The Requirement of a Positive Charge at the Amino Terminus Can be Compensated for by a Longer Central Hydrophobic Stretch in the Functioning of Signal Peptides

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A variety of model presecretory proteins, proOmpF-Lpps, possessing different numbers of lysine residues (0, 2, and 4) and as positively charged amino acid residues and different numbers of leucine residues (7, 8, and 9) as hydrophobic amino acid residues in their signal peptides were constructed. The effect of positive charges on the in vitro translocation efficiency markedly differed with the number of leucine residues. Positive charges were strongly required for translocation when the hydrophobic region comprised 7 or 8 leucine residues, whereas the translocation of proOmpF-Lpps possessing 9 leucine residues took place efficiently even in the absence of positive charges and the introduction of positive charges did not significantly enhance the translocation efficiency. The translocation of all the proOmpF-Lpps, including one possessing no positive charge, was ATP-, protonmotive force-, and SecA-dependent and accompanied by signal peptide cleavage, indicating that they are translocated via the usual secretory pathway. It is likely that the requirement of positive charges can be compensated for by a longer hydrophobic stretch in the functioning of the signal peptide.

Signal peptides that are attached to the amino termini of secretory proteins play indispensable roles in the process of protein translocation across the cytoplasmic membranes of prokaryotic cells or the endoplasmic reticulum membrane of eukaryotic cells. Signal peptides are characterized by a positively charged amino-terminal region and a hydrophobic central region (1, 2).

The importance of amino-terminal positive charges in protein translocation has been demonstrated both in vivo (3-6) and in vitro (7). The interaction of this charged region with SecA, an essential component of the secretory machinery of Escherichia coli (8, 9), in the process of protein translocation has been suggested (10); an increase in the number of positively charged residues at this position results in an increase in the extent of cross-linking of a presecretory protein with SecA, with a concomitant increase in the in vitro translocation rate.

The importance of the central hydrophobic region has also been demonstrated in vivo through the deletion of this region, the insertion of a charged amino acid residue, and the creation of a new hydrophobic domain (11-13). Biophysical studies also demonstrated the importance of a particular conformation of this region in protein translocation (14, 15). Furthermore, artificial signal peptides possessing different lengths of one or two species of hydrophobic amino acid residues have been systematically constructed on a DNA level, and their functions in protein translocation were studied both in vivo (16-19) and in vitro (20). The results demonstrated the existence of a critical size for individual hydrophobic stretches. They also showed that total hydrophobicity is an important factor that determines the function of the hydrophobic region.

Thus, so far, the role of the amino-terminal positively charged region and that of the central hydrophobic region in protein translocation have been studied rather independently. On the other hand, a genetic study suggested that the functions of these two regions may be interrelated (21). In the present work, based on the results of our previous studies on model presecretory proteins, proOmpF-Lpps, possessing different numbers of lysine residues as amino-terminal positively charged residues (7) or different numbers of leucine residues as hydrophobic amino acid residues (20) in their signal peptide domains, we have constructed a variety of model secretory proteins possessing different combinations of charged regions and hydrophobic regions. The model presecretory proteins were then examined as to in vitro translocation. The effect of positive charges on the translocation efficiency markedly differed with the number of leucine residues in the hydrophobic region. Positive charges were strongly required for translocation when the number of leucine residues was 7 or 8, whereas they were not required when the number was 9. It is suggested that the function of the amino-terminal positively charged residues and that of the hydrophobic region are interrelated.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA-modifying enzymes and SP6 RNA polymerase were purchased from Takara Shuzo Co. Proteinase K was from Merck. Tran32P-S-label, a mixture of 70% [35S] methionine (1000 Ci/mmol; 1 Ci = 37 GBq) and 29% [35S]cysteine, was obtained from ICN Radiochemicals. ProOmpF-Lpp, a hybrid presecretory protein (22), and mutant proteins derived from it through gene manipulation were used as substrates for in vitro translocation.

