To flip or not to flip: lipid–protein charge interactions are a determinant of final membrane protein topology

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The molecular details of how lipids influence final topological organization of membrane proteins are not well understood. Here, we present evidence that final topology is influenced by lipid–protein interactions most likely outside of the translocon. The N-terminal half of *Escherichia coli* lactose permease (LacY) is inverted with respect to the C-terminal half and the membrane bilayer when assembled in mutants lacking phosphatidylethanolamine and containing only negatively charged phospholipids. We demonstrate that inversion is dependent on interactions between the net charge of the cytoplasmic surface of the N-terminal bundle and the negative charge density of the membrane bilayer surface. A transmembrane domain, acting as a molecular hinge between the two halves of the protein, must also exit from the membrane for inversion to occur. Phosphatidylethanolamine dampens the translocation potential of negative residues in favor of the cytoplasmic retention potential of positive residues, thus explaining the dominance of positive over negative amino acids as co- or post-translational topological determinants.

Introduction

The final topology of a polytopic membrane protein is determined by a complex interplay (Bowie, 2005; Mackenzie, 2006; von Heijne, 2006) between the topogenic sequences of the protein, the membrane insertion machinery, and the properties of the lipid bilayer (Dowhan et al., 2004). How the sum of these factors determines final topology of individual membrane proteins is not completely understood. This paper focuses on the role of lipid–protein interactions as one determinant of topological organization of the polytopic membrane protein lactose permease (LacY) of *Escherichia coli*, which is a paradigm for membrane transport proteins throughout nature.

The orientation of transmembrane domains (TMs) of bacterial proteins is largely determined by charged residues in extramembrane domains flanking TMs and can in most cases be predicted by the "positive inside rule" (von Heijne, 1989), based on the fact that cytoplasmic domains relative to periplasmic domains are fourfold enriched in positively charged residues. In eukaryotic cells, orientation is more dependent on an equal but opposite contribution of positive and negative residues (Zhang et al., 1995). The positive inside rule is not absolute. Cytoplasmic domains with a net negative charge are found (Allard and Bertrand, 1992; Pi et al., 2002). The positive inside rule can be overridden when negatively charged residues are present in high numbers (Nilsson and von Heijne, 1990), flank a marginally hydrophobic TM (Delgado-Partin and Dalbey, 1998), or lie within a window of six residues from the end of a highly hydrophobic TM (Rutz et al., 1999). However, the molecular mechanism underlining the positive inside rule and the apparent dominance of positively over negatively charged residues is not understood.

Large protein segments (Kida et al., 2007; Ismail et al., 2008) may adopt an initial topology for the N-terminal TM according to the positive inside rule by direct charge interactions between the translocon and the protein (Goder and Spiess, 2003;

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Abbreviations used in this paper: aTC, anhydrotetracycline; IPTG, isopropyl-β-D-thiogalactoside; LacY, lactose permease; MPB, 3-(*N*-maleimidylpropionyl) biocytin; PE, phosphatidylethanolamine; SCAM; substituted cysteine accessibility method applied to transmembrane domains; TM, transmembrane domain, TMG, methyl-β-D-galactopyranoside.

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Goder et al., 2004). However, irrespective of the number of TMs accommodated by the translocon pore (Hamman et al., 1997; Van den Berg et al., 2004), final protein topology and organization after exit from the translocon must follow thermodynamically driven routes involving direct interaction of the TMs and associated extramembrane domains within the protein and with the lipid bilayer (Hessa et al., 2005), which may further decode the topogenic signals within nascent polypeptides.

Although protein sequence appears to be the primary determinant of final organization, the topology of several twelve-TM spanning secondary transporters of *E. coli* is dramatically influenced by the membrane lipid composition. The N-terminal six-TM helical bundle of LacY (Bogdanov et al., 2002) (see Fig. 2 A vs. Fig. 1 A) and the N-terminal two-TM hairpins of phenylalanine permease (PheP) (Zhang et al., 2003) and γ-aminobutyrate permease (GabP) (Zhang et al., 2005) are inverted with respect to the membrane bilayer when assembled in membranes lacking the major lipid phosphatidylethanolamine (PE). Introduction of PE post-assembly of these proteins results in complete reversal of the aberrant topological organization for PheP (Zhang et al., 2003) and at least the cytoplasmic domain C6 of LacY (Bogdanov et al., 2002). The above permeases maintain a compact folded state in the absence of PE as indicated by retention of energy independent downhill transport function and resistance to degradation (Bogdanov and Dowhan, 1995; Bogdanov et al., 2002; Zhang et al., 2003, 2005).

The requirement for zwitterionic PE in supporting native topology of the permeases appears to be as a diluent of the high negative surface charge due to the exclusive content of anionic lipids, mainly phosphatidylglycerol and cardiolipin, in −PE mutants. When *E. coli* was engineered to synthesize the neutral lipid α-monoglucosyl diacylglycerol (Xie et al., 2006) in place of PE, native topology of LacY was observed. Similarly, reconstitution of LacY into proteoliposomes containing PE or phosphatidylcholine along with anionic phospholipids, but not with anionic phospholipids alone, resulted in wild-type topology (Wang et al., 2002).

The above results strongly indicate that the collective charge nature of the bilayer surface influences final membrane protein topology. However, the structural features of LacY that make it sensitive to lipid environment are not known. We now report the previously unknown disposition of TMVII in −PE cells and the complete topology of LacY after post-assembly synthesis of PE. By varying the positive and negative charges in the normally cytoplasmic domains of LacY and determining topology as a function of membrane lipid composition, we determined the topogenic signals within LacY that require the presence of PE to establish native topology. Our novel results explain why positive residues are more potent topological determinants than negative residues under physiological conditions and establish lipid–protein net charge balance as one of several physiologically important determinants of final protein topology.

Results

Experimental rationale

The ability to regulate membrane lipid composition coupled with determination of orientation of proteins with changes in putative topogenic signals is a powerful approach to determine the coupled influence of membrane lipid environment and protein sequence on membrane protein topology. A plasmid copy of OP_{tac}-*lacY* (isopropyl-β-D-thiogalactoside [IPTG] controlled expression of LacY) and a chromosomal copy of OP_{tet}-*pssA* (anhydrotetracycline [aTc] controlled expression of phosphatidylserine synthase [initiates PE synthesis]) were combined in the same *lacY* null cell (strain AT2033). As previously demonstrated using arabinose to regulate OP_{araB}-*pssA* expression (Bogdanov et al., 2002), strain AT2033 grown without aTc had < 3% PE with the remainder being cardiolipin and phosphatidylglycerol and had wild-type phospholipid composition after 3 h growth with aTc (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1). To determine the effect of a change in membrane lipid composition on topology of LacY, cells were grown in the presence of IPTG without aTc to allow membrane assembly of LacY in the absence of PE. Then cells were switched to growth without IPTG in the presence of aTc to permit biosynthesis of PE in the absence of newly synthesized LacY. The orientation of extramembrane domains of LacY relative to the membrane bilayer were determined before (−PE) and after (+PE) growth in the presence of aTc by the accessibility of engineered cysteine residues to a membrane impermeable sulfhydryl reagent (3-(*N*-maleimidylpropionyl) biocytin [MPB]) using the substituted cysteine accessibility method applied to TMs (SCAM) as described in Materials and methods. To identify the molecular basis for lipid-sensitive topogenesis of specific protein domains, plasmid copies of LacY with altered amino acid sequences were expressed in −PE strain AL95 (*lacY* and *pssA* null) and +PE strain AL95/pDD72 (carries plasmid copy of *pssA*) followed by SCAM analysis.

