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Monoclonal Antibodies to Distinct Regions of Human Myelin Proteolipid Protein Simultaneously Recognize Central Nervous System Myelin and Neurons of Many Vertebrate Species

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Myelin proteolipid protein (PLP), the major protein of mammalian CNS myelin, is a member of the proteolipid gene family (pgf). It is an evolutionarily conserved polytopic integral membrane protein and a potential autoantigen in multiple sclerosis (MS). To analyze antibody recognition of PLP epitopes *in situ*, monoclonal antibodies (mAbs) specific for different regions of human PLP (50–69, 100–123, 139–151, 178–191, 200–219, 264–276) were generated and used to immunostain CNS tissues of representative vertebrates. mAbs to each region recognized whole human PLP on Western blots; the anti-100–123 mAb did not recognize DM-20, the PLP isoform that lacks residues 116–150. All of the mAbs stained fixed, permeabilized oligodendrocytes and mammalian and avian CNS tissue myelin. Most of the mAbs also stained amphibian, teleost, and elasmobranch CNS myelin despite greater diversity of their pgf myelin protein sequences. Myelin staining was observed when there was at least 40% identity of the mAb epitope and known pgf myelin proteins of the same or related species. The pgf myelin proteins of teleosts and elasmobranchs lack 116–150; the anti-100–123 mAb did not stain their myelin. In addition to myelin, the anti-178–191 mAb stained many neurons in all species; other mAbs stained distinct neuron subpopulations in different species. Neuronal staining was observed when there was at least approximately 30% identity of the PLP mAb epitope and known pgf neuronal proteins of the same or related species. Thus, anti-human PLP epitope mAbs simultaneously recognize CNS myelin and neurons even without extensive sequence identity. Widespread anti-PLP mAb recognition of neurons suggests a novel potential patho-

physiologic mechanism in MS patients, *i.e.*, that anti-PLP antibodies associated with demyelination might simultaneously recognize pgf epitopes in neurons, thereby affecting their functions. © 2006 Wiley-Liss, Inc.

Key words: demyelination; evolution; M6 proteins; multiple sclerosis; rhombex

Proteolipid protein (PLP), the major protein of mammalian CNS myelin, is an intrinsic membrane protein of oligodendrocytes with proposed roles in the formation and maintenance of myelin, intracellular transport, interactions with axons, and integrin signaling (Griffiths et al., 1998; Campagnoni and Skoff, 2001; Yool et al., 2001; Gudz et al., 2002). It is highly conserved among mammals; the sequences of murine and human PLP are identical. This conservation suggests that

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both the primary structure of PLP and its conformation *in situ* are critical for its functions. DM-20, the smaller isoform of PLP, is a minor component of mammalian CNS myelin. The DM protein members of the PLP/DM-20 gene family (pgf; also referred to as “lipophilins”; Gow, 1997) are found in CNS myelin in bony fish and sharks, but they exhibit greater sequence diversity, suggesting their greater plasticity (Kitagawa et al., 1993; Geltner et al., 1998). Members of the pgf are also expressed in nonmyelinating cells, including neurons, in developing and mature vertebrate CNS tissues (Kitagawa et al., 1993; Yan et al., 1993, 1996; Roussel et al., 1998; Werner et al., 2001; Jacobs et al., 2003, 2004). To our knowledge, however, evolutionary conservation of pgf protein epitopes *in situ* has not been investigated.

PLP is a CNS autoantigen in demyelinating diseases (Greer et al., 1996a). We previously demonstrated that both naturally occurring and synthetic amino acid substitutions in PLP epitopes can dramatically alter T-cell responses and the induction of experimental autoimmune encephalomyelitis (Nicholson et al., 1995; Greer et al., 1997). Thus, small alterations of PLP epitopes can have profound effects on CNS autoimmunity. T-cell responses to PLP epitopes in patients with multiple sclerosis (MS) have been associated with clinical relapses (Pelfrey et al., 2000; Pender et al., 2000), and MS patients may have anti-PLP antibodies in their sera and cerebrospinal fluid (CSF). However, there currently is little information on anti-PLP antibody recognition of different PLP epitopes in CNS tissue or the potential pathophysiological consequences of this recognition.

