The Initial Events in Myelin Synthesis: Orientation of Proteolipid Protein in the Plasma Membrane of Cultured Oligodendrocytes

Lynn D. Hudson, Victor L. Friedrich, Jr., Toby Behar, Monique Dubois-Dalcq, and Robert A. Lazzarini

Laboratory of Viral and Molecular Pathogenesis, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Proteolipid protein (PLP) is the most abundant transmembrane protein in myelin of the central nervous system. Conflicting models of PLP topology have been generated by computer predictions based on its primary sequence and experiments with purified myelin. We have examined the initial events in myelin synthesis, including the insertion and orientation of PLP in the plasma membrane, in rat oligodendrocytes which express PLP and the other myelin-specific proteins when cultured without neurons (Dubois-Dalcq, M., T. Behar, L. Hudson, and R. A. Lazzarini. 1986. *J. Cell Biol.* 102:384–392). These cells, identified by the presence of surface galactocerebroside, the major myelin glycolipid, were stained with six anti–peptide antibodies directed against hydrophilic or short hydrophobic sequences of PLP. Five of these anti–peptide antibodies specifically stained living oligodendrocytes.

Staining was only seen ∼10 d after PLP was first detected in the cytoplasm of fixed and permeabilized cells, suggesting that PLP is slowly transported from the RER to the cell surface. The presence of PLP domains on the extracellular surface was also confirmed by cleavage of such domains with proteases and by antibody-dependent complement-mediated lysis of living oligodendrocytes. Our results indicate that PLP has only two transmembrane domains and that the great majority of the protein, including its amino and carboxy termini, is located on the extracellular face of the oligodendrocyte plasma membrane. This disposition of the PLP molecule suggests that homophilic interactions between PLP molecules of apposed extracellular faces may mediate compaction of adjacent bilayers in the myelin sheath.

THE myelin sheath in the central nervous system is a specialized extension of the oligodendrocyte plasma membrane that enwraps axons, thereby permitting fast saltatory conduction of nerve impulses (for review see 51). An oligodendrocyte extends as many as 20 processes, each of which contacts and repeatedly envelops a stretch of axon with subsequent condensation of the encircled membranes to form a compact sheath (51). The sheath has an atypical abundance of lipid, with a lipid-to-protein ratio of 80:20 (wt/wt) in myelin vs. 55:45 in the oligodendrocyte plasma membrane and a limited repertoire of proteins (28, 42). Moreover, the two proteins that comprise the bulk of myelin, myelin basic protein (MBP)[1] and proteolipid protein (PLP), each contain covalently attached lipid on at least a fraction of the molecules (5, 60, 61, 64, 70). MBP is a peripheral membrane protein localized on the cytoplasmic face of the myelin membrane that functions in the compaction of the sheath, as

suggested by the absence of a compact major dense line in shiverer mice mutants unable to synthesize MBP (35, 49, 53). PLP is an extremely hydrophobic protein with 75% α-helical content (7) that spans the myelin membrane (10, 25, 27, 28, 48, 62). The primary sequence of PLP is strikingly conserved among species: human, mouse, and rat PLP are identical and differ from bovine PLP at only three amino acids (11, 14, 21, 22, 34, 38, 39, 50). Although the function of PLP in the myelin sheath is not clear, a structural role is likely simply because PLP constitutes half of the myelin protein.

The initial events in myelin assembly, including the trafficking of myelin-specific proteins and lipids to the oligodendrocyte plasma membrane and the subsequent segregation of myelin components within the plasma membrane which must occur before sheath formation, are largely unknown (for reviews see Benjamins [3] and Morell and Toews [36]). As suggested for other systems of membrane assembly (for review see Pfeffer and Rothman [47]), various pathways may be involved in targeting myelin constituents to sites of assembly. For example, myelin constituents synthesized in the RER, such as PLP (8) and myelin-associated glycoprotein (MAG), may be packaged as precursor vesicles for constitutive (bulk flow) or regulated transport (as described in 47)

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1. *Abbreviations used in this paper:* GC, galactocerebroside; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; PLP, proteolipid protein.

to the myelin membrane. Vesicular transport of PLP has been suggested previously by the subcellular fractionation studies of Pereyra et al. (45, 46) and the ability of monensin to disrupt PLP transport to myelin (63). Other myelin components could be synthesized at or very close to the myelin membrane for direct insertion. MBP is a candidate for this pathway because the message for MBP, which is translated on free ribosomes (8), is present in oligodendrocyte processes (72) and in subcellular fractions comprising compact myelin (8, 33). Thus, nascent MBP might be targeted to sites of myelin assembly in the immediate vicinity of the translating ribosome shortly after the covalent addition of phosphatidylinositol bisphosphate (70). Most of the myelin-specific lipids, including the myelin marker galactocerebroside (GC), are synthesized on intracytoplasmic membranes and may be transported in vesicles or individually by some of the putative carrier proteins in brain shown to bind to sulfatides, cerebrosides, or phospholipids (for review see Morell and Toews [36]).

The simplicity of the myelin membrane and the availability of cultured oligodendrocytes affords a unique system to approach questions of membrane assembly. We have previously observed that rat oligodendrocytes cultured without neurons express myelin-specific constituents and that the initial stages of myelin assembly can be studied in such cells (16). To examine myelin architecture and assembly at a molecular level, we have analyzed the structure of the major myelin protein, PLP, in the oligodendrocyte plasma membrane. PLP was the focus of these studies because the topology of this abundant protein is likely to dominate the structure of myelin and possibly organize the other components of myelin. In addition, knowledge of which PLP epitopes reside on the oligodendrocyte surface may be important in unraveling the pathology of demyelinating diseases, including models such as experimental allergic encephalomyelitis which can be induced by purified PLP (57, 68). The advantages of the oligodendrocyte culture system to probe PLP structure include the ability to readily stain by immunofluorescence for myelin proteins and lipids, unlike the situation for compact myelin in which PLP and other proteins are relatively inaccessible unless the myelin is fixed or disrupted by procedures that can induce artifacts. Cultured oligodendrocytes also afford the opportunity to observe PLP in its native conformation, unlike the case for PLP reconstituted into artificial membranes (30). Our approach has been to probe cultured oligodendrocytes with a panel of antibodies to peptides located mostly in hydrophilic regions of the molecule. The presence of PLP epitopes on the extracellular surface was assayed by three criteria: immunofluorescent labeling of living, nonpermeabilized oligodendrocytes, cleavage of extracellular epitopes with protease, and lysis of oligodendrocytes in the presence of the appropriate antibody and complement. Contrary to computer predictions based on the primary sequence and other models of PLP topology (27, 30, 62), our results suggest that PLP has only two transmembrane domains, with the great majority of the protein located on the extracellular face of the oligodendrocyte membrane. PLP appears to be transported from the cell body to the processes via vesicles that eventually fuse with the plasma membrane after a long delay, suggesting that PLP delivery is a regulated, signal-mediated process.

Materials and Methods

Preparation of Affinity-purified Antibodies

The preparation of anti-PLP I antibody has been described earlier (16). Peptides were constructed by the Merrifield solid-phase technique with a Beckman 990 (Beckman Instruments, Inc., Palo Alto, CA) or an Applied Biosystems (Foster City, CA) automated peptide synthesizer. After synthesis, peptides were desalted on a Sephadex G-10 column, then analyzed, and, if necessary, purified by high pressure liquid chromatography (Waters Associates, Milford, MA). Synthetic peptides were coupled to the carrier keyhole limpet hemocyanin (Calbiochem-Behring Corp., La Jolla, CA) in 0.1% glutaraldehyde; 0.5 mg of each coupled peptide in Freund's incomplete adjuvant was distributed by subcutaneous injection to 10-15 sites per rabbit for each boost. Freund's complete adjuvant was used for the initial immunization.

