Anionic lipids stimulate Sec-independent insertion of a membrane protein lacking charged amino acid side chains

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We have investigated the influence of the different lipid classes of *Escherichia coli* on Sec-independent membrane protein insertion, using an assay in which a mutant of the single-spanning Pf3 coat protein is biosynthetically inserted into liposomes. It was found that phosphatidylethanolamine and other non-bilayer lipids do not have a significant effect on insertion. Surprisingly, the anionic lipids phosphatidylglycerol and cardiolipin stimulate N-terminal translocation of the protein, even though it has no charged amino acid side chains. This novel effect is general for anionic lipids and depends on the amount of charge on the lipid headgroup. Since the N-terminus of the protein is at least partially positively charged due to a helix dipole moment, apparently negatively charged lipids can stimulate translocation of slightly positively charged protein segments in a direction opposite to the positive-inside rule. A mechanism is proposed to explain these results.

INTRODUCTION

Biological membranes contain a diversity of lipids which differ in the nature of their headgroups. The composition of the inner membrane of *Escherichia coli* is relatively simple, containing ~75% of the zwitterionic lipid phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG) and 5% cardiolipin (CL); the latter two lipids are both negatively charged (Raetz, 1978). Because of this simplicity and the possibility of modulating the lipid composition of this organism, much is known about the functions of these lipids in *E. coli*, for example in membrane protein translocation.

The most abundant lipid of *E. coli*, the zwitterionic PE, has a small headgroup and therefore has a tendency to form non-bilayer structures. By replacing PE either with lipids that prefer to

form bilayers or with lipids prone to form non-lamellar structures, it was found that a certain non-bilayer propensity is crucial for protein translocation in *E. coli* (Rietveld *et al.*, 1995) as well as for stimulation of the activity of reconstituted Sec-translocase (Van der Does *et al.*, 2000). Furthermore, and most likely also related to its small headgroup size, it was found that PE stimulates partial insertion of the catalytic domain of the enzyme leader peptidase into the membrane (Van Klompenburg *et al.*, 1998; E. Van den Brink-Van der Laan, R.E. Dalbey, J.A. Killian and B. de Kruijff, submitted). PE can also act as a molecular chaperone in the folding of the integral membrane protein LacY, which could be related to packing properties of the membrane (Bogdanov and Dowhan, 1998).

Negatively charged lipids, which constitute the remaining part of the *E. coli* inner membrane, are essential for protein translocation via the translocase (De Vrije *et al.*, 1988; Kusters *et al.*, 1991, 1994; Van der Does *et al.*, 2000). Due to electrostatic interactions between anionic lipids and positively charged amino acid residues, these lipids are important in the membrane interaction of SecA (Lill *et al.*, 1990; Hendrick and Wickner, 1991; Breukink *et al.*, 1992) and signal sequences (Keller *et al.*, 1992, 1996; Phoenix *et al.*, 1993a,b; Sankaram and Jones, 1994). Anionic lipids are also essential for the Sec-independent translocation of M13 coat protein (Gallusser and Kuhn, 1990; Kusters *et al.*, 1994; Soekarjo *et al.*, 1996). Negatively charged lipids can even affect the topology of proteins, as was shown with constructs of the well studied membrane protein leader peptidase (Van Klompenburg *et al.*, 1997), and are thus at least partially responsible for the positive-inside rule.

The observed effects of these two different lipid classes, non-bilayer and negatively charged lipids, on protein insertion or translocation described above can in most cases be ascribed to

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effects on components of the Sec-translocation machinery. In this study, we have investigated direct effects of the different *E. coli* lipids on insertion of an integral membrane protein. For this purpose, a mutant of the Pf3 major coat protein was used. This small, 5 kD single-spanning protein does not need the Sec-machinery for its insertion (Rohrer and Kuhn, 1990; Kiefer and Kuhn, 1999). Although wild-type Pf3 depends on YidC for efficient membrane insertion, mutants with more hydrophobic transmembrane segments are YidC- and pmf-independent (M. Chen, F. Jiang, D. Kiefer, R. Dalbey and A. Kuhn, in preparation) and can thus efficiently insert co-translationally into pure lipid vesicles using an *in vitro* translation/translocation protocol (Ridder *et al.*, 2000). This allows us to investigate systematically for the first time the effect of lipids on biosynthetic insertion of a protein in the bilayer without making use of artificial approaches using purified protein.

Using a Pf3 mutant without any charged amino acid side chains, we investigated the influence of different lipid classes on its membrane insertion. Surprisingly, the *E. coli* lipids PG and cardiolipin, as well as other anionic lipids, were shown to substantially stimulate N-terminal translocation of this protein. A mechanism for this novel effect of anionic lipids on membrane protein insertion is discussed.

