Structural Determinants for Signal Sequence Function in the Mammalian Endoplasmic Reticulum*

(Received for publication, August 31, 1999, and in revised form, October 3, 1999)

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Signal sequences function in protein targeting to and translocation across the endoplasmic reticulum membrane. To investigate the structural requirements for signal sequence function, chimeras of the Escherichia coli LamB signal peptide and prolactin were prepared. The LamB signal peptide was chosen by virtue of the extensive biophysical and biological characterization of its activity. In vitro, nascent prolactin chains bearing the LamB signal peptide (LamB) were targeted in a signal recognition particle (SRP)-dependent manner to rough microsomes but remained protease- and salt-sensitive and translocated at low efficiency. Full translocation activity was obtained in a gain of function mutant (LamB*) in which three hydrophobic residues in the LamB hydrophobic core were converted to leucine residues. Cross-linking studies demonstrated that the LamB* signal sequence displayed markedly enhanced interactions with SRP and integral membrane proteins. In contrast, chemically denatured LamB and LamB*-precurors bound with identical efficiencies and in a salt-resistant manner to rough microsomes, suggesting that during de novo synthesis the signal sequence of LamB-bearing precursors assumes a conformation refractory to translocation. These data indicate that a leucine-rich signal sequence is necessary for optimal interaction with SRP and suggest that SRP, by maintaining the signal sequence in a conformation suitable for membrane binding, performs a chaperone function.

Protein translocation in the endoplasmic reticulum (ER) requires a signal sequence, an amino-terminal 12–30-amino acid precursor domain that functions in both targeting to and vectorial transfer across the ER membrane. A targeting function for the signal sequence was defined coincident with the identification of the signal recognition particle (SRP) (1–4). In these pioneering studies, it was observed that targeting of ribosome-nascent chain complexes to the ER requires SRP binding to the signal sequence and that biosynthetic substitution of β-hydroxyleucine for leucine blocked SRP activity. In addition to identifying a targeting function for SRP, these studies established the importance of the hydrophobic core region of the signal sequence in SRP/signal sequence interactions. Subsequent studies have provided extensive molecular insights into the signal recognition process, with the identification of the 54-kDa SRP subunit (SRP54) as the site of signal peptide/SRP interactions being of particular interest (5–8). Structural analyses of SRP54 have identified three primary domains, a carboxyl-terminal methionine-rich domain, which serves as the site of signal sequence recognition, an aminoterminal N domain, and a central GTPase domain (9–12).

SRP-mediated targeting of ribosome-nascent chain complexes to the ER occurs very early in protein synthesis and is necessary for efficient targeting of ribosome-nascent chain complexes to the ER membrane. The functional basis for early recognition of the nascent chain by SRP was identified in studies demonstrating that the affinity of SRP for signal sequence-bearing nascent chains decreases dramatically with continued elongation (13–15). On the basis of these studies, it is thought that the inability of SRP to direct ribosome-nascent chain targeting at late stages of protein synthesis reflects a protein folding problem common to mammalian precursor proteins. And it is for this reason that protein translocation is obligatorily co-translational in the mammalian ER. The cotranslational nature of protein translocation has, however, made the analysis of signal sequence function occurring subsequent to targeting difficult. In experimental scenarios in which the SRP-dependent targeting phase of protein translocation is bypassed, it is clear that the signal sequence is necessary for efficient vectorial transport across the ER membrane (16–20). At present, however, the molecular basis for signal sequence function in the ER membrane is not understood. By analogy with bacteria, the signal sequence may function to regulate or gate the conductance state of a protein conducting channel (21).

Are the physical characteristics of the signal sequence that define its activity in the targeting phase of translocation identical to those required for binding to components of the ER membrane? It is well established that isolated signal peptides can spontaneously insert into lipid membranes and in so doing assume a predominately α-helical structure (22–24). Mutations that block activity in vivo disrupt such structural interconversions, so it is apparent that the capacity for spontaneous membrane insertion and α-helix formation correlates well with signal sequence function (25). However, signal sequences physically associate with SRP; thus, it is reasonable to expect that direct interactions with integral membrane protein components of the ER would be necessary for signal sequence function in the ER membrane. In fact, near-neighbor interactions of the signal sequence with TRAPα, TRAM, and Sec61p, all resident integral membrane protein components of the ER, have been identified by cross-linking techniques (26–31). The question remains unresolved, however, as to whether signal sequences function through direct insertion into the membrane bilayer, binding to membrane protein components of the ER, or both.

