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The Hydrophobic Region of Signal Peptides Is a Determinant for SRP Recognition and Protein Translocation across the ER Membrane[1]

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Newly synthesized mammalian secretory proteins such as preprolactin are translocated across the endoplasmic reticulum (ER) in a signal recognition particle (SRP)-dependent manner. Recent studies revealed that there are two recognition steps for signal peptides during this translocation. The first step is recognition by SRP, which results in elongation arrest, and the second step is interaction between signal peptides and the translocation channel embedded in the ER membrane. To determine the roles of the hydrophobic region of signal peptides in the recognition by SRP and the membrane-embedded translocation machinery, we constructed chimeric proteins consisting of the mature region of preprolactin and signal peptides containing different numbers of leucine residues. The translocation of these chimeric proteins was completely dependent on SRP, and the efficiency increased as the number of leucine residues increased up to 10 and then decreased. Although the efficiency of elongation arrest also increased as the number of leucine residues increased up to 10, it only slightly decreased as the number increased up to 20. Similar results were obtained when the hydrophobic region was replaced by alternate leucine and alanine residues, except that the most efficient translocation occurred when the number was 14. Taken together, the present results suggest that the total hydrophobicity of the hydrophobic region of signal peptides is a determinant for recognition by both SRP and the membrane-embedded translocation machinery, although the specificities of the two signal recognition steps are slightly different from each other.

Key words: ER translocation, hydrophobic region, presecretory protein, signal peptide, SRP.

Secretory proteins are generally synthesized as precursor forms with an amino-terminal signal peptide. Many prokaryotic and eukaryotic signal peptides are interchangeable, implying that the functions of signal peptides have been conserved during evolution (*1*). Although signal peptides show no amino acid sequence homology, they have a common structural motif (*2*). In general, they consist of a positively charged amino-terminal region, a hydrophobic core region (H region), and a polar carboxyl-terminal region that usually contains the processing site for signal peptidase.

In *Escherichia coli*, secretory proteins are post-translationally translocated across the cytoplasmic membrane. Using a model secretory protein, proOmpF-Lpp, and an *in vitro* translocation system, we have extensively characterized the signal peptide of *E. coli* (for a review, see Ref. *3*). Our results suggested that the positively charged amino-terminal and H regions of the signal peptide are important for the translocation reaction (*4-6*). The importance of these regions is accounted for by the fact that they are both recognized by SecA (*7*)[3], which drives preproteins across the membrane using ATP and the proton motive force as energy sources (*8-10*).

In mammalian cells, secretory proteins are co-translationally translocated across the ER membrane. When a signal peptide emerges from the large ribosomal subunit, it is associated with the 54-kDa subunit of SRP, which results in elongation arrest of the nascent chain (for a review, see Ref. *11*). SRP mediates the targeting of the nascent chain-ribosome complex to the ER membrane *via* interaction with the SRP receptor. The docking of the complex to the ER membrane releases the elongation arrest, and the nascent chain is translocated into the ER through a channel that consists of the Sec61p complex and other proteins. Jungnickel and Rapoport (*12*) recently showed that the Sec61p complex is essential and sufficient for SRP-independent translocation. Thus, in the overall translocation reaction, there are at least two steps of recognition of signal peptides: recognition by SRP in the cytosol and subsequent recognition by the translocational machinery in the ER membrane.

Previous studies on the structural features of mammalian signal peptides revealed that the functional effi-

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Abbreviations: proOmpF-Lpp, a model secretory protein composed of proOmpF and the major lipoprotein of *E. coli*; proOmpF-PL, a model presecretory protein composed of proOmpF and prolactin; ER, endoplasmic reticulum; prePL, preprolactin; RM, rough microsome; ekRM, 25 mM EDTA and 0.5 M potassium acetate-washed rough microsome; SRP, signal recognition particle.

ciency of a signal peptide is directly related to its hydrophobicity (*13-16*), as in the case of *E. coli* signal peptides (*5, 6, 17-19*). However, it was not clear in those studies whether the hydrophobicity of the H region is important for the recognition by SRP or the membrane-embedded translocation machinery. In the present study, we constructed a series of proOmpF-PL proteins, *i.e.* chimeric proteins containing the mature region of preprolactin and the signal region of proOmpF with an H region of various lengths, and investigated their translocation efficiencies *in vitro*. We found that the total hydrophobicity, rather than the length of the hydrophobic stretch, of the signal peptide is an important factor for recognition by SRP and for subsequent translocation across the ER membrane.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA-modifying enzymes and SP6 RNA polymerase, were purchased from Takara Biomedicals. Proteinase K was obtained from Merck. EXPRE^{35}S^{35}S (1,175 Ci/mmol), a mixture of 73% [^{35}S]methionine and 22% [^{35}S]cysteine, was obtained from Du Pont-New England Nuclear. Dog pancreas RM membranes and wheat germ cell-free extracts were prepared as described previously (*20, 21*). Plasmid pK125 carries the *ompF-lpp* gene, which is under the control of the SP6 RNA polymerase promoter (*22*). L-series proOmpF-Lpp plasmids carry *ompF-lpp* genes encoding mutant proOmpF-Lpps, of which the signal peptide region has been changed so as to possess different numbers of leucine residues as the hydrophobic stretch (*5*). AL-series proOmpF-Lpp plasmids carry *ompF-lpp* genes encoding proOmpF-Lpps, of which the signal peptide region has been changed so as to possess different numbers of lysine residues at the amino terminus, and different numbers of alternate leucine and alanine residues as the hydrophobic stretch (*5, 6*). Plasmid pSPBP4 contains a cDNA insert for bovine prePL (*23*).

