL MEMBRANE

ABSTRACT

Signal recognition particle (SRP) is a ribonucleoprotein consisting of six distinct polypeptides and one molecule of small cytoplasmic 7SL RNA. It was previously shown to promote the cotranslational translocation of secretory proteins across the endoplasmic reticulum by i) arresting the elongation of the presecretory nascent chain at a specific point, and ii) interacting with the SRP receptor, an integral membrane protein of the endoplasmic reticulum which is active in releasing the elongation arrest. Recently a procedure was designed by which the particle could be disassembled into its protein and RNA components.

We have further separated the SRP proteins into four homogeneous fractions. When recombined with each other and with 7SL RNA, they formed fully active SRP. Particles missing specific proteins were assembled in the hope that some of these would retain some functional activity. SRP(-9/14), the particle lacking the 9 kd and 14 kd polypeptides, was fully active in promoting translocation, but was completely inactive in elongation arrest. This implied that elongation arrest is not a prerequisite for protein translocation. SRP receptor was required for SRP(-9/14)-mediated translocation to occur, and thus must play some role in the translocation process in addition to releasing the elongation arrest.

INTRODUCTION

Signal Recognition Particle (SRP) has been shown to couple the cytoplasmic protein synthesis machinery with the membrane bound protein translocation machinery of the endoplasmic reticulum. The function of SRP is well established and can be readily assayed *in vitro*. Experiments performed in a wheat germ *in vitro* translation system that was

supplemented with purified SRP and SRP-depleted microsomal membranes, and programmed with total pituitary RNA, coding primarily for the secretory protein preprolactin, and/or total reticulocyte RNA, coding primarily for the cytoplasmic protein globin, have led to the following model for SRP activity (called the "SRP cycle"; for review see Chapter 1): First, SRP binds with high affinity to ribosomes that are synthesizing secretory proteins (Walter et al., 1981) and arrests their synthesis at a specific point in the nascent chain (Walter & Blobel, 1981b), corresponding to that point where the signal sequence is fully exposed on the surface of the ribosome. Second, SRP interacts with its own receptor in the endoplasmic reticulum, called SRP receptor (Gilmore et al., 1982a,b) or docking protein (Meyer et al., 1982a,b). This interaction results in a release of the translation arrest (Gilmore et al., 1982b; Meyer et al., 1982a), and a weakening of the high affinity binding between SRP and the ribosome (Gilmore and Blobel, 1983). The nascent chain then traverses the membrane cotranslationally (Walter & Blobel, 1981b, Blobel & Dobberstein, 1975a); several models have been proposed for the mechanism of the passage (Blobel & Dobberstein, 1975b, Steiner et al., 1980, VonHeijne & Blomberg, 1979), but the details remain unclear. After the formation of the functional ribosome-membrane junction, SRP and SRP receptor are free to re-enter the cycle.

SRP is an 11S particle composed of four proteins (two monomers composed of a 19 kd polypeptide and a 54 kd polypeptide, and two heterodimers, one composed of a 9 kd and a 14 kd polypeptide, and the other composed of a 68 kd and a 72 kd polypeptide, respectively) (Walter & Blobel, 1980, Walter & Blobel, 1983a), and one 300 nucleotide molecule of 7SL RNA (Walter & Blobel, 1982). The RNA is composed of both unique

and repetitive genomic sequences (Weiner, 1980, Li et al., 1982, Ullu et al., 1982, Ullu & Tschudi, 1984). Approximately 100 nucleotides at the 5' end and 45 nucleotides at the 3' end are homologous to the human Alu right monomer sequence. The central "S segment" of 155 nucleotides shows no homology to Alu DNA and is unique to 7SL RNA. It has been possible to disassemble SRP into its protein and RNA components, and to reconstitute from them a functional SRP (Walter & Blobel, 1983a). Preliminary subfractionation of the SRP proteins has indicated that all the proteins except the 54 kd protein can bind to the RNA directly, and that the 54 kd protein probably joins the assembly through the 19 kd protein (Walter & Blobel, 1983a). In this study we have further fractionated the protein component into four homogeneous subfractions, and have used these subfractions to assemble partial SRPs. Our purpose in doing so was to attempt i) to assign functional domains on the particle, and ii) to address questions concerning the dependency relationships of the various SRP activities that can be assayed in vitro, i.e., whether the completion of a given event in the SRP cycle is required in order for subsequent events to occur.

MATERIALS AND METHODS

Materials

^{35}S-methionine (1500 Ci/mmol), "translation grade," was purchased from Amersham; ^{125}I-Bolton Hunter reagent (1900 Ci/mmol) from NEN; Nikkol (octa-ethyleneglycol-mono-n-dodecyl ether, a nonionic detergent shown to stabilize SRP activity) from Nikko Chemicals Co., Ltd, Tokyo, Japan; Trasylol (the protease inhibitor aprotinin, sold as a liquid at 10,000 U/ml) from FBA Pharmaceuticals; elastase from Boehringer Mannheim; aminopentyl agarose from Sigma. Most other reagents were from

Baker. All preparative procedures were carried out at 4°C, except where noted. All glassware was siliconized.

Preparation of salt-extracted microsomal membranes and SRP

Microsomal membranes were prepared according to Walter and Blobel (1983b), with the following modification. The column-washing step was replaced by two consecutive washes of the membranes by pelleting (90,000 x g(av), 45 min) and resuspending the membranes in twice the original volume of 50mM TEA, 1mM $Mg(OAc)_2$, 0.5mM EDTA, pH 7.5, 1mM DTT, 0.5mM PMSF, and 0.1% Trasylol.

SRP was prepared from these membranes as described (Walter & Blobel, 1983c). We found that the purification of SRP from the microsomal salt extract was dependent on the particular batch of the aminopentyl agarose used, being optimal, under the conditions described (Walter & Blobel, 1983c), at a density of 5.7 µmoles diaminopentane coupled per ml of resin.

Disassembly of SRP and Separation of SRP Polypeptides

SRP was disassembled into protein and RNA components essentially as described previously (Walter & Blobel, 1983a). A solution of 180 µg (0.5 nmol) gradient-purified SRP in 1 ml of 50mM TEA, 500mM KOAc, 5mM $Mg(OAc)_2$, 1mM DTT, 0.01% Nikkol, was diluted with 1 ml of a solution containing 50mM TEA, 12.5mM EDTA, 1mM DTT, 0.01% Nikkol, and added to a pellet of ~0.8 ml pre-equilibrated DEAE cellulose (DE53, Whatman). The slurry was incubated 10 min on ice and 10 min at 37°C, with mixing by inversion once each min. The resin was then pelleted in a microfuge for 2 min and the supernatant removed. The pellet was resuspended in 2 ml of a solution containing 50mM TEA, 250mM KOAc, 5mM EDTA, 1mM DTT, 0.01% Nikkol. The slurry was incubated at 37°C for 10 min, and the resin

removed by centrifugation. The two supernatants were pooled and constitute the SRP protein fraction.

In order to separate the individual proteins, the SRP protein fraction was adjusted to 20mM NaP_i and loaded onto a 0.5 ml hydroxylapatite column (HTP, BioRad) at 4oC. After washing with two volumes of 20mM Hepes/KOH, pH 7.5, 0.1mM EDTA, 1mM DTT, 0.01% Nikkol (Buffer A), containing 250mM KOAc and 20mM NaP_i, proteins were sequentially eluted from the column with 2 ml steps of Buffer A containing the following concentrations of NaP_i and KOAc, respectively: a) 50mM NaP_i, 250mM KOAc, b) 160mM NaP_i, 100mM KOAc, c) 250mM NaP_i, no KOAc, and c) 400mM NaP_i, no KOAc. Fractions of 0.5 ml were collected at 4^oC, except during the 400mM NaP_i elution step, when we moved the column to room temperature to prevent the phosphate buffer from precipitating.

An aliquot (50 µl) of each fraction was TCA precipitated and prepared for SDS-PAGE as described (Blobel & Dobberstein, 1975b, Lingappa et al., 1977). The SRP polypeptides were separated on 10-15% polyacrylamide gradient gels and visualized by staining with Coomassie Blue in order to locate the peaks. At this stage the proteins were separated into three fractions, comprised of the 19 kd protein (eluting at 160mM NaP_i), the 9/14 kd and 54 kd proteins (eluting at 250mM NaP_i), and the 68/72 kd protein (eluting at 400mM NaP_i), respectively. We found it necessary to monitor the elution profile of the hydroxylapatite column by SDS-PAGE, since the exact position of the protein peaks with respect to the NaP_i concentration was slightly variable from column to column.

We further fractionated and concomitantly concentrated the SRP proteins by ion-exchange chromatography. The three peak fractions were

diluted 8 fold with Buffer A, and were loaded onto separate 100 µl CM Sepharose Cl-6B columns, poured in nylon mesh-sealed 200 µl glass capillary tubes and equilibrated in Buffer A containing 50mM KOAc. The columns were eluted with two steps of Buffer A containing 0.4M and 1M KOAc, respectively. One 30 µl fraction and six 50 µl fractions were collected at each step. Aliquots of 5 µl of each fraction were TCA precipitated, and the polypeptides separated and displayed by SDS-PAGE as described above. The 54 kd protein eluted at 0.4M KOAc in Buffer A, and the other proteins eluted at 1M KOAc in Buffer A.

The described fractionation scheme resulted in four fractions containing essentially homogeneous SRP proteins. The proteins were concentrated enough for subsequent reconstitution of SRP and were in a buffer that was compatible with both reconstitution and subsequent activity assays. Aliquots of the SRP protein fractions were frozen in liquid nitrogen and stored at -80°C. They could be thawed and rapidly refrozen at least twice with no measurable loss of activity (as measured by the reconstitution of a functional SRP).

The concentration of the SRP proteins was estimated by comparing the band intensity after SDS-PAGE and Coomassie Blue staining to that of an SRP standard of known concentration. A typical preparation (as in Fig. 1, Panel A) yielded the following fractions: a) 50 µl of 68/72 kd protein (Fig. 1A, lane b) at 5 µM in Buffer A containing 1M KOAc (50% of theoretical yield). b) 50 µl of 54 kd protein (Fig. 1A, lane c) at 2.5 µM in Buffer A containing 0.4M KOAc (25% of theoretical yield); and c) 50 µl of 19 kd protein (Fig. 1A, lane d) at 2.5 µM in Buffer A containing 1M KOAc (25% of theoretical yield); d) 50 µl of 9/14 kd protein (Fig. 1A, lane e) at 2.5 µM in Buffer A containing 1M KOAc (25% of theoretical yield).

Iodination of SRP and SRP Proteins

A benzene solution containing 25-50 uCi ^{125}I-Bolton-Hunter reagent (NEN, 1900 Ci/mmol) was dried down with a gentle stream of liquid nitrogen. Then 10 µl SRP or fractionated SRP proteins were added and the mixture was incubated 2-3 hr on ice, with gentle vortexing every 30 min. The reaction was stopped by adding Tris/HOAc (pH 8.0) to a final concentration of 50mM. No attempt was made to separate the unincorporated Bolton-Hunter reagent, as previous attempts to remove it by gel filtration on a variety of resins resulted in a substantial loss of labeled protein, presumably caused by irreversible binding to the gel filtration column.

Purification of 7SL RNA

Gradient-purified SRP (150 µg in 1 ml) was precipitated with 2.5 volumes of ethanol by freezing the samples in liquid nitrogen for at least 30 sec, and then spinning at top speed in a microfuge for 15 min at 4°C. The pellet was resuspended in a solution containing 25mM TEA, 2.4% SDS, 100mM NaCl, 15mM EDTA, 200 µg/ml proteinase K (Boehringer), and incubated 30 min at 37°C. This solution was then extracted with an equal volume of phenol, followed by an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and finally with an equal volume of chloroform/isoamyl alcohol (24:1). NaOAc was added to 300mM and the solution was ethanol precipitated as above. The resulting pellet was resuspended twice more in 50 µl 300mM NaOAc, 1mM EDTA and reprecipitated with 125 µl ethanol. The final pellet was dried in a Speed-Vac Concentrator (Savant) and then resuspended in sterile, diethyl pyrocarbonate-treated water. The concentration was calculated assuming 20 A_{260} units=1 mg RNA. We typically obtained 30 µg of 7SL RNA from 150 µg SRP (60% of theoretical yield).

Reconstitution of Complete and Partial SRPs

Reconstitutions were performed under standard conditions as described (Walter & Blobel, 1983a). Proteins were recombined in approximately stoichiometric amounts on ice. For the partial reconstitutions, a buffer equivalent in composition to that containing the missing protein was added so that the concentration of components in the different reconstitutions would be identical. The ionic conditions were adjusted by the addition of another buffer solution so that the final concentrations in the reconstitution mix were 20mM Hepes, 500mM KOAc, 5mM $Mg(OAc)_2$, 1mM DTT, and 0.01% Nikkol. RNA was added last, also in stoichiometric amounts with respect to the proteins. The concentration of proteins and RNA was ~400nM. The mixture was incubated for 10-15 min on ice and 10-15 min at 37°C. The reconstitutes were then returned to ice if they were to be used immediately in an activity assay, or frozen in liquid nitrogen and stored at -80°C. A single freeze-thaw cycle resulted in no detectable change in activity.

Sucrose gradient analysis (data not shown) demonstrated that ~50% of the protein assembled with the RNA in each case. In the experiments described in this communication, no attempt was made to separate unassembled proteins and RNA from assembled particles.

Activity Assays

Total bovine pituitary RNA, coding primarily for the secretory protein preprolactin, and total rabbit reticulocyte RNA, coding primarily for the cytoplasmic protein globin, were translated together in a wheat germ cell-free system (25 µl final volume) in the presence or absence of salt-extracted microsomal membranes (Erickson & Blobel, 1983). The ionic conditions of this assay were kept constant at 144mM

KOAc, 2.4mM $Mg(OAc)_2$ in all cases. Temperature was kept constant at 26°C. The translation products were displayed by PAGE in SDS, and bands were localized by autoradiography and quantitated by densitometer scanning of preflashed film, using a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Inc, marketed by LKB).

Two activities of SRP (or partial SRPs) were monitored: i) promotion of the translocation of presecretory protein (preprolactin) across the microsomal membrane (thus allowing the processing of preprolactin to prolactin by signal peptidase), and ii) site-specific elongation arrest of preprolactin synthesis in the absence of microsomal membranes. Varying amounts of SRP or partial SRPs were added to <u>in vitro</u> translation reactions either containing SRP-depleted membranes (at 2 eq per 25 µl (Walter et al., 1981)), to measure percent processing of preprolactin to prolactin, or not containing membranes, to measure percent inhibition of preprolactin synthesis. Translations were allowed to proceed for 1 hr and were stopped by chilling on ice. The reactions were TCA-precipitated, resuspended in DTT Sample Buffer, denatured, and alkylated with iodoacetamide. Samples were then submitted to PAGE in SDS on 12% gels, and visualized by autoradiography on preflashed film. The intensities of the preprolactin, prolactin, and globin bands were quantitated by densitometer scanning.

$$\text{Percent processing} = \frac{(8/7)\text{prolactin}}{(8/7)\text{prolactin} + \text{preprolactin}} \times 100$$

$$\text{Percent inhibition at concentration a} = 1 - \frac{[\text{preprolactin(a)} \times \text{globin(0)}]}{[\text{preprolactin(0)} \times \text{globin(a)}]} \times 100$$

Synchronized Translations

Protein synthesis was initiated by the addition of RNA to 100 µl of a prewarmed extract (containing all components necessary for protein synthesis except mRNA). After 30 sec further initiation was inhibited by the addition of the cap analog 7-methylguanosine-5'-monophosphate (7MeG) (Walter & Blobel, 1981b) to 4mM. To test translocation capability of the nascent chain as a function of elongation time, 10 µl aliquots of these extracts were added at various time points to tubes containing 1 µl of microsomal membranes, and incubation was continued for a total of 30 min. To measure the time of completion of the preprolactin nascent chain, 10 µl aliquots of these extracts were TCA precipitated at various time points after the initiation of translation to halt further protein synthesis. The products of the in vitro protein synthesis reactions were submitted to PAGE in SDS, the gels were fluorographed (Walter & Blobel, 1981b), and the bands visualized by autoradiography and quantitated as described above.

Purification of a 60 kd Elastase Fragment of SRP Receptor

The 60 kd elastase fragment of SRP receptor was purified by a modification of the procedure described by Meyer et al. (1982b); however, the pre-proteolytic detergent extraction was omitted. Purification was monitored by following the fragment's ability to restore the translocation activity of trypsinized microsomal membranes as described (5), as well as by Western blotting (Fisher et al., 1982) after SDS-PAGE.

Salt-extracted microsomes (750 ml at 1 eq/µl (as defined in Walter et al., 1981) were further extracted with EDTA (Walter & Blobel, 1983b) and resuspended in half the original volume in a buffer containing 50mM TEA, 500mM KOAc, 5mM $Mg(OAc)_2$, 1mM DTT, and 1% Trasylol (to inhibit hydrolases other than elastase). Elastase was added to 1 µg/ml and the mixture was incubated for 1 hr at $0^{o}C$. PMSF was added to 0.5mM final concentration and the proteolyzed microsomes were pelleted for 1 hr at 140,000 x g(av). The pelleted microsomes were resuspended in the same buffer as above and were digested a second time with 1 µg/ml elastase. The supernatants obtained from both digestions were combined, diluted with 2.5 volumes of a solution containing 50mM TEA, 1mM DTT, and loaded onto a 20 ml CM-Sephadex A50 column. The column was developed with a 150 ml linear gradient of KOAc in the above buffer from 150mM to 500mM KOAc. The 60 kd SRP receptor fragment eluted at ~370mM KOAc. The peak fractions were pooled, adjusted to 10mM NaP_i, and loaded onto a 1 ml hydroxyl apatite column. The column was eluted with a step of 300mM NaP_i, 250mM KOAc, 0.1mM EDTA, 1mM DTT. The eluted material was chromatographed on Biogel P200 (1.6 x 80 cm) to separate the 60 kd fragment from high molecular weight contaminants, reconcentrated on a small (0.3 ml) hydroxylapatite column as described above, and finally fractionated on a 5-20% sucrose gradient containing 50mM TEA, 500mM KOAc, 0.1mM EDTA, 1mM DTT (15 hr at 60,000 rpm in the Beckman SW60 rotor). At this stage the fragment was essentially homogeneous. It was active in restoring translocation activity to trypsinized membranes and showed no contamination with intact SRP receptor as judged by Western blotting. Starting with 750 ml of a rough microsome preparation at 50 A_{280} units/ml, we obtained ~200 µg of 60 kd SRP receptor fragment.

RESULTS

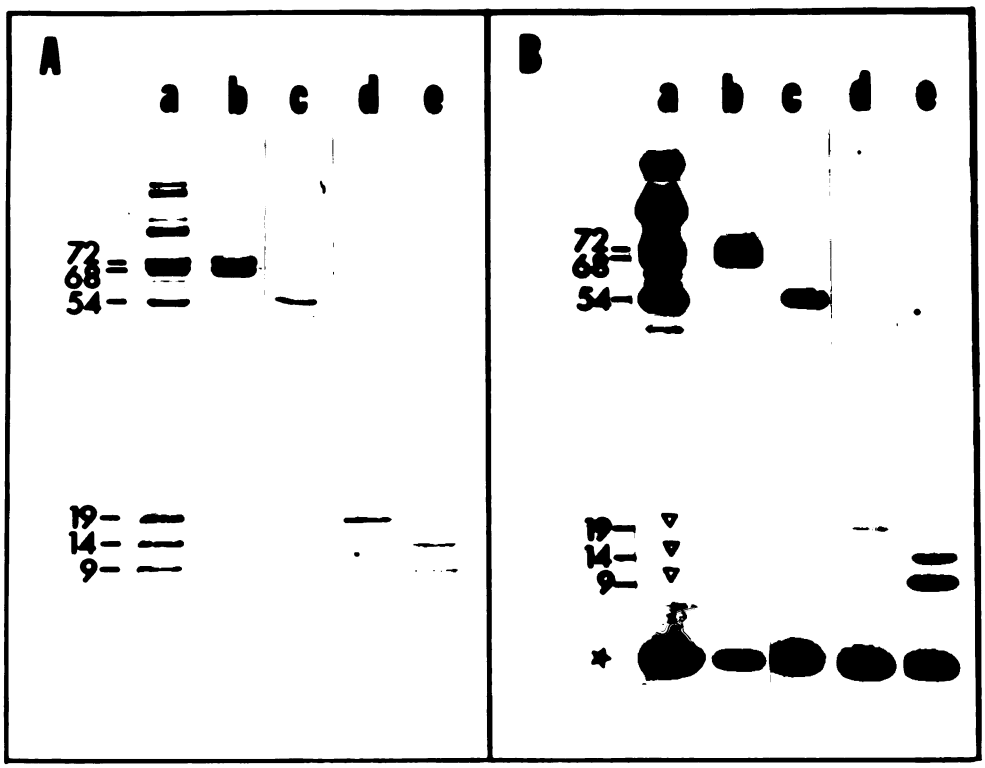
Separation of SRP Proteins

In the absence of divalent cations, SRP can be dissociated into RNA and protein components by a brief incubation with polycationic substances. This disassembly reaction is mild and nondenaturing as judged by the fact that fully active SRP can easily be reconstituted from these, by themselves inactive, subfractions (Walter & Blobel, 1983a). Having such a disassembly/reconstitution assay in hand, we decided to further fractionate the SRP protein fraction into its individual components.

We designed a fractionation scheme using a combination of hydroxylapatite and CM-Sepharose chromatography that allowed us to separate the SRP proteins into four homogeneous fractions (Fig. 1A). During this fractionation we noted that two SRP polypeptides appeared to act as monomeric proteins (namely, the 19 kd and 54 kd proteins; Fig. 1A, lanes c and d), while two pairs of SRP polypeptides under all of our conditions precisely co-chromatographed in approximately stoichiometric proportions and appeared to constitute dimeric proteins (the 68/72 kd protein and the 9/14 kd protein; Fig. 1A, lanes b and e) as previously described (Walter & Blobel, 1983a). The described fractionation procedure is relatively fast and the resulting SRP proteins are obtained in good yields. They can readily be reconstituted into active SRP when they are recombined and mixed with stoichiometric amounts of 7SL RNA under reconstitution conditions (Fig. 2A, open squares; Fig. 2B, open squares). Note that the reconstituted SRP was active in both of our activity assays, namely i) promotion of the translocation of nascent secretory proteins across the microsomal membrane (Fig. 2A), and ii)

Figure 1. Separation of SRP proteins

SRP (180 µg in 1 ml) was disassembled into protein and RNA, and the proteins were further fractionated by hydroxylapatite and CM-Sepharose chromatography as described in Materials and Methods. Panel A: The panel shows a 10-15% SDS polyacrylamide gel stained in Coomassie Blue of 50 µl SRP (5% of total, lane a) and 5 µl of peak fractions from CM-Sepharose columns (10% of total, lanes b-e). Molecular weights of the SRP polypeptides are indicated. Higher molecular weight bands are contaminants in the starting SRP preparation. Panel B: 10 µl SRP or 2 µl (diluted to 10 µl) peak fractions shown in Panel A were labeled with ^{125}I-Bolton-Hunter reagent. 1% of each sample was directly denatured in Sample Buffer and submitted to PAGE in SDS as above. The bands were visualized by autoradiography. Exposure was for 5 hr with an intensifying screen. Lanes correspond to those in Panel A. The asterisk indicates unincorporated Bolton-Hunter reagent.


A
a b c d e
72
68
54
19
14
9
B
a b c d e
72
68
54
19
14
9

specific elongation arrest during presecretory protein synthesis (Fig. 2B). We concluded from this that none of the essential proteins were substantially inactivated during their purification.

In order to assess the purity of the SRP protein fractions, we radiolabeled the proteins and subjected them to analysis by SDS-PAGE followed by autoradiography. Even upon prolonged exposure we did not detect cross-contamination of SRP polypeptides between the fractions (Fig. 1B, lanes b-e), except a trace amount (<5%) of the 54 kd protein in the 9/14 kd fraction (not visible at exposure shown in Fig. 1B, lane e). Also note that, during the disassembly and fractionation, all of the polypeptides that were contaminating our starting SRP preparation (mostly the high molecular weight bands in Fig. 1A,B, lanes a) were completely removed and are absent in the four SRP protein fractions.

SRP(-9/14) is Active in Translocation, But at a Reduced Efficiency When Compared with Complete SRP

After we had convinced ourselves of the purity of the separated SRP proteins and of their ability to reassemble into active particles, we proceeded to reconstitute partial SRPs by omitting specific SRP proteins from the reconstitution reactions. We reasoned that SRPs lacking certain proteins might exhibit partial functions and thereby allow us to map functional properties to specific SRP polypeptides.

We assembled the following partial SRPs: SRP(-9/14) (that is, the reconstitute lacking the 9/14 protein), SRP(-54), and SRP(-68/72). In our activity assays, we found that SRP(-54) and SRP(-68/72) were completely inactive in both translocation and elongation arrest (data not shown). Thus far, we have not assembled an SRP(-19). However, it was previously shown (Walter & Blobel, 1983a) that the binding of the 54 kd

protein was dependent on the presence of the 19 kd protein. Therefore SRP(-19) would essentially be an SRP(-19,-54), and thus is likely to be inactive as well.

In contrast to these overall defective SRPs, we observed that an SRP(-9/14) reconstitute was active in promoting the translocation of preprolactin across microsomal membranes (Fig. 2A, closed squares), albeit at reduced (~50%) efficiency, under these assay conditions. We noted that SRP(-9/14) exhibits this reduced activity with respect to the complete particle over the entire concentration range, and that both curves plateau at about the same SRP concentrations. SRP(-9/14) is therefore qualitatively different in its behavior from a complete SRP in that it appears to allow a certain percentage of the nascent chains to escape the translocation process at any given SRP concentration, and thereby reduces the overall efficiency of the process. This behavior is not consistent with the alternative possibility that the absence of the 9/14 kd protein leads to a mixture of fully active and inactive particles (due for example to a defect in reconstitution), since if this were the case we would expect, as total RNA concentration was increased, that the activity would eventually reach the same level as that of the complete particle.

SRP(-9/14) Does Not Inhibit the Elongation of Presecretory Proteins

Very much to our surprise, we found that although SRP(-9/14) was active in promoting cotranslational translocation of preprolactin across the endoplasmic reticulum, it did not arrest preprolactin synthesis. Fig. 2B (closed squares) demonstrates that the amount of preprolactin synthesized in 60 min in the presence of SRP(-9/14) (or in the presence of any of the other partial particles, data shown only for SRP(-9/14) in

Figure 2. Activity assay of SRP and SRP(-9/14)

Panels A and B: 7SL RNA and SRP proteins were mixed to ~400nM final concentration of each component, and reconstituted under standard conditions. Varying amounts of SRP and SRP(-9/14) were added to elongation arrest and translocation assays, and the results quantitated as described (see Materials and Methods). Panel A: percent processing as a function of SRP (open squares) and SRP(-9/14) (closed squares) concentration. Panel B: percent inhibition as a function of SRP (open squares) and SRP(-9/14) (closed squares) concentration. Panel C: titration of 9/14 kd protein back on SRP(-9/14). SRP(-9/14) was assembled at 600nM final concentration of each component. Then, varying amounts of the 9/14 kd protein were added, and the mixture diluted to 400nM final concentration of the other components. The temperature was elevated to 37°C for 10 min and the samples then returned to ice. Then 6 µl of each sample was added to a 25 µl translation reaction to assay percent inhibition. Samples were processed and results quantitated as above.

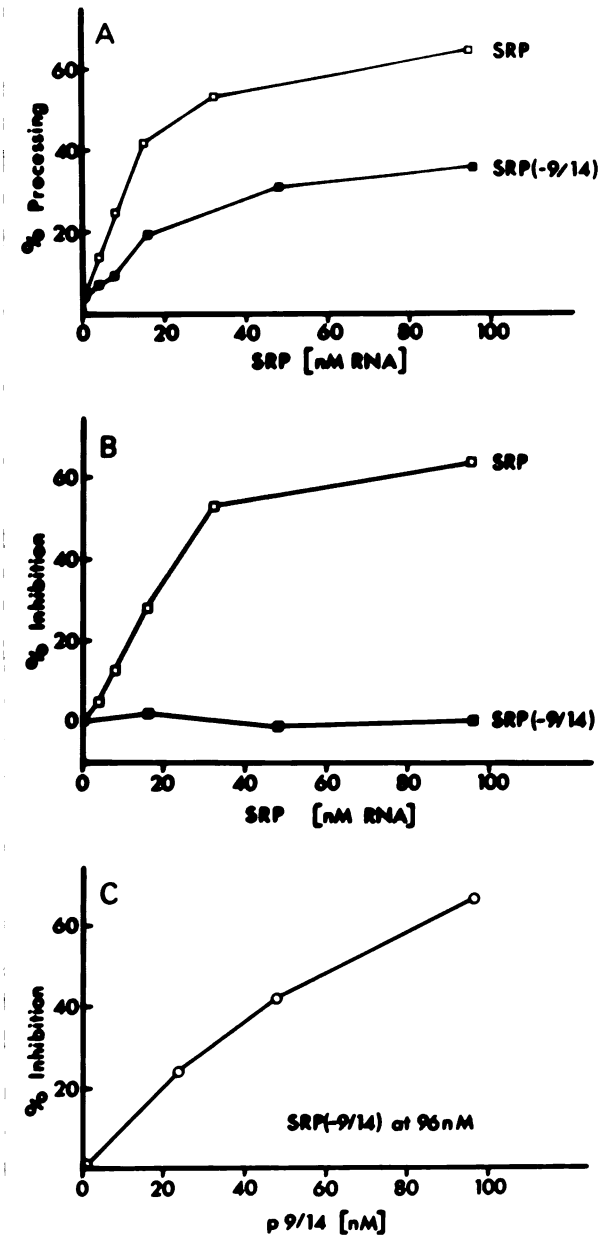

A
SRP
SRP(-9/14)
% Processing
0
20
40
60
80
100
SRP [nM RNA]
B
SRP
SRP(-9/14)
% Inhibition
SRP [nM RNA]
C
% Inhibition
SRP(-9/14) at 96nM
p 9/14 [nM]

Fig. 2B) was the same as that synthesized in the absence of SRP.

The elongation arresting activity of the particle could be restored by titrating back the purified 9/14 kd protein to an already assembled SRP(-9/14), as shown in Fig. 2C. SRP(-9/14) was first assembled at a slightly higher concentration (600nM of each component) than was used for the experiments in Fig. 2A and 2B. Then varying amounts of the 9/14 kd protein were added, the concentration of SRP(-9/14) adjusted to 400nM, and the mixtures returned to elevated temperature. The samples were submitted to an elongation arrest assay, and the results are plotted in Figure 2C. The elongation arresting activity increased as the concentration of the 9/14 kd protein increased, and, as expected, reached the same level as that of the complete reconstitute at stoichiometric proportions. We noted, however, that the elongation arresting activity at intermediate concentrations of the 9/14 kd protein is lower than it is at the same concentration of complete particle (compare Figs. 2B and 2C, see below).

