Biochemistry (Moscow), Vol. 67, No. 9, 2002, pp. 978-985. Translated from Biokhimiya, Vol. 67, No. 9, 2002, pp. 1182-1190.

Study of Interaction of Export Initiation Domain of *Escherichia coli* Mature Alkaline Phosphatase with Membrane Phospholipids during Secretion

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Received February 1, 2002

Abstract—The efficiency of secretion of alkaline phosphatase from *Escherichia coli* depending on the primary structure of its N-terminal region and the content of zwitterionic phospholipid phosphatidylethanolamine and anionic phospholipids in membranes has been studied in this work to establish the peculiarities of interaction of mature protein during its secretion with membrane phospholipids. It has been shown that the effect of phosphatidylethanolamine but not anionic phospholipids on the efficiency of alkaline phosphatase secretion is determined by the primary structure of its N-terminal region. The absence of phosphatidylethanolamine appreciably reduces the efficiency of secretion of wild type alkaline phosphatase and its mutant forms with amino acid substitutions in positions +5+6 and +13+14. In contrast, secretion of the protein with amino acid substitutions in positions +2+3, significantly decreased as a result of such mutation, in the presence of phosphatidylethanolamine, reaches the level of wild type protein secretion in the absence of phosphatidylethanolamine. The results suggest an interaction of the N-terminal region of the mature protein under its translocation across the membrane with phosphatidylethanolamine.

Key words: alkaline phosphatase, protein secretion, phospholipids, amino acid substitutions, SecA, *Escherichia coli*

Most secreted proteins of *Escherichia coli* are known to be synthesized as cytoplasmic precursors containing an additional N-terminal extension called a signal peptide that is essential for protein membrane translocation and is cleaved after the translocation is completed (for review see [1, 2]). It contains information for the interaction with protein components of the secretory machinery [1, 2], membrane phospholipids [3, 4], and signal peptidase [5, 6]. The theory of protein topogenesis [7] suggests, however, that the information about protein secretion is contained not only in the signal peptide but also in other "topogenic" sequences, localized in the mature portion of secreted proteins. However, much less is known about the role of such sequences in protein secretion. In our previous work, an export initiation domain of 16-18 amino acid residues in size was revealed in the N-terminal region of mature alkaline phosphatase (PhoA) [8]. The effect of substitutions for amino acid residues in different positions of this domain (positions +2+3; +5+6; +13+14; +19+20) on protein secretion was studied in detail [8, 9]. Substitutions for amino acid residues close to the signal peptide (in positions +2+3) depressed protein secretion most significantly. It is assumed that the export initiation domain is involved in PhoA secretion due to its interaction with components of the secretory machinery. Indeed, the dependence of PhoA secretion on a cytoplasmic chaperone—the protein SecB—was first shown [9]. This export-specific chaperone was also suggested to interact with the export initiation domain of mature PhoA close to the signal peptide, and probably in concert with translocational ATPase (the protein SecA).

Phospholipids also play an important role in protein translocation across the cytoplasmic membrane of *E. coli* [10-12]. The requirement of both anionic phospholipids (APL) [3, 4, 13-15] and zwitterionic phosphatidylethanolamine (PE) [16-19] for efficient protein membrane translocation *in vivo* and *in vitro* has been clearly demonstrated. However, the studies addressing the mechanism of their involvement in protein membrane translo-

Abbreviations: PhoA and prePhoA) *Escherichia coli* alkaline phosphatase and its precursor, respectively; PE) phosphatidylethanolamine; APL) anionic phospholipids; CL) cardiolipin; PG) phosphatidylglycerol; PA) phosphatidic acid; SDS-PAGE) sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG) isopropyl-β-D-thiogalactopyranoside.

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0006-2979/02/6709-0978$27.00 ©2002 MAIK "Nauka/Interperiodica"

cation are limited to experiments where the interaction between APL and the signal peptides was investigated [4, 20-24]. Meanwhile, there are large gaps in the study of the interaction between phospholipids and the mature portion of a protein in the course of its secretion.