Plasmid Construction—ProOmpF-PL, a chimeric preprotein consisting of the signal peptide of proOmpF-Lpp and the mature region of bovine PL (residues 33-199), was prepared as follows. Plasmid pSPBP4, encoding prePL, was digested with *Sma*I and *Eco*RI, blunt-ended with T4 DNA polymerase, and then self-ligated. Deletion of the *Hin*dIII site of this plasmid was accomplished by digestion with *Hin*dIII, and then the plasmid was blunt-ended and ligated with an *Eco*RI linker (pd-GGAATTCC) to yield pSPBP4-1. A *Sac*I site was introduced before codon 84 of prePL by PCR using 5′-TTTCCGAGCTCAGGTCATG-3′ (the *Sac*I site is underlined) as the 5′ primer and an oligonucleotide corresponding to the SP6 promoter of pSBP4-1 as the 3′ primer. The resulting PCR product was digested with *Eco*RI and *Sac*I, and then purified by polyacrylamide gel electrophoresis. The resulting fragment is referred to as Sac-1. PCR was performed again using pSPBP4-1, as the template, and two primers (5′-CATGACCTGAGCTCGGAAA-3′ and 5′-CCCCGTACCTCGGTGAC-3′; the *Sac*I site is underlined). The PCR product was digested with *Sac*I and *Nco*I, and then gel-purified (Sac-2). Both Sac-1 and Sac-2 were subcloned into the *Eco*RI-*Nco*I site of pSBP4-1 to yield pSBP4-2. The sequences of the PCR amplified regions were confirmed by the dideoxy method (*24*). The plasmids for various mutant proOmpF-Lpps, which were used previously (*5*), were digested with *Eco*RI and *Sac*I. The *Eco*RI-*Sac*I fragments encoding signal peptides were inserted into the corresponding sites of pSBP4-2 to yield plasmids for various mutant proOmpF-PLs. The construction of a plasmid for a mutant proOmpF-PL lacking a signal peptide was carried out as follows. Two oligonucleotides (5′-AATTCCATGGCGGAGAGCT-3′ and 5′-CTCCGCCATGG-3′), which each contain an ATG codon, and both *Eco*RI and *Sac*I sites, were annealed and ligated to the *Eco*RI-*Sac*I site of pSBP4-2 to yield a plasmid encoding ΔSP-PL.

In Vitro Translation and Post-Translational Proteinase K Treatment—*In vitro* transcription and translation were carried out as described by Erikson and Blobel (*21*). Messenger RNAs were synthesized from plasmids that had been linearized with *Bam*HI (*25*). Translation was conducted at 26°C for 30 min in a wheat germ cell-free system in the absence or presence of RM membranes (1 equivalent per 15 μl; for a definition, see Ref. *20*). When ekRM membranes were used, mRNAs were first translated at 26°C for 5 min in the presence of SRP (final, 0.107 A_{280} unit/ml), and then ekRM membranes were added to the reaction mixtures, followed by incubation at 26°C for 20 min. ekRM membranes were prepared by washing RM membranes with 25 mM EDTA and 0.5 M potassium acetate as described previously (*20, 26*). After translation, the mixtures were incubated with proteinase K (final, 0.2 mg/ml) at 20°C for 10 min in the presence or absence of 1% Triton X-100. The protease treatment was terminated by adding 200 ml of 10% trichloroacetic acid, and the proteins were recovered by centrifugation. The precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis on 15% gels, followed by fluorography. The intensities of the precursor and mature bands of OmpF-PLs were quantified by densitometer scanning. The processing efficiency (%) was calculated as follows:

$$\text{processing efficiency (\%)} = [(8/6)\,\text{mature}/((8/6)\,\text{mature}+\text{precursor})] \times 100.$$

Elongation Arrest Analysis—SRP was purified from RM by sucrose density gradient centrifugation (*26, 27*). mRNAs were translated in the presence or absence of purified SRP (final, 0.107 A_{280} unit/ml) at 26°C for 20 min. To correct for variability in translational efficiency in the absence (−) or presence (+) of SRP, the relative levels of preproteins were determined from the respective densitometric intensities by multiplying by the ratio of ΔSP-PL synthesized in the absence (−) and presence (+) of SRP. The elongation arrest efficiency (%) was calculated as follows:

$$\text{elongation arrest efficiency (\%)} = \{1-[\text{precursor}\,(+)\times\Delta\text{SP-PL}\,(-)]/[\text{precursor}\,(-)\times\Delta\text{SP-PL}\,(+)]\}\times 100.$$

RESULTS

ProOmpF-PL Is Translocated across the ER Membrane—In previous studies, we constructed a large number of mutants of the proOmpF-Lpp protein to examine the roles of the positively charged amino-terminal region and the H region of the signal peptide in the translocation across the *E. coli* cytoplasmic membrane (*4-6*). In the present study, we first examined whether or not these proteins are applicable to an *in vitro* mammalian translocation system.

