The myelin proteolipid plasmolipin, forms oligomers and induces liquid ordered membranes in the Golgi apparatus

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Abstract
Myelin is comprised of a compactly stacked massive surface area of protein-poor, thick membrane that insulates axons to allow fast signal propagation. Increasing levels of the myelin protein plasmolipin (PLLP) were correlated with post-natal myelination. However its function is unknown. Here, the intracellular localization and dynamics of PLLP was characterized in primary glial and cultured cells using fluorescent protein (FP) tagged PLLP and anti-PLLP antibodies. PLLP localized to and recycled between the plasma membrane (PM) and the Golgi apparatus. In the Golgi apparatus PLLP forms oligomers based on fluorescence resonance energy transfer (FRET). PLLP oligomers blocked Golgi to PM transport of the secretory protein VSVG, but not a VSVG mutant with an elongated transmembrane domain. Laurdan staining analysis showed that this block is associated with PLLP-induced proliferation of liquid-ordered membranes. These findings show the capacity of PLLP to assemble potential myelin membrane precursor domains at the Golgi apparatus via its oligomerization and attraction of liquid ordered lipids. These data support a model whereby PLLP functions in myelin biogenesis by organization of myelin liquid ordered membranes in the Golgi apparatus.
Introduction

Myelin is composed of stacked tightly layered membranes that surround axons multiple times. These membranes are essentially a very large domain within the surface membrane of oligodendrocytes or Schwann cells in the central and the peripheral nervous systems, respectively (Aggarwal et al., 2011a). Hence, each myelin-forming cell has to synthesize and later maintain the enormous mass of membranes comprising the myelin. The main function of myelin is to insulate axons and limit sodium channels to distinct clusters known as nodes of Ranvier (Eshed-Eisenbach and Peles, 2013). This allows rapid advance of action potential between the nodes. This structure of myelin is fundamental for the ability of the nervous system to rapidly process and respond to signals in a compact and dense environment. The fundamental importance of myelin is emphasized by the severe consequences of various demyelinating diseases such as multiple sclerosis and other neuropathies (Simons et al., 2002). The lipid and protein composition of the myelin membrane is unique although it has some similarities with epithelial apical membranes (Frank et al., 1998). Unlike most biological membranes myelin is relatively protein poor (Aggarwal et al., 2011c). Myelin is highly enriched in sphingolipids and glycosphingolipids of the galactosylceramides and sulfatides species (Aggarwal et al., 2011a). Another key feature of myelin lipids is that the N-linked fatty acyl moieties are usually fully saturated and belong to the very-long-chain fatty acids type (22-26 carbons chain length) (Yurlova et al., 2011). Myelin membranes are highly enriched with the plasmalogen form of phosphatidylethanolamine. These have been suggested to contribute to the rigidity of the myelin membrane as well as to protection against fatty acid oxidation. A significant part of myelin integral-membrane proteins include tetra-spanning proteins. Of those, the most characterized are the myelin proteolipid protein PLP1, its splice variant DM20 (Van Dorselaer et al., 1987) and the MARVEL family proteins myelin and lymphocyte associated protein (MAL) and plasmolinopin (PLLP) (Perez et al., 1997). Like other MARVEL family proteins MAL and PLLP are also found in epithelial cells (Bosse et al., 2003). MAL is associated with the establishment and maintenance of the polarized phenotype in epithelial and other cells. The mechanism of MAL function was demonstrated to be the formation of membrane platforms via oligomerization and interaction with the surrounding membrane lipids (Magal et al., 2009). The function of plasmolinopin is essentially unknown. Sequence analysis shows 29 % identity and 49 % similarity between human PLLP and MAL. As in all other MARVEL proteins PLLP is very hydrophobic as it partitioned with lipid fractions in myelin tissue extracts. Post-natal increased levels of PLLP
were correlated with myelin development in both the central and peripheral nervous systems (Sapirstein et al., 1992a).

In this study, we test the hypothesis that the function of PLLP in myelin biogenesis is the generation of myelin precursor membrane domains in the Golgi apparatus. To this end, we have expressed PLLP-tagged with fluorescent proteins in primary Schwann cells co-cultured with neurons or in COS7 cells. We found that endogenous PLLP localizes to the PM and the Golgi apparatus. Fluorescence recovery after photobleaching (FRAP) analysis demonstrated that PLLP recycles between the Golgi and the PM. FRET analysis indicates that PLLP forms oligomers. Mutagenesis of conserved aromatic amino acids within the MARVEL motif affected FRET efficiency. Coexpression of PLLP-FP with the myelin proteolipid PLP1, revealed distinct segregation of these two proteins in the PM: PLP1 had a strong preference to high curvature PM sections from which PLLP was almost excluded. Finally, expression of PLLP in COS7 cells blocked the secretory transport of vesicular stomatitis virus G protein (VSVG) at the Golgi entry stage but not of a mutated VSVG with an extended transmembrane domain. In polarized Madin-Darby canine kidney (MDCK) cells, this VSVG mutant was sorted to the apical membrane. Thus, we propose that PLLP functions in myelin biogenesis by the assembly at the Golgi apparatus of myelin liquid ordered membrane domains.
Results