Antiserum was purified first by passage through a protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with PBS. The immunoglobulin fraction containing predominantly IgG was eluted in 10% dioxane (J. T. Baker Chemical Co., Pillipsburg, NJ), 90% citrate phosphate buffer, pH 2.4 (90 mM citric acid, 20 mM Na_2HPO_4), and dialyzed in PBS. The antibody was next applied to an affinity column prepared by coupling 10-20 mg peptide/ml of Affigel 10 as described by the manufacturer (Bio-Rad Laboratories, Rockville Centre, NY); coupling of the hydrophobic peptide PLP VI was carried out with the addition of 0.1% SDS to the coupling buffer (0.1 M $NaHCO_3$, pH 8). After an extensive PBS wash, columns were also washed in citrate phosphate buffer, pH 6 (37 mM citric acid, 93 mM Na_2HPO_4), before the anti-peptide antibody was eluted in 10% dioxane, 90% citrate phosphate buffer, pH 2.4. Purified antibodies were dialyzed against PBS and concentrated to 1 mg protein/ml with a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA). PLP VI antisera was additionally purified by absorption to a monolayer of type 1 astrocytes.

Purified antibodies were titered by a dot immunobinding assay as previously detailed (16) and were tested for reactivity to PLP on immunoblots of SDS-polyacrylamide gels (12.5% acrylamide; 30:1 acrylamide/bis) transblotted in 0.1% SDS, 25 mM Tris, 192 mM glycine, 20% methanol. An alkaline phosphatase-conjugated anti-rabbit IgG (Promega Biotec, Madison, WI) was used to detect PLP-specific bands in samples of guinea pig or human myelin. Some preparations were also tested by ELISA (1), using chloroform-methanol extract of human myelin (gift of R. Quarles, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD) or purified aqueous bovine PLP (gift of N. Potter, Department of Neurobiology, Harvard Medical School, Cambridge, MA) as antigen and detection with peroxidase-conjugated goat anti-rabbit IgG followed by 2,2'-Azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma Chemical Co., St. Louis, MO).

Immunostaining of Cultured Oligodendrocytes

Primary brain cultures devoid of neurons and cultures enriched for oligodendrocytes were prepared from newborn Sprague-Dawley rats as previously described (16, 72), except that cerebral hemispheres were soaked in L15 medium (Gibco Laboratories, Grand Island, NY) instead of MEM with 25 mM Hepes. Most experiments were carried out with enriched oligodendrocyte cultures. The ages mentioned in this paper are not the "in vitro" age but the postnatal rat age; for example, if a 1-d-old rat was killed and the brain dissociated and cultured for 17 d, the rat postnatal age would be 18 d.

Immunostaining of living cells was carried out at 23°C in PBS or MEM-Hepes buffer as follows: buffer rinse, 0.5-1 min; anti-peptide primary antibody, 20-30 min; buffer rinse, 0.5-1 min; fluorescein conjugated-goat anti-rabbit IgG antibody (Cooper Biomedical, Inc., Malvern, PA or ICN Biomedicals, Lisle, IL); buffer rinse, 1-2 min; 2% formaldehyde, 5-10 min; coverslip under Tris-glycerol.

Double staining for surface PLP and GC was performed in two ways: (procedure 1) simultaneous live staining with anti-peptide and anti-GC antibodies; and (procedure 2) sequential staining. Procedure 1 was carried out as described above for anti-peptide antiserum alone, except that the primary mixture contained anti-GC (52) in addition to the anti-peptide antibody and the secondary mixture contained rhodamine-conjugated goat anti-mouse IgG in addition to the anti-rabbit secondary. For procedure 2, living cells were stained with anti-peptide antiserum alone as described above. Next, after formaldehyde fixation, the PLP-stained cells were treated with: buffer, 3 h; anti-GC in buffer with 10% goat serum and 10% rabbit serum, 1 h; buffer, 30 min; 2% formaldehyde, 20 min; buffer, 20 h; biotinylated goat anti-mouse

IgG (Cappel Laboratories, Malvern, PA) with 10% rabbit serum and 0.5% biotin, 30 min; buffer, 10 min; rhodamine-conjugated streptavidin (Molecular Probes Inc., Eugene, OR) with 1% BSA, 30 min; buffer, 10 min; 2% formaldehyde, 5 min; and coverslipped as above.

Living cells incubated with either mouse anti-GC or rabbit anti-peptide antibodies followed by the inappropriate conjugate did not show any stain. The latter control was extremely critical, as a number of commercially available lots of fluorescein-conjugated goat anti-rabbit IgG antibody were found to react with the mouse monoclonal anti-GC antibody.

In experiments aimed at detecting intracytoplasmic PLP, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized by a 5-min 0.05% Triton X-100 treatment before staining.

Peptide competition experiments were carried out by including each peptide (final concentration of 1 mg/ml MEM with 25 mM Hepes) in the 20-min incubation with the corresponding anti-peptide antibody (14 μg/ml) and anti-GC antibody. To control for nonspecific competition, each anti-peptide antibody was also tested for its ability to stain oligodendrocytes in the presence of an unrelated PLP peptide; for example, PLP I peptide was added to the anti-PLP IV antibody and did not abrogate PLP IV immunofluorescence. When cells were double labeled for PLP and MAG, cultures were fixed with 1% formaldehyde and 0.05% saponin for 20 min and then labeled with mouse monoclonal antibody to MAG (kindly provided by R. Quarles; reference 15) and rabbit anti-PLP peptide antibody followed by goat anti-mouse IgG rhodamine and goat anti-rabbit fluorescein conjugates.

Protease Treatment of Cultured Oligodendrocytes

Primary rat brain cultures grown in 75-cm² Falcon flasks were prepared in two different manners for the protease experiments: (a) cells were first scraped with a rubber policeman and then washed and put in suspension containing 0.05% trypsin and 0.02% EDTA in Hanks' Ca- and Mg-free medium for 25 min at 37°C; or (b) monolayers were rinsed and directly incubated with 0.05% trypsin and 0.02% EDTA in the same medium for 25 min at 37°C. In either case, serum-containing medium was added and cells in suspension were washed before incubation with antibodies. The first procedure resulted in more efficient proteolytic cleavage. Living cells in suspension were stained with anti-GC and anti-peptide antibodies as described above. The total number of GC-positive cells as well as the total number of GC-positive cells that were also stained with PLP antibodies were counted. In some experiments chymotrypsin (0.05% in Hanks' balanced salt solution with 0.02% EDTA; Worthington Biochemical Corp., Freehold, NH) was substituted for trypsin. To test whether protease treatment exposed internal protein to antibody, living cells in suspension treated as described above were stained with rabbit anti-peptide antibody directed against actin (Miles-Yeda, Rehovot, Israel) or a myelin basic protein sequence encoded by exon 6 of the MBP gene (13).

Complement-dependent Cytotoxicity Assays

Cultures enriched for oligodendrocytes were harvested by scraping and counted. 6 × 10⁴ cells were incubated with anti-peptide or anti-GC antibody and rabbit complement (Cooper Biomedical, Inc.) for 15 min at 37°C. Cells were washed and resuspended in MEM with 25 mM Hepes and stained with 0.4% trypan blue in PBS. Since living cells exclude trypan blue, the number of stained dead cells was counted and the ratio to total cells calculated.