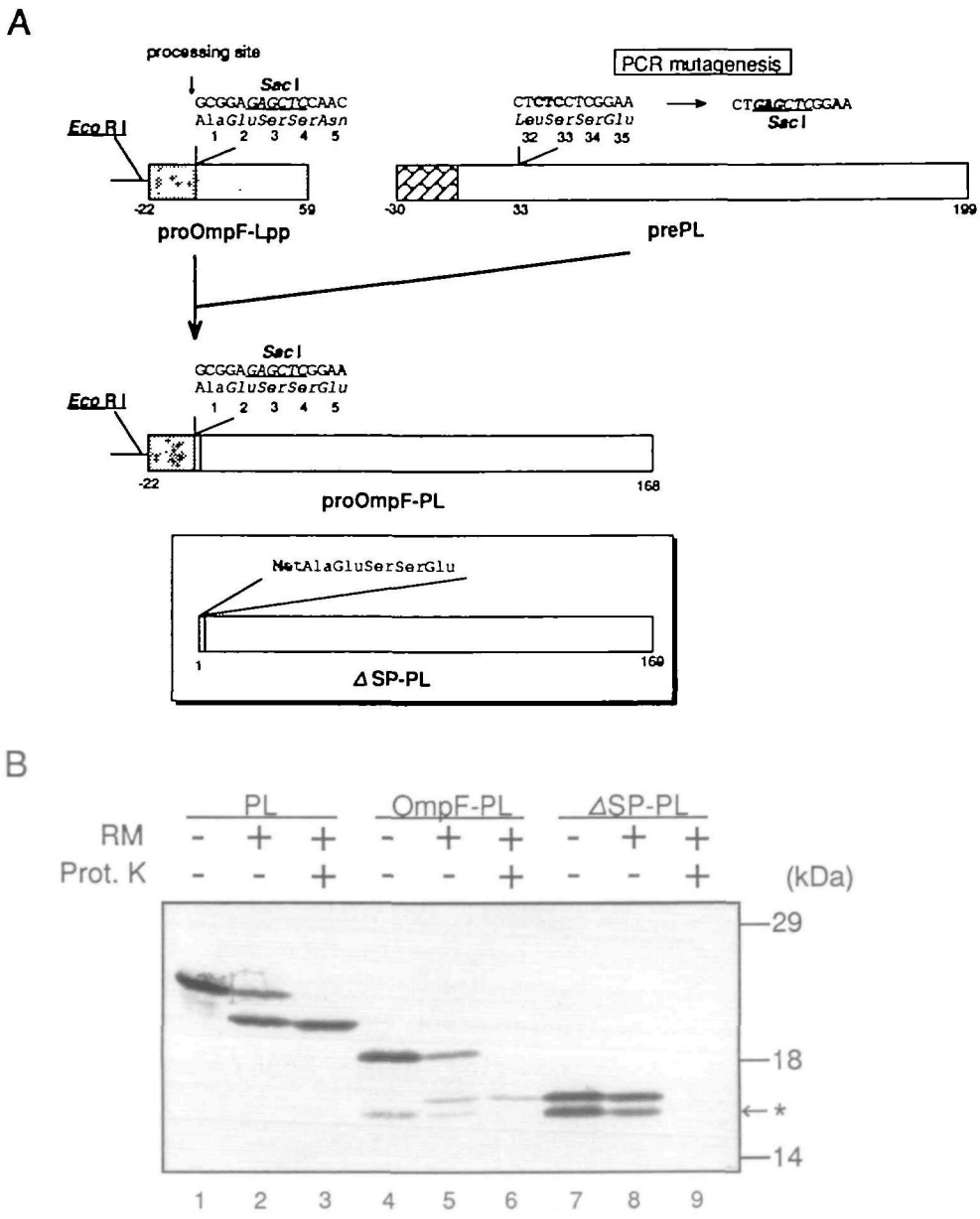

Fig. 1. **Schematic representation of chimeric proOmpF-PL construction (A), and *in vitro* translation, processing, and translocation across RM membranes (B).** A: At the cDNA level, the N-terminus (33 amino acids) of bovine prePL, which was altered by PCR mutagenesis, was replaced with a signal peptide containing the N-terminus (25 amino acids) of proOmpF-Lpp. The resulting chimeric proprotein was named proOmpF-PL. ⊿SP-PL, which lacks the signal peptide of proOmpF-PL but contains an initiation methionine, was also constructed. B: mRNAs encoding prePL (lanes 1-3), proOmpF-PL (lanes 4-6), and ⊿SP-PL (lanes 7-9) were translated in a wheat germ cell-free system in the absence (−) or presence (+) of RM (1 eq/15 μl). After incubation at 26°C for 30 min, proteinase K (final concentration, 0.2 mg/ml) treatment (+) was performed. The proteins were precipitated, separated by SDS-polyacrylamide gel electrophoresis (15% gel), and then analyzed by autoradiography with a Fujix Bioimage Analyzer BAS-2000II. The minor band arising from the translation of proOmpF-PL series mRNAs is indicated by an asterisk (lanes 4, 5, 7, and 8).

