MEMBRANE ASSEMBLY AND TRANSLATION PAUSING OF THE SIGNAL RECOGNITION PARTICLE RECEPTOR α SUBUNIT

By

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ASSEMBLY OF SRP RECEPTOR

ABSTRACT

The α subunit of the signal recognition particle receptor (SRα) is targeted to the endoplasmic reticulum membrane by a mechanism independent of the signal recognition particle-mediated translocation pathway. The membrane binding of SRα polypeptides was examined using a cell-free translation and targeting system. A membrane binding domain consisting of the amino-terminal 140 residues of SRα was identified. This domain forms a protease-resistant folded unit, and is the major site of tight binding to the β subunit of the receptor (SRβ). SRα was also shown to be a peripheral membrane protein, suggesting that it is bound to the membrane largely via interactions with the integral membrane SRβ. Co-translational targeting of SRα was correlated with a translation pause site on the SRα mRNA. An mRNA sequence at this pause site that resembles a class of retroviral frameshift sites was shown to be necessary and sufficient for a strong translation pause. SRα polypeptides synthesized from a mutant non-pausing mRNA were impaired in co-translational membrane binding, and translation-dependent localization of polysomes synthesizing SRα. These data resolve a discrepancy in earlier reports concerning the membrane attachment of SRα. Moreover, a novel pathway for polypeptide targeting and mRNA localization is proposed, and a previously undescribed signal for translation pausing is identified. Translation pausing may be general mechanism to coordinate co-translational folding, oligomeric assembly and targeting of other proteins.

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for my parents

"The future is chaos and beyond it is freedom;

Confusion is next, and next after that comes the truth."

Sonic Youth

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ABBREVIATIONS USED IN THIS THESIS

7mG	7-methylguanosine-5'-phosphate
ATA	aurintricarboxylic acid
ATP	adenosine 5'-triphosphate
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CRMs	canine pancreatic rough microsomes, Sepharose CL-2B chromatography extracted
CTAB	cetyltrimethylammonium bromide
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetate, sodium salt
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra-acetate, sodium salt
ER	endoplasmic reticulum
Gpp(NH)p	5'-guanylylimidodiphosphate

GTP	guanosine 5'-triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HTLV	human T-cell leukemia virus
IgG	immunoglobulin G
kD	kilodalton
KRMs	CRMs extracted with 0.5 M KOAc
mRNA	messenger ribonucleic acid
OAc	acetate
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
rpf	ribosome protected mRNA fragment
RRL	rabbit reticulocyte lysate
SDS	sodium dodecyl sulfate
SRα	signal recognition particle receptor α subunit
SRβ	signal recognition particle receptor β subunit
SRP	signal recognition particle
SRP-R	signal recognition particle receptor
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
UTP	uridine 5'-triphosphate

CHAPTER I

General Introduction

Protein targeting in mammalian cells

In order for an eukaryotic cell to maintain and expand the various organelles, the proteins responsible for the structure and function of the organelles must be synthesized, sorted and transported to appropriate locations within the cell. To fully understand the targeting pathway taken by a particular protein, we must consider not only the final destination which may be intended only for that protein, but also the point of synthesis on a common ribosome machinery shared by all other proteins. The subject of this thesis is the targeting of one polypeptide, the signal recognition particle receptor (SRP-R) α subunit (SRα), from the site of initial synthesis in the cytosol to the endoplasmic reticulum membrane. The conclusion of this thesis reveals a previously undescribed mechanism in which protein synthesis and assembly at the final destination may be intimately linked.

In general, organelle-specific protein targeting mechanisms require specific signals on the targeted protein. The co-translational sorting of secretory proteins at the rough endoplasmic reticulum (ER) membrane is mediated by a specialized motif on the nascent polypeptides, a secretory signal sequence (Blobel and Dobberstein, 1975). After translocation across the ER membrane, many proteins are carried along the secretory pathway from the ER to the Golgi apparatus and eventually the plasma membrane, by a non-specific transport mechanism (reviewed by Palade, 1975). However, additional targeting signals can divert proteins to different compartments of the vesicular system. For example, mannose 6-phosphate modified polypeptides in the Golgi apparatus are removed

into a separate pre-lysosomal compartment by the action of mannose 6-phosphate receptors and an intricate process of vesicle formation and transport (reviewed by Pryer et al, 1992).

Signal-mediated mechanisms have also been identified that transport polypeptides from the cytoplasm into other organelles, such as mitochondria and peroxisomes. However, targeting of proteins into these organelles is thought to occur mostly after translation is complete (reviewed by Verner and Schatz, 1988). The folding of proteins into conformations with secondary and tertiary structure must be considered in these cases, since chaperone-mediated folding of polypeptides begins before release from the ribosome (Frydman et al, 1994). For example, import of some proteins into mitochondria has been shown to require unfolding of the polypeptide chain and is facilitated by protein folding chaperones (Verner and Schatz, 1988).

There are still many proteins for which the targeting signals and pathways are largely uncharacterized. Many of these proteins, such as SRα (Andrews et al, 1989), are targeted within the cytoplasmic compartment, in this case to the ER membrane (Meyer et al, 1982; Gilmore et al, 1982). Various intracellular structures including the surfaces of membrane-bound organelles and the cytoskeletal network are directly accessible to newly synthesized proteins in the cytoplasm. Targeting may in some cases be primarily dependent upon biophysical characteristics, such as the spontaneous insertion of rat liver cytochrome b5 into membranes via a hydrophobic polypeptide sequence at the carboxyl terminus (Rachubinski et al, 1979). In other cases, the mRNA coding for the polypeptide may be localized within the cell prior to translation, as reported for the localization of

mRNA encoding *bicoid* protein to the anterior pole of *Drosophila* blastoderms (Macdonald and Struhl, 1988). Together, these processes illustrate three stages at which novel polypeptides may be targeted within the cytoplasm: first, prior to translation by localization of the mRNA (*bicoid*); second, during translation of the nascent polypeptide (secretory proteins); and third, after synthesis of the polypeptide is complete (cytochrome b5).

Organelle-specific targeting of a newly synthesized polypeptide within the cytoplasm is expected to require interactions with unique proteins at the intracellular destination. Folding of the targeted polypeptide into a conformation capable of forming the appropriate protein-protein interactions is therefore important if not essential for targeting. Elucidation of such a targeting pathway can be approached by analysing (1) the protein-protein interactions at the destination, (2) the sequences and structures of the targeted polypeptide required to form these protein-protein interactions, and (3) the relationship in timing between polypeptide synthesis, protein folding and protein-protein assembly. The experiments in this thesis investigated the targeting of newly synthesized and nascent SRα polypeptides in an *in vitro* system using this analytical approach.

In the following sections of this chapter, the biological role of SRα in protein translocation across the ER membrane and previous data on the targeting of SRα will be reviewed.

Targeting of secretory proteins to the ER membrane

Translocation of secretory proteins across the ER membrane is the initial step of the secretion process (reviewed by Palade, 1975). Early studies demonstrated that a fraction of rat liver lysate containing both membrane bound and cytoplasmic ribosomes incorporated radiolabelled amino acids into proteins *in vitro* (eg. Littlefield et al, 1955). Membrane bound polysomes recovered from rat liver were found to be preferentially synthesizing secretory proteins as opposed to cytoplasmic proteins (Hicks et al, 1969; Redman, 1969). The secretory polypeptides synthesized by ribosomes attached to ER membrane microsomes from rat liver were released directly into the lumen of the microsome, and were protected by the membrane from externally added protease (Redman and Sabatini, 1966; Sabatini and Blobel, 1970). Therefore, the initial evidence indicated there was a close connection in timing between synthesis, sorting and translocation of secretory proteins.

Membrane bound ribosomes from rat liver were found to be essentially identical to cytoplasmic ribosomes when analysed by SDS-PAGE and the exchange of small ribosomal subunits (Borgese et al, 1973). Thus, secretory proteins are not synthesized by a specialized class of ribosomes. Furthermore, a secretory protein synthesized by free wheat germ ribosomes programmed *in vitro* with a specific mRNA was translocated across the membrane during, but not after translation (Warren and Dobberstein, 1978). Polysome binding to microsomes *in vitro* was blocked by an inhibitor of translation initiation, aurintricarboxylic acid (Borgese et al, 1974). Sorting of nascent secretory

proteins therefore occurs co-translationally and does not require targeting of the mRNA to membrane bound ribosomes independent of translation. The determinant of secretory protein sorting must then reside on the nascent polypeptide chain and was found to be a specialized polypeptide motif termed a secretory signal sequence. This signal sequence was postulated to trigger a specific mechanism responsible for membrane binding of ribosomes and co-translational translocation of the nascent polypeptide across the membrane (Milstein et al, 1972; Blobel and Dobberstein, 1975). Sorting and targeting of nascent secretory polypeptides was eventually found to require a proteinaceous machinery in which SRα is an essential subunit.

Structure and function of SRP and SRP-R

Nascent polypeptides containing secretory signal sequences are sorted by a non-ribosomal component necessary for translocation *in vitro*. The active component could be extracted from canine pancreatic rough microsomes (CRMs) at high ionic strength and thus was peripherally bound to the membranes (Warren and Dobberstein, 1978). Upon purification, this component was called signal recognition particle, or SRP (Walter and Blobel, 1980). In a wheat germ cell-free translation system, SRP bound preferentially to ribosomes synthesizing polypeptides with signal sequences (Walter et al, 1981). The binding of SRP onto ribosomes caused a translation arrest that could be released by adding ER microsomes (Walter et al, 1981; Walter and Blobel, 1981a and 1981b).

Purified SRP was found to be a ribonucleoprotein made up of six different polypeptides with apparent molecular mass 72 kD, 68 kD, 54 kD, 19 kD, 14 kD and 9 kD, and a 7S RNA molecule (Walter and Blobel, 1980 and 1982). These subunits were arranged to form a long, narrow structure, presumably allowing SRP to bind a signal sequence at the polypeptide exit site of a ribosome and interact with the A and/or P site of the same ribosome to cause translation arrest (Andrews et al, 1985 and 1987). SRP with the 9 kD and 14 kD subunits specifically removed could not arrest translation, but could still mediate a reduced level of protein translocation *in vitro* (Siegel and Walter, 1985). The 54 kD subunit of SRP was shown by chemical cross-linking to directly bind signal sequences emerging from ribosomes (Krieg et al, 1986; Kurzchalia et al, 1986). However, complete SRP could not activate polypeptide translocation after the polypeptide had been released from the ribosome (Perara et al, 1986) unless the polypeptide was unfolded in high concentrations of a denaturant (Sanz and Meyer, 1988).

Extraction of CRMs with limited protease digestion and high ionic strength revealed a membrane component distinct from SRP that was required for protein translocation *in vitro* (Walter et al, 1979; Meyer and Dobberstein, 1980a). This component was determined to be a 58 kD apparent molecular mass proteolytic fragment (variously reported to be between 52 kD and 60 kD) of a larger 72 kD membrane-bound protein, the SRP receptor or docking protein (Meyer and Dobberstein, 1980b; Meyer et al, 1982). Purified 72 kD protein was sufficient to release the SRP-induced translation arrest of wheat germ ribosomes (Gilmore et al, 1982). Proteolysis of the purified protein generated the 58 kD fragment and an additional 14 kD fragment that was postulated to

contain the membrane binding domain (Hortsch et al, 1985). A β subunit of the receptor (SRβ), with apparent molecular mass 30 kD, was found to copurify with the 72 kD receptor protein (SRα) by antibody or SRP-affinity chromatography and to cosediment with SRα in sucrose density gradients. SRα and SRβ exist in close to 1:1 stoichiometry on ER membranes, and were proposed to function as a heterodimer (Tajima et al, 1986). Because both subunits of the SRP-R were resistant to high pH extractions of microsomes and could only be solubilized by a combination of nonionic detergent and moderate ionic strength (at least 200 mM KCl), the subunits were characterized as integral membrane proteins (Meyer and Dobberstein, 1980a; Hortsch et al, 1985; Tajima et al, 1986). Therefore, SRP-R is the first constitutively membrane-bound component of the ER protein translocation pathway.

Figure 1. Predicted amino acid sequence of canine SRα. Hydrophobic regions are underlined, and charged regions are double underlined (Lauffer et al, 1985). The GTP binding consensus sequence elements are shown above the corresponding SRα sequences (Connolly and Gilmore, 1989; Romisch et al, 1989; Althoff et al, 1994). Amino acids are represented in the standard one-letter code; X, similar amino acid; x, any amino acid.

```
MLDFFTIFSK GGLVLWCFQG VSDSCTGPVN ALIRSVLLQE RGGNNSFTHE   50

ALTLKYKLDN QFELVFVVGF QKILTLTYVD KLIDDVHRLF RDKYRTEIQQ  100

QSALSLLNGT FDFQNDFLRL LREREESSKI RAPTTMKKFE DSEKAKKPVR  150

SMIETRGEKP KEKAKNSKKK GAKKESSDGP LATGKAVPAE KSGLPAGPEN  200

GVELSKEELI RRKREEFIQK HGRGLEKSSK STKSDAPKEK GKKAPRVWAL  250

GGCANKEVLD YSAPTTNGAP DAAPPEDINL IRGTGPGGQL QDLDCSSSDD  300

EETAQNASKP SATKGTLGGM FGMLKGLVGS KSLSREDMES VLDKMRDHLI  350

AKNVAADIAV QLCESVANKL EGKVMGTFST VTSTVKQALQ ESLVQILQPQ  400

                        GXXGxG KXTxxxK
RRVDMLRDIM DAQRHQRPYV VTFCGVNGVG KSTNLAKISF WLLENGFSVL  450

    DxXRxx AxxQL
IAACDTFRAG AVEHVRTHTR RLSALHPPEK HAGPTMVQLF EKGYGKDAAG  500

              XXXXXD TXGR
IAMEAIAFAR NQGFDVVLVD TAGRMQDNAP LMTALAKLIT VNTPDLVLFV  550

                                    XXKx D
GEALVGNEAV DQLVKFNRAL ADHSMAQTPR LIDGIVLTKF DTIDDKVGAA  600

ISMTYITSKP IVFVGTGQTY CDLRSLNAKA VVAALMKA               638
```

Canine cDNA sequences have been obtained for both SRα and SRβ (Lauffer et al, 1985; Miller et al, 1995). The 638 amino acid predicted SRα polypeptide sequence reveals two short stretches of hydrophobic residues (amino acids 1 to 22 and 64 to 79), followed by three stretches of polar residues with a net positive charge (amino acids 84 to 97, 129 to 175 and 205 to 243; Fig. 1; see also Chapter II, Fig. 1). The cytoplasmic 58 kD fragment was found to contain amino acids 151 to the carboxyl terminus of the polypeptide (Lauffer et al, 1985). A GTP binding consensus sequence was identified near the carboxyl terminus, consisting of four conserved sequence elements (Fig. 1) (Connolly and Gilmore, 1989; Romisch et al, 1989; Althoff et al, 1994). The predicted polypeptide sequence of SRβ contains a short amino terminal section followed by a single long stretch of hydrophobic residues and a large carboxyl terminal section that also contains a GTP binding sequence (Miller et al, 1995). Proteolysis data suggested that the hydrophobic sequence of SRβ spans the ER membrane with the amino terminus in the lumen and the carboxyl terminus in the cytoplasm (Andrews et al, 1989; Miller et al, 1995). The GTP binding sequences of both SRα and SRβ are most closely related to each other and to a GTP binding sequence on the 54 kD SRP polypeptide, or SRP54 (Romisch et al, 1989; Althoff et al, 1994; Miller et al, 1995).

Binding and hydrolysis of GTP plays a central role in the function of SRP and SRP-R. GTP or the non-hydrolyzable analog Gpp(NH)p was found to be required for tight binding to CRMs of wheat germ ribosomes translating a secretory protein and for translocation of the protein (Connolly and Gilmore, 1986). In the absence of guanine nucleotides, SRP recognized signal sequences and localized ribosomes to the membrane

(Rapiejko and Gilmore, 1994), but SRP-R was unable to displace SRP from the ribosome-nascent chain complex and release the translation arrest (Connolly and Gilmore, 1989). With Gpp(NH)p, the interaction between SRP and SRP-R was stabilized and one round of translocation could proceed, but SRP and SRP-R were not recycled (Connolly et al, 1991; Rapiejko and Gilmore, 1992). SRα was shown to bind GTP by photoactivated crosslinking, and a mutation within SRα that disrupted the GTP binding sequence abolished translocation activity (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1992). The GTP binding domain of SRP54 is also necessary for translocation (Zopf et al, 1993), and both SRP54 and SRβ could be crosslinked to GTP (Miller et al, 1993 and 1995). In addition, while SRα and SRβ appeared to hydrolyze GTP at a basal rate in the absence or presence of SRP54 as assayed by photoactivated crosslinking, GTP hydrolysis by SRP54 was found to be stimulated by the presence of SRP-R (Miller et al, 1993). Filter-binding experiments to determine the stoichiometry of GTP bound by the complex of SRP and SRP-R indicated a roughly 1:1 mole ratio of GTP to SRP-R (Connolly and Gilmore, 1993), suggesting that one cycle of GTP hydrolysis by SRP54 is sufficient for the action and recycling of SRP and SRP-R. However, recent experiments using the purified bacterial homologs of SRα and the SRP54/7S RNA complex suggested that the GTPase activities of both SRα and SRP54 may be stimulated when the polypeptides are added together. Thus, GTP hydrolysis by SRα and perhaps SRβ may have an as yet unknown function in the translocation cycle (Powers and Walter, 1995).

SRP and SRP-R act catalytically to initiate polypeptide translocation since both components dissociate after translocation is initiated (Gilmore and Blobel, 1983). This

is consistent with the relatively low abundance of SRP-R on the ER membrane. Canine rough microsomes are generally quantified in terms of "membrane equivalents", one equivalent defined as producing an absorption of 0.05 units at 250 nm wavelength when solubilized in 1 mL buffer (Walter and Blobel, 1983). One membrane equivalent, after extraction with high salt, contains approximately 100 fmol or 10 ng of SRP-R (Gilmore et al, 1982; Tajima et al, 1986), as compared to 1.7 μg total protein (J. Young, unpublished). The population of SRP-R on the ER is about ten-fold smaller than the population of ribosomes and other components of the translocation machinery (Gilmore et al, 1982).

Membrane binding of ribosomes and polypeptide translocation

The catalytic action of SRP-R in the translocation process indicates that the receptor cannot contribute directly to the tight membrane binding of ribosomes. Several different proteins have been proposed as ribosome receptors, including p180 and Sec61p (Savitz and Meyer, 1990; Gorlich et al, 1992b). The p180 protein, an ER resident membrane protein (Wanker et al, 1995), was sufficient for binding of of HeLa cell ribosomes to liposomes in low salt conditions (Savitz and Meyer, 1990). Translocation activity and low salt ribosome binding of proteoliposomes reconstituted with ER membrane proteins was abolished by immunodepleting the proteoliposomes of p180, and restored by adding purified p180 (Savitz and Meyer, 1992). Also, expression of p180 in

Saccharomyces cerevisiae enabled binding of mammalian ribosomes (from HeLa cells) to yeast ER membranes that normally could not bind the ribosomes (Wanker et al, 1995). Sec61p, an integral membrane heterotrimer, cofractionated on sucrose density gradients with solubilized canine membrane-bound ribosomes in a high salt resistant but puromycin sensitive manner (Gorlich et al, 1992b). Depletion of Sec61p from ER proteoliposomes also abolished translocation activity and high salt resistant ribosome binding, and both functions were restored by adding back purified Sec61p (Kalies et al, 1994). High salt resistant membrane binding of ribosomes is dependent upon the activation of translocation by SRP and SRP-R (Connolly and Gilmore, 1986), suggesting the Sec61p-ribosome interaction is regulated.

Nascent secretory polypeptides are translocated through an aqueous pore in the ER membrane that is usually accessed only through the ribosome (Simon and Blobel, 1991; Crowley et al, 1993). Sec61p has been proposed to function with the integral membrane protein TRAM (TRanslocation Associated Membrane protein) to form the translocation pore (Gorlich et al, 1992a and 1992b). TRAM was identified as a possible pore component by chemical cross-linking to a nascent secretory protein, and was determined to be a resident ER protein with eight predicted transmembrane domains (Gorlich et al, 1992a). Sec61p could also be cross-linked to a nascent secretory polypeptide, suggesting that it may be a pore component in addition to binding ribosomes. Furthermore, the α subunit of Sec61p is highly homologous to the SEC61p protein of *S. cerevisiae* and the SECY protein of *E. coli*, both of which are important for translocation in the respective organisms (Gorlich et al, 1992b). Another integral membrane protein associated with the

translocation machinery is signal peptidase, a multisubunit complex containing up to six polypeptides (Evans et al, 1986) that proteolytically removes the signal sequences of secretory polypeptides during the translocation process (Blobel and Dobberstein, 1975). However, no function of signal peptidase in translocation, except for signal sequence cleavage, has been directly demonstrated.

Translocation activity has been reconstituted in proteoliposomes containing total ER membrane proteins (Nicchitta and Blobel, 1990; Zimmerman and Walter, 1990). A minimal translocation competent proteoliposome system has been reported containing only purified SRP-R, Sec61p and the integral membrane proteins TRAM and signal peptidase (Gorlich and Rapoport, 1993). However, this system showed only limited transfer of nascent polypeptides to the protease-protected lumenal compartment, and ER lumenal proteins were required for efficient translocation in the complete proteoliposome system (Nicchitta and Blobel, 1993). Proteins within the ER lumen including the protein chaperone BiP are thought to aid translocation by preventing retrograde movement through the translocation pore (Nicchitta and Blobel, 1993).

In common with other intracellular compartments, the ER lumen contains various resident protein chaperones that mediate protein folding and stability. The ER resident chaperones, such as BiP, Grp94 and calnexin, bind nascent secretory polypeptides during or after translocation (reviewed in Hartl et al, 1994; Bergeron et al, 1994; Jakob and Buchner, 1994). BiP and Grp94 are homologous to the cytoplasmic chaperone protein Hsc70 and Hsp90 respectively, and may act similarly to fold proteins and prevent aggregation (Hartl et al, 1994; Jakob and Buchner, 1994). Other chaperones appear to be

unique to the ER, such as the integral membrane protein calnexin (Bergeron et al, 1994). Interestingly, there is no known ER chaperone that corresponds to the "ring" complex chaperones typified by Gro-EL in *E. coli*, Hsp60 in mitochondria, and the Tcp-1 complex in the eukaryotic cytoplasm (Hartl et al, 1994). These compartment-specific chaperones do not perform analogous functions, but instead may be adapted to specific requirements of their intracellular locations. For example, the Tcp-1 complex appears to aid polypeptides to form tertiary structure (Hartl et al, 1994), while calnexin is involved in the assembly of some multisubunit membrane proteins (Bergeron et al, 1994). However, the cytoplasmic chaperones have been shown to mediate protein folding co-translationally (Frydman et al, 1994). Thus, the putative binding of ER lumenal protein chaperones to nascent secretory polypeptides (Nicchitta and Blobel, 1993) suggests that chaperone-mediated protein folding may also occur co-translationally in the ER lumen.

Translation arrest and translation pausing

Binding of SRP to wheat germ ribosomes induces an almost complete arrest of translation elongation (Walter et al, 1981). In contrast, SRP in the rabbit reticulocyte lysate translation system does not arrest translation of nascent secretory polypeptides but instead appears to cause a transient pause in elongation (Wolin and Walter, 1989). Since secretory polypeptides synthesized by wheat germ or rabbit reticulocyte lysate are translocated across microsomal membranes with comparable efficiency (Warren and

Dobberstein, 1978; Walter et al, 1979), the complete translation arrest observed in the wheat germ system may not be required for optimal translocation activity. However, SRP that was unable to cause translation arrest in wheat germ was also less active in promoting polypeptide translocation (Siegel and Walter, 1985). This suggests that the SRP-mediated translation arrest or translation pause, while not essential for translocation, contributes to the efficiency of the translocation mechanism.

The SRP-mediated translation arrest or pause is only one example of transient ribosome pausing during translation. Translation pausing has also been observed in connection with initiation and termination of translation (Wolin and Walter, 1988), frameshifting on retroviral mRNAs (reviewed by Hatfield et al, 1992) and other uncharacterized situations (Wolin and Walter, 1988; Krasheninnikov et al, 1991; see chapter three for further references). The mechanisms of pausing during translation elongation are in general still poorly understood. The presence of rare codons on the mRNA has been postulated to mediate translation pausing (Purvis et al, 1987), but this has not yet been directly demonstrated. Translation pausing has been observed at an mRNA frameshift sequence containing a pseudoknot (Somogyi et al, 1993), although an extended mRNA stem-loop structure alone has been shown not to modulate the elongation rate (Lingelbach and Dobberstein, 1988). For many other observed translation pause sites, the determinant for translation pausing is not known.

The physiological functions of translation pausing are also relatively unexplored. Ribosome pausing observed during translation initiation or termination may simply reflect the time required for these processes, and have no further significance. However, pausing

during translation elongation has been hypothesized to contribute to co-translational protein folding by allowing different protein domains to be synthesized and folded separately (Purvis et al, 1987). There is evidence that modulating a predicted translation pause contributes significantly to enzymatic stability of the TRP3 protein in *Saccharomyces cerevisiae* (Crombie et al, 1992), although translation pausing was not demonstrated. In chapter three of this thesis, an hypothesis was developed in which a translation pause during synthesis of SRα allows folding and co-translational targeting via an amino terminal domain.

Membrane targeting of SRα

Early evidence suggested that SRα could form two types of interactions with the ER membrane. As mentioned above, SRα was initially characterized as an integral membrane protein since the polypeptide is tightly anchored to the membrane (Hortsch et al, 1985). Microsomes that were protease treated and salt stripped to remove the 58 kD cytoplasmic fragment of SRα could not translocate polypeptides synthesized on rabbit reticulocyte lysate ribosomes. However, the purified 58 kD fragment restored translocation activity when added back to the reaction. This suggested the unanchored cytoplasmic domain of SRα is still able to form functional contacts with the ER membrane as well as the SRP-ribosome complex (Meyer et al, 1980b).

