# Sorting and trafficking of proteins in oligodendrocytes during myelin membrane biogenesis

Klunder, Lammert

# *Chapter 4*

# Involvement of PLP-sulfatide microdomains and transcytosis in myelin sheet assembly

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Part of this chapter has been submitted for publication

**Abstract**

During the process of myelination of axons by oligodendrocytes (OLGs) in the central nervous system, myelin-specific proteins and lipids are sorted and transported from their site of synthesis, the OLG cell body, to their site of destination, the myelin sheath. Insight into the mechanism of these events is crucial for the development of therapeutic approaches in devastating demyelinating diseases, like multiple sclerosis. Here, we demonstrate in cultured OLGs, grown on different substrates of extracellular matrix proteins, that transcytotic transport of the major myelin resident protein, PLP, from cell body plasma membrane to myelin sheet is a key step in the mechanism of proper myelin assembly. By carrying out pulse chase experiments, in conjunction with biotinylation to determine and characterize its surface pool, our data reveal that following biosynthesis, PLP is transported to the cell surface of the cell body in microdomains that were operationally defined as Triton X-100 resistant. The latter was confirmed by in situ detergent extraction. The involvement of sulfatide in this process was suggested by the observation of plasma membrane-directed PLP transport in the oligodrendoglia derived cell line OLN-93, overexpressing ceramide sulfatide transferase but not in cells overexpressing ceramide galactosyl transferase. Interestingly, PLP only transiently associated with the Triton X-100 resistant microdomains at the plasma membrane, its lateral dissipation being followed by subsequent re-internalization and transport towards the sheet. We propose a model in which PLP traffics to the myelin sheet via syntaxin 3 mediated docking at the cell body plasma membrane, involving a transcytotic transport mechanism, consistent with the polarized nature of oligodendrocytes.

**Introduction**

Oligodendrocytes (OLGs), the glial cells in the central nervous system (CNS), synthesize a multilamellar membrane structure called the myelin sheath, which wraps around the axons thereby facilitating rapid saltatory conduction. Myelin, which is essential for proper functioning of the nervous system, is a specialized membrane structure, being enriched in glycosphingolipids and cholesterol and containing myelin-specific proteins (Lees and Brostoff, 1984; Norton and Cammer, 1984; Simons *et al.*, 2000; de Vries and Hoekstra, 2001). Since myelin proteins, except MBP, are synthesized in the OLG cell body, followed by subsequent transport to the myelin sheet, it is evident that sorting and transport of proteins and lipids to the myelin membrane must be carefully regulated. In previous work we have shown (Klunder *et al.*, submitted; chapter 3) that the t-SNAREs syntaxin 3 and 4, as part of the docking and fusion machinery of transport vesicles for membrane proteins (Low *et al.*, 1996; Madison *et al.*, 1999), are intimately involved in intracellular transport and sheet delivery of the major myelin protein PLP and MBP mRNA, respectively, the latter being locally expressed in the sheet upon arrival. In OLGs, which can be considered as polarized cells (de Vries *et al.*, 1998; Krämer *et al.*, 2000; Kroepfl and Gardinier, 2001), syntaxin 3 was found to localize largely at the plasma membrane of the OLG cell body and the primary processes, whereas syntaxin 4 was enriched at the myelin sheet (Klunder *et al.*, submitted; chapter 3). Particularly intriguing, this work indicated that transport of PLP which is synthesized at the rough endoplasmic reticulum (RER), processed through the Golgi apparatus (Colman *et al.*, 1982; Nussbaum *et al.*, 1983; Schwob *et al.*, 1985), depends on syntaxin 3. In fact, the data suggested that PLP transport to the sheet could rely on a transcytotic mechanism. Intriguingly, the previous data also suggested that in this transport process, PLP transiently associated with microdomains that displayed Triton X-100 (TX-100) resistance. Hence, this transient association of PLP with TX-100 resistant domains may (at least partly), in conjunction with a gradually expanding panel of non-ionic detergents, used to identify such microdomains (Simons *et al.*, 2000), explain the controversy as to the extent to which PLP transport is coupled to glycosphingolipid transport, the lipid being considered as a typical component of TX-100 resistant micordomains (Pasquini *et al.*, 1989; Brown *et al.*, 1993; van der Haar *et al.*, 1998; Bansal *et al.*, 1999).

Thus far, knowledge as to how myelin biogenesis is regulated and maintained has been scanty. Obviously, such insight will be essential in improving our insight in the etiology

of several neurological diseases. In particular, failure of remyelination seems to be an underlying cause of the detrimental consequences of such diseases. Accordingly, at least for therapeutic purposes, fundamental insight into molecular events related to the onset and maintenance of myelin sheet biogenesis are crucial. The present work was undertaken to further corroborate previous observations on the potential involvement of a transcytotic transport mechanism in myelin assembly.

## Materials and Methods

### Materials

Dulbecco's Modified Eagle's Medium (DMEM, with 4500 mg/l Glucose and L-glutamine), L-glutamine, penicillin/streptomycin and G418 were purchased from Gibco Invitrogen Corporation (Paisley, UK). Fetal calf serum (FCS) was obtained from Bodinco (Alkmaar, The Netherlands). Growth factors FGF-2 and PDGF-AA were supplied by PeproTech Inc. (London, UK). Protease inhibitor cocktail tablets (Complete Mini) were obtained from Roche Diagnostic Corp (Mannheim, Germany). Nonidet P40 (NP40) was purchased from Fluka BioChemica (Buchs, Zwitserland). Sulfo-NHS-LC-Biotin was obtained from Pierce (Rockford, IL). Streptavidin was obtained from Upstate Lake Placid (New York, NY). Paraformaldehyde was supplied by Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Chemical Co. (St. Louis. MO).

### Antibodies

The polyclonal antibody against rat syntaxin -3 was a generous gift of Dr. Thomas Weimbs (Department of Molecular Medicine, Cleveland). The monoclonal antibodies against PLP (Greenfield *et al.*, 2006) and TGN-38 (IgG1, Luzio *et al.*, 1990) were generously provided by Dr. V. Kuchroo (Center of Neurological Diseases, Harvard Medical School, Boston, MA) and Dr. George Banting (Bristol, UK), respectively. The monoclonal antibody anti-GFP was purchased from Roche Diagnostic Corp (Mannheim, Germany). The O4 hybridoma was a kind gift of Guus Wolswijk (NIBR, Amsterdam, the Netherlands). Fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) and Cy-3 conjugated secondary antibodies were supplied by Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Secundary horseradish peroxidase (HRP)-conjugated antibodies were provided by Amersham Biosciences (Buckinghamshire, UK).

**Constructs**

General procedures for cloning and DNA manipulations were performed as described by Sambrook *et al.* (1989). The cDNAs encoding galactosyl ceramide 3'-sulfotransferase (cst) and ceramide galactosyltransferase (cgt) were kind gifts of Drs. Matthias Eckhardt and Brian Popko (Chapel Hill, NC), respectively. For cloning, the cst and cgt genes were inserted into the retroviral vector pLXIN (Clontech Biosciences, Mountain View, CA). Recombinant plasmids were grown in TOP10 cells, and plasmids with the cDNA insert in the correct orientation with respect to transcription were identified by restriction analysis. The orientation and the integrity of the obtained pLXIN constructs were confirmed by DNA sequencing.

