Palmitoylation is a sorting determinant for transport to the myelin membrane

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Summary

Myelin is a specialized membrane enriched in glycosphingolipids and cholesterol that contains a restricted set of proteins. The mechanisms by which oligodendrocytes target myelin components to myelin are not known. To identify the sorting determinants for protein transport to myelin, we used a primary oligodendrocyte culture system in which terminal differentiation is synchronized and there is excessive deposition of myelin-like membranes (MLMs). Because several myelin proteins are palmitoylated, we explored the role of acylation in protein transport to MLMs. We found that palmitoylation-deficient mutants of a major myelin protein, proteolipid protein (PLP/DM20), were less efficiently targeted to MLMs. The N-terminal 13 amino acids of PLP/DM20, which are palmitoylated at three sites, were sufficient to direct a fluorescent fusion protein to MLMs. Mutagenesis of the N-terminal palmitoylation motif abolished the transport of the fusion protein to MLMs, indicating that palmitoylation is required for sorting to myelin. Similar results were obtained in myelinating co-cultures of oligodendrocytes and neurons. Furthermore, the combined farnesylation/palmitoylation signals from c-Ha-Ras and the N-terminal consensus sequence for dual palmitoylation from neuromodulin were sufficient for the transport of fluorescent fusion proteins to MLMs. Thus, we conclude that palmitoylation is a sorting determinant for transport to the myelin membrane.

Key words: Oligodendrocytes, Myelin, Proteolipid protein, Palmitoylation, Membrane trafficking, Sorting determinants

Introduction

Specialized glial cells, oligodendrocytes and Schwann cells, synthesize myelin as a multilamellar, compact membrane that ensheathes and insulates axons (Pedraza et al., 2001; Salzer, 2003). The structure and molecular composition of myelin is unique. In contrast to most plasma membranes, myelin is enriched in glycosphingolipids. The major glycosphingolipids in myelin are galactosylceramide and its sulfated derivative sulfatide (20% of lipid dry weight) (Morell and Jurevics, 1996). Myelin contains a restricted set of proteins, which are in most cases exclusively found in myelin. The major central-nervous-system myelin proteins, the myelin basic protein (MBP) and the proteolipid proteins (PLP/DM20), are low-molecular-weight proteins found in compact myelin and constitute 80% of total myelin proteins. PLP and its alternatively spliced isoform DM20 are acylated integral-membrane proteins, containing four transmembrane α-helices with cytoplasmic N- and C-termini, and molecular masses of 26 kDa and 20 kDa, respectively (Griffiths et al., 1998).

The mechanisms by which oligodendrocytes target myelin components to myelin are not known. Studies performed in epithelial cells demonstrated that polarized protein transport to distinct apical and basolateral membrane compartments, which are separated by tight junctions, occurs after sorting in the trans-Golgi network into distinct vesicular carriers (Kreitzer et al., 2003). A polarized membrane composition and distinct trafficking routes have also been proposed for oligodendrocytes, because asymmetric membrane growth (oligodendroglial plasma membrane versus myelin membrane) requires polarized transport (de Vries et al., 1998). This hypothesis has been supported by the identification of claudin11/OSP-based tight junctions in the interlamellar strands of myelin sheaths at the border between compact and non-compact myelin. Tight junctions could serve as a diffusion barrier and maintain the asymmetric protein distribution between the different myelin subdomains and the plasma membrane (Bronstein et al., 2000; Gow et al., 1999; Morita et al., 1999). For epithelial cells, two major sorting principles have been described. Transport to the basolateral surface requires the interaction of a cytoplasmic protein transport machinery with transmembrane proteins (Mellman, 1996; Mostov et al., 2003; Nelson and Yeaman, 2001). Transport to the apical membrane involves the association of proteins with glycosphingolipid- and cholesterol-enriched membrane

platforms in the trans-Golgi network, followed by vesicular transport to the apical membrane (Rodriguez-Boulan and Zurzolo, 1993; Simons and Ikonen, 1997). Given the similarities between the lipid compositions of apical and myelin membranes, which are both highly enriched in glycosphingolipids and cholesterol, it seems likely that oligodendrocytes and epithelial cells use common mechanisms to form the specialized membranes. Indeed, there is accumulating evidence that oligodendrocytes use a raft-like transport for directing proteins to myelin (Decker et al., 2004; Kim et al., 1995; Kramer et al., 1997; Simons et al., 2000; Taylor et al., 2002). The aim of this study was to identify sorting determinants for transport of proteins to the myelin membrane.

Materials and Methods

Plasmid construction and preparation of recombinant Semliki Forest virus

The 13-amino-acid N-terminal peptide of PLP was constructed with oligonucleotides encoding the appropriate wild-type or palmitoylation-deficient mutant sequences, in which cysteines were exchanged for serines. The oligonucleotides were subcloned into EYFP-N1 vector (Clontech). We used PCR to generate myc-tagged constructs of PLP [PLP(C5,6,9S)-myc] and DM20 [DM20(C5,6,9,108S)-myc] in which cysteines 5, 6, 9 and 108 were exchanged for serines. cDNAs encoding DM20-myc, PLP-myc, DM20(C5,6,9S)-myc, PLP(C5,6,9S)-myc, DM20(C5,6,9,108S)-myc, the wild-type N-terminal 13 amino acids of PLP fused to enhanced yellow fluorescent protein (1-13PLP-EYFP) and 1-13PLP(C5,6,9S)-EYFP were cloned into the *Sma*I restriction site of pSFV1 vector and recombinant Semliki Forest virus (SFV) was prepared according to Liljestrom and Garoff (Liljestrom and Garoff, 1991).