While antibody affinity purified SRα was able to bind to an SRP affinity column,

the purified cytoplasmic fragment of SRα could not, suggesting the remainder of the polypeptide contributes to receptor function (Lauffer et al, 1985). When the 58 kD proteolytic fragment was generated after purification of SRα, the preparation was found to release the SRP-induced translation arrest of wheat germ ribosomes (Hortsch et al, 1985). However, a 14 kD proteolytic fragment was also detected in the proteolytic preparation and the apparent molecular weight suggested that it formed the remainder of the SRα polypeptide after removal of the 58 kD fragment (Hortsch et al, 1985). The presence of the 14 kD fragment, as well as a 30 kD species that was probably SRβ, may have allowed the otherwise nonfunctional 58 kD fragment to interact with SRP.

The 14 kD proteolytic fragment of SRα was proposed to form the membrane binding domain although the fragment could not be detected on protease treated microsomes with the available antibodies (Hortsch et al, 1985). The SRα cDNA sequence and protein sequencing of the 58 kD fragment revealed the putative membrane binding fragment contained at most the 151 amino terminal residues of SRα, and the two stretches of hydrophobic amino acids near the amino terminus of SRα were identified as possible membrane anchors (Lauffer et al, 1985). Insertion of both these sequences into the ER membrane suggested that amino acids 1 through 79 would be protected from protease by the membrane, resulting in a fragment with a predicted molecular mass of about 9 kD. While this contradicted the evidence that the amino terminal 14 kD of SRα forms a relatively protease resistant unit (Hortsch et al, 1985), the apparent integral membrane character of SRα suggested that one or both of the hydrophobic sequences spanned the ER membrane.

SRα synthesized *in vitro* on wheat germ ribosomes was reported to bind post-translationally and therefore independently of SRP to microsomes in a manner resistant to high pH extraction (Hortsch and Meyer, 1988). This surprising result was confirmed by the post-translational anchoring and functional assembly of SRα synthesized in rabbit reticulocyte lysate onto microsomes (Andrews et al, 1989). Post-translational membrane anchoring of SRα in the reticulocyte lysate system was shown to be resistant to 2 M urea, independent of nucleotide triphosphates, and using affinity purified cell-free translation product, independent of factors in the reticulocyte lysate. Urea-resistant anchoring was abolished by previous digestion of the microsomes with low levels of trypsin but not by alkylation of the microsomes with N-ethyl maleimide. This suggested that there was a necessary specific protein target for SRα on the membrane and was supported by the inability of SRα polypeptides to anchor onto mitochondria or pure lipid vesicles. Furthermore, the anchoring of SRα onto microsomes was saturable at about 5 fmol SRα per membrane equivalent (Andrews et al, 1989). This amount of binding was consistent with the known excess of endogenous SRβ over SRα on microsomes (93 fmol of SRα to 107 fmol of SRβ per membrane equivalent; Tajima et al 1986), suggesting that SRβ may be the protein target of SRα. However, levels of trypsin digestion that abolished SRα anchoring had no apparent effect on SRβ. As expected, a polypeptide corresponding to the 58 kD cytoplasmic fragment of SRα was not able to anchor onto microsomes in a urea-resistant manner (Andrews et al, 1989).

Post-translationally targeted SRα in the reticulocyte system was also demonstrated to function in translocation (Andrews et al, 1989). An assay was developed in which the

translocation activity of microsomes was abolished by low levels of trypsin, and *in vitro* translation reactions of SRα were added to these microsomes followed by *in vitro* translation reactions of a secretory proteins. Using this assay, SRα and the 58 kD cytoplasmic fragment of SRα were shown to mediate translocation activity. These results were in agreement with the experiments with purified SRα mentioned above (Meyer and Dobberstein, 1980b; Gilmore and Blobel, 1982). However, combined with the anchoring data, this suggested a two-step model of SRα targeting in which targeting of the translocation active cytoplasmic domain provided functional interactions with the ER translocation machinery, and anchoring via the amino terminus of SRα conferred urea-resistant membrane binding (Andrews et al, 1989).

Treatment of membrane vesicles with buffer at pH 11 has been shown to extract peripheral and lumenal proteins without releasing integral membrane proteins from the lipid bilayer (Fujiki et al, 1982). While SRα remains attached to membranes at pH 11 (Hortsch et al, 1985), a recent study reported that SRα was preferentially extracted from CRMs compared to SRβ using buffers at pH 12 and pH 13 (Miller et al, 1995). Triton X-114 cloud point extraction is another assay designed to separate peripheral and integral membrane proteins, in which hydrophobic proteins solubilized in detergent are recovered by phase partitioning at temperatures above which the detergent becomes insoluble (Bordier, 1981). Using this assay, roughly half of both SRα and SRβ populations were recovered with the hydrophobic membrane protein phase. After specific proteolysis of SRα, SRβ was found almost entirely in the hydrophobic phase after cloud point extraction. On the basis of this data, it was proposed that SRα was a peripheral

membrane protein attached to the ER membrane by interactions with SRβ (Miller et al, 1995).

This conclusion was weakened by problems with the procedures used in these extraction assays. The solubilization conditions used in the phase partitioning did not fully solubilize the SRP-R (Meyer and Dobberstein, 1980a; also, chapter two of this thesis) and the effect on membranes of extraction at pH above 11.5 is not well characterized. Therefore, experiments reported in chapter two of this thesis re-examined the interactions between SRα and the ER membrane. Membrane extractions were performed at pH 11.5 supplemented with chaotropic agents in a previously characterized assay (Andrews et al, 1992). Furthermore, phase partitioning conditions were adjusted to fully solubilize the SRP-R subunits, producing clearer and more interpretable data. The results of these experiments confirmed the peripheral membrane nature of SRα, and provided additional information on the membrane binding of SRα.

The work presented in this thesis used the reticulocyte lysate system combined with experiments on endogenous microsomal SRα to further probe the targeting of the amino terminal membrane binding region of SRα. As mentioned above, the general approach taken was to examine the structures within SRα and the interactions required for targeting, as well as the timing of membrane binding. In chapter two of this thesis, the sequences within SRα required for membrane binding were identified and shown to form an independently folded domain. Also, further experiments suggested that SRα is in fact a peripheral membrane protein bound via both polar and nonpolar interactions to a protein target on the ER membrane, and that binding to SRβ is sufficient for SRα

binding. In chapter three, co-translational membrane binding of SRα was demonstrated to initiate during a translation pause. An mRNA sequence resembling a retroviral frameshift site was shown to be necessary and sufficient for translation pausing. The level of translation pausing affected the timing of SRα targeting and membrane binding of ribosomes translating SRα mRNA. A model of SRα targeting during a pause in translation was developed, and discussed in relation to other targeted proteins in chapter four. Because chapters two and three were written as independent publications (Young et al, 1995; Young and Andrews, 1995), they have been left essentially in the journal format. Certain experiments in chapter two, Fig. 5*a*, and Fig. 6, were not performed by the author but were important for the interpretation of the data and have been included in this thesis.

References

Althoff, S., Selinger, D. and Wise, J.A. (1994) Molecular evolution of SRP cycle components: functional implications. *Nucl. Acids Res.* **22**, 1933-1947

Andrews, D.W., Walter, P. and Ottensmeyer, F.P. (1985) Structure of the signal recognition particle by electron microscopy. *Proc. Natl. Acad. Sci. USA* **82**, 785-789

Andrews, D.W., Walter, P. and Ottensmeyer, F.P. (1987) Evidence for an extended 7SL RNA structure in the signal recognition particle. *EMBO J.* **6**, 3471-3477

Andrews, D.W., Lauffer, L., Walter, P. and Lingappa, V.R. (1989) Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. *J. Cell Biol.* **108**, 797-810

Andrews, D.W., Young, J.C., Mirels, L.F. and Czarnota, G.C. (1992) The role of the N region in signal sequence and signal-anchor function. *J. Biol. Chem.* **267**, 7761-7769

Bergeron, J.J.M, Brenner, M.B., Thomas, D.Y., and Williams, D.B. (1994) Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* **19**, 124-128

Blobel, G. and Dobberstein, B. (1975) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* **67**, 835-851

Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114. *J. Biol. Chem.* **256**, 1604-1607

Borgese, D., Blobel, G. and Sabatini, D.D. (1973) *In vitro* exchange of ribosomal subunits between free and membrane-bound ribosomes. *J. Mol. Biol.* **74**, 415-438

Borgese, D., Mok, W., Kreibich, G. and Sabatini, D.D. (1974) Ribosomal-membrane interaction: *in vitro* binding of ribosomes to microsomal membranes. *J. Mol. Biol.* **88**, 559-580

Connolly, T. and Gilmore, R. (1986) Formation of a functional ribosome-membrane junction during translocation requires the participiation of a GTP-binding protein. *J. Cell Biol.* **103**, 2253-2261

Connolly, T. and Gilmore, R. (1989) The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. *Cell* **57**, 599-610

Connolly, T. and Gilmore, R. (1993) GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particle receptor. *J. Cell Biol.* **123**, 799-807

Connolly, T., Rapiejko, P.J. and Gilmore, R. (1991) Requirement for GTP hydrolysis for dissociation of the signal recognition particle from its receptor. *Science* **252**, 1171-1173

Crombie, T., Swaffield, J.C. and Brown, A.J.P. (1992) Protein folding within the cell is influenced by controlled rates of polypeptide elongation. *J. Mol. Biol.* **228**, 7-12

Crowley, K.S., Reinhart, G.D. and Johnson, A.E. (1993) The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. *Cell* **73**, 1101-1115

Evans, E.A., Gilmore, R. and Blobel, G. (1986) Purification of microsomal signal peptidase as a complex. *Proc. Natl. Acad. Sci. USA* **83**, 581-585

Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum membrane. *J. Cell Biol.* **93**, 97-102

Frydman, J., Nimmesgern, E., Ohtsuke, K. and Hartl, F.U. (1994) Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* **370**, 111-117

Gilmore, R., Blobel, G and Walter, P. (1982) Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* **95**, 470-477

Gilmore, R. and Blobel, G. (1983) Transient involvement of signal recognition particle and its receptor in the microsomal membrane prior to protein translocation. *Cell* **35**, 677-685

Gorlich, D. and Rapoport, T.A. (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell* **75**, 615-630

Gorlich, D., Hartmann, E., Prehn, S. and Rapoport, T.A. (1992a) A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature* **357**, 47-52

Gorlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. and Rapoport, T.A. (1992b) A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell* **71**, 489-503

Hartl, F.-U., hlodan, R. and Langer, T. (1994) Molecular chaperones in protein folding:

the art of avoiding sticky situations. *Trends Biochem. Sci.* **19**, 20-25

Hatfield, D.L., Levin, J.G., Rein, A. and Oroszlan, S. (1992) Translational suppression in retroviral gene expression. *Adv. Vir. Res.* **41**, 193-239

Hicks, S.J., Drysdale, J.W. and Munro, H.N. (1969) Preferential synthesis of ferritin and albumin by different populations of liver polysomes. *Science* **164**, 584-585

Hortsch, M. and Meyer, D.I. (1988) The human docking protein does not associate with the membrane of the rough endoplasmic reticulum via a signal or insertion sequence-mediated mechanism. *Biochem. Biophys. Res. Comm.* **150**, 111-117

Hortsch, M., Avossa, D. and Meyer, D.I. (1985) A structural and functional analysis of the docking protein. *J. Biol. Chem.* **260**, 9137-9145

Jakob, U. and Buchner, J. (1994) Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. *Trends Biochem. Sci.* **19**, 205-211

Kalies, K.-U., Gorlich, D. and Rapoport, T.A. (1994) Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. *J. Cell Biol.* **126**, 925-934

Krasheninnikov, I.A., Komar, A.A. and Adzhubei, I.A. (1991) Nonuniform size distribution of nascent globin peptides, evidence for pause localization sites, and a cotranslational protein-folding model. *J. Prot. Chem.* **10**, 445-453

Krieg, U.C., Walter, P. and Johnson, A.E. (1986) Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle. *Proc. Natl. Acad. Sci. USA* **83**, 8604-8608

Kurzchalia, T.V., Wiedmann, M., Girshovich, A.S., Bochkareva, E.S., Bielka, H. and Rapoport, R.A. (1986) The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. *Nature* **320**, 634-636

Lauffer, L., Garcia, P.D., Harkins, R.N., Coussens, L., Ullrich, A. and Walter, P. (1985) Topology of signal recognition particle receptor in endoplasmic reticulum membrane. *Nature* **318**, 334-338

Lingelbach, K. and Dobberstein, B. (1988) An extended RNA/RNA duplex structure within the coding region of mRNA does not block translational elongation. *Nucl. Acids Res.* **16**, 3405-3414

Littlefield, J.W., Keller, E.B., Gross, J. and Zamecnick, P.C. (1955) Studies on cytoplasmic ribonucleoprotein particles from the liver of the rat. *J. Biol. Chem.* **217**, 111-123

Macdonald, P.M. and Struhl, G. (1988) *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila* embryos. *Nature* **336**, 595-598

Meyer, D.I. and Dobberstein, B. (1980a) A membrane component essential for vectorial translocation of nascent proteins across the endoplasmic reticulum: requirements for its extraction and reassociation with the membrane. *J. Cell Biol.* **87**, 498-502

Meyer, D.I. and Dobberstein, B. (1980b) Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. *J. Cell Biol.* **87**, 503-508

Meyer, D.I., Louvard, D. and Dobberstein, B. (1982) Characterization of molecules involved in protein translocation using a specific antibody. *J. Cell Biol.* **92**, 579-583

Miller, J.D., Wilhelm, H., Gierasch, L., Gilmore, R. and Walter, P. (1993) GTP binding and hydrolysis by the signal recognition particle during inittiation of protein translocation. *Nature* **366**, 351-354

Miller, J.D., Tajima, S., Lauffer, L. and Walter, P. (1995) The ß subunit of the signal recognition particle receptor is a transmembrane GTPase that anchors the α subunit, a peripheral membrane GTPase, to the endoplasmic reticulum membrane. *J. Cell Biol.* **128**, 273-282

Milstein, C., Brownlee, G.G., Harrison, T.M. and Mathews, M.B. (1972) A possible precursor of immunoglobulin light chains. *Nature New Biol.* **239**, 117-120

Nicchitta, C.V. and Blobel, G. (1990) Assembly of translocation competent proteoliposomes from detergent-solubilized rough microsomes. *Cell* **60**, 259-269

Nicchitta, C.V. and Blobel, G. (1993) Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell* **73**, 989-998

Palade, G. (1975) Intracellular aspects of protein secretion. *Science* **189**, 347-358

Perara, E., Rothman, R.E. and Lingappa, V.R. (1986) Uncoupling translocation from translation: implications for transport of proteins across membranes. *Science* **232**, 348-352

Powers, T. and Walter, P. (1995) Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. *Science* **269**, 1422-1424

Pryer, N.K., Wuestehube, L.J. and Schekman, R. (1992) Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* **61**, 471-516

Purvis, I.J., Bettany, A.J.E., Chinappan Santiago, T., Coggins, J.R., Duncan, K., Eason, R. and Brown, A.J.P. (1987) The efficiency of folding of some proteins is increased by controlled rates of translation *in vivo*: a hypothesis. *J. Mol. Biol.* **193**, 413-417

Rachubinski, R.A., Verma, D.P.S. and Bergeron, J.J.M. (1979) Synthesis of rat liver microsomal cytochrome b5 by free ribosomes. *J. Cell Biol.* **84**, 705-716

Rapiejko, P.J. and Gilmore, R. (1992) Protein translocation across the ER requires a functional GTP binding site in the α subunit of the signal recognition particle receptor. *J Cell Biol.* **117**, 493-503

Rapiejko, P.J. and Gilmore, R. (1994) Signal sequence recognition and targeting of ribosomes to the endoplasmic reticulum by the signal recognition particle do not require GTP. *Mol. Biol. Cell* **5**, 887-897

Redman, C.M. (1969) Biosynthesis of serum proteins and ferritin by free and attached ribosomes of rat liver. *J. Biol. Chem.* **244**, 4308-4315

Redman, C.M. and Sabatini, D.D. (1966) Vectorial discharge of peptides released by puromycin from attached ribosomes. *Proc. Natl. Acad. Sci. USA* **56**, 608-615

Romisch, K., Webb, J., Herz, H., Prehn, S., Frank, R., Vingron, V. and Dobberstein, B. (1989) Homology of 54K protein of signal-recognition particle, docking protein and two *E. coli* proteins with putative GTP-binding domains. *Nature* **340**, 478-486

Sabatini, D.D. and Blobel, G. (1970) Controlled proteolysis of nascent polypeptides in rat liver cell fractions. II. Location of the polypeptides in rough microsomes. *J. Cell Biol.* **45**, 146-157

Sanz, P. and Meyer, D.I. (1988) Signal recognition particle (SRP) stabilizes the translocation -competent conformation of pre-secretory proteins. *EMBO J.* 7, 3553-3557

Savitz, A.J. and Meyer, D.I. (1990) Identification of a ribosome receptor in the rough endoplasmic reticulum. *Nature* **346**, 540-544

Savitz, A.J. and Meyer, D.I. (1993) 180-kD ribosome receptor is essential for both ribosome binding and protein translocation. *J. Cell Biol.* **120**, 853-863

Siegel, V. and Walter, P. (1985) Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. *J. Cell Biol.* **100**, 1913-1921

Simon, S.M. and Blobel, G. (1991) A protein-conducting channel in the endoplasmic reticulum membrane. *Cell* **65**, 371-380

Somogyi, P., Jenner, A.J., Brierley, I. and Inglis, S.C. (1993) Ribosome pausing during translation of an RNA pseudoknot. *Mol. Cell. Biol.* **13**, 6931-6940

Tajima, S., Lauffer, L., Rath, V.L and Walter, P. (1986) The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. *J. Cell Biol.* **103**, 1167-1178

Verner, K. and Schatz, G. (1988) Protein translocation across membranes. *Science* **241**, 1307-1313

Walter, P. and Blobel, G. (1980) Purification of membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* 77, 7112-7116

Walter, P. and Blobel, G. (1981a) Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of *in-vitro*-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* **91**, 551-556

Walter, P. and Blobel, G. (1981b) Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *J. Cell Biol.* **91**, 557-561

Walter, P. and Blobel, G. (1982) Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. *Nature* **299**, 691-698

Walter, P. and Blobel, G. (1982) Preparation of microsomal membrane for cotranslational protein translocation. *Meth. Enz.* **96**, 84-93

Walter, P., Jackson, R.C., Marcus, M.M., Lingappa V.R. and Blobel, G. (1979) Tryptic dissection and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes. *Proc. Natl. Acad. Sci. USA* **76**, 1795-1799

Walter, P., Ibrahimi. I. and Blobel, G. (1981) Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to *in-vitro*-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* **91**, 545-550

Wanker, E.E., Sun, Y., Savitz, A.J. and Meyer, D.I. (1995) Functional characterization of the 180-kD ribosome receptor in vivo. *J. Cell Biol.* **130**, 29-39

Warren, G. and Dobberstein, B. (1978) Protein transfer across microsomal membranes reassembled from separated components. *Nature* **273**, 569-571

Wolin, S.L. and Walter, P. (1988) Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J.* 7, 3559-3569

Wolin, S.L. and Walter, P. (1989) Signal recognition particle mediates a transient elongation arrest of preprolactin in reticulocyte lysate. *J. Cell Biol.* 109, 2617-2622

Young, J.C., Ursini, J., Legate, K.R., Miller, J.D., Walter, P. and Andrews, D.W. (1995) An amino-terminal domain containing hydrophobic and hydrophilic sequences binds the signal recognition particle receptor α subunit to the β subunit on the endoplasmic reticulum membrane. *J. Biol. Chem.* **270**, 15650-15657

Young, J.C. and Andrews, D.W. (1995) The signal recognition particle receptor α subunit assembles co-translationally on the endoplasmic reticulum membrane during an mRNA encoded translation pause *in vitro*. *EMBO J.*, in press.

Zimmerman, D.L. and Walter, P. (1990) Reconstitution of protein translocation activity from partially solubilized microsomal vesicles. *J. Biol. Chem.* **265**, 4048-4053

Zopf, D., Bernstein, H.D. and Walter, P. (1993) GTPase domain of the 54kD subunit of the mammalian signal recognition particle is required for protein translocation but not for signal sequence binding. *J. Cell Biol.* **120**, 1113-1121

CHAPTER II

An Amino-terminal Domain Containing Hydrophobic and Hydrophilic Sequences Binds the Signal Recognition Particle Receptor α Subunit to the β Subunit on the Endoplasmic Reticulum Membrane.

adapted from

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SUMMARY

The signal recognition particle receptor consists of two subunits of 72 kD (SRα) and 30 kD (SRβ). Assembly of SRα on the endoplasmic reticulum membrane can occur independent of the signal recognition particle-mediated translocation pathway. To identify the sequences within SRα necessary for membrane anchoring, a series of amino terminal and internal deletion mutants was constructed and translated in a cell-free system. In addition, nascent SRα polypeptides of varying lengths were generated by cycloheximide treatment of translation reactions. Microsome binding assays performed on these polypeptides revealed a membrane binding domain consisting of the amino terminal 140 residues of SRα. This domain includes the two hydrophobic sequences originally proposed to bind to membranes and a highly charged region not previously implicated in membrane assembly. Furthermore, the domain forms a protease-resistant folding unit that after proteolysis can target and anchor onto microsomes. Extraction of microsomal SRα at high pH supplemented with 1 M NaSCN suggests that SRα and the membrane binding domain are not integrated in the ER membrane. The membrane binding domain is also the major site of tight binding with SRβ, suggesting that SRβ plays a role in the membrane anchoring of SRα.

INTRODUCTION

In mammalian cells, secretory signal sequences of nascent polypeptide chains are bound by the ribonucleoprotein signal recognition particle (SRP) as they emerge from the ribosome. Targeting to polypeptide translocation sites on the endoplasmic reticulum (ER) membrane then occurs via the interaction of SRP with the SRP receptor on the cytoplasmic face of the ER membrane (1, 2). The major components of this targeting pathway are conserved in eukaryotes, and possibly in prokaryotes (for review, see ref. 3).

The SRP receptor has been isolated as a heterodimer of two polypeptides which migrate in SDS-PAGE as 72 kD (SRα) and 30 kD (SRβ) species (4). Both subunits are resistant to extraction from the membrane with urea or high salt and have been characterized as integral membrane proteins by resistance to extraction at high pH (2, 4, 5). Protease dissection of SRα on microsomes or purified by affinity chromatography revealed a translocation active cytoplasmic fragment of about 58 kD and a fragment of about 14 kD containing a putative membrane anchor (5-7). The cDNA for SRα encodes a 638 residue polypeptide containing two stretches of hydrophobic amino acids (residues 1-22 and 64-79) near the amino terminus that were proposed to serve as membrane anchors, as well as three clusters of charged (mostly basic) residues between residues 84 and 243 (8). The cytoplasmic elastase fragment of SRα was shown to consist of the sequence from residue 152 to the carboxyl terminus, and contains a GTP binding site (8, 9). The cytoplasmic elastase fragment can assemble on trypsin-digested membranes to restore translocation activity, suggesting that it may bind SRβ directly (10). SRβ is

predicted from the primary amino acid sequence to have a single transmembrane domain near the amino terminus and a GTP binding site near the cytoplasmic carboxyl terminus (11).

SRα has previously been shown to target and anchor onto the ER membrane *in vitro* by a mechanism independent of the SRP-mediated pathway (10). Membrane assembly and functional reconstitution of SRα can occur post-translationally and in the absence of GTP or ATP. Cell-free synthesized SRα can also restore SRP-mediated translocation activity to microsomes in which the endogenous SRα has been inactivated by digestion with trypsin or by alkylation of free sulfhydryls. The binding of SRα onto trypsin digested microsomes is labile to urea, suggesting the subunit is not assembled on the membrane by spontaneous insertion into the lipid bilayer (10).

The exact mechanism by which SRα assembles on the membrane is unknown. Furthermore, the sequences within SRα required for interaction with SRß have not been identified. To investigate these issues, we have assayed deletion mutants of SRα translated in a cell-free system for anchoring onto ER microsomes. An amino terminal domain of SRα including amino acids 1 to 140 was found to be necessary for membrane anchoring. Immunoprecipitation experiments indicate the domain is also responsible for binding to SRβ. The SRα anchoring domain appears to be an independent folding unit that is tightly bound to SRβ but not integrated into the ER membrane. A new model of SRα membrane assembly is proposed in which both hydrophobic and hydrophilic regions of SRα anchor the protein to the membrane primarily by interacting with the transmembrane SRβ.

METHODS

Materials and General Methods

General chemical reagents were obtained from either Fisher, Sigma or Life Technologies, Inc. SURE™ *Escherichia coli* cells used for plasmid construction were purchased from Stratagene. Except where specified, restriction enzymes, other molecular biology enzymes, and reagents were from New England Biolabs. ^{35}S-labelled methionine was from DuPont-NEN. SP6 polymerase was purchased from Epicentre Technologies. Creatine kinase, staphylococcal nuclease and various proteases were from Boehringer Mannheim, and RNAguard (an RNase inhibitor) was from Pharmacia Biotech Inc.

Transcription reactions with SP6 polymerase were performed as described previously (14). Cell-free translation reactions were performed in rabbit reticulocyte lysate (RRL) and labelled with [^{35}S]methionine as described previously (12); translation products were analyzed by SDS-PAGE (15, 16) followed by fluorography. Canine pancreatic rough microsomes were obtained as described, and either extracted with 0.5 M KOAc (KRMs) or washed by Sepharose CL-2B gel exclusion chromatography (CRMs) (13).

Polyclonal antisera against SRα and SRβ were raised in rabbits injected with purified bacterially overexpressed fusion proteins. Plasmid pMAC142 contained the sequence encoding amino acids 39-295 of SRα inserted into pRIT-2T (Pharmacia) resulting in a fusion protein with *S. aureus* Protein A. Plasmid pMAC359 encoded amino acids 208-265 of canine SRβ fused to glutathione *S*-transferase in the vector pMAC241, a modification of pGEX-2T (Pharmacia) with an enhanced polylinker. The SRα and SRβ

fusion proteins were purified using IgG-Sepharose and glutathione-Sepharose columns, respectively. Other antisera were kind gifts of J.J.M. Bergeron, R. Gilmore and T. Rapoport.

