Distinct endocytic recycling of myelin proteins promotes oligodendroglial membrane remodeling

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Summary

The central nervous system myelin sheath is a multilayered specialized membrane with compacted and non-compacted domains of defined protein composition. How oligodendrocytes regulate myelin membrane trafficking and establish membrane domains during myelination is largely unknown. Oligodendroglial cells respond to neuronal signals by adjusting the relative levels of endocytosis and exocytosis of the major myelin protein, proteolipid protein (PLP). We investigated whether endocytic trafficking is common to myelin proteins and analyzed the endocytic fates of proteins with distinct myelin subdomain localization. Interestingly, we found that PLP, myelin-associated glycoprotein (MAG) and myelin-oligodendrocyte glycoprotein (MOG), which localize to compact myelin, periaxonal loops and abaxonal loops, respectively, exhibit distinct endocytic fates. PLP was internalized via clathrin-independent endocytosis, whereas MAG was endocytosed by a clathrin-dependent pathway, although both proteins were targeted to the late-endosomal/lysosomal compartment. MOG was also endocytosed by a clathrin-dependent pathway, but in contrast to MAG, trafficked to the recycling endosome. Endocytic recycling resulted in the association of PLP, MAG and MOG with oligodendroglial membrane domains mimicking the biochemical characteristics of myelin domains. Our results suggest that endocytic sorting and recycling of myelin proteins may assist plasma membrane remodeling, which is necessary for the morphogenesis of myelin subdomains.

Supplementary material available online at
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Key words: Endocytosis, Recycling, Membrane remodeling, Oligodendrocytes, Myelin domains

Introduction

Oligodendroglial cells synthesize the central nervous system myelin sheath by wrapping multiple layers of a specialized membrane around the axon, permitting saltatory impulse conduction. They simultaneously ensheath several axonal segments, exhibiting an extraordinarily high production of membrane during active myelinogenesis (Pfeiffer et al., 1993), which requires a sophisticated membrane-trafficking machinery (Anitei and Pfeiffer, 2006; Kramer et al., 2001; Larocca and Rodriguez-Gabin, 2002). The mature myelin sheath is characterized by a highly organized domain structure with distinct protein-lipid domains, which can broadly be divided into compact and non-compact regions (Salzer, 2003). In compact myelin, the membranes are condensed, whereas the non-compact areas comprise a loop-like structure filled with cytoplasm. The non-compact areas are named after their topological location in the sheath: the periaxonal loop is in direct apposition to the axon, the abaxonal loop at the outer surface and the lateral paranodal loops face the nodes of Ranvier. Tight junctions composed of claudin-11/OSP serve as physical barriers between compact and non-compact myelin (Gow et al., 1999; Morita et al., 1999). Studies with knockout mice have demonstrated that the precise localization of proteins and lipids to myelin subdomains is critical for myelin morphogenesis and neuronal function (Popko, 2000; Schaeren-Wiemers et al., 2004; Sherman et al., 2005). How myelinating cells control domain assembly and which membrane sorting and trafficking mechanisms operate is currently unresolved.

The temporal and spatial coordination of myelin formation is achieved by bidirectional communication between neurons and glia (Barres and Raff, 1999; Sherman and Brophy, 2005; Simons and Trajkovic, 2006). Interestingly, trafficking of proteolipid protein (PLP), which is the major protein of compact myelin, is controlled by neuronal signals (Boiko and Winckler, 2006; Trajkovic et al., 2006). Oligodendrocytes grown in the absence of neurons accumulate PLP in late endosomal/lysosomal (LE/Lys) stores, whereas in the presence of neurons or neuron-conditioned medium, the LE/Lys pool is mobilized and exocytosis of PLP to the plasma membrane is stimulated.

In this study, we asked whether endocytic recycling is a common feature of myelin membrane traffic and whether endosomal sorting of myelin components assists myelin domain formation. We focused on the myelin proteins PLP, myelin-associated glycoprotein (MAG) and myelin-oligodendrocyte glycoprotein (MOG) as model proteins exemplifying localization to compact myelin, periaxonal loops or abaxonal loops of non-compact myelin, respectively (Arroyo and Scherer, 2000). Our results demonstrate that these three proteins follow distinct endocytic sorting and recycling pathways. We show that endocytic recycling is associated with remodeling of the oligodendroglial plasma membrane, resulting in association of PLP, MAG and MOG with specific membrane domains sharing biochemical characteristics of myelin subdomains.

Results

Endocytosis is common to myelin proteins

In light of the fact that the main myelin protein PLP is enriched in LE/Lys compartments and undergoes regulated endocytosis and exocytosis (Kramer et al., 2001; Trajkovic et al., 2006), we asked whether endocytic recycling is common to myelin proteins. We first analyzed the steady state localization of myelin proteins in

endosomal compartments by co-labeling primary cultured oligodendrocytes with transferrin-FITC or antibodies against LAMP1, which serve as markers for recycling endosomes or LE/Lys, respectively. Although PLP strongly colocalized with LAMP1 in LE/Lys (Fig. 1, top row, magenta in overlay pictures), we observed only a little overlap of MAG and MOG with LAMP1 or transferrin-FITC in endocytic compartments (Fig. 1, middle and bottom row, magenta and yellow color in overlays, respectively). Thus, among the studied myelin proteins, only PLP accumulates in endosomal compartments. However, a transient endosomal localization of MAG and MOG may be missed or underestimated with this type of steady-state analysis.

We further examined endocytosis in oligodendrocytes by following the internalization of cell-surface-labeled myelin proteins. In addition to primary cultured oligodendrocytes, which are heterogeneous in their composition of different maturation stages and generally exhibit a slow metabolism, we made use of Oli-neu cells. This cell line has characteristics of immature oligodendrocytes and can be induced to express myelin proteins in a subpopulation of the cells by treatment with di-butyryl-cyclic (dbc)-AMP (Jung et al., 1995). Upon transplantation into demyelinated lesions of the CNS, Oli-neu cells specifically ensheath axons, although a compacted myelin sheath is not formed. To allow quantitative analyses, we transiently expressed the myelin proteins PLP, MAG and MOG in Oli-neu cells, which upon ectopic expression exhibit steady-state localization similar to that of endogenous proteins in primary oligodendrocytes. Transiently transfected Oli-neu cells or primary oligodendrocytes (cultured for 3-4 days) were cell-surface biotinylated using a reducible biotin analogue and subsequently incubated at 37°C to allow endocytic internalization of biotinylated plasma membrane proteins. Subsequently, the remaining cell-surface biotin was cleaved under reducing conditions and endocytosed myelin proteins were detected by NeutrAvidin-mediated precipitation followed by western blotting (Fig. 2A). In both transiently transfected Oli-neu cells and primary oligodendrocytes, we observed a selective endocytic uptake of all three myelin proteins PLP, MAG and MOG, whereas other plasma-membrane-associated proteins, such as the Na^+/K^+-ATPase were not endocytosed under these conditions. The two splice isoforms of MAG – L-MAG and S-MAG – were internalized in a similar fashion when individually expressed in Oli-neu cells (not shown). Endocytosis of endogenous myelin proteins in primary oligodendrocytes was not as efficient as that of ectopically expressed proteins in Oli-neu cells, and the endocytic rate varied between experiments, but endocytosis per se was observed in any single experiment. Variation in endocytosis rates probably mirror the heterogeneity of primary cells, in terms of the relative composition of different maturation stages. When primary oligodendrocytes cultured for more than 5 days were subjected to endocytosis assays, internalization of myelin proteins was virtually absent (not shown), indicating that endocytosis is a feature of maturing oligodendrocytes whereas terminally differentiated oligodendrocytes exhibit reduced endocytic activity. Moreover, primary cells generally exhibit a slower metabolism, resulting in slower kinetics of endocytosis compared with Oli-neu cells. By densitometric quantification of internalized biotinylated myelin proteins in primary oligodendrocytes (Fig. 2Ac), we calculated that 12±6% of the total surface-biotinylated PLP, 7±2% of MAG and 14±4% of MOG were endocytosed within 1 hour (P=0.013 for PLP, P=0.033 for MAG and P=0.022 for MOG, paired t-test).