RESULTS AND DISCUSSION

Translocation assay

In order to investigate the lipid dependency of direct membrane insertion of a protein, a Pf3 mutant called 3L-4N was used (Figure 1A). Both the N- and C-terminal tails of this mutant are neutral and translocation of either terminus is about equally efficient (Kiefer and Kuhn, 1999). The transmembrane segment of the 3L-4N protein is lengthened by three leucine residues and it inserts independently of the proton motive force into *E. coli* membranes (Kiefer and Kuhn, 1999). Therefore, it is also able to insert into pure lipid vesicles that do not have a proton motive force (Ridder *et al.*, 2000). The strategy used to determine the amount of N-terminal translocation is depicted in Figure 1B. The translocation experiments were conducted by adding large unilamellar vesicles (LUVs) to an *E. coli* translation system to a final concentration of 2 mM (phospho)lipid. Since this mixture contains [³⁵S]methionine, all synthesized protein contains a radioactive label at its N-terminus. After 30 min incubation at 37°C to allow translation and translocation to take place, an aliquot is withdrawn to serve as 25% standard (Figure 1C, lane 1). The LUVs with associated proteins are subsequently pelleted by centrifugation. The supernatant is removed and contains typically ~20% of the radiolabeled protein (lane 2). Approximately 70% of the labeled protein can be pelleted together with the LUVs (lane 3). This pellet is divided into two equal portions, one of which is treated with proteinase K. Treatment with proteinase K yields a radiolabelled fragment with a slightly lower molecular weight (lane 4), corresponding to protein inserted with its N-terminus into the vesicles and its C-terminus cleaved off by proteinase K. When no LUVs are present in the translation mixture, ~10% of the protein is pelleted, but no protease-protected fragment is observed (data not shown). Throughout this study, the translocation efficiency is expressed as the fraction of bound protein

Fig. 1. (A) Amino acid sequences of Pf3 and the 3L-4N mutant. The hydrophobic transmembrane segment is shown in italics. The charged residues in Pf3, which are changed to asparagines in the 3L-4N mutant, are shown in bold, as well as the three additional leucines in the hydrophobic segment of 3L-4N. The underlined residues show the position of the tag introduced to make the C-terminus protease-cleavable (Kiefer *et al.*, 1997). (B) Schematic representation of the translocation assay (see text for details). For sake of clarity only transmembrane orientations of the proteins are drawn. (C) Translocation assay of 3L-4N with LUVs composed of 75% DOPC and 25% DOPG. Lane 1, 25% standard; lane 2, supernatant after centrifugation; lane 3, pellet fraction after centrifugation; lane 4, pellet fraction after proteinase K (pK) treatment.

that exhibits this shift in molecular weight upon proteinase K treatment.

Effect of PE and other non-bilayer lipids

First, we investigated the influence of the most abundant lipid of *E. coli*, the non-bilayer lipid PE. This lipid is essential for Sec-dependent protein translocation (Rietveld *et al.*, 1995), but its effect on Sec-independent translocation is unknown. We investigated this by employing liposomes containing 75% zwitterionic lipids and 25% of the negatively charged dioleoyl phosphatidyl glycerol (DOPG), thus mimicking the lipid composition of the *E. coli* inner membrane. The zwitterionic

Fig. 2. (A) Influence of DOPE on translocation of 3L-4N. Translocation experiments were conducted with LUVs containing 25% DOPG and 75% DOPC, DOPE or a mixture of these two lipids. The points are the averages of three or four experiments and the error bars indicate the standard deviation. (B) Influence of DOG or MGDG on translocation of 3L-4N. For DOG, DOPC LUVs with increasing amounts of DOG were used. For MGDG, the experiments were conducted with LUVs containing 25% DOPG and 75% DOPC, which was partially replaced by MGDG. In both cases, the situation without non-bilayer lipid was set at 100%. The experiment was performed twice and both values are shown.

bilayer-lipid dioleoyl phosphatidylcholine (DOPC) in the LUVs was gradually replaced by dioleoyl phosphatidylethanolamine (DOPE) and N-terminal translocation efficiencies of 3L-4N were determined (Figure 2A). Inclusion of 25% PE results in a slight increase in translocation efficiency, but at higher PE concentrations translocation decreases slightly. To investigate whether this effect of PE is significant and can be related to its non-bilayer forming properties, we also investigated whether the same effects occur with lipids with stronger non-bilayer propensities. For this purpose, dioleoylglycerol (DOG) and the plant lipid monogalactosyl diacylglycerol (MGDG) were used, of which less can be incorporated in the bilayer of unilamellar vesicles (De Boeck and Zidovetzki, 1989; Pinnaduwege and Bruce, 1996; Hinch *et al.*, 1998). As can be seen in Figure 2B these two lipids have no significant effect on the translocation efficiency of 3L-4N. Therefore, we conclude that the non-bilayer propensity of the membrane has no significant influence on Sec-independent protein insertion. The importance of PE in protein translocation was observed previously (Rietveld *et al.*, 1995; Van der Does *et al.*, 2000) is, therefore, most likely to be due to effects on the translocase.