To characterize the intracellular localization of PLLP, a Green fluorescent protein (GFP) tagged version was inserted into the pMX retroviral vector. Retroviruses containing PLLP-GFP were used to infect Schwann cells in a glia-neuron co-culture. Figure 1A shows a three-week-old culture, 10 days after induction of myelin. Several PLLP-GFP expressing elongated membrane structures are visible, some of which are co-labeled with myelin-associated glycoprotein (MAG), an early and non-compact myelin marker. A similar colocalization is observed with a compact myelin marker, myelin basic protein (MBP). Figure 1B shows that in young co-cultures prior to myelin induction, PLLP-GFP is found in the PM with a significant fraction localized to internal membranes. This fraction at least in part colocalizes with the Golgi apparatus marker p115. We found that in COS7 cells as well as in other cell types (not shown), PLLP-GFP localizes to the PM and the Golgi apparatus. To further confirm this localization we used CFP-tagged galactosyltransferase (GalT-CFP), a marker for Golgi membranes. Fig. 1C shows colocalization of PLLP-mCherry with GalT-CFP (Pearson’s coefficient for Golgi area only is 0.75). The cell surface PLLP fraction was colocalized with Alexa488-modified cholera toxin B subunit.

Next, we asked to examine the localization of endogenous PLLP. Fig. 2 shows results for immunofluorescence analysis using a specific anti PLLP antibody. Figures 2A through C show localization of PLLP to myelin membranes as well as localization to p115-positive Golgi membranes. Golgi localization was more prominent in undifferentiated Schwann cells. Also, myelin staining of PLLP was seen at early stages of differentiation. MBP-positive myelin was strongly labeled with PLLP as well. Analysis of PLLP in sciatic nerve from a MAG-GFP transgene mouse shows colocalization of MAG and PLLP in a population of smaller diameter myelinated axons (Figure 2D). Moreover, concentration of PLLP to structures associated with non-compact myelin is in line with the finding that PLLP may localize to myelin membrane zones that maintain the ability to circulate with endomembranes.

A fraction of PLLP-GFP is found in dynamic tube-shaped membrane structures that seem to follow microtubule tracks (Fig. 3A). These tube shaped membranes depend on polymerized microtubules as they disappear upon addition of nocodazole, a microtubule polymerization inhibitor. The tubular PLLP-GFP membrane structures did not colocalize with clathrin light chain (not shown), early endosome antigen 1 (EEA1) or caveolin (Fig. 3B). However, some colocalization was observed with flotillin (Figure 3C) a cytosolic PM peripheral protein that is associated with the formation of cholesterol-rich microdomains as well as with a unique
endocytic pathway (Glebov et al., 2006). We next asked to verify the nature of the Golgi localization of the PLLP-GFP. To distinguish between saturation of secretory transport due to overexpression and steady-state localization due to recycling we used FRAP. The Golgi population of PLLP-mCherry was photobleached in a cell coexpressing the Golgi protein GalT-CFP. The Golgi localized population of PLLP recovered within a timescale of 125 minutes based on a fit to an exponential equation (Fig. 4A and B). This time scale is comparable to overexpressed glucosylphosphatidylinositol (GPI)-anchored anchored GFP that has a PM to Golgi recycling time of about 200 min (Nichols et al., 2001). Thus, the steady state Golgi localization of PLLP is an outcome of its constant recycling from the PM. 

