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Topology of Proteolipid Protein in the Myelin Membrane of Central Nervous System

A Study Using Antipeptide Antibodies

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Summary: Peptides according to amino-acid sequences of the N- and C-terminus of lipophilin (proteolipid protein, PLP) (Gly^{1}-Phe^{15} = **1**; Thr^{261}-Phe^{276} = **6**) and of the other four hydrophilic domains (Glu^{37}-Leu^{60} = **2**; Arg^{97}-Leu^{112} = **3**; Gly^{119}-Gly^{127} = **3A**; Trp^{144}-Tyr^{156} = **3B**; Lys^{191}-Ala^{203} = **4**; Asn^{222}-Phe^{232} = **5**) have been synthesized by the solid-phase Fmoc method, linked covalently to keyhole limpet hemocyanin (KLH) and used as antigens. Monospecific antibodies against these antigens were isolated by affinity chromatography. Each antibody recognized its epitope in isolated partially delipidated PLP with the ELISA technique, western blot, thin sections of paraffin embedded rat brains and in the plasma membrane of appropriately fixed/permeabilized rat oligodendrocytes in culture. After fixation with formaldehyde antipeptide 3A antibody stained intact non-permeabilized cells. Therefore the epitope 3A must be located on the extracellular surface of the membrane. This is in full support of our previous biochemical results on the orientation of lipophilin in the myelin membrane.

Topologie des Proteolipidproteins in der Myelinmembran des Zentralen Nervensystems – Untersuchung mit Antikörpern gegen synthetische Peptide

Zusammenfassung: Mit Hilfe der Fmoc-Festphasensynthese wurden Peptide des Lipophilins (Proteolipid-Protein, PLP) synthestisiert, die die Aminosäuresequenzen des N- und C-Terminus des PLP (Gly^{1}-Phe^{15} = **1**; Thr^{261}-Phe^{276} = **6**) und vier hydrophile Domänen des PLP darstellen: (Glu^{37}-Leu^{60} = **2**; Arg^{97}-Leu^{112} = **3**; Gly^{119}-Gly^{127} = **3A**; Trp^{144}-Tyr^{156} = **3B**; Lys^{191}-Ala^{203} = **4**; Asn^{222}-Phe^{232} = **5**). Sie wurden kovalent an „keyhole limpet hemocyanin" (KLH) gekoppelt und als Antigene eingesetzt. Monospezifische Antikörper wurden gegen diese Antigene durch Affinitätschromatographie isoliert. Alle Antikörper erkennen ihre Epitope in isoliertem partiell delipidiertem PLP im ELISA und Westernblot sowie an Paraffin-Dünnschnitten von Rattengehirn. Oligodendrozyten in Primärkultur ließen sich nach geeigneter Fixierung/Permeabilisierung spezifisch markieren. Antipeptid-3A-Antikörper reagieren als einzige mit intakten, nicht-permeabilisierten Zellen nach Fixierung mit Formaldehyd. Das entsprechende Epitop 3A muß also auf der extrazellulären Seite der Membran lokalisiert sein. Dies ist in voller Übereinstimmung mit unseren früheren biochemischen Ergebnissen.

Abbreviations:
CNS, central nervous system; PNS, peripheral nervous system; Fmoc, fluorenylmethyloxycarbonyl; KLH, keyhole limpet hemocyanin; MBP, myelin basic protein; PLP, proteolipid protein, lipophilin; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; NCS, newborn calf serum; DMEM, Dulbecco's modified Eagle's medium; PAP, peroxidase-antiperoxidase; DAB, diaminobenzidine; $NaCl/P_i$, phosphate-buffered saline, pH 7.4; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; MDL, main dense line; IDL, intraperiod dense line; DCC, *N,N'*-dicyclohexylcarbodiimide.

Key words: Myelin, proteolipid protein, antipeptide antibodies, oligodendrocyte, immunogenic determinants, membrane topology.

Myelin is a multilayered, compacted membrane system spirally wrapping the axons in CNS and PNS. In CNS the oligodendrocyte synthesizes during myelinogenesis the lipid constituents and the proteins and sequesters them into the sheath-like processes of its plasma membrane[1,2]. The Schwann cell shares this function in PNS. Ultrastructurally the lamellae system reveals a 15–16 nm periodicity between two main dense lines (MDL) in which two 5-nm lipid bilayers are separated by a 3-nm intraperiod dense line (IDL)[3]. It is generally accepted that the MDL represents the cytosolic cleft and the IDL the apposition of the external surfaces of the plasma membrane processes. In hypotonic, $Ca^{2\oplus}$-free solutions the extracellular space expands with the abolishment of the IDL except the area of the radial components[4–7].

The two major myelin proteins in CNS are myelin basic protein (MBP) and lipophilin (proteolipid protein, PLP) comprising up to 90% of the total protein. MBP occurs as a peripheral, water-soluble protein in up to four isoforms of 21.5, 18.5, 17.2, 14.5 kDa of which the 170 amino-acid residues (18.5 kDa) containing form is the predominant one. All isoforms are expression products of one gene with seven exons. Alternative splicing leads to the respective transcripts[8–10]. PLP amounts to approximately 55%. The primary structure of this 276 amino-acid residues containing, extremely hydrophobic integral membrane protein with 30 kDa of bovine and human myelin origin has been established in this laboratory[11–14] (partial sequences reported by other laboratories[16,17] confirmed our sequence data). It revealed clearly separated domains. This suggested to us the topology of PLP in the myelin lipid bilayer indicated in Fig. 1[15]. In this model the hydrophilic N-terminus (Gly^1-Ala^9) is positioned together with the hydrophilic loops 3, 4 and 5 on the extracytoplasmic surface. Each of these extracytoplasmic loops carries surplus positive charges and most cysteine residues present in hydrophilic loops. No cysteine residues are located in loop 2 and the C-terminal sequence 6.

