Positively charged lysine at the N-terminus of the signal peptide of the *Escherichia coli* alkaline phosphatase provides the secretion efficiency and is involved in the interaction with anionic phospholipids

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Abstract Positively charged amino acid residues at the N-terminus of the signal peptide (SP) have been proposed to play a significant role in the initial step of protein secretion in bacteria. To test this hypothesis, Lys(−20) of the *Escherichia coli* alkaline phosphatase SP was replaced by other amino acid residues, and the effect of these substitutions on protein maturation was studied. The introduction of negatively charged and hydrophobic amino acids resulted in a decrease in secretion efficiency and impaired the SP-APL interaction, whereas His and Tyr had no significant effect. A structural analysis of the SP-APL interaction suggests that the positively charged Lys(−20) determines the stability of the complex.

Key words: Alkaline phosphatase; Signal peptide; Anionic phospholipid; Protein secretion; Stereochemical analysis; *Escherichia coli*

1. Introduction

Most secreted proteins of bacteria are known to be synthesized as cytoplasmic precursors containing an additional N-terminal extension called the signal peptide that is essential for protein membrane translocation. After translocation, the SP is cleaved by a membrane-bound signal peptidase (for review, see [1]). Various bacterial SPs have no extensive sequence homology but are rather similar in their properties. The canonical SP contains three characteristic regions: the N-terminal domain positively charged due to one or two basic residues (n-region), the hydrophobic core (h-region), and the polar C-terminal domain (c-region) containing the SP cleavage site [2]. Due to the hydrophobic core, SP tends to adopt an α-helix conformation that facilitates its insertion into the hydrophobic interior of the membrane lipid bilayer [3]. The role of positively charged residues in the SP N-terminus is not completely understood. It is known that they are important for the efficient secretion of lipoprotein [4], and of OmpA [5] and OmpF-Lpp proteins [6]. The positively charged SP N-terminus is supposed to contribute to the specific net charge distribution in the region comprising SP and the mature protein N-terminal domain. This 'dipolar' structure is probably important for the function of the SP [7]. Another hypothesis ('loop model') suggests that the positively charged SP N-terminus is significant by itself at the initial step of protein membrane translocation [8]. Our data on the interrelation between the secretion of the *E. coli* PhoA and phospholipid exchange led us to suggest the involvement of the protein-lipid interaction in protein secretion [9,10]. This hypothesis presumes an electrostatic interaction of positively charged amino acid residues of the SP and APL followed by their joint translocation as an SP-APL complex across the cytoplasmic membrane. In fact, APL are shown to be required for protein membrane translocation both in vivo [11,12] and in vitro [13,14]. However, the mechanism of their involvement in protein translocation is obscure. Here we present data on the role of the positively charged Lys(−20) of the *E. coli* prePhoA in secretion and involvement of APL in this process.

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Abbreviations: SP, signal peptide; APL, anionic phospholipids; PG, phosphatidylglycerol; PhoA and prePhoA, *Escherichia coli* alkaline phosphatase and its precursor, respectively.

2. Materials and methods

2.1. Introduction of amino acid substitutions into SP of the PhoA

Various amino acid substitutions for Lys(−20) in prePhoA were obtained by amber-suppression mutagenesis in vivo as described [15]. The amber codon instead of Lys(−20) codon was introduced by oligonucleotide-directed mutagenesis using oligonucleotide 5′-GAAAATAAAGTGTAGCAAAGCACTATT-3′, and replacement of the Lys(−20) codon with Ala and Glu codons was achieved using oligonucleotides 5′-GAAAATAAAGTGGCACAAAGCACTATT-3′ and 5′-AATAAAGTGGAACAAAGCACT-3′, respectively. Wild-type and mutant *phoA* genes were cloned in p15SK(−) (Fischer, R. and Hengstenberg, W., unpublished). The resulting plasmids bore a chloramphenicol resistance marker and p15A *ori* of replication allowing their usage in a two-plasmid system in combination with the plasmids with Co1E1 *ori* of replication carrying the cloned genes of amber suppressor tRNAs. Collection of the *E. coli* amber suppressors (Su3 (Tyr), Ala2, GluA, Phe, Gly1, HisA, ProH and Cys) [16] and phage T5 amber suppressor specific to Lys (Ksenzenko, V.N., unpublished) were used.

