



Published in final edited form as:

J Phys Chem B. 2020 July 16; 124(28): 5930–5939. doi:10.1021/acs.jpcc.0c03028.

Myelin-associated MAL and PLP are unusual among multi-pass transmembrane proteins in preferring ordered membrane domains

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Abstract

Eukaryotic membranes can be partitioned into lipid-driven membrane microdomains called lipid rafts, which function to sort lipids and proteins in the plane of the membrane. As protein selectivity underlies all functions of lipid rafts, there has been significant interest in understanding the structural and molecular determinants of raft affinity. Such determinants have been described for lipids and single-spanning transmembrane proteins; however, how multi-pass transmembrane proteins (TMPs) partition between ordered and disordered phases has not been widely explored. Here we used cell-derived Giant Plasma Membrane Vesicles (GPMVs) to systematically measure multi-pass TMP partitioning to ordered membrane domains. Across a set of 24 structurally and functionally diverse multi-pass TMPs, the large majority (92%) had minimal raft affinity. The only exceptions were two myelin-associated four pass TMPs, Myelin and Lymphocyte protein (MAL) and ProteoLipid Protein (PLP). We characterized the potential mechanisms for their exceptional raft affinity and observed that PLP requires cholesterol and sphingolipids for optimal association with ordered membrane domains and that PLP and MAL appear to compete for cholesterol-mediated raft affinity. These observations suggest broad conclusions about the composition of ordered membrane domains in cells and point to previously unrecognized drivers of raft affinity for multi-pass transmembrane proteins.

Keywords

lipid raft; microdomain; plasma membrane; myelin; cholesterol; sphingolipid; MAL; PLP

INTRODUCTION

The PMs of eukaryotic cells are believed to be comprised of a mosaic of compositionally and structurally distinct lateral nanodomains¹. One of the most prominent, but least well understood, examples of such domains are lipid rafts^{2,3}. Lipid rafts were originally defined operationally, for example by being resistant to solubilization in non-ionic detergents (i.e. detergent resistant membranes, DRMs^{4,5}), or rich in glycolipids, sphingolipids⁶, or

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cholesterol⁷. However, the general principle underlying all of these properties is that lipid interactions can enforce lateral membrane organization. Specifically, preferred interactions between sterols, saturated lipids, and sphingolipids form tightly packed (i.e. liquid ordered⁸) membrane regions that are capable of selectively sorting lipids and proteins to facilitate cell functions⁹. Despite this simple definition and much supporting evidence^{10–17}, there remains significant controversy about the existence and functionality of raft domains in living cells³. In large part, the controversies persist because raft domains have not been directly, microscopically observed in living cell PMs, although liquid lipid-driven domains have been imaged directly in yeast vacuoles^{17, 18}. PM nanodomains may be difficult to image due to the limits on spatial and temporal resolution of currently available technologies. However, other possibilities must be considered, including the dearth of validated, consistent probes for raft domains and indeed even the non-existence of rafts in some, most, or all PMs.

A key piece of supporting evidence for the raft hypothesis is the direct observation of phase separation in isolated plasma membrane vesicles known as Giant Plasma Membrane Vesicles (GPMVs)^{19, 20}. These vesicles separate into coexisting ordered and disordered lipid phases, like biomimetic model membranes^{21, 22}, but retain the compositional complexity and high protein content of cell membranes. It remains unresolved why this phase separation is easily observed in isolated PMs, but not in vivo. One possibility, supported by experimental^{23–25} and theoretical^{26–28} studies is that the cytoskeleton and transmembrane proteins prevent macroscopic phase separation by pinning/scaffolding membrane components. Another possibility is that macroscopic phase separation is manifested in nanodomains²⁹, due to critical fluctuations^{30, 31} or other mechanisms^{32, 33}. In any case, phase separation in GPMVs provides a practical and tractable model for investigation of raft domains in biological membranes.

The major purpose of membrane domains is to selectively recruit specific proteins. Thus, an essential question is how and why certain proteins are sorted to these domains. For many peripheral proteins, the properties of their lipid-anchor determines their raft residence³⁴, with saturated lipid or sterol anchors preferring ordered membrane regions³⁵. For transmembrane proteins, a series of studies in GPMVs has revealed the structural determinants of raft affinity, which largely rely on structural features of the TM α -helix and its interaction with the surrounding bulk membrane environment^{36, 37}. However, this model is not easily extensible to multi-pass proteins. Firstly, the model was constructed entirely using single-pass proteins, making extrapolation inherently questionable. Moreover, it is difficult to estimate the precise structure of the TMD bundle for multi-pass proteins because it is usually not known how multiple transmembrane helices (TMHs) pack together and which residues face the lipid environment.

Here, we used GPMVs to test the ordered phase affinity of a panel of 24 different multi-pass proteins, spanning from two to twelve TMHs. Consistent with predictions based on single TMH proteins³⁷, almost all multi-pass proteins were excluded from more ordered raft domains. However, we report the notable exceptions of two 4-TM proteins of similar topologies, MAL (Myelin And Lymphocyte; otherwise known VIP17, Vesicle Integral Protein 17 kDa)³⁸ and PLP (ProteoLipid Protein)³⁹ which have a high affinity for ordered

raft phases. Because these proteins do not have the typical features associated with raft affinity, we investigated the origins of their ordered phase preference. Our observations suggest that lipid-protein binding is involved in the raft affinity of these multi-pass TMPs, as depletion of cholesterol and sphingolipids reduced their raft affinity while having no effect on single-pass TMP partitioning. These observations reveal novel determinants of raft affinity for multi-pass TMPs and suggest that specific lipids play essential roles in recruiting specific proteins to raft domains.

MATERIALS AND METHODS

Materials.

The following materials were purchased for this study: Methyl- β -cyclodextrin (M β CD, Sigma); Fast DiO (F-Dio), Fast DiI (F-DiI) and DiD (Life Technologies, Carlsbad, CA); myriocin (Cayman Chemical).

DNA constructs.