Viral infection and transfection of primary oligodendrocytes

Green fluorescent protein (GFP)-expressing SFV (SFV-GFP) was kindly provided by P. Macchi (Max-Planck Institute for Developmental Biology, Tübingen, Germany). Primary oligodendrocytes were infected with wild-type SFV, recombinant SFV, vesicular stomatitis virus (VSV) or wild-type fowl plague virus (FPV). For viral infection, the relevant virus was diluted in maintenance medium and cells were incubated for 1 hour before replacing the virus with fresh medium. Infection was allowed to continue for a further 1-3 hours (FPV, VSV, SFV) or 5-9 hours (recombinant SFV). Primary oligodendrocytes were transfected using Nupherin™-neuron kit (Biomol, PA) according to the manufacturer's protocol. p-EGFP-F (farnesylated and double palmitoylated EGFP) and pEYFP-Mem (double palmitoylated EYFP) were from Clontech and EGFP-LDL-CD46 was from P. Keller (Max-Planck Institute for Cell Biology and Genetics, Dresden, Germany).

Primary cell cultures

Primary cultures of mouse oligodendrocytes were prepared as described previously except of a few modifications (Simons et al., 2000). After shaking, cells were plated in minimum essential medium (MEM), containing B27 supplement, 1% horse serum, L-thyroxine, tri-iodothyronine, glucose, glutamine, gentamycine, pyruvate and bicarbonate on poly-L-lysine-coated dishes or glass-coverslips. The resulting cultures were enriched in oligodendrocytes, although they still contained astrocytes. Cultures were kept without addition of growth factors for 5-8 days before virus infection, transfection or metabolic labelling.

Myelinating co-cultures of neurons and oligodendrocytes were prepared according to a modified protocol of Lubetzki et al. (Lubetzki et al., 1993). Cultures were grown on glass coverslips in oligodendrocyte growth medium as described above and virus infection was done after 24 days in culture.

Immunofluorescence and electron microscopy

Immunofluorescence and electron microscopy were performed using a standard protocol. Primary antibodies were against: haemagglutinin (HA) (Harder et al., 1998); VSV glycoprotein (VSVG) (Harder et al., 1998); MBP [monoclonal IgG (Sternberger) or polyclonal antibody (DAKO)]; PLP [polyclonal antibody P6 (Linington and Waehneldt, 1990); O10, monoclonal mouse IgM]; galactosylceramide (monoclonal IgM, O1); galactosylsulfatide (monoclonal IgM, O4); tubulin-βIII (Promega); neurofilament (MN2E4, monoclonal IgM, Alexis); and myc tag (monoclonal IgG, Cell Signaling). For filipin labelling, cells were incubated for 20 minutes at room temperature with filipin (Sigma) diluted in PBS (0.5 mg ml^{-1}) and washed twice for 5 minutes in PBS. For quantification of transport to MLMs, more then 50 cells were counted from two to five independent experiments. For quantification of transport to myelin in oligodendroglial and neuronal co-cultures, more than 30 myelinating cells were evaluated from three or four independent experiments. Data are reported as dichotomized categories (colocalization vs no colocalization). Statistical differences were determined with Fischer's exact test.

Immunoblotting

Proteins were separated on 15% SDS-PAGE and detected by using the ECL western-blotting system (BioRad). Anti-GFP antibody was obtained from Santa Cruz Biotechnology.

MLM preparation

MLMs were prepared from enriched oligodendrocyte cultures 8 hours after infection with SFV according to Norton and Poduslo (Norton and Poduslo, 1973). A postnuclear supernatant was subjected to 12 minutes of centrifugation (JA20, 4°C) at 12,250 ***g***. The membrane-containing pellet was resuspended in 0.85 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and subjected to discontinuous sucrose-gradient centrifugation (11.5 ml 0.85 M sucrose overlaid with 0.5 ml 0.25 M sucrose) using a SW41 Ti rotor at 50,000 ***g*** at 4°C for 30 minutes. The light membrane was collected from the interface and washed twice with buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 2 mM PMSF by centrifugation with SW41 Ti rotor at 50,000 ***g*** at 4°C for 30 minutes. The purified membrane fraction was resuspended in lysis buffer (1% Triton X-100, 0.1% SDS in PBS).

Viral production rates were determined by immunoblotting of 1% total cell lysates with antibodies against the myc or GFP tags. The amount of endogenous PLP in each myelin preparation was measured by immunoblotting with anti-PLP (P6) antibody. In case of virally produced PLP, the amount of endogenous DM20 served as a measure for the preparation yield. The ratio of virus-derived PLP or DM20 mutants compared with the corresponding virus-derived wild-type protein in the myelin fraction was calculated by adjusting the for different production levels and preparation yields. For quantification, blots were scanned and evaluated with the ScionImage. Values are shown as means ± SD. Statistical differences were determined with unpaired, two-tailed Student's *t* tests.

Results

Enriched oligodendrocyte cultures form MLMs

One precondition for studying membrane trafficking to the myelin compartment in vitro is the establishment of a culture system in which terminal differentiation of oligodendrocytes

into myelin-producing cells occurs. Various oligodendrocyte-neuron co-culture systems have been described, such as dorsal-root-ganglia cells with oligodendrocytes and mixed brain cultures (Lubetzki et al., 1993; Shaw et al., 1996; Svenningsen et al., 2003). However, the selective production of exogenous reporter proteins in myelinating oligodendrocytes is difficult to achieve in these co-cultures. We therefore used primary cultures of oligodendrocytes, in which terminal differentiation is synchronized and excessive deposition of MLMs is observed. Oligodendrocytes, when enriched in culture, rapidly differentiate to a stage at which they elaborate membrane sheets that contain both PLP and MBP (Fig. 1A). After further differentiation, the cells form thin processes that contain many annular membrane profiles (Fig. 1B) (Dubois-Dalcq et al., 1986). By immunofluorescence microscopy, we found that these structures contained MBP, PLP (Fig. 1B,D) and major myelin lipids such as galactosylceramide (O1) (Fig. 1C), galactosylsulfatide (O4) (Fig. 1E) and cholesterol (shown by filipin staining) (Fig. 1C-E). Because the colocalization of major myelin components in distinct membrane profiles suggests the deposition of myelin membrane, we performed electron-microscopy analysis of these cultures (Fig. 1F-H). The electron micrographs confirmed abundant formation of multilamellar membrane stacks apposed to or emanating from processes of oligodendrocytes. As shown previously, these multilamellar membranes frequently contain multiple dense layers with a 7 nm periodicity, in contrast to the alternate dense and intraperiod lines of 12 nm periodicity found in situ (Arvanitis et al., 1992; Dubois-Dalcq et al., 1986). Because the multilamellar membrane formations observed in our culture system do not display myelin in its full complexity, we called them MLMs.

