Topologically Diverse Human Membrane Proteins Partition to Liquid-Disordered Domains in Phase-Separated Lipid Vesicles

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S Supporting Information

ABSTRACT: The integration of membrane proteins into "lipid raft" membrane domains influences many biochemical processes. The intrinsic structural properties of membrane proteins are thought to mediate their partitioning between membrane domains. However, whether membrane topology influences the targeting of proteins to rafts remains unclear. To address this question, we examined the domain preference of three putative raft-associated membrane proteins with widely different topologies: human caveolin-3, C99 (the 99 residue C-terminal domain of the amyloid precursor protein), and peripheral myelin protein 22. We find that each of these proteins are excluded from the ordered domains of giant unilamellar vesicles containing coexisting liquid-ordered and liquid-disordered phases. Thus, the intrinsic structural properties of these three topologically distinct disease-linked proteins are insufficient to confer affinity for synthetic raft-like domains.

Many biochemical processes are believed to be mediated by the partitioning of integral membrane proteins into so-called "lipid raft" nanodomains enriched with saturated lipids, sphingolipids, cholesterol, and assorted proteins.[1] These membrane domains are thought to persist in a state resembling the liquid-ordered (L_o) phase. Preferential interactions between certain lipids and proteins are believed to drive the condensation of lipid rafts.[2] However, emerging evidence suggests that, unlike those in model membranes, L_o domains are small and transient in cellular membranes,[1,3,4] which complicates their direct characterization.[5] Nevertheless, it seems clear that biochemically defined lipid rafts contain a specific subset of proteins,[6] which is indicative of an underlying physical mechanism for membrane protein integration. Experimental evidence has suggested that certain structural and biochemical properties of membrane proteins help modulate their partitioning between coexisting liquid-disordered (L_α) and L_o membrane domains.[7−9] However, the structural basis for this selectivity remains poorly understood.

A wealth of insight has emerged from investigations of synthetic liposomes, the precise composition of which can be controlled and manipulated. Ternary liposomal membranes provide a stringent system for the exploration of membrane protein partitioning between chemically pure, coexisting L_o and L_α domains at equilibrium.[10,11] Several previous investigations

have revealed that membrane proteins are often excluded from synthetic L_o domains.[11] However, these investigations have been restricted to a limited number of integral and peripheral membrane proteins amenable to purification and reconstitution,[12−19] many of which are bacterial, viral, or synthetic peptides. Furthermore, several putative raft-associated proteins have distinctive structural features that have been proposed to facilitate their raft partitioning.[7−9] Therefore, in the current study we characterized the phase partitioning of membrane proteins with unusual topological features in order to determine whether these motifs can function as specific raft targeting mechanisms.

We focused on three human integral membrane proteins with distinct topologies (Supplementary Figure 1), which are known to reside in lipid rafts under physiological conditions: caveolin-3 (Cav3),[20] C99—the 99-residue C-terminal domain of the amyloid precursor protein,[21] and peripheral myelin protein 22 (PMP22).[22] Here, we characterize the partitioning of non-post-translationally modified forms of these proteins in phase-separated giant unilamellar vesicles (GUVs). In addition to the biophysical relevance of these studies, each of these proteins are directly related to human disease (Cav3—muscle disorders,[23] C99—Alzheimer's disease,[24] PMP22—Charcot-Marie-Tooth disease[25]). Therefore, these results may also illuminate disease mechanisms and inform the development of novel therapeutics.

The partitioning of membrane proteins between coexisting membrane domains can be directly assessed in phase-separated GUVs. We first generated GUVs exhibiting robust separation of liquid phases of known composition. The ternary phase diagram of membranes containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), N-palmitoyl-D-erythro-sphingosylphosphorylcholine (PSM), and cholesterol has been characterized in great detail.[26,27] Macroscopic phase separation was consistently observed in GUVs composed of 2:2:1 POPC:PSM:cholesterol. This composition lies at the center of a tie line within the portion of the phase diagram in which L_o and L_α phases coexist.[27] Therefore, equal fractions of L_o and L_α phases should persist at equilibrium in these membranes. Indeed, confocal images of these membranes containing trace amounts of fluorescent lipid marker Rh-DOPE confirm the coexistence of two phases with roughly equal