2.2. Alkaline phosphatase maturation and localization

Pulse-chase experiments were used to analyze the prePhoA maturation. *E. coli* cells incubated for 30 min in medium without orthophosphate to induce PhoA synthesis [17] were labeled with L-[^{35}S]methionine and chased for various periods of time. PhoA and prePhoA were immunoprecipitated using affinity-purified rabbit antibodies against denatured PhoA and separated by 10% SDS-PAGE followed by autoradiography and densitometry.

To determine whether the mutant PhoA precursors were localized in the cytoplasm or periplasm, proteolysis experiments were performed [18]. An aliquot of spheroplasts from pulse-labeled cells was treated with 50 μg/ml proteinase K (Boehringer Mannheim) in the presence of 0.2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Serva). Proteolysis was halted by the addition of 5 mM phenylmethylsulfonyl fluoride. After precipitation of proteins with trichloroacetic acid, PhoA and prePhoA were immunoprecipitated and analyzed by 10% SDS-PAGE.

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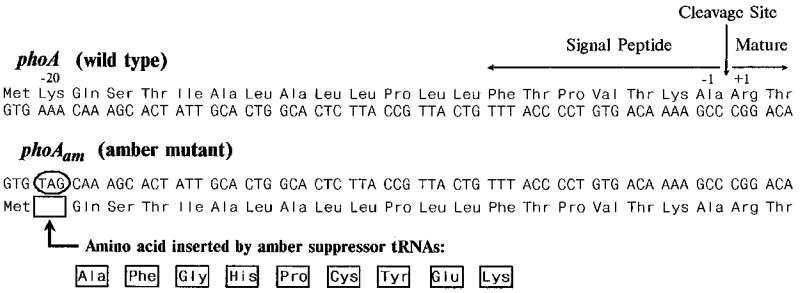

Fig. 1. Position of amber mutation in the *phoA* gene (circled) and amino acid substitutions in mutant proteins (boxed). Portions of the wild-type and mutant *phoA* genes encoding residues in position −21 to position +2 of prePhoA and amino acid residues introduced by amber suppressors are shown.

2.3. Phospholipid analysis

For the phospholipid assay cells were grown to the mid-log phase as described above in the presence of 5 μCi/ml of [^{32}P]orthophosphoric acid followed by incubation for 1 h under orthophosphate starvation. Lipids were extracted according to Ames [19] and chromatographed on TLC plates with Kieselgel 60 (Merck) in chloroform/methanol/water/ammonium hydroxide (65:37.5:3:0.62). The plates were impregnated with 1.2% boric acid in 50% ethanol and dried before use. Individual phospholipid spots were cut out, and their radioactivity was counted in toluene scintillator in a gamma counter (SL-30, Intertechnique, France).

To examine glycerophosphate transfer from phosphatidylglycerol to arbutin (*p*-hydroxyphenyl-β-*o*-glucoside), the latter was added to the medium and the arbutinphosphoglycerol formed was sorbed on activated charcoal (Norit A, 50 mg/ml). The charcoal was then washed three times with water, and arbutinphosphoglycerol was eluted with 0.25 ml of 15% (v/v) pyridine for 1 h with agitation. The radioactivity of 0.2 ml of the pyridine extract was determined by Cerenkov counting.

2.4. Stereochemical analysis

The atomic coordinates of a three-dimensional structure of the SP-APL complex revealed in the previous work [20] were used for stereochemical analysis of the effects of SP mutations. Complexes of PG with SP of different mutant prePhoAs were constructed using the TURBO-FRODO computer program. These structures were further refined by the X-PLOR program (version 3.1).