We tried to access this suggested structure in a biochemical approach by tryptic cleavage of hyposmotically dissociated myelin membranes. PLP was cleaved into three large fragments[15], which were sequenced. Trypsin, which does not penetrate the myelin membrane, cleaved within loops 3 and 4.

The tryptic treatment of the myelin membrane also proved that MBP is protected by the lipid bilayers in the cytosolic cleft, which is in agreement with the electron microscopic immunocytochemical localization of MBP in the MDL[18] and its synthesis on free ribosomes[19].

In this paper we report the results of immunocytochemical studies targeting the topology of lipophilin in the myelin membrane and the plasma membrane of oligodendrocytes in culture. Monospecific antibodies against synthetic peptides with sequences within putative antigenic epitopes were obtained and the monospecific IgG fractions isolated by affinity chromatography. These epitopes are parts of hydrophilic domains protruding on the extracytoplasmic and cytosolic surfaces of the lipid bilayer of the myelin membrane. The results provide further important support for the molecular arrangement of proteolipid protein in the myelin membrane previously proposed in our model[15].

Materials and Methods

All immunochemicals were purchased from Sigma, München, Fmoc amino acids from Bachem AG, CH-4416 Bubendorf, Affigel 10 from Bio Rad, München, cell culture media and sera from GIBCO, D-7514 Eggenstein, all other cell culture biochemicals from Sigma, München.

Peptide synthesis

Solid-phase peptide synthesis following the standard procedure[20] was carried out with 4-alkoxybenzyl resin as solid phase (degree of derivatization 0.63 mmol/g) and Fmoc protected α-amino acids[21–23]. The following side chain-protected L-amino acids were used: *t*-butylester of Asp and Glu, *t*-butylether of Ser, Thr, Tyr, Mtr (4-methoxy-2,3,6-trimethylbenzolsulfonyl derivative) of Arg, *t*-butyloxycarbonyl-protected Lys and tritylthioether of Cys.

All amino acids were coupled with DCC in the presence of 1-hydroxybenzotriazol according to Stewart and Young[20] using the semiautomatic peptide synthesizer SP640, Labortec AG, CH-4416 Bubendorf.

Peptides cleaved from the solid support were purified by Biogel P2 chromatography with 0.1M acetic acid as solvent and by isocratic reversed-phase HPLC with acetonitrile/water/0.1% trifluoroacetic acid and detection at 220 nm.

The sequence of the peptides was confirmed by amino-acid analysis, liquid-phase and gas-phase sequencing using a Beckman peptide sequencer, Model 890C and an Applied Biosystems Analyser Model 477A respectively.

Immunization

Peptides were coupled to keyhole limpet hemocyanin (KLH)[24]. Rabbits were immunized with 1.5 mg peptide-KLH-conjugate dispersed in 750 µl NaCl/P_i, pH 7.4, and 750 µl complete Freund's adjuvant. For production of PLP-antisera reductively carboxymethylated protein was used (500 µg protein for each immunization). Animals were boostered at two-week intervals by an intramuscular injection of the antigen suspended in incomplete Freund's adjuvant. The titer was determined 7 days later and the blood taken by cardiac puncture.

Antibody purification

Antiserum was adjusted with $(NH_4)_2SO_4$ to 33% (w/v), the precipitate collected after 30 min dissolved in 0.0175M phosphate buffer, pH 6.3, equivalent to half the volume of the serum, and dialysed 2–3 days against five changes of the same buffer. Antibodies were separated by DEAE-Sephacel chromatograhy in the same buffer, lyophilized, dissolved in NaCl/P_i and chromatographed on a peptide-Affigel 10 column. Unspecific proteins were eluted with NaCl/P_i, NaCl/P_i/1M NaCl and the monospecific antipeptide IgG desorbed with 0.1M glycine/6M urea, pH 2.8. The eluate was dialysed against four changes of NaCl/P_i.

ELISA

Falcon microtiter plates were coated with PLP (1 mg/ml solubilized in 1% $NaDodSO_4$) or peptide at a concentration of 10 µg/ml in borate-buffered saline (0.025M disodium tetraborate, 0.9% NaCl pH 8.3) for 4 h at room temperature or overnight at 4 °C. The plates were then incubated with 1% BSA in NaCl/P_i for 2 h at room temperature, washed twice with phosphate buffer (0.1M sodium phosphate pH 6.5, 1% Tween 20) and incubated with antiserum in the respective dilutions for 90 min at 37 °C or overnight at 4 °C. Plates were washed three times with phosphate buffer. A complex of alkaline

