Proper fatty acid composition rather than an ionizable lipid amine is required for full transport function of lactose permease from *Escherichia coli*

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Running title: Lipid-protein interaction as structure-function determinant

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**Background:** Principles governing functional reconstitution of membrane proteins remain largely empirical.

**Result:** Native lactose permease structure and function are dependent on head group and fatty acid composition of the lipid environment.

**Conclusion:** In vivo and in vitro studies are necessary to address lipid-protein interactions in structure/function analysis of membrane protein.

**Significance:** Short- and long-range changes in lipid-protein interactions affect membrane protein structure and function.

**SUMMARY**

Energy-dependent uphill transport but not energy-independent downhill transport by lactose permease (LacY) is impaired when expressed in *Escherichia coli* cells or reconstituted in liposomes lacking phosphatidylethanolamine (PE) and containing only anionic phospholipids. Absence of PE results in inversion of the N-terminal half and mis-folding of periplasmic domain P7, which are required for uphill transport of substrates. Replacement of PE in vitro by lipids with no net charge (phosphatidylcholine (PC), monoglucosyl diacylglycerol (GlcDAG), or diglucosyl diacylglycerol (GlcGlcDAG)) supported wild type transmembrane topology of the N-terminal half of LacY. The restoration of uphill transport in vitro was dependent on LacY native topology and proper folding of P7.

Support of uphill transport by net neutral lipids in vitro (PE > PC >> GlcDAG ≠ GlcGlcDAG provided PE or PC contained one saturated fatty acid) paralleled the results observed previously in vivo (PE = PC > GlcDAG ≠ GlcGlcDAG). Therefore, a free amino group is not required for uphill transport as previously concluded based on lack of in vitro uphill transport when fully unsaturated PC replaced *E. coli*-derived PE. A close correlation was observed in vivo and in vitro between the ability of LacY to carry out uphill transport, the native conformation of P7, and the lipid head group and fatty acid composition. Therefore, the head group and the fatty acid composition of lipids are important for defining LacY topological organization and catalytically important structural features, further illustrating the direct role of lipids, independent of other cellular factors, in defining membrane protein structure/function.

Knowledge of the role of lipid-protein interactions with respect to the nature of lipid head groups, lipid acyl chain composition and activity of membrane proteins is central to understand structure-function relationships in biological membranes. Lipids have a strong
influence on folding (1,2), topology (3,4) and function of integral membrane proteins (5,6) interacting either transiently or permanently with membrane proteins to elicit dynamic or steady state effects on structure and activity.

By taking advantage of a simple yet well-characterized *Escherichia coli* system, we can easily manipulate the lipid composition in genetically altered strains to determine the *in vivo* involvement of individual lipids in such complex processes as folding, structure and function of membrane proteins (2,7). However, *in vivo* studies cannot distinguish between effects due to direct lipid-protein interactions and secondary effects due to alterations in other cellular processes. Although the effects of changes in phospholipid head group composition can be monitored using the above strains, the effect of changes in both fatty acid and head group composition is more feasible in *in vitro* studies. On the other hand *in vitro* studies of membrane proteins are often compromised by artifacts due to the necessity of removing membrane proteins from their nature lipid environment, the ineffectiveness of detergents as a substitute for lipids, or the use of unnatural lipid environments. Therefore, a combination of *in vivo* and *in vitro* studies is required to more precisely determine the role or site of action of lipids in a particular process.

Lactose permease (LacY), a member of the Major Facilitator Superfamily, of *E. coli* has served as a paradigm for studying the function and biogenesis of a large number of secondary transporters composed of twelve α-helical transmembrane domains (TMs) connected by extramembrane loops. The structural organization and function of LacY, as well as several other secondary transporters, is sensitive to the membrane lipid composition (2,8). The topological organization and function of LacY *in vivo* has been extensively studied as a function of lipid head group composition in wild type *E. coli* cells containing the normal levels of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) as well as in mutants either lacking PE (4,9) or in which PE was substituted by either phosphatidylcholine (PC) (10), monoglucosyl diacylglycerol (GlcDAG) (11) or diglucosyl diacylglycerol (GlcGlcDAG) (12).

Early studies demonstrated that PE is required for energy-dependent uphill substrate accumulation but not for energy-independent downhill substrate equilibration carried out by LacY reconstituted in liposomes (13,14). The PE requirement for uphill transport but not for downhill transport was subsequently verified *in vivo* using strains of *E. coli* either with or without PE. (15,16). The lack of PE *in vivo* results in the topological inversion of the N-terminal six TM bundle of LacY (16), exposure of TM VII to the periplasm (4) and misfolding of periplasmically exposed domain P7 (9,10) (see Fig. 1). The proper folding of domain P7 is linked to the ability of LacY to effect uphill transport (17). The conformation-specific monoclonal antibody (mAb) 4B1 recognizes native LacY via an epitope in domain P7 (18,19). The mis-orientation of the N-terminal six-TM bundle of LacY in PE-lacking cells disrupts the native structure of P7 (16). In addition there is a strong correlation between the presence of PE, proper folding of P7 and the ability of LacY to mediate uphill transport (9,10,16).

Previous studies established that PC supports proper topological organization of LacY in proteoliposomes (20) but not uphill transport by LacY (13,20). Unfortunately, the recognition by mAb 4B1, which only recognizes properly oriented and functional protein, was not tested in these *in vitro* studies. Since phosphatidylycerine supports uphill transport and progressive methylation of the amine of PE resulted in a parallel decrease in the level of uphill transport observed *in vitro*, its was suggested that an ionizable amine was required to support uphill transport (13). PC also did not substitute for PE in proteoliposome studies of proline transport by PutP from *E. coli* (21), multidrug transport by LmrP from *Lactococcus lactis* (41,42), leucine by the leucine permease from *Pseudomonas aeruginosa* (43), branched chain amino acid transport by the transporter from *Streptococcus cremoris* (44), and the ABC transporter HorA from *Lactococcus lactis* (45). The failure of PC to substitute for PE in the case of LacY as well as several other secondary transporters has led to the general conclusion that an ionizable lipid amine may be either involved directly in the transport mechanism or required for proper assembly. However, LacY expressed in mutant cells in which PE was replaced by either PC or GlcDAG...
displayed either a high level of or a lower but significant level of uphill transport, respectively, suggesting no requirement for an ionizable amine in support of uphill transport. LacY in these cells was also properly oriented in the membrane and recognized by mAb 4B1 (10). All in vitro reconstitution studies employed either PC from soybeans, which is highly enriched in unsaturated fatty acids, or synthetic dioleoyl PC (DOPC). Since PC and PE synthesized by E. coli are primarily saturated in the 1-position and unsaturated in the 2-position (10), the failure of PC to support uphill transport in vitro might have been due to the fatty acid composition of PC used rather than a difference in head group chemistry between PC and PE as was previously concluded (13) or differences in the physical properties between PC and PE. Since a combined study in which both head group and fatty acid composition of lipids are simultaneously varied in vivo is not feasible, the resolution of this discrepancy was addressed by additional in vitro reconstitution experiments. More precise understanding of the lipid requirement for uphill transport will further define the nature of lipid-protein interactions that determine native structure and function of LacY and other membrane proteins. In addition reliance solely on in vivo results does not rule out indirect pleiotropic effects of lipids on the membrane protein insertion/assembly machinery or molecular chaperones.

