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The hydrophobic region of signal peptides is involved in the interaction with membrane-bound SecA

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Abstract

The positive charges of signal peptides are important for the interaction with SecA, a translocation ATPase. To examine whether or not the hydrophobic region of signal peptides also interacts with SecA, we constructed model preproteins, proOmpF-Lpps, possessing no positively charged amino acid residues at the amino-terminus and different numbers of alanine/leucine residues in the hydrophobic region of signal peptides. When the hydrophobic stretch was sufficiently long, amino-terminal positively charged residues were not required for the translocation of preproteins across the cytoplasmic membrane of *Escherichia coli* both in vitro and in vivo. Chemical cross-linking between SecA and preproteins possessing no positively charged residues at the amino-terminus was observed only in the presence of liposomes containing acidic phospholipids. The degree of cross-linking increased as the length of the hydrophobic stretch increased irrespective of whether positively charged residues were present or not. A preprotein possessing no positively charged residues at the amino-terminus, which is competent in the presence of liposomes, competitively inhibited the cross-linking of wild-type proOmpF-Lpp with SecA under the same conditions. It is concluded that both the amino-terminal positive charges and central hydrophobic domains are involved in the interaction with SecA in the initial stage of translocation in addition to their possible roles in transmembrane movement of preproteins.

Keywords: SecA; Signal peptide; Protein translocation; Presecretory protein; Cross-linking; Acidic phospholipid; Hydrophobic region

1. Introduction

Presecretory proteins possess a signal peptide, which comprises amino-terminal positively charged amino acid residues and a stretch of hydrophobic amino acid residues [1,2]. The importance of the amino-terminal positively charged groups has been demonstrated in *Escherichia coli* both in vivo [3–5] and in vitro [6]. In vitro experiments demonstrated that the positive charge is essential for translocation

Abbreviations: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; proOmpF-Lpp, a model presecretory protein composed of proOmpF and the major lipoprotein of *Escherichia coli*; $\Delta\bar{\mu}H^+$, proton motive force; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; DTT, dithiothreitol; TCA, trichloroacetic acid

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and the rate of translocation increases as the positive charge number increases, irrespective of the amino acid residues that donate the charge [6].

The positive charge plays a role in the interaction of presecretory proteins with SecA [7], a translocation ATPase [8], that exists in both the cytoplasmic membrane and the cytosol [9,10]. The interaction was enhanced as the positive charge number increased, as in the case of translocation, indicating that the interaction thus demonstrated represents an essential step of the translocation reaction [7]. The possible role of the positive charge in the interaction of signal peptides with the cytoplasmic membrane in the process of protein translocation was also suggested [11].

Although the importance of central hydrophobic region of the signal peptides in protein translocation has been demonstrated [12–14], a critical study on the role of this region was rather difficult because of heterogeneity in amino acid composition. To overcome this difficulty, presecretory proteins possessing simple amino acid compositions in this region were constructed at the DNA level, and their function in translocation was examined in vivo [15–18] and in vitro [19]. An important conclusion drawn from these studies was that the total hydrophobicity is important for the functioning of the hydrophobic domain, and this importance has been discussed in terms of the functioning of this domain in the membrane. The in vitro studies, furthermore, showed that the requirement of the amino-terminal positive charge can be partly compensated for by a longer (stronger) hydrophobic stretch [20]. The latter findings suggest that the functions of the two regions are interrelated.

In the present work, we constructed, at the DNA level, a series of presecretory proteins possessing repeated alanine/leucine residues as the hydrophobic stretch. We found that preproteins possessing no positively charged amino acid residues at the amino-terminus are translocated when the hydrophobic stretch is sufficiently long. Cross-linking and competition studies revealed that the hydrophobic stretch also plays a role in the interaction of preproteins with SecA in the presence of liposomes containing acidic phospholipids. Based on these observations, the roles of the positive charge and the hydrophobic stretch in protein translocation will be discussed with special reference to SecA function.

2. Materials and methods

2.1. Materials

Restriction endonuclease, DNA-modifying enzymes and SP6 RNA polymerase were purchased from Takara Shuzo Co. Proteinase K was from Merck. Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine (1000 Ci/mmol; 1 Ci = 37 GBq), was obtained from ICN Radiochemicals, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), a cross-linking reagent, was from Sigma. *Staphylococcus aureus* V8 protease was purchased from ICN. Phosphatidylglycerol (PG) (egg yolk), phosphatidylethanolamine (PE) (*Escherichia coli*), phosphatidylcholine (PC) (soybean), and cardiolipin (CL) (*Escherichia coli*) were purchased from Sigma. ProOmpF-Lpp, a hybrid preprotein [21], and mutant proteins derived from it in the present study were used as substrates for in vitro translocation. SecA purified as described [7] was used. The method for the purification of *E. coli* deformylase encoded by the *def* gene will be described elsewhere.

2.2. Bacterial strains

The *E. coli* strains used were K003 (Lpp$^-$ *uncB-C*-Tn10) for the preparation of everted membrane vesicles and S100 [22], MM66 (F$^-$, *Δlac169*, *araD139*, *rpsL*, *relA*, *thi*, *secA^{am}*, *Tn19*, *su3^{ts}*, *trp^{am}*) [23] for the preparation of SecA-depleted S100, MH1160 *recA* (F$^-$, *Δlac169*, *araD139*, *rpsL*, *relA*, *thyA*, *flbB*, *recA*, *ompR*) [24] for DNA manipulation, and JM103 Lpp$^-$ [22] for in vivo labeling with Tran^{35}S-label and the purification of various proOmpF-Lpps.

2.3. In vitro transcription and translation

In vitro transcription of genes encoding proOmpF-Lpp and mutants derived from it was carried out with SP6 RNA polymerase as described [25], and the translation reaction was performed as described [6].

2.4. Translocation reaction

The translocation reaction (20 μl/tube) was initiated by adding the mixture after translation to 9

volumes of the translocation mixture (50 mM potassium phosphate (pH 7.6), 1 mM ATP, 5 mM $MgSO_4$, 5 mM succinate, 100 ng/ml creatine kinase, 30 mM creatine phosphate, 20 ng/μl purified SecA and 0.3 μg/μl total protein of everted membrane vesicles) [26] 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 translocated OmpF-Lpps were detected on an SDS-polyacrylamide gel. The amount of protein in the OmpF-Lpp band on the gel was determined by either fluorography which was followed by densitometrical scanning with a Shimadzu CS-9300PC chromatoscanner or a Fujix bioimage analyzer BAS-2000II. The efficiency of translocation was expressed as the ratio of the intensity of protein bands on the gel before and after the proteinase K treatment.