proportions under these conditions (Figure 1A). Rh-DOPE consistently exhibited a strong preference for one of the two
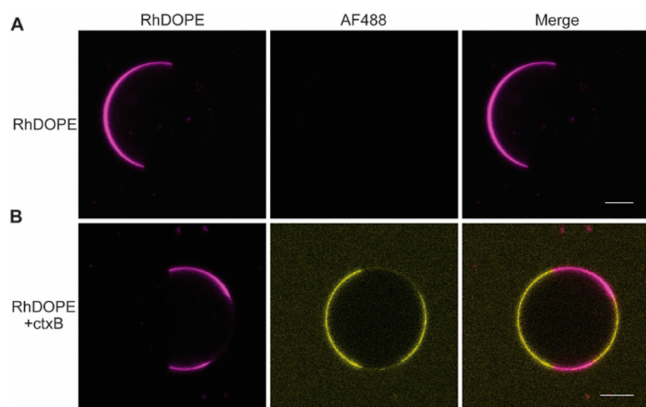

Figure 1. Phase-separated giant unilamellar vesicles. GUVs were formed from liposomes containing a 2:2:1 molar ratio of POPC:PSM:cholesterol. (A) To detect phase separation, 0.1 mol % of the fluorescent lipid tracer rhodamine DOPE (magenta) was incorporated to detect phase separation using confocal fluorescence microscopy. A representative image along with a 10 μm scale bar are shown for reference. (B) To identify the L_o phase, 0.1 mol % of the fluorescent lipid tracer rhodamine DOPE along with 0.1 mol % GM1 ganglioside were incorporated into the liposomes prior to GUV formation. GUVs were then mixed with 1 μg/μL of AF488-labeled cholera enterotoxin subunit B (yellow) in order to mark the L_o phase. A representative image along with a 10 μm scale bar is shown for reference.

phases—likely the L_α phase. To confirm the identity of this phase we incorporated 0.1 mol % ganglioside GM1, which exhibits a strong preference for the L_o phase, into the membrane in the presence of fluorescently labeled GM1-binding protein cholera enterotoxin subunit B (ctxB). Consistent with previous observations,[28] CtxB binds GM1 in the phase opposite of that marked by Rh-DOPE (Figure 1B), confirming that Rh-DOPE specifically marks the L_α phase. Together, these findings illustrate the robust phase separation and probe partitioning in these GUVs.

We first surveyed the partitioning of full-length human Cav3 in its non-lipidated form. Cav3 exhibits an uncommon topology featuring a single kinked helical hairpin that enters and exits the inner membrane leaflet (Supplementary Figure 1). Caveolins are critical components of caveolae,[29] which are small raft-like membrane invaginations. Furthermore, Cav3 contains a CRAC motif[30] and is highly enriched in biochemically extracted lipid raft preparations known as detergent resistant membranes (DRM).[20] To determine whether Cav3 exhibits a thermodynamic preference for L_o domains, we first reconstituted fluorescently labeled Cav3 into proteoliposomes containing 2:2:1 POPC:PSM:cholesterol and the L_α phase marker Rh-DOPE at a molar lipid:protein ratio (LPR) of 400:1. Proteoliposomes were then electroswelled into GUVs and imaged using confocal fluorescence microscopy (see Supplementary Methods). We observed consistent colocalization of Cav3 and Rh-DOPE (3 trials, $n = 38$) (Figure 2A), which indicates the protein exhibits a strong preference for the L_α phase. Indeed, the partition coefficient of Cav3 could not be accurately determined due to the absence of detectable protein signal in the L_o domain of the vast majority of these GUVs. Similar results were obtained even when an alternative
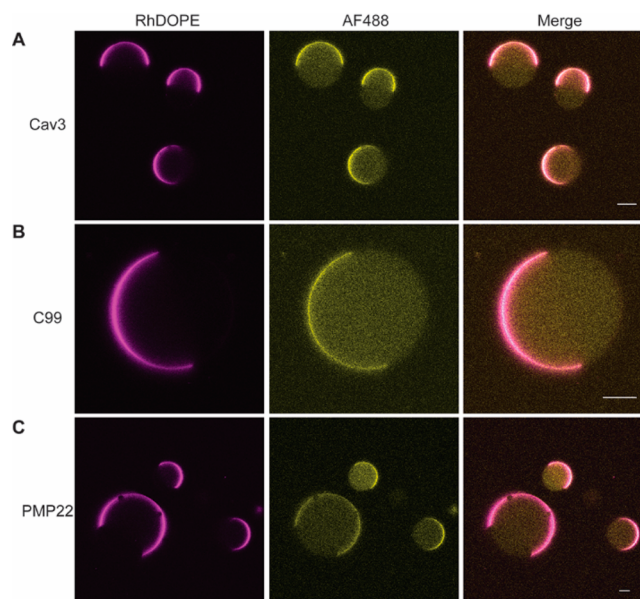