The goal of the current work was to elucidate how the effect of anionic and zwitterionic phospholipids on alkaline phosphatase secretion depends on the primary structure of the export initiation domain of mature protein and whether the activity of SecA affects the enzyme secretion and its dependence on phospholipids. The main strategy for addressing the above questions was to analyze the secretion of wild type and mutant PhoAs with amino acid replacements in the N-terminal export initiation domain of mature PhoA in strains of *E. coli* with different compositions of membrane phospholipids. The results of the current work have first shown that the effect of PE but not APL on PhoA secretion is determined by the primary structure of the export initiation domain, which suggests an interaction of the N-terminal domain of mature PhoA with PE.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strain AD93 (*pss9::kan recA srl::Tn10 nadB*$^+$) with and without plasmid pDD72 carrying the *pss* gene [25] and strain HDL11 with a controlled synthesis of anionic phospholipids [14] were used in this study. Plasmid pSAP10 carrying the wild type *phoA* gene and plasmids pSAP12, pSAP13, and pSAP14 carrying mutant *phoA* genes were constructed in the current work by cloning the *phoA* genes in plasmid vector pBAD18 under the control of *ara* P_{BAD} promoter [26]. For this purpose, the wild type and mutant *phoA* genes were amplified by PCR using the following pair of primers: 5′-GCAAGCTAGCTTTGTACATGGAGAAA-ATAAAGTG-3′ and 5′-GCTCTAGATTTCAGCCCCA-GAGCGG-3′, with the sites of restriction endonucleases *Nhe*I and *Xba*I introduced into these primer sequences, respectively. PCR products were cloned into the *Nhe*I-*Xba*I sites of the vector pBAD18. Mutations were confirmed by DNA sequencing [27].

Bacterial strains *E. coli* AD93 and *E. coli* HDL11 and strains containing a temperature sensitive plasmid pDD72 were grown at 37 and 30°C, respectively, in a mineral medium [28] supplemented with tryptophan (100 μg/ml). Because of the requirement of divalent cations for growth of the strain *E. coli* AD93, the latter was grown in the presence of $MgCl_2$ (50 mM) [25]. As required, kanamycin (50 μg/ml), tetracycline (14 μg/ml), and IPTG (100 μM) were added to the growth medium. Chloramphenicol (25 μg/ml) and ampicillin (100 μg/ml) were added to support plasmids pDD72 and pSAP, respectively. To inhibit the activity of SecA, 2 mM sodium azide was added when required. To induce PhoA synthesis, 0.5% of arabinose was added to the culture grown in the mineral medium with P_i to the mid-log phase. The *phoA* gene expression from the chromosome is completely repressed under these conditions. Biosynthetic processes were stopped by 0.02% Merthiolate.

Alkaline phosphatase secretion. The protein secretion was assessed by two approaches: analysis of the alkaline phosphatase activity in culture, since the enzyme becomes enzymatically active only after translocation of the polypeptide chain across the cytoplasmic membrane into the periplasm [29], and analysis of the dynamics of conversion of pulse labeled PhoA precursor into a mature form (maturation) [30].

Alkaline phosphatase maturation. Pulse-chase experiments were used to analyze prePhoA maturation. *E. coli* cells from the mid-log phase of growth were incubated for 10 min with 0.5% arabinose to induce PhoA synthesis, labeled with L-[^{35}S]methionine (50 μCi/ml) for 60 sec and then chased for various periods of time. Culture samples were taken at certain time intervals, proteins were precipitated by 10% TCA, prePhoA and PhoA were immunoprecipitated using antibodies against denatured alkaline phosphatase and analyzed by electrophoresis followed by autoradiography.

Phospholipid analysis. Lipids were extracted according to Ames et al. [31] and chromatographed on boric acid-impregnated TSL plates with Kieselgel 60 (Merck, Germany) in chloroform–methanol–water–ammonium hydroxide (60 : 37.5 : 3 : 1 v/v) [32]. Individual phospholipid spots were detected in iodine vapor, cut out, extracted from the silica gel with chloroform–methanol–water (5 : 5 : 1 v/v), and then lipid phosphorus content quantified spectrophotometrically at 660 nm after staining with malachite green as described [33].