2.5. Removal of SecA from everted membrane vesicles

Everted membrane vesicles prepared from K003 [27] were suspended in 50 mM potassium phosphate (pH 7.6), mixed with 3 volumes of 8 M urea, and then placed on ice water for 1 h. The mixture was then centrifuged at $150\,000 \times g$ for 30 min at 4°C, and the pelleted membrane vesicles were suspended in 50 mM potassium phosphate (pH 7.6) [28]. More than 80% of the SecA was removed from the membranes through this treatment [29].

2.6. 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 [6].

2.7. Construction of plasmids carrying mutant ompF-lpp genes

These plasmids carry ompF-lpp genes encoding proOmpF-Lpps, in which the signal peptide region has been changed so as to possess different numbers of lysine residues (K) or arginine residues (R) at the amino terminus and different numbers of alanine/leucine residues (AL) as the hydrophobic stretch. The total number of amino acid residues at the positively charged amino terminal domain was fixed to four by filling the space with asparagine residues (N). The outline of the construction and the structures of the encoded mutant proOmpF-Lpps are given in Fig. 1 and Table 1. pK125 carries the *ompF-lpp* gene, which is under the control of the SP6 promoter [25]. pK125 was digested with *Eco*RI and *Sac*I to prepare the *Eco*RI-*Sac*I large fragment. The *Eco*RI and *Sac*I sites were located in the upstream region and at the carboxyl end of the signal peptide, respectively. Separately, synthetic DNA fragments encoding the carboxyl-terminal region of the signal peptide, and ones encoding various lengths of alanine/leucine polymers, were prepared as described previously [20], as shown in Table 1. The synthetic DNA fragments were then ligated with the *Eco*RI-*Sac*I large fragment of pK125 to construct plasmids carrying mutant *ompF-lpp* genes. The DNA sequences of all mutant *ompF-lpp* genes thus constructed were confirmed as described [30].

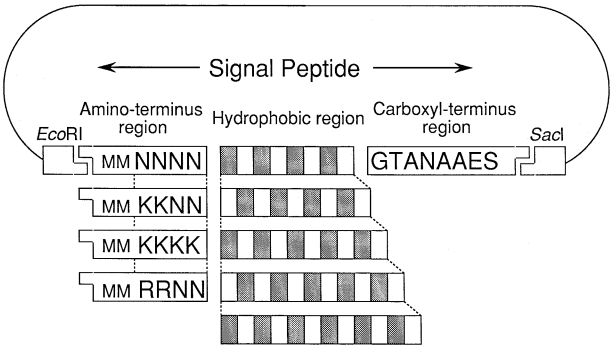

Fig. 1. Structure of the signal peptide regions of proOmpF-Lpp derivatives. Principal structures around the signal sequence region are shown. For convenience, the cleavage sites for *Eco*RI and *Sac*I, used for gene manipulation, are shown together. *Closed bars* and *open bars* represent alanine and leucine residues, respectively.

2.8. Construction of p4N12AL^{lac} and p2K2N12AL^{lac}

To construct these plasmids, p4N12AL and p2K2N12AL were treated with *Eco*RI and *Hin*dIII to isolate fragments carrying the *ompF-lpp* genes. They were then ligated with the *Eco*RI-*Hin*dIII fragment of pK025 [31], carrying the *lpp-lac* promoter-operator, to construct p4N12AL^{lac} and p2K2N12AL^{lac}, respectively.

2.9. In vivo translocation

The medium used was M9 supplemented with 50 μg/ml ampicillin. Cultures were incubated at 37°C to the exponential growth phase, labeled with 5 μCi of Tran^{35}S-label for 30 s, and then chased. The translocation reaction was stopped by adding an equal volume of 10% trichloroacetic acid (TCA), and the precipitate was analyzed by gel electrophoresis, followed by fluorography.

2.10. Cross-linking reaction

SecA was preincubated in the presence or absence of different phospholipid species for 10 min at 25°C and then mixed with 6 μl of the gel-filtered translation mixture containing ^{35}S-labeled proOmpF-Lpp derivatives (2×10^5 dpm), followed by further incubation for 10 min. A 0.11 volume of 25 mM EDAC was added, and then the incubation was continued for an additional 50 min for cross-linking. The cross-linking reaction was quenched for 10 min by the addition of 200 mM Tris-HCl (pH 7.5). All reactions were carried out at 25°C. Cross-linked complexes were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography.

2.11. Purification of proOmpF-Lpp, 4N12AL, 2K2N12AL and mature OmpF-Lpp

The purification was carried out as described [32] with some modification. ProOmpF-Lpp and 2K2N12AL were purified from JM103Lpp$^-$ harboring pK025 and p2K2N12AL^{lac}, respectively. The cytoplasmic membrane fraction prepared from these cells was solubilized with 1% (wt/vol) SDS containing 10 mM sodium phosphate (pH 7.2). An equal volume of 30% (wt/vol) TCA was added to the solution. After centrifugation at $8500 \times g$ for 10 min, the supernatant was dialyzed against 10 mM sodium phosphate (pH 7.2) and then mixed with 9 volumes of acetone. The precipitate was recovered by centrifugation at $8500 \times g$ for 10 min, and then solubilized with 8 M urea containing 1 mM dithiothreitol (DTT) and 50 mM potassium phosphate (pH 7.5).

4N12AL was purified from JM103Lpp$^-$ harboring p4N12AL^{lac}. Cells were suspended in 10 mM sodium phosphate (pH 7.2), 1 mM DTT, and then passed

Table 1
Structure of the signal peptide region of AL-series proOmpF-Lpp derivatives

Plasmid	Gene product (proOmpF-Lpp derivative)	Signal peptide			
		Amino terminus	Positively charged region	Central hydrophobic region	Carboxyl terminus to cleavage site [a]
	Wild-type	MM	KRN	ILAVIVPALLVA	GTANA↓AES
p4N8AL	4N8AL	MM	NNNN	ALALALAL	GTANA↓AES
p2K2N8AL	2K2N8AL	MM	KKNN	ALALALAL	GTANA↓AES
p4K8AL	4K8AL	MM	KKKK	ALALALAL	GTANA↓AES
p4N9AL	4N9AL	MM	NNNN	LALALALAL	GTANA↓AES
p2K2N9AL	2K2N9AL	MM	KKNN	LALALALAL	GTANA↓AES
p2R2N9AL	2R2N9AL	MM	RRNN	LALALALAL	GTANA↓AES
p4K9AL	4K9AL	MM	KKKK	LALALALAL	GTANA↓AES
p4N10AL	4N10AL	MM	NNNN	ALALALALAL	GTANA↓AES
p2K2N10AL	2K2N10AL	MM	KKNN	ALALALALAL	GTANA↓AES
p4K10AL	4K10AL	MM	KKKK	ALALALALAL	GTANA↓AES
p4N11AL	4N11AL	MM	NNNN	LALALALALAL	GTANA↓AES
p2K2N11AL	2K2N11AL	MM	KKNN	LALALALALAL	GTANA↓AES
p4K11AL	4K11AL	MM	KKKK	LALALALALAL	GTANA↓AES
p4N12AL	4N12AL	MM	NNNN	ALALALALALAL	GTANA↓AES
p2K2N12AL	2K2N12AL	MM	KKNN	ALALALALALAL	GTANA↓AES
p2R2N12AL	2R2N12AL	MM	RRNN	ALALALALALAL	GTANA↓AES

