Assembly of Myelin by Association of Proteolipid Protein with Cholesterol- and Galactosylceramide-rich Membrane Domains

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Abstract. Myelin is a specialized membrane enriched in glycosphingolipids and cholesterol that contains a limited spectrum of proteins. We investigated the assembly of myelin components by oligodendrocytes and analyzed the role of lipid–protein interactions in this process. Proteolipid protein (PLP), the major myelin protein, was recovered from cultured oligodendrocytes from a low-density CHAPS-insoluble membrane fraction (CIMF) enriched in myelin lipids. PLP associated with the CIMF after leaving the endoplasmic reticulum but before exiting the Golgi apparatus, suggesting that myelin lipid and protein components assemble in the Golgi complex. The specific association of PLP with myelin lipids in CIMF was supported by the finding that it was efficiently cross-linked to photoactivable cholesterol, but not to phosphatidylcholine, which is underrepresented in both myelin and CIMF. Furthermore, depletion of cholesterol or inhibition of sphingolipid synthesis in oligodendrocytes abolished the association of PLP with CIMF. Thus, PLP may be recruited to myelin rafts, represented by CIMF, via lipid–protein interactions. In contrast to oligodendrocytes, after transfection in BHK cells, PLP is absent from isolated CIMF, suggesting that PLP requires specific lipids for raft association. In mice deficient in the enzyme ceramide galactosyl transferase, which cannot synthesize the main myelin glycosphingolipids, a large fraction of PLP no longer associates with rafts. Formation of a cholesterol- and galactosylceramide-rich membrane domain (myelin rafts) may be critical for the sorting of PLP and assembly of myelin in oligodendrocytes.

Key words: proteolipid protein • cholesterol • galactocerebroside • myelin • rafts

Introduction

Differentiated oligodendrocytes synthesize large amounts of myelin, a multilamellar membrane that ensheathes and insulates the axons of the central nervous system (Pfeiffer et al., 1993). The specific molecular organization of myelin is essential for the function of the nervous system, and the disturbance of this architecture leads to severe neurological symptoms observed in diseases such as multiple sclerosis. How oligodendrocytes form and maintain this specialized membrane is not known.

In contrast to most plasma membranes, myelin has a specialized lipid composition and contains a restricted set of proteins. Two main proteins comprise the majority (~85%) of the myelin protein: the cytoplasmic protein myelin basic protein (MBP)[1] and the integral membrane protein proteolipid protein (PLP) (Braun, 1984; Lees and Brostoff, 1984). PLP and its alternatively spliced isoform DM20 (Nave et al., 1987) are very hydrophobic proteins spanning the membrane four times with a molecular mass of 26 and 20 kD, respectively (Milner et al., 1985; Popot et al., 1991). The lipophilic character of both isoforms increases during transport through the cell due to the addition of several acyl chains during the final stages of biosynthesis (Weimbs and Stoffel, 1992).

Myelin has an unusual lipid composition. In contrast to most plasma membranes, myelin is enriched in the glycosphingolipids, galactosylceramide and sulfatide. This

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[1]*Abbreviations used in this paper:* CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CGT, ceramide galactosyl transferase; CIMF, CHAPS-insoluble membrane fraction; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; HS, horse serum; mβCD, methyl-β-cyclodextrin; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NCAM, neural cell adhesion molecule; PLP, proteolipid protein; SFV, Semliki Forest virus; VSV G, vesicular stomatitis virus G protein.

high level of glycosphingolipids is accompanied by a low level of phosphatidylcholine, the main lipid of plasma membranes of most cells. The ratio of glycosphingolipid/phospholipid/cholesterol is very similar to that of the apical brush border membrane of epithelial cells (Simons and van Meer, 1988). This led us to postulate that oligodendrocytes and epithelial cells may utilize common mechanisms to form these specialized membranes.

Two major sorting principles have been described in epithelial cells. For transport to the basolateral surface, the interaction of a cytoplasmic protein machinery with transmembrane proteins is essential (Mellman, 1996). In contrast, lipid–lipid and protein–lipid interactions play a major role in apical transport (Rodriguez-Boulan and Zurzolo, 1993; Simons and Ikonen, 1997). According to this model, glycosphingolipid and cholesterol segregate from bulk lipids within the TGN and form so called lipid rafts that recruit a specific set of proteins, which are transported via vesicular carriers to the apical surface membrane. The formation of rafts is thought to occur by self-association of sphingolipids via their long saturated acyl chains (Brown, 1998). Hydrogen bonding between the glycosphingolipid headgroups of rafts is not required, but might additionally stabilize rafts (Ostermeyer et al., 1999). These interactions lead to the formation of a less fluid, liquid-ordered phase, separating from a phosphatidylcholine-rich liquid-disordered phase within the exoplasmic leaflet (Brown and London, 2000). It has been shown that molecules within the liquid-ordered phase remain as an insoluble membrane fraction after extraction with detergent under mild conditions (Brown and Rose, 1992; Schroeder et al., 1994). Due to their high lipid content, detergent-insoluble membrane fractions float in a low-density fraction after density gradient centrifugation and are sequestered from other detergent-insoluble material such as the majority of the cytoskeleton. An interesting feature of proteins that associate with rafts is the presence of specific post-translational modifications. For example, glycosylphosphatidylinositol (GPI)-anchored proteins are anchored via a lipid moiety, whereas Src-family kinases, nitric oxide synthase, influenza virus hemagglutinin (HA), and caveolin are acylated (Simons and Ikonen, 1997). Cholesterol has been shown to be essential for association of these proteins with rafts (Cerneus et al., 1993; Scheiffele et al., 1997). Thus, the behavior of proteins in response to cholesterol depletion and their resistance to extraction with mild detergent can be used as criteria for raft association.

Rafts are thought to be small and highly dynamic and thus are not resolved on cultured cells by conventional microscopic techniques (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998; Pralle et al., 2000). However, the patching of membrane components of rafts with antibodies leads, to the coalescence of small rafts into larger domains that can be readily visualized (Harder et al., 1998). Proteins present in similar lipid phases tend to cocluster during patching experiments, while proteins present in immiscible lipid phase remain separate (Harder et al., 1998). Hence, the patching behavior of a protein can be used as a morphological criterion for raft association complementary to the biochemical criteria.

Here, we have studied the mechanism of myelin assembly and asked the question whether lipid–protein interactions are involved in the generation of myelin.

Materials and Methods

Reagents

Unless otherwise stated, all chemicals were obtained from the sources described previously by Krämer et al. (1997). 3-[(3-cholamidopropyl) dimethylammonia]-1-propanesulfonate (CHAPS), fumonisin B1, and methyl-β-cyclodextrin (mβCD) were from Sigma-Aldrich; Optiprep was from Nycomed Pharma. The following rabbit pAbs were used: antibodies recognizing neural cell adhesion molecule (NCAM) (Trotter et al., 1989), HA (Harder et al., 1998), E2, the COOH-terminal peptide of PLP (Linington and Waehneldt, 1990), and myelin-associated glycoprotein (MAG; a gift from F. Kirchhoff, Max-Planck-Institute of Experimental Medicine, Göttinger, Germany). The following mAbs were used: murine mAb 27-11-111 made against chick F11 (Bruemmendorf et al., 1993), provided by F. Rathjen (Max Delbrick Center for Molecular Medicine, Berlin-Buch, Germany), which cross-reacts with mouse F3; murine mAb against myelin oligodendrocyte glycoprotein (MOG) (clone 818C5) and mouse mAb against MBP provided by C. Linington (Max-Planck-Institute of Neurobiology, Planegg-Martinstied, Germany); murine mAb O10 (Kuhlmann-Krieg et al., 1988), which recognizes PLP (Jung et al., 1996). Secondary antibodies were from Dianova.