Particularly in eukaryotic systems the systematic alteration of membrane lipid composition in vivo is not possible so that a clear demonstration of the validity of in vitro approaches reflecting in vivo physiological function of lipids is required. Building on our earlier work on in vivo lipid-assisted membrane protein folding, we report an in vitro comparative study of the lipid-dependent topogenesis, folding and function of LacY using (i) substituted the cysteine accessibility method to assess TM orientation (22,23), (ii) monoclonal antibody to establish the proper folding of domain P7, and (iii) direct monitoring of transport activity after reconstitution of LacY into proteoliposomes made of native and synthetic lipids with different head group and fatty acid compositions. Our results demonstrate that the phospholipid head group and the fatty acid composition play a crucial role in determining the proper orientation, folding and function of LacY, and that their involvement in these events after reconstitution into proteoliposomes mirrors results obtained in vivo. The close correlation between effects of lipids on LacY observed in vitro with the effects observed in vivo further supports a direct effect of membrane lipid composition on LacY properties rather than an indirect effect on the membrane protein assembly machinery or other cellular factors.

EXPERIMENTAL PROCEDURES

Materials—E. coli total lipid extract, E. coli PG, E. coli CL, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids. [14C]Lactose was purchased from Moravek. HiTrap columns and Vivaspin concentrators (50 kDa molecular weight cut off) were purchased from GE Healthcare. GFTS (0.22 µm) filters were purchased from Millipore. The mouse anti-His antibody was purchased from GenScript. The ECL kit, Imperial protein stain solution, HRP-labeled secondary antibody, micro BCA protein reagent assay, Slide-A-Lyzer G2 Dialysis Cassettes and avidin-HRP were purchased from Pierce. β-D-Dodecylmaltoside (DDM) and n-octyl-β-D-glucopyranoside (OG) were purchased from Anotrace. Complete® protease inhibitor was purchased from Roche Molecular Biochemicals. Site-directed polyclonal antibody (pAb 1043) directed against the C-terminal dodecapeptide of LacY was made by ProSci Inc. Monoclonal antibody against LacY 4B1 epitope (mAb 4B1) and plasmids pT7-5/C-less lacY/H205C and pT7-5/C-less lacY/F250C were provided by Dr H. R. Kaback (University of California, Los Angeles). 4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), 3-(N-maleimidopropionyl) biocytin (MPB) and 3,3'-dipropylthiadicarbocyanine iodide (DiSC3(5)) were purchased from Molecular Probes. Bio-spin 6 columns were purchased from Bio-Rad. Valinomycin, nigericin, DNase and all other reagents were purchased from Sigma.
**Bacterial Strains, Plasmids and Growth Conditions**—Strain AL95 (pss93::kan<sup>R</sup> lacY::Tn9) was used as the host strain (16). This strain cannot make PE and is not viable without either plasmid pDD72GM (pssA<sup>A</sup> gen<sup>R</sup> and pSC101 temperature-sensitive replicon) (16) or growth media containing 50 mM MgCl<sub>2</sub> (24). Strain AL95 (grown at 37°C) lacks PE, and strain AL95/pDD72GM (grown at 30°C) contains the normal *E. coli* complement of phospholipids including PE. LacY was expressed from plasmids (amp<sup>R</sup>, ColE1 replicon) encoding a single cysteine replacement in cysteine-less LacY at either H205 (pT7-5/C-less lacY/H205C) in the C6 cytoplasmic domain connecting TMs VI and VII or F250 (pT7-5/C-less lacY/F250C) in the periplasmic domain P7 connecting TMs VII and VIII (see Fig. 1A). LacY was engineered with a His<sub>6</sub> tag at the C-terminus to facilitate purification and was expressed under control of OP <sub>lac</sub> by growth of cells in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown in LB-rich medium containing ampicillin (100 µg/mL) as required and 50 mM MgCl<sub>2</sub>.

Strain AD93 (pss93::kan<sup>R</sup>) with plasmid pTMG3 (GlcDAG in place of PE) (25), AD93 with plasmid pTMG3 and pACYC177DGs (GlcGlcDAG in place of PE) (12) or AL95 was plasmid pACPCSpGm (PC in place of PE) was grown as previously described and used to prepare lipid extracts containing *E. coli*-synthesized GlcDAG, GlcGlcDAG or PC, respectively.

**Preparation of Total Lipid Extracts**—Total lipid extracts were prepared from cells grown to OD<sub>600</sub> of 0.7-0.8 in LB medium with appropriate additives. Cells were harvested by centrifugation, and phospholipids and glycolipids were quantitatively extracted together, as previously described (11), from cell pellets by vigorously vortexing in chloroform/methanol (2:1, v/v). After centrifugation, the pellet was resuspended for a second extraction in methanol containing 0.1 N acetic acid. Supernatants from the two extractions were pooled and concentrated under vacuum using a SpeedVac concentrator (Savant) and stored at -20°C in chlorofom/methanol (9:1) until further use.

**LacY Expression and Purification**—*E. coli* cells were grown to an OD<sub>600</sub> of 0.6, induced by addition of IPTG and grown until cell arrest occurred. Purification of LacY was carried out at 4°C or on ice by a modification of a published procedure (26). Cells were harvested by centrifugation, and the cell pellet was suspended in 50 mM sodium phosphate (NaPi; pH 7.5)/200 mM NaCl/10 mM dithiothreitol (DTT)/Complete®/30 mg/mL DNase I. Cells were disrupted with a French press and unbroken cells removed by centrifugation at 13,000 g<sub>av</sub> for 20 min. Membranes were collected by centrifugation at 100,000 g<sub>av</sub> and washed once with 50 mM NaPi (pH 7.5)/200 mM NaCl/Complete®. Membranes (at 15-25 mg/mL) were solubilized with 2% DDM and affinity purified on a Co(II)-HiTrap affinity column. The resin was equilibrated with 50 mM NaPi (pH 7.5)/5 mM imidazole/200 mM NaCl/0.01% DDM/5%/glycerol (column buffer). Imidazole, glycerol and NaCl were added to the DDM solubilized extract to final concentrations of 5 mM, 5% and 200 mM, respectively, and the pH was adjusted to 7.5. LacY was absorbed to the resin at a flow rate of 1 mL/min. The column was washed with column buffer, then exhaustively with the same buffer containing 50 mM imidazole. LacY was eluted with column buffer containing 200 mM imidazole. The purified protein was dialyzed overnight against 20 mM Tris-HCl (pH 7.4)/0.01% DDM and fractions containing LacY were pooled and concentrated by means of a Vivaspin-20 concentrator with 50 kDa cutoff. Purified samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Blue staining and Western blot analysis.