To confirm the ability of protease to cleave extracellular PLP domains (as detailed above) and leave only residual amounts of PLP epitopes on the cell surface, parallel experiments were carried out on cells treated with trypsin before addition of antibody and complement. Cells were incubated with 0.05% trypsin at 37°C for 25 min and washed thoroughly before continuing with antibody and complement incubations.

All preparations for immunofluorescence were examined on a microscope (photoscope III; Carl Zeiss, Inc., Thorwood, NY) equipped with epifluorescence and appropriate filters for rhodamine and fluorescein as well as a 63-oil objective.

Results

Most Anti-peptide Antibodies Stain Living Oligodendrocytes in Culture

A hydropathy plot of PLP (Fig. 1) revealed four extremely hydrophobic sections of sufficient length to serve as transmembrane domains (A-D) alternating with hydrophilic stretches of amino acids. To probe the orientation of PLP in the oligodendrocyte plasma membrane, six peptides were chemically synthesized (Fig. 2; peptides were numbered by the order in which they were synthesized). Most of these peptides correspond to hydrophilic segments of PLP with the exception of peptide VI, which is composed of a hydrophobic stretch from domain C. Antibodies to each peptide were raised in rabbits and affinity purified on peptide columns.

Each anti-peptide antibody reacted with PLP on immunoblots, as shown for PLP I in an earlier publication (50); however, while anti-PLP I reacted strongly, antibodies to the other peptides reacted only weakly. When assayed by ELISA, both anti-PLP I and anti-PLP V exhibited substantial immunoreactivity to purified aqueous PLP and to chloroform-methanol extract of isolated myelin, but anti-PLP II, IV, and VI did not. Remarkably, anti-PLP III bound extremely weakly to untreated antigen, but strongly to antigen treated after adsorption by weak trypsin or chymotrypsin digestion or by fixation with glutaraldehyde. All the anti-peptide antibodies stained the cytoplasm of cultured oligodendrocytes strongly. While not definitive, these results suggest that the antibody-binding activity of PLP depends on the milieu in which the molecule presents itself and that PLP, when removed from biological membrane, may fold in ways which severely limit the availability of particular epitopes.

Five of the anti-peptide antibodies (anti-PLP I, III, IV, V, and VI) labeled the surface of rat brain oligodendrocytes in culture at a time corresponding to the postnatal rat age of 20-37 d (Figs. 3 and 4). The oligodendrocytes could be identified in phase contrast by their size and the characteristic branching pattern of their processes; their identity was verified by double staining cultures with antibody against GC in addition to the anti-peptide antibodies. Each anti-peptide antibody produced a patchy pattern of surface label over oligodendrocyte cell bodies and processes (Fig. 3, a and c-e), visible in both singly and doubly stained preparations. A different pattern, seen in oligodendrocyte cell bodies stained after fixation and permeabilization, included focal intracytoplasmic and perinuclear fluorescence suggestive of an association with the Golgi and ER membrane systems (Fig. 3 b).

Immunostaining with each anti-peptide antibody was completely abolished in the presence of excess homologous peptide but was unaffected by a nonhomologous peptide (as shown in Fig. 4 for PLP IV), demonstrating the specificity of the surface label.

Only the anti-PLP II antibody was unable to immunostain intact oligodendrocytes, although it readily reacted with oligodendrocytes that were first fixed and permeabilized with Triton X-100 (Fig. 5). Unlike the focal cytoplasmic stain of all other PLP antibodies, the PLP II antibody produced a diffuse pattern of cytoplasmic stain. Presumably PLP II was cross reacting with other rat intracellular proteins and these interactions were masking the patchy pattern of PLP staining; PLP II antibody, as well as another peptide antibody directed against only the first eight amino acids of the PLP II peptide, did react with other rat brain proteins in addition to PLP on immunoblots.

A critical factor in the detection of PLP on the extracellular face of the oligodendrocyte plasma membrane was the age
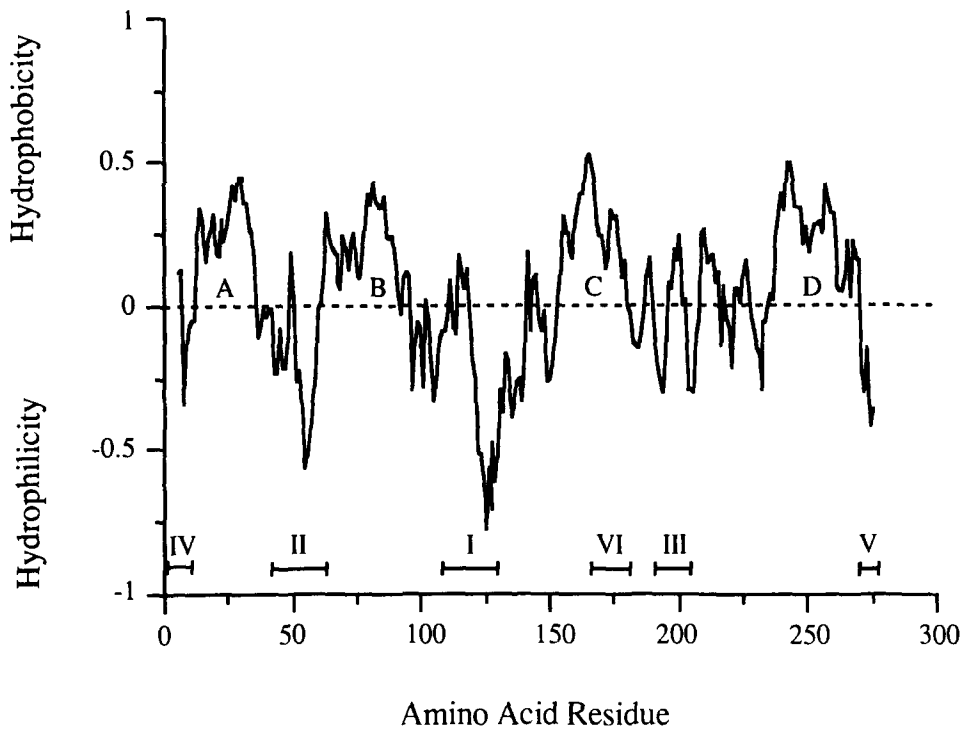
Figure 1. Hydropathy plot of PLP. The consensus hydrophobicity scale of Eisenberg (20) was used. The four hydrophobic domains are indicated as *A–D*; the positions of the synthetic peptides I–VI are shown below their respective hydrophobic/hydrophilic segment.

of the oligodendrocyte culture. In preliminary experiments to determine the time course of PLP expression in cultures established from 1-2-d-old rats, we found that anti–PLP I antibody could label intact oligodendrocytes corresponding to a minimal rat postnatal age of 20 d (18–19 d of culture). Shorter periods of culture failed to yield reproducible surface staining of PLP, although PLP was clearly present throughout the cell body and processes of permeabilized oligodendrocytes as early as 14 d. Similar results were obtained with the other anti–peptide antibodies, so subsequent experiments were done with cultures >20 d old.

The ability of anti–peptide I antibody to label the surface of oligodendrocytes (Fig. 3 *a*) contradicts the work of

Trifilieff et al. (66), who constructed a synthetic peptide (117–129) that overlaps with our peptide I (109–128). The failure of their anti–peptide antibody to stain intact oligodendrocytes is most likely attributable to the use of 18–19-d-old cultures that were not yet expressing PLP on the surface of the plasma membrane. As discussed above and in our previous work (16), a time course of PLP expression in oligodendrocytes cultured from newborn rat brain reveals that PLP is first detected in the cell body at 7–9 d (rat postnatal age), is spread throughout the processes by 14–15 d, and can be reliably detected on the surface of oligodendrocytes only after 20 d.