**Production of retroviral particles**

The production of retroviral particles and the subsequent infection of OLN-93 cells were performed according to Relvas *et al.* (2001). Briefly, for production of recombination-deficient retroviruses, the constructs were transfected into the GP+E86-packaging cell line (Genetix Pharmaceuticals, Inc. Cambridge, MA), using lipofectamine 2000 (Invitrogen, Breda, The Netherlands). Two days after transfection, cells were collected, diluted 5-fold and cultured under selection in packaging cells medium supplemented with 1 mg/ml G418 (corrected for inactivity) until resistant clones appeared (70 % confluent). The cells were subsequently washed with PBS, and packaging cells medium without G418 was added. The conditioned medium was collected after 24 hrs, filtered (Schleicher and Schuell, Dassel, Germany, 0.45 μm pore size), and either used immediately or stored frozen at -80 °C.

**Cell culture**

Primary cultures of OLGs were prepared from brains of 1-2 day old Wistar rats as described previously (Baron *et al.*, 1998), with slight modifications. Briefly, after decapitating the rats, the forebrains were removed and the cells were dissociated, first mechanically and then with papaine (30 U/ml) in the presence of L-cysteine (0.24 mg/ml) and DNase (10 μg/ml) for 1 hour at 37 °C. A single cell suspension was prepared by repeated pipetting in a trypsin inhibitor solution (1 mg/ml). After centrifugation the cells were resuspended in DMEM supplemented with 10 % FCS and seeded into 75 $cm^2$ flasks (Nalge Nunc International, Roskilde, Denmark), which were precoated with poly-L-Lysine

(PLL, 5 μg/ml), at approximately 1.5 brain per flask. The OLG progenitor cells appear as round-shaped, phase dark cells on top of a monolayer of flat type-1 astrocytes. After 11-12 days in culture, the OLG progenitor cells were isolated by mechanical shaking at 240 rpm and 18-20 hrs as described by McCarthy and de Vellis (1980). Contaminating astrocytes and microglia were subsequently removed by differential adhesion for 20 min at 37°C.

For biochemical analysis, the OLG progenitor cells were plated in proliferation medium (SATO medium (Buttery *et al.*, 1999) supplemented with the growth factors FGF-2 (10 ng/ml) and PDGF-AA (10 ng/ml)) on PLL or laminin-2 (Ln2, 10 μg/ml) coated dishes (Nalge Nunc, Naperville, IL) at a density of 1 X $10^6$ cells per 100 mm dishes. To differentiate the cells, the cells were cultured on SATO medium supplemented with 0.5 % FCS for 3 days (GC-stage), 7 days (MBP-stage) or 10 days ($MBP^+$-stage). For immunocytochemical studies the OLG progenitor cells were seeded at a density of 2 X $10^4$ cells per well on PLL- or Ln2-coated 8 well permanox chambers (Nunc) and treated as described above.

**Infection of OLN-93 cells**

The oligodendroglia derived cell line OLN-93, a kind gift of Dr. Christiane Richter-Landsberg (University of Oldenburg, Germany; Richter-Landsberg and Heinrich, 1996), were cultured in DMEM supplemented with 10% heat-inactivated FCS, L-glutamine, and the antibiotics penicillin and streptomycin. Cells were trypsinized when they reached near-confluency. GalCer and sulfatide expressing OLN-93 cells (OLN-G and OLN-GS, respectively) were obtained via retroviral infections with the plasmids pLXIN-cgt and pLXIN-cgt/pLXIN-cst respectively. A mock-OLN-93 cell-line was obtained by retroviral infection with vector only, pLXIN. Retroviral infections of OLN-93 cells were carried as described previously (Maier *et al.*, 2006). Briefly, OLN-93 cells at 50% confluency were exposed to retroviral particles (see above) and 8 μg/ml polybrene for 16-18 hrs. The cells were cultured for another 24 hrs, trypsinized and cultured in the presence of 2 mg/ml G418 (corrected for inactivity) for 14 days. Colonies were isolated and selected for the appearance of GalCer and/or sulfatide on the cell surface. Since cgt and cst mRNAs in OLN-93 are hardly detectable, for the sulfatide-expressing OLN-93 cells (OLN-GS), a retroviral infection with pLXIN-cgt followed by a second infection with pLXIN was necessary.

**Expression of PLP-GFP**

OLN-mock, OLN-G and OLN-GS (see above) cells were transfected with pEGFP-N1-PLP using lipofectamin 2000 as a transfection reagent. In short, cells cultured on an 8 well permanox chamber slides at a density of 10000 cells per well for 4 hrs, were incubated with lipofectamine 2000 and pEGFP-N1-PLP (pEGFP-N1-PLP was a kind gift of Niels Hellings, Biomedisch Onderzoeksinstituut and Transnationale Universiteit Limburg, Diepenbeek, Belgium) for 16-20 hrs. The cell were analysed by immunofluorescence with anti-GFP antibodies 48 hrs after transfection.

**Antibody perturbation experiment**

The hybridoma cells producing O4 monoclonal antibodies were grown in heat-inactivated FCS/DMEM, upon antibody production cells were grown without medium change for 2 weeks in the absence FCS. Antibodies were concentrated from the hybridoma culture supernatants by ammonium sulfate precipitation and dialysis to the appropriate buffer. OLG were cultured till the onset of myelin sheet formation, i.e. 3 days in differentiation medium (GC-stage). The cells were then further cultured in either control or antibody-containing (O4, 10 µg/ml) media for another 4 days (MBP-stage).

**Immunocytochemistry and in situ extraction**

Antibody staining of cell surface components was performed on live cells at 4°C. After blocking non-specific binding with 4% BSA in PBS, cells were incubated with O4 (anti-sulfatide antibody) for 30 min, washed three times and incubated for 25 min with FITC-conjugated goat-anti-mouse IgM. The cells were fixed with 4 % paraformaldehyde (pfa) in PBS for 20 min at room temperature (RT). For double or single staining of intracellular antigens, cells were fixed with 4% pfa, and permeabilized and blocked with 0.1 % TX-100 and 4% BSA in PBS for 30 min at RT. The cells were incubated for 1-2 hours with anti-PLP antibodies or anti-GFP antibodies at RT. The cells were washed with PBS and incubated with appropriate TRITC-conjugated secondary antibodies and DAPI for 25 min at RT. After washing with PBS, the cells were covered with 2.5 % 1,4-diazobicyclo[2.2.2]octane (DABCO) in 90 % glycerol/ 10 % PBS, to prevent image fading. For in situ extraction, cells were exposed to cold 0.5 % TX-100 in PBS for 2 min at 4 °C, followed by pfa fixation at 4 °C. The samples were analyzed with an immunofluorescence microscope (Olympus AX70),

equiped with analySIS software. Pictures were processed with Paint Shop Pro and/or Adobe Photoshop.

**OptiPrep density gradient centrifugation**

The cells were washed with PBS, harvested by scraping with a rubber policeman in 350 µl TNE-lysis buffer (20 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA supplemented with 1 % TX-100 and a cocktail of protease inhibitors), and pressed 18 times through a 21-gauge needle. Lysis was done on ice for 30 min and the protein content was determined by a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Herculas, CA), using BSA as a standard. For density gradient centrifugation a discontinous OptiPrep gradient (2.25 ml 10 %, 2.25 ml 30 %, and 750 µl 40 %) was prepared. For preparing 40 % OptiPrep 250 µl of total cell lysates (equal protein) were added to 500 µl of 60 % OptiPrep. This mixture was then overlaid with 30 % and 10 % OptiPrep, respectively. Gradients were centrifuged overnight at 40000 rpm (SW55 Beckman, 4 °C) and 750 µl gradient fractions were collected from the top (fraction 1) to the bottom (fraction 7). For Western blot analysis, equal fraction volumes were TCA precipitated, resuspended in SDS reducing sample buffer and analyzed by SDS-PAGE and Western blotting.

