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Anionic phospholipids are determinants of membrane protein topology

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The orientation of many membrane proteins is determined by the asymmetric distribution of positively charged amino acid residues in cytoplasmic and translocated loops. The positive-inside rule states that loops with large amounts of these residues tend to have cytoplasmic locations. Orientations of constructs derived from the inner membrane protein leader peptidase from *Escherichia coli* were found to depend on the anionic phospholipid content of the membrane. Lowering the contents of anionic phospholipids facilitated membrane passage of positively charged loops. On the other hand, elevated contents of acidic phospholipids in the membrane rendered translocation more sensitive to positively charged residues. The results demonstrate that anionic lipids are determinants of membrane protein topology and suggest that interactions between negatively charged phospholipids and positively charged amino acid residues contribute to the orientation of membrane proteins.

Keywords: anionic phospholipids/membrane proteins/topology

Introduction

In most cases, all molecules of each membrane protein share the same orientation. How is this achieved? Features of both the pre-existing membrane and the newly synthesized proteins are likely to be involved. The orientation of many proteins is dictated by arginyl and lysyl residues in short loops (<60 residues) connecting the hydrophobic transmembrane helices (von Heijne and Gavel, 1988; Gavel *et al.*, 1991; Gavel and von Heijne, 1992; Sipos and von Heijne, 1993). Loops that do not translocate across the membrane contain more of these positively charged residues than do translocated loops (the 'positive-inside' rule). In addition, positively charged residues are able to block the translocation of larger domains if they are placed immediately downstream of a signal peptide or transmembrane helix (Yamane and Mizushima, 1988). How the positively charged residues exert their influence on membrane protein topology is largely unknown. Evidence for a role for the protonmotive force (pmf) was published (Andersson and von Heijne, 1994), and involvement of lipid composition hypothesized (Andersson *et al.*, 1992; Krishtallik and Cramer, 1995). In the present study, we report on the involvement of negatively charged phospholipids in preventing transmembrane passage of positive charges. Anionic phospholipids are present in all membranes (Zambrano *et al.*, 1975; Shibuya *et al.*, 1985; Douce and Joyard, 1990; Hovius *et al.*, 1990) and fulfill diverse functions, often based on electrostatic interactions with positively charged protein domains (Van der Waart *et al.*, 1983; Sixl *et al.*, 1984; Yang and Glaser, 1995). We reasoned that charge interactions between arginyl and lysyl residues and anionic phospholipids might determine the orientation of membrane proteins. To test this hypothesis, lipid biosynthetic *Escherichia coli* mutants, in which the anionic phospholipid content could be manipulated between 10 and 100%, were employed. In these strains, the transmembrane orientations of constructs derived from leader peptidase (Lep, also called signal peptidase I) were determined. Lep is a well-characterized inner membrane protein from *E.coli* which removes signal peptides from translocated precursor proteins (Wolfe *et al.*, 1985). Lep is often used as a model to study membrane protein topogenesis. The orientation of Lep can be altered by the addition or removal of positively charged residues in appropriate locations, as was predicted by the positive-inside rule (von Heijne, 1989). Analysis of the orientations of these constructs in the lipid mutant strains demonstrates that negatively charged phospholipids can inhibit translocation of positively charged domains, thereby contributing to the control of membrane protein topology.

Results

In order to test whether anionic phospholipids in the inner membrane of *E.coli* influence the orientation of membrane proteins, constructs derived from Lep were expressed in strain HDL11. In this strain, expression from the *pgsA* gene which encodes the key enzyme for the synthesis of anionic phospholipids is under control of the *lac* promoter (Kusters *et al.*, 1991). When cells are grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), the inner membrane contains wild-type levels of the anionic phospholipids phosphatidylglycerol (PG, 16%) and cardiolipin (CL, 3%) (Kusters *et al.*, 1991). In the absence of IPTG, the levels of PG and CL drop to 2 and 1% respectively. However, this is partially compensated by accumulation of the precursor lipid phosphatidic acid such that a level of 9% acidic phospholipids remains (Kusters *et al.*, 1991).

