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Molecular Convergence of Bacterial and Eukaryotic Surface Order*

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Background: Living cells maintain a fluid membrane at their surface.
Results: Bacteria and eukaryotes display comparable surface order. Transmembrane proteins order cell membranes in the absence of sterol (Bacteria) and disorder in its presence (Eukarya).
Conclusion: Bidirectional ordering may provide a means to achieve similar barrier properties despite compositional differences.
Significance: Nature may use different protein/lipid combinations to standardize cell surface order.

The conservation of fluidity is a theme common to all cell membranes. In this study, an analysis of lipid packing was conducted via C-laurdan spectroscopy of cell surface membranes prepared from representative species of *Bacteria* and *Eukarya*. We found that despite their radical differences in composition (namely the presence and absence of membrane-rigidifying sterol) the membrane order of all taxa converges on a remarkably similar level. To understand how this similarity is constructed, we reconstituted membranes with either bacterial or eukaryotic components. We found that transmembrane segments of proteins have an important role in buffering lipid-mediated packing. This buffering ensures that sterol-free and sterol-containing membranes exhibit similar barrier properties.

Cell membranes are among the few structures in biology that are not shaped by the intrinsic attraction of their molecular building blocks. Instead, the bilayer composite arises through the hydrophobic effect, wherein lipids and hydrophobic proteins are excluded from the dense hydrogen-bonding network of the surrounding water (1). This means of assembly engenders the membrane with crucial fluidity, allowing for shape flexibility as well as functional dynamics. However, as a hydrophobic layer alone does not equate to the capacity to encapsulate life, the importance of understanding the additional cell membrane-building specificities becomes apparent (2). Here its function as a selectively permeable barrier requires mechanical and chemical robustness, *i.e.* rigidity to resist rupture and leakage. Such tightening can be achieved by increasing the molecular packing in the hydrophobic core of the membrane (3, 4). Regulation of lipid composition is one means to influence packing and thereby balance rigidity and fluidity (5, 6). However, despite the increasing understanding of the physicochemical properties of model membranes (7–9), structural studies of cell membranes have begun to identify clear discrepancies between the model and the cell (10, 11). Here integral membrane proteins account for one-third of the proteome (12), meaning that, unlike model systems, cell membranes, both eukaryotic and bacterial, are most appropriately understood as lipid-protein composites in which membrane protein occupies a substantial surface area (13, 14). This large protein content has been proposed to influence many physiochemical properties of the membrane such as bilayer thickness (10), translational diffusivity (15), and membrane heterogeneity (11, 16), leaving us with the question of how to properly define lipid-protein architecture at the cell surface.

To gain more insight into the structure of cell membranes, we focused on membrane order, a parameter encompassing conformational packing of bilayer constituents (17). We began by measuring this parameter in surface membranes purified from a number of species representing members of the eukaryotic and bacterial domains of life. Surface membrane was defined as the cell membrane directly encapsulating the cytosol. We found that, despite their lack of membrane-rigidifying cholesterol, bacterial membranes exhibited a strikingly similar level of order as their eukaryotic counterparts. Membrane protein was identified as the basis for this convergence. Our data suggest that lipids and proteins act synergistically in the absence of sterol and

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antagonistically in its presence. This translates to a bifunctional capacity by which membrane proteins may tune robustness and strength at the cell surface.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise stated, all reagents were from Sigma. POPC,[2] POPG, POPE, SM (d18:1/C18:0), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). DDM was obtained from Glycon Biochemicals (Luckenwalds, Germany). For membrane reconstitution experiments, a synthetic transmembrane (TM) peptide (sequence = KKW-WLLLLLLLALLLLLLWWKK; a poly-leucine-hydrophobic peptide with tryptophans at the water-bilayer boundary and flanking lysines that readily form stable TM helices when reconstituted from organic solvent into bilayers (18–20)) was ordered from Genscript (Piscataway, NJ). The peptide was obtained at 98% purity, and stocks were prepared in ethanol. C-laurdan was a gift from Prof. B. R. Cho (Seoul, Korea). All stock concentrations of dyes were measured by spectroscopy, and all lipid stocks were measured by phosphate assay (Invitrogen).