**Preparation of Proteoliposomes Containing LacY—E. coli** total lipid extracts (prepared from lab-grown cells) or lipids from a commercial source were dissolved in chloroform/methanol (9:1, v/v). The solvent was removed first under a stream of oxygen-free nitrogen and then under a vacuum to obtain a thin layer of dry lipids. The lipids were suspended in 50 mM potassium phosphate (KPi; pH 7.4)/0.01% DDM and fractions containing LacY were pooled and concentrated by means of a Vivaspin-20 concentrator with 50 kDa cutoff. Purified samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Blue staining and Western blot analysis.
(when necessary), OG was added to 1.5%, mixed with purified LacY in a 500:1 lipid to protein ratio (w/w with LacY at 100 µg/mL), incubated at 30°C with gentle agitation for 10 min and then placed on ice for 20 min. To remove OG, the mixture was diluted at least 30-fold into 50 mM KPi (pH 7.5)/50 mM MgCl2/2 mM βME, and centrifugated at 190,000 g av for 45 min to recover proteoliposomes. Proteoliposomes (final concentration of 50 mg/mL phospholipid and 100 µg/mL LacY) were suspended in 50 mM KPi (pH 7.5)/50 mM MgCl2 and either used immediately (transport activity and epitope folding assessment) or stored at -80°C (for topology assessment at a later time). Prior to use, proteoliposomes were subjected to three cycles of freeze/thaw/sonication (30s sonication) in a bath sonicator.

**Determination of Membrane Potential**–Dilution of liposomes containing K+ into Na+ in the presence of valinomycin generates a positive outward membrane potential (ΔΨ). Addition of the positively charged fluorescent probe DiSC3(5) to the liposomes results in a transient fluorescence that is self quenched as the dye concentrates within the liposome (27,28). Addition of nigericin dissipates ΔΨ with the magnitude of the increase in fluorescence due to the released DiSC3(5) being proportional to ΔΨ. Proteoliposomes (5 µL) prepared in 50 mM KPi (pH 7.5)/50 mM MgCl2 were diluted into 995 µL of 50 mM NaPi (pH 7.5)/50 mM MgCl2/5 µM valinomycin/1 µM DiSC3(5). Addition of 5 µM nigericin was used to dissipate the membrane potential to measure the increased fluorescence of DiSC3(5), and ΔΨ was monitored at an excitation wavelength of 650 nm and an emission wavelength of 675 nm, both with a 0.5-nm band pass. The fluorescence measurements were performed using a QuantaMaster model QM3-SS (Photon Technology International), a cuvette-based fluorescence spectrometer. Using a Peltier TE temperature controller, the sample was held at a constant 20°C. Data were collected and analyzed using Felix 32 software.

**Sugar Transport Assays**–To measure membrane potential-driven uphill transport, 10 µL of proteoliposomes containing 50 mM KPi (pH 7.5)/50 mM MgCl2 were diluted into 990 µL of 50 mM NaPi (pH 7.5)/50 mM MgCl2 containing 0.22 mM [14C]lactose (2.7 Ci/mol) and 10 µM valinomycin. Addition of 10 µM nigericin was used to dissipate the membrane potential and perform measurements within de-energized proteoliposomes. Aliquots of 150 µL were removed at various times, quenched with 3 mL of ice-cold 50 mM NaPi (pH 7.5)/50 mM MgCl2/100 mM LiCl, immediately filtered through GFTS filters and washed using 5 mL of the same buffer. Filters were dried and counted by liquid scintillation. A background value was determined by assaying liposomes lacking LacY and was subtracted from all measurements. All incubations were at 30°C.

**Determination of TM Orientation of LacY in Proteoliposomes**–The following procedure was performed as previously described (20). Briefly, proteoliposomes were incubated in 200 µM MPB at room temperature for 20 min to biotinylate LacY, or in 200 µM AMS for 10 min to block the single cysteine in LacY exposed to the exterior of liposomes. Cysteine labeling was quenched by addition of 1 mM βME. OG (at 1.5%) was used to disrupt proteoliposomes and label cysteine exposed to the interior of proteoliposomes. Centrifuging the reaction mixture through a Bio-spin column was used to change the solution surrounding proteoliposomes and/or to remove AMS/βME. Samples were subjected to SDS-PAGE followed by Western blot analysis after incubation with avidin-HRP to assess biotinylation or pAb and HRP-conjugated secondary antibody for total LacY recovery.

**Determination of Properly Oriented LacY by Antibody Precipitation**–Properly oriented LacY was isolated by immunoprecipitation with conformation-specific mAb 4B1 (which only recognizes properly oriented and functional LacY (9,10) as previously described (29,30) with minor modifications. Total membranes were obtained from whole cells by sonication. Supernatants obtained by ultracentrifugation at 4°C and 65,000 x g for 10 min were solubilized in 50 mM Tris-HCl, pH 7.5/150 mM NaCl/1.0 mM EDTA/2% DDM and incubated further with either mAb 4B1 or pAb 1043 (detects LacY irrespective of orientation or functionality), both previously crosslinked to Protein A/G agarose beads, in the same buffer on a rocking platform overnight at 4°C. The affinity resin were collected by a 1 min centrifugation at 20,800 x g and then washed four
times using Spin Columns with wash buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.2% DDM) followed by resuspension in 1% DDM and 1 mM DTT. After mixing with SDS sample buffer, the supernatants obtained from a brief centrifugation were analyzed by SDS-PAGE. LacY was visualized in gels stained with Coomassie blue. The relative amounts of LacY in each lane were quantified using ImageJ software.

**Determination of Protein Concentration**—Protein content during purification and the concentration of LacY in proteoliposomes were determined by the micro BCA protein assay, according to the manufacturer’s instructions.

**RESULTS**

**Expression and Purification of LacY**—In this study, two cysteine-less mutants of LacY carrying a single cysteine at position H205 (C6) or F250 (P7) (see Fig. 1A) were purified from either PE-containing (AL95/pDD72GM) or PE-lacking (AL95) cells. Protein purification was monitored by SDS-PAGE followed by either staining with Imperial protein stain solution or detection by Western blot analysis (Fig. 1B and C). A broad band centered at an apparent molecular mass of ~40 kDa was detected from either PE-containing (Fig. 1B) or PE-lacking cells (Fig. 1C) in the various steps of the procedure. Few or no higher molecular weight aggregates of LacY were detected in samples purified from PE-containing background while more aggregation was observed in LacY samples purified from PE-lacking background. About 800 µg of LacY was obtained from 10 g wet weight of cells. Similar results were obtained for each of the four strains.

**Proteoliposome Characterization**—The ability of proteoliposomes to maintain a membrane potential over the time course of the transport assay was measured using the membrane potential-sensitive fluorescent dye DiSC3(5). Stable membrane potential was taken as an indication that the vesicles were sealed and capable of providing energy to support uphill transport of substrate by reconstituted LacY. Generation of a membrane potential across the bilayer was achieved by treating proteoliposomes containing 50 mM KPi (pH 7.5) buffer with valinomycin, a cation ionophore with high affinity toward K+ ions, and then diluting them 200-fold into buffer containing 50 mM NaPi (pH 7.5).