Plasmids

Construction of plasmids, DNA sequencing and site-directed mutagenesis was performed using standard techniques (36). Unless otherwise stated, all constructs were inserted following the SP6 RNA polymerase promoter in pSPUTK (37). The deletion mutants of SRα and the relevant restriction sites are outlined in fig. 1 and briefly described below. Full construction details for each of the plasmids are included in the Appendix to this chapter.

Plasmid pMAC191 contains the full-length cDNA sequence of canine SRα (8), with a C→G point mutation at nucleotide 4 of the open reading frame, in the plasmid vector pSPUTK (37). The mutation introduces an NcoI site at the start codon of SRα. The overall translation efficiency of SRα in the cell-free system is increased by this mutation, but the resulting leucine to valine substitution does not affect the membrane targeting behaviour or translocation activity of the polypeptide (data not shown). The mutant polypeptide is termed SRαN to distinguish it from polypeptides with the wild-type sequence and microsomal SRα. Plasmid pMAC42 encoding the polypeptide SR-EF, corresponding to the soluble elastase fragment of SRα, has been reported previously (10).

Plasmid pMAC3 encodes the polypeptide SRD1, containing amino acids 79 to the stop codon of SRα and therefore having the two hydrophobic regions deleted from the

amino terminus of SRα. Plasmid pMAC456 encodes the polypeptide SRD3 in which residues 156-250 of SRαN are deleted, removing part of the second and all of the third charged regions of SRα. Plasmid pMAC55 encodes the polypeptide SRD4 containing an initial methionine followed by a glycine residue and residues 28 to the stop codon of SRα, deleting the first hydrophobic region of SRα.

Plasmid pMAC205 encodes the first 176 amino acids of SRαN followed by Ser-Asn-Tyr-Ser-Arg-stop codon. This polypeptide, SRX2, includes the two hydrophobic regions and the first two charged regions of SRα. Plasmid pMAC268 encodes SRX3, containing the polypeptide sequence Met-Gly-Ala-Pro followed by amino acids 28 to the stop codon of SRX2 and deleting the first hydrophobic region from SRX2. Plasmid pMAC135 encodes SRX6, containing residues 1-38 of SRα, Asn-Ser and residues 79 to the end of SRX2, thereby deleting the second hydrophobic region of SRX2. Plasmid pMAC362 encodes SRX7, containing residues 1-79 and 103 to the stop codon of SRX2 and deleting the first charged region of SRX2.

Plasmid pMAC459 encodes SRD6, containing the sequence of SRαN with amino acids 39-79 replaced with Asn-Ser and thus deleting the second hydrophobic region from SRαN. Plasmid pMAC494 encodes SRD7, having the sequence of SRαN with amino acids 79-103, and thus the first charged region, deleted.

Plasmid pMAC455 codes for a chimaeric SRβ polypeptide (SRβ-MD), containing the first 29 residues of mouse SRβ followed by the predicted transmembrane and cytoplasmic domains of canine SRβ. The chimaeric polypeptide was used because the cDNA sequence of canine SRβ was incomplete and the encoded protein was missing the

initiation site and an unknown number of amino-terminal residues. However, the missing residues were predicted to be in the ER lumen (11) and less likely to interact with SRα. The lumenal domain of canine SRβ was therefore replaced with the complete amino terminal lumenal domain of mouse SRβ, and the DNA sequence encoding this polypeptide was inserted into the vector pSPUTK. For immunoprecipitation experiments, plasmid pMAC690 was constructed encoding SRβ-MD with two copies of the influenza hemeagglutinin epitope tag at the amino terminus (HASRβ-MD). The sequence of the epitope tag was provided by inserting the DNA sequence encoding SRβ-MD into the plasmid pG7SCTHA2 (35). The resulting coding sequence was inserted behind the SP6 promoter of plasmid pMAC334, a version of pGEM3 with the 5'-untranslated region of pSPUTK and the 3'-untranslated region of bovine preprolactin. Plasmic pMAC508 encoding the integral membrane protein S_LS_TgPA has been previously reported (38).

Cell-Free Translations and Membrane Targeting

For post-translational targeting reactions, translation was terminated by chilling on ice, and then ribosomes were removed by centrifugation at 30 psi (180,000 x g) for 5 min in an Airfuge. A 20 μL aliquot of the supernatant was incubated with either 10 equivalents of CRMs or an equal amount of buffer for 5 min at 24°C. The mixture was then loaded onto a 0.5 mL column of Sepharose CL-2B in a 1-mL syringe equilibrated with 500 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc pH 7.5, 2.5 mM $MgCl_2$ and 1 mM dithiothreitol. The column was eluted with equilibration buffer and fractions (single drops) from the column were collected. 1.5 μL samples of these fractions were analyzed

by SDS-PAGE. The excluded volume of each column was calibrated by passing CRMs over the columns and identifying microsomal SRα by immunoblot analysis. The included volume was identified by the red colour of the globin from the RRL.

Incomplete nascent SRαN polypeptides of different lengths were generated by terminating cell-free translation reactions at various times with 1 mM cycloheximide. To assay membrane targeting of these polypeptides, a 20 μL aliquot of each reaction was incubated with 5 equivalents of KRMs for 5 min at 24°C. An equal volume of buffer containing 1 M NaCl, 50 mM EDTA and 20 mM Tris-Cl pH 8.0 was added at 4°C. The mixture was layered over a 100 μL sucrose step gradient containing 500 mM sucrose, 500 mM NaCl, 25 mM EDTA and 20 mM Tris-Cl pH 8.0, and the membranes were pelleted by centrifugation in an Airfuge at 20 psi (100,000 x g) for 10 min. The top 75 μL (supernatant) was recovered and peptidyl-tRNA precipitated by adding 500 μL of 2% cetyltrimethylammonium bromide (CTAB) and 500 μL of 0.5 M NaOAc pH 5.0 (17). Equivalent portions of the pellet and supernatant fractions were analysed by SDS-PAGE.

Proteolytic Digestions

Controlled proteolysis of RRL translation products was performed by adding proteinase K at a final concentration of 10 μg/mL to a completed 25 μL translation reaction and incubating on ice. Digestion was terminated after 30 min with 1 mM phenylmethylsulfonyl fluoride and 2 μg/mL aprotinin, and the reaction was incubated with 2.5 equivalents of KRMs for 5 min at 24°C. The mixture was adjusted to 2 M urea and the membranes pelleted as described previously (10). The supernatant and pellet fractions

were analysed by SDS-PAGE.

KRMs at 1 equivalent/μL were digested for 1 h at 4°C with either 0 or 10 μg/mL proteinase K. The microsomes were adjusted to 500 mM NaCl and pelleted in an Airfuge at 20 psi (100,000 x g) for 10 min. Immunoblots were probed for SRα and visualized using an alkaline phosphatase colour reaction.

Membrane Extractions and Immunoprecipitations

The Triton X-114 (TX114) cloud point partitioning assay (18) was adapted to enhance solubilization of SRα by the addition of 5% glycerol to the solubilization buffer and 1% glycerol to the sucrose cushion (19). The immunoblot was probed with monoclonal antibodies against both SRα and SRβ, and visualized using a two-colour enzymatic system to permit unambiguous identification of the polypeptides (20). Immunoblots probed for other proteins were visualized using an alkaline phosphatase colour reaction.

Microsomes were extracted with high pH following a modified procedure based on the published assay (21). 2 mL of KRMs at 1 equivalent/μL were loaded onto a 100 mL Sepharose CL-2B gel exclusion column equilibrated and eluted with 1 M NaSCN, 0.2 M Na_2CO_3 pH 11.5 and 10 mM dithiothreitol. 1.5 mL fractions were collected and concentrated by trichloroacetic acid precipitation for SDS-PAGE analysis. Immunoblots were visualized as above.

For immunoprecipitations of the SRα mutants with SRβ-MD, 10 μL RRL translation reactions were mixed with 10 μL reactions of SRß-MD after translation was

complete, and incubated at 24°C for 30 min. The mixtures were then diluted in 500 µL of buffer (100 mM Tris-Cl pH 8.0, 100 mM NaCl, 1% Triton X-100) at 4°C and the translation products isolated using a monoclonal-Sepharose affinity matrix. To prepare the affinity matrix, IgG against SRα was purified from ascites fluid (4) and coupled to CNBr-activated Sepharose. As controls, 10 µL translation reactions of SRαN, the deletion mutants and SRβ-MD were immunoprecipitated using the same monoclonal-Sepharose.

To co-precipitate various SRα mutants with HASRβ-MD, RRL translation reactions synthesizing HASRβ-MD were carried out in the presence of KRMs. A 5 µL aliquot of the HASRβ-MD reaction was incubated with a 30 µL translation reaction of each SRα mutant at 24°C for 30 min. The mixture was loaded onto a 0.8 mL Sepharose CL-2B column equilibrated and eluted with buffer containing 250 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc, pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. Fractions containing the excluded volume of the column were pooled, adjusted to 350 mM NaCl, 5% glycerol, and 1% Triton X-100, and HASRβ-MD was recovered using monoclonal antibodies against the hemeagglutinin epitope and Protein G Affi-Gel (Pharmacia).

RESULTS

Sequences within SRα Required for Membrane Anchoring

A plasmid was constructed encoding SRαN, a mutant of SRα (Leu replaced with Val) that has increased translational efficiency in our cell-free system but the same functional and membrane targeting behaviour as wild-type SRα (data not shown). Plasmid vectors encoding deletion mutants of SRαN (Fig. 1) were constructed to investigate the membrane anchoring of the receptor subunit. Previous experiments indicated that some portion of the amino terminal region of the polypeptide, containing two relatively hydrophobic sequences, was involved in anchoring SRα to the ER membrane (5, 8, 10). Therefore, a series of plasmids were made containing deletions in the region encoding the two hydrophobic regions (SRD1, SRD4 and SRD6) and an adjacent region of charged amino acids (SRD7). A construct coding for the amino terminal 176 amino acids of SRαN plus four additional residues (SRX2) was also made. Additional deletions were made within the SRX2 sequence (SRX3, SRX6 and SRX7). A broad deletion was also made in a central region of the SRαN sequence that was not expected to affect membrane anchoring (SRD3).

In a previous study of the membrane assembly of SRα, anchored and loosely bound molecules could be separated by a simple pelleting assay in the presence of 2 M urea (10). However, this assay could not clearly distinguish membrane-bound polypeptides from large insoluble aggregates. Therefore, to assay the deletion mutants for tight membrane binding, translation reactions containing microsomes were fractionated

by Sepharose CL-2B gel exclusion chromatography at high ionic strength. Endogenous microsomal SRα was identified by immunoblotting and found to elute solely in the excluded volume of the columns (fraction 4, marked with arrowhead for all columns used in Fig. 2, data not shown). The included volume (fractions 7-12 in all assays) was determined using the endogenous hemoglobin in the RRL reaction. Because the included volume eluted as a broad peak, fractions 7, 9 and 11 are shown in Fig. 2 as representative fractions.

In the absence of microsomes, SRαN synthesized in RRL fractionated in the included volume (Fig. 2 *a*, lanes 3-6). As expected, after incubation with membranes, much of the SRαN fractionated in the excluded volume together with the microsomes (Fig. 2 *a*, compare lane 1 with lane 7), indicating that these polypeptides were tightly bound on the membranes. SR-EF (which lacks the amino terminal 151 residues of SRα) has been shown to behave as a peripheral membrane protein (2, 5, 8, 10). Consistent with this, SR-EF was found only in the included volume of the columns in either the absence or presence of membranes (data not shown).

The two hydrophobic stretches in SRα were deleted in SRD1 and as expected, this polypeptide did not fractionate with microsomes as it was recovered only in the included volume (Fig. 2 *b* compare lanes 1-6 to lanes 7-12). Constructs that removed only the first (SRD4) or second (SRD6) of the hydrophobic sequences were also assayed. Although a fraction of SRD6 aggregated in RRL and therefore is recovered in fractions 5 and 6, the aggregates were clearly resolved from fraction 4 containing membranes (Fig. 2 *d*, compare lanes 8 and 9 with lane 7). Neither SRD4 nor SRD6 were able to bind efficiently onto

microsomes (Fig. 2 *c* and *d*, compare lanes 1 and 7). Surprisingly, a construct (SRD7) that left both the hydrophobic regions intact but deleted an adjacent section of strongly charged residues (amino acids 79-103) was also unable to bind efficiently onto microsomes (Fig. 2 *e*, compare lanes 1 and 7). Although analysis of this molecule was complicated by the presence of large aggregates (Fig. 2 *e*, lane 1), there was still a large portion of unaggregated polypeptide (Fig. 2 *e*, lanes 10-12) that was expected to be targeted to the membrane. As a control, a construct with a deletion in a region of the SRα sequence containing numerous basic amino acids (residues 156 - 250) but containing an intact amino terminus (SRD3) was found to fractionate with microsomes as expected (Fig. 2 *f*, compare lanes 1 and 7).

These results suggested that sequences beyond the predicted membrane anchor of SRα (8) may be required for membrane assembly. The carboxyl terminal domain of SRα (residues 152 to 638) has been shown to target to translocation sites on the ER but not anchor to the membrane in a manner resistant to high salt or urea concentrations (1, 10). To directly examine the membrane assembly of the amino terminal region of SRα, a construct (SRX2) containing the first 176 amino acids of SRαN was assayed. Although the putative carboxyl terminal targeting domain was deleted from SRX2, the polypeptide anchored on microsomes (Fig. 2 *g*, compare lanes 1 and 7). To analyze the sequence of SRX2 further, plasmids were constructed with deletions within the SRX2 coding region (SRX3, SRX6 and SRX7, Fig. 1). However, cell-free synthesized SRX3, SRX6 and SRX7 were unable to clearly bind membranes and formed very large aggregates in the presence or absence of microsomes (data not shown).

The positively charged amino acid sequence deleted in SRD7 may be specifically required for anchoring. However, it is also possible that the deleted sequence is not itself involved in anchoring but adversely affects protein folding around an adjacent membrane anchor sequence. To address this issue, cell-free translation reactions of SRαN were terminated with cycloheximide at different times after initiation to generate a series of ribosome-bound peptidyl-tRNA translation intermediates with a range of lengths. Ribosome-bound nascent polypeptides prepared in this manner should be free of aggregates. The reactions were incubated with microsomes to allow anchoring of the nascent chains, and then adjusted to 500 mM NaCl and 25 mM EDTA. The membranes were separated by centrifugation and analysed for the presence of anchored polypeptides. Peptidyl-tRNA was precipitated from the supernatant with CTAB (17) to recover nascent chains not anchored to the microsomes. It was expected that if the amino terminal hydrophobic regions of SRαN (up to around residue 80) were sufficient for membrane assembly while attached to ribosomes then polypeptides of molecular weight greater than or equal to 13 kD (corresponding to about residue 120, presuming 40 amino acids at most are sequestered within the ribosome (22, 23)) would be detected in the membrane fraction. On the other hand, if anchoring required sequences beyond the hydrophobic regions, then only larger polypeptides (approximately 190 amino acids, for a 150 residue anchoring domain) would be recovered with the microsomes.

Nascent SRαN polypeptides of discrete sizes from 10 kD upwards (estimated by migration in SDS-PAGE) could be detected after precipitation with CTAB (Fig. 3, lanes 7-12). The CTAB precipitated products reflected the polypeptides present in the total

translation reaction (Fig. 3, lane 13). However, no polypeptides smaller than a 23 kD translation intermediate were recovered with microsomes (Fig. 3, lanes 1-6). This 23 kD product was chased to full-length SRαN when the translation reactions were allowed to proceed for 1 h, and is therefore a true translation intermediate (data not shown). The deletion mutant SRX2 containing 180 amino acids also migrates as a 23 kD band, suggesting the 23 kD nascent polypeptide contains a similar number of residues. This is too large to consist of the hydrophobic regions of SRαN alone, but is consistent with an anchoring domain of approximately 140 amino acids. These data therefore support the hypothesis that sequences carboxyl terminal to the hydrophobic regions of SRα are necessary for membrane assembly.

Figure 1. Mutants of SRα. Diagram of the SRα coding region (top bar) with restriction enzyme sites in the DNA used to construct mutants. Amino acid residues are numbered below bar. Hydrophobic sequences are shown in black and charged sequences are shaded. Deletion mutants are diagrammed below with solid bars indicating the region(s) expressed in each.

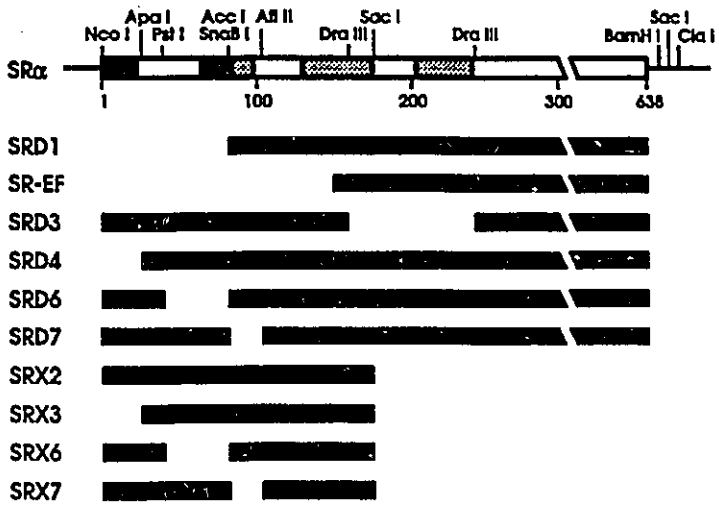


Figure 2. Membrane Anchoring of SRα Deletion Mutants. RRL translation reactions of SRαN and selected deletion mutants (lanes 1-6) or reactions incubated with microsomes (lanes 7-12) were loaded on 0.5 mL Sepharose CL-2B columns equilibrated and eluted in buffer containing 500 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc pH 7.5, 2.5 mM $MgCl_2$ and 1 mM DTT. Membranes eluted in the excluded volume (fraction 4, arrowheads) while hemoglobin eluted as a broad peak in the included volume (fractions 7 - 12).

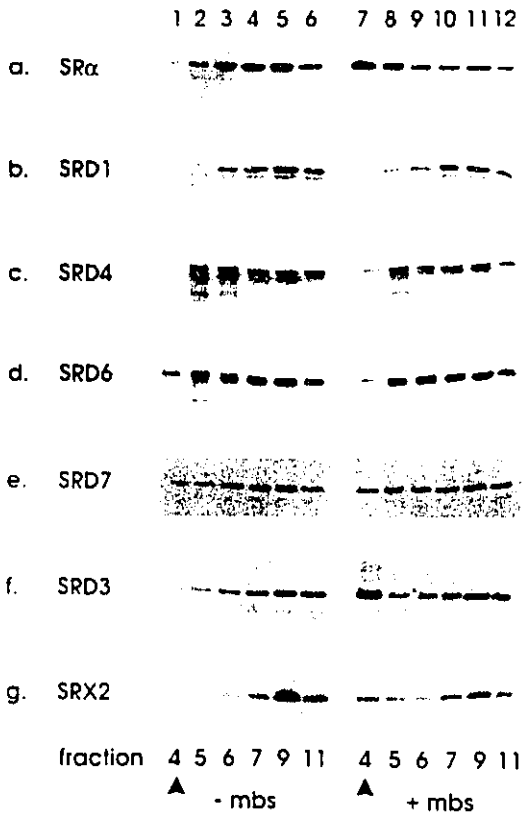
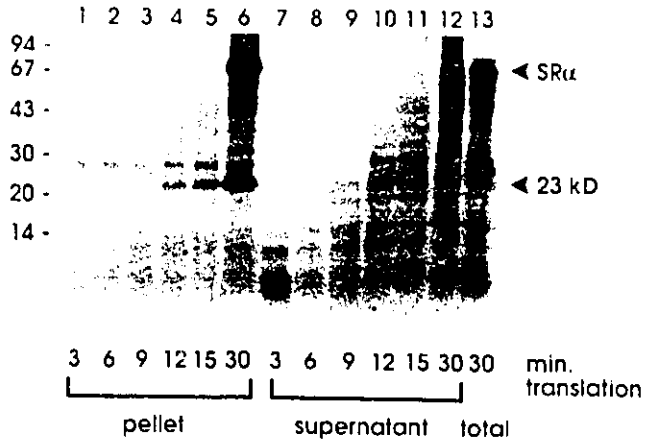


Figure 3. Membrane Anchoring of SRα Nascent Chains. RRL translation reactions of SRαN were terminated with 1 mM cycloheximide at various times after initiation. The terminated reactions were incubated with microsomes and then adjusted to 500 mM NaCl and 25 mM EDTA. Membranes were pelleted by centrifugation in an airfuge (lanes 1 - 6), and peptidyl-tRNA precipitated from the resulting supernatant with CTAB (lanes 7 - 12). A sample of the total translation reaction terminated after 30 min. was also analysed (lane 13). A 23 kD translation intermediate that anchored onto membranes is marked.



Domain Structure of the SRα Membrane Binding Sequence

To determine whether the membrane anchoring sequence of SRα forms an independently folded protein domain, we examined the sensitivity of SRα and several deletion mutants to protease digestion. Elastase dissection of purified SRα revealed a 14 kD amino terminal fragment presumed, but not directly demonstrated, to bind onto membranes (5, 8), as the fragment could not be detected on microsomes digested with elastase using SRα antisera (5). Therefore, to identify folding units within SRα that are competent in membrane targeting, we assayed proteolysis fragments of cell-free synthesized SRα for membrane anchoring. Cell-free translation reactions of SRαN were digested on ice with 10 μg/mL proteinase K and then incubated with KRMs to allow membrane assembly. The reactions were adjusted to 2 M urea, and microsomes were recovered by centrifugation. Both the supernatant (Fig. 4 *a*, lane 1) and the pellet (Fig. 4 *a*, lane 2) were analyzed for the presence of proteolytic fragments. Proteolytic fragments with a range of sizes were detected in the membrane fraction (Fig. 4 *a*, lane 2), and the smallest of these fragments had an apparent molecular size of 16 kD as estimated by migration in SDS-PAGE (Fig. 4 *a*, lane 2). Since the amino terminal deletion mutants SRD1 and SRD4 were unable to associate with microsomes (see Fig. 2), it is likely that the membrane-anchored proteolytic fragment contained intact amino terminus.

Our polyclonal antisera recognizes this region of SRα on immunoblots. Therefore, to confirm that the 16 kD membrane binding fragment includes the amino terminal domain, microsomes were digested with 10 μg/mL proteinase K for 1 h, adjusted to 500 mM NaCl, and recovered by centrifugation. Immunoblots of the digested microsomal

proteins as well as proteins from mock-digested membranes were probed with the antisera against the amino terminus of SRα. As expected, full length SRα was detected in the membrane pellet from mock digests (Fig. 4 *a*, lane 3) and as predicted, a 16 kD fragment generated by proteolysis also pelleted with membranes (Fig. 4 *a*, lane 4). This suggests that the 16 kD membrane binding fragment in Fig. 4 *a*, lane 2, consists of the amino terminal membrane binding domain. The apparent molecular size of the 16 kD fragments produced by proteolysis of both cell-free synthesized or endogenous microsomal SRα is consistent with the 140 residue amino terminal domain suggested by Fig. 3.

The comparative resistance of the amino terminal fragment to proteinase K digestion whether or not the protein is attached to membranes suggests that the anchoring domain forms a folded unit. To test this directly, the deletion mutants SRX2, SRX3, SRX6 and SRX7 (see Fig. 1) were assayed for resistance to proteinase K. SRX2 contains the complete amino terminal domain of SRαN, and the other polypeptides have deletions within the SRX2 sequence. Cell-free translation reactions of the polypeptides were digested with 10 μg/mL proteinase K on ice for up to 1 h, and analysed at intermediate time points. The SRX3, SRX6 and SRX7 polypeptides were rapidly degraded under these conditions, with less than 30% of the initial populations remaining after 20 min. (Fig. 4 *b*). In contrast, more than 60% of the initial population of SRX2 polypeptide remained after 40 min. of digestion (Fig. 4 *b*). Interestingly, these data are reflected in the membrane anchoring behaviour of SRX3, SRX6 and SRX7 reported above. In addition, the deletion mutants SRD4, SRD6 and SRD7 containing deletions in the SRX2 sequence of SRαN cannot anchor on membranes (Fig. 2). Taken together, these data suggest that

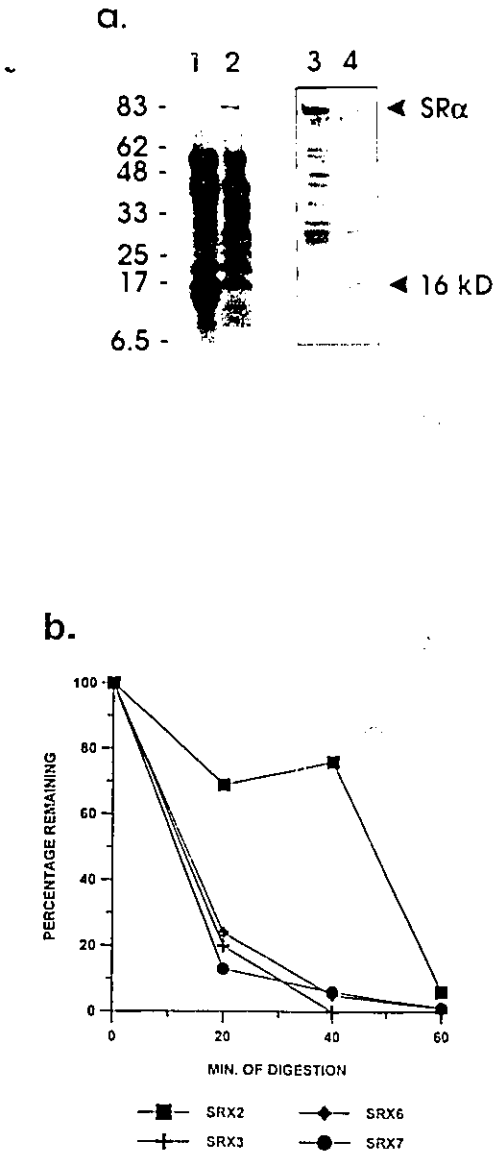
the deletions within SRX2 lead to misfolding, and that SRX2 forms an independently folded protein domain.