Surface Membrane Preparation—Surface membranes were defined as the limiting cell barrier responsible for encapsulation of the cytosol. This organelle was prepared from both eukaryotic and bacterial cells according to published procedures (supplemental Methods and supplemental Figs. S1 and S2). For bacteria, surface membranes were obtained from Gram-negative (*Escherichia coli* inner membrane), Gram-positive (*Acetobacterium woodii* and *Bacillus subtilis*), and cyanobacteria (*Synechococcus* sp.) species. The eukaryotic counterparts included plasma membranes prepared from yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyes pombe*), plants (*Lactuca sativa*), and mammalian sources (red blood cells (*Homo sapiens*), Rat basophil leukaemia (RBL) cells (*Rattus norvegicus*)). Eukaryotic internal membrane preparations served as non-surface membrane controls: endoplasmic reticulum, mitochondrial outer membrane, and mitochondrial inner membrane (supplemental Figs. S3 and S4).

Liposome/Proteoliposome Composition and Preparation—To scale the order of cell membranes, samples were compared with the extrema of liquid ordering in model membranes (11): liposomes of pure liquid ordered (Lo) phase (SM/cholesterol, molar ratio 1:1) and liposomes of pure liquid disordered (Ld) phase (POPC). To reconstitute protein/lipid specificity of bacteria, vesicles were assembled with bacterial inner membrane lipids (21) with or without TM peptide: POPG; POPG/POPE (molar ratio 1:1); POPG + 3 mol% TM peptide; POPG/POPE (molar ratio 1:1) + 3 mol% TM peptide. Eukaryotic lipid/protein specificity was reconstituted with plasma membrane lipids (22) with or without TM peptide: POPC; POPC/cholesterol (molar ratio 2:1); POPC/cholesterol/SM (molar ratio 1:1:1); POPC/cholesterol (molar ratio 2:1) + 3 mol% TM peptide; POPC/cholesterol/SM (molar ratio 1:1:1) + 3 mol% TM peptide. To assess the contribution of lipid *versus* protein in the cell surface membranes themselves, *E. coli* inner membrane and RBC plasma membranes were extracted for total lipids according to the two-step extraction procedure recently established for quantitative lipidomics (22–24) and formed into protein-free liposomes as described below.

For each composition, large unilamellar vesicles were prepared according to Kalvodova *et al.* (25), and proteoliposomes were formed by organic solvent reconstitution as described previously (11). Lipid or lipid/peptide mixtures were adjusted to the appropriate composition, evaporated under nitrogen, and then left under vacuum for 1 h. The dry film was then rehydrated in large unilamellar vesicles buffer (50 mM HEPES/150 mM NaCl, pH 7.4) and shaken for 40 min, all the time being heated above the T_m of the lipid mixture. The resulting homogeneous suspension was subjected to ten freeze-thaw cycles and then extruded through 100 nm pore diameter polycarbonate membrane using the Avanti mini-extruder. For proteoliposomes, TM peptide insertion was confirmed using a proteinase protection assay. Here, 10 μl of a 100 μg/ml large unilamellar vesicle suspension was incubated with proteinase K at 100 μg/ml in the presence or absence 0.5% SDS (w/v) and 0.5% Triton X-100 (w/v) for 3 h at 37 °C. Then PMSF was added to 100 mM. One volume of ethanol was added, and the sample was immediately heated for 2 min at 98 °C. The whole sample was applied to a silica TLC plate and run in the system *n*-butanol/acetic acid/water (3.5:1:2, v/v). Plates were dried, briefly stained with Coomassie Blue, and washed with water.

C-laurdan Spectroscopy—Cell membrane, liposome, and proteoliposome amounts were standardized to scattering fluorescence emission at 425 nm (λ_{ex} 385 nm) (26). This emission intensity relates directly to membrane amount as judged by phosphate assay (supplemental Fig. S5). Following standardization to 30,000 intensity units, membranes were stained with 100 μM C-laurdan and incubated for 15 min at room temperature to equilibrate (11). C-laurdan was then excited at 385 nm. All spectra were recorded twice, averaged, and background-subtracted. This was repeated three times per sample. The general polarization (GP) value was calculated from the following emission bands: (Ch1) 400–460 nm and (Ch2) 470–530 nm according to Parasassi *et al.* (27):

$$GP = \frac{I_{Ch1} - I_{Ch2}}{I_{Ch1} + I_{Ch2}} \quad \text{(Eq. 1)}$$

All spectra were recorded with 1 nm resolution on a fluorescence spectrometer (Fluoromax-3, Horriba, Kyoto, Japan) with a Thermo-Haake thermostat (Karlsruhe, Germany) at 23 °C.