Changes of membrane potential across liposomes were followed by fluorescence changes of DiSC3(5). After the addition of DiSC3(5), equilibration was nearly complete within 30 s (Fig. 2A) and fluorescence was quenched in response to the membrane potential (ΔΨ, positive outwards). Addition of nigericin resulted in the inward diffusion of potassium in exchange for a proton, dissipation of the membrane potential (high buffering capacity prevents ΔpH formation) and release of the membrane-sensitive probe with an associated rapid increase in fluorescence.

When potassium-loaded liposomes were diluted in 50 mM KPi (instead of 50 mM NaPi), no effect of valinomycin was observed. When nigericin was added to proteoliposomes before the addition of DiSC3(5) (Fig. 2B), no quenching of fluorescence was observed, confirming the need for a membrane potential to quench DiSC3(5) fluorescence. In addition, DiSC3(5) fluorescence was proportional to the membrane potential. A nearly identical membrane potential was generated for the same amount of proteoliposomes energized independent of the lipid composition (Fig. 2A, C and D). In all cases, the membrane potential was maintained for a minimum of 5 min, as indicated by the stable low fluorescence after DiSC3(5) addition. The results shown in Fig. 2 are representative of all proteoliposomes used in the experiments to follow.

**Dependence of LacY Transport on Lipid Head Group Composition**—Functional characterization of LacY reconstituted in proteoliposomes of various lipid compositions was assessed by measurement of [14C]lactose uptake by energized proteoliposomes. When a membrane potential is present, uphill transport can be measured as the accumulation of substrate within the proteoliposomes, which is coupled to the inward movement of protons made available by the positive outward membrane potential (see cartoon in Fig. 3). In the presence of nigericin any substrate accumulation is energy independent downhill transport. The radioactivity taken up by the proteoliposomes was converted to lactose concentration and normalized to the amount of LacY in the sample. Fig. 3 shows the transport function of LacY in different proteoliposomes made of total lipid extracts from cells (Fig. 3A, B,
D and E) or synthetic commercially available lipids (Fig. 3C). Average results representative of 3 to 5 experiments are shown for LacY (C6, H205C) isolated from PE-containing (Fig. 3A to C) and PE-lacking cells (Fig. 3D and E); the same results were obtained LacY (P7, F250C) from both cell sources.

As previously shown (20), LacY reconstituted using a total lipid extract from PE-containing cells exhibited a robust uphill transport (Fig. 3A and D) irrespective of the lipid composition of cells from which LacY was isolated. In the presence of nigericin, accumulation of lactose was observed at the level of liposomes lacking PE, confirming that uphill transport in PE-containing proteoliposomes was being measured. When reconstituted in a total lipid extract from PE-lacking cells (Fig. 3A and D), LacY exhibited no uphill transport with values being the same as that observed with added nigericin indicative of downhill transport. When reconstituted in a total lipid extract from cells in which PC replaced PE (Fig. 3A and D), LacY also exhibited very significant uphill transport, which was also inhibited by addition of nigericin. This result is in good agreement with previous observations made in vivo where cells containing 70% PC or PE displayed the same level of uphill transport (10) while cells containing only PG and CL exhibited only downhill transport (15,16).

When reconstituted in total lipid extracts from cells containing glycolipids in place of PE (Fig. 3B and E), a marked reproducible difference between GlcDAG and GlcGlcDAG was observed. LacY reconstituted into proteoliposomes made from the GlcGlcDAG total lipid extracts showed little or no uphill transport, while the GlcDAG total lipid extract supported a low but significant uphill transport, since addition of nigericin reduced uptake to the level observed for the total lipid extract lacking PE. These results are also in good agreement with experimental observations made in vivo where significant uphill transport was observed in GlcDAG-containing cells lacking PE (25), while no uphill transport was observed in GlcGlcDAG-containing cells lacking PE (12).

Therefore, LacY reconstituted into liposomes composed of lipids extracted from cells displayed the same dependence on lipids for supporting uphill transport as observed in vivo. Most notably the new in vitro data showed that PC and GlcDAG supported uphill transport while GlcGlcDAG did not. The results with E. coli-derived PC were significantly different from the results previously reported for proteoliposomes composed from DOPC (13,20). This in vitro result using lipids including PC derived from E. coli confirms a direct lipid-LacY interaction in supporting LacY function and eliminates an indirect effect on other cellular processes involved in proper assembly of LacY.

Dependence of LacY Transport on Lipid Fatty Acid Composition—In order to further dissect the molecular requirements for LacY activity in proteoliposomes, we used proteoliposomes containing synthetic PE or PC at physiologically equivalent amounts (both at 70% relative to E. coli-derived PG + CL at 30%) consisting of homo- or hetero-fatty acid composition (DO or PO, respectively). The results are presented in Fig. 3C and were the same for LacY derivatives derived either from PE-containing or PE-lacking cells. For the same fatty acid composition a higher uphill transport activity was observed when using PE-based compared to PC-based phospholipids. However, significant uphill transport activities were observed in the following order with POPE > POPC > DOPE >> DOPC liposomes; in multiple experiments the uptake of substrate using DOPC vesicles was not significantly above the nigericin control. These results are in agreement with previous results (13,20) demonstrating that proteoliposomes prepared with DOPC did not exhibit any uphill transport activity. However, PC liposomes support uphill transport provided the PC contains at least one saturated fatty acid, which is consistent with conclusions drawn from in vivo experiments with E. coli cells in which PE was substituted by PC (10). In a similar fashion, when DOPE was used, a very low uphill transport was observed that was barely above the downhill transport level. This result demonstrates that the fatty acid composition also influences the activity of LacY reconstituted in proteoliposomes. The presence of both saturated and unsaturated fatty acids in same molecule of PE or PC was required to observe significant uphill transport and accumulation to a significant level while the presence of zwitterionic phospholipids with fully unsaturated fatty acids (this work and (13,20)) did not support LacY uphill activity. Our new in vitro results explains the previous lack of support by...
PC for uphill transport and eliminates any mechanistic involvement of an ionizable lipid amine in LacY function.