**TCA precipitation**

To concentrate proteins, the fractions were adjusted to a final volume of 1 ml with TNE and treated with DOC (25 mg/ml) for 5 min at 4 °C, followed by precipitation with 6.5 % trichloric acid (TCA) for 15 min at 4 °C. Precipitates were centrifuged for 20 min at 10000 rpm at 4°C. The pellets were dryed and resuspended in 2 X SDS reducing sample buffer. After the pH was adjusted to 6.8 by exposure to ammonia, the samples were heated for 2 min at 95 °C or 30 min at 37 °C and subjected to SDS-PAGE and Western blotting.

**Surface biotinylation**

Cells were differentiated for 3 or 10 days, washed twice with ice-cold PBS, and incubated for 1 hr with Sulfo-NHS-L-C-Biotin (0.1 mg/ml in PBS) at 4 °C. The cells were washed three times for 5 min with cell wash buffer (CWB, 65 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) to remove excess biotin and twice with PBS. The cells were harvested by scraping with a rubber policeman in 350 µl TNE-lysis buffer and pressed 18 times through a 21-gauge needle. Lysis occured on ice for 30 min and the protein

content was determined by a Bio-Rad DC protein assay (Bio-Rad, Herculas, CA), using BSA as a standard. Equal amounts of protein were subjected to OptiPrep density gradient centrifugation. Biotinylated proteins from equal volumes of the fractions were immunoprecipitated with streptavidin (SA)-agarose for 16-18 hrs at 4 °C. After centrifugation the SA-agarose beads (biotinylated proteins) were washed four times with CWB supplemented with 1% NP-40 and 0.35 M NaCl and once with PBS. Non-biotinylated proteins (supernatants) were concentrated by TCA precipitation. Samples from SA-agarose and supernatant fractions were mixed with SDS sample buffer, heated for 2 min at 95 °C or 30 min at 37 °C and were subjected to SDS-PAGE and Western blotting.

**SDS-PAGE, Western Blotting and dotblots**

Samples were loaded onto 10 % SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA) by semi-dry blotting. The membranes were blocked with 5 % nonfat dry milk in PBS to inhibit nonspecific binding, washed with buffer (0.1% Tween-20 in PBS), and incubated overnight at 4°C with primary antibody in 0.5 % nonfat dry milk in buffer, followed by a 2 hrs incubation with the appropriate horseradish peroxidase (HRP)-conjugated antibodies (1:2000 in 0.5 % nonfat dry milk in buffer). For GM1-dotblots, 5 μl of each fraction was spotted onto a nitrocellulose membrane (BioRad). The membranes were blocked with 5 % nonfat dry milk in Tris buffered saline (TBS) for 1 hr at RT, washed with 0.1% Tween-20 in TBS, and incubated with CTB-HRP (1:1000, Calbiochem-Novabiochem Corporation, La Jolla, CA) for 1 hr at RT. The signals were visualized by ECL (Amersham, Pharmacia Biotech) and films were processed with Adobe Photoshop and quantified with Scion Image Software.

**Pulse-chase experiment**

OLGs were cultured on 10 cm PLL-coated dishes for 3 days. The cells were pre-incubated in DMEM minus methionine (Invitrogen, Breda, the Netherlands) for 1 hour. Cells were labelled for 10 min in this medium with the addition of 200 μCi/dish Tran$^{35}$S-label (Amersham, BioSciences, Buckinghamshire, UK). The cells were then chased by replacing the labeling medium supplemented with 10 mM methionine (Merck, Darmstaft, Germany). The chase periods were 0, 15, 30 and 60 min. Cells were scraped in PBS, extracted with TNE-lysis buffer at 4°C, and separated by centrifugation into TX-100-soluble supernatants (S) and –insoluble pellets (I). PLP was immunoprecipitated with Protein G-sepharose beads

(Amersham, BioSciences, Buckinghamshire, UK) overnight at 4 °C from the TX-100-soluble and –insoluble fractions. Protein G-sepharose beads were washed four times with TNE-lysis buffer supplemented with 0.2 % SDS and once with PBS. Washed Protein G-sepharose beads were resuspended in SDS sample buffer, incubated 30 min at 37° C, and counted for 2 min in a microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT).

**Results and Discussion**

**Intracellular distribution and dynamic partitioning of PLP in detergent resistant membranes depends on cell development**

The multi-spanning membrane protein PLP is the major myelin protein and reaches this specialized membrane domain in OLGs by vesicular transport (Simons *et al.*, 2000). Its biosynthesis becomes distinctly apparent in newly formed OLGs, and rapidly increases when the cells further develop into mature myelinating cells. To accurately define its membrane trafficking pathway, following biosynthesis, we first determined the intracellular localization of PLP at various stages of OLG development in cells, grown on the inert PLL. Examination by immunofluorescence microscopy revealed (fig. 1) that in newly formed OLGs (3 days, GC stage), when processes develop, PLP is abundantly localized in vesicular structures in the perinuclear region of the cell (fig. 1A2), often showing a punctuate appearance when associated with primary processes. Furthermore, in a small subset of the cells, a prominent labeling of the plasma membrane was observed (fig. 1A1 and 1A3), suggesting a transient association of PLP with the plasma membrane of the cell body. In mature, well differentiated OLGs (7-10 days), the protein is present in the cell body, but particularly the processes and membrane sheets showed extensive PLP labeling with a diffuse appearance, suggesting its proper membrane integration (fig. 1B and C).

The primarily intracellular localization of PLP in newly formed OLGs was further confirmed by a biotinylation experiment, allowing a distinction to be made between surface localized (i.e., biotinylated) PLP versus intracellular (i.e., non-biotinylated) PLP by means of streptavidine precipitation (see Materials and Methods). To simultaneously obtain insight into the lateral membrane distribution of PLP, the biotinylated cells were extracted with TX-100 and the lysates were applied to an OptiPrep density gradient to determine whether PLP partitioned in detergent-resistant microdomains, as subsequently revealed by Western blotting analysis.

