Oligodendrocytes Direct Glycosyl Phosphatidylinositol-anchored Proteins to the Myelin Sheath in Glycosphingolipid-rich Complexes

(Received for publication, December 3, 1996)

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The myelin sheath synthesized by oligodendrocytes insulates central nervous system axons and is a specialized subdomain of the plasma membrane, containing a restricted pattern of proteins and lipids. Myelin is enriched in glycosphingolipids and cholesterol, a lipid environment favored by glycosphosphatidylinositol (GPI)-anchored proteins, which associate with these lipids in detergent-insoluble complexes in many cell types. Since proteins regulating oligodendroglia-neuron interaction are largely unknown and GPI-anchored proteins are often involved in cell-cell interactions, we examined oligodendrocytes and myelin for their expression of these proteins. Oligodendrocyte precursors and maturing oligodendrocytes express a similar pattern of GPI-anchored proteins, which unlike the majority of oligodendrocyte plasma membrane proteins, accumulate in myelin. To elucidate mechanisms underlying the expression of GPI-anchored proteins in myelin, we analyzed detergent-insoluble complexes from cells and myelin using TX-100 extraction and sucrose density gradients. In precursor cells, the GPI-anchored proteins are not incorporated in detergent-insoluble complexes. In contrast, GPI-anchored proteins from maturing oligodendrocytes and from myelin were isolated as complexes associated with glycosphingolipids and cholesterol. These results show a specific association of GPI-anchored proteins with glycosphingolipids and cholesterol during oligodendrocyte maturation and suggest sorting of these macromolecular complexes to myelin.

Myelin is the multilamellar sheath insulating selective axonal processes in higher animals, facilitating fast propagation of the action potential. In the central nervous system (CNS) it is synthesized by oligodendrocytes, and in the peripheral nervous system it is synthesized by Schwann cells as a specialized domain of the plasma membrane (1). In contrast to most plasma membranes, myelin is enriched in lipids, which comprise 70% of the dry weight, and contains a restricted pattern of proteins when compared with the oligodendrocyte (2, 3). During myelination, large diameter axons are encircled by the myelinating glial cell process. This recognition event is assumed to involve specific adhesion molecules (4), which may be contained in the restricted fraction of myelin proteins. These molecules are still largely unknown, although the main protein components of the myelin sheath are defined and cloned. The relatively normal development and myelination of mice deficient in the myelin-associated glycoprotein (MAG), which was previously thought to be essential for glia-axon recognition during the early phases of myelination, has underscored this point (5–7). Hence, as yet unknown and possibly minor oligodendrocyte/myelin proteins and their direction to specialized membrane domains may play important roles in the onset and maintenance of myelination. The cell biological processes underlying the segregation of specific membrane components to myelin are thus of functional importance.

GPI-anchored proteins represent a diverse and growing group of membrane proteins, which includes parasite and lymphocyte surface antigens, cell adhesion molecules, membrane-associated ectoenzymes, and receptors (8–10). They are inserted into the outer leaflet of the lipid bilayer via covalent linkage to phosphatidylinositol. The insolubility of GPI-anchored proteins at 4 °C in nonionic detergents such as TX-100 has been ascribed to their incorporation into large macromolecular complexes at the level of the trans-Golgi network by agglomeration with glycosphingolipids and cholesterol (11). As shown for many cell types, such detergent-insoluble glycosphingolipid-enriched complexes (DIGs; Ref. 12) can be isolated by flotation in low density fractions on sucrose gradients. The complexes also include cytoplasmic signaling molecules such as src family tyrosine kinases, heterotrimeric GTP-binding proteins and small GTPases (13–16). It is thought that glycosphingolipid-rich complexes represent functional microdomains within the plasma membrane of intact cells (15, 17, 18), which are sorted to the apical surface in polarized epithelial cells (19) or participate in transmembrane signaling in lymphocytes (20, 21).