In addition to cell-surface biotinylation, we performed antibody-internalization experiments to visualize endocytosis morphologically. Briefly, antibodies specific for one of the myelin proteins were bound to the cell surface, followed by Cy3-coupled secondary antibodies. After endocytosis, remaining cell-surface antibodies were counterstained with Cy2-coupled secondary antibodies. In overlay images, endocytosed proteins thus appear colored red, whereas proteins localized at the cell surface appear yellow. We observed increasing internalization of PLP, MAG and MOG over 1 hour of endocytosis in transfected Oli-neu cells (Fig. 2B), as well as of endogenous proteins in primary oligodendrocytes (see Fig. S1A in supplementary material). After incubation for 1 hour at 37°C, endocytosis was observed in 80±6%, 90±4%, or 92±4% of the PLP-, MAG- or MOG-expressing Oli-neu cells, respectively. In primary oligodendrocytes cultured for 3 days 53±6% of the PLP-, 86±4% of the MAG-, and 67±1% of the MOG-expressing cells exhibited internalization of the respective protein from the plasma membrane within 1 hour. Notably, internalized MOG localized to the perinuclear region (Fig. 2B, arrowheads), whereas endocytosed PLP and MAG were randomly scattered throughout the cytoplasm. Myelin protein endocytosis was prominent over that of other oligodendroglial plasma membrane proteins not located in myelin, such as NG2, which was not internalized under these conditions (Fig. 2B and Fig. S1A

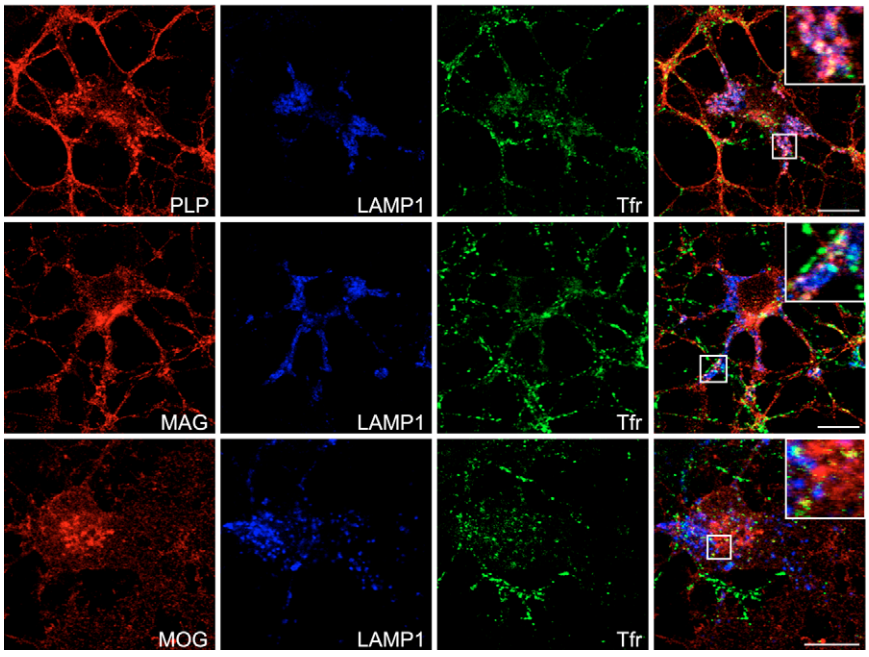

Fig. 1. Localization of myelin proteins in endocytic compartments. Primary oligodendrocytes differentiated for 4 days in culture were incubated with transferrin-FITC (green) to label recycling endosomes. Cells were stained using antibodies specific for the myelin proteins PLP, MAG or MOG (red), and antibodies against the LE/Lys marker LAMP1 (blue). Localization of myelin proteins in the LE/Lys appears magenta and localization in the recycling endosomes appears yellow. Confocal planes are shown, insets show enlarged areas. More than 50 cells were analyzed per condition in each of 3-5 experiments. Scale bars: 10 μm.

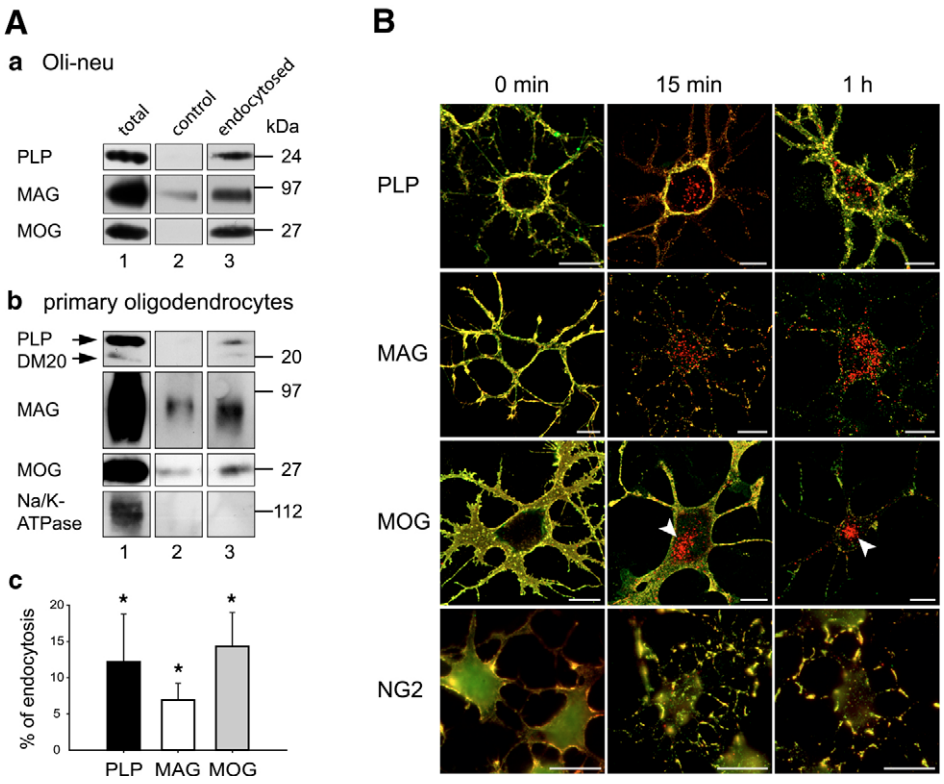

Fig. 2. Endocytic uptake of myelin proteins in oligodendrocytes. (A) Oli-neu cells transiently expressing the myelin proteins PLP, MAG or MOG (a) and primary cultured oligodendrocytes (b) were cell-surface biotinylated using a reducible biotin analogue. Cells were either lysed immediately after biotinylation (lane 1, total biotinylation), kept on ice to block endocytosis followed by cleavage of biotin with the reducing agent DTT (lane 2, control), or incubated at 37°C to allow endocytosis followed by biotin cleavage (lane 3). After cell lysis, biotinylated proteins were precipitated with NeutrAvidin beads and analyzed by western blotting. Endocytosed PLP, MAG and MOG, but not Na^+/K^+-ATPase, were recovered from cells, which were subjected to endocytosis and biotin cleavage (lane 3). Densitometric quantification of endocytosis of biotinylated proteins of 3-5 independent experiments was performed with primary cultured oligodendrocytes (c). Error bars indicate s.e.m.; $*P<0.05$ (paired *t*-test). (B) Antibody-uptake experiments using Oli-neu cells transiently expressing the myelin proteins PLP, MAG or MOG and cells expressing endogenous NG2. Live cells were incubated on ice with primary antibodies recognizing cell-surface epitopes of PLP, MAG, MOG or NG2 followed by Cy3-coupled secondary antibodies. After endocytosis for 0 minutes, 15 minutes or 1 hour at 37°C, surface-localized antibody complexes were counterstained with Cy2-coupled antibodies. Endocytosed proteins appear red, cell-surface-localized proteins appear yellow. Internalized MOG localizes to the perinuclear region (arrowheads). Scale bars: 10 μm.

in supplementary material). Internalization of myelin proteins was also observed in nontransfected Oli-neu cells, which occasionally exhibited endogenous PLP, MAG or MOG expression (not shown). Protein clustering as a result of crosslinking with secondary antibodies was not responsible for the internalization, because omitting secondary antibodies led to identical results (see Fig. S2 in supplementary material). However, secondary-antibody-mediated clustering occasionally led to patches of nonendocytosed proteins at the plasma membrane (see Fig. 2B, patchy appearance of surface-localized proteins after incubation at 37°C).

Myelin proteins are sorted to distinct endocytic trafficking pathways

The selective endocytosis of myelin proteins from the plasma membrane of oligodendrocytes raises the question as to whether they are subsequently sorted and recycled back to the cell surface. To assess the endosomal destination of PLP, MAG and MOG, we performed antibody-uptake assays similar to those described above and co-immunostained the endocytosed proteins using markers for different endocytic compartments (Fig. 3A and Fig. S1B in supplementary material). Interestingly, endocytosed PLP and MAG were both targeted to LAMP1-positive LE/Lys. In contrast to PLP and MAG, MOG co-endocytosed with transferrin-FITC to a juxtanuclear compartment – probably the recycling endosome. When simultaneously visualized during endocytosis, PLP and MAG were initially localized in distinct early endocytic compartments and started to colocalize only at later stages of endocytosis (probably reflecting LE/Lys), indicating utilization of distinct endocytic pathways by the two proteins (Fig. 3B).