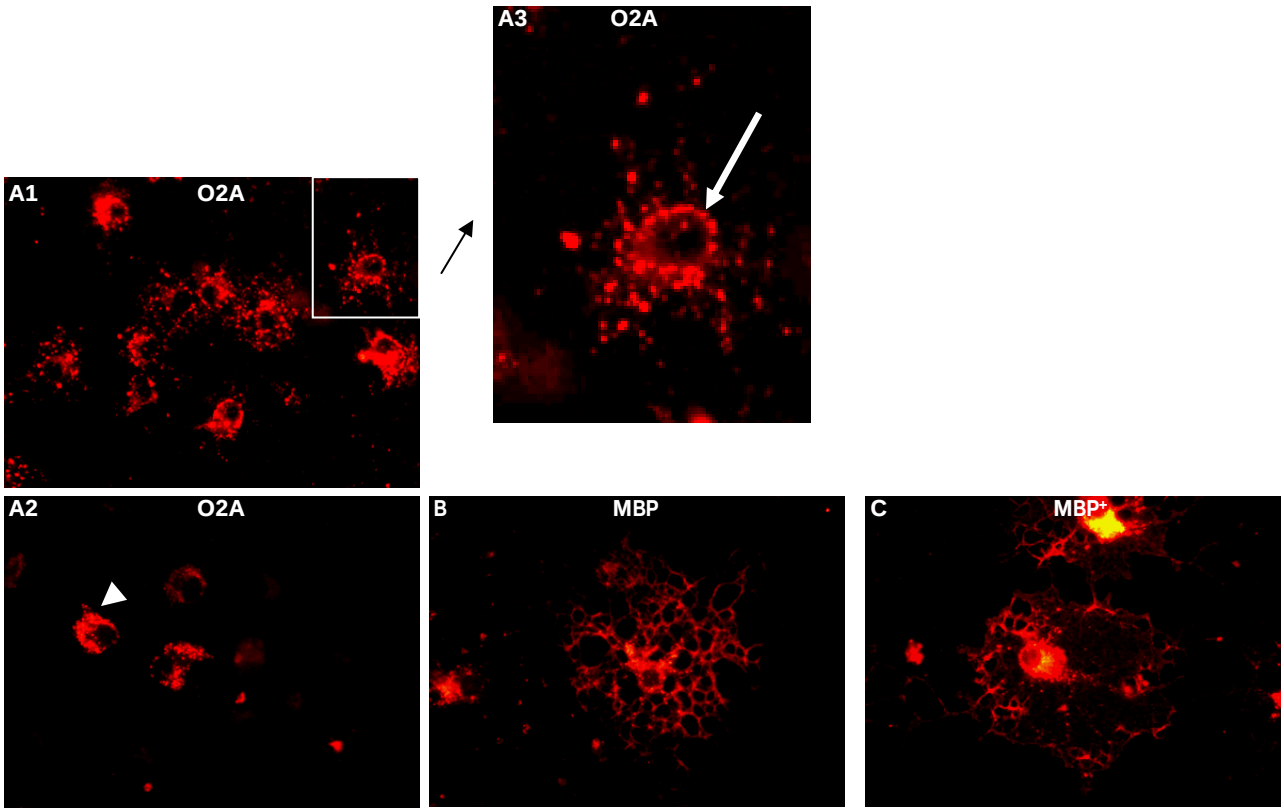


**Figure 1:** Localization of PLP at various stages of oligodendrocyte development. Newly formed (A1, A2, A3), mature (B) and myelinating (C) OLGs were cultured on PLL and differentiated for 3 days, 7 days and 10 days, respectively. Cells were fixed, permeabilized and the localization of PLP was analyzed via immunostaining as described in Materials and Methods. For clear presentation a segment of A1 is enlarged in A3. The arrow shows plasma membrane localization of PLP, whereas the arrowhead points to PLP-containing structures in the perinuclear region, showing a punctuate appearance when associated with primary processes. Scale bar = 20 µM. Representative pictures of at least 5 independent experiments are shown.

As shown in figure 2, biotinylated PLP was not detected in newly formed OLGs cultured on PLL, implying that at steady state conditions the residence time of PLP surface localization, if occurring at all, is negligible. In addition, the entire PLP fraction, thus largely localized intracellularly, was solubilized by TX-100. In contrast, in mature cells, biotinylated PLP was present on the surface (approx. 40 %), and this surface pool partitioned roughly equally between a detergent-resistant and detergent soluble fraction. Of the intracellular fraction, less than 15 % was resistant to solubilization by TX-100.

The foregoing data suggested a dynamic behavior of PLP as reflected by distinct cell development dependent differences in its intracellular distribution as well as its ability to partition into different lateral membrane pools, characterized by TX-100 soluble and insoluble fractions. However, the apparent absence of such a partitioning in newly formed

OLGs, i.e., relatively early in development, would be consistent with previous observations on the developmental expression of such domains in neurons (Ledesma *et al.*, 1999), although a transient association with such domains can as yet not be excluded.

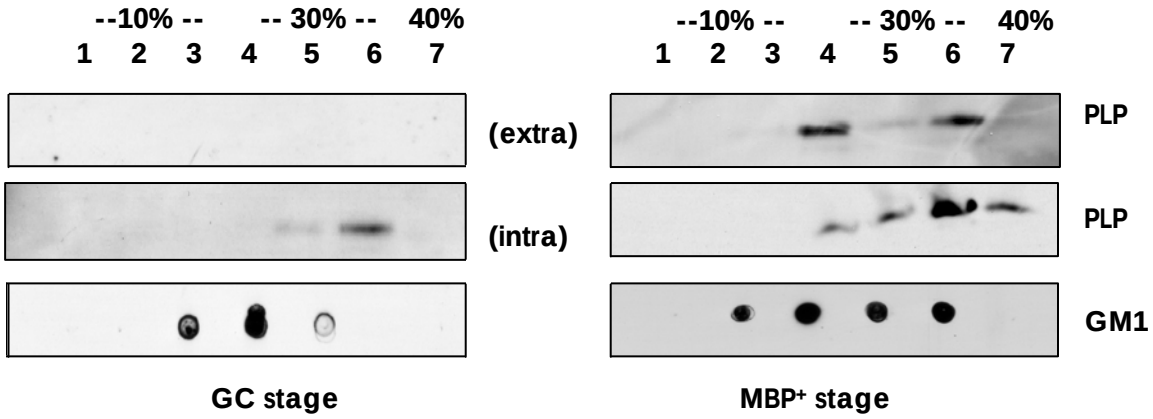


**Figure 2:** Association of PLP with TX-100-resistant membrane microdomains during oligodendrocyte differentiation on PLL. Newly formed OLGs (GC-stage) and myelinating OLGs (MBP$^+$-stage) were cultured on PLL and differentiated for 3 and 10 days, respectively. Cell surface proteins were biotinylated at 4°C, lysed, and subjected to OptiPrep density gradient centrifugation (40 %, 30 % and 10 %). Of each fraction (equal volumes), the pools of biotinylated, i.e. surface localized (extra) and non-biotinylated, i.e., intracellularly localized (intra) protein, were determined as described in Materials and Methods. PLP distribution was analyzed by SDS-PAGE, followed by Western blotting with anti-PLP antibodies. As a control for microdomain distribution along the gradient GM1 dotblots were performed (Materials and Methods).

These data thus raise several questions, including when and where PLP integrates into such detergent resistant microdomains (DRM), particularly since previous observations indicated that in both isolated myelin and cultured (mature) OLGs, the majority of PLP is solubilized upon extraction with 1 % TX-100 (Pereyra *et al.*, 1998; Krämer *et al.*, 1997; van der Haar *et al.*, 1998; Kim and Pfeiffer, 1999; Simons *et al.*, 2000). Likely, because of the relatively small absolute size of the pool residing in DRM, the significance of such a fraction may well have been underestimated. However, given PLP's seemingly dynamic behavior, the present observations may also imply that localization in these domains is only transient, which could have largely precluded detection at steady state conditions. This issue was therefore addressed next.

**Following biosynthesis, PLP transiently associates with Triton X-100-resistant microdomains**

To investigate the potentially transient association of PLP with a given microdomain, operationally distinguished here as a TX-100 soluble and insoluble domain(s), we carried

out pulse chase experiments. Newly formed and mature OLGs were cultured on PLL and metabolically labeled with Tran$^{35}$S for 10 min at 37 °C. Subsequently, excess non-radioactive methionine was added, and the cells were chased for 0, 15, 30 and 60 min, followed by extraction with 1 % TX-100 and analysis of PLP in pellet (insoluble) and supernatant (soluble) by counting of radioactive label. As shown in figure 3 for newly formed OLGs (GC-stage), during the first 15 min, essentially all PLP was present within a detergent resistant fraction, while over the next 45 min, PLP gradually partitioned in a TX-100 solubilized fraction.