3. Results

3.1. Lys(−20) of the prePhoA signal peptide determines the secretion efficiency

Eight mutant PhoAs with amino acid substitutions for Lys(−20) were obtained (Fig. 1). All *E. coli* strains producing these mutant phosphatases exhibited high enzymatic activities (not shown). Consequently, all mutant PhoAs are capable of being secreted, since the PhoA becomes active only after

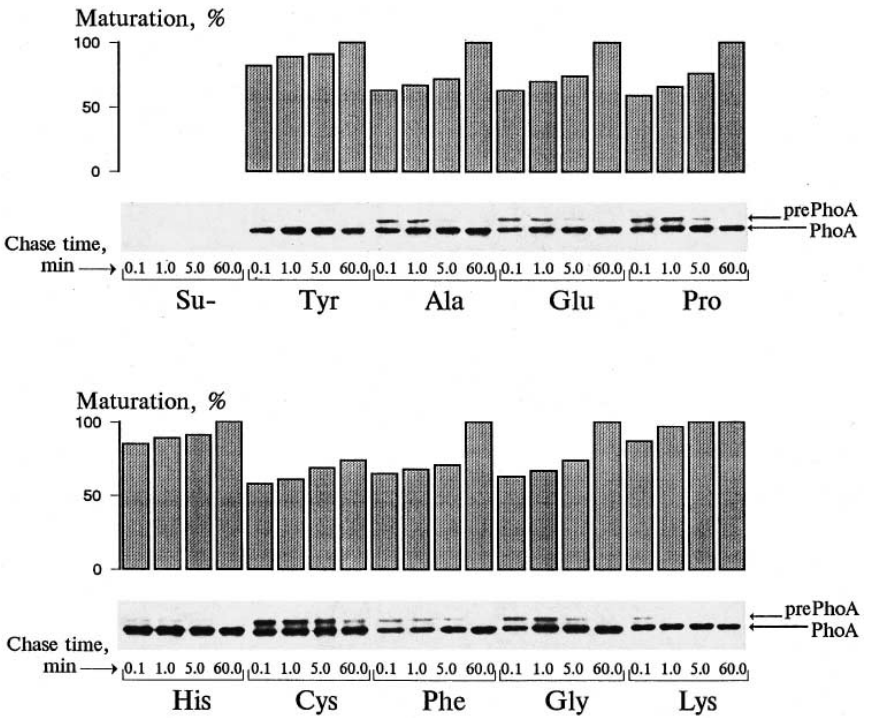

Fig. 2. Maturation of mutant PhoAs with amino acid substitutions in position −20. Cells were pulse-labeled with L-[^{35}S]methionine (50 μCi/ml) for 60 s, and radioactivity was chased for 0.1, 1.0, 5.0, or 60 min by addition of unlabeled methionine to a final concentration of 0.05%. Samples were immunoprecipitated and analyzed by 10% SDS-PAGE with subsequent autoradiography and densitometry. Autoradiograms and densitometry results are presented for each PhoA mutant with an amino acid substitution (indicated). *E. coli* E15 transformed with pPHOA21 was used as a control (indicated as Su-). The total amount of PhoA and prePhoA was calculated with adjustments of additional methionine residues in the prePhoA and set to 100% for each point of the chase time.

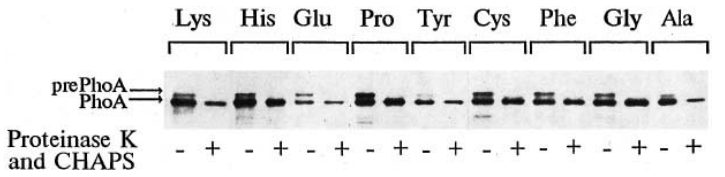

Fig. 3. Proteinase K treatment of mutant PhoAs. Pulse-labeled *E. coli* cells were treated with lysozyme and EDTA to obtain spheroplasts which then were subjected to proteinase K treatment in the presence of detergent CHAPS (marked as +). Untreated spheroplasts (marked as −) were used as a control. Immunoprecipitated PhoA and prePhoA were analyzed by SDS-PAGE followed by autoradiography. Amino acid residues in position −20 are indicated.

translocation across the cytoplasmic membrane [21]. However, some amino acid substitutions affect the rate of PhoA maturation, i.e. conversion of the prePhoA into the mature form (Fig. 2). The wild-type and mutant protein precursors with His or Tyr in position −20 are converted into the mature form at the greatest rate, while the replacement of Lys(−20) with Glu, Ala, Phe, Pro, Gly and Cys reduces the rate of protein maturation.