Cell Culture and Transfection

Primary cultures of oligodendrocytes were prepared from embryonic day 14–16 mice as described (Trotter et al., 1989). Oligodendrocytes growing on top of a layer of astrocytes were shaken off and cultured further in modified Sato medium supplemented with 1% horse serum (HS) on poly-L-lysine–coated dishes. The cells were fed with 10 ng/ml human recombinant PDGF and 5 ng/ml basic FGF immediately and 24 h after the shake. The cells were left for at least 5 d in culture before using in experiments. For inhibition of sphingomyelin synthesis, fumonisin B1 was added from a 1 mM stock in 20 mM Hepes, pH 7.4, to oligodendrocytes 24 h after plating at a final concentration of 50 μM. Fumonisin B1 was subsequently added every 24 h at a concentration of 50 μM for a total of 5 d.

BHK-21 cells were maintained at 37°C in 5% CO_2 in a humidified atmosphere in G-MEM (GIBCO BRL), 10% FCS, supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml) and 2 mM glutamine (all from GIBCO BRL).

Transfection of BHK cells was performed with FuGENE Transfection reagents (Roche Molecular Biochemicals) according to the manufacturer's protocol.

Myelin Preparation

Myelin was prepared from adult mice as described previously (Krämer et al., 1997). In brief, brains were homogenized in ice-cold 10.5% sucrose using an Ultra-Turrax T25 (IKA) and centrifuged in a sucrose step gradient. Myelin was recovered from the 10.5 and 30% interface and further purified by two rounds of hypoosmotic shock by resuspension in a large volume of ice-cold water, followed by a second round of centrifugation in a sucrose step gradient. Purified myelin was collected from the interface, washed twice with ice-cold water, resuspended in a small volume of water, and frozen in small aliquots at −80°C. The protein content of the myelin preparations was determined using a protein assay from BioRad Laboratories.

Preparation of Detergent-insoluble Membrane Fractions and Total Membrane Fraction

Detergent extraction with CHAPS and Triton X-100 was performed as detailed by Fiedler et al. (1993).

Primary oligodendrocytes cultured in a 5-cm dish were washed once in PBS and scraped into 300 μl 50 mM Tris-HCl, pH 7.4, 5 mM EDTA (TE), or 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA (TNE) buffer supplemented with protease inhibitors (Complete Mini; Roche Molecular Biochemicals). Cells were sheared by resuspending 20 times with a blue pipette tip and 25 times with a 25 G needle and centrifuged for 5 min at 3,000 rpm. The postnuclear supernatant was subjected to detergent extraction for 30 min at 4°C by adding CHAPS (20 mM final) to TE buffer or Triton X-100 (1% final) to TNE buffer. For extraction of myelin, 1–10 μg of myelin was added to 300 μl of 20 mM CHAPS in TE, or 1% Triton X-100 in TNE, supplemented with protease inhibitors, and incubation was performed for 30 min at 4°C. For density gradient centrifugation, we prepared a discontinous gradient with different dilutions of Iodixanol (Optiprep; Nycomed Pharma), which has a density of 1.32 g/ml. After the extraction, 250 μl of the lysates was adjusted to 40% Optiprep by adding 500 μl of Op-

tiprep solution (60%), overlaid with 1.2 ml of 30% Optiprep in extraction buffer, and 200 μl of extraction buffer. The samples were centrifuged for 2 h at 55,000 rpm in a TLS 55 rotor (Beckman) and six fractions of equal volume were collected from the top. In some experiments, the top fraction was subjected to a second round of detergent extraction, adjusted to 40% Optiprep, and centrifuged in a density gradient as described above.

An equal volume of each fraction was either directly processed further for SDS-PAGE, or fractions were concentrated by adding an equal volume of 20% TCA, centrifuging for 30 min at 15,000 *g* at 4°C, and washing the pellets with acetone at −20°C.

Total membranes were prepared as described above except that the extraction step was omitted and preparation of cleared lysates and flotations in density gradients were performed with a buffer containing 10 mM Hepes, pH 7.4, and 2 mM EGTA. Total cellular membranes were recovered from the 0–30% interface.

Immunoblotting and Immunoprecipitation

For immunoblotting, samples were incubated at 95°C for 5 min or at 55°C for 10 min in Laemmli sample buffer and proteins were resolved on SDS-PAGE and transferred to polyvinylidene difluoride. Membranes were blocked in PBS/4% milk powder/0.2% Tween and incubated overnight at 4°C with primary antibody diluted in blocking buffer. Blots were incubated with appropriate horseradish peroxidase–labeled secondary antibody for 1 h. The blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). For pulse-labeling experiments, oligodendrocytes were incubated with 1 mCi/ml [^{35}S]methionine/cysteine for 5 min and chased in the presence of 150 μg/ml unlabeled methionine and cysteine before detergent extraction and density gradient flotation. Immunoprecipitation was performed from the collected Optiprep fractions. The samples were incubated overnight at 4°C with anti-PLP antibody (Linington and Waehneldt, 1990) and immunocomplexes were recovered with protein A–Sepharose. Washed samples were solubilized in Laemmli buffer, incubated for 10 min at 55°C, subjected to SDS-PAGE, and followed by fluorography.

Viral Infection and Antibody-induced Patching

For infection with HA influenza virus or Semliki Forest virus (SFV), virus was diluted in culture medium and virus absorption was performed for 1 h. Infection was allowed to continue for a further 1–3 h. For antibody-induced patching, cells were incubated with polyclonal anti-HA or polyclonal anti-E2 antibodies in combination with monoclonal O10 anti-PLP antibody (Jung et al., 1996) or monoclonal NCAM antibody BSP2 for 1 h at 12°C. After washing briefly in PBS, cells were incubated with the respective FITC- and Cy-3–coupled secondary antibodies for 1 h at 12°C. Cells were washed, then fixed for 4 min in 4% PFA at 8°C and for 5 min with methanol at −20°C. The coverslips were mounted in moviol, and immunofluorescence pictures of the FITC and rhodoamine stainings were taken separately using a Zeiss Axiophot microscope coupled to a Photonic Science 8-bit CCD color camera (Photonic Science Ltd.). Digital images were processed using Photoshop® software (Adobe Systems).

Cholesterol Depletion and Labeling with Photoactivable Lipids

Cholesterol depletion was performed as detailed by Scheiffele et al. (1997). In brief, oligodendrocytes were washed once with Sato medium, treated with 5 mM mβCD in Sato medium for 30 min at 37°C, and washed again with Sato medium before further processing. Photoaffinity labeling was performed exactly as described by Thiele et al. (1999).

Cells cultured in 5-cm dishes were washed with Sato supplemented with 1% lipid-free HS and incubated with 100 μl ^{3}H-photocholesterol–mβCD inclusion complex (for preparation of the inclusion complex see Thiele et al., 1999) in 2 ml Sato/1% lipid-free HS for 16 h. Cells were washed with PBS/1% lipid-free HS and irradiated for 20 min with UV light at 4°C. The cells were washed, lysed in lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and protease inhibitors) and cleared of nuclei by a brief centrifugation. Lysates were either directly processed for SDS-PAGE or first subjected to immunoprecipitation with anti-PLP antibodies.

For labeling with photophosphatidylcholine, cells (5-cm dish) were washed with labeling medium (phenol red–free DMEM, without choline, supplemented with 1% lipid-free HS) and incubated with 3 ml of labeling medium and 100 μl serum loaded with 10-ASA (for preparation of serum containing 10-ASA see Thiele et al., 1999). After 20 min incubation, 200 μCi of [^{3}H]choline was added and incubation continued for 16 h. Cells were washed with labeling medium, irradiated for 20 min with UV light at 4°C, and further processed as described above.