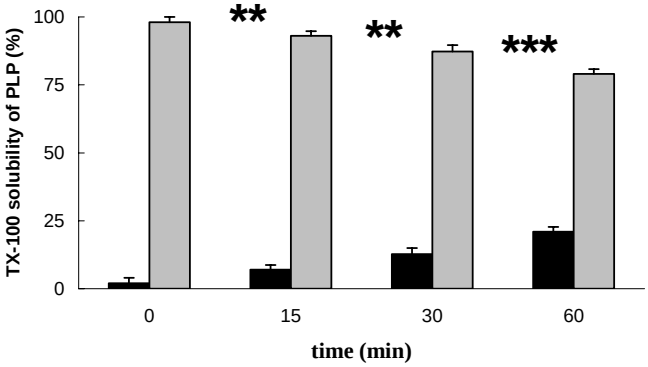


**Figure 3:** Intercalation of PLP in TX-100 resistant microdomains, analyzed by pulse-chase analysis. Newly formed OLGs cultured on PLL were pulse-labeled with Tran$^{35}$S for 10 min, which was followed by incubation in chase medium for the indicated time intervals. Subsequently, the cells were extracted with 1% TX100 at 4 °C as described in Materials and Methods. Soluble (S; supernatant) and insoluble (I; pellet) fractions were obtained by centrifugation, after extracting equal protein amounts of total cell lysates. PLP was immunoprecipitated and the amount in the soluble and insoluble fraction was analyzed by radioactive counting. Black bars represent soluble protein and grey bars represent insoluble protein. Data were obtained from three independent experiments. Statistical significance between O2A stage and the other developmental stages is shown (**$P$ < 0.01, ***$P$ < 0.001). Note that following biosynthesis PLP primarily associates with TX-100 resistant membrane microdomains, but subsequently acquires TX-100 solubility, as becomes apparent after 30-60 min.

Very similar data were obtained when analogous experiments were carried in mature OLGs, grown on PLL. Intriguingly, Simons *et al.* (2000) reported that in cultured mature OLG PLP acquires significant CHAPS detergents resistance only after 30-60 min. Thus, the kinetics of PLP's acquirement of Triton-***solubility*** versus CHAPS ***insolubility*** are very similar and it is therefore tempting to suggest that these kinetics reflect the transient partitioning of PLP into different membrane domains at different stages of its processing after de novo biosynthesis. Specifically, these results thus indicated that de novo synthesized PLP localizes to membrane microdomains that are characterized by TX-100 detergent insolubility. However, in contrast to these initial developments, at steady state a

major fraction of the total PLP fraction, which appears to be largely localized intracellularly, has become TX-100 soluble (fig. 2), whereas the insoluble fraction is relatively enriched at the cell surface. Accordingly, the observations led us to consider the following scenario. After synthesis at the ER, PLP likely integrates into a membrane domain, typified by TX-100 resistance, which presumably occurs at the Golgi, analogously as observed for other membrane spanning proteins (Kundu *et al.*, 1996; Millan *et al.*, 1997; Aït Slimane *et al.*, 2003). The kinetics of TX-100 solubilization of PLP, seen in figure 3, would be consistent with the time likely required (30-60 min) for PLP-containing transport vesicles to reach the cell surface (cf. Aït Slimane *et al.*, 2003). After reaching the cell surface, PLP redistributes, as reflected by an increasing fraction that acquires TX-100 solubility in mature OLGs (fig. 2). In newly formed OLGs, the transition is even more dramatic in that initially, like in mature cells, all newly synthesized PLP is detergent resistant, while at steady state essentially all PLP is detergent soluble, and localized almost exclusively intracellularly. Yet the kinetics of solubilization (fig. 3) was very similar as those observed in mature cells. Thus, in conjunction with the data in mature cells ($MBP^+$-stage) this could imply that after reaching the surface, as part of detergent resistant domain of a transport vesicle, PLP, after dissipating to a detergent-soluble region, is internalized again in vesicles (fig. 1) that no longer contain domain components that convey TX-100 detergent resistance to PLP. Presumably, the major glycosphingolipids present in OLGs, GalCer and sulfatide, both being expressed in newly formed and mature OLGs, likely co-determine this property, in conjunction with cholesterol (Lees and Brostoff, 1984; Norton and Cammer, 1984; de Vries and Hoekstra, 2000; Simons *et al.*, 2000), which was subsequently examined. In addition, a transient localization of PLP at the cell body plasma membrane followed by its subsequent internalization would, as a matter of mechanistic principle, be consistent with recent observations reported by Trajkovic *et al.* (2006). These authors suggested that myelin compounds might be stored into endocytic compartments prior to signal-mediated delivery towards the myelin sheet.

**PLP transiently localizes in sulfatide domains at the plasma membrane of the oligodendrocyte cell body**

In preliminary work, we have observed by TX-100 extraction in situ that GalCer positive microdomains are largely confined to the myelin sheets, whereas sulfatide

containing microdomains are restricted to the cell body and primary processes (Baron *et al.*, manuscript in preparation).