The myelin sheath synthesized by oligodendrocytes and Schwann cells has a similar lipid composition to the detergent-insoluble microdomains described above. It has a high glycosphingolipid content (approximately 30% of total lipid weight) and is rich in cholesterol (26% of total lipid weight). This prompted us to analyze the expression of GPI-anchored proteins by oligodendrocyte lineage cells and myelin; such molecules could be candidates for the adhesion molecules involved in glial cell-neuron interaction and in the synthesis and maintenance of the myelin sheath. We report here that oligodendrocytes, both precursors and more mature cells, synthesize a broad spectrum of GPI-anchored proteins. Furthermore, we show that oligodendrocytes and myelin express the same pattern of GPI-anchored proteins. We investigated the flotation of detergent-insoluble complexes from precursor cells, maturing oligodendrocytes, and myelin on sucrose density gradients and
observed that with maturation of the cells, GPI-anchored proteins become associated with glycosphingolipids and cholesterol in DIGs. This association may be crucial for the sorting of GPI-anchored proteins to the myelin sheath.

**EXPERIMENTAL PROCEDURES**

**Materials**—Biotinylating reagents were from Pierce; growth factors were from Collaborative Research (Bedford, MA); [3H]glucosamine, [3H]ethanolamine, [3H]glycerol, [3H]inositol, [3H]glutamate, [3H]threonine, and [3H]phosphate were purchased from Amersham-Buchler (Braschnau, Germany); recombiant phosphatidylinositol-specific phospholipase C from Boecillus thuringiensis was purchased from Oxford Glycosystems; Triton X-100 and lipids were from Sigma (Deisenhofen, Germany); polyvinylidene difluoride membrane was from Millipore Corp. (Bedford, MA); Silica Gel 60 F254 TLC plates were from Merck (Darmstadt, Germany).

**Antibodies**—The following rabbit polyclonal antibodies were used: rat monoclonal antibody 324 against the neuronal cell surface glycoprotein L1 (26); secondary antibodies were from Dianova (Hamburg, Germany). Monoclonal antibodies used were as follows: murine monoclonal antibody against the myelin protein MOG (clone S185C5) and mouse monoclonal antibody against myelin basic protein (MBP) (clone 9–8) kindly provided by C. Linington; murine monoclonal antibody against 180-kDa isoform of NCAM (clone 9–8) kindly provided by C. Linington; murine monoclonal antibody against the cell adhesion molecule L1 and the 180-kDa isoform of NCAM. In addition, mAbs against the ganglioside GM1, the 180-kDa isoform of NCAM, and GTP-binding protein were purchased from Chemicon International (Temecula, CA). Secondary antibodies were from Dianova (Hamburg, Germany).

**Primary Cultures and Cell Lines**—Primary cultures of oligodendrocytes were prepared as described (22, 27). Oligodendrocytes growing on top of astrocyte monolayers were shaken off and plated in modified Sato medium (22) containing 1% charcoal-stripped fetal calf serum. Oligodendrocytes were maintained in vitro without further growth factor additions before they were harvested. Cortical astrocytes described were derived from NMRI mice of both sexes according to standard procedures (32, 33).

**Preparation of Oligodendrocyte Membranes**—For preparation of oligodendroglial membranes, cultures of primary oligodendrocytes (2 days after shaking off) were rinsed twice with PBS and harvested with a cell scraper. The cell pellet was resuspended in 10 mM Tris, pH 7.4 and left for 10 min on ice, and cells were disrupted using a 22-gauge syringe. Nuclei were removed by centrifugation for 10 min at 2000 g and 4 °C. The pellet was resuspended in SDS-PAGE sample buffer.

**Myelin Preparation**—Myelin was prepared as described (39). In brief, detergent-insoluble complexes pelleted from detergent extracts were pre-resuspended in a small volume of water, and immediately used or frozen in small aliquots at −80 °C. The myelin preparations were anulohxed in an Ultra-Turrax T25 (IKA, Staufen, Germany). Myelin was removed from the interface between 10.5 and 30% sucrose gradients and subjected to two rounds of hypotonic shock by resuspension in a large volume of ice-cold water and resolubilization on the step gradient; this separates myelin membranes from axolemmata. Purified myelin was collected from the final sucrose interface, washed twice with cold water, resuspended in a small volume of water, and immediately used or frozen in small aliquots at −80 °C. The myelin preparations were analyzed by SDS-PAGE and stained either with Coomassie blue or silver stain. Membrane fragments by Western blot analysis using antibodies against the cell adhesion molecule L1 and the 180-kDa isoform of NCAM. In addition, the absence of contaminating astrocyte cytoskeletal material was monitored with antibodies against glial fibrillary acidic protein.