Characterization of LacY expression and function

LacY expressed and radiolabeled during growth in the presence of IPTG was only slightly reduced (Fig. S2, lane 1 vs. lane 2; available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1) during a 3-h chase of radiolabel in the absence of IPTG and the presence of aTc to induce PE synthesis. There was no detectible radiolabeled LacY expressed during 3 h of growth in the presence of aTc but without IPTG (lane 3) while LacY was produced when both aTc and IPTG were present during growth (lane 4). Therefore, during PE induction, as previously shown using the *araB* promoter to regulate PE levels (Bogdanov et al., 2002), LacY synthesized in the absence of PE was stable and no new LacY was synthesized in the absence of IPTG.

Previous studies showed loss of energy dependent uphill transport of LacY substrates in −PE cells, which was regained upon induction of PE synthesis using the OP_{araB}-*pssA* system (Bogdanov et al., 2002); energy independent downhill transport was independent of lipid composition. Consistent with previous results, LacY expressed in −PE cells (AT2033, plus IPTG and minus aTc) displayed downhill transport of lactose but did not carry out uphill accumulation of the nonhydrolyzable substrate analogue methyl-β-D-galactopyranoside (TMG) (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1). However, after recovery of PE levels (plus aTc) in the absence of IPTG (see previous paragraph), uphill transport of TMG was

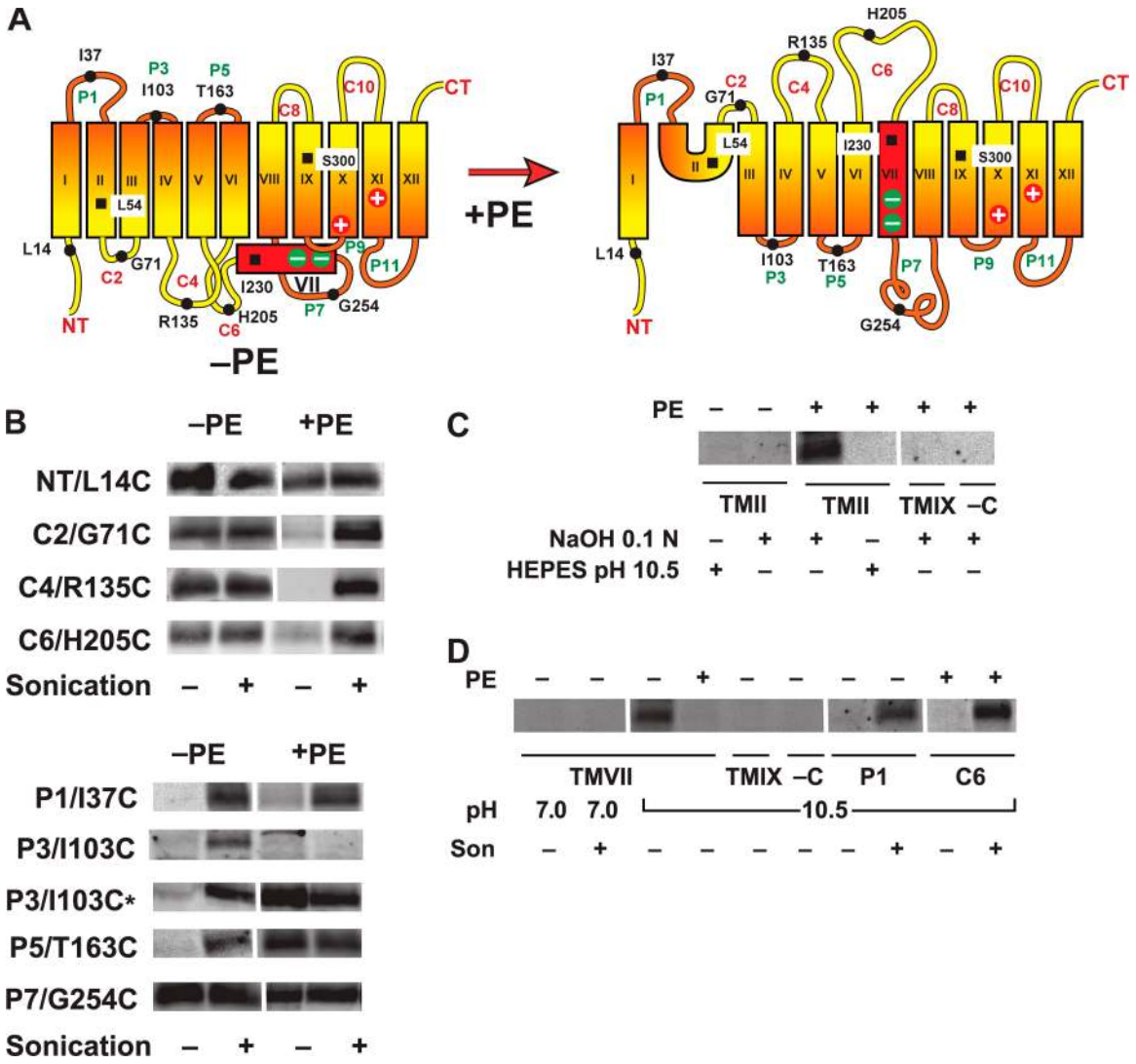

Figure 1. **PE-induced post-insertion topological reorganization of LacY.** (A) Change in topology of LacY assembled in −PE cells (left panel; Bogdanov et al., 2002) after post-assembly synthesis of PE (right panel; this paper) in strain AT2033. Rectangles define the TMs (Abramson et al., 2003) oriented with the cytoplasm above the figure. TMs (roman numerals), extramembrane domains (P for periplasmic and C for cytoplasmic as oriented in +PE cells), N terminus (NT), and C terminus (CT) are indicated. The locations of negatively charged and positively charged residues involved in salt bridges between TMs are indicated. The locations and names of amino acids substituted by cysteine and used for SCAM analysis are indicated near circles (extramembrane) or squares (within TMs). TMVII is indicated in a periplasmic location (left) and a membrane-inserted location (right). (B–D) AT2033 cells without (−) or with (+) sonication were treated with MPB as described in Materials and methods. Labeling was performed on samples either after initial assembly of LacY in −PE cells (−PE, with IPTG induction but before addition of aTc) or after removal of IPTG and induction of PE synthesis (+PE) for 3 h (maximum PE level) during logarithmic growth (OD_{600} increased from 0.4 to 1–1.4). Western blotting was used to detect biotinylation of diagnostic cysteines that were accessible to MPB; domain and/or substitution positions are indicated. Images are horizontal strips of the LacY (33 kD) position. See Materials and methods for details of image acquisition. (B) MBP labeling was performed at pH 7.5 except for P3/I103C*, where labeling was done at pH 10.5. (C and D). Labeling pH is indicated, and the cysteine substitutions in TMII, TMVII, and TMIX were L54C, I230C, and S300C, respectively. The substitutions in P1 and C6 are those noted in (B), and "−C" refers to cysteine-less LacY. Sonication of the TMVII samples from −PE and +PE cells followed by SCAM analysis at pH 10.5 gave the same result as shown in lanes 3 and 4 from the left in D, respectively (not depicted).

restored as confirmed by inhibition of accumulation of TMG by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. LacY performed downhill transport of lactose in the absence of PE and after restoration of PE levels as shown in the inset to Fig. S3.