Figure 2. Partitioning of raftophillic integral membrane proteins in giant unilamellar vesicles. GUVs were formed from liposomes containing a 2:2:1 molar ratio of POPC:PSM:cholesterol along with 0.1 mol % of the fluorescent tracer lipid rhodamine-DOPE (magenta, L_α phase) and various membrane proteins at a bulk molar lipid: protein ratio of 400:1. (A) A representative image of GUVs containing AF488-labeled caveolin-3 (yellow) are shown along with a 10 μm scale bar for reference. (B) A representative image of GUVs containing AF488-labeled C99 (yellow) are shown along with a 10 μm scale bar for reference. (C) A representative image of GUVs containing AF488-labeled PMP22 (yellow) are shown along with a 10 μm scale bar for reference.

fluorophore, labeling position, and reconstitution protocol were employed (Supplementary Figure 2). We recognize that the oligomerization state of Cav3 is another variable that may be critical for regulating its phase preference. However, our studies were carried out at a bulk LPR of 400:1, which is almost certainly higher than the physiological concentration and potentially sufficient to promote oligomerization. Taken together, these results demonstrate that non-lipidated Cav3 is excluded from L_o domains.

We next assessed the partitioning of C99, which consists of a single helical transmembrane domain flanked by short N- and C-terminal surface-associated helices (Supplementary Figure 1).[31] C99 is found in DRMs.[21] Additionally, the proteolytic processing of C99 is thought to depend on its association with lipid rafts.[31−35] C99 is also known to bind cholesterol,[31,36] which might promote partitioning into cholesterol-rich membrane domains. To determine whether C99 exhibits a thermodynamic preference for L_o domains, we reconstituted fluorescently labeled C99 into GUVs composed of 2:2:1 POPC:PSM:cholesterol and trace amounts of Rh-DOPE at an LPR of 400:1. Similar to our observations for Cav3, we also found C99 to consistently colocalize with the L_α domain marker (3 trials, $n = 24$) (Figure 2B). Again, the partition coefficient could not be accurately determined due to a lack of detectable C99 in the L_o phase in all cases. The partitioning of C99 was found to be identical even when an alternative fluorophore and lipid tracer were employed (Supplemental Figure 3). Together, these results demonstrate that C99 is excluded from L_o domains under these conditions.

Given its propensity to bind cholesterol, the exclusion of C99 from cholesterol-enriched L_o domains is somewhat surprising. However, such domains are only moderately enriched with cholesterol relative to the disordered phase. On the basis of the position of the tie line in the phase diagram,[27] we expect cholesterol concentrations of 38 mol % in the L_o phase versus 10 mol % in the L_α phase under this condition. Furthermore, the difference in the concentration of free cholesterol in the two phases may be minimal due to favorable interactions between sphingomyelin and cholesterol in the L_o phase. Even if the activity coefficient for cholesterol approached unity in each phase, the difference in the free energy of binding would still only amount to roughly 0.6 kcal/mol given the experimentally determined equilibrium dissociation constant of 5 mol %.[31] Our results suggest that this energetic contribution must be insignificant relative to the intrinsic transfer free energy of the protein. In this regard, it should also be noted that C99 likely persists as a mixture of monomers and dimers under this condition, the latter of which competes with cholesterol binding.[36] Though we reconstituted C99 at a bulk LPR of 400:1, a condition under which C99 is predominantly monomeric,[31,36] the effective LPR is likely to be closer to 200:1 because it is excluded from the lipids in the L_o phase. On the basis of our previous measurements,[36] we expect C99 to be approximately 50% monomeric under this condition. Nevertheless, considering the absence of detectable signal in the L_o phase, it seems likely that both species exhibit a pronounced thermodynamic preference for the L_α phase.