Bacterial Strains—The E. coli strains used were K003 (LppΔunjB-C-Δn10) for preparations of everted membrane vesicles and

1 The abbreviations used are: proOmpF-Lpp, a model presecretory protein composed of proOmpF and the major lipoprotein of Escherichia coli; SDS, sodium dodecyl sulfate; AMP-PNP, adenosine 5'-diphosphate; ΔH⁰, protonmotive force; Δψ, membrane potential.
Domain Functions of Signal Peptide

S100 (23), MM66 (F-, ΔlacI69, araP139, rplA, relA, thi, sec444, rnx19, ssp3, trpl98) (24) for preparations of SecA-depleted membrane vesicles and SecA-depleted S150, and MH1160recA (F-, ΔlacI69, araD139, rpsL, relA, thyA, fimB, recA, ompR) (25) for DNA manipulation.

In Vitro Transcription and Translation—In vitro transcription of genes encoding proompF-Lpps and mutants derived from it was carried out with SP6 RNA polymerase as described (26), and the translation reaction was performed as described (7).

Sonication of Translation Products in 6 M Urea and Translocation Reaction—The mixture after translation was mixed with an equal volume of 12 M urea dissolved in 50 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol and then subjected to sonication to keep the translation products competent as to translocation, as described previously (20). The translocation reaction was started by adding this mixture to 9 volumes of the translocation mixture (50 mM potassium phosphate (pH 7.6), 1 mM ATP, 5 mM succinate, 100 μg/ml creatine kinase, 30 mM creatine phosphate, 20 ng/μl partially purified SecA (9), and 0.3 μg/μl everted membrane vesicles), which had been preincubated at 37°C for 5 min. The translocation mixture was then treated with 1 mg/ml proteinase K at 25°C for 10 min, and the translocates were detected by SDS-polyacrylamide gel electrophoresis followed by fluorography (20). The amount of protein in the OmpF-Lpp band on a gel was determined densitometrically with a Shimadzu CS-930 chromato-scanner. The efficiency of translocation was expressed as the ratio of the intensities of protein bands on fluorograms before and after the proteinase K treatment.

Removal of Small Molecules from the Translation Mixture by Gel Filtration—The removal of small molecules from the translation mixture was carried out as described previously (20).

Removal of SecA from Everted Membrane Vesicles with 6 M Urea—Everted membrane vesicles prepared from K003 (27) were suspended in 50 mM potassium phosphate (pH 7.6), mixed with 0.75 volume of 8 M urea and then placed in ice water for 1 h. The mixture was then centrifuged at 150,000 × g for 30 min at 4°C and the pellet was suspended in 50 mM potassium phosphate (pH 7.6). More than 80% of the SecA was recovered from the membranes through this treatment (29).

Removal of Small Molecules from the Translation Mixture by Gel Filtration—The removal of small molecules from the translation mixture was carried out as described previously (20).

Construction of KL-series Plasmids—These 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 leucine residues as the hydrophobic stretch. An outline of the construction and the structures of the encoded mutant proompF-Lpps are given in Fig. 1 and Table I, respectively. pk127 carries the ompF-lpp gene (uncleavable), which is under the control of the SP6 promoter (26), pK127 was digested with EcoRI and SstI to prepare an EcoRI-SstI large fragment. The EcoRI and the SstI site were located in the upstream region and at the carboxyl end of the signal peptide, respectively. Separately, synthetic DNA fragments encoding the carboxy-terminal region of the signal peptide, one encoding the amino-terminal region with different numbers of lysine residues, and ones encoding various lengths of leucine polymers were prepared as described previously (7, 20). The synthetic DNA fragments were then ligated with the EcoRI-SstI large fragment of pk127 to construct KL-series plasmids. The DNA sequences of all the mutant ompF-lpp genes thus constructed were confirmed as described (30).