Membrane Solubilization Analysis—Because scattering fluorescence emission intensity at 425 nm (λ_{ex} 385 nm) reports membrane amount (supplemental Fig. S5), it also decreases as a function of membrane solubilization by detergent. Resistance to detergent solubilization is known to correlate membrane order and robustness (28) and was in this way measured for bacterial and eukaryotic surface membranes (with or without

[2] The abbreviations used are: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; SM, sphingomyelin; DDM, dodecyl maltoside; laurdan, 6-dodecanoyl-2-dimethylaminonapthalene; C-laurdan, 6-dodecanoyl-2-methylcarboxymethylaminonapthalene; GP, generalized polarization; Lo, model membrane liquid-ordered phase; Ld, model membrane liquid disordered phase; TM, transmembrane.

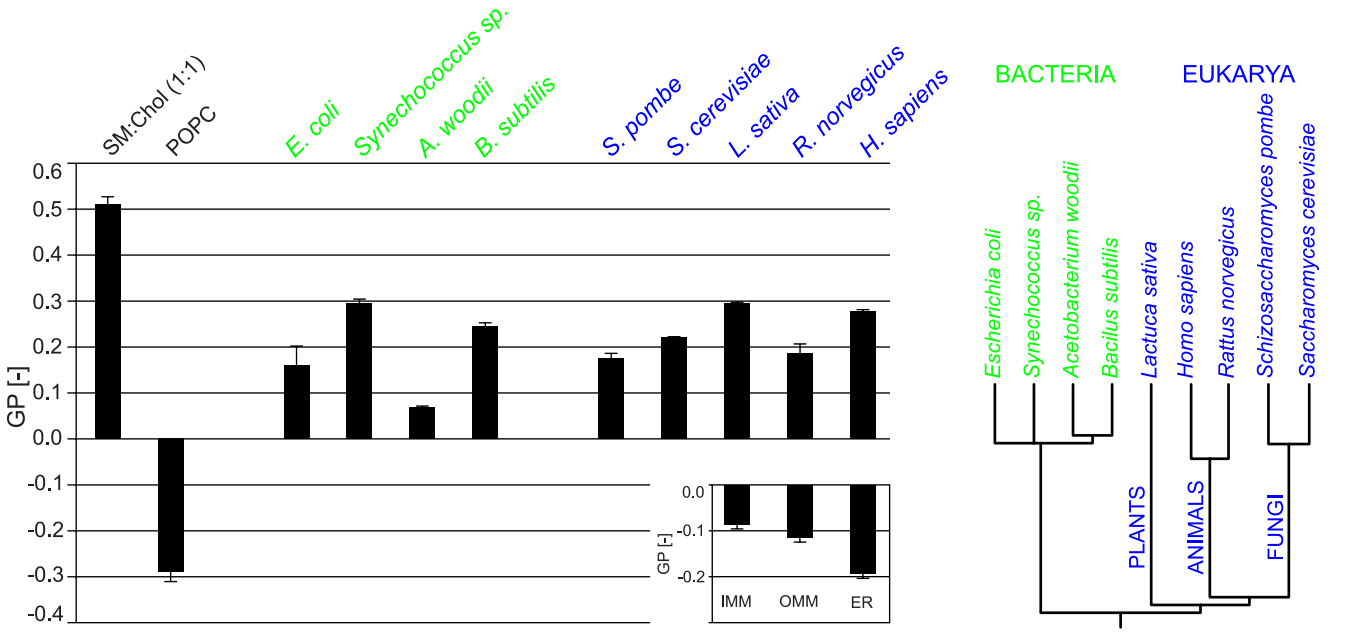

FIGURE 1. **Evolutionary convergence of cell surface order.** The limiting membranes of bacteria and sterol-containing eukaryotes were prepared and evaluated by C-laurdan spectroscopy. This index of lipid packing is presented as a GP value (mean ± S.D., $n = 3$), ranging from +1 as most ordered and −1 as least ordered. Samples are scaled against liposome standards for Lo phase (*SM/Chol, 1:1*), Ld phase (*POPC*), and internal eukaryotic membranes (*inset*: inner mitochondrial membrane (*IMM*); outer mitochondrial membrane (*OMM*); and endoplasmic reticulum (*ER*)). The order of the cell surface converges to a positive GP range, independent of taxonomic distinction and evolution of membrane-rigidifying cholesterol.

protein) along with their counterpart-reconstituted systems. *E. coli* inner membrane and RBC plasma membrane preparations were extruded to 100 nm vesicles and then adjusted in concentration to match the scattering fluorescence given by the 100 nm liposomes made from their extracted lipids (for bacterial membrane, starting emission intensity = 25,000 units; for erythrocyte membrane, starting emission intensity = 40,000 units). For their reconstituted counterpart systems (bacterial = POPG ± TM peptide; eukaryotic = POPC/cholesterol/SM ± TM peptide) membranes were standardized to 80,000 intensity units. Increasing concentrations of DDM were then added. For RBC membranes (with or without protein), starting DDM concentration = 0.008% (w/v) and was sequentially increased by 0.008%; for *E. coli* membranes (with or without protein), starting DDM concentration = 0.004% (w/v) and was sequentially increased by 0.004%; for model membranes (liposomes *versus* proteoliposomes), starting DDM concentration = 0.016% (w/v) and was sequentially increased by 0.016%. Detergent titrations were repeated three times for each sample.