Finally, we examined the partitioning of the multispan integral membrane protein PMP22. PMP22 forms a transmembrane four helix bundle that is highly abundant in the peripheral myelin sheath (Supplementary Figure 1).[37,38] Its transmembrane helices are unusually long and hydrophobic. Like Cav3 and C99, PMP22 is found within cellular DRMs.[22] Furthermore, its native membranes in the myelin sheath are highly enriched in both sphingolipids and cholesterol.[39] To determine whether PMP22 partitions into L_o domains, we reconstituted fluorescently labeled PMP22 into GUVs containing 2:2:1 POPC:PSM:cholesterol and trace amounts of Rh-DOPE at a bulk LPR of 400:1. Again, PMP22 was only present at detectable levels in the L_α domains (three trials, $n = 51$) (Figure 2C). Similar results were obtained even when an alternative fluorophore and lipid tracer were employed (Supplemental Figure 3). These results demonstrate that PMP22 does not exhibit an intrinsic affinity for L_o domains under these conditions. This is particularly interesting in light of the fact that the "hydrophobic matching" of long transmembrane helices to thicker membranes has been found to mediate raft partitioning in some cases.[9] Nevertheless, this protein is still apparently incompatible with the thicker L_o phase membranes within these GUVs.

The results of this study very clearly reveal that Cav3, C99, and PMP22 exhibit little tendency to partition into the L_o domain of phase-separated GUVs, despite their unusual structural features. This is contrary to an abundance of evidence suggesting that these proteins preferentially reside in the raft domains of cellular membranes: PMP22 in myelin membranes, Cav3 in membranes of caveolae, and C99 in raft-like membrane nanodomains.[20−22] We suggest three non-mutually exclusive hypotheses to account for these GUV results.

First, the lack of appropriate post-translational modifications could potentially account for the difference between observa-tions in GUVs relative to those in cellular membranes. It has been established that raft-association of several proteins is promoted by thiopalmitoylation.[11,40] While C99 is not lipidated, both Cav3 and PMP22 are known to be natively thiopalmitoylated; Cav3 has three palmitoylation sites and PMP22 has one.[41,42] The recombinant forms of Cav3 and PMP22 used in these studies were not lipidated. Thus, it is possible that the partitioning of the thiopalmitoylated forms of Cav3 and PMP22 may be distinct from that of the unmodified forms of these proteins. Indeed, the reversibility of palmitoylation makes it a potentially versatile biochemical mechanism for the dynamic remodeling of biological membranes. While the isolation and characterization of palmitoylated PMP22 and Cav3 are beyond the scope of this paper, this represents an important hypothesis for future investigations.

A second possible explanation involves differences in the physical and chemical properties of synthetic L_o domains relative to those of cellular lipid rafts. Because of their homogeneous lipid compositions, these liposomal L_o domains are more ordered than those found within natural mem-branes.[10,11] It may be that the interactions between sphingomyelin and cholesterol are too strong in these synthetic membranes to accommodate the disruptive influence of a flexible polypeptide.[43] In synthetic membranes, the character-istic features of L_o domains are also likely to diverge from those of native membranes in several ways including but not limited to their material properties,[44] stoichiometric ratio of sphingomyelin and cholesterol,[45] symmetric distribution of lipids in the inner and outer leaflets,[4,46] and lack of a cytoskeletal scaffold.[47]

A third possibility is that integration of these proteins into cellular lipid rafts hinges upon specific binding interactions that are not present under these conditions. Cav3, C99, and PMP22 each form complexes with other proteins in the cell.[48−51] Additionally, cellular lipid rafts contain a host of chemically distinct lipids that are not present in the ternary lipid mixture utilized herein. It cannot be excluded that raft association of Cav3, C99, and PMP22 is dependent on association with specific raft-resident lipids and/or proteins in the cell.

From our results we can conclude that the intrinsic structural properties of these topologically diverse proteins are insufficient to endow them with an intrinsic affinity for a simplified liquid-ordered membrane domain. Further studies on the targeting mechanisms of Cav3, C99, and PMP22 are needed to gain insight into the regulation of their spatiotemporal distribution in biological membranes. Such insights will also fortify our understanding of both the native functions and the disease mechanisms associated with each protein, insight that could potentially translate to new therapeutic strategies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01154.

Supplemental Methods, Supplemental References, and Supplemental Figures 1−3 (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Cav3, caveolin 3; C99, the 99-residue C-terminal fragment of the amyloid precursor protein; APP, the amyloid precursor protein; PMP22, peripheral myelin protein 22; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PSM, N-palmitoyl-D-erythro-sphingosylphosphorylcholine; GUV, giant unilamellar vesicle

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