Influence of negatively charged lipids

Next, we investigated whether the negatively charged lipids of *E. coli* have an effect on direct membrane insertion of the

Fig. 3. (A) Translocation of 3L-4N in DOPC LUVs with increasing amounts of DOPG. Only the pellet fractions are shown, which were treated with proteinase K (pK) where indicated. (B) Translocation of 3L-4N in DOPC LUVs with increasing amounts of cardiolipin (CL).

protein. For this purpose, the amount of N-terminal translocation of the 3L-4N mutant was determined in LUVs composed of the zwitterionic lipid DOPC and different amounts of negatively charged lipids.

In 100% phosphatidylcholine (PC) vesicles, two protease-protected fragments are observed (Figure 3). Apparently a portion of the protein is inaccessible to proteinase K. Since it is not known how these proteins are associated with the membrane, this fragment was not included in our calculation of the N-terminal translocation efficiency. Upon incorporation of negatively charged lipids in the LUVs, this uncleaved fragment disappears (Figure 3). As is shown in Figure 3A, increasing amounts of DOPG lead to a remarkable increase in the amount of N-terminal translocation of 3L-4N. Surprisingly, the translocation efficiency of this protein, which does not contain any charged side chains, increases >2-fold from ~20% in pure DOPC to >50% in vesicles containing 75% DOPG, where maximal translocation is observed (Figure 4A). In vesicles of 100% DOPG, the translocation efficiency is somewhat lower, suggesting that there is a certain optimal charge density of the membrane.

We compared the behaviour of PG with that of cardiolipin, the other negatively charged lipid of *E. coli*. As shown in Figure 3B, cardiolipin also stimulated N-terminal translocation of 3L-4N. A similar large increase in translocation efficiency to ~60% is observed as for PG (Figure 4A). Even when the upper band is included in the calculation, the translocation efficiency still increases almost 2-fold when as little as 5–10% of CL is included in liposomes (data not shown). For CL the optimal concentration is lower than for PG, since maximal translocation is already observed at ~20% CL, while at higher CL concentrations the translocation efficiency decreases again. The maximal translocation efficiency of 50–60% in liposomes is similar to that observed in *E. coli* membrane vesicles (Ridder *et al.*, 2000), suggesting that a similar insertion mechanism is used in both cases.

The difference in the optimal concentration of PG and CL could be caused by the fact that their amount of charge is

Fig. 4. (A) Influence of DOPG and CL on translocation of 3L-4N. (B) Influence of DOPA and DOPS on translocation of 3L-4N. Translocation in 100% DOPC LUVs is $22.07 \pm 3.4\%$ ($n = 8$). The other values were determined twice and both are indicated in the figure.

different. The PG headgroup contains one negative charge, while CL consists of two PG molecules coupled together via a glycerol and therefore has two charged groups.

To gain insight into the role of the amount of charge, we also investigated the influence of two other negatively charged lipids on insertion of 3L-4N. For this purpose, we used dioleoyl phosphatidic acid (DOPA) and dioleoyl phosphatidylserine (DOPS), which have different headgroup sizes and different charge densities. These two lipids were incorporated into LUVs and the extent of N-terminal translocation of the mutant 3L-4N was determined. As shown in Figure 4B, these two negatively charged lipids are also able to stimulate translocation and again the maximal translocation efficiency is 50–60%, demonstrating that the stimulatory effect is general for anionic phospholipids. The amount of phosphatidic acid (PA) needed for maximal translocation is much lower than of phosphatidylserine (PS), for which the optimal amount is more similar to that of PG. Since at pH 8.0 PA contains ~1.5 negative charge, while PG and PS have only one net charge, it can be concluded that the amount of negatively charged lipid needed for maximal translocation depends on the amount of charge on the lipid headgroup: if a lipid is more highly charged, less of it is needed. These results confirm that there is indeed a certain charge density of the membrane that is optimal for N-terminal translocation.