We considered the possibility that although SRP(-9/14) did not exhibit a detectable elongation arrest when measured after 60 min of incubation, it might induce a transient one. We have tested this idea by measuring directly the amount of preprolactin synthesized with time in a synchronized translation system. Synthesis was started by addition of mRNA to a prewarmed translation mix, and then synchronized after a 30 sec initiation period by the addition of 7MeG (a cap analog preventing further initiation, see Walter & Blobel, 1981b).

We detected no measurable difference in the rate of preprolactin synthesis with (Fig. 3, closed squares) or without SRP(-9/14) (Fig. 3, open squares); we detected the first completed preprolactin chains at 7

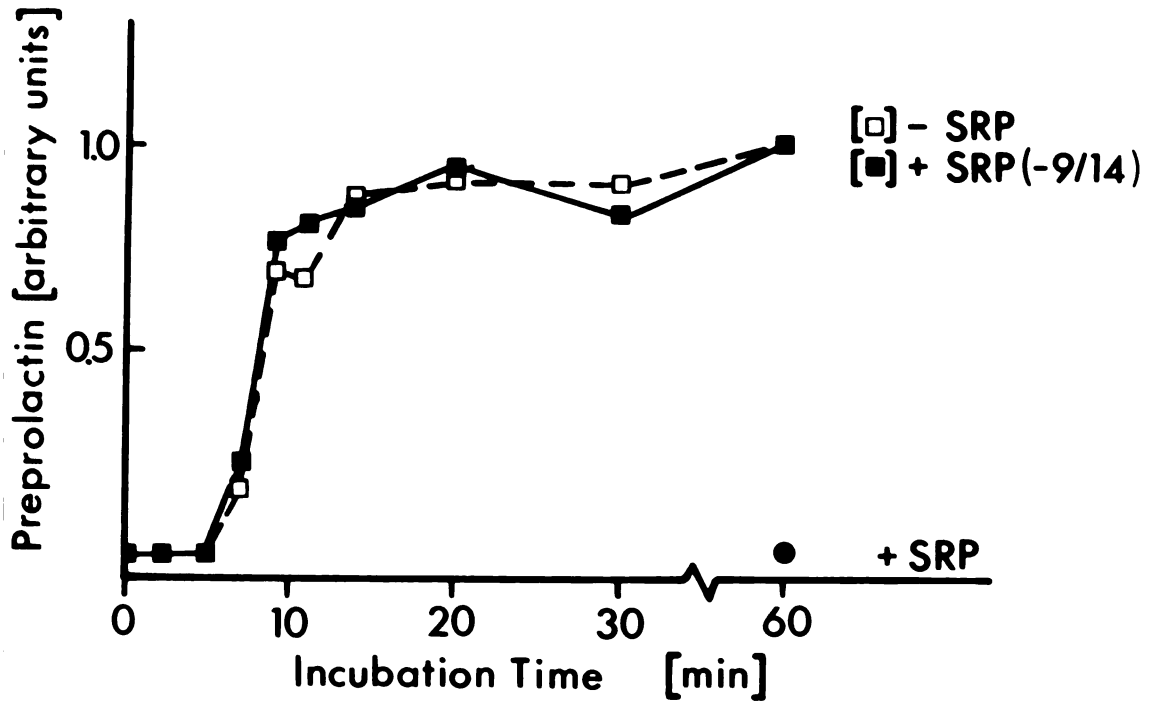
min, and ~70% of the total number of chains at 9 min. In the presence of complete SRP essentially no preprolactin was formed (due to the SRP-mediated elongation arrest) (Fig. 3, closed circle). We concluded from these data that SRP(-9/14) did not measurably delay or arrest protein synthesis, and that therefore elongation arrest was not a prerequisite for the protein translocation demonstrated in Fig. 2A.

SRP and SRP(-9/14) Recognize Nascent Secretory Proteins with Similar Affinity

Although SRP and SRP(-9/14) are qualitatively different with respect to their ability to arrest presecretory protein synthesis, they must share the ability to recognize signal sequences and thus promote protein translocation across the microsomal membrane. We were therefore interested to know whether both particles would recognize signal sequences with equal efficiency. This could be tested by assaying the ability of SRP(-9/14) to compete with SRP and inhibit elongation arrest. Data addressing this question are contained in the experiment described in Fig. 2C. When we demonstrated that arresting activity could be restored by readdition of the 9/14 kd protein to SRP(-9/14), we noted that at intermediate concentrations of 9/14 kd protein, there actually exists a mixture of complete SRP and SRP(-9/14). In this titration, SRP(-9/14) was held constant at 96nM. It follows that at the point where 24nM 9/14 kd protein was added back, we were actually assaying arresting activity of a mixture consisting of 24nM SRP and 72nM SRP(-9/14). Note that at 24nM of 9/14 kd protein (Fig. 2C), the arresting activity of this mixture of SRP and SRP(- 9/14) is 24%, whereas completely reconstituted SRP exhibits 24% inhibition at only 14nM (Fig. 2B). In other words, a three-fold molar excess of SRP(-9/14)

Figure 3. Preprolactin synthesis in a synchronized translation assay

Translation reactions (50 µl) contain SRP (closed circle) or SRP(-9/14) (closed squares) at 96nM, or no SRP (open squares). The amount of preprolactin synthesized at each time point was determined by densitometer scanning of fluorographed gels as described in Materials and Methods.


Preprolactin [arbitrary units]
1.0
0.5
0
10
20
30
60
Incubation Time [min]
[□] - SRP
[■] + SRP(-9/14)
● + SRP

effectively halved the arresting activity of SRP. We conclude from these results that SRP(-9/14) does compete with complete SRP with similar (same order of magnitude) affinity.

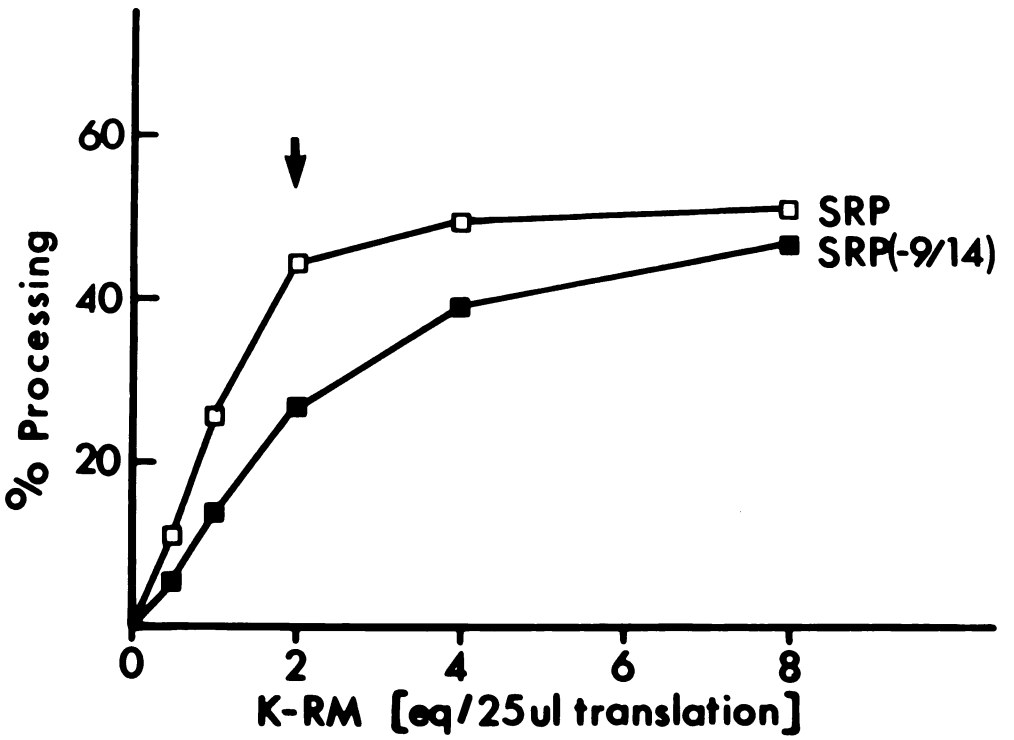
SRP(-9/14)-Mediated Translocation is Time Dependent

The absence of an elongation arrest offered a possible explanation for the decreased translocation activity of SRP(-9/14) (Fig. 2A). We reasoned that now that protein synthesis was no longer arrested, the preprolactin-synthesizing ribosome had only a finite time window within which it must interact with the membrane of the endoplasmic reticulum in order for translocation to occur. If elongation were to proceed too far, then the nascent chain would assume a state that could no longer lead to the formation of a functional ribosome-membrane junction and no translocation would be observed. This hypothesis could be tested by measuring the percent translocation by SRP(-9/14) as a function either of membrane concentration or of the time of membrane addition in a synchronized system.

We titrated SRP-depleted microsomal membranes into a translocation assay, keeping the concentration of SRP (open squares) or SRP(-9/14) (closed squares) constant at 24nM. We observed (Fig. 4) that the processing efficiency of SRP(-9/14) relative to complete SRP increased as the concentration of membranes was increased. If translocation with SRP(-9/14) is dependent on forming a productive membrane junction within a finite period of time, then the translocation capacity of SRP(-9/14) should more closely approach that of SRP as the concentration of membranes is increased, since the probability of forming such a junction within a given time window would be higher. The data in Figure 4 demonstrate that this is indeed the case. In fact, the translocation acti-

Figure 4. Translocation as a function of membrane concentration

Varying amounts of SRP-depleted membranes (K-RM) were added to 25 µl translation reactions containing SRP (open squares), or SRP(-9/14) (closed squares) at 24 nM. translation was for 1 hr at 26°C. Samples were processed and quantitated as described in Materials and Methods. The arrow indicates the "standard" microsome concentration used for the experiments shown in Figure 2, which is saturating for the assay with SRP. For technical reasons we could not test any membrane concentration higher than that of the last point indicated.


60
40
20
% Processing
0
2
4
6
8
K-RM [eq/25ul translation]
SRP
SRP(-9/14)

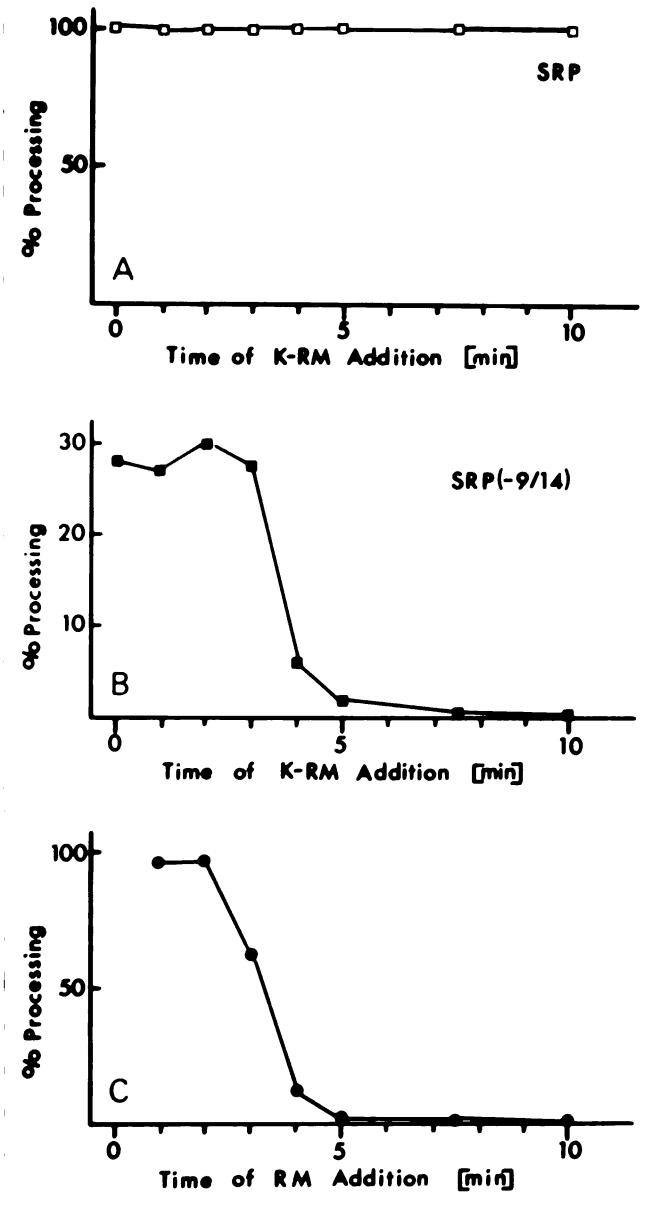
vity of SRP(- 9/14) approached that of SRP at the highest concentration of membranes assayed. We conclude from this result that SRP(-9/14) is fully functional with respect to translocation.

In a second experiment, we tested directly the time dependence of the protein translocation process. In a synchronized translation experiment (see Materials and Methods), microsomal membranes were added at various times after the initiation of protein synthesis, and synthesis was allowed to continue for a total of 30 min.

As expected (Fig. 5A), if SRP was present throughout the translation, membranes could be added at any subsequent time point tested and productive translocation would still occur. On the other hand, if SRP(-9/14) was present (Fig. 5B), processing was strictly dependent on the time at which membranes were added; translocation (measured as percent processing) was decreased by 50% at ~3.6 min, and essentially abolished when microsomes were added after 4 min of elongation. A similar "cut-off time", after which translocation of the nascent chain could no longer take place, was also observed when complete SRP was not present throughout the translation, but rather was added together with microsomal membranes (as rough microsomes) at different time points (Fig. 5C). In this case, percent processing was halved at ~3.3 min, and further elongation beyond this point no longer allowed SRP to recognize and/or promote translocation of the nascent chain. Since preprolactin is fully translated with a $t_{1/2}$ of ~8 min (see Fig. 3), this cut-off time corresponds to a stage in elongation where less than half of a preprolactin molecule has been polymerized, and agrees well with the size of the elongation-arrested fragment of preprolactin synthesized in the presence of SRP (Walter & Blobel, 1981b).

Figure 5. Translocation as a function of time of membrane addition

SRP or SRP(-9/14) was added to 16nM in a 75 µl synchronized translation reaction. At various times, 5 µl aliquots were added to 0.5 µl of microsomal membranes (2 eq/µl) contained in separate tubes. The translations were allowed to proceed for a total of 30 min. Samples were analyzed as described in Materials and Methods. Panel A. SRP present throughout, SRP-depleted microsomes (K-RM) added at different time points. Panel B. SRP(-9/14) present throughout, SRP-depleted microsomes (K-RM) added at different time points. Panel C. No SRP in translation, rough microsomes (containing SRP) added at different time points.


100
50
% Processing
SRP
A
0
5
10
Time of K-RM Addition [min]
30
20
10
% Processing
SRP(-9/14)
B
0
5
10
Time of K-RM Addition [min]
100
50
% Processing
C
0
5
10
Time of RM Addition [min]

We therefore concluded that because SRP(-9/14) failed to arrest nascent preprolactin synthesis and thereby to hold the nascent chain in a translocation-competent state, the efficiency of protein translocation was now the result of a race between protein elongation and the time required to form a functional ribosome-membrane junction.

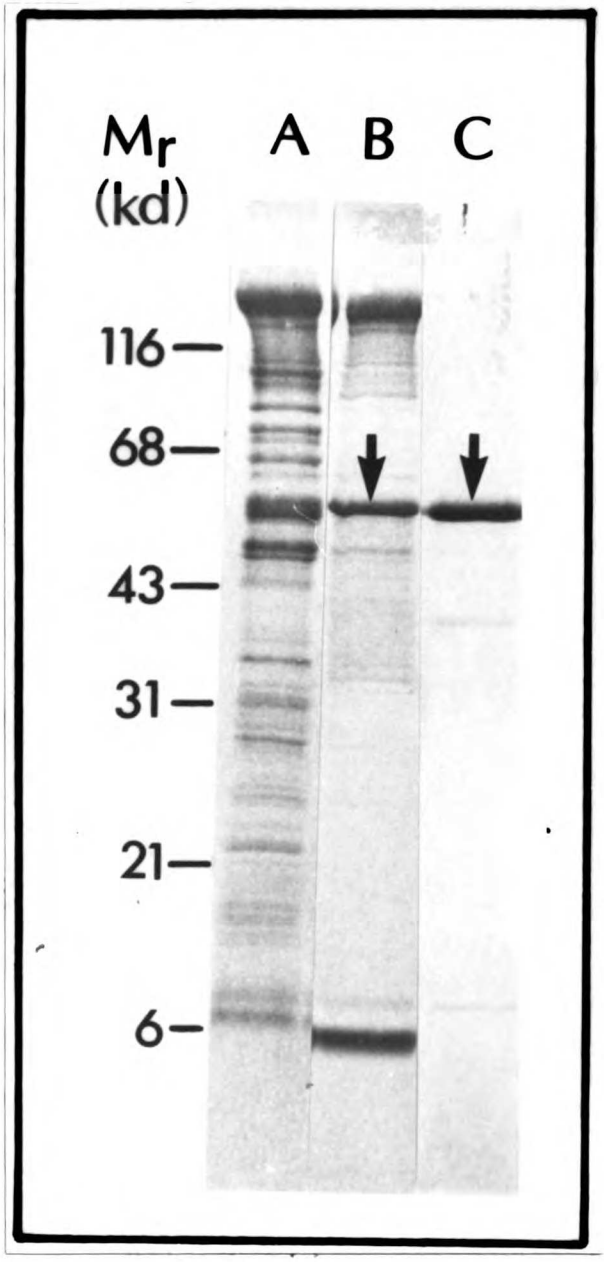
SRP Receptor is Required for Translocation Mediated by SRP(-9/14)

The SRP receptor has been shown to be required for the translocation process in vitro (Gilmore et al., 1982a,b; Meyer et al., 1982a,b), and to release the SRP-induced elongation arrest (Gilmore et al., 1982b; Meyer et al., 1982a). In this context, the interesting question arose as to whether, in a case where translocation occurred without any prior arrest in protein synthesis, mediated by SRP(-9/14), there would still be an absolute requirement for SRP receptor. The assay that allowed us to address this question directly was based on the observation that the cytoplasmic domain of the SRP receptor can be proteolytically severed from the membrane and then added back to it to reconstitute SRP receptor function (Gilmore et al., 1982b).

We purified a 60 kd elastase fragment of SRP-receptor (Fig. 6) as described in Materials and Methods. Adding this pure protein fraction back to trypsinized microsomal membranes allowed us to restore their translocation activity in the presence of SRP (see Table I). Note that neither the purified SRP receptor fragment nor trypsinized membranes alone release the SRP-induced elongation arrest. However, arrest-release can be restored by reconstitution of the receptor fragment with the membranes. In doing such a reconstitution, because the 60 kd receptor fragment is essentially pure, the only activity that has been added back to the membranes is indeed that of SRP receptor. Hence, if

Figure 6. Characterization of the 60 kd elastase fragment of SRP-receptor

The cytoplasmic domain of SRP receptor was purified as described in Materials and Methods. The figure shows a 10-15% SDS polyacrylamide gel stained in Coomassie Blue of the proteolytic extract (lane a), the fraction after CM-Sephadex chromatography (lane b), and the purified 60 kd fragment after sucrose gradient centrifugation (lane c).


Mr
(kd)
A
B
C
116
68
43
31
21
6

the 60 kd fragment is required for SRP(-9/14)-mediated (i.e., elongation arrest independent) translocation across trypsinized membranes, then the SRP-receptor must be utilized in the translocation process.

We compared the translocation activity of SRP and SRP(-9/14) in the presence of i) SRP-depleted membranes, ii) of trypsinized SRP-depleted membranes, and iii) of trypsinized membranes reconstituted with the purified 60 kd SRP receptor fragment (Table I). Neither SRP nor SRP(-9/14) promoted translocation in the presence of trypsinized membranes alone; both did when the 60 kd proteolytic fragment of SRP receptor was added back to the system. These data demonstrate that the SRP receptor is required for protein translocation across microsomal membranes even in the absence of elongation arrest.

DISCUSSION

Our view of ribonucleoproteins has evolved over the years from that of rather static, multicomponent assemblies, to that of incredibly dynamic structures. In particular, studies on the ribosome have revealed that it exhibits many long range interactions. Compared to the ribosome, the signal recognition particle is a rather simple structure, consisting of six polypeptides (organized in four SRP proteins) and one 300 nucleotide RNA molecule. The particle has been purified to homogeneity and the RNA sequenced. Recent EM studes have depicted SRP as a rod-like particle of a length of about 24 nm and a diameter of about 5 nm (Andrews et al., 1985). In spite of this simple composition and the apparently rather extended physical structure of the particle, SRP is also likely to be governed by allosteric interactions between its constituents. This was most clearly demonstrated by the highly cooperative reconstitution of SRP from separated proteins and RNA (Walter &

Table I: SRP receptor is required for translocation mediated by SRP(-9/14).

ADDITIONS			ACTIVITY	
SRP	Membranes	SRP Receptor	% Processing	% Inhibition
--	RM	--	76	< 10
--	K-RM	--	9.6	< 10
--	T5-K-RM	--	1	< 10
SRP	--	--	n.d.	90
SRP	--	60k	n.d.	90
SRP	K-RM	--	94	< 10
SRP	T5-K-RM	--	4	90
SRP	T5-K-RM	60k	75	35
SRP(-9/14)	K-RM	--	47	< 10
SRP(-9/14)	T5-K-RM	--	2	< 10
SRP(-9/14)	T5-K-RM	60k	48	< 10

Wheat germ translation reactions were programmed with pituitary RNA as described in Materials and Methods. SRP or SRP(-9/14) was added to 96nM. Membranes, where added, were at 2 eq (eq is defined in Walter et al., 1981) per 25 µl translation. (RM: rough microsomes; K-RM: SRP-depleted microsomes; T5-K-RM: trypsinized SRP-depleted microsomes, the T5-K-RM fraction used corresponds precisely to that described by Gilmore et al. (Fig. 5 in Gilmore et al., 1982a). 60 kd receptor fragment was added to 27nM (2 µl of a 20 µg/ml solution per 25 µl translation). *In vitro* translations were allowed to proceed for 1 hr at 26°C, and samples were processed and quantitated as described in Materials and Methods. In the reactions where membranes were omitted, percent processing was not determined (n.d.).

Blobel, 1983a).

"Single omission experiments" (the omission of one specific component in the reconstitution of an RNP) have been extensively used to study the function of ribosomes (Nierhaus, 1980, Nomura & Held, 1974, Held et al., 1973, Schulze & Nierhaus, 1982, Tate et al., 1983). In order to perform similar experiments on SRP, we have fully separated the SRP proteins into its four components and have shown these component fractions to be essentially free of cross-contamination. We then used these fractions to assemble partial and complete SRPs, and assayed them in in vitro elongation arrest and in vitro protein translocation assays. The complete particle was active in both assays, indicating that the separation process did not substantially inactivate any of the components.

Single omission experiments, when performed on the ribosome, were often difficult to interpret. Many different proteins, when omitted from the assembly, have been shown to affect the same function, but their omission tended to decrease rather than abolish activity (Nomura & Held, 1974, Held et al., 1973); conversely, a single omission has affected different functions (Nomura & Held, 1974, Held et al., 1973, Schulze & Nierhaus, 1982, Tate et al., 1983). We were not surprised, therefore, to find that omission of an individual SRP protein in the in vitro reconstitution of the particle led in two of the three cases (see Results) to defective SRPs for which we were unable to demonstrate any activity in either of our relatively stringent assays. For both assays we asked that a complex activity, rather than merely binding affinity, be retained. Possible explanations for those negative results are plentiful, and we cannot necessarily conclude that the omitted protein

constitutes an essential functional domain, since its omission could simply result from an overall incorrect or severly altered assembly.

In marked constrast, we discovered that one of the partial particles, SRP(-9/14), was active in the translocation assay, although at a reduced level, under our standard assay conditions, relative to that of the complete particle. Surprisingly, this particle inhibited neither the rate nor the degree of preprolactin synthesis; the decreased translocation activity by SRP(-9/14) could be accounted for by the observation that without elongation arrest, the functional interaction of the polysome with the microsomal membrane had acquired a strict time dependence. In addition, we could more closely approach the translocation activity of complete SRP simply by increasing the membrane concentration. We concluded from this result that we had not in fact effected the translocation activity of the particle by removing these polypeptides. In other words, we have completely abolished one of the assayable activities of SRP (elongation arrest) without noticeably altering the other.

This uncoupling of elongation arrest and translocation by removal of a specific protein domain from SRP has allowed us i) to assign a functional domain to the particle, and ii) to ascertain the dependency relationships of partial reactions occurring in the SRP cycle.

SRP Contains an Elongation Arrest Domain

It seems reasonable that the portion of SRP comprised of the 9/14 kd protein and the RNA it binds to is contained in a structural domain separate from the rest of the particle. Limited micrococcal nuclease digestion of SRP (Gundelfinger et al., 1983) resulted in two separately sedimenting species in sucrose gradients, one containing the 9/14 kd

protein and the Alu portion of 7SL RNA (with its 3' and 5' regions basepaired to each other), and the other containing the remaining proteins and the S fragment of 7SL RNA. Since it is the Alu-like portion of 7SL RNA that interacts with the 9/14 kd SRP protein which in turn is responsible for the elongation arrest activity of SRP, it seems likely that these separate structural domains represent separate functional domains as well. It remains to be tested whether a truncated SRP, lacking both the 9/14 kd protein and the Alu portion of 7SL RNA, like SRP(-9/14), is defective in arrest but not in signal recognition and translocation promoting activity. Furthermore, since the elongation arrest activity of SRP seems to reside in the portion of the molecule containing the Alu-like sequence of 7SL RNA, the provocative possibility arises that Alu transcripts in general may function in some aspect of translational control.

Elongation Arrest is not a Prerequisite for Protein Translocation

Since SRP(-9/14) is fully active in promoting secretory protein translocation but does not measurably arrest presecretory protein synthesis, it follows that elongation arrest is not itself a prerequisite for translocation, but rather, at least in our *in vitro* assays, that elongation arrest increases the efficiency of the translocation process, since it allows for an essentially infinite time window for the attachment of the polysome to the microsomal membrane. We can further conclude that SRP(-9/14) undergoes all biochemical and conformational changes that are necessary for translocation to occur. For example, if an increase in affinity of SRP to the ribosome is a prerequisite to translocation, then this particle must exhibit it; indeed, our data indicate that both SRP and SRP(-9/14) recognize signal peptides with

approximately equal (same order of magnitude) affinities (see Fig. 2 and Results). This indicates that while the temporal sequence of events in the SRP cycle described earlier (see Introduction and Walter et al., 1984) are confirmed, the dependency relationships need to be modified, and in particular elongation arrest is not an obligatory step.

SRP Receptor is Required for Protein Translocation, Even in the Absence of Elongation Arrest

SRP receptor releases the SRP-induced arrest of presecretory protein synthesis. It seemed possible that it was solely on the basis of the arrest-releasing activity that SRP receptor was required for translocation. Since SRP(-9/14) did not arrest protein synthesis, we were able to address the question of whether, in the absence of translation arrest, translocation could occur in the absence of SRP receptor.

Such translocation was not observed (see Table I), and we concluded therefore that SRP receptor was required for translocation, even in the absence of elongation arrest. This absolute requirement for SRP receptor may reflect merely the affinity between SRP and SRP receptor, i.e., that SRP receptor is required solely to correctly target the ribosome to the microsomal membrane. Alternatively, SRP receptor may be involved in the initation of the translocation process itself, either directly or by organizing in its proximity whatever components are required for the translocation process.

In summary, the construction and assays of SRP(-9/14) have enabled us to gain considerable insight not only into structure/function relationships in SRP, but also into the role of the elongation arrest reaction. Given that elongation arrest is not absolutely required for protein translocation *in vitro*, the question about its function and

importance *in vivo* becomes an even more interesting one. Elongation arrest clearly could serve a fidelity function to prevent synthesis of precursors in the cytoplasmic compartment, and in addition to improve the efficiency of the translocation reaction by retaining the nascent chain in a translocation competent state. It may have been added as an evolutionary refinement to a more primitive SRP cycle, and in this regard it may be significant that arrest resides in a separate RNP domain. Elongation arrest could also be exploited as a regulatory step, providing the cell with a fast, possibly selective, on/off switch modulating specific secretory or membrane protein synthesis at the level of elongation.

There is some evidence that elongation arrest may be variable among different secretory or membrane proteins. For example, at a given SRP concentration preprolactin synthesis is arrested better than pregrowth hormone synthesis (P. Garcia & P. Walter, unpublished), which in turn is arrested better than ovalbumin, a secretory protein containing an uncleaved signal sequence. Also, a variety of membrane proteins containing uncleaved signal sequences failed to show demonstrable arrest *in vitro* (Anderson et al, 1983). It is possible that the nascent chain itself plays a direct role in elongation arrest; alternatively, it may affect the affinity of SRP for the ribosome, and thus indirectly affect both elongation arrest and translocation. Using SRP(-9/14) to compare the translocation efficiencies of these proteins in a system where elongation arrest is not a factor will enable us to determine at what level this variability is exerted.

CHAPTER 3

SIGNAL RECOGNITION PARTICLE WILL PROMOTE SECRETORY PROTEIN TRANSLOCATION AFTER THE REMOVAL OF A STRUCTURAL DOMAIN CONTAINING THE ALU-LIKE SEQUENCES OF 7SL RNA

ABSTRACT

Micrococcal nuclease digestion of signal recognition particle (SRP) cleaves the 7SL RNA molecule into five distinct fragments, one of which contains the entire unique S sequence of 7SL RNA and virtually none of the 150 nucleotides which are homologous to the human Alu sequences (Gundelfinger et al., 1983). We have purified from micrococcal nuclease treated material a subparticle of SRP which contains only the S fragment of 7SL RNA. It contains the 72/68 kd, 54 kd, and 19 kd proteins tightly bound, but lacks the 9/14 kd protein. In vitro activity assays demonstrated that the subparticle could still promote secretory protein translocation across the microsomal membrane, but could no longer trigger an arrest of presecretory protein synthesis. Thus the subparticle is phenotypically similar to a partial SRP composed of intact 7SL RNA that is lacking the 9/14 kd protein (Siegel & Walter, 1985 (Chapter 2)). In contrast to this partial reconstitute, however, the addition of even a 10-fold molar excess of the 9/14 kd protein to the subparticle did not restore the elongation arrest. We conclude that the region of SRP comprised of the Alu-like RNA and the 9/14 kd protein exists in a distinct structural domain which is not required for the protein translocation promoted by SRP but apparently confers elongation arresting activity to the particle.

INTRODUCTION

Alu-like elements comprise the most abundant family of interspersed repetitive sequences in primates and rodents (Schmid & Jelinek, 1982), with about 500,000 copies per human genome. Human Alu DNA is a head to tail dimer of two similar sequences of ~130 bp long, the right monomer of which contains an insert not present in the left monomer. Alu-like

elements contain many features of processed genes (Sharp, 1983), suggesting that they were initially derived by reverse transcription of processed RNA transcripts. Transcripts which contain Alu family members are represented in hnRNAs, cytoplasmic mRNAs, and small RNAs (Schmid & Jelinek, 1982; Calabretta et al., 1981). Although nothing is known about their function, it is possible that some of the RNA transcripts containing these elements have retained activities of their parent RNA. There is strong evidence based on evolutionary grounds (Walter & Blobel, 1982; Ullu & Tschudi, 1984) that the parent RNA for the Alu-like elements is the highly conserved 7SL RNA, which is an essential component of signal recognition particle (SRP), a small cytoplasmic ribonucleoprotein. SRP functions in the targeting of nascent secretory and membrane proteins to the rough endoplasmic reticulum (for review see Walter et al., 1984).