Lipid triggered transbilayer reorganization of LacY

Previous results established that cytoplasmic domain C6, initially misoriented to face the periplasm in −PE cells, reorients to face the cytoplasm after post-assembly synthesis of PE (Fig. 1 A), but the orientation of the remaining TMs was not established. Cysteine replacements in otherwise cysteine-less LacY were expressed in +PE and −PE cells, and reactivity with MPB in intact cells (periplasmic exposure) or only after cell disruption by sonication (cytoplasmic exposure) was used to establish TM orientation (Bogdanov et al., 2002, 2005).

Consistent with previous results (Bogdanov et al., 2002) (Fig. 1 B, −PE), cysteines residing within normally (+PE) cytoplasmic domains (NT-C6), when analyzed in AT2033 grown in

the absence of aTc (< 3% PE), were labeled whether or not cells were disrupted, indicating periplasmic exposure. A cysteine in normally periplasmic domain P1, P3, or P5 was labeled only after cell disruption, indicating cytoplasmic exposure.

Next, topology of LacY initially assembled in −PE cells was examined after induction of PE synthesis in the absence of new LacY synthesis. A cysteine in domains C2, C4, or C6 initially exposed to the periplasm in −PE cells was only biotinylated after cell disruption (Fig. 1 B, +PE), consistent with a return to normal topology. However, NT (L14C) remained in its abnormal periplasmic orientation. A cysteine in domain P5 was accessible to MPB either with or without sonication. However, the labeling of domain P1 was observed only after cell disruption, consistent with retention of a cytoplasmic location. Lack of a mixed topology and retention of the topology observed in −PE cells for the extramembrane domains flanking TMI after restoration of PE content further support flipping of "old" LacY. Domain P7 was biotinylated without sonication in −PE and +PE cells. The cysteine residue in domain P3 (I103C), which in −PE cells was accessible only after sonication, was not accessible to MPB at pH 7.5 either with or without sonication after growth in aTc; Western blotting analysis showed a full complement of LacY (unpublished data). Lack of biotinylation of a single cysteine residue can be due to its location within a TM or proximal environmental effects, which affect the thiol pKa or sterically restrict access (Bogdanov et al., 2005). Increasing the solution pH should favor alkylation of an extramembrane cysteine as well as disrupt local restrictive secondary structure while truly membrane imbedded cysteines should not react. Indeed, P3 cysteine labeling proportionately increased with increasing pH with full labeling at pH 10.5 without sonication (Fig. 1 B, I103C*). Controls for retention of membrane impermeability to MPB at pH 10.5 are presented in Fig. 1 D.

Organization of TMII in cells with restored PE levels

If NT and P1 remain periplasmic and cytoplasmic, respectively, while C2 adopts the correct cytoplasmic location after restoration of PE levels (Fig. 1 A), then a large structural rearrangement must occur in TMII. The most probable arrangement in this case would be a "U"-shaped membrane-dipping mini-loop (Lasso et al., 2006). Under strongly alkaline conditions (pH >11), biological membranes are converted to open membrane sheets (Ito and Akiyama, 1991). Peripheral membrane proteins are released in a soluble form and presumably so would domains such as mini-loops that do not span the membrane bilayer. TMs of integral membrane proteins remain embedded in the lipid bilayer. This empirical method has been extensively used to differentiate between integral and peripheral membrane proteins and is the basis for the following approach to probe the location of unreactive cysteine residues.

L54C (TMII) in −PE membranes was not labeled after exposure to NaOH, as would be expected for a cysteine within a TM (Fig. 1 C, −PE). After induction of PE synthesis, L54C was not labeled at pH 10.5 even after sonication, but was readily labeled after NaOH treatment (Fig. 1 C, +PE). No labeling of cysteine-less (−C) LacY or LacY containing S300C in TMIX after alkali treatment established specificity for cysteine labeling and inaccessibility of cysteines within a TM, respectively. Strong alkaline conditions lyse cells while treatment at pH 10.5 does not permeabilize cells to MBP (see following paragraph and Fig. 1 D). Differential susceptibility to increased alkaline conditions viewed within the context of other structural features of a protein can be used to establish differences in the environment of a particular cysteine, which in this case suggests a progression from a solvent-exposed domain (exposed at pH 7.5) to an extramembrane-hindered domain (exposed at pH 10.5), to a mini-loop domain (exposed after NaOH treatment) as opposed to a true TM (not exposed by NaOH). Therefore, TMII appears to form a flexible hinge between P1 and C2 to allow a differential response of these domains to a change in lipid composition.

Organization of TMVII dependent on PE

Previous results (Bogdanov et al., 2002) did not establish the location of TMVII either in −PE cells or after restoration of PE levels. An I230C substitution near the cytoplasmic end of TMVII, which is not alkylated by *N*-ethyl maleimide in +PE membranes (Venkatesan et al., 2000), was chosen as a diagnostic residue to assess location of TMVII. In –PE cells I230C was not labeled by MPB at pH 7.0 or 7.4 even after sonication of cells, but was moderately labeled at pH 9.0 (unpublished data) and highly labeled at pH 10.5 (Fig. 1 D) without sonication, consistent with TMVII being a hindered extramembrane domain exposed to the periplasm in −PE cells. Cell integrity is maintained at pH 10.5, as indicated by labeling of domains P1 (−PE cells) and C6 (+PE cells) only after sonication. Cysteine-less LacY and LacY with an intramembrane cysteine (S300C) were not labeled at pH 10.5. Because residue I230C of LacY lies close to the cytoplasmic end of TMVII in wild-type cells, its exposure to the exterior of the cell under elevated pH conditions supports a structural rearrangement that brings the N-terminal end of TMVII in close proximity to the periplasm in −PE cells rather than being a TM. The most likely location of the TMVII is in the periplasm (but a mini-loop is possible) due to its low hydrophobicity (Bogdanov et al., 2002), the presence of two charged residues near its C-terminal end, and external exposure of its N-terminal end at pH 10.5. Restoration of normal PE levels resulted in protection of I230C from labeling at pH 10.5 in whole cells, consistent with TMVII returning to its normal TM location after reorganization of LacY (Fig. 1 D).

Proposed molecular basis for lipid-dependent topogenesis of LacY

LacY belongs to the 45-member major facilitator superfamily (Saier, 2003). Sequence alignment of LacY with its four most similar sugar permeases (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1) revealed strong conservation of negatively and positively charged residues within domains C2, C4, and C6, except for C6 of raffinose permease. Although the positive inside rule is strictly followed, a noteworthy feature of the cytoplasmic domains flanking TMs I-VI is the high content of negatively charged residues when compared with the cytoplasmic domains of the C-terminal five-TM bundle. For LacY (Fig. S4 and Fig. 2 A) C2 contains one, C4 contains three, and

C6 contains two negatively charge residues. The abundance of these negatively charged residues led us to postulate a critical role for these residues in lipid-dependent topogenesis of the N-terminal bundle of LacY. However, why are these residues only topologically active in −PE cells? If interactions in the membrane-aqueous interfacial region between charged extramembrane domains and the collective charge of the membrane surface are a determinant of TM orientation, then alterations in the charge nature of either the lipid headgroups, as already demonstrated (Bogdanov et al., 2002; Xie et al., 2006), or the protein domains, should affect final protein topology.