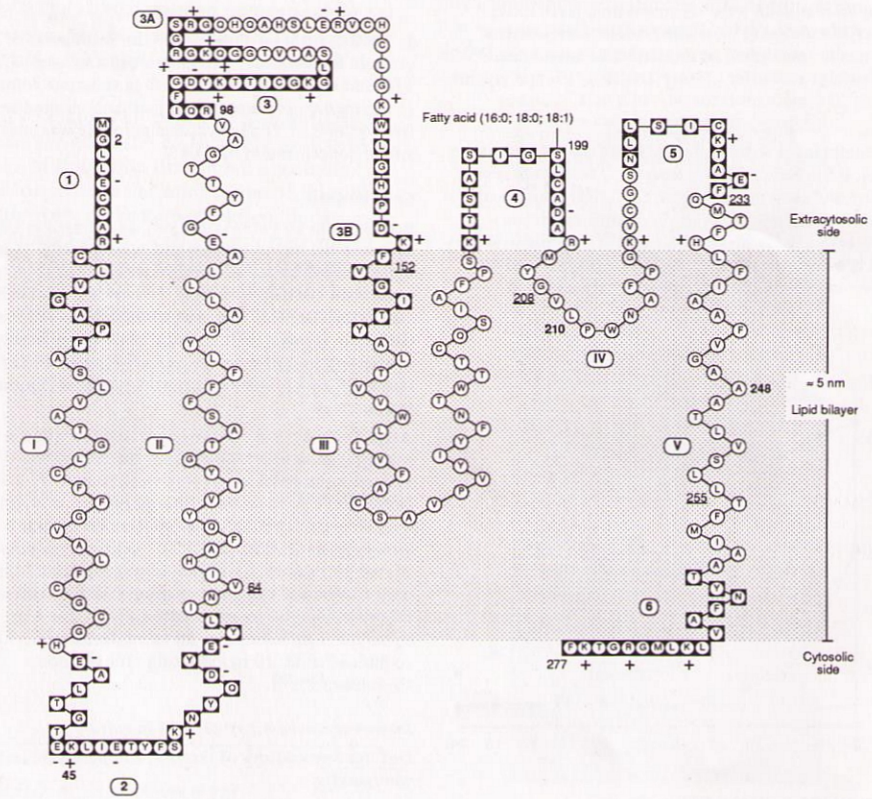


Fig. 1. Proposed model for the molecular orientation and arrangement of lipophilin (proteolipid protein, PLP) in the lipid bilayer of the CNS myelin membrane.

The hydrophobic lipid bilayer is indicated as stippled area, *trans*- and *cis*-membrane hydrophobic domains of PLP are marked with Roman numbers, hydrophilic sequences with arabic numbers and sequences which were synthesized are inserted in black boxes.

phosphatase coupled to goat anti rabbit IgG, diluted 1:1000 in NaCl/P_i/5% BSA was added and incubated for 2 h at 37 °C. Microtiter plates were washed three times with NaCl/P_i and the substrate (4-nitrophenyl-phosphate 25mM) in diethanolamine buffer (1M diethanolamine, 0.5mM $MgCl_2$, pH 9.8) was added and incubated. The reaction was stopped with 3M NaOH and the extinction measured at 405 nm in an ELISA reader (SLT EAR 400 AT).

Competitive ELISA

Stock solutions of peptides and PLP (4 mg/ml 1% $NaDodSO_4$) were diluted with NaCl/P_i/1% BSA to final concentrations between 0.1–1000 µg/ml. A five-fold excess of Triton X-100 over $NaDodSO_4$ was adjusted before the addition of antiserum in this competition assay. Antisera dilutions of 1:100 in NaCl/P_i/1% BSA were incubated with the respective synthetic peptides at 4 °C overnight and assayed for reactivity.

Western blot

The proteins in the chloroform/methanol extract of bovine brain myelin were separated on a $NaDodSO_4$ polyacrylamide gel (15%). The proteins were electrophoretically transferred to nitrocellulose membrane[25] in a Tris/glycine buffer (25mM Tris/HCl, 192mM glycine pH 8.3, 20% methanol) for 24–48 h at 4 °C and at 30 V.

The membrane was treated with TBS (Tris/HCl 10mM, pH 7.4, 0.9% NaCl) and 3% BSA for 2 h at room temperature and then incubated with a 1:200 dilution of PLP antibodies or 1:50 dilution of antipeptide antibodies, respectively in TBS-BSA at 4 °C overnight. Afterwards it was washed three time with TBS 0.1% NP40 and peroxidase-coupled second antibody added in 1:1000 dilution in TBS-BSA for an incubation of 2 h at room temperature. The membrane was washed with TBS and incubated for detection in substrate solution (3 mg/ml 4-chloronaphthol in methanol diluted with five volumes of TBS). Hydrogenperoxide was added to a final concentration of 0.01%.

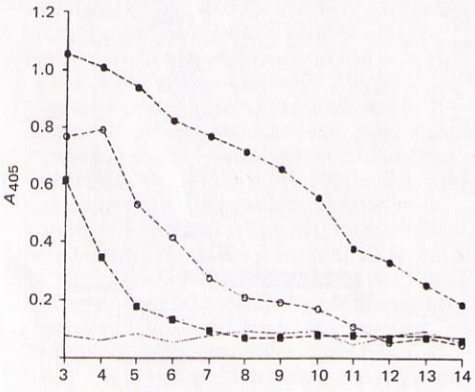


Fig. 2. ELISA with anti PLP antiserum.
Antigens: PLP (●), peptide **6** (○), peptide **2** (■). Dotted line: Level of nonimmune serum and of peptides **3**–**5**. All peptides except **3A** could be used for coating of microtiter plates without being coupled to a carrier, **3A** coupled to BSA.

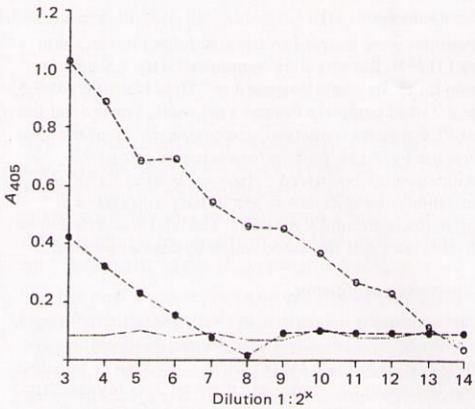


Fig. 3. ELISA with antipeptide **3A** antiserum.
Antigens: PLP (●), BSA-peptide **3A** (○).

Cell culture

Brains of one to two day-old rats were rinsed in DMEM, squeezed through a 100-µm and subsequently 28-µm nylon sieve. The cell suspension was diluted to 40 ml/brain with DMEM, 10% NCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and to this was added 2mM Gln, 1mM pyruvate[26]. Fifteen ml of the cell suspension was seeded in polylysine (5 µg/cm^2) coated Falcon flasks (75 cm^2). The medium was exchanged in three-day intervals.