Lep was chosen as a model in our studies because its assembly and orientation are well established. The wild-type protein has two hydrophobic transmembrane helices (H1 and H2) separated by a positively charged cytoplasmic loop (P1), and a large periplasmic domain (P2) (similar to 0K/K in Figure 1). Previously, several constructs based

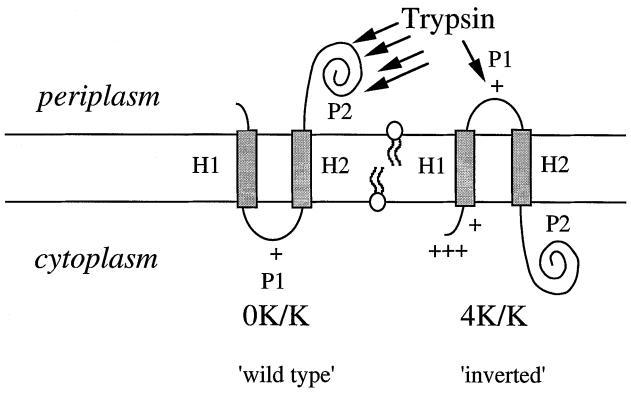

Fig. 1. Schematic representation of the topology of Lep constructs. 0K/K (left) and 4K/K (right) with wild-type and inverted topology are shown. Apart from the varying number of lysines inserted between residues 4 and 5, these constructs differ from wild-type Lep by a replacement of the highly positively charged segment 30–52 (in the P1 loop) with a nine residue spacer and the substitution of Lys56 by asparagine. The resulting *n*K/K series (one lysine in the P1 loop, varying numbers of lysines in the N-terminal tail) has been described previously (Nilsson and von Heijne, 1990).

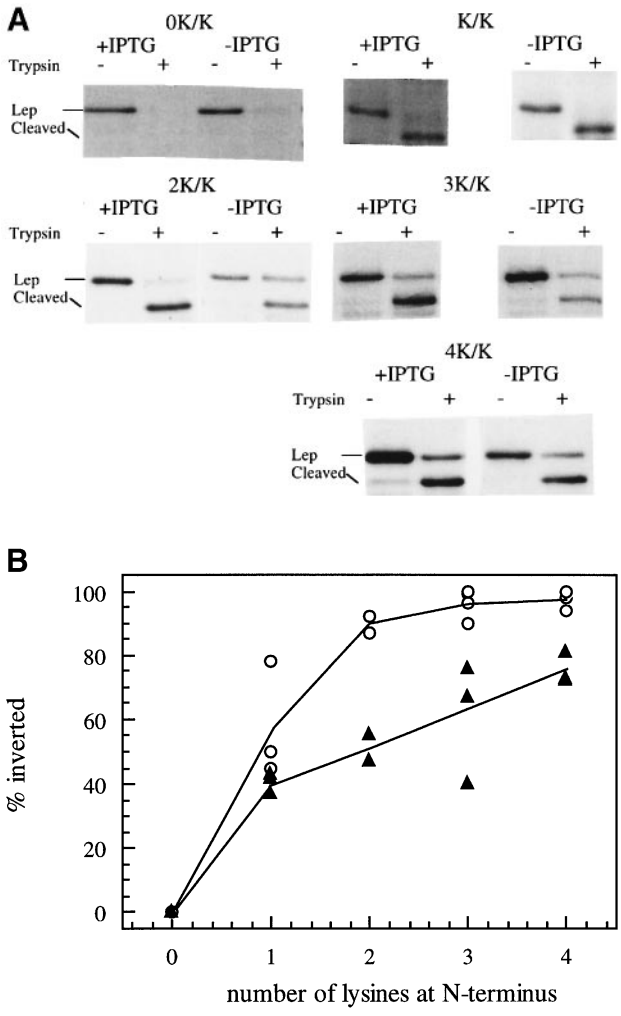

Fig. 2. Influence of negatively charged lipids on the fraction of inverted molecules of the *n*K/K series in HDL11. (**A**) Trypsin treatment of HDL11 spheroplasts with different Lep constructs. Overnight cultures were diluted into mimimal medium without (–IPTG) or with (+ IPTG) 50 μM IPTG to induce phosphatidylglycerol synthesis. The topologies of the constructs were determined as described in Materials and methods. (**B**) Quantitation of the topologies of each individual point in cells grown with (○) and without (▲) IPTG.