Lipid Analysis

Oligodendrocytes were labeled overnight with 50 μCi/ml [^{14}C]acetate and a total membrane fraction was prepared as described above. Floated membranes were split in two fractions, treated with detergent (either 20 mM CHAPS or 1% Triton X-100) or buffer alone, and floated in a density gradient. For analysis of lipids from myelin, 10 μg (protein weight) of myelin was extracted with 20 mM CHAPS, 1% Triton X-100, or buffer alone, before flotation in a density gradient. Lipids were extracted from the top fraction by the method of Bligh and Dyer (1959). Dried samples were dissolved in 20 μl of chloroform/methanol (1:1, vol/vol) and spotted on activated Silica Gel 60 F 254 plates. After resolution of lipids in chloroform/methanol/water (65:25:4, by volume), the plates were air-dried and lipids were visualized either by autoradiography or by exposure of the plates to 20% sulfuric acid and charring.

Quantification of lipids was performed by densitometric scanning (Arcus II; Agfa) using Adobe Photoshop® and MacBAS. Results from three independent experiments are given as an average ± SD.

Results

Myelin Is Resistant to Extraction with CHAPS

To investigate whether components of myelin associate to form rafts, we prepared myelin from adult mice and subjected it to extraction with various detergents. The sample was adjusted to 40% Optiprep and loaded at the bottom of a step gradient (40, 30, and 0%). After centrifugation, six fractions were collected starting at the top. Proteins were separated by SDS-PAGE followed by Western blotting using antibodies against specific myelin proteins.

As shown previously (Pereyra et al., 1988; Krämer et al., 1997; van der Haar et al., 1998; Kim and Pfeiffer, 1999), extraction with 1% Triton X-100 leads to solubilization of the majority of PLP/DM20, MAG, MOG, and MBP (Fig. 1 A). Only a minor fraction of these proteins was recovered from the low-density, detergent-insoluble membrane fraction (0–30% interface) (Fig. 1 A). However, when myelin was extracted with 20 mM CHAPS, the difference compared with extraction with Triton X-100 was striking. PLP/DM20, MAG, and MOG were almost exclusively recovered from the detergent-resistant interface (Fig. 1 A). In contrast, a prominent fraction of the major cytosolic protein, MBP, was found at the bottom of the gradient (Fig. 1 A).

To determine the lipid composition of the detergent-insoluble membrane fractions, myelin was extracted with detergents, or was treated with buffer alone as a control, and centrifuged in a density gradient. Analysis of the lipid composition of the floating membrane fraction showed that Triton X-100– and CHAPS-extracted membranes were composed of similar lipids (Fig. 1 B). While a fraction of the glycerophospholipids was solubilized, the majority of galactosylceramide and cholesterol was found to be resistant to extraction with Triton X-100 or CHAPS (Fig. 1 B).

We investigated whether the CHAPS-insoluble membrane fraction (CIMF) was generated in primary cultures of oligodendrocytes. After several days in culture, these cells produce an extensive network of branching processes followed by the formation of myelin-like membrane sheets. The morphological changes are accompanied by the expression of the major components of myelin, such as the lipids galactosylceramide and sulfatide, and the proteins PLP, MBP, and MOG (Pfeiffer et al., 1993). Lysates of these cells were extracted with either 1% Triton X-100 or 20 mM CHAPS at 4°C, mixed with Optiprep, and subjected to centrifugation and SDS-PAGE. Western blot-

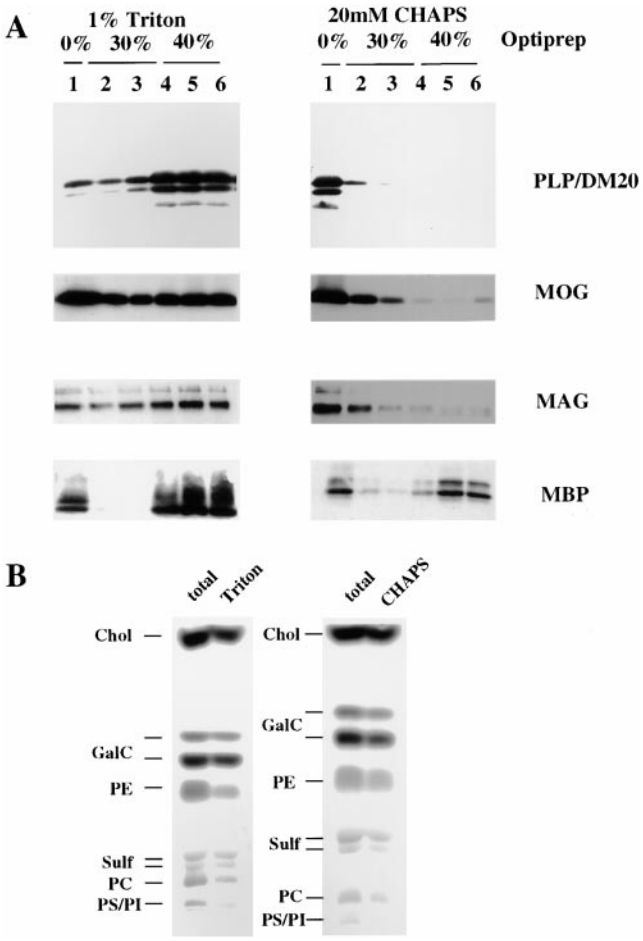

Figure 1. Myelin is resistant to extraction with 20 mM CHAPS. (A) Myelin was prepared from adult mice and 1 μg (protein weight) of myelin was extracted with 1% Triton X-100 or 20 mM CHAPS for 30 min at 4°C followed by flotation in an Optiprep gradient (40, 30, and 0%). Equal fractions were collected from the top (lane 1) to the bottom (lane 6). Fraction 1 (0–30% interface) represents low-density, insoluble membrane. Proteins were resolved on SDS-PAGE and, after Western blotting, were detected with anti-PLP, anti-MOG, anti-MBP, or anti-MAG antibodies. (B) For lipid analysis, 10 μg (protein weight) of myelin was extracted with Triton X-100, 20 mM CHAPS, or with respective buffer alone for 1 h at 4°C, and floated in a density gradient. Lipids were extracted from the floating fraction (0–30% interface), resolved by TLC, and visualized by exposure to sulfuric acid and charring. Myelin treated with buffer alone (total) is shown next to detergent-extracted myelin (Triton and CHAPS). The position of the reference lipids is indicated on the left. Chol, cholesterol; GalC, hydroxylated and nonhydroxylated galactosylceramide; PE, phosphatidylethanolamine; Sulf, hydroxylated and nonhydroxylated sulfatide; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