7SL RNA is composed of both unique and Alu-like sequences (Ullu et al., 1982). Approximately 100 nucleotides at the 5' end and 45 nucleotides at the 3' end are homologous to the human Alu right monomer sequence, and, in proposed secondary structures, the 5' and 3' segments base pair with each other such as to form one end in the folded RNA (see below). The central "S segment" of 155 nucleotides shows no homology to Alu DNA and is unique to 7SL RNA. Structurally, SRP is a rod-shaped particle (Andrews et al., 1985) which, in addition to its RNA, contains 4 proteins (two monomers composed of a 19 kd and a 54 kd polypeptide, and two heterodimers, one composed of a 9 kd and a 14 kd polypeptide, and the other composed of a 68 kd and a 72 kd polypeptide, respectively) (Walter & Blobel, 1980, 1983a). The RNA moiety of the particle has been shown to be required for SRP activity, as well as for structural inte-

grity of the particle (Walter & Blobel, 1982). In order to address the question of whether the Alu-like segments of 7SL RNA have a specific role in SRP activity, we have purified and analyzed a subparticle of SRP that is created upon extensive digestion with micrococcal nuclease and is entirely lacking the Alu-like sequences.

MATERIALS AND METHODS

Purification of SRP(S)

SRP(S) was purified after micrococcal nuclease digestion by a combination of DEAE-Sepharose chromatography and sucrose gradient sedimentation as follows. Various steps in the purification are shown in Figure 1.

Gradient purified SRP (200 µg in 1 ml, purified from canine pancreas as described (Walter & Blobel, 1983c) was digested with 4,000 units of Staphylococcus aureus nuclease (Boehringer) in a solution containing 50mM triethanolamine/HOAc, pH 7.5, 250mM KOAc, 2mM Mg(OAc)2, 1mM $CaCl_2$, 1mM dithiothreitol (DTT), and 0.01% Nikkol (octa-ethyleneglycol-mono-n-dodecyl ether, Nikko Chemicals, Japan). The digestion was allowed to proceed for 1 hr at 30°C and was then stopped by the addition of EGTA to 3.8mM. Panel A of Figure 1 shows an ethidium bromide stained 7M urea/ 6% polyacrylamide gel of RNA prepared from SRP incubated without and with micrococcal nuclease. Note that the major product of the digestion is the 150 nucleotides long "S" fragment.

The remainder of the purification was performed at 0-4°C. The nuclease digested material was loaded directly onto a 250 µl DEAE-Sepharose CL-6B column. The column was washed with ten column volumes of 20mM Hepes/KOH, pH 7.5, 2mM $Mg(OAc)_2$, 2mM EGTA, and 0.01% Nikkol (Buffer A), containing 250mM KOAc. It was then sequentially eluted with

530 µl aliquots of Buffer A containing the following concentrations of KOAc: 480mM, 800mM, and 1000mM. One 80 µl fraction and six 125 µl fractions were collected at each step. Fractions 2,3, and 4 were pooled, and the protein and RNA components displayed by polyacrylamide gel electrophoresis followed by Coomassie Blue or ethidium bromide staining, respectively. Panels B and C of Figure 1 show the RNA and protein compositions of the eluted fractions at the indicated KOAc concentrations. Note that the S fragment elutes at a lower ionic strength than any of the other fragments, probably as a result of the particle having a higher protein to RNA ratio. All four proteins are present in both the 480mM and the 800mM salt steps, although the 9/14 kd protein is present in lower amounts in the 480mM step than in the starting SRP, and no protein is detectable in the 1000mM step (see below).

The peak from the 480mM KOAc elution was loaded onto a 5-20% sucrose gradient (12 ml) in 20mM Hepes/KOH, pH 7.5, 500mM KOAc, 5mM $Mg(OAc)_2$, 1mM EGTA, 1mM DTT, and 0.01% Nikkol. The gradients were centrifuged in a Beckman SW40 swinging bucket rotor for 24 hr at 40,000 rpm. Gradients were fractionated with an Isco Gradient Fractionator. 0.5 ml fractions were collected. Panel D of Figure 1 shows the absorbance profile of the gradient monitored at 254 nm. The left side is the top of the gradient.

Panel E shows the polypeptide profile of an analytical gradient spun and fractionated under identical conditions as above, but using as starting material in the preparation SRP that had been radioactively labelled with ^{125}I-Bolton Hunter reagent (Siegel & Walter, 1985). The polypeptide components of each of the fractions were separated by SDS-

PAGE on 10-15% gradient gels and visualized by autoradiography. A preparative gradient run in parallel was monitered by SDS-PAGE and Coomassie Blue staining and shows an identical profile. Note that the 9/14 kd protein stays at the top of the gradient, whereas the 68/72 kd, 54 kd, and 19 kd proteins cosediment with the RNA peak. This indicates that during the fractionation on the DEAE-Sepharose column either that the 9/14 kd protein gratuitously co-eluted at the same ionic strength as SRP(S), or that the protein bound to the particle, but with a substantially decreased affinity that led to its dissociation on the sucrose gradient. The small amount of 54 kd protein that dissociated from SRP(S) seems not to result from the nuclease treatment, as a similar fraction of the 54 kd protein sediments at this position when intact labelled SRP is fractionated on a sucrose gradient. The 19 kd protein shows the same relative intensity compared to the higher molecular weight polypeptides as in the starting material (data not shown, but a similarly labelled SRP can be seen in Fig. 1 of Chapter 2), indicating that the 19 kd protein remains bound stoichiometrically. This is seen much more clearly in Panel F, where the peak fraction from the sucrose gradient shown in Panel D is visualized by SDS-PAGE followed by staining with Coomassie Blue.

No protein could be detected in the 1000mM salt eluate from the DEAE-Sepharose column, which is the step in which the Alu-like RNA elutes (Fig. 1, Panels B and C). It is possible, however, that the RNA-protein complex is unstable at the high salt concentrations used in the elution procedure. Indeed, when the peak from the 800 mM salt step was subjected to sucrose gradient analysis (data not shown), the 9/14 kd protein cosedimented with the fragments of Alu-like RNA in that fraction.

The RNA from SRP(S) was isolated by phenol/chloroform extraction and ethanol precipitation. Its 5' end was labelled with γ-^{32}P-ATP using T4 polynucleotide kinase and its length determined on polyacrylamide sequencing gels (data not shown). The major species were 149 and 150 nucleotides long with minor species which were 147 and 148 nucleotides long. Using an oligonucleotide complementary to a sequence within the S segment, the precise 5' end of the fragment was determined by primer extension (data not shown). It occurs predominantly at A102, and to a minor degree at G101 and T103. These analyses define both boundaries of the fragment and are indicated schematically in Fig. 4; they coincide almost precisely with those of the S segment defined by boundaries of homology (see Fig. 4). The primer extension experiment also confirmed that the SRP(S) preparation was not contaminated with undigested RNA.

Activity Assays

SRP or SRP(S) was titrated into wheat germ *in vitro* translations (Erickson & Blobel, 1983) that were programmed with a mixture of pituitary RNA, coding primarily for the secretory protein preprolactin, and reticulocyte RNA, coding primarily for the cytoplasmic protein globin. One set of titrations also contained SRP- depleted microsomal membranes (Walter & Blobel, 1983b) at 2 eq. per 25 µl translation, a concentration that had previously been shown to saturate the translocation activity (measured by percent processing from preprolactin to prolactin) of a given concentration of SRP (Siegel & Walter, 1985). Translation reactions were incubated at 26°C for 1 hr and stopped by chilling on ice followed by TCA precipitation. Samples were processed and percent processing and percent inhibition quantitated as described (Siegel & Walter, 1985).

Synchronized translation assays

SRP, SRP(S), or SRP(S) supplemented with 9/14 kd protein (see Results) were added to a wheat germ *in vitro* translation reaction at a final concentration of 14 nM. One tube contained no SRP. The translations were prewarmed to 26°C for 2 min and then a mixture of pituitary and reticulocyte RNA were added to initiate protein synthesis. After 45 sec, all further initiation was blocked by the addition of 7-methylguanosine-5'-monophosphate to a final concentration of 4mM. At various times thereafter, 5 µl aliquots were added either to 5 µl of 20% TCA, to measure preprolactin synthesis, or to 0.5 µl of SRP-depleted microsomal membranes, to measure translocation as a function of the time of membrane addition. In the case of the membrane addition experiments, synthesis was allowed to continue for a total of 20 min. Samples were processed as above except that gels were subjected to fluorography prior to autoradiography.

Sequence determination of canine 7SL RNA

We sequenced canine 7SL RNA by a combination of RNA sequencing (Walter & Blobel, 1982) and primer extension in the presence of dideoxy nucleotides (data not shown). The sequence differs from human 7SL RNA (Ullu et al., 1982) at only four positions. The nucleotide present in the canine RNA at these positions is as follows: C19, A53, C69, and C87. None of these differences alter the predicted secondary structure.

RESULTS

It had previously been shown that the RNA in SRP can be rather selectively cleaved with micrococcal nuclease such that the S segment remains largely intact and is separated from the Alu-like segments (Gundelfinger et al., 1983). SRP was digested with a 10-fold molar

excess of micrococcal nuclease to yield five major RNA fragments (Fig. 1A). We employed DEAE-Sepharose chromatography and sucrose gradient sedimentation to purify to homogeneity a subparticle containing the major 150 nucleotide fragment (see Fig. 1). We demonstrated that the sucrose gradient fraction containing this fragment was pure with respect to RNA content both by gel analysis of end-labelled RNA and by primer extension to map the 5' end of the molecule (see Materials and Methods); we found that a single RNA was present in this fraction and that it consisted entirely of the unique S sequences of 7SL RNA. In addition, the fraction contains the 68/72 kd, 54 kd, and 19 kd proteins, but has lost the 9/14 kd protein. We shall henceforth refer to the subparticle as SRP(S).

SRP has been shown to have two separable activities which can be assayed in a wheat germ cell free translation system (Walter & Blobel, 1981b; Walter et al., 1984). First, it promotes the translocation of secretory proteins across the membrane of SRP-depleted microsomes ("translocation promoting" activity), which can be evidenced by signal peptidase cleavage; without SRP, such translocation and processing do not occur. Second, SRP selectively inhibits the synthesis of presecretory proteins at the level of elongation ("elongation arresting" activity).

We have measured the translocation promoting and elongation arresting activities of SRP(S). The data in Figure 2 demonstrate that SRP(S) was active in promoting the translocation of preprolactin across the microsomal membrane (Panel A), although at ~40% the efficiency of the same concentration of undigested SRP. At this point in time we do not know whether the decreased activity is a result of an inactivation that

Figure 1. Purification of SRP(S).

See Materials and Methods.

(A) 200 µg gradient purified SRP was digested with 4,000 units of Staphylococcus aureus nuclease for 1 hr at 30°C. Shown here is an ethidium bromide stained urea/polyacrylamide gel of RNA prepared from SRP incubated without and with micrococcal nuclease. Note that the major product of the digestion is the 150 nucleotides long "S" fragment. Micrococcal nuclease digested SRP was loaded directly onto a 250 µl DEAE-Sepharose CL-6B column and eluted with the following steps of KOAc: 480mM, 800mM, and 1000mM.

(B) The RNA composition of the eluted fractions at each step.

(C) The protein composition of the eluted fractions at each step.

The peak from the 480mM KOAc elution was loaded onto a 5-20% sucrose gradient (12 ml) and centrifuged in a Beckman SW40 swinging bucket rotor for 24 hr at 40,000 rpm.

(D) The absorbance profile of the gradient monitored at 254 nm. The left side is the top of the gradient.

(E) The polypeptide profile of an analytical gradient spun and fractionated under identical conditions as above, but using as starting material in the preparation SRP that had been radioactively labelled with ^{125}I-Bolton Hunter reagent.

(F) The peak fraction from the sucrose gradient shown in Panel D is visualized by SDS-PAGE followed by staining with Coomassie Blue. Approximate length in nucleotides of the RNA fragments are indicated alongside the figures in Panels A and B. Positions of the six SRP polypeptides are indicated by lines in Panels C, E, and F, and their approximate molecular weights in kd are given in Panel C.

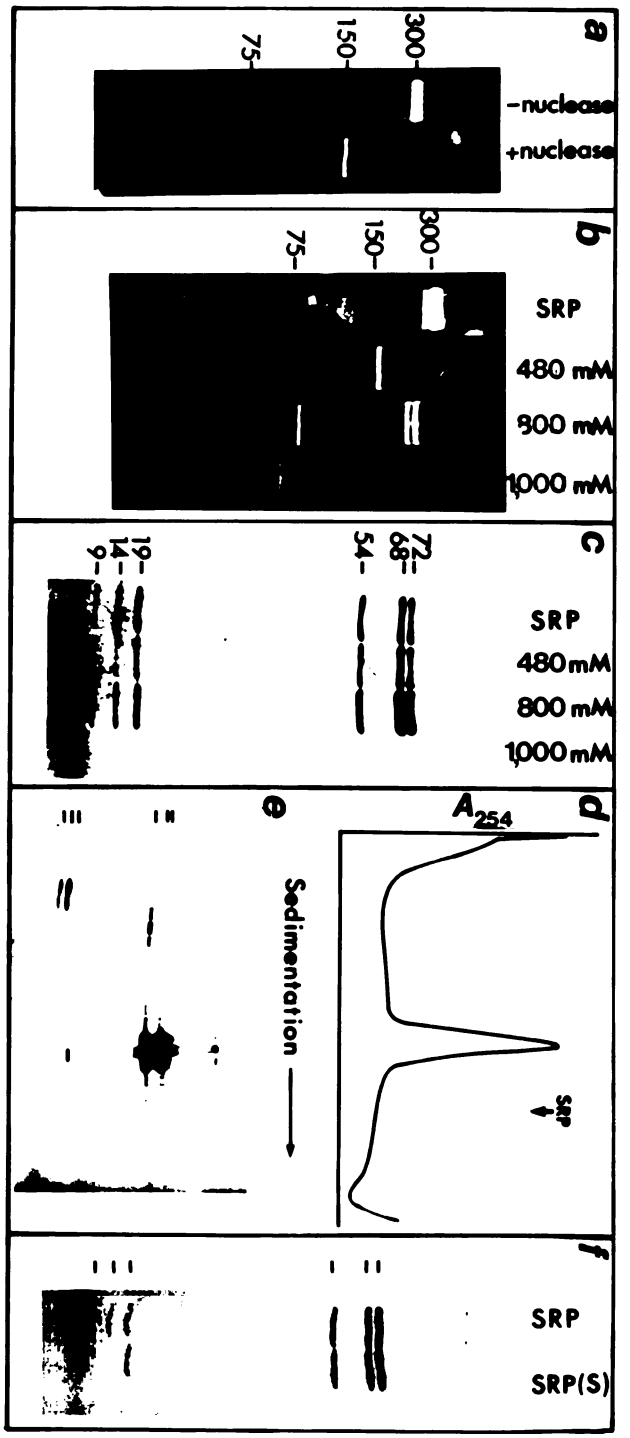
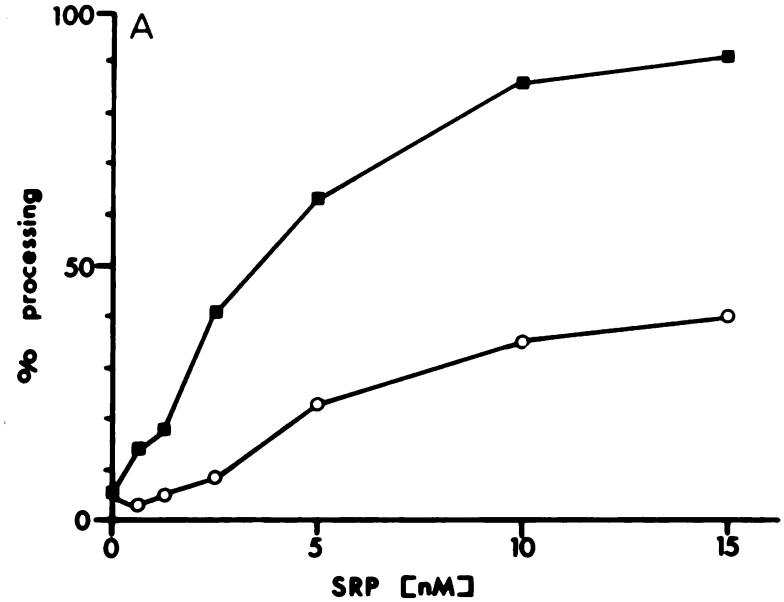

a
75-
150-
300-
-nuclease
+nuclease
b
75-
150-
300-
SRP
480 mM
800 mM
1000 mM
c
19-
14-
9-
72-
68-
54-
SRP
480 mM
800 mM
1000 mM
d
A_{254}
Sedimentation
SRP
e
f
SRP
SRP(S)

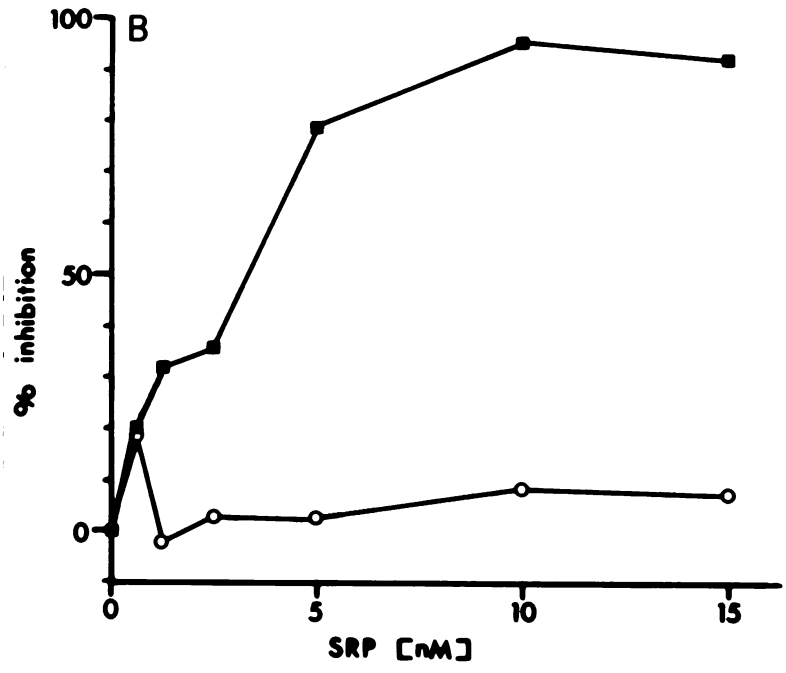
Figure 2. Activity assays for SRP and SRP(S).

SRP (closed squares) or SRP(S) (open circles) was titrated into wheat germ in vitro translations (Erickson & Blobel, 1983) that were programmed with a mixture of pituitary and reticulocyte RNA. The set of titrations shown in Panel A also contained SRP- depleted microsomal membranes (Walter & Blobel, 1983b) at 2 equivalents per 25 µl translation. Translation reactions were incubated at 26°C for 1 hr and stopped by chilling on ice followed by TCA precipitation. Samples were processed and percent processing and percent inhibition quantitated as described (Siegel & Walter, 1985).

(A) Percent processing as a function of SRP or SRP(S) concentration.

(B) Elongation arrest as a function of SRP or SRP(S) concentration.


A
100
50
0
% processing
0
5
10
15
SRP [nM]


B
100
50
0
% inhibition
0
5
10
15
SRP [nM]

occurred during the additional fractionation, or if it is an intrinsic property of the subparticle. In marked contrast to its ability to promote secretory protein translocation, however, SRP(S) was completely unable to trigger a detectable arrest of preprolactin synthesis (Panel B). These results are further confirmed by the data shown in Figure 3.

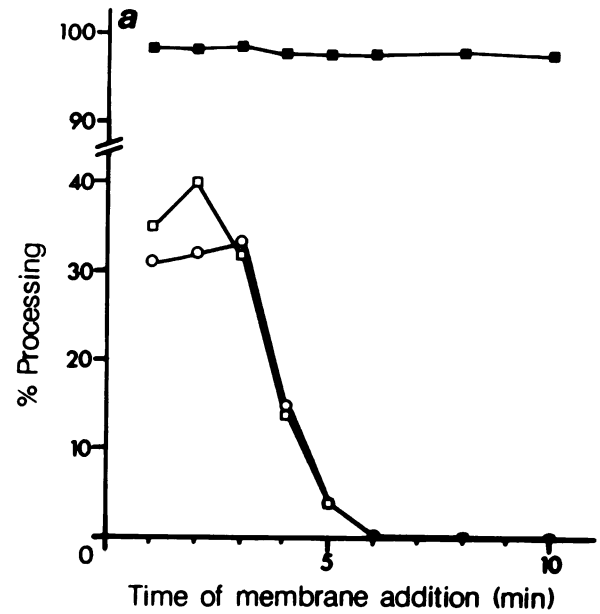
Translocation across the membrane of the endoplasmic reticulum appears to occur in a co-translational manner, and the elongation-arresting activity of SRP has been shown in vitro to maintain the nascent chain in a translocation competent state; i.e., if SRP was present throughout the experiment (Fig 3A, closed squares), because of its elongation arresting activity, membranes could be added at any time point tested and productive translocation would still occur. However, if SRP(S) was present (Fig. 3A, open circles), processing was strictly dependent on the time at which membranes were added; translocation (measured by percent processing) was reduced 50% at ~3.9 min, and was completely abolished when membranes were added after 5 min of elongation. This corresponds to a stage of elongation in which approximately half of the preprolactin molecule has been polymerized (see Panel B) and agrees well with the size of the arrested fragment synthesized in the presence of SRP (Walter & Blobel, 1981b). Thus SRP(S) lacks the elongation arresting activity required to maintain the translocation competence of the nascent chain. Indeed, by measuring the appearance of preprolactin in a synchronized system (Fig. 3B), we have shown that SRP(S) (open circles), again in marked contrast to intact SRP (closed square), causes no detectable delay in the synthesis of the presecretory protein when compared to a translation reaction in which no SRP was added (closed circles).

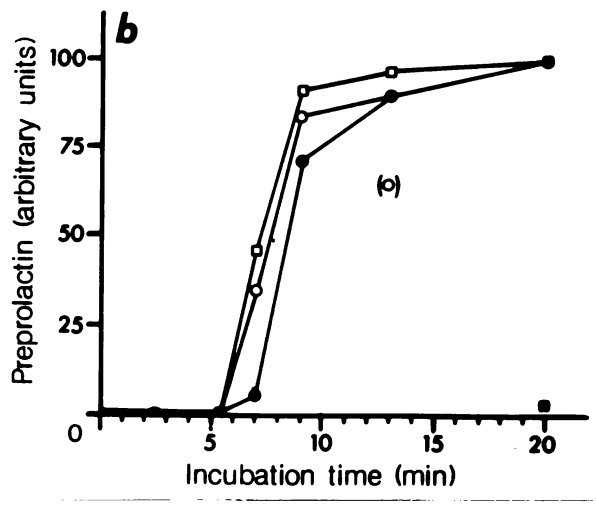
Figure 3. Synchronized translation assays.

SRP (closed squares), SRP(S) (open circles), or SRP(S) supplemented with 9/14 kd protein (open squares) (see below) were added to a synchronized wheat germ *in vitro* translation reaction at a final concentration of 14 nM. One tube contained no SRP (closed circles). At various times, 5 µl aliquots were added either to 5 µl of 20% TCA, to measure preprolactin synthesis, or to 0.5 µl of SRP-depleted microsomal membranes, to measure translocation as a function of the time of membrane addition.

(A) Percent processing as a function of the time of membrane addition.

(B) Preprolactin synthesis as a function of time.


a
100
90
40
30
20
10
0
5
10
% Processing
Time of membrane addition (min)


b
100
75
50
25
0
5
10
15
20
(o)
Preprolactin (arbitrary units)
Incubation time (min)

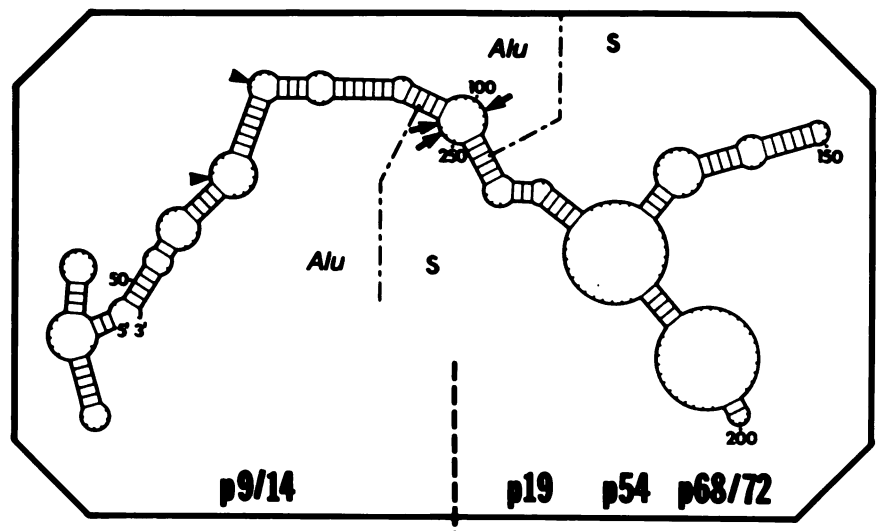
In all these respects, the subparticle is phenotypically similar in activity to an SRP composed of intact 7SL RNA and lacking the 9/14 kd protein ("SRP(-9/14)" was created by reconstituting SRP from purified components and omitting the 9/14 kd protein, as discussed in Siegel & Blobel, 1985). Because the purified SRP(S) also no longer contained the 9/14 kd protein, we expected its loss of elongation arresting activity. We were surprised, however, to find that a full half of 7SL RNA appears to be dispensable as well.

It seemed possible that SRP(S) retained some, albeit decreased, affinity for the 9/14 kd protein (see Fig. 1), and that by supplementing the subparticle with an excess of this protein, we might be able to restore the elongation arresting activity. The 9/14 kd protein was preincubated with SRP(S) under ionic conditions used to reconstitute SRP from RNA and proteins (Siegel & Walter, 1985) in order to facilitate any functional rebinding of the 9/14 kd protein to SRP(S). We found that, in contrast to the case of SRP(-9/14), addition of even a 10-fold molar excess of the 9/14 kd protein to SRP(S) in no way restored the elongation arrest activity, as evidenced from the rate and the degree of preprolactin synthesis (Fig. 3B, open squares), or from the time window within which membranes could still be added for translocation to occur (Fig. 3A, open squares).

We conclude that SRP(S) contains all the essential components involved in signal recognition and the promotion of secretory protein translocation, and that the 9/14 kd protein and the Alu-like RNA exist in a separate and separable structural domain which, although not required for the protein translocation stimulated by SRP, confers elongation arresting activity to the particle (Fig. 4). In addition, we

Figure 4. SRP is composed of two separable domains.

A possible secondary structure for human 7SL RNA is shown (similar secondary structures have been proposed by Gundelfinger et al. (1984), E. Ullu (personal communication) and C. Zwieb (1987). Connecting lines indicate base pairs; G-U base pairs are included. Micrococcal nuclease cleaves the particle at the arrows, removing the elongation arrest-promoting domain, which is comprised of the Alu-like RNA and the 9/14 kd protein (the thin dashed lines indicate the boundaries of homology (Ullu & Tschudi, 1984; Ullu et al., 1982)). SRP(S) retains signal recognition and protein translocation promoting functions. The major 5' and 3' ends of the S fragment are indicated by arrows. Additonal cuts by micrococcal nuclease at positions determined by Gundelfinger et al. (1983) yield the RNA fragments shown in Figure 1A and are indicated by arrow head.



Elongation arrest domain

Signal recognition domain

SRP(S)

have shown that regions required for the tight binding of the 9/14 kd protein must be contained within the Alu-like segments of 7SL RNA (see legend to Fig. 1). Because portions of the Alu-like segments were degraded by the micrococcal nuclease, we were not able to test whether this domain has any activity by itself, or whether it could restore elongation activity when added "in trans" to SRP(S).

DISCUSSION

7SL RNA has been highly conserved through evolution (Ullu & Tschudi, 1984) across the entire length of the molecule and *Drosophila* and human 7SL RNA can be folded into phylogenetically conserved secondary structures. This implies that "Alu" sequences, although not present as a repetitive genomic sequence family in *Drosophila*, are part of *Drosophila* 7SL RNA. The dispensability of the "Alu domain" *in vitro* therefore raises the question of its role *in vivo*. We have shown here that once SRP has been assembled, this portion of the particle can be removed and the subparticle retains function. It is possible, then, that the Alu-like sequences are required for folding or stabilizing the RNA in the secondary structure necessary for proper assembly. This is plausible because in the proposed secondary structure of 7SL RNA (see Fig. 4), the Alu-like RNA portion contributes substantially to the folding energy of the molecule by forming an extended stem structure. Because Alu-like elements contain RNA polymerase III promoter function (Sakonju et al., 1980), another not necessarily exclusive possibility is that these sequences are required for the transcription of 7SL RNA. Alternatively, these sequences may be conserved because it is advantageous for the particle *in vivo* to retain elongation arresting activity, either because arrest serves a fidelity function, preventing synthesis

of preproteins in the cytoplasmic compartment, or because it can be exploited as a regulatory step, providing the cell with a means of selectively controlling secretory or membrane protein synthesis at the level of elongation. Thus far, however, elongation arrest has only been documented in the heterologous wheat germ in vitro translation system. Attempts to demonstrate elongation arrest in heterologous systems comprised of mammalian components have failed (Meyer, 1985). It should be noted, however, that no mammalian system studied thus far has responded to the addition of exogenous SRP either by elongation arrest or by increased levels of translocation; elongation arrest in these systems may become apparent once they are dependent on exogenously added SRP. Ultimately, however, due to the in vitro nature of these approaches, the relevance of elongation arrest still remains to be demonstrated in vivo.

Alu-like sequences are present in other cytoplasmic RNAs (Schmid & Jelinek, 1982; Calabretta et al., 1981) (e.g., the 3' untranslated region of the LDL receptor mRNA (Yamamoto et al., 1984). It is possible that these sequences, now out of the context of SRP RNA, form similar structural domains which could bind the 9/14 kd or related proteins. Indeed, two of the Alu sequences in the LDL receptor mRNA can be folded into secondary structures closely resembling the Alu "end" of 7SL RNA (P. Walter, unpublished). If these domains are functional, then in analogy to their role in SRP, Alu transcripts that are transported to the cytoplasmic compartment may play some role in translational control.

CHAPTER 4

BIOCHEMICAL MUTAGENESIS OF SRP:

ASSIGNMENT OF FUNCTION TO PROTEIN DOMAINS

ABSTRACT

Signal recognition particle (SRP), a small ribonucleoprotein composed of six distinct polypeptides and one molecule of small cytoplasmic 7SL RNA, has been shown to facilitate ribosome-coupled targeting of secretory proteins to the endoplasmic reticulum (ER) membrane of higher eukaryotic cells. It performs this function as the result of at least three distinct activities that can be assayed in vitro: i) high affinity binding to ribosomes synthesizing secretory proteins (signal recognition); ii) elongation arrest of presecretory nascent chains; and iii) targeting of secretory proteins to the ER membrane. Recently it has become possible to separate SRP into its constituents and then recombine them to form fully active SRP. In single omission experiments, it was shown that the 9/14 kd protein of SRP is required for its elongation arrest activity.