Membrane Binding of SRα Correlates with Binding to SRβ

SRα has been previously described as an integral membrane protein since solubilization could be achieved only in the presence of detergent and high salt concentrations (2). In addition, SRα was detected in the membrane pellet after extractions of microsomes at pH 11 (5). Interestingly, SRα has recently been shown to become largely extracted at pH 13, along with roughly half of the SRβ population (11). Furthermore, SRα and SRβ were found in the aqueous and hydrophobic phases after TX114 cloud point extractions of membranes (11). To extend and clarify these results, the behaviour of microsomal SRα in high pH and cloud point extractions was re-examined.

Figure 4. Proteinase K Dissection of SRα. *a*, SRαN synthesized in RRL was digested with 10 μg/mL proteinase K for 30 min and incubated with microsomes after digestion was terminated. The reaction was adjusted to 2 M urea and separated into supernatant (lane 1) and pellet (lane 2) fractions by centrifugation in an Airfuge. The entire pellet and 25% of the supernatant were analysed. A fragment with an apparent molecular size of 16 kD (marked) produced by the proteolysis was observed to pellet with microsomes. Also, microsomes digested with 0 (lane 3) and 10 μg/mL proteinase K (lane 4) were adjusted to 500 mM NaCl and the membranes recovered for immunoblot analysis by centrifugation in an Airfuge. Immunoblots were probed with polyclonal antisera raised against an amino terminal segment of SRα. A 16 kD proteolysis product immunoreactive with SRα antisera (marked) remained on membranes. *b*, RRL translation reactions of SRX2, SRX3, SRX6 and SRX7 were digested with 10 μg/mL proteinase K, and samples were analysed at 20 min intervals by SDS-PAGE and fluorography. The amount of translation product remaining was quantified for three independent experiments and plotted as a percentage of the amount present before digestion. The average standard deviation was ±5.2%.

Figure 4.


a.
1 2 3 4
83 -
62 -
48 -
33 -
25 -
17 -
6.5 -
SRα
16 kD
b.
PERCENTAGE REMAINING
100
80
60
40
20
0
0
20
40
60
MIN. OF DIGESTION
SRX2
SRX3
SRX6
SRX7

In studies of the translocation and membrane integration of proteins in cell-free systems, it has been observed that extraction with high pH alone was not always sufficient to distinguish peripherally bound proteins from integrated polypeptides (24). To increase the stringency of the high pH extractions, microsomes were extracted in buffer containing 1 M NaSCN, 0.2 M Na_2CO_3 pH 11.5 and 10 mM dithiothreitol, and membranes separated from extracted material by Sepharose CL-2B gel exclusion chromatography. Fractions were analysed for the presence of SRα and SRβ by immunoblot analysis. As controls, the immunoblots were also probed for the integral membrane proteins SSRα and the 48 kD subunit of oligosaccharyl transferase (OST48), the cytosolic protein actin, and the 54 kD subunit of the peripheral membrane SRP (SRP54) (25 - 30). This experiment, shown in Fig. 5 *a*, was performed by D.W. Andrews.

Under these conditions, microsomal SRβ was detected solely in the predetermined excluded volume of the column, corresponding to fractions 4-6 (Fig. 5 *a*, lanes 1-3). While a fraction of microsomal SRα eluted in fractions 4 and 5 (Fig. 5 *a*, lanes 1-2), the majority of the SRα polypeptides eluted in a broad peak between fractions 24 and 32 (represented by fractions 24, 28 and 32, Fig. 5 *a*, lanes 8-10). Visual inspection of this experiment and replicate trials indicated that approximately 20% or less of the SRα population remained on membranes in the excluded volume. As expected, the integral membrane control proteins SSRα and OST48 were also observed almost exclusively in the membrane fractions (Fig. 5 *a*, lanes 1-3), while actin and peripherally bound SRP54 eluted in a broad peak centred around fraction 30 (Fig. 5 *a*, lanes 9-11). It appears that while perturbation of the membrane at pH 11 was not sufficient to extract SRα (5), most

SRα polypeptides can be clearly separated from integral membrane ER proteins under the conditions used here.

SRα and SRβ have been previously observed to partition into both phases following cloud point separation (11). However, SRα is known to be fully solubilized only in the presence of detergent and moderately high ionic strength (250 mM KOAc and above) (2), and the cloud point assay uses solubilization conditions at physiologic ionic strength (150 mM NaCl) (18). As expected, we discovered that a large portion of microsomal SRα and SRβ remained insoluble in the original cloud point solubilization buffer. However, both subunits became fully solubilized when the buffer was supplemented with 5% glycerol (data not shown). We therefore assayed microsomes solubilized in this manner by cloud point separation, to confirm and extend previous results. Immunoblots were probed for SRα and SRβ and as controls for the integral membrane protein OST48 and the lumenal protein calreticulin (39).

Surprisingly, both SRα and SRβ were now detected only in the aqueous supernatant (Fig. 5 *b* compare lanes 1 and 2). The partitioning of SRα into the aqueous phase is consistent with its strongly hydrophilic primary sequence (8) and the apparently anomalous membrane interaction as demonstrated in Fig. 5 *a*. While SRβ appears to be integral membrane in high pH extractions supplemented with 1 M NaSCN (Fig. 5 *a*), it is possible that the tight interaction between the receptor subunits (4) causes SRβ to partition in the aqueous phase with SRα. We therefore digested microsomes with 5 μg/mL trypsin for 1 h at 4°C to proteolyze SRα while leaving SRβ unaffected (10), and then solubilized the membranes as above. After partitioning, tryptic fragments of SRα

(Fig. 5 *b*, lane 3) but no full-length protein were detected in the aqueous phase, and SRβ was detected solely in the detergent phase (Fig. 5 *b*, compare lanes 3 and 4). This agrees with recent results confirming the integral membrane nature of SRβ (11). As expected, in our solubilization conditions, OST48 was observed almost entirely in the detergent phase after cloud point separation (Fig. 5 *b*, compare lanes 5 and 6), and calreticulin partitioned solely into the aqueous phase (Fig. 5 *b*, compare lanes 7 and 8).

These results suggest that SRα is anchored largely by binding to the transmembrane SRβ polypeptide. To determine if this interaction is mediated by the membrane binding domain of SRα, we assayed the SRα deletion mutants used to map the SRα anchoring domain for the ability to bind SRβ in co-immunoprecipitations. These experiments, shown in Fig. 6 *a* and *b*, were performed by J. Ursini and K.R. Legate.

A cDNA encoding canine SRβ was available but lacked a complete amino terminus (11). However, a complete cDNA of mouse SRβ was available (11), so a plasmid coding for a hybrid murine/canine SRβ (SRβ-MD) was constructed. The mouse and dog sequences are highly homologous (88% identity, 93% similarity), both having a single putative transmembrane domain and a carboxyl terminal GTP-binding consensus sequence predicted to be on the cytoplasmic side of the ER (11). The SRβ-MD hybrid was constructed to contain the amino terminal lumenal domain of mouse SRβ and the transmembrane and carboxyl terminal cytoplasmic domains of canine SRβ. The junction between the sequences was selected because the binding site for SRα was expected to be in the transmembrane or cytoplasmic domain of SRβ.

RRL translation reactions of SRβ-MD were mixed with translation reactions of

various SRα deletion mutants and immunoprecipitated using monoclonal antibodies against SRα (4). A fraction of SRβ-MD was observed to co-precipitate with SRαN (Fig. 6 *a*, lanes 1 and 4) and SRD3 polypeptides (Fig. 6 *a*, lane 3), but not with SR-EF (Fig. 6 *a*, lane 2). Furthermore, SRβ-MD did not co-precipitate with SRD4 or SRD1 (Fig. 6 *a*, lanes 5 and 6), nor with SRD6 or SRD7 (data not shown). Control experiments (data not shown) suggest that the relatively poor co-precipitation of SRβ-MD with SRαN is likely due to inefficient formation of dimers in the absence of membranes. As expected in control immunoprecipitations of translation reactions containing only SRαN, the deletion mutants or SRβ-MD, no protein bands corresponding to SRβ-MD were observed (data not shown). These results suggest that the polypeptide sequences involved in the membrane anchoring of SRα are sufficient for interaction with SRβ in the absence of membranes.

To examine the assembly of SRα-SRβ dimers on microsomes, selected deletion mutants of SRα were assayed for co-precipitation with a version of SRβ-MD tagged at the amino terminus with an influenza hemeagglutinin epitope (HASRβ-MD). As a control, SRαN was tested for co-precipitation with the integral membrane protein S_LS_TgPA (38). RRL translation reactions for SRαN, SRD4, SRD6, SRD7 and SRX2 were incubated with microsomes containing HASRβ-MD or S_LS_TgPA. To recover polypeptides associated with, although not necessarily anchored to, the membrane, the completed reactions were fractionated by Sepharose CL-2B chromatography at moderate ionic strength. After the microsomes were solubilized, immunoprecipitation using antibodies against the hemeagglutinin epitope recovered HASRβ-MD in each case (Fig. 6 *b*, lanes

1-5). As predicted, SRαN was co-precipitated with HASRβ-MD (Fig. 6 *b*, lane 1). Although significant amounts of SRD4, SRD6 and SRD7 fractionated with membranes after chromatography (data not shown), SRD4, SRD6 and SRD7 were co-precipitated very poorly with HASRβ-MD (Fig. 6 *b*, lanes 2-4). Also, when membranes containing HASRβ-MD were added to translation reactions of SRD7 and the mixture was solubilized and immunoprecipitated with the same antibody, no SRD7 was co-precipitated even when the polypeptide was present in large excess (data not shown). In contrast, SRX2 was co-precipitated with HASRβ-MD at a level comparable with full-length SRαN (Fig. 6 *b*, lane 5). As expected, no SRαN was detected in immunoprecipitations of the integral membrane protein S_LS_TgPA (Fig. 6 *b*, lane 6), suggesting that the co-precipitation of SRαN and SRX2 with HASRβ-MD was not due to nonspecific aggregation of the hydrophobic sequences. PhosphorImager quantification revealed that after correction for the number of labelled methionine residues in the polypeptides, the ratio of SRαN to HASRβ-MD in the co-precipitation was approximately 0.23:1, and the ratio of SRX2 to HASRβ-MD was approximately 0.45:1. These ratios are reasonable given the expected low probability of contact between the SRα and HASRβ-MD polypeptides. The ratios of co-precipitated SRD4, SRD6 and SRD7 to HASRβ-MD were at least an order of magnitude lower than for SRαN. Therefore, these results indicate that the polypeptide sequences within the SRα membrane anchoring domain also mediate binding to SRβ. Taken together, our data suggest that SRα is bound to the ER membrane largely by interactions between the folded amino terminal domain and the SRβ subunit.

Figure 5. Membrane Extraction of the SRP Receptor. *a*, Microsomes were loaded onto a Sepharose CL-2B gel exclusion column equilibrated and eluted with 1 M NaSCN, 0.2 M Na_2CO_3 pH 11.5 and 10 mM dithiothreitol. Fractions were collected for SDS-PAGE and immunoblot analysis. Immunoblots of selected fractions were probed with monoclonal antibodies against SRα and polyclonal antibodies against SRβ, SSRα, OST48, actin , and SRP54. This experiment was performed by D.W. Andrews. *b*, Membranes either before (lanes 1-2 and 5-8) or after (lanes 3-4) digestion with 5 μg/mL trypsin were partitioned by cloud point extraction after solubilization with Triton X-114. The aqueous phases (lanes 1, 3, 5 and 7, marked A) and detergent phases (lanes 2, 4, 6 and 8, marked D) were resolved by SDS-PAGE followed by immunoblot identification of SRα and SRβ using specific antibodies and a two-colour dye reaction (lanes 1-4) or antibodies against OST48 (lanes 5-6) and calreticulin (lanes 7-8).

Figure 5.

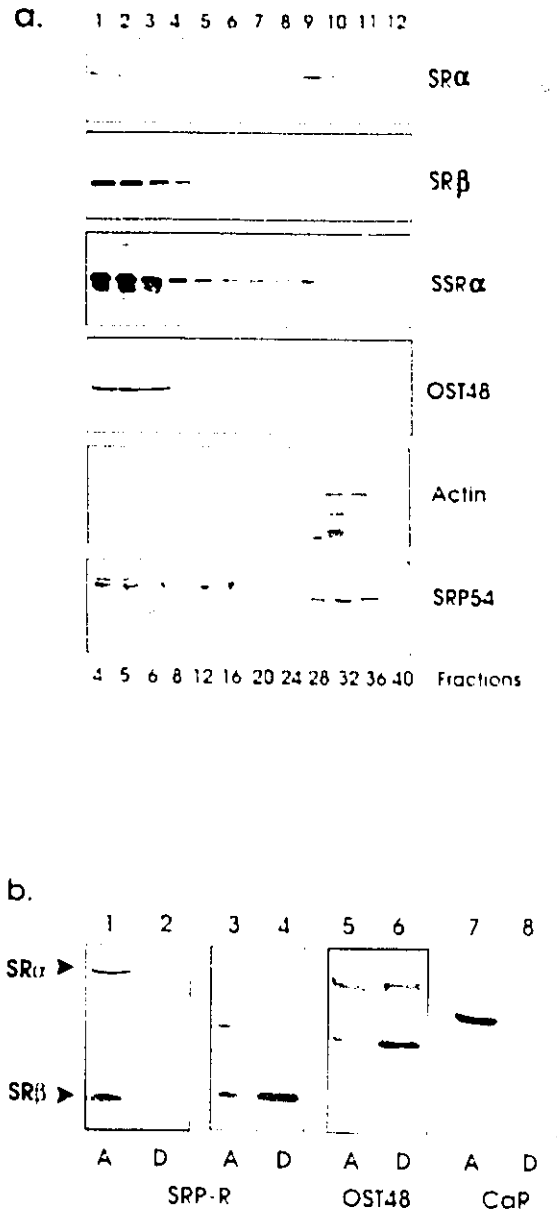
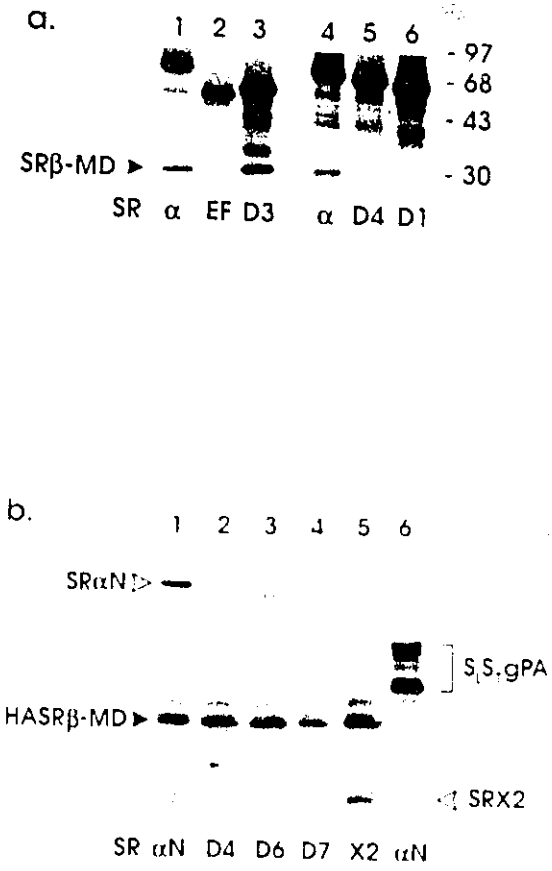

a.
1 2 3 4 5 6 7 8 9 10 11 12
SRα
SRβ
SSRα
OST48
Actin
SRP54
4 5 6 8 12 16 20 24 28 32 36 40 Fractions
b.
1 2 3 4 5 6 7 8
SRα
SRβ
A D A D A D A D
SRP-R
OST48
CaP

Figure 6. Co-Precipitation of SRα Mutants with SRβ. *a*, Cell-free translation reactions of SRαN (lanes 1 and 4), SR-EF (lane 2), SRD3 (lane 3), SRD4 (lane 5) and SRD1 (lane 6) were mixed with translation reactions of SRβ-MD and then immunoprecipitated with monoclonal antibodies against SRα. The SRβ-MD protein band in indicated with an arrowhead. This experiment was performed by J. Ursini. *b*, Translation reactions of SRαN (lane 1), SRD4 (lane 2), SRD6 (lane 3), SRD7 (lane 4), SRX2 (lane 5) and S_LS_TgPA (lane 6) were incubated with microsomes populated with HASRβ-MD. The mixtures were loaded onto Sepharose CL-2B columns equilibrated and eluted with buffer containing 250 mM NaCl, 100 mM KOAc, 10 mM Tris-OAc pH 7.5, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. Fractions containing membranes were pooled, solubilized, and immunoprecipitated using antibodies against the hemeagglutinin epitope tag (lanes 1-5) or using IgG-Sepharose (lane 6). Because SRX2 contains three labelled methionine residues compared to 17 for SRαN, lane 5 shows a 5-fold longer exposure. The SRαN and SRX2 bands are indicated with a solid arrowhead. The glycosylated, unglycosylated and signal-cleaved protein bands of S_LS_TgPA are indicated by the bracket. This experiment was performed by K.R. Legate.

Figure 6.


a.
1 2 3 4 5 6
- 97
- 68
- 43
- 30
SRβ-MD ▶
SR α EF D3 α D4 D1
b.
1 2 3 4 5 6
SRαN ▷
S,S,gPA
HASRβ-MD ▶
◁ SRX2
SR αN D4 D6 D7 X2 αN

DISCUSSION

We have shown here that a folded amino terminal membrane binding domain of SRα containing hydrophobic and charged amino acids is required for tight binding to SRβ. The SRα membrane binding sequence contains approximately 140 residues and forms an independently folded protein domain (Fig. 4 *b*). Membrane binding is observed when this region of SRα is generated by proteolysis of intact molecules either before or after targeting to microsomes (Fig. 4 *a*) or by cell-free synthesis as an isolated polypeptide (Fig. 2 *g* and Fig. 3). Furthermore, the SRα membrane binding domain binds directly to SRβ in the absence of other membrane proteins or lipids (Fig. 6 *a*). Despite the presence of a membrane targeting signal within the carboxyl terminal domain of SRα (10), deletion of either hydrophobic or charged sequences from the amino terminal domain abolished tight binding to the membrane (Fig. 2) and to SRβ (Fig. 6 *b*). Our results therefore suggest that the membrane binding domain of SRα is not inserted into the membrane (Fig. 5), but the entire domain is involved in binding to SRβ. The remarkably strong interaction between the subunits is resistant to 1% nonionic detergent and high ionic strength (Fig. 6 *b*), pH 11, and 2 M urea (2, 4, 5, 10) and most likely requires both hydrophobic and nonhydrophobic interactions. While the exact sequences within the SRα membrane binding domain that are in direct contact with SRβ remain to be determined, we expect they will include polar and nonpolar amino acids.

This model is not contradicted by the primary sequence of SRα, as the two hydrophobic regions within the anchoring domain are of comparatively low hydrophobicity and are both broken by lysine residues (8). Although the data cannot

entirely discount the possibility of interactions between the membrane lipids and SRα, the relative extractibility of membrane bound SRα in Fig. 5 *a* suggests these interactions are not typical of a membrane protein with even a single transmembrane domain. Our results are more consistent with the hydrophobic regions of SRα contributing to intersubunit contacts.

Hydrophobic interactions alone are not sufficient for receptor dimer assembly, as the deletion mutant SRD7 that has the same hydrophobic sequences as full-length SRα was unable to bind SRβ (Fig. 6 *b*). The importance of nonhydrophobic protein-protein interactions is further demonstrated by the cosegregation of both subunits as a complex in the aqueous phase after cloud point extraction (Fig. 5 *b*). Moreover, SRα could be dissociated from membrane-bound SRβ by the combined disruption of polar and hydrophobic interactions with pH 11.5 and 1 M NaSCN, without solubilizing the microsomal lipid bilayer (Fig. 5 *a*).

A slight molar excess of SRβ over SRα on the ER membrane has been reported (1.1 mol SRβ/mol of SRα) (4). In our model, novel SRα polypeptides targeted to the ER membrane would be anchored via these unpaired SRβ molecules. Anchoring of novel SRα is saturable at a concentration similar to that of excess SRβ on the membrane (10). The identity of the trypsin sensitive membrane component required for anchoring of novel SRα (10) is still unresolved. However, despite the apparent resistance of SRβ to protease digestion (10, 11), our results suggest that SRβ is the required factor. The SRα anchoring domain within SRX2 is necessary for co-precipitation of SRα and SRβ (Fig. 6), and similar to full-length SRα the binding of SRX2 onto trypsin treated microsomes is labile

to urea (data not shown).

The domain of SRα required for membrane anchoring and tight binding to SRβ has been demonstrated to be unnecessary for functional assembly of the receptor on the ER membrane (10). Therefore, tight binding between the receptor subunits is not required for receptor activity. This suggests a dual role for SRβ, as a membrane anchor for SRα and as a part of the translocation machinery. A specific role for SRβ in translocation has not been directly demonstrated, but the GTPase activity of SRP54 requires binding to the SRP receptor (31), and SRβ has been shown to be labelled *in vitro* with GTP (11).

The two-domain structure of SRα is likely evolutionarily conserved. The sequence of a homologue of SRα has been obtained from yeast, and contains a complete amino terminal sequence (32). A yeast homologue of SRβ has now been identified (11), and we predict a similar pattern of interactions between the proteins. The polypeptide sequence of the *E. coli* homolog of SRα, FtsY, begins at residue 126 of the canine sequence (32, 33) and thus corresponds closely to the carboxyl terminal domain of mammalian SRα. Interestingly, a bacterial homologue of SRβ has not been identified. Since the mammalian SRα anchoring domain that mediates binding to the β subunit is absent in FtsY, there may not be a homologue of SRβ in *E. coli*. However, FtsY has recently been reported to be resistant to high pH extraction despite the absence of hydrophobic domains (34) suggesting it may also bind to an integral membrane protein.

As demonstrated in Fig. 3, nascent SRα polypeptides can assemble on microsomes while still attached to ribosomes. Since membrane anchoring appears to require a folded amino terminal domain to interact with SRβ, targeting in this manner would still be

essentially post-translational. However, this suggests that folding of SRα and receptor dimer assembly can occur cotranslationally, at least *in vitro*. While post-translational targeting of SRα molecules has been demonstrated *in vitro* (10), the subunit may assemble co-translationally *in vivo*. We have therefore begun to investigate the possibility that SRα assembles onto the membrane during a pause in translation.

REFERENCES

1. Gilmore, R., Blobel, G. and Walter, P. (1982a) *J. Cell Biol.* **95,** 463-469
2. Gilmore, R., Walter, P. and Blobel, G. (1982b) *J. Cell Biol.* **95,** 470-477
3. Wolin, S. L. (1994) *Cell* 77, 787-790
4. Tajima, S., Lauffer, L., Rath, V. L. and Walter, P. (1986) *J. Cell Biol.* **103,** 1167-1178
5. Hortsch, M., Avossa, D. and Meyer, D. I. (1985) *J. Biol. Chem.* **260,** 9137-9145
6. Meyer, D. I. and Dobberstein, B. (1980a) *J. Cell Biol.* **87,** 498-502
7. Meyer, D. I. and Dobberstein, B. (1980b) *J. Cell Biol.* **87,** 503-508
8. Lauffer, L., Garcia, P. D., Harkins, R. N., Coussens, L., Ullrich, A. and Walter, P. (1985) *Nature* **318,** 334-338
9. Römisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingron, M. and Dobberstein, B. *Nature* **340,** 478-482
10. Andrews, D. W., Lauffer, L., Walter, P. and Lingappa, V. R. (1989) *J. Cell Biol.* **108,** 797-810
11. Miller, J.D., Tajima, S., Lauffer, L. and Walter, P. (1995) *J. Cell Biol.* **128**, 273-282
12. Jackson, R.J. and Hunt, T. (1983) *Meth. Enz.* **96,** 50-74
13. Walter, P. and Blobel, G. (1983) *Meth. Enz.* **96,** 84-93
14. Gurevich, V.V., Pokrovskaya, I. D., Obukhova, T. A. and Zozulya, S. A. (1991) *Anal. Biochem.* **195,** 207-213

15. Lämmli, U.K. (1970) *Nature* **227,** 680-685

16. Schagger, H. and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368-379

17. Hobden, A. N. and Cundliffe, E. (1978) *Biochem. J.* **170,** 57-61

18. Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604-1607

19. Andrews, D. W., Gupta, J. and Abisdris, G. (1993) *Mol. Cell Biol.* **13,** 4703-4713

20. Theisen, N., Schulze Lohoff, E., von Figura, K. and Hasilik, A. (1986) *Anal. Biochem.* **152,** 211-214

21. Fujiki, Y., Hubbard, A. D., Fowler, S. and Lazarow, P. B. (1982) *J. Cell Biol.* **93,** 97-102

22. Malkin, L. I. and Rich, A. (1967) *J. Mol. Biol.* **26,** 329-346

23. Blobel, G. and Sabatini, D. D. (1970) *J. Cell Biol.* **45,** 130-145

24. Andrews, D. W., Young, J. C., Mirels, L. F. and Czarnota, G. J. (1992) *J. Biol. Chem.* **267,** 7761-7769

25. Wiedmann, M., Görlich, D., Hartmann, E., Kurzchalia, T.V. and Rapoport, T. A. (1989) *FEBS Lett.* **257,** 263-268

26. Görlich, D., Prehn, S., Hartmann, E., Herz, J., Otto, A., Kraft, R., Wiedmann, M., Knespel, S., Dobberstein, B. and Rapoport T. A. (1990) *J. Cell Biol.* **111,** 2283-2294

27. Wada, I., Rindress, D., Cameron, P. H., Ou, W.-J., Doherty, J. J. II, Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y. and Bergeron, J. J. M. (1991) *J. Biol. Chem.* **266,** 19599-19610

28. Kelleher, D. J., Kreibich, G. and Gilmore, R. (1992) *Cell* **69,** 55-65

29. Silberstein, S., Kelleher, D. J. and Gilmore, R. (1992) *J. Biol. Chem.* **267,** 23658-23663

30. Walter, P. and Blobel, G. (1982) *Nature* **299,** 691-698

31. Miller, J. D., Wilhelm, H., Gierasch, L., Gilmore, R. and Walter, P. (1993) *Nature* **366,** 351-354

32. Ogg, S.C., Poritz, M. A. and Walter, P. (1992) *Mol. Biol. Cell.* **3,** 895-911

33. Gill, D. R., Hatfull, G. F. and Salmond, G. P. C. (1986) *Mol. Gen. Genet.* **205,** 134-145

34. Luirink, J., ten Hagen-Jongmann, C. M., van der Weijden, C. C., Oudega, B., High, S., Dobberstein, B. and Kusters, R. (1994) *EMBO J.* **13,** 2289-2296

35. Frydman, J., Nimmesgern, E., Ohtsuka, K. and Hartl, F. U. (1994) *Nature* **370,** 111-117

36. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press.