[a] ↓ denotes the signal peptide cleavage site.

through an Aminco French pressure cell at 780 kg/cm^2. The cell lysate was centrifuged at 8500 × *g* for 10 min. The pellet was treated with 1% Triton X-100 containing 10 mM sodium phosphate (pH 7.2) to solubilize cytoplasmic membrane proteins and then the solution was centrifuged at 8500 × *g* for 5 min. Then the pellet was solubilized with 6 M guanidine-HCl containing 10 mM sodium phosphate (pH 7.2) and centrifuged at 540 000 × *g* for 30 min to remove outer membrane proteins. The supernatant was dialyzed with 10 mM sodium phosphate (pH 7.2) containing 1 mM DTT and then mixed with a one-ninth volume of 10% SDS and an equal volume of 30% TCA. After centrifugation at 8500 × *g* for 10 min, the supernatant was dialyzed against 10 mM sodium phosphate (pH 7.2) and then mixed with 9 volumes of acetone. The precipitate was recovered by centrifugation at 8500 × *g* for 10 min, and then solubilized with 8 M urea containing 1 mM DTT and 50 mM potassium phosphate (pH 7.5).

Mature OmpF-Lpp was purified from JM103Lpp$^-$ harboring pK025. To the cytosolic fraction prepared from the cells were added a one-ninth volume of 10% SDS and an equal volume of 30% TCA. After centrifugation at 8500 × *g* for 10 min, the supernatant was dialyzed against 10 mM sodium phosphate (pH 7.2) and then mixed with 9 volumes of acetone. The precipitate was recovered by centrifugation at 8500 × *g* for 10 min, and then solubilized with 8 M urea containing 1 mM DTT and 50 mM potassium phosphate (pH 7.5).

2.12. Binding of preproteins to acidic phospholipids

A binding assay solution (150 μl) containing 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, 1 mM ATP and 0.16 μg/μl PG in the presence of SecA or bovine serum albumin was preincubated at 37°C for 5 min and then mixed with 10 μl of translational products containing ^{35}S-labeled proOmpF-Lpp derivatives (3×10^5 dpm). After incubation at 37°C for 10 min, the mixture was chilled on ice for 10 min, diluted with 150 μl 50 mM potassium phosphate (pH 7.5), placed on the top of a 200-μl 0.2 M sucrose cushion and centrifuged at 150 000 × *g* for 1 h. The precipitate, which contained PG-associated proOmpF-Lpp derivatives, was analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography.

Table 2
Synthetic oligonucleotide linkers encoding the amino-terminal, hydrophobic, and carboxy-terminal regions of the signal peptides shown in Fig. 1

	Linker Sequence
Amino-terminal region	
4N	5'-AATTCTAGGAGGTTTAAATTTATGATGAACAACAACAAC-3' 3'-GATCCTCCAAATTTAAATACTACTTGTTGTTGTTG-5'
2K2N	5'-AATTCTAGGAGGTTTAAATTTATGATGAAAAAAAACAAC-3' 3'-GATCCTCCAAATTTAAATACTACTTTTTTTTGTTG-5'
4K	5,-AATTCTAGGAGGTTTAAATTTATGATGAAAAAAAAAAA-3' 3'-GATCCTCCAAATTTAAATACTACTTTTTTTTTTTT-5'
2R2N	5'-AATTCTAGGAGGTTTAAATTTATGATGCGTCGTAACAAC-3' 3'-GATCCTCCAAATTTAAATACTACGCAGCATTGTTG-5'
Hydrophobic region	
8AL	5'-GCACTGGCCTTAGCTCTGGCACTG-3' 3'-CGTGACCGGAATCGAGACCGT-5'
9AL	5'-CTGGCTCTTGCACTGGCCTTAGCTCTG-3' 3'-GACCGAGAACGTGACCGGAATCGA-5'
10AL	5'-GCACTGGCCTTAGCTCTGGCACTTGCGCTG-3' 3'-CGTGACCGGAATCGAGACCGTGAACGC-5'
11AL	5'-CTGGCTCTTGCACTGGCTCTGGCCTTAGCACTG-3' 3'-GACCGAGAACGTGACCGAGACCGGAATCGT-5'
12AL	5'-GCACTGGCCTTAGCTCTGGCACTTGCTCTGGCACTG-3' 3'-CGTGACCGGAATCGAGACCGTGAACGAGACCGT
Carboxyl-terminal region	5'-GGTACTGCAAACGCCGCGGAGAGCT-3' 3'-GACCCATGACGTTTGCGGCGCCTC-5'

3. Results

3.1. Structures of proOmpF-Lpp derivatives possessing different numbers of lysine or arginine and alanine / leucine residues in the amino-terminal and central regions of the signal peptide, respectively

Using proOmpF-Lpps possessing hydrophobic stretches comprising polyleucine residues, we previously showed that the translocation of proOmpF-Lpps possessing no positively charged amino acid residues at the amino-terminus of the signal peptides occurs when the hydrophobic stretch is sufficiently long [20]. Since polyleucine stretches are strongly hydrophobic and hence tend to aggregate, such preproteins are not adequate for the analysis of the SecA-preprotein interaction. In a previous study, we showed that signal peptides possessing Lys-Arg residues at the amino-terminus and 8 to 12 alanine/leucine residues as the hydrophobic stretch are functional in translocation and do not cause aggregation of presecretory proteins in the conventional reaction mixture