SRP has been shown to be exquisitely sensitive to the sulfhydryl alkylating reagent N-ethylmaleimide (NEM). We have taken advantage of the disassembly and reconstitution protocol to assemble particles in which a single protein component has been modified with NEM. In this way we hoped to inactivate only those functions for which the modified component was responsible. SRP(68/72^{N}), the particle containing a modification on the 68/72 kd protein, was active in elongation arrest, but was completely inactive in promoting translocation. SRP(54^{N}) has lost the signal recognition function. With this approach, we have been able to demonstrate the requirement for a protein component for each of the activities exhibited by SRP, and to map each of the activities to specific protein domains.

INTRODUCTION

The role of SRP in the targeting of nascent secretory proteins to the endoplasmic reticulum has been reviewed earlier (see Chapter 1). Three distinct activities of the particle can be assayed in the wheat germ cell free translation system. The first activity is "signal recognition," by which SRP binds to ribosomes that are synthesizing secretory proteins with high affinity (~10^{-9}M, Walter et al., 1981), and which can be measured in cosedimentation experiments. Concomitant with "signal recognition," SRP specifically blocks further elongation of the presecretory nascent chain (Walter & Blobel, 1981b). This elongation arrest can be assayed in vitro as an inhibition of full-length presecretory chain synthesis, and as the appearance of an "arrested fragment" in synchronized translation systems. Finally, SRP "promotes translocation" (Walter & Blobel, 1980); this activity can be measured in a number of ways, such as the removal of the signal sequence from presecretory proteins by signal peptidase, a lumenal ER protein (signal cleavage), or cosedimentation of nascent or full length secretory proteins with microsomal membranes, or protection of these proteins from exogenously added proteases.

SRP is comprised of a number of different components. It contains four different proteins (two monomers composed of a 19 kd and a 54 kd polypeptide, and two heterodimers, one composed of a 9 kd and a 14 kd polypeptide, and the other composed of a 68 kd and a 72 kd polypeptide, respectively), and one 300 nucleotide molecule of 7SL RNA (Walter & Blobel, 1983a, Chapter 2). The RNA is composed of both unique and repetitive sequences (Ullu et al., 1982). Approximately 100 nucleotides at the 5' end and 45 nucleotides at the 3' end are homologous to the

human Alu right monomer sequence. The central "S" segment of 155 nucleotides shows no homology to Alu DNA and is unique to 7SL RNA.

It has been our goal to understand the structural and functional organization of SRP, i.e., the relationship between its multiple components and its multiple activities. Our approach has been to create biochemically the equivalent of mutations in the particle; our hope was to create particles that were lacking a single activity, and in so doing i) to assign functional domains on the particle, and ii) to address questions concerning the dependency relations of the various SRP activities that could be assayed in vitro.

We described in Chapters 2 and 3 two such "mutations." In Chapter 2, we described experiments in which protein and RNA components were fully fractionated into five homogeneous subfractions. Because fully active SRP was obtained upon recombining these fractions, we could ask what would happen if one of the five fractions was left out of the reconstitution protocol. In four out of five cases, the particle obtained was completely inactive. However, in one case, we obtained a particle, SRP(-9/14), which could promote translocation even though it had completely lost its elongation arrest activity. We learned from these experiments that elongation arrest was not a prerequisite for protein translocation and that SRP receptor was required for protein translocation even in the absence of elongation arrest.

In Chapter 3, we described the use of micrococcal nuclease to generate a subparticle, which was missing, in addition to the 9/14 kd protein, essentially all of the Alu-like sequences of the RNA. We found that this subparticle, SRP(S), was stable to ion-exchange chromatography and sucrose gradient sedimentation and thus could be thought of as an

independent structural domain in the particle. SRP(S) had the same activity as SRP(-9/14), namely, it was active in promoting translocation but could not induce an elongation arrest. Thus in our model of SRP, we have divided it into two domains: an "Alu domain", which contains the elongation arrest activity of SRP, and an "S domain", which contains its signal recognition and protein translocation functions.

In assigning the other known functions of SRP to domains on the particle, these two approaches had failed. However, single omission and nuclease digestion represent rather severe structural alterations of the particle, and it is not surprising that in most cases we have simply inactivated it. For this reason, we sought a way to introduce more subtle domain specific lesions into SRP.

We chose for this approach the sulfhydryl specific alkylating agent N-ethylmaleimide (NEM). SRP was known to be exquisitely sensitive to this agent (Walter & Blobel, 1980), losing even its ribosome binding activity (Walter et al., 1981). Furthermore, when ^{3}H-NEM was used to label SRP, several of the polypeptides were labelled (Walter & Blobel, 1980). It was our hope that if we could modify specific proteins in SRP with NEM, then we would inactivate only the functions encoded by the modified protein.

MATERIALS AND METHODS

Materials

^{125}I-Bolton-Hunter reagent (diiodo form, 4000 Ci/mmol) was purchased from Amershem; ^{125}I-protein A was from ICN; ^{3}H-N-ethylmaleimide (40 Ci/mmol) from NEN; N-ethylmaleimide, glutathione and Hepes from Sigma; other reagents as described previously.

Purification of SRP and SRP Proteins

SRP was purified according to Walter and Blobel (1983c) and was frozen after the DEAE concentration step. SRP was disassembled as in Chapter 2 with the following modification. The ionic strength of the SRP fraction was determined by conductivity and adjusted to 250mM potassium acetate (KOAc), 5mM EDTA prior to disassembly. Proteins derived from 1 mg of SRP could be purified using a 1 ml hydroxylapatite column, followed by three 50 µl CM Sepharose columns. DTT was omitted from the wash and elution buffers of the CM Sepharose columns. 10% glycerol was included in these buffers as a cryoprotectant and to protect from hydroxyl radical damage.

Reconstitution of Partial SRPs

Reconstitution was performed as described (Walter and Blobel, 1983a and Chapter 2). Reconstitution buffer (SRP buffer) consisted of 20 mM Hepes, pH 7.5, 500mM KOAc, 5mM magnesium acetate ($Mg(OAc)_2$), 0.01% Nikkol. DTT was generally omitted from the reconstitution reaction.

Labelling of SRP and Reconstitutes With ^{125}I-Bolton-Hunter Reagent

500 µCi diiodo-Bolton-Hunter reagent was transferred to 50 µl SRP buffer. 10 µl of this solution was then added to 5 pmol of SRP. The mixture was incubated for 2 hr at 4°C before the addition of Tris, pH 8.0 to 100mM to stop the reaction. Labelled SRP was purified by sucrose gradient sedimentation. A one ml gradient was formed containing steps of 5%, 10%, 15%, and 20% sucrose in SRP buffer and allowed to diffuse overnight at 4°C. The gradients were spun in the TLS-55 swinging bucket rotor in the Beckman TL-100 Tabletop Ultracentrifuge for 3 hr at 55,000 rpm (g_{av}=201,247 g). The gradients were fractionated by hand into 75 µl fractions, and the peak visualized following resolution of the proteins

by PAGE in SDS using autoradiography on X-ray film.

Labelling of SRP Proteins and Reconstitutes With ^{3}H-NEM

SRP proteins were reconstituted in the presence of a 10 fold mol-excess of 7SL RNA. 60μCi ^{3}H-NEM in pentane was mixed with 5 pmol SRP proteins or reconstitutes and the pentane evaporated out of the mixture using a gentle stream of N_2 gas. The NEM modification was then performed as above. After labelling, the samples were TCA precipitated and resolved by polyacrylamide gel electrophoresis.

Generation of SRPs in Which a Single Protein is Modified with NEM

The protein to be modified (2 μl of a 2.5 μM solution in 2X SRP buffer) was first mixed with a two-fold molar excess of 7SL RNA (2 μl 5μM in H_2O) and reconstituted. If protein alone was modified, H_2O was added in place of RNA. Then 1 μl NEM in SRP buffer was added to a final concentration of 1mM. After 30 min at 25°C, 1 μl 50mM DTT or 1 μl 50mM glutathione (both in SRP buffer) was added to quench any unreacted NEM. In control reactions DTT or glutathione was added prior to the addition of NEM. All remaining SRP components were then added in SRP buffer to a final volume of 10 μl and a final concentration of 1 μM. The reconstitution reaction (10 min on ice, 10 min 37°C) was then repeated.

In Vitro Transcription

Plasmid pSPBP4 encoding bovine preprolactin was constructed by members of our laboratory (Hansen et al.) in the following manner. Plasmid pSP64T, which contains the 5' and 3' untranslated regions of the xenopus β-globin gene separated by a BglII linker was obtained from Doug Melton (Harvard University). These regions were cloned between the HindIII and PstI sites of the vector pSP64 marketed by ProMega Biotech. The BglII site of pSP64T was filled in with Klenow DNA polymerase and

converted to an NcoI site which conforms to Kozak's optimal initiation context (ACCATGG, Kozak, 1986). The resulting vector was cleaved with NcoI and PstI and then the NcoI (partial digest)-PstI fragment from the bovine prolactin cDNA clone pBPRL 72 (Sasavage et al., 1982) was inserted. In this construction, the 5' untranslated region of preprolactin has been deleted; the 3' untranslated region, polyA and polyG tails have been retained. pSPBP4 was cut with EcoRI and transcribed with SP6 polymerase according to Hansen et al. (1986), with a 5:1 ratio of GpppG to GTP to cap the 5' end. Transcription was for 1 hr at 40°C. The transcription reaction was then frozen in liquid nitrogen and stored at -80°C until use.

In Vitro Translations

Translations were performed as described previously (Erickson and Blobel, 1983) with the following modifications. Wheat germ was prespun for 2 min at 30 psi in the Beckman airfuge A-110 rotor to remove contaminating membranes. Translation reactions contained 0.1 U/µl RNAse inhibitor and 8 µM S-adenosyl methionine. The ionic conditions of this assay were kept at 144mM KOAc and 3mM $Mg(OAc)_2$.

Activity Assays

Elongation arrest and translocation promoting activities were assayed as described previously (Chapter 2) with the following modifications. Synthetic preprolactin mRNA was used in place of bovine pituitary RNA. Translations were performed in a final volume of 10 µl.

SRP was titrated into the translation reaction in the absence or presence of 1 equivalent (eq, defined in Walter et al.,1981) SRP-depleted membranes per 10 µl to measure elongation arrest and translocation, respectively. The amount of preprolactin, prolactin, and globin

in each lane was quantitated using an LKB Ultroscan XL Laser densitometer (LKB Instruments, Gaithersberg, MD). The amount of elongation arrest was calculated as before (Chapter 2), but the amount of translocation was calculated differently, so that the inhibition of preprolactin synthesis by SRP(68/72^{N}) would not give abberantly high numbers for percent processing. This new "percent translocation" was calculated according to Garcia and Walter (1987) as follows.

$$\% \text{ translocation(a)} = \frac{(8/7)\text{prolactin(a)} \times \text{globin(0)} \times 100}{\text{globin(a)} \times [\text{preprolactin(0)} + (8/7)\text{prolactin(0)}]}$$

Elongation arrest in the presence of membranes was calculated using the following equation:

$$\% \text{ inhibition(a)} = \frac{[\text{preprolactin(a)} + (8/7)\text{prolactin(a)}] \times \text{globin(0)} \times 100}{\text{globin(a)} \times [\text{preprolactin(0)} + (8/7)\text{prolactin(0)}}$$

Cotranslational targeting assays (Connolly & Gilmore, 1986) were performed by adding SRP to 120nM in a 50 µl translation. The translation reaction was allowed to incubate for 15 min at 26^{o}C to allow the accumulation of arrested chains. Then cycloheximide was added to 1mM to block further elongation. 15 µl of the reaction was mixed with 4 eq of SRP depleted membranes and allowed to incubate for 5 min at 22^{o}C. Stability of the targeted chains to high salt was tested by adding KOAc and $Mg(Oac)_2$ to 500mM and 5mM final, respectively. The mixture was layered over a 50 µl cushion comprised of 250mM sucrose, 20mM Hepes, 1mM DTT and either 140mM KOAc, 2.5mM $Mg(OAc)_2$ (physiological salt), or 500mM KOAc, 5mM $Mg(OAc)_2$ (high salt). Samples were spun for 3 min (physiological salt) or 4 min (high salt) at 20 psi in the Beckman A-100/30 rotor in the Beckman airfuge, not counting acceleration or deceleration times. The entire supernatant, including the cushion, was added to 250 µl 2% CTABr. Likewise, the pellet was dissolved in 250 µl 2% CTABr. Then 50

µg calf liver tRNA and 250 µl 0.5 M sodium acetate (NaOac), pH 5.4 were added, and the samples were incubated for 10 min at 30°C. Samples were spun in a microfuge for 5 min, the pellets rinsed twice with 500 µl acetone/HCl, dried in a Speed-Vac, and resuspended in 25 µl SDS-PAGE sample buffer. After 1 hr at 55°C, nascent chains were resolved by SDS-PAGE, the gels were fluorographed (Chapter 2), and the bands visualised by autoradiography.

"Posttranslational" targeting assays (described in greater detail in Chapter 6) were performed as for the cotranslational assay with the following exception. Nascent chains were allowed to achieve "steady state" by incubation of the translation reaction at 22°C for 14 min prior to addition of cycloheximide to 1mM. A single translation reaction was used for all the assays. The translation reaction was divided and various SRPs were added to 80nM and incubated for 10 min at 22°C. The remainder of the assay was as above.

Signal recognition was assayed by a polysome binding experiment (see below).

SRP Receptor Affinity Chromatography

A monoclonal antibody against the α-subunit of SRP receptor (Tajima et al., 1986) was coupled to CNBr activated Sepharose as described (ibid.). 10 ml solubilized membranes (~1 eq per µl, representing a total of about 1 nmol SRα) was mixed with 0.2 ml of resin. After 2 hr of mixing in batch, a column was poured from this resin, which was then washed with 2 ml of Buffer A (20mM Hepes, pH 7.4, 0.5mM glutathione, 0.5% Nikkol) containing 500mM KOAc and 5mM $Mg(OAc)_2$. The column was then washed with 2 ml of Buffer A containing 50mM KOAc, 2mM $Mg(OAc)_2$ before an SRP preparation (7.5 pmol) or a mixture of SRP proteins (about

60 pmol each protein) was bound to it. These preparations were adjusted to a buffer composition equal to the 50mM KOAc wash buffer (a 10-fold dilution) and then loaded onto the SRP receptor-anti receptor Sepharose column. 1 ml of load buffer followed and was pooled with the flowthrough fraction. The column was eluted with 1 ml each of buffer A containing the following concentrations of potassium and magnesium: 100mM KOAc, 2mM $Mg(OAc)_2$; 200mM KOAc, 2mM $Mg(OAc)_2$; and 500mM KOAc, 5mM $Mg(OAc)_2$. 1 ml of the 50mM KOAc wash buffer was then passed over the column to prepare it for the next sample to be loaded.

Western Blotting

After resolution of the protein fractions by SDS PAGE, the proteins were transferred to nitrocellulose using 0.75 Amp current for 2 hr. The nitrocellulose was blocked with 4% BSA in 10mM sodium phosphate buffer, pH 7.5, 150mM NaCl, 1mM EGTA, 0.2% TX-100, 0.02% NaN_3 (BSA buffer) overnight. The blot was then incubated with a polyclonal serum against the 68 kd protein diluted 1:200 in BSA buffer for 6 hr. The blot was washed 5 x 3 min with 50mM triethanolamine-HCl, pH 7.5, 100mM NaCl, 2mM EDTA, 0.5% TX-100, 0.1% SDS (SDS buffer), rinsed with BSA buffer and then incubated with BSA buffer for 5 min. 2.5 µCi ^{125}I-Protein A in 50 ml BSA buffer was incubated with the blot for 30 min. The blot was rinsed again 5 x 3 min in SDS buffer, 3 times with Tris buffered saline (25mM Tris-HCl, pH 8.0, 144mM NaCl), and blotted dry. Exposure was for 24 hr with an intensifying screen. The amount of 68 kd protein in each fraction was quantitated by densitometry using a Zeineh Soft Laser Scanning densitometer (Biomed Instruments Inc, Fullerton, CA).

Polysome Binding Analysis

24 µl of gradient purified ^{125}I-SRP or ^{125}I-SRP(54^N) (~200 fmol)

was included in a 100 µl wheat germ translation reaction programmed with either total reticulocyte RNA or synthetic preprolactin mRNA and incubated for 15 min at 26°C. The samples were then loaded onto 12.5 ml 5-20% sucrose gradients (50mM triethanolamine-OAc, pH 7.5, 100mM KOAc, 2.5mM $Mg(oac)_2$, 1mM DTT, 10 µg/ml insulin) that were poured in gelatin-coated polyallomer SW40 ultracentrifuge tubes. Gradients were spun for 2 hr at 39,000 rpm (Acc 7 Dec 7) at 4°C. Gradients were fractionated by underlayering with 60% sucrose using an Isco gradient fractionator. Forty-nine 0.25 ml fractions were collected from each gradient. Fractions were counted with a Beckman 4000 gamma counter.

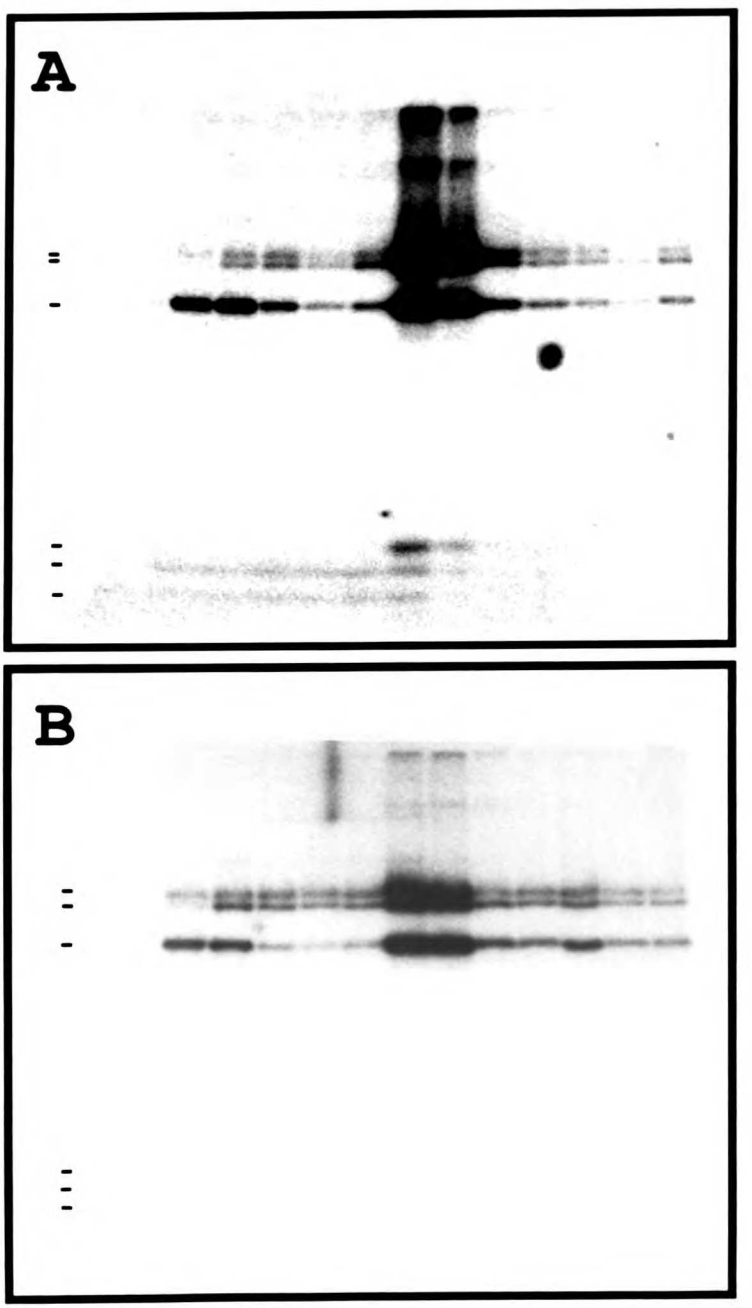
RESULTS

NEM Does Not Cause the Disassembly of SRP

One explanation for the complete inactivation of SRP by NEM is that NEM causes SRP to disassemble. We tested this possibility by modifying ^{125}I-SRP with NEM and then analysing the integrity of the particle by sucrose gradient sedimentation. In Figure 1 we show a sucrose gradient profile of unmodified (Panel A) and modified (Panel B) SRP. We found that some of the 54 kd protein has been lost from the particle and can be seen sedimenting less far into the gradient; however, this is true whether or not the particle was modified with NEM and probably represents an alteration of the 54 kd protein by the labelling procedure. A small effect on the binding of the 9/14 kd protein (compare the levels of 9/14 kd in the top fraction of the two gradients) to the particle is seen upon modification. In addition, the increased level of the small polypeptides in fraction 6 in the NEM-trated sample may reflect a small amount of SRP(-68/72) due to disassembly of those polypeptides. However, a majority of the labelled protein sediments as a single peak at 11S,

Figure 1. Sucrose gradient analysis of unmodified and NEM-treated SRP.

50 µl ^{125}I-SRP (~150 fmol) was treated with 1mM NEM for 30 min at 25°C prior to addition of DTT to 10mM. In the unmodified sample, DTT was added prior to the addition of NEM. Samples were loaded onto 13 ml 5-20% sucrose gradients, spun for 20 hr at 40,000 rpm in an SW40 rotor in the Beckman L8 ultracentrifuge, and fractionated into thirteen 1 ml fractions. Samples were resolved by SDS PAGE and visualised by autoradiography with an intensifying screen. Exposure time was 7 days. Panel A is unmodified and Panel B modified SRP. The left hand side represents the top of the gradient. The lines alongside the autoradiograms indicate the positions of the SRP polypeptides.


A
B

and thus represents fully assembled SRP. Therefore the inactivation of the particle by NEM must result from something other than its disassembly.

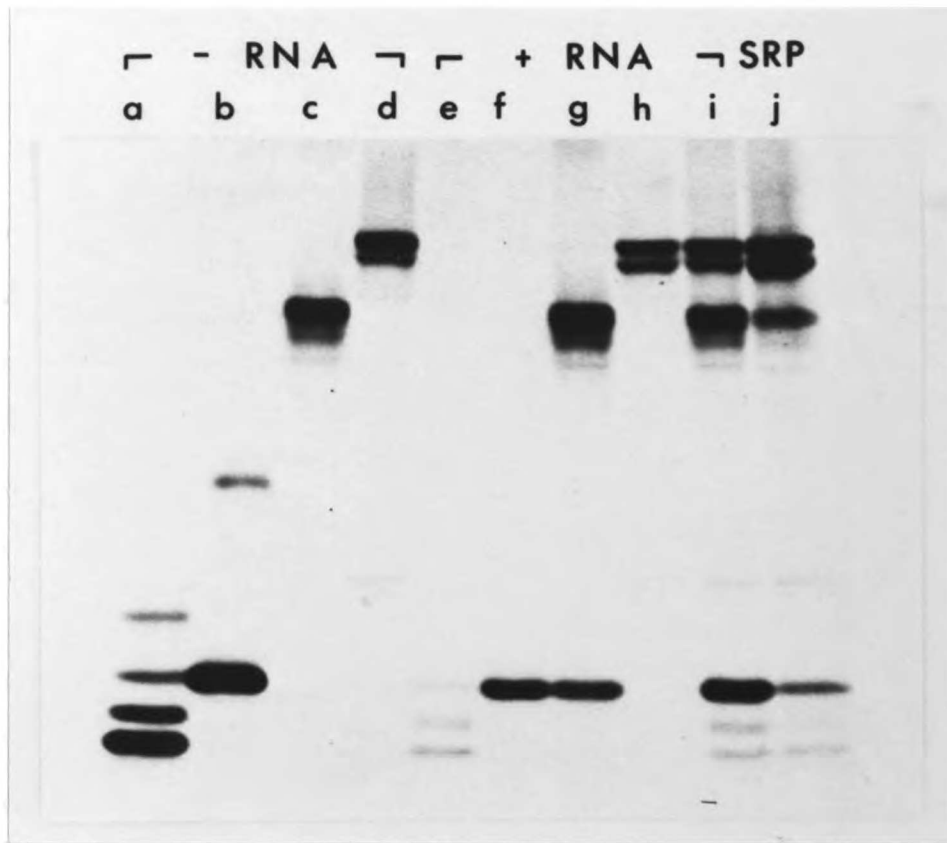
Several SRP Proteins are Potential Targets for NEM Inactivation

In order to determine which SRP proteins could be modified with the sulfhydryl reactive agent N-ethylmaleimide (NEM) and thus be potential targets for NEM inactivation experiments, individual SRP proteins were labelled with ^{3}H-NEM either alone or after being bound to SRP RNA under standard reconstitution conditions. The result of such a labelling experiment is shown in Figure 2. As can be seen in lanes a-d, when the proteins are free in solution, all of the SRP polypeptides can be labelled (when comparing the intensities of labelling, note that the 9 kd protein contains two cysteine residues (K. Strub & P. Walter, unpublished)). However, when the RNA has been bound to the proteins in a reconstitution reaction prior to labelling, protection of the proteins to NEM modification was seen. Most strikingly, the 9 kd polypeptide was almost completely protected from modification by the RNA (compare Fig. 2, lanes a and e). In addition, the labelling of most of the other proteins by ^{3}H-NEM was diminished in the presence of the RNA. One explanation for the diminution of protein labelling in the presence of the RNA is that the modification sites lie within regions of the protein involved in RNA binding. If such an explanation is correct, modification of these proteins in isolation may influence their ability to subsequently bind to the RNA in a reconstitution reaction.

With this in mind, we designed a reconstitution scheme to generate complete SRP particles in which a single protein contains the NEM modification, and this scheme is depicted in Figure 3. The salient

Figure 2. ^{3}H-NEM labelling of SRP proteins and reconstitutes.

5 pmol of each SRP protein in the absence (lanes a-d) or in the presence (lanes e-i) of a 10 fold molar excess of RNA was incubated with 60 µCi ^{3}H-NEM for 30 min at 25^{o}C prior to TCA precipitation and SDS-PAGE analysis. Lanes a,e: 9/14 kd; lanes b,f: 19 kd;lanes c,g: 54 kd; and lanes d,h: 68/72 kd proteins. Lane i is a total reconstitute and lane j gradient purified SRP.


- RNA
+ RNA
SRP
a b c d e f g h i j

feature of this method is that the protein is modified in the context of the SRP RNA under conditions of RNA excess. The protein in this way is driven into the bound state, and any regions involved in RNA binding are hopefully protected. First, the protein to be modified was incubated with SRP RNA under standard reconstitution conditions (Fig. 3, Step 1). Next, NEM was added and allowed to react for 30 min at 25°C (Fig. 3, Step 2), after which time DTT or glutathione was added to inactivate any residual NEM (Fig. 3, Step 3.1). Finally, the other SRP proteins were included in the mixture (Fig. 3, Step 3.2) and the particle was subjected to another round of reconstitution, thus generating complete particles with single protein components modified.

Phenotypes of the NEM-Modified Particles

We assayed the activity of particles thus generated, and compared it to ones in which the isolated protein was modified with NEM prior to reconstitution with the RNA. Using the well established *in vitro* translation/translocation reactions, we determined the ability of these particles to inhibit synthesis of the preprolactin nascent chain (elongation arrest) and to promote the translocation and subsequent signal cleavage of preprolactin to prolactin (protein translocation). The results are summarized in Table I.

We found that SRP was exquisitely sensitive to NEM modification of the 54K protein, losing both elongation arrest and translocation functions (experiments addressing the reason for this inactivation are presented towards the end of this chapter). In contrast, SRP could tolerate a modification of the 19K protein, and retained wild type activity. In two cases (9/14 and 68/72) we found that the activity of the particle differed depending on whether the protein was modified or

Figure 3. Generation of singly modified SRPs.

The scheme for protecting RNA binding domains from inactivation by NEM is shown here. See results section for discussion.

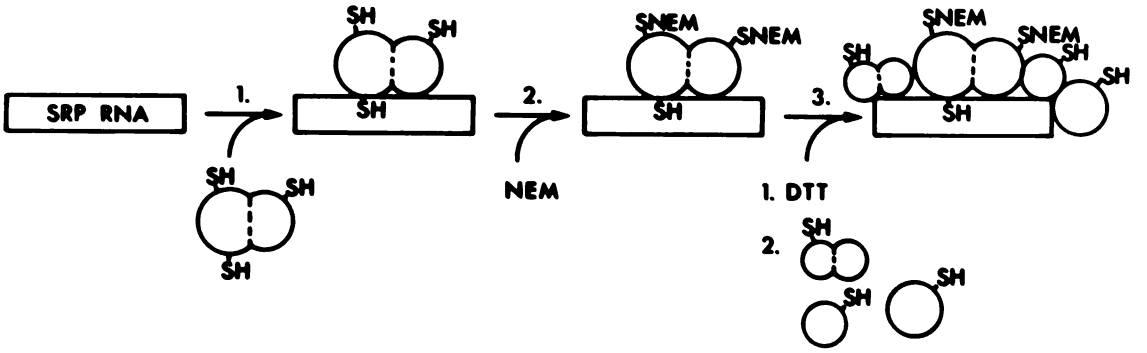

SRP RNA
1.
SH
SH
SH
SH
SH
2.
NEM
SNEM
SNEM
SH
3.
1. DTT
2.
SH
SH
SH

TABLE I: Phenotype of NEM-Modified SRPs

Protein Modified	RNA present During Modification?	Elongation Arrest	Protein Translocation
54	no	-	-
19 & 54	yes	-	-
19	no	+	+
19	yes	+	+
*9/14	no	-	+
*9/14	yes	+	+
*68/72	no	-	-
*68/72	yes	+	-

Proteins were modified with NEM either as isolated proteins or after reconstitution onto SRP RNA as depicted in Figure 3. Complete reconstitutes containing these modified proteins were then generated, and the activity of these reconstitutes in elongation arrest and translocation assays is shown here. * denotes particles in which the activity differs depending on whether the modification was performed on proteins in isolation or on partial reconstitutes.

the single reconstitute was modified, and in both these cases when the protein alone was modified the activity was equivalent to that of a reconstitute lacking that protein (loss of elongation arrest for the 9/14 and loss of both activities for the 68/72), consistent with the idea that modification of these proteins affected their binding. When the 9/14 kd protein was modified in the presence of the RNA, the activity of this particle was wild type; this is consistent with the result in Fig. 2 that the labelling of these polypeptides with ^{3}H-NEM is almost completely abolished in the presence of the RNA.

A rather intriguing phenotype was found when the 68/72 kd protein was modified as a single reconstitute: the particle was able to arrest preprolactin synthesis, but could not promote its translocation across the microsomal membrane. We wished to determine the "primary defect" resulting in this phenotype, and so we studied its activity in greater detail.

