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Steric Pressure between Membrane-Bound Proteins Opposes Lipid Phase Separation

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Supporting Information

ABSTRACT: Cellular membranes are densely crowded with a diverse population of integral and membrane-associated proteins. In this complex environment, lipid rafts, which are phase-separated membrane domains enriched in cholesterol and saturated lipids, are thought to organize the membrane surface. Specifically, rafts may help to concentrate proteins and lipids locally, enabling cellular processes such as assembly of caveolae, budding of enveloped viruses, and sorting of lipids and proteins in the Golgi. However, the ability of rafts to concentrate protein species has not been quantified experimentally. Here we show that when membrane-bound proteins become densely crowded within liquid-ordered membrane regions, steric pressure arising from collisions between proteins can destabilize lipid phase separations, resulting in a homogeneous distribution of proteins and lipids over the membrane surface. Using a reconstituted system of lipid vesicles and recombinant proteins, we demonstrate that protein−protein steric pressure creates an energetic barrier to the stability of phase-separated membrane domains that increases in significance as the molecular weight of the proteins increases. Comparison with a simple analytical model reveals that domains are destabilized when the steric pressure exceeds the approximate enthalpy of membrane mixing. These results suggest that a subtle balance of free energies governs the stability of phase-separated cellular membranes, providing a new perspective on the role of lipid rafts as concentrators of membrane proteins.

From the structure of trafficking vesicles and signaling complexes to the assembly of cytoskeletal protrusions and the budding of enveloped viruses, cellular processes require that biological membranes take on a high degree of local order. Coat proteins during endocytosis,[1] G-protein-coupled receptors during signaling,[2] nucleation promotion factors during cytoskeletal assembly,[3] and matrix proteins during viral assembly[4] each must be concentrated at the right place and time to ensure the formation of functional protein complexes on cellular membrane surfaces. Similarly, the development of biomimetic materials for drug delivery[5] and biodetection[6] requires increased control of biomolecular assemblies.

There is considerable evidence that integral and membrane-associated proteins are distributed into phase-separated assemblies known as lipid rafts.[7] These liquid-ordered structures, which are thought to be enriched in sphingolipids and cholesterol,[8] provide a favorable environment for proteins with structured transmembrane domains,[9,10] proteins with affinity for cholesterol,[11] and lipid-conjugated proteins.[12] Although the existence of lipid-driven phase separation in cellular membranes is debated,[13] rafts are of great interest in both biology[14,15] and materials science,[16] largely because they suggest a physical mechanism for locally concentrating and organizing biomolecules.

The formation and stability of phase-separated membrane regions depends on a balance of free energies. Lipid contributions to the stability of rafts have been well-explored[7] and are known to arise from the immiscibility of saturated lipids and cholesterol with unsaturated lipids at biologically relevant temperatures. Protein contributions to the formation and stability of lipid rafts are considerably less well understood. In particular, the capacity of rafts to concentrate proteins locally, which is thought to be one of their primary roles in cellular membranes,[7] has not been measured experimentally. Here we report that increasing the protein density on the surfaces of liquid-ordered membrane regions creates a lateral steric pressure that competes with phase separation, significantly altering the stability of lipid domains.

To create a simple model of protein interactions within phase-separated lipid membrane domains, we employed a reconstituted system of giant unilamellar vesicles (GUVs) and membrane-bound reconstituted proteins. The GUVs consisted of a ternary lipid mixture containing 45% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 20% 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 35% cholesterol.[17] This mixture separates at room temperature into liquid-ordered (L_o) phases that concentrate DPPC and cholesterol and liquid-disordered (L_d) phases that concentrate DOPC. To attach recombinant proteins to the surfaces of the raftlike L_o phases, DPPC was partially replaced by a synthetic lipid, 9-[2,3-bis(hexadecyloxy)propyl]-3,6,9-trioxanonyl-1-iminodiacetic acid (DPIDA).[18] Since DPIDA has the same palmitoyl tails as DPPC, it concentrates in L_o phases. Its iminodiacetic acid−Cu headgroup has a high affinity for histidine, providing a strong binding site (K_d = 1−10 nM) for recombinant proteins with histidine tags.[19,20] By varying the DPIDA concentration, protein size, and membrane phase-transition temperature (T_m), we explored the impact of the concentration of membrane-bound proteins on the stability of phase-separated L_o membrane domains.