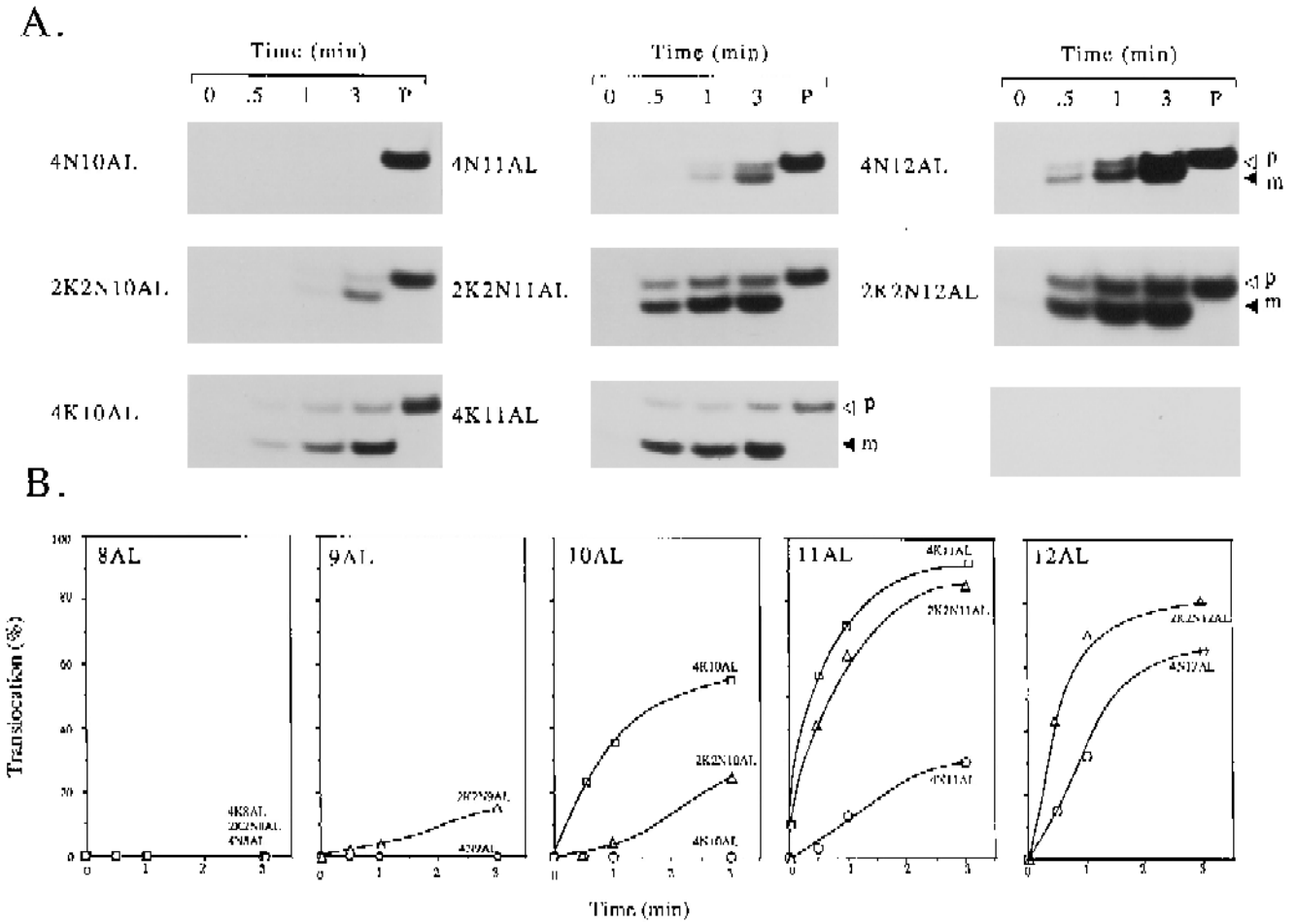

Fig. 2. A longer hydrophobic domain of signal peptides compensates for the requirement of amino-terminal positively charged amino acid residues for in vitro translocation of proOmpF-Lpp derivatives. Preproteins were subjected to in vitro translocation at 37°C. At the indicated time, aliquots (20 μl) were withdrawn and the reaction was terminated on ice, followed by treatment with 5 μl of 5 mg/ml proteinase K for 10 min at 25°C. The translocated proteins, which were proteinase K-resistant, were then detected on an SDS-polyacrylamide gel by means of fluorography. In A, fluorograms (except those for the 8AL-, and 9AL-series) are shown with the translocation times. One-third amounts of the proOmpF-Lpps added to the individual reaction mixture are also shown (P). The positions of the precursor (p) and mature (m) forms of the OmpF-Lpps are also shown. B, the translocation at each time was quantified by densitometric scanning and expressed as a percentage of the total input OmpF-Lpp.

for translocation [19]. We therefore constructed, in the present study, proOmpF-Lpps possessing repeated alanine/leucine residues in place of leucine (Fig. 1). The names of these proOmpF-Lpps and synthetic oligonucleotide linkers used for constructing genes for these proteins are summarized in Fig. 1, Tables 1 and 2.

3.2. Requirement of amino-terminal positively charged amino acid residues for translocation of proOmpF-Lpp derivatives is compensated for by a longer hydrophobic stretch

ProOmpF-Lpp derivatives were subjected to in vitro translocation. As shown in Fig. 2A,B, translocation was observed even in the absence of the positively charged residues, when the length of the hydrophobic stretch was sufficiently long (4N11AL, 4N12AL) although translocation was further stimulated upon introduction of positively charged residues. These results are similar to those obtained with signal peptides possessing polyleucine stretches [20], indicating that the previous results are not due to a peculiar nature of polyleucine stretches. The translocation observed as resistance to proteinase K was accompanied by signal peptide cleavage (Fig. 2A). As in the case of polyleucine-possessing proOmpF-Lpps, the translocation of all alanine/leucine-possessing ones was SecA-, ATP- and $\Delta\bar{\mu}H^+$-dependent (data not shown). SecA and ATP were obligatory, whereas $\Delta\bar{\mu}H^+$ was not.

The length of the hydrophobic stretch required for active translocation was larger with alanine/leucine than with polyleucine, indicating the importance of total hydrophobicity of the stretch for translocation competency as previously discussed [18,19].

3.3. In vivo translocation of 4N12AL

All of the signal peptides of *E. coli* presecretory proteins so far reported possess at least one positively charged amino acid residue at the amino-terminus [10]. Therefore, we examined whether or not 4N12AL can be translocated across the cytoplasmic membrane