Viral surface glycoproteins HA and VSVG but not E2, are transported to myelin

To gain insight into the mechanisms involved in trafficking of proteins to MLM, we analysed the targeting of different viral membrane proteins. Oligodendrocytes were infected with FPV to express the HA, which associates with detergent-resistant membranes (DRMs) and is transported to the apical membrane in epithelial cells (Mostov et al., 2000). To evaluate the targeting of HA to MLMs, cells were stained for HA, MBP and PLP by indirect immunofluorescence labelling (Fig. 2A). Colocalization of MBP, PLP and HA in large membrane patches of cellular processes was regarded as completed transport to MLMs. Quantification revealed that HA is efficiently targeted to MLMs (in 70% of the cells). When cells were infected with VSV in order to produce VSVG, a marker for transport to the basolateral membrane in MDCK cells (Musch et al., 1996), we found that VSVG was also transported to MLMs (in 90% of the cells) (Fig. 2B). The similar distribution of both apical and basolateral markers in MLMs could lead to the assumption that transport to myelin is not regulated but rather occurs by default. However, when we infected oligodendrocytes with SFV, its spike protein E2 [localization of which to the apical or to the basolateral compartment is dependent on the cell type (Zurzolo et al., 1992)] was homogeneously distributed in cellular processes but largely excluded from MLMs (localized to MLM in 30% of cells) (Fig. 2C). For expression of all viral proteins, short post-infection times (1-3 hours) were used to avoid oversaturation of the sorting machinery and thus unselective transport to MLMs.

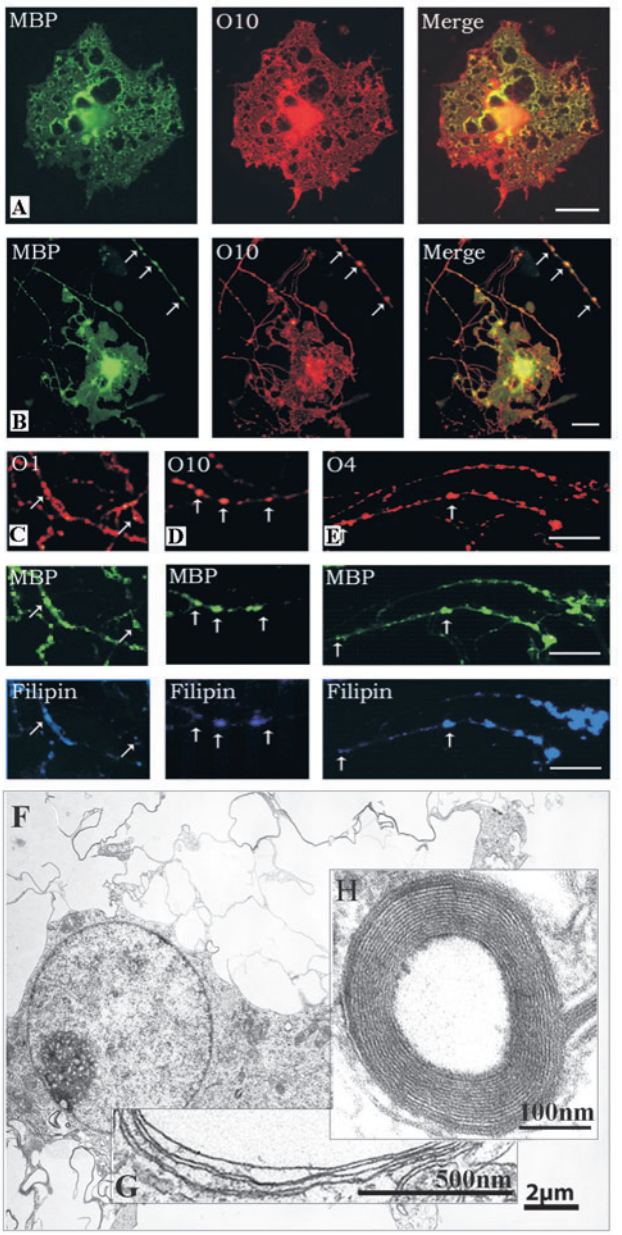

Fig. 1. Enriched oligodendrocyte cultures form MLMs even in the absence of neurons. (A) Primary cultures of oligodendrocytes with membrane sheets at an early stage of differentiation were stained for MBP and PLP. (B) Immunofluorescence analysis of the same culture at a later stage of differentiation. Notice the many thin processes, which contain pearl-like membrane patches (arrows). These patches are recognized by antibodies directed at both major myelin proteins MBP and PLP. Membrane patches also contain the myelin lipids cholesterol (C-E), galactosylceramide (C) and sulfatide (E), as shown by reactivity with filipin (C-E) and antibodies against the O1 (C) and O4 (E) epitopes. (F) Electron micrograph of differentiated oligodendrocyte shows multiple thin processes containing MLMs. (G,H) Multilamellar membranes at different stages of compaction. MLMs were seen in almost all of the cells. The interior of these structures were most often electron lucent and did not contain axons. Bar, 20 μm or as indicated.