The concentration of DPIDA was gradually increased within the phase-separated regions of GUVs from 0 to 40 mol % of the

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total 20 mol % saturated lipid concentration, defined as the sum of the DPPC and DPIDA concentrations. Prior to the addition of proteins, 60−80% of the GUVs contained phase-separated regions, which appeared dark, excluding the lipid dye Texas Red−DHPE, which is known to partition into L_d phases (Figure 1A).[21] When GUVs were exposed to histidine-tagged green

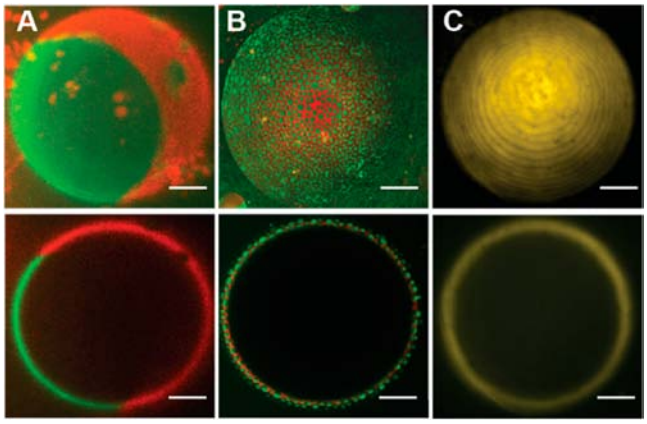

Figure 1. As the protein concentration on the surface of the GUV increases, domain mixing occurs. Shown are spinning-disc confocal images (top) and 3D reconstructions (bottom) of GUVs with Texas Red−DHPE incorporated into the L_d phase and His−GFP (1 μM concentration in solution) bound to the DPIDA lipids in the L_o domain: (A) GUV with demixed domain (15% DPIDA); (B) GUV with partially mixed domains (20% DPIDA); (C) GUV with a mixed membrane (25% DPIDA). Scale bars: (A) 5 μm; (B) 10 μm; (C) 5 μm.

fluorescent protein (His−GFP), L_o domains containing smaller fractions of DPIDA (<20%) were covered by the protein, while those containing larger fractions of DPIDA (>25%) experienced a rapid mixing transition [<4 min; Figure S1 in the Supporting Information (SI)] after which both His−GFP and Texas Red−DHPE were present over the entire GUV surface (Figure 1C). Less than 3% of the GUVs contained domains after proteins were added. Membranes containing ∼20% DPIDA appeared to be in transition from a phase-separated to a mixed morphology. These vesicles contained numerous small, stable domains with diameters of a few micrometers, even after observation periods of greater than 60 min (movie S1 in the SI). Figure 1 and Figures S2 and S3 in the SI depict the progression from large domains (Figure 1A) to small domains (Figure 1B) to a fully mixed membrane (Figure 1C) that occurred with an increase in the percentage of DPIDA in the membrane, where the concentration of membrane-bound proteins increased linearly with binding lipid concentration before reaching saturation (Figure S4). In the absence of proteins, we also observed a decrease in the frequency of phase separation as the concentration of DPIDA increased, though the magnitude of the decrease was considerably smaller (∼10%). We attribute this decrease to the increase in electrostatic repulsion with increasing concentration of negatively charged DPIDA lipids.[22]