37. Falcone, D. and Andrews, D. W. (1991) *Mol. Cell Biol.* **11,** 2656-2664

38. Janiak, F., Glover, J.R., Leber, B., Rachubinski, R.A. and Andrews, D.W. (1994) *Biochem. J.* **300**, 191-199

39. Michalak, M., Milner, R.E., Burns, K., and Opas, M. (1992) *Biochem. J.* **285**, 681-692

APPENDIX

Construction of Plasmids

Construction of plasmids, sequencing and site-directed mutagenesis were performed using standard techniques (36). Unless otherwise stated, all constructs were inserted following the SP6 RNA polymerase promoter in pSPUTK (37). The deletion mutants and the relevant restriction sites are outlined in Fig. 1. Construction details for each of the plasmids are given below.

Plasmid pMAC191 (Fig. 7) contains the full-length cDNA sequence of canine SRα (8), with a C to G point mutation at nucleotide 4 of the open reading frame, in the plasmid vector pSPUTK (37). The mutation introduces an NcoI site at the start codon of SRα. The overall translation efficiency of SRα in the cell-free system is increased by this mutation, but the resulting Leu→Val substitution does not affect the membrane targeting behaviour or translocation activity of the polypeptide (data not shown). The mutant polypeptide is termed SRαN to distinguish it from polypeptides with the wild-type sequence and microsomal SRα. Plasmid pMAC42 encoding the polypeptide SR-EF, corresponding to the soluble elastase fragment of SRα, has been previously reported (10).

SRD1. Plasmid pMAC3 encodes SRα with both of the hydrophobic domains deleted from the amino terminus (SRD1). This plasmid was constructed by digesting plasmid pMAC2 (encoding SRα) with AccI, followed by end repair with the Klenow fragment of DNA polymerase and insertion of a linker (5'-AGATCTACCATGG) containing an NcoI

site. The DNA between this new NcoI site and an endogenous NcoI site near the 3' end of the SRα coding region was excised and exchanged into the corresponding sites in plasmid pMAC191. The resulting plasmid codes for an initial methionine followed by amino acids 79 to the stop codon of SRα.

SRD3. Plasmid pMAC456 codes for the polypeptide SRD3 in which residues 156 to 250 of SRαN are deleted. The deleted region includes part of the second and all of the third charged regions of SRα. To construct pMAC456, plasmid pMAC191 was digested with DraIII at two sites within the coding region, the ends were repaired with T4 DNA polymerase and the plasmid was religated.

SRD4. The SRD4 polypeptide encoded by plasmid pMAC55 contains an initial methionine followed by a glycine residue and residues 28 to the stop codon of SRα, thus deleting the first hydrophobic segment of the SRα polypeptide. The region of the DNA encoding SRα between an ApaI site corresponding to amino acid 27 and a BamHI site 3' to the stop codon was ligated into ApaI and BamHI sites in the polylinker of pSPUTK. This plasmid was cut with ApaI again, the ends repaired with the Klenow fragment of DNA polymerase and religated to adjust the reading frame of the SRα sequence with that of the pSPUTK start codon.

SRX2. Plasmid pMAC205 encodes the first 176 amino acids of SRαN followed by Ser-Asn-Tyr-Ser-Arg-stop codon. This polypeptide (SRX2) includes the two hydrophobic

regions and the first two charged regions of SRα. To construct plasmid pMAC205, the sequence of pMAC191 between a SacI site within the coding region of SRα and another in the pSPUTK polylinker 3' to the SRα stop codon was excised. The remaining plasmid sequence was then religated. To introduce a stop codon, the plasmid was digested at a ClaI site in the pSPUTK polylinker, the ends repaired with the Klenow fragment of DNA polymerase and the plasmid religated.

SRX3. Plasmid pMAC268 codes for the second hydrophobic region and the first two charged regions of SRαN (SRX3). To construct pMAC268, plasmid pMAC205 (encoding SRX2) was cleaved with NcoI and ApaI, the ends repaired with the Klenow fragment of DNA polymerase, an ApaI linker was inserted and the plasmid was religated. The resulting plasmid encodes the polypeptide sequence Met-Gly-Ala-Pro followed by amino acids 28 to the stop codon of SRX2.

SRX6. Plasmid pMAC135 encodes the first hydrophobic region and the first two charged regions of SRαN (SRX6). To construct this plasmid, pMAC205 was digested at the PstI site within the coding region, the ends were repaired with the Klenow fragment of DNA polymerase, the DNA redigested at the SnaBI site and an EcoRI linker was inserted to maintain the reading frame. The final open reading frame codes for residues 1 to 38 of SRα, followed by Asn-Ser and residues 79 to the end of SRX2.

SRX7. Plasmid pMAC362 codes for the two hydrophobic regions and the second charged

region of SRαN (SRX7). To construct this plasmid from pMAC205 (encoding SRX2), the DNA sequence between the SnaBI and AflII sites was excised, the AflII end blunted with the Klenow fragment of DNA polymerase and the plasmid religated. The resulting plasmid codes for residues 1 to 79 and 103 to the stop codon of SRX2.

SRD6. Plasmid pMAC459 encodes SRαN with amino acids 39 to 79 replaced with Asn-Ser (SRD6), thus deleting the second of the two amino terminal hydrophobic regions from SRαN. To construct this plasmid, the DNA sequence of plasmid pMAC191 (encoding SRαN) between a SacI site in the open reading frame and another SacI site 3' to the stop codon was excised and ligated into the SacI site of plasmid pMAC135 (encoding SRX6).

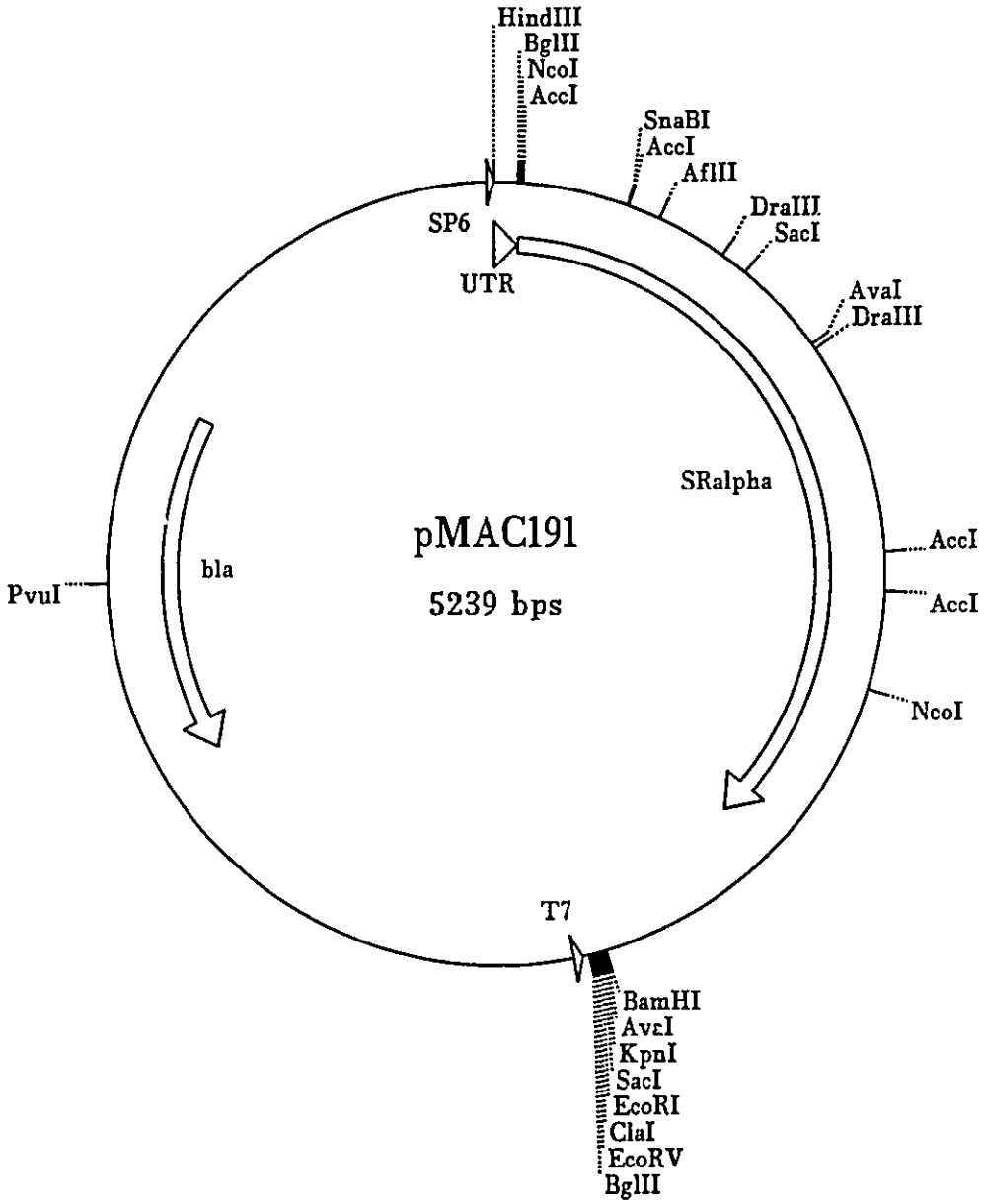
SRD7. Plasmid pMAC494 codes for the SRαN polypeptide with amino acids 79 to 103 (including the first charged region) deleted (SRD7). This plasmid was constructed analogously to pMAC459 (SRD6) by ligating the SacI to SacI fragment of plasmid pMAC191 (SRα) into the SacI site of plasmid pMAC362 (SRX7).

SRβ-MD. An incomplete cDNA sequence of canine SRβ was available as an insert in the plasmid vector pBluescript II (plasmid pMAC276). The encoded protein was missing the initiation site and an unknown number of amino terminal residues. However, the missing residues were predicted to be in the ER lumen (11) and less likely to interact with SRα. The lumenal domain of canine SRβ was therefore replaced with the complete amino terminal lumenal domain of mouse SRβ. The resulting plasmid (pMAC455) codes

for a chimaeric polypeptide (SRβ-MD) containing the first 29 residues of mouse SRβ followed by the predicted transmembrane and cytoplasmic domains of canine SRβ. To construct this chimaera, the canine SRβ cDNA was ligated between the XbaI and EcoRI sites of pSPUTK (plasmid pMAC438). In parallel, the mouse sequence was amplified by the polymerase chain reaction and inserted between the NcoI and SalI sites of pSPUTK (plasmid pMAC433). A PstI site at amino acid 29 of the mouse SRβ sequence is in a corresponding position and the same reading frame in the canine sequence. Therefore, the DNA sequence of plasmid pMAC438 between the PstI site and a SmaI site in the polylinker 3' to the stop codon, encoding the desired region of canine SRβ, was excised and ligated into the corresponding sites of plasmid pMAC433 to produce pMAC455.

HASRβ-MD. Plasmid pMAC690 was constructed encoding SRβ-MD with two copies of the influenza hemeagglutinin epitope tag at the amino terminus (HASRβ-MD). To provide the sequence of the epitope tag, the DNA sequence encoding SRβ-MD was excised from pMAC455 between the NcoI and SphI sites and ligated between the NcoI and SphI sites of plasmid pG7SCTHA2 (35). The resulting plasmid was digested with BamHI and SphI to produce a DNA fragment encoding HASRβ-MD. This fragment was ligated between the BglII and SphI sites of plasmid pMAC334, a modified version of pGEM3. The final plasmid (pMAC690) contained an SP6 RNA polymerase promoter, the 5' untranslated region of pSPUTK (37), the sequence encoding HASRβ-MD, and the 3' untranslated region of bovine preprolactin.

Figure 7. Map of plasmid pMAC191. This plasmid encodes SRαN (labelled SRalpha) behind an SP6 RNA polymerase promoter (labelled SP6) and the 5' untranslated region of pSPUTK (labelled UTR). An antisense T7 RNA polymerase promoter (labelled T7) and the β-lactamase gene (labelled bla) providing ampicillin resistance is also shown. Restriction sites used in the construction of SRα deletion mutants are marked (see also Fig. 1).



CHAPTER III

The Signal Recognition Particle Receptor α Subunit Assembles Co-translationally on the Endoplasmic Reticulum Membrane During an mRNA Encoded Translation Pause *in Vitro.*

adapted from

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SUMMARY

Many proteins including the α subunit of the signal recognition particle receptor (SRα) are targeted within the cell by poorly defined mechanisms. A 140 residue amino terminal domain of SRα targets and anchors the polypeptide to the endoplasmic reticulum membrane by a mechanism independent of the pathway involving the signal recognition particle. To investigate the mechanism of membrane anchoring, translation pause sites on the SRα mRNA were used to examine the targeting of translation intermediates. A strong pause site at nucleotide 507 of the mRNA open reading frame corresponded with the shortest nascent SRα polypeptide able to assemble on membranes. An mRNA sequence at this pause site that resembles a class of viral -1 frameshift sequences caused translation pausing when transferred into another mRNA context. Site-directed mutagenesis of the mRNA greatly reduced translation pausing without altering the polypeptide sequence, demonstrating unambiguously a role for this mRNA sequence in translation pausing. SRα polypeptides synthesized from the non-pausing mRNA were impaired in cotranslational membrane anchoring. Furthermore, cotranslational membrane assembly of SRα appears to anchor polysomes translating SRα to membranes.

INTRODUCTION

A large variety of proteins are targeted within the cytoplasm to specific sites on intracellular membranes or structures. In general, targeting requires some form of localization signal to specify the intracellular destination. This signal may be contained within the sequence of the targeted protein (reviewed in Verner and Schatz, 1988), or on the sequence of the mRNA encoding the polypeptide (reviewed in Wilhelm and Vale, 1993). However, for many proteins the mechanisms responsible for localization are poorly defined. One such protein is the α subunit of the signal recognition particle receptor (SRα). In a cell-free system, SRα is targeted to the endoplasmic reticulum (ER) membrane by a mechanism apparently unrelated to other known pathways (Andrews et al, 1989). The amino acid sequences responsible for targeting and anchoring SRα to the endoplasmic reticulum (ER) membrane have recently been identified (Young et al, 1995). In this paper, we have further characterized this targeting pathway by examining the membrane assembly event. We now demonstrate a novel mode of targeting in which nascent polypeptides are localized during a pause in translation.

SRα is essential for the translocation of nascent polypeptides across the ER membrane via a mechanism mediated by the signal recognition particle (SRP) (Gilmore et al, 1982a and 1982b). The SRα polypeptide (638 residues, 72 kD apparent molecular weight) is bound to the the cytoplasmic face of the ER membrane largely by strong interactions with the transmembrane β subunit of the receptor (SRß) (Gilmore et al, 1982b; Hortsch et al, 1985; Lauffer et al, 1985; Tajima et al, 1986; Miller et al, 1995;

Young et al, 1995). In a cell-free system, novel SRα polypeptides are assembled onto ER microsomes in a urea and salt resistant manner by an SRP independent pathway (Andrews et al, 1989). An independently folded amino terminal domain of SRα, containing about 140 residues, is necessary and sufficient for targeting and tight binding onto membranes (Young et al, 1995). Furthermore, SRα translation intermediates with discrete apparent molecular weights as low as 23 kD can anchor onto membranes while attached to ribosomes in cell-free translation reactions. Synthesis of these 23 kD nascent polypeptides coincides with the emergence of the amino terminal anchoring domain from the ribosome, indicating that SRα can assemble on membranes cotranslationally (Young et al, 1995).

The discrete size of the nascent 23 kD SRα polypeptide suggested it was produced by a translation pause. Translation pausing has been demonstrated for several different mRNAs both *in vitro* and *in vivo*, but in most cases the mechanisms and biological importance of pausing are unknown (Doohan and Samuel, 1992; Kim et al, 1991; Kim and Hollingsworth, 1992; Wolin and Walter, 1993). In this paper, we have identified an mRNA element responsible for ribosome pausing at the site on the SRα mRNA corresponding to synthesis of the 23 kD membrane binding translation intermediate. The assay we used was previously employed to examine the translation pausing of secreted polypeptides during SRP-mediated membrane translocation (Wolin and Walter, 1988; Wolin and Walter, 1989; Wolin and Walter, 1993). The mRNA sequence at the SRα pause site resembles a class of viral and bacterial frameshift sites, and was sufficient to mediate pausing in an unrelated mRNA context. Moreover, site-directed mutagenesis of

the putative pause sequence abolished pausing and demonstrated the importance of pausing for co-translational membrane binding of SRα. Finally, co-translational binding of SRα onto microsomes was shown to play a role in polysome targeting. From these experiments, the localization of the SRα mRNA at steady state translation *in vitro* was determined.

METHODS

Materials and general methods

General chemical reagents were obtained from either Fisher, Sigma or Gibco BRL. SURE™ *E. coli* cells used for plasmid construction and preparation of single-stranded DNA were purchased from Stratagene. Unless otherwise stated, restriction enzymes and other molecular biology enzymes and reagents were from New England Biolabs. ^{35}S-labelled methionine and UTP were from Dupont-NEN. SP6 polymerase was purchased from Epicentre Technologies. Creatine kinase, cycloheximide and Staphylococcal nuclease were from Boehringer Mannheim. RNAguard (an RNase inhibitor) and VI nuclease were from Pharmacia.

Plasmid construction and sequencing, electrophoresis of RNA and single-stranded DNA preparation using M13 R408 helper phage were accomplished using standard techniques (Sambrook et al, 1989). Transcription reactions with SP6 polymerase were performed as previously described (Gurevich et al, 1991). To radiolabel mRNA, the reactions were supplemented with α[^{35}S]-UTP at 12.5 μCi per 10 μL reaction. Rabbit reticulocyte lysate (RRL) was prepared and transcription-linked cell-free translation reactions were performed as published (Jackson and Hunt, 1983). Canine pancreatic rough microsomes were prepared as described and further purified by Sepharose CL-2B gel exclusion chromatography (CRMs) or extracted with 0.5 M KOAc (KRMs) (Walter and Blobel, 1983). The RNAfold program (Scientific & Educational Software, State Line, PA USA), based on the methods of Freier et al, 1986, was used for predictions of RNA

secondary structure.

Plasmids

Plasmid pMAC191 has been previously reported, and encodes a point mutant of SRα termed SRαN (Falcone and Andrews, 1991). The C→G substitution at nucleotide 4 of the coding region increases the translation efficiency of the polypeptide in the cell-free system, but the resulting Leu2→Val mutation does not affect the membrane targeting, translocation activity or translation pausing of SRαN compared to wild-type SRα (data not shown, and Young et al, 1995). All nucleotides within the SRα coding region are numbered relative to the initial AUG rather than the start of the reported cDNA sequence (Lauffer et al, 1985).

A plasmid (pMAC623) encodes a globin-protein A fusion protein (Janiak et al, 1994) with a pause site from SRα inserted between the chimpanzee α-globin and *S. aureus* protein A domains (gSRpa-1). To assemble this plasmid, a synthetic oligonucleotide linker containing PstI and SacI sites was ligated between the BglII and NcoI sites 5' of the coding region of the protein A Fc-binding domain. DNA coding for a fragment of globin was then inserted at the BglII site. This allowed a synthetic oligonucleotide linker coding for amino acids 160 to 183 of SRα (nucleotides 478 - 530) to be inserted between the PstI and SacI sites without altering the reading frame of the fusion protein. The coding region of gSRpa-1 followed an SP6 RNA polymerase promoter and the pSPUTK 5' untranslated sequence (Falcone and Andrews, 1991).

Plasmid pMAC714 contains the sequence of SRαN with point mutations at

nucleotides 504 (A→G), 507 (A→G), 510 (G→A), 513 (G→C) and 516 (C→G) of the coding region. The mutations were designed to alter the mRNA structure at a translation pause site without changing the polypeptide sequence. For clarity, the protein encoded by the mutant mRNA is termed SRαNP. To construct plasmid pMAC714, a fragment of DNA was amplified from plasmid pMAC191 by the polymerase chain reaction using an oligonucleotide primer with the sequence 5'-GCCATCAGAGCTCTCCTTCTTCTCGCC-TTTCTTCTTGCTG and another primer complementary to the SP6 promoter. The amplified fragment was cleaved at a HindIII site 3' of the SP6 promoter and at a SacI site 3' of the introduced mutations. The fragment was inserted into the corresponding HindIII and SacI sites of plasmid pMAC191.

To obtain single-stranded DNA for primer extension assays, sequences between the NheI and EcoRI sites of plasmid pMAC191 and pMAC623 were ligated into the XbaI and EcoRI sites of the plasmid pGC1 containing an M13 origin of replication (Myers et al, 1985). The resulting plasmids, pMAC573 and pMAC646, contain the SP6 RNA polymerase promoter, the 5' untranslated sequence and the complete coding regions of SRαN and gSRpa-1 respectively.

The plasmid vector pSPUTKf(-) was constructed by adding an f1 origin of replication to the plasmid pSPUTK (Falcone and Andrews, 1991). To assemble this plasmid, an NdeI linker was inserted into the HpaI site of pSPUTK, so that the T7 promoter was flanked by the NdeI site and an EcoRI site in the polylinker. A DNA fragment containing an f1 origin and T7 promoter was excised from pGEM3Zf(-) (Promega) between the NdeI and EcoRI sites, and ligated into the corresponding sites in

the modified pSPUTK to produce pSPUTKf(-). To insert the coding region of SRαNP into pSPUTKf(-), the DNA sequence of pMAC714 between the NcoI sites (at nucleotides -1 and 1505) was inserted into the NcoI site of pSPUTKf(-). This plasmid was cleaved at SacI sites 3' of the silent mutations and in the polylinker 3' of the stop codon, and the corresponding region of plasmid pMAC191 (encoding SRαN) digested between similarly positioned Sac I sites was inserted. The resulting plasmid pMAC745 contains the 5' untranslated region and coding sequence of SRαNP in pSPUTKf(-).

Plasmid pMAC35 coding for chimpanzee α-globin was as published (Andrews et al, 1989).

Isolation and mapping of ribosome protected fragments

A published procedure for identifying ribosomal pause sites (Wolin and Walter, 1989) was adapted as follows. To obtain monosomes from 25 μL RRL cell-free translation reactions terminated at steady state (25 min for SRαN and SRαNP, 15 min for gSRpa-1), reactions were digested with Staphylococcal nuclease at a final enzyme concentration of 0.75 units/μL. Digestion was terminated by adding EGTA to 5 mM and ribonucleoside-vanadyl complex to 10 mM. Total ribosome protected mRNA fragments (rpfs) from translations in the absence of microsomes were obtained as published (Wolin and Walter, 1989). To obtain membrane bound rpfs, 25 μL translations containing 2.5 equivalents of CRMs were digested with nuclease as above. The reactions were adjusted to 150 mM KOAc and cytoplasmic monosomes were isolated as published (Wolin and Walter, 1993), or by centrifugation in a Beckmann Instruments TLA-100 rotor at 100,000

rpm (436,000 x g) for 90 min. The microsomal fraction (120 µL supernatant) was solubilized by the addition of 6 µL of 20% CHAPS (final concentration 1% detergent). Solubilized monosomes were pelleted through a 60 µL 1.8 M sucrose step containing 1% CHAPS, and rpfs were isolated from both monosome pellets as described (Wolin and Walter, 1993). Cytoplasmic and membrane bound polysomal mRNA were obtained by the same procedure but with the omission of the nuclease digestion. To abolish translation, the RRL translation reactions were preincubated with 4 mM 7-methylguanosine-5'-phosphate (7mG) and 0.1 mM aurintricarboxylic acid (ATA). Isolated rpfs and polysomal mRNA were resuspended in 10 µL of sterile water following precipitation with ethanol.

To analyze the size of the rpfs, radiolabelled transcripts were added to the cell-free translation reactions and rpfs were isolated as above. 1 µL of rpfs were added to 9 µL of deionized formamide and analyzed by electrophoresis on denaturing 8 M urea/10% acrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) and visualized by autoradiography. To determine the distribution of rpfs on the original mRNA, aliquots of rpfs were annealed to a single-stranded DNA template and mapped by primer extension using a modification of the published procedure (Wolin and Walter, 1989). A mixture containing 2 µL of 10X T4 polymerase buffer (New England Biolabs), 2 µg acetylated bovine serum albumin, 50 ng of single-stranded DNA, 0.5 ng of a 5'-[^{32}P] end-labelled primer, 1 µL of rpfs (unless otherwise indicated) and water for a final volume of 17 µL was heated to 65°C for 5 min and allowed to cool to 42°C on the benchtop. 2 µL of a solution containing 5 mM each of dATP, dCTP, dGTP and dTTP

was added followed by 3 units (1 μL) of T4 DNA polymerase (New England Biolabs). The reaction was incubated at 37°C for 30 min., nucleic acids were precipitated with ethanol and the primer extension products analyzed on sequencing gels as previously described (Wolin and Walter, 1989). In comparisons of pausing on the SRαN and SRαNP mRNA, approximately equal amounts of rpfs (determined by electrophoresis) were used in the primer extension assays. The toeprint band at pause site *b* was used to control for differences in loading or labelling of the primer extension reactions. Further details of the isolation and analysis of rpfs are presented in the Appendix to this chapter.