All single pass protein constructs were based on the trLAT (LAT) backbone previously described^{35–37}. The amino acid sequence of WT trLAT is NH₂-MEEAILVPCVLGLLLLLPILAMLMALCVHCHRLP followed by a short linker (GSGS) and monomeric RFP (mRFP). TMD mutants (LIME, CD4, PAG, LAX, LDLR) were generated by synthesizing the gene of interest (Genscript) and subsequent cloning of the mutant sequence into the LAT construct. Several multi-pass protein constructs were purchased from Addgene: mCherry-CD36-C10 (CD36, 55011), mCherry-Occludin-N-10 (Occludin, 55112), mCherry-CD9–10 (CD9, 55013), mCherry-CD81–10 (CD81, 55012), YFP-CD82 (CD82, 1819), pFCK(1.3)GFP-SynaptophysW (Synaptophysin, 27235), pEGFP-N-Drd1 (DRD1, 104358), Kv7.1/pcDNA3.1 (KCNQ1, 111452), pcDNA3.1(+)-mGAT1–0-GFP (GAT1, 41662). Remainder of constructs were kindly gifted: hMOG α , hMOG β , mMOG, rMOG and Beta2AR (Marcus Reindl, Medical University of Innsbruck); cMAL (Kai Simons, MPI-CBG); hMAL, BENE and MAL2 (Miguel A. Alonso, Universidad Autonoma de Madrid); TRPA1, TRPM8 and TRPV1 (Michael X. Zhu, UTHealth); AC9 (Carmen Dessauer, UTHealth); NKCC1 (Eric Delpire, Vanderbilt University); GPM6a (Michihiro Igarashi, Niigata University). MAL Ct mutants were all based on cVIP17-mRFP and point mutations were performed following the QuickChange II XL Site-directed mutagenesis kit (Agilent).

Cell culture and transfection.

Rat basophilic leukemia (RBL) and HEK-293 (HEK) cells were purchased from ATCC and cultured in medium containing 89% Eagle's Minimum Essential Medium (EMEM), 10% FCS, and 1% penicillin/streptomycin at 37 °C in humidified 5% CO₂. Transfection was done by nucleofection (Amaxa) using the protocols provided with the reagents. 4–6 h after transfection, cells were washed with PBS and then incubated with serum-free medium overnight. To synchronize the cells, 1 h before preparation of GPMV, the cells were given full-serum medium. Cholesterol depletion was accomplished by incubating the cells with 3 mM M β CD for 30 min in MEM before GPMV isolation⁴⁰. To inhibit sphingolipid metabolism, the medium was changed six hours after transfection to include 25 μ M

myriocin and to remove the transfection reagent. GPMVs were isolated after 24 hours of myriocin addition⁴¹.

Partitioning measurements in Giant Plasma Membrane Vesicles (GPMVs).

Cell membranes were stained with 5 $\mu\text{g}/\text{ml}$ of FAST-DiO, FAST-DiI or DiD (Invitrogen), respectively, green, red or far-red fluorescent lipid dyes that strongly partition to disordered phases^{42, 43}. Following staining, GPMVs were isolated from transfected RBLs, HEK-293, or HeLa cells as described¹⁹ (cell type had no effect on results). Briefly, GPMV formation was induced by 2 mM N-ethylmaleimide (NEM) in hypotonic buffer containing 100 mM NaCl, 10 mM HEPES, and 2 mM CaCl_2 , pH 7.4. To quantify protein partitioning, GPMVs were observed on an inverted epifluorescence microscope (Nikon) at 4°C after treatment with 200 μM DCA to stabilize phase separation; this treatment has been previously demonstrated not to affect raft affinity of various proteins⁴⁴. The partition coefficient ($K_{p,\text{raft}}$) for each protein construct was calculated from fluorescence intensity of the construct in the raft and non-raft phase for >10 vesicles/trial (Fig. 1), with multiple independent experiments for each construct. The results are displayed as $\log(K_{p,\text{raft}})$, in analogy with $\log P$ values oil-water partitioning, to avoid appearance of compression for values less than 1.

RESULTS

Quantitative characterization of raft affinity in GPMVs

Membrane domains are functionalized by their selective recruitment of membrane proteins and lipids. However, until recently, the structural principles underlying protein recruitment to ordered membrane domains have not been widely explored. Such investigations are now enabled by direct measurements of raft affinity in GPMVs. GPMVs are intact cell-derived PMs that macroscopically separate into coexisting phases of distinct physical properties and compositions²⁰. One of these phases exhibits many of the features associated with raft domains in cells: it is cholesterol-dependent⁴⁵, more tightly packed^{43, 46}, less diffusive, enriches sterols, sphingolipids, and saturated lipids and excludes unsaturated lipids⁴⁷, recruits GPI-anchored and palmitoylated proteins, and excludes many predicted non-raft proteins³⁵. Protein partitioning to this “raft phase” in GPMVs has thus been widely validated as a robust tool for quantifying ordered phase affinity in cell-derived membranes⁴⁸.

Recent investigations into the raft partitioning of single-pass transmembrane proteins have demonstrated that the length and surface area of membrane-spanning α -helices, together with post-translational palmitoylation, comprise a set of independent, predictive determinants of raft affinity^{36, 37}. The principle of the assay is demonstrated in Figure 1. Briefly, a protein of interest with a fluorescent tag (e.g. RFP) is expressed in a cultured mammalian cell line (here, RBLs), followed by isolation of GPMVs following standard protocols¹⁹ (see Materials and Methods). Phase separation in these GPMVs is visualized by staining with a marker that partitions strongly to one of the two phases, e.g. FAST-DiO in Fig. 1A–B, which has been shown to strongly label the non-raft phase⁴². Some proteins enrich in the opposing (raft) phase, as shown for a widely characterized model

transmembrane peptide, the TMD from the linker for activation of T-cells (LAT) (Fig. 1A), while others are almost wholly excluded from the raft domains, as for the TMD of the Low Density Lipoprotein Receptor (LDLR) (Fig. 1B). This behavior can be quantified by fluorescence intensity line scans across the two phases, wherein a ratio between the phases defines the raft partition coefficient ($K_{p, \text{raft}}$).

We confirmed previously published results that single-pass proteins with relatively long and thin TMHs (e.g. LAT, LIME, CD4, and PAG) partition efficiently to the raft phase, while those with shorter or fatter TMHs (LAX and LDLR) are generally excluded (Fig. 1C). Next, we explored the determinants of raft affinity for proteins with multiple membrane spanning domains. Multi-spanning TMPs would inherently have larger lipid-accessible surface areas than single-pass and would thus be predicted to be excluded from raft phases³⁷. On the other hand, many multi-pass proteins are found in detergent-resistant fractions (i.e. DRMs), which have been previously used as a proxy for isolated lipid rafts^{49–52}. Several of these DRM-resident multi-pass proteins were excluded from the raft phase in GPMVs (Fig 1C), leading us to undertake a more systematic analysis of raft affinity across several representative classes of multi-pass TMPs in mammalian PMs.