Microsomal Membranes Fail to Release the Arrest Induced by SRP(68/72^{N})

Varying amounts of SRP and SRP(68/72^{N}) were added to elongation arrest and translocation assays, and the results are depicted in Figure 4. Confirming the results of Table I, we found that SRP(68/72^{N}) was able to arrest preprolactin synthesis (Fig. 4A, filled triangles), indicating that the signal recognition and elongation arrest domains were not affected by the modification. The amount of arrest shown here reached a maximum of about 70%. In other reconstitution reactions, we have been able to improve the arrest demonstrated by SRP(68/72^{N}) and its control reconstitute by increasing the amount of 9/14 kd protein in the reconstitution. In some cases we found that the modified particle was somewhat less active in elongation arrest activity than the control recon-

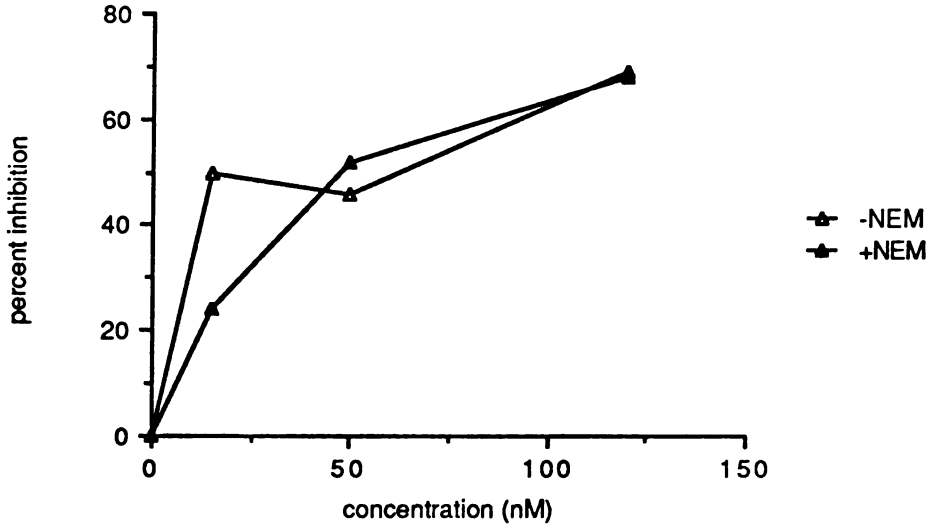
FIGURE 4. Activity assay of SRP and SRP(68/72^{N}).

SRP and SRP(68/72^{N}) were assembled to a final concentration of 500nM p68/72. Varying amounts of these particles were added to elongation arrest and translocation assays, and the results quantitated by densitometry, and percent inhibition and percent translocation calculated as described (see Materials and Methods).

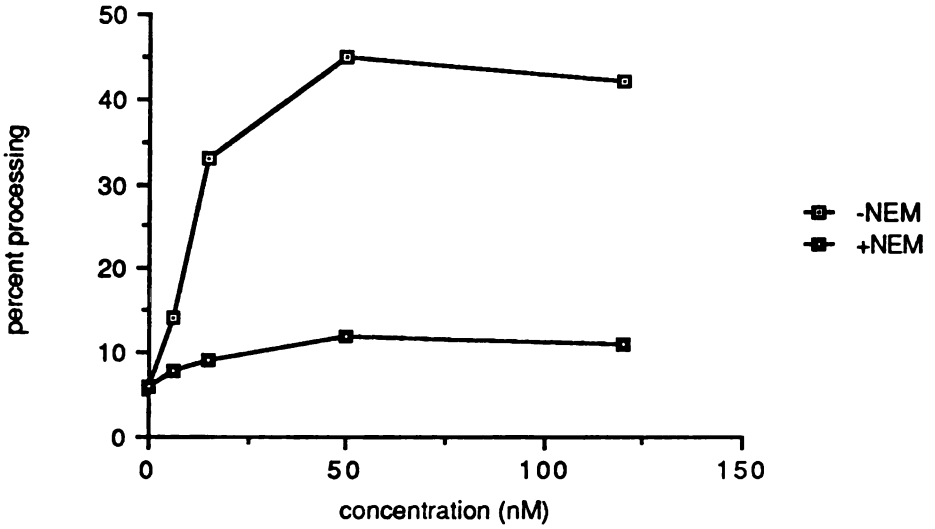
(A) Percent inhibition as a function of SRP (open triangles) and SRP(68/72^{N}) (filled triangles) concentration.

(B) Percent translocation as a function of SRP (open squares) and SRP(68/72^{N}) (filled squares) concentration.

A


80
60
40
20
0
percent inhibition
0
50
100
150
concentration (nM)
-NEM
+NEM

B


50
40
30
20
10
0
percent processing
0
50
100
150
concentration (nM)
-NEM
+NEM

stitute (see Fig. 6A, lanes b and c), which may be the result of some of the protein being free in solution during the modification reaction. In contrast to its ability to inhibit the elongation of preprolactin, SRP(68/72^{N}) was completely unable to promote the translocation of preprolactin across the microsomal membrane (Fig. 4B, filled squares and Fig. 6A, lane g).

Another measure of the functional interaction of SRP-arrested nascent complexes with SRP receptor in the microsomal membrane is the release of the SRP-induced arrest (Walter & Blobel, 1981b). When membranes were included in translation reactions containing SRP or SRP(68/72^{N}), we found that the inhibition of synthesis induced by the control reconstitute was reduced ~3-fold (compare open triangles and open diamonds in Fig. 5A), indicating that the membranes were able to release the arrest induced by SRP. Such an arrest releasing activity was <u>not</u> seen when SRP(68/72^{N}) was included in the reaction (compare filled diamonds and triangles in Fig. 5B). In other words, in contrast to the case for SRP, which upon interaction with SRP receptor is released from the ribosome/nascent chain complex and is free to recycle (Gilmore & Blobel, 1983), SRP(68/72^{N}) remains bound to the complex even in the presence of microsomal membranes.

<u>The Failure of Microsomal Membranes to Release Arrest Does Not Explain the Translocation Defective Phenotype of SRP(68/72^{N})</u>

We wondered whether the translocation defective phenotype of SRP(68/72^{N}) was the result of the failure of SRP receptor to release the arrest induced by this particle. If such a hypothesis is correct, we would expect translocation promoting activity to be restored by inactivating the elongation arrest function. We did this by omitting the 9/14

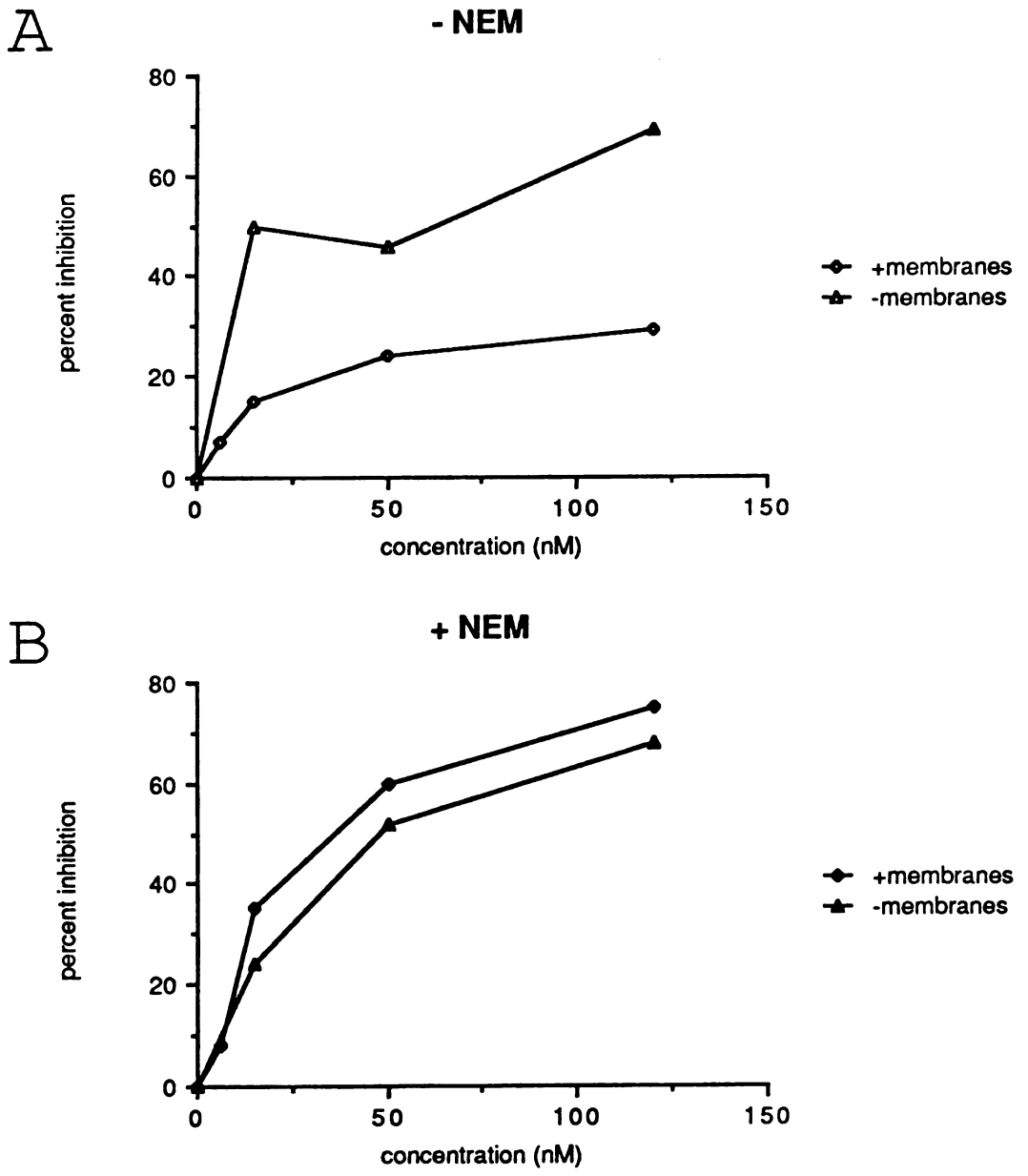
Figure 5. Elongation arrest in the absence and presence of microsomal membranes.

Titrations of SRP and SRP(68/72^{N}) were performed as in Figure 4.

(A) Percent inhibition as a function of SRP concentration in the absence (open triangles) and the presence (open diamonds) of microsomal membranes.

(B) Percent inhibition as a function of SRP(68/72^{N}) concentration in the absence (filled triangles) and the presence (filled diamonds) of microsomal membranes.

The curves generated in the absence of microsomal membranes consist of the same data points as Panel A of Figure 4.


A
- NEM
percent inhibition
80
60
40
20
0
0
50
100
150
concentration (nM)
+membranes
-membranes
B
+ NEM
percent inhibition
80
60
40
20
0
0
50
100
150
concentration (nM)
+membranes
-membranes

kd protein from the reconstitution reaction (see Chapter 2).

When we titrated SRP(68/72^{N})(-9/14) into a translocation assay (Fig. 6B), we found that, as expected, the elongation arrest was relieved by removal of the 9/14 kd protein (lanes b and c). However, even in the absence of elongation arrest, the particle was unable to promote translocation (lanes g and h). It seemed rather, as was the case in translation reactions containing SRP(68/72^{N}), that the pattern of chains synthesized in the presence of microsomal membranes differed in no way from those synthesized in their absence. We wondered whether these nascent chains were in fact ever targeted to the membrane.

SRP(68/72^{N}) Fails to Target Nascent Preprolactin to the Microsomal Membrane

Simple assays for the targeting of preprolactin to microsomal membranes are described in greater detail in Chapter 6. In brief, it has recently been shown that SRP will mediate the targeting only of chains associated with the ribosome (Mueckler & Lodish, 1986, Perara et al., 1986; Garcia & Walter, in preparation; Chapter 6). The assays take advantage of this fact by enriching for ribosome associated chains with the positively charged detergent cetyltrimethylammoniumbromide (CTABr), which precipitates the peptidyl-tRNA by virtue of the tRNA linkage (Hobden & Cundliffe, 1978). By looking only at "nascent" chains, it has been possible to synthesize chains either in the presence or absence of SRP (the former yielding the characteristic "arrested fragment", and the latter yielding a distribution of chains of various lengths), and then to target these chains to microsomal membranes in an SRP-dependent manner.

In Figure 7A, translation extracts were programmed with synthetic

Figure 6. Arrest and translocation assays for SRP, SRP(68/72^{N}), SRP(-9/14), and SRP(-9/14)(68/72^{N}).

p68/72 was reconstituted onto 7SL RNA prior to NEM modification as described. When the additional protein components were added in the final reconstitution step, p9/14 was included or was replaced with compensating buffer to generate SRP(68/72^{N}) and SRP(-9/14)(68/72^{N}), respectively, and their control particles.

(A) Elongation arrest and translocation assay for SRP and SRP(68/72^{N}). lanes a-d, - membranes; lanes e-h, + membranes. Lanes a,e: no SRP; Lanes b,f: control reconstitute; lanes c,g: SRP(68/72^{N}); lanes d,h: gradient purified SRP.

(B) Elongation arrest and translocation assays for SRP(-9/14) and SRP(-9/14)(68/72^{N}). lanes a-c, - membranes; lanes d-h, + membranes; lanes a,d: no SRP; lanes b,e,f: SRP(-9/14) at 120nM, 5nM, and 120nM, respectively; lanes c,g,h: SRP(-9/14)(68/72^{N}) at 120nM, 5nM, and 120nM, respectively.

The major bands, in order of decreasing molecular weight, are preprolactin, prolactin, and globin.

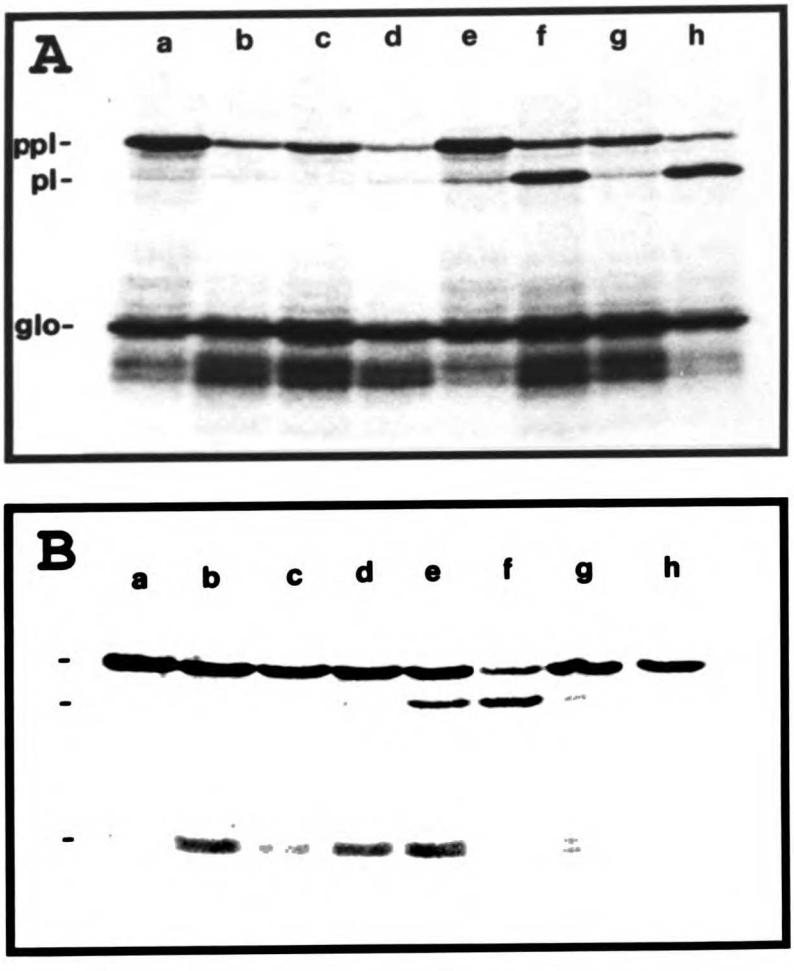
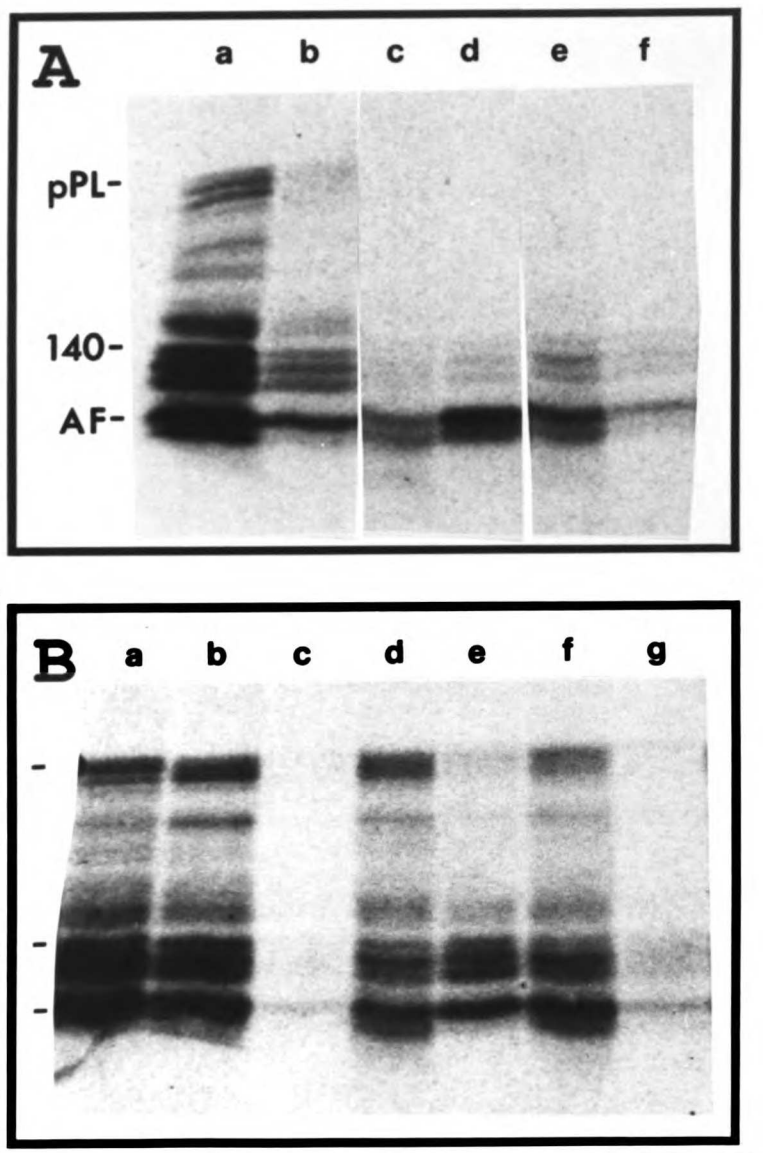

A
a b c d e f g h
ppl-
pl-
glo-
B
a b c d e f g h

FIGURE 7. Targeting of preprolactin to microsomal membranes.

(A) No SRP was added, or SRP or SRP(68/72^{N}) was added to 120nM prior to the addition of mRNA to initiate protein synthesis. After a 20 min synthesis, cycloheximide was added, and then microsomal membranes were added and incubated for 5 min at 22^{o}C. Samples were spun for 3 min at 20 psi in the Beckman airfuge A-100/30 rotor over a 50 µl sucrose cushion. Supernatants and pellets were analysed by CTABr precipitation. lanes a,c,e: supernatant; lanes b,d,f: pellet. Lanes a,b, -SRP; lanes c,d, +SRP; lanes e,f, +SRP(68/72^{N}).

(B) After synthesis of preprolactin for 14 min at 22^{o}C, cycloheximide was added. Then SRP was added to 80nM and incubated for 10 min, followed by incubation with microsomal membranes for 5 min as in Panel A. Lane a, total; lanes b,d,f, supernatant; lanes c,e,g, pellet; lanes b,c, - SRP; lanes d,e, + SRP; lanes f,g, + SRP(68/72^{N}).

Positions of preprolactin (229 amino acids), a 140 amino acid length chain, and the "arrested fragment" (70 amino acids) are indicated.


A
a
b
c
d
e
f
pPL-
140-
AF-
B
a
b
c
d
e
f
g

preprolactin mRNA in the absence or presence of SRP and allowed to synthesize chains for about 20 min. Then cycloheximide was added to inhibit further elongation. Note that in the absence of SRP (lanes a and b), chains ranging from about 50 amino acids in length to chains approximately full length are detected. However, when SRP or SRP(68/72^{N}) is added to the translation (lanes c-f), only the shorter chains are seen, indicating that these SRPs inhibit elongation.

In Figure 7B, nascent chains were allowed to accumulate, cycloheximide was added, and the translation extract was divided for the sedimentation assay. Samples were incubated either in the absence or presence of SRP prior to the addition of membranes. In this case the nascent chain distribution in each of the targeting assays was identical.

In the absence of SRP (Fig. 7A, lanes a and b, Fig. 7B, lanes b and c), most of the chains were found in the supernatant. In the presence of SRP (Fig. 7A, lanes c and d, Fig. 7B, lanes d and e), most of the chains (~80% of the chains in Panel A and ~60% of the chains in Panel B) were targeted to the membrane. When SRP(68/72^{N}) was included in the assay (Fig 7A, lanes e and f, Fig 7B, lanes f and g), a distribution similar to that seen in the absence of SRP was found, i.e., most of the chains were found in the supernatant.

To summarize, we found that the targeting of the preprolactin nascent chain was markedly reduced. This was true whether SRP was present during the synthesis of the nascent chain or only added after further elongation was inhibited, or whether the targeting assay was performed in high salt (500mM) (not shown), physiological salt (140mM) (Fig. 7), or low salt (50mM) (not shown), suggesting that the reaction

is blocked at the initial stage of targeting, i.e., in the interaction of SRP with SRP receptor.

We wanted to test whether this particle was in fact hindered in its ability to bind to SRP receptor, and for this purpose we established a system in which to perform SRP receptor affinity chromatography.

SRP(68/72^{N}) Binds to an SRP Receptor Column with Reduced Affinity

Monoclonal antibodies have been generated against the α-subunit of SRP receptor (Tajima et al., 1986). These antibodies have been useful in purifying SRP receptor from preparations of solubilized membranes. Attempts to use these antibodies to block the arrest releasing activity of SRP receptor have failed, suggesting that these antibodies bind to a region of the receptor molecule that is not important for its interaction with SRP. We therefore used these antibodies to generate an SRP receptor column. First, anti-SRα antibodies were coupled to CNBr activated Sepharose. Then this resin was mixed with solubilized membranes under conditions that have been shown to immunopurify SRP receptor. Such a receptor-antibody complex is stable to high salt and thus is a reasonable way to couple SRP receptor to a resin. It has advantages over coupling the receptor directly to the resin: first, such a preparation has never been subjected to harsh conditions, such as the 4.5M $MgCl_2$ elution step used to immunopurify receptor (Tajima et al., 1986), nor to the lengthy purification procedure used to isolate the complex using SRP-Sepharose chromatography (Gilmore et al., 1982b); second, each receptor molecule on this column is identical, since it is bound to a monoclonal antibody. If receptor were directly purified to CNBr Sepharose, it would be coupled in a number of different positions, some of which may influence binding of SRP to the column.

A column was poured from this receptor-anti-receptor resin, and SRP and SRP(68/72^{N}) were bound and eluted from the column with various steps. The elution profiles for SRP and SRP(68/72^{N}) are shown in Figure 8. When SRP was bound to such a receptor column, approximately equal amounts were eluted at 200mM and 500mM KOAc. This profile is identical to that found when SRP receptor was bound and eluted from an SRP Sepharose column (Gilmore, 1982b). However, when SRP(68/72^{N}) was bound to this column, the majority of the particle eluted at 100mM KOAc. We conclude from this result that SRP(68/72^{N}) is diminished in its ability to interact with SRP receptor.

We also wished to test whether the 68/72 kd protein could interact with SRP receptor directly. We passed a mixture of SRP proteins (in the absence of RNA) over the column (Fig. 9). We found that the 68/72 kd protein was specifically depleted from the flow through fraction (lane b). The majority of the protein eluted at 100mM salt (lane c), a lower salt than that required for elution of SRP from the column. This may indicate that the RNA and/or the other proteins in the particle play a role in the interaction with SRP receptor, either directly or by an overall charge effect.

SRP(54^{N}) Has Lost its Ability to "Recognise" Signal Sequences

We were interested in the reason for the loss of both elongation arrest and translocation promoting activities when the 54 kd moiety of SRP is modified. Because the 54 kd protein has been implicated in signal recognition by virtue of the cross linking of this protein to signal sequences (Kurzchalia, et al., 1986, Kreig et al., 1986), one explanation for this inactivation would be a loss of the signal recognition activity of the particle. We tested this hypothesis by per-

Figure 8. Binding of SRP and SRP(68/72^{N}) to an SRP-receptor column.

SRP receptor was bound to anti-SRα Sepharose as described in Materials and Methods. First SRP and then SRP(68/72^{N}) was bound and eluted from this column. Binding conditions were 50mM KOAc, 2mM $Mg(OAc)_2$. Elution was performed with step of increasing ionic strength. The percent recovered in each step was calculated by densitometer scanning of a Western blot probed with an anti-68 kd polyclonal serum and ^{125}I-protein A. The total integrated area in the two experiments in arbitrary units was 654 for SRP and 695 for SRP(68/72^{N}).

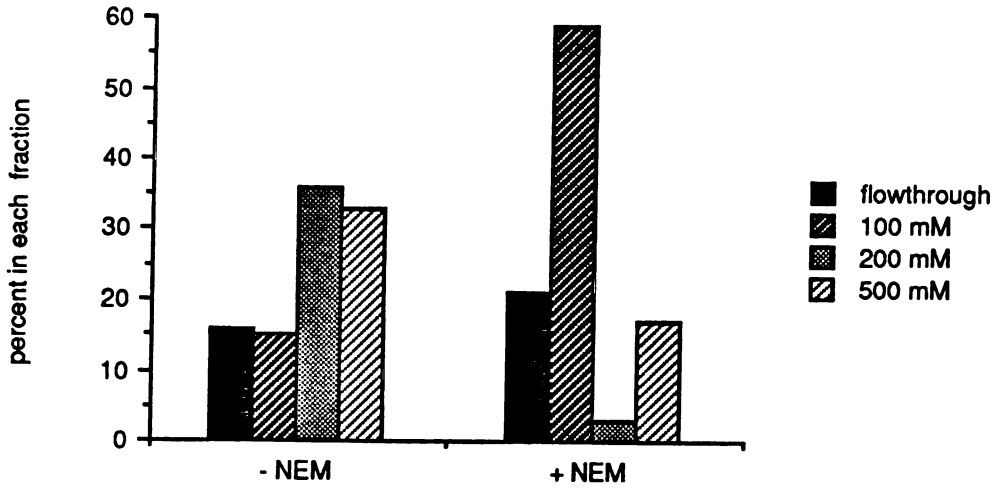

60
50
40
30
20
10
0
percent in each fraction
- NEM
+ NEM
flowthrough
100 mM
200 mM
500 mM

Figure 9. Binding of SRP proteins to an SRP receptor column.

A mixture of SRP proteins (see Materials and Methods) was passed over the column described in Figure 8 and eluted under the same conditions. lane a, load; lane b, flow through; lane c, 100 mM eluate; lane d, 200mM eluate; lane e, 500mM eluate. Lanes b-e contain 2.5 times as much sample as lane a. The two bands appearing in the column fractions but not in the load fraction are immunoglobulin heavy and light chain.

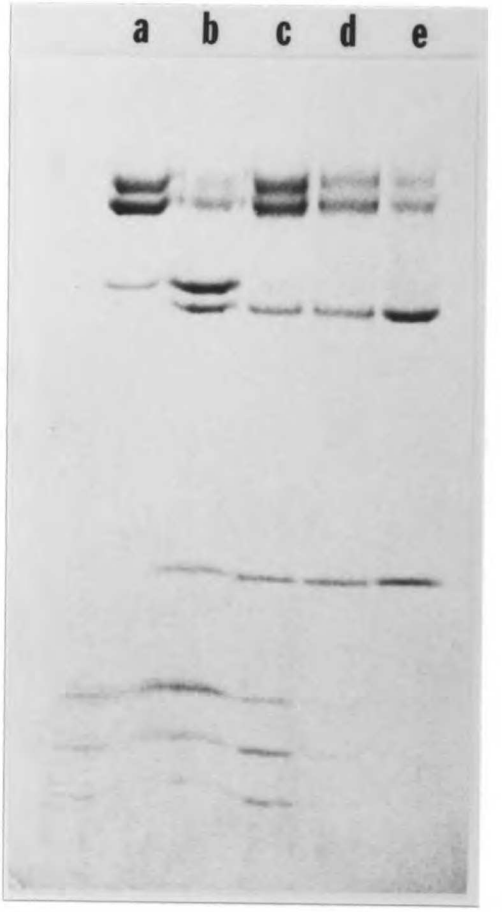
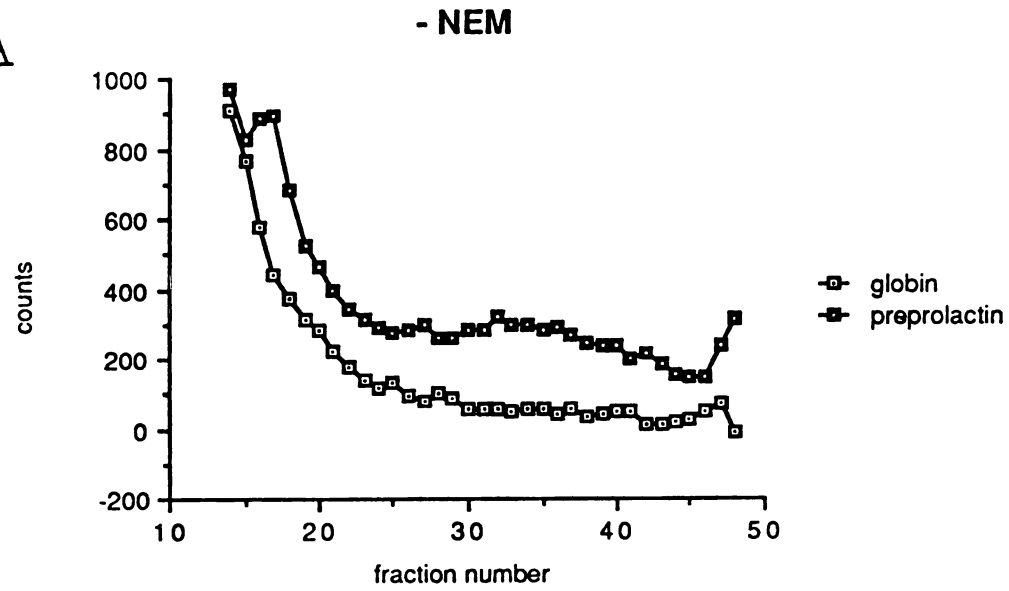

a
b
c
d
e

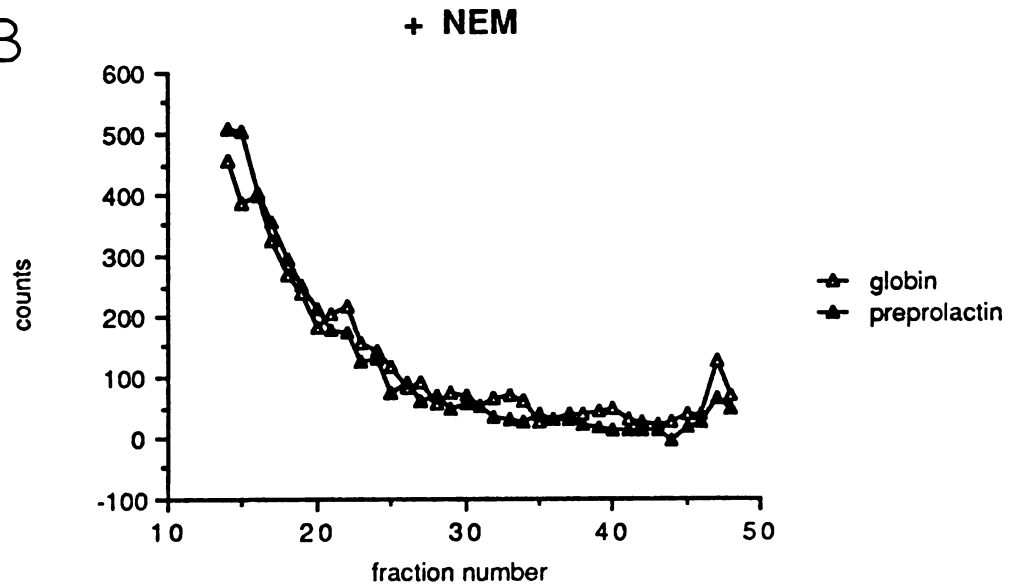
Figure 10. Binding of SRP and SRP(54^N) to polysomes synthesizing globin and prolactin.