Analytical methods. Proteins were separated in 10% SDS-PAGE [34]. The PhoA activity was assayed by the rate of *p*-nitrophenylphosphate hydrolysis in 50 mM Tris-HCl, 5 mM $MgCl_2$ buffer, pH 8.5 [28], taking the amount of the enzyme hydrolyzing 1 μmol of substrate per 1 min at 37°C as a unit (U) of enzymatic activity. The PhoA activity was measured in whole culture (cells + culture liquid), because PhoA encoded by the gene cloned in plasmid is partially secreted into the medium [35]. Protein was assayed by the Lowry method [36].

RESULTS

Effect of PE but not APL on PhoA secretion is determined by the primary structure of the export initiation domain of the mature protein. Secretion of wild type (wt) PhoA and its mutant forms (K(2,3), K(5,6), K(13,14)) with amino acid substitutions of two Lys for the residues in positions +2+3, +5+6, +13+14 of the mature enzyme, respectively, was studied in the current work. The least effect was observed when Lys residues were placed in

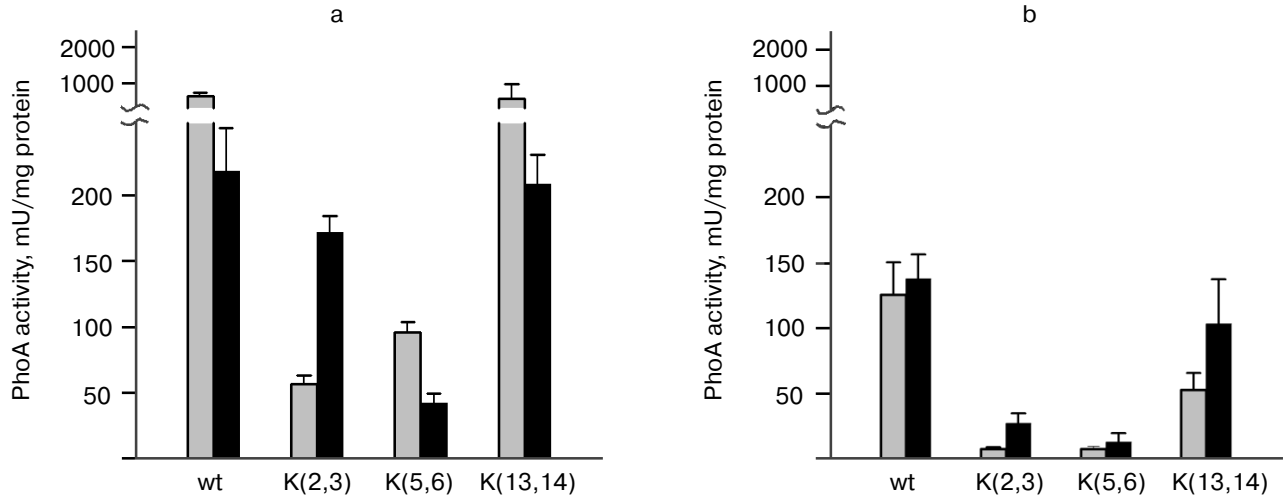

Fig. 1. Effect of PE depletion on secretion of wild type (wt) and mutant PhoAs in the *E. coli* AD93 (−PE) (dark column) and AD93/pDD72 (+PE) (light column) in the absence (a) and presence (b) of 2 mM sodium azide. The data given are the mean values ± S.D. of six independent experiments.

positions +13+14. Amino acid substitutions close to the signal peptide (positions +2+3), in contrast, were shown to have the greatest effect on PhoA secretion (Fig. 1a, light columns), and this mutant protein was studied in more detail later in this work.