on Lep with one positive charge in the P1 loop and varying amounts of positive charges at the N-terminus were made (Nilsson and von Heijne, 1990). This series is called the *n*K/K series where *n* indicates the number of lysines at the N-terminus. These constructs were expressed in strains with wild-type lipid composition and used to demonstrate that membrane proteins obey the positive-inside rule. A construct without positive charge at the N-terminus (0K/K) resides in the membrane with the same orientation as Lep; referred to hereafter as the 'wild-type' topology (Figure 1). A construct with four lysines at the N-terminus (4K/K) resides in the membrane with a so-called inverted topology (Figure 1), which is in accordance with the positive-inside rule. The orientation of different Lep constructs in the inner membrane is usually assayed by protease treatment of spheroplasts (von Heijne, 1989). The P2 domains of molecules with 'wild-type' orientation are degraded such that no immunoprecipitable material remains. The inverted orientation of Lep is detected by a shift in apparent molecular weight as a result of cleavage by trypsin in the P1 loop.

The constructs of the *n*K/K series were expressed in HDL11 cells, which were grown in the presence of IPTG and therefore had wild-type lipid composition. After conversion to spheroplasts, the topologies were determined by trypsin treatment and immunoprecipitation, and typical examples of such experiments are shown in Figure 2A. 0K/K inserts in the wild-type orientation (Figure 2A), as shown by the complete loss of immunoprecipitable material after trypsin treatment. Construct K/K has equal amounts of charges at the N-terminus and in P1. Half of the population of K/K molecules inserts in the inverted orientation, as shown by trypsin cleavage in the P1 loop, and the other half inserts in the wild-type orientation. As expected from the positive-inside rule, increasing amounts of positively charged residues at the N-terminus cause the constructs to insert with an 'inverted' orientation (Figure 2A, lanes marked '+IPTG'). When constructs insert in the inverted orientation, usually a small portion of undigested molecules is also observed (Nilsson and von Heijne, 1990). The relative amount of inverted molecules could be calculated from the intensity of the bands of the trypsin-treated and untreated lanes after correction for lysis and loss of one methionine after cleavage in the P1 loop. Lysis was corrected for by making use of the cytosolic proteins AraB and BandX (de Gier *et al.*, 1996). Because of the addition of magnesium to the spheroplasts, very little lysis (<10%) was observed. The percentage of inverted molecules as a function of the amount of N-terminal lysines was calculated from several experiments and is depicted by the open symbols in Figure 2B. Under these conditions, the topologies of the constructs of the *n*K/K series are comparable with those previously published for a wild-type strain (Nilsson and von Heijne, 1990). When HDL11 cells were grown in the absence of IPTG, the anionic lipid contents dropped below 10%. In this situation, constructs 0K/K and K/K insert with the same orientations as in membranes with wild-type lipid composition (Figure 2). On the other hand, constructs