**Detection of GPI-anchored Proteins by Biosynthetic Radiolabeling—Oli-neu cells or primary cultures of oligodendrocytes 2 days after plating were incubated for 18 h with either 100 μCi/ml [3H]ethanol-1-ol-2-amino hydrochloride (28 Ci/mmol) or 100 μCi/ml [3H]glucosamine hydrochloride (20 Ci/mmol). The cell monolayer was then incubated for a further hour at 37 °C in RPMI medium containing 10 μM HEPES either with or without the addition of 1 unit/ml PI-PLC. The culture supernatants were then harvested, and proteins were precipitated with cold acetone. The total cell pellet as well as the proteins precipitated from the supernatants were subject to SDS-PAGE and autoradiography.

**Protein Biotinylation—**Biotinylating cell surface proteins were carried out as described (34, 35). After repeated washings with ice-cold PBS containing 1 mM CaCl2 and 1 mM MgCl2 (PBS-CM), cells were incubated twice for 30 min with membrane-impermeable Sulfo-NHS-LC-Biotin (1 mg/ml; approximately 2.5 mg of biotin/106 cells) at 4 °C. They were then washed three times with cold PBS-CM, harvested with a cell scraper, and pelleted.

Myelin diluted to a final protein concentration of 200 μg/ml was lightly sonicated to disrupt compacted lamellae and to facilitate access of biotin. Proteins were biotinylated with two additions of 1 mg/ml membrane-permeable NHS-LC-Biotin II for 45 min with shaking. Free biotin was removed by pelleting myelin membranes by ultracentrifugation for 30 min at 100,000 × g and 4 °C.

**Isolation of GPI-anchored Proteins Using Triton X-114 Phase Separation—**TX-114 phase separation was performed according to a protocol established by Dyer (37). Dried samples were solubilized at 4 °C in 2 ml of Tris-buffered saline containing 1 mM EDTA, 1% TX-114, and a mixture of protease inhibitors. This mixture was shaken for 30 min at 37 °C and centrifuged (5 min, 15,000 × g) to separate detergent and aqueous phases. Detergent phases were reextracted twice with 1 ml Tris-buffered saline/EDTA. After dilution with Tris-buffered saline/EDTA to a final TX-114 concentration of 2%, the detergent phase was incubated without (control), or with 2 units/ml PI-PLC for 1 h at 37 °C with shaking to specifically release GPI-anchored proteins into the aqueous phase. After additional separation of the phases (centrifugation for 5 min at 13000 × g) the second aqueous phase was reextracted twice with 2% TX-114. The proteins of the second aqueous and detergent phases were precipitated with cold acetone and analyzed by SDS-PAGE followed by autoradiography in the case of radiolabeling experiments or followed by Western blotting and detection of biotinylated proteins with 125I-labeled streptavidin (0.12 μCi/ml, 1 h at room temperature) and autoradiography. PI-PLC-treated and untreated second aqueous phases were compared to identify GPI-anchored proteins.

**Preparation of Detergent Extracts—**Detergent extracts were prepared as described (11, 15). In brief, cells (3–4 × 107) or sonicated myelin (0.5 mg/ml protein) were solubilized at 4 °C in 2 ml of buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2% TX-100 (TX/100, 1000-fold dilution). The extracts were then incubated for 30 min at either 4 or 37 °C. Soluble and insoluble membrane proteins were separated by centrifugation for 10 min at 13,000 × g and 4 °C or room temperature, respectively, in a microcentrifuge. Pellets and supernatants were analyzed by SDS-PAGE and Western blotting.

**Sucrose Density Gradients—**0.5–1 ml of total detergent extracts were adjusted to 40% sucrose by adding equal volumes of 80% sucrose in TNE without TX-100 and placed into an ultracentrifuge tube. A linear gradient from 5 to 30% sucrose (in TNE without TX-100) was layered over the lysate. Gradients were centrifuged for 16–20 h at 33,000 rpm at 4 °C in a Beckmann SW 40 Ti rotor. Fractions of 1 ml were harvested from the top, and the density was determined by measurement of the refractive index. Proteins and lipids in each fraction were analyzed by SDS-PAGE followed by Western blot and TLC.

**Immunoblotting—**Proteins blotted onto polyvinylidene difluoride membrane were detected by incubation with primary antibodies overnight at 4 °C. In the case of primary monoclonal antibodies, the blots were incubated with a second rabbit anti-species antibody for 1 h at room temperature. Bound antibodies were detected with 125I-labeled protein A (0.12 μCi/ml, 1 h of incubation at room temperature).