Almost all multi-pass TM proteins have low raft affinity

Figure 2 shows the results of raft affinity measurements for proteins with 2, 4, 6, 7, and >10 TMHs, each in comparison to the raft-preferring LAT and non-raft LDLR. The most prominent finding across this set of 24 structurally and functionally diverse multi-pass TMPs is that the vast majority (92%) had minimal raft affinity. This finding is consistent with the predictions from the single-pass model³⁷, but in many cases inconsistent with previous reports of raft residence for these proteins. Possible reasons for this discrepancy and details of these proteins are expanded upon in Discussion; however, the broad conclusion is clearly that most multi-pass transmembrane proteins are excluded from the raft phase in GPMVs.

Two 4-TM proteins of similar topology associated with myelin have anomalously high raft affinity

The only exceptions in our dataset to the overall exclusion of multi-pass TM proteins from the raft phase were two 4-TM proteins, MAL and myelin PLP (Fig. 3A–C). As their names imply, both of these proteins are functionally implicated with the structure and physiology of myelin^{39, 53}. Despite their general topological and functional similarity, a BLAST search revealed no homology between these proteins.

The strikingly high raft affinity of these two proteins notably diverges with the model for single-pass TMPs, in which lipid-accessible surface area (ASA) and TMD palmitoylation are key drivers of raft affinity. While calculating ASA of a multi-pass TMP cannot be accurately done without a structural model, certainly any 4-TM protein has a larger ASA than any single TMH. Furthermore, several other similar or smaller proteins (e.g. the 2-TM proteins in Fig. 2B) had minimal raft affinity. Additionally, while PLP can be palmitoylated at three sites in the cytoplasmic N-terminus, with these modifications being a major determinant for its distribution to myelin-like membranes⁵⁴, MAL is not known to be palmitoylated, and topological modeling revealed no cytoplasmic cysteine residues that

would be good candidates for palmitoylation. Thus, despite a relatively large TMD and no palmitoylations, MAL partitions into raft domains in GPMVs with $K_{p,raft}$ of ~ 3.5 (Fig. 3C), greater than any previously reported TMPs^{36, 37, 55}, and similar to the lipid-anchored raft markers like GPI-anchored proteins and cholera toxin^{35, 44, 56}. Notably, a recent unpublished report also found high raft affinity for another protein with similar topology to MAL and also associated with myelin, Peripheral Myelin Protein 22 (PMP22)⁵⁷.

Other MARVEL family members do not partition to raft phase

These surprising observations led us to explore the possible mechanisms for the raft affinity of these 4-TM proteins. MAL is reported to be a member of a protein family named MARVEL (MAL and related proteins for vesicle trafficking and membrane link)^{58, 59}, which includes occludin, tricellulin, and synaptophysin and has been implicated in mediating membrane apposition⁶⁰. We tested the ordered phase affinity of several other MARVEL family members and observed minimal raft preferences for all of them, including two closely MAL-related proteins MAL2 and BENE (also called MAL-like) (Fig. 2B). These results were surprising because of the evolutionary relationship between these proteins and MAL and because MAL2 has been previously implicated in raft-related cellular functions, namely apical transport in polarized cells⁵². To ensure consistency of partitioning measurements, we tested a canine homolog of MAL (cMAL) and found that it also partitioned efficiently to raft phases. Thus, it appears that MAL contains unique features that impart very high raft affinity, even compared to closely related proteins.

LIRW peptide is important for ER exit of MAL but not raft affinity

MAL has been previously reported to be a raft-associated protein, with the possible determinants of this behavior sporadically explored. In particular, a short peptide (LIRW, Fig 3A) located at a cytoplasmic interface of the protein with the lipid bilayer⁶¹ has been implicated in MAL recruitment to detergent-resistant membranes, with mutations of this sequence completely abrogating DRM residence. We thus tested whether these mutations also abrogated raft affinity in GPMVs. However, mutations of this C-terminal peptide lead to near absolute ER retention (Fig 3D), consistent with previously reported trafficking disruptions^{61, 62}. While this ER localization almost completely precluded measurements of these mutants in GPMVs (which are derived from the PM), the few GPMVs with detectable protein signal still showed partitioning preference for the raft phase (Fig 3E). Thus, we conclude that the LIRW motif is important for MAL export from the ER, rather than a *bona fide* raft partitioning determinant. The absence of the protein from DRMs is likely explained by the trafficking defect.

MAL and PLP compete for raft affinity—The surprising finding that only two multi-pass proteins, MAL and PLP, showed lipid raft affinity and that these possessed some general functional and structural similarities led us to hypothesize that they share characteristics that impart raft affinity. Because both are myelin-related proteins, we tested the raft affinity of other myelin-related proteins, but found that all four isoforms of Myelin Oligodendrocyte Glycoprotein (MOG), a prominent myelin-associated protein⁶³, were raft-excluded (Fig 2A and B). Similarly, MAL-related proteins MAL2 and BENE were not raft-preferring. Thus, we conclude that myelin-association *per se* is not a determinant of raft

affinity, although the connection to myelin for both raft-preferring proteins found here and another one recently reported⁵⁷ is intriguing.

Post-translational lipidations have been widely implicated as raft-sorting determinants³⁴, but MAL is not known to be lipidated, whereas several of the raft multi-pass proteins in Figure 2 (CD9, CD81, CD82) can be palmitoylated. Thus, palmitoylation is neither necessary nor sufficient for raft affinity of multi-pass proteins, unlike single-pass TMDs.

We next hypothesized that both MAL and PLP may acquire raft affinity by binding to a common raft-preferring component (e.g. a protein or lipid with intrinsic ordered phase affinity). To evaluate this possibility, we conducted a ‘competition’ experiment by co-transfecting the two proteins and measuring the effect of co-transfection on raft affinity. The rationale behind this experiment was that if both proteins share a saturable binding partner required for their raft residence, then co-transfection should deplete the availability of this component and reduce raft affinity of MAL and/or PLP. This effect was indeed observed for PLP, whose raft residence was significantly reduced by co-transfection with MAL (Fig. 4A&C). This effect was also dose-dependent, as PLP raft affinity was negatively correlated with the relative abundance of MAL to PLP (quantified as MAL/PLP fluorescence intensity, Fig 5E). MAL raft affinity was also somewhat, though not significantly, reduced by co-transfection. Importantly, PLP raft affinity was not reduced by co-transfection with LAT, which resides in rafts solely due to the physical properties of its TMD, rather than binding to a separate component (Fig. 4C–D).