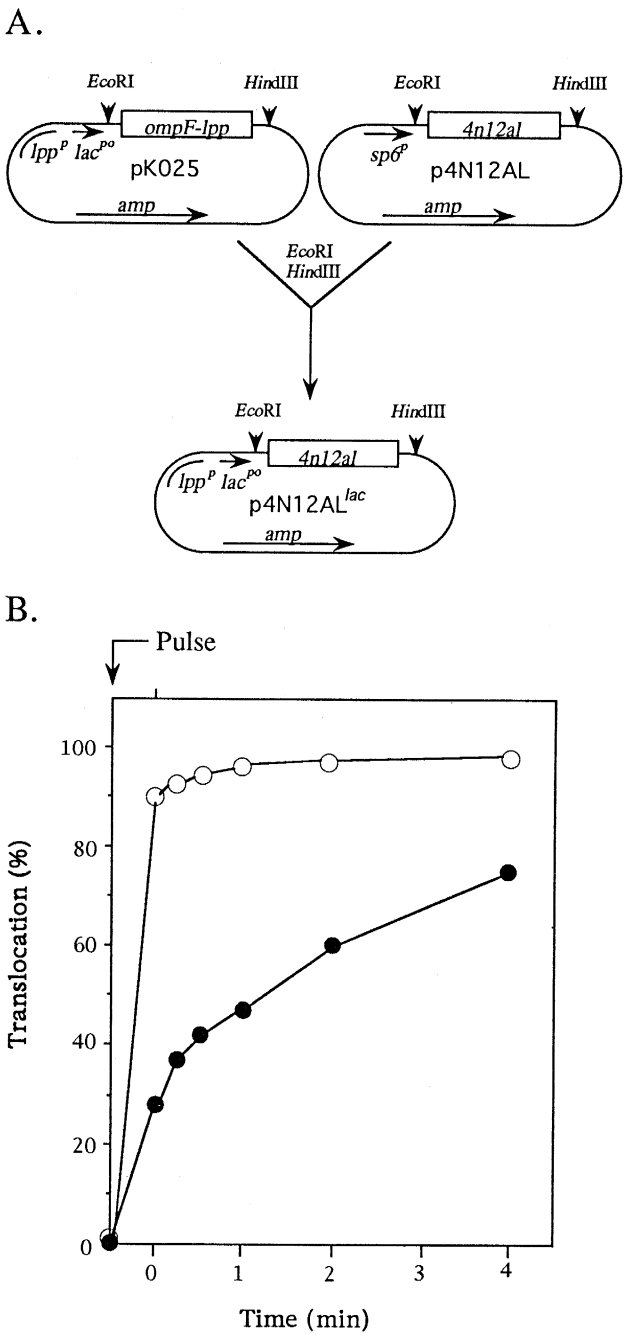

Fig. 3. Translocation of 4N12AL across the cytoplasmic membrane in vivo. A, the construction scheme for the in vivo expression plasmid encoding 4N12AL is shown. The open boxes labeled *ompF-lpp* and *4n12al* represent the DNAs encoding wild type proOmpF-Lpp and 4N12AL, respectively. The *lpp promoter* (lpp^p) and *lac promoter-operator* (lac^{po}) are also indicated. Only relevant restriction sites are indicated. See text for details. B, transformed cells possessing individual plasmids [$p4N12AL^{lac}$ (●), and $p2K2N12AL^{lac}$ (○)] were subjected to a pulse-chase experiment as described in Section 2. The translocation was expressed as a fraction (%) of the labeled proOmpF-Lpp that was processed at each time.

in vivo as well. The 4N12AL and 2K2N12AL genes were isolated and placed after *lpp*^{p}-*lac*^{po} to construct p4N12AL^{lac} and p2K2N12AL^{lac}, respectively (Fig. 3A). JM103Lpp^{-} was then transformed with these plasmids and subjected to a pulse-chase experiment. 2K2N12AL appeared as the mature form even during the pulse, showing rapid translocation, whereas 4N12AL mostly appeared as the precursor form during the pulse and was converted to the mature form during the chase (Fig. 3B). These results show that although the positively charged residues at the amino-terminus of the signal peptide are not absolutely required, even for in vivo translocation, they significantly enhanced translocation. The enhancement was seemingly more significant in vivo than in vitro.

3.4. The amino-terminal positively charged amino acid residues determine the binding of preproteins to the free form of SecA

The SecA-preprotein interaction was examined by means of a chemical cross-linking reagent, EDAC. This reagent is capable of condensing amino and carboxyl groups of proteins. We previously showed that there is a good correlation between cross-linking and in vitro translocation efficiencies [7]. Therefore, cross-linking is a good method for evaluating the SecA-preprotein interaction. To eliminate possible cross-linking via the amino-terminal lysine residue, preproteins possessing arginine residues in place of lysine residues at the amino-terminus of the signal peptide were also constructed at the DNA level (Table 1). The results obtained with 2R2N9AL and 2R2N12AL with respect to the in vitro translocation rate, energy requirement and SecA-dependence were the same as those with 2K2N9AL and 2K2N12AL, respectively (data not shown).

Consistent with our previous observation [7], SecA cross-linked with preproteins possessing positively charged residues at the amino-terminus, and the degree of cross-linking was independent of the amino acid species that donated the positive charge, i.e., either Lys or Arg (Fig. 4A). The result also indicates that the amino-terminal lysine residues of signal peptides are not involved in the cross-linking with SecA. Curiously, the cross-linking was rather independent of the length of the hydrophobic stretch. No significant difference in the degree of cross-linking was observed between 2K2N9AL and 2K2N12AL (Fig. 4A), of which the activity in translocation differed