Testing the lipid–protein charge balance hypothesis

To investigate whether the presence of mixed proximal topogenic signals, i.e., both negative and positive residues, is the basis for lipid-dependent topogenesis, charged residues within the cytoplasmic domains of the N-terminal bundle were altered and topology was studied as a function of lipid composition. Acidic residues within the cytoplasmic face of the N-terminal bundle were converted to their corresponding neutral amides. Derivatives with D68N (C2), E126Q (C4), or E215Q (C6) and containing a H205C in C6 for SCAM analysis were expressed in −PE cells (strain AL95). The elimination of each negatively charged residue increased the net charge of each respective cytoplasmic domain and the cytoplasmic face of LacY by +1 (Fig. 2 A). H205C in "wild-type" LacY, which was biotinylated (cytoplasmic) in +PE cells (AL95/pDD72) only after sonication (Fig. 2 B, lanes 3 and 4), was labeled (periplasmic) whether or not −PE cells (AL95) were sonicated (lanes 5 and 6). H205C in each replacement mutant was also cytoplasmic in +PE cells (only the D68N derivative is shown in Fig. 2 A, lanes 1 and 2), but now remained cytoplasmic when expressed in −PE cells (Fig. 2 C), indicating a wild-type topology for domain C6 in both types of cells. In addition, the above replacement mutants containing G13C (NT) and H205C (C6) replacements, which flank the N-terminal bundle, were also only biotinylated after cell disruption (Fig. 2 D), consistent with retention of whole bundle orientation. Because the H205C substitution is cytoplasmic, it did not interfere with determination of a possible periplasmic exposure of G13C. Therefore, elimination of any one of the negative residues on the cytoplasmic surface of the N-terminal bundle, which increases the net charge by +1, prevented the misorientation of the whole bundle in −PE cells in a position-independent manner. Inversion was also prevented in −PE cells by adding a single positive charge (L72K) to the N-terminal bundle (Fig. 2 E, lanes 1 and 2). Finally, eliminating of both a negative (D68N) and positive (K69N) charge from C2, thus not changing net charge, allowed inversion in −PE cells (Fig. 2 E, lanes 3 and 4).

Inversion of topology in +PE cells

If the net charge of these domains coupled with the presence of negative residues is a determinant of TM orientation, then increasing the negative charge density should result in inversion of topology in +PE cells (strain AL95/pDD72). To test this hypothesis, a series of mutants carrying diagnostic H205C in C6 were constructed in which positive charges were replaced

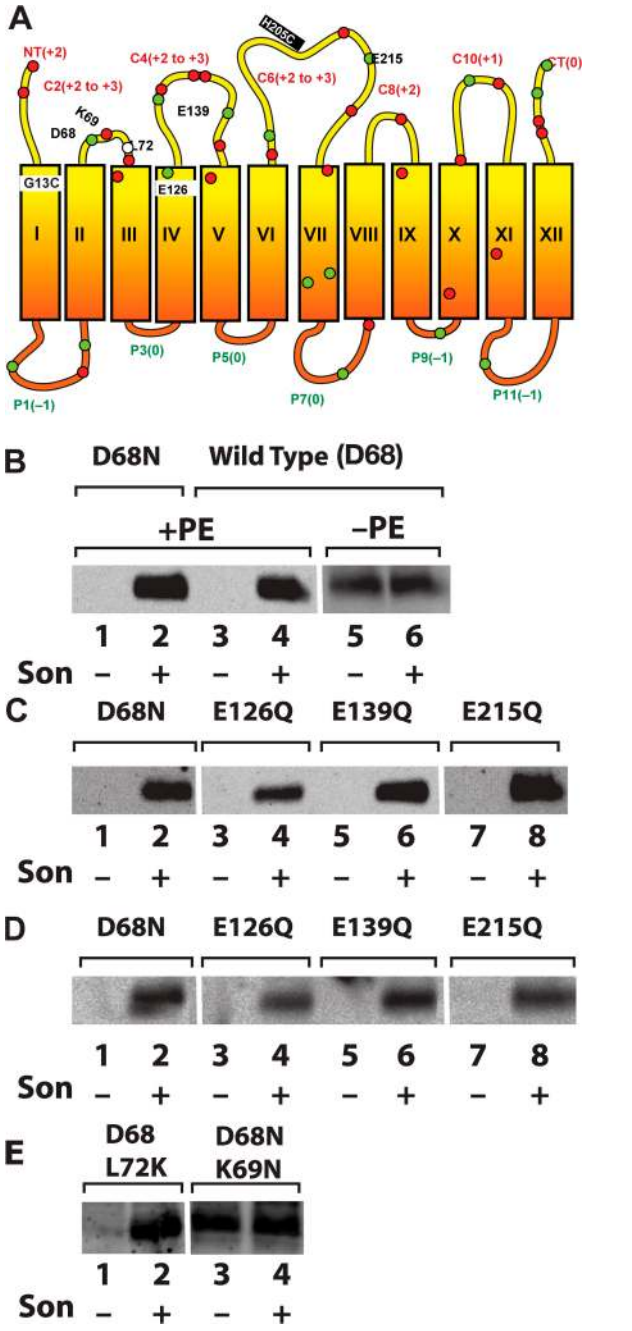

Figure 2. **Effect of net positive charge of the N-terminal bundle cytoplasmic domains on TM orientation in −PE cells.** (A) TM orientation of LacY in +PE cells (Bogdanov et al., 2002) (see Fig. 1 A for TM orientation in −PE cells and other details). Locations of positively (red) and negatively (green) charged residues are indicated (see Fig. S4 for exact locations). Name and number of residues changed in B–E are indicated, as well as the net charge of each extramembrane domain. LacY contained either a single cysteine replacement (B, C, and E) at H205 (C6) or an additional cysteine replacement (D) at G13 (NT). SCAM analysis with (+) or without (−) sonication (Son) is shown for LacY with a change of net charge from +2 ("wild type") in each cytoplasmic domain C2, C4, or C6 to +3 for domain C2 (D68N) or C4 (E126Q) or C4 (E139Q) or C6 (E215Q). The combination of D68N and K73N resulted in no change in net charge for C2, and addition of L72K but retaining D68 increased C2 charge by +1. The presence (strain AL95/pDD72) or absence of PE (strain AL95) is indicated in B, and all derivatives in C–E were expressed in −PE cells (AL95). Images are horizontal strips of the LacY (33 kD) position. See Materials and methods for details of image acquisition.