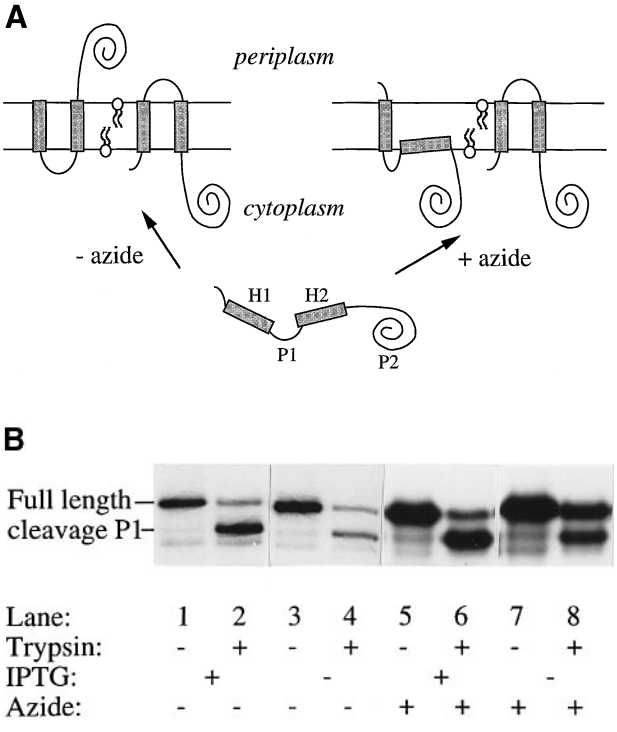

Fig. 3. Effects of azide on topology. (**A**) Schematic representation of the effects of azide on the topology of Lep. (**B**) Azide pre-treatment confirms the mixed topology of construct 2K/K. HDL11 cells grown in the presence (lanes 1, 2, 5 and 6) or absence of IPTG (lanes 3, 4, 7 and 8) were treated without (lanes 1–4) or with 2 mM sodium azide (lanes 5–8) 1 min prior to labeling, to block the Sec-dependent translocation of the P2 domain. Labeling (2 min pulse, 30 s chase) and protease protection assays were carried out as described in Materials and methods.

with two, three or four lysines at the N-terminus show less increase in the fraction of inverted molecules (Figure 2A, lanes marked '–IPTG') when compared with the situation '+IPTG'. After correction for lysis, the percentage of inverted molecules was calculated and plotted in Figure 2B (closed symbols). Figure 2B shows that in cells with wild-type lipid composition, positively charged amino acid residues are much stronger topological determinants than in cells with reduced anionic lipid contents. This implies that with decreased anionic phospholipid contents, positive charges at the N-terminus can pass the membrane more readily.

The two possible orientations of Lep derivatives are achieved by different mechanisms; while translocation of the P2 domain is catalyzed by the proteinaceous secretion (Sec) machinery (Lee *et al.*, 1992), membrane passage of the P1 loop does not require functionality of this machinery (Andersson and von Heijne, 1993). To show that the effects of anionic lipids as observed in Figure 2 are not due to an altered functioning of the Sec machinery, the Sec machinery was blocked by azide. The rationale of this experiment is displayed in Figure 3A. Azide blocks the SecA-dependent translocation of the P2 domain, while insertion of P1 is unaffected under these conditions. Therefore, the ratio between fully protected molecules and those cleaved in P1 is a measure of the ratio between molecules inserting in the wild-type and the inverted orientations.

Cells were grown in the presence of IPTG and the topology of construct 2K/Kwas analyzed. More than 90% of the 2K/K molecules can be cleaved in the P1 domain (Figure 3B, lanes 1, 2, 5 and 6) both in the absence and presence of azide. In the absence of IPTG, only 55% of 2K/K is cleaved in P1 (Figure 3B, lanes 3, 4, 7 and 8) in the presence and absence of azide. In the absence of anionic phospholipid synthesis, azide gives rise to a large increase in fully protease-protected material. This shows that in the absence of IPTG and azide, HDL11 cells can indeed translocate the P2 loops of 45% of the 2K/K molecules, making use of a functional Sec machinery. Neither with wild-type nor lowered levels of anionic lipids does the azide treatment result in a change in the amount of inverted molecules. It should be concluded, therefore, that the effects of lowered anionic lipids content on the orientation of membrane proteins cannot be attributed to a decreased functioning of the Sec machinery.