In this role, the study of the signal sequence in precursor targeting and vectorial transport was investigated. As a model...
Signal Sequence Function in Translocation

**EXPERIMENTAL PROCEDURES**

**Construct Preparation**—A chimera containing the LamB signal sequence in frame with the mature domain of preprolactin was prepared as follows. A PCR product containing the LamB signal sequence was obtained using the primers 5'-GCC-GGA-TCC-ACC-CCG-TGT-GTC-AGC-3' (sense primer) and 5'-GCC-GGA-TCC-ACC-CCG-TGT-GTC-AGC-3' (antisense primer) and a full-length LamB cDNA as template. A PCR product containing the mature domain of prolactin was obtained with the primers 5'-GCC-GGA-TCC-ACC-CCG-TGT-GTC-AGC-3' (sense primer) and 5'-GCC-GGA-TTC-TTA-GCA-GTT-GTT-3' (antisense primer). PCR products were gel-purified and used in a triple ligation reaction with gel-purified, XhoI- and EcoRI-digested pGEMBP1 (30) as the vector host. Ligation products were transformed into E. coli strain DH5α, and positive clones were selected by ampicillin resistance. All cloning steps were sequenced to confirm the intended mutation (V95L, V11L, and A11L).

**Preparation of the LamB Signal Peptide**—A chimera containing the LamB signal sequence in frame with the mature domain of preprolactin (preprolactin) or 81 (LamB-prolactin chimera) amino acids. The transmembrane precursor fraction was recovered by the addition of 0.5 molar sucrose, 20 mm K-HEPES, 2.5 mm Mg(OAc)₂, high salt buffer (0.5 molar K-HEPES, pH 7.2), or physiological salts buffer with the indicated concentrations of EDTA. Following incubation on ice for 20 min, samples were overlaid onto a 70-μl cushion of 0.5 molar sucrose, 20 mm K-HEPES, pH 7.2, and centrifuged in the Beckman TLS100 rotor for 5 min at 60,000 rpm (4 °C). The supernatant and cushion fractions were processed as the supernatant fraction. The membrane precursor fraction was recovered by the addition of 20 μl of 0.5 molar Trias base, 5% SDS, 0.1 molar DTT and heating to 55 °C for 15 min.

**Protease Protection Studies**—Completion of translation reactions were chilled on ice and diluted to 50 μl in 110 mm KOAc, 20 mm K-HEPES, 2.5 mm Mg(OAc)₂. Proteinase K was added from freshly prepared stocks in water to the indicated concentrations. Protease digestion reactions were performed for 30 min on ice and quenched by the addition of phenylmethylsulfonyl fluoride to a final concentration of 5 mM. Following fractionation with ammonium sulfate, pellet fractions were washed with 10% trichloroacetic acid and prepared for SDS-PAGE as described below.

**RESULTS**

**The LamB Signal Peptide Targets Precursor Proteins to the ER**—The LamB signal peptide has been the subject of extensive structural, biochemical, and biological investigation and is known to participate in both protein/lipid and protein/protein...
interactions (22, 25, 32, 34). Given these precedents and the intent of the present study to investigate the structural requirements for signal sequence function in both the targeting and membrane transport stages of protein translocation, a fusion protein was prepared consisting of the signal sequence of LamB in frame with the mature domain of preprolactin. To investigate the behavior of the chimera in the targeting phase of translocation, a series of experiments were performed to compare the targeting activities of truncated forms of preprolactin and the LamB-prolactin fusion. In these assays, truncated forms of the two proteins (pPl86 and pLamBP81) were synthesized in vitro in the presence or absence of canine pancreas rough microsomes (RM), and the association of ribosomal-nascent chain complexes with the RM was assayed by centrifugation. Operationally, the formation of a translocation-competent junction of the ribosomal-nascent chain complex with RM can be assayed as resistance to extraction with high salt or EDTA or protection of the bound nascent chain from digestion with exogenous proteases (20, 35, 36).