**Lipid Analysis—**Lipid was extracted by the method of Bligh and Dyer (37). Dried samples were dissolved in 10–20 μl of chloroform/methanol (2:1, v/v) and subjected to TLC analysis on polyethyleneimine plates. After resolution of the lipids in methanol, chloroform, methanol, 0.25% (w/v) KCl (25:25:25:10.9, by volume; Ref. 38), the plates were dried, and lipids were visualized after exposure of the plates to 10% sulfuric acid, 5% methanol and charring.

**Electron Microscopy—**Electron microscopic analysis was performed as described (39). In brief, detergent-insoluble complexes pelleted from
Oligodendroglial Cells Synthesize a Variety of GPI-anchored Proteins—To investigate the expression of GPI-anchored proteins by oligodendrocyte lineage cells, we took advantage of the selective incorporation of \(^{3}H\)ethanolamine into GPI anchors. The cell line Oli-neu was generated by immortalization of mitotic oligodendrocyte precursor cells with retroviral vectors containing the t-neu oncogene (30). It adheres to and ensheathes axons in vitro and in vivo in an oligodendroglial specific manner. In vitro metabolic labeling of Oli-neu cells and subjection of the total cell lysate to SDS-PAGE yielded a variety of distinct bands on the autoradiograph with prominent components of 135 and 120 kDa and a broad band at 50–70 kDa probably consisting of several components (Fig. 1A, lane 2). Several minor components were also visible. PI-PLC, which selectively cleaves GPI-anchored proteins from the lipid anchor (9), was used to examine the susceptibility of the \(^{3}H\)ethanolamine-labeled proteins expressed by Oli-neu to enzyme-dependent release prior to cell lysis. Although cleavage was not complete (compare with Fig. 1A, lane 1), the majority of the different \(^{3}H\)ethanolamine-labeled proteins was released into the culture supernatant after treatment of the cells with PI-PLC (Fig. 1A, lane 3), confirming the presence of the GPI anchor. The heavily labeled band at 45 kDa not cleavable by PI-PLC is probably the cytoplasmic protein synthesis elongation factor 1a (40). Not all of the ethanolamine-labeled protein is released under these conditions. In order to visualize the GPI-anchored proteins present in the culture supernatant (S) without (+, lane 4) or after (+, lane 3) incubation of the radiolabeled cells with PI-PLC, the figure shows the \(^{3}H\)ethanolamine-labeled proteins as visualized by SDS-PAGE and autoradiography in the second detergent phase (lanes 11 and 12) and the second aqueous phase (lanes 13 and 14) with (+) or without (−) PI-PLC treatment. The band pattern in lane 13 is similar to that in Fig. 1A, lane 3, in which the \(^{3}H\)ethanolamine-labeled proteins released from intact cells by PI-PLC treatment are shown. Not all of the ethanolamine-labeled protein is released from the detergent phase into the second aqueous phase by PI-PLC treatment under these conditions. C, cells; S, supernatant.

**RESULTS**

Oligodendroglial Cells Synthesize a Variety of GPI-anchored Proteins—To investigate the expression of GPI-anchored proteins by oligodendrocyte lineage cells, we took advantage of the selective incorporation of \(^{3}H\)ethanolamine into GPI anchors. The cell line Oli-neu was generated by immortalization of mitotic oligodendrocyte precursor cells with retroviral vectors containing the t-neu oncogene (30). It adheres to and ensheathes axons in vitro and in vivo in an oligodendroglial specific manner. In vitro metabolic labeling of Oli-neu cells and subjection of the total cell lysate to SDS-PAGE yielded a variety of distinct bands on the autoradiograph with prominent components of 135 and 120 kDa and a broad band at 50–70 kDa probably consisting of several components (Fig. 1A, lane 2). Several minor components were also visible. PI-PLC, which selectively cleaves GPI-anchored proteins from the lipid anchor (9), was used to examine the susceptibility of the \(^{3}H\)ethanolamine-labeled proteins expressed by Oli-neu to enzyme-dependent release prior to cell lysis. Although cleavage was not complete (compare with Fig. 1A, lane 1), the majority of the different \(^{3}H\)ethanolamine-labeled proteins was released into the culture supernatant after treatment of the cells with PI-PLC (Fig. 1A, lane 3), confirming the presence of the GPI anchor. The heavily labeled band at 45 kDa not cleavable by PI-PLC is probably the cytoplasmic protein synthesis elonga-
When [3H]glucosamine was used to metabolically label cells of the line Oli-neu, the total cell homogenate contained many more [3H]-labeled proteins (Fig. 1B, lane 6) than in the ethanolamine-labeling experiments (Fig. 1A). This result was expected, since this metabolite is not specific for the GPI anchor and after acetylation will also be incorporated into the carbohydrate side chains of many glycoproteins. However, when the GPI-anchored proteins were released from intact cells by PI-PLC treatment prior to lysis, analysis of such supernatants yielded a pattern of labeled proteins similar to that seen in the [3H]ethanolamine experiments (Fig. 1B, lane 7). In particular, the dominant signals at 135 and 120 kDa are clearly visible.