To estimate the interaction of PE with the export initiation domain, AD93 strains either containing PE (with plasmid pDD72) or completely lacking PE (without plasmid pDD72) were used. AD93 carries a null allele of the *pss* gene that is required for the synthesis of PE precursor [25]. Plasmid pDD72 carries a functional copy of the *pss* gene and restores the wild type phospholipid composition [25], which was confirmed in the current work (Table 1). PE depletion was shown to reduce about 2.5-3-fold the secretion efficiency of both wild type and mutant PhoAs (Fig. 1a), with the exception of the mutant protein K(2,3) having amino acid substitutions close to the signal peptide (positions +2+3). Its secretion was, in contrast, 3-fold higher in PE-depleted than in PE-containing cells and reached that of the wild type under these conditions. The data obtained indicate that PE depletion partially suppresses the efficiency of protein secretion, which significantly decreases as a result of such mutation. The dynamics of maturation of pulse-labeled wild type and mutant K(2,3) prePhoAs supports this conclusion (Fig. 2a). In the cells of *E. coli* AD93, the wild type prePhoA could be still observed 30 sec after the chase, while the control strain AD93/pDD72 had only mature PhoA already in the zero point (Fig. 2, Ia). Mutant PhoA K(2,3) matured more slowly (Fig. 2, IIa) as expected: its precursor was present in the control strain for even 5 min and the mature protein appeared 1 min after the chase. In PE-depleted strain this mutant protein matured, on the contrary, somewhat more quickly than in the presence of PE, and its mature form appeared already at the zero point. Thus, the effect of PE on PhoA secretion is determined by the primary structure of the export initiation domain.

Table 1. Phospholipid composition of *E. coli* strains depending on cell growth conditions (the data given are the mean values ± S.D. of three independent experiments)

Strain	Growth conditions	Phospholipid content, mole %			
		PE	PG	CL	PA
AD93/pSAP10	Mg^{2+}	0.0	61.6 ± 3.6	32.1 ± 3.0	6.3 ± 0.7
AD93/pDD72/pSAP10	—	78.6 ± 4.5	14.8 ± 1.7	4.0 ± 0.5	2.7 ± 0.3
HDL11/pSAP10	+ IPTG	83.6 ± 2.1	10.7 ± 3.1	3.9 ± 1.3	1.8 ± 0.5
	− IPTG	93.6 ± 0.9	0.4 ± 0.2	1.3 ± 0.7	4.7 ± 0.7