The results of these experiments are depicted in Fig. 1. Following translation in the absence of RM and centrifugation, both precursor proteins were recovered in the supernatant fraction (Fig. 1, lanes 1–4). Following translation in the presence of RM, both pPl86 and pLamBP81 were recovered in the pellet (membrane) fraction (Fig. 1, lanes 5–8). The two precursors could, however, be readily distinguished by treatment of the completed binding reactions with either 0.5 M KOAc or 10 mM EDTA prior to centrifugation (Fig. 1, lanes 9–16). Under these conditions, the pPl86 nascent chains were recovered in the pellet fraction, whereas the pLamBP81 precursors were recovered in the supernatant fraction. These data indicate that the LamB signal peptide displayed activity in the targeting phase of translocation but was ineffective in the formation of a tight ribosome-membrane junction.

To further investigate the activity of the LamB signal peptide in the targeting stage of translocation, the capacity of ribosome-LamBP81 nascent chain complexes to form a protease-resistant membrane junction was determined. Depicted in Fig. 2 are the results of an experiment in which the protease accessibility of the truncated chains was determined as a function of the RM concentration present during translation. In Fig. 2A, the results with the 86-amino acid pPl precursor are shown. When translated in the absence of RM and subjected to proteolysis, a labeled fragment of higher mobility, representing the ribosome-protected fragment of the nascent chain, was recovered (Fig. 2A, lanes 1–3). When translated in the presence of 0.5, 1.0, or 1.5 eq of RM/20-μl translation, pPl86 was efficiently targeted to the RM and formed a tight junction, with the nascent chain being protected from proteolysis in the presence of either 0.1 or 0.5 mg/ml proteinase K (Fig. 2A, lanes 4–9). In contrast to the pPl86, the pLamBP81 nascent chains, although targeted to the RM, formed a protease-resistant membrane junction only inefficiently (Fig. 2B, lanes 4–9). Importantly, the efficiency with which the pLamBP81 precursors achieved the protease-resistant stage of targeting was essentially independent of the RM concentration present during the translation (Fig. 2B, lanes 4–9). These data indicate that the RM concentration is not limiting in these experiments and suggest that the targeting defect seen with the LamB signal sequence is a property of the signal sequence itself.

Identification of a Gain of Function LamB Signal Peptide Mutant—Signal sequences have a well-established canonical structure consisting of a positively charged N terminus, an central hydrophobic core of 7–15 amino acids, and a polar C terminus containing a consensus motif for cleavage by signal peptidase (40). The LamB signal peptide satisfies all available criteria for signal sequence structure and has been demonstrated to bind to SRP54 (32, 34). Thus, the relative inability of the LamB signal peptide to elicit formation of a tight ribosome-membrane junction was unexpected. To determine if the activity of the LamB signal sequence in translocation could be enhanced, a number of LamB signal sequence mutants were prepared. Increasing the positive charge at the N terminus by converting methionine (position −24) to lysine was without effect (data not shown). It is well established that the hydrophobic regions of prokaryotic signal sequences have a relatively high prevalence of alanine residues, whereas eukaryotic signal

FIG. 1. The LamB signal peptide supports targeting of ribosome-nascent chain complexes to mammalian microsomes. pPl86 and pLamBP81 nascent chains were translated in the presence or absence of canine pancreas rough microsomes (1 eq/20 μl) in a reticulocyte lysate translation system. Following translation, reactions were diluted in physiological salts buffer, physiological salts buffer supplemented with 0.5 M KOAc, or physiological salts buffer supplemented with 20 mM EDTA and incubated on ice for 20 min. Free and membrane-bound nascent chains were subsequently fractionated by centrifugation, and pellet (P) and supernatant (S) fractions were collected. Samples were processed for SDS-PAGE and resolved on 12.5% Tris-Tricine gels. Gels were fixed and analyzed by phosphor imager detection using a Fuji MacBas1000 phosphorimager. A digital image of the gel is depicted.