Some cell death was apparent over time in cultured oligo-


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            Peptide IV                   .                          .
  1  |G L L E C C A R| C L V G A P F A S L V A T G L C F F G V A L F C G C G H E A L T   40
                                                         A
            Peptide II                                  .                          .
 41  G T |E K L I E T Y F S K N Y Q D Y E| Y L I N V I H A F Q Y V I Y G T A S F F F L   80
                                                         B
                          .                             .              Peptide I
 81  Y G A L L L A E G F Y T T G A V R Q I F G D Y K T T I C |G K G L S A T V T G G Q   120
                                                            
121  |K G R G S R| G Q H Q A H S L E R V C T C L G K W L G H P D K F V G I T Y A L T V   160
                   .           Peptide VI       .                      .      Peptide III
161  V W L L V F A C |S A V P V Y I Y F N T W T T| C Q S I A F P S |K T S A S I G S L C   200
            C                                                      
               .                     .                        .
201  |A D A R| M Y G V L P W N A F P G K V C G S N L L S I C K T A E F Q M T F H L F I   240
                       .                     .                Peptide V
241  A A F V G A A A T L V S L L T F M I A A T Y N F A V L |K L M G R G T K F|          276
                       D
```
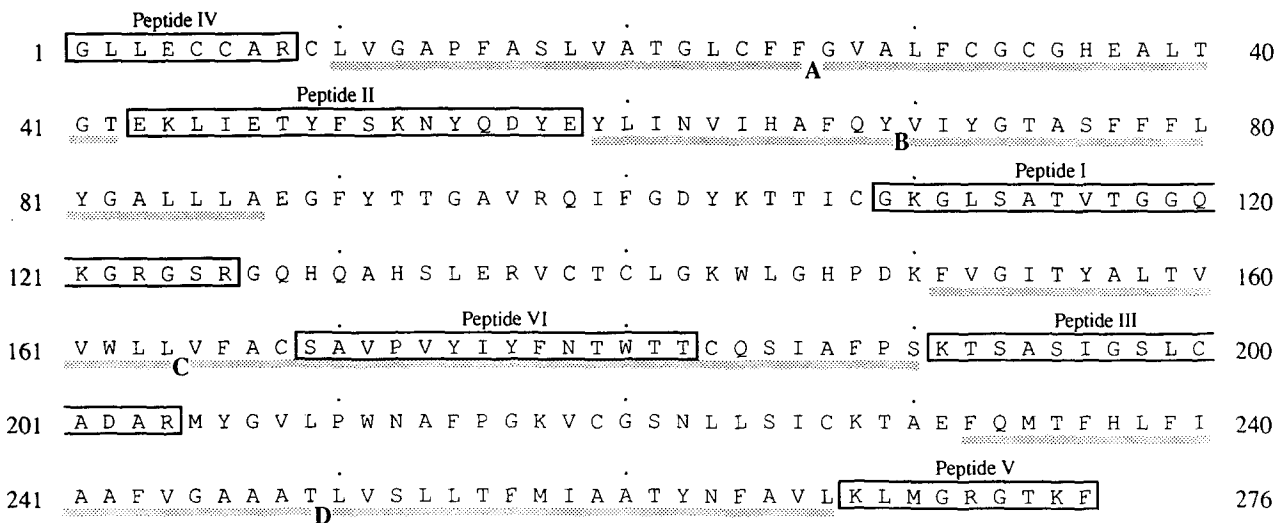
Figure 2. Location of the synthetic peptides in the PLP protein. The amino acid sequence of murine PLP (21) is shown. Peptide sequences are boxed; hydrophobic stretches A–D are underlined. The bovine sequence at position 198 has a threonine instead of serine (27, 38); PLP III was synthesized with a threonine at 198 since the human or rodent sequence was not available at the time these studies were initiated.
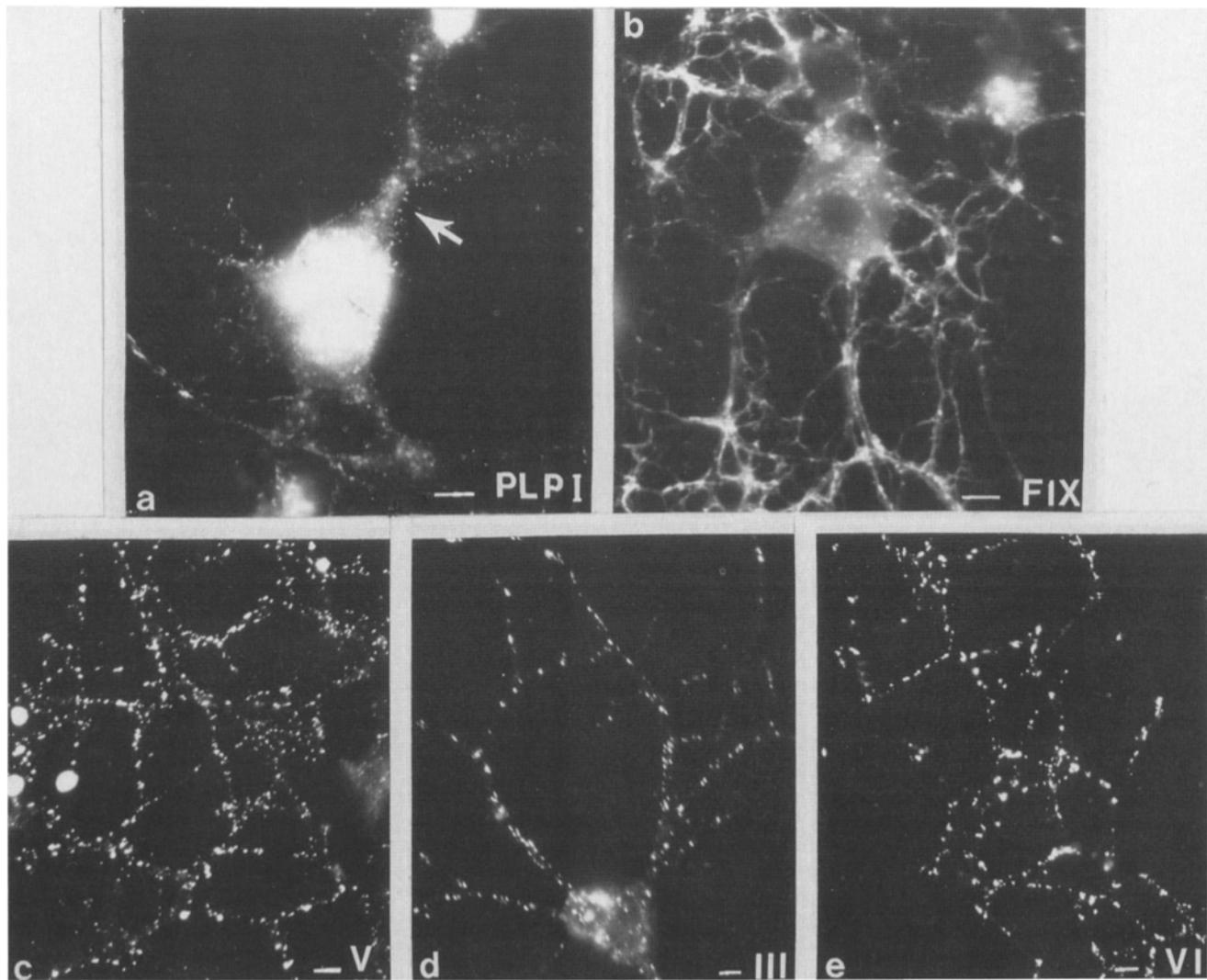
Figure 3. Immunofluorescent staining of cultured oligodendrocytes with various anti–PLP peptide antibodies. With PLP I antibody (*a* and *b*), the patterns of staining on living (*a*) and fixed (*b*) cells are compared. After staining of living cells, numerous small dots are seen on the surface of the cell body (very bright fluorescence) and processes (*a, arrow*). When staining is performed after fixation and permeabilization, PLP is seen in small dots and larger clusters along the processes as well as in the cytoplasm of the perinuclear region. In *c–e*, staining of living oligodendrocytes with three other anti–peptide antibodies (*V, III,* and *VI,* respectively) is shown. Bar, 10 µm.

dendrocytes, raising the possibility that membrane fragments containing PLP could interact with the surface of living cells. This, however, would create a variable and irregular pattern of surface staining which was not observed. In fact, surface staining always revealed a regular dotted pattern over the cell body and processes. A curious phenomena observed in some cultures enriched in astrocytes was the presence of PLP immunoreactivity in glial fibrillary acidic protein–positive cells (not illustrated). Although the astrocytic staining could result from phagocytosis of cellular debris, our recent discovery that nonmyelinating Schwann cells synthesize PLP (44) raises the intriguing possibility that the PLP gene may be activated in astrocytes upon culturing.

Protease Cleavage of Extracellular PLP Domains

To confirm the presence of PLP domains on the extracellular face of the plasma membrane, cultures were treated with pro-

tease and subsequently dual labeled with anti–GC and anti–PLP antibodies. For these experiments, cells had to be stained in suspension because the enzyme treatment detached oligodendrocytes from their substrate. Potential trypsin cleavage sites within the PLP molecule are depicted in Fig. 6. As shown in Table I, trypsin treatment resulted in a marked reduction in the number of living oligodendrocytes with surface-labeled PLP I, III, and IV domains. The hydrophobic domain VI was variably affected but never eliminated by trypsin despite being flanked by potential trypsin cleavage sites (Fig. 6). The persistence of the PLP VI epitope on the oligodendrocyte surface after trypsin treatment could be due to an association of this hydrophobic domain with the oligodendrocyte membrane. Therefore, we treated the cultures with chymotrypsin, which could cleave within the PLP VI domain at any of its numerous aromatic residues, and obtained a 78% reduction of staining of living oligodendrocytes (Table I).
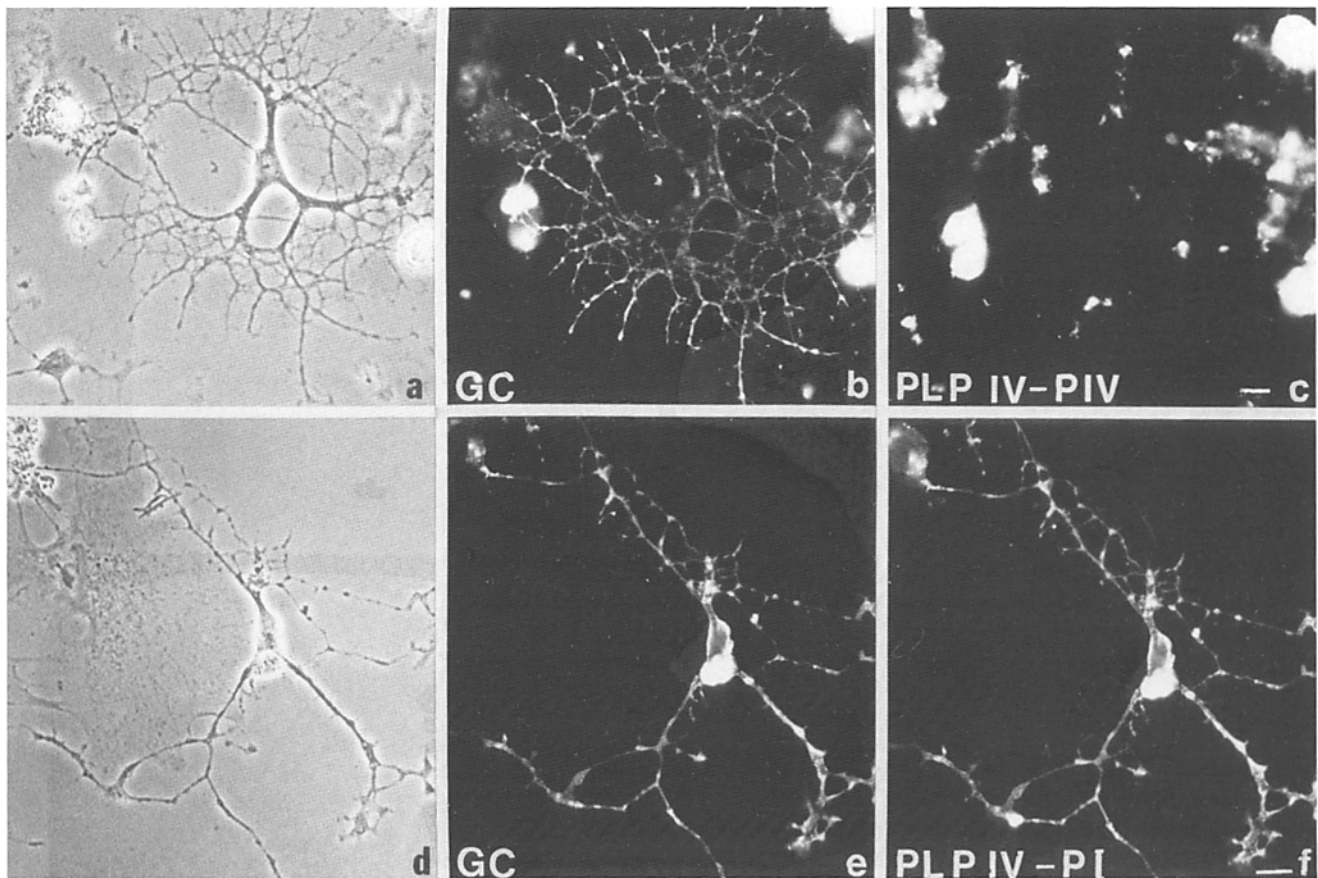