To understand PLP epitope binding, a panel of antibodies recognizing its distinct intra- and extracellular regions is needed (Greer and Lees, 2002). In the present study, PLP knockout (PLP^{-/-}) and wild-type mice were immunized with PLP and PLP peptides, and a large panel of monoclonal antibodies (mAbs) with reactivities to different PLP regions was generated. Selected mAbs were characterized for their reactivities to 1) whole PLP and PLP peptides by ELISA, 2) PLP and DM-20 on Western blots, and 3) live and fixed oligodendrocytes and normal human CNS tissue by immunohistochemistry. The results demonstrated the epitope specificity of the mAbs and their applicability to immunohistochemical studies of paraffin-embedded tissue samples.

To analyze mAb recognition of PLP epitopes *in situ* and assess their evolutionary conservation, archival CNS tissue samples from representative vertebrates were immunostained with the mAbs. In addition to staining CNS myelin, most of the mAbs also stained multiple neuron populations in each species. To understand the basis for this unexpected result, the occurrence of myelin and neuronal staining was then correlated with the degree of identity of the anti-PLP mAb epitope sequences and sequences of known major pgf myelin and neuronal proteins in the same or most closely related species. Myelin staining was frequently observed when there was as little as 40% sequence identity of the PLP mAb epitope and a known pgf myelin protein; neuronal staining

was frequently observed when there was as little as 30% sequence identity with a known pgf neuronal protein. These results suggest that anti-PLP mAbs may recognize evolutionarily conserved epitopes present not only in CNS myelin but also in mature neurons. Insofar as an mAb to pgf M6 neuronal proteins inhibits neuronal development and function *in vitro*, the results raise the possibility that, in MS patients with anti-PLP antibodies associated with demyelination, similar recognition of neuronal pgf protein epitopes might contribute to dysfunction, degeneration, or impaired regeneration of neurons *in vivo*. Thus, the cross-reactivity of PLP mAbs suggests a new potential pathogenetic mechanism in MS.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 4–8 weeks old, were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). PLP^{-/-} mice were obtained from Klaus-Armin Nave (Max-Planck Institute of Experimental Medicine, Göttingen, Germany) (Klugmann et al., 1997) and were bred onto the SJL background. (SJL × B10.BR)F₁ mice were bred at The Children's Hospital of Philadelphia Laboratory Animal Facility. All animals were acquired and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committees of Harvard Medical School, E.K. Shriver Center, and The Children's Hospital of Philadelphia.

PLP and PLP Peptides

PLP was isolated from frozen samples of human post-mortem brain tissue and bovine brain, as previously described (Greer et al., 1996a, 1997). All PLP preparations contain both PLP and DM-20. All peptides were synthesized by using F-moc chemistry and were greater than 90% pure by HPLC analysis. Peptides either were synthesized in the laboratory of Dr. Richard Laursen, Department of Chemistry, Boston University, or were a generous gift of Dr. David Hafler, Department of Neurology, Harvard Medical School (Marcovic-Plese et al., 1995).

Generation of PLP Antibodies

One hundred micrograms of human PLP were suspended in Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY) and emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Mice were immunized by injection of the emulsion at three subcutaneous sites. Fourteen days after the initial immunization, the mice were given a booster immunization *i.p.* with 100 µg human PLP suspended in PBS and emulsified with an equal volume of incomplete Freund's adjuvant. A second booster was given after another 14 days. Ten days later, a small amount of blood was collected by retroorbital bleeding, and the serum activity against PLP was titered by ELISA. Mice were selected for cell fusion when the titer exceeded 1:24,300. Rat anti-PLP 264–276 mAb AA3 (IgG_{2b}) or AB3 (IgG_{2a}; Yamamura et al., 1991) were used as positive controls.

The selected mice were rested for 4 weeks after the last immunization and then boosted by intravenous injection with

either 50 μg PLP or synthetic PLP peptide in PBS. Four days later, the mice