After 7–10 days in culture the O2A-progenitor cells as upper layer were separated from the underlying type-I astrocytes by horizontal rotation (250 min^{-1}, 37 °C overnight)[27]. They were filtered through a 20-µm nylon sieve to eliminate the few clusters of type-1 astrocytes and seeded at 4 x 10^4 cells/cm^2 in polylysine-coated petridishes. The medium was replaced 24 h later with a chemically defined medium (DMEM, 100 units/ml penicillin, 100 µg/ml streptomycin, 2mM Gln, 1mM pyruvate, 5 µg/ml insulin, 50 µg/ml transferrin, 30nM sodium selenite, 10 ng/ml biotin, 30nM triiodothyronine)[28,29].

Immunocytochemistry on cells in culture

Different procedures of fixation and permeabilization were used:

1) Cell surface labelling of living cells according to Roussel et al.[30].
2) Fixation in 4% formaldehyde (30 min at room temperature).
3) Fixation as above, followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min.

4) Fixation and permeabilization with ethanol-acetic acid (95:5, v/v) for 20 min at 4 °C.

The PAP method[31] was applied in immunochemistry for light and electron microscopy. The peroxidase reaction was enhanced for electron microscopy by a 30-min postfixation with 1% OsO_4. Cells were fixed with formaldehyde (4%, 30 min) for immunogold staining and incubated with antipeptide 3A antiserum (dilution 1:50) for 4 h and colloidal gold (5 nm) coupled to goat anti rabbit IgG (dilution 1:20) for 1 h. In situ embedding and sectioning of oligodendrocytes was performed as described previously[32].

Results

Characterization of antibodies to synthetic peptides recognizing linear epitopes of lipophilin

Peptide sequences within the six hydrophilic domains indicated as black boxes in Fig. 1 were synthesized by solid-phase synthesis and purified by HPLC. They were coupled to keyhole limpet hemocyanin. Antibodies against PLP and every single KLH-conjugated peptide were raised in rabbits.

Titers of PLP and PLP peptides 1–6 antisera were determined in the ELISA. The water-insolubility of lipophilin demanded a suitable method for coating the solid support: lipophilin was reductively carboxymethylated, denatured and solubilized in 1% $NaDodSO_4$ (1 mg/ml) at 100 °C. A protein concentration of 10 µg/ml and of $NaDodSO_4$ to 0.01% was adjusted with buffer. Antilipophilin antibodies reacted with a titer of > 1:10 000 with lipophilin. Peptides 6 and 2 reacted with anti-PLP antibodies with a titer of 1:1 024 and 1:64, respectively. We conclude

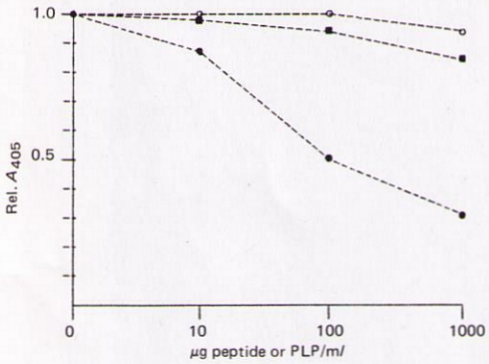


Fig. 4. Competitive ELISA with antipeptide **3A** antiserum.

Antigens: peptide 3A-BSA conjugate. Competing antigens: peptide **3A** (●), PLP (■), control peptide (○) (MBP-specific).

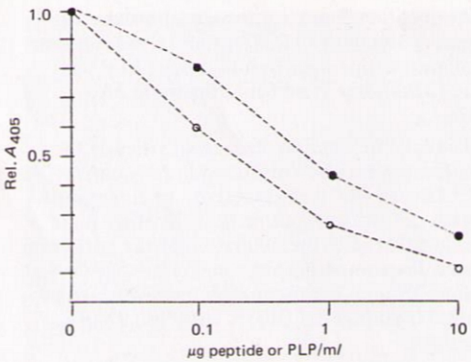


Fig. 5. Competitive ELISA with anti-PLP antiserum.

Antigen: peptide 2. Competing antigens: peptide 2 (●), PLP (○).

that these linear domains are essential continuous immunogenic determinants of lipophilin. Fig. 2 summarizes the results of ELISA with lipophilin and the peptides as antigens.

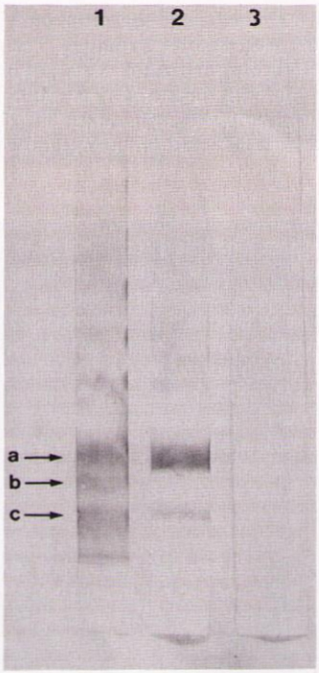


Fig. 6. Western blot analysis of proteins in chloroform/methanol extract of bovine brain myelin using 1) anti-PLP antibodies (1:200); 2) antipeptide **3A** antibodies (1:50) and 3) nonimmune serum (1:50) 15% $NaDodSO_4$ polyacrylamide gel (a. PLP; b. DM20; c. low-molecular mass proteolipid proteins).

Antipeptide 3 and 3A antisera showed cross reactivities with PLP. The titer of the reaction of antipeptide 3 antiserum against PLP was 1:10000, but 1:64 for antipeptide 3A, Fig. 3.