Dependence of LacY Topology on Lipid Composition—LacY with single cysteine replacements in otherwise cysteine-less LacY (Fig. 1A) and purified from either PE-containing or PE-lacking cells was reconstituted in proteoliposomes of various lipid compositions. In order to establish the topology of the hydrophilic domains C6 and P7, LacY reactivity to MPB in intact or solubilized proteoliposomes (addition of OG) was probed. AMS, another fully membrane-impermeable but “transparent” non-biotinylated maleimide, was used to block exposed cysteines prior to MPB treatment, thus preventing biotinylation of outward facing cysteines. After SDS-PAGE and transfer onto nitrocellulose membranes, LacY biotinylation was visualized using avidin-HRP. The results obtained for the four LacY derivatives reconstituted in different total lipid extracts from cells are presented in Fig. 4. As previously shown (20), when LacY was assembled in proteoliposomes made of PE-containing total lipid extracts, the domains C6 and P7 were located on opposite sides of the membrane as observed in whole cells (4,16); C6 was accessible to MPB and AMS without solubilization by OG and P7 was labeled only after solubilized by OG. Additionally, the same topological arrangement was observed irrespective of the source of LacY (PE-containing or PE-lacking cells). In proteoliposomes made of a total lipid extract from cells containing only anionic phospholipids, both C6 and P7 domains were located on the same side (luminal side) of liposomes, their labeling being only possible after solubilization by OG. The presence of an equal amount of protein was demonstrated in each sample by Western blot analysis of an equal volume of each reaction mixture (bottom panel, Fig. 4) and is representative of all samples presented in Fig. 4.

When assembled in total lipid extracts from cells where PE was replaced by either PC, GlcDAG or GlcGlcDAG, LacY exhibited the same “wild-type” topology as when assembled in PE-containing liposomes; domain C6 was located outside while domain P7 was on the inside of liposome, irrespective of the source of LacY mimicking in vivo mapping of topology in the recombinant cells expressing these foreign phospholipids in the absence of PE (10,12,25). The results of topological mapping of LacY in E. coli-derived PC are consistent with the observation of an uphill transport activity (Fig. 3A and D), as well as the in vitro (using DOPC (20)) and the in vivo determination of LacY topology (10). We also confirmed that LacY displays the same topology when assembled in proteoliposomes consisting of synthetic PC (DOPC and POPC), as well as synthetic PE (DOPE and POPE), as summarized in Table 1. Thus the lack of net charge of the zwitterionic PE and PC or neutral lipids GlcDAG and GlcGlcDAG, irrespective of their fatty acid composition, is sufficient in vivo and in vitro for proper topological organization of LacY in membranes containing the anionic lipids PG and CL. Therefore, the effect of lipid composition on the topological organization of LacY is solely due to a direct interaction of LacY with its lipid environment independent of any effects on the membrane insertion and assembly machinery.

Effect of Lipid Composition on Folding 4B1 Epitope—The native structure of the periplasmic domain P7 connecting TMs VII and VIII (Fig. 1A) contains a continuous epitope that is recognized by the conformation-specific mAb 4B1. This epitope is recognized in spheroplasts containing LacY made from PE-containing but not PE-lacking strains of E. coli (9); the same is true for Western blotting after SDS-PAGE (9,31,32). The proper folding of this epitope is correlated with uphill transport (9,19,31) of lactose in intact cells. We anticipated that the recognition by mAb 4B1 could be used as an indicator of the proper topological and structural organization of LacY in the vicinity of domain P7. Thus, we investigated the conformation of the 4B1 epitope in proteoliposomes of different lipid compositions as a possible indicator of functional LacY. Since domain P7 is located on luminal side of the proteoliposomes, the experiments were performed after solubilizing of the liposomes with DDM, a detergent that has been shown useful for immunoprecipitation (IP) of LacY without aggregation (30). Binding of mAb 4B1 to LacY assembled in proteoliposomes of different lipid compositions exhibits significant differences when analyzed by IP (Fig. 5). Examples of Coomassie-stained gels
after SDS-PAGE are presented for the various total lipid extracts used in reconstituted proteoliposomes. As a control, pAb 1043 (which detects LacY irrespective of orientation or functionality) was used to assess IP of total LacY regardless of 4B1 epitope folding. By comparing the amount of LacY immunoprecipitated using pAb (lane 1043) to LacY starting material (lane input, with no IP), the overall IP efficiency was determined. The histogram presented in Fig. 5A depicts the ratio 1043/input after densitometric quantification of the bands. The linearity of the band quantification by densitometry was verified in the range of LacY concentrations used in our study (Fig. 5F). The overall efficiency was determined to be constant throughout the various lipid compositions used and above 86% for the 12 lipid compositions tested. All the experiments were performed at least 3 times and the standard deviation determined for the IP recovery was ≤ 4.5%.

The histograms presented in Fig. 5B depict the ratio 4B1/input after densitometric quantification of the bands. This ratio accounts for the ability of mAb 4B1 to pull-down LacY assembled in proteoliposomes of a particular lipid composition and should be a direct measure of the proportion of LacY with a properly folded domain P7. LacY reconstituted in proteoliposomes lacking PE (-PE and PG/CL) was not immunoprecipitated by mAb 4B1, which was similar to purified LacY in the presence of DDM alone (LacY) (<5% recovery). In contrast, LacY reconstituted in PE-containing (+PE) and PC-containing (-PE+PC) total lipid extracts was immunoprecipitated (>90% recovery) by mAb 4B1 with very high efficiency. This efficiency was also observed in LacY samples reconstituted with commercial total lipid extract from E. coli (EcPE). On the other hand, when LacY was assembled in proteoliposomes containing glycolipids (GlcDAG and GlcGlcDAG), an intermediate yield of recovery was observed (~40%). All the experiments were performed at least 3 times and the standard deviation determined for the 4B1 epitope immunoprecipitation (compared to the input) was ≤ 6.5%.

When dissecting the effect of fatty acid composition on 4B1 epitope native conformation, the results obtained showed that heterogeneous fatty acid composition (PO) resulted in a significantly better epitope folding than fully unsaturated (DO) phospholipids. In addition, DOPE supported partial 4B1 folding (~35% recovery) while DOPC immunoprecipitation was barely detectible (<5% recovery) (Fig. 5B). These results are in good agreement with the transport activity observed in vitro (Fig. 3C). LacY refolding efficiency was also studied by Eastern-Western blots (10,32) where phospholipids with at least one saturated fatty were necessary to refold partially denatured LacY as determined by mAb 4B1 recognition. In the Eastern-Western procedure, LacY is partially denatured by SDS solubilization and then subjected to SDS-PAGE followed by transfer to a solid support where different phospholipids are preblotted prior the protein transfer. As the SDS is removed during electrobloctting of proteins, LacY refolds and Western blotting using conformation-specific mAb 4B1 is used to assess the efficiency of native refolding.