Cholesterol is important for PLP raft affinity—The most prominent components of lipid rafts are sphingolipids and cholesterol⁶⁴. Thus, we hypothesized that these lipids may be responsible for recruiting MAL and/or PLP to raft domains. To test this possibility, we assayed whether depletion of cholesterol or sphingolipids had an effect on raft affinity of the multi-pass TMPs. To deplete cholesterol, cells were treated prior to GPMV isolation with methyl- β -cyclodextrin (M β CD), a common method for acute depletion of cholesterol⁶⁵. Importantly, phase separation into raft and non-raft domains was still observable after M β CD treatment (Fig. 5A–C), consistent with previous reports and explainable by the fact that only a fraction of PM cholesterol is removed by this treatment^{40, 66}. M β CD treatment significantly reduced the raft affinity of PLP (Fig. 5A&D), with a smaller and not significant effect on MAL (Fig. 5B&D). As a control, we tested the cholesterol sensitivity of raft partitioning for the single-pass LAT, whose raft affinity was not reduced by M β CD (Fig. 5E). The effect of cholesterol depletion on PLP partitioning was further exacerbated by co-transfection with MAL, with PLP becoming quite depleted from the raft phase under the co-transfected+M β CD condition (Fig. 5C–D). Again, this effect was dependent on MAL expression, with the correlation between PLP partitioning and MAL-to-PLP abundance strongly exacerbated by cholesterol depletion (Fig. 5G). No trend was observed for MAL partitioning as a function of PLP levels (not shown). These observations suggest that MAL competes with PLP for cholesterol binding, which appears to be required for PLP to partition to the raft phase. This hypothesis is consistent with the previously reported interaction of PLP with cholesterol, which has been implicated in PLP transport to myelin in neurons⁶⁷. It is important to note that M β CD-mediated cholesterol extraction likely has side-effects and therefore direct binding of cholesterol to these proteins and its effect on raft

affinity is a provisional conclusion that will require confirmation by direct experiments and/or computational modeling.

PLP, but not MAL, requires sphingolipids for raft residence—To evaluate whether sphingolipids could similarly promote PLP raft affinity, we depleted sphingolipids in cells using myriocin, a potent inhibitor of serine palmitoyltransferase, which catalyzes the first step in *de novo* sphingolipid synthesis^{68, 69}. Myriocin treatment significantly inhibited the raft affinity of PLP (Fig 6A&C), with a subtle and not significant effect on MAL partitioning (Fig 6B–C). Co-transfection had no additive effect in the presence of myriocin (Fig 6D). These observations suggest that sphingolipid interactions are important for PLP raft affinity. This effect is likely mediated by glycosylated sphingolipids (GSLs), as it was previously reported that galactosyl transferase is essential for PLP to associate with DRMs⁷⁰.

In summary, we conclude from these observations that PLP uses both cholesterol and sphingolipids for its recruitment to raft domains. It appears to compete with MAL for the free cholesterol available in the PM, with MAL likely having greater affinity to cholesterol. Finally, the raft affinity of MAL was not strongly dependent on either sphingolipids or cholesterol abundance, revealing a yet-unidentified mechanism of raft affinity.

Discussion

Minimal raft affinity of multi-pass proteins.

The major finding of our study is that the large majority of multi-spanning TMPs are excluded from raft phases in GPMVs. This observation is particularly striking because the 24 proteins in our study were not chosen randomly, but rather many of them had been previously implicated in raft-associated functions or shown to be in DRMs (biochemical preparation enriching for raft components). Included among these proteins are glycosylated and palmitoylated proteins, features that are thought to confer raft affinity^{35, 71}. Fig 2A shows that several 2-TM glycoproteins Myelin-Oligodendrocyte Glycoprotein (MOG) and the Platelet Glycoprotein (CD36) were raft-excluded. These were tested because glycosylations were implicated in raft affinity for single-pass proteins including P-selectin Glycoprotein Ligand-1 (PSGL1), Leukosialin (CD43), and CD44 Antigen (CD44)⁷². All 6-TM and 7-TM receptors were completely excluded from the raft phase (Fig 2C). We tested several members of the TRP family receptors, a large group of calcium-selective ion channels that are likely palmitoylated to regulate their function⁷³. Again, the fact that these proteins may be palmitoylated was insufficient for their raft affinity, as was also the case for the G Protein-Coupled Receptors DRD1 and β 2-AR, which are believed to be palmitoylated in their C-termini^{74, 75}. It is important to note that the presence of documented palmitoylation sites does not imply that these proteins are necessarily palmitoylated in any given context.

We also analyzed several proteins with more than 10 TM helices, and again all were excluded from the raft phase. Among these was the scramblase TMEM16F, involved in the externalization of phosphatidylserine in the cell PM. The other 10+ TM proteins tested (Solute Carrier Family 12 Member 2 (SLC12A2 or NKCC1), Potassium Voltage-gated Channel Subfamily KQT Member 1 (KCNQ1), and Sodium-and Chloride-dependent GABA

Transporter 1 (SLC6A1 or GAT)) also included several other proteins belonging to the sodium transporter family. Finally, we found that neither myelin residence nor 4-TM domains were sufficient to explain raft affinity. The myelin point was discussed above for MOG and MAL2. We also tested several known ‘tetraspanin’ proteins (CD9, CD81, and CD82), a family with specific characteristics including 2 extracellular loops, one short and one large, and highly conserved cysteine-rich domains that are likely palmitoylated. In our experiments, neither CD9^{76, 77}, CD81⁷⁸, nor CD82⁷⁹ were raft preferring.

These experiments show a broad survey of partitioning for multi-pass TMPs. A related but separate point of interest is the behavior of protein complexes, from homodimers to more complex multi-subunit assemblies⁸⁰. Is raft affinity of such complexes predictable from the raft affinities of individual components or are there non-ideal effects arising from interactions? Such questions remain to largely unexplored.

Cholesterol as a regulator of raft affinity

Our measurements implicate cholesterol as a potential regulator of partitioning for PLP, and to a lesser extent MAL. This inference is based on the observations that these proteins appear to compete for a saturable component and that the partitioning of PLP is strongly sensitive to cholesterol depletion. A role for cholesterol in PLP’s raft affinity would also be consistent with previously suggested cholesterol interaction of PLP via its Cholesterol Recognition Amino acid Consensus (CRAC) motif in the cytosolic interface of its TMD2 and a Sterol Sensing Domain (SSD) in the extracellular interface of its TMD3⁸¹. None of these motifs have been reported in MAL and our bioinformatics analysis also did not identify any such motifs. Obviously, the known cholesterol-binding modes are not exhaustive, and it is possible that MAL (and PLP) interacts with lipids through as-yet-undefined mechanisms.