The specificities and cross reactivities of the antisera were also confirmed in the competitive ELISA, in which the reactivity of an antiserum with the antigen coating the microtiter plate is quantified after preincubation of the antiserum with the competing antigen. Preincubation of anti- 3A and 3 antisera with the respective peptides inhibited the ELISA with lipophilin as antigen.

Antipeptide 3 and 3A antisera do not recognize its epitope on lipophilin in solution but only in its conformation on the microtiter plate. This is demonstrated in Fig. 4. The antipeptide antiserum 3A was preincubated with lipophilin but still reacted with the same titer with its respective antigen.

When peptides 2 and 6 are used as coating antigens PLP antiserum can be inhibited by competing peptides 2 and 6 and PLP, as shown in Fig. 5.

We further characterized the antipeptide antisera by the Western blot technique. The proteins in the chloroform/methanol extract of bovine brain myelin were separated by $NaDodSO_4$ polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose[25].

Anti-PLP antiserum, antipeptide 3 and 3A antibodies combined with the peroxidase-conjugated antibody clearly recognized their distinct epitopes: whereas anti-PLP stained PLP, DM20 (the second main component of the proteolipid fraction, with a molecular mass of 20 kDa) and the low molecular mass proteolipid proteins[33] (fragments of PLP with molecular masses of 14–16 kDa), identical with the antipeptide 3 IgG (Fig. 6, lane 1). Antipeptide 3A IgG bound only to PLP and to low-molecular mass proteolipid proteins, not to DM20, in agreement with a previous report[34] and due to the deletion of Val^{116} to Lys^{150}[35] in DM20 caused by alternative splicing within exon III[36].

Anti-lipophilin peptide antibodies as tools in immunocytochemistry

Thin sections of paraffin-embedded rat brains were stained with each of the antipeptide IgGs by the PAP procedure[31]. Myelinated axons and white matter were intensively stained with all antibodies, Fig. 7.

Orientation of lipophilin domains in the plasma membrane of oligodendrocytes

Our attempts to label the domains of lipophilin in the intraperiod dense line of the myelin membrane with the different antipeptide antibodies by either the pre- or postembedding immuno-

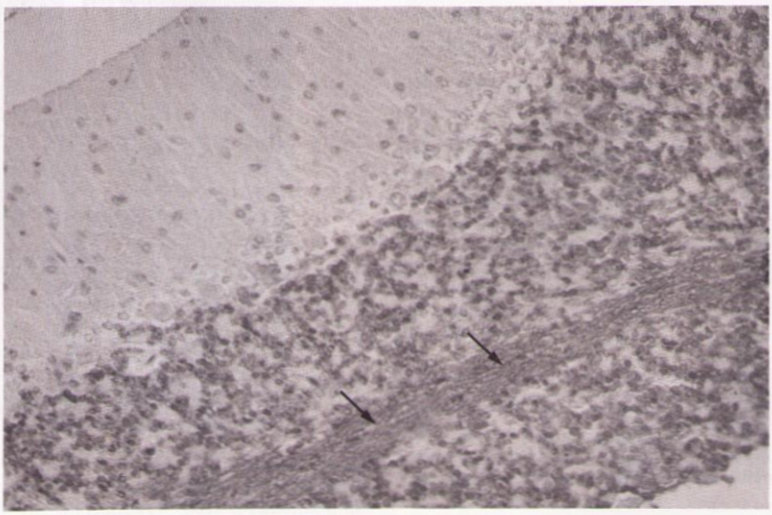
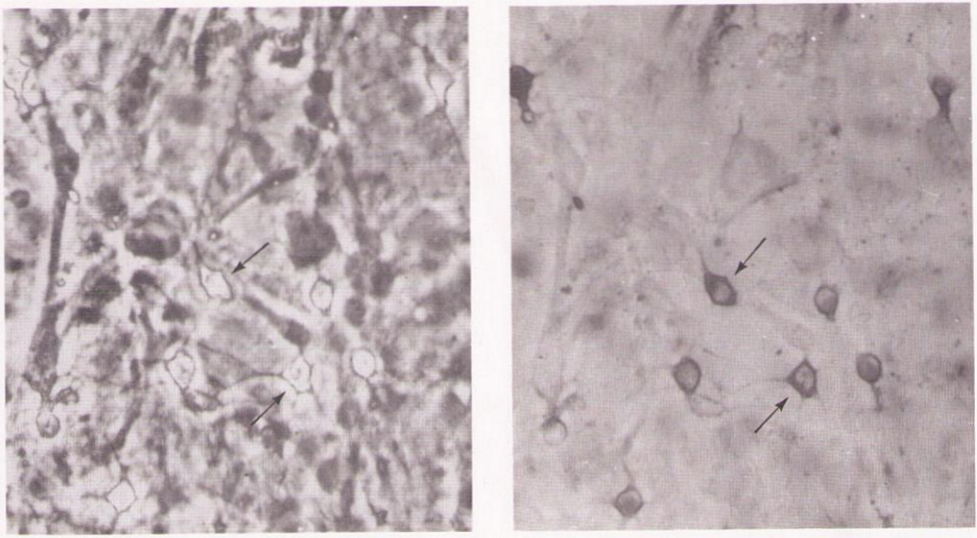