Primary cultures of murine oligodendrocytes (2 days after plating) consisting of a spectrum of differentiation stages were then analyzed. Metabolic labeling with [3H]glucosamine followed by incubation of the cells with PI-PLC yielded a pattern of radio-labeled proteins in the supernatant (Fig. 1C) similar to that seen with Oli-neu (Fig. 1B).

Purification of Oligodendrocyte GPI-anchored Proteins—GPI-anchored proteins that are susceptible to PI-PLC cleavage can be specifically enriched in the detergent phase of a TX-114 phase separation protocol (34–36). They are subsequently cleaved from their hydrophobic lipid anchors and partition into the aqueous phase after a second extraction. Ethanolamine-labeled Oli-neu cells were subjected to this purification protocol. Labeled proteins were separated into the TX-114 detergent phase (Fig. 1D, lane 11) and could be specifically although not completely released into the second aqueous phase with PI-PLC (Fig. 1D, lane 13). Aqueous phases derived from untreated detergent phases did not yield any ethanolamine-labeled protein bands (Fig. 1D, lane 14). It was important to determine the pattern of proteins seen when [3H]ethanolamine-labeled cells were subjected to this technique, since non-GPI-anchored proteins that are associated strongly with a GPI-anchored protein will also be co-purified. Such proteins would be visible in the biotinylation experiments described below but would not be visible when ethanolamine labeling is used.

The Expression Pattern of GPI-anchored Proteins Is Specific for Oligodendroglial Cells—We used biotinylation to detect protein components independent of their metabolic turnover. Oli-neu cells and primary oligodendrocytes were subjected to cell surface biotinylation followed by purification of the GPI-anchored proteins according to the TX-114 phase separation protocol and detection of individual proteins with iodinated streptavidin. The results reproduced the data of the radiolabeling experiments shown previously (Fig. 2A, lanes 2 and 4). The 135-kDa protein was also found in the PI-PLC untreated second aqueous phase, but the signal was of much lower intensity. Precursor cells and more mature cultures showed the same pattern of bands (data not shown). Similar experiments conducted with other cells such as primary astrocytes from mouse cortex, NIH-3T3 fibroblasts, and PC12 cells yielded a different pattern of GPI-anchored proteins cleavable by PI-PLC for each of the cell types tested, although a few proteins may be common to several cell types. Thus, oligodendrocyte lineage cells express a specific and distinct set of GPI-anchored proteins that are susceptible to PI-PLC cleavage.

The GPI-anchored Proteins Expressed by Oligodendrocytes Are Also Found in Myelin—To test whether the GPI-anchored proteins expressed by oligodendrocytes are included in the restricted fraction of proteins that are present in the myelin sheath, CNS myelin was prepared from adult mice and subjected to biotinylation, TX-114 phase separation, and PI-PLC treatment. The pattern of GPI-anchored proteins observed for myelin was strikingly similar to that seen for primary oligodendrocytes (Fig. 3A, lanes 2 and 4) and for the Oli-neu cell line (Fig. 2A, lane 2). Again, clear protein bands were discernible at 135, 120, and 100 kDa, a broad band probably consisting of several proteins between 50 and 70 kDa, and several minor components of lower molecular mass. This suggests that all GPI-anchored proteins expressed by oligodendrocytes are also located in myelin. Comparison of total biotinylated oligodendrocyte plasma membrane proteins and biotinylated myelin proteins (Fig. 3A, lanes 5 and 6), reveals a totally different overall protein pattern. The majority of the oligodendrocyte plasma membrane proteins are not visualized in the myelin fraction as previously reported (3). Silver staining of total oligodendrocyte membrane proteins and myelin proteins (Fig. 3A, lanes 7 and 8) also shows the same point. The fact that all
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GPI-anchored proteins are located in myelin suggests that the GPI anchor may be an address signal for the myelin sheath.