RESULTS AND DISCUSSION

Order of Eukaryotic and Bacterial Plasma Membranes—The fluorescence spectroscopy of membranes stained with the lipid dye laurdan, and in particular with its more water-soluble analog C-laurdan (29), has emerged as a robust method to measure order, both for model membrane systems and cell membrane preparations (11, 30, 31). Reported as a GP value, this index of lipid packing is of arbitrary units and theoretically ranges from +1 as most ordered and −1 as least ordered (27).

We used this technique to measure lipid packing in plasma membranes prepared from a number of eukaryotic and bacterial sources. Live cell staining was not employed due to photoselectivity effects (11). Membrane content was measured by the scattering of fluorescence light at 425 nm (λ_{ex} 385 nm) (Ref. 26 and supplemental Fig. S5) and standardized to the amount of C-laurdan added (11). The resultant GP values were scaled against the known extrema of order in wholly liquid membranes (11): pure liquid-ordered/Lo phase (SM/cholesterol, 1:1, v/v; GP = 0.5 ± 0.017) and pure liquid disordered/Ld phase (POPC; GP = −0.29 ± 0.020) (supplemental Fig. S6). We find that, irrespective of whether the surface membranes contained sterol, all converged on similar values with a positive GP value (Fig. 1). This level of ordering was notably greater than the pure POPC bilayer and eukaryotic internal membranes, consistent with densely packed plasma membranes that confer robustness at all cell boundaries. At the other extreme, membrane order was well below that of the SM/cholesterol bilayer. This indicates that, although high order seems to be an important surface feature, it is kept below the maximum order achievable for fluid lipid-only membranes, perhaps indicative of the fact that it must not exceed levels that may compromise functional membrane fluidity. This is consistent with the fact that Lo membrane reduces inclusion of most transmembrane proteins (32). Taken together, it appears that eukaryotic and bacterial surface membrane orders have converged on a similar level. As the conservation of fluidity is considered a universal attribute of cell membrane functionality (5, 6, 33–36) evolutionary convergence of membrane order is functionally predictable. Structurally, however, membrane-rigidifying cholesterol is unique to eukaryotes, and work from model systems predicts that bilayers with or without sterol are incapable of producing similar membrane order (3, 37).