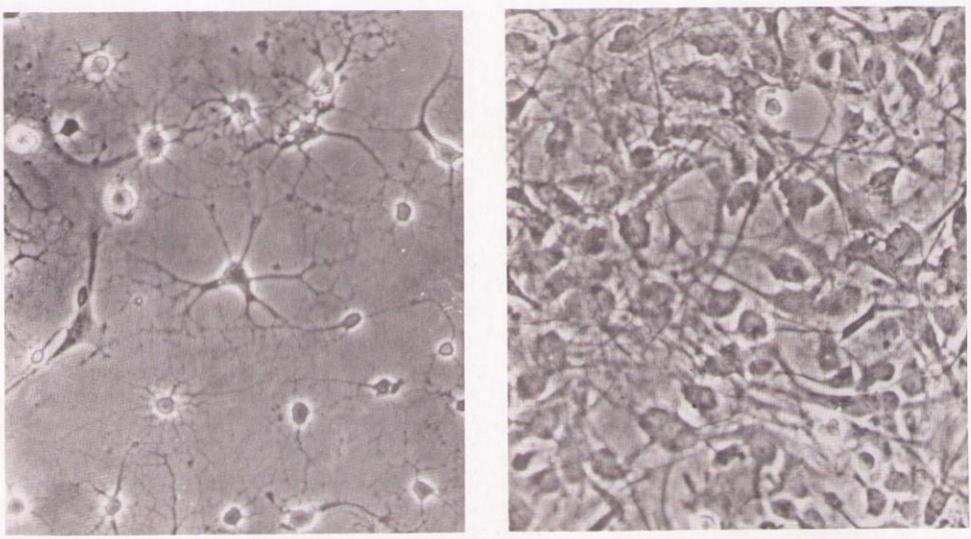
Fig. 7. Immunostaining of a thin section of rat cerebellum with antipeptide 3A antiserum and the PAP technique. Myelinated axons are stained brownish (arrow). Magnification 260x.



a) b)

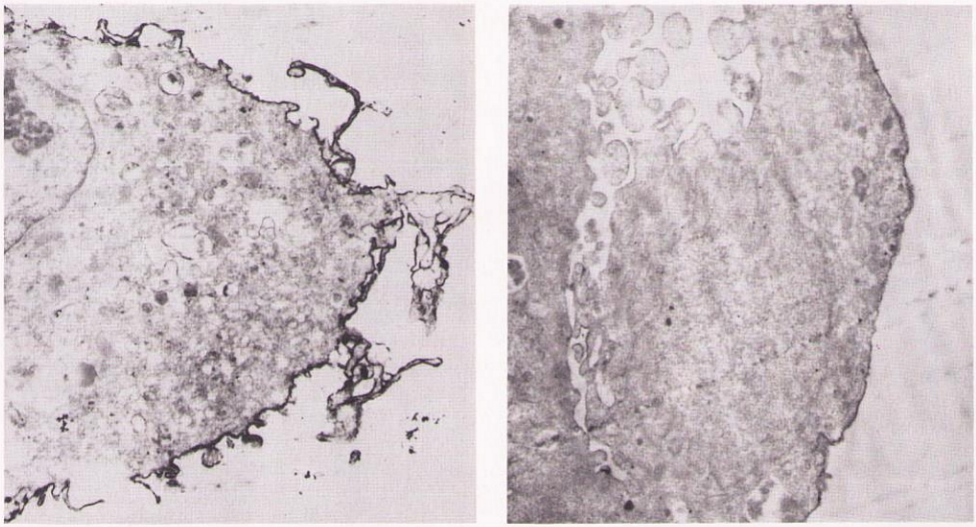
Fig. 8. Mixed glial cell culture pretreated with formaldehyde, stained with antipeptide 3A/PAP.
a) Phase contrast; b) transmission microscopy. Magnification 540x.

labelling technique have been unsuccessful so far. Therefore we chose primary oligodendrocytes cultured through the period of myelinogenesis starting around day 10 with its peak around days 20 to 25 after birth. Lipophilin was probed in the oligodendrocytes by the sensitive and specific PAP method[31]. Starting at day 10 after birth oligodendrocyte cultures were probed for lipophilin expression immunocytochemically with the antipeptide IgGs.



a) b)

Fig. 9. a) Rat brain oligodendrocytes and b) astrocytes in culture, 16 days after isolation from new-born rat brain. Magnificatin 320x.



a) b)

Fig. 10. Electron microscopy of a rat brain oligodendrocyte in culture (day 20).

In situ flat embedding in petridishes; a) immunostaining with antipeptide **3A** antibody combined with PAP/DAB technique and postfixation with OsO_4. Magnification 9600x. b) Nonimmune IgG, same preparation as above. 9600x. The OsO_4 precipitate is visible only on the surface of the oligodendrocyte probed with the antipeptide **3A** antibodies.

Light microscopic analysis of mixed glial cell cultures

Living oligodendrocytes showed no reaction on the plasma membrane with any of the antibodies.

Fixation of oligodendrocytes with 4% formaldehyde in phosphate-buffered saline, a procedure which does not permeabilize the plasma membrane[39], made the domain of peptide **3A** accessible for antipeptide **3A** IgG.

Pretreatment of the oligodendrocytes with 4% formaldehyde and 0.1% Triton X-100 uncovers the epitope of lipophilin corresponding to the peptides **3** and **4** sequence (Fig. 1) which was detected by its respective antibody.

Antilipophilin antibodies and antipeptide antibodies **2** and **6** reacted with lipophilin of the oligodendrocyte plasma membrane only after ethanol/acetic acid fixation. Fig. 8 shows the PAP staining with antipeptide **3A** antibodies of a mixed astrocyte/oligodendrocyte primary culture.