The Identity of GPI-anchored Proteins Expressed by Oligodendrocytes and Myelin—Western blotting and peptide sequencing was used to identify some of the different GPI-anchored proteins synthesized by oligodendrocytes. NCAM 120 and 5'-nucleotidase were confirmed as the 120- and 65-kDa bands, respectively (data not shown). It is well established that both the GPI-anchored isoform of NCAM (NCAM 120; Ref. 41) and 5'-nucleotidase are synthesized by oligodendrocytes and found in myelin (3, 22, 42–44).

Tryptic digests of the 135-kDa protein from Oli-neu after separation by two-dimensional gel electrophoresis yielded sequence data corresponding to the neuronal cell adhesion molecule F3 (data not shown; Ref. 45). In Western blots with antibodies against F3, the expression of this protein was confirmed in myelin and oligodendrocytes. Tryptic digests of the 67-kDa band yielded sequences identical to the GPI-anchored proteins NCAM 120 and F3, respectively (data not shown). It is well established that the GPI-anchored proteins NCAM 120 and F3 are partially insoluble in TX-100 at 4 °C, while the transmembrane NCAM isoform (NCAM 140) and other oligodendroglial transmembrane proteins MAG, MOG, and PLP are released into the supernatant. Solubilization of myelin is incomplete at 4 °C for both GPI-anchored and the main myelin proteins. Extraction at 37 °C solubilizes the oligodendrocyte and myelin proteins almost completely. B, the cell line Oli-neu was analyzed as described in A. GPI-anchored NCAM 120 is resistant to complete solubilization with TX-100 at 4 °C, while transmembrane NCAM 140 is completely solubilized. P, pellet; SN, supernatant.

A small fraction of PLP remained in the pellet at 4 °C. Extraction at 37 °C or extraction with n-octyl glucoside (data not shown) completely solubilized all of the proteins. The precursor cell line Oli-neu again demonstrates the different behavior of the two NCAM isoforms, with a small fraction of the NCAM 120 but no NCAM 140 remaining insoluble in TX-100 at 4 °C.

Extraction of myelin yielded slightly different results. As for oligodendrocyte, NCAM 120 and F3 were partially insoluble and substantially enriched in the pellet after detergent extraction at 4 °C. However, differing proportions of the other myelin proteins MAG, MOG, and PLP also remained associated with the pellet, although the bulk of these proteins was found in the supernatant. Repeated extractions of the pellet did not solubilize further material. Extraction of myelin at 37 °C revealed that a minor fraction of NCAM 120 and PLP remained insoluble, whereas all other proteins examined were completely solubile (Fig. 4). Lipid analysis of the TX-100 insoluble pellet from myelin confirmed the presence of glycosphingolipids and cholesterol in the pellet (data not shown).

The insolubility of the GPI-anchored proteins described above could be due to the association of the proteins with glycosphingolipids and cholesterol. It is well established that the expression of galactocerebroside and sulfatide is up-regulated during the differentiation of oligodendrocytes from pre-
**DISCUSSION**

**Identification of Oligodendrocyte and Myelin GPI-anchored Proteins**—Biochemical studies demonstrated that oligodendrocytes and myelin contain many different GPI-anchored proteins. Some are known proteins, including cell adhesion molecules of the Ig superfamily and proteins involved in cell-cell interactions. These include NCAM 120, and 5′-nucleotidase,
which have previously been reported to be expressed by oligodendrocytes and to be present in myelin. In addition, we identified two proteins as F3 and CD55. The oligodendrocyte myelin glycoprotein has a molecular mass of 120 kDa, which is reduced to 105 kDa after PI-PLC cleavage (50, 51) and may also be included in our preparations. Other candidate molecules for some of the observed components are the ciliary neurotrophic factor receptor α subunit and prion protein, which have been reported to be expressed by oligodendrocyte lineage cells (29, 52).