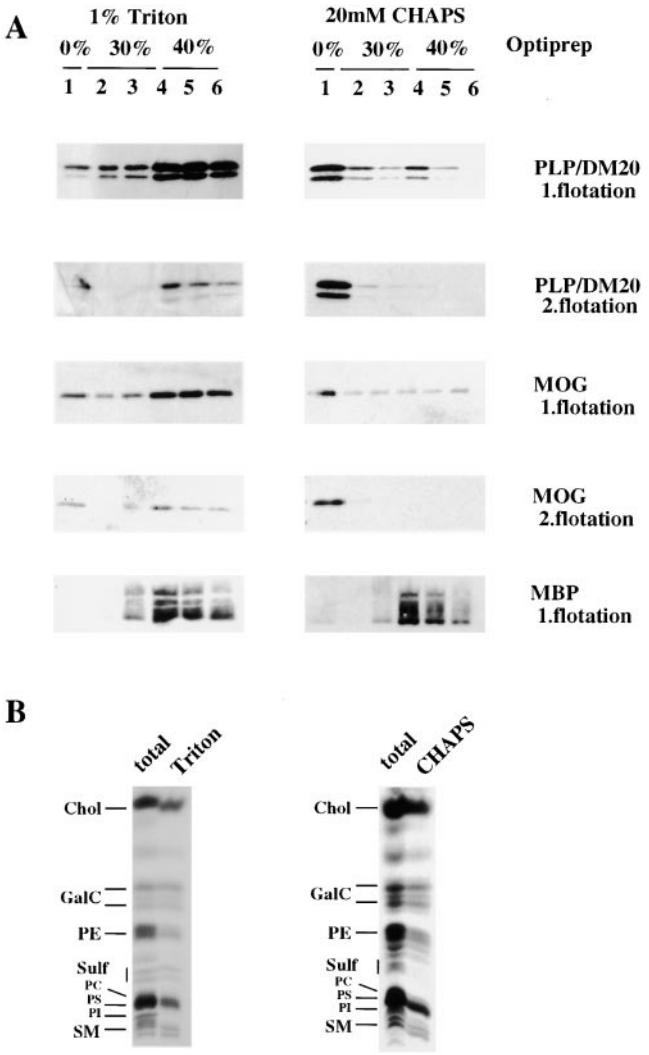

Figure 2. Identification of a CIMF in primary cultures of oligodendrocytes. (A) Oligodendrocytes were extracted with 1% Triton X-100 or 20 mM CHAPS for 30 min at 4°C and floated in a density gradient (1.flotation). Six fractions were collected from the top (lane 1) to the bottom (lane 6). Proteins were separated by SDS-PAGE and, after Western blotting, were detected with the respective antibodies. The detergent-insoluble membrane fraction from the 1.flotation was reextracted, refloated in a density gradient (2.flotation), and proteins were visualized as described above. (B) Lipids were labeled overnight with [^{14}C]acetate. Total floated membranes were incubated with 20 mM CHAPS, 1% Triton X-100, or buffer alone (total) for 30 min at 4°C and centrifuged in a density gradient. Lipids were extracted from the 0–30% interface and analyzed by TLC. Labeled lipids were detected by autoradiography and quantified by densitometric scanning. The position of the reference lipids is indicated on the left (abbreviations as in the legend to Fig. 1).

ting showed that the majority of PLP/DM20 was recovered from the CIMF (Fig. 2 A). When this fraction was reextracted and refloated in a density gradient, it remained completely associated with CIMF (Fig. 2 A). In contrast, extraction with Triton X-100 leads to almost complete solubilization of PLP after two rounds of detergent extraction. MOG was also shown to be resistant to extraction with CHAPS, but not with Triton X-100, whereas MBP was solubilized by both Triton X-100 and CHAPS (Fig. 2 A).

To determine the lipid composition of the CIMF from oligodendrocytes, cells were labeled with [^{14}C]acetate and the total membrane fraction was incubated either with 20 mM CHAPS or with buffer alone and centrifuged in a density gradient. Lipids were extracted from the gradient fractions and separated by TLC. We found that the majority of galactosylceramide (63 ± 3%) and cholesterol (63 ± 3%) was insoluble, whereas most of the glycerophospholipids were solubilized (23 ± 1%; Fig. 2 B). For compari-

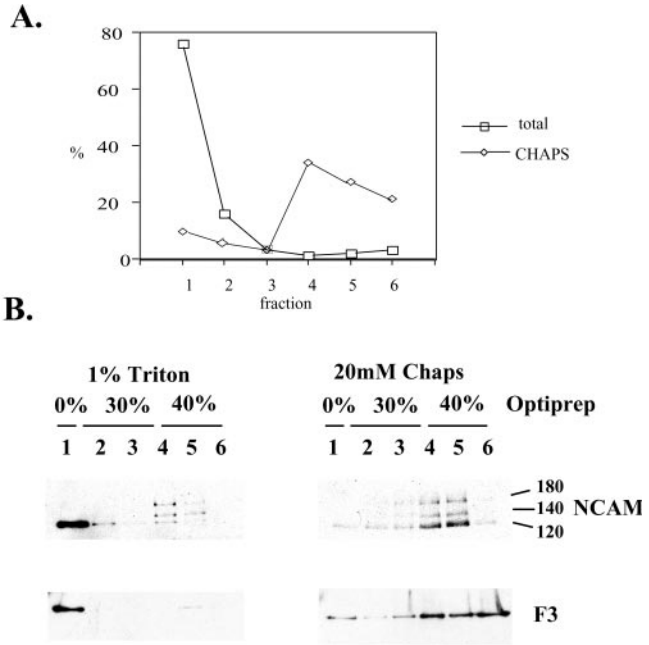

Figure 3. The CIMF represents a subdomain of the membrane in oligodendrocytes. (A) Oligodendrocytes were labeled overnight with 100 μCi/ml [^{35}S]methionine/cysteine; a total membrane fraction was then prepared and treated with 20 mM CHAPS or buffer alone (total). After centrifugation in a density gradient, six equal fractions were collected from the top (lane 1) to the bottom (lane 6) and the radioactive counts were determined in each fraction. The percent of radioactivity in each fraction is shown. (B) Oligodendrocytes were extracted with 1% Triton X-100 or with 20 mM CHAPS and centrifuged in a density gradient as described in the text. The GPI-anchored proteins NCAM120 and F3 are enriched in the Triton X-100, but not in the CHAPS–insoluble membrane fraction.

son, 83 ± 6% of galactosylceramide, 78 ± 5% of cholesterol, and 34 ± 3% of the glycerophospholipids remained insoluble when extractions were performed with Triton X-100 (Fig. 2 B). Comparison of the ratio of cholesterol/phospholipid/glycosphingolipid of the detergent-insoluble membrane fractions (4:4:2) with the ratio of the respective lipids in myelin (4:4:2–4:3:2) (Norton and Cammer, 1984) reveals a striking similarity.

GPI-anchored Proteins Resist Extraction with Triton X-100 but Are Largely Solubilized by CHAPS

Having shown that the major components of myelin were resistant to extraction with CHAPS, it was important to rule out that the conditions for detergent extraction were so mild that large fragments of the plasma membrane remained completely intact. Oligodendrocytes were subjected to metabolic radiolabeling with ^{35}S-Translabel, and the total floated membrane fraction was either extracted with 20 mM CHAPS or left untreated; fractions were then separated on a density gradient. The majority of the radioactivity was recovered at the 0–30% interface from membranes that had not been incubated with CHAPS (Fig. 3 A). In contrast, the radioactivity was mainly found in the bottom fractions (especially fraction 4) from membranes that had been incubated with CHAPS, demonstrating that CIMF at the 0–30% interface represents a subdomain of the oligodendrocyte membrane (Fig. 3 A).

Subsequently, we further characterized the CIMF. The fact that GPI-anchored proteins are highly enriched in a Triton X-100–insoluble complex in oligodendrocytes (Krämer et al., 1997) prompted us to investigate if GPI-anchored proteins also associate with CIMF. To address this question, we compared the distribution of two major GPI-anchored proteins of oligodendrocytes, F3 and NCAM120, on density gradients from CHAPS- and Triton X-100–extracted cells. As shown previously, we found that F3 and NCAM120 were highly enriched in the Triton X-100–insoluble membrane, whereas a large fraction of F3 and NCAM120 was solubilized by CHAPS (Fig. 3 B). The high molecular weight forms of NCAM, NCAM140, and NCAM180, which lack a GPI anchor, were exclusively found in the soluble fraction with both detergents (Fig. 3 B).

These results demonstrate that the CIMF represents a subdomain of the oligodendrocyte membrane, which is highly enriched in myelin components.

PLP Associates with CIMF in the Golgi Apparatus

To determine if PLP associates with CIMF during the biosynthetic pathway, we subjected primary oligodendrocytes to a 5-min labeling "pulse" of ^{35}S-Translabel. After various times of "chase," cells were lysed, incubated with CHAPS, and fractions were separated on a density gradient, as described previously. PLP/DM20 was immunoprecipitated from the low-density, detergent-insoluble fraction (fraction 1) and from the soluble fraction (fraction 4), which contains the majority of soluble PLP/DM20. PLP/DM20 was mainly found in the soluble fraction up to 30 min after the pulse, but started to become insoluble at later time points (Fig. 4 A).