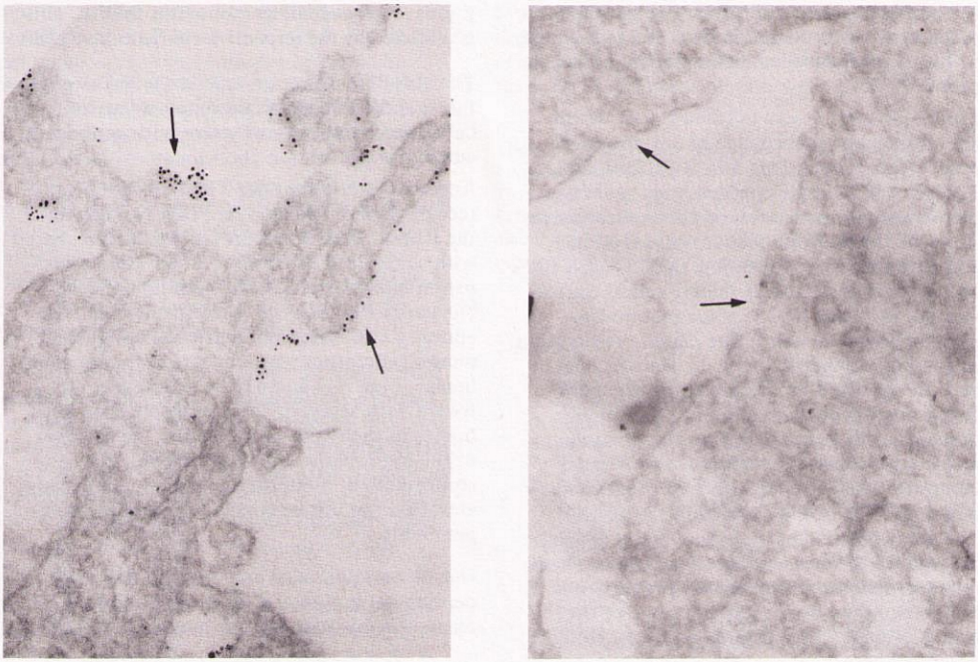
Immunoelectron microscopic labelling of lipophilin in isolated oligodendrocytes

Oligodendrocyte progenitor cells (O2A)[37,38] differentiated as an upper layer on top of large, flat type-I astrocytes in a medium supplemented with 10% calf serum and could easily be separated and seeded for further studies (Fig. 9).

Oligodendrocytes 20 days in culture were probed for the extracytosolic antigenic domains of lipophilin with antipeptide **3A** antibodies and monitored with the PAP/DAB and immunogold technique. After postfixation with OsO_4 which reacts with the osmiophilic diaminobenzidine (DAB) cells were prepared for in situ flat embedding in Petri dishes[32]. The electron microscopic appearance of a non-permeabilized oligodendrocyte in which the lipophilin domain corresponding to peptide **3A** has been immunostained is shown in Fig. 10a, the control treated with nonimmune serum and OsO_4 in Fig. 10b and Fig. 11a and b.

The oligodendrocyte-specific morphology was apparent: the heterochromatin-containing nucleus with its excentric position, the numerous large Golgi cisternae, fibrillar structures characteristic for astrocytes were missing.

The electron dense OsO_4 and the 5 nm gold second antibody against antipeptide **3** antibody precipitated only on the surface of the plasma membrane of the oligodendrocyte pretreated with antipeptide **3A** IgG and no other structure was labelled within the cell.



a) b)

Fig. 11. Electron microscopy of a rat brain oligodendrocyte in culture (day 20); immunostaining with rabbit anti peptide 3A antiserum and goldcoupled goat anti rabbit IgG. Magnification 82 000x.

Discussion

Myelin lipophilin (proteolipid protein) is the major protein component (> 50%) of CNS myelin and is present as integral membrane protein. The insolubility of PLP in aqueous solutions prohibited the structural elucidation of this important protein but yielded only very fragmentary sequence data over many years[16,17,40–43].

Despite its extreme hydrophobicity the amino-acid sequence of bovine and human lipophilin with 276 residues has been elucidated and its almost complete homology reported[13,14].

The hydrophilic N-terminus of lipophilin is followed by clusters of strongly hydrophobic residues with 27 amino acids corresponding to 7.5 α-helical turns, 42 Å long, 29 residues (8 turns, 45 Å), 40 residues (11 turns, 62 Å) and 30 residues (8.3 turns, 47 Å). They are linked by hydrophilic sequences.

Our sequencing data were confirmed by rat[44,46], mouse[47], bovine[48] and human[36] cDNA and genomic DNA data.

Our sequence data also gave clues to studies on the molecular arrangement and orientation in the lipid bilayer of the myelin membrane with the aim to understand PLP functions during myelination and its function in compacting the multilayer system. Furthermore the orientation or exposure of the potential immunogenic epitopes of PLP might be of importance for demyelinating processes of the multilayer system.

We therefore concentrated on studies of the orientation of PLP in the myelin membrane[15] which led to the proposal of our model shown in Fig. 1. It is based on experimental data, trypsinization of hyposmotically dissociated myelin and sequencing the resulting PLP fragments and theoretical considerations as well. Hyposmotic shock dissociates the multilayer system at the intraperiod dense line and exposes the extracellular surfaces. Proof for this lies in the full protection of myelin basic protein which as an external protein and is syntheiszed on free ribosomes in the cytosol. The location of MBP in the main dense line has also been derived from other immunocytochemical studies[8] and

the partial exposure of PLP on the membrane surface has been concluded from the accessibility of PLP to radiolabelled membrane-impermeable probes[49,50].

Lipophilin contains 14 cysteines, 9 of which are on the extracellular side in our proposed model (Cys 5, 6, 9, 108, 138, 140, 200, 219, 227), five in the hydrophobic *trans*- and *cis*-membrane domains, none on the cytosolic side. Three of the hydrophobic domains (I, II and III) have the size for spanning the 45–50 Å thick lipid bilayer if we accept their α-helical conformation. Domains II and IV are pictured as *cis*-membranal domains. The planar presentation of the PLP orientation (Fig. 1) is provisional in view of the disulfide bridges in the sequence. Regarding the conformation of hydrophilic domains of PLP protruding on the cytosolic and extracytoplasmic surface we performed computer-assisted calculations of the secondary structure[51,52] of the hydrophilic sequences. The overwhelming part of them reveal a high probability to form amphipathic helices. These might contribute to the surface properties of the myelin sheaths and explain some of the results obtained by our immunological studies described here.