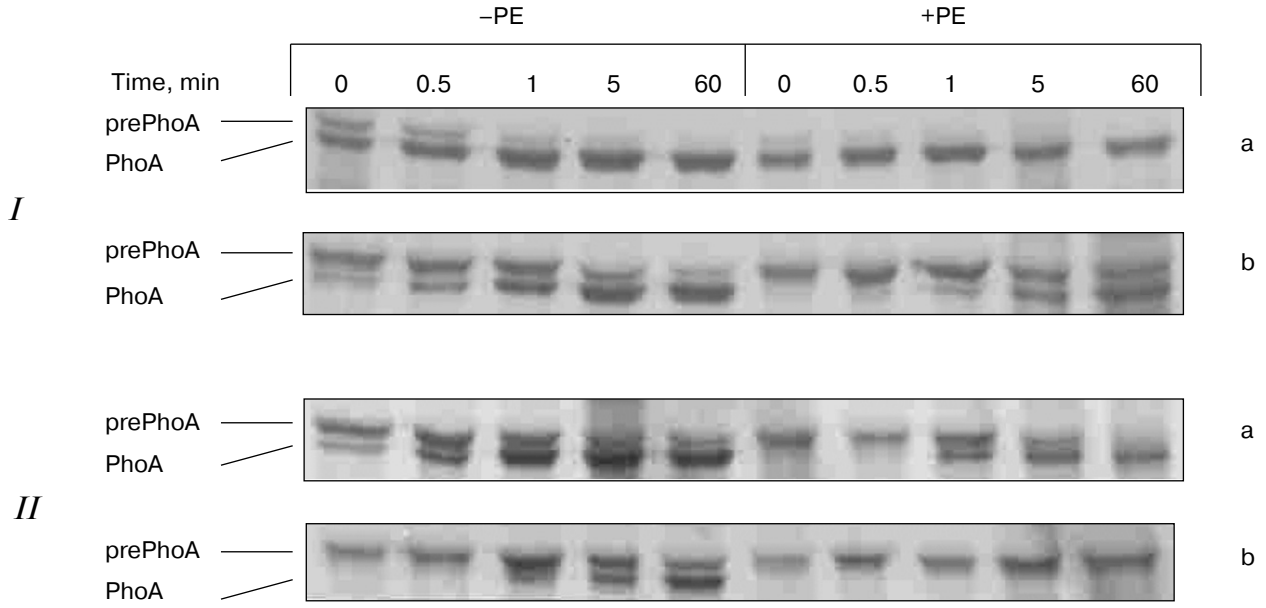

Fig. 2. Effect of PE depletion on maturation of the wild type (I) and mutant K(2,3) (II) PhoAs in the absence (a) and presence (b) of 2 mM sodium azide. After PhoA induction in AD93 (–PE) and in AD93/pDD72 (+PE) by arabinose, cells were pulse-labeled with L-[^{35}S]methionine. At the indicated times aliquots were taken, pulse-labeled PhoA and its precursor were immunoprecipitated and resolved by 10% SDS-PAGE followed by autoradiography.

To estimate the effect of anionic phospholipids on secretion of the mutant PhoAs, strain *E. coli* HDL11 was used with the APL content strongly regulated by the presence of IPTG in the medium [14]. The content of APL (PG, CL, and PA) in this strain grown to the mid-log phase in the mineral medium was shown to be about 6% in the absence and 16% in the presence of IPTG (Table 1). The content of APL, however, had no significant effect on secretion efficiency of both the wild type and mutant PhoAs (Fig. 3a). A minor effect of APL was revealed only by the analysis of the wild type protein maturation in pulse chase experiments (Fig. 4, Ia). The effect of amino acid substitutions close to the signal peptide was the most crucial in this strain as well, independent of the

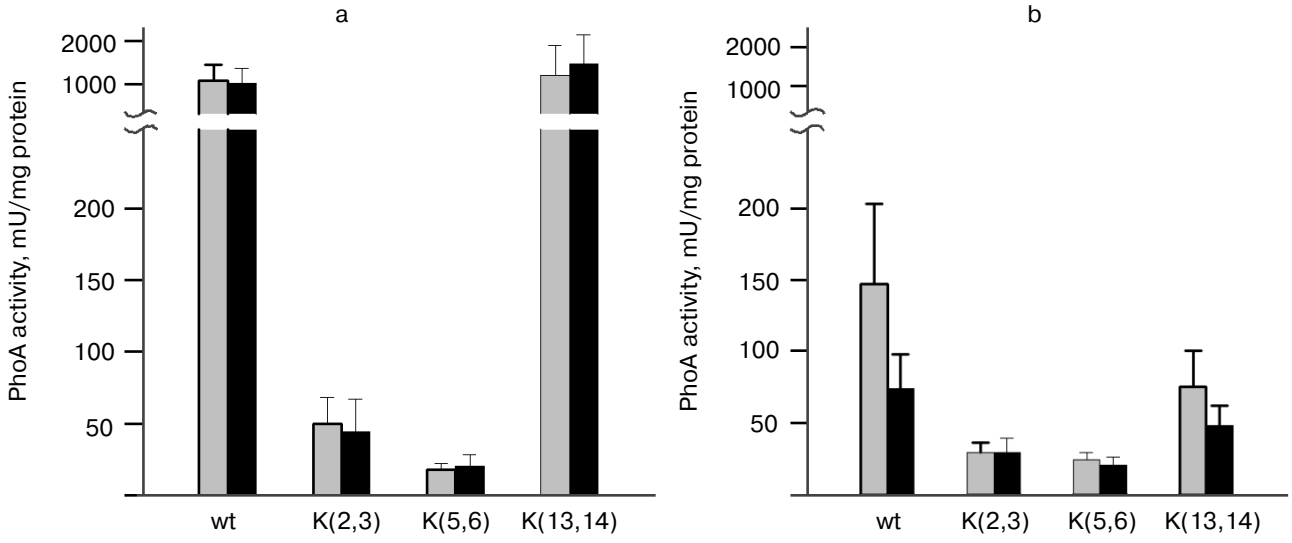

Fig. 3. Effect of the content of anionic phospholipids on secretion of PhoAs (wild type and mutant) in the *E. coli* HDL11 strain grown in the presence (light column) and absence (dark column) of IPTG, in the absence (a) and presence (b) of 2 mM sodium azide. The data given are the mean values ± S.D. of four independent experiments.