Since PLP does not acquire carbohydrate modifications after translation, which could be used to trace the transit

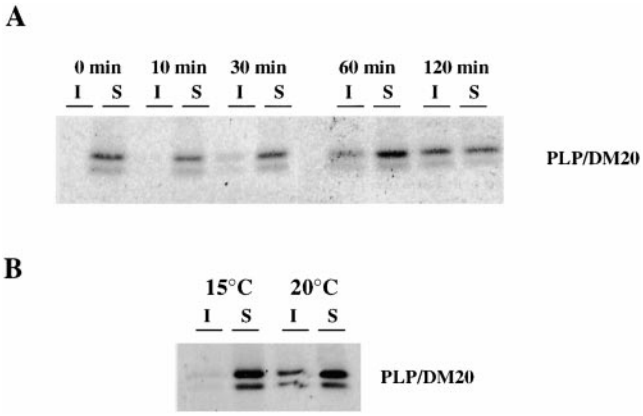

Figure 4. Association of PLP/DM20 with CIMF occurs during transport through the biosynthetic pathway. (A) Oligodendrocytes were pulse labeled for 5 min with 1 mCi/ml [^{35}S]methionine/cysteine and chased in chase medium for the times indicated. Cells were extracted with 20 mM CHAPS and centrifuged in a density gradient. PLP/DM20 was immunoprecipitated from fraction one (I) or four (S) from the gradient, separated by SDS-PAGE, and visualized by autoradiography. (B) Oligodendrocytes were pulse labeled for 10 min with 1 mCi/ml [^{35}S]methionine/cysteine and chased for 120 min at the temperature indicated.

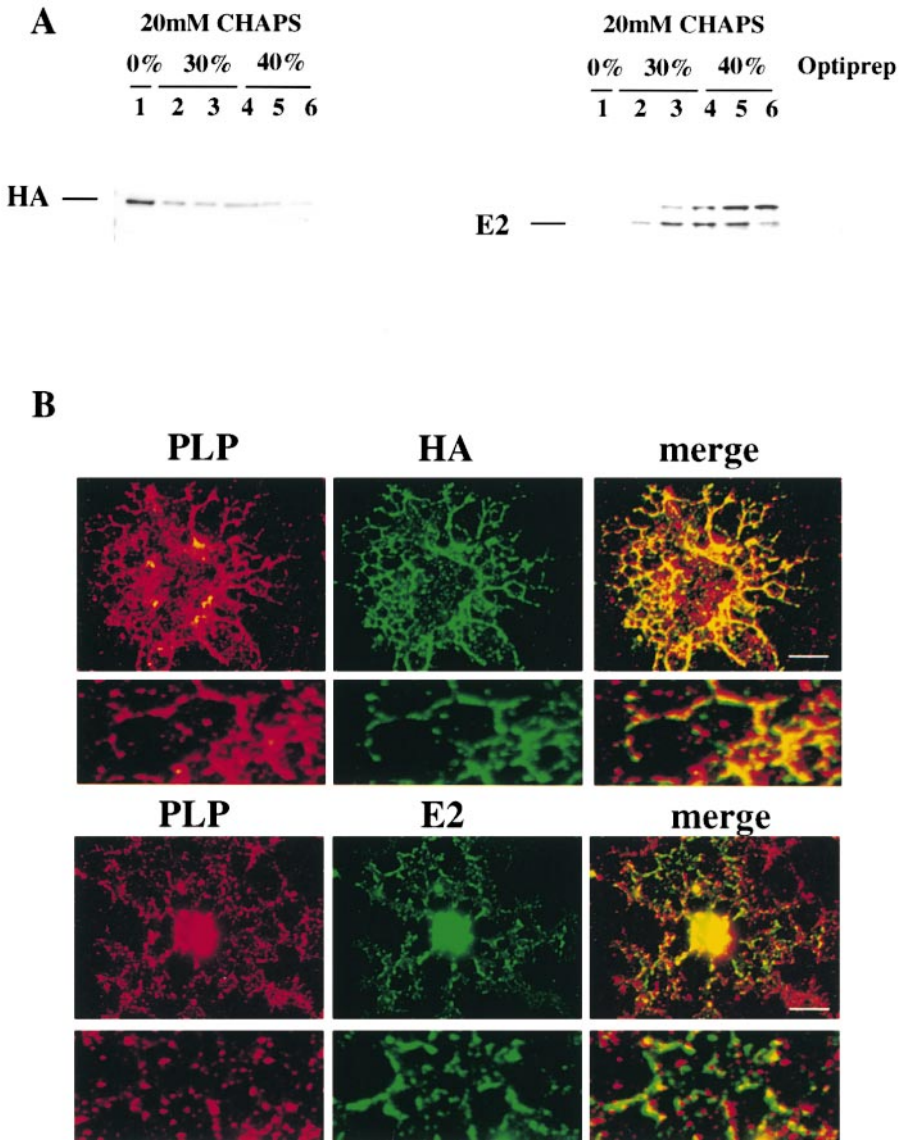

Figure 5. PLP copatches with influenza HA on the surface of living oligodendrocytes. Oligodendrocytes were infected for 1 h with the influenza virus or with SFV and incubated for a further 1 or 2 h, respectively. (A) Cells were extracted with 20 mM CHAPS and the distribution of influenza HA and SFV protein E2 on a density gradient was analyzed as described in the text. (B) Cells were incubated simultaneously with anti-HA and O10 anti-PLP or with anti-E2 and O10 anti-PLP followed by the respective secondary antibodies. The small bottom panels are enlargements of areas of the upper panels. Bars, 30 μm.

of the protein through the biosynthetic pathway, we took advantage of the fact that newly synthesized proteins accumulate in the ER at 15°C and in the Golgi apparatus at 20°C to determine where PLP/DM20 first associates with CIMF. Primary oligodendrocytes were labeled with a 10-min pulse and chased for 120 min at 15°C or 20°C. While PLP/DM20 remained soluble during the entire chase period at 15°C, CHAPS-insoluble complexes containing a fraction of PLP/DM20 could be isolated when the chase was performed at 20°C (Fig. 4 B).

These data show that PLP/DM20 becomes CHAPS insoluble during passage through the biosynthetic pathway and suggest that the insoluble complexes are generated after the protein has left the ER but before exit from the TGN.

HA Colocalizes with PLP on the Surface of Oligodendrocytes

Our biochemical data using detergents suggest that PLP/DM20 is present in a membrane fraction with properties similar to lipid rafts. If this were indeed the case, PLP should colocalize on the surface of oligodendrocytes with other components of CIMF and should segregate from components that are not part of CIMF. It has been shown previously that the influenza virus HA protein is recovered to a greater extent from CHAPS-insoluble membrane than from Triton X-100–insoluble membrane (Kurzchalia et al., 1992) and thus might be a useful marker for CIMF. We confirmed that HA was also insoluble in CHAPS in primary oligodendrocytes (Fig. 5 A). HA was largely insoluble in Triton X-100 (data not shown) as well. For a non-CIMF marker, we used the SFV spike protein, E2, which we found to be soluble in CHAPS in oligodendrocytes (Fig. 5 A).