When placed immediately downstream of a transmembrane segment, positively charged residues can also control topology by blocking the SecA-dependent translocation of large periplasmic domains (Andersson and von Heijne, 1991). To investigate the effects of increased amounts of anionic phospholipids, the experiments were carried out in *E.coli* strain AD93 (De Chavigny *et al.*, 1991). This strain lacks an intact *pss* gene and therefore does not synthesize the only zwitterionic lipid class of *E.coli*, phosphatidylethanolamine. This results in a membrane with only negatively charged lipids. For growth and survival, this strain requires divalent cations. Therefore, conventional EDTA–lysozyme treatment does not result in stable spheroplasts. Moreover, pulse labeling of these cells is severely hampered because of their inability to grow on minimal media. For these technical reasons, we switched to a different approach to see whether anionic lipid levels can control passage of positively charged residues in this situation. To determine the localization of the P2 domain of Lep *in vivo*, we made use of constructs in which the mature part of the periplasmic marker alkaline phosphatase (Manoil and Beckwith, 1985) was fused to amino acid 253 of Lep (Figure 4A). Enzymatic activity of alkaline phosphatase (PhoA) depends on periplasmic localization and is therefore a good marker for the periplasm. Activity can be determined on whole cells and is therefore also useful in strains from which no stable spheroplasts can be obtained. To determine the influence of positive charges on topology of the fusion constructs, 0–3 arginines were placed immediately downstream of H2 of Lep (Figure 4A). These constructs are called //*n*R where *n* indicates the number of arginines. All constructs of the //*n*R series were expressed in strain JM109 with wild-type lipid composition and in AD93 with 100% anionic lipids. Constructs with or without positive charges behind H2 are synthesized in comparable amounts in JM109 and AD93, as shown by Western blotting (Figure 4B). In strain JM109 with wild-type lipid composition, it was observed that constructs //0R and //1R display high alkaline phosphatase activity while constructs //2R and //3R are inactive (Figure 5A, circles). The amount of alkaline phosphatase activity is most often related to the optical density of the cell suspensions (Manoil and Beckwith, 1985). However, since the shape of AD93 cells is different from that of wild-type cells (Rietveld *et al.*,

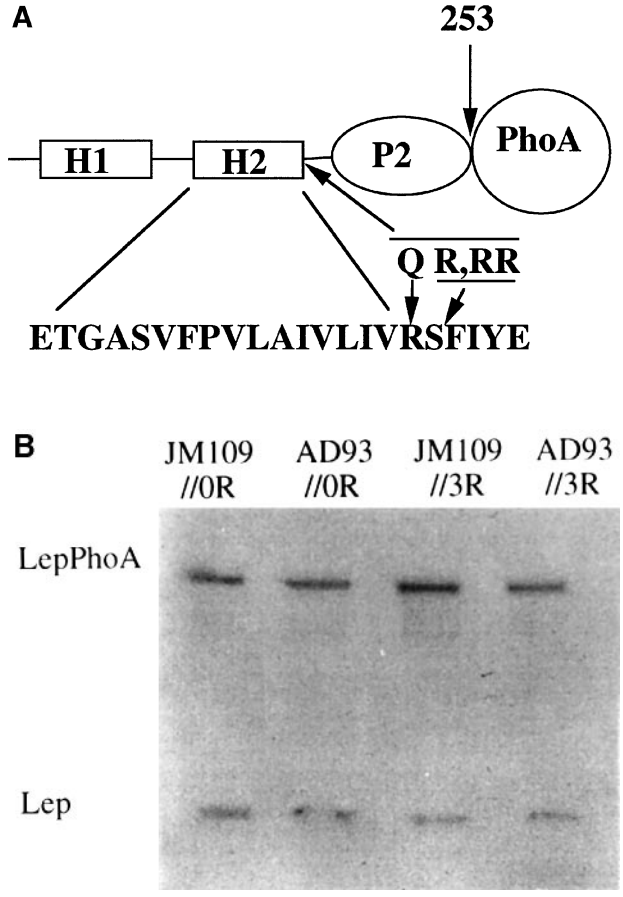

Fig. 4. Construction and expression of Lep–PhoA fusion constructs. (**A**) Domain structure and partial amino acid sequence of Lep–PhoA fusion constructs. The position of the positively charged amino acids and the fusion point are indicated. (**B**) Expression of fusion constructs //0R and //3R in JM109 and AD93. Samples were treated as described in Materials and methods. Fusion constructs were visualized by immunoblotting with Lep antibodies, followed by an enhanced chemiluminescence detection method.