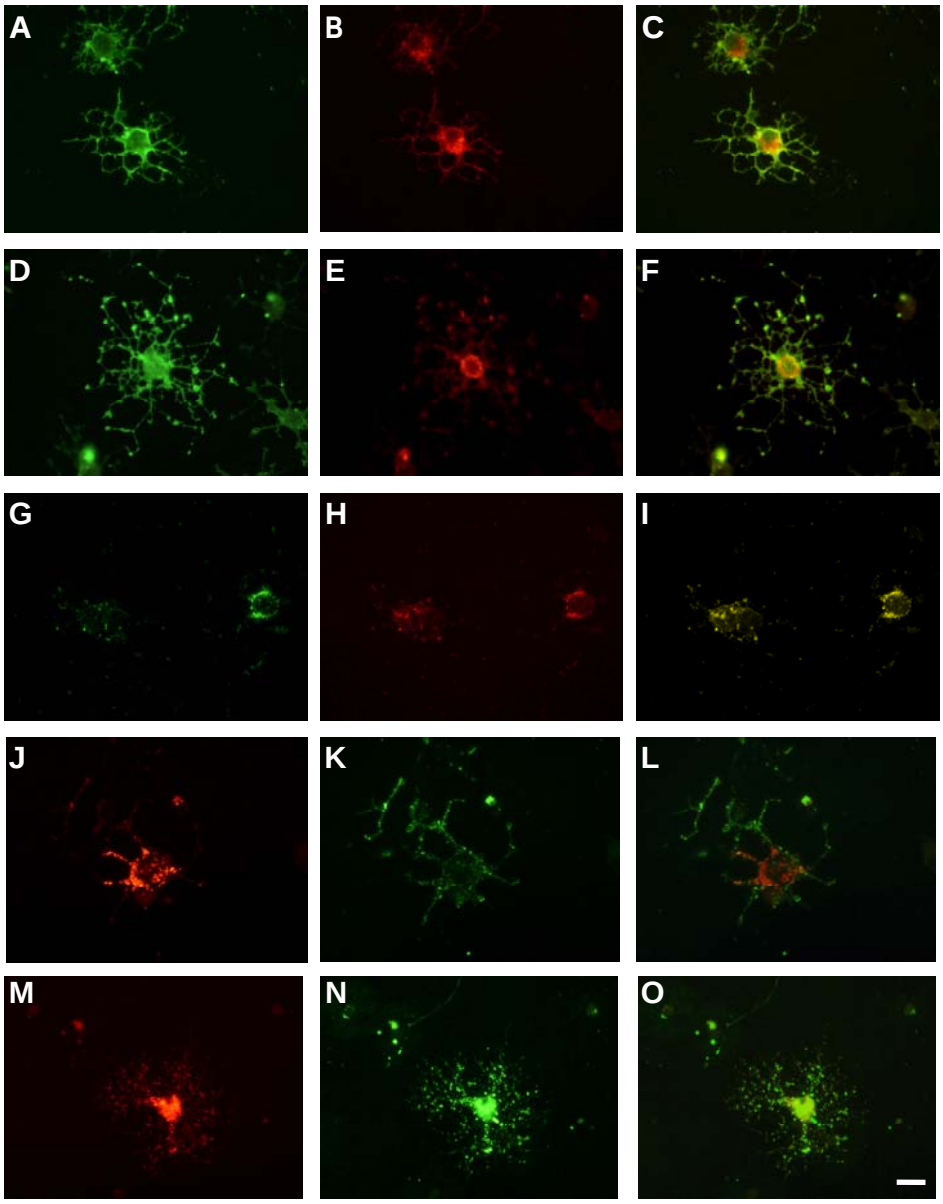


**Figure 4:** Colocalization of PLP with GalCer and sulfatide in oligodendrocytess. Newly formed (A-L, 3 days of differentiation) and mature OLGs (M-O, 10 days of differentiation) were cultured on PLL (A-C, J-O) or Ln2 (D-I). Colocalization of PLP (B, E, H, K, N) with sulfatide, visualized by staining with O4 (A, D, G, J) or GalCer, stained with O1 (M), was carried out as described in Materials and Methods. Live surface labeling with the O4 antibody was performed in A and D, whereas in J and M, PLP colocalization was carried out with O4 (J) or O1 (M) in fixed and permeabilized cells. In G-H, in situ detergent extraction prior to fixation was performed (see Materials and Methods). Merged pictures (C, F, I, L, O) were obtained using Adobe Photoshop software. Representative pictures of at least three independent experiments are shown. Scale bar = 20 µm**.** Note that PLP colocalized with sulfatide (O4) at the plasma membrane in TX-100 resistant microdomains when OLG were cultured on Ln2 (D-I). PLP and GalCer (O1) primarily colocalized in intracellular vesicular structures in the cell body (M-O).

Accordingly, further insight into the glycosphingolipid microenvironment of PLP could provide insight into the role of either lipid in the dynamics of PLP transport. Since the

transient localization of PLP in distinct domains was particularly apparent over the first hour after biosynthesis, sulfatide, being largely localized to the cell body and resistant to TX-100 extraction, was considered the major sphingolipid player in early PLP processing. To determine whether PLP and sulfatide were present in the same micodomains, double immunfluorescence staining with the anti-sulfatide antibody O4 and anti-PLP were performed. Little if any co-staining was observed in the plasma membrane by performing live staining with the anti-sulfatide antibody (fig. 4A-C), consistent with the biotinylation experiments (fig. 2). However, when newly formed OLGs were grown on laminin-2 (Ln2), an ECM substrate that strongly promotes myelin membrane formation (Buttery *et al.*, 1999; Šišková *et al.*, 2006) and interacts with sulfatide (Baron *et al.*, manuscript in preparation), both PLP and sulfatide evidently colocalized at the plasma membrane of the OLG cell body (fig. 4 D-F). Moreover, this fraction was at least in part resistant to TX-100 solubilization, as reflected by in situ extraction (Fig.4 G-I), implying detergent resistant microdomain localization within the cell body plasma membrane. Surface biotinylation experiments confirmed the plasma membrane localization of PLP on Ln2 in newly-formed OLGs (data not shown, cf. fig. 6). In contrast, both in newly formed and mature OLGs at steady state, very little if any intracellular colabeling of PLP and sulfatide was visible (fig. 4 J-L). Interestingly, GalCer did colocalize intracellularly with PLP (fig. 4 M-O), but in this case (at steady state in newly formed OLGs) the protein is detergent soluble, as revealed by in situ extraction. Together, these results would suggest that PLP is present in the plasma membrane of the cell body in sulfatide containing microdomains which conveys TX-100 detergent resistance to PLP. Evidently, PLP does not remain localized in this membrane domain, likely reflected by the time-dependent enhancement in TX-100 detergent solubilization (fig. 3), and the protein apparently becomes internalized as part of a sheet-directed vesicular transport process (fig. 1) in which sulfatide and PLP no longer colocalize. Rather, the evidence may suggest that at these conditions the protein may have acquired access to GalCer-enriched domains.

To obtain further support for PLP/sulfatide cotransport to the plasma membrane during *initial* post-biosynthetic transport of PLP, the following experiment was carried out. The oligodendroglia derived cell line, OLN-93 (Richter-Landsberg and Heinrich, 1996), was transfected with a PLP-GFP construct and constructs that either expressed ceramide galactosyl transferase (CGT), the enzyme catalyzing the final step in GalCer biosynthesis and/or ceramide sulfatide transferase (CST), which catalyzes the biosynthesis of sulfatide.

As shown in figure 5, in both mock infected cells (A) and CGT+ cells (B), the GFP-tagged PLP was randomly distributed throughout the cell, and no apparent plasma membrane localization was seen. In contrast, in cells that also expressed sulfatide (CGT+/CST+), PLP translocated to regions near and at plasma membrane (fig. 5C). Hence, these data suggested the need for sulfatide as a determining (co-)factor in plasma membrane-directed transport for PLP. This mechanistic feature, as an essential step in PLP transport to the sheet, was further illustrated and emphasized by adjustment of the nature of the ECM on which the cells grow. Thus, we exploited the fact that Ln2 promotes myelin biosynthesis, and indeed, as suggested by the observation in figure 4, in cells grown on Ln2 plasma membrane delivery of PLP in mature OLGs is strongly facilitated. The persistent association of PLP with sulfatide, promoted by Ln2 (fig. 4 d-f), potentially governing a plasma membrane directed transport step of PLP prior to its delivery to the sheet, was supported by determination of the PLP surface pool by biotinylation. In conjunction with PLP biotinylation, we also established the protein's lateral domain distribution by means of TX-100 extraction, followed by OptiPrep gradient analysis and Western Blotting. As shown in figure 6, in the $MBP^+$ stage next to a substantial PLP fraction at the cell surface (60-70%) localized to TX-100-resistant microdomains, such a fraction (approx. 20-30 %) could also be retrieved from the intracellular pool. The latter is in marked contrast to the virtual absence of such a fraction in case cells were grown on PLL (fig. 2). Possibly, this ECM dependent distinction in detergent resistant pools reflected the relatively enhanced demand for de novo sheet biogenesis, as promoted by Ln2. In this context, Ln2 has been shown to be associated with developing axons (Colognato *et al.*, 2002), implying that axons could direct intracellular traffic in oligodendrocytes via Ln2-sulftatide interactions. As a final piece of evidence in support of PLP's transport in sulfatide-enriched membrane domains to the plasma membrane of the OLG cell body prior to its delivery to the sheet, we took into account previous work in which we demonstrated that the t-SNARE syntaxin 3 localizes in OLGs to the plasma membrane and primary processes, rather than to the sheet. In this study (Klunder *et a*l., submitted; chapter 3) we demonstrated that syntaxin 3 is required for the eventual PLP delivery into the sheet, but, given the absence of this t-SNARE from the sheet, deposition of the protein in the sheet itself is clearly independent of syntaxin 3. Interestingly, syntaxin 3 distributes roughly equally in TX100-soluble and insoluble domains, which appears to match the distribution of PLP detergent solubility and insolubility at the cell surface of mature OLGs (fig. 2b).