Next, we asked to compare the intracellular localization of PLLP with an abundant myelin protein, the myelin proteolipid PLP1. PLP1 is also a tetraspanning protein that is found only in myelin. Like PLLP, PLP1 was reported to partition to detergent resistant membranes (Simons et al., 2002). Coexpression of PLLP and PLP1 resulted in both proteins efficiently arriving at the PM although PLP1-positive intracellular membranes did not colocalize with PLLP (Fig. 5A). At the PM, PLLP and PLP1 displayed different preference with regard to PM domains: While PLLP was evenly distributed throughout the entire PM, PLP1 is seen concentrating at the contour of the cell as well as in tubular PM protrusions. Figure 5B shows a line scan analysis showing the specific increase of PLP1 membrane concentration and not of PLLP. The increase of PLP1 in the contour of the cell is clearly demonstrated in Figure 5C. The partitioning of PLP1 to high curvature PM areas was not facilitated by PLLP as it occurred when expressed alone or with other PM markers (not shown). These data indicate that PLLP has no preference for high curvature membranes as in the case of PLP1. Despite a high level of homology to human MAL (46%) and their similar localization to epithelial apical and myelin membranes, the intracellular localization of these proteins differs considerably. MAL localizes to the cell surface and to Rab11-positive endosomal sorting compartments, while PLLP localizes to the PM, a tubular endocytic compartment and the Golgi apparatus. Oligomerization plays an important role in the function of MAL. The propensity of MAL to form oligomers is at least in part facilitated by aromatic amino acids within \( \Phi XX \Phi \) motifs, known to mediate intra-membrane helix-helix interactions (Sal-Man et al., 2007). Interestingly, PLLP contains multiple \( \Phi XX \Phi \) motifs as well as a high content of aromatic amino acids that are highly conserved throughout evolution (see alignment in Fig. 6A). We tested if PLLP could form oligomers as well. To this end, we applied FRET analysis using a stepwise acceptor photobleaching technique. Principally, the acceptor protein is
successively and moderately photobleached within a region of interest (ROI) and the donor fluorescence channel is recorded after every bleach cycle. As shown in Figure 5B, a rectangle ROI over the Golgi apparatus was repeatedly photobleached in a cell expressing PLLP-GFP as a donor and PLLP-mCherry as the acceptor. The upper panel shows the entire cell with the bleach box while the lower panel shows the separate donor and acceptor channels. The increase in fluorescence observed post bleach in the donor channel converted to a pseudocolor lookup table is indicative of FRET. Figure 6C shows a typical quantitative analysis of the donor and acceptor FRET efficiency and acceptor residual fluorescence values, respectively. To demonstrate that the FRET is not an artifact of the formaldehyde fixation, we carried out FRET experiments in living unfixed cells (Fig. 6D). Principally, similar FRET values were obtained for both fixed and unfixed cells. However, it was difficult to perform a reproducible quantitative analysis in living unfixed cells due to the fast membrane diffusion. These data demonstrate the self-association of PLLP. To demonstrate that the FRET interaction represents specific interactions and is not a result of simple crowding of donor and acceptor molecules, we compared the FRET efficiency of PLLP pairs with PLLP and either VSVG or VSVGln4 (Figure 6E). The later is a VSVG mutant with extra 4 amino acids added to its TMD (Magal et al., 2009). The FRET efficiency of PLLP with VSVG was over an order of magnitude lower than PLLP donor and acceptor pairs. For PLLP and VSVGln4, FRET efficiency was higher than VSVG but still about 6 fold lower than PLLP pairs. These data suggest that the FRET between PLLP donor and acceptor denotes specific interactions. In MAL, elimination of \(\Phi XX\Phi\) motifs using mutagenesis resulted in a significant decrease in oligomerization (Magal et al., 2009). While mutagenesis of aromatic amino acids in the occludin MARVEL motif had a minor effect on oligomerization (Yaffe et al., 2012). We therefore tested if PLLP oligomerization is facilitated by aromatic amino acids. Figure 6F shows a predicted structural model of PLLP generated using the Rosseta software package. In the model, two mutant proteins each with a set of 4 mutated aromatic amino acids are shown labeled in yellow and red. The mutant marked M1 in Fig. 6G contains the mutations W68A/F71A/Y162A/W165A, (Fig. 6F, amino acids labeled with yellow) which comprise of two pairs of conserved \(\Phi XX\Phi\) motifs. The mutant M2 (Fig. 6G) containing 4 conserved yet scattered aromatic amino acids (Fig. 6F, red) W52A/F75A/F119A/F146A. All of the abovementioned aromatic amino acids were substituted by alanines. FRET analysis between a wild type donor or acceptor and a mutant acceptor or donor, respectively, was carried out. FRET efficiency was reduced by 20 % and
60% for pairs consisting a wild type and either M1 or M2 respectively. Surprisingly, FRET efficiency of an M1 mutant donor and acceptor pairs was about 60% higher than control. A plausible explanation is that the mutated ϕXXϕ aromatic amino acids are pointed outwards. Consequently, they contribute to the oligomerization interaction, but at the same time impede direct helix-helix contact. In other words, the absence of the motifs in both donor and acceptor (M1-M1) resulted with a tighter conformation of oligomerization yielding higher FRET efficiency values. These data establish the formation of PLLP oligomers at the Golgi and the PM. We have previously shown that the direct consequence of MARVEL protein oligomerization is the formation and stabilization of liquid ordered thick membrane domains (Aranda et al., 2011; Magal et al., 2009). Thus, we asked if PLLP oligomerization affected the membrane environment of the Golgi apparatus. To this end, we followed the secretory transport of the thermoreversible VSVG protein mutant VSVGtsO45-YFP (Hirschberg et al., 1998; Presley et al., 1997) in cells coexpressing PLLP. VSVG is a transport competent membrane protein that is excluded from liquid ordered domains as well as from Triton-X-100 detergent resistant membranes (Dukhovny et al., 2006; van Meer and Simons, 1982). It is targeted to the basolateral membrane in polarized epithelia. After overnight accumulation in the endoplasmic reticulum (ER) membranes at the non-permissive temperature of 39.5 °C, cells were transferred to permissive temperature (32 °C). At this temperature VSVG is properly folded leading to its release from the ER. As shown in Fig. 7A and in the supplementary movies 1 and 2, in cells coexpressing PLLP, VSVG-YFP secretory transport was completely blocked from passing through the Golgi. As a consequence, VSVG accumulated in membranes at the Golgi circumference as well as in pre-Golgi compartments (Fig. 7B-C). This was confirmed by quantitative analysis of Golgi fluorescence intensity. In a cell not expressing PLLP a clear peak of VSVG could be seen at about 20 min after the beginning of the experiment. However in a cell expressing PLLP the Golgi fluorescence intensity accumulated and did not decline for the duration of the experiment (Fig. 7C). Throughout the experiment VSVG remained at this localization and apparently did not arrive at the PM. We hypothesized that PLLP oligomerization and accumulation in the Golgi as well as its association with lipids may create a thick liquid-ordered lipid environment. This lipid environment blocks the passage of VSVG. VSVG is targeted to the basolateral PM pole in polarized MDCK cells (Compton et al., 1989; Thomas et al., 1993). We thus asked if elongation of the VSVG TMD by 4 hydrophobic amino acids (VSVGIn4) may also allow its partitioning into thick liquid-ordered membrane domains in the Golgi thereby redirecting it to
the apical instead of the basolateral PM domain in polarized epithelia. To this end, the sorting of VSVGIn4 was tested in polarized MDCK cells grown on Transwell filters. Figure 7D shows that in a MDCK line stably expressing the apical marker GPI-YFP, VSVGIn4 colocalizes with GPI-YFP at the apical PM domain. Next, we tested our hypothesis that PLLP induce proliferation of thick liquid ordered lipid domains by analyzing its effect on secretory transport of VSVGIn4. We anticipated that PLLP-mediated proliferation of liquid ordered domains would not block VSVGIn4. The effect of PLLP on VSVGIn4 is shown in Figure 6E and F as well as in supplementary movie 3. Unlike wild type VSVG, VSVGIn4 effectively transports through the Golgi apparatus despite the presence of PLLP in the Golgi.