**DISCUSSION**

Newly synthesized α-helical membrane proteins must be integrated into the membrane with the correct TM topology and proper folding of extramembrane domains in order to acquire a functional three-dimensional TM bundle assembly. Here, LacY isolated from cells with different phospholipid compositions was assembled into proteoliposomes with controlled lipid composition to determine the chemical and structural properties of lipids necessary for native steady-state TM orientation, folding of a functionally important extramembrane domain and display of uphill transport activity. Consistent with previous reconstitution results (20), the final properties of LacY were completely dependent on the lipid composition of the liposomes into which LacY was reconstituted and not on the properties of LacY in the cells (PE-containing or PE-lacking cells) from which it was isolated. For internal consistency some experiments employing E. coli lipid extracts from PE-containing or PE-lacking cells) from which it was isolated. However, the novel contributions of the present work have several important implications and can be summarized as follows:

Substitution of PE by PC, GlcDAG or
GlcGlcDAG (remainder being PG + CL) either in vivo (10-12), PC in vitro (20) or now the two glycolipids in vitro results in a native topology for LacY in cell membranes or liposomes (Fig. 4). The lack of net charge of PE, PC, GlcDAG or GlcGlcDAG strongly indicates that the charge character of the membrane surface is the most important lipid-dependent topological determinant since all four of these lipids, irrespectively to their head group and fatty acid composition or their differences in physical and chemical properties, dampen the negative charge of the membrane surface. These results fully support our previous conclusions based on in vivo data (3) that direct interaction of LacY with the lipid bilayer provides the thermodynamic driving force for TM orientation and is not due to an indirect effect of lipids on other cellular components (translocon, molecular chaperones, etc.) that might contribute to the orientation process. The shared capacity of lipids with net zero charge to support proper topology in vivo and in vitro may explain why the proportion of neutral and anionic lipids in all biogenic membranes is physiologically important and kept constant.

The effect of lipids on LacY activity is more complex and involves both the hydrophilic head group domain and the hydrophobic fatty acid domain. A correlation was observed in vivo (10-12,16,25) and now in vitro (as summarized in Fig. 6) between the ability of LacY to carry out uphill transport (Fig. 3), adopt a proper TM topology (Fig. 4), the nature of lipid head group/fatty acid composition (Fig. 3) and the conformation of the 4B1 epitope in domain P7 (Fig. 5). The correlation diagram obtained for in vitro experiments depicted in Fig. 6 displays several characteristics for LacY reconstituted in proteoliposomes of different lipid compositions. The types can be differentiated depending on their levels of uphill transport (high, intermediate or none) and 4B1 epitope folding (full, partial or none). The presence of cell derived PE at physiological levels supports both optimal LacY uphill transport and complete 4B1 epitope folding. The absence of a net neutral lipid (when only PG and CL are present) in the proteoliposomes resulted in no uphill transport and lack of 4B1 epitope folding. Interestingly, when assembled in E. coli-derived PC, high uphill transport was observed along with near-complete 4B1 epitope folding consistent with lack of requirement for an ionizable amine. At intermediate levels of 4B1 epitope folding either no or marginal uphill transport (GlcDAG, GlcGlcDAG and DOPE) or intermediate uphill transport (POPC and POPE) was observed. GlcDAG appears to support marginal but still nigericin-sensitive and therefore uphill transport activity in vitro consistent with a similar activity observed in vivo while GlcGlcDAG does not support uphill transport in either case (11,12,25).

The independent influence of both lipid head group and fatty acid composition is now clear. E. coli-made PE and PC were more potent than neutral glycolipids in supporting LacY proper 4B1 epitope structure while anionic PG + CL membranes exhibited misfolded 4B1 epitope, inverted topology and no uphill LacY. DOPC did not support uphill transport, as previously reported (Wang et al., 2002), while DOPE supported only marginal uphill transport consistent with no and marginal yield of proper periplasmic domain P7 folding, respectively. More complex and diverse fatty acid combinations in the phospholipids (POPE and POPC on one hand and E. coli-derived fatty acid composition on the other hand) simultaneously support 4B1 folding and uphill transport. Therefore, previous results suggesting a role for the amine of PE in supporting uphill transport is not the case. Although an ionizable amine is not required for uphill transport, a balance between net neutral lipids and anionic lipids still appears to be required. Proper folding of the P7 domain using the Eastern-Western method did not occur with POPC unless E. coli anionic lipids were also included (10).

The correlation diagram obtained for in vivo experiments depicted in the insert in Fig. 6 represents 3 major groups: full uphill transport and complete 4B1 epitope folding (PE-containing cells and cells in which PC replaced PE), intermediate uphill transport but near complete 4B1 folding (GlcDAG cells) and no uphill transport but low or no 4B1 epitope folding (GlcGlcDAG or PE-lacking cells, respectively). Neighboring cysteines introduced one at the time into periplasmic loop P7 of LacY expressed in GlcDAG-containing cells exhibited altered accessibility and periodicity of labeling by sulfhydryl reagents compared to alkylation
patterns obtained in PE-containing cells (11), suggesting a change in structure in this domain. The results are also in good agreement with in vitro data where marginal uphill transport is observed with GlcDAG and no uphill transport with GlcGlcDAG. In particular, these results indicate that both in vivo and in vitro E coli-derived PC can substitute for PE in establishing the correct structure and function of LacY, while glycolipids can only support partial LacY structural and/or functional features.

The conformation-specific mAb 4B1 recognizes properly oriented and fully functional LacY. The differences in IP yield with different lipids may be due to differences in the proportion of properly folded domain P7. Therefore, this antibody could be used not only to isolate a properly oriented and functional LacY, but also to assess the structural organization of LacY in the vicinity of domain P7. The epitope recognized by mAb 4B1 consists of F247, F250, and G254 and is located on one face of a putative α-helical segment in the periplasmic loop between helices VII and VIII (19). This epitope is strongly recognized in PE-containing and PC-containing cells and to a lesser degree in cells in which PE is replaced by the glycolipids (10-12,16,25). Similar results were seen in liposomes. The ability of such diverse head group structures to support the proper folding of the P7 domain is not surprising since the primary amine of PE-NH₃⁺ and quaternary amine of PC-N(CH₃)₃⁺ interact with aromatic amino acids (tryptophan, tyrosine and phenylalanine) through the partial negative character of the quadrupole moments arising from the π-electron cloud of their aromatic rings (33). The formation of such adducts has been documented between the primary amine of PE and the aromatic ring of phenylalanine in a highly resolved structure of cytochrome c oxidase (34) and in a similar way by the choline head group of PC with the faces of three aromatic residues within the highly resolved structure of human PC transfer protein (35).

Protein-carbohydrate interactions typically also rely on aromatic stacking interactions of amino acid aromatic side chains with the sugar rings (36,37). The database of crystal structures with complexed carbohydrate moieties also reveals interactions between the carbohydrate CH groups and the π-electron cloud of aromatic residues. In this case, the aromatic ring provides a geometrically complementary apolar surface for interactions with carbohydrates and its electron cloud interacts favorably with the aliphatic protons of the saccharide that carry a partial positive charge. The adjacent stacking interaction with both F183 and F259 onto the sugar ring is essentially conserved among glycosyltransferases (38). The role of the carbohydrate/aromatic CH-π interaction with Y100, F259 and F183 seems to fix the pyranose ring of substrates in appropriate positions and to determine their orientation, thus stabilizing interactions between sugar substrates and the proteins.