FIG. 2. The LamB signal peptide participates inefficiently in the formation of a tight ribosome-membrane junction. pPl86 and pLamBP81 nascent chains were translated in a homologous translation/translocation system consisting of reticulocyte lysate and the indicated quantities of canine pancreas rough microsomes for 20 min at 25 °C. After translation, reactions were diluted 2.5-fold in physiological salts buffer and placed on ice. Proteolysis reactions were performed for 30 min at 4 °C at the indicated concentrations of proteinase K, quenched by the addition of 5 mM phenylmethylsulfonyl fluoride, and processed for SDS-PAGE. Samples were resolved on 12.5% Tris-Tricine gels and analyzed as described in the legend to Fig. 1.
sequence hydrophobic regions are relatively enriched in leucine residues (40). To test the hypothesis that the inability of the LamB signal sequence to efficiently direct translocation across the ER membrane was a consequence of its alanine/valine-rich hydrophobic region, a mutant form of the LamB signal sequence was created in which valine residues (40) were either treated with 250 μM puromycin for 15 min, to determine translocation activity (lanes 1 and 2), or diluted to 150 μl in either physiological salts buffer or physiological salts buffer adjusted to 0.5 M KOAc, for analysis of free and membrane-bound nascent chains by centrifugation (lanes 3 and 4). Samples were processed as described in the legends to Figs. 1 and 2. Note that for the mature LamB*P81 nascent chains, 67% of the isotopically labeled amino acids are located in the signal sequence, and thus the efficiency of processing, by visual estimation, is abnormally low. Precursor (p) and mature (m) forms are indicated to the left.

Although the hydrophobicity profiles are similar, the functional properties of nascent chains bearing either of the two forms of the LamB signal sequence differ markedly. In Fig. 4, the activities of the pLamBP81 and pLamB*P81 chimeras were investigated in vitro, with the pPl86 serving as a positive control. As shown in lanes 1 and 2, the addition of puromycin to membrane-bound pPl86 nascent chains yields the release of the nascent chain from the ribosome and cleavage by signal peptidase to yield mature truncated prolactin. Whereas puromycin addition to membrane-bound pLamBP81 nascent chains yielded barely detectable levels of signal peptide processing, the pLamB*P81 nascent chains were efficiently processed. Note that for the pLamB*P81 nascent chains, 67% of the radio-labeled amino acids are located in the signal sequence, and thus cleavage of the signal peptide is accompanied by a substantial apparent loss of the mature prolactin. Correction for the distribution of methionine residues in the signal sequence and the mature domain of the pLamB*P81 precursors indicates that processing occurs at 75% efficiency. As would be expected from the gain of function activity of the pLamB*P81 signal sequence in the translocation assay, the data in Fig. 4 also indicate that this form of the LamB signal sequence is fully functional in the targeting assay. Thus, as shown in Fig. 4, lanes 3 and 4, the targeting behavior of pLamB*P81 nascent chains following targeting and extraction of the bound nascent chains with physiological (0.15 M) or high (0.5 M) salt mirrors that of pPl86 nascent chains and is distinct from that seen with the pLamBP81 precursors. From these data, it can be concluded that efficient activity of the LamB signal sequence in a mammalian translocation system requires the presence of leucine residues in the signal sequence hydrophobic region.