To rule out that the effect is a result of different levels of PLLP in the Golgi, VSVG-CFP and VSVGIn4YFP were coexpressed in PLLP-mCherry expressing cells. Supplementary figure S1A shows that while VSVG is restricted to the periphery of the Golgi apparatus VSVGIn4 and PLLP overlap over all Golgi membranes. Supplementary figure S1B shows the Pearson coefficient values for all pair combinations of the three proteins. Here, the coefficients were obtained for the Golgi ROI. PLLP and VSVGIn4 had a high value of 0.83-0.89 for the duration of the experiment. Finally to demonstrate that PLLP block of VSVG is via induction of liquid ordered membranes we carried out staining with the Laurdan dye to observe change in Golgi membrane degree of order. Fig. 7G shows that in PLLP-mCherry expressing cells a significant increase in Golgi membrane generalized polarization (GP) values (from 0.05 ± 0.01 to 0.15 ± 0.01 S.E.M.) is observed.

In summary, we propose that the self-association and induction of liquid ordered membrane domains in the Golgi apparatus point to PLLP as a key protein in myelin membrane biogenesis. In the Golgi apparatus, PLLP assembles myelin membrane domains to be delivered as membrane carriers to the rapidly growing myelin.
Discussion

PLLP is a typical MARVEL protein: It is small, hydrophobic, has four transmembrane spanning helices, high content of conserved aromatic amino acids some in ΦXXΦ motifs, forms oligomers and associates and attracts liquid ordered lipids. All these attributes, the high identity/homology to MAL and the expression in myelin and epithelia amount to the function of generation of functional protein-lipid membrane domains. We propose that PLLP forms oligomers at the Golgi apparatus and attracts liquid-ordered lipids such as cholesterols and long saturated fatty-acyl chain sphingolipids. In Schwan cells, these protein-lipid domains are essentially myelin membrane domains. These are then delivered to the cell surface creating the massive flux of membranes required during myelin biogenesis. PLLP is then recycled to the Golgi apparatus for additional rounds of myelin membrane domain assembly and delivery. Another non-exclusive proposed function might be that PLLP is a myelin membrane thickness sensor as was suggested for MAL (Magal et al., 2009; Mitra et al., 2004).

Myelin proteolipids were discovered over 60 years ago (Folch and Lees, 1951). One myelin proteolipid was later named lipophilin (Boggs and Moscarello, 1978) and than PLP1 after the sequencing of the gene (Naismith et al., 1985). PLLP was primarily identified in kidney samples and then found to be part of the myelin proteolipid proteins (Cochary et al., 1990). Despite all the data accumulated, very little is known about the function of PLLP or any other proteolipid in myelin biogenesis. Here we used expression of fluorescently labeled PLLP in both COS7 cells and in primary Schwan cells-neurons cocultures. The later allows induction of myelin formation and specific expression of proteins in the Schwann cells using retroviral vectors. PLLP is mainly a PM protein that recycles through Golgi membranes via a tubular endocytic organelle. Its recycling from the PM is supported by the findings that it serves as a murine viral receptor (Miller et al., 2008) and that it co-isolates with endocytic markers (Sapirstein et al., 1992b). Recently PLLP was demonstrated to regulate epithelial development through regulation of endocytosis in Zebra fish (Rodriguez-Fraticelli et al., 2015). Golgi labeling was apparent in young Schwann cells and to a lesser extent in older cultures. It is conceivable that the Golgi recycling is more intense during myelin biogenesis and less during its maintenance. Another explanation may be that the Golgi population of FP-PLLP is masked by the PM-myelin localized bright fluorescence. Nevertheless, the recycling of PLLP is a hallmark of a protein that is associated with orchestrating a biosynthetic flux of membrane. The partial colocalization of PLLP with flotillin may suggest a non-chlathrin,
non-caveolin PM to Golgi pathway (Otto and Nichols, 2011). Flotillin is associated with adhering to the inner leaflet of cholesterol rich domains. Thus, the interaction with a MARVEL family protein is imaginable.