**GPI-anchored Proteins Are Included in Detergent-insoluble Complexes in Mature Oligodendrocytes and Myelin but Not in Oligodendroglial Precursor Cells**—We observed a clear difference between the behavior of GPI-anchored proteins of the myelin sheath and other myelin proteins, reminiscent of the behavior of GPI-anchored proteins included in glycosphingolipid-rich microdomains (11). Simple detergent extraction studies of oligodendroglial cells and myelin revealed that a fraction of the GPI-anchored NCAM 120 and F3 was insoluble in the detergent TX-100 at 4 °C, whereas transmembrane proteins such as NCAM 140 and other oligodendroglial or myelin proteins were almost completely solubilized. In contrast, GPI-anchored proteins could be completely solubilized in extracts prepared at 37 °C or with the detergent n-octyl glucoside. It is known that myelin is poorly soluble in detergents compared to classical plasma membranes and that the cholesterol and glycosphingolipid component remain largely insoluble (53).

Analysis of detergent extracts on sucrose density gradients showed that low density complexes, containing GPI-anchored proteins, could be isolated from mature cells and myelin but not from precursor cells. The very small amount of DIGs that could occasionally be isolated from precursor cell cultures may result from the low percentage of more mature cells in these cultures (7% O1-positive cells). The appearance of DIGs thus correlates with maturation of the cells. The DIGs from myelin were of lower density, indicating a higher lipid:protein ratio. The complete pattern of GPI-anchored proteins found in myelin and oligodendrocytes is, however, already present in precursor cells. GPI-anchored proteins are not organized in DIGs in precursor cells, which are migrating cells establishing initial axon contacts, but are recruited to DIGs by myelinating cells and are present in DIGs in myelin. This may reflect a change in function of GPI-anchored proteins during oligodendrocyte development where the association in DIGs may regulate the signal transduction capacities of the GPI-anchored molecules by association with signaling molecules. In support of this concept, we have observed that the DIGs contain significant amounts of fyn kinase. It is well established in lymphocytes that cross-linking GPI-anchored proteins with antibodies can transmit signals inside the cell (20), possibly mediated by src family kinases.

Interestingly, Olive et al. (47) have reported the isolation of DIGs from adult mouse cerebellum. They isolated two fractions of microdomains floating at different buoyant density in the sucrose gradient, both containing F3. Association of F3 with...
the neuronal cell adhesion molecule L1, and *fyn* tyrosine kinase was shown. However, the authors reported the "contamination" of the lighter fraction with myelin and assumed that the F3 signal was exclusively of neuronal origin. Our data show that myelin and oligodendrocyte membranes include such DIGs containing NCAM 120, F3, and *fyn* kinase.

**Myelin May Contain Several Different Populations of Microdomains**—Different subpopulations of detergent-insoluble microdomains may exist in myelin, consistent with the view that myelin consists of several distinct compartments, e.g. paranodal loops and compact myelin, with specific biological functions (4). In contrast to the GPI-anchored proteins, which were exclusively localized in the DIGs, the proteins MAG and PLP were found predominantly at high densities at the bottom of the sucrose density gradients from myelin. However, significant fractions of each of these proteins were also found in floating fractions containing the DIGs. The DIGs from myelin appear to be more heterogeneous than those from oligodendrocytes and include a population with a multilamellar structure.

**Specific Lipid Association of GPI-anchored Proteins in Oligodendrocytes and Myelin Suggests Sorting Mechanism**—The biological relevance of the GPI anchor as a mode of attachment of cell surface proteins is still a matter of debate (10). It has been suggested that it is involved in intracellular targeting (11, 15, 18, 34, 54). Thus, in differentiated hippocampal neurons in culture, it has been proposed that the GPI anchor sorts Thy-1 to the axonal domain and excludes it from the somatodendritic compartment (55). The original postulate that GPI-anchored proteins are sorted in rafts in association with glycosphingolipids and cholesterol (19, 56) has been demonstrated in the polarized epithelial line Madin-Darby canine kidney cells (11). It was shown that the rafts are generated in the Golgi apparatus.