^{125}I labelled SRP or SRP(54^N) was included in translation reactions programmed with total reticulocyte RNA (open squares and open triangles) or synthetic prolactin mRNA (filled squares and filled triangles). After allowing synthesis for 15 min, samples were layered on top of 13 ml 10-30% sucrose gradients and spun at 39,000 RPM in a Beckman SW40 rotor for 2 hr. The gradients were fractionated into 0.25 ml fractions. The first fraction (numbered from the top of the gradient) shown on these curves is fraction number 13. Panel A, SRP; Panel B, SRP(54^N).

A


- NEM
1000
800
600
400
200
0
-200
counts
10
20
30
40
50
fraction number
globin
preprolactin

B


+ NEM
600
500
400
300
200
100
0
-100
counts
10
20
30
40
50
fraction number
globin
preprolactin

forming a "polysome binding experiment" (Walter & Blobel, 1981a). SRP and SRP(54^N) were labelled with ^{125}I-Bolton-Hunter reagent and then added to a translation reaction synthesizing either globin or preprolactin. The amount of SRP bound to ribosomes synthesizing these messages was determined by sedimenting such a translation reaction in a sucrose gradient, and determining the amount of ^{125}I-SRP in the polysome region of the gradient. The result of this analysis is shown in Figure 10.

When SRP was added to a translation reaction synthesizing preprolactin (Fig. 10A), the amount of SRP found in both the monosome (arrow) and polysome region of the gradient was greater than that found when SRP was added to a translation reaction synthesizing globin. This increase reflects an increase in affinity of SRP for the translating ribosome (Walter, 1981a). When SRP(54^N) was added to such translation reactions (Fig. 10B), no such increase in SRP in these regions of the gradient was found. We conclude from this result that SRP(54^N) is no longer able to respond to the presence of a signal sequence on the ribosome, and that without this signal recognition event, both elongation arrest and translocation promotion are abolished.

DISCUSSION

Signal recognition particle is comprised of a number of different components and exhibits a number of different activities. We have sought to understand the relationship between the structure of SRP and its functions, and for this reason we embarked upon a project of biochemical mutagenesis.

It was not clear from the outset that such a project would be successful. Similar attempts have been made to analyse the function of

ribosomes (Nierhaus, 1980; Nomura & Held, 1981; Held et al., 1973; Schulze & Nierhaus, 1982; Tate et al., 1983), but with little success. In particular, single omission experiments were tried and were difficult to interpret. Many different proteins, when omitted from the assembly, affected the same function, and conversely, single omissions often affected a number of different functions. It was never clear in these experiments whether the activity tested in fact resided in a domain built up from a number of different proteins or whether the overall structure of the ribosome was severely altered, yielding for example active sites in the wrong geometry.

It was with the caveats of single omission experiments in mind that we designed a scheme for modifying single protein components of SRP with the sulfhydryl reactive agent NEM. We felt that such an inactivation procedure would yield particles that were assembled approximately normally, and we were encouraged by the result that NEM modification does not cause the disassembly of SRP (Fig. 1).

One incentive for using the reagent NEM in these experiments was that each polypeptide had a site that could potentially be modified, as can be seen when the isolated proteins were modified with ^{3}H-NEM (Fig. 2). However, some of these sites of modification appear to have been protected when RNA was allowed to bind to the protein prior to modification. Most strikingly, the 9 kd protein, which was labelled rather strongly in isolation (lane a), was almost completely protected from modification when the RNA was present (lane e). There are several possible reasons for this protection. One is that the sulfhydryl groups lie along an RNA binding region of the protein, and the RNA is directly protecting these groups. A second is that the RNA stabilizes the

protein in a conformation that sequesters the sulfhydryl groups, perhaps in a disulfide bridge. It is reasonable to conclude from the protection that the 9 kd protein contains an RNA binding domain.

Because of the potential for interfering with assembly of the particle by NEM modifying the proteins in isolation, our scheme entailed reconstituting the proteins onto the RNA prior to modification. Using this approach, we were able to generate biochemically every "mutant" we could have hoped for in a genetic screen (see Table II). In addition to the elongation arrest defective phenotype we had isolated earlier, we generated particles that were active in elongation arrest, but could not promote translocation, and ones that had lost both activities, which we show here to be the result of a signal recognition defective phenotype. In other words, each of the activities of SRP that we have measured can be destroyed by removing or altering a protein component.

The picture that we get from this series of experiments is that the activities of the particle reside in the protein domains, and that the RNA serves primarily as a structural lattice, holding the proteins in specific geometry. It should be noted, however, that a similar picture would have been seen for RNAse P (Guthrie & Athison, 1980, Kole & Altman, 1979) in which it was later shown that the RNA contains the enzymatic activity of the particle (Guerrier et al., 1983). Only when electrostatic repulsion of the two RNAs was diminished (by the inclusion of high concentrations of spermidine in the reaction) was it possible to uncover the enzymatic activity of the RNA alone. These ionic conditions are incompatible with the standard translocation assay (because they would inhibit protein synthesis). Because of recently established assays in which SRP-mediated targeting is uncoupled from elongation (Fig. 7B,

TABLE II: Summary of "mutants" obtained by removal or alteration of specific protein or RNA domains within SRP

Mutant Phenotype	Particle Displaying The Phenotype	Reference
Elongation Arrest$^-$ Translocation$^+$	SRP(-9/14), SRP(S), p9/14 modified in isolation	Chapter 2, 3 This Chapter
Elongation Arrest$^+$ Translocation$^-$	SRP(68/72^N)	This Chapter
Elongation Arrest$^-$ Translocation$^-$	SRP(54^N)	This Chapter

See Discussion.

also cf. Chap 6), it should now be possible to assay for targeting under high concentrations of spermidine and perhaps uncover an activity for SRP RNA alone.

Signal Recognition, Elongation Arrest and Translocation Promotion are Independent Activities

We found that SRP(-9/14) and SRP(68/72^{N}) have converse phenotypes. SRP(-9/14) has lost the ability to arrest elongation but can still promote translocation, while SRP(68/72^{N}) has lost the ability to promote translocation but can still arrest elongation. Both these particles must still be able to recognise signal sequences. We can conclude from this i) that signal recognition can be uncoupled from elongation arrest, and ii) that signal recognition can be uncoupled from translocation promotion. Therefore, it follows that all three activities are distinct.

p68/72 Interacts With SRP Receptor

Microsomal membranes fail to release the arrest induced by SRP(68/72^{N}) (Fig. 5B), demonstrating that SRP receptor cannot interact functionally with this SRP and cause its release from the ribosome. The continued arrest of preprolactin by SRP(68/72^{N}) does not explain the translocation defective phenotype because removal of the elongation arrest domain does not restore translocation (Fig. 6B). Rather, nascent chains of preprolactin bound to SRP(68/72^{N}) fail to be targeted to microsomal membranes under any salt conditions (Fig. 7), suggesting that the initial interaction of the nascent chain-ribosome-SRP intermediate with SRP receptor did not occur. Finally, NEM inactivation of p68/72 reduced the binding of SRP to an SRP receptor affinity column (Fig. 8), and p68/72 could be specifically depleted from an SRP protein fraction

when it was passed over such a column (Fig. 9). Together these data strongly support the hypothesis that p68/72 interacts directly with SRP receptor to target secretory proteins to the membrane.

The binding of p68/72 to the receptor column was somewhat reduced when compared to the binding of SRP. This suggests that the other proteins and/or the RNA play a role in the interaction, either by interacting directly with SRP receptor, or by changing the overall charge of the molecule. It should be noted that both 68/72 and SRP receptor are positively charged proteins, which should lead to electrostatic repulsion. However, SRP has an overall negative charge, which may help to overcome the electrostatic repulsion of the two proteins, thereby increasing the apparent affinity. Finally, it has been seen by footprint analysis (see Chapter 5) that SRP receptor and p68/72 protect similar regions of 7SL RNA from nuclease digestion. It is not clear whether such an interaction of SRP receptor with SRP RNA has functional significance, because none of the sites protected by SRP receptor are accessible in SRP, but it is tempting to speculate that after an initial interaction of p68/72 with SRP receptor, the SRP RNA becomes involved in the interaction, leading to a change in the conformation of SRP and to its release from the ribosome.

It is interesting to note in this context that SRP receptor is able to release the arrest induced by SRP even when SRP is cross linked to the signal sequence (Wiedmann et al., 1987). In other words, even though SRP continues to interact with the signal because it is cross-linked to it, this interaction no longer leads to elongation arrest. The result suggests that SRP receptor releases SRP from the ribosome by inducing a conformational change in the particle.

p54 is Required for Signal Recognition

When we tested SRP(54^N) for its ability to bind to ribosomes synthesizing preprolactin, we found that the affinity was not detectably increased over its affinity for ribosomes synthesizing globin (Fig. 10). Such a result suggests that p54 is required for signal recognition per se. This result is consistent with the findings of Tom Rapaport's group (Kurzchalia et al., 1986) and Art Johnson's group (Krieg et al., 1986) that the 54 kd protein of SRP interacts directly with the signal sequence in photoaffinity cross linking experiments.

It is intruiging that the 54 kd protein, which is absolutely essential for the activity of SRP, is the one that is associated least tightly in the particle. It does not bind directly to the RNA either by cosedimentation (Walter & Blobel, 1983a) or footprint (Chapter 5) analysis; rather it requires the 19 kd protein for its association with SRP. Whether this indirect association of p54 with the RNA reflects the evolution of this particle from a peripheral membrane protein that acted directly as a signal receptor (p54 in an earlier form) to a ribonucleoprotein that acts as an adapter between the cytoplasmic translational machinery and the membrane-bound translocational machinery, or whether it reflects a multiplicity of p54s that have specificities for different classes of signal sequences, awaits further study.

In summary, the use of NEM to selectively inactivate single proteins in the signal recognition particle has allowed us to map the functions of SRP to particular protein domains. Such a method may be of general utility in the study of the structure and function of ribonucleoproteins and other complex biological structures.

CHAPTER 5

MAP OF SRP PROTEIN BINDING SITES BY NUCLEASE PROTECTION

ABSTRACT

We have used the nuclease α-sarcin to map the binding sites of the 19 kd and the 68/72 kd proteins on SRP RNA. We found that the regions of protection to nuclease afforded by the two proteins were distinct. p19 protected primarily the two tips in the RNA secondary structure, suggesting that these regions might be involved in tertiary interactions. p68/72 protected a large region, and altered the nuclease pattern in the regions that p19 would bind, suggesting that these two proteins may be in close proximity in the particle. The protection afforded by the two proteins in combination was equal to the sum of the individual protections. We have not observed cooperativity in the binding of these two proteins; nor do we have any evidence that the structure becomes more compact as it assembles.

The map derived from this footprint analysis places the signal recognition domain (p54 bound to the RNA via the 19 kd protein) and the elongation arrest domain (associated with the Alu end of the particle) on opposite ends of the particle. Thus it is possible that SRP recognizes signals by the direct interaction of p54 with the signal sequence at the nascent chain exit site and simultaneously blocks elongation by the entrance of p9/14 into the aminoacyl tRNA site 16 nm away.

INTRODUCTION

Signal recognition particle is a small cytoplasmic ribonucleoprotein composed of one molecule of 7SL RNA and four proteins. The RNA within SRP is highly resistant to micrococcal nuclease (see Chap. 3), suggesting that most of it is involved in protein contact. Conversely, three of the four SRP proteins have been shown to bind independently to the RNA (Walter & Blobel, 1983a, Chap. 2).

Electron spectroscopic imaging has been used to localize the RNA within the particle. The RNA was found to be concentrated at the two ends of the particle, suggesting that the RNA spans the length of SRP, forming an extended stem structure which serves as a backbone for SRP assembly (Andrews et al., submitted).

One function of the RNA in SRP is clearly to form a strucural lattice. When SRP was disassembled into RNA and protein components, the proteins no longer sedimented together as a particle of 250,000 molecular weight, but rather sedimented as monomers or dimers (Walter & Blobel, 1983a). Furthermore, these proteins, which in the presence of the RNA formed a tightly bound particle which was stable up to 1M salt, could be separated into four distinct groups under nondenaturing conditions (Chap. 2).

In order for SRP to target proteins to the endoplasmic reticulum, it must maintain a number of different activities. These activities have been mapped to distinct protein domains by the biochemical mutagenesis experiments described in Chapters 2-4. It is reasonable to assume that in order for these proteins to be active, they must be oriented in the particle in a particular relative geometry. For this reason, we sought to determine the protein binding sites of the SRP proteins on SRP RNA.

Micrococcal nuclease digestion of SRP yielded two distinct particles that cosedimented on sucrose gradients (Gundelfinger et al., 1983). p9/14 cosedimented with the Alu-like sequences of SRP RNA and the remaining proteins cosedimented with the S-sequences of the RNA. We showed in Chapter 3 that this latter subparticle, which we termed SRP(S), contained the signal recognition and translocation promoting

activities of the particle, and hence contained all the essential functions that enable SRP to mediate targeting. Thus the elongation arrest domain has been mapped to the "Alu end" of the RNA, and the signal recognition and protein translocation functions have been mapped to the "S" end.

α-Sarcin, a small basic protein purified from Aspergillus giganteus, is a ribonuclease with sequence homology to ribonuclease U_2 (for review see Wool, 1984) which cuts on the 3' side of most adenines and guanines. More specifically, although there appears to be some preference for particular purines within an RNA molecule, that preference is not based on secondary structure (Endo et al., 1983). It was shown to be a useful enzyme for footprinting RNA by Ira Wool and colleagues, who chose as substrates the complexes of E. coli 5S rRNA and ribosomal proteins. They succeeded in determining the binding sites for each of the ribosomal proteins (L5, L18, and L25) by this method (Huber & Wool, 1984). We have used α-sarcin to map on the SRP RNA the proteins responsible for signal recognition (p19 together with p54) and protein translocation (p68/72).

MATERIALS AND METHODS

Materials

Nuclease free BSA, proteinase K, and calf liver tRNA were purchased from Boehringer Mannheim; ribonuclease α-sarcin was a kind gift of Ira Wool; T_4 RNA ligase and ribonucleases T_1 and U_2 were purchased from Pharmacia; γ-^{32}P-ATP was purchased from ICN; T_4 polynucleotide kinase was from U.S. Biochemical Corp; dimethylsulfoxide (glass-distilled and filtered through 0.5 micron filters) was from Aldrich. All other reagents were purchased as described previously.

Purification of SRP, SRP Proteins and SRP RNA

These procedures are described in Chap. 2.

Synthesis of ^{32}P-pCp

pCp was synthesized according to England et al. (1980). One millicurie γ-labelled 5'-^{32}P-ATP (7000 Ci/mmol) was incubated for 1 hr at 37°C in 20µl 25mM Tris-HCl, pH 8.0, 5mM $Mg(Cl)_2$, 5mM DTT, 50 µg/ml nuclease free BSA, 2mM 3'-CMP, and 250 U/ml T_4-polynucleotide kinase. The sample was then incubated for 15 min at 65°C before dilution to 50 µl for storage.

3'-Labelling of 7SL RNA

Labelling was performed essentially as described (ibid.). 2µg 7SL RNA in 2µl was mixed with 2 µl DMSO, heated for 3 min at 65°C, and chilled in ice water. 100 µCi ^{32}P-pCp was evaporated to dryness in a Savant Speed-Vac concentrator. The final volume of the ligation reaction was 10 µl and contained 50mM Hepes, pH 7.5, 20mM $MgCl_2$, 10 µg/ml nuclease free BSA, 6 µM ATP, the DMSO/RNA mixture, and 8U T_4-RNA ligase. The ligation reaction was incubated overnight at 4°C, and was stopped by bringing the mixture to 1% SDS, 20mM EDTA, 50mM TRIS, pH, 7.5, and 600 µg/ml calf liver tRNA. Proteinase K was then added to 200 µg/ml and incubated at 37°C for 30 min. RNA was then purified by perchlorate extraction as described (Walter and Blobel, 1982), or by phenol extraction followed by ethanol precipitation.

Footprinting with α-Sarcin

3'-labelled 7SL RNA (0.6 pmol) was mixed with purified SRP protein at varying stoichiometries (a 2-fold and a 10-fold excess of protein to RNA is shown here) under standard reconstitution conditions. One tube contained SRP protein compensating buffer rather than protein. The RNA

concentration during the reconstitution ranged from 100 to 150nM. After reconstitution, the sample was diluted to final buffer conditions of 20mM Hepes, pH 7.5, 100mM KOAc, 1mM $Mg(OAc)_2$, 10 µg/ml calf liver tRNA, and 50 µg/ml nuclease free BSA. The tRNA and BSA were added prior to the final dilution, so that competitor would be present as soon as the sample was in low ionic strength.

α-Sarcin was then added to 0.2 or 2 µM and the samples incubated for 15 min at 30°C. After digestion, the sample was diluted to 50 µl and 5mM EDTA. Samples were extracted with hot (60°C) phenol, ethanol precipitated, and resuspended in 10 µl formamide sample buffer (95% formamide, 10mM EDTA, 0.1% (w/v) each xylene cyanol and bromphenol blue). Samples were heated to 95°C for 5 min and quick chilled in ice water. Digestion products were displayed on a 5% acrylamide, 50% urea wedge gel which was run at a constant power of 50 watts. Gels were rinsed for 20 min with 5% methanol, 5% acetic acid and then dried at 80°C for 30 min. Bands were visualized by autoradiography.

Digestion with Nucleases T_1 and U_2

3'labelled 7SL RNA (0.3 pmol) was mixed with 1 µg calf liver tRNA and evaporated to dryness. It was then respended in 2 µl 25mM NaOAc, 1mM EDTA, 7M urea, containing 10 or 100 U/ml T_1, or 100 U/ml U_2 (Donis-Keller et al., 1978), and incubated for 15 min at 55°C. 2 µl loading buffer was then added, samples heated to 95°C, quick chilled, and loaded onto gels as above.

Alkaline Hydrolysis

0.3 pmol 3'-labelled 7SL RNA was mixed with 1 µg calf liver tRNA and evaporated to dryness. It was then resuspended in 2 µl 50mM NaOH, 1mM EDTA and boiled for 40 sec. 2 µl loading buffer was added, and the

sample was loaded directly onto the gel without further denaturation.

RESULTS

SRP RNA was labelled with ^{32}P-pCp using RNA ligase. After elution of the labelled RNA from a 7M urea, 6% acrylamide gel, the RNA was reconstituted under standard conditions with one or a combination of SRP proteins at either a 2- or a 10-fold excess of protein over RNA. Because α-sarcin is inhibited by both monovalent and divalent cation, we diluted the reconstitute in the presence of competitor RNA (calf liver tRNA) and protein (nuclease free BSA) prior to digestion. We have tried other competitor RNAs (total oocyte RNA and total ribosomal RNA) and have found the same results in our footprinting experiments (data not shown).

We found that α-sarcin was remarkably inactive even at the relatively low concentrations of monovalent and divalent cations used in these experiments (100mM KOAc, 1mM $Mg(OAc)_2$), requiring an excess of nuclease over RNA to see a sequencing ladder (in Fig. 1 we show 2 μM sarcin on 0.6 μM RNA). This is consistent with the results found for 28S ribosomal RNA, in which the concentration required to cleave 50% of the RNA was 4.2 μM (Endo et al. 1983), and in that case even lower monovalent (50mM KCl) and divalent (0) cation was used. However, the digestion at these high concentrations was sufficient to detect protection by the protein, as can be seen in Figure 1.

Lane 1 shows the digestion pattern of naked SRP RNA in the region of the S sequences. Many but not all purines in this region have been cleaved by the nuclease. This nucleolytic pattern represents the part of the RNA on which the footprinting of the proteins can be read, and is shown in nucleotide sequence in Figure 2A (as capitol letters in the sequence), and along the secondary structure diagram in Figure 2B (as

Figure 1. Nuclease digestion pattern of SRP RNA in the absence and presence of SRP proteins.

SRP proteins, singly or in combination, were reconstituted with 3'-labelled SRP RNA as described in Materials and Methods. The reconstitutes were then diluted and digested with 0.2 or 2 μM sarcin for 15 min at 30°C. The digested particle was phenol extracted, ethanol precipitated, and resolved on 7M urea 6% acrylamide wedge gels.

Lane 1: naked RNA

Lane 2: p19, 2-fold excess over RNA

Lane 3: p19, 10-fold excess

Lane 4: p54, 2-fold

Lane 5: p54, 10-fold

Lane 6: p19+p54, 2-fold

Lane 7: p19+p54, 10-fold

Lane 8: p68/72, 2-fold

Lane 9: p68/72, 10-fold

Lane 10: p19+p68/72, 2-fold

Lane 11: p19+p68/72, 10-fold

Lane 12: p19+p54+p68/72, 2-fold

Lane 13: p19+p54+p68/72, 10-fold

Last lane: uncut RNA

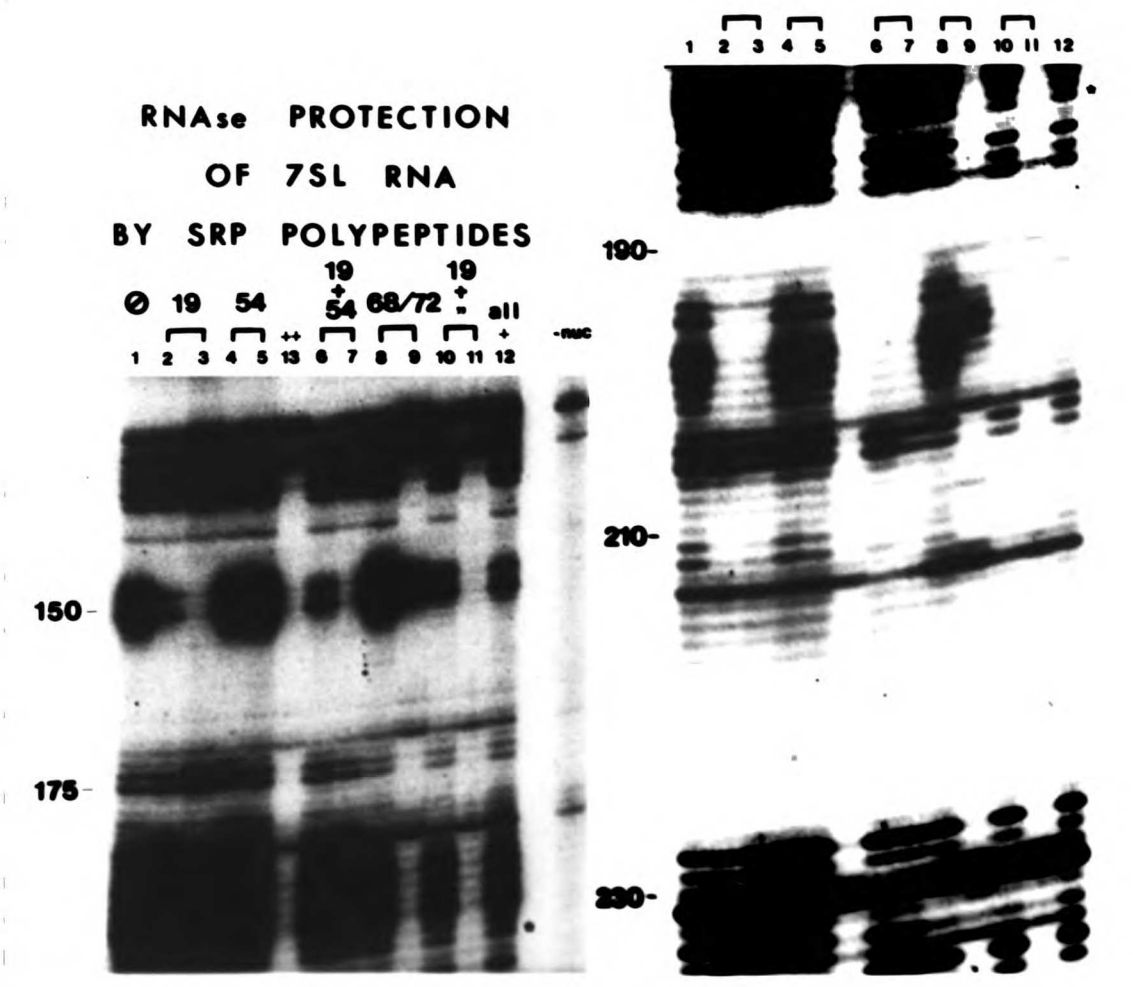

RNAse PROTECTION
OF 7SL RNA
BY SRP POLYPEPTIDES
Ø
19
54
19 + 54
68/72
19 + -
all
+
-muc
1 2 3 4 5 13 6 7 8 9 10 11 12
150
175
1 2 3 4 5 6 7 8 9 10 11 12
190
210
230

Figure 2. Sites on naked SRP RNA that are susceptible to nuclease cleavage.

Panel A: The nucleotides that are cut by α-sarcin are shown in capitol letters.

Panel B: The nucleotides that are cut by α-sarcin are shown as doubled lines in the secondary structure diagram.

A

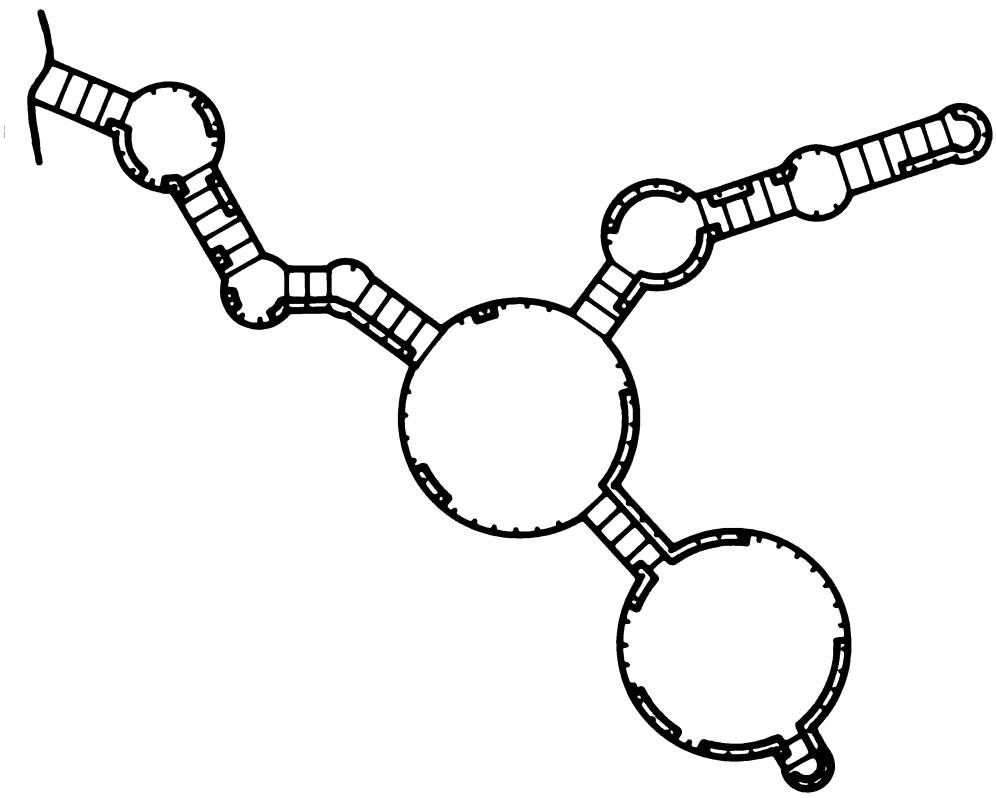
c_{100} GAtcGGgtgtccgcactaaGttcggcatCAANAtGGtGacctcccgGGAG

CGGgggaccaccAGGTTGcctAAGGAGGGGTGAaccggcccAGGTCGGAA

AcGGAGcAGGtcaaAActccngtgctGAtcagtAGTGGGAtcGcGcctGt

GAAtAG c_{257}

B



doubled lines in the structure).

The pattern of nucleolytic cuts in the presence of each of the proteins and of the proteins in combination is shown in Figure 1. The protection that results from adding these proteins is depicted schematically in Figure 3. p19 (lanes 2 and 3) protects the RNA from digestion primarily at nucleotides 147-153 and nucleotides 192-201, and lightly at nucleotides 207-209. The regions constitute the tips in the secondary structure of the RNA (Fig.3A).

p54 does not protect the RNA on its own (lanes 4 and 5), consistent with the result that p54 does not bind to the RNA, as assessed by sucrose gradient analysis. Because p54 has been shown to bind via p19 (Walter & Blobel, 1983a), we reconstituted these two proteins together on the RNA, and found the footprint to be identical to that found for p19 alone (compare lanes 2 and 3 with lanes 7 and 8). Thus p54 does not alter the binding of p19 to the RNA.

p68/72 protects the RNA at sites distinct from p19. The sites of protection by this protein are extensive (see lanes 8 and 9, and Fig.B), though perhaps the most striking protection (because of the strong nucleolytic cleavage on naked RNA in this region) is at nucleotides 171-183. Note also that although p68/72 does not completely protect the sites protected by p19, it alters the pattern of digestion seen in those regions (giving an enhancement of digestion at C_{196}, for example, and an incomplete protection at nucleotides 147-153). This alteration in the cutting pattern may result from p68/72 sterically hindering the entrance of α-sarcin into these regions.

The protection pattern seen when p68/72 and p19 are reconstituted together on the RNA (lanes 10 and 11) is the sum of the patterns seen

Figure 3. Protection of SRP RNA by SRP proteins.

The nucleotides that are protected by protein are indicated by circles along the secondary structure diagram. Filled circles indicate strong protection, open circles indicate weaker protection, and arrows indicate enhancement.