When the gene encoding proOmpF-Lpp was transcribed with SP6 RNA polymerase and then translated in a wheat germ cell-free system in the presence of RM membranes, only a small amount of a translocated polypeptide was observed (data not shown). Since proOmpF-Lpp consists of only 81 amino acid residues, it may be released from the ribosomes before the signal peptide in the nascent polypeptide is recognized by SRP. We therefore constructed a gene encoding a chimeric proprotein (proOmpF-PL) that contains the signal peptide region of proOmpF-Lpp and a part of the mature region of preprolactin (residues 33-199) (Fig. 1A). Preprolactin has been used as a substrate for ER-translocation analyses. Transcription-translation of the proOmpF-PL gene gave a major product of an apparent molecular mass of 20 kDa and a minor one of 15.5 kDa (Fig. 1B, lane 4). The mass of the former species was in good agreement with that of the expected precursor. These two species were susceptible to proteinase K, suggesting that

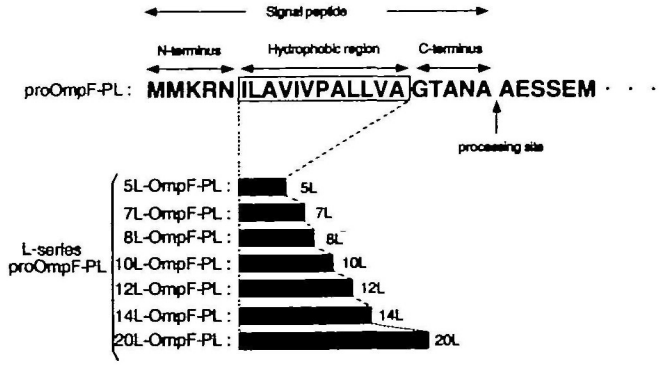

Fig. 2. **Structures of the signal peptide regions of primary and L-series proOmpF-PLs.** The hydrophobic region of proOmpF-PL, from amino acid 6 to 17, was replaced with clusters of various numbers of leucine residues, as shown in the figure. The closed bars represent leucine residues. The numbers at the ends of the bars are the total numbers of leucine residues comprising the hydrophobic region.

they represent cytosolic forms of the chimeric protein. When RM membranes were included in the assay mixture, a 16-kDa product was detected. The difference in mass between the 20- and 16-kDa species corresponded to the mass of the signal peptide. The 16-kDa species was resistant to proteinase K, suggesting that it is the translocated mature form. This translocation was completely dependent on the presence of the signal peptide derived from proOmpF. No proteinase K-resistant species was observed when the signal was omitted from proOmpF-PL (ΔSP-PL) (Fig. 1B, lane 9).

Effect of the Polyleucine Stretch in the H Region of the Signal Peptide on the Efficiency of Translocation—We previously constructed proOmpF-Lpp proteins with various numbers of leucine residues (L-series) to determine the function of the H region of *E. coli* signal peptides (*5*). In the present study, we constructed an L-series, proOmpF-PLs (5L-, 7L-, 8L-, 10L-, 12L-, 14L-, and 20L-OmpF-PL) (Fig. 2), and examined their translocation efficiencies. As shown in Fig. 3, A and B, the translocation efficiency increased as the number of leucine residues increased up to 10, and then decreased. It seemed that the impaired translocation of 20L-OmpF-PL was not due to the aggregation of the precursor peptide on the RM membranes because it was proteinase K-sensitive even in the absence of Triton X-100.

Translocation of L-Series proOmpF-PLs across the ER Membrane Is Dependent on SRP—We next examined whether or not the translocation of L-series proOmpF-LPs is dependent on SRP. As shown in Fig. 4, A and B, no significant translocation was observed of either mutant protein when SRP was not added to the assay mixture, suggesting that the reaction is completely dependent on SRP. As observed in translocation assays involving RM membranes (Fig. 3B), the processing efficiency increased as