We next investigated whether myelin proteins are endocytosed by clathrin-dependent or clathrin-independent pathways. To interfere with clathrin-dependent endocytosis, we overexpressed a truncated form of the AP180 adaptor protein (AP180C), which blocks recruitment of clathrin to the plasma membrane (Ford et al., 2001). In AP180C-expressing Oli-neu cells, the uptake of transferrin – a marker for clathrin-dependent endocytosis – was almost completely blocked (Fig. 4A). We determined levels of endocytosis of PLP, MAG and MOG in AP180C-expressing Oli-neu cells by antibody uptake (Fig. 4B). While endocytosis of PLP was only slightly affected, a strong reduction in endocytosis of MAG as well as MOG was observed upon inhibition of the clathrin-dependent pathway. To block clathrin-independent, cholesterol-dependent endocytosis pathways, PLP-, MAG- and MOG-expressing Oli-neu cells were treated with the cholesterol-sequestering agent filipin during the antibody-uptake procedure. However, a strong reduction of plasma membrane cholesterol is known to additionally affect the clathrin-dependent pathway (Kirkham and Parton, 2005). To exclude this side effect, we simultaneously allowed internalization of transferrin during the antibody uptake and only considered cells where transferrin uptake was intact (Fig. 5). Specific inhibition of the clathrin-independent, cholesterol-dependent pathway blocked the endocytosis of PLP and did not affect the internalization of MAG and MOG. Taken together, these data show that MAG and MOG are internalized via clathrin-dependent endocytosis, but are subsequently sorted to LE/Lys and recycling endosomes, respectively. PLP is taken up by a clathrin-independent cholesterol-sensitive pathway and accumulates in LE/Lys.

Cultured oligodendrocytes form distinct membrane domains

Why do myelin proteins undergo such complex endocytic trafficking? It is possible that cultured oligodendrocytes, which do not form a

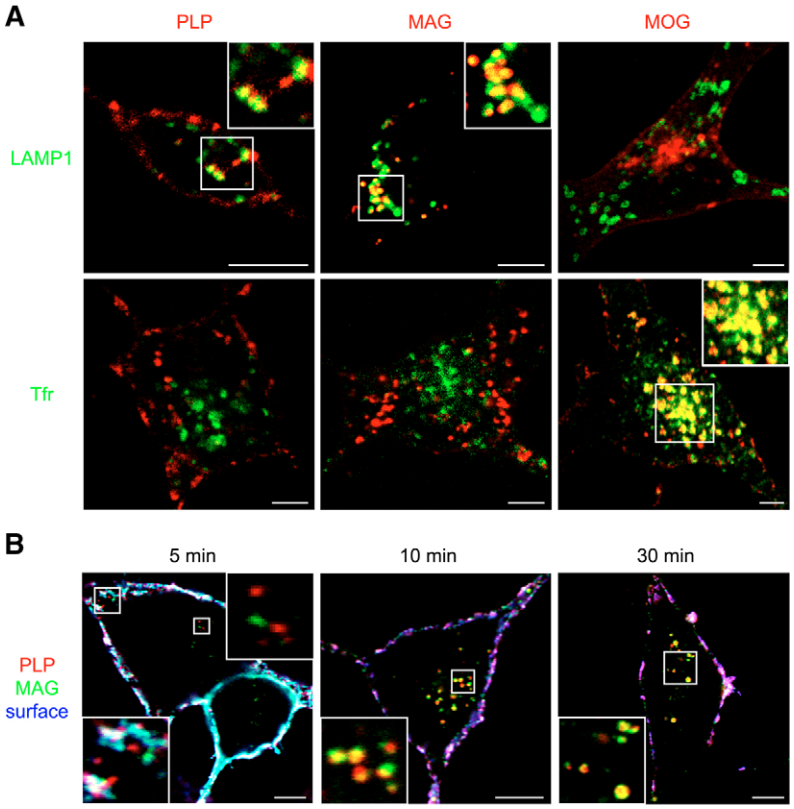

Fig. 3. Differential endocytic targeting of PLP, MAG and MOG. (A) Colabeling of endocytosed proteins with endocytic markers. Transfected Oli-neu cells were allowed to endocytose surface-bound antibodies for 1 hour at 37°C. During the endocytosis period, labeling of recycling endosomes was achieved by co-endocytosis of transferrin-FITC (bottom images, green). To label LE/Lys, cells were immunostained using anti-LAMP1 antibodies (top images, green). Confocal images show colocalization of PLP and MAG with LAMP1 in LE/Lys (top images, yellow). MOG colocalized with transferrin-FITC in the recycling endosomes (bottom image, yellow). (B) Co-endocytosis of PLP (red) and MAG (green), performed by simultaneous antibody uptake. Cell-surface-localized proteins were counterstained in blue and thus appear cyan, magenta or white. After 5 minutes of endocytosis PLP and MAG are localized to distinct populations of early endosomes (red and green dots). Colocalization of endocytosed PLP and MAG increases over 10 and 30 minutes of endocytosis (yellow dots). Insets show enlarged areas. Scale bars: 5 μm.

fully developed myelin sheath, carry out increased turnover of redundant myelin components. However, in this case the myelin proteins would probably enter the same endocytic degradation pathway, inconsistent with our results showing sorting to distinct endocytic pathways and compartments. We thus hypothesized that endocytic sorting of myelin proteins precedes recycling of the proteins back to the oligodendroglial plasma membrane, hence promoting the morphological differentiation of distinct membrane domains. Oligodendrocytes in culture extend myelin-like membrane sheets and even multilamellar membranes (Dubois-Dalcq et al., 1986; Schneider et al., 2005). Costaining of primary cells and confocal analysis showed that PLP, MAG and MOG exhibit a surprisingly low degree of colocalization at the oligodendroglial plasma membrane and thus associate with distinct membrane domains (Fig. 6A). MAG was preferentially localized to primary processes and at the distal fringe of membrane sheets (arrows in a′). By contrast, PLP was present throughout the sheets (asterisk), whereas MOG was enriched in definite areas of the sheets (arrows in a″). Thus, myelin membrane sorting and trafficking mechanisms operate even in these cultured cells, resulting in the generation of defined membrane domains. To isolate these domains, we made use of a classic density gradient centrifugation protocol that was developed and applied to separate myelin membrane subdomains (Matthieu et al., 1973; Rios et al., 2000). Owing to their distinct protein and lipid composition, compact myelin domains are recovered from gradient fractions of low density (‘light’ membranes), whereas noncompact myelin domains predominantly associate with high density (‘heavy’ membranes) and/or medium-density fractions. We subjected oligodendroglial membranes and myelin from postnatal day 10 (P10) mouse brain to this protocol and compared the fractionation patterns of PLP, MAG and MOG (Fig. 6B). We chose to isolate myelin from P10 mice because this represents an in vivo developmental stage analogous to that of the oligodendrocytes in culture. At this early stage of myelination, the compact myelin domain is beginning to develop and was thus underrepresented in the light fraction compared with fractionations of myelin prepared from adult mice in which compact myelin is fully established (see Fig. S3 in supplementary material). The fractionation pattern of oligodendroglial and P10 myelin membranes as indicated by the overall distribution of proteins between heavy, medium and light fractions was strikingly similar. MAG and OSP (a component of tight junctions located at the interface between compact and non-compact myelin) are exclusively present in high-density fractions, whereas PLP and MOG emerged in light fractions with a tendency of MOG to be additionally located in medium-density fractions. The SNARE protein syntaxin 6 was used as a marker to ensure that oligodendroglial light fractions were free of Golgi-derived and endosomal membranes, which might be misinterpreted as plasma membrane domains. Thus, cultured oligodendrocytes evolve morphologically differentiated plasma membrane domains that resemble the biochemical composition of compact and non-compact myelin domains.