pLamBP81 Targeting to RM Requires SRP Receptor—The data presented in Figs. 1–4 indicate that signal sequence function in ribosome-nascent chain targeting to the ER can be distinguished from signal sequence function in translocation. To gain further insights into this observation, the targeting of pLamBP81 and pLamB*P81 precursors was studied under conditions in which the SRP receptor was inactivated (36, 41, 42). In these experiments, depicted in Fig. 5, A and B, the targeting of pLamBP81 and pLamB*P81 precursors was compared in native RM and RM in which SRP receptor activity was inactivated by treatment of the RM with N-ethylmaleimide (36, 41–43). In Fig. 5A are shown the results with the pLamBP81 precursor. When translated in the absence of RM, pLamBP81 is recovered in the supernatant fraction, and subsequent digestion with proteinase K yields the ribosome-protected fragment (Fig. 5A, lanes 1 and 2). When translated in the presence of RM, a substantial quantity of the nascent chains are targeted to the membrane, yet binding of the signal sequence to the membrane was inefficient (Fig. 5A, lanes 3 and 4). When translated in the presence of N-ethylmaleimide-treated RM, the targeting activity is approximately that seen when translations were performed in the absence of RM, indicating that the targeting of the pLamBP81 precursor requires a functional SRP receptor. The activity of the pLamB*P81 precursor in these assays is
shown in Fig. 5B. As can be seen upon comparison of lanes 1–4, the pLamB*P81 precursor efficiently targets to native RM and forms a translocation-competent junction. As with the pLamBP81 precursors, when translation was performed in the presence of N-ethylmaleimide-treated RM, little or no targeting to the RM was observed, and the nascent chains were unable to associate with the RM. These data further demonstrate that the wild type LamB signal sequence is capable of supporting the targeting of nascent chains to the ER membrane, yet is unable to efficiently interact with the ER membrane components that mediate translocation.

Wild-type LamB Signal Sequence Associates with SRP and Integral ER Membrane Proteins—To investigate the physical environment of the LamB signal sequence during the targeting and membrane association stages of translocation, a series of chemical cross-linking experiments were performed. First, to determine if targeted pLamBP81 precursors reside in physical proximity to membrane protein components of the ER, pPl86, pLamBP81, and pLamB*P81 precursors were synthesized in the presence of RM, the membrane-bound intermediates were collected by centrifugation through a sucrose cushion, and the nascent chain complexes (A) or in the presence or absence of membranes (B). Following translation, ribosome-membrane complexes (A and B) and ribosome-nascent chain complexes (B) were collected by centrifugation and resuspended in physiological salts buffer. In the experiment depicted in A, ribosome-membrane complexes were cross-linked with increasing concentrations of MBS for 30 min on ice, the reaction was quenched by the addition of 50 mM DTT, and the samples were processed for SDS-PAGE, as described in the legend to Fig. 1. In B, either ribosome-membrane complexes or ribosome-nascent chain complexes were cross-linked with 100 μM MBS for 30 min on ice, the reactions were quenched in 50 mM DTT, and the samples were processed identically. The asterisk indicates the appearance of a cross-linked species of 68 kDa and the horizontal line indicates a cross-linked product of 45–48 kDa. Studies of pLamBP81 and pLamB*P81 synthesized in vitro in the presence or absence of RM. As shown in Fig. 6B, when ribosome-bound pLamBP81 nascent chains, synthesized in the absence of RM, were treated with MBS, a number of prominent products were observed, with the major cross-linked species having a relative molecular mass of 40 kDa (Fig. 6B, lanes 1 and 2). When membrane-bound pLamBP81 nascent chains were cross-linked, the pattern was quite different. Under these conditions, the yield of the major 40-kDa product was decreased, coincident with an increased yield of the 47-kDa species (Fig. 6B, lanes 3 and 4). These data indicate that the 40-kDa pLamBP81 cross-linking product represents a cross-link to a ribosomal component and that the efficiency with which this near-neighbor interaction can be captured is diminished when synthesis is performed in the presence of RM. A prominent 40-kDa cross-linking product was also observed when pLamB*P81 nascent chains, synthesized in the absence of RM, were treated with MBS (Fig. 6B, lanes 6–8). For the pLamB*P81 precursors synthesized in the absence of RM, treatment of the ribosome-nascent chain complexes with MBS
yielded two prominent cross-linked products of 40 and 68 kDa (Fig. 6B, lanes 6–8). When pLamB*P81 synthesis was performed in the presence of RM, the yield of these two products was markedly diminished, whereas the yield of the 47-kDa product was greatly enhanced. With respect to Fig. 6A, these data indicate that the prominent 40- and 68-kDa cross-linked products represent cross-linking to ribosomal components, whereas the 47-kDa product represents cross-linking to ER membrane components.