Oligomer formation was demonstrated in this work by using FRET. In the case of PLLP the FRET signal was rather robust. Oligomerization of MARVEL proteins may be driven at least in part by hydrophobic mismatching interactions between TMDs and fatty acyl chains of lipids (Milovanovic et al., 2015). However, it was shown that an intrinsic propensity to self-associate is a prerequisite for mismatching-driven oligomerization. We have previously shown this by mutagenesis of MAL and modifications of the lipid content by the addition of cholesterol as well as short and extra long chain ceramides to its surrounding membrane (Magal et al., 2009). Here we show that mutagenesis of conserved aromatic amino acids affected the oligomerization. The attraction of lipids with long and saturated fatty acyl chains promoting liquid ordered membrane formation is another manner by which integral membrane proteins reduce the hydrophobic mismatching driven tension. Thus, the final product of mismatching-driven oligomerization and attraction of distinct lipid species is the formation of a large and stable membrane domain that may have various biological functions. PLLP expression in COS7 cells resulted in a significant proliferation of liquid ordered membranes that resulted in blocking of the transport of VSVG-YFP through the Golgi apparatus. This was shown by the fact that elongation of the VSVG TMD by four amino acids eliminated the effect. Thus, we assert that PLLP recycling, oligomerization and proliferation of liquid ordered membranes in the Golgi serve as evidence supporting its function in myelin biogenesis, a process where massive amounts of membranes is synthesized, sorted and delivered.

Co-expression of PLLP with PLP1 demonstrated distinct distributions within the PM. It was reported that as for PLLP, PLP1 was isolated in detergent resistant membranes (Schneider et al., 2005). PLP1 and PLLP are mainly PM proteins in COS7 cells however within the cell surface they clearly segregate at the contour of the cell where PLP1 is enriched. Tubular PM protrusions were dominated by high concentrations of PLP1 and reduced PLLP. Membrane tubes and the contour of the cell have increased curvature compared to the near zero curvature of the COS7 flat PM. This partition of PLP1 was independent of PLLP. Besides its identical topology to PLLP, PLP1 has no sequence similarity. The function of the myelin proteolipid PLP1 is unknown as well. However its partitioning to high curvature PM zones suggests a role in the stabilization of the myelin sheets in its tightly rolled form or alternatively in stabilization of distinct myelin high curvature subdomains. Nevertheless, the
function of PLP1 is distinct from that of PLLP. An exciting testable hypothesis is that the tetraspanning myelin proteins PLLP and PLP1 function in establishing the composition and the shape of myelin, respectively.
Materials and Methods

Reagents and constructs
Plasmolipin-GFP was kindly provided by Prof. Miguel Angel Alonso, CBMSO Madrid. Human plasmolipin (NP_057077) was subcloned into pmCherry-C1, pECer-C1 or pEYFP-C1 (Clontech), using BspEI and BamHI restriction sites, and verified by sequencing.

Cell culture and transfections
To produce stably expressing mCherry-PLLP clone, 10 μg of the neomycin resistance-containing expression constructs were transfected to MDCK using lipofectamine 2000. Selection medium containing 0.8 mg/mL G418 (Gibco) was added 48 h after transfection. The cells were incubated with sodium butyrate for 2 to 6 h prior to the experiment. MDCK cells stably expressing mCherry-PLLP were grown on transwell filters (Corning) for 4 to 12 days.

Dissociated dorsal root ganglia cultures were prepared from rat embryos at day 15.5 of gestation. Dorsal root ganglia were dissociated and plated at a density of 4X10^4 per 13 mm slide, coated with matrigel (BD biosciences) and poly-L-lysine (Sigma). A day after plating, cultures were incubated with retrovirus-containing media supplemented with 5μg/ml polybrene (Sigma) for 2 h, for three consecutive days. During the first two days after plating cultures were grown in neurobasal medium (Sigma) supplemented with B-27, glutamax, penicillin/streptomycin (all from Gibco) and 50ng/ml nerve growth factor (NGF, Alomone Labs). Cultures were then grown for 8 additional days in BN medium containing Basal medium-Eagle (Sigma) insulin, transferrin and selenium (ITS) supplement (Sigma), glutamax (Gibco), 0.2 % bovine serum albumin (BSA, Sigma), 4 mg/mL D-glucose (Sigma), 50 ng/mL NGF and antibiotics. To induce myelination, cultures were grown in BNC medium, namely a BN medium supplemented with 15% heat inactivated fetal calf serum (replacing the BSA) and 50 μg/mL L-ascorbic acid (sigma). Cultures were fixed after 10 additional days with 4% paraformaldehyde (PFA) for 10 minutes at room temperature.