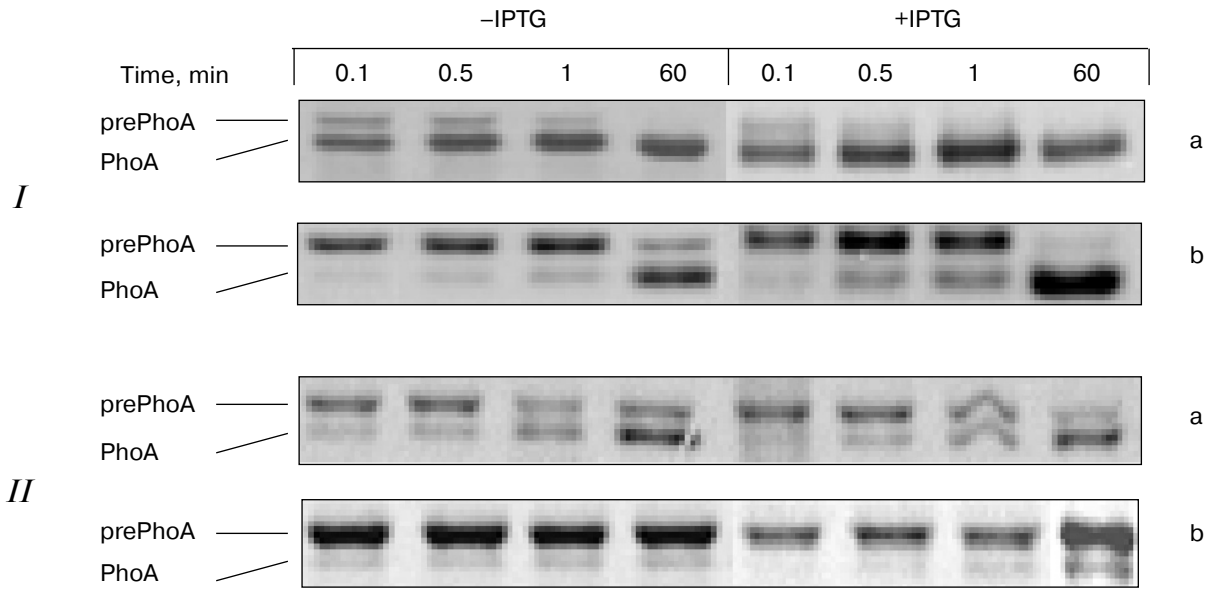

Fig. 4. Effect of APL content on maturation of the wild type (I) and mutant K(2,3) (II) PhoAs, in the absence (a) and presence (b) of 2 mM sodium azide. After PhoA induction in HDL11 strain grown in the presence (+IPTG) and absence (–IPTG) (marked) by arabinose, cells were pulse-labeled and analyzed as described in Fig. 2.

APL content. Precursor of this mutant protein K(2,3) was found even 60 min after chase (Fig. 4, IIa).

Activity of translocational ATPase (protein SecA) affects the phospholipid dependence of PhoA secretion. Inactivation of the translocational ATPase, protein SecA, with sodium azide leads to a significant decrease in secretion efficiency both in PE-containing and PE-depleted cells of the AD93 strain (Figs. 1b and 2b) and in the HDL11 strain with different content of APL (Figs. 3b and 4b). Sodium azide is known as an ATPase inhibitor, and SecA is the most azide-sensitive ATPase of *E. coli* [37]. However, the effect of SecA activity on secretion efficiency of PhoA depends on the phospholipid content of membranes. So, SecA dependence significantly decreases in the absence of PE and, on the contrary, increases at the lower APL content, especially in case of secretion of wild type and mutant K(13,14) PhoA (Table 2). On the other hand, inactivation of protein SecA significantly changes the dependence of PhoA secretion on phospholipids (Table 2). Thus, the secretion of wild type PhoA under these conditions is less dependent on the presence of PE and even, as is seen from Figs. 1b and 2b, becomes more efficient in the absence of this phospholipid. The dependence of PhoA secretion on the content of anionic phospholipids under SecA inactivation, on the contrary, increases (Table 2 and Figs. 3b and 4b) almost 2-fold, judging from the PhoA activity.