Recycling of PLP, MAG, and MOG to oligodendroglial membrane domains with characteristics of myelin domains

Based on the above observation, we designed a biotinylation pulse-chase protocol followed by cell subfractionation, enabling the fate of surface-biotinylated myelin proteins to be followed through endocytosis and recycling to specific membrane domains. Briefly, primary cultured oligodendrocytes at an intermediate maturation stage were subjected to surface biotinylation and cultured further for 4 or 24 hours at 37°C to allow endocytosis and plasma membrane recycling of labeled proteins. At the indicated times, oligodendroglial membrane domains were separated into light, medium and heavy fractions as described above, and the distribution

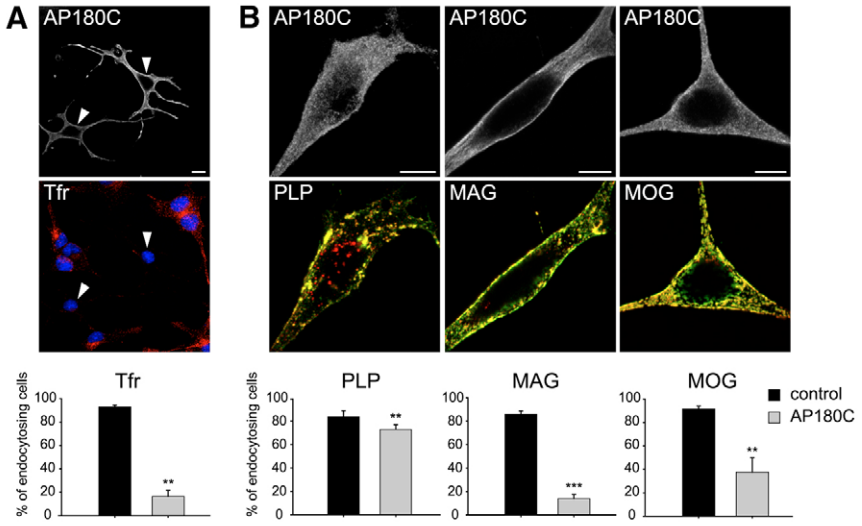

Fig. 4. Analysis of clathrin-dependent endocytosis. The clathrin-sequestering protein AP180C-myc was overexpressed in Oli-neu cells to interfere with clathrin-mediated endocytosis. AP180C-expressing cells were identified by staining with anti-myc antibodies (top row). Color images (bottom row) depict endocytosis (red) and cell surface localization (green/yellow). The graphs show the percentage of endocytosing AP180C-negative cells (black bars, control) and the percentage of endocytosing AP180C-expressing cells (gray bars). (A) The clathrin-mediated uptake of transferrin Alexa Fluor 594 (red) is blocked specifically in AP180C-overexpressing Oli-neu cells (arrowheads). Blue color depicts DAPI-stained nuclei. (B) Coexpression of AP180C-myc and PLP, MAG or MOG. Transfected cells were subjected to antibody-uptake experiments as described in Fig. 2B. Stacked confocal images of representative cells are shown. Overexpression of AP180C strongly reduced the internalization of MAG and MOG. More than 50 cells were counted per three (PLP and MAG) or four (MOG) independent experiments. Error bars represent s.e.m.; $^{**}P<0.01$; $^{***}P<0.001$ (paired *t*-test). Scale bars: 5 μm.

of surface-biotinylated PLP, MAG and MOG between these fractions was determined after NeutrAvidin precipitation. A shift of a surface-biotinylated protein between the fractions within 24 hours of chase thus indicates recycling to biochemically distinct membrane domains. In parallel, a fraction of the cells was stained with streptavidin-FITC to visualize the subcellular distribution of biotinylated proteins at each of time points analyzed.

Directly after biotinylation, streptavidin labeling of permeabilized cells was restricted to the cell surface and largely confined to the cell soma and primary processes (Fig. 7A, arrow, see also Fig. S4 in supplementary material; note that 3D stacks of confocal planes are shown and thus almost complete cells are displayed). After 4 hours, the initial surface staining of streptavidin was transformed into a scattered distribution throughout the cells (Fig. 7A, arrowheads) and intracellular colocalization of streptavidin with myelin proteins was detectable, indicating endocytosis of biotinylated proteins (see Fig. S4 in supplementary material). After 24 hours, biotinylated proteins had redistributed to the cell periphery where they associated with myelin-like membrane sheets (Fig. 7A, asterisks). NeutrAvidin precipitation of biotinylated proteins from density gradient fractions demonstrated that directly after biotinylation, surface-biotinylated PLP, MAG and MOG were preferentially associated with heavy fractions (Fig. 7Ba,C). The distribution of biotinylated myelin proteins between the density gradient fractions had not significantly changed within 4 hours (not shown). However, after 24 hours, a significant proportion of biotinylated PLP and MOG had shifted from heavy to light fractions, demonstrating a change in association to membrane domains of lower density. Light fractions are free of intracellular membranes derived from Golgi or endosomes and thus reflect domains of the plasma membrane as demonstrated by the absence of the marker protein syntaxin 6 (Fig. 6B, Fig. 7Bb). By contrast, biotinylated MAG was constantly associated with heavy fractions throughout the experiment. Unlike the biotinylated fraction of proteins, the distribution of total PLP, MAG and MOG between the fractions remained unchanged during the 24 hour chase (Fig. 7Bb). Together, these data suggest that endocytic sorting and recycling of myelin proteins leads to rearrangement of the

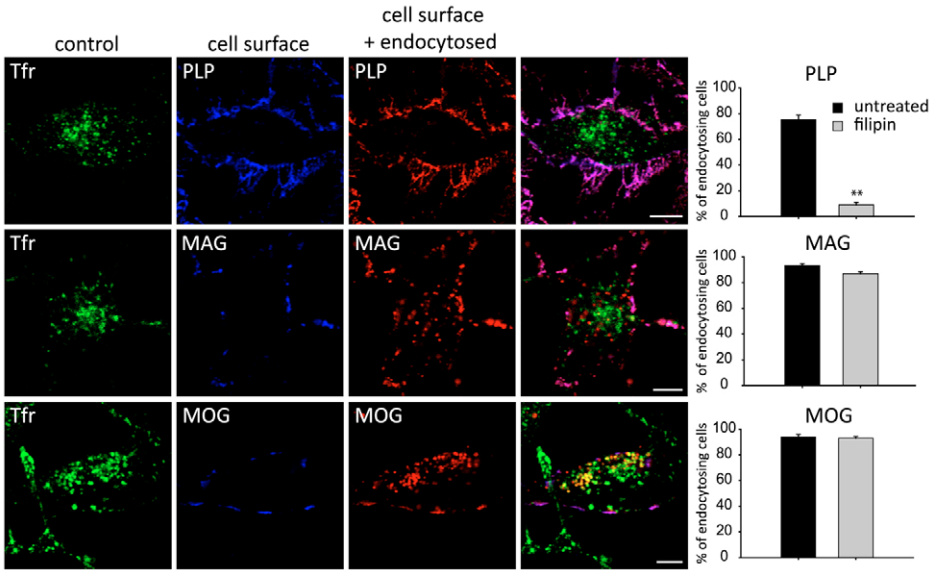

Fig. 5. Analysis of clathrin-independent endocytosis. To inhibit clathrin-independent cholesterol-dependent endocytosis, Oli-neu cells transiently expressing PLP, MAG and MOG were treated with the cholesterol-depleting agent filipin (1 μg/ml) during antibody uptake. To exclude that cholesterol depletion affects clathrin-dependent endocytosis, transferrin-FITC (green) was co-endocytosed with the myelin proteins and cells internalizing transferrin were exclusively analyzed. Cell-surface PLP, MAG or MOG was counterstained in blue. In merged images, surface-localized proteins thus appear magenta, whereas endocytosed proteins appear red (or yellow in case of colocalization with transferrin-FITC). Confocal images of representative cells are shown. The percentage of cells internalizing PLP, MAG or MOG was calculated from the population of transferrin-internalizing cells (graphs: black bars, untreated cells; grey bars, filipin-treated cells). More than 50 cells were counted per three (PLP and MAG) or five (MOG) independent experiments. PLP endocytosis was inhibited by filipin treatment, whereas endocytosis of MAG and MOG was unaffected. Error bars represent s.e.m.; $^{**}P<0.01$ (paired *t*-test). Scale bars: 5 μm.