Site-directed mutagenesis
Mutagenesis was carried out on all FP-tagged plasmids using the QuickChange (Stratagene) mutagenesis kit. All of the forward primer sequences are listed in Primers List.

Retrovirus production
cDNA of PLLP-GFP was subcloned into a pMX-EGFP (Clontech), while excluding the EGFP cassette. For retroviral stock preparation, helper virus free Phoenix-Eco packaging cells were transfected with the pMX-PLLP-GFP construct using the CaPO4 method. Medium
was changed a day after transfection and collected after additional 24 h. Virus-containing media were centrifuged to remove cells and stored at −80°C until use.

**Immunofluorescent labeling**

Fixed cultures were permeabilized in methanol for 5 minutes at -20°C. Following 3 washes in phosphate buffered saline (PBS), cultures were incubated in blocking solution (PBS, 1% glycine, 5% normal goat serum, 0.1% Triton X-100) for 45 minutes at room temperature. Cultures were subsequently incubated over-night at 4°C with primary antibodies diluted in blocking solution. After extensive washing with PBS, cultures were incubated with fluorophore-conjugated secondary antibodies for 45 minutes at room temperature, and were next washed and mounted in elvanol (DuPont). Primary antibodies rabbit anti PLLP was from abcam. Primary antibodies rat anti MBP; rat anti Neurofilament-H; mouse anti MAG were all from Merck Millipore; Secondary antibodies Alexa Fluor 647 donkey anti rat and Cy3-donkey anti-mouse from Jackson Immunoresearch Laboratories.

**Confocal microscopy, time-lapse imaging, FRAP analysis and image processing**

Cells were imaged in Dulbecco's Modified Eagle's medium (DMEM) without Phenol Red but with supplements, including 20 mM HEPES, pH 7.4 or with Hybernate A medium (Gibco). Transfection and imaging were carried out in Lab-Tek chambers (Nunc). Fluorescence images were obtained using a confocal microscope (LSM model PASCAL or 510 META with an Axiovert 200 microscope; Carl Zeiss MicroImaging). Fluorescence emissions resulting from Ar 458 nm, 488 nm, 514 nm and 543 nm laser lines for ECFP, EGFP, EYFP and or mCherry respectively, were detected using filter sets supplied by the manufacturer. The confocal and time-lapse images were captured using a Plan-Apochromat 63°— NA 1.4 objective (Carl Zeiss MicroImaging). Image capture was carried out using the standard time-series option (Carl Zeiss MicroImaging). Temperature on the microscope stage was monitored during time-lapse sessions using an electronic temperature-controlled airstream incubator. Images and movies were generated and analyzed using the Zeiss LSM software, and ImageJ software (W. Rasband, NIH, Bethesda, MD). Long time-lapse image sequences were captured using the autofocusing function integrated into the ‘advanced time series’ macro set (Carl Zeiss MicroImaging, Inc.). For quantitative FRAP measurements, a 63°— 1.4 NA Plan-Apochromat objective was used. Fluorescence recovery in the bleached region during the time series was quantified using Zeiss LSM software. For presentation purposes, confocal images were exported in TIFF and their contrast and brightness optimized in Adobe Photoshop. For FRET analysis, cells were washed three times in PBS, fixed in 2% PFA (Merck) in PBS for 15 min and then washed two times with imaging buffer.
**FRET analysis**

For acceptor photobleaching FRET, cells grown on glass coverslips. The cells were fixed by addition of PFA to the medium to a final concentration of 4% for 15 min at room temperature. The cells were washed with PBS containing 1% FCS twice, and then with PBS and mounted onto the microscope. The mCherry–tagged acceptor was photobleached in a region of interest (ROIs) over the Golgi apparatus. The 488 nm or 514 nm laser line was used for the acceptor-photobleaching. FRET efficiency \( E \) was calculated from the CFP channel images according to:

\[
E = \frac{(F_{\text{post}} - F_{\text{re}})}{F_{\text{post}}}
\]

Where \( F \) is the fluorescence intensity of the GFP using 488-nm laser before \((\text{pre-bleach})\) and after \((\text{post-bleach})\) photobleaching of the mCherry using a high power 543-nm laser.

**Colocalization**

Colocalization was carried out using the Zeiss colocalization module. Values were obtained after thresholding background pixel values. As control an image of a cell expressing two FP-PLLp variants was used yielding a Pearson’s coefficient of 0.98.

**Site-directed mutagenesis**

Mutagenesis was carried out on GFP and mCherry tagged PLLP plasmids using the QuickChange (Stratagene) mutagenesis kit. Forward primer sequences are listed:

- **W68A/F71A**: 5’-CCTATGCGCGATGGCCGTCGCTGCTTC-3’;
- **Y162A/W165A**: 5’-CTTCTTCCAGGCACAGGCAGGCAGCGAGGAGTAG-3’;
- **W52A**: 5’-CTGGGGCTGCTGGTGGCTGCGCTGATTG-3’;
- **F75A**: 5’-GTTCGTCGCTGCTGCTGCGCGCTGCTGATTG-3’;
- **F119A**: 5’-CTCTACATCACCAGCGCGCATCGCCTGCTCTG-3’;
- **F146A**: 5’-GCTGCCTCGTTCGCTGCTGCTGCTGCTGATG-3’.