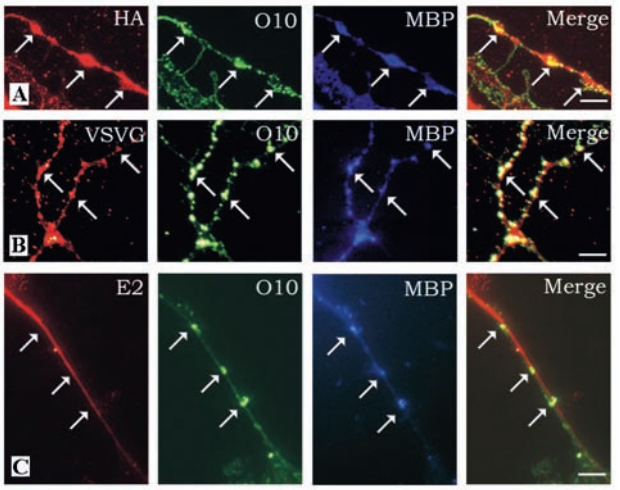

Fig. 2. The viral surface glycoproteins HA and VSVG, but not E2, are transported to MLMs. After infection of primary oligodendrocytes with FPV, VSV or SFV, cells were fixed and processed for immunofluorescence microscopy. Cells were triple stained for PLP (O10), MBP and the viral glycoproteins. Both HA (A) and VSVG (B) were found in MLMs (arrows), from which E2 (C) was largely excluded. Bar, 10 μm.

In a previous study that used a similar experimental approach (de Vries et al., 1998), VSVG but not HA was transported to 'membrane sheets' of cultured oligodendrocytes. However, whether or not these membrane sheets correspond to myelin remains to be determined. In fact, we found that all ectopically produced viral markers (HA, VSVG and E2) were eventually transported to membrane sheets (data not shown). Our finding that both VSVG and HA are targeted to MLMs suggests that myelin sorting might not be analogous to a basolateral or an apical sorting pathway. However, it is in line with the concept that membrane transport to myelin is mediated by rafts (Decker and ffrench-Constant, 2004; Kim et al., 1995; Kramer et al., 1997; Simons et al., 2000; Taylor et al., 2002). Despite its solubility in Triton X-100, evidence has recently been provided that VSVG is a raft-associated protein that, upon antibody cross-linking, forms patches with raft markers, and that partitions into a raft-like virus envelope during virus budding from the host cell plasma membrane (Harder et al., 1998; Pickl et al., 2001). An interesting feature of proteins that associate with lipid rafts is the presence of post-translational lipid modifications. Glycosylphosphatidylinositol (GPI) anchors, combined myristoylation-palmitoylation and dual palmitoylation are essential for the association of many proteins with rafts (Brown and London, 1998; Zacharias et al., 2002). Because palmitoylation is a frequent post-translational modification of myelin proteins (e.g. PLP/DM20, CNP, P0) (Agrawal et al., 1990; Bizzozero et al., 1994; Weimbs and Stoffel, 1992), we hypothesized that palmitoylation is a sorting signal for targeting proteins to myelin.

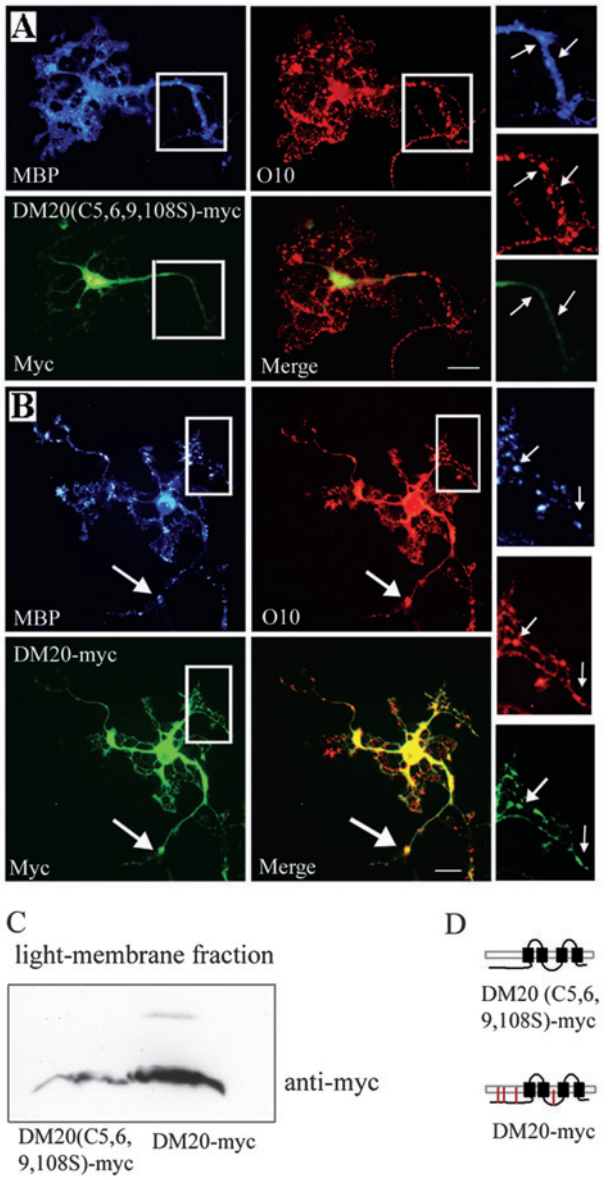

Fig. 3. Palmitoylation enhances transport of PLP and DM20 to MLMs. Primary oligodendrocytes were infected with recombinant SFV to express the palmitoylation-deficient form of DM20, DM20(C5,6,9,108S)-myc (A), and DM20-myc (B). Cells were stained for PLP (O10) and MBP to visualize MLM (arrows). Staining with anti-myc antibody revealed preferential targeting of DM20-myc to MLMs compared with DM20(C5,6,9,108S)-myc. Primary oligodendrocytes were infected with SFV-DM20-myc or SFV-DM20(C5,6,9,108S)-myc and light-membrane fractions were isolated according to the myelin-isolation protocol to enrich for MLMs. The amount of virus-derived DM20-myc and DM20(C5,6,9,108S)-myc in the myelin preparations was determined by immunoblotting with an anti-myc antibody (C). (D) DM20-myc and DM20(C5,6,9,108S)-myc with palmitoylation sites are shown in red. Bar, 20 μm.