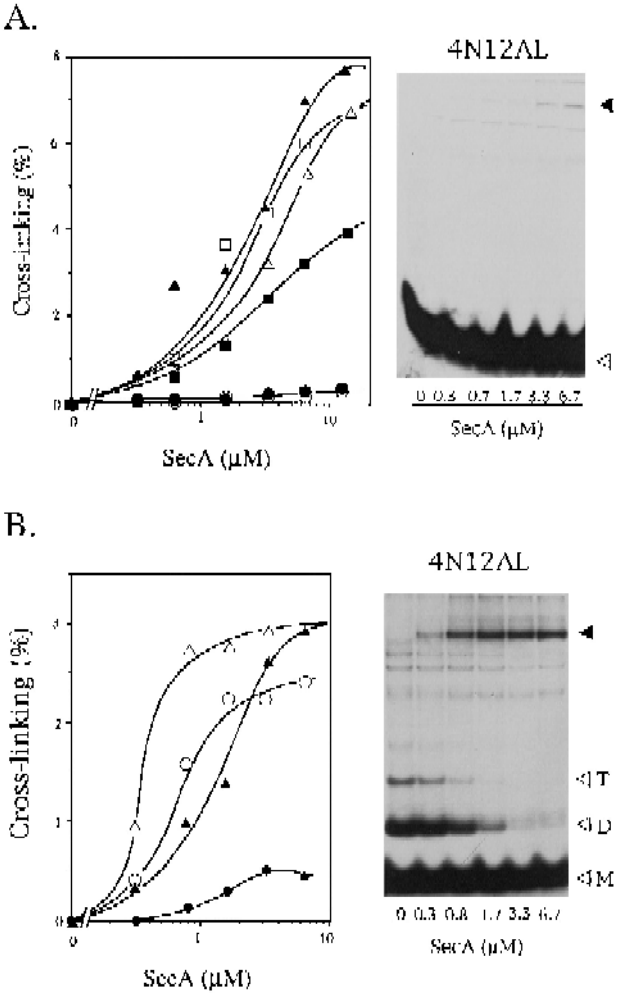

Fig. 4. Phospholipids affect chemical cross-linking of proOmpF-Lpp derivatives with SecA. [^{35}S]ProOmpF-Lpps translated in the presence of a SecA-deficient S-100 were gel-filtered. Cross-linking of these labeled precursors, 2R2N9AL (■), 2K2N9AL (▲), 4N9AL (●), 2R2N12AL (□), 2K2N12AL (△), and 4N12AL (○) (about 2×10^{5} dpm per 15 μl reaction mixture), was performed with different concentrations of SecA in the absence (A) or presence (B) of PG (2.5 μg/reaction mixture) as described in Section 2. Cross-linked products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The fluorograms obtained were subjected to densitometric scanning, and the densities of cross-linked bands relative to those of uncross-linked proOmpF-Lpp are expressed as percentage. The original fluorograms for 4N12AL are also shown in A and B. The positions of monomers (M), cross-linked dimers (D), and cross-linked trimers (T) of 4N12AL are shown. The position of the cross-linked SecA-4N12AL complex (◄) is also shown.

significantly (Fig. 2). In the case of preproteins possessing no positively charged residues at the amino-terminus, no significant cross-linking was observed, even though one of them (4N12AL) is functionally active in translocation in a SecA-dependent manner. It should be noted that the SecA concentration employed in the cross-linking studies was sufficiently high to ensure translocation of 4N12AL.

3.5. The hydrophobic stretch is involved in interaction of preproteins with SecA in the presence of phospholipids

We wondered why the cross-linking was independent of the length of the hydrophobic stretch. Since the ATPase activity of SecA is markedly enhanced by acidic phospholipids [33], we carried out cross-linking experiments in the presence of acidic phospholipids. As shown in Fig. 4B, 4N12AL cross-linked with SecA when liposomes consisting of phosphatidylglycerol (PG) were added. The cross-linking was also observed in the presence of an *E. coli* phospholipid mixture (data not shown). The intensity of bands, probably representing cross-linked dimers (D) and trimers (T) of 4N12AL on the gel (Fig. 4B), decreased as the amount of SecA in the reaction mixture increased, also indicating the occurrence of interaction between the preprotein and SecA. Only a slight cross-linking was observed with the signal peptide possessing a shorter hydrophobic stretch, 4N9AL (Fig. 4B). 4N9AL was inactive in translocation as well, suggesting that the observed cross-linking represents functional interaction.

Although 4N12AL contains no positively charged amino acid residues at the amino-terminus of the signal peptide, the formyl group of the initiator fMet might be cleaved off by deformylases in the cell extracts that were used to synthesize preproteins. When translated 4N12AL was treated with purified deformylase, no stimulation of cross-linking was observed. This may imply that the formyl group was cleaved off during protein synthesis. Alternatively, the formyl group might not be cleaved off during protein synthesis, but a positive charge at the α-amino group of the initial Met emerged as a consequence of the deformylase treatment is not important for the interaction with SecA. Although we could not determine which possibility is correct, it is obvious that

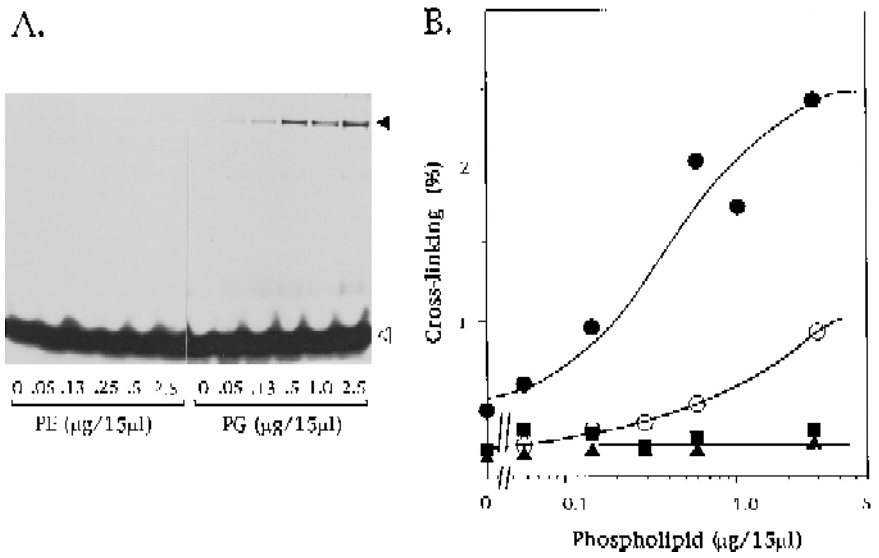

Fig. 5. The cross-linking of 4N12AL with SecA is acidic phospholipid-dependent. Gel-filtered 4N12AL (about 2×10^5 dpm) was subjected to cross-linking with different concentrations of phospholipids, PG (●), PE (▲), CL (○), and PC (■), in the presence of 5 μg SecA, 50 mM potassium phosphate (pH 7.5), 1 mM $MgSO_4$, 2 mM ATP per 15 μl reaction mixture. Cross-linked products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. A, The original fluorogram obtained in the presence of PG or PE is shown. The positions of the precursor proteins (◁) and cross-linked products (◀) are shown. B, The cross-linked bands were quantified by the procedure described in the legend to Fig. 4.

the cross-linking efficiency depended on the length of the hydrophobic stretch (Fig. 4B).

It should also be noted that the degree of cross-linking (Fig. 4B) as well as that of translocation (Fig. 2) depended on the length of the hydrophobic stretch even in the case of preproteins possessing positively charged signal peptides, both cross-linking and translocation being more efficient with 2K2N12AL than with 2K2N9AL. In the case of preproteins possessing positively charged amino acid residues at the amino-terminus of the signal peptides, the degree of cross-linking was rather lower in the presence of phospholipids (Fig. 4A,B). This may be due to a phospholipid-induced change of SecA conformation [33,34]. Alternatively, the signal peptides may be sequestered into the phospholipids [35]. Cross-linking