Figure 4. Specific peptide competition of PLP antibody binding. Living cultures were stained simultaneously with anti–PLP IV and anti–GC, in the presence of peptide IV (*a–c*) or peptide I (*d–e*). Shown are phase (*a* and *d*), GC (*b* and *e*), and PLP IV (*c* and *f*) immunofluorescence of one cell from each condition. GC staining is not affected by the presence of either peptide. PLP IV immunostaining is abolished by the specific peptide (*c*) but is undiminished in the presence of the unrelated peptide (*f*). Bar, 20 μm.

The carboxy terminus of PLP (domain V) was unusually resistant to trypsin cleavage despite the presence of three arginine and lysine residues in this 9–amino acid domain. The immunofluorescent staining of this domain was even enhanced after trypsin treatment; all GC-positive cells could be stained with anti–PLP V antibody, and the fluorescence was much more intense than observed for nontrypsinized cells. This suggests that the carboxy terminus of PLP is inac-
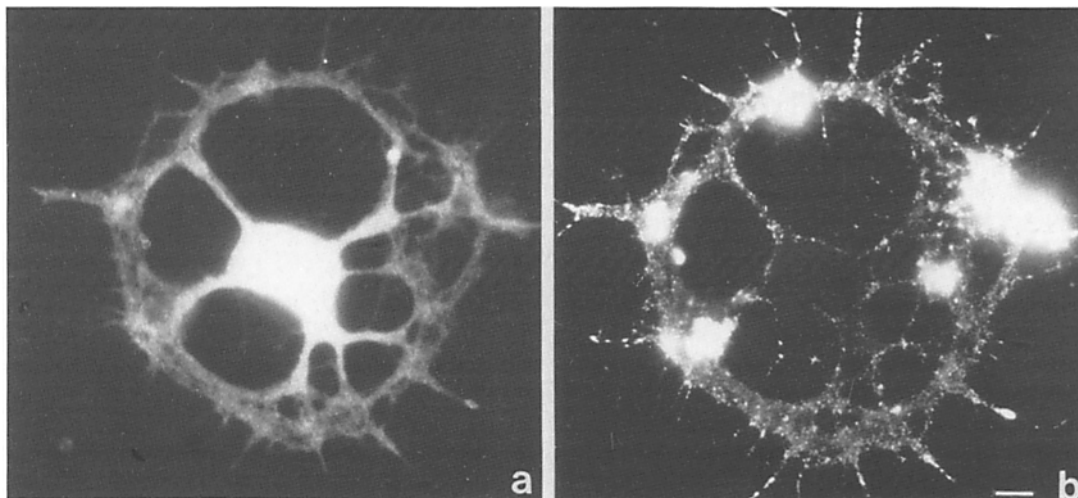
Figure 5. Distribution of cytoplasmic PLP II immunoreactivity. This oligodendrocyte was stained for GC, fixed, permeabilized, and stained with PLP II antibody. PLP II immunoreactivity (*a*) exhibits a diffuse cytoplasmic distribution. Marked GC immunostaining (*b*) in dots and patches clearly identifies this cell as an oligodendrocyte. Bar, 10 μm.

PLP Domains: A - - - B C D

Anti-peptide Antibodies: IV II I VI III V

Arginine/Lysine Residues:
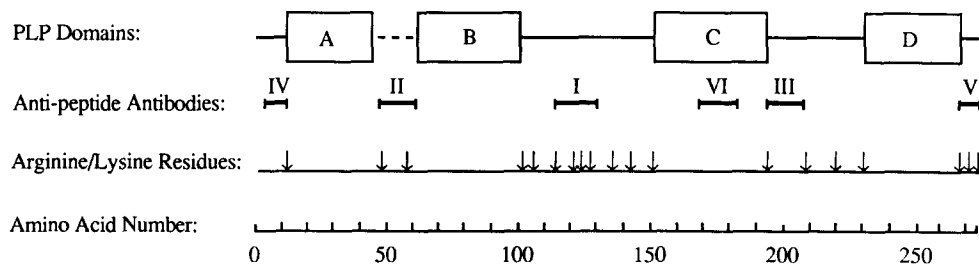
Amino Acid Number:

0   50   100   150   200   250

Figure 6. Mapping of the potential trypsin sites along the different extracellular PLP domains.

cessible to trypsin cleavage due to interactions with other extracellular domains and these interactions are partially disrupted by trypsin treatment. The addition of low levels of detergent slightly facilitated trypsin cleavage of the amino acid stretch encompassing PLP V. PLP V staining was markedly reduced (by 45%) with chymotrypsin treatment.

Importantly, neither the trypsin or chymotrypsin treatment breached the cytoplasmic membrane or exposed any cytoplasmic proteins, as evidenced by the lack of staining of actin or the cytoplasmic protein MBP in parallel experiments.

Complement-dependent Lysis of Oligodendrocytes Mediated by Antibody to Extracellular PLP Domains

To further verify that the carboxy terminus of PLP resides on the extracellular face, despite its partial resistance to protease cleavage, cells were incubated with this anti–peptide V antibody in the presence of complement and examined for lysis. PLP V was compared with two other trypsin-sensitive extracellular domains, PLP I and PLP III. Table II shows that anti–PLP I, III, and V antibodies can each induce complement-mediated lysis of oligodendrocytes at the appropriate dilution of antibody. These results indicate that the PLP V epitope as well as PLP I and III are present on the extracellular surface of the oligodendrocyte plasma membrane and can mediate complement-dependent lysis of oligodendrocytes. When cells were pretreated with trypsin, the ability of anti–PLP I and III antibodies to lyse cells in a complement-depen-

dent fashion was markedly reduced, indicating that the protease was effective in removing much of these PLP epitopes from the extracellular surface. Trypsin cleavage had little effect on the amount of lysis detected in cells treated with PLP V antibody plus complement; this suggests that the PLP V domain remained on the surface after trypsin treatment, in agreement with the surface labeling of this epitope on trypsinized oligodendrocytes described above.

Distribution of PLP Compared with Other Myelin Constituents

When living oligodendrocytes were labeled with mixtures of anti–GC and anti–peptide antibodies, the GC immunofluorescence exhibited a patched surface pattern like that of the PLP immunofluorescence, and the GC and PLP patches were often coincident (Fig. 4, *e* and *f*). However, GC immunofluorescence did not show patching when staining was performed on intact fixed cells. When cells were stained live with anti–PLP peptide antiserum, fixed, and then stained for GC, we saw patches of PLP immunofluorescence distributed along processes and cell bodies that were uniformly stained for GC (Fig. 7, *a* and *b*). It thus appears that patched GC

Table I. Cleavage of Extracellular PLP Domains with Protease

Antibody	Protease	Reduction of oligodendrocytes expressing surface PLP*
		%
PLP I	Trypsin	67 ± 12
	Chymotrypsin	40
PLP III	Trypsin	100
PLP IV	Trypsin	80 ± 28
PLP V	Trypsin	0 ± 0
	Trypsin‡	9
	Chymotrypsin	45
PLP VI	Trypsin	18 ± 31
	Trypsin*	23
	Chymotrypsin	78

* For each experiment, ~100 untreated and 100 protease-treated cells from a 20–37-d (rat age) culture were scored for GC and PLP. Results are expressed as the reduction in the percentage of cells stained for GC that also stain for PLP after protease treatment. Multiple experiments were carried out with trypsin-treated cultures of different ages and the results expressed as the mean ± SD.