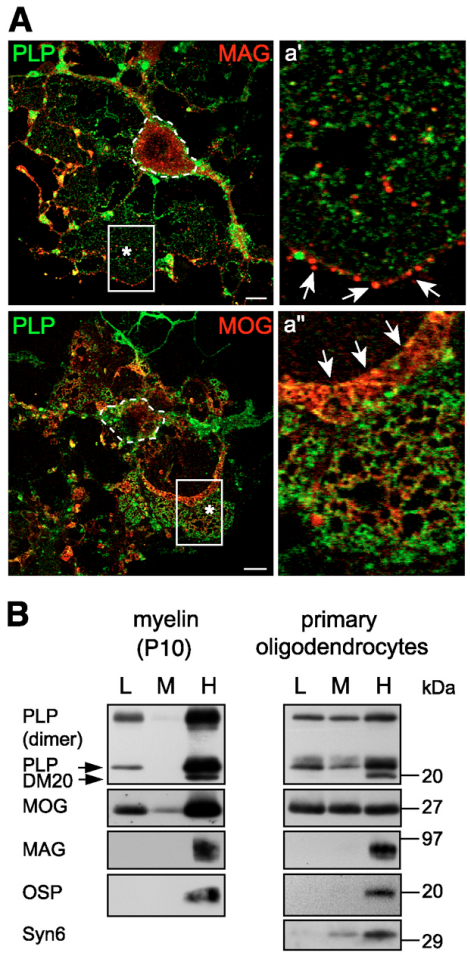

Fig. 6. Cultured oligodendrocytes develop morphologically and biochemically distinct membrane domains. (A) Confocal analysis of co-stained primary oligodendrocytes demonstrates association of PLP (green), MAG (red, top images) and MOG (red, bottom images) with distinct membrane domains. Dashed circles delineate cell bodies and boxed areas are enlarged in a′ and a″. Asterisks indicate membrane sheets. Arrows indicate the typical localization of MAG (a′) and MOG (a″). Scale bars: 5 μm. (B) Subfractionation of myelin and oligodendroglial membranes by discontinuous density gradient centrifugation in heavy (H), medium (M) and light (L) membrane fractions, analyzed by western blotting (nonreducing conditions, thus the dimeric form of PLP is prominent). Myelin was isolated from postnatal day 10 (P10) mouse brain at a developmental stage analogous to that of the cells in culture. Note that the compact myelin domain (indicated by PLP in light fractions) is beginning to develop at this early state of myelination and is thus underrepresented in the light fraction. Claudin11/OSP, which delineates the non-compact domain, is used as an additional marker. Syntaxin 6 was used as a marker of Golgi and endosomal membranes.

oligodendroglial cell surface resulting in association of the myelin proteins PLP, MAG and MOG with membrane domains sharing biochemical characteristics of myelin subdomains.

Discussion

We show that the myelin proteins PLP, MAG and MOG, which exhibit specific subdomain localization in the mature myelin sheath, undergo distinct endocytic sorting in oligodendroglial cells. Furthermore, we demonstrate recycling of these proteins to different membrane domains of cultured oligodendrocytes, suggesting a role of endocytic recycling in remodeling of the oligodendroglial plasma membrane. Intriguingly, the oligodendroglial membrane domains share biochemical characteristics of myelin subdomains, raising the concept that endocytic recycling may assist morphogenesis of the myelin sheath by sorting and redirecting myelin components. What may be the benefit of trafficking myelin components through endocytic compartments? Myelination requires rapid mobilization of lipids and proteins to the growing myelin membrane that can be balanced by endocytosis and exocytosis from endocytic pools, which serve as membrane reservoirs and sorting stations. Endocytic recycling pathways allow local membrane remodeling, essential for oligodendrocytes myelinating multiple axonal segments simultaneously, and in addition, may support the longitudinal expansion of the myelin membrane as the axons lengthen during development. Recent work has emphasized the importance of endocytic recycling for cell morphogenesis in developmental processes, especially where rapid mobilization of plasma membrane and polarized membrane growth occurs (Lecuit and Pilot, 2003). This includes membrane insertion at the leading edge of migrating cells, or the biogenesis of the epithelial membrane domains (Bretscher and Aguado-Velasco, 1998; Lu and Bilder, 2005; Perret et al., 2005). In polarized neurons, endosomal membrane trafficking is required for specific axonal targeting of the cell adhesion molecule NgCAM (Wisco et al., 2003), and for activity-induced growth and remodeling of dendritic spines (Park et al., 2006). Moreover, neurite extension is mediated by regulated exocytosis of a specialized endocytic compartment (Alberts and Galli, 2003; Arantes and Andrews, 2006). It is important to emphasize that an endocytic contribution to myelin morphogenesis does not exclude direct transport pathways from the Golgi, rather both routes probably cooperate to synthesize myelin, similarly to the generation of basolateral and apical membrane domains in epithelial cells (de Vries and Hoekstra, 2000; Rodriguez-Boulan et al., 2005).

In vitro endocytosis assay systems

We used two independent methods to study endocytosis in oligodendroglial cells: cell surface biotinylation and antibody uptake. Treatment of oligodendrocytes with MOG antibodies has been shown to increase intracellular Ca^{2+} levels and to activate the MAP-kinase/Akt signaling pathway; moreover, secondary-antibody-mediated clustering of MOG favored its partitioning in lipid rafts, resulting in cytoskeletal changes and altered oligodendrocyte morphology (Marta et al., 2005; Marta et al., 2003). During our antibody-uptake experiments, oligodendroglial cell morphology remained unaltered, although we occasionally observed patches of nonendocytosed proteins at the plasma membrane, which may reflect partitioning of crosslinked proteins into lipid rafts. It is nonetheless possible that antibody-evoked signals influenced the behavior of the cells during endocytosis. However, internalization of surface-biotinylated proteins conclusively demonstrated that endocytosis occurs independently of antibody stimulation. Moreover, myelin proteins were preferentially endocytosed compared with other membrane proteins.

It is important to highlight that endocytic trafficking of ectopically expressed myelin proteins in Oli-neu cells is identical to that of the endogenous proteins in primary oligodendrocytes. Overexpression of high levels of PLP results in sequestration of cholesterol to endocytic compartments and reduced incorporation of MBP in myelin, probably contributing to the dysmyelinating pathology observed in the case of PLP gene duplication in vivo (Karim et al., 2007; Simons et al., 2002). In Oli-neu cells, the level of transiently expressed PLP is not sufficient to induce cholesterol sequestration

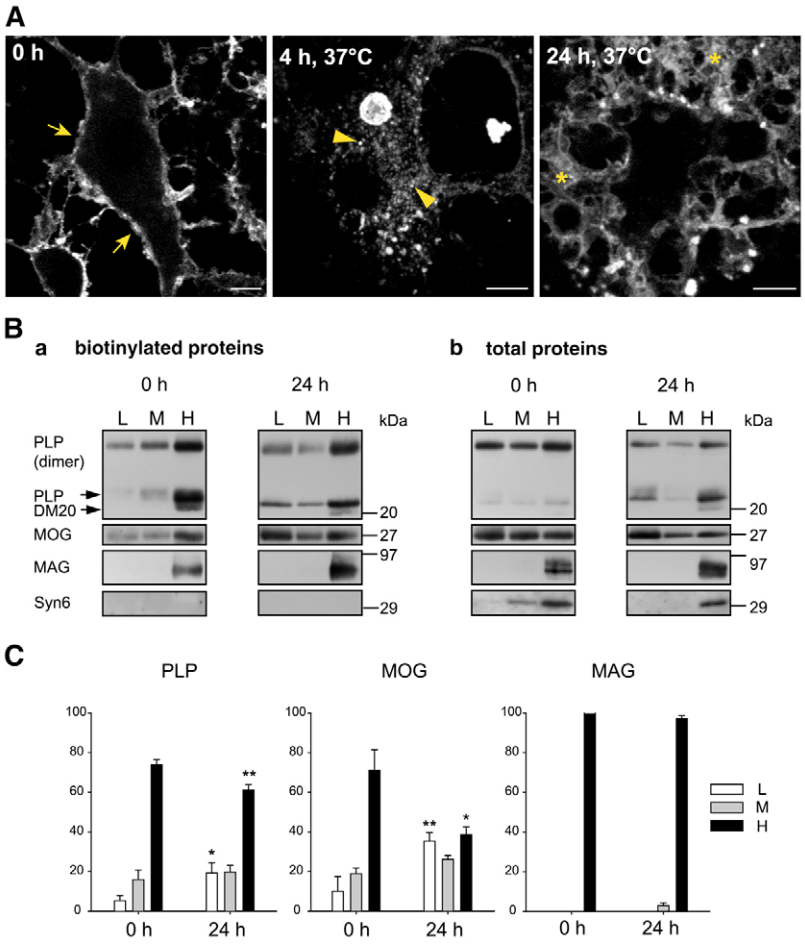

Fig. 7. Chase of surface biotinylated PLP, MAG and MOG through endocytosis and recycling. Primary cultured oligodendrocytes were cell-surface biotinylated at 4°C and incubated for 0, 4 and 24 hours at 37°C. (A) Streptavidin-FITC staining of permeabilized cells to visualize cell-surface localization (0 hours, arrow), endocytosis (4 hours, arrowhead), and recycling of biotinylated proteins (24 hours, asterisks; see also supplementary material Fig. S4). Within 24 hours, biotinylated proteins recycle from the cell body to the periphery where they localize to myelin-like membrane sheets (asterisks). 3D stacks of confocal planes are shown. Scale bars: 5 μm. (B) Subfractionation of primary oligodendrocytes as described in Fig. 6 directly after biotinylation (0 hours) and after a chase period of endocytosis and recycling for 24 hours at 37°C. Biotinylated proteins were precipitated from the fractions using NeutrAvidin-beads and analyzed by western blotting (a). Within 24 hours, biotinylated PLP and MOG significantly shift from heavy (H) to light fractions (L). MAG remains associated with the heavy (H) fraction. As expected, endosomal syntaxin 6 is not biotinylated and does not precipitate with NeutrAvidin (negative control). Western blots of the total fractions are also shown (b). The distribution of total PLP, MAG and MOG between the fractions does not significantly change over 24 hours at 37°C. Note that gels were run under nonreducing conditions, favoring dimeric PLP. (C) Densitometric quantification of NeutrAvidin-precipitated proteins from three independent experiments. The relative distribution of biotinylated PLP (monomeric + dimeric), MAG and MOG between the fractions is shown as a percentage. Error bars represent s.e.m.; **P*<0.05; ***P*<0.01 (paired *t*-test).

in endocytic compartments (Kramer-Albers et al., 2006). We are thus confident that ectopic expression of myelin proteins in Oli-neu cells is a suitable system to analyze endocytic trafficking. However, our studies are limited by the fact that cultured oligodendroglial cells do not form a mature myelin sheath with a three-dimensional arrangement of the myelin domains. Nevertheless, our results illustrate fundamental principles of oligodendroglial cell biology that can ultimately be verified in carefully designed in vivo test systems. Owing to the essential role of endocytosis in cells, it is difficult to study the role of endocytosis in myelination in vivo.