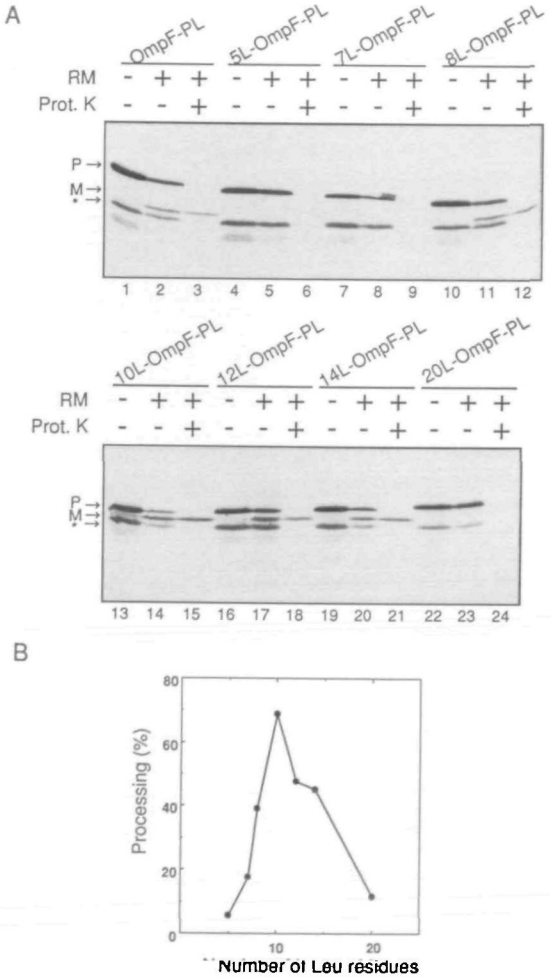

Fig. 3. **Effects of the number of leucine residues on the processing and the translocation across RM membranes.** A: mRNAs were translated in the absence (−) or presence (+) of RM, and then the translates were treated with proteinase K (+). The positions of the precursor (P) and mature (M) forms of L-series proOmpF-PLs are indicated, and the asterisk indicates the minor band arising on translation of mRNAs. B: The efficiency of processing was dependent on the number of leucine residues. After quantification of each band on the gel (A: lanes 5, 8, 11, 14, 17, 20, and 23) with a Fujix Bioimage Analyzer BAS-2000II, the efficiency of processing was calculated using the formula given in the text.

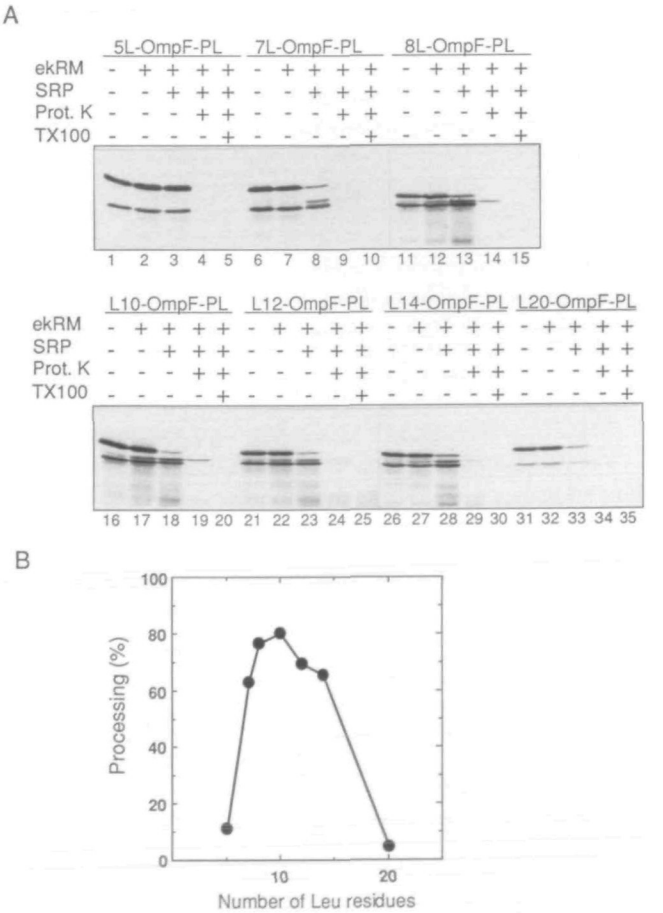

Fig. 4. **Translation of L-series OmpF-PLs in assays involving ekRM membranes in the presence of SRP.** A: mRNAs were translated in the absence (−) or presence (+) of SRP (final concentration, 0.107 A_{280} unit/ml). The translation mixtures were incubated in the absence (−) or presence (+) of ekRM (1 eq/15 μl) at 26°C for 20 min. The translation products were digested with proteinase K (Prot. K) in the absence (−) or presence (+) of 1% Triton X-100 (TX100). B: The effect of the number of leucine residues on the processing of proOmpF-PLs. After quantification of each band on the gel (A, lanes 3, 8, 13, 18, 23, 28, and 33) with a Fujix Bioimage Analyzer BAS-2000II, the efficiency of processing was calculated using the formula given in the text.

the number of leucine residues increased up to 10, and then gradually decreased.

Effect of SRP on the Elongation of L-Series proOmpF-PLs—Translocation across the ER membrane can be divided into two steps; the recognition step by SRP, which results in the elongation arrest of the nascent polypeptide chain, and the step of insertion of the polypeptide chain into the lumen of ER through the translocation channel (*11*).

To examine the interaction between SRP and the signal peptide region of proOmpF-PL, we examined the effect of SRP on the elongation of L-series proOmpF-PLs. Although the mechanism underlying the elongation arrest by SRP is not yet fully understood, it is a good means of evaluating the interaction between signal peptides and SRP because the interaction is strictly specific. As shown in Fig. 5A, no significant elongation arrest was observed for ΔSP-PL or 5L-OmpF-PL. The efficiency of the arrest increased as the number of leucine residues increased and became maximum for 10L-OmpF-PL (Fig. 5, A and B). The efficiency of elongation arrest only slightly decreased as the number of leucine residues increased from 10 to 20. These results indicate that the impaired translocation of proOmpF-PLs with long hydrophobic stretches is not due to the low affinity of SRP for their signal peptide regions.