**RESULTS**

**Structures of ProompF-Lpp Derivatives Possessing Different Numbers of Polyleucine and Polyleucine Residues in the Amino-terminal and Central Regions of the Signal Peptide Domain, Respectively**—In a previous study (20), we showed that the proompF-Lpp possessing 7, 8, or 9 leucine residues as the hydrophobic domain of the signal peptide exhibit in vitro translocation activity. The amino terminus of these proteins possesses two positively charged residues, lysine and arginine. In the present work, we introduced different numbers of lysine residues into the amino-terminal regions of these polyleucine-possessing proompF-Lpps and examined them as to their in vitro translocation. The primary structures and names of these proompF-Lpp derivatives are summarized in Table I.

**Requirement of Amino-terminal Positive Charges for Translocation Differs with the Length of the Hydrophobic Polyleucine Stretch**—Kinetic studies as to in vitro translocation indicated that the effect of positively charged residues at the amino terminus of signal peptides on the rate of their translocation markedly differed with the number of leucine residues comprising the hydrophobic stretch (Fig. 2). When the hydrophobic region comprised 8 leucine residues, the positive charge was almost completely essential, as in the case of proompF-Lpp derivatives possessing the wild-type hydrophobic region (Fig. 2A). Although the rate of translocation was very slow when the hydrophobic domain comprised 7 leucine residues, the rate of translocation was also positive charge-dependent (Fig. 2A). With 9 leucine residues, on the other hand, appreciable translocation activity was observed even in the absence of positively charged residues, and the activity was not enhanced significantly when the number of positively charged residues was introduced. In all cases, the translocation was accompanied by the appearance of mature forms of proompF-Lpps (Fig. 2B). The appearance was translocation-dependent (Fig. 2B) and independent of proteinase K-treatment (data not shown), indicating that the observed translocation was genuine.

The distance on the gel between the precursor and mature forms markedly differed with the number of amino-terminal positively charged residues (Fig. 2B). This is most likely due to the retardation effect of positive charges on the rate of migration on SDS-gel electrophoresis as described (31). The charge-dependent difference supports the view that these proompF-Lpps differed only with the number of positive charges at the amino terminus. Migration positions of mature proteins were the same throughout all proompF-Lpps.

It should be mentioned that the rate of translocation of 2KL was significantly slower than that of 8L with the wild-type amino-terminal sequence, which also possesses two positively charged residues, Lys and Arg (20). 7L with the wild-type amino-terminal sequence exhibited appreciable translocation (20), whereas 2KL was almost incompetent. It should be noted in this respect that 8L and 7L with the wild-type amino-terminal sequence have only one Asn between the basic
proteins, which were proteinase K-resistant, were then detected on cine residues in the previously reported that the number of positive charges is the of 1 mg/ml proteinase K for 10 min at 25 °C. The translocated mature translocation of KL-series proOmpF-Lpps. The reaction was terminated on ice, followed by treatment with 15 μl of urea, 10-fold diluted with the translocation mixture, and then subjected to the translocation assay at 57 °C. At the indicated times, aliquots (60 μl) were withdrawn, and the reaction was terminated on ice, followed by treatment with 15 μl of 1 mg/ml proteinase K for 10 min at 25 °C. The translocated mature proteins, which were proteinase K-resistant, were then detected on an SDS-polyacrylamide gel by means of fluorography followed by densitometric scanning. In B, fluorograms (except those for 0K7L, 2K7L, and 4K7L) are shown with translocation times (min). One-sixth amounts of the proOmpF-Lpps added to the individual reaction mixtures is at the right (P). The assay was also carried out in the absence of everted membrane vesicles (−MV). The positions of the precursor (p) and mature (m) forms of the OmpF-Lpps are also shown.

region and the hydrophobic stretch, whereas 2K8L and 2K7L have two Asn. 4K8L and 4K7L have no such residue between them. The distance between the two regions may be a critical factor that determines translocation efficiency, although we previously reported that the number of positive charges is the factor that determines the functional role of the aminoterminal region (7). This must be reexamined.