Endocytic trafficking of myelin proteins

It has been demonstrated recently that soluble factors secreted from neurons induce a reduction in clathrin-independent endocytosis and a stimulation of exocytosis of PLP in cultured oligodendrocytes, which is regulated by Rho-GTPase activity levels (Kippert et al., 2007; Trajkovic et al., 2006). Thus, neuron-to-glia signaling regulates endocytic trafficking of the major myelin protein allowing target-oriented tuning of myelin membrane traffic. Our results demonstrate that in addition to their role as a storage compartment for PLP, oligodendroglial endosomes act as sorting stations for proteins with distinct myelin domain localization.

MAG and MOG, localized to periaxonal and abaxonal loops of myelin, respectively, are both internalized via clathrin-dependent endocytosis, but are specifically sorted to LE/Lys (MAG) or recycling endosomes (MOG), whereas the compact myelin component PLP is endocytosed by a clathrin-independent cholesterol-sensitive pathway and targeted to LE/Lys. Although both MAG and PLP are sorted to LE/Lys, only PLP accumulates, whereas MAG localization in LE/Lys appears to be transient, suggesting different half-lives of MAG and PLP in the LE/Lys. MAG may be degraded in the lysosome or is constitutively recycled to the plasma membrane. Previous ultrastructural studies utilizing immuno-electronmicroscopy have detected MAG in multivesicular bodies in situ within the oligodendroglial soma and processes exclusively during development at stages of active myelination (Trapp et al., 1989). It was suggested that this observation reflects retrograde transport of the L-MAG isoform, which is predominantly expressed during development. The L-MAG sequence exhibits two tyrosine-based sorting signals, whereas the adult isoform S-MAG contains a single tyrosine motif (Bo et al., 1995), and differential sorting of the two isoforms has been observed in epithelial cells (Minuk and Braun, 1996). It has thus been proposed that exclusively L-MAG undergoes endocytosis and is removed from the periaxonal space upon myelin maturation (Bo et al., 1995). However, when we individually expressed L- or S-MAG in Oli-neu cells, both isoforms exhibited identical endocytosis and trafficking behavior. Interestingly, upon ectopic expression in polarized MDCK cells, PLP, MAG and MOG exhibit distinct trafficking pathways to apical and basolateral membrane domains (Kroepfl and Gardinier, 2001b). MOG has been demonstrated to contain a tyrosine-based sorting signal as well as a di-leucine motif, responsible for its basolateral targeting in MDCK cells (Kroepfl and Gardinier, 2001a). The same sorting signals driving polarized trafficking in epithelial cells thus probably determine the individual endocytic fate of PLP, MAG and MOG in oligodendroglial cells. The regulatory mechanisms of endocytic trafficking in oligodendrocytes are unknown. It may be of interest that among the Rab-GTPases regulating specific membrane-trafficking steps, Rabs involved in endocytic recycling are strikingly prominent in oligodendrocytes (Bouverat et al., 2000; Rodriguez-Gabin et al., 2004; Rodriguez-Gabin et al., 2001).

Oligodendroglial membrane domains and surface remodeling

Oligodendrocytes cultured in the absence of neurons express all myelin genes and form extensive myelin-like membrane sheets, which in some instances form compacted lamellae with the periodicity of normal myelin, indicating that these cultured cells are intrinsically myelination competent (Dubois-Dalcq et al., 1986; Pfeiffer et al., 1993; Schneider et al., 2005). Although the morphological differentiation of a fully assembled myelin sheath is dependent on extrinsic factors, such as interaction with neurons, it has been demonstrated that myelin membrane-trafficking pathways act in cultured cells affecting the formation of myelin-like domains in the oligodendroglial plasma membrane (Anitei et al., 2006; de Vries et al., 1998; Siskova et al., 2006). As a consequence of specific protein sorting and trafficking, PLP, MAG and MOG exhibit unique plasma membrane localization and furthermore, membrane domains that share the biochemical characteristics of domains isolated from myelin can be isolated.

When chasing cell-surface-biotinylated PLP and MOG in primary oligodendrocytes, we observed a relocalization from membrane domains of high density to membrane domains of low density, with characteristics of compact myelin isolated from brain. Hence, PLP and MOG undergo recycling to domains of the plasma membrane that mimic compact myelin. MOG translocation to low-density membrane domains either reflects a low-density abaxonal membrane or indicates additional localization of MOG to compacted regions of the myelin membrane. The constant association of MAG with high-density membranes has two possible interpretations: (1) it does not reappear at the cell surface after endocytosis, or (2) it recycles to membrane domains with similar characteristics to the domains with which it was originally associated, but different to the recycling domains of PLP and MOG. However, the total amount of biotinylated MAG had not diminished after 24 hours, arguing against the degradation of endocytosed MAG and thus favoring the recycling hypothesis. The association of MAG with high-density membranes, even after recycling, is consistent with its association with the noncompacted periaxonal domain, because MAG is always present in high-density fractions from myelin.

Taken together, the distinct endocytic trafficking of myelin proteins in oligodendrocytes indicates a broader role of myelin protein recycling in myelin formation than previously recognized. We propose that endocytic recycling supports simultaneous myelinogenesis of multiple axonal segments by facilitating local membrane remodeling and furthermore assists the morphogenesis of the compact, periaxonal, abaxonal and paranodal domains of the mature myelin sheath.

Materials and Methods

Reagents, antibodies and plasmids

General chemicals were purchased from Roth (Karlsruhe, Germany) and Sigma (Munich, Germany). Antibodies used were: for PLP, AA3 (M. B. Lees, Waltham, MA) and O10 (Jung et al., 1996); MOG, 8-18-C5 (C. Linington, Aberdeen, Scotland); MAG, 513; myc-tag (Cell Signaling, Danvers, MA); NG2, AN2 (Niehaus et al., 1999); anti-OSP/claudin11 (J. Bronstein, Los Angeles, CA), LAMP1, CD107a (BD Biosciences); Syntaxin 6 (Synaptic Systems, Göttingen, Germany); Na^+/K^+-ATPase, 464.6 (Novus Biologicals, Littleton, CO). Secondary antibodies were from Dianova (Hamburg, Germany). Expression vectors used were: AP180C-myc (Harvey McMahon, MRC LMB, Cambridge, UK) (Ford et al., 2001), pCMV-PLP (Kramer-Albers et al., 2006), pcDNA1.1-S-MAG/-L-MAG (S. Kelm, Bremen, Germany) and pcDNA3.1-MOG (C. Linington).

Cell culture, transfections and immunofluorescence

Primary cultures of mouse oligodendrocytes were prepared as described previously (Kramer et al., 1997). Purified oligodendrocytes were grown in Sato medium with 1% horse serum (HS), supplemented with B27 (Invitrogen, Karlsruhe, Germany) or astrocyte-conditioned medium. The purity of the oligodendroglial cultures ranges between 90% and 97%, contaminating cells were astrocytes, neurons or microglial cells. The oligodendroglial precursor cell line Oli-neu was cultured in Sato, 1% HS. The cell line is characterized by expression of the oligodendroglial precursor marker NG2 (100% of the cells) and at least 50% of the cells expressed the oligodendrocyte marker O4 (Jung et al., 1995). Oligodendroglial differentiation of these cells can be supported by culturing the cells in the presence of 1 mM di-butyryl-cyclic (dbc)-AMP. Transient transfections (electroporation) and immunofluorescence staining were performed as described (Kramer-Albers et al., 2006). In brief, plasmid DNA (5-10 μg) was added to 3×10^6 cells in 600 μl Sato-medium and cells were electroporated at 220 V and 950 μF using the Bio-Rad Gene Pulser Xcell (exponential decay program). Dbc-AMP (1 mM) and sodium-butyrate (2 mM) were added 2 hours after plating and experiments were performed 24 hours after the transfection. The transfection efficiency was in the range of 50-70% for MAG and MOG and 40-50% for PLP. The transiently expressed myelin proteins exhibited similar steady-state distribution as endogenously expressed proteins in primary oligodendrocytes. Images were acquired with a Leica TCS SP5 confocal microscope using a 63×, 1.4 NA lens and LAS AF (Leica, Wetzlar, Germany) software and processed with Adobe Photoshop software (linear adjustments of histograms). Stacked confocal images were processed by ImageProPlus 4.5 software (Media Cybernetics, Bethesda, MD).