Our observation that the domains mixed as the number of protein binding sites increased could be explained by either the interaction of histidine tags with the membrane surface or the interactions of increasingly crowded proteins with each other. To determine which type of interaction caused the domains to mix, we examined a family of His-tagged recombinant proteins with increasing molecular weights: ubiquitin (8 kDa), GFP (26 kDa), transferrin (Tf) (77 kDa),[23] and the ectodomain of Tf receptor dimer (TfR) (180 kDa).[24] If interactions between the histidine tags and the lipids were responsible for mixing of the domains, then we would expect smaller proteins to drive domain mixing more easily since their small size would permit a higher density of histidine−lipid binding events. In contrast, if protein−protein interactions were responsible for the mixing of domains, we would expect that larger proteins would drive domain mixing more efficiently since they could cover the membrane surface and come into contact with one another at a lower density of lipid binding sites (lower DPIDA concentration). We found that as the protein size increased, a lower percentage of DPIDA was required to mix L_o domains upon protein binding. Figure 2A

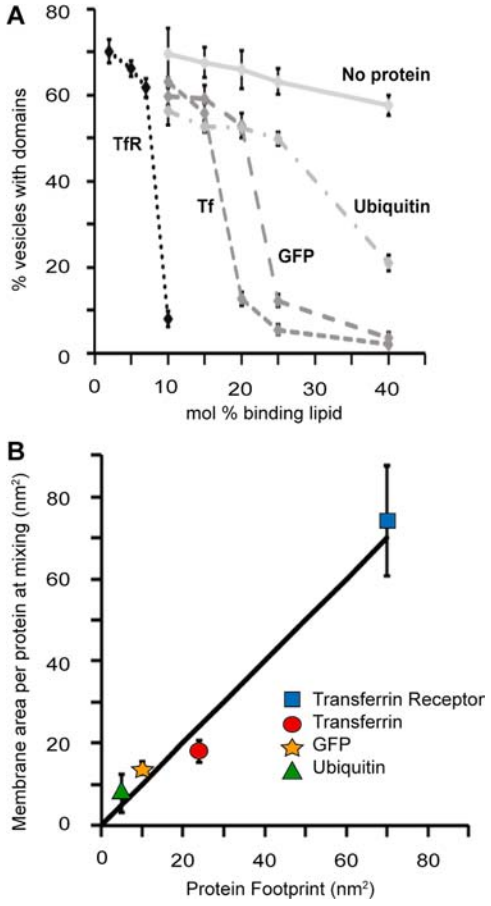

Figure 2. Domains dissolve as proteins concentrate on membrane surfaces. (A) Percentage of vesicles with domains after addition of proteins vs percentage of DPIDA within L_o domains. For each protein at each DPIDA concentration, 100 vesicles were counted. The error bars represent standard deviations of three trials. (B) Membrane area per protein at mixing vs projected area occupied by the protein. The area per protein was estimated from the average of the values before and after the mixing transition, assuming 2.5 lipids per protein binding site (see the SI). Error bars represent the differences between these values.

displays the trend in domain dissolution for the four protein sizes. We found that at measured DPIDA concentrations of ∼40, ∼25, ∼20, and ∼10%, ubiquitin, GFP, Tf, and TfR, respectively, were sufficient to mix the L_o domains (similar to Figure 1C). We also found that at the highest measured DPIDA concentrations before membrane mixing (25, 20, 15, and 7%, respectively), the domains broke up into multiple small domains with diameters of a few micrometers. By averaging the DPIDA concentrations at which the domains were completely mixed with the highest

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DPIDA concentration before the domains mixed, we approximated the transition concentration at which membrane mixing occurred. Figure 2B demonstrates the linear relationship between the projected area occupied by each protein and the coverage of membrane by each type of protein at mixing, which was found by assuming that 2−3 DPIDA lipids (a value of 2.5 was used in the calculations) constitutes a binding site for a His_6 tag (see the SI).[25,26] This analysis illustrates that domains dissolve when proteins occupy a large fraction of the membrane surface. Furthermore, qualitative analysis of fluorescence images of mixed and demixed membranes suggested that the local concentration of membrane-bound proteins decreases during membrane mixing (Figure S5).