Although stimulatory effects of negatively charged lipids on protein translocation were found previously, these could be

ascribed to effects on the translocase, or to increased membrane binding of a protein due to electrostatic interactions between the anionic lipids and positively charged residues as, for example, in the case of M13 (De Vrije *et al.*, 1988; Gallusser and Kuhn, 1990; Kusters *et al.*, 1991, 1994; Soekarjo *et al.*, 1996). However, the stimulatory effect of anionic lipids on translocation of a Sec-independent protein lacking charged residues is a novel observation. What is the explanation for this effect? We propose that even though the 3L-4N protein does not contain any charged amino acid side chains, electrostatic interactions are nevertheless involved. It is not known whether the N-terminus of the protein remains formylated *in vitro* and consequently is uncharged, but if not, both the N- and the C-terminus will contain one charge. An additional contribution might come from a helix dipole moment. Interaction of a protein with the bilayer surface generally promotes the formation of secondary structure (White and Wimley, 1999). Indeed, Pf3 coat protein was shown to already contain 40% α -helix in buffer, while in membranes the helical content increased to 75% (Thiaudière *et al.*, 1993). Therefore, it is very likely that the protein will have a substantial amount of helical structure when it is bound to the membrane, resulting in a helix dipole moment, which is equivalent to ~0.5 positive charge on the N-terminal side and 0.5 negative charge on the C-terminal side. Overall, the N-terminal part of the protein will thus be positively charged, while the C-terminal part is negative. When the membrane contains increasing amounts of negatively charged lipids, this charge distribution in the protein possibly results in attraction of the N-terminus and repulsion of the C-terminus by the bilayer, thereby orienting the protein and thus facilitating translocation of the slightly positively charged N-terminus. This is surprising, since positively charged amino acids are 4-fold more abundant on the cytosolic side of the membrane and can control the topology of membrane proteins—the so-called positive-inside rule (Von Heijne, 1986, 1989). This rule originates at least partially from electrostatic interactions of positively charged amino acids with negatively charged lipids at the *cis*-side of the membrane (Van Klompenburg *et al.*, 1997). However, our results demonstrate that when the positive charge on a protein segment is small enough, such a segment can be translocated across the membrane in a process that is stimulated by negatively charged lipids. At high concentrations of anionic lipids, translocation of the N-terminus of our protein becomes hampered, presumably because the electrostatic interactions are now strong enough to cause retention of the positive charge on the *cis*-side of the membrane, in accordance with the positive-inside rule.

In conclusion, we have observed a novel effect of negatively charged lipids on protein translocation, namely stimulation of translocation of a slightly positively charged protein segment in a direction opposite to the positive-inside rule. This effect could also play a role in the membrane insertion of other small proteins or peptides.

METHODS

Materials. The lipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), cardiolipin (CL), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) were supplied by

Avanti Polar Lipids. The 1,2-dioleoyl-*sn*-glycerol (DOG) was from Doohan Serdary Research Laboratories. Monogalactosyl diglyceride (MGDG) was obtained from Larodan Lipids.

The Pf3 mutant used throughout this study is the neutral 3L-4N protein (Kiefer and Kuhn, 1999; Figure 1A).

Preparation of LUVs. Dry lipid films were prepared by mixing the appropriate amounts of lipids dissolved in chloroform and evaporation of the solvent under a stream of nitrogen. These films were dried overnight under vacuum to remove all solvent. They were then rehydrated at room temperature in LUV buffer (104 mM Na₂SO₄, 40 mM HEPES pH 8.0) to a final concentration of 10 mM phospholipid and vortexed. After 10 freeze-thawing cycles, the vesicle suspensions were extruded 10× through 0.4 µm polycarbonate filters. The LUVs were stored under nitrogen at 4°C for a maximum of one day before use.

In vitro translocation. *In vitro* transcriptions and translations were carried out as described (Ridder *et al.*, 2000). Translation/translocation reactions included 5 µCi [³⁵S]methionine, 6 µl of S-135 *E. coli* extract, 8 µl of transcription mix and 10 µl of the appropriate LUV-suspension and had a total volume of 50 µl. This reaction mixture was incubated at 37°C for 30 min to allow translation and translocation to proceed, and afterwards an aliquot of 10 µl was withdrawn to serve as 25% standard. The LUVs were subsequently re-isolated by centrifugation. In order to pellet the LUVs, the salt concentration was lowered by the addition of 120 µl of H₂O and subsequently the mixture was centrifuged in a TLA100 rotor at 100 000 r.p.m. for 60 min at 4°C. The supernatant was removed and the pellets were resuspended in 40 µl of LUV buffer. The pellet fraction was divided into two equal aliquots, one of which was treated with 40 µl of proteinase K (pK, 1 mg/ml) for 30 min at room temperature. All samples were trichloroacetic acid precipitated and analyzed on 16.5% SDS-tricine gels containing 6 M urea (Schägger and von Jagow, 1987). Protein bands were quantified on a Phosphor-Imager (Molecular Dynamics).

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