It is reasonable to suppose that the decrease in maturation rate may result from the slowing down of protein translocation across the cytoplasmic membrane. To assess this assumption, proteolytic treatment was employed. It is known that the PhoA is assembled after translocation to the periplasm and becomes strongly resistant to proteolysis even in the presence of detergent [18]. The prePhoA is also resistant to proteolysis when translocated across the cytoplasmic membrane. In contrast, the prePhoA remaining in the cytoplasm cannot be folded properly and is sensitive to proteolytic attack when detergent is added. We have found that all mutant prePhoAs including those with a decreased maturation rate are sensitive to proteinase K in the presence of the detergent CHAPS (Fig. 3). This provides evidence of the cytoplasmic localization of the prePhoAs and demonstrates the effect of amino acid substitutions in position −20 on protein membrane translocation.

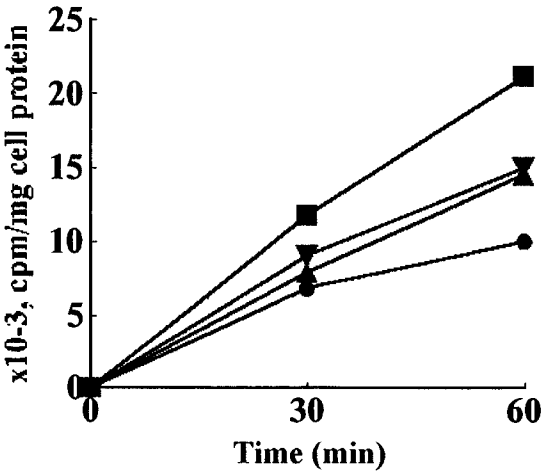

Fig. 4. Radioactivity of arbutinphosphoglycerol in the growth medium of *E. coli* cells producing wild-type (circle) and mutant PhoAs with Ala(−20) and Pro(+1) (triangle), Glu(−20) and Pro(+1) (wedge), Pro(+1) (square). Arbutin was added to 17 mM to cells grown on a medium containing 5 μCi/ml [^{32}P]orthophosphoric acid. Aliquots were taken at 30 and 60 min, cells were pelleted, and the supernatant was shaken with Norit A charcoal for 30 min. The charcoal was then washed with water, and the adsorbed arbutinphosphoglycerol was eluted with 15% pyridine (v/v). Radioactivity of the pyridine extract was counted. Each data point is the mean of 4 experiments.

3.2. *Lys(−20) of the prePhoA signal peptide is involved in SP-APL interaction*

Previously we found that substitution of Val for Ala(−1) or Pro for Arg(+1) completely inhibited signal peptide cleavage without preventing prePhoA translocation across the cytoplasmic membrane [12]. This resulted in anchoring of the mutant proteins in the cytoplasmic membrane accompanied by