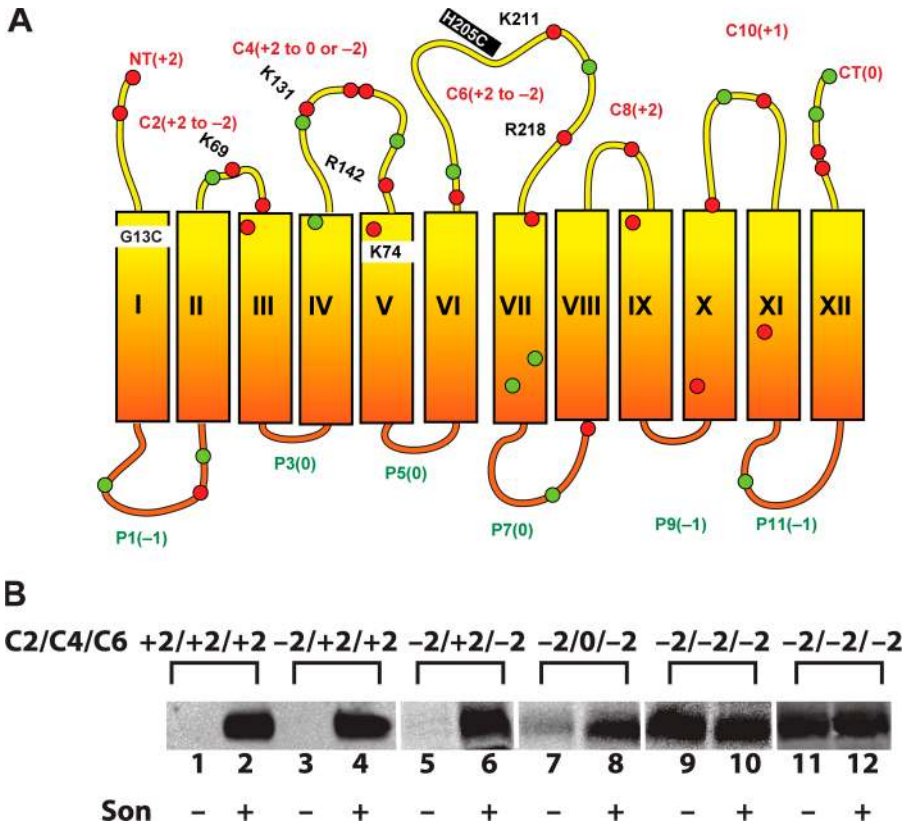

Figure 3. **Effect of net negative charge of the N-terminal bundle cytoplasmic domains on TM orientation in +PE cells.** (A) TM orientation of LacY in +PE cells (see Fig. 1 A for other details). Name and number of residues changed are indicated, as well as the net charge and change in net charge of each domain C2, C4, or C6. (B) LacY derivatives contained a single diagnostic cysteine at H205 (C6) (lanes 1–10) or G13 (NT) (lanes 11 and 12) were expressed in +PE cells (strain AL95/pDD72). SCAM analysis with (+) or without (−) sonication (Son) is shown for LacY with a change in net charge from +2 (lanes 1 and 2) to −2 for domain C2 either separately (K69E and K74E, lanes 3 and 4) or in combination with a change in net charge of domain C6 from +2 to −2 (K211E and R218E, lanes 5 and 6). The changes in domains C2 and C6 were combined with a change in domain C4 from +2 to 0 (K131E, lanes 7 and 8) or from +2 to −2 (K131E and R142E lanes 9–12). Images are horizontal strips of the LacY (33 kD) position. See Materials and methods for details of image acquisition.

by negative charges (Fig. 3 A). A change in net charge (Fig. 3 B and Fig. S4) from +2 to −2 for C2 separately (K69E and K74E, lanes 3 and 4) or in combination with a +2 to −2 change in C6 (K211E and R218E, lanes 5 and 6) had no effect on orientation of C6. Similarly, combining the changes in C2 and C6 with a change in C4 from +2 to 0 (K131E, lanes 7 and 8) had little effect on orientation, although a small level of exposure of H205C in cells before sonication (lane 7) was observed, suggesting possible mixed topology. Also, making domain C4 −2 alone (K131E and R142E) had no effect (unpublished data). However, combining the changes in C2 and C6 with a R142E substitution in domain C4 (lanes 9 and 10), making a +2 to −2 change in each of the three cytoplasmic domains, resulted in a periplasmic exposure of H205C. Finally, a G13C (NT) substitution (normally cytoplasmic) as the only cysteine in the −2/−2/−2 derivative was periplasmic in +PE cells (lanes 11 and 12). Because G13 and H205 flank the N-terminal helical bundle, the results are consistent with complete inversion of the bundle in the −2/−2/−2 derivative.

TMVII as a molecular hinge between two halves of LacY

Common to many sugar permeases (Fig. S4) is the very hydrophilic TMVII, which is not predicted by topology algorithms as a TM, but has been verified in LacY to span the membrane (Wolin and Kaback, 1999). TMVII contains D237 and D240, which are in salt bridges (Fig. 4 A) with K358 (TMXI) and K319 (TMX), respectively (Abramson et al., 2004). We previously postulated that the low hydrophobicity of TMVII is necessary for it to act as a reversible molecular hinge to allow independent response of the flanking domains to the lipid environment (Bogdanov et al., 2002). To test this hypothesis, we increased the overall hydrophobicity of TMVII by introducing D240I in a LacY with H205C in domain C6. When expressed in +PE (strain AL95/pDD72) or −PE cells (strain AL95), this cysteine was biotinylated only after sonication of cells, consistent with lack of inversion (Fig. 4 B, lanes 1–4). The same result in −PE cells (lanes 9 and 10) was obtained with a LacY derivative in which G13C (NT) and H205C (C6) (both facing the cytoplasm in +PE cells) flanked the N-terminal helical bundle, consistent with lack of misorientation of the whole bundle. In addition, the diagnostic I230C substitution in TMVII, which was shown in Fig. 1 D to be membrane embedded in +PE cells and exposed to the periplasm in −PE cells, when combined with a D240I substitution was not biotinylated at pH 10.5 either with or without sonication in +PE or −PE cells (lanes 5–8). Furthermore, domain C6 (H205C) of LacY (D240I) remained accessible from only the cytoplasm after post-assembly synthesis of PE in −PE cells (AT2033 plus aTc; unpublished data).

A D240I substitution in TMVII and an H205C substitution in C6 were introduced into the LacY −2/−2/−2 mutant to determine whether exit of TMVII was also required for inversion of topology in +PE cells. This derivative was biotinylated with and without sonication (periplasmic and inverted) when TMVII did not have a D240I substitution (Fig. 5, top right half panel). However, the D240I substitution in the −2/−2/−2 mutant prevented periplasmic exposure and inversion of C6 (top left half panel). An I230C substitution in TMVII of wild-type LacY (+2/+2/+2) was inaccessible to MPB with and without sonication at pH 10.5 (bottom left half panel), consistent with it

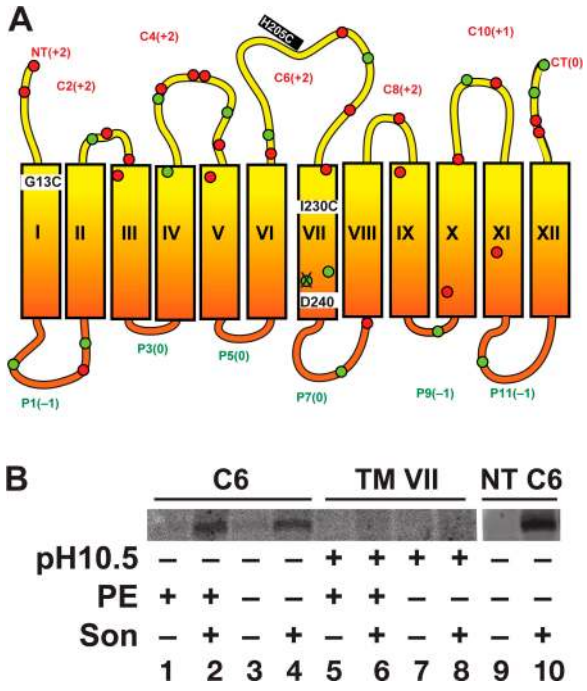

Figure 4. **Effect of increasing hydrophobicity of TMVII.** (A) See Fig. 1 A for additional details. Positions of cysteine replacements are indicated by black and white rectangles. The "X" in TMVII indicates the position of the D240I substitution. (B) LacY with a D240I substitution in TMVII and a cysteine substitution in either C6 (H205C) or TMVII (I230C) or NT (G13C) and C6 (H205C) was expressed in +PE (+, strain AL95/pDD72) or −PE (−, strain AL95) cells. Cells were treated with MPB without (−) or after (+) sonication (Son) at either pH 7.5 (−) or 10.5 (+) and analyzed by SCAM. Additional controls are shown elsewhere as follows: accessibility of C6 (H205C) at pH 7.5 without the D240I substitution in +PE and −PE cells is shown in Fig. 1 B; accessibility of TMVII at pH 10.5 (I230C) without the D240I substitution in +PE and −PE cells is shown in Fig. 1 D. Images are horizontal strips of the LacY (33 kD) position. See Materials and methods for details of image acquisition.