Thus not only the positively charged amine or choline head group of the lipids but also a carbohydrate moiety can be engaged in cation- or CH-π interactions within a cage formed by the faces of several aromatic residues. This may explain why the glucose residues of GlcDAG and GlcGlcDAG support partial folding of P7 domain in vivo (10) or as shown now in vitro (Fig. 5). The head group of GlcGlcDAG would be expected to bind weakly at this site because of steric clashes with the more bulky disaccharide group. Differences in the ability to support uphill transport may also be related to flexibility of the P7 domain. Immobilization of P7 by mAb 4B1 inactivates uphill transport (17,19). Binding by mAb 4B1 changes the conformation of several adjacent TMs, which results in a lowering of the abnormally high pKa of E325, which is part of the proton wire responsible for coupling proton movement to substrate transport. Therefore, lack of tighter correlation between reactivity with 4B1 epitope and uphill transport activity (especially for the glycolipids) may be due to differences in LacY flexibility or subtle changes in the packing of TMs. Supporting this idea is the observation that cysteines within the same extramembrane loop P7 comprising the epitope for mAb 4B1 also displayed different reactivity and labeling patterns when LacY is assembled in cells making GlcDAG instead of PE (11).

At present the best documented examples for a specific balance between anionic lipids and lipids with no net charge for proper TM orientation and to support full function are among the secondary transporters of E. coli, namely LacY and the permeases for phenylalanine (39), γ-
aminobutyrate (40), sucrose (8) and possibly tryptophan (25). These are all membrane transport proteins with high degrees of conformational flexibility. In addition the following transporters from other sources show a requirement for PE after reconstitution into liposomes in order to display uphill transport, which is not satisfied by PC: the multidrug transporter (LmrP) of Lactococcus lactis (41,42), the leucine permease of Pseudomonas aeruginosa (43), the branched chain amino acid transporter of Streptococcus cremoris (44), and the ABC transporter HorA from Lactococcus lactis (45). Since in all cases the dioleoyl form of PC was used, the lipid dependence for supporting uphill transport needs to be reinvestigated in the above cases using a variety of PC species in vitro and expression in cells containing PC. PC does not substitute for PE for in vivo uphill transport by the sucrose permease of E. coli (H. Vitrac and W. Dowhan, personal communication), which emphasizes the need to carry out in vivo and in vitro experiments to assess lipid function.

Lipids have been found integrated into the three-dimensional structure and bound at specific sites on the surface of membrane proteins. The conformation of the P7 domain may involve such specific surface interactions in determining its folding as was suggested (46). However, proper folding may also be influenced by the property of the annular lipid shell surrounding and in loose association with membrane proteins. POPE was much more effective than DOPE in disrupting the interaction of PG with LacY in proteoliposomes as measured by FRET between LacY-W151 and pyrene-labeled PG (47). Therefore, the preference of LacY for POPE and POPC, rather than DOPE and DOPC that we observed may be explained by the capacity of hetero-fatty acid phospholipids to exclude non-supportive anionic lipids from annular shell surrounding LacY.

In summary, it appears that membrane proteins have been under strong evolutionary pressure to maintain a delicate balance between structural features required for proper function, folding, topogenesis and the membrane lipid environment. Particularly in eukaryotic cells where many membrane proteins encounter large changes in their local lipid environment during intracellular trafficking and laterally within one membrane, proteins have evolved to be immune to such changes. However, our results also strongly suggest that such changes in lipid environment could be utilized to dynamically change membrane protein structure and function post-assembly (29). A correct understanding of lipid-protein structure-function relationships should be based not only on the knowledge of the physical and chemical properties of phospholipid bilayers but also on a comparison of in vivo and in vitro results. As exemplified here, sole dependence on in vivo or in vitro results or reliance on non-native lipids in reconstitution experiments can result in the wrong conclusions about the importance and nature of lipid-protein interactions. Although water-exposed and lipid-exposed domains of membrane proteins have co-evolved to maintain gross correct structure and function to tolerate drastic changes in lipid composition (48), subtle changes in short- and long-range lipid-protein interactions can greatly affect both structure and function.

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FOOTNOTES

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1The abbreviations used are: LacY, lactose permease; TM, transmembrane domain; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; GlcDAG, monoglucosyl diacylglycerol; GlcGlcDAG, diglucosyl diacylglycerol; DOPC, dioleoyl PC; DOPE, dioleoyl PE; POPE, palmitoyl, oleoyl PE; POPC, palmitoyl, oleoyl PC; DDM, β-D-dodecylmalto side; OG, N-octyl-β-D-glucopyranoside; pAb, polyclonal antibody; mAb, monoclonal antibody; DTT, dithiothreitol; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; MPB, 3-(N-maleimidylpropionyl) biocytin; DiSC3(5), 3,3′-dipropylthiadicarbocyanine iodide; PAGE, polyacrylamide gel electrophoresis; βME, β-mercaptoethanol; ΔΨ, membrane potential; IP, immunoprecipitation.

FIGURE LEGENDS

Figure 1: Topological organization and purification of LacY. (A) Topological organization of LacY in *vivo* as a function of membrane lipid composition (4). The topology of LacY initially assembled in *E. coli* with wild-type phospholipid composition is shown (top). Rectangles define the TMs, oriented with the cytoplasm above the figure. TMs (Roman numerals), extramembrane domains, N terminus (NT), and C terminus (CT) are indicated. A star and the single-letter amino acid code of the replaced amino acid followed by the residue number indicate the position of single cysteine replacements in a cysteine-less derivative of LacY used in this study. The approximate localization of the 4B1 epitope in the periplasmic loop P7 connecting TM VII to TM VIII is shown. The topology of LacY assembled in *E. coli* cells lacking PE is shown (bottom). TMs I–VI are inverted with respect to TMs VIII–XII, which still exhibit native topology. TM VII (gray) is exposed to the periplasm. (B) SDS-PAGE analysis of purified LacY from PE-containing cells (top) and PE-lacking cells (bottom). Proteins were resolved by SDS-PAGE (4-12% gels) and visualized by Coomassie Blue staining (lanes 1-4) or Western blot analysis with mAb.
raised against the His-Tag (lanes 5-8). The results shown are for LacY H205C purified from PE-containing cells (top) or PE-lacking cells (bottom), 1 and 2, membranes; 3 and 4, LacY eluted from a Co(II)-NTA column with 200 mM imidazole; 5 and 6, LacY after dialysis; 7 and 8, LacY after dialysis and concentration. The 22-kDa band visualized on the Western blot corresponds to a non-specific reaction since no such band is visualized by Coomassie Blue staining or with pAb 1043 directed against LacY.

Figure 2: Detection of membrane potential generated in proteoliposomes. In all cases, LacY was C6 (H205C substitution) from PE-containing cells. Proteoliposomes (5 µL) of the indicated lipid composition containing 50 mM KPi pH 7.5 were diluted 200-fold into 50 mM NaPi pH 7.5 containing 5 µM valinomycin at time zero. DiSC3(5) (1 µM) or nigericin (5 µM) was added as indicated by arrows, and fluorescence was recorded as described in EXPERIMENTAL PROCEDURES. The lipid composition is as follows: (A) and (B) proteoliposomes made of total lipid extract from PE-containing cells; (C) proteoliposomes made of total lipid extract from PE-lacking cells (dotted black line) and of total lipid extract from PC-containing cells (solid gray line); (D) proteoliposomes made of total lipid extract from GlcDAG-containing cells (solid gray line) and of total lipid extract from GlcGlcDAG-containing cells (dotted black line).