In comparing the cross-linking profiles of both the ribosome-associated and membrane-bound pLamBP81 and pLamB*P81 nascent chains, the patterns are quite similar, with the marked exception of the 68-kDa cross-linked species, indicated by the asterisk in Fig. 6B. This cross-linked product was far more efficiently recovered in the pLamB*P81 cross-linking reactions, suggesting a possible explanation for the gain of function behavior of the LamB* mutations. To further examine this hypothesis, the cross-linking patterns of ribosome-associated pPl86, pLamBP81, and pLamB*P81 precursors, synthesized in the absence of RM, were directly compared. These data indicate that the prominent 40- and 68-kDa cross-linked products mediate targeting. In these experiments, ribosome-nascent chain complexes were collected by centrifugation, resuspended in physiological salts buffer, and subsequently treated with increasing concentrations of MBS for 30 min on ice (A). Cross-linking reactions were quenched by the addition of 50 mM DTT, and protein was concentrated by trichloroacetic acid precipitation. Samples were resuspended in sample buffer, processed for SDS-PAGE, and analyzed as described in the legend to Fig. 1.

signal sequence/membrane interactions in the absence of SRP-mediated targeting. In these experiments, ribosome-nascent chain complexes were synthesized in the absence of RM and collected by centrifugation. The ribosome pellets were denatured in guanidinium/HCl, and the denatured nascent chains were diluted into physiological salts buffer in the presence or absence of RM (5 eq/100 μl). Binding reactions were conducted for 15 min at 25 °C, chilled on ice, and, where indicated, adjusted to 0.5 M KOAc. Free and membrane-bound nascent chains were separated by flotation in discontinuous Nycodenz gradients, as described under “Experimental Procedures.” Gradients were harvested into five fractions, the proteins were collected from reticulocyte lysate. To assess membrane binding, completed binding reactions were mixed with 50% Nycodenz, in either physiological salts or high salts buffers, overlaid with a three-step Nycodenz gradient, and centrifuged as described under “Experimental Procedures.” Under these conditions, the RM float to the uppermost interface. The results of these experiments are shown in Fig. 8. When binding reactions were performed in the absence of RM, the precursor proteins were predominately recovered in the load, or bottom, fractions (Fig. 8A, −RM). When binding reactions were performed in the presence of RM and centrifuged in gradients containing physiological salts or 0.5 M KCl, pPl86, pLamBP81, and pLamB*P81 nascent chains bound in a salt-resistant manner and with nearly identical efficiencies to RM (Fig. 8B). These data indicate that when presented to RM in an unstructured state, the signal sequences of the pPl86, pLamBP81, and pLamB*P81 nascent chains bind to RM, yet when presented in the context of the physiological targeting machinery, only the pPl86 and pLamB*P81 nascent chains were efficiently bound to the ER membranes. Thus, it is not only the capacity of the signal sequence to interact with SRP that governs insertion into the
ER membrane, but also the ability of such an interaction to maintain the signal sequence in a structural state competent for membrane binding.

Although efficient binding of the pPl86, pLamBP81, and pLamB*P81 nascent chains to RM was observed when the nascent chains were presented in a chemically denatured form, the bound nascent chains did not undergo translocation. To determine if the capacity of the RM to support post-translational translocation was inhibited in the described assay conditions, similar experiments were performed with a precursor protein, preprocecropin, known to undergo SRP- and ribosome-independent post-translational translocation (44, 45). As shown in Fig. 8B, when provided to RM in either a co- or post-translational format, preprocecropin was efficiently processed to procecropin in the presence of both low (1.5 eq) and high (4.0 eq) concentrations of RM (Fig. 8B, lanes 1–6). The capacity of preprocecropin to undergo post-translational translocation is strictly dependent upon the structural characteristics of the mature region (44, 45), and thus by inference it is probable that rapid folding of the mature region is a likely explanation for the lack of translocation of the preprolactin and probable that rapid folding of the mature region is a likely explanation for the lack of translocation of the preprolactin and LamB chimera used in this study. Nonetheless, these data clearly indicate that the signal sequences can, when presented in an unstructured state, bind in a salt-resistant manner to membrane components of the RM.