The mutant protein K(2,3) with amino acid substitutions close to the signal peptide is an exception. First, the dependence of its secretion on SecA activity is less determined by the content of phospholipids, particularly APL (Table 2). Second, the dependence of its secretion on both PE and APL, in contrast to other proteins, is not determined by SecA activity (Table 2). Thus, mutations in the N-terminal domain of the mature protein, critical for its secretion, change the dependence of the protein secretion on certain components of the secretory machinery and apparently affect the interaction of the protein with these components.

DISCUSSION

The current work has first studied by the example of *E. coli* alkaline phosphatase the interaction between the export initiation domain of the mature portion of a secreted protein and both zwitterionic and anionic phospholipids of membranes. The role of individual phospholipids in specific membrane functions including protein membrane translocation has not been completely understood to date. Mutants with defects in biosynthesis of particular classes of phospholipids provide a powerful tool to study different membrane-associated processes and specifically protein translocation across the membranes. The *pss93* mutant of *E. coli* AD93 lacks PE due to interruption of the *pss* gene [25] that encodes phosphatidylserine (PS) synthase. It was shown with the use of this mutant that PE acts as a molecular chaperone in the folding of a polytopic membrane protein—lactose permease [38]—and also in correction of its incorrect folding [39]. In *E. coli*, PE is the major constituent of the cytoplasmic membrane as well as the major lipid that strongly prefers

Table 2. Dependence of PhoA secretion on phospholipid composition, activity of SecA, and the primary structure of the export initiation domain

Dependence of PhoA secretion	Conditions of PhoA secretion	APL content, mole %	PhoA			
			wt	K(2,3)	K(5,6)	K(13,14)
PE dependence	– azide		3.0	0.3	2.3	2.7
	+ azide		0.9	0.3	0.6	0.5
APL dependence	– azide		1.1	1.2	0.9	0.8
	+ azide		2.0	1.0	1.2	1.6
SecA dependence	+ PE	21.5	8.5	7.7	12.0	11.0
	– PE	100.0	1.6	5.4	3.0	2.0
	+ IPTG	16.4	7.3	1.7	0.8	16.0
	– IPTG	6.4	13.5	1.5	1.0	31.0

Note: PE, APL, and SecA dependences are derived from the data on the alkaline phosphatase activity and presented as a ratio of PhoA activity in PE-containing and PE-depleted cells (PE dependence); in cells grown with and without IPTG (APL dependence); in cells grown with and without sodium azide (SecA dependence). The data presented are the mean values of four to six independent experiments.