Cholesterol is highly abundant in mammalian PMs (up to 40 mol%⁸²), thus it may be surprising that co-transfection of two putatively cholesterol-interacting proteins could deplete a sufficient fraction of cholesterol to effect such large changes on raft affinity. Even in the case of MBCD depletion, there are almost certainly more remaining cholesterol molecules than PLP and MAL combined, regardless of the extent of overexpression. A possible answer to this seeming paradox comes from the extensive literature that reveals that a relatively small fraction of the cholesterol present in the PM is actually available for bioactivity, known as the active cholesterol hypothesis⁸³. While most of the cholesterol in the PM is “hidden” by its interactions with membrane phospholipids, some small “active” fraction is available for interaction with proteins and it is presumably this small fraction that can be saturated.

Other potential modulators of raft affinity

The reduction in PLP raft affinity by myriocin treatment reveals that lipid raft affinity of some proteins could be affected by their binding sphingolipids. Protein motifs that bind sphingolipids⁸⁴ or glycolipids^{85, 86} have been extensively reported and some of these may have a significant role in conferring raft affinity. MAL itself has been shown to bind

sulfatide⁸⁷, a glycosphingolipid enriched in myelin sheath. Thus, the interaction of MAL and/or PLP with glycolipids may be involved in their unique raft affinity.

Why is partitioning in GPMVs so different from DRMs?

The fact that so many proteins previously associated with rafts show minimal ordered phase affinity in GPMVs demands critical examination. It should be noted that almost all previous such studies have relied on detergent resistance to infer raft residence. The many caveats associated with detergent resistance have been discussed at length^{88, 89} and will not be recapitulated here, except to point out that DRM compositions are rather unpredictable and highly dependent on specific experimental conditions, and that protein appearance in detergent resistant fractions cannot be used as *bona fide* evidence of raft affinity. However, pointing out the issues with DRMs should not be construed as absolute validation of GPMVs as a system for evaluating raft affinity. Many essential factors of live cell membranes are perturbed in GPMVs, including lipid asymmetry, out-of-equilibrium energy consumption, assembled cytoskeleton, and protein-protein interactions that are disrupted by the isolation procedure, among others previously discussed⁴⁸. Any of these factors may be necessary for a given protein to partition to raft domains *in vivo*. Furthermore, the specific relationships between phase separation in GPMVs and the nanodomains presumed to be present in living membranes remain incompletely understood²⁹. Ultimately, low raft affinity in GPMVs seems to be the default condition for many proteins and the very large majority of multi-pass TMPs. However, whether this accurately represents their *in-situ* association with ordered domains remains to be resolved.

Acknowledgements

Funding for this work was provided by the NIH/National Institute of General Medical Sciences (GM114282, GM124072, GM120351, GM134949), the Volkswagen Foundation (grant 93091), and the Human Frontiers Science Program (RGP0059/2019). All authors have no competing interests.

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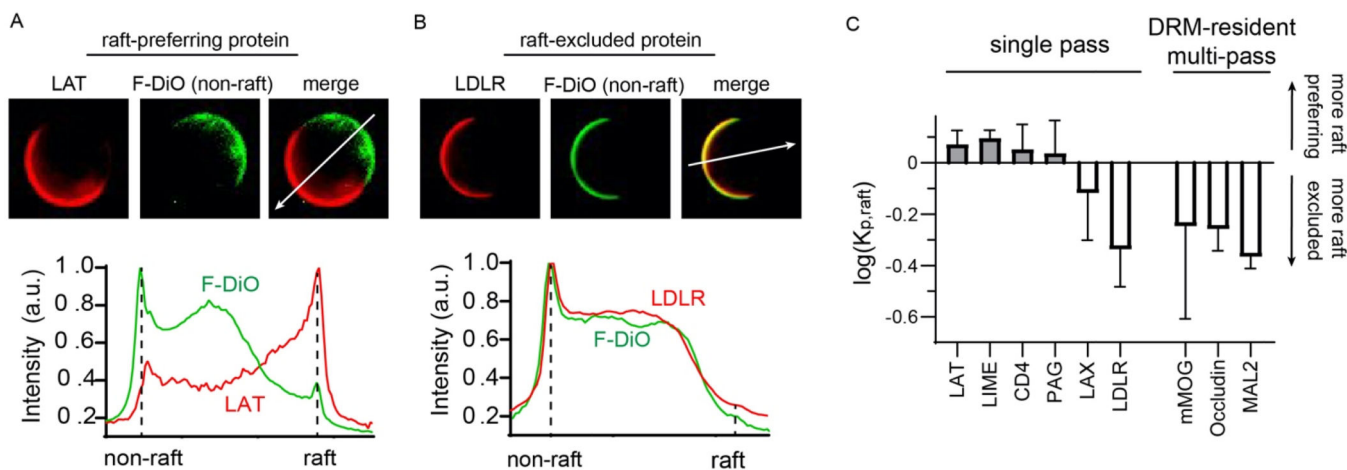


Figure 1 – GPMVs as a biomimetic model of lipid raft affinity for PM proteins.

(A) Example of quantification of raft affinity for a raft-prefering protein. A protein of interest (LAT= TMD of Linker for Activation of T-Cells, red) is expressed in cells, which are stained with a lipid dye with a known phase preference (F-DiO, green, non-raft). The intensity of the protein in the raft versus non-raft phase yields raft partition coefficient ($K_{p,raft}$). LAT has $K_{p,raft} > 1$, and is thus a raft phase preferring protein. (B) Example of quantification of a non-raft preferring protein. Same principle as panel A, but with the TMD of LDLR, which is largely excluded from the raft phase. (C) Comparison of raft affinity for several single pass proteins and three multi-pass TMPs. Single TMD raft affinity is in good agreement with predictions³⁷, whereas multi-pass proteins that have been previously associated with rafts have minimal raft phase affinity. Average \pm st. dev. for at least 10 vesicles for each protein, representative of three independent trials.