**DISCUSSION**

The data presented herein demonstrate that SRP performs both a targeting and chaperone function in the initiation of protein translocation across the mammalian endoplasmic reticulum and that SRP function is critically dependent upon a leucine-rich signal peptide hydrophobic core. These conclusions are drawn from three primary observations. First, in assays of co-translational translocation, the LamB signal peptide supports the targeting of ribosome-nascent chain complexes to the ER membrane but does not elicit efficient formation of a translocation-competent ribosome-membrane junction, indicating that the processes of ribosome-nascent chain targeting and signal sequence insertion into the ER membrane represent two distinct aspects of signal sequence function. Second, a small increase in the mean hydrophobicity of the hydrophobic core domain of the LamB signal sequence marginally affected the efficiency of targeting to the ER membrane, yet greatly enhanced the ability of the signal sequence to tightly associate with components of the ER membrane. Last, when nascent chains bearing either the LamB or a gain of function (LamB*) signal sequence were presented to RM in a chemically denatured form, both sequences bound in a stable, salt-resistant manner, suggesting that the inability of the wild type LamB signal peptide to bind to the ER membrane was a consequence of inappropriate folding, which was itself a consequence of weak interactions between the wild type signal sequence and SRP.

The wild type LamB signal peptide functions in the targeting or ribosome-nascent chain complexes to the ER but is unable to elicit formation of a translocation-competent ribosome-membrane junction. At which stage of translocation is LamB signal peptide function deficient? Two series of experiments suggest that the defect in LamB signal peptide function is due to its inability to insert into the translocon when presented to the ER membrane by the physiological targeting machinery. Previous studies of SRP/LamB signal peptide interactions demonstrated that the LamB signal peptide productively interacts with the 54-kDa subunit of SRP (32, 34). Yet, membrane-bound ribosome-LamB nascent chain complexes were readily extracted with high salt buffers, and the membrane-bound LamB signal peptide-bearing nascent chains remained accessible to digestion with exogenous proteases (Figs. 1 and 2). Hence, although the LamB ribosome/nascent chain complexes utilize the SRP/SRP receptor dependent pathway for membrane targeting, the LamB signal peptide is unable to support the conversion of the membrane-bound ribosomes to the tightly bound, translocation-competent state (20, 35, 36). Since the deficit in LamB signal peptide function is clearly post-targeting, these data suggest that the LamB signal peptide fails in the second membrane signal sequence recognition stage identified by Jungnickel and Rapoport (20) and subsequently by Belin et al. (46).

A series of point mutations were made to identify a gain of function LamB signal peptide mutant that would display translocation activity in a mammalian system. Although the substitution of positively charged residues in the amino-terminal or N-domain of the signal sequence was without effect, a gain of function mutant (LamB*) was obtained following conversion of valine residues at the −12 and −14 positions and an alanine residue at the −11 position to leucine residues. In comparing the amino acid distribution in the hydrophobic region, or hydrophobic region, of prokaryotic and eukaryotic signal sequences, it is clear that prokaryotic signal sequence hydrophobic regions display a relatively higher frequency of alanine residues and a lower frequency of leucine residues than that seen in eukaryotic signal sequences and therefore a somewhat lower mean hydrophobicity (40). In comparing the optimal hydrophobicity by the Engelman hydrophobicity scale (47), the gain of function mutant displayed a small increase in the mean hydrophobicity of the hydrophobic region (−0.66 kJ/mol) (Table I) and an absolute increase in hydrophobicity of −6.7 kJ/mol, equivalent to 1.6 kcal/mol or the free energy of transfer of a single alanine residue. Despite such seemingly minor changes in hydrophobicity, nascent chains bearing the LamB* signal peptide were efficiently targeted to and translocated across the ER membrane. That such a relatively small change in the physical properties of the signal peptide would yield such a dramatic functional difference was unexpected and suggested that the gain of function mutant differed in the protein/protein interactions experienced by the signal sequence in its interactions with the translocation machinery.