Preparation of rpfs from nuclease released monosomes

A 100 μL translation reaction supplemented with 10 equivalents of KRMs were terminated by adding cycloheximide to 1 mM and then mixed with 25 μL of 2.5 M KOAc, 20 mM HEPES-KOH pH 7.5, 10 mM $MgOAc_2$ and 2 mM dithiothreitol). This was layered on 75 μL of BK (500 mM KOAc, 20 mM HEPES-KOH pH 7.5, 10 mM $MgOAc_2$, and 2 mM dithiothreitol) containing 1.8 M sucrose and fractionated by centrifugation in a Beckman Instruments TLA-100 rotor at 100,000 rpm (436,000 x g) for 90 min. The top 160 μL (containing membrane bound polysomes) was removed and divided into 35 μL aliquots. An aliquot was mixed with 5 μL of BK containg 25 mM $CaCl_2$, and digested with Staphylococcal nuclease at 0.5 unit/μL for 30 min at 24°C. Digestion was terminated by adding ribonucleoside-vanadyl complex to 10 mM and 10 μL of BK containing 25 mM EGTA. The mixture was layered on a 100 μL step gradient

of BK containing 1.8 M sucrose and 5 mM EGTA and fractionated by centrifugation in a TLA-100 rotor at 100,000 rpm (436,000 x g) for 90 min. The top 120 µL (containing membrane bound monosomes) was removed and solubilized with CHAPS as above. Nuclease released monosomes in the pellet were resuspended in the remaining 30 µL of the cushion and rpfs were extracted as above. The membrane bound monosomes were then pelleted and the rpfs isolated as above.

RESULTS

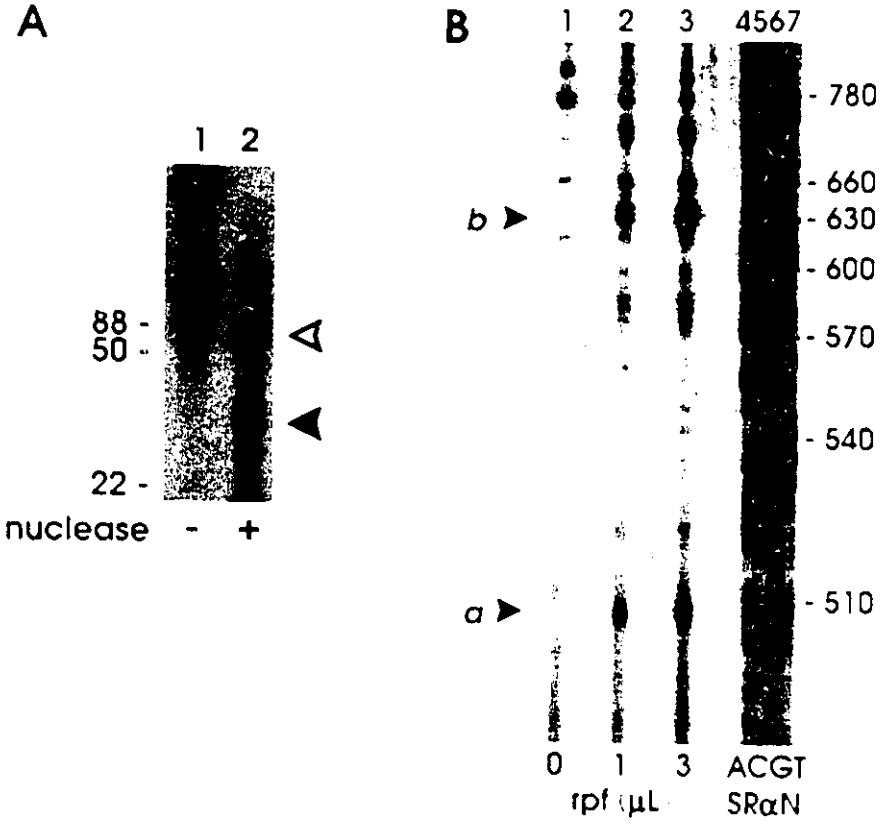
Translation pause sites within the SRα mRNA

SRαN, a Leu→Val point mutant of SRα, is translated at higher levels in the rabbit reticulocyte lysate (RRL) cell-free system than wild-type SRα, without affecting the targeting or translocation activity of the protein (Falcone and Andrews, 1991; Young et al, 1995). Nascent SRαN polypeptides that have discrete molecular weights and that can bind to ER microsomes are observed in RRL translation reactions (Young et al, 1995). To identify specific translation pause sites on the SRαN mRNA and investigate the possible relationships of these pause sites to membrane targeting, a previously developed assay (Wolin and Walter, 1988; Wolin and Walter, 1989) was used to map the distribution of ribosomes on *in vitro* transcribed mRNAs encoding SRαN. Cell-free translation reactions of α[^{35}S]-UTP labelled SRαN mRNA were terminated with the elongation inhibitor cycloheximide during steady-state synthesis. The reactions were digested with Staphylococcal nuclease to produce monosomes, and mRNA fragments protected from the nuclease by the ribosomes were isolated. These ribosome protected fragments (rpfs) were analysed by electrophoresis and as expected are approximately 30 nucleotides in length (Fig. 1 A. lane 2, solid arrowhead) (Wolin and Walter, 1989). The isolated rpfs were distinct from the undigested mRNA (Fig. 1 A. lane 1) and an unidentified species derived from the RRL reaction (Fig. 1 A. open arrowhead, and data not shown). The rpfs were then annealed onto a single stranded SRα DNA template and their positions "toeprinted" using a primer extension reaction with T4 DNA polymerase (Wolin and Walter, 1988) and

a primer within the SRα coding region (nucleotides 325 - 346).

The positions of the rpfs revealed a series of putative translation pause sites on the mRNA (Fig. 1 B. lanes 2 and 3). Two exceptionally strong and consistent pause sites were identified, site *a* around nucleotide 507 (Fig. 1 B. marked *a*, and Fig. 3 A, insert) and site *b* around nucleotide 627 (Fig. 1 B. marked *b*) using a dideoxy sequencing ladder from the same primer to provide size markers (Fig. 1 B. lanes 4 - 7). Few toeprint bands could be attributed to aberrant termination of the T4 polymerase on the single stranded template (Fig. 1 B. lane 1). As expected, when rpfs obtained from translation reactions of an unrelated mRNA (chimpanzee α-globin) were added to the SRαN single stranded DNA as a control, no toeprint bands were observed other than those produced in the absence of rpfs (data not shown). Moreover, although there is a possibility of mRNA secondary structure near pause site *a* (discussed below) the toeprint band at site *a* is not due to incompletely digested base-paired mRNA, since supplementing the Staphylococcal nuclease with the double-strand RNA specific nuclease V1 (Wolin and Walter, 1993) did not affect this toeprint band (data not shown).

Figure 1. Translation pause sites on the SRα mRNA. A. Ribosome-bound [^{35}S]-labelled mRNA was isolated from RRL translation reactions of SRαN either before (lane 1) or after (lane 2) nuclease digestion. Ribosome protected fragments (rpfs) are marked with a solid arrowhead, and an unidentified non-RNA species derived from the RRL reaction is marked with an open arrowhead. The lengths in nucleotides of RNA molecular weight standards are marked on the left. B. Either 0 μL (lane 1), 1 μL (lane 2) or 3 μL (lane 3) of rpfs were annealed to a single-stranded DNA template of SRαN and mapped by primer extension with T4 polymerase using a primer within the coding region of SRα. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 4-7). The two strong pause sites identified are marked *a* and *b*.



An mRNA pause sequence resembling a frameshift site

Ribosomes paused at site *a* (nucleotide 507) will have synthesized roughly 170 amino acids of SRα. This polypeptide chain likely corresponds to a 23 kD translation intermediate previously estimated to contain about 180 amino acid residues (Young et al, 1995). The 23 kD translation intermediate is the minimum length of polypeptide required to bind to microsomes in a urea resistant manner while still attached to ribosomes treated with cycloheximide (Young et al, 1995). Pause site *a* therefore appears to be closely correlated with the membrane anchoring of nascent SRα, and the predicted sequence of the surrounding mRNA (Lauffer et al, 1985) was examined for structures that may cause the translation pause.

The primer extension band at pause site *a* lies within a series of three lysine codons with the sequence AAA-AAA-AAG, starting at nucleotide 502. A potential stem-loop structure is located immediately 3' on the mRNA of the lysine codons (Fig. 2). Interestingly, this mRNA sequence closely resembles a class of ribosomal frameshift structures. These structures, typified by the human T-cell leukemia virus (HTLV) type I *gag/pro* -1 ribosomal frameshift and conserved in the HTLV type II, bovine leukemia virus and equine infectious anemia virus frameshifting mRNAs, consist of a heptamer sequence of A-AAA-AAC followed by a stem-loop structure of varying size and distance from the heptamer (fig. 2) (reviewed in Hatfield et al, 1992). Both the heptamer sequence and the stem-loop structure have been shown to be necessary and sufficient for frameshifting on the HTLV type II mRNA *in vitro* (Falk et al, 1993). A similar group of -1 frameshift structures is found in the *E. coli* mRNA coding for the *dnaX*, IS150 and

IS911 proteins and consists of the heptamer sequence A-AAA-AAG followed by a stem-loop (Blinkowa and Walker, 1990; Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Polard et al, 1991; Vogele et al, 1991).

The thermodynamic stability of the predicted RNA stem-loop at the SRα pause site is calculated to have a ΔG of -10.3 kcal (Freier et al, 1986), comparable to the stability of the viral frameshift structures (between -19.0 and -7.9 kcal) (Hatfield et al, 1992). However, unlike the viral frameshift structures the SRα heptamer-like sequence has a G in the final position and as predicted (Brierley and Jenner, 1992; Chamorro et al, 1992; Garcia et al, 1993), frameshifted premature termination products are not prominently observed in optimized RRL translation reactions of SRαN (data not shown). The *gag/pro* -1 frameshift structure of the mouse mammary tumour virus containing an A-AAA-AAC heptamer has been shown to require a pseudoknot for efficient frameshifting (Chen et al, 1995) and there is no predicted pseudoknot structure at the SRα pause site.

To determine if the signal for translation pausing at site *a* resides on the SRα mRNA and not on the encoded polypeptide, point mutations were introduced into the plasmid encoding SRαN that do not change the encoded amino acid sequence. In the *in vitro* transcribed mutant mRNA (referred to as encoding SRαNP), the frameshift-like structure is disrupted by changing the heptamer-like sequence from AAA-AAA-AAG to AAG-AAG-AAA, and the putative stem-loop was interrupted with G→C and C→G substitutions. (Fig. 2). The remainder of the coding region and both the 5' and 3' untranslated regions are identical to that of SRαN. Rpfs were isolated from RRL

translation reactions of SRαN and SRαNP in parallel and mapped by primer extension to assay the level of translation pausing at the mutated site relative to the wild-type sequence.

Rpfs isolated from translation reactions of SRαN produced toeprint bands at the two strong pause sites as expected (Fig. 3 A. lane 1, marked *a* and *b*). However, rpfs from SRαNP translation reactions revealed a considerably fainter toeprint band near the mutated pause site compared to the SRαN toeprint band at site *a*, while the toeprint band at pause site *b* remained prominent on the SRαNP mRNA (Fig. 3 A. compare lane 3 to lane 1). The toeprint band near the mutated site of SRαNP was close in intensity to the surrounding background pause sites, and moreover was shifted by about 3 nucleotides 3' of the original position (Fig. 3 A. lane 3, and inserts). A dideoxy sequencing ladder of SRαNP is shown (Fig. 3 A. lanes 4 - 7) as size markers. The changes in intensity and position of the primer extension band suggest that the mutations in SRαNP have abolished the strong translation pause on the mRNA. Densitometry of the autoradiograms from three different experiments revealed that the toeprint band near the mutated site was reduced to below 35% of the original intensity at site *a* in the SRαN mRNA. As expected, few primer extension bands were due to the SRαNP single-stranded DNA (Fig. 3 A. lane 2). Therefore, these results suggest that the specific sequence of the SRα mRNA at this site, possibly including the frameshift-like structure, is required for the specific strong translation pause.

To determine if the isolated SRα sequence sequence is sufficient for translation pausing, this sequence was inserted into the mRNA encoding a fusion protein containing

a fragment of chimpanzee α-globin and the Fc-binding region of *S. aureus* protein A (gPA) (Janiak et al, 1994). The plasmid vector was constructed to code for the globin fragment, the short segment of SRα and the protein A domain in one continuous open reading frame (gSRpa-1). The sequence derived from SRα (nucleotides 478 - 530) included the mRNA heptamer-like sequence and putative stem-loop of pause site *a*. When rpfs were isolated from RRL translations of this construct and mapped as above, a strong toeprint band was observed at about nucleotide 120 of the fusion protein coding region (Fig. 3 B. lane 2, marked a). This pause site is within the section of mRNA derived from SRα. The primer extension band is close to the position in the sequence corresponding to SRα pause site *a*, although shifted 3' by about 2 nucleotides (Fig. 3 B. insert). Therefore, the site *a* mRNA sequence containing a frameshift-like structure is both necessary and sufficient for a strong translation pause in RRL translation reactions. Experiments to determine the exact sequence requirements for translation pausing at site *a* are currently underway.

Figure 2. The mRNA at pause site *a* resembles a frameshift sequence. The predicted secondary structure of the SRα mRNA at ribosome pause site *a* is compared to the mutated pause site of the SRαNP mRNA and a frameshift signal in the HTLV *gag/pro* sequence (adapted from Falk et al, 1993). The position of the pause site *a* toeprint band is marked with an arrowhead, the frameshift heptamer and the heptamer-like sequences are underlined, and mutated residues on the SRαNP mRNA are marked with dots.

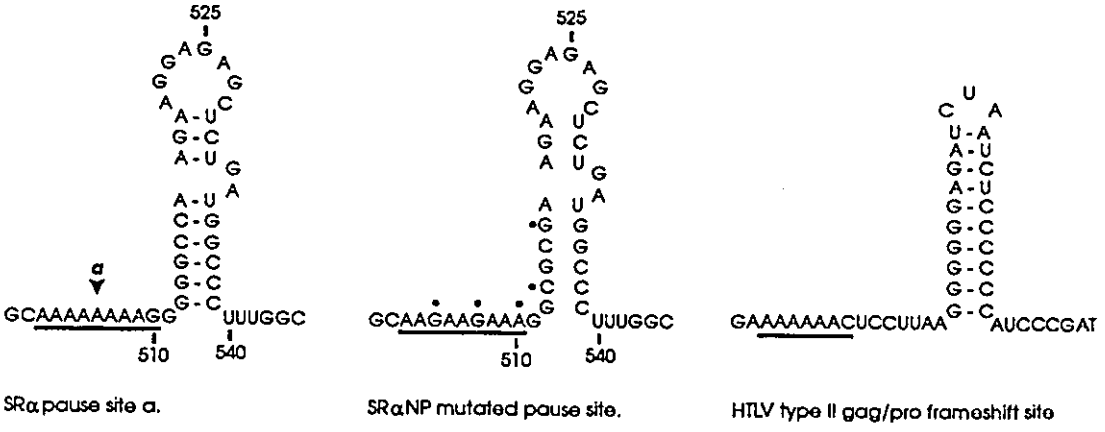
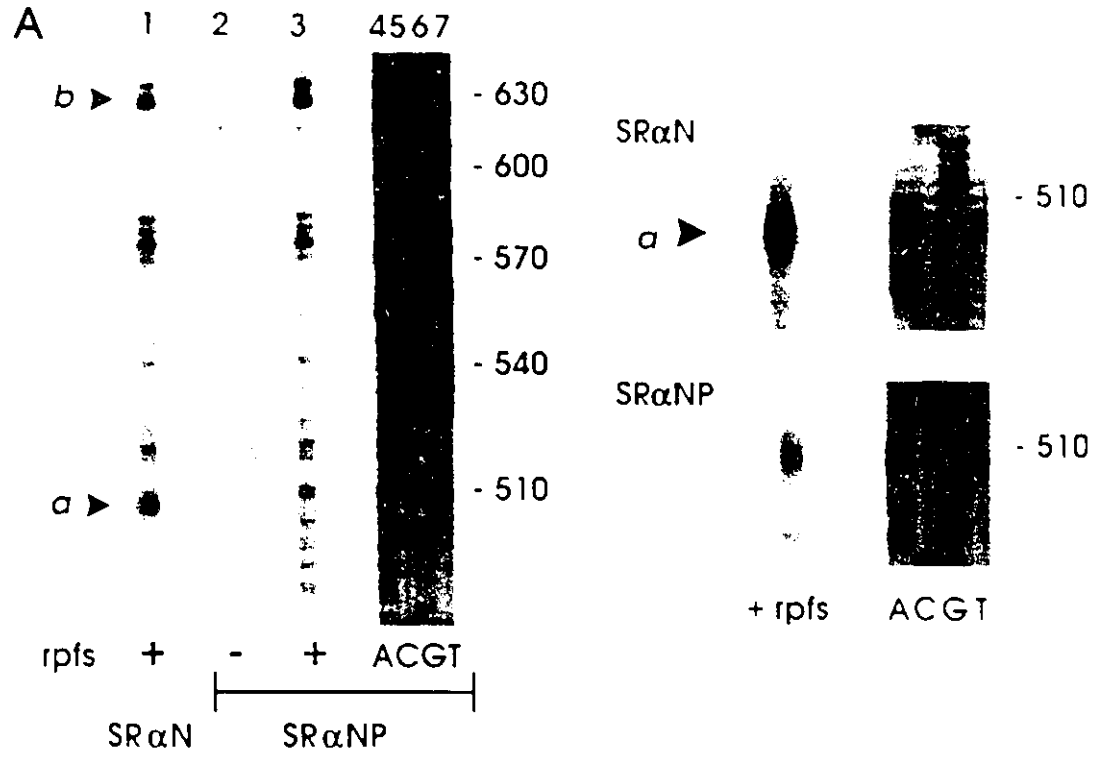


Figure 3. An mRNA sequence necessary and sufficient for translation pausing. A. Rpfs were isolated from RRL translation reactions of SRαN and the pause site mutant SRαNP. Primer extension assays were performed in the presence of rpfs from SRαN reactions (lane 1), or in the absence (lane 2) or presence (lane 3) of rpfs from SRαNP reactions. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 4-7). Pause sites *a* and *b* of SRα are marked. Inserts on right, magnification of primer extension assays at pause site *a* for SRαN (from Fig. 1 B) and SRαNP (from left panel). B. Rpfs were isolated from RRL translation reactions of the fusion polypeptide gSRpa-1 containing the sequence of SRα at pause site *a* between protein sequences derived from chimpanzee α-globin and *S. aureus* protein A. Primer extension assays were performed in the absence (lane 1) or presence (lane 2) of these rpfs. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 3-6). A pause site corresponding to SRα pause site *a* is marked. Insert on right, magnification of primer extension assay at the SRα sequence of gSRpa-1 (from left panel). Nucleotide 120 of the gSRpa-1 sequence, the first G residue following 8 A residues (reading upwards), corresponds to nucleotide 510 of the SRα sequence.

Figure 3. A.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2
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b
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- 630
- 600
- 570
- 540
- 510
rpfs
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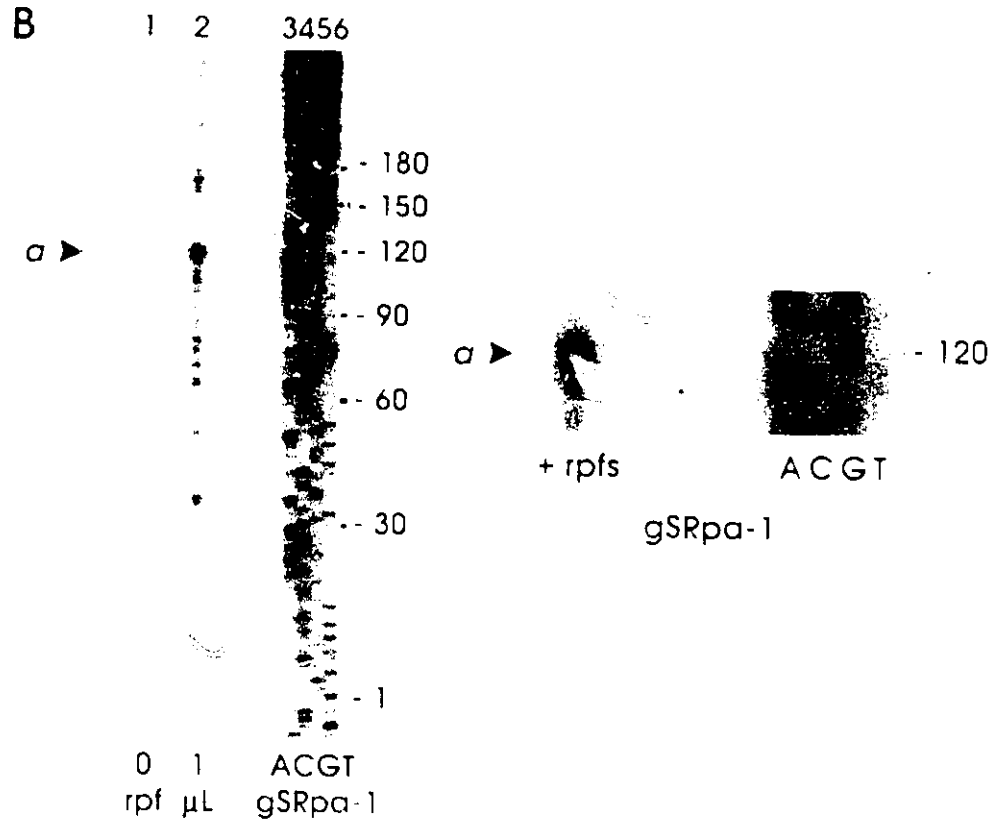
+ rpfs
ACGT

Figure 3. B.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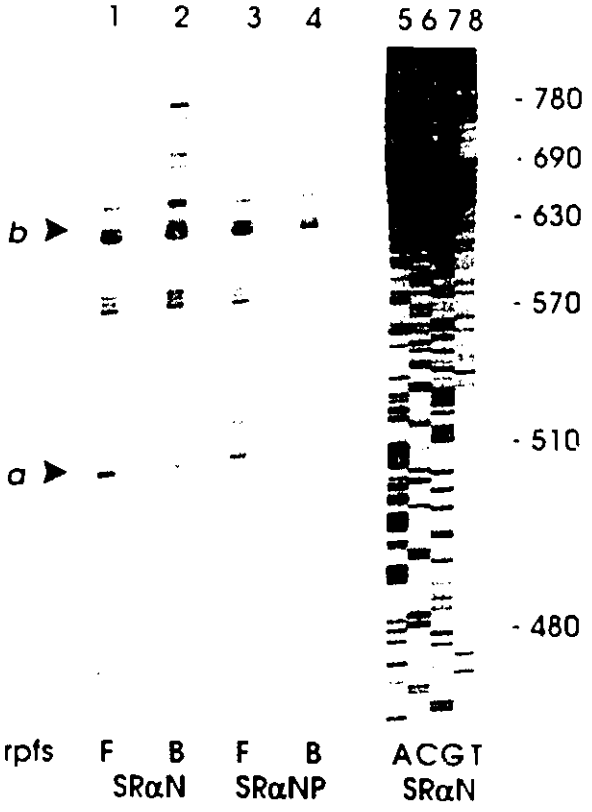
Translation pausing facilitates co-translational membrane binding of SRα

Translation pausing at site *a* on the SRα mRNA is closely correlated with membrane anchoring of nascent SRα (Young et al, 1995). By isolating cytoplasmic and membrane-bound ribosomes from RRL translation reactions of SRαN supplemented with ER microsomes (Wolin and Walter, 1993), we used the pause sites at nucleotides 507 and 627 as markers to confirm that nascent SRα polypeptides can be targeted to the ER membrane during translation. As expected, the pause sites observed were similar to those in Fig. 1 B including the two strong toeprints noted above (Fig. 4 lanes 1 - 2, marked *a* and *b*). Furthermore, toeprint bands 5' of pause site *a* were mostly produced by rpfs from free ribosomes (Fig. 4 lane 1), whereas toeprint bands 3' of pause site *b* were primarily due to rpfs from bound ribosomes (Fig. 4 lane 2). Minor toeprint bands representing rpfs that mapped to the region between the two strong pause sites were distributed between the free and bound ribosome fractions (Fig. 4 lanes 1 and 2). These results indicate that ribosomes translating the SRα polypeptide become anchored to the membrane during the translation of the region of the mRNA containing the two strong ribosome pause sites. This more precisely locates ribosomes that have synthesized the minimum amino terminal sequence necessary for assembly on the membrane and is in general agreement with earlier evidence (Young et al, 1995).

To determine whether pausing at site *a* contributes to the co-translation targeting of SRα, we repeated this experiment using translation reactions of the non-pausing SRαNP mRNA supplemented with microsomes. As predicted, the pause sites observed matched those in Fig. 3 A, including a prominent toeprint band at site *b* and a fainter

band near the mutated sequence of site *a* (Fig. 4 lanes 3 - 4). However, rpfs isolated from membrane bound ribosomes on the SRαNP mRNA produced significant toeprint bands only 3' of pause site *b* (Fig. 4 lane 4) compared to 3' of pause site *a* on the SRαN mRNA (Fig. 4 lane 2). Also, rpfs from cytoplasmic ribosomes on the SRαNP mRNA produced a considerably stronger toeprint band at pause site *b* compared to rpfs from membrane bound ribosomes (Fig. 4 compare lanes 3 and 4). In contrast, free and membrane bound ribosomes on the SRαN mRNA produced roughly equal bands at pause site *b* (Fig. 4 compare lanes 1 and 2). Therefore, ribosomes translating the non-pausing SRαNP mRNA bind to membranes after they are 3' of pause site *b* while ribosomes translating SRαN mRNA bind to membranes 3' of pause site *a*. This suggests that a significantly longer nascent polypeptide is required to bind ribosomes translating SRαNP mRNA onto the membrane than for ribosomes translating SRαN mRNA. Therefore, nascent polypeptides synthesized from the non-pausing SRαNP mRNA are temporally impaired in co-translational membrane assembly. Since the polypeptide sequence encoded by the SRαNP and SRαN mRNAs is identical, these results suggest that the translation pause at site *a* directly contributes to co-translational membrane binding of SRα.