Palmitoylation of PLP/DM20 is important for transport to myelin

We designed palmitoylation-deficient PLP and DM20 mutants, which allowed us to study the particular effects of distinct palmitoylation sites on the targeting of PLP. Palmitoylation of PLP occurs N-terminally at C5, C6 and C9, and in the cytoplasmic loop at C108, C138 and C140. DM20, the minor isoform of PLP, lacks 35 amino acids in the cytosolic loop and

therefore lacks cysteines 138 and 140. We exchanged the N-terminal palmitoylation sites of PLP (C5, C6 and C9) for serines and added a myc tag to its C-terminus to obtain a mutant form of PLP that is still palmitoylated in the cytosolic loop [PLP(C5,6,9S)-myc]. Furthermore, we constructed a mutant form of DM20 that lacks C5, C6, C9 and C108, and is therefore devoid of all palmitoylation sites [DM20(C5,6,9,108S)-myc]. All constructs were cloned into an SFV vector. To ensure that the proteins were folded properly and transported to the plasma membrane, we performed surface stainings of living BHK cells with a conformation-dependent antibody against PLP/DM20 (O10) (Jung et al., 1996). All of the above proteins were stained on the cell surface with O10, demonstrating their transport through the biosynthetic pathway (data not shown). Next, we tested their targeting to MLMs. Immunofluorescence microscopy revealed that targeting of PLP(C5,6,9S)-myc and DM20(C5,6,9,108S)-myc to MLMs was not completely abolished. However, compared with their respective wild-type constructs, both proteins appeared to be more restricted to the soma and fewer MBP/PLP patches were positive for DM20(C5,6,9,108S)-myc compared with DM20-myc (localization to MLMs in 67% of cells for DM20-myc and in 38.5% of cells for DM20(C5,6,9,108S)-myc; $P<0.005$) (Fig. 3A,B). The same applies to PLP(C5,6,9S)-myc compared with PLP-myc (localization to MLMs in 90% of the cells for PLP-myc and in 69% of the cells for PLP(C5,6,9S)-myc; data not shown; $P<0.05$).

To obtain further support for the different targeting to MLMs, a biochemical approach was used. Primary cultures of oligodendrocytes were infected with recombinant SFV and a light membrane fraction was isolated according to the myelin isolation protocol of Norton and Poduslo (Norton and Poduslo, 1973) to enrich MLMs. Consistent with the immunocytochemical results, we repeatedly found less DM20(C5,6,9,108S)-myc and PLP(C5,6,9S)-myc in the MLM-enriched fraction than we did of the respective wild-type forms (Fig. 3C). Quantification revealed a reduced incorporation of DM20(C5,6,9,108S)-myc (reduced to 48±14%, $n=4$, $P<0.01$) and PLP(C5,6,9S)-myc (reduced to 32±31%, $n=3$, $P<0.05$; data not shown) into the light membrane fraction.

Both morphological and biochemical experiments indicate that transport to MLMs is facilitated by palmitoylation of PLP/DM20. However, targeting of the palmitoylation-deficient PLP/DM20 to MLM still occurs, albeit less efficiently than wild-type PLP/DM20. This points to the presence of additional transport signals in PLP/DM20 that can substitute for the absent palmitoylation sites. Another explanation could be that the mutant form of PLP is routed to MLMs by associating with wild-type endogenous PLP/DM20. To test this possibility, enriched primary oligodendrocyte cultures were prepared from PLP knockout animals. As in the wild-type cultures, patches of MBP and galactosylceramide (O1) were observed along processes, which is consistent with the finding that myelin assembly occurs despite the absence of PLP (Klugmann et al., 1997). Targeting of PLP(C5,6,9S)-myc to MLM in PLP knockout cultures was not completely abolished (data not shown), indicating that the transport of palmitoylation-deficient PLP is not facilitated by co-transport with endogenous wild-type PLP.

Palmitoylation of the N-terminal 13 amino acids of PLP is required for sorting into myelin

A previous study has shown that the N-terminal 13 amino acids of PLP fused to LacZ are sufficient for targeting the