Antibody internalization assay

Living Oli-neu cells or primary oligodendrocytes were incubated on ice for 45 minutes with antibodies recognizing extracellular epitopes of PLP (O10), MAG (513), MOG (8-18-C5) or NG2 (AN2) in DMEM, 10% HS, followed by incubation with goat Cy3 antibodies (or in the case of co-endocytosis, with goat Cy3 and goat Cy2) for 30 minutes on ice. The cells were either left on ice (control) or incubated at 37°C to allow endocytosis and subsequently incubated with tertiary anti-goat Cy2 or Cy5 antibody to stain cell-surface-localized proteins. The percentage of endocytosing cells was determined from three to nine independent experiments (between 60 and 100 cells counted per experiment). Transferrin-FITC (Invitrogen, Karlsruhe, Germany) was added to the endocytosis medium as a marker for clathrin-dependent endocytosis and the recycling endosomes . To interfere with clathrin-independent endocytosis, cells were treated with 1 μg/ml filipin III (Sigma, Munich, Germany) during the secondary antibody incubation at 37°C. Finally, cells were fixed with 4% paraformaldehyde and immunostained where indicated.

Biotin endocytosis assay

Primary oligodendrocytes or Oli-neu cells were cell-surface biotinylated for 30 minutes at 4°C using 0.25 mg/ml reducible Sulfo-NHS-SS-LC-Biotin (Pierce, Rockford, IL) in PBS. Free biotin was quenched with 50 mM glycine in PBS, followed by incubation of the cells for 1 hour at 4°C (control) or 37°C to allow endocytosis. Remaining cell-surface biotin was cleaved with 100 mM DTT and free DTT was quenched with 5 mg/ml iodacetamide in PBS. Cells were lysed in PBS, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin and endocytosed biotinylated proteins were precipitated using NeutrAvidin-beads (Pierce, Rockford, IL) according to the manufacturer's instructions. Precipitates were analyzed by western blotting. Densitometric analysis was carried out with the help of Aida Image Analyzer v.3.28 (Raytest, Straubenhardt, Germany). The recovery of biotinylated proteins after endocytosis (Fig. 2Aa,b lane 3) was corrected by the value of the cleavage control (Fig. 2Aa,b lane 2) and related to the total recovery directly after biotinylation (Fig. 2Aa,b lane 1).

Recycling analysis of biotinylated cell-surface proteins

Primary oligodendrocytes (3 days in vitro) were cell-surface biotinylated for 30 minutes at 4°C, using 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in PBS. Free biotin was quenched with 50 mM glycin in PBS. Biotinylated cells were either directly subjected to membrane fractionation (0 hours, control), or cultured for 4 or 24 hours at 37°C in astrocyte-conditioned Sato-medium with 1% HS containing 100 μg/ml leupeptin to allow endocytosis and recycling. Subsequently, heavy, medium and light membrane fractions were isolated as described below. Biotinylated proteins were precipitated from the solubilized membrane fractions using NeutrAvidin-beads and analyzed by SDS-PAGE (nonreducing conditions) and western blotting. To visualize biotinylated proteins during the recycling analysis, cells cultured on glass coverslips were treated as described above and stained with streptavidin-FITC (Invitrogen, Karlsruhe, Germany).

Fractionation of membrane subdomains

Subfractions of oligodendroglial membranes were prepared as described (Matthieu et al., 1973; Rios et al., 2000). Briefly, cells were scraped in 10 mM Tris, 5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin, pH 7.3 and homogenized by repeated shearing through a 22-gauge syringe. Nuclei were pelleted at 300 ***g*** for 10 minutes and the resulting postnuclear supernatant was subjected to centrifugation for 15 minutes at 20,000 ***g*** at 4°C. The oligodendroglial membrane pellet or isolated myelin (Kramer et al., 1997) were overlaid with a 0.8/0.62/0.32 M discontinuous sucrose gradient and centrifuged for 30 minutes at 75,000 ***g*** and 4°C. Three fractions were collected: the 0.32/0.62 M interface (light membranes), the 0.62/0.8 M interphase (medium membranes) and the pellet (heavy membranes). The membranes were pelleted from the fractions by centrifugation for 30 minutes at

100,000 ***g*** and 4°C, resuspended in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin, and either analyzed directly or subjected to precipitation using NeutrAvidin beads. The samples were analyzed by nonreducing SDS-PAGE and western blotting.

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References

Alberts, P. and Galli, T. (2003). The cell outgrowth secretory endosome (COSE): a specialized compartment involved in neuronal morphogenesis. *Biol. Cell* **95**, 419-424.

Anitei, M. and Pfeiffer, S. E. (2006). Myelin biogenesis: sorting out protein trafficking. *Curr. Biol.* **16**, R418-R421.

Anitei, M., Ifrim, M., Ewart, M. A., Cowan, A. E., Carson, J. H., Bansal, R. and Pfeiffer, S. E. (2006). A role for Sec8 in oligodendrocyte morphological differentiation. *J. Cell Sci.* **119**, 807-818.

Arantes, R. M. and Andrews, N. W. (2006). A role for synaptotagmin VII-regulated exocytosis of lysosomes in neurite outgrowth from primary sympathetic neurons. *J. Neurosci.* **26**, 4630-4637.

Arroyo, E. J. and Scherer, S. S. (2000). On the molecular architecture of myelinated fibers. *Histochem. Cell Biol.* **113**, 1-18.

Barres, B. A. and Raff, M. C. (1999). Axonal control of oligodendrocyte development. *J. Cell Biol.* **147**, 1123-1128.

Bo, L., Quarles, R. H., Fujita, N., Bartoszewicz, Z., Sato, S. and Trapp, B. D. (1995). Endocytic depletion of L-MAG from CNS myelin in quaking mice. *J. Cell Biol.* **131**, 1811-1820.

Boiko, T. and Winckler, B. (2006). Myelin under construction – teamwork required. *J. Cell Biol.* **172**, 799-801.

Bouverat, B. P., Krueger, W. H., Coetzee, T., Bansal, R. and Pfeiffer, S. E. (2000). Expression of rab GTP-binding proteins during oligodendrocyte differentiation in culture. *J. Neurosci. Res.* **59**, 446-453.

Bretscher, M. S. and Aguado-Velasco, C. (1998). Membrane traffic during cell locomotion. *Curr. Opin. Cell Biol.* **10**, 537-541.

de Vries, H. and Hoekstra, D. (2000). On the biogenesis of the myelin sheath: cognate polarized trafficking pathways in oligodendrocytes. *Glycoconj. J.* **17**, 181-190.

de Vries, H., Schrage, C. and Hoekstra, D. (1998). An apical-type trafficking pathway is present in cultured oligodendrocytes but the sphingolipid-enriched myelin membrane is the target of a basolateral-type pathway. *Mol. Biol. Cell* **9**, 599-609.

Dubois-Dalcq, M., Behar, T., Hudson, L. and Lazzarini, R. A. (1986). Emergence of three myelin proteins in oligodendrocytes cultured without neurons. *J. Cell Biol.* **102**, 384-392.

Ford, M. G., Pearse, B. M., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R. and McMahon, H. T. (2001). Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* **291**, 1051-1055.

Gow, A., Southwood, C. M., Li, J. S., Pariali, M., Riordan, G. P., Brodie, S. E., Danias, J., Bronstein, J. M., Kachar, B. and Lazzarini, R. A. (1999). CNS myelin and sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. *Cell* **99**, 649-659.

Jung, M., Kramer, E., Grzenkowski, M., Tang, K., Blakemore, W., Aguzzi, A., Khazaie, K., Chlichlia, K., von Blankenfeld, G., Kettenmann, H. et al. (1995). Lines of murine oligodendroglial precursor cells immortalized by an activated neu tyrosine kinase show distinct degrees of interaction with axons in vitro and in vivo. *Eur. J. Neurosci.* **7**, 1245-1265.