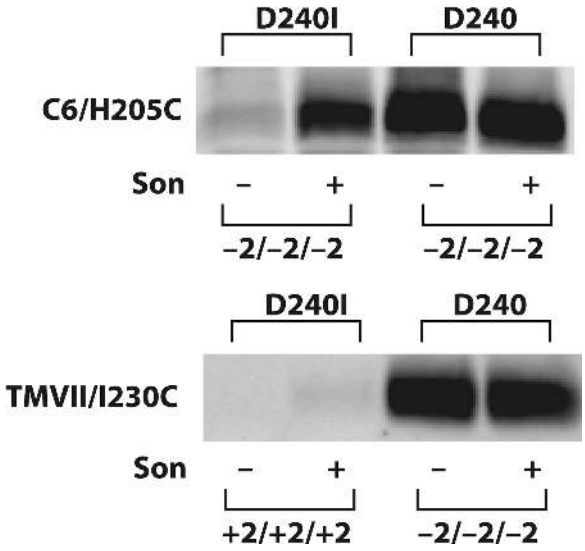

Figure 5. **Effect of TMVII hydrophobicity on topology of LacY mutants in +PE cells.** SCAM analysis is shown for C2/C4/C6 (+2/+2/+2) or C2/C4/C6 (−2/−2/−2) LacY (see Fig. 3 B) expressed in +PE cells (AL95/pDD72). The domain containing the indicated diagnostic cysteine replacement is shown on the left. The amino acid at position 240 was either aspartic acid or isoleucine (see Fig. 4 A), as indicated. Biotinylation was performed at pH 7.5 (top panel) or pH 10.5 (bottom panel). Images are horizontal strips of the LacY (33 kD) position. See Materials and methods for details of image acquisition.

being a membrane embedded in +PE cells (Fig. 1 D). However, I230C in the −2/−2/−2 derivative was accessible with and without sonication at pH 10.5, consistent with periplasmic exposure of TMVII as previously shown for wild-type LacY in −PE cells (Fig. 1 D). Therefore, inversion of the N-terminal helical bundle of the −2/−2/−2 derivative in +PE cells and wild-type LacY in −PE cells displays the same dependence on a flexible molecular hinge (TMVII), linking it to the lipid-insensitive C-terminal bundle.

Discussion

In the present study, we analyzed the molecular determinants that specify retention or translocation of extramembrane domains connecting TMs of the polytopic membrane protein LacY in the context of different lipid compositions, thereby significantly advancing our understanding of the rules that govern membrane protein topogenesis. We established that large topological reorganization involving six TMs (II–VII) of a protein is possible post-assembly. Therefore, topological organization, once established, is not static but is dynamic in response to changes in lipid environment. This result strongly implies that post-translational TM orientation dependent on the lipid environment can be established outside the translocon. Our results support the following conclusions: cooperative and cumulative interactions between charged residues of the cytoplasmic surface of the N-terminal bundle and the collective charge of the membrane lipid headgroups is a determinant of TM orientation; final topological decisions may be delayed to after exit from the translocon and are determined by short- and long-range interactions within the protein that balance these charge interactions with necessary interactions between TMs and of TMs with the membrane bilayer; the translocation potential of negatively charged amino acids working in opposition to the positive inside rule is largely dampened by the presence of PE, thus explaining the dominance of positive residues as retention signals; TM orientation maybe initially influenced by the translocation machinery, but final topology is determined outside the translocon by the above interactions.

The N-terminal six-TM bundle appears to behave as a single unit in response to changes in protein topogenic signals, hydrophobicity of TMVII, and membrane lipid composition, consistent with the independent folding of the two halves of LacY (Nagamori et al., 2003). Co-translational orientation of the N-terminal bundle as a whole was based on exposure of the flanking NT and C6 domains. However, the more demanding post-assembly reorientation indicated that NT-TMI would be the most likely not to properly orient during cotranslational insertion. Because NT properly oriented, we concluded the whole bundle properly oriented cotranslationally. Even if some of the middle TMs did not properly orient, this would not compromise the final conclusions with respect to the effects of charged residues on topology. Therefore, we can treat the N-terminal bundle mostly as a single TM unit tethered to a molecular hinge (TMVII), the properties of which also govern upstream topology.

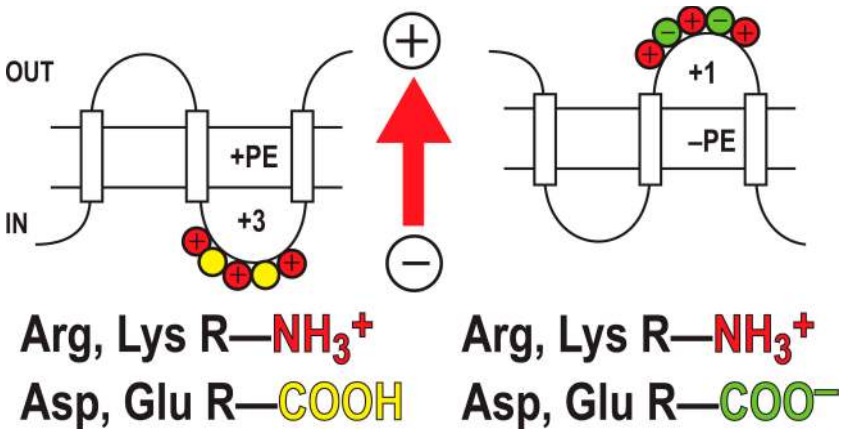

Figure 6. **PE and the positive inside rule.** In the left panel a cytoplasmic domain is shown containing a mixture of negative and positive amino acids. PE suppresses or neutralizes the presence of negative residues (yellow circles), which increases the effective positive charge potential, thus favoring cytoplasmic retention of the domain. In the absence of PE (right panel) negative residues (green circles) exert their full potential, resulting in translocation of the domain with a lower effective net positive charge. The proton motive force (arrow) positive outward determines domain directional movement depending on the domain effective net charge as influenced by the lipid environment.

In contrast to the numerous and continuing reports on the role of extramembrane charged residues in determining TM topology, only one report investigated the role of lipid–protein charge interactions in determining topology (van Klompenburg et al., 1997). In this study, positively charged residues were varied in a chimeric bitopic membrane protein that was expressed in an *E. coli* mutant with normal to reduced anionic phospholipid content. Retention of the cytoplasmic domain was proportional to both the number of positively charge residues and the membrane content of anionic phospholipids, thus indicating lipid–protein charge interaction as a determinant of topology. However, this observation appears to be in conflict with the results we report. Because all the cytoplasmic domains of LacY follow the positive inside rule, lack of PE resulting in only anionic phospholipids in the membrane would be expected to stabilize native topology rather than cause inversion. Therefore, the role of lipid–protein interactions and PE in particular in determining final topology is more complex than previously recognized.