‡ 0.01% Triton X-100 included in trypsin incubation.

Table II. Complement-dependent Lysis of Oligodendrocytes with Anti–peptide Antibodies

Anti–peptide antibody	Antibody dilution*	Amount of lysis detected by trypan blue staining‡	
		−Trypsin	+Trypsin
		%	%
PLP V	1:2	92	90
	1:10	54	65
	1:50	46	55
	1:100	31	40
PLP I	1:2	69	20
	1:30	69	7
	1:100	38	0
PLP III	1:30	94	20
	1:100	0	0

* Undiluted antibody was 1 mg/ml.
‡ Lysis is expressed as

(% of cells treated with anti–PLP antibody + complement that are trypan blue positive)	−	(% of cells treated with complement that are trypan blue positive)
(% of cells treated with anti–GC antibody + complement that are trypan blue positive)	−	(% of cells treated with complement that are trypan blue positive)
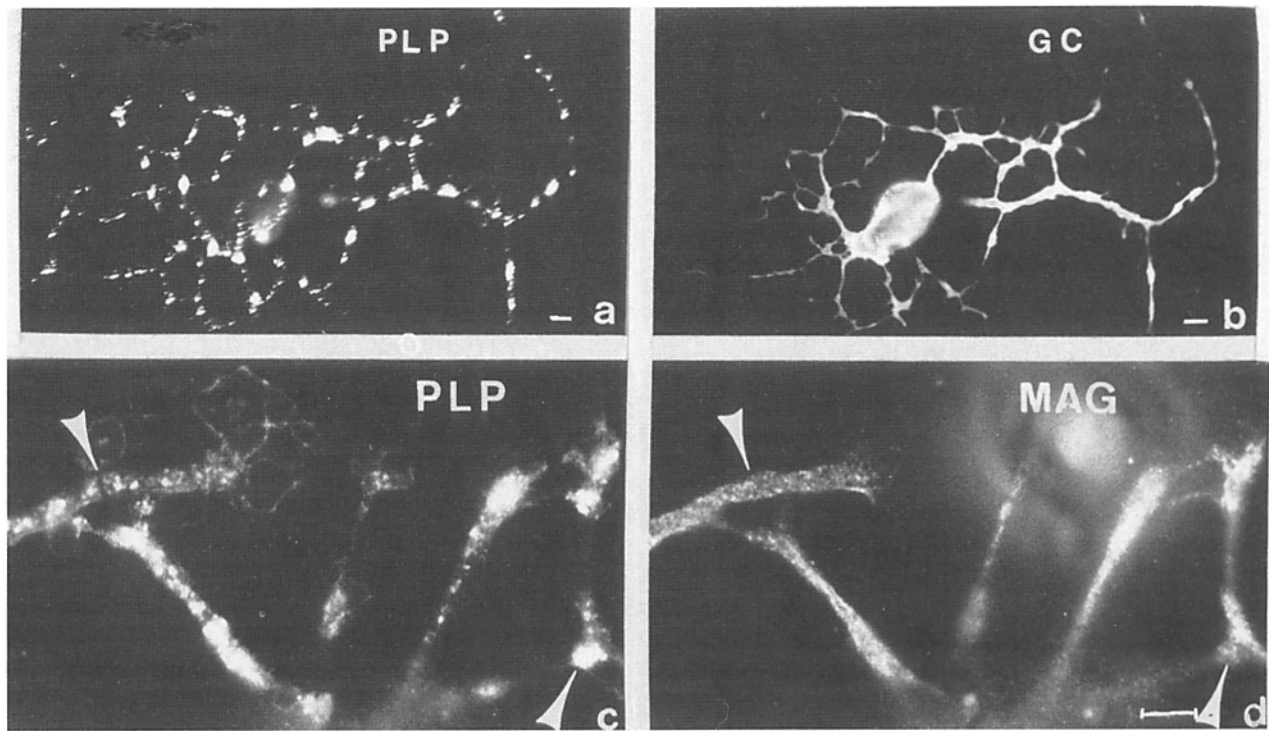
Figure 7. Distribution of surface PLP (*a*) compared with GC (*b*) and of cytoplasmic PLP (*c*) compared with cytoplasmic MAG (*d*). In *a* and *b*, staining was carried out by procedure 2 of Materials and Methods. PLP IV immunoreactivity is seen in clusters on the living cell membrane (*a*), while GC shows a uniform distribution (*b*). Inside the processes of fixed and permeabilized oligodendrocytes, PLP I (*c*) and MAG (*d*) appear localized in different dots and patches and the PLP clusters are larger. Bar, 10 μm.

immunofluorescence, seen only with GC staining of living cells, reflects the aggregation of bound antibody molecules before fixation and not the native distribution of the GC antigen, as also noted by Dyer and Benjamins (18, 19). PLP was always distributed in clusters in our surface-stained preparations; however, these preparations involved exposure of living cells to PLP antibodies. The clustered pattern of PLP surface staining illustrated here may also result from aggregation secondary to antibody binding, and our data do not exclude a uniform distribution of PLP in the unperturbed native membrane.

We previously found MBP and cytoplasmic PLP to be colocalized in clusters within oligodendrocyte processes (16). Furthermore, cytoplasmic staining for both antigens preceded the appearance of PLP on the external surface of the oligodendrocyte plasma membrane. In the present study

we have found that MAG staining was not colocalized with cytoplasmic PLP (Fig. 7, *c* and *d*). Within oligodendrocyte processes double stained by two-color immunofluorescence, MAG was visualized as numerous small dots, possibly transport vesicles, that rarely coincided with dots and larger clusters stained by PLP I antibody (Fig. 7, *c* and *d*). In addition, PLP also formed larger cytoplasmic clusters at the very extremities of the processes, where MAG was not seen (Fig. 7, *c* and *d*). This segregation suggests that MAG and PLP are transported to the oligodendrocyte membrane in different populations of vesicles.

Discussion

In the present study, we have probed cultured oligodendrocytes with antibodies to various domains of PLP and have
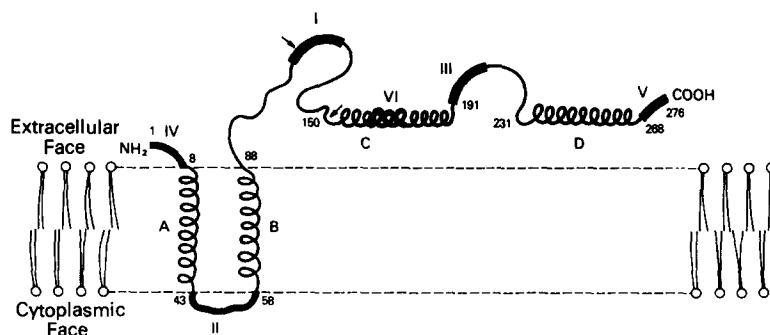
Figure 8. Orientation of PLP in the oligodendrocyte plasma membrane. A linear representation of the PLP molecule is drawn to scale, with the location of the synthetic peptides (*I–VI*) depicted by the thick lines and the four hydrophobic regions (*A–D*) shown as α-helices. Both the amino (*NH₂*) and carboxy (*COOH*) termini are shown on the extracellular face of the lipid bilayer (*dashed line*). The amino acid residues marking the borders of the hydrophobic regions are numbered (see Fig. 2 for the precise location of the synthetic peptides and the hydrophobic regions). The region deleted in DM20 (amino acids 116–150) is bounded by arrows.

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found that all but one of the anti-peptide antibodies labeled living oligodendrocytes. These observations allow us to propose the model for the orientation of PLP in the oligodendrocyte plasma membrane presented in Fig. 8. Most of the hydrophilic portions of the protein, including the amino and carboxy termini, were detected on the extracellular surface of the plasma membrane and were accessible to exogenous protease. The only negatively charged domain resided on the cytoplasmic face. Contrary to computer predictions (Fig. 1; reference 27), of the four hydrophobic segments large enough to cross the membrane, only two (A and B) are likely to be membrane-spanning domains. Domain C does not appear to loop in and out of the membrane as proposed by Stoffel et al. (62), since it was immunostained by anti-peptide VI antibody and cleaved by chymotrypsin. The extramembrane location of domain C is supported by the observation of Kahan and Moscarello that a photoactivated