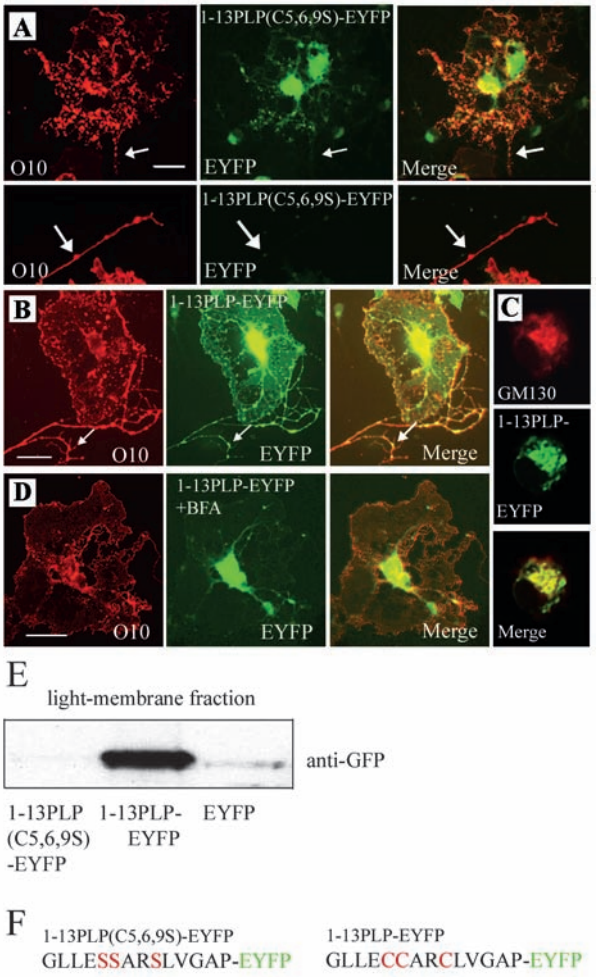

Fig. 4. The 13 amino acid N-terminus of PLP is sorted into MLMs in a palmitoylation-dependent way. Primary oligodendrocyte cultures were infected with recombinant SFV producing 1-13PLP-EYFP or its palmitoylation-deficient mutant 1-13PLP(C5,6,9S)-EYFP, and processed for immunofluorescence 5 hours after infection. Cells were stained for PLP (O10) to visualize MLMs (arrows). 1-13PLP(C5,6,9S)-EYFP showed a diffuse staining of the oligodendroglial soma and some processes (A), whereas 1-13PLP-EYFP was distributed over the entire oligodendrocyte membrane and was detectable in MLMs (B). 1-13PLP-EYFP was also found on intracellular membranes in the Golgi region. Confocal microscopy analysis showed colocalization of 1-13PLP-EYFP with GM130, a marker for the Golgi apparatus (C). Treatment with BFA (10 μg ml^{-1}) for the last 4 hours during post-infection time resulted in a redistribution of 1-13PLP-EYFP from the distal membrane extensions into the soma and proximal part of some processes (D). Primary oligodendrocytes were infected with SFV-1-13PLP-EYFP, SFV-1-13PLP(C5,6,9S)-EYFP or SFV-EYFP, and light-membrane fractions were isolated according to the myelin-isolation protocol to enrich MLMs. The amounts of virus-derived 1-13PLP-EYFP, 1-13PLP(C5,6,9S)-EYFP and EYFP in the myelin preparations were determined by immunoblotting with an anti-GFP antibody (E). The sequence of the N-terminal 13 amino acids is shown for 1-13PLP-EYFP and 1-13PLP(C5,6,9S)-EYFP (F). Bars, 20 μm.

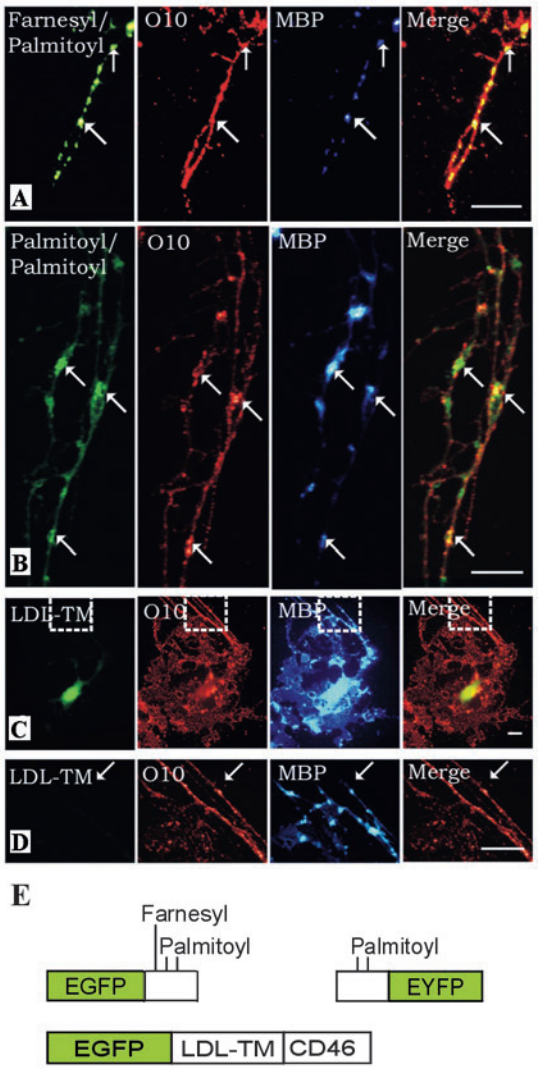

Fig. 5. Transport of acylated GFP fusion proteins to MLMs. Primary oligodendrocytes were transiently transfected with plasmids coding for various fluorescent fusion proteins containing different acylation sites. We used a EGFP fusion protein with the C-terminal 20 amino acids of H-Ras (EGFP-tH), which contain a combined farnesylation/palmitoylation signal, and an EYFP fusion protein with the N-terminal 20 amino acids of neuromodulin (tN-EYFP), which contain a consensus sequence for dual palmitoylation. To visualize MLMs, cells were stained for PLP (O10) and MBP. Both EGFP-tH (A) and tN-EYFP (B) were found in MLMs. By contrast, an LDL-receptor construct in which the cytoplasmic domain of the LDL receptor had been replaced by the cytoplasmic domain of CD46 to remove its endocytic sorting determinant and the extracellular LDL receptor domain had been replaced by EGFP was not localized to MLMs (C,D). (E) The constructs used. Bar, 10 μm.

cytoplasmic fusion protein PLP-LacZ to the myelin membrane (Wight et al., 1993). Its sequence harbours three cysteine residues, which are palmitoylated in full-length PLP in vivo. Because palmitoylation-deficient PLP/DM20 was targeted less efficiently to MLMs, we studied whether transport of the N-terminal PLP peptide was also dependent on palmitoylation. For this purpose, we constructed recombinant SFV either with the wild-type N-terminal 13 amino acids of PLP fused to EYFP (1-13PLP-EYFP) or with a mutant form in which the cysteines had been replaced by serines [1-13PLP(C5,6,9S)-EYFP]. To find out whether the N-terminal 13 amino acids of PLP were still palmitoylated when fused to EYFP, cells were infected with recombinant SFV and metabolically labelled with [^{3}H]-labelled palmitic acid. The autoradiography revealed palmitoylation of 1-13PLP-EYFP but not of 1-13PLP(C5,6,9S)-EYFP (data not shown). To evaluate the subcellular localization of 1-13PLP-EYFP and 1-13PLP(C5,6,9S)-EYFP, primary cultures of oligodendrocytes were infected with recombinant SFV and processed for immunofluorescence 5 hours after infection. Whereas 1-13PLP(C5,6,9S)-EYFP showed a diffuse staining of the oligodendroglial soma and some processes (Fig. 4A), 1-13PLP-EYFP was distributed over the entire oligodendrocyte membrane and was readily detectable in MLMs (Fig. 4B). In addition, it was found on intracellular membranes in the Golgi region. Confocal microscopy analysis proved colocalization of 1-13PLP-EYFP with GM130, a marker for the Golgi apparatus (Fig. 4C). To test whether 1-13PLP-EYFP transport to oligodendroglial membrane extensions occurred by a Golgi-dependent pathway, cells were treated with the Golgi-disrupting agent, brefeldin A (BFA) (Fig. 4D). Treatment with BFA resulted in a redistribution of 1-13PLP-EYFP from the distal membrane extensions into the soma and some processes, suggesting that 1-13PLP-EYFP traffics by the exocytic pathway to the plasma membrane (targeting of 1-13PLP-EYFP to MLM was reduced by 62.5% in BFA-treated cells; $P<0.0001$).