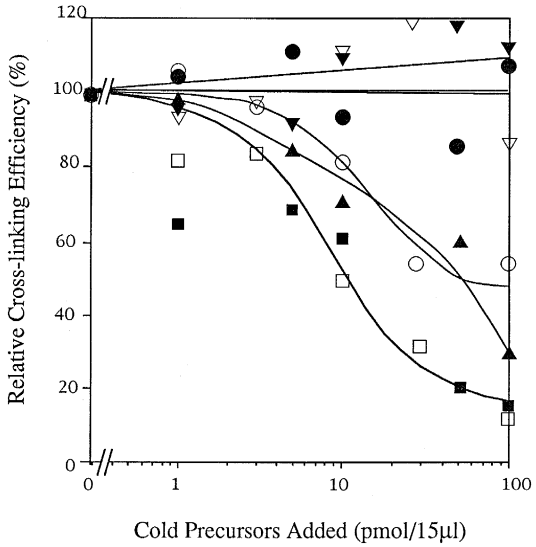

Fig. 6. 4N12AL competes with the wild-type preprotein for cross-linking with SecA only in the presence of acidic phospholipids. All reaction mixtures (15 μl) contained 50 mM potassium phosphate (pH 7.5), 1 mM $MgSO_4$, 2 mM ATP, gel-filtered [^{35}S]proOmpF-Lpp (about 2×10^5 dpm). They also contained the indicated amounts of nonradiolabeled 4N12AL (●), 2K2N12AL (▲), proOmpF-Lpp (■), and mature OmpF-Lpp (▼) in the absence of acidic phospholipids, or 4N12AL (○), proOmpF-Lpp (□), and mature OmpF-Lpp (▽) in the presence of PG (2.5 μg/reaction mixture). They were incubated at 25°C for 50 min, subjected to the cross-linking reaction, and then analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. Fluorograms were densitometrically scanned to determine the degree of cross-linking. 100% indicates the degree of cross-linking in the absence of nonradiolabeled preproteins.

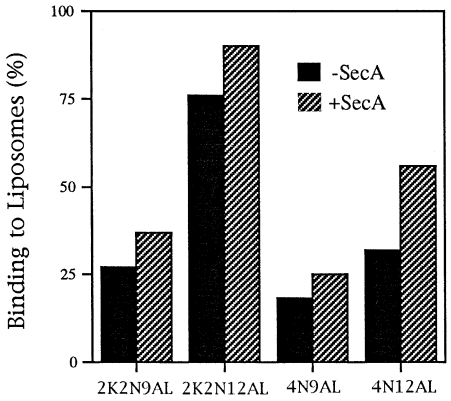

Fig. 7. SecA stimulates binding of preproteins possessing longer hydrophobic region to acidic phospholipid liposomes. [^{35}S] proOmpF-Lpp derivatives were incubated with PG liposomes. PG-associated [^{35}S]proOmpF-Lpp derivatives were analyzed with SDS-polyacrylamide gel electrophoresis and fluorography. The relative binding efficiency was calculated.

with SecA of 2K2N12AL and that of 2K2N9AL were more efficient than that of 4N12AL and 4N9AL, respectively, suggesting that the positively charged animo-terminal residues are also involved in the interaction with SecA in the presence of liposomes as well.

3.6. Acidic phospholipids are essential for interaction of SecA with 4N12AL

4N12AL was subjected to cross-linking with SecA in the presence of different phospholipid species (Fig. 5). Stimulation of cross-linking was only observed in the presence of acidic phospholipids (PG (Fig. 5A) or CA). On the other hand, no cross-linking was observed in the presence of PE (Fig. 5A) or PC. Moreover, PG stimulated the cross-linking when it was added to PE liposomes, whereas PE exhibited no stimulatory effect on PG liposomes (data not shown).

3.7. Interaction of 4N12AL with SecA in the presence of acidic phospholipids is specific

There is the possibility that the phospholipid-dependent cross-linking of 4N12AL with SecA is a nonspecific one mediated by acidic phospholipids which are able to interact with both the longer hydrophobic stretch of preproteins and SecA. To ex-

clude this possibility, the following competition experiment was performed. The cross-linking of ^{35}S-labeled wild-type proOmpF-Lpp with SecA in the presence or absence of PG was carried out in the presence of different concentrations of cold 4N12AL, 2K2N12AL, wild-type proOmpF-Lpp or mature OmpF-Lpp (Fig. 6). In the absence of PG, 2K2N12AL prevented the cross-linking as effectively as wild-type proOmpF-Lpp did, whereas 4N12AL as well as mature OmpF-Lpp did not inhibit it at all. In the presence of PG, on the other hand, 4N12AL prevented the cross-linking, whereas mature OmpF-Lpp did not. It should be noted that the 4N12AL molecule in the reaction mixture (100 pmol at maximum) was roughly equivalent to that of SecA (50 pmol), implying that 4N12AL binds to a specific site(s) of SecA. It is most probable, therefore, that the observed PG-dependent interaction between 4N12AL and SecA is a specific one.

3.8. SecA stimulates the binding efficiency of a preprotein to liposomes

One possibility to explain the increased cross-linking of SecA with precursors by acidic phospholipids is that they provide a milieu for signal peptides and SecA to interact in (i.e., a concentration effect). To exclude this possibility, we determined how much preprotein containing different signal peptides is bound to PG-liposomes. As shown in Fig. 7, about 20% 4N9AL was bound to the liposomes, and the rest was not. When the hydrophobic stretch became longer (4N12AL), the binding efficiency increased only 1.5-fold. It should be noted that the about 5-fold increased cross-linking of 4N12AL versus 4N9AL was observed (Fig. 4B). Therefore, the increased cross-linking efficiency for 4N12AL cannot be simply explained by a PG-mediated concentration effect. When SecA was present, binding of 4N12AL to the