Recently, several studies have explored the ability of collisions between membrane-bound proteins to produce a steric pressure that favors expansion of the membrane surface. Our experimental studies with collaborators[19,27] as well as computational studies by others[28,29] have demonstrated that this steric pressure, which arises from the entropic cost of concentrating the proteins, is capable of driving dramatic increases in membrane curvature. However, the impact of crowding on the stability of lipid phases has not been considered to date. If steric pressure is responsible for mixing of domains, it should be possible to prevent mixing by increasing T_m, a measure of domain stability. As shown in Figure S6, when we changed the composition to increase T_m from approximately 27 to 35 °C as described in the literature[17,18] while keeping the percentage of DPIDA constant, domains were no longer mixed.

Here we propose a simple thermodynamic model to determine whether or not steric pressure between proteins can be expected to drive mixing of membrane domains. Lipid contributions lead to an energetic barrier favoring membrane phase separation that is approximately equal to the enthalpy of mixing of the lipids within the L_o and L_d phases (~4.8 mJ/m^2 on the basis of temperature integration of heat capacity measurements through transition points of a phase-separated system found in the literature[30,31]). Additionally, the mismatch between the hydrocarbon chain lengths of saturated and unsaturated lipids results in partial exposure of the longer, saturated hydrophobic tails at the domain edge. This energetic contribution is proportional to the domain perimeter, where the constant of proportionality is the line tension (~1 pN).[29,32] For a domain with a diameter of ~10 μm, as is common in our system, the energy per unit area is ~4×10^{-7} J/m^2. Since this energetic contribution is several orders of magnitude less than the enthalpy of mixing, we assume that the line tension contribution to domain stability can be neglected.

The energetic cost of concentrating proteins per unit area of membrane surface can be estimated as the lateral pressure produced by collisions among freely diffusing proteins confined within a two-dimensional (2D) domain on the membrane surface. The Carnahan−Starling equation of state can be used to estimate the pressure of crowding as a function of the density of molecules[33,34] and has recently been used to estimate the protein−protein steric pressure required to bend membranes.[19] The values of the protein areas (A_p) were 70 nm^2 for TfR (PDB entry 1CX8), 24 nm^2 for Tf (1H76), 10 nm^2 for GFP (1GFL), and 5 nm^2 for ubiquitin (1UBQ). According to the Carnahan−Starling equation, the pressure (p), an energy per unit area, should increase nonlinearly with increasing coverage of proteins on the membrane surface (η) as follows:

$$p = \frac{\eta(A_p)}{A_p}\left\{1 + 2\eta(A_p)\frac{1 - 0.44\eta(A_p)}{[1 - \eta(A_p)]^2}\right\}k_BT$$

in which k_B is Boltzmann's constant and T is the absolute temperature. For a given vesicle composition, there is a fixed density of binding lipids per unit membrane area. Therefore, if it is assumed that a protein occupies each binding site, larger proteins will occupy a larger fraction of the membrane surface area than small ones. Specifically, η is expected to increase linearly with A_p. In Figure 3A, the energy per unit area (2D pressure) produced by each protein is plotted as a function of the fraction of the membrane surface area covered by proteins. The black horizontal line represents the approximate enthalpy of membrane mixing. Therefore, the membrane coverage for which the pressure generated by protein collisions equals the enthalpy of mixing provides a prediction of the minimum membrane coverage required to drive mixing of phase-separated membrane domains. A plot of the measured transition points (Figure 2A) versus these predictions (Figure 3B) reveals an agreement between our measurements and this simple analytical model, which suggests that the steric pressure created by protein−protein crowding on the surface of our membranes is sufficient to