To determine whether the proteins are found in a similar environment on the surface of oligodendrocytes as PLP, we performed copatching experiments. Cells were infected either with influenza virus or SFV and subsequently incubated with anti-HA and O10 anti-PLP antibodies, or anti-E2 and O10 anti-PLP antibodies respectively, at 12°C. This was followed by incubation with the appropriate secondary antibodies for an additional 1 h at 12°C. Microscopic analysis showed that HA and PLP appeared in the same patches in the majority of cells, suggesting that both molecules are present in the same domains of the plasma membrane (Fig. 5 B). In contrast, E2

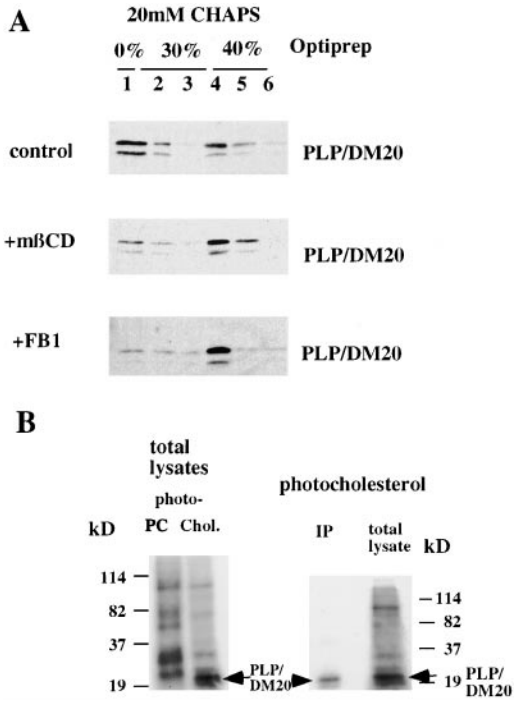

Figure 6. Cholesterol interacts with PLP/DM20 and both cholesterol and sphingolipids are required for integrity of CIMF. (A) Oligodendrocytes were incubated for 30 min with 5 mM mβCD, for 5 d with 50 μM fumonisin B1 (+FB1), or left untreated, before extraction with 20 mM CHAPS and centrifuged in a density gradient. PLP/DM20 was visualized by Western blotting. (B) Oligodendrocytes were grown in the presence of ^{3}H-photocholesterol or 10-azisteric acid and [^{3}H]-choline (PC), as indicated. Cells were UV irradiated for cholesterol and PC cross-linking and were prepared as described in the legend to Fig. 4 A. Cell lysates were either directly applied to SDS-PAGE (5–20%) or first immunoprecipitated (IP) with anti-PLP antibody. Labeled proteins were visualized by fluorography. Note that photoaffinity labeling reveals binding of PLP/DM20 to photocholesterol, but not to PC.

and PLP only partially colocalized on the surface of oligo dendrocytes (Fig. 5 B). When antibody against NCAM was used to localize the GPI-anchored protein NCAM120, the major NCAM isoform in more mature oligodendrocytes, it was apparent that HA and NCAM were largely colocalized (data not shown).

Since proteins present in similar lipid phases tend to cocluster, while proteins present in immiscible lipid phases separate (Harder et al., 1998), these data support the premise that PLP is found in a similar lipid environment to HA at the surface of oligodendrocytes.

Cholesterol Interacts with PLP/DM20 and Is Required for the Formation of CIMF

To study the involvement of cholesterol in the formation of CIMF, we analyzed whether PLP/DM20 was still associated with CIMF in cholesterol-depleted membranes. To deplete oligodendrocytes of cholesterol, we used mβCD, which is known to extract cholesterol with a higher specificity than other lipids (Klein et al., 1995). Oligodendrocytes were cultured in the presence of 5 mM mβCD for 30 min, extracted with CHAPS, and run on a density gradient gel, where the distribution of PLP/DM20 was compared with untreated cells. After cholesterol depletion, PLP/DM20 was no longer associated with CIMF to the same extent as in control cells (Fig. 6 A). These results demonstrate that cholesterol is an essential component of CIMF.

To investigate whether PLP/DM20 interacts directly with cholesterol, we used a recently developed photocholesterol probe (Thiele et al., 1999). In this compound a photoactivable diazirine ring replaces the Δ5 double bond and the hydrogen bond at C-6. Use of this probe has recently identified synaptophysin as a major cholesterol-interacting protein in neuroendocrine cells (Thiele et al., 1999). To determine whether PLP/DM20 interacts with cholesterol, oligodendrocytes were cultured for 16 h in the presence of ^{3}H-photocholesterol complexed to mβCD. Subsequently, proteins interacting with cholesterol were cross-linked by irradiation with UV light. The autoradiography of proteins separated by SDS-PAGE shows a major radiolabeled low molecular weight protein with an apparent molecular mass of 25 kD (Fig. 6 B). To determine if this protein is indeed PLP, the oligodendrocytes were immunoprecipitated with an anti-PLP antibody after the photolabeling. As shown in Fig. 6 B, immunoprecipitation clearly identifies the 25-kD photolabeled protein as PLP.

To demonstrate that the interaction of PLP with cholesterol is specific and not merely due to the high abundance of these two components in the plasma membrane of oligodendrocytes, we compared the labeling of proteins by photocholesterol with the labeling of proteins by another lipid that is present at high levels in the plasma membrane. Photoactivable phosphatidylcholine is an ideal control; however, this lipid cannot be readily introduced into the membrane by exogenous addition. Thus, we used the nonradioactive fatty acid analogue 10-azisteric acid, which bears the same photoactivable carbene-generating azi group as photocholesterol, and [^{3}H]choline. Both compounds are taken up simultaneously and metabolized to photoactivable phosphatidycholine (Thiele et al., 1999). Cells were cultured for 16 h in the presence of these two compounds and UV irradiated. Radiolabeled proteins were separated by SDS-PAGE and visualized by fluorography. When the pattern of labeled proteins was compared with those proteins labeled after cross-linking to photocholesterol, a specific photolabeling of different proteins with each compound was strikingly apparent (Fig. 6 B). Whereas photocholesterol was largely cross-linked to PLP, photophosphatidylcholine was cross-linked to other proteins, but not to PLP (Fig. 6 B). Additionally, immunoprecipitation experiments confirmed that phoshatidylcholine does not interact with PLP (data not shown). Together, our results identify PLP as a major cholesterol-interacting protein in oligodendrocytes.

Association of PLP with CIMF Is Reduced in CGT Knockout Mice

We have demonstrated that cholesterol interacts with PLP and is required for its association with CIMF. To determine whether sphingolipids are also required for CIMF formation, we cultured primary cultures of oligodendrocytes 1 d after plating for 5 d in the presence of 50 μM fumonisin B1 (Harel and Futerman, 1993). This treatment

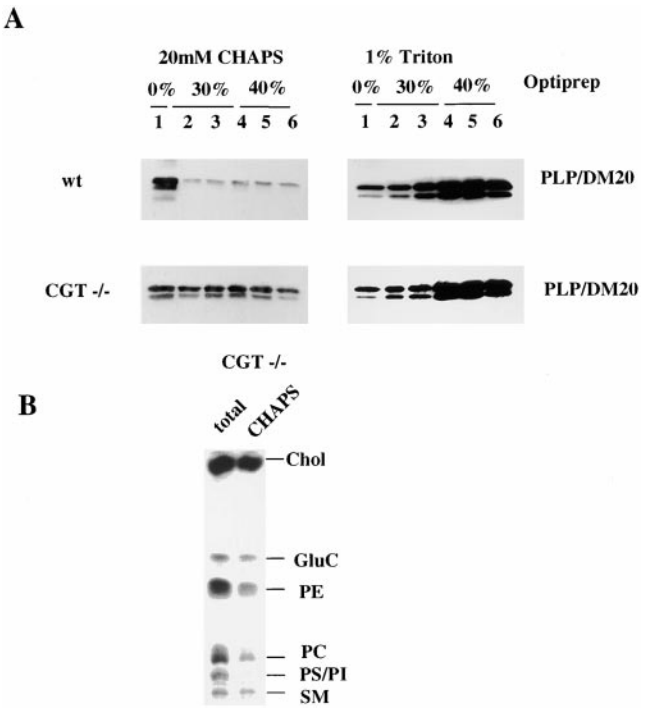

Figure 7. Association of PLP/DM20 with CIMF is reduced in myelin from CGT knockout mice. (A) Myelin was prepared from CGT knockout and wild-type mice and 1 μg (protein weight) of each was extracted with 20 mM CHAPS or 1% Triton X-100 for 30 min at 4°C. Density centrifugation, SDS-PAGE, and Western blotting were performed as in the legend to Fig. 1. (B) 10 μg (protein weight) of myelin from CGT knockout mice was extracted with 20 mM CHAPS for 30 min at 4°C, or with buffer alone, and floated in a density gradient. Lipids were extracted from the floating fraction (0–30% interface) and resolved by TLC. Myelin treated with buffer alone (total) is shown next to detergent-extracted myelin (CHAPS). The position of the reference lipids is indicated on the left. GluC, glucosylceramide; SM, sphingomyelin; additional abbreviations as in the legend to Fig. 1.