Molecular Origins of Membrane Order—To address this issue, we investigated in more detail the structure of two bacterial and eukaryotic surface membrane preparations for which

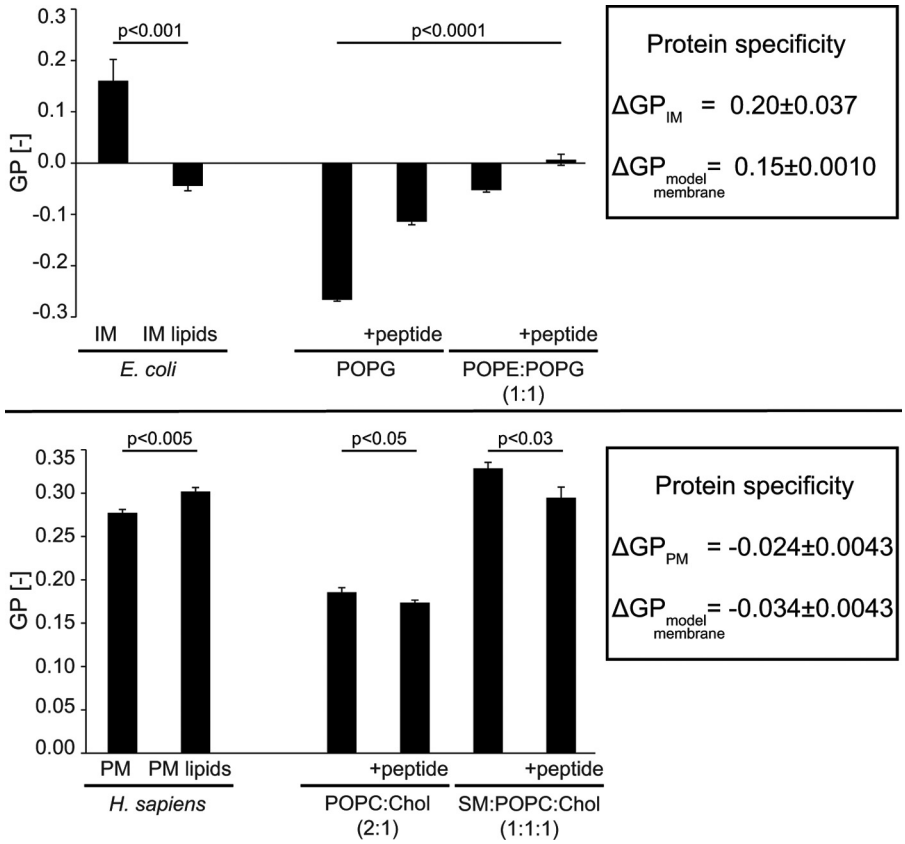

FIGURE 2. **Organization of order in *E. coli* inner membrane (*IM*) (*upper panel*) and human RBC plasma membrane (*lower panel*).** For both cases, order of the intact surface membrane was compared with membranes formed from their extracted lipids (two sided *t*-tests). The corresponding bidirectional effect of a model peptide was then reconstituted into membranes formed from lipids of inner membrane (PG/PE) (ANOVA: $p < 0.0001$; with Tukey's test: POPG *versus* POPG/peptide, $p < 0.01$; POPG *versus* POPG/POPE, $p < 0.01$; POPG *versus* POPG/POPE/peptide, $p < 0.01$; POPG/peptide *versus* POPG/POPE, $p < 0.05$; POPG/peptide *versus* POPG/POPG/peptide, $p < 0.05$; POPG/POPE *versus* POPG/POPE/peptide, $p < 0.05$) and plasma membrane (SM/PC/cholesterol) (two sided *t*-tests). This contribution of protein to membrane order is summarized as ΔGP_{PM} or ΔGP_{IM} = GP intact cell membrane minus GP cell membrane lipids only and $\Delta GP_{model\ membrane}$ = GP proteoliposome (TM peptide + POPG or SM/POPC/Chol) minus GP liposome (POPG or SM/POPC/Chol). GP values represent mean ± S.D. ($n = 3$).