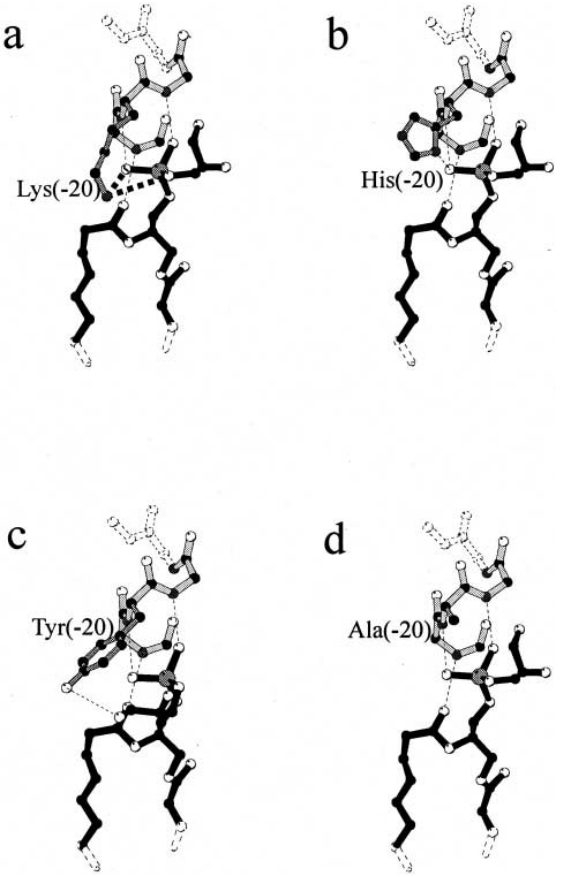

Fig. 5. Ball-and-stick representation of the structural model of the SP-PG complex in the region of intermolecular interactions. The N-terminal turn of SP which interacts with the polar head of PG is represented by white sticks, PG by black sticks. The next turn of the α-helix is marked by broken lines. Side chains of SP, except the residue in position −20, and also all the hydrogen atoms are not shown. Carbon atoms are in black, oxygen atoms in white, and nitrogen atoms in gray. Hydrogen bonds between the peptide groups and polar head of PG are denoted by thin dotted lines, and ionic pairs between the positively charged residue in position −20 and the negatively charged phosphate group are marked by thick dotted lines. (a) Wild-type complex with the ionic pair between Lys(−20) and the phosphate group of PG; (b) complex with substitution of His(−20) for Lys; (c) complex with substitution of Tyr(−20) for Lys; (d) complex with substitution of Ala(−20) for Lys.

Table 1
Phospholipid content in *E. coli* cells producing wild-type and mutant PhoAs

Produced protein	PG		CL		PEA		PA		D.f.	*n*
	Content (%)	S.L.	Content (%)	S.L.	Content (%)	S.L.	Content (%)	S.L.		
Wild-type	13.2±0.3	1.00	3.0±0.1	1.00	82.0±0.3	1	1.8±0.2	1.00	1, 22	13
P(+1)	15.8±0.6	$<10^{-5}$	3.7±0.2	0.01	78.7±0.7	$<10^{-5}$	1.8±0.2	0.86	1, 22	11
A(−20)P(+1)	14.5±0.3	0.05	2.9±0.2	0.13	80.9±0.1	0.13	1.7±0.1	0.85	1, 15	4
G(−20)P(+)	14.3±0.3	0.10	2.9±0.2	0.21	81.1±0.2	0.21	1.7±0.2	0.78	1, 15	4
A(−20)	13.5±1.0	0.66	2.8±0.3	0.93	82.0±0.9	0.93	1.7±0.3	0.91	1, 15	4
G(−20)	13.9±0.5	0.29	3.0±0.2	0.35	81.3±0.3	0.35	1.8±0.3	0.88	1, 15	4

Results are given as mean (±) standard deviation of mean. Phospholipid content in *E. coli* cells producing mutant PhoAs was compared with that in the cells producing the wild-type by Fisher test. Significance level (S.L.), degree of freedom (D.f.), and number of independent experiments (*n*) are indicated.

an increase in the content and rate of synthesis of the anionic phospholipids, phosphatidylglycerol and cardiolipin. These data allow us to suggest the immobilization of APL by uncleaved SP anchored in the cytoplasmic membrane.

To verify whether Lys(−20) is involved in the SP-APL interaction, double amino acid substitutions were introduced into the PhoA. In addition to the substitution of Pro for Arg(+1) [12], Lys(−20) was replaced by uncharged Ala or negatively charged Glu. These mutant proteins were also found anchored in the cytoplasmic membrane, but the phosphatidylglycerol and cardiolipin contents in the cells producing these mutant proteins turned out to be lower than those in the cells producing the PhoA with the single amino acid substitution of Pro for Arg(+1) (Table 1). A decrease in PG content of the cytoplasmic membrane of these cells is also demonstrated by another approach. Plasma membrane-bound phosphoglyceroltransferase I catalyses the transfer of a phosphoglycerol moiety from the membrane PG to the nascent chain of the periplasmic membrane-derived oligosaccharides or model substrate arbutin, freely diffusable through the pores of the outer membrane [22]. We have found that the replacement of Lys(−20) with Ala or Glu in the prePhoA with uncleaved SP results in a sufficient lowering of the amount of arbutinphosphoglycerol in the growth medium of the *E. coli* cells producing these proteins (Fig. 4). The data suggest the involvement of Lys(−20) in the interaction with PG during protein translocation across the cytoplasmic membrane.

4. Discussion

The SP carries information about both electrostatic and hydrophobic interactions with the membrane components of the secretion machinery. APL are good candidates for this interaction. It is known that: (i) membrane vesicles without APL do not support preprotein translocation [14]; (ii) SPs are not inserted into the phospholipid monolayers containing no APL [23]; (iii) APL stimulate the ATPase activity of SecA [24] and SecA insertion into the phospholipid monolayer [25]; and (iv) APL are found in the membrane site bound with the translation complex [26].