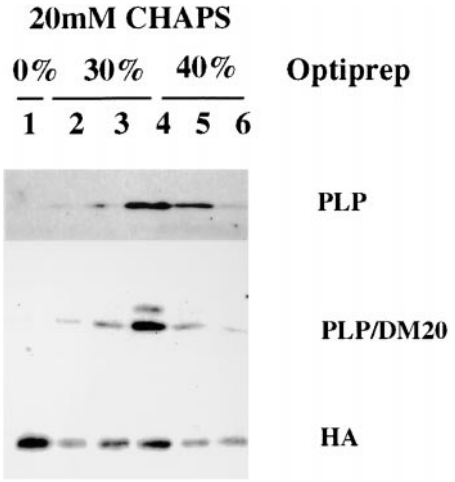

Figure 8. PLP does not associate with CIMF in BHK cells. Cells were transiently transfected for expression of PLP or PLP/DM20, or infected with influenza virus for expression of HA. Cells were extracted with 20 mM CHAPS, subjected to density gradient centrifugation, and proteins were analyzed from each fraction.

completely abolished the association of PLP/DM20 with CIMF (Fig. 6 A). To investigate more specifically the requirement of the major myelin glycosphingolipids, galactosylceramide and sulfatide, we took advantage of a ceramide galactosyl transferase (CGT) knockout mouse (Bosio et al., 1996). Mice that lack CGT, the key enzyme of galactolipid biosynthesis, are completely deficient in galactosylceramide and sulfatide (Bosio et al., 1996; Coetzee et al., 1996). Myelin from CGT knockout mice was prepared, extracted with 20 mM CHAPS, and floated in a density gradient.

When the density gradient distribution of PLP/DM20 from CGT knockout mice was compared with PLP/DM20 from wild-type mice, the difference was striking. Whereas PLP/DM20 from wild-type mice was mainly found associated with CIMF, a significant fraction of PLP/DM20 from CGT knockout mice was no longer associated with CIMF (Fig. 7 A). When extractions were performed with Triton X-100, no substantial differences were observed (Fig. 7 A). Lipid analysis of myelin from CGT knockout mice showed the absence of galactosylceramide and sulfatide concomitant with an upregulation of glucosylceramide, which was mainly recovered from CIMF (Fig. 7 B). In contrast, a large fraction of the myelin glycerophospholipids of CGT knockout mice was soluble in CHAPS (Fig. 7 B).

The above results suggest that galactosylceramide and/or sulfatide are required for the association of PLP with CIMF. We tested whether PLP is associated with CIMF in cells that generate rafts, but do not synthesize galactosylceramide and sulfatide as major sphingolipids (Scheiffele et al., 1999). For this purpose, we transiently transfected BHK cells with PLP and prepared CIMF as described above. In contrast to oligodendrocytes, PLP was not associated with CIMF in BHK cells (Fig. 8 A). Exogeneously expressed HA was recovered from CIMF in BHK cells (Fig. 8 C), thus showing that this cell type does indeed generate rafts. In case the transport of PLP is dependent on the presence of the DM20 isoform, we cotransfected cells with both PLP and DM20. However, PLP still did not associate with the isolated CIMF (Fig. 8 B). We ensured that exogenously expressed PLP had reached the plasma membrane surface by labeling living BHK cells at 4°C with O10 mAb, which recognizes an extracellular epitope of PLP (Jung et al., 1996). The positive staining with O10 of transfected BHK cells confirmed that PLP had reached the cell surface and suggests that the protein was correctly folded (data not shown). Hence, we conclude that association of PLP with CIMF is not an intrinsic property of PLP, but depends on the specialized lipid environment generated by oligodendrocytes.

Discussion

The myelin sheath has a very restricted composition containing specific lipids and proteins. How myelin lipids and proteins are assembled to generate the myelin sheath in oligodendrocytes is not known. Here, we provide evidence that specific lipid–protein interactions are involved in the generation of myelin.

We show that PLP/DM20, the major myelin protein, is recovered in a low-density CIMF enriched in myelin lipids from primary cultures of oligodendrocytes. Several lines of

evidence led us to conclude that the association of PLP/DM20 with CIMF is specific and due to distinct lipid–protein interactions. First, we show that insolubility of PLP/DM20 in CHAPS is acquired during biosynthetic transport. When PLP in cultured oligodendrocytes was labeled with a short pulse of radioactivity and chased at a temperature (15°C) at which proteins are trapped in the ER (Saraste et al., 1986), PLP was soluble in CHAPS. However, when the chase temperature was raised to 20°C, a temperature at which proteins accumulate in the Golgi complex (Matlin and Simons, 1983) where the majority of glycolipids are synthesized, PLP became insoluble. Hence, insolubility is not an inherent characteristic of PLP, but depends on the lipid environment.

Second, removal of cholesterol or inhibition of sphingolipid synthesis of cultured oligodendrocytes renders PLP CHAPS-soluble. Cholesterol is thought to be essential for the generation and stability of sphingolipid–cholesterol rafts in biological membranes (Cerneus et al., 1993; Scheiffele et al., 1997) and enhances the detergent insolubility of sphingolipids in model membranes (Schroeder et al., 1994). Third, PLP was efficiently cross-linked to cholesterol, but not to another abundant lipid, phosphatidylcholine, which was not associated with the CHAPS-insoluble membrane fraction. Fourth, PLP clusters with the influenza HA on the surface of oligodendrocytes in overlapping patches more than with the SFV spike protein E2. The patching behavior of the two proteins is thought to be mediated by the miscibility of their lipid environments (Harder et al., 1998). Copatching of PLP and HA is probably caused by a preference of both proteins for similar lipid environments. Taken together, these results suggest that the association of PLP/DM20 with CIMF within the Golgi apparatus represent an important sorting step in the biogenesis of myelin. We propose that CIMF represents specialized lipid rafts, myelin rafts, generated by myelin-producing cells.

These results are at variance with a previous study using a different experimental strategy. The vesicular stomatitis virus G protein (VSV G), a marker of the basolateral pathway of vesicle traffic in epithelial cells, was found within myelin-like membrane extensions of cultured oligodendrocytes. HA in contrast, a marker of the apical pathway in epithelial cells, was excluded from this domain. These observations lead the authors to conclude that the myelin membrane is the target of a basolateral-type pathway (de Vries et al., 1998). In contrast, we found that all exogenously expressed viral markers (SFV-E2, influenza-HA, and VSV G) were found within the membrane extensions of primary cultures of oligodendrocytes (unpublished observation). The reason why we did not observe differential sorting of HA and VSV G in our oligodendrocytic culture remains to be determined. We assume that primary oligodendrocytes in our cultures are not yet terminally differentiated and therefore the sorting processes that lead to the formation of mature myelin are not yet fully developed. This behavior is reminiscent of what has been observed in stage three cultured hippocampal neurons (Ledesma et al., 1999). In all likelihood, maturing oligodendrocytes require axonal contact to become fully polarized and to segregate markers into different domains. The solubility of the major myelin proteins in cold Triton X-100 has also argued against the association of the main myelin proteins with lipid rafts (Krämer et al., 1997; van der Haar et al., 1998).