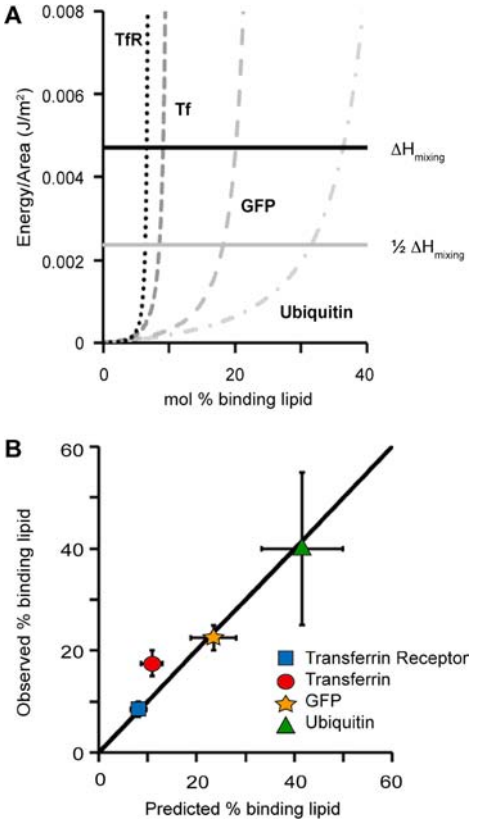

Figure 3. Protein−protein crowding drives domain mixing. (A) Theoretical prediction of steric pressure as a function of binding lipid concentration. The pressure generated by protein−protein crowding is expected to exceed the enthalpy of membrane mixing (horizontal lines). (B) Observed experimental values of the DPIDA concentration at the mixing transitions vs the predicted percentages from (A). The x-axis error was determined from the predicted transition with proteins binding 2−3 lipids. The y-axis error was determined by the difference between the concentrations of binding lipid before and after the mixing transition, where the reported value is the mean of these two values. The black line represents ideal agreement between the model and the data.

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drive the observed mixing of membrane domains. In view of the high protein concentrations present on the surfaces of biological membranes,[35] we expect steric pressure to play an important role in determining the stability of membrane rafts. If our membranes were crowded by proteins on both surfaces, similar to biological membranes, the enthalpic barrier that crowding on each surface would have to overcome would be reduced by approximately half (gray horizontal line in Figure 3A).

We observed that steric interactions between membrane-bound proteins can dissolve domains (Figure 4). This protein–

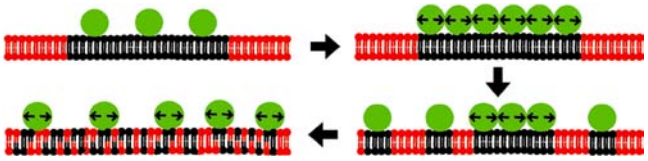

Figure 4. Schematic illustrating the steric pressure created by protein crowding on the surface of a domain, resulting in domain dissolution.

protein steric pressure has not been broadly recognized in discussions of the formation and stability of cellular lipid rafts. Our findings highlight the energetic cost of concentrating proteins on membrane surfaces, which lipid rafts, protein coats, and other cellular structures must overcome in order to organize cellular membranes dynamically. On the basis of a comparison of energetic contributions, we expect the line tension to become an important factor in determining domain stability for domains of nanometer diameter. The imaging results we have presented are limited by diffraction, so we could not determine whether nanoscale domains persisted after mixing of the micrometer-scale domains. Similarly, cellular lipid rafts are thought to exist on the nanometer scale, while micrometer-scale lipid rafts have not been observed in living cells. Therefore, our results point to a possible explanation for the instability of micrometer-scale rafts but leave open the possibility that lipid domains remain stable and able to concentrate proteins on the nanoscale. On the basis of our findings, we speculate that protein oligomers such as caveolin, connexin, and viral matrix proteins that are frequently found in raftlike structures[4,11] may play an important role in stabilizing these structures. In general, our results highlight the delicate balance of energetic contributions that underlies membrane organization. A detailed understanding of this dynamic equilibrium will enable the development of materials that are capable of specifically manipulating the organization of cellular membranes, leading to new, molecular-level strategies for biodetection and drug delivery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods and supporting figures, video, and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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