Elongation Arrest and Translocation of KAL-Series proOmpF-PLs—We showed in Fig. 3 that the rate of translocation is determined by the number of leucine residues comprising the H region of signal peptides. This may suggest that the length of the hydrophobic polypeptide is important for efficient translocation. Another possibility is that the total hydrophobicity, rather than the number of hydrophobic residues, is important for translocation. To determine which of these possibilities is correct, we next examined the efficiencies of the elongation arrest and translocation of KAL-series proOmpF-PLs, in which two (2K) or no (0K) lysine residues preceded the hydrophobic region consisting of alternate alanine and leucine residues (Fig. 6). Since alanine is less hydrophobic than leucine, it is expected that KAL-series proOmpF-Lpps have less hydrophobic signal regions than the corresponding L-series ones. Figures 7 and 8 show the results for the elongation arrest and processing of KAL-series proOmpF-PLs. The results were very similar to those for L-series proOmpF-PLs, except that a larger number of alternate alanine and leucine residues is required for maximal elongation arrest and processing, suggesting that the total hydrophobicity, rather than the length of the hydrophobic stretch, is important for efficient translocation. Consistent with the previous finding that a positively charged residue is not required for translocation (*28*), the presence or absence of lysine residues in

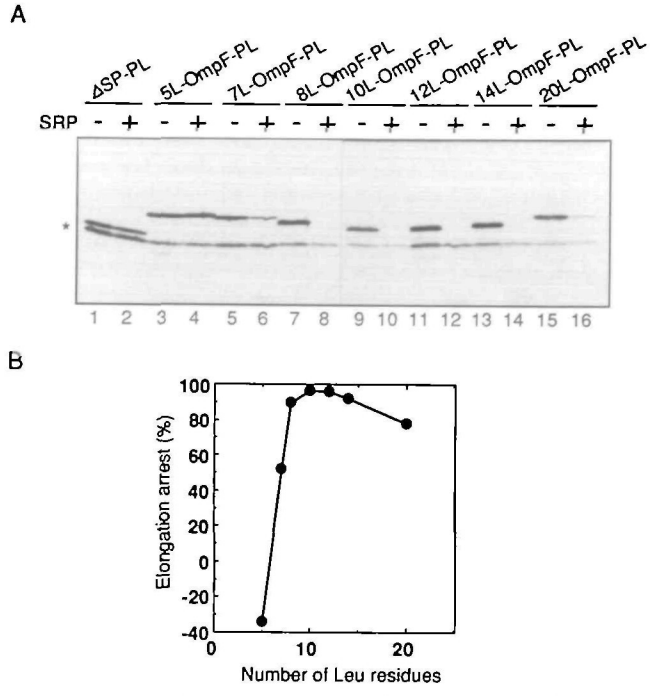

Fig. 5. **Effect of SRP on the elongation of proOmpF-PLs.** A: mRNAs encoding L-series proOmpF-PLs and ΔSP-PL were translated in a wheat germ cell-free system in the absence (−) or presence (+) of the purified SRP (final concentration, 0.107 A_{280} unit/ml) at 26°C for 20 min. B: The efficiency of elongation arrest was dependent on the number of leucine residues up to 10. After quantification of each band on the gel with a Fujix Bioimage Analyzer BAS-2000II, the efficiency of translation inhibition was calculated using the formula given in the text.