The involvement of lipid rafts in the generation of membrane compartments has been demonstrated in many cell types (Brown and Rose, 1992; Ledesma et al., 1999). Our work suggests that oligodendrocytes employ a rafting process for myelin assembly. However, our results suggest that myelin rafts, represented by the CIMF in our study, are a specialized form of lipid rafts. When PLP was expressed in BHK cells, PLP was CHAPS soluble. This might be due to the lack of specific lipids in the rafts derived from BHK cells, which are present in oligodendrocytes. The most abundant sphingolipid in the CIMF in oligodendrocytes was galactosylceramide, whereas sphingomyelin represents the major sphingolipid in most cell types, including BHK cells. The requirement of galactosylceramide for association of PLP/DM20 with CIMF is supported by our finding that PLP/DM20 is recovered to a lesser extent from CIMF of myelin from CGT knockout mice than from myelin from wild-type mice. CGT knockout mice, are unable to synthesize galactosylceramide and sulfatide, but compensate partially by upregulating the synthesis of glucosylceramide (Bosio et al., 1996; Coetzee et al., 1996). However, production of glucosylceramide in CGT knockout mice accounts for ~30% of the galactosylceramide and sulfatide level in wild-type mice (Bosio et al., 1996; Coetzee et al., 1996). Hence, the glucosylceramide level may not suffice to generate enough membrane in a raft phase to recruit all of the PLP, the major myelin protein. Another perhaps more likely explanation is that the lack of galactosylceramide and/or sulfatide in CGT knockout mice leads to generation of lipid rafts that are not fully competent to recruit PLP/DM20. Although CGT knockout mice produce myelin with apparently normal ultrastructure of compact myelin, alterations of the paranodal and periaxonal myelin domains are observed (Dupree et al., 1999). It will be interesting to determine if the underlying cause may be the disturbance of the normal molecular organization of these domains by missorted PLP.

Taken together, our results show that PLP requires a specific lipid matrix for raft association. The direct interaction of PLP with cholesterol and galactosylceramide in the Golgi complex may be a critical step in the sorting of components destined for the myelin membrane.

The existence of several different myelin subdomains with different molecular composition (compact myelin, paranodal loop, and periaxonal and abaxonal membrane) adds an additional level of complexity to the sorting process. Segregation of these different subdomains is unlikely to be driven by the immiscibility of different lipid phases alone; additional sorting determinants (for example, defined protein–protein interactions) must also contribute. This is exemplified in caveolae, considered to be subsets of lipid rafts, which function as signal transduction platforms in many cell types (Anderson, 1993). Homooligomeric interactions of caveolin proteins are intrinsic to the formation of this specific subdomain (Monier et al., 1995). In the case of myelin, it is important to address which protein and lipid molecules are involved in the assembly of the different myelin subdomains. Biochemical dissection of these subdomains, isolation of specific components, and their in

vivo localization will yield information regarding molecular interactions driving the formation of compact myelin, paranodal loops, and periaxonal and abaxonal membranes.

We have reported previously that a Triton X-100–insoluble membrane fraction from primary cultures of oligodendrocytes is enriched in molecules involved in signal transduction, such as GPI-anchored proteins and the nonreceptor tyrosine kinases of the Src family, Fyn and Lyn, but devoid of PLP and MAG (Krämer et al., 1997). Interestingly, these kinases are maximally active, reflected by their phosphorylation status, during myelination and are downregulated in adult myelin (Krämer et al., 1999). A dominant negative form of Fyn was shown to inhibit morphological differentiation, reflected by process outgrowth of oligodendrocytes (Osterhout et al., 1999). We have shown previously that these molecules are purified with myelin, but are minor components (Krämer et al., 1999). The precise localization within the myelin membrane remains to be determined. However, we do not expect them to be located within compact myelin, which is not accessible to signals from neighboring cells.

The different extractability of the above molecules and PLP by CHAPS- and Triton X-100 raises the question as to whether these detergents allow the isolation of different myelin subdomains. Fiedler et al. (1993) have shown previously that the CHAPS- and Triton X-100–insoluble membrane fractions in MDCK cells are qualitatively similar, but differ only quantitatively in their individual components. The CHAPS complex is depleted of GPI-anchored proteins and retains a minor fraction of lipids, the latter similar in composition to that of the Triton X-100–insoluble complex (Fiedler et al., 1993). Furthermore, we have shown that HA is insoluble in both Triton X-100 and CHAPS in oligodendrocytes (data not shown). HA copatches both with PLP (enriched in CIMF) and the GPI-anchored protein NCAM120 (enriched in the Triton X-100–insoluble membrane fraction in Fig. 3; data not shown), demonstrating the location of these proteins in overlapping membrane domains. In individual cells, the GPI-anchored proteins may play a role in the early phases of axon–glial wrapping (Trotter et al., 2000), whereas PLP is synthesized late in oligodendrocyte development and is located throughout the compact myelin sheath. Thus, the generation of rafts containing GPI-anchored proteins may be earlier and thus largely temporally separated from the synthesis of rafts containing PLP.

Other experiments using lymphoid cells have shown that certain multichain recognition receptors, such as FcεRI or the T cell receptor, are highly sensitive to Triton X-100 extraction, but fulfill all other known criteria for raft association (Brown and London, 2000). In these cells the detergent Brij 58 appears to preserve proteins in lipid rafts better than Triton X-100 (Bohuslav et al., 1993). The extractability of a protein by detergents presumably depends on the level and the specific content (e.g., acyl chain length) of sphingolipids and cholesterol in a given cell type. Detergents differ in their structure; this results in differential partitioning into the plasma membrane and the disruption of specific protein–lipid and protein-protein interactions in rafts by individual detergents. However, it is unlikely that specific subdomains of lipid rafts can be isolated based on differing extractability with individual detergents, unless these domains form different lipid phases.

An interesting feature of the CIMF in oligodendrocytes is the high proportion of glycosphingolipids (63%) and cholesterol (60%). In BHK cells, for example, only 30% of the cholesterol and 40% of sphingomyelin (the major sphingolipid in these cells) were detergent insoluble (Scheiffele et al., 1999). These differences probably reflect the abundance of raft lipids in oligodendrocytes. These cells are specialized to form myelin rafts.

Recent experiments have demonstrated that in unpolarized BHK and PTK2 cells, rafts are small (50 nm), dispersed, liquid-ordered domains in an otherwise fluid (i.e., liquid-disordered phase) membrane (Pralle et al., 2000). However, in the plasma membrane of oligodendrocytes, as the mass fraction of sphingolipids and cholesterol continuously increases, the liquid-ordered phase may become continuous. Therefore, the myelin membrane is likely to be a percolating raft domain (Vaz and Almeida, 1993). Generation of macrodomains is thought to be facilitated by trapping or clustering of microdomains by factors which are intrinsic or extrinsic to the cell (Vaz and Almeida, 1993). For example, clustering of caveolin-3–containing caveolae in developing T tubules promotes the formation of large raft macrodomains and thereby serves as a starting point for T tubule biogenesis (Parton et al., 1997). Ligation of myelin rafts by cytosolic components (for example, MBP) or via extracellular factors (axonal contact) may drive the formation of a continuous myelin raft membrane.

Perturbation of the equilibrium of lipid phases might occur by an alteration in the turnover of components. In this regard, it is interesting to note that several lipidoses result in dysmyelination and demyelination with accumulation of specific lipids in oligodendrocytes (Adams et al., 1997). The extreme susceptibility of oligodendrocytes to accumulating lipids might be due to destabilization of the myelin raft phase equilibrium. Oligodendrocytes may be exquisitely sensitive to an imbalance in the synthesis and turnover rate of their protein and lipid components due to their long in vivo life and the very high rate of myelin synthesis.

In summary, we show here that sorting of myelin proteins via myelin rafts is a major pathway of membrane assembly in oligodendrocytes and that lateral lipid–protein interactions represent an important mechanism in the assembly of myelin by these cells. The challenge for the future is to elucidate further components of this sorting machinery.

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