**Membrane labeling with cholera toxin B subunit (CTXB488)**

Cells at 60% confluence in Lab-Tek chambered glass coverslips (NalgeNunc International) were transfected with PLLP-mCherry using FuGENE6 and after 18 to 24 h, labeled with 1 \( \mu \)g/mL CTXB488.

**Laurdan analysis**

COS7 cells were treated with 5 \( \mu \)M Laurdan (Invitrogen, D250) in cell culture medium and incubated for 45 min at 37°C. Subsequently cells were fixed in 4% PFA and then imaged using a confocal laser-scanning microscope (SP5; Leica Microsystems) using a 63× glycerol-immersion objective with a numerical aperture (NA) of 1.3. Laurdan excitation at 780 nm was achieved using a femtosecond-pulsed titanium sapphire laser (Mai-Tai; Spectra-Physics).
Laurdan fluorescence of was detected simultaneously in two channels of 400–460 nm and 470-530 nm. GP images were constructed using an Image J plugin (Owen and Gaus, 2010). For each pixel, the GP was calculated from the two channel intensity values as

\[ GP = \frac{I_{400-460} - GI_{470-530}}{I_{400-460} + GI_{470-530}} \]

where \( I \) represents the intensity in each pixel acquired in the indicated channel and \( G \) is the G-factor or calibration factor. The G factor is then calculated using the following equation:

\[ G = \frac{GP_{\text{ref}} + GP_{\text{ref}}GP_{\text{mes}} - GP_{\text{mes}} - 1}{GP_{\text{mes}} + GP_{\text{ref}}GP_{\text{mes}} - GP_{\text{ref}} - 1} \]

Here \( GP_{\text{mes}} \) is the measured GP value of the reference sample Laurdan in dimethyl sulfoxide (DMSO).