Figure 4. Pausing contributes to targeting of SRα nascent chains. Rpfs from free cytoplasmic and membrane bound ribosomes were isolated from RRL translation reactions of SRαN (lanes 1 and 2) and SRαNP (lanes 3 and 4) supplemented with CRMs. Primer extension asays were performed in the presence of rpfs from the free (lanes 1 and 3, marked F) and membrane bound (lanes 2 and 4, marked B) ribosome fractions. A dideoxy sequencing ladder of SRαN using the same primer was used as size markers (lanes 5-8). Pause sites *a* and *b* on the SRαN mRNA are marked.



Membrane binding of polysomes translating SRα

Polysomes bound to the membrane by nascent SRα polypeptides should contain ribosomes at various stages of translation. As shown in Fig. 4, many ribosomes 5' of the pause sites are not directly associated with the membrane. Therefore, it was predicted that some of these ribosomes would be tethered to the ER membrane via the mRNA attached to membrane bound ribosomes at later stages of translation. However, the relatively small population of such tethered ribosomes is difficult to detect in unfractionated translation reactions. To identify these ribosomes, RRL translation reactions of SRαN supplemented with microsomes were terminated at steady state with cycloheximide and the membranes were isolated. Membrane bound polysomes were then digested with nuclease, fractionated by centrifugation as above, and the rpfs from both membrane bound and nuclease released monosomes were isolated and mapped by primer extension.

Toeprint bands in the region bounded by the two strong pause sites (Fig. 5 A. marked *a* and *b*) were examined. Consistent with the prediction that some ribosomes are tethered to the membrane by the mRNA, a number of toeprint bands were produced by rpfs from nuclease released ribosomes (Fig. 5 A. lane 1). Moreover, toeprint bands produced by rpfs from membrane bound ribosomes increased in intensity 3' of pause site *a* (Fig. 5 A. lane 2). At pause site *b*, the primer extension bands revealed roughly equal populations of nuclease released and membrane bound ribosomes, and 3' of pause site *b* comparatively more membrane bound ribosomes were detected (Fig. 5 A. compare lane 1 and 2). Thus, as expected, the distribution of paused ribosomes is similar to that

observed in Fig. 4. Furthermore, the selective nuclease release of ribosomes 5' of the strong pause sites suggested that entire polysomes are bound to membranes by targeted nascent SRα polypeptides.

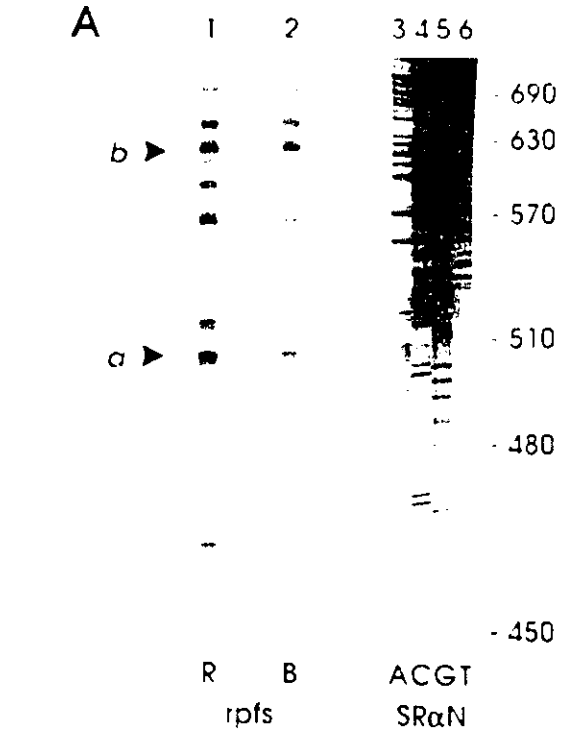
To investigate this possibility, polysomes translating SRαN were assayed for binding onto microsomal membranes. If polysomes are bound to the membrane primarily by the nascent polypeptides, then the impaired membrane binding of SRαNP polypeptides should result in reduced membrane binding of polysomes translating the non-pausing SRαNP mRNA. In addition, preventing new synthesis of SRαN with the initiation inhibitors 7-methylguanosine-5'-phosphate (7mG) and aurintricarboxylic acid (ATA) should prevent membrane binding of polysomes. Cytoplasmic and membrane bound polysomes were isolated from RRL translation reactions of SRαN or SRαNP terminated at steady state with cycloheximide or incubated prior to initiation with a combination of 7mG and ATA. To reveal the location of the ribosomes, the polysomal α[^{35}S]-UTP labelled mRNA was isolated and analyzed by electrophoresis.

As expected, after adding cycloheximide a large proportion, 56%, of the SRαN polysomal mRNA was recovered from the membrane bound polysome fraction (Fig. 5 B). Also as predicted, the proportion of membrane bound non-pausing SRαNP mRNA in cycloheximide-terminated translation reactions, 28%, was considerably lower (Fig. 5 B). Furthermore, the proportion of SRαN mRNA in the membrane bound fraction was reduced to 31% when 7mG and ATA were used to prevent translation initiation (Fig. 5 B). In control assays without membranes, 21% of the SRαN mRNA was recovered after the free cytoplasmic fraction was removed (Fig. 5 B), and may result from

uncharacterized interactions with components of the translation system. In additional control asays using microsome-supplemented translation reactions of chimpanzee α-globin, a protein not expected to interact with membranes 24% of the mRNA was recovered in the membrane bound fraction (Fig. 5 B). Therefore, this amount of mRNA represents the membrane-independent background in this assay. These results confirm that the association of the nascent SRα polypeptides with the ribosomes and with the ER membrane is largely responsible for the membrane binding of the polysomes. In addition, the delay observed in membrane binding of ribosomes translating SRαNP mRNA is reflected in the total membrane binding of the polysomes. Taken together with the results of Fig. 5 A, this suggests that the SRα mRNA is targeted by the membrane binding of the nascent polypeptides synthesized on ribosomes rather than by a specific mRNA targeting mechanism.

Figure 5. Anchoring of polysomes via nascent SRα chains. A. Membrane bound polysomes were digested with nuclease, and rpfs were isolated from monosomes released from membranes by the nuclease and from membrane bound monosomes. Primer extension assays were performed in the presence of rpfs from the nuclease released (lane 1, marked R) and membrane bound (lane 2, marked B) monosome fractions. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 3-6). Pause sites *a* and *b* are marked. B. α[^{35}S]-UTP labelled mRNA from free cytoplasmic and membrane bound polysomes was isolated from cycloheximide (marked chx) terminated RRL translation reactions of SRαN, SRαNP or globin supplemented with CRMs (marked mbs). One set of translation reactions of SRαN were incubated with 7mG and ATA, and another set of translation reactions were performed in the absence of microsomes. Isolated mRNA was analysed by electrophoresis and quantified using a PhosphorImager. The percentage of membrane bound mRNA was calculated using the sum of free and bound populations as the total.

Figure 5.


A
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a
R
B
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ACGT
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PERCENT mRNA BOUND
60
40
20
0
mbs
chx
7mG/ATA
SRαN
SRαNP
SRαN
globin

DISCUSSION

The model of SRα targeting suggested by our data is depicted in Fig. 6. As the amino terminal anchoring domain of SRα emerges from the ribosome, translation is paused at site *a* on the mRNA (Fig. 1 B). At this stage, membrane targeting of SRα is initiated (Fig. 4), most likely by folding of the nascent polypeptide into a conformation capable of assembling onto the SRβ subunit on the ER membrane (Young et al, 1995). While our data do not rule out interactions between the nascent SRα polypeptide and other membrane proteins, our previous data suggests that the interaction between SRα and SRβ is sufficient for membrane binding of SRα (Young et al, 1995). As synthesis of the SRα polypeptide continues beyond the pause site, the nascent chains remain membrane bound and thereby attach the polysome to the membrane surface (Fig. 5 A and B). The novel aspect of this model is the connection between the translation pause and the membrane assembly event, suggested by the impaired membrane binding of the nascent polypeptide (Fig. 4) and of polysomes (Fig. 5 B) when the level of translation pausing was reduced (using SRαNP mRNA). Previously, ribosome pausing has been detected in connection with other regulatory events during translation, including initiation, termination, recognition of signal sequences by SRP, and frameshifting (Wolin and Walter, 1988; Somogyi et al, 1993). Our data suggests a functional role for the regulation of protein synthesis by translation pausing, in folding and/or targeting of nascent proteins.

The resemblance between pause site *a* and a class of frameshift sites suggest that the mechanism of frameshifting on the viral mRNAs is related to or even adapted from

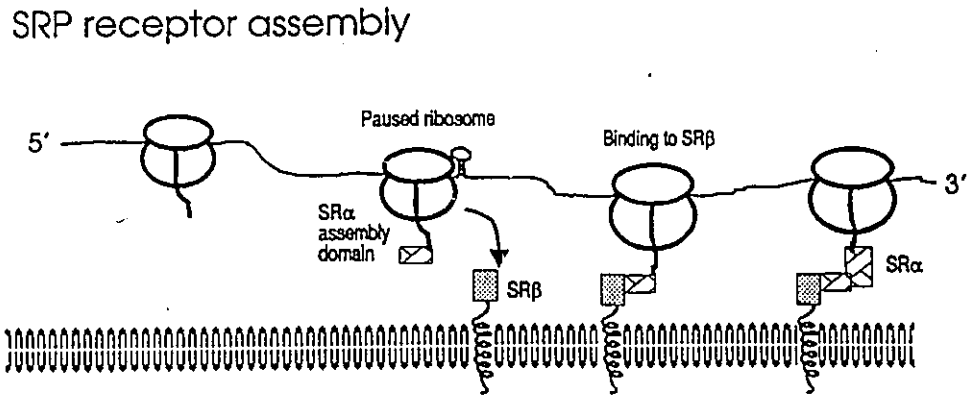
a general mechanism for translation pausing. However, since the primer extension toeprint band lies within the heptamer-like sequence on the SRα mRNA (Fig. 1 B and Fig. 3 A, insert), ribosomes paused at this site appear to have already passed over at least part of the heptamer-like sequence. On the other hand, the presence of an mRNA stem-loop structure alone has been shown not to affect the rate of polypeptide elongation (Lingelbach and Dobberstein, 1988). Therefore, the exact mechanism of pausing at this site remains to be determined.

How does ribosome pausing facilitate the co-translational targeting of SRα? Evidence has been published that a nascent polypeptide can acquire secondary and even tertiary structure as it emerges from the ribosome (Crombie et al, 1992; Krashenninikov et al, 1991), in some cases with the aid of heat shock proteins (Frydman et al, 1994; reviewed in Gething and Sambrook, 1992, and Hartl et al, 1994). Since membrane assembly of SRα appears to require an independently folded domain (Young et al 1995), the cotranslational targeting of the molecule demonstrated here indicates that the membrane anchoring domain folds soon after synthesis. Therefore, the delayed co-translational targeting of non-pausing SRαNP polypeptides may be due to a reduction in the efficiency of protein folding. For example, in the absence of a translation pause at site *a* on the SRα mRNA, polypeptide sequences carboxyl terminal to the anchoring domain may interfere with folding. However, misfolded polypeptides synthesized from the non-pausing mRNA would probably be folded into the proper final conformation by chaperone proteins over time and would then assemble onto membranes later in translation as observed in Fig. 4, or post-translationally. We therefore hypothesize that

translation pausing facilitates or complements the action of protein chaperones in the co-translational folding of SRα and perhaps of other proteins. Similarly, SRP-mediated translation pausing increases the efficiency of, but is not essential for the transition from free to membrane bound ribosomes (Siegel and Walter, 1986).

Although ribosome pausing may contribute in general to polypeptide folding, we predict a more specific role for pausing in co-translational targeting events. In addition to secretory proteins, certain cytoskeletal proteins have been reported to target cotranslationally. Myosin heavy chain, titin and in certain cases vimentin can be assembled onto the appropriate cytoskeletal networks during translation *in vivo* (Isaacs and Fulton, 1987; Isaacs et al, 1989a and 1989b). The appearance of nascent polypeptides of discrete lengths during synthesis of these cytoskeletal proteins (Isaacs and Fulton, 1987; Isaacs et al, 1989a) suggests that translation pausing may be occuring at specific sites on the mRNAs encoding these polypeptides. The coordination between ribosome pausing and cotranslational assembly we have demonstrated for SRα may also contribute to the targeting of one or more of these proteins. Indeed, translation pausing may be a general mechanism for regulating the folding and/or targeting of a variety of nascent polypeptides. This hypothesis is discussed further in Chapter IV of this thesis.

Figure 6. Model of SRα membrane assembly. A ribosome synthesizing SRα reaches a translation pause site on the mRNA. At this point, the amino terminal membrane anchoring domain of SRα has been synthesized and has emerged from the ribosome. The anchoring domain is targeted to the ER membrane where it assembles onto SRβ. Other proteins may participate in the assembly reaction but are omitted for clarity. Translation of the remainder of the SRα polypeptide resumes. Some ribosomes translating sequences 5' of the pause site are tethered to the membrane by the mRNA.



REFERENCES

Andrews, D.W., Lauffer, L., Walter, P. and Lingappa, V.R. (1989) Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. *J. Cell Biol.* **108**, 797-810

Blinkowa, A.L. and Walker, J.R. (1990) Programmed ribosomal frameshifting generated the *Escherichia coli* DNA polymerase III γ subunit from within the τ subunit reading frame. *Nucl. Acids Res.* **18**, 1725-1729

Brierley, I. and Jenner, A.J. (1992) Mutational analysis of the "slippery-sequence" component of a coronavirus ribosomal frameshifting signal. *J. Mol. Biol.* **227**, 463-479

Chamorro, M., Parkin, N. and Varmus, H.E. (1992) An RNA pseudoknot and an optimal heptameric shift site are required for highly efficient ribosomal frameshifting on a retroviral messenger RNA. *Proc. Natl. Acad. Sci. USA*. **89**, 713-717

Chen, X., Chamorro, M., Lee, S.I., Shen, L.X., Hines, J.V., Tinoco, I.Jr. and Varmus, H.E. (1995) Structural and functional studies of retroviral RNA pseudoknots involved in ribosomal frameshifting: nucleotides at the junction of the two stems are important for efficient ribosomal frameshifting. *EMBO J.* **14**, 842-852

Crombie, T., Swaffield, J.C. and Brown, A.J.P. (1992). Protein folding within the cell is influenced by controlled rates of polypeptide elongation. *J. Mol. Biol.* **228**, 7-12

Doohan, J.P. and Samuel, C.E. (1992) Biosynthesis of reovirus-specific polypeptides: ribosome pausing during the translation of reovirus S1 mRNA. *Virology* **186**, 409-425

Falcone, D. and Andrews, D.W. (1991) Both the 5' untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation *in vitro*. *Mol. Cell Biol.* **11**, 2656-2664

Falk, H., Mador, N., Udi, R., Panet, A. and Honigman, A. (1993) Two *cis*-acting signals control ribosomal frameshift between human T-cell leukemia virus type II *gag* and *pro* genes. *J. Virol.* **67**, 6273-6277

Flower, A.M. and McHenry, C.S. (1990) The γ subunit of DNA polymerase III holoenzyme of *Escherichia coli* is produced by ribosomal frameshifting. *Proc. Natl. Acad. Sci. USA* **87**, 3713-3717

Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA* **83**, 9373-9377

Frydman, J., Nimmesgern, E., Ohtsuke, K. and Hartl, F.U. (1994) Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* **370**, 111-117

Garcia, A., van Duin, J. and Pleij, C.W.A. (1993) Differential response to frameshift signals in eukaryotic and prokaryotic translational systems. *Nucl. Acids Res.* **21**, 401-406

Gething, M.-J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33-45

Gilmore, R., Walter, P. and Blobel, G. (1982a) Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. *J. Cell Biol.* **95**, 463-469

Gilmore, R., Blobel, G and Walter, P. (1982b) Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* **95**, 470-477

Gurevich, V.V., Pokrovskaya, I.D., Obukhova, T.A. and Zozulya, S.A. (1991) Preparative *in vitro* mRNA synthesis using SP6 and T7 RNA polymerases. *Anal. Biochem.* **195**, 207-213

Hartl, F.-U., Hlodan, R. and Langer, T. (1994) Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.* **19**, 20-25

Hatfield, D.L., Levin, J.G., Rein, A. and Oroszlan, S. (1992) Translational suppression in retroviral gene expression. *Adv. Vir. Res.* **41**, 193-239

Hortsch, M., Avossa, D. and Meyer, D.I. (1985) A structural and functional analysis of the docking protein. *J. Biol. Chem.* **260**, 9137-9145

Isaacs, W.B. and Fulton, A.B. (1987) Cotranslational assembly of myosin heavy chain in developing cultured skeletal muscle. *Proc. Natl. Acad. Sci. USA* **84**, 6174-6178

Isaacs, W.B., Cook, R.K., Van Atta, J.C., Redmond, C.M. and Fulton, A.B. (1989a) Assembly of vimentin in cultured cells varies with cell type. *J. Biol. Chem.* **264**, 17953-17960

Isaacs, W.B., Kim, I.S., Struve, A. and Fulton, A.B. (1989b) Biosynthesis of titin in cultured skeletal muscle cells. *J. Cell Biol.* **109**, 2189-2195

Jackson, R.J. and Hunt, T. (1983) Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Meth. Enz.* **96**, 50-74

Janiak, F., Glover, J.R., Leber, B., Rachubinski, R.A. and Andrews, D.W. (1994) Targeting of passenger protein domains to multiple intracellular membranes. *Biochem. J.* **300**, 191-199

Kim, J., Gamble Klein, P. and Mullet, J.E. (1991) Ribosomes pause at specific sites during synthesis of membrane-bound chloroplast reaction center protein D1. *J. Biol. Chem.* **266**, 14931-14938

Kim, J.-K. and Hollingsworth, M.J. (1992) Localization of in vivo ribosome pause sites. *Anal. Biochem.* **206**, 183-188

Krashenninikov, I.A., Komar, A.A. and Adzhubei, I.A. (1991) Nonuniform size distribution of nascent globin peptides, evidence for pause localization sites, and a cotranslational protein-folding model. *J. Protein Chem.* **10**, 445-453

Lauffer, L., Garcia, P.D., Harkins, R.N., Coussens, L., Ullrich, A. and Walter, P. (1985) Topology of signal recognition particle receptor in endoplasmic reticulum membrane. *Nature* **318**, 334-338

Lingelbach, K. and Dobberstein, B. (1988) An extended RNA/RNA duplex structure within the coding region of mRNA does not block translational elongation. *Nucl. Acids Res.* **16**, 3405-3414

Miller, J.D., Tajima, S., Lauffer, L. and Walter, P. (1995) The ß subunit of the signal recognition particle receptor is a transmembrane GTPase that anchors the α subunit, a peripheral membrane GTPase, to the endoplasmic reticulum membrane. *J. Cell Biol.* **128**, 273-282

Myers, R.M., Lerman, L.S. and Maniatis, T. (1985) A general method for saturation mutagenesis of cloned DNA fragments. *Science* **229**, 242-247

Perara, E., Rothman, R.E. and Lingappa, V.R. (1986) Uncoupling translocation from translation: implications for transport of proteins across membranes. *Science* **232**, 348-352

Polard, P., Prere, M.F., Chandler, M. and Fayet, O. (1991) Programmed translation frameshifting and initiation at an AUU codon in gene expression of bacterial insertion sequence IS*911*. *J. Mol. Biol.* **222**, 465-477

Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press.

Siegel, V. and Walter, P. (1986) Removal of the *Alu* structural domain from signal recognition particle leaves its protein translocation activity intact. *Nature* **320**, 81-85

Somogyi, P., Jenner, A.J., Brierley, I. and Inglis, S.C. (1993) Ribosome pausing during translation of an RNA pseudoknot. *Mol. Cell Biol.* **13**, 6931-6940

Tajima, S., Lauffer, L., Rath, V.L and Walter, P. (1986) The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. *J. Cell Biol.* **103**, 1167-1178

Tsuchihashi, Z. and Kornberg, A. (1990) Translational frameshifting generates the γ subunit of DNA polymerase III holoenzyme. *Proc. Natl. Acad. Sci. USA* **87**, 2516-2520

Verner, K. and Schatz, G. (1988) Protein translocation across membranes. *Science* **241**, 1307-1313

Vogele, K., Schwartz, E., Welz, C., Schiltz, E. and Rak, B. (1991) High-level ribosomal frameshifting directs the synthesis of IS*150* gene products. *Nucl. Acids Res.* **19**, 4377-4385

Walter, P. and Blobel, G. (1983) Preparation of microsomal membranes for cotranslational protein translocation. *Meth. Enz.* **96**, 84-93

Wilhelm, J.E. and Vale, R.D. (1993) RNA on the move: the mRNA localization pathway. *J. Cell Biol.* **123**, 269-274

Wolin, S.L. and Walter, P. (1988) Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J.* 7, 3559-3569

Wolin, S.L. and Walter, P. (1989) Signal recognition particle mediates a transient elongation arrest of preprolactin in reticulocyte lysate. *J. Cell Biol.* **109**, 2617-2622

Wolin, S.L. and Walter, P. (1993) Discrete nascent chain lengths are required for the insertion of presecretory proteins into microsomal membranes. *J. Cell Biol.* **121**, 1211-1219

Young J.C., Ursini, J., Miller, J.D., Walter, P. and Andrews, D.W. (1995) An amino-terminal domain containing hydrophobic and hydrophilic sequences binds the signal recognition particle receptor α subunit to the β subunit on the endoplamsic reticulum membrane. *J. Biol. Chem.* **270**, 15650-15657

APPENDIX

Translation Pause Assay

Isolation of ribosome protected mRNA fragments (rpfs)

A 25 μL RRL translation reaction of SRαN or SRαNP was incubated at 24°C for 25 min, then terminated on ice by adding 1.25 μL of 20 mM cycloheximide. 15 μL of CBnuc (100 mM KOAc, 10 mM Tris-Cl pH 7.5, 8 mM $CaCl_2$ and 6 mM $MgCl_2$) was added to the reaction, followed by 2 μL of 15 units/μL Staphylococcal nuclease. The reaction was incubated 3 min at 0°C and then 30 min at 24°C. Nuclease digestion was terminated by adding 2 μL of 0.1 M EGTA and 2 μL of 0.2 M ribonucleoside-vanadyl complex (New England Biolabs) on ice. The reaction was mixed with 60 μL of BT (150 mM KOAc, 20 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA and 2 mM dithiothreitol), and layered over 60 μL of BT containing 0.25 M sucrose in a 200 μL pollyallomer Beckman Instruments Airfuge tube. The reaction was centrifuged at 30 psi (180,000 x g) for 30 min at 4°C to pellet ribosomes. The top 120 μL of the supernatant was removed and discarded. The pellet and remaining 40 μL was mixed with 100 μL of BPD (50 mM NaCl, 50 mM Tris-Cl pH 7.5, 5 mM EDTA and 0.5% SDS) prewarmed to 37°C, and the pellet was resuspended. 7 μL of 4 mg/mL Proteinase K was added, and the reaction was incubated for 30 min at 37°C. The reaction was transferred to a microfuge tube and extracted with 140 μL of phenol buffered with TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). The aqueous supernatant was transferred to a fresh microfuge

tube and rpfs were ethanol precipitated after adding 1 μL of 10 mg/mL yeast tRNA as a carrier. The pelleted rpfs were resuspended in 10 μL of sterile water or TE. This method was adapted from Wolin and Walter, 1989.

Electrophoresis of rpfs

Radiolabelled mRNA was produced by supplementing 10 μL SP6 RNA polymerase transcription reactions (Gurevich et al, 1991) with 1 μL of α[^{35}S]-UTP (12.5 mCi/mL, ~10 μM UTP; DuPont-NEN). This mRNA was used in RRL translation reactions and rpfs were isolated from the reactions as above. After isolation, 1 μL of the rpfs was added to 9 μL of deionized formamide containing 0.01% xylene cyanol and 0.01% bromophenol blue, and the mixture heated to 72°C for 2 min before electrophoresis. A 10% acrylamide gel containing 8 M urea and TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) was prepared in a BioRad Mini Protein gel apparatus and pre-run at 25 W constant power for 5 min using TBE as the electrode buffers. The samples were loaded and electrophoresis continued at 25 W constant power until the bromophenol blue dye migrated about 2/3 the length of the gel. The gel was fixed in 35% methanol and 10% acetic acid, dried and visualized by autoradiography. To provide RNA size markers, plasmid pMAC369 was digested with SacI, NcoI and AvaII and end repaired with the Klenow fragment of DNA polymerase to produce DNA templates truncated 23, 50 and 110 nucleotides respectively from the SP6 RNA polymerase promoter. The truncated templates were used in transcription reactions containing α[^{35}S]-UTP as above, and 0.5 μL of each transcript was mixed with deionized formamide and loaded on the gel

alongside the rpf sample.

Primer extension mapping of rpfs

Single-stranded template DNA was purified using standard procedures (Sambrook et al, 1989) from SURE™ *E. coli* cells grown in the presence of R408 M13 helper phage. A synthetic oligonucleotide primer corresponding either to nucleotides 325-346 of SRα or to the SP6 RNA polymerase promoter was 5'-[^{32}P] end-labelled with T4 polynucleotide kinase (New England Biolabs) in a reaction with γ[^{32}P]-ATP (10 mCi/mL; DuPont-NEN). A mixture containing 2 μL of 10X T4 polymerase buffer (New England Biolabs), 2 μg acetylated bove serum albumin, 50 ng of single-stranded DNA, 0.5 ng of 5'-[^{32}P] end-labelled primer, 1 μL of rpfs and water for a final volume of 17 μL was heated to 65°C for 5 min and allowed to cool to 42°C on the benchtop. 2 μL of a solution containing 5 mM each of dATP, dCTP, dGTP and dTTP was added followed by 3 units (1 μL) of T4 DNA polymerase (New England Biolabs). The reaction was incubated at 37°C for 30 min, and nucleic acids were ethanol precipitated. The pellet was resuspended in 10 μL of deionized formamide and analyzed on 8% acrylamide, 8 M urea, TBE sequencing gels. Dideoxy sequencing reactions using the same primer and DNA template were used as size markers.