Translocation of KL-series ProOmpF-Lpps, Including One Possessing No Positive Charge at the Amino Terminus of the Signal Peptide, is ATP- and µH+−dependent—We examined the primary sequences of all the signal peptides of E. coli presecretory proteins so far reported with reference to the NBRF protein data base 28.0 (National Biomedical Research Foundation). All of them possess at least one positively charged amino acid residue at the amino terminus. This positively charged region plays an important role in the protein translocation, which requires ATP and ΔµH+ as sources of energy (3–7). We examined whether or not proOmpF-Lpps constructed in the present work, especially 0K9L that has no positive charge at the amino terminus, require ATP and ΔµH+ for their translocation. The translocation of all proOmpF-Lpps was completely inhibited in the presence of 10 mM AMP-PNP, suggesting the essential requirement of ATP (data not shown). The ATP requirement was further directly demonstrated for the proOmpF-Lpps, including 0K9L (Fig. 3, lanes 5 and 6–8). Fig. 3 also indicates that the translocation of 0K9L, as well as that of other proOmpF-Lpps, was ΔµH+−dependent (lanes 2–4 and 6–8). The role of ΔµH+ in protein translocation has been discussed in connection with the inside amino-terminal orientation of the signal peptide in the cytoplasmic membrane (32). The ΔµH+−dependent translocation of 0K9L, however, suggests that this is not the principal role of ΔµH+. SecA Is Also Involved in the Translocation of 0K9L but Its Effect on Translocation Differs between 0K9L and 4K9L—In the course of translocation, presecretory proteins are recognized by SecA, and the amino-terminal positively charged residues of the signal peptide play an important role in the recognition (10). Therefore, we examined whether the translocation of 0K9L is SecA-dependent or not, using urea-treated membrane vesicles, which are SecA-deficient. SecA enhanced the in vitro translocation of all proOmpF-Lpp derivatives, including 0K9L (Fig. 4A). The translocation was accompanied by signal peptide cleavage. Thus, it is most likely that the in vitro translocation observed here represents genuine translocation, and that even the signal peptide possessing no positive charge requires SecA in addition to ATP and ΔµH+ for it to function.

Although the rate of translocation of 0K9L into native membrane vesicles was comparable with that of 4K9L, the translocation of 0K9L into urea-treated (SecA-depleted) membrane vesicles was considerably slower than that of 4K9L, even in the presence of the sufficient amount of SecA (Fig. 4A). This was observed over a wide concentration range of SecA (Fig. 4B). This suggests the involvement of a urea-extractable factor, in addition to SecA, in the translocation of 0K9L. Identification of such a factor is now under way in this laboratory. It should be noted in this respect that the

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**TABLE I**

**Structures of the signal peptide regions of KL-series proOmpF-Lpp derivatives**

| Plasmid     | Gene product (proOmpF-Lpp derivative) | Amino terminus | Positively charged region | Central hydrophobic region | Carboxyl terminus to cleavage site |
|-------------|---------------------------------------|----------------|---------------------------|---------------------------|----------------------------------|
| pCH0K7L     | Wild-type 0K7L                         | MM             | KRN                       | ILAVYPALLVA                | GTANAAS                          |
| pCH2K7L     | 2K7L                                  | MM             | NNNN                      | LLLLLLLL                  | GTANASS                          |
| pCH4K7L     | 4K7L                                  | MM             | KKKK                      | LLLLLLLLL                 | GTANASS                          |
| pCH0K8L     | 0K8L                                  | MM             | NNNN                      | LLLLLLLL                  | GTANASS                          |
| pCH2K8L     | 2K8L                                  | MM             | KKKK                      | LLLLLLLLL                 | GTANASS                          |
| pCH4K8L     | 4K8L                                  | MM             | KKKK                      | LLLLLLLL                  | GTANASS                          |
| pCH0K9L     | 0K9L                                  | MM             | NNNN                      | LLLLLLLLL                 | GTANASS                          |
| pCH2K9L     | 2K9L                                  | MM             | KKKK                      | LLLLLLLLL                 | GTANASS                          |
| pCH4K9L     | 4K9L                                  | MM             | KKKK                      | LLLLLLLL                  | GTANASS                          |