To obtain more evidence for the palmitoylation-dependent targeting, we biochemically isolated and analysed the amount of mutant and wild-type constructs targeted to the MLM fraction. A membrane fraction prepared according to the myelin isolation protocol was obtained from cells infected with the different recombinant forms of SFV. As shown in Fig. 4E, 1-13PLP-EYFP was clearly retrieved from myelin preparations, in contrast to 1-13PLP(C5,6,9S)-EYFP, which was virtually absent from MLM preparations.

Palmitoylation is a general sorting determinant for transport to myelin

To test whether palmitoylation can act as a general targeting motif for transport to MLMs, primary oligodendrocytes were transiently transfected with plasmids coding for various fluorescent fusion proteins containing different acylation sites. For transfection, a peptide that combines a non-classical nuclear localization sequence (M9) with a nucleotide-binding domain was used to improve delivery of plasmids into differentiated oligodendrocytes (Subramanian et al., 1999). The distribution of enhanced green fluorescent protein (EGFP) fused to the C-terminal 20 amino acids of H-Ras (EGFP-tH) was investigated first. This 20-residue sequence contains a combined farnesylation/palmitoylation signal, and is sufficient to target the cognate full-length protein to lipid rafts (Prior et al., 2001; Zacharias et al., 2002). We found that EGFP-tH was efficiently transported to MLM (Fig. 5A). In a similar way, the N-terminal 20 amino acids of neuromodulin (which contain a consensus sequence for dual palmitoylation) fused to EYFP (tN-EYFP) were highly enriched in MLMs (Fig. 5B). By contrast, EYFP lacking any additional targeting information

was absent from MLMs (data not shown). As an additional control and to rule out unspecific targeting, we expressed a low density lipoprotein (LDL) receptor construct in which the cytoplasmic domain of the LDL receptor had been replaced by the cytoplasmic domain of CD46 to remove its endocytic sorting determinant. Moreover, the extracellular LDL receptor domain had been replaced by EGFP. This fusion protein was not targeted to MLMs, demonstrating selective transport to MLM (Fig. 5C).

Palmitoylation-deficient mutants of PLP/DM20 are less efficiently targeted to myelin in myelinating co-cultures of oligodendrocytes and neurons

Having identified a role for palmitoylation in the targeting to MLMs, we next characterized the function of this targeting motif in the context of myelinating co-cultures. Co-cultures were infected with recombinant SFV and processed for immunofluorescence 8 hours after infection. The myelin sheaths and axons were identified by labelling with anti-MBP antibody and with an antibody specific for neuronal tubulin or neurofilament, respectively. We found that only 1-13PLP-EYFP but not 1-13PLP(C5,6,9S)-EYFP was efficiently delivered to myelin [localization in myelin in 65% of the cells for 1-13PLP-EYFP and 35% of the cells for 13PLP(C5,6,9S)-EYFP; $P<0.05$] (Fig. 6A,B). Next, we tested the targeting of the palmitoylation-deficient mutant of DM20 to myelin. Immunofluorescence microscopy revealed that DM20(C5,6,9,108S)-myc appeared to be more restricted to the soma and was less efficiently targeted to myelin than wild-type DM20 [localization in myelin in 79% cells for DM20-myc; in 50% cells for DM20(C5,6,9,108S)-myc; $P<0.05$] (Fig. 6C,D). These results strengthen our conclusion that palmitoylation is a sorting determinant to myelin.

Discussion

The aim of this study was to identify sorting determinants for the targeting of proteins from oligodendrocytes to myelin. Taken together, we provide evidence that palmitoylation is a sorting determinant for transport to myelin. This conclusion is supported by several findings. First, a 20-amino-acid sequence with a combined farnesylation/palmitoylation signal from c-Ha-Ras and the N-terminal 20 amino acids of neuromodulin, which contain a consensus sequence for dual palmitoylation, fused to fluorescent proteins, were both transported to MLMs. Second, EYFP was transported to MLMs only when it was coupled to the wild-type sequence of the N-terminal 13 amino acids of PLP but not when the palmitoylation sites had been deleted. Third, palmitoylation-deficient mutants of PLP/DM20 were less efficiently transported to MLMs than their wild-type forms.

Our data are supported by a previous in vivo study that revealed targeting of β-galactosidase to the myelin membrane of transgenic animals expressing a PLP-LacZ fusion gene that contained the portion of the PLP sequence coding for the first 13 amino acids of PLP (Wight et al., 1993). By contrast,