there is the most structural and compositional information: the inner membrane of *E. coli* and human RBC ghosts. We began by comparing membrane order in the intact preparation to membranes made from their extracted lipids only. In the bacterial condition, lipid composition alone failed to confer positive GP (Fig. 2), suggesting that proteins contribute significantly to structural robustness. Indeed, inflexible TM protein segments are known to increase membrane order by limiting conformational movement of lipids (38, 39). Moreover, it has been suggested that the large oligomeric protein complexes of the bacterial inner membrane provide an additional source of membrane structure and rigidity (13). However, in the eukaryotic condition, the order of the lipid membranes without protein exceeded that of the intact plasma membrane preparation (Fig. 2), indicating that in this case the protein reduced the membrane order potentiated by the eukaryotic lipids. RBC membranes are likely comparable to bacteria in terms of their high protein density (13, 40), suggesting that the rigidifying property of membrane proteins is context-specific. This destabilizing effect, although small in the RBC membranes, was confirmed to a much larger extent in ergosterol-containing yeast plasma membranes (supplemental Fig. S8) suggesting that it may be a general feature of sterol membranes. Interestingly, experiments employing electron spin resonance and measures of phase transition temperatures have also revealed disruption of eukaryotic lipid ordering by plasma membrane protein (41, 42).

Reconstitution of Ordered Membranes—To investigate the molecular basis for this bifunctionality in membrane ordering, we used a synthetic hydrophobic peptide as a generic TM protein substitute that forms a TM helix when reconstituted into bilayers (43, 44). It was reconstituted to physiological concentrations (3 mol% (Refs. 39 and 40)) into liposomes made from the lipids of either bacterial inner membrane (IM) (phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (21)) or eukaryotic plasma membrane (POPC/SM/cholesterol) (22). In both cases verification of peptide integration was obtained by a proteinase K protection assay (supplemental Fig. S7). We found that in the sterol-free case, the peptide increased membrane order (Fig. 2), consistent with model membrane studies (16, 45, 46). Here, the peptide integration accounts for almost the same order difference we observe for the inner membrane preparation with removed protein ($\Delta GP_{IM} = 0.20 \pm 0.04$ *versus* $\Delta GP_{model\ membrane} = 0.15 \pm 0.01$). This indicates that the addition of a generic TM protein segment to a simple PG bilayer can contribute an increase in lipid packing as seen in the isolated bacterial membrane. Interestingly, the combination of PG and PE also increases membrane order compared with PG alone