*↓ denotes the signal peptide cleavage site.*
Concentration of SecA was 20 ng/μl. The translocation reaction was carried out in the presence of different concentrations of SecA for 3 min and the rate of translocation was expressed as the fraction (%) of the substrate that was translocated and processed in 3 min.

The translocation reaction was carried out using everted membrane vesicles from which SecA had been removed by the urea treatment. The translocation was carried out in the presence or absence of SecA, as indicated. B, translocation was carried out in the presence of different concentrations of SecA for 3 min and the rate of translocation was expressed as the fraction (%) of the substrate that was translocated and processed in 3 min.

Discussion

In the present work, we demonstrated that proOmpF-Lpp possessing 9 leucine residues as the hydrophobic stretch in the signal peptide was efficiently translocated even in the absence of amino-terminal positive charges (OK9L), and the translocation was not significantly enhanced upon the introduction of positively charged residues at the amino terminus. In contrast, the translocation of proOmpF-Lpps possessing 7 or 8 leucine residues as the hydrophobic stretch strongly depended on the presence of positively charged residues at the amino terminus. We conclude, therefore, that the requirement of positive charges can be compensated for by a longer hydrophobic stretch. The translocation of OK9L was ATP-, ΔH⁺-, and SecA-dependent and accompanied by signal peptide cleavage like that of other proOmpF-Lpps and naturally occurring presecretory proteins, suggesting that OK9L is translocated via the usual secretory pathway. Although we did not determine the amino-terminal amino acid residue of the precursor proteins, those synthesized in vitro most likely retain the initiator Met. It is probable, therefore, that the signal peptide of OK9L, which is functional, does not possess any positive charge at the amino terminus. We also constructed a similar series of proOmpF-Lpp derivatives containing polylysine and/or polyleucine, of which signal peptides are uncleavable. The requirement of amino-terminal positive charges for signal peptides possessing 8 leucine residues but not for those possessing 9 leucine residues was also observed (data not shown).

How can the functions of the two regions be interrelated? The positively charged region is recognized by SecA at the initial stage of the translocation reaction (10). Since the translocation of OK9L is SecA-dependent, it is probable that SecA recognizes the hydrophobic region as well. Chemical cross-linking studies have not proven the interaction so far, however. Alternatively, the membrane may play some role in the interrelation. The interaction of positive charges with the acidic residues of phospholipids on the inner surface of the membrane has been suggested (33-35). The involvement of Δψ, which is inside negative, in protein translocation also favors such an interaction. For a signal peptide possessing stronger hydrophobicity, such an electrostatic interaction may not be needed.

The compensation of the positive charge requirement by a longer hydrophobic stretch has been suggested genetically (21). A statistical study on eukaryotic presecretory proteins suggested that signal peptides possessing hydrophobic stretches, which are stronger in hydrophobicity, are more likely not to possess positive charges at their amino termini (36). We have performed a more extensive search as to this matter with reference to the NBRF protein data base. The results did not support this view, however (data not shown).
Consistent with our present results, some gene-engineered signal peptides possessing no amino-terminal positively charged residues were reported to be functional in vivo (3, 4, 6), suggesting that signal peptides do not necessarily possess an amino-terminal positive charge for their in vivo function. Interestingly, on the other hand, all signal peptides of E. coli listed in the NBRF protein data base possess at least one positive charge at their amino termini. The reason for this apparent discrepancy is unclear. Signal peptides possessing an amino-terminal positive charge may have some functional advantage.

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