Jung, M., Sommer, I., Schachner, M. and Nave, K. A. (1996). Monoclonal antibody O10 defines a conformationally sensitive cell-surface epitope of proteolipid protein (PLP): evidence that PLP misfolding underlies dysmyelination in mutant mice. *J. Neurosci.* **16**, 7920-7929.

Karim, S. A., Barrie, J. A., McCulloch, M. C., Montague, P., Edgar, J. M., Kirkham, D., Anderson, T. J., Nave, K. A., Griffiths, I. R. and McLaughlin, M. (2007). PLP overexpression perturbs myelin protein composition and myelination in a mouse model of Pelizaeus-Merzbacher disease. *Glia* **55**, 341-351.

Kippert, A., Trajkovic, K., Rajendran, L., Ries, J. and Simons, M. (2007). Rho regulates membrane transport in the endocytic pathway to control plasma membrane specialization in oligodendroglial cells. *J. Neurosci.* **27**, 3560-3570.

Kirkham, M. and Parton, R. G. (2005). Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim. Biophys. Acta* **1746**, 349-363.

Kramer, E. M., Koch, T., Niehaus, A. and Trotter, J. (1997). Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. *J. Biol. Chem.* **272**, 8937-8945.

Kramer, E. M., Schardt, A. and Nave, K. A. (2001). Membrane traffic in myelinating oligodendrocytes. *Microsc. Res. Tech.* **52**, 656-671.

Kramer-Albers, E. M., Gehrig-Burger, K., Thiele, C., Trotter, J. and Nave, K. A. (2006). Perturbed interactions of mutant proteolipid protein/DM20 with cholesterol and lipid rafts in oligodendroglia: implications for dysmyelination in spastic paraplegia. *J. Neurosci.* **26**, 11743-11752.

Kroepfl, J. F. and Gardinier, M. V. (2001a). Identification of a basolateral membrane targeting signal within the cytoplasmic domain of myelin/oligodendrocyte glycoprotein. *J. Neurochem.* **77**, 1301-1309.

Kroepfl, J. F. and Gardinier, M. V. (2001b). Mutually exclusive apicobasolateral sorting of two oligodendroglial membrane proteins, proteolipid protein and myelin/oligodendrocyte glycoprotein, in Madin-Darby canine kidney cells. *J. Neurosci. Res.* **66**, 1140-1148.

Larocca, J. N. and Rodriguez-Gabin, A. G. (2002). Myelin biogenesis: vesicle transport in oligodendrocytes. *Neurochem. Res.* **27**, 1313-1329.

Lecuit, T. and Pilot, F. (2003). Developmental control of cell morphogenesis: a focus on membrane growth. *Nat. Cell Biol.* **5**, 103-108.

Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat. Cell Biol.* **7**, 1232-1239.

Marta, C. B., Taylor, C. M., Coetzee, T., Kim, T., Winkler, S., Bansal, R. and Pfeiffer, S. E. (2003). Antibody cross-linking of myelin oligodendrocyte glycoprotein leads to its rapid repartitioning into detergent-insoluble fractions, and altered protein phosphorylation and cell morphology. *J. Neurosci.* **23**, 5461-5471.

Marta, C. B., Montano, M. B., Taylor, C. M., Taylor, A. L., Bansal, R. and Pfeiffer, S. E. (2005). Signaling cascades activated upon antibody cross-linking of myelin oligodendrocyte glycoprotein: potential implications for multiple sclerosis. *J. Biol. Chem.* **280**, 8985-8993.

Matthieu, J. M., Quarles, R. H., Brady, R. O. and Webster, H. d. F. (1973). Variation of proteins, enzyme markers and gangliosides in myelin subfractions. *Biochim. Biophys. Acta* **329**, 305-317.

Minuk, J. and Braun, P. E. (1996). Differential intracellular sorting of the myelin-associated glycoprotein isoforms. *J. Neurosci. Res.* **44**, 411-420.

Morita, K., Sasaki, H., Fujimoto, K., Furuse, M. and Tsukita, S. (1999). Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. *J. Cell Biol.* **145**, 579-588.

Niehaus, A., Stegmuller, J., Diers-Fenger, M. and Trotter, J. (1999). Cell-surface glycoprotein of oligodendrocyte progenitors involved in migration. *J. Neurosci.* **19**, 4948-4961.

Park, M., Salgado, J. M., Ostroff, L., Helton, T. D., Robinson, C. G., Harris, K. M. and Ehlers, M. D. (2006). Plasticity-induced growth of dendritic spines by exocytic trafficking from recycling endosomes. *Neuron* **52**, 817-830.

Perret, E., Lakkaraju, A., Deborde, S., Schreiner, R. and Rodriguez-Boulan, E. (2005). Evolving endosomes: how many varieties and why? *Curr. Opin. Cell Biol.* **17**, 423-434.

Pfeiffer, S. E., Warrington, A. E. and Bansal, R. (1993). The oligodendrocyte and its many cellular processes. *Trends Cell Biol.* **3**, 191-197.

Popko, B. (2000). Myelin galactolipids: mediators of axon-glial interactions? *Glia* **29**, 149-153.

Rios, J. C., Melendez-Vasquez, C. V., Einheber, S., Lustig, M., Grumet, M., Hemperly, J., Peles, E. and Salzer, J. L. (2000). Contactin-associated protein (Caspr) and contactin form a complex that is targeted to the paranodal junctions during myelination. *J. Neurosci.* **20**, 8354-8364.

Rodriguez-Boulan, E., Kreitzer, G. and Musch, A. (2005). Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* **6**, 233-247.

Rodriguez-Gabin, A. G., Cammer, M., Almazan, G., Charron, M. and Larocca, J. N. (2001). Role of rRAB22b, an oligodendrocyte protein, in regulation of transport of vesicles from trans Golgi to endocytic compartments. *J. Neurosci. Res.* **66**, 1149-1160.

Rodriguez-Gabin, A. G., Almazan, G. and Larocca, J. N. (2004). Vesicle transport in oligodendrocytes: probable role of Rab40c protein. *J. Neurosci. Res.* **76**, 758-770.

Salzer, J. L. (2003). Polarized domains of myelinated axons. *Neuron* **40**, 297-318.

Schaeren-Wiemers, N., Bonnet, A., Erb, M., Erne, B., Bartsch, U., Kern, F., Mantei, N., Sherman, D. and Suter, U. (2004). The raft-associated protein MAL is required for maintenance of proper axon-glia interactions in the central nervous system. *J. Cell Biol.* **166**, 731-742.

Schneider, A., Lander, H., Schulz, G., Wolburg, H., Nave, K. A., Schulz, J. B. and Simons, M. (2005). Palmitoylation is a sorting determinant for transport to the myelin membrane. *J. Cell Sci.* **118**, 2415-2423.

Sherman, D. L. and Brophy, P. J. (2005). Mechanisms of axon ensheathment and myelin growth. *Nat. Rev. Neurosci.* **6**, 683-690.

Sherman, D. L., Tait, S., Melrose, S., Johnson, R., Zonta, B., Court, F. A., Macklin, W. B., Meek, S., Smith, A. J., Cottrell, D. F. et al. (2005). Neurofascins are required to establish axonal domains for saltatory conduction. *Neuron* **48**, 737-742.

Simons, M. and Trajkovic, K. (2006). Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. *J. Cell Sci.* **119**, 4381-4389.

Simons, M., Kramer, E. M., Macchi, P., Rathke-Hartlieb, S., Trotter, J., Nave, K. A. and Schulz, J. B. (2002). Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus-Merzbacher disease. *J. Cell Biol.* **157**, 327-336.

Siskova, Z., Baron, W., de Vries, H. and Hoekstra, D. (2006). Fibronectin impedes "myelin" sheet-directed flow in oligodendrocytes: a role for a beta 1 integrin-mediated PKC signaling pathway in vesicular trafficking. *Mol. Cell. Neurosci.* **33**, 150-159.

Trajkovic, K., Dhaunchak, A. S., Goncalves, J. T., Wenzel, D., Schneider, A., Bunt, G., Nave, K. A. and Simons, M. (2006). Neuron to glia signaling triggers myelin membrane exocytosis from endosomal storage sites. *J. Cell Biol.* **172**, 937-948.

Trapp, B. D., Andrews, S. B., Cootauco, C. and Quarles, R. (1989). The myelin-associated glycoprotein is enriched in multivesicular bodies and periaxonal membranes of actively myelinating oligodendrocytes. *J. Cell Biol.* **109**, 2417-2426.

Wisco, D., Anderson, E. D., Chang, M. C., Norden, C., Boiko, T., Folsch, H. and Winckler, B. (2003). Uncovering multiple axonal targeting pathways in hippocampal neurons. *J. Cell Biol.* **162**, 1317-1328.