We found that increasing the net positive charge within the negative amino acid–rich cytoplasmic face of the N-terminal bundle of LacY by either eliminating negatively charged residues or introducing positively charged residues in a position-independent manner prevented inversion in −PE cells. Alterations resulting in no change in net charge did not prevent inversion. Moreover, the effects of a change in charge on topology were the same whether these resulted from the lipid or the protein, i.e., either an increase in the net positive charge of the cytoplasmic protein surface or reduction of net negative charge density of the membrane by the presence of lipids with net zero charge resulted in wild-type topology. Similarly, increasing the negative charge of the cytoplasmic surface of the N-terminal bundle resulted in topological inversion in +PE cells, but a large increase in the net negative charge was required. Although these results might be expected from a general consideration of the positive inside rule, they uncover an unrecognized role for membrane surface charge properties in attenuating and enhancing the topogenic signals stemming from charged residues. Our results are consistent with PE reducing the effectiveness of negative residues in destabilizing the cytoplasmic retention potential of positive residues, which explains why basic amino acids are generally dominant retention signals over acidic amino acids as translocation signals. However, in the absence of PE, negative residues appear to act as dominant translocation signals. These results suggest an important physiological role for PE, as well as possibly neutral glycolipids that substitute in the absence of PE (Xie et al., 2006), in maintaining TM orientation by accentuating the positive inside rule while allowing insertion of negative residues in cytoplasmic domains for functional purposes without destabilizing cytoplasmic retention.

A possible explanation for the less potent translocation potential of negative residues is provided by the effect of PE on the pKa of negatively charged amino acids in a multidrug transporter (LmrP) of *Lactococcus lacits* (which contains both PE and glycolipids) with homology to LacY (Gbaguidi et al., 2007; Hakizimana et al., 2008). When LmrP was reconstituted into liposomes lacking PE, acidic amino acids exhibited the expected pKa values of 4–5. However, the presence of PE raised the pKa values to 6–7, making these residues prone to protonation. Therefore, the pKa shift caused by PE would selectively neutralize negatively charged residues.

PE resides within both leaflets of the membrane bilayer, so lipid composition alone cannot impart directionality to TM orientation. However, the positive outward proton motive force across the cytoplasmic membrane was demonstrated to drive negatively charged domains outward (Andersson and von Heijne, 1994; Cao et al., 1995) and counter the translocation of positively charged domains (Cao and Dalbey, 1994). Therefore, PE (Fig. 6) would increase the positively charged amino acid retention potential of domains containing both negatively and positively charged residues, whereas the absence of PE would increase the negatively charged amino acid translocation potential, which may even be dominant. Introduction of PE post-assembly of LacY would return the effective charge properties of the N-terminal bundle to normal, and the proton motive force may provide the driving force for flipping the bundle.

Flip-flop of large TM bundles in vivo challenges the dogma that once TM orientation is established it is not subject to post-insertional topological editing. However, the activation energy for a reversible orientation of LacY in response to lipid composition appears easily overcome, and the −11 kcal/mol of orientation driving force provided by the proton motive force (Cao et al., 1995) may be sufficient. Changing the lipid environment would establish new energy minima for the two halves of LacY, which can be attained because of the TMVII molecular hinge. Given the

highly flexible properties of the N-terminal half relative to the C-terminal half of LacY (Nagamori et al., 2003; Bennett et al., 2006), spontaneous flipping of the former is a distinct possibility. The TMs of the N-terminal bundle are highly hydrated (Lomize et al., 2006), which might lower the activation energy for flipping. TMI is more exposed to the membrane hydrophobic core, which may explain retention of its orientation and the formation of a second hinge region represented by TMII. Moreover, increasing the low hydrophobicity of TMVII by the D240I replacement is sufficiently energetically unfavorable to counter the favorable inversion of the N-terminal bundle in −PE cells.

A direct role for the translocon in determining final topological orientation of TMs has not been established, but models of cotranslational insertion of polytopic proteins suggest an initial influence of the translocon on orientation (Goder and Spiess, 2003; Goder et al., 2004) in a sequential manner (Hartmann et al., 1989) for TMs before exiting the translocon (Sadlish et al., 2005). However, the topology of LacY reconstituted into liposomes is determined solely by the lipid composition and is independent of other protein factors or the topological history of LacY (Wang et al., 2002). Because LacY exists in a compact folded state in −PE cells (Bogdanov et al., 2002) and is in large molar excess over the number of functional translocons (Urbanus et al., 2002), it is very unlikely that the translocation machinery is recruited for the reorientation process. Short-range and long-range interactions governed by topological determinants of the lipid bilayer and topogenic signals within the protein sequence are more likely as dominant determinants of final topology. The cooperative topological response of the N-terminal bundle to changes in the charge in a position-independent manner and the effect of membrane environment on global topology changes in this large domain are unlikely due to a specific property of the translocon. Similarly, the ability of TMVII to exist in or out of the membrane bilayer, dependent on the lipid environment, further supports factors other than the translocation machinery as a primary determinant. These results are consistent with the N-terminal bundle remaining in a non-native and presumably uncommitted topological state until TMVIII is synthesized, resulting in an integral protein (Nagamori et al., 2003). Although the translocon may be involved in establishing initial orientation of TMs during cotranslational insertion, the post-assembly TM lipid-dependent flipping of LacY indicates that final topology is under thermodynamic rather than kinetic control (Bogdanov et al., 2002; Mackenzie, 2006), as determined by the protein sequence and the lipid environment. The balance of net charge due to the presence of opposing topogenic signals (opposite charges) can either represent a retention or driving force, which either supports or overrides the positive inside rule depending on the presence of PE and magnitude of negative charge density of the membrane surface.

Lipid-dependent topogenesis appears to depend on integration of responses to at least three parameters: charge of cytoplasmic domains containing conflicting acidic and basic topological signals, charge of the membrane surface, and the presence of hinge region. Sequence comparison within the closely related sugar permeases (Fig. S4) reveals a high content of negatively charged residues in the cytoplasmic face of the N-terminal bundles. TMVII of all but the sucrose permease contains two negatively charged residues. The C2 domain, which is misoriented in PheP and GabP assembled in −PE cells (Zhang et al., 2003, 2005), has a net negative charge, as do closely related amino acid permeases (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1). TMIII appears to be the hinge region that allows the N-terminal hairpins of PheP and GabP to misorient in −PE cells. TMIII in these permeases is highly enriched in interface-prone aromatic residues, which might allow TMIII to assume a mini-loop structure in order to act as a hinge. Therefore, our results would predict that permeases with the above types of cytoplasmic domains and putative TM molecular hinges would also require PE to establish native topology. Preliminary results show that decreasing the net negative charge of the extramembrane domains flanking the N-terminal hairpin of PheP forces 60% of molecules to assume wild-type orientation in −PE cells (unpublished data).

Changes in protein sequence resulting in a change from an even to odd number of TMs generally results in inversion of protein topology (Saier, 2003), as we report for LacY. The thirteen-TM *Pseudomonas aeruginosa* ChrA protein is composed of two homologous orientationally opposed six-TM bundles that are separated by a hydrophobic TM (TMVII). However, the homologous two six-TM bundles of the twelve-TM *Cupriavidus metallidurans* ChrA are arranged in the same orientation with respect to each other (Jimenez-Mejia et al., 2006). TMVII of ChrA from *P. aeruginosa* contains no charged amino acids, whereas the corresponding region from *C. metallidurans* contains two positively charged residues and is not a TM. This suggests that both proteins arose from the same intragenic duplication of an ancestral six-TM protein followed by the insertion of an intervening sequence whose hydrophobicity determined the relative orientation of the duplicated domains.