In chemical cross-linking experiments, it was observed that the LamB* signal peptide interacted substantially more efficiently than the wild type LamB with both SRP and membrane protein components of the ER. In interpreting these data, two hypotheses were considered: (i) that the increase in signal sequence/SRP interactions displayed by LamB* was alone responsible for the gain of function activity, or (ii) that the gain of

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**TABLE I**

Mean hydrophobicity of the hydrophobic core of a subset of signal sequences

| Protein               | Mean hydrophobicity | Sequence               |
|-----------------------|---------------------|------------------------|
| LamB wild type        | 8.96                | LAVAVAAAGVM            |
| LamB*                 | 9.62                | LALALLAGVM             |
| VSV-G                 | 9.84                | CILYLALFLFID          |
| plpG                  | 10.80               | IWIFLFLSG             |
| Preprolactin          | 10.35               | LLLLIVS                |
| Ovalbumin             | 9.96                | IAIMSALAMV             |
| Growth hormone        | 9.78                | WLLIFSILCLLW           |

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2 T. Zheng and C. V. Nicchitta, unpublished observations.
function occurred through an increased efficiency of interaction with both cytosolic (SRP) and ER membrane protein signal recognition components (TRAM and Sec61p). To discern between these two possibilities, experiments were performed in which the SRP/SRP receptor-dependent targeting stage was bypassed, and the interaction of both the LamB and LamB* signal peptides with the membrane was studied directly. To accomplish this, LamB and LamB*-bearing nascent chains were synthesized and denatured in guanidinium/HCl, and the denatured nascent chains were diluted into a reaction mix containing ER microsomes. In principle, this experimental system is identical to that used to identify post-translational translocation of the yeast precursor, prepro-o-factor, into yeast microsomes (48, 49). In these experiments, the LamB and LamB* signal peptides bound to ER membrane with similar efficiencies. In addition, the membrane-bound nascent chains were insensitive to extraction in high salt buffers, indicating that binding was occurring through the activity of the signal sequence. These data indicate that the LamB and LamB* signal sequences can associate with components of the ER membrane, if presented in the appropriate structural form. On the basis of these data and the arguments presented above, we propose that the inability of the wild type LamB signal peptide to initiate translocation is due to a conformational flaw generated through consequence of its relatively weak interactions with SRP. In this hypothesis, the affinity between the LamB signal peptide and SRP is sufficient to support targeting but not sufficient to prevent the signal peptide from assuming a conformation that eliminates or greatly reduces its ability to bind to membrane components of the ER. Thus, SRP is proposed to function as both a targeting factor and a signal sequence-specific molecular chaperone. As a chaperone, SRP will maintain the signal sequence in a conformation suitable for binding to membrane components of the ER.

Interpreting SRP/signal sequence interactions in context of both a targeting and a chaperone activity may provide an additional explanation for previous studies demonstrating that the translocation of secretory protein precursors into mammalian microsomes is dependent on the properties of the hydrophobic region of the signal sequence (20, 46, 50). In particular, the study of Belin et al. (46) noted that the interaction of SRP with the peptide sequence, as determined in elongation arrest assays, did not correlate with the ability of the signal peptide to initiate translocation. That signal sequence function can be clearly segregated into targeting and translocation functions raises a fundamental question. If the structure and physical properties of the signal sequence determine access to the translocon, is the second stage of signal sequence recognition (20) in actuality a measure of signal sequence conformation, as determined by the particular steric characteristics of the signal sequence/SRP interaction?

As noted previously, eukaryotic signal sequences differ notably from their prokaryotic relatives in their relatively high statistical abundance of leucine in the hydrophobic core domain (40). Nonetheless, the amino acid side chains of leucine, isoleucine, and to a lesser degree valine, display very similar physical properties and are on a phylogenetic basis interchangeable (47, 51, 52). Why then, if hydrophobicity is the primary determinant for a functional hydrophobic domain, is leucine statistically overrepresented? The data presented herein argue that the relative abundance of leucine residues in the hydrophobic core is of clear functional significance. We propose that a leucine-rich hydrophobic domain allows the signal sequence to interact with SRP54 in a manner that maintains the unfolded structure necessary for subsequent binding to components of the ER membrane.