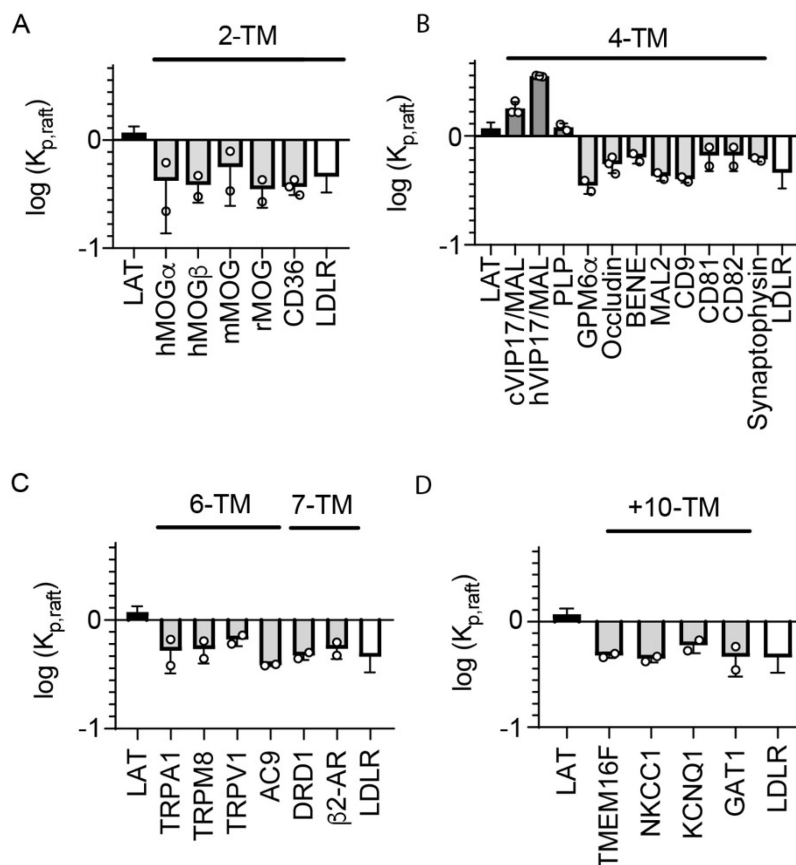


Figure 2 –. Almost all multi-pass TMDs have low raft affinity in GPMVs.

Shown are the raft affinity of multi-pass TMDs with varying number of TMHs. Raft affinity is shown in log scale, with 0 representing equal partitioning between the phases and positive and negative values representing enrichment in or depletion from the raft phase, respectively. Included in each panel is the value for LAT (a raft-prefering single pass TMD, black) and LDLR (a non-raft TMD, white). (A) 2-TM proteins. Three homologs of MOG from different species (h:human; m:mouse; r:rat) and both isoforms (alpha and beta) of the human protein are excluded from rafts, as is the unrelated CD36. (B) Two 4-TM proteins are highly raft-prefering, namely MAL and PLP. In contrast, several other members of the MARVEL family (BENE, MAL2, occludin, and synaptophysin) and other tetraspanin family proteins (CD9, CD81, CD82), as well as the neuronal membrane glycoprotein (GPM6a) are almost completely excluded. Two analogs of MAL (c:canine; h:human) were tested and both had significantly greater raft affinity than LAT. (C) All tested 6-TM and 7-TM proteins are raft excluded. Several proteins from the TRP family (TRPA1, TRPM8, TRPV1), Adenylyl Cyclase 9 (AC9), and G Protein-Coupled Receptors (DRD1, β 2AR) were not significantly different from LDLR. (D) All tested proteins with 10 or more TMDs - PM scramblase TMEM16F and several transporters (NKCC1, KCNQ1 and GAT1) - were raft excluded. Data points represent the means of individual experiments of >10 vesicles each.

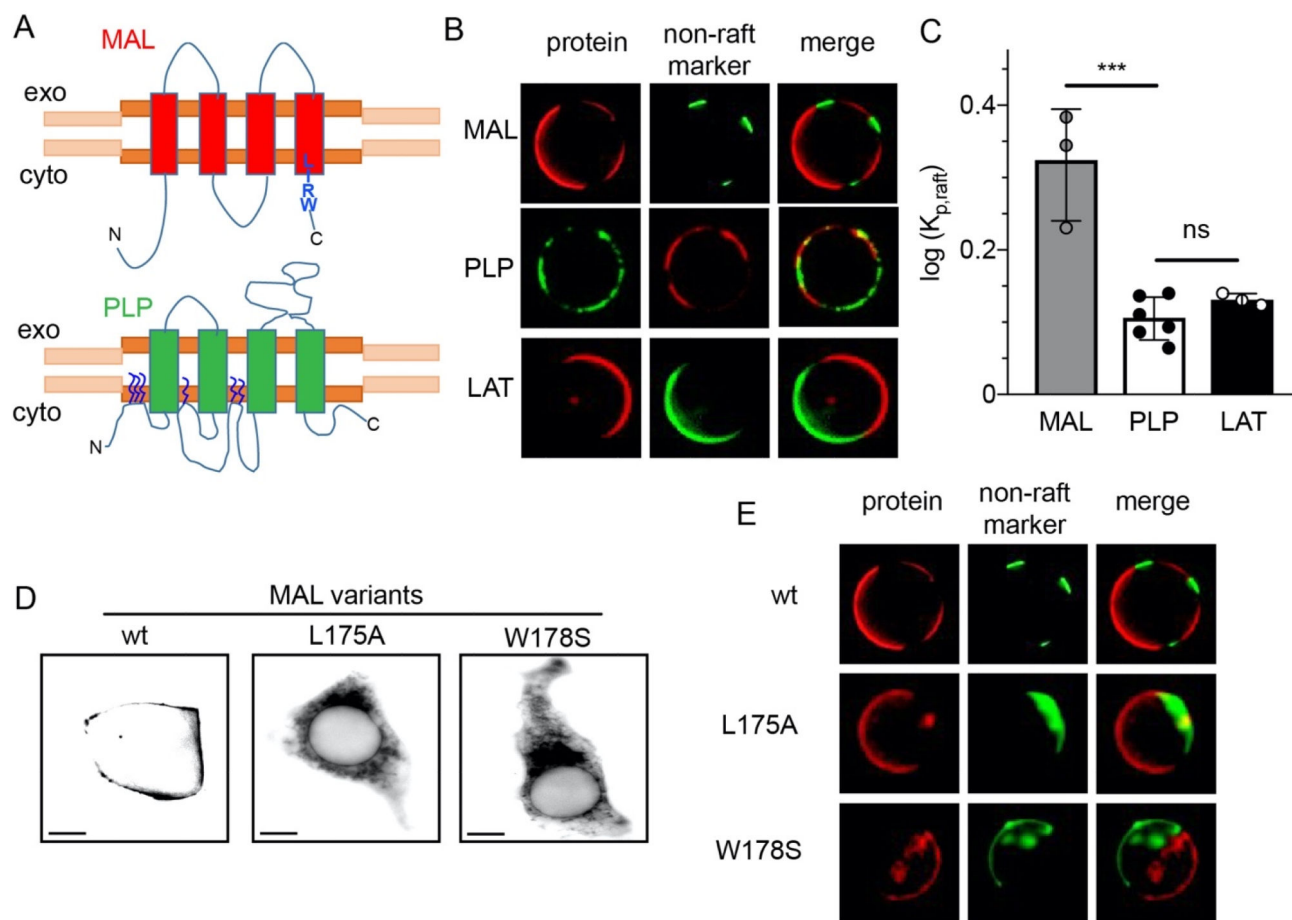


Figure 3 –. LIRW peptide is important for MAL ER exit but not raft affinity.