hydrophobic reagent labeled the transmembrane helices A and B but did not interact with domain C (24). The disposition of the hydrophobic domain D is not precisely known since an anti-peptide antibody was not prepared to this segment, but it probably resembles and possibly interacts with the similarly hydrophobic, highly α-helical domain C. If domain D was buried within the lipid bilayer, the extracellular location of the carboxy terminus dictates that domain D would enter and exit the bilayer on the same face of the membrane. The unreliability of computer-generated predictions for the transmembrane orientation of PLP has been likewise observed for other membrane proteins, notably the acetylcholine receptor (9, 71) and the 32-kD chloroplast thylakoid membrane protein, which Sayre et al. found to contain five instead of the predicted seven transmembrane helices (58). Among proteins with multiple transmembrane helices (for review see Wickner and Lodish [67]), PLP may not be unique in orienting both its amino and carboxy termini on the extracellular face of the plasma membrane, as Schofield et al. speculate that the γ-aminobutyric acid receptor may also position both termini extracellularly (59).

It is of note that the structure of PLP we propose, with the majority of the molecule on the extracellular face and only a small cytoplasmic domain, resembles the predicted structure of the peripheral nervous system counterpart of PLP, the P₀ glycoprotein (29). Our results also predict that the DM20 protein, which is produced by alternative splicing at the PLP locus (21, 40) and is deleted for the small stretch encompassing epitope I (66) (Fig. 8), should display the structural features of PLP and function in a similar manner. Finally, our model indicates that if PLP and MBP do specifically interact, as has been suggested by cross-linking and binding studies (for review see Braun [6]), this interaction must involve domain II (Fig. 8), the only PLP domain present on the cytoplasmic surface where MBP is located.

The topology of PLP in the plasma membrane (Fig. 8) resembles the orientation of PLP in compact myelin proposed by Stoffel et al. (62) only in the amino-terminal half of the protein. In both the plasma membrane and in myelin, the hydrophilic domains of PLP stained by anti-peptide antibodies I, III, and IV reside on the extracellular face, which forms the intraperiod line in compact myelin, and the hydrophilic domain II resides on the cytoplasmic face. The two models differ, however, in the disposition of the hydrophobic domains C and D and the carboxy terminus. These differences are explicable in view of the methods used to ana-

lyze PLP structure in compact myelin. Hyposmotically shocked compact myelin, which was presumably dissociated at the intraperiod line, was trypsin treated and the largest three tryptic fragments were partially sequenced (62). Since the hydrophobic domains C and D lack trypsin cleavage sites, trypsin fragments analyzed would be identical whether C and D were in or out of the bilayer. Moreover, the carboxy terminus was intractable to trypsin digestion (see Results) under conditions similar to those used by Stoffel et al. (62) despite the presence of arginine and lysine in this domain. As the hyposmotic shock method assigned domains that were not cleaved by trypsin to the major dense line, the carboxy terminus in the Stoffel model was shown on the cytoplasmic face. The inaccessibility of PLP's carboxy terminus in compact myelin, which would complicate analyses dependent on protease treatment, was also demonstrated by Nussbaum et al. (43). The disparity between the two models of PLP topology could also be reconciled if PLP rearranges during its transition from plasma membrane to compact myelin. While conformational changes due to aggregation, acylation, or disulfide bridge formation are anticipated during myelin assembly, it is unlikely that these events could radically alter the orientation of PLP in the membrane. Nevertheless, the PLP topology we propose here should be further explored in myelinating oligodendrocytes.

Our model is supported by the electron density profiles of the cytoplasmic and extracellular spaces calculated from measurements of central nervous system myelin by Inouye and Kirschner (23). The location of the majority of the PLP molecule on the extracellular face of the membrane (Fig. 8) is consistent with the observed electron density profile of the intraperiod line, which is much higher than either the Stoffel et al. (62) or Laursen et al. (27) models would predict (23).