We synthesized eight peptides by solid-phase peptide synthesis with sequences of the six hydrophilic domains of PLP indicated in Fig. 1 as black squares. KLH-peptide conjugates were used as antigens. The affinity-purified antipeptide antibodies isolated from a rabbit antiserum were used as probes for the molecular orientation or accessibility of their respective immunogenic epitopes. They were compared with PLP antibodies which were also raised in rabbits.

It is noteworthy that one rabbit out of two immunized by intramuscular injection and two out of two by direct application of PLP into the popliteal lymph nodes developed severe neurological symptoms with paralysis of their hind legs. Light microscopy of different areas of the CNS showed severe perivascular lymphocyte infiltration, a picture identical with that of MBP-induced experimental allergic encephalitis (EAE) (not shown).

The KLH-conjugated peptides investigated here for their antigenic properties, however, did not cause symptoms of EAE.

The anti-PLP antiserum (titer $> 1:10\,000$) crosireacted in the ELISA only with peptide **6** (titer 1:1 024) and peptide **2** (titer 1:64) coated as free peptide or conjugated. These two domains on the cytosolic surface in our model must therefore be regarded as the two essential contínous immunogenic determinants in PLP. This is validated by the respective competitive ELISA.

The anti-PLP antiserum reaction with the peptide **2** and **6** antigens is inhibited by preincubation of the PLP antiserum with peptides **2** and **6** (Fig. 5).

Antisera against peptides **3** and **3A** specifically recognize their epitopes on PLP as antigen in the ELISA. Anti **3** and **3A** antisera preincubated with solubilized PLP however show an unimpaired binding to its surface-bound antigen. Obviously PLP does not present the **3** and **3A** epitopes in solution but when coated on solid phases (microtiter plates or nitrocellulose membranes, Figs. 3, 4 and 6) it is extensively unfolded with access of the aforementioned antibodies to their epitopes. Whether the epitopes of the hydrophilic loop 3 are cryptic due to conformation, dimerisiation or even further aggregation can not be concluded from these experiments.

Our observations also demonstrate that antipeptide antibodies of PLP can be used for diagnostic purposes only with great restriction.

The orientation of the hydrophilic domains 2 and 3 of PLP has been deduced from biochemical experiments. The immunochemical approach with specific antibodies against synthetic peptides mimicking surface located epitopes as probes described here gave further support of the model proposed before[15].

The application of anti-PLP and antipeptide antibodies in pre- and postembedding immunostaining of isolated myelin for electron microscopy was unsuccessful so far. It is reasonable to assume that PLP targeting the plasma membrane of the oligodendrocyte is integrated in the lipid bilayer in its final orientation before it segregates with the myelin-specific lipid into the sheath-like plasma membrane extensions. Therefore the topochemical analysis of the oligodendrocyte in vitro with our antibody probes bypasses the difficulties encountered with the compacted membrane system of isolated myelin.

Living rat oligodendrocytes in culture neither reacted with anti-PLP nor with any antipeptide antibody. Only after a brief exposure to formaldehyde known not to permeabilize the plasma membrane for immunoglobulins but only for molecules < 80 kDa[39] the lipophilin in the plasma membrane strongly reacted with antipeptide **3A** IgG but not with any other antipeptide immunoglobulin (Fig. 8).

Peptide 2 and 6 antibodies shown to react strongly with PLP in solution and in the ELISA had no access to their epitopes in the oligodendrocyte plasma membrane. The combined action of formaldehyde and Triton X-100 exposes in addition epitopes 3 and 4 of the hydrophilic domain to its antibody but no other epitopes become accessible. If epitopes 2 and 6 were exposed on the extracytosolic surface they would not have been missed by this technique. Their antibodies and anti-PLP antibodies lead only after complete permeabilisation and denaturation with ethanol/acetic acid to the positive immunostaining of the complete cell.

The surface location of epitope 3A was further assessed by labelling of formaldehyde exposed oligodendrocytes with antipeptide 3A IgG combined with the PAP/DAB and immunogold technique. Postfixation with OsO_4 led to electron dense deposits on the surface of a PLP – positive oligodendrocyte which were completely absent after staining with IgG from nonimmune serum (Fig. 10).

We can only speculate why the N-terminus and loops 4 and 5 of PLP in the nonpermeabilized oligodendrocyte plasma membrane are not reactive with their respective antibodies and why domain 3A is accessible for specific antibodies only upon exposure to formaldehyde. Extensive parts of the hydrophilic domains form with a high probability amphipathic helices. We favor the idea that their hydrophilic sides with surplus cationic charges may interact with the polar head groups of the lipid bilayer rich in anionic polar head groups and thereby forming the hydrophobic side to the surface. Such yet unproven orientation might explain the high tendency of hydrophobic aggregation of PLP and also the cryptic orientation of antigenic sequences in these domains.

These epitopes become accessible for their antibodies when PLP is unfolded by hydrophobic interactions with hydrophobic surfaces (microtiter plates in ELISA or nitrocellulose in Western blot).

In summary the immunological probes specifying epitopes of potential and putative antigenic hydrophilic loops of lipophilin were applied in studies on the molecular orientation of this main integral membrane protein of myelin in the plasma membrane of oligodendrocytes in vitro. The results strongly support the proposed model for lipophilin integration in the myelin lipid bilayer shown in Fig. 1. They are at variance with a model proposed by others[54], and for which no experimental data are available.

It was reported as a short note that oligodendrocytes could be stained by immunofluorescence with a peptide sequence of domain 3, but again no experimental details were given[55].

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