(A) Schematics for MAL (red) with LIRW motif close to the cytosolic C-terminal and PLP (green) with numerous palmitoylation at cytosolic loops. (B) Images of GPMVs containing cMAL-RFP (MAL), PLP-GFP (PLP) and trLAT, with their respective complementary non-raft marker, showing that all three proteins prefer the raft phase. (C) Raft affinity ($\log K_{p,raft}$) measurements show MAL has higher raft affinity than PLP and LAT, which have similar raft affinity. (D) Representative images for HEK-293 cells transfected with cMAL and C-terminal mutants. Wild-type cMAL (wt) accumulates in the PM with some punctate structures while single point mutations of the LIRW motif lead to clear accumulation in the ER. (E) Mutations in LIRW motif do not change lipid raft affinity of the protein. Both mutants, L175A and W178S, showed a retention in the ER, but the minimal amount of protein present in PM still partitioned into the raft phase in GPMVs. Scale bar corresponds to 10 μm . *** $p < 0.001$ by t-test

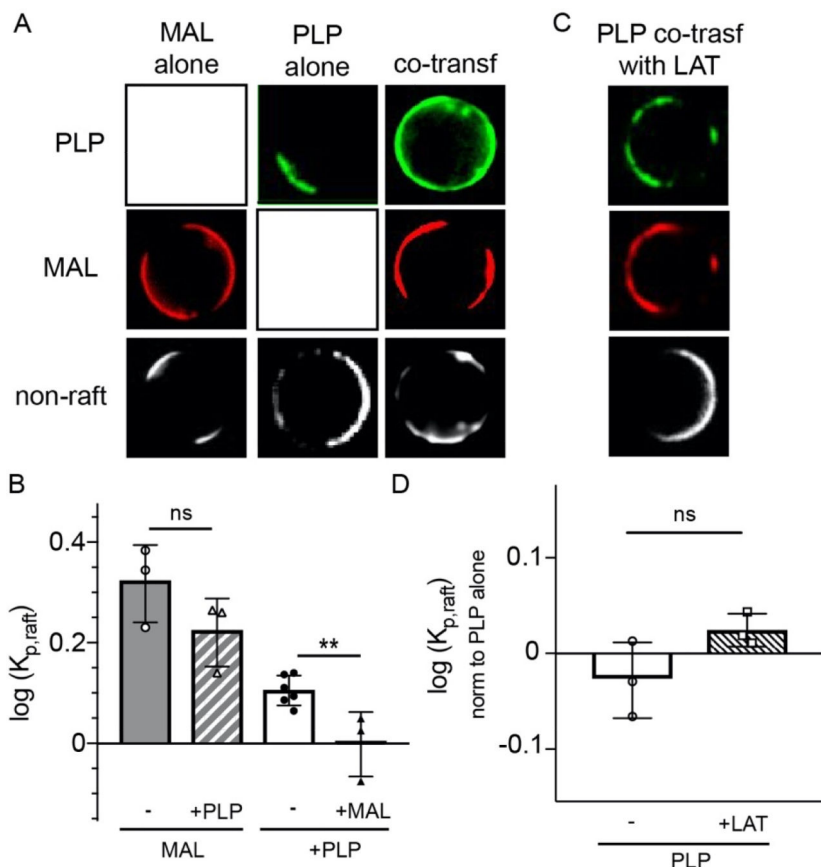


Figure 4 –. MAL displaces PLP from raft phase.

(A) Single transfected PLP and MAL both have preference for raft phase (non-raft labeled with F-DiO, white). Co-transfection reduces PLP raft preference to approximately equal partitioning between phases, but does not significantly affect MAL. (B) PLP partitioning is unaffected by co-transfection with LAT. (C) Raft partitioning of MAL (open symbols) and PLP (closed symbols) as a function of co-transfection (triangles) compared with single transfected (circles). PLP raft affinity is significantly reduced by co-transfection with MAL, (D) but not by the co-transfection with LAT. Partitioning in panel D shown as normalized by PLP alone. Bars represent average \pm s.e.m.; circles represent means of individual experiments (>10 vesicles each). $**p < 0.01$ by t-test.