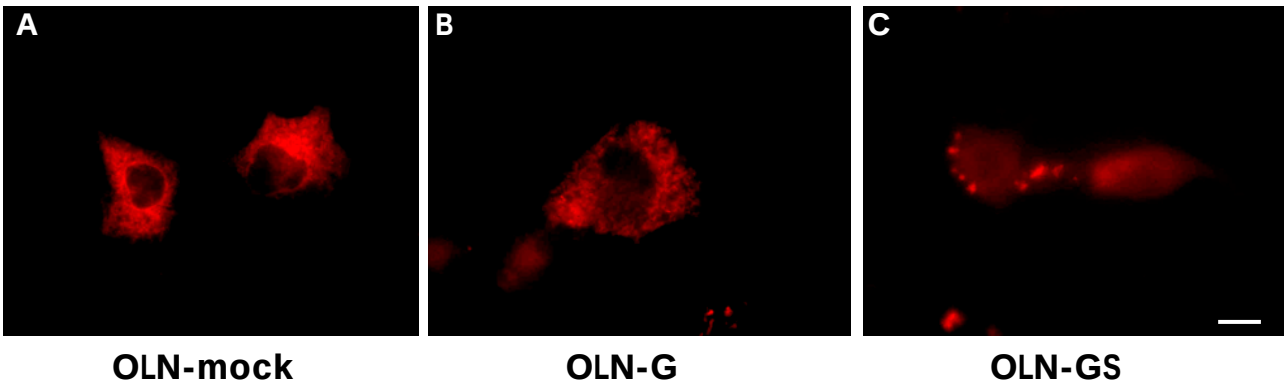


**Figure 5:** Localization of PLP-GFP in transfected OLN-93 cells. Mock infected (pLXIN) and OLN-93 cells expressing GalCer (CGT), or GalCer and sulfatide (CGT/CST) were transfected with PLP-GFP as described in Materials and Methods. The localization of PLP-GFP was determined with immunofluorescence, using anti-GFP antibodies. A. OLN-mock, B. OLN-G and C. OLN-GS. Scale bar = 20 µm. The image is representative of at least three independent experiments. Note that in OLN-GS cells PLP-GFP is plasma membrane localized, whereas in OLN-mock and OLN-G cells, PLP-GFP is mainly localised intracellularly.

To examine whether a causal relationship existed between syntaxin 3 and sulfatide membrane localization, and hence, that the plasma membrane was the initial target site for PLP transport prior to sheet delivery, we determined their potential interaction.

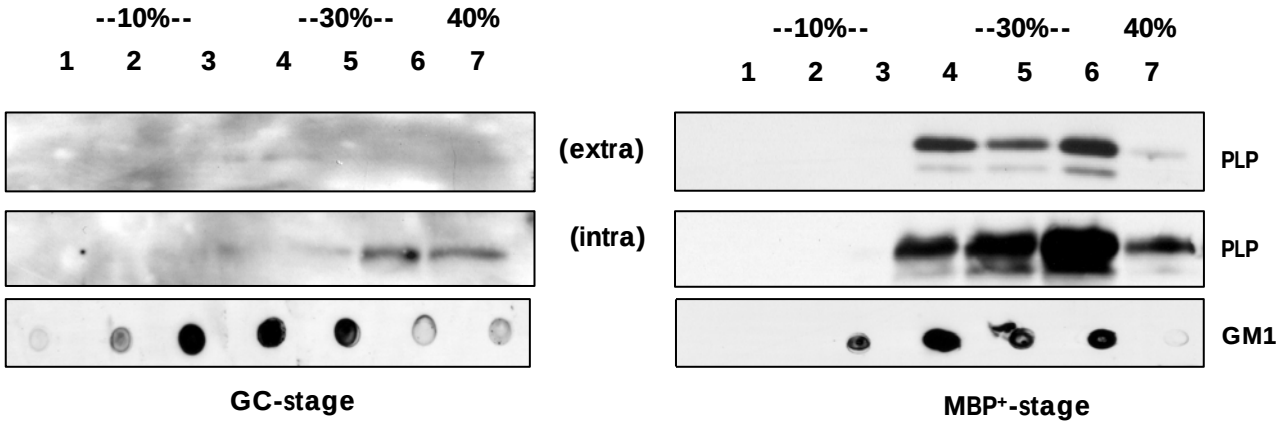


**Figure 6:** Association of PLP with TritonX-100-resistant membrane microdomains during oligodendrocyte differentiation on laminin 2. Newly formed OLGs (GC-stage) and mature OLGs ($MBP^+$-stage) were cultured on Ln2 and differentiated for 3 and 10 days, respectively. Cell surface proteins were biotinylated at 4 °C, lysed, and subjected to OptiPrep density gradient centrifugation (40 %, 30 % and 10 %). Of each fraction (equal volumes), biotinylated proteins, i.e. surface localized, were separated from non-biotinylated proteins, i.e. intracellular localized, as described in Materials and Methods. PLP distribution was analyzed by SDS-PAGE, followed by Western blotting with anti-PLP antibodies. As a control for microdomain distribution along the gradient, GM1 dotblots were performed (Materials and Methods).

Newly formed OLGs were grown in the presence of the anti-sulfatide antibody for 4 days. Control cultures were grown under the same conditions but without adding anti-sulfatide antibody O4. After harvesting, the cells were lysed and the homogenates were fractionated on an OptiPrep density gradient and analyzed by Western blotting. In figure 7 it is demonstrated that the membrane localization of syntaxin 3 altered after antibody addition.

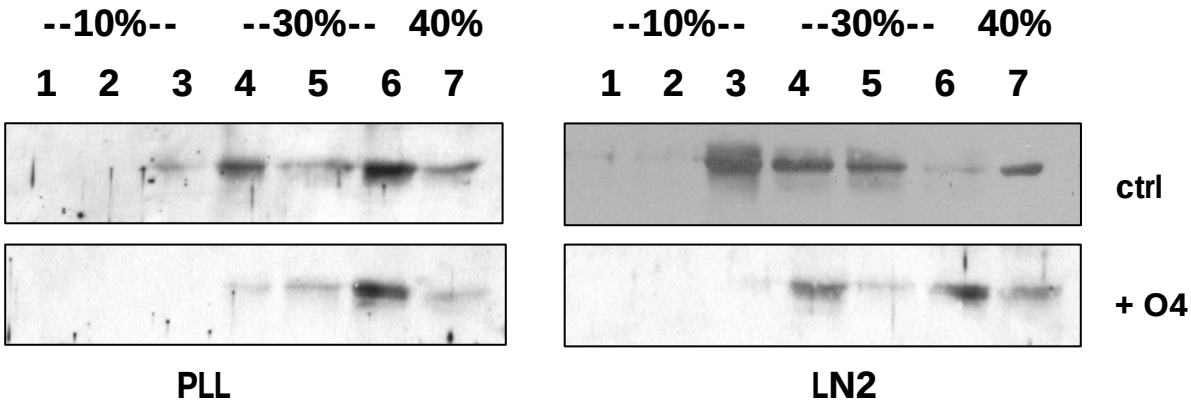


**Figure 7:** Effect of anti-sulfatide antibody O4 on the association of syntaxin 3 with TritonX-100 resistant microdomains. Newly formed OLGs cultured on PLL or Ln2 were treated without (ctrl)) or with O4 (+ O4)) for 4 days. Cells were lysed, and subjected to OptiPrep density gradient centrifugation (40 %, 30 % and 10 %) as described in Materials and Methods. Equal volumes of each gradient fraction were analyzed for syntaxin 3 by SDS-PAGE and Western blotting. Representative blots of three independent experiments are shown. Note the reduced association of syntaxin 3 with TX-100 resistant membrane microdomains upon treatment with O4 on PLL and Ln2.