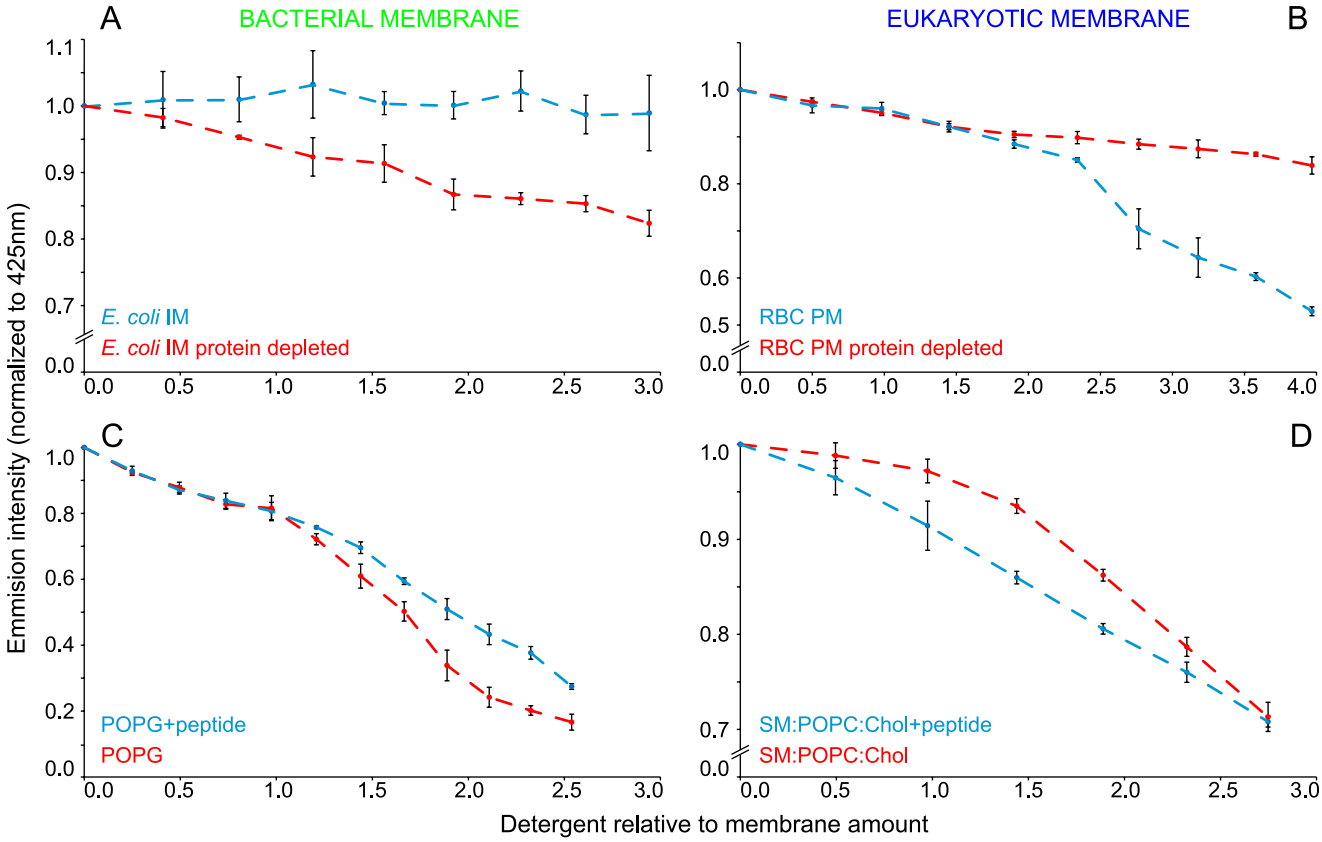

FIGURE 3. **Membrane protein bifunctionality revealed by differential resistance to detergent.** To determine the nature by which membrane protein influences mechanical robustness at the cell surface, susceptibility to solubilization by DDM was measured for membranes extruded to 100 nm: *E. coli* inner membrane with or without protein (*A*) and RBC plasma membrane with or without protein (*B*). These cases were also reconstituted by incorporating a synthetic TM peptide into 100 nm membranes formed from the sterol-free lipids of bacterial inner membrane (*C*) or sterol-containing eukaryotic plasma membrane (*D*). Data are presented as unsolubilized membrane amount (*ordinate*; scatter intensity normalized to emission at 425 nm (λ_{ex} 385 nm)) *versus* relative detergent concentration (*abscissa*; (%DDM/initial membrane scatter intensity) * 100). The values are mean ± S.D., $n = 3$.

(Fig. 2). This is suggestive of a headgroup-limited spacing, where the small ethanolamine slips underneath the large glycerol headgroup, perhaps akin to the eukaryotic "umbrella effect" in which cholesterol tightly packs under membrane lipids with large headgroups (47). As such, lipid and protein specificities appear additive, suggesting that the conserved regime of surface ordering seen in sterol-lacking bacteria may be achieved via the cooperation between lipid and protein-derived ordering. In contrast, incorporation of the same peptide to cholesterol-containing lipid systems resulted in a decrease in sterol-derived membrane order (Fig. 2). Membrane ordering both by sterol and by sterol-sphingolipid combination has been well documented (7–9, 11); however, the disordering effect of peptide incorporation into such systems is beginning to emerge as a new theme. This work suggests that disorder may arise from disruption of lipid packing either by hydrophobic mismatch between protein transmembrane segments and the bilayer or by protruding side chains on the helix surface (16, 17, 48, 49). Reconstitution of the model peptide into cholesterol-containing model membranes also generated a similar protein specificity to that observed for the native red blood cell system, albeit now eliciting a reduction in GP ($\Delta GP_{PM} = -0.024 \pm 0.0042$ *versus* $\Delta GP_{model\ membrane} = -0.034 \pm 0.0043$).

Our data indicate that, unlike bacteria, the source of eukaryotic membrane ordering is mainly via lipid interactions involving sterol and sphingolipid; this view is consistent with both model and cell membrane studies (9, 50, 51). In this context, lipid-derived order is prevented from exceeding the functionally relevant surface order membrane regime by transmembrane protein-dependent disordering. This suggests that membrane proteins can "buffer" bacterial and eukaryotic lipid composition to the surface membrane order regime.