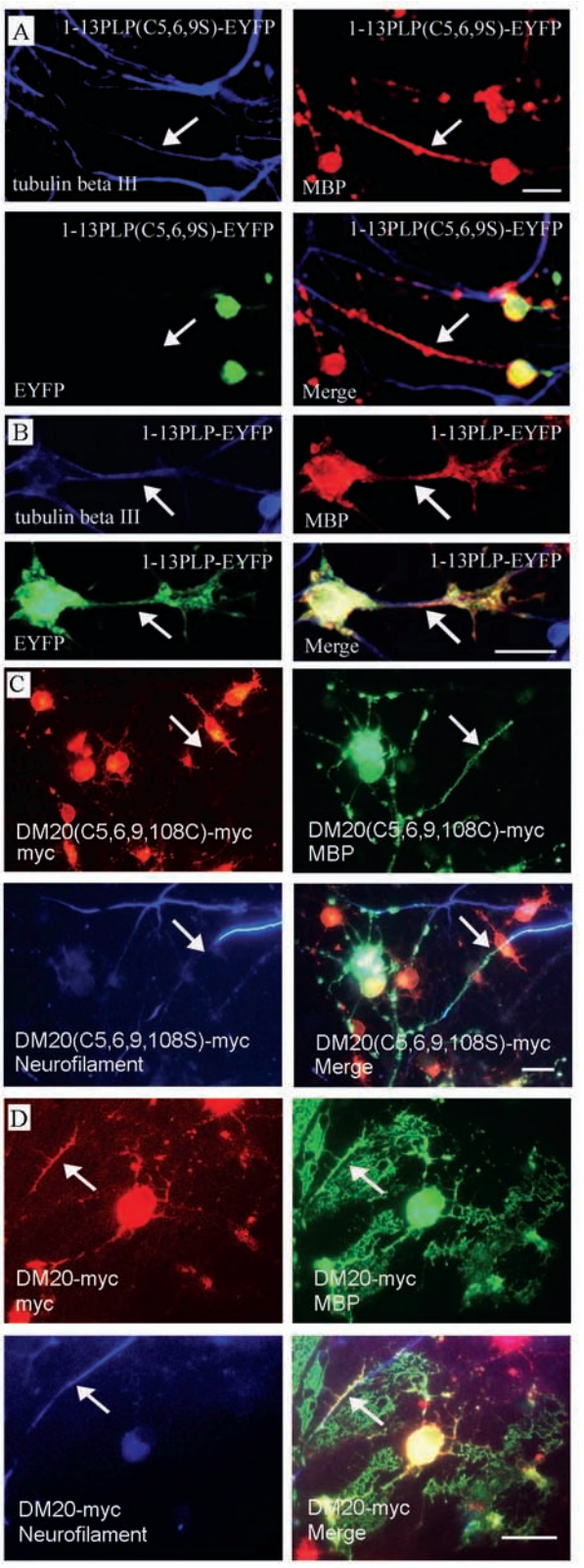

Fig. 6. Palmitoylation-deficient mutants of PLP/DM20 are less efficiently targeted to myelin in myelinating co-cultures of oligodendrocytes and neurons. Myelinating co-cultures of oligodendrocytes and neurons were infected with recombinant SFV to produce 1-13PLP(C5,6,9S)-EYFP (A), 1-13PLP-EYFP (B), DM20(C5,6,9,108S)-myc (C) and DM20-myc (D). The myelin sheaths and axons were identified by labelling with anti-MBP antibody and with an antibody specific for neuronal tubulin or neurofilament, respectively. Bars, 20 μm.

transgenic mice producing GFP under the control of the same PLP promoter but lacking the sequence coding for the N-terminal 13 amino acids of PLP produced GFP in the cell body of oligodendrocytes (Fuss et al., 2000). Taken together with our findings that define the palmitoylated cysteines as the crucial sorting determinants of these 13 amino acids, these data clearly indicate that palmitoylation acts as a sorting signal to myelin that probably also operates in vivo. It is interesting to compare our findings using genetic tools with a previous report in which palmitoylation of PLP in brain slices was partially inhibited by various drugs (DeJesus and Bizzozero, 2002). Here, a reduction of PLP palmitoylation by less then 50% was probably too low to have a significant effect on PLP targeting. It is possible that PLP contains additional sorting determinants and palmitoylation acts together with these signals to enhance the fidelity of sorting to myelin. We have previously shown that PLP tightly associates with the major myelin lipids galactosylceramide and cholesterol, and that this step might be crucial for sorting of PLP (Simons et al., 2000). Association of transmembrane proteins with lipids also involves direct protein-lipid interactions, as has been shown for HA, in which hydrophobic amino acid residues in contact with the exoplasmatic leaflet of the membrane play a crucial role in mediating protein interaction with an ordered lipid environment (Scheiffele et al., 1997). It is therefore conceivable that association of PLP with the myelin membrane is also governed by direct interaction of the transmembrane domains of PLP with myelin lipids. Palmitoylation might be important to stabilize these protein-lipid interactions. The preferential association of palmitate, a 16-carbon saturated acyl chain, with ordered lipid environments might be the molecular basis for the affinity to the myelin membrane.

It is important to realize, however, that palmitoylation is not sufficient to transport all proteins to myelin. For example, the SFV glycoprotein E2 is palmitoylated close to its transmembrane domain (Scharer et al., 1993) but is not transported to MLMs. Palmitoylation might therefore be overruled by additional sorting signals in proteins that are not destined for myelin. This is reminiscent to sorting in epithelial cells, in which, for example, *N*- and *O*-glycosylation (a sorting determinant for transport to the apical membrane) is recessive to basolateral sorting signals (Scheiffele et al., 1995). However, we found that palmitoylation of the peripheral membrane protein 1-13PLP-WT-EYFP, which is diffusely localized through the cytoplasm, is sufficient for its targeting to the myelin membrane; this clearly shows that palmitoylation can act as the sole sorting determinant to myelin in some cases. This is supported by the sorting of palmitoylated fusion proteins of EYFP to MLMs. Given the complexity of the myelin membrane with subdomains containing different proteins (e.g. abaxonal, adaxonal and periaxonal myelin), a range of sorting determinants are likely to operate in oligodendrocytes. General post-translational modifications, such as protein palmitoylation, might be important for enhancing the affinity of proteins for the myelin membrane. However, additional molecular interactions are required for the generation of myelin in its full complexity, including its different subdomains. For example, ligation of NF-155 to the axonal contactin/Caspr complex is crucial for the formation of paranodal myelin and leads to the recruitment of NF-155 into oligodendroglial lipid rafts (Schafer et al., 2004; Tait et al., 2000). Myelin biogenesis must be considered to be a multistep process involving hierarchies of sorting events. The challenge for the future will be to define additional molecular interactions and to identify the underlying sorting machinery required for the formation of the myelin membrane.

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