to adopt a non-bilayer structure [40]. It has been proposed that the non-bilayer structure of membranes, in particular the balance of bilayer and non-bilayer forming lipids, facilitates protein translocation across the membrane [10, 41]. This idea was first confirmed in experiments *in vitro* with membrane vesicles isolated from the strain AD93 [16] and in our previous work *in vivo* with the use of the same PE-depleted strain [18, 19]. This work has shown for the first time that the character of the PE effect on PhoA secretion is determined by the primary structure of the export-initiation domain of its mature part. Under PE depletion, the secretion of wild type and most mutant proteins with amino acid substitutions in positions far from the signal peptide significantly decreases. However, the secretion of a protein with a mutation close to the signal peptide, which leads to a considerable decrease in secretion efficiency of the strain with the wild type phospholipid composition, is partially restored under PE depletion. Under these conditions, the level of secretion of this mutant protein reaches the level of secretion of the wild type protein. Thus, the defect of the protein structure affecting the efficiency of its secretion is suppressed by the defect in phospholipid composition of membranes. This is apparently evidence of the interaction between the protein and phospholipids, in this case between the N-terminal domain of the mature protein and phosphatidylethanolamine. It is known that most prokaryotic secreted proteins have a negative or electroneutral net charge in the region immediately downstream of the signal sequence, and the net N-C charge imbalance is important for signal sequence function and for protein secretion in bacteria [42]. Introduction of basic amino acid residues immediately downstream of the signal peptide inhibits protein membrane translocation due to the "stop transport" function of such charged residues [43]. In case of interaction of this domain with the membranes during the protein secretion, the zwitterionic phospholipid is more preferable. Anionic phospholipids were shown [4, 20-24] to interact with the positively charged N-terminal domain of the signal peptide. Mutations in the N-terminal region of mature protein (replacements of amino acid residues by two positively charged Lys close to the signal peptide) prevent this interaction with PE, which seems to be important for secretion. The correct protein secretion probably needs neutralization of the positive charge of introduced residues, and this in turn needs APL in the site of prePhoA interaction with the membrane. Such possibility occurs under complete PE depletion. The membrane of the PE-lacking strain AD93 was shown to contain only anionic phospholipids (Table 1), and this seems to be very important for partial restoration of secretion efficiency of mutant proteins with positively charged residues close to the signal peptide. A change in the content of anionic phospholipids only within 6 to 16% in the strain *E. coli* HDL11 has no restoring effect on secretion of this protein. It is important to mention, however, that the secretion of such mutant PhoA under PE depletion is restored only partially, reaching the level of wild type protein secretion under the same conditions of PE depletion, which significantly decreases the efficiency of secretion. In the absence of PE, cells are unable to quite effectively translocate the protein, although cardiolipin in the presence of 50 mM Mg^{2+} can functionally replace PE [16, 19]. Thus, the suggestion that PE is involved in protein secretion, first of all as a non-bilayer-forming lipid, is supported once again.

Since the N-terminal region of mature protein contains hydrophilic and uncharged residues, it is not improbable that just the non-bilayer-forming potential of PE determines the translocation of this region across the membrane and thus provides its entering the hydrophilic SecYEG channel, which is called a translocon and supposed to be open with its flank towards the lipid interior of membranes. This initial step of translocation—the hydrophilic N-terminal region of mature protein entering the secretory pathway—is still unclear.

As regards the effect of anionic phospholipid deficiency on protein secretion, it is less expressed and does not depend on the primary structure of the export initiation domain. However, under PE depletion APL partially restores the secretion efficiency depressed due to the introduction into the protein of a stop-transport signal—positively charged residues close to the signal peptide. Besides, their requirement for PhoA secretion increases under inactivation of SecA, particularly for the secretion of wild type protein. Its secretion is somewhat more dependent on SecA activity when the content of anionic phospholipids decreases, e.g., in the strain HDL11 in the absence of IPTG. On the contrary, this SecA dependence decreases as the content of anionic phospholipids increases, particularly under PE depletion, indicating that the APL increase in the absence of PE compensates for the effect of SecA inactivation. The current work has revealed a definite correlation (see SecA dependence in Table 2) between the content of APL and dependence of PhoA secretion on SecA activity. The results allow us to conclude that SecA and APL can be partially interchangeable to provide secretion. However, APL have never been able to restore secretion efficiency to the level corresponding to that in the presence of active SecA protein. This indicates that SecA is more important for secretion than anionic phospholipids, though they are probably involved in the same mechanism.

In conclusion it may be said that the N-terminal region of the mature secreted protein, localized immediately after the signal peptide, is able to interact with membranes through the zwitterionic phospholipid PE, and this interaction is in agreement with structural and charge features of this region of the mature protein and probably provides the hydrophilic protein entering the secretory pathway. The revealed interrelation between the requirement for anionic phospholipids and protein SecA for secretion is evidence of possible cross-reciprocal usage of these components of the secretory machinery in protein secretion.

We are grateful to W. Dowhan for kindly providing us with AD93 and HDL11 strains with controlled phospholipid synthesis, to M. Bogdanov for assisting us with optimization of the strain cultivation and fruitful discussion, to J. Beckwith for pBAD-18 plasmid, and to N. Mikhaleva and M. Shilov for assistance in providing some experiments.

This work was supported by the Russian Foundation for Basic Research (grant Nos. 99-04-48153, 00-15-97851, 02-04-49765 and 02-04-06304) and by an Award of the CRDF (the US Civilian Research and Development Foundation for the Independent States of the Former Soviet Union) (grant RB1-2038).

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