During compaction of the myelin sheath, the apposed extracellular faces of adjacent lipid bilayers fuse to form the intraperiod line. The presence of the majority of the PLP molecule (residues 1-8, 88-276; Fig. 8) in the intraperiod region indicates that this abundant protein may function to maintain the correct spacing between bilayers. A structural role for PLP as a strut that preserves the intraperiod structure was originally proposed by Kirschner et al. (25) and is substantiated by recent work on the jimpy mouse mutant. In jimpy mice, aberrant splicing of PLP mRNA results in the absence of normal PLP protein (12, 21, 22, 31, 37, 39, 41, 54). The myelin sheaths formed by jimpy oligodendrocytes have a much condensed intraperiod line (17) consistent with a role for PLP as a strut. Homophilic interactions of PLP molecules could contribute significantly to the intraperiod structure of the myelin sheath. Two posttranslational events may be critical in mediating these homophilic interactions: PLP molecules in apposing bilayers and/or within a bilayer could be cross-linked by disulfide bridges between loops IV, I, and III as previously characterized (27, 61, 62) or PLP molecules could aggregate via the hydrophobic, α-helical domains C and D. Another posttranslational modification, the attachment of palmitic acid to domain III shortly before or during the insertion of PLP into myelin, could also act to maintain spacing, especially if the covalently bound fatty acid intercalates into the apposing lipid bilayer as proposed by Laursen et al. (27). The other two models of PLP topology (27, 62) are also consistent with homophilic interactions of PLP molecules within and between bilayers.

PLP is synthesized in the RER and can be detected in vivo

in a myelin fraction after a lag of 30–60 min (4, 8). Analogous to other membrane-spanning proteins (55, 67), PLP must have at least one internal signal sequence (uncleaved) that directs its insertion into the RER membrane and one stop–transfer sequence (within domain A). One of the rate-limiting steps in the transport of proteins from the RER is their correct folding or the assembly of protein monomers with their cognate partners (e.g., heavy and light chains of IgM) or with themselves (e.g., trimers of influenza virus hemagglutinin), as reviewed by Pfeffer and Rothman (47). The capacity of PLP to aggregate in vitro even in the presence of strong denaturants (6, 28) suggests that PLP monomers may assemble before exiting the ER in transport vesicles for the Golgi region.

Protein sorting occurs chiefly in the Golgi stacks (47), where PLP may be directed by either bulk flow to the plasma membrane in vesicles that are nonselective with regard to their contents or be packaged in a signal-dependent fashion into vesicles whose transport to the myelin membrane is regulated. Our finding of prolonged delay between the accumulation of PLP in the cytoplasm of cultured oligodendrocytes and the appearance of PLP on the surface of the plasma membrane (see Results; reference 16) suggests that PLP transport follows a regulated pathway. Since MAG and PLP do not colocalize within the cytoplasm, it appears that the transport of PLP occurs via vesicles different from those that transport MAG, a conclusion anticipated by their disparate kinetics of transport (16) and by their ultimate location in distinct compartments of the myelin sheath (32, 65). Cytoplasmic movement of these two populations of vesicles seems independently regulated, since MAG moves from perikarya to processes of cultured oligodendrocytes days earlier than PLP does (16).

The transport of PLP to the cell processes appears to occur independently of the other myelin constituents that were monitored by immunostaining: GC, MBP, and MAG. GC is established throughout the extracellular surface of the oligodendrocyte processes days before PLP synthesis initiates (16). MBP is sometimes colocalized with internal PLP clusters and dots as shown earlier (16); this initial association of MBP and PLP may be mediated by the single cytoplasmic domain of PLP (Fig. 8). As discussed above, MAG and PLP appear to be transported by distinct, independently regulated vesicular transport systems. This segregation of MAG from PLP is consistent with the proposed role for MAG, which is structurally similar to cell adhesion molecules (2, 26, 56), in mediating contacts between oligodendrocyte processes and axons before being excluded from compact myelin (32, 65). Thus, an initial event in myelin assembly could be the sorting of myelin constituents based on their ultimate destination in the myelin sheath. Such events may be enhanced when oligodendrocytes are presented with their target neurons and, therefore, constitute the initial events in myelin assembly. The complete elucidation of these events will require coculture with neurons in a myelinating system (69) as well as ultrastructural analysis of immunolabeled myelinating cells.

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