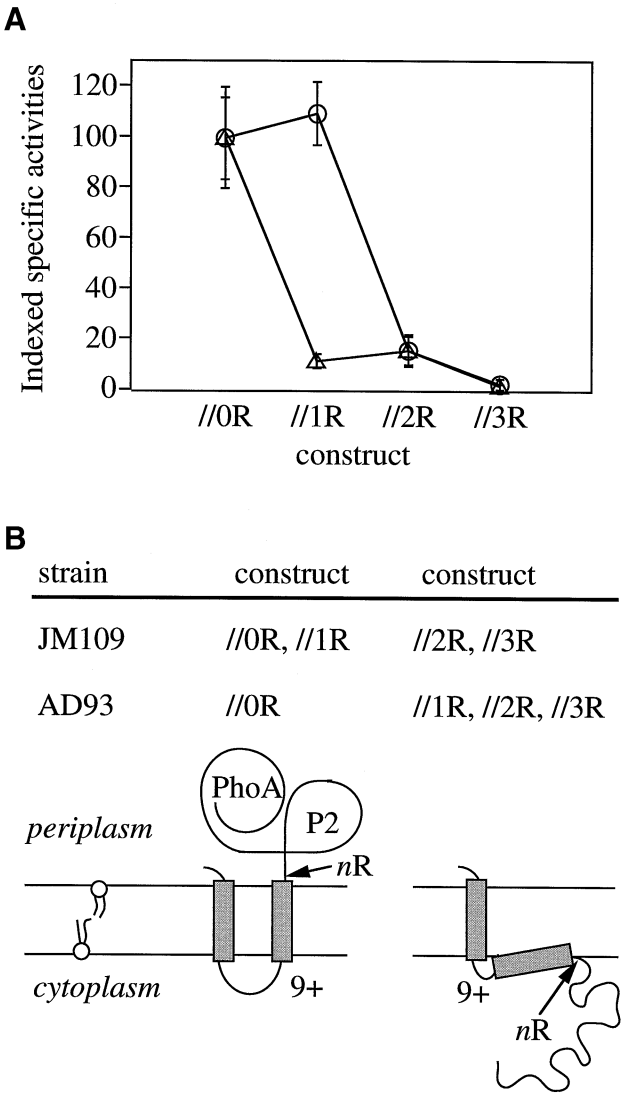

Fig. 5. Topology and activity of Lep–PhoA fusions. (**A**) Specific alkaline phosphatase activities of Lep–PhoA constructs in JM109 cells with wild-type lipid composition (○) and AD93 with only anionic phospholipids (△). The indexed specific alkaline phosphatase activities were determined as in Materials and methods. Averages and standard deviation ($n = 4$) are plotted against the amount of charges behind H2. (**B**) Schematic representation of the topologies of constructs //0R, //1R, //2R and //3R in the inner membrane of *E.coli* cells.

1996), we chose to relate phosphatase activity to the amount of fusion construct which was present in the assay, i.e. calculate specific activities. The alkaline phosphatase activity results establish the topologies depicted in Figure 5B. The localizations of the P2-PhoA domain in constructs //0R and //3R were confirmed by trypsin treatment of spheroplasts (results not shown). It appears that, in a strain with wild-type lipid composition, one positive charge directly following a transmembrane helix can be translocated whereas two positive charges are sufficient for a complete block of translocation. Next, the activities of the fusion proteins in AD93 were determined (Figure 5A, triangles). In this case also, high activity was observed for //0R and low activity for //2R and //3R. However, a big difference from JM109 is observed for //1R. In AD93, this construct is completely inactive. Apparently, in this strain, a single arginine is sufficient to block translocation (Figure 5A, triangles). Thus, membrane integration of a hydrophobic segment is more sensitive to positively charged residues placed immediately downstream of a transmembrane segment at high densities of anionic phospholipids in the membrane.