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References


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**Figure 1. The intracellular localization of PLLP-FP.** A. Immunofluorescence analysis of a mouse co-cultured Schwann cells and neurons infected with the pMX retrovirus containing the PLLP-GFP (green) two weeks after induction of myelin formation. Cells were fixed, permeabilized and labeled with primary antibodies against the early myelin marker myelin associated glycoprotein MAG (Red, insert) or myelin basic protein MBP (red) and secondary fluorescently labeled antibodies as described in the methods section; arrowheads point to co-labeled myelin; B. Immunofluorescence analysis of mouse Schwann cells and neuron co-culture infected with PLLP-GFP (green) before induction of myelin. Cells were fixed, permeabilized and labeled antibodies against P115 (red) or neurofilament (blue) C. Fluorescence microscopy analysis of cells co-expressing GalT-CFP (top, left and green) and PLLP-mCherry (middle and red) or expressing PLLP-mCherry and labeled with Alexa488 modified cholera toxin B subunit (CTXb488, bottom, left panel and green) Bars=10 μm.
Figure 2. The intracellular localization of endogenous plasmolinip. A. Immunofluorescence analysis of mouse co-cultured Schwann cells and neurons two weeks after induction of myelin formation. Cells were fixed, permeabilized and labeled with primary antibodies against PLLP (green), the Golgi marker P115 (red), myelin basic protein MBP (blue) and secondary fluorescent labeled antibodies as described in the methods section; Images for separate channels are inverted. Arrowheads point to co-labeled Golgi membranes. Area within the rectangle is enlarged in B. C. Same as in A. rectangles surrounding Golgi-labeled areas are inverted to show localization of endogenous PLLP in the Golgi apparatus. D. Localization of PLLP in Sciatic nerve slices of a MAG-GFP (green) transgenic mouse labeled as described in A with anti-PLLP antibody (red). Arrows point to non-compact myelin structures (Schmidt-Lanterman incisures) co-labeled with MAG and PLLP. Arrowheads in inserts point to paranodal loops.
Figure 3. Characterization of the PLLP-FP tubular endocytic compartment. A. PLLP tubular endocytic compartment is aligned with polymerized microtubules. A living cell cotransfected with tubulin-GFP (green) and PLLP-mCherry (red) B. Colocalization of PLLP-mCherry (red) with GFP-tagged EEA1 (green top panel, Pearson’s colocalization coefficient 0.42) or Caveolin (green bottom panel, Pearson’s colocalization coefficient 0.87) C. Coexpression of PLLP-mCherry (Red) with Flotillin-GFP (green) Pearson’s colocalization coefficient 0.87. Inserts are enlarged and inverted areas showing colocalization Bars = 10 µm. Control for Pearson’s coefficient was coexpressed PLLP-mCherry and PLLP-GFP (0.98)
Figure 4. Cycling of PLLP-FP between the PM and the Golgi apparatus. A. FRAP of PLLP-mCherry in the Golgi apparatus of a cell coexpressing GalT-CFP (green) and PLLP-mCherry (red and inverted lower panel) Bars = 10 µm B. Quantitative analysis of the recovery of PLLP-mCherry. Average fluorescence intensity data (dots) was fitted with a single exponential equation $F_i = Mf \times (1 - e^{-kt})$. Time constant was 2.08 min.
Figure 5. Partitioning at the PM of the myelin proteolipids PLLP and PLP1. A. Segregation at the PM of PLLP and PLP1. Coexpression of PLLP-mCherry (red) and PLP1-YFP (green) in COS7 cells. Bars = 10 µm B. PLP1-YFP and not PLLP-mCherry partitions to high curvature PM edges. Line-scan analysis of fluorescence intensity of PLLP-mCherry (red line) and PLP1-YFP (green line). Graphs I and II correspond to lines I and II in the enlarged insert from A. Bars = 3 µm C. An enlarged insert from A showing the actual concentration of PLP1 at the rims and tubes of the PM. Bars = 3 µm.
Figure 6. Oligomerization of PLLP in the Golgi apparatus. A. Sequence alignment and transmembrane domain (TMD) predictions of PLLP proteins: 7 PLLP paralogs were aligned by MUSCLE and color-coded according to amino acids charge groups. Transmembrane (TM1-4) helix predictions for the human PLLP sequence were obtained by four different webservers (red lines under the alignment). Bars = 10 µm B. Stepwise acceptor photobleaching to detect FRET between GFP (green) and mCherry (red) tagged PLLP. Top panel is a typical cell showing the bleached rectangle. Donor and acceptor channels are separated and shown in the middle and lower panel, respectively. Arrow points to an area of increased fluorescence demonstrating FRET. C. Quantification of a typical FRET experiment. Red columns are the fraction of residual fluorescence intensity of the bleached acceptor. Green dots and line are the calculated FRET efficiency. D. Same as in C except that the experiment was carried out on a living unfixed cell. E. Specificity of FRET between
PLLP molecules. Dot plot analysis of FRET efficiency between overexpressed proteins at the PM. FRET was measured as described for PLLP-YFP and PLLP-mCherry (filled circles, n=10), VSVG-YFP and PLLP-mCherry (filled triangles, n=10) and VSVGIn4-YFP and PLLP-mCherry (filled diamonds, n=4). F. *Ab initio* structure determination for human PLLP: TM1 - gray, TM2 - cyan, TM3 - blue and TM4 - dark blue. Phenylalanines W68A/F71A and Y162A/W165A are shown by yellow colored sticks. Phenylalanines W52A, F75A, F119A and F146A are shown by red colored sticks. Upper panel is a view of the two structural models from outside the cell. G. The effect of mutagenesis of two mutant PLLP molecules (M1, labeled yellow in 6F and M2, labeled red in 6F) on the FRET efficiency between GFP and mCherry-tagged PLLP. In each mutant four aromatic amino acids were replaced by alanine. Data shown is average ± standard deviation (n = 10-12). FRET efficiency is calculated as described in the Methods section. Green columns represent FRET efficiency. Black columns represent control FRET efficiency out of bleach box and red columns represent average residual acceptor.
Figure 7. PLLP blocks Golgi to PM transport of VSVG but not transport of VSVGIn4 with a longer transmembrane domain, by inducing proliferation of liquid ordered membranes. A. VSVG-YFP is accumulating at the Golgi periphery in PLLP-mCherry expressing cells. VSVG-YFP (green) and PLLP-mCherry (red) were coexpressed in COS7 cells. Cells were imaged after overnight incubation at 39.5°C to accumulate VSVG in the ER and shift to 32 °C. Shown is a single image from the time-lapse sequence movie 1 in the supplementary information section. Bars = 10 µm. B. Golgi to PM transport of VSVG-YFP (green) is blocked in cells expressing PLLP-mCherry (red). Time-lapse analysis of a living cell expressing VSVG-YFP with or without coexpression of PLLP-mCherry (time-lapse sequence movie 2 in the supplementary information section). Fluorescence intensity in the yellow circles surround the Golgi apparatus in each cell are shown in C. Top is the left hand cell that does not express PLLP and bottom graph is the cell coexpressing PLLP-mCherry. D.
The VSVGIn4-mCherry mutant with an elongated transmembrane domain is sorted to the apical membrane in polarized MDCK cells. VSVGIn4-mCherry (red) was expressed in filter grown polarized MDCK cells stably expressing the apical marker GPI-YFP (green). Shown is a three dimensional reconstruction with three sections YZ (Side) and XY at the apical and lateral plains. Bars = 10 µm E. Secretory transport of VSVGIn4-mCherry is not blocked by PLLP-mCherry. Coexpression of VSVGIn4-YFP (green) and PLLP-mCherry (red). Selected images from a time-lapse sequence (Movie 3, supplementary information section) at the designated times after shift from 39.5°C to 32°C. Lower panel showing VSVG moving from the ER through the Golgi to the PM. Bars = 10 µm F. Quantitation of the Golgi fluorescence in E. G. Analysis of membrane order using Laurdan analysis. COS7 cells expressing (red filled circles) or not expressing (black filled circles) PLLP-mCherry were labeled with laurdan, imaged and processed as described in the Methods section. Shown are values ± standard error of mean.