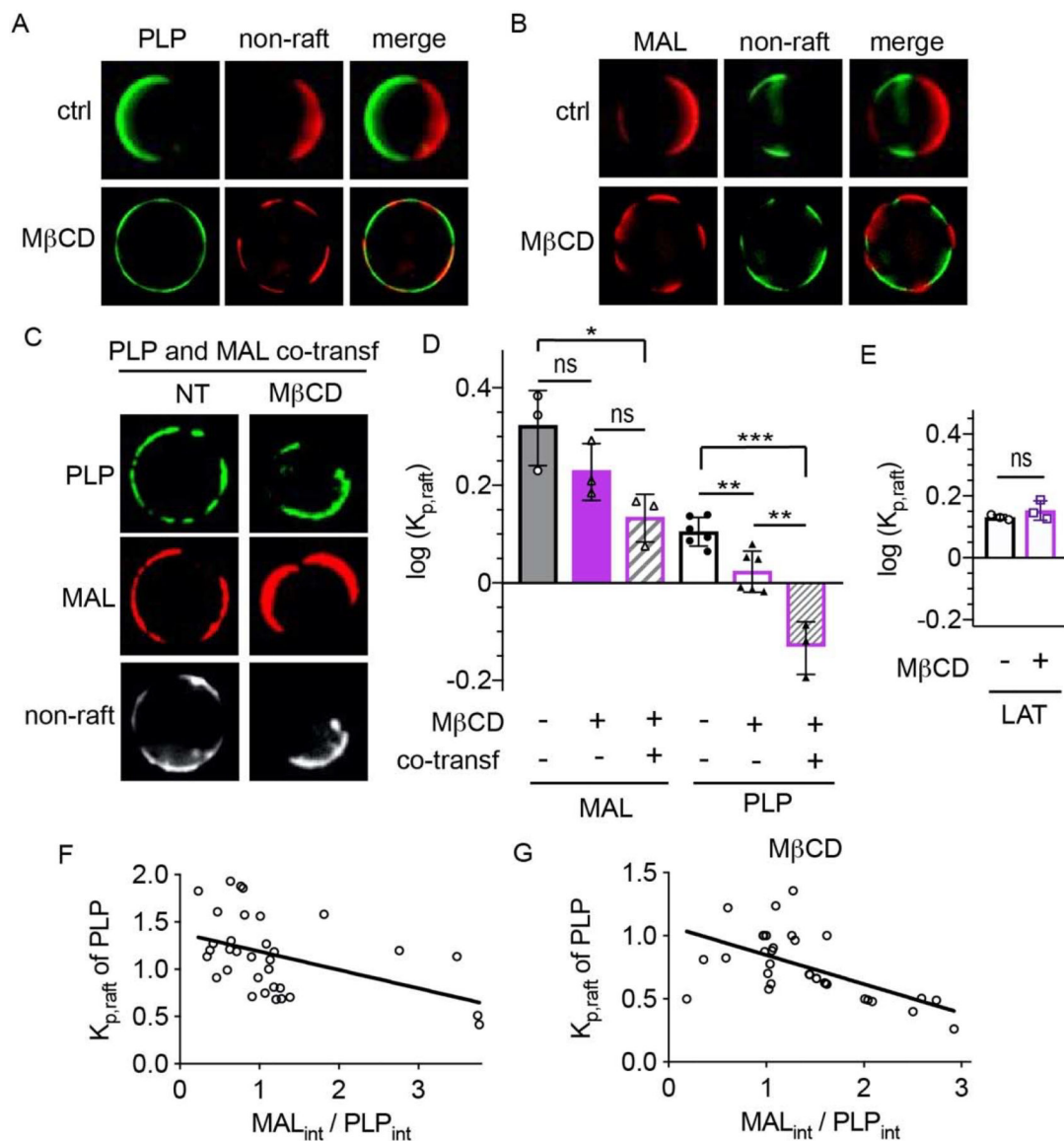


Figure 5 –. MAL and PLP may compete for cholesterol.

(A) Cholesterol depletion does not significantly affect MAL partitioning, which still enriches in raft phase upon cholesterol depletion with MβCD. (B) PLP partitioning is reduced by cholesterol depletion, becoming approximately homogeneously distributed between phases. (C) Cholesterol depletion severely reduces PLP raft affinity when co-expressed with MAL. Under this condition, PLP is excluded from rafts domains and co-enriches with the non-raft marker (F-DiD: white). (D) Quantifications of all conditions. (E) LAT partitioning is not affected by MβCD treatment. Bars represent average \pm s.e.m.; circles represent means of individual experiments (>10 vesicles each). * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ by t-test. (F) PLP partitioning negatively correlates with the abundance of MAL. (i.e. relative intensity of MAL to PLP). (F) The effect is enhanced by cholesterol depletion with MβCD.

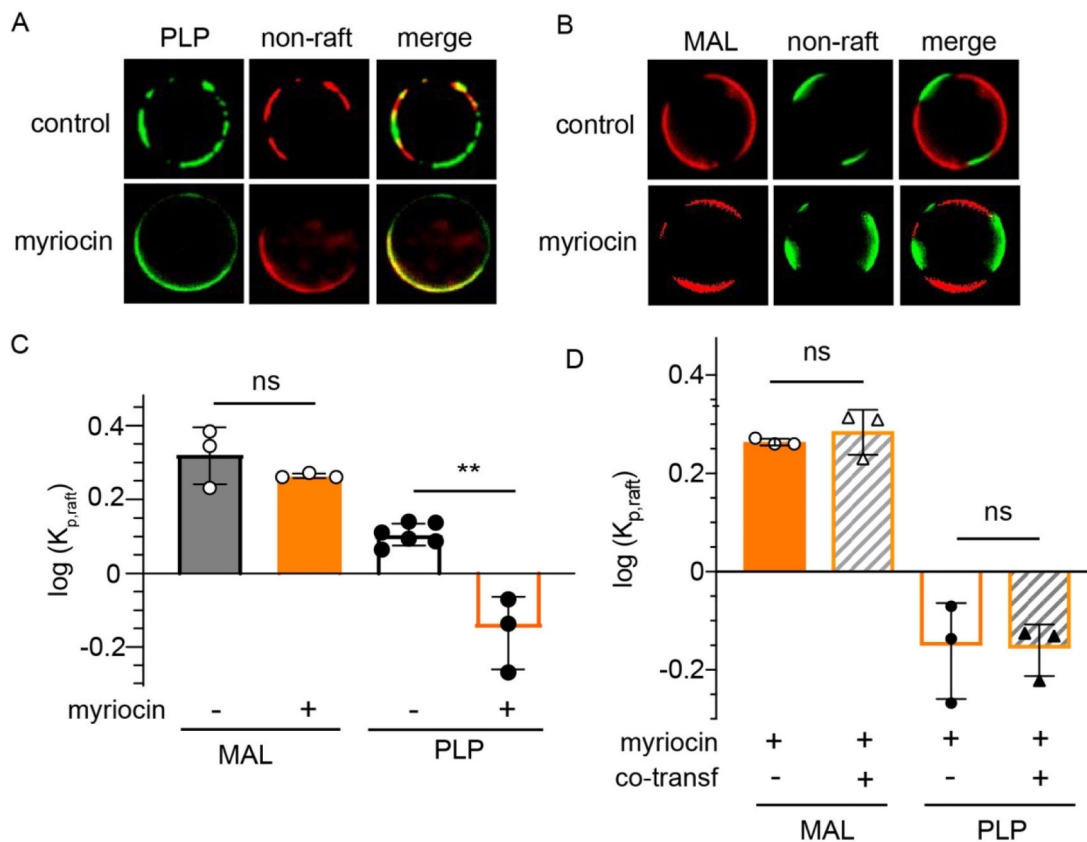


Figure 6 – Spingolipids are required for PLP raft enrichment.

(A) MAL raft affinity is not significantly affected by sphingolipid depletion. (B) In contrast, PLP enriched in non-raft phase upon myriocin treatment. (C) Quantification of myriocin-dependent partitioning. (D) Myriocin effect on PLP is not affected by co-transfection with MAL. Bars represent average \pm s.e.m.; circles represent means of individual experiments (>10 vesicles each). $**p < 0.01$.