Discussion

Two aspects of membrane protein topology are sensitive to the positioning of positive charges. The orientation of a whole protein can be inverted and the membrane integration of a hydrophobic segment prevented. Here it is shown that in both cases anionic phospholipids play a role.

For several constructs of Lep with defined distributions of lysines around the first membrane-spanning segment, transmembrane orientation in the *E. coli* inner membrane was found to be dependent on the concentration of the negatively charged lipids. Lowering this concentration below wild-type levels allows the positive charges to move more readily across the membrane. The anionic lipids might exert their effect on membrane protein topology directly by interacting with the incoming protein

or indirectly, possibly by influencing the translocation machineries. This latter possibility should be seriously considered because Lep achieves a wild-type orientation by making use of the Sec machinery, which mediates membrane passage of the periplasmic domain. However, by blocking the activity of SecA by azide (Figure 3), it could be demonstrated that the fraction of the Lep molecules with an inverted orientation depends on the anionic lipids but not on the function of the Sec machinery. In addition, it was observed that a Lep construct with one lysine on both sides of H1 inserts in a random orientation in the membrane independently of the phospholipid composition. This shows that with decreased anionic lipid levels, the Lep constructs do not insert with an intrinsic preference for either wild-type or inverted topology. Another factor which could, in principle, affect the interpretation of our experiments is the pmf. Previously, the pmf was shown to be involved in membrane protein topology (Andersson and von Heijne, 1994). The effects reported in the present study are not likely to be caused by changes in the pmf, since the energetic parameters of HDL11, such as $\Delta\psi$, were quantitated and found to be comparable with wild-type strains and independent of IPTG (Van der Goot *et al.*, 1993; results not shown). Because of the facts summarized above, it is concluded that a direct interaction between the newly synthesized protein and the membrane lipids is responsible for the observed effects. The results suggest that the anionic lipids prevent membrane passage of positive charges.

When positive charges are present at both sides of a hydrophobic segment, they can prevent it from assuming a transmembrane orientation. In Lep, there are nine positive charges upstream of H2 and, in cells with wild-type lipid composition, two arginines downstream of H2 are required to prevent H2 from spanning the membrane (Figure 5). Increasing the anionic lipid content to 100% rendered one positive charge sufficient to produce this effect. This finding corroborates the suggestion that anionic lipids can prevent the membrane passage of positive charges. An attractive explanation for both results is the occurrence of direct, probably electrostatic, interactions between newly synthesized membrane proteins and anionic lipids.

In *E.coli*, both the pmf and anionic phospholipids have now been shown to exert control on membrane protein orientation. It was observed (Andersson and von Heijne, 1994) that membrane passage of one or two lysines at the N-terminus was possible in the absence of a pmf, but not in its presence. No effect of the pmf was found for constructs with more positive charges at the N-terminus. Reduction of the anionic lipid content from 25% to <10% resulted in facilitated membrane passage of up to four positive charges. On the basis of these results, it is not possible to gain quantitative insight into the contributions of the pmf and the anionic lipids to membrane protein orientation. However, it is clear that they can both have very pronounced effects. Interestingly, for some membrane systems to which the positive-inside rule applies, such as the eukaryotic endoplasmic reticulum and the thylakoids of chloroplast, no or only a small $\Delta\psi$ is found. In these cases, the anionic lipids may be the major determinants of membrane protein topology.

Materials and methods

Enzymes and chemicals

TPCK-treated trypsin, soybean trypsin inhibitor, chicken egg white lysozyme, phenylmethylsulfonyl fluoride (PMSF) and IPTG were from Sigma; *p*-nitrophenylphosphate (PNPP) was obtained from Serva.