CHAPTER IV

Discussion

A new model of protein targeting

It is generally accepted that all of the information required for protein folding is contained within the amino acid sequence of the polypeptide or polypeptides involved (reviewed by Anfinsen, 1973). Aspects of the information transfer that are still controversial include the order and timing of folding events, the role of accessory factors such as protein chaperones, and the assembly of more than one polypeptide into a stable structure (reviewed by Tsou, 1988; Jaenicke, 1991; Gething and Sambrook, 1992). Since targeting information is also carried by a polypeptide sequence in the context of the cell, the processes of protein folding and protein targeting are linked at a fundamental level.

The data presented in this thesis suggests a new model of SRα targeting to the ER membrane that illustrates this link. The folded amino terminal domain of SRα mediates binding to the ER membrane, and to the integral membrane SRβ subunit (Chapter II). A nascent SRα polypeptide can bind onto membranes during translation after the amino terminal domain has emerged from the ribosome (Chapters II and III). Translation pausing at a specific site on the SRα mRNA facilitates co-translational targeting of SRα, since reducing the level of pausing at this site impairs co-translational SRα targeting (Chapter III). It is proposed that translation pausing, possibly together with the action of protein chaperones, promotes co-translational folding and therefore targeting of SRα. This model of SRα targeting is consistent with previous ideas of translation pausing, co-translational folding and protein localization. The novel concept introduced in the model is a direct link between translation pausing and SRα targeting. Furthermore, this model may be applicable to the targeting of other proteins.

Co-translational protein folding

There is evidence that the folding of a polypeptide begins during synthesis on the ribosome. Experiments with structural probes such as conformation-specific antibodies suggest that a nascent polypeptide can take on considerable secondary structure (eg. Fedorov et al, 1992). The final tertiary structure cannot be formed until the entire polypeptide has been synthesized. However, proteins reach a native conformation much more rapidly after release from ribosomes than after dilution out of denaturing solutions, suggesting that the conformation of an almost-complete nascent polypeptide is close to the final protein conformation (eg. Kolb et al, 1994). It has been proposed that longer nascent polypeptides form folded modules which are assembled to the final conformation very quickly (reviewed by Jaenicke, 1991).

The results of Chapter II suggest that SRα begins to fold co-translationally. Two lines of evidence support the hypothesis that folding of the SRα amino terminal domain is required for membrane binding. First, deletion mutations that reduced the protease resistance of the amino terminal domain (Chapter II, Fig. 4*b*) abolished membrane binding of SRα (Chapter II, Fig. 2). Second, binding to SRβ of the SRα amino terminal domain (Chapter II, Fig. 6) is consistent with a folded structure capable of forming specific protein-protein interactions. The peripheral nature of SRα and integral membrane nature of SRβ suggest that these protein-protein interactions are responsible for SRα membrane anchoring (Chapter II, Fig. 5). Therefore, membrane anchoring of nascent SRα polypeptides while still ribosome-attached (Chapter II, Fig. 3; also Chapter III, Fig. 4) argues for co-translational folding of SRα. Although the amino terminal membrane

binding domain of SRα may not fold into the final native conformation during translation, it is likely folded into a structural module that can bind to the target on the ER membrane.

Translation pausing and co-translational folding

Translation pausing has been proposed to aid the co-translational folding of proteins by allowing sections of a nascent polypeptide to fold without interference from carboxyl terminal sequences (Purvis et al, 1987). A hypothetical scheme for the co-translational folding of rabbit α-globin compared apparently paused translation intermediates to the crystallographic structure of the native protein. It was suggested that translation pausing aided the formation of the secondary structure units, and the progressive folding of these units into the final globular conformation (Krasheninnikov et al, 1991). However, the globin polypeptide folds into a single 14 kD globular domain, so the hypothetical folding scheme has limited relevance to the model proposed for SRα in which a translation pause separates large domains able to fold independently.

Experiments with the *TRP3* gene product of *Saccharomyces cerevisiae* suggest that translation pausing between two protein domains contributes to correct folding *in vivo*. The TRP3 polypeptide forms a bifunctional enzyme with anthranilate synthase II activity in the amino terminal region and indoleglycerol phosphate synthase activity in the carboxyl terminal region. The mRNA sequence encoding the start of the carboxyl terminal region contains a series of ten rare codons, predicted to cause a translation pause because of the low population of matching tRNAs. When these codons were mutated to

commonly used codons, both of the enzymatic specific activities of the *in vivo* expressed protein were significantly lower than those of the protein expressed from the original mRNA. While translation pausing during synthesis of TRP3 was not directly demonstrated, these experiments strongly suggested a connection between translation pausing and co-translational folding (Crombie et al, 1992 and 1994). TRP3 protein was also expressed in a yeast strain deficient in functional *SSA* gene product, the homologue of the Hsp70 protein chaperone. The specific enzymatic activities of TRP3 protein synthesized from the original mRNA were lower in the mutant yeast strain than in the wild-type strain expressing functional chaperones. Therefore, translation pausing was postulated to act together with protein chaperones to aid protein folding (Crombie et al, 1994).

The function of the putative TRP3 translation pause is analogous to that in SRα, except that folding of the SRα amino terminal domain, facilitated by translation pausing, supports protein targeting and not simply enzymatic stability. The data in Chapter III extends the work of Crombie et al, 1994, by unambiguously demonstrating a translation pause after synthesis of the amino terminal domain (Chapter III, Fig. 1). Furthermore, while a reduction in translation pausing was only predicted on the mutated TRP3 mRNA (Crombie et al, 1992), the reduction in translation pausing on the mutated non-pausing SRα mRNA was directly observed and quantified (Chapter III, Fig. 3). However, the lower specific activities of TRP3 protein in the *SSA*-deficient yeast suggest that in parallel, the co-translational folding of SRα may be examined using an expression system, *in vitro* or *in vivo*, that is deficient in protein chaperones.

Co-translational oligomer assembly

Many polypeptides, like SRα, function as subunits of an oligomeric complex, in this case the heterodimeric SRP receptor. Oligomer assembly may occur co-translationally in addition to protein folding, since a nascent polypeptide should be able to form appropriate intermolecular contacts as easily as it forms appropriate intramolecular contacts (Jaenicke, 1991). Nascent globin polypeptides were shown to bind heme during translation and the α and β polypeptide subunits of bacterial luciferase assembled co-translationally (Komar et al, 1993; Fedorov and Baldwin, 1995). Consistent with these reports, the data in this thesis suggests that a nascent SRα polypeptide forms a dimeric complex with SRβ during translation. A connection between translation pausing and oligomeric assembly has also been proposed for the barley chloroplast reaction centre protein D1. Translation pause sites, estimated from the discrete apparent molecular masses of translation intermediates, were correlated with different putative stages in chlorophyll binding and thylakoid membrane insertion (Kim et al, 1994).

The results in Chapter III provide more direct evidence that translation pausing during synthesis of SRα promotes oligomer assembly as well as protein folding. The targeting of SRα can be viewed as a special case of co-translational oligomer assembly, since the molecules (such as SRβ) to which the nascent polypeptide binds are already localized within the cell. In order to test this hypothesis, it must first be demonstrated that binding to SRβ is sufficient for the urea-resistant membrane binding of novel SRα polypeptides. The immunoprecipitation experiments in Chapter II (Fig. 6) already suggest this may be the case as intersubunit binding was stable in high ionic strength and 1%

nonionic detergent. Experiments using SRβ coupled to a solid matrix or reconstituted into proteoliposomes may reveal whether or not interactions with lipids or other microsomal components are required for SRα binding. The same experimental systems can then be used to examine co-translational binding of SRα onto SRβ.

It has been suggested that ligand or intersubunit binding may fix the nascent polypeptide onto a folding pathway to the correct conformation (reviewed by Jaenicke, 1991). For example, while folding of the SRα amino terminal domain supports binding to SRβ, binding to SRβ may in turn support the folding of SRα. Therefore, the folding and targeting of nascent SRα that is coordinated by the translation pause may be cooperative events. Cooperativity between folding and targeting was not observed in the cell-free system, since the efficiency of co-translational and post-translational SRα membrane binding could not be directly compared. However, other factors such as the presence of protein chaperones or the inherent stability of the SRα polypeptide may make cooperativity undetectable in the cell-free system. In Chapter II, Fig. 5*a*, SRα was shown to be extracted from membranes and from SRβ in chaotropic conditions known to disrupt hydrophobic interactions. Therefore, as expected for internal protein contacts (Jaenicke, 1991), hydrophobic interactions are involved in binding SRα to SRβ and the ER membrane. Protein chaperones like Hsc70 that recognize hydrophobic polypeptides (reviewed by Hartl et al, 1994) may sequester the hydrophobic binding face of nascent SRα polypeptides. By acting transiently as surrogate targets, protein chaperones may allow stable folding in the absence of the actual SRβ target and without cooperativity between folding and subunit assembly. Also, the isolated amino terminal domain of SRα

forms a protease resistant unit when expressed as a polypeptide (SRX2, Chapter II, Fig. 4*b*), in the absence of SRβ or microsomes. This suggests that the folded SRα amino terminal domain is relatively stable thermodynamically, and cooperativity between SRα folding and targeting may be difficult to detect.

Co-translational targeting and translation pausing of other proteins

Co-translational targeting via oligomer assembly has also been hypothesized for the intermediate filament proteins myosin heavy chain, vimentin and titin based on *in vivo* polysome binding experiments. Polysome-bound mRNA encoding these protein but not others remained attached to the cytoskeletal matrix of cultured cells after detergent extraction. The attachment was sensitive to puromycin (which releases nascent polypeptides from ribosomes) suggesting that polysome binding was mediated by the nascent polypeptides (Isaacs and Fulton, 1987; Isaacs et al, 1989a and 1989b). New cytoskeletal filaments are assembled at distinct locations within the cell, implying that the nascent polypeptides were targeted to these sites where they were incorporated co-translationally into new cytoskeleton (reviewed by Fulton and L'Ecuyer, 1993). While the presence of discrete-sized translation intermediates suggests there may be translation pausing during synthesis of these proteins (Fulton and L'Ecuyer, 1993), this possibility and the relation to targeting is as yet unexplored.

A frameshift-like mRNA sequence resembling the sequence at the SRα pause site is found on the mRNA encoding human protein 4.1, a component of the plasma membrane cytoskeleton. Similar to the SRα mRNA, the protein 4.1 mRNA sequence

predicted from the cDNA (Conboy et al, 1986) contains the heptamer-like sequence A-AAA-AAG followed by a predicted stem-loop structure (Fig. 1; compare with Chapter III, Fig. 2). Based on the data in Chapter III, these mRNA structure elements may be sufficient to cause translation pausing on the SRα mRNA and therefore the protein 4.1 sequence is also predicted to mediate a translation pause. This sequence is in a region of the mRNA common to the various alternatively spliced isoforms expressed in different cell types (Conboy et al, 1991). The putative translation pause sequence begins at the codon for amino acid 62 of the isoform in mature erythroid cells. However, other isoforms expressed in lymphoid cells and in pre-erythroid cells have amino terminal extensions of up to 209 amino acids preceding the initial methionine of the mature erythroid protein (Conboy et al, 1991). While the shorter isoform is localized to the plasma membrane, the isoforms with the amino terminal extensions are localized in the cytoplasm with a punctate, perinuclear pattern (Chasis et al, 1993). Thus, the cytoplasmic localization may be mediated by targeting information contained in the amino terminal extensions. Furthermore, similar to the targeting mechanism of SRα, the putative protein 4.1 translation pause may facilitate co-translational folding and localization of the amino terminal extension.

The co-translational targeting of SRα is analogous to the targeting of nascent secretory polypeptides, although the mechanisms involved are unrelated. In each case, there is a pause in translation after a polypeptide encoded targeting sequence has been synthesized and has emerged from the ribosome. For secretory proteins the targeting polypeptide is the signal sequence (Walter et al, 1981), while for SRα it is the complete

membrane binding domain. In both cases, the translation pause is followed by a targeting event. The similarity between the pathways may also indicate a similar function of the translation pause. One possibility is that in parallel with SRα synthesis, the SRP-induced translation pausing of a secretory protein allows some conformation change to occur as an early event in translocation. Such a conformation change may not involve the signal sequence as it remains bound to SRP during the translation pause. Therefore, the hypothetical conformation change would be in some other component of the complex, either on the ribosome in preparation for membrane binding or on SRP itself, perhaps as part of the GTPase cycle. SRP-induced translation arrest facilitates but is not essential for translocation, suggesting that as for SRα, the pause facilitates a thermodynamically favoured but kinetically slow event in the pathway. Translation pausing may thus provide a general mechanism to coordinate protein synthesis with slow co-translational events.

Mechanism of translation pausing on the SRα mRNA

The results presented here demonstrate a previously unknown mechanism of translation pausing. The SRα pause site is encoded by an mRNA structure that outwardly resembles a class of frameshift sites used by viral and bacterial mRNAs (listed in Chapter III). On a representative viral mRNA, such as that found in human T-cell leukemia virus type II, the frameshift site follows the mRNA sequence encoding the viral *gag* protein. Most ribosomes do not frameshift but maintain the *gag* reading frame and reach a stop codon. The fraction of ribosomes that frameshift encounter a second reading frame and

synthesize a *gag/pro* fusion protein (reviewed by Hatfield et al, 1992). A related frameshift sequence containing an RNA pseudoknot has been shown to produce paused translation intermediates in the rabbit reticulocyte lysate system (Somogyi et al, 1993). It is possible that translation pausing is a common phenomenon at the viral frameshift sites and, in parallel with the TRP3 protein (Crombie et al, 1992) and SRα, contributes to the folding of the viral fusion proteins.

The resemblance between the viral mRNA frameshift structures and the mRNA sequence at the SRα pause site (Chapter III, Fig. 2) suggests the mechanisms of frameshifting and translation pausing may be related. Frameshifting is thought to occur at the "slippery site" heptamer where the same amino acid can be coded by an overlapping mRNA sequence in two different reading frames (reviewed by Hatfield et al, 1992). A similar mechanism may cause the translation pause on the SRα mRNA, such that the ribosome reading the heptamer-like sequence either begins to slip but continues, or slips out of and then back into the reading frame. The three point mutations in the non-pausing SRα mRNA that disrupted the heptamer-like sequence may interfere with this mechanism. On the other hand, the ribosome decoding site was predicted to be about 15 nucleotides on the mRNA from each side of the ribosome, based on primer extension experiments using ribosomes paused at start codons (Wolin and Walter, 1988; Anthony and Merrick, 1992). The heptamer-like SRα sequence ends about 3 nucleotides 5' of the pause site *a* primer extension band (chapter three, Fig. 2), suggesting the decoding site of the paused ribosome has already passed over the potential slippery site. However, the role of the mRNA secondary structure in frameshifting is less well defined (Hatfield et

al, 1992), and it is possible that the combined effect of decoding the SRα heptamer-like sequence and melting the predicted stem-loop is necessary for the translation pause. The non-pausing mutations disrupted the predicted stem-loop as well as the heptamer-like sequence, and the reduction in pausing is consistent with this hypothesis. Further substitution mutations combined with quantitation of the pause site primer extension band should reveal the mRNA structures required for translation pausing on the SRα mRNA.

An alternative mechanism of translation pausing is sequence-specific binding to the mRNA of a factor that induces the pause in translation elongation. This factor may be part of the ribosome, either a constituent protein or ribosomal RNA, or it may be a non-ribosomal protein. There is no evidence as yet to judge this hypothesis, but the mutations introduced on the non-pausing mRNA suggest two possible recognition sites for a pausing factor. First, the hypothetical pausing factor may bind the mRNA 5' of the ribosome decoding site and recognize the heptamer-like sequence. Second, the factor may bind the mRNA 3' of the decoding site at the top of the stem-loop, and the non-pausing mutations within the stem-loop disrupted a distal structure required for binding. Another interesting possibility raised by a hypothetical pausing factor is regulation by the cell to control the level of translation pausing. Since translation pausing may be a general mechanism to support protein folding, expression of the pausing factor may be induced under heat shock conditions like the protein chaperones. Also, if the pausing factor is a ribosomal component, recognition of the pause sequence may be regulated by a covalent modification such as phosphorylation.

Targeting of SRα mRNA

In the cell-free system, mRNA encoding SRα was bound to microsomes primarily by ribosomes synthesizing co-translationally targeted nascent SRα polypeptides (Chapter III, Fig. 5). This suggests that the mRNA may be localized in this manner to the ER membrane *in vivo*, and that a specialized targeting mechanism for this mRNA is not required. Targeting sequences for some mRNAs have been located in the 3' untranslated regions, but no ER-specific targeting signals have been identified (reviewed by St. Johnston, 1995). However, the SP6 polymerase transcribed SRα mRNA used for the membrane binding experiments contained about 400 nucleotides of the 970 nucleotides in the 3' untranslated region predicted from the SRα cDNA (Lauffer et al, 1985). Thus, the presence of an mRNA targeting signal in the remainder of 3' untranslated region cannot be ruled out.

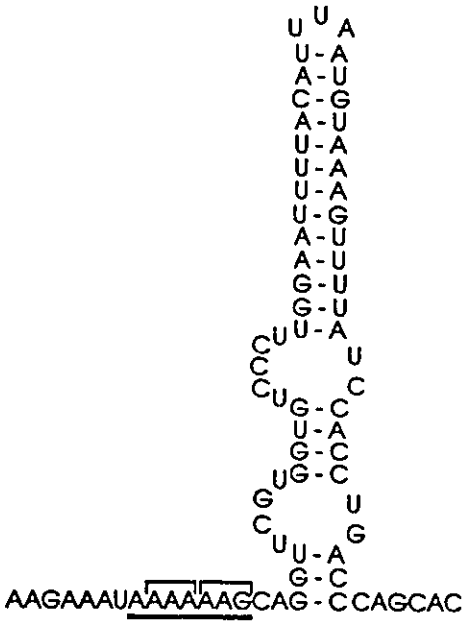
As mentioned above, there is evidence that mRNAs coding for certain cytoskeletal proteins are also localized by polysomes synthesizing co-translationally targeted nascent polypeptides (Fulton and L'Ecuyer, 1993). Other mRNAs are known to be localized within the cell, but most of the targeting mechanisms are still poorly defined. Sequences within the 3' untranslated regions of some mRNAs have been shown to support localization within the cytoplasm and possibly vectorial export from the nucleus (reviewed by St. Johnston, 1995). However, mRNA targeting mediated by these untranslated sequences is expected to be independent of translation. In parallel with protein targeting, a variety of mRNA targeting mechanisms likely operate on different mRNAs, or on the same mRNA at sequential stages of targeting.

Future prospects

The work presented in this thesis forms a basis for the further study of SRα targeting in the context of other cellular processes. While the membrane binding domain of SRα was identified in chapter two, the molecules on the ER membrane to which it binds must be unambiguously determined. If SRβ is indeed sufficient for SRα targeting and tight membrane binding, the receptor heterodimer provides an interesting model system to examine protein assembly, because of the exceptionally strong intermolecular contacts and the possibility of cooperativity between folding and assembly. Translation pausing and co-translational membrane binding of SRα must be demonstrated *in vivo*, most likely by adapting the methods used in the cell-free system. With translation pausing established as a truly physiological phenomenon, the mechanisms of pausing can be more closely analyzed *in vitro*. The sequence determinant of translation pausing at site *a* on the SRα mRNA can be addressed by further site-directed mutagenesis, and the data interpreted in light of the hypotheses discussed above. Also, the translation pause at site *b* on the SRα mRNA appears to be unrelated to site *a* and may use a different pausing mechanism. If the mRNA sequence at pause site *b* is shown to promote translation pausing in an unrelated mRNA context (eg. in the globin-Protein A fusion used for pause site *a*), a site-directed mutagenesis approach can also be used to investigate this pause site. It is possible that pause site *b* supplements the function of pause site *a* in SRα targeting. Once the requirements for translation pausing have been determined, pause sites on the mRNAs encoding other proteins can be identified. By abolishing pausing during synthesis of a varitey of proteins, a better understanding can be gained of the

physiological importance of translation pausing in general. Finally, the connections discovered between mRNA targeting, translation regulation, polypeptide folding and protein sorting advocates an integrated experimental approach to cellular processes.

Figure 1. A predicted translation pause site on the protein 4.1 mRNA. The mRNA sequence predicted from the cDNA of human protein 4.1 (Conboy et al, 1986) was analysed, and a sequence resembling the SRα mRNA pause site was identified. The RNAfold program, based on the methods of Freier et al, 1986, was used to predict secondary structures. A heptamer-like sequence is underlined with the reading frame indicated by brackets above.



References

Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science* **181**, 223-230

Anthony, D.D. and Merrick, W.C. (1992) Analysis of 40 S and 80 S complexes with mRNA as measured by sucrose density gradients and primer extension inhibition. *J. Biol. Chem.* **267**, 1554-1562

Conboy, J., Kan, Y.W., Shohet, S.B. and Mohandas, N. (1986) Molecular cloning of protein 4.1, a major structural element of the human erythrocyte membrane skeleton. *Proc. Natl. Acad. Sci. USA* **83**, 9512-9516

Conboy, J.G., Chan, J.Y., Chasis, J.A., Kan, Y.W. and Mohandas, N. (1991) Tissue- and development-specific alternative RNA splicing regulates expression of multiple isoforms of erythroid membrane protein 4.1. *J. Biol. Chem.* **266**, 8273-8280

Chasis, J.A., Coulombel, L., Conboy, J., McGee, A., Andrews, K., Kan, Y.W. and Mohandas, N. (1993) Differentiation-associated switches in protein 4.1 expression. *J. Clin. Invest.* **91**, 329-338

Crombie, T., Swaffield, J.C. and Brown, A.J.P. (1992). Protein folding within the cell is influenced by controlled rates of polypeptide elongation. *J. Mol. Biol.* **228**, 7-12

Crombie, T., Boyle, J.P., Coggins, J.R. and Brown, A.J.P. (1994) The folding of the bifunctional TRP3 protein in yeast is influenced by a translational pause which lies in a region of structural divergence with *Escherichia coli* indoleglycerol-synthase. *Eur. J. Biochem.* **226**, 657-664

Fedorov, A.N., Friguet, B., Djavadi-Ohaniance, L., Alakhov. Y.B. and Goldberg, M.E. (1992) Folding on the ribosome of *Escherichia coli* tryptophan synthase β subunit nascent chains probed with a conformation-dependent monoclonal antibody. *J. Mol. Biol.* **228**, 351-358

Fedorov, A.N., Baldwin, T.O. (1995) Contribution of cotranslational folding to the rate of formation of native protein structure. *Proc. Natl. Acad. Sci. USA* **92**, 1227-1231

Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA* **83**, 9373-9377

Fulton, A.B. and L'Ecuyer, T. (1993) Cotranslational assembly of some cytoskeletal proteins: implications and prospects. *J. Cell Sci.* **105**, 867-871

Gething M.-J. and J. Sambrook. (1992) Protein folding in the cell. *Nature* **355**, 33-45

Hartl, F.-U., Hlodan, R. and Langer, T. (1994) Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.* **19**, 20-25

Hatfield, D.L., Levin, J.G., Rein, A. and Oroszlan, S. (1992) Translational suppression in retroviral gene expression. *Adv. Vir. Res.* **41**, 193-239

Isaacs, W.B. and Fulton, A.B. (1987) Cotranslational assembly of myosin heavy chain in developing cultured skeletal muscle. *Proc. Natl. Acad. Sci. USA* **84**, 6174-6178

Isaacs, W.B., Cook, R.K., Van Atta, J.C., Redmond, C.M. and Fulton, A.B. (1989a) Assembly of vimentin in cultured cells varies with cell type. *J. Biol. Chem.* **264**, 17953-17960

Isaacs, W.B., Kim, I.S., Struve, A. and Fulton, A.B. (1989b) Biosynthesis of titin in cultured skeletal muscle cells. *J. Cell Biol.* **109**, 2189-2195

Jaenicke, R. (1991) Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry* **30**, 3147-3161

Kim, J., Gamble Klein, P. and Mullet, J.E. (1994) Synthesis and turnover of photosystem II reaction center protein D1. Ribosome pausing increases during chloroplast development. *J. Biol. Chem.* **269**, 17918-17923

Kolb, V.A., Makeyev, E.V. and Spirin, A.S. (1994) Folding of firefly luciferase during translation in cell-free system. *EMBO J.* **13**, 3631-3637

Komar, A.A., Kommer, A., Krasheninnikov, I.A. and Spirin, A.S. (1993) Cotranslational heme binding to nascent globin chains. *FEBS Lett.* **326**, 261-263

Krashenninikov, I.A., Komar, A.A. and Adzhubei, I.A. (1991) Nonuniform size distribution of nascent globin peptides, evidence for pause localization sites, and a cotranslational protein-folding model. *J. Protein Chem.* **10**, 445-453

Lauffer, L., Garcia, P.D., Harkins, R.N., Coussens, L., Ullrich, A. and Walter, P. (1985) Topology of signal recognition particle receptor in endoplasmic reticulum membrane. *Nature* **318**, 334-338

Purvis, I.J., Bettany, A.J.E., Chinappan Santiago, T., Coggins, J.R., Duncan, K., Eason, R. and Brown, A.J.P. (1987) The efficiency of folding of some proteins is increased by controlled rates of translation *in vivo*: A hypothesis. *J. Mol. Biol.* **193**, 413-417

St. Johnston, D. (1995) The intracellular localization of messenger RNAs. *Cell* **81**, 161-170

Somogyi, P., Jenner, A.J., Brierley, I., and Inglis, S.C. (1992) Ribosomal pausing during translation of an RNA pseudoknot. *Mol. Cell. Biol.* **13**, 6931-6940

Tsou, C.-L. (1988) Folding of the nascent peptide chain into a biologically active protein. *Biochemistry* **27**, 1809-1812

Wolin, S.L. and Walter, P. (1988) Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J.* 7, 3559-3569

Walter, P., Ibrahimi, I. and Blobel, G. (1981) Translocation of proteins across the endoplasmic reticulum I. Signal recognition protein (SRP) binds to *in-vitro*-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* **91**, 545-550