In summary, the influence of the lipid environment on protein topology was only revealed through manipulation of lipid composition and protein sequence. These relationships are not always evident under normal conditions because lipids and proteins have coevolved so that final structural organization supports function. Our results significantly extend the understanding of the complex process of insertion and folding of membrane proteins by providing new insights into how the membrane lipid matrix interacts with defined protein motifs to determine final protein folding. The results show that PE is required to fulfill the positive inside rule as applied to N-terminal bundle of LacY and explains why positively charged residues are more potent topological determinants than negatively charged residues under physiological conditions. PE and an appropriate charge density for the membrane surface maintain the charge balance between translocation and retention signals required to achieve proper TM topology while allowing the presence of negative residues in the cytoplasmic face of proteins for other purposes. Sequence comparisons between proteins whose topology is lipid sensitive and related homologues indicate a specific role for PE. Polytopic membrane proteins containing competing opposite charges within their cytoplasmic domains may share a common mechanism for topogenesis dependent on PE. Moreover, significant topological decisions can be made outside and most likely independent of the translocation machinery.

Materials and methods

Plasmids

Amp^R plasmids expressing LacY derivatives under OP_{tac} regulation and containing single or multiple amino acid replacements in a derivative of LacY in which endogenous cysteines were replaced by serine (Frillingos et al., 1998) were constructed by site-directed mutagenesis (Ho et al., 1989). These amino acid replacements did not affect steady-state LacY levels as determined by Western blot analysis as previously shown (Bogdanov et al., 2002).

Bacterial strains and growth conditions

Strain AT2033 (*$P_{LtetO-1}$-pssA*$^+$ *pss93*::*kan*^{R} *lacY*::*Tn9 recA srl*::*Tn10*) in which the PE content of the cell can be regulated by the level of aTc (Spectrum Chemical Corp.) in the growth medium was constructed as follows. The spectinomycin resistance gene was isolated from plasmid pZS4int-1, which is integrated into the genome of strain DH5aZ1 (Lutz and Bujard, 1997), by PCR using primers 5′ (TCGAGGTGAAGACGAAAGGG) and 3′ (TGCTGTTCAGCAGTTCCTGC). The PCR product was digested with AatII and SacI, as was plasmid pZE21-mcs1 (Lutz and Bujard, 1997; to remove the *kan*^{R} gene), and both were ligated to create plasmid pZE41-1. The *P_{N25}-TetR* (regulatory protein TetR under N25 promoter control) locus of DH5aZ1 was isolated using the PCR primers 5′ (TTTTTGACGTCGGCCGATTCATTAATGCAGC, adds an AatII site) and 3′ (TTTTCCGGATTAAGACCCACTTTCACATTTAAGTTG, adds a BspEI site). The PCR product and plasmid pZE41-mcs1 were digested with AatII and BspEI and ligated resulting in plasmid pZT41. The λ phage attachment site (*attP*) and a *cam*^{R} gene were excised from plasmid pLDR10 (Diederich et al., 1992) by digestion with SacI and HincII and ligated with plasmid pZT41 digested with SacI and BsrBI to create plasmid pZTL41. The *pssA* gene of *E. coli* (DeChavigny et al., 1991) was isolated using PCR primers 5′ (GGGCTGCAGGAACAGAGAAGAAATGCACTGTG, adding a PstI site) and 3′ (GCGGATCCTGAATATTCATTTCCGGCG, adding a BamHI site). The PCR product and pZTL41 were digested with PstI and BamHI and ligated to place the *pssA* gene under $P_{LtetO-1}$ control. The *oriC* locus and the *cam*^{R} gene were removed by NotI digestion of this plasmid and the remaining DNA ligated and used to integrate the origin-less plasmid at the *attB* site of strain W3899 (DeChavigny et al., 1991) carrying plasmid pLDR8 (Diederich et al., 1992), a thermosensitive plasmid encoding the λ phage integrase and a *kan*^{R} cassette, followed by growth at 42°C. The *pss93*::*kan*^{R} null allele of strain AD93/pDD72 (DeChavigny et al., 1991) was introduced into the resulting strain by P1 transduction. The strain was made *lacY* null and *recA* null by P1 transduction using strains AL95/pDD72 (*lacY*::*Tn9*) (Bogdanov et al., 2002) and AD93/pDD72 (*recA srl*::*Tn10*), respectively, as donors, resulting in strain AT2033.

The −PE strain AL95 (*pss93*::*kan*^{R} *lacY*::*Tn9*) was grown at 37°C, whereas the +PE strain (AL95/pDD72 (*pssA*$^+$ *cam*^{R})) was grown at 30°C because plasmid pDD72 contains a temperature-sensitive replicon (Bogdanov et al., 2002). Strain AT2033 was grown at 37°C in the presence (+PE) or absence (−PE) of 1 µg/ml aTc. All cells were grown in Luria–Bertani medium containing 50 mM $MgCl_2$ (necessary for growth in the absence of PE) supplemented with ampicillin (100 µg/ml) to maintain LacY plasmids and IPTG (1 mM) when LacY expression was induced.

SCAM

SCAM, based on the controlled membrane permeability of the thiol-specific reagent MPB (Invitrogen), was used to probe the TM topology of LacY, as previously described (Bogdanov et al., 2002, 2005). Reaction with MPB was performed at pH 7.5 or 10.5. Treatment under strong alkaline conditions before SCAM analysis was accomplished by mixing an equal volume of cells in reaction buffer with cold 0.2 N NaOH followed by incubation for 5 min on ice and isolation of a pellet by centrifugation at 40,000 rpm (TLA-100; Beckman Coulter) for 10 min. The pellet was homogenized by sonication and washed three times with reaction buffer before reaction with MPB. Biotinylation of exposed cysteine residues was detected after isolation of biotinylated LacY using precipitation by antibody specific for the C-terminus of LacY (prepared by ProSci, Inc.), followed by SDS-PAGE and Western blotting using avidin linked to horseradish peroxidase and chemiluminescent reagents (Pierce Biotechnology). In all cases, equal amounts of cells were processed from samples before and after sonication, and the same amount of sample was applied to SDS gels. Figures show the presence or absence of signal in the Western blot region at the apparent molecular mass for LacY (33 kD). The results presented are representative of two or more determinations.

Image acquisition and processing

Western blots were imaged using a Fluor-S Max MultiImager (Bio-Rad Laboratories) equipped with a CCD camera and a Nikon 50-mm 1:1.4 AD (F 1.4) at the ultrasensitive chemiluminescence setting, which cools the camera to −33°C. Quantity One versions 4.6.5.094 and 4.4.1 (Bio-Rad Laboratories) were used to collect and store the images as TIFF files, which were later imported into Adobe Illustrator CS to construct the figures. Images were expanded or reduced so that the horizontal strip containing LacY (apparent molecular mass of 33 kD) on all images within the same figure was approximately the same size. Images were then masked to only show the LacY strips, which were then aligned and labeled. The only valid comparison in intensity is between whole cell and sonicated sets (images treated identically) run on the same gel. Pairs of images from different gels or from discontinuous regions of the same gel are separated by white spaces on the figures. Final figures were saved at 300 dpi as EPS files.

Online supplemental material

Fig. S1 shows phospholipid composition as a function of *pssA* gene induction. Fig. S2 shows stability and read-through expression of LacY. Fig. S3 shows transport function of LacY dependent on membrane PE content. Fig. S4 shows distribution of charged amino acids in homologous sugar permeases. Fig. S5 shows distribution of charged amino acids in homologous amino acid permeases. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200803097/DC1.

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