Thus, it was apparent that the detergent-resistant fraction of syntaxin 3 was relatively enhanced (approx. 80 % vs 45 %) when cells had been grown on Ln2, i.e., at conditions that promote myelin sheet biogenesis. Hence, these data imply a correlation between enhanced biogenesis and the lateral membrane localization of syntaxin 3. Moreover, sulfatide appeared to affect this lateral organization as the presence of the antibody shifted the localization of syntaxin 3 from a detergent resistant to a detergent soluble localization in both PLL (from approx. 50 % to 15 %) and Ln2 (from 80 % to 40 %) cultured cells. In this context, we (Baron *et al.*, manuscript in preparation) and others (Bansal *et al.*, 1998) have shown that anti-sulfatide antibody O4, but not anti-galactocerebroside antibody O1, blocks terminal differentiation of OLGs. Our data thus suggest that addition of anti-sulfatide antibody O4 interferes with the localization of syntaxin 3, which, as noted, is enriched in the cell body plasma membrane. The most straightforward explanation

would thus be that a mislocalization of syntaxin 3 interferes with the proper delivery of PLP to the plasma membrane. This impediment, in turn, interferes with the subsequent delivery of PLP to the myelin sheet, implying that sheet development might be hampered. In line with this is the observation that overexpression of syntaxin 3 persevers PLP's localization in TX-100 resistant microdomains and indeed impedes delivery of PLP towards the sheet (Klunder *et al.*, submitted).

**Concluding remarks**

In summary, in the present work, we have shown, by exploiting the effect that of Ln2 exert on sheet development in OLGs, and by carrying out pulse chase experiments next to steady state analysis in cells at different stages of development, that myelin biogenesis in OLGs involves a transcytotic transport mechanism, as exemplified by sheet-directed transport of PLP. Thus our data support a model (fig. 8) in which PLP, early after biosynthesis, integrates into TX-100 resistant microdomains for transport to the plasma membrane in likely a sulfatide dependent manner of the OLG cell body (red structures), involving syntaxin 3 mediated docking (squiggle). Here, PLP is apparently redistributed into domains that are characterized by TX-100 solubility but CHAPS insoluble, as we propose based upon earlier work by Simons *et al.* (2000). These domains subsequently pinch off as transport vesicles (blue), which thus mediate the eventual trafficking of PLP into the sheet. Obviously, the molecular mechanisms underlying PLP's lateral redistribution and internalization remain to be determined. However, an interesting possibility could be that as part of this myelin sheet-directed transport process, PLP has become integrated in GalCer-enriched microdomains, thereby explaining the time-dependent shift from a TX-100 to an CHAPS insolubility. Nevertheless, as such, this mechanism would be entirely in line with our previous contentions that OLGs can be envisioned as polarized cells, displaying properties analogously to those reported for typical polarized cells like epithelial cells. In fact, in such cells, like hepatocytes, the major epithelial cell in the liver, similar transcytotic events occur in the transport of apical resident proteins, which proceeds via the basolateral surface, involving molecular mechanisms that remain to be clarified (Hoekstra *et al.*, 2004). Clearly, defining the processes of cellular sorting and transport, in which lipid-protein interactions with environmental signals regulate OLG development, is essential for an understanding of both normal OLG development and the process of remyelination in demyelinating diseases such as multiple sclerosis (MS), which relies on a disturbance of myelin membrane

organization. Particularly sulfatide is a key compound in regulating terminal OLG differentiation (Bansal *et al.*, 1999; Hirahara *et al.*, 2004). Here, we demonstrated its intimate role in PLP transport, as a crucial intermediate step in subsequent transport to and from the plasma membrane to the sheet. Sulfatide interacts with several key adhesion and ECM proteins, including laminin, secreted by astrocytes and neurons (Mcloon *et al.*, 1988; Roberts and Ginsburg, 1988; O'Shea *et al.*, 1990).

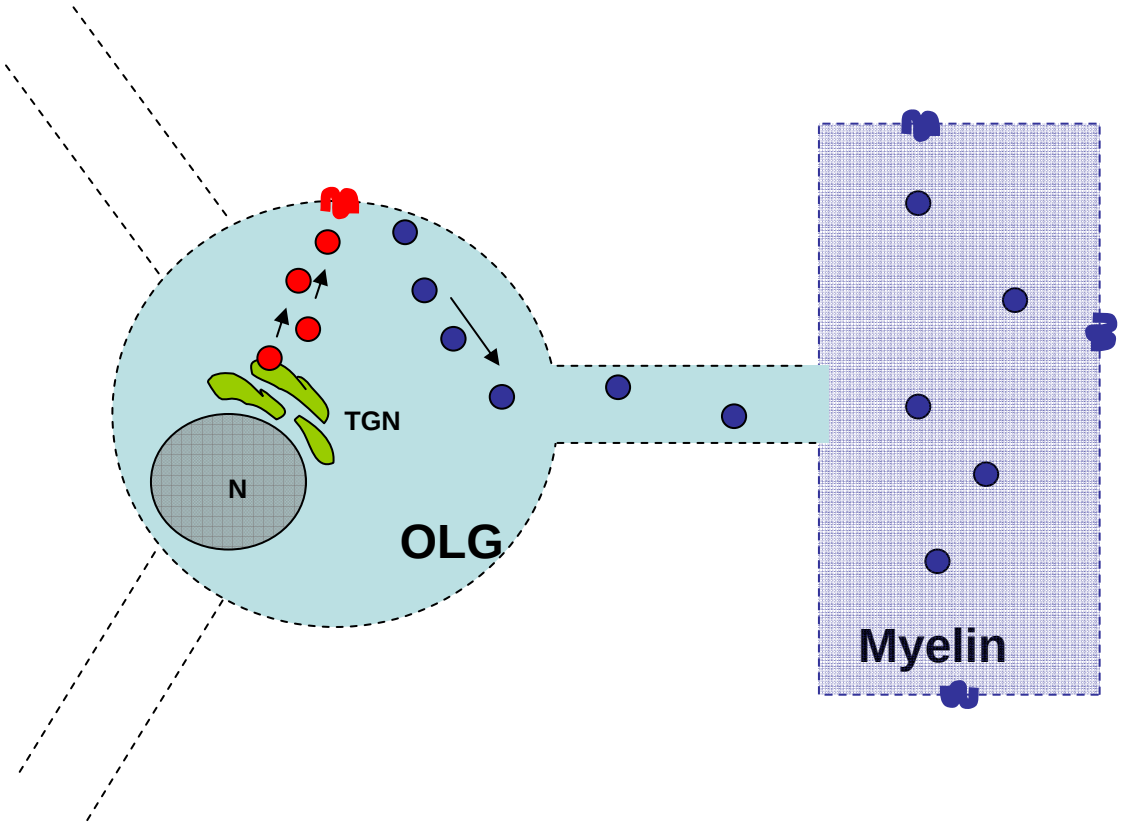


**Figure 8:** Schematic overview of the trafficking of de novo synthesized PLP to the myelin sheet. (a) Transport of PLP from *trans*-Golgi network to the plasma membrane of the cell body occurs as part of TX-100 insoluble, CHAPS soluble membrane microdomains (red); (b) Transport from the plasma membrane to the myelin sheet occurs by means of TX-100 soluble, CHAPS insoluble microdomains (blue). N. nucleus; TGN, *trans*-Golgi-network, squiggle, PLP. See text for further details.

However, in this interaction with the ECM, additional cell surface adhesion receptors very likely play a role as well, such as the α6β1 intergrin (Blaschuk *et al*, 2000; Baron *et al*, 2003), which interacts with Ln2 and promotes myelin biogenesis (Relvas *et al.*, 2000). The principle of such a mechanism could be consistent with our observations (fig. 4), showing an

enhanced recruitment of PLP at the plasma membrane, presumably mediated by sulfatide. These issues are currently investigated in our laboratory.

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