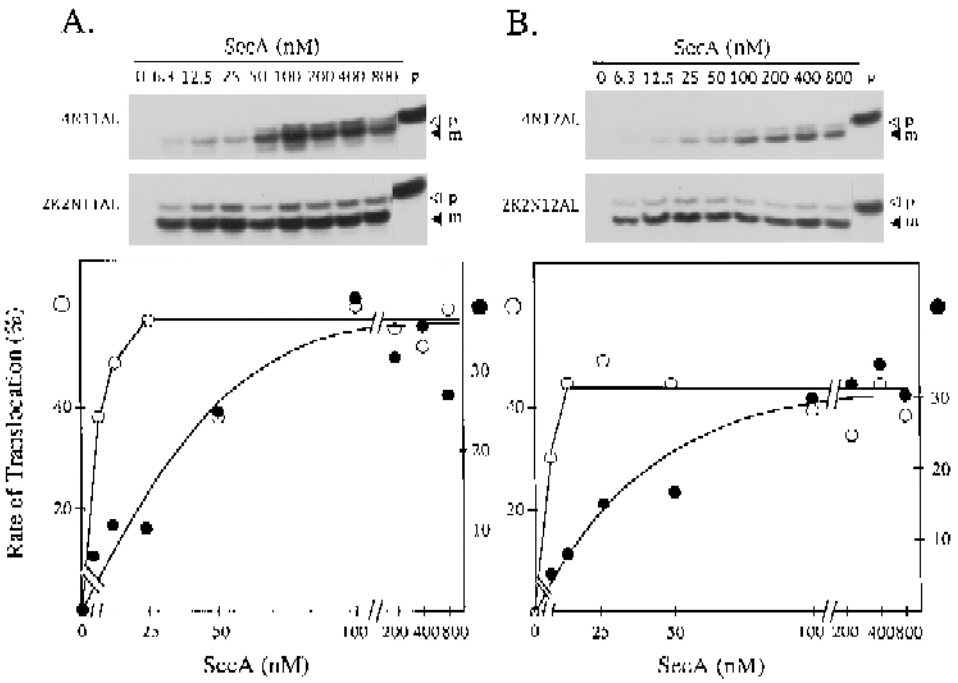

Fig. 8. Translocation of positive charge-minus proOmpF-Lpps requires a higher concentration of SecA than that of positively charged ones. The translocation of [^{35}S]proOmpF-Lpps (A; 11AL-series, B; 12AL-series) was carried out using the SecA-deficient S-100 fraction and SecA-depleted membrane vesicles in the presence of different concentrations of SecA for 3 min. The original fluorograms are shown at the top. One-third amounts of the proOmpF-Lpps added to the individual reaction mixture is at the right (P). The positions of the precursor (*p*) and mature (*m*) forms of the OmpF-Lpp are also shown. The rate of translocation of these proteins [2K2N-series (○) and 4N-series (●)] were expressed as the fraction (%) of the processed substrate.

liposomes increased 1.8-fold compared with its absence, whereas binding of 4N9AL less increased (Fig. 7). These results may suggest that acidic phospholipids promote a conformational change in SecA to reveal a binding site for the hydrophobic core of the signal peptides. Alternatively, acidic phospholipids may induce conformational changes in the signal peptides themselves, as suggested [35,36], to allow SecA interaction.

3.9. Translocation of 4N-series proOmpF-Lpps requires a higher concentration of SecA than that of 2K2N-series ones does

The urea treatment of membrane vesicles, which removes most SecA from the membrane [28,29], resulted in a more severe defect on the translocation of 4N-series preproteins than positive charge-possessing ones, suggesting that the SecA requirement differs with the presence and absence of the amino terminal positive charges of the signal peptide. In one experiment, of which the results are shown in Fig. 8, the in vitro translocation was carried out with urea-treated membrane vesicles and the cytosol from MM66 (*secA*^{am}) grown at a nonpermissive temperature in the presence of different amounts of SecA. For both 11AL- and 12AL-series proOmpF-Lpps, an appreciably higher concentration of SecA was required for preproteins possessing no positively charged amino acid residues at the amino-terminus of the signal peptide in order to be translocated. The SecA concentration for the half-maximal rate of translocation was about 0.5 ng/μl for 2K2N11AL and 2K2N12AL, and about 5 ng/μl for 4N11AL and 4N12AL.

4. Discussion

The SecA-signal peptide interaction has been assumed to represent the initial stage of protein translocation in *E. coli* cells. Involvement of the amino-terminal positively charged region in the interaction has already been reported [7]. In the present work, we further showed that the hydrophobic region of signal peptides is also involved in the interaction when liposomes containing PG or CL are present. This conclusion was based on the following observations: (1) 4N12AL, that is functional in translocation, was capable of cross-linking with SecA in the presence of PG or CL, whereas only a slight cross-linking was observed for 4N9AL, that is translocationally incompetent. (2) 4N12AL competed with wild-type proOmpF-Lpp in cross-linking when PG or CL was present. (3) The competition of 4N12AL with wild-type proOmpF-Lpp was observed in a translocation experiment as well (data not shown). (4) Acidic phospholipids, PG or CL, also stimulated cross-linking with SecA, even in the case of preproteins possessing positively charged residues at the amino-termini of their signal peptides. (5) SecA stimulated binding of preproteins possessing a long hydrophobic stretch to acidic liposomes. The possibility that the interaction is a nonspecific one mediated by phospholipids which interact with both SecA and hydrophobic polypeptide chains is unlikely, as has already been discussed in section 3. Overall, we conclude that the hydrophobic stretch of functionally active signal peptides is involved in recognition of preproteins by SecA in the presence of PG or CL and that this represents a process of translocation of secretory proteins.

How does SecA interact with preproteins possessing no positively charged amino acid residues at the amino-terminus of the signal peptides? One possible explanation is that SecA changes its conformation upon interaction with acidic phospholipids so as to be able to recognize the hydrophobic stretch of the signal peptide domain of preproteins. The SecA-acidic phospholipid interaction has been demonstrated in several experiments as follows: PG and CL play important roles in preprotein translocation [34,37], stimulate SecA ATPase activity [33], promote the insertion of SecA into membranes [38,39] and induce a conformational change of SecA as revealed by V8 protease sensitivity [40]. We observed the same conformational change under the conditions we employed for cross-linking experiments (data not shown). Since acidic phospholipids are important components of membranes and only exist in membranes, this hydrophobic recognition must take place only with SecA molecules on the cytoplasmic membrane. Another possibility is that acidic phospholipids induce conformational changes in the signal peptides themselves, as suggested [35,36], to allow SecA in-

teraction. Further study is required to distinguish these possibilities.

We previously observed that about 80–90% of SecA is localized in the cytosol and the rest on the inner surface of the cytosolic membrane [10]. Another group reported that 50% of SecA is localized in the cytosol [9]. Several lines of evidence suggest that preproteins first interact with cytosolic SecA to initiate translocation [10]. Results by other groups also support this idea. Chun and Randall [41] showed that SecA can be immunoprecipitated from the cytosol in a complex with both fully elongated and nascent species of preproteins. Dolan and Oliver [42] revealed that autoregulation of the *secA* gene by SecA is probably depressed by preprotein accumulation. As shown in the present study, however, when preproteins do not possess positively charged residues at the amino terminus of the signal peptides, the first interaction of preproteins with SecA for initiating translocation occurs on the membrane.

Signal peptides are peculiar in that they possess an amino-terminal positively charged domain and a central hydrophobic domain. Signal peptides have been thought to take on a membrane spanning structure (inside amino-terminus) during translocation [43], and the roles of both domains have been discussed in relation to this structure; for example, the positively charged domain interacts with negatively charged phospholipids, PG or CL, to keep the amino-terminus of the signal peptide inside the membrane [44], and the hydrophobic domain plays a role in the membrane as a membrane-spanning structure. In the present and previous works [7], it was demonstrated that both domains are required for the interaction with SecA as well. It is likely, therefore, that the two domains play dual functions in the translocation reaction.

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