Panel A. Protection by p19.

Panel B. Protection by p68/72.

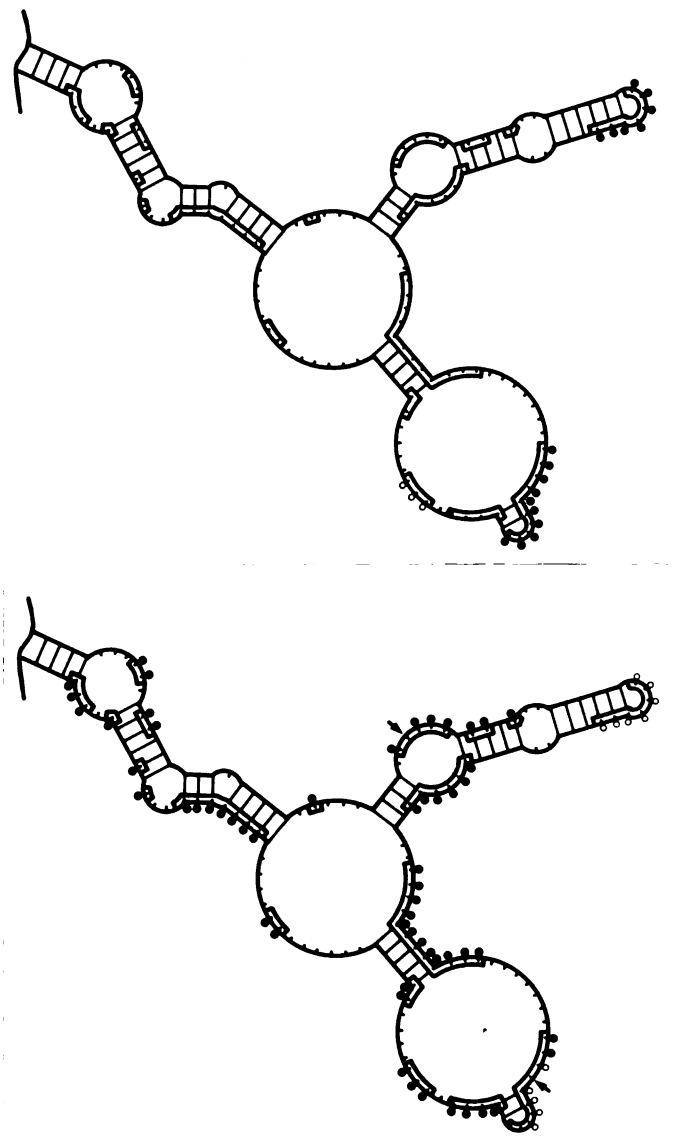
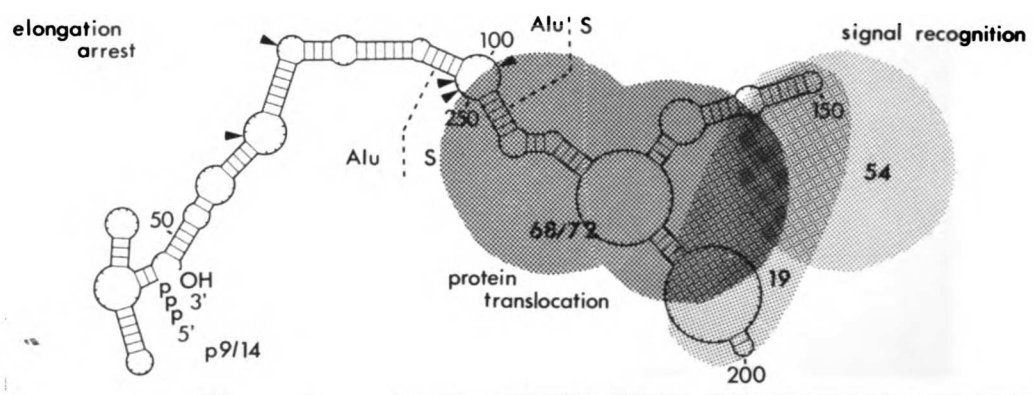


Figure 4. Map of the relative geometry of the protein domains with respect to the RNA secondary structure.

See Chapters 2-4 for assignment of functions to specific protein domains.


elongation
arrest
100
Alu' S
signal recognition
250
150
Alu S
54
50
68/72
OH
protein
translocation
19
3'
5'
p9/14
200

when each of the proteins is bound individually. In particular, the regions that were altered in their digestion pattern by 68/72 but fully protected by p19 are fully protected when the proteins are added in combination. In this case, the only nucleolytic sites remaining in the S region are those found in the undigested RNA (last lane). This is consistent with the results from nuclease digestion studies on intact SRP, in which the entire S region was found to be nuclease resistant (micrococcal nuclease digestion was shown in Chapter 3; α-sarcin seems to cut SRP only once, approximately at nucleotide 75 (data not shown)).

A higher concentration of p68/72 (a 10-fold excess) than of p19 (a 2-fold excess) was required to completely protect the RNA from nuclease. This result suggests that p19 has a higher affinity for the RNA than does p68/72, which may indicate that this protein serves as an initiator of assembly. However, when the two proteins were bound in combination, the affinity of p68/72 was not demonstrably increased; again a 10-fold excess of protein was required to completely protect the RNA. Thus we have no evidence as yet for any cooperativity of binding, which has been implicated by reconstitution experiments (Walter & Blobel, 1983a). It is possible that the cooperativity of binding is brought in by another component (p9/14), or that we have lost the cooperativity in the further purification of these proteins (Chap. 2), or that we have simply not tested the cooperativity at a concentration of protein where the effect would be detected.

DISCUSSION

We have mapped by nuclease protection the binding sites of the 19 kd and the 68/72 kd proteins of SRP on the SRP RNA. We found that the protection of SRP RNA by p19 is very specific: only the tips of the

molecule are altered in their digestion pattern. The region of protection includes the most highly conserved region of the molecule (Gundelfinger et al., 1984). When secondary structures are drawn for putative SRP RNAs from the two yeast species Schizosaccharomyces pombe and Yarrowia lipolytica, the sequences in these tips are conserved, in contrast to the overall lack of primary sequence homology in the rest of the molecule (M. Poritz & P. Walter, unpublished). These findings suggest that p19 binding is sequence specific. Therefore it should be possible to identify SRP RNAs in other organisms by their ability to bind canine p19.

In contrast, p68/72 protects a large region of the RNA, the conservation of which is more strongly seen in secondary rather than primary structure. In addition, SRP receptor has been seen to footprint a very similar region of the SRP RNA (unpublished), the functional significance of which has yet to be determined. It seems possible that p68/72 (and SRP receptor) binds primarily to double stranded regions of the RNA and is positioned properly along the particle by the structure of the RNA rather than by its sequence. It would be interesting to see whether this protein could bind to double stranded RNAs in general.

When added together to the RNA, the proteins protect the sum of the regions they protect individually, suggesting that the RNA does not become more compact as SRP assembles. Furthermore, we have failed to detect an increase in affinity of the proteins when they are added in combination, which one would expect from reconstitution experiments. This cooperativity in binding may become apparent when intermediate concentrations of the proteins are tested in the footprinting assay.

In conclusion, we have determined the relative geometry of the various functional domains of SRP (see Fig. 4). We showed in Chapter 3 that the elongation arrest domain, which requires p9/14 for activity, resides at the Alu end of the particle. In the footprinting studies delineated in this Chapter, we have shown that the protein translocation domain, which requires p68/72 for activity (Chap. 4), extends across much of the length of the S region of the RNA, and may be thought of as sitting in the middle of the particle, and that the signal recognition domain, which requires p54 for activity (Chap. 4), associates with the particle by binding to p19 (Walter & Blobel, 1983a), which in turn binds to the tips of the RNA secondary structure, and thus may be thought of as being at the end (opposite to the Alu end) of SRP.

We have therefore placed the elongation arrest domain and the signal recognition domain at opposite ends of the particle. SRP has been shown to be 24 nm long (Andrews et al., 1985), and the aminoacyl tRNA site and the nascent chain exit site 16 nm apart (Bernabeau et al., 1983). The positioning of the elongation arrest domain and the signal recognition domain at opposite ends of SRP is consistent with the model that SRP physically bridges the distance between the nascent chain exit site and the aminoacyl tRNA site, and that these protein domains on SRP perform their functions by a direct interaction with the two regions on the ribosome, as opposed to by some long range conformational effect.

CHAPTER 6

SUBSTRATE REQUIREMENTS FOR SRP MEDIATED TARGETING

ABSTRACT

We have developed an assay for SRP-mediated targeting of preprolactin nascent chains to the endoplasmic reticulum (ER) membrane that is not dependent on concommitant elongation. This assay has allowed us to determine the relationship between the sharp "cut-off" point in elongation beyond which preprolactin can no longer be translocated across the microsomal membrane and the actual nascent chain length. We found that chains up to about 140 amino acid residues in length were efficiently targeted to the membrane, and that beyond this length a drop in affinity of SRP for the nascent chain-ribosome complex occurred. If the SRP concentration was raised high enough, all chains associated with the ribosome could be targeted. We propose that the drop in affinity reflects a sequestering of the signal sequence within the folding preprolactin chain. Chains that are too long to be efficiently targeted at a "low" SRP concentration can be brought to the microsomal membrane along polysomes that contain shorter chains. However, these longer chains do not engage the translocation machinery, suggesting that SRP is required to initiate each translocation event.

INTRODUCTION

When signal recognition particle (SRP) was first purified, a detailed analysis was made of its effects on secretory protein synthesis and translocation (Walter et al., 1981; Walter & Blobel, 1981a,b). This analysis led to the model for its role in the targeting of secretory proteins to the endoplasmic reticulum membrane reviewed in Chapter 1. The view was that SRP was specialized to couple the cytoplasmic translational machinery with the membrane bound translocational machinery. In particular, SRP was found to bind with high affinity to ribosomes

synthesizing the precursor to the secretory protein prolactin (Walter et al., 1981) and to arrest the synthesis of this protein at a specific point, leading to the formation of an arrested fragment (Walter & Blobel, 1981b). This elongation arrest was released upon interaction of SRP with an integral membrane component of the endoplasmic reticulum termed SRP receptor (Gilmore et al., 1982b) or docking protein (Meyer et al., 1982a). The interaction of SRP with its receptor led to the release of SRP from the nascent preprolactin-ribosome complex (Gilmore & Blobel, 1983), and to the translocation of preprolactin by a mechanism that is unknown, but which contains a GTP requiring step (Connolly & Gilmore, 1986).

The SRP-mediated targeting of secretory proteins to the ER membrane was strictly cotranslational. In other words, there existed a sharp cut-off point in the elongation of the secretory protein chain beyond which the protein could no longer be translocated. It was not clear whether this cut off point reflected an incompatability of more completely folded chains with the translocation machinery or a loss of affinity of SRP for ribosomes synthesizing these chains. The time in elongation at which this cut-off point occurred roughly agreed with the time of synthesis of the arrested fragment (Chap. 2, Walter & Blobel, 1981c), giving the impression that the nascent chain could be targeted only at this precise point. The elongation arrest activity was therefore thought to serve a fidelity function, preventing the synthesis of secretory proteins in the cytoplasmic compartment.

The view that SRP can interact with the nascent chain only at a precise point in its synthesis has been called into question. In particular, although elongation arrest has been demonstrated for a

number of presecretory proteins, the arrested fragment has been successfully isolated only in a subset of these cases (for review see Hortsch & Meyer, 1984), and seemed to require that the translation reaction be synchronous. In addition, other workers found that SRP could productively interact with the nascent chain quite late in its synthesis (Ainger & Meyer, 1986).

In order to address these questions, we assayed the ability of incomplete preprolactin chains of varying lengths to be targeted to the ER membrane. To do this, we developed an assay in which the targeting of these chains was uncoupled from elongation.

MATERIALS AND METHODS

Materials

Micrococcal nuclease from Staphylococcus aureus was purchased from Boehringer Mannheim. Other materials were described previously.

Purification of SRP and Microsomal Membranes

Purification was as described previously (Chap. 2).

In Vitro Transcription

pSPBP4 was transcribed using SP6 polymerase as described (see Chap. 4).

In Vitro Translations

Generation of a distribution of preprolactin nascent chains was achieved by translating full length preprolactin synthetic mRNA in a wheat germ cell-free translation system (Erickson & Blobel, 1983) for 14 min at 22°C. Truncated nascent chains were allowed to accumulate over a 30 min synthesis at 26 °C. Cycloheximide was then added to 1 mM to block further elongation and to encourage the nascent chains to remain bound to the ribosome.

Targeting Assays

All targeting assays were performed under physiological salt conditions. 10 µl of a wheat germ translation reaction (see above) was mixed with 4 eq (Walter et al., 1981) of microsomal membranes and incubated for 5 min at 22°C. This mixture was then layered on top of a 50 µl 0.5M sucrose cushion containing 50mM triethanolamine, pH 7.5, 140mM KOAc, 2.5mM $Mg(OAc)_2$, and 1mM DTT in a polyallomer airfuge tube. These tubes were spun for 3 min at 20 psi in an 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 250 µl 2% CTABr and nascent chains were precipitated using 50 µg calf liver tRNA according to Gilmore and Blobel (1985).

Micrococcal Nuclease Digestion of Polysomes

After synthesis, $CaCl_2$ was added to 1mM and micrococcal nuclease was added to 10 U/ml and allowed to react for 10 min at 25°C. Digestions were stopped by the addition of EGTA to 2mM.

RESULTS

The "Arrested Fragment" of Preprolactin Represents an SRP-Independent Stutter Point in the Synthesis of the Nascent Chain

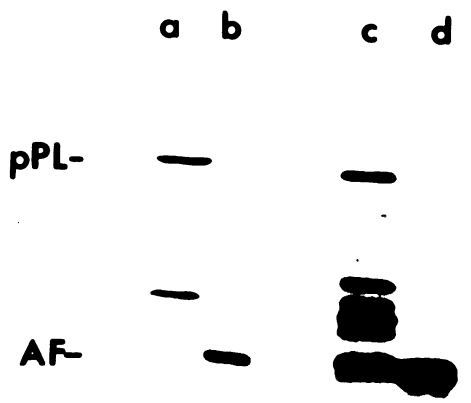
Experiments done in synchronized translation assays (in which only a short period of initiation is allowed) programmed with pituitary RNA give the impression that signal recognition particle (SRP) interacts with nascent preprolactin at a precise point: in the presence of SRP a novel band appears (compare Fig. 1, lanes a and b), which can be chased upon addition of microsomal membranes into mature prolactin (Walter & Blobel, 1981b) This band has been termed the "arrested fragment". We attempted to visualise this arrested fragment in nonsynchronized systems

Figure 1. The arrested fragment of preprolactin is a stutter point in its synthesis.

Pituitary RNA was used to program a wheat germ translation reaction either in the absence (lane a) or presence (lane b) of 50nM SRP. After 1 min, a mixture of 7-methyl-guanosine-5'monophosphate and edeine was added to block further initiation. After 40 min the translation was stopped by addition of TCA to 10%.

Synthetic preprolactin RNA was used to program the translation reactions in lanes c (no SRP) and d (50nM SRP). No initiation inhibitors were added. After 40 min the translation was stopped by addition of cycloheximide to 1mM. These samples were then CTABr precipitated according to Gilmore & Blobel (1985).

The bands that correspond to preprolactin (pPL) and the arrested fragment (AF) are indicated.


a
b
c
d
pPL-
AF-

by using synthetic preprolactin mRNA and by enriching for nascent chains with the positively charged detergent cetyltrimethylammoniumbromide (CTABr), which precipitates these chains by virtue of their covalent linkage to tRNA (Hobden & Cundliffe, 1978). We compared these chains with those synthesized in the synchronized system.

In a nonsynchronized translation of synthetic preprolactin message (Fig. 1, lanes c and d), we found a nascent chain that comigrated with the arrested fragment. Note that this band represents the predominant chain synthesized in the presence of SRP. That SRP has interacted with a chain of this length and blocked all further protein synthesis is evident from the disappearance of chains higher up the gel (compare lane c with lane d). Surprisingly, this band also appears in the absence of SRP (lane c), and thus represents a stutter point in the synthesis of preprolactin.

The "arrested fragment" is not the only stutter point in the synthesis of preprolactin, which is evident in lane c. Several discrete bands appear of varying length, ranging from chains shorter than the "arrested fragment" to what appears to be unterminated preprolactin. We can calculate the lengths of these chains by comparing them with chains of known length generated by truncation of the preprolactin mRNA. We show a series of chains generated in this way in Figure 2; the length of each chain is indicated.

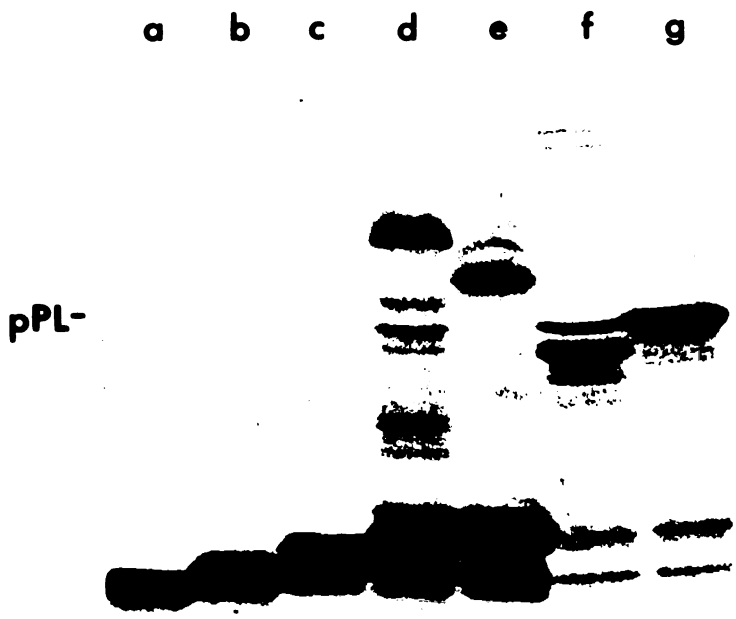
SRP Can Interact With Nascent Chains in the Absence of Elongation

The translocation of preprolactin across the microsomal membrane was found to be strictly cotranslational. In other words, a sharp cut-off point in elongation was observed after which translocation no longer occurred. We wondered whether we could take advantage of the

Figure 2. Synthesis of truncated preprolactin chains.

Plasmid pSPBP4 was cut with the following endonucleases and then transcribed with SP6 polymerase and translated in a wheat germ extract to yield chains of the following sizes:

Lane	Enzyme	# of Amino Acids	Molecular Weight
a	FokI	55	5818
b	PvuII	87	9539
c	MboII	100	11964
d	HinFI	114	12581
e	RsaI	132	14689
f	FspI	205	22898
g	EcoRI	229	25797


a b c d e f g
pPL-

distribution of nascent chain lengths to ask directly whether the cut off point seen for translocation reflected the loss of the ability of the chain to interact with SRP, and if so, to determine at what length of chain this "cut-off" occurred. To do this, we needed to know first whether SRP could interact "post-translationally" with any of these chains. A targeting assay was chosen to detect this interaction.

Preprolactin was translated for a short time allowing the accumulation of nascent chains. Then further protein synthesis was inhibited by addition of cycloheximide. Finally SRP and membranes were added and the mixture was centrifuged in the airfuge yielding a membrane bound (pellet) and an unbound (supernatant) fraction. The result of this experiment is shown in Figure 3.

We found that SRP stimulated the binding of incomplete chains to the membrane (compare lanes d and f). Furthermore, certain quite predominant chains failed to be targeted and were recovered almost completely in the supernatant fraction (lane e). Interestingly, these chains corresponded to ones that were inefficiently precipitated by CTABr (compare total (lane a), CTABr precipitable (lane b), and lane e), and thus probably represented chains that were no longer associated with the ribosome. We conclude from this result that SRP can target only those chains that are associated with the ribosome.

Because of the requirement for ribosome coupling, it seemed possible that we could increase the percentage of chains targeted to the membrane subsequent to elongation by programming the translation reaction with incomplete mRNAs. These RNAs would lack a termination codon and thus be held onto the ribosome after synthesis. Figure 4 shows some of the truncations we had generated (displayed in Fig. 2) used in a

Figure 3. Targeting of incomplete preprolactin chains after inhibition of elongation by cycloheximide.

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 (lane a) and one was CTABr precipitated (lane b). One aliquot (lanes e and f) received SRP (10nM final) and one (lanes c and d) an SRP compensating buffer. After a 10 min incubation at 22°C, 4 eq SRP-depleted membranes were added to the latter two samples, which were then incubated 5 more minutes at 22°C. The samples were then layered onto sucrose cushions and spun in an airfuge to obtain a supernatant (unbound, lanes c and e) and a pellet (membrane bound, lanes d and f) fraction. The lines alongside the figure, in order of decreasing molecular weight, indicate preprolactin, 140 amino acids in length, and the arrested fragment, respectively.

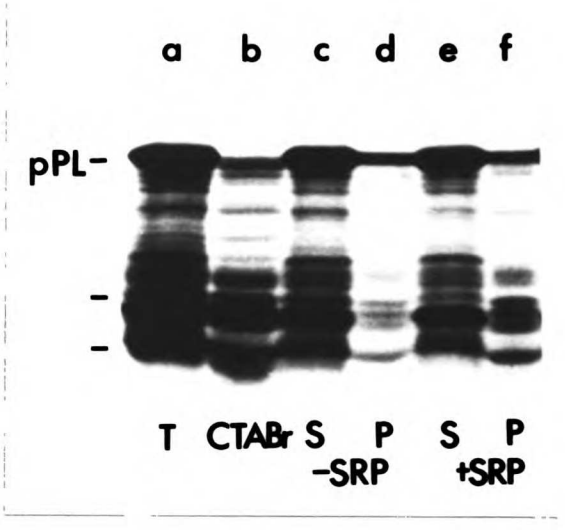
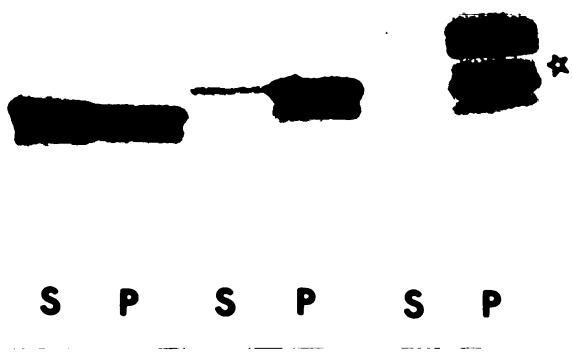

a b c d e f
pPL-
T CTABr S P S P
-SRP +SRP

Figure 4. Targeting of truncated preprolactin chains to microsomal membranes.

The FokI- (lanes a and b), PvuII- (lanes c and d), and RsaI- (lanes e and f) cut plasmids (see Fig. 2) were transcribed and translated as described. Then SRP (10 nM) and membranes (4 eq 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 Figure 3, which were then CTABr precipitated. The asterisk indicates the signal cleavage product of the RsaI derived nascent chain.


a b c d e f
S P S P S P

targeting assay. As can be seen by comparing supernatant and pellet fractions for each of the truncations, both the PvuII cut plasmid (lanes c and d) and the RsaI cut plasmid (lanes e and f), when used in transcription and translation reactions, yielded nascent chains that were very efficiently targeted to the membrane. The chain synthesized as the result of a FokI digestion (lanes a and b) was less efficiently targeted, consistent with its length (55 amino acids) being too short for the signal sequence to be fully exposed on the surface of the membrane. The asterisk next to the lane showing targeted chains derived from RsaI-digested plasmid (lane f) denotes a chain generated by signal cleavage (data not shown) which is still CTABr precipitable, thus suggesting that the RsaI generated translation product can cross the membrane while it is still attached to the ribosome.

In Figure 5, we compare the efficiency of targeting when SRP was present during elongation of the nascent chain to that when SRP was added subsequent to elongation. We found the percentage of chains associated with the membrane in the two cases to be approximately equal (compare lanes e and j). Thus the elongation independent assay in a reasonable one for studying the length dependence of targeting.

<u>There is a Cut-Off Point in the Ability of Nascent Chains to be Targeted</u>

We noticed in this experiment (see lane j) that quite long chains were efficiently targeted to the membrane. We wondered whether this was the result of being carried "piggyback" on polysomes containing shorter chains. In order to test this hypothesis, micrococcal nuclease was added subsequent to chain elongation in order to digest the mRNA between the ribosomes, thus cleaving the polysome into monosomes and yielding chains that should be targeted to the membrane independently of one

Figure 5. Comparison of targeting efficiency when SRP is present during synthesis and when it is added after cycloheximide addition.

Synthetic preprolactin RNA was used to program a translation extract, which was incubated for 14 min at 22°C in the presence (lanes a-e) or absence (lanes f-j) of 10nM SRP. SRP or SRP compensating buffer was then 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 4 eq SRP depleted membranes and one received membrane compensation buffer. The standard targeting assay was then performed, and supernatant and pellet fractions were CTABr precipitated.

Lanes a, f: CTABr precipitate

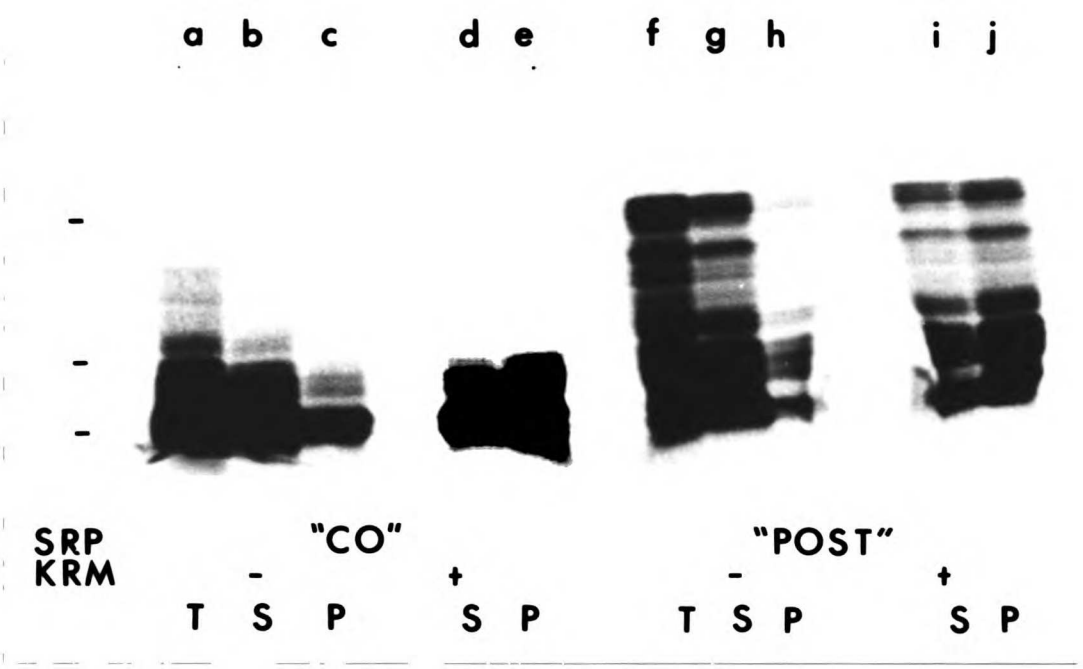
Lanes b, g: supernatant (- membranes)

Lanes c, h: pellet (- membranes)

Lanes d, i: supernatant (+ membranes)

Lanes e, j: pellet (+ membranes)

The lines alongside the figure indicate the positions of preprolactin, 140 amino acids, and the arrested fragment, in order of decreasing molecular weight.


a b c d e f g h i j
SRP
KRM
"CO"
"POST"
- + - +
T S P S P T S P S P

another. We then compared the distribution of chains targeted to the membrane without and with micrococcal nuclease digestion. The results of this analysis are shown in Figure 6.

We found that up to a certain length, the addition of micrococcal nuclease had no effect on the efficiency of targeting, but as chains became longer than this length, the ability of SRP to target them after micrococcal nuclease digestion diminished (Fig. 6, compare lanes f and h; also note the increase in higher molecular weight chains in the supernatant (lane g) of the micrococcal nuclease treated sample). The cut off point between the two states was at approximately 140 amino acids. This cut-off point probably reflects the cut off point seen in the translocation assays. Note that the size of the chain that can still be efficiently targeted (140 amino acids) is significantly longer than the size of the arrested fragment (70 amino acids). We conclude from this that SRP need not interact with nascent preprolactin at a precise point, but rather can interact over a rather long range.

The Cut-Off Point Represents a Decrease of Affinity of Nascent chains for SRP

There are two possible reasons for the existence of a cut-off point. One is that SRP can no longer interact with the nascent chain, and therefore it will not be targeted. The second is that a nascent chain of a particular length has a structure that is incompatible with targeting (i.e., at a stage after the initial SRP interaction), although SRP can interact with it.

We compared the ability of longer chains to be targeted under various SRP concentrations (Fig. 6). If we increased the SRP concentration high enough (lanes i-l show targeting reactions containing 50nM

Figure 6. Effect of micrococcal nuclease on targeting of preprolactin nascent chains at various SRP concentrations.

Preprolactin nascent chains were allowed to accumulate over a 14 min period as described. The sample was then divided into two: in one of these samples micrococcal nuclease was added to 10 U/ml and incubated for 10 min at 25 ^{o}C (see Materials and Methods) before addition of EGTA to inactivate the nuclease. The samples were further divided into 15 µl aliquots. SRP was added to varying concentrations and incubated for 10 min. Then 4 eq SRP-depleted membranes were added and incubated for 5 min. Finally, samples were divided into supernatant and pellet fractions and CTABr precipitated as above.

Lane	SRP Concentration	Micrococcal Nuclease	Supernatant/Pellet
a	0	-	S
b	0	-	P
c	0	+	S
d	0	+	P
e	17nM	-	S
f	17nM	-	P
g	17nM	+	S
h	17nM	+	P
i	50nM	-	S
j	50nM	-	P
k	50nM	+	S
l	50nM	+	P
m	270nM	-	S
n	270nM	-	P
o	270nM	+	S
p	270nM	+	P

The lines alongside the figure indicate preprolactin, 140 amino acids, and the arrested fragment.