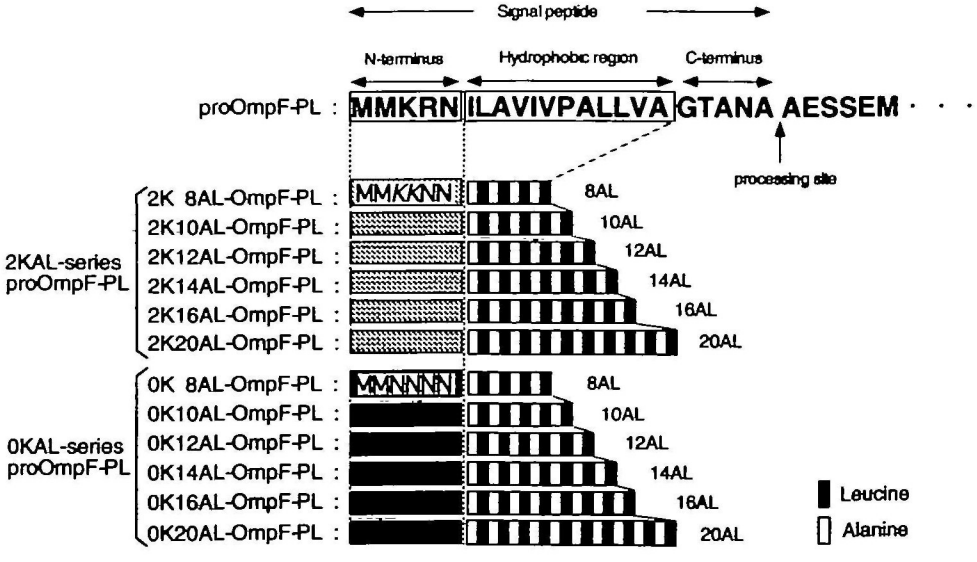

Fig. 6. **Structures of the signal peptide regions of primary and KAL-series proOmpF-PLs.** The hydrophobic region of proOmpF-PL, from amino acid 6 to 17, was replaced with clusters of alternate alanine and leucine residues, and the amino acid residues of the N-terminal region, from amino acid 1 to 5, were replaced by the residues indicated, which included two lysine residues (2K) or no positively charged residue (0K), as shown in the figure. The closed and open bars represent leucine and alanine residues, respectively. The numbers at the ends of the bars are the total numbers of leucine/alanine residues comprising the hydrophobic region.

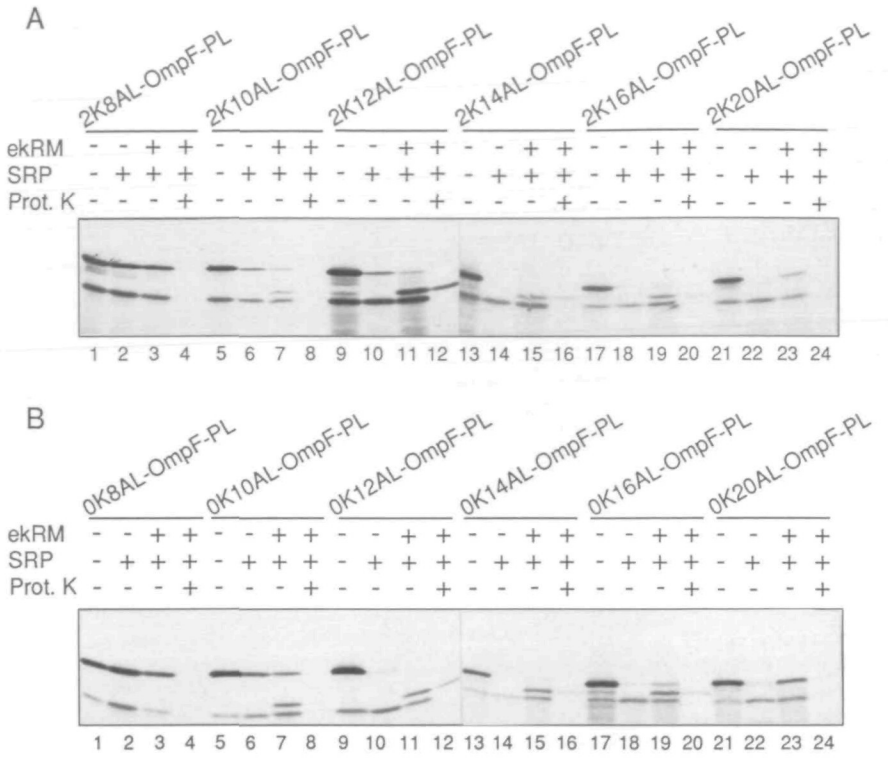

Fig. 7. **Translation inhibition, processing, and translocation of KAL-series proOmpF-PLs.** The experimental conditions were the same as given in the legends to Figs. 4A and 5A. 2KAL-series (A) and 0KAL-series (B) are shown.

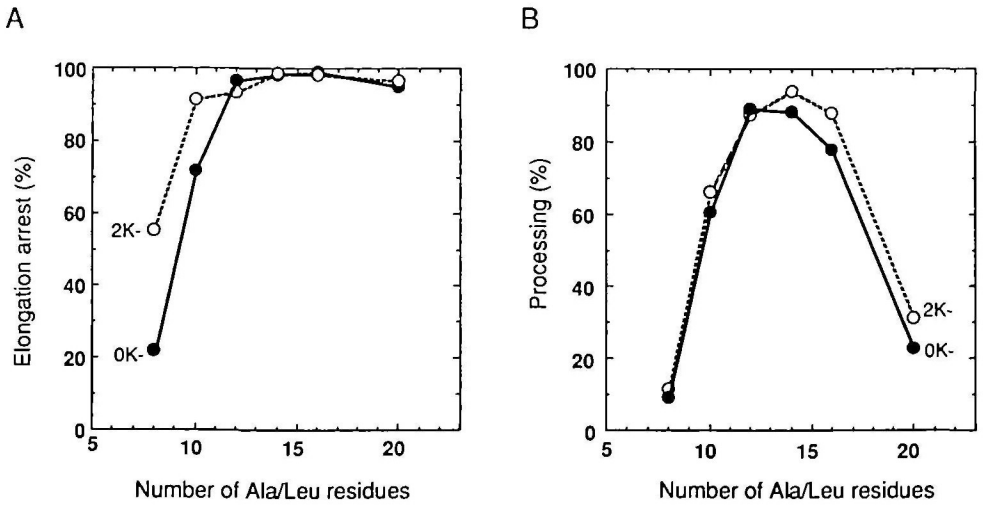

Fig. 8. **N-terminal positively charged amino acid residues had no effect on either binding to SRP or signal peptide processing.** The efficiencies of elongation arrest (A) and processing (B) of KAL-series proOmpF-PLs were calculated using the formula given in the text. The data for 2KAL- (○) and 0KAL- (●) series proOmpF-PLs were taken from Fig. 7.

the amino-terminal region had little effect on either reaction (Fig. 8).