Strains, growth conditions and plasmids

JM109 [*endA1 recA1 gyrA96 thi hsdR17 recA1 supE44* Δ*(lac-proAB)/* F′ *traD36 proAB lacIqZ* Δ(M15)] (Yanisch-Perron *et al.*, 1985) and AD93 (*pss93:: kan recA srl*::Tn*10 nadB*$^+$) (De Chavigny *et al.*, 1991) were grown in LB supplemented with 50 mM $MgCl_2$ and 10 mM KPi, pH 7.3. HDL11 [*pgsA::kan* Φ(*lacOP-pgsA*$^+$) *lacZ′ lacY*::Tn*9 lpp2 zdg*::Tn*10*] (Kusters *et al.*, 1991) was grown in M9 medium supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml) and tetracycline (10 μg/ml) and, when appropriate, with 50 μM IPTG. These strains were used to express constructs derived from Lep from the pING1 plasmid after induction by arabinose.

Assays of membrane topology for constructs of the nK/K series

HDL11 cells were grown overnight in the presence of 50 μM IPTG, to synthesize wild-type levels of PG and CL, and ampicillin (50 μg/ml) to select for the presence of plasmids. Overnight cultures were washed twice in physiological salt and diluted 1:40 in fresh medium with or without 50 μM IPTG to induce anionic phospholipid synthesis. Induction, radiolabeling, protease treatment of spheroplasts and immunoprecipitations with antisera to Lep, OmpA (an outer membrane protein as control for complete conversion of cells to spheroplasts, data not shown) and AraB (a cytoplasmic protein as a marker for intactness of the inner membrane, data not shown) were performed essentially as described (Nilsson and von Heijne, 1990). Cells were pulse-labeled for 2 min and chased for 30 s and, during protease treatment, 10 mM $MgSO_4$ was included to improve intactness of spheroplasts. The percentage of inverted molecules (P_i) was calculated as $P_i = 100 \times (7/6) \times (C_{inv}/C_{tot})$. C_{inv} and C_{tot} are the background and lysis-corrected counts after quantification on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) of, respectively, the trypsin-cleaved species indicative of N_{in}–C_{in} orientation and the full-length molecules before trypsin treatment. Lysis (<10%) was corrected for by normalizing all counts from Lep in a lane to the counts of the cytoplasmic marker AraB or bandX. BandX is an unknown cytoplasmic protein that fortuitously precipitates with AraB antibody (de Gier *et al.*, 1996). The factor 7/6 corrects for the loss of one methionine upon trypsinization. The phospholipid composition of HDL11 cells was checked after lipid extraction (Bligh and Dyer, 1959) and thin-layer chromatography with the eluent mixture choroform: methanol:acetic acid (65:25:10) and found to be in accordance with the results of Kusters *et al.* (1991).

Assays for membrane topology of the Lep–PhoA fusion proteins

JM109 and AD93 cells were grown until $OD_{660} = 0.8$. Arabinose was added (1%) to express the fusion constructs, and after 1 h cells were harvested and alkaline phosphatase activity was measured and calculated as described (Manoil and Beckwith, 1985). To calculate specific activities, cell contents were separated by SDS–PAGE and transferred to nitrocellulose, after which the amount of Lep–PhoA fusion construct was quantified by making use of Lep antibodies and enhanced chemiluminescence (ECL) detection as described for Lep (Van Klompenburg *et al.*, 1995). The specific activity of //0R in both strains was indexed at 100 and the other activities were related to this value. The topologies of constructs //0R and //3R in JM109 were confirmed by trypsinization assays on spheroplasts. The topologies of constructs //0R and //3R in AD93 were confirmed by alkaline phosphatase assays performed on inverted vesicles of AD93 bearing //0R in the absence or presence of detergent and by trypsin treatment of vesicles bearing //0R and //3R (results not shown).

Acknowledgements

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