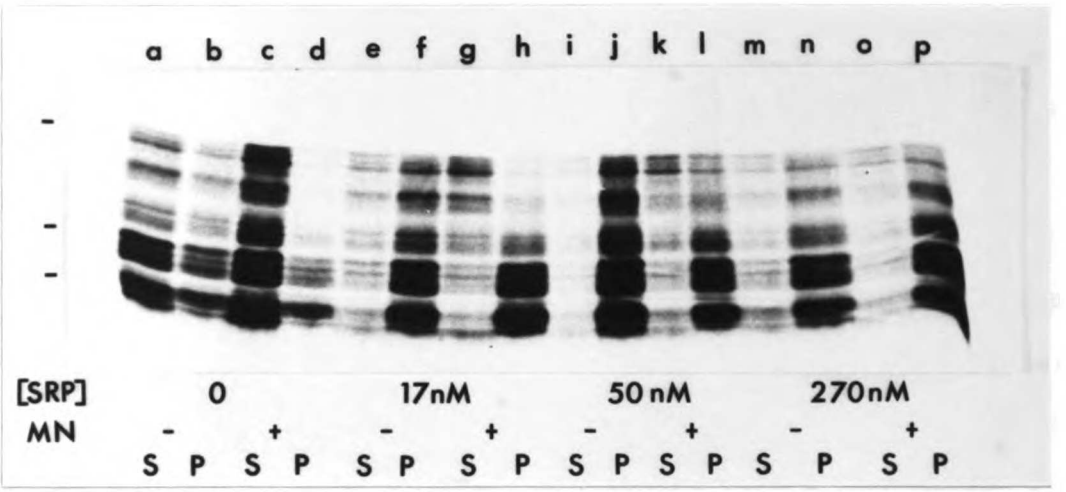
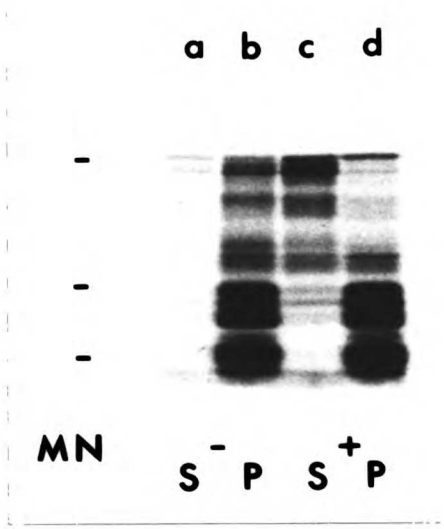

a b c d e f g h i j k l m n o p
[SRP]
0
17nM
50nM
270nM
MN
- + - + - +
S P S P S P S P S P S P S P S P

Figure 7. Effect of microccal nuclease digestion on already targeted chains.

Nascent chains were allowed to accumulate, SRP was added to 10nM, and membranes were added under conditions that all chains would be targeted. Then the reaction mixture was divided into two, and micrococcal nuclease was added to one sample (lanes c and d) as in Fig. 6. 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. The lines alongside the figure indicate preprolactin, 140 amino acids, and the arrested fragment, respectively.


a b c d
MN
S - P S + P

SRP, lanes m-p show reactions containing 270 nM SRP), we found that the longer nascent chains could also be targeted to the membrane after micrococcal nuclease digestion. We conclude from this that there is no intrinsic incompatability of the longer chains with the targeting machinery. Instead, the cut off point for targeting must represent a decrease in the affinity of SRP for the nascent chain, and that at the lower concentrations of SRP, the longer chains do not have SRP bound.

SRP is Required to Target Each Chain to the Membrane

The role of SRP in promoting translocation across the ER membrane is not fully understood. One possibility is that SRP is required solely to bring the chain in close proximity with the microsomal membrane, thus increasing the likelihood that it can interact with some membrane component. If this were the case, we would expect that longer chains, brought to the membrane along polysomes containing shorter chains, might also engage the translocation machinery. In order to test this, we added micrococcal nuclease subsequent to incubation of the nascent chain complexes with microsomal membranes (Fig. 7). If the longer chains became engaged in translocation, then we would expect them to remain associated with the membrane after digestion. We found instead that these longer chains were released from the membrane and were recovered in the supernatant fraction (lane c). We conclude from this result that proximity to the membrane is not sufficient to initiate translocation, and that each nascent chain requires SRP to engage the translocation machinery.

DISCUSSION

When we analyzed nascent chains synthesized in the presence and absence of SRP (Fig. 1), we found that the "arrested fragment" was not a

novel chain synthesized as the result of SRP arrest at a precise point in elongation, but rather was a "natural" stutter point in the synthesis of the preprolactin chain. Because SRP can interact with nascent chains the length of the "arrested fragment", ribosome stalling at this position may account for the high degree of efficiency with which preprolactin is translocated in both the wheat germ and reticulocyte lysate systems, since this stalling will increase the time window with which SRP can interact productively (Rapaport et al., 1987).

It is not clear what causes the ribosome to stall at specific points. When synthetic preprolactin mRNA was synthesized in the reticulocyte lysate translation system (data not shown), similar stutter points were seen. Also, Ainger and Meyer (1986) found positions of ribosome stalling in the synthesis of IgG light chain precursor in the wheat germ system. These findings suggest that ribosome stalling must reflect a feature that is common to the two proteins and the two translation systems, such as the level of a particular charged tRNA. Other stutter points in the synthesis of preprolactin can be seen, and we have taken advantage of this phenomenon to create an assay for targeting that does not depend on the continued elongation of the targeted substrate. With such an assay in hand, we have been able to directly assess the substrate requirements for this process.

In particular, we found three features that were important for targeting. First, the chain needed to be associated with the ribosome. Chains that were not precipitable with the positively charged detergent CTABr were found predominantly in the unbound fraction (Fig. 3). This finding is consistent with all published examples of SRP dependent "post-translational" targeting of proteins in systems utilizing

mammalian components (Mueckler & Lodish, 1986, Perara et al., 1986, Caulfield et al., 1986). In each case the targeting seemed to require continued association of the secretory protein chain with the ribosome, which could be enhanced by truncating the mRNA coding for these proteins.

The requirement for ribosome coupling seems to be intrinsic to the targeting machinery rather than to the substrate. In particular, the "post-translational" translocation of the yeast protein prepro-α-factor, which, in the homologous yeast system could occur after it was released from the ribosome and did not seem to require an SRP-like component (Hansen et al., 1986, Rothblatt & Meyer, 1986, Waters & Blobel, 1986), was dependent on SRP and required ribosome association (Garcia & Walter, in preparation) in the wheat germ system.

It appears, then, that the mammalian translocation machinery has specialized to accept as substrates only those chains that are still ribosome associated. Hence, although the targeting of these chains does not require concommitant elongation, it is not "post-translational" in the true sense of the word, because these chains would not be targeted if the final step of translation, termination, had occurred. We would like to suggest the term "ribosome-coupled translocation" for these events, and that SRP, with its micromolar affinity for all ribosomes (Walter et al., 1981), has evolved to mediate this process.

We found that as long as the chain was ribosome associated ("nascent") that targeting was equally efficient when SRP was added during nascent chain elongation and when it was added after the inhibition of elongation by cycloheximide (Fig. 5). We conclude from this that SRP does not derive any energy from nascent chain elongation in

order to bind to the nascent chain-ribosome complex. Furthermore, since in the presence of cycloheximide the peptidyl tRNA remains in the P-site, the A site will continue to scan tRNAs, and will therefore at least occasionally be empty. The targeting of cycloheximide arrested chains is consistent with the model that SRP sits partially in the A site when it is bound (Gilmore & Blobel, 1985).

Second, the efficiency of targeting seemed to depend on the length of the nascent chain. Chains 55 amino acids in length (Fig. 4) were not efficiently targeted, consistent with the hypothesis 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 about 70 amino acids in length to about 140 amino acids in length were efficiently targeted at 17nM SRP (Fig. 6), chains longer than this length were not targeted. The window in elongation during which targeting could occur is much larger than had previously been supposed (when the arrested fragment was thought to be the only length chain capable of interacting with SRP), but agrees well with results of Ainger and Meyer (1986), from which it was calculated that up to 150 amino acids of the preprolactin chain could be synthesized prior to interaction with SRP and subsequent translocation.

The cut-off in targeting efficiency reflected a decrease in apparent affinity of these nascent chain-ribosome complexes for SRP; as the SRP concentration was increased, the longer chains also became membrane bound (Fig. 6). This result is consistent with the finding of Kurzchalia et al. (1986) that chains longer than the arrested fragment could still be cross-linked to the 54 kd polypeptide of SRP, but that the cross-linking efficiency dropped off as the chains increased in

length. We suggest that the decrease in apparent affinity reflects a sequestering of the signal sequence within the growing preprolactin chain. We envisage that a component of the translation extract (perhaps an "unfoldase") allows the sequestering to be reversible (and hence we can now think of the chain 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.

Finally, we found that although at the lower SRP concentrations longer nascent chains can be brought to the membrane along polysomes synthesizing shorter chains, these chains could not engage the translocation machinery. Thus when micrococcal nuclease was added to the translation extract after targeting, the longer chains were released from the membrane (Fig. 7). From the SRP titration experiment shown in Figure 6, we concluded that the longer chains did not have SRP bound. This experiment suggests that each chain requires SRP in order to initiate translocation, and rules out the model that SRP is required solely to bring the chain in close proximity to the microsomal membrane.

With respect to the mechanism of targeting, a number of questions remain unanswered. It is not known, for example, whether SRP is sufficient to target the nascent chain-ribosome complex to the membrane, or whether additional soluble factors, such as the "unfoldase" we had hypothesized earlier, are required. Furthermore, the search for a catalytic activity for the RNA has been frustrated by the stringent ionic requirements of a cotranslational assay. The development of an assay for targeting that does not depend on ongoing protein synthesis, and the relative ease with which nascent chain-ribosome complexes can be purified, should make it possible to address these and other questions.

CHAPTER 7

SUMMARY AND FUTURE PROSPECTS

ANALYSIS OF SRP MUTANTS

Using a variety of biochemical approaches, we have generated a series of altered SRPs that retain a subset of activities. From the analysis of these mutants, we have been able i) to assign each of the activities of SRP to particular domains within the particle, and ii) to ascertain the dependency relationships of each of these activities within the framework of the "SRP cycle".

Single Omission Experiments

A simple and nondenaturing method had been devised to separate SRP into a protein fraction and an RNA fraction. We further separated the proteins by a combination of hydroxylapatite and CM Sepharose chromatography into four homogeneous subfractions. These fractions could be recombined together with the RNA to form a fully active SRP. With these separated proteins in hand, we proceeded to generate particles in which one of the protein fractions was omitted from the reconstitution reaction.

When subjected to elongation arrest and protein translocation assays, most of the particles generated in this way were completely inactive. However, one particle, SRP(-9/14) was fully active in translocation promoting activity but was completely inactive in elongation arrest. The isolation of this particle i) allowed us to assign the elongation arrest domain to the part of SRP containing the 9/14 kd protein, and ii) established that elongation arrest was not an obligatory step in the targeting of secretory proteins to the microsomal membrane. However, although the translocation promoting domain was apparently unaffected by the omission of p9/14 from the particle, translocation itself was less efficient at the membrane concentrations

initially used in the assay. This finding suggested to us that elongation arrest, although not absolutely necessary for protein translocation process per se, has evolved to serve a fidelity function, preventing the synthesis of secretory proteins in the cytoplasmic compartment.

Furthermore, we could use this particle to address the role of SRP receptor in the translocation process. Because SRP receptor was found to have arrest releasing activity, it was possible that the role of this molecule was solely to release the arrest induced by SRP. If this were the case, then SRP receptor would not be required for protein translocation mediated by SRP(-9/14). In our experiments, we found that SRP receptor was required, and that therefore it must play a role in addition to arrest release.

Nuclease Digestion Experiments

Micrococcal nuclease digestion had been shown (Gundelfinger et al., 1983) to cleave SRP at the boundary between the Alu-like sequences of the SRP RNA and the S-sequences. On sucrose gradients the 9/14 kd protein was found associated with the Alu-like region of the RNA, and all the other SRP proteins with the S region. We took advantage of this finding to purify, by DEAE-Sepharose chromatography and sucrose gradient sedimentation, the subparticle containing the S-region of SRP RNA. We found that this subparticle, which we called SRP(S), was stable to these purification conditions and thus could be considered an independent structural domain within SRP. Furthermore, we found that SRP(S) had the same activity as SRP(-9/14); in other words, it was fully active in translocation promoting activity even though it had lost a full half of its RNA component.

SRP RNA is highly conserved across its entire sequence, suggesting that the entire molecule is important for the function of SRP in vivo. The finding that after SRP had been assembled, a full half of the RNA was dispensible, was therefore perplexing to us. We could think of three reasons why this might be the case. The Alu region of the RNA might be important for folding the S region into its correct structure. This is plausible because in the proposed secondary structure of the RNA, the Alu-like portion contributes substantially to the folding energy of the protein by forming an extended stem structure. Because Alu-like elements contain polymerase III promoter function, another possibility is that these sequences are required for the transcription of the RNA. A third possibility is that these sequences are conserved because it is advantageous for the cell in vivo to retain elongation arresting activity, either as a fidelity function, or as a potential regulatory step.

We showed that the addition of p9/14 in 10-fold molar excess to a translation reaction contanining SRP(S) did not restore elongation arresting activity, suggesting that the RNA was also required, either because it had some activity of its own (see "What about the RNA?" section of this chapter) or because it was required to bind the 9/14 kd protein and correctly position it on the ribosome. We had been unable at this stage in our studies to ask whether the Alu-like region of the RNA, together with the 9/14 kd protein, could be added back "in trans" to restore elongation arrest activity. The Alu-region of SRP RNA has recently been cloned behind a T7 promoter (K. Strub, unpublished), and so it now should be possible to address this important question of SRP function.

Alu-like sequences have been found as part of other RNAs; for example, three such sequences are found in the 3' untranslated region of the LDL receptor mRNA. If these sequences can fold into a structure similar to that of the Alu-like region of SRP RNA, the intriguing possibility arises that p9/14 or a related protein might bind to these sequences and affect the translation of the messages containing them. A relatively easy experiment that would implicate a role for these sequences in translational control would be to fuse the coding sequence for a cytoplasmic protein such as globin in front of the 3' untranslated region of the LDL receptor gene, and ask whether the translation of the protein is now inhibited by the addition of p9/14 to the translation reaction.

NEM Inactivation Experiments

Both single omission and nuclease digestion result in severe alterations in the structure of SRP, and so it is not surprising that by these methods we had failed to assign the other two assayable functions of this particle (signal recognition and translocation promotion) to specific domains. In order to introduce more "subtle" alterations in the particle, we exploited the ease of SRP disassembly and reconstitution to modify single proteins with the sulfhydryl reactive agent NEM.

By this approach, we isolated a particle, which we called SRP(68/72^{N}), which had the converse activity to the mutant SRPs we had isolated earlier. Namely, it was active in elongation arrest, but was completely inactive in promoting translocation. We showed that this particle failed to target nascent preprolactin to the microsomal membrane under any conditions, and that membranes failed to release the arrest induced by it. In other words, it had apparently lost the

ability to interact functionally with SRP receptor. We further showed that SRP(68/72^{N}) was reduced in its ability to biochemically interact with an SRP receptor column, and that p68/72 was specifically depleted from the flow-through fraction when a mixture of SRP proteins was passed over the column. Together these data support the hypothesis that SRP targets nascent secretory proteins to the endoplasmic reticulum via a direct interaction of p68/72 with SRP receptor.

The isolation of two particles with converse phenotypes has led us to conclude that the three activities of SRP which we can assay in vitro reside in distinct domains in the particle. This idea has been further substantiated by the isolation of SRP(54^{N}), which was found to have lost both elongation arrest and protein translocation activities. When we measured the affinity of SRP(54^{N}) for ribosomes synthesizing either globin or preprolactin chains, we found them to be indistinguishable. In other words, in contrast to the wild type SRP, which upon emergence of the preprolactin signal sequence, increases in its affinity for the translating ribosome by approximately three orders of magnitude, this mutant SRP does not detectably increase in affinity, suggesting that the signal recognition activity has been abolished. This result suggests that p54 is responsible for the signal recognition activity of SRP, and is consistent with the results of cross-linking studies, in which the 54 kd protein was found cross-linked to the preprolactin signal sequence.

WHAT ABOUT THE RNA?

We have shown in our analysis of the SRP mutants presented here that a protein component is required for each of the activities we have assayed in vitro. This give a picture of RNP organization in which the proteins do all the work and the RNA serves as a structural lattice,

maintaining the proteins in a specific geometry.

Such a picture is rather disappointing to students of ribonucleoprotein assembly and evolution. In particular, since the novel and spectacular findings of Tom Cech and colleagues (Kruger et al., 1982) that a Tetrahymena ribosomal RNA could undergo self-splicing, there has been a flurry of activity aimed at demonstrating enzymatic activity of RNA components.

Because it was initially a surprise that SRP contained an RNA (Lewin, 1982), the question was raised from the beginning: "What does it do?" Indeed, for each of the activities that SRP was found to possess, the RNA component has been hypothesized to play a role. For example, Christian Zwieb noticed a complementarity between a region of SRP RNA (within the S sequence) and 5S RNA (Zwieb, 1985) that he postulated played a role in the elongation arrest activity of the particle (that idea came into slight disfavor when it was found that the Alu-domain rather than the S domain of SRP was required for elongation arrest). A role for the Alu-like region of SRP RNA in elongation arrest was indeed suggested when it was noted that the 5' end of 7SL RNA could be folded into a structure that was very much like a tRNA missing the anticodon loop (K. Strub et al., unpublished), and thus may bind SRP to the aminoacyl tRNA site of the ribosome. Elisabetta Ullu found sequence complementarity between a region of SRP RNA and a number of secretory protein mRNAs (the "Ullu box", unpublished), which she proposed had a role in signal recognition. Zwieb and Ullu (1986) noticed that the RNA was dynamic in the sense that it could exist in a number of conformations, and that this dynamic state seemed to depend on alternative base pairing within the S region. The possibility therefore arose that a

conformational change in the RNA was important for the progression of SRP through the SRP cycle. Finally, it was noted when the α-subunit of SRP receptor was cloned and sequenced that the region of the molecule that was essential for SRP binding contained a number of mixed charge residues, similar to sequences found in nucleic acid binding proteins (Lauffer et al., 1985). This led to the suggestion that the RNA is involved in the targeting of the nascent chain to the membrane.

Reconstitution of Active SRPs from In Vitro Transcripts of 7SL RNA

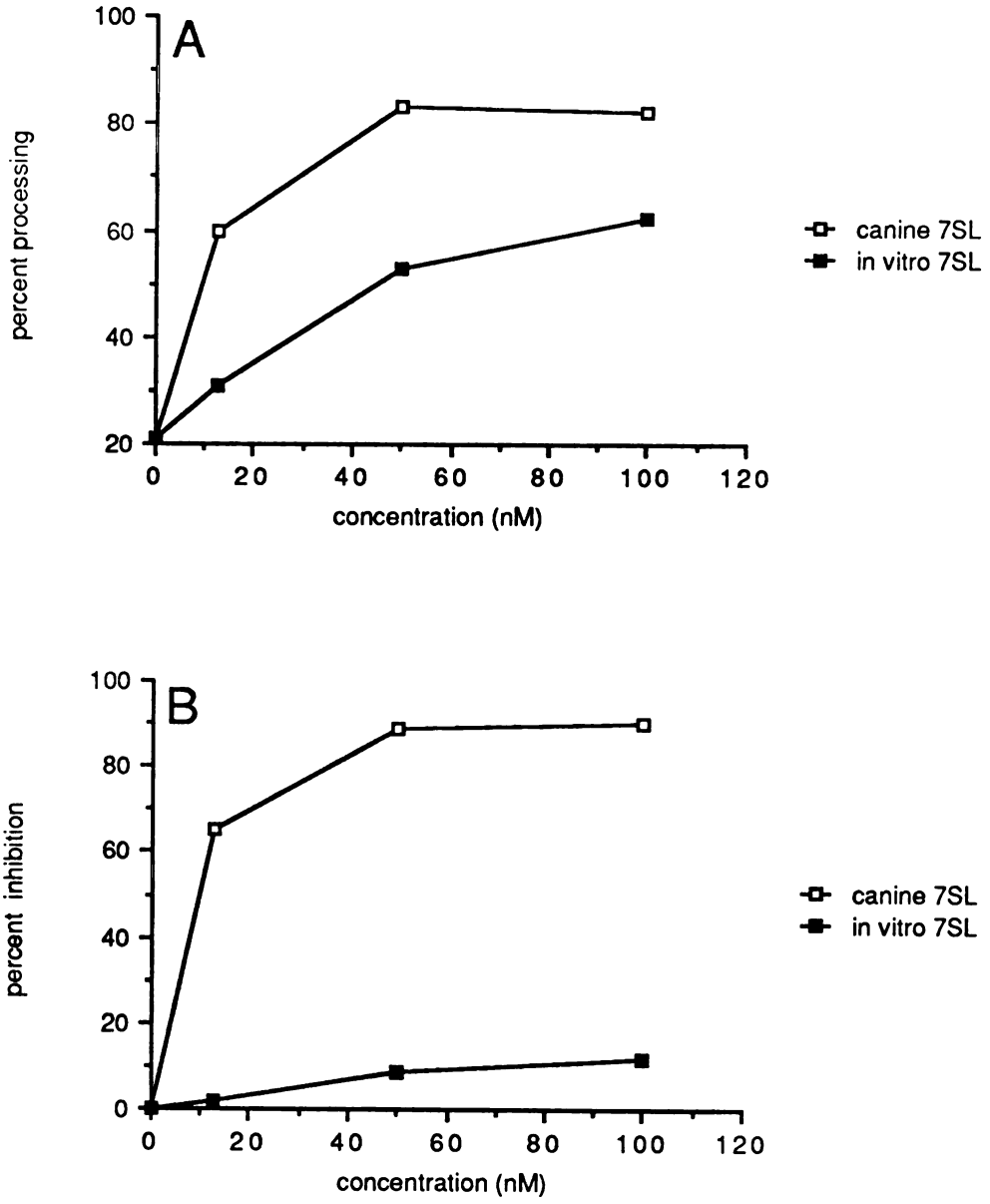
While it is all very well to speculate, in order to demonstrate an actual role of the RNA in any of these functions, it would be important to introduce mutations in the RNA and analyze them in vitro. Thus it would be useful to be able to reconstitute the particle from an in vitro transcript of 7SL RNA. As a starting point for these experiments, Christian Zwieb inserted the human gene for 7SL RNA behind a T_7 promoter. When linearized with EcoRI and transcribed in vitro (according to US Biochem Corp specifications, Chamberlin & Ring, 1973a,b), a transcript of ~400 nucleotides was synthesized. Because of the way the DNA was inserted into the transcription vector, the synthetic 7SL RNA contained extra sequences at both the 5' (~50 nt) and 3' (~30 nt) ends (compare the size of the in vitro transcript, Fig. 2, lane a, with canine 7SL RNA, lane e). After phenol extraction and ethanol precipitation, this RNA was tested for its ability to reconstitute an active SRP. As can be seen in Figure 1, a reconstitute generated from this in vitro transcript was active in promoting translocation (Panel A), but could not arrest elongation (Panel B). We found by sucrose gradient analysis (not shown) that p9/14 failed to bind to such a transcript, probably because the extra sequences at the ends affected the folding of

Figure 1. Activity of pT_7-7SL *in vitro* transcript.

The human 7SL RNA gene was cloned behind the T_7 promoter by Christian Zwieb. It was then transcribed with T_7 polymerase for 1 hr at 37°C according to the recommendations of US Biochemical Corp. After phenol extrction and ethanol precipitation, the RNA was used directly in a reconstitution reaction. As a control, canine 7SL RNA was used in a reconstitution. Particles generated with these two RNAs were titrated into elongation arrest and translocation assays.

(A) Percent processing as a function of the concentration of SRP reconstituted from canine 7SL RNA (open squares) or from the *in vitro* transcript (filled squares).

(B) Percent inhibition as a function of SRP concentration.


A
100
80
60
40
20
percent processing
0
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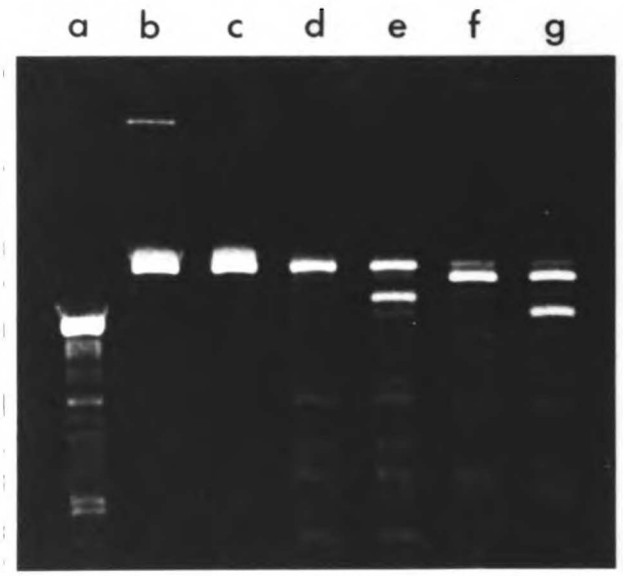
40
60
80
100
120
concentration (nM)
canine 7SL
in vitro 7SL
B
100
80
60
40
20
0
percent inhibition
0
20
40
60
80
100
120
concentration (nM)
canine 7SL
in vitro 7SL

Figure 2. RNAse H cleavage of 7SL in vitro transcript.

Oligonucleotides complementary to the regions flanking the authentic 7SL RNA sequence on the 5' and/or the 3' end were annealed to the in vitro 7SL transcript according to Donis-Keller (1979). A 5-fold excess of nucleotide to transcript was used. RNAse H was added, and the reaction incubated for 30 min at 37°C. After phenol extraction and ethanol precipitation, the RNAs were run on 6% polyacrylamide, 7M urea gels. Shown here is an ethidium stained gel of RNAse H digested RNAs. Lane a, canine 7SL RNA; lane b, in vitro transcript of 7SL RNA; lane c, in vitro 7SL after digestion with RNAse-free DNAse to hydrolyze the plasmid prior to RNAse H digestion; lane d, RNAse H digestion in which no oligonucleotide was added; lane e, RNAse H digestion in which an oligonucleotide complementary to the 5' flanking sequence was added; lane f, RNAse H digestion in which an oligonucleotide complementary to the 3' flanking sequence was added; lane g, RNAse H digestion in which both oligonucleotides were added.


a
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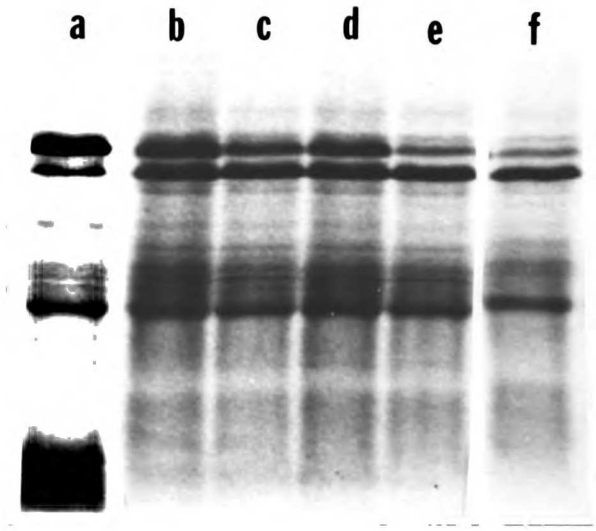
the RNA.

In order to generate precise ends in the RNA, oligonucleotides complementary to the first 15 nucleotides flanking either end of the authentic 7SL sequence were synthesized. These oligonucleotides were annealed to the in vitro transcript and then RNAse H (Donis-Keller, 1979) was used to cleave the RNA in the RNA/DNA hybrid. As can be seen in Figure 2, this method resulted in the cleavage of the RNA at the 5' and 3' ends (lanes b-d). The RNA thus cleaved was tested for activity, and again was inactive in elongation arrest. However, when the RNA was purified on a denaturing gel, we found that cleavage of the ends improved the activity of the particle (Fig. 3). In particular, we found that it was important for the 5' end of the RNA to be precise, and that removal of flanking sequences from this end improved the activity of the particle. This is consistent with the idea that the structure of the 5' end (which contains the tRNA like region) is important for elongation arrest activity. Because the RNAse H cleaved transcript was active, we can further conclude that a 5' triphosphate is not required for activity.

Recently, Katharina Strub has synthesized by ligation of oligonucleotides a synthetic 7SL RNA gene, which when appropriately linearized can be transcribed with T7 polymerase to form a precise SRP RNA molecule. This molecule is fully active in both elongation arrest and protein translocation activities. She is now in the process of testing the role of the tRNA like structure in elongation arrest by introducing single point mutations that should destroy an important tertiary interaction (if indeed the molecule is folded like a tRNA) and a revertant that should restore that interaction. Other interesting mutations would

Figure 3. Activity of RNAse H cleaved transcripts after gel purification.

RNAse H digested transcripts such as those shown in Figure 2 were eluted from a 6% polyacrylamide, 7M urea gel. After precipitation of the RNA, canine SRP proteins were added and the mixture subjected to standard reconstitution conditions. The reconstitutes were then added to translation reactions containing microsomal membranes to test their relative activities. Lane a, no SRP was added; lane b, full length *in vitro* transcript was used in the reconstitution; lane c; an *in vitro* transcript in which the 5' end was precise was used in the reconstitution; lane d, the 3' end of the transcript was precise; lane e, both ends were precise; lane f, gradient purified SRP was added.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include a strengthening of one of the potential secondary structures in the S region of the molecule (Zwieb & Ullu, 1986), in order to test whether and at what stage conformational changes in the RNA are important.

RELATIVE POSITION OF FUNCTIONAL DOMAINS

We have mapped within the S region of SRP RNA the relative positions of p68/72 and p19. In conjunction with micrococcal nuclease studies on SRP, in which p9/14 was found to be associated with the Alu-like sequences of the RNA, we have positioned the functional domains along the RNA. We found that the signal recognition domain was on the opposite end of the particle from the elongation arrest domain. This finding is consistent with the hypothesis that SRP concomitantly performs these two functions by physically bridging the distance between the nascent chain exit site and the aminoacyl tRNA site.

We found that p19 bound to a region of extremely high primary sequence conservation. The footprint of the canine protein on 7S RNAs from different species may indicate whether these RNAs are in fact the SRP RNAs for those species.

We further found (not shown) that SRP receptor protects a very similar sequence on the SRP RNA as that protected by p68/72. This finding demonstrates that SRP receptor has RNA binding properties and raises that intriguing possibility that after an initial interaction of SRP receptor with p68/72 (which was demonstrated in Chap. 4), that the RNA is somehow involved in the interaction, perhaps leading to a conformational change in the particle.

SUBSTRATE REQUIREMENTS FOR SRP-MEDIATED TARGETING

We took advantage of ribosome stalling at a number of positions in

the preprolactin chain to analyze directly the substrate requirements for targeting. We found three factors to be important. First the chain needed to be associated with the ribosome. This requirement may reflect the affinity that SRP has for all ribosomes, or may result from the ribosome keeping the nascent chain in a somewhat more unfolded state. Second, we found a length dependence in targeting. Certain chains were too short to have the signal sequence fully exposed on the surface of the ribosome, and these chains were not efficiently targeted. Other chains were too long. We found that the cut-off point occurred at about 140 amino acids and that it reflected a decrease in affinity of the nascent chain-ribosome complex for SRP. Finally, we found that although these longer chains could be brought to the membrane along polysomes synthesizing shorter chains, these chains did not engage the translocation machinery, suggesting that SRP was required to initiate each translocation event.

The development of an assay for targeting that is not dependent on concomitant elongation will allow a more complete dissection of the targeting process. It should be possible to purify the nascent chain-ribosome complex away from soluble factors in the translation reaction and to ask whether SRP is sufficient to target these complexes to the membrane. In addition, it should now be possible to alter ionic conditions and to control the composition of nucleotides in the assay. Analysis of targeting under these conditions may lead to the elucidation of an activity within the RNA, and a complete dissection of the energy requiring steps in the pathway.

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