Figure 3: Measurement of uphill transport by proteoliposomes. Lactose uptake was measured in energized proteoliposomes of various lipid compositions. In all cases, LacY was C6 (H205C substitution) either from PE-containing cells (panels A to C) or from PE-lacking cells (panels D and E). The lipid composition was as follows: (A) and (D) +PE, total lipid extract from PE-containing cells; -PE, total lipid extract from PE-lacking cells; -PE+PC, total lipid extract from PC-containing cells; (B) and (E) GlcDAG, total lipid extract from GlcGlcDAG-containing cells; (C) DOPC, POPC, DOPE, POPE indicate liposomes made of 70% of the indicated synthetic lipid with the remainder being PG + CL from E. coli. In all cases, Nigericin indicates the uptake of lactose occurring in de-energized proteoliposomes. Cartoon: Schematic representation of LacY activity assay. Proteoliposomes containing purified permease are equilibrated in 50 mM KPi containing no lactose. After treatment with valinomycin (generating a positive-outward gradient), aliquots of the suspension are then diluted into iso-osmotic reaction mixture containing NaPi buffer (sodium or potassium) with radiolabeled lactose. Uphill transport of lactose is driven by ΔΨ (interior negative) when [K+]in > [K+]out at the time of dilution. Addition of nigericin dissipates the K+ gradient, therefore dissipating uphill transport. In latter condition only energy-independent downhill transport is measured.

Figure 4: Determination of the orientation of domains C6 and P7 of LacY in proteoliposomes. Proteoliposomes containing total lipid extracts from cells with the indicated lipid composition (above panels), LacY with a single cysteine in the C6 or P7 domain and isolated from either PE-containing (+) or PE-lacking (-) cells were treated with MPB directly (biotinylatin of exterior cysteine), after pre-blocking with AMS (+, blocking of exterior cysteine), or after solubilization with OG (+, biotinylation of all cysteines). Samples were dissolved in 1% SDS and subjected to SDS-PAGE. Biotinylation with MPB was detected using avidin-HRP (above the continuous line) and sample content in LacY was detected using pAb raised against LacY (below the continuous line). Deduced orientation of C6 and P7 is summarized at bottom of figure.

Figure 5: Effect of lipids on the folding of 4B1 epitope of LacY reconstituted in proteoliposomes. (A) LacY with a single cysteine in the C6 domain prepared from PE-lacking (LacY(-)) or from PE-containing (LacY(+) cells) was reconstituted into proteoliposomes composed of phospholipids extracted from cells containing the indicated phospholipid composition as noted in Fig. 3 and 4. After reconstitution, proteoliposomes were solubilized by 2% DDM, and overnight IP was performed using either the conformation-sensitive mAb 4B1 or the pAb 1043, both previously crosslinked to Protein A/G agarose beads. Input indicates the total amount of LacY in proteoliposomes before IP. After SDS-PAGE analysis,
visualization was performed by Coomassie Blue staining. Estimation of band intensities was performed using ImageJ software. Histogram presentation of band intensity ratios depicts protein recovery after IP for the different lipid compositions: total IP recovery with pAb 1043 (B, ratio 1043/input) versus total recovery with mAb 4B1 (C, ratio 4B1/input). Black bars represent results obtained for LacY purified from PE-lacking cells, and gray bars are for LacY purified from PE-containing cells. Composition of liposomes is indicated by the total extract from cells or synthetic lipids and E. coli-derived PE (EcPE) with 30% PG + CL. LacY indicates IP of purified protein in DDM without added phospholipid. (D) LacY with a single cysteine in the C6 domain was overexpressed in cells with the indicated lipid compositions. Total membranes were isolated and dissolved in DDM followed by SDS-PAGE of immunoprecipitates by pAb 1043 (total LacY) or mAb 4B1 (properly oriented LacY) from samples with equal amounts of total protein as described in EXPERIMENTAL PROCEDURES. (E) Quantification of results from D. Black bars represent results obtained for IP efficiency with mAb 4B1, and gray bars indicate efficiency for IP performed with pAb 1043. (F) Calibration of the zymographic analysis of 4B1 IP assay. Top panel: increasing amounts (from 1 to 20 µg) of purified LacY were loaded on a 4-12% SDS-PAGE gel as described and underwent a zymographic analysis. Lower panel: the two bands visualized on the gel are taken into account and were quantified by densitometry and plotted against the loaded amount. The calibration curve shows the linear relationship between densitometric signal and protein content in the range of protein concentration used for the assay.

Figure 6: Correlation between LacY transport activity and 4B1 epitope folding for different lipid compositions as observed in vitro and in vivo (insert). Lactose and TMG uptake values are taken from transport assay kinetics time points at 180 s and 5 min for in vitro and in vivo (9-12,25) experiments, respectively. The dotted line marks the limit between downhill (lower values) and uphill transport (higher values). 4B1 folding (in %) values derive from IP experiments (in vitro results) or immunoreactivity to mAb 4B1 after SDS-PAGE performed on total membranes samples (in vivo results). Closed diamond: +PE cells total lipid extracts; dark grey diamond: commercial E. coli total lipid extract; light gray diamond: POPE; open diamond, DOPE; closed circle; -PE total lipid extracts; open circle; PG/CL; closed triangle: -PE+PC total lipid extracts; dark gray triangle: POPC; open triangle: DOPC; closed square; GlcDAG total lipid extracts; open square: GlcGlcDAG total lipid extracts. No error bars indicate error within the symbol.
|                     | Uphill transport | C6 Topology | P7 Topology |
|---------------------|------------------|-------------|-------------|
| +PE*                | +++              | OK          | OK          |
| PG/CL (-PE)*        | -                | Inverted    | OK          |
| Ec total lipids#    | ++               | OK          | OK          |
| Ec PE# + PG/CL#     | ++               | OK          | OK          |
| Ec PE# + PG/CL*     | ++               | OK          | OK          |
| POPE# + PG/CL#      | +                | OK          | OK          |
| POPE# + PG/CL*      | +                | nd          | nd          |
| DOPE# + PG/CL#      | ±                | OK          | OK          |
| DOPE# + PG/CL*      | ±                | nd          | nd          |
| -PE+PC*             | ++               | OK          | OK          |
| POPC# + PG/CL#      | ±                | OK          | OK          |
| POPC# + PG/CL*      | ±                | nd          | nd          |
| DOPC# + PG/CL#      | -                | OK          | OK          |
| DOPC# + PG/CL*      | -                | OK          | OK          |
| -PE+GlcDAG*         | ±                | OK          | OK          |
| -PE+GlcGlcDAG*      | -                | OK          | OK          |

* - Total lipids extract from the indicated cells
# - Commercial lipids from Avanti Polar Lipids
nd - Not determined

Ec - Escherichia coli
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