DISCUSSION

In spite of the accumulation of a large amount of evidence that the hydrophobic region of signal peptides is important for translocation across the ER membrane (*13-16, 29*), the basic principles of the hydrophobic region, which is essential for translocation, remained unclear. This is most likely due to the fact that this region consists of a variety of amino acids and shows very great sequence diversity. To overcome this problem, we constructed L-series proOmpF-PLs containing polyleucine stretches, and KAL-series proOmpF-PLs containing alternate alanine and leucine residues, and investigated their translocation efficiencies.

The maximal translocation of L-series proOmpF-PLs in assays involving RM membranes and ekRM membranes was observed for 10L-OmpF-PL. However, it should be noted that more than 60% processing was observed for 7L-OmpF-PL in an assay involving ekRM membranes and exogenously added SRP, whereas less than 20% processing of the same precursor was observed with RM membranes. This difference may be due to the limited amount of SRP in our RM membrane preparation. In the case of 7L-OmpF-PL, the elongation arrest by SRP was not complete, suggesting that SRP exhibits low affinity for this precursor. It is therefore reasonable to assume that the amount of SRP

in the RM preparation is critical for elongation arrest and subsequent translocation of a precursor protein containing a small number of leucine residues in its signal peptide region. Belin *et al.* (*29*) also reported that RM membranes prepared according to a commonly used protocol do not contain a sufficient amount of SRP for the translocation of plasminogen activator inhibitor-2.

For KAL-series proOmpF-PLs, the maximal translocation was observed for 0K12AL-OmpF-PL and 2K14AL-OmpF-PL. Obviously, a larger number of hydrophobic residues (alanine and leucine) in the H region was required for efficient translocation compared with in the case of L-series proOmpF-PLs. Since alanine is less hydrophobic than leucine, this result suggests that the total hydrophobicity, rather than the number of hydrophobic residues, is important for translocation. This is also true for the elongation arrest by SRP.

One important observation in the present study is that the efficiencies of elongation arrest and translocation show different dependencies on the number of hydrophobic residues in the H region. For both L- and KAL-series proOmpF-PL, the efficiency of elongation arrest increased as the number of hydrophobic residues increased, and reached a maximum at 8–12 residues. The efficiency did not decrease as the number of hydrophobic residues increased up to 20. On the other hand, the translocation efficiency also increased as the number of hydrophobic residues increased up to 10–14 residues, but decreased over 14 residues. The fact that the elongation arrest of 20L- and 20AL-OmpF-PL was relieved on the addition of ekRM membranes implies that SRP normally docks at the ER membrane through the SRP receptor, although processing does not occur. In addition, the translocated precursors may not be aggregated, as judged by the proteinase K-sensitivity in the absence of Triton X-100. Therefore, the impaired processing of precursors containing longer hydrophobic stretches may be due to the failure of recognition of the precursors by the translocation machinery involved in the processes occurring after the nascent chain-ribosome-SRP complex binds to the membrane. Another interpretation is that the too hydrophobic signal peptide may function as a signal-anchor (SA) sequence. The SA sequence mediates the translocation of the following portion of a polypeptide without being cleaved by signal peptidase, and anchors the protein in the membrane (*30–32*). Judging from the proteinase K-sensitivity of precursors, the signal peptides of 20L- and 20AL-OmpF-PL may be anchored in the N_{exo}/C_{cyt} orientation (type I signal-anchor) in the membrane through recognition by the translocation machinery, although we did not directly examine this possibility in the present study. Our finding that SRP and the membrane-embedded translocation machinery exhibit different specificities for the H region in the signal peptide is consistent with the recent finding that signal recognition occurs not only in the SRP-recognition step, but also in the step of insertion through the membrane-embedded channel (*12, 29*).

Another interesting finding is that the optimal number of hydrophobic residues required for translocation differs between prokaryotic and eukaryotic cells. In *E. coli*, L-series and AL-series proOmpF-Lpps exhibit a sharp response in the rate of translocation to the number of hydrophobic amino acid residues (*5*). The optimal numbers of hydrophobic residues are 8 and 10 for the translocation of L-series and AL-series proOmpF-Lpps, respectively. When the number of hydrophobic residues is 2 greater or smaller than the optimal number, no significant translocation occurs. On the other hand, as shown in the present study, 10 and 14 hydrophobic residues for L-series and AL-series proOmpF-PLs, respectively, were required for the optimal translocation across the ER membrane. In addition, these precursors show a broader response in the efficiency of translocation to the number of hydrophobic residues. These differences between prokaryotic and eukaryotic systems probably reflect the different features of the translocation machineries.

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