Membrane Robustness—Having identified lipid context as the basis for ordering and disordering by protein, we tested whether this bifunctionally could be confirmed by resistance to membrane detergent. Detergent resistance is a measure that relates directly to lipid packing (28) and would be predicted to correlate with the C-laurdan ordering results of the *E. coli* inner membrane and RBC plasma membrane. To this end we measured DDM-mediated solubilization of uniform, 100 nm vesicles (extruded from native membranes and membranes reconstituted from their extracted lipids) as a decrease in the scattering of fluorescence light at 425 nm (λ_{ex} 385 nm) (26). The scattering signal correlated well with vesicle concentration and was used to adjust membrane amounts to the same level (supplemental Fig. S5). In the applied detergent concentration ranges, we observed resistance to solubilization for native *E. coli* membranes but not for protein-free *E. coli* membranes (Fig. 3). In contrast, the native RBC membranes exhibited a faster onset of solubilization than did their protein-free coun-

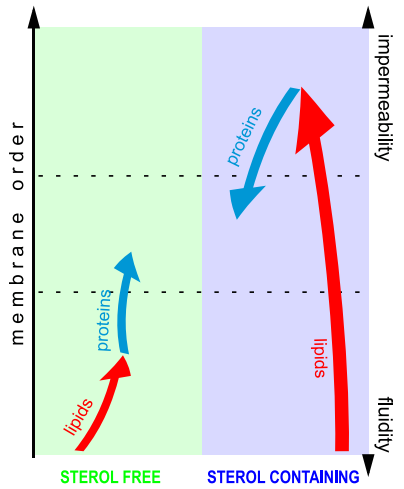

FIGURE 4. **Transmembrane protein as a buffer for membrane order and robustness at the cell surface.** The functional range of order seen at the cell surface may represent a balance between enhancing packing in the hydrocarbon core to resist rupture and leakage and maintaining the level of fluidity needed to support membrane bioactivity. Convergence to this order regime could be achieved in two ways. In the absence of sterol, lipids and proteins act cooperatively to drive hydrocarbon chain packing, with transmembrane protein providing a rigid surface upon which acyl chains are ordered. In sterol-containing membranes, the same protein input now disrupts sterol-acyl chain alignment, placing an upper limit on ordering by eukaryotic lipids.

terparts (Fig. 3). This suggested that the packing state of the membrane indeed reflects structural robustness and that the dual nature by which membrane proteins organize order also regulates resistance to solubilization. Moreover, we were able to reconstitute the same protein bifunctionality in our minimal model membrane systems: sterol-free liposomes were more efficiently solubilized in the absence of model TM peptide, whereas sterol-containing POPC/SM/cholesterol liposomes were more efficiently solubilized if the same TM peptide was incorporated (Fig. 3). This bi-directionality in protein effects supports our previous assertion that proteins can act oppositely to tune order, and therefore structural robustness.

Conclusions and Perspectives—In the eukaryotic and bacterial domains of life there exists a similar degree of lipid packing at the cell surface. Evolutionary convergence to this surface order regime likely reflects the preservation of a functional condition: a densely packed hydrocarbon core endowing the membrane with reduced permeability and higher resistance to mechanical forces, all at a level of order that is still compatible with the lateral mobility needed to support membrane bioactivity. Our data suggest that this convergence could involve the action of transmembrane proteins, albeit in two distinct roles (Fig. 4). In the absence of sterol, transmembrane proteins drive lipid packing, very likely acting in a similar way to cholesterol: providing a rigid surface in a sea of highly flexible acyl chains. In sterol-containing membranes, there now appears to be an antagonistic relationship in which transmembrane proteins place an upper limit on eukaryotic lipid order, most likely via the breaking of sterol-acyl chain alignment. Bidirectional ordering by membrane protein is a physical principle that, although it does not account for variations in lipid subclass or protein oligomerization behaviors, it emphasizes cell membranes as a protein-lipid composites, which despite radically distinct compositions (*e.g.* inter-species/cell type variation) can be tuned to yield similar barrier properties.

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