The possibility of the direct interaction of the prePhoA SP with *E. coli* phospholipids was earlier analyzed using molecular modeling of their three-dimensional structure [20]. Stereochemical analysis revealed that the N-cap of the SP α-helix can complementarily interact with APL via the network of hydrogen bonds between the peptide groups of SP and oxygen atoms of the phosphate and carbonyl groups of APL. These bonds are most important and sufficient for the formation of the SP-APL complex being able to be inserted into the membrane bilayer and translocated across it with lower energy expenditure. Since SPs of the wild-type proteins are cleaved before translocation is completed, and then destroyed by a specific protease [27], the lifetime of the wild-type preprotein-APL complex is supposed to be extremely short. The anchoring of the preprotein with uncleavable SP in the cytoplasmic membrane may provide a kind of a 'stop frame' of the process. In fact, we have found that the processing block of the prePhoA due to the substitution of Pro for Arg(+1) or Val for Ala(−1) leads to the simultaneous accumulation of the prePhoA and APL in the cytoplasmic membrane. However, the replacement of Lys(−20) with Ala or Glu in addition to the substitution of Pro for Arg(+1) in the prePhoA suppresses the accumulation of APL, indicating that APL immobilization by uncleaved SP is prevented. The data suggest the involvement of Lys(−20) in the SP-APL interaction, supporting the possible electrostatic interaction of the positively charged amino acid residues of preproteins and the negatively charged head group of APL during secretion [9,10].

As for the prePhoA, it contains three positively charged residues which may be involved in the protein-lipid interaction at the initial stage of the PhoA secretion. They are Lys(−20) and Lys(−2) of SP, and Arg(+1) of the mature protein N-terminus. However, we have shown that different substitutions for Arg(+1) and Lys(−2) (unpublished data) have no appreciable effect, and only substitutions of the negatively charged or hydrophobic amino acid residues for Lys(−20) reduce the translocation efficiency of PhoA. Unfortunately, the way Lys(−20) contributes to the secretion efficiency and the reason for the different effects of Lys-replacing amino acid residues are obscure. To elucidate these questions, the influence of amino acid substitutions on the SP-APL interaction was examined using the structural model of the SP-APL complex proposed earlier [20].

The stereochemical analysis of SP-PG complexes has shown (Fig. 5a) that Lys(−20) of the wild-type SP could form an ionic pair with the negatively charged phosphate group of the lipid molecule (in this case PG is considered). This stabilizes the complementary hydrogen bond interactions in the SP α-helix-PG complex. His(−20) and Tyr(−20) in SP are shown, in contrast, to be able to form additional hydrogen bonds with the polar head of the lipid molecule (Fig. 5b,c). They also stabilize the SP-APL complex and somehow compensate the absence of electrostatic interaction. The substitutions of Ala, Gly, Glu, Phe and Pro for Lys(−20) eliminate the favorable electrostatic interaction and bring about neither new ion pairs nor hydrogen bonds (Fig. 5d). In agreement with the

model, these complexes are less stable than the wild-type complex. The data suggest that the positively charged amino acid residue of SP N-terminus provides the secretion efficiency via stabilization of the SP-APL complex. It is noteworthy that at least some secreted proteins have on their SP N-terminus His, Tyr or Thr instead of the positively charged amino acid residues. This observation supports the similarity of the functions of these amino acid residues with that of Lys and the possible involvement of all residues mentioned above in the stabilization of the SP-APL complex. The electrostatic interaction between SP of OmpF-Lpp and PG has been proved experimentally [28].

It is known that a number of membrane Sec proteins are involved in protein membrane translocation forming an ATP-dependent preprotein translocase (for review, see [1]). We suggest that some of them, e.g. SecA, are able to catalyze the formation of the SP-APL complex and to stabilize it. For instance, if the signal peptide freely scans the membrane surface, the assembly of the SP-APL complex competent for translocation can be impeded by a small concentration of APL in the membrane and instability of the SP-APL complex, since its lifetime is not sufficient for SP to be inserted into the membrane. In those cases where any protein molecule specifically binds both the lipid molecule required for translocation and SP, the formation of the SP-APL complex is more probable. SecA is quite suitable for this purpose, since its function depends on APL and it can interact with SP [29].

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