Recent studies on sorting in Madin-Darby canine kidney cells show however, that not all glycosphingolipids are directed apically (57). The lipids of oligodendrocyte and myelin DIGs consist largely of the glycosphingolipids galactocerebrosides, sulfatide, and sphingomyelin, as well as cholesterol, lipids that are enriched in myelin. Oligodendrocyte precursor cells as well as mature oligodendrocytes also express low levels of gangliosides, but these are not all sorted to myelin (58). Oligodendrocyte precursor cells do not associate with the GPI-anchored proteins in DIGs; concomitant with the appearance of the myelin lipids galactocerebrosides and sulfatide during maturation of the precursor cell to an oligodendrocyte, DIGs are up-regulated. We suggest that the association of GPI-anchored proteins with these glycosphingolipids and cholesterol in oligodendrocytes is responsible for the direction of these proteins to myelin. The complexes isolated from oligodendrocytes were shown to contain specifically the 120-kDa GPI-anchored but not the 140-kDa transmembrane isoform of NCAM. Since these isoforms have the same extracellular region but are differently anchored in the membrane, this observation supports the targeting hypothesis in which the GPI anchor is responsible for directing the proteins to DIGs and thus to myelin. Accordingly, myelin contains only NCAM 120. In addition, the GPI-anchored protein F3, galactocerebroside and sulfatide appear to be co-localized in double immunofluorescence stainings of cultured oligodendrocytes (45). Two groups have recently generated mice deficient in the enzyme ceramide galactosyl transferase (59, 60). These mice are unable to synthesize galactocerebrosides and sulfatide. Although they synthesize myelin around appropriate axons, they show severe clinical symptoms and have a shortened life span. It will be interesting to analyze the DIGs from such animals and, in particular, to see if the transport and location of GPI-anchored proteins is abnormal.

The prerequisites for the formation of DIGs are still unresolved. VIP 21/caveolin, which was initially thought to play a role in the formation of such complexes, has been reported to be absent from brain (17, 47) and expressed neither in oligodendrocytes nor myelin. For several cell types, including neuroblastoma cells, lymphocytes, and epithelial cells, glycosphingolipid-rich, detergent-insoluble microdomains have been shown to exist independently of caveolin and the expression of VIP 21/caveolin (16, 47, 48, 61, 62). For such caveolin-independent microdomains, it is feasible that other unknown proteins might be involved in their formation. A 17-kDa protein has been isolated from detergent-insoluble complexes of oligodendrocytes and myelin (MVP 17 or rMAL; Refs. 63 and 64) that belongs to the family of proteolipid proteins. It was proposed by Kim et al. (65) that it may be involved in regulating protein sorting in myelinating oligodendrocytes. However, the association of GPI-anchored proteins with specific glycosphingolipids and cholesterol may be sufficient to generate detergent-insoluble...
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...uble complexes (65); the saturated character of the fatty acyl chains appears to be very important.

During myelogenesis the oligodendrocyte synthesizes copious amounts of myelin membrane and segregates specific proteins and lipids to the myelin compartment (1, 2). For the cytoplasmic myelin protein MBP, it has been shown that the messenger RNA is targeted to the oligodendroglial processes forming the myelin sheath (66). Additionally, the mechanism for the segregation of myelin proteins and lipids may include a combination of selective signals on proteins destined for myelin as well as retention of specific components in the oligodendroglial cell body.

We show here that oligodendrocyte precursor cells, oligodendrocytes, and central nervous system myelin display a strikingly similar pattern of GPI-anchored proteins, whereas oligodendrocyte plasma membrane and myelin exhibit a markedly different pattern of total proteins. As proposed for epithelial cells, we suggest that the GPI anchor is responsible for the selective association of GPI-anchored proteins with glycosphingolipid-rich microdomains during maturation of oligodendrocytes and targets these molecules to the myelin sheath, thus acting as a myelin sorting signal. GPI-anchored proteins, often involved in cell-cell interactions, are expressed at the external face of the plasma membrane in oligodendrocytes, which in myelin also opposes the axonal membrane. They are thus candidates for molecules involved in recognition and signal transduction between axons and the ensheathing glial cells, a process still largely unresolved at the molecular level. Their insertion in the external leaflet of the lipid bilayer may permit fluidity within the plane of the membrane, thus retaining adhesive contacts during the spiralling of the glial process around the axon and the laying down of the multilamellar sheath.

Acknowledgments—We thank D. Kendell and I. Bünzli-Ehret for excellent technical assistance, A. Hellwig for the electron microscopy, and A. Summerfield for photography. We thank Drs. K. Simonis, J. Garwood, Matthew Hannah, and Ilse Sommer for helpful comments on the manuscript. J. T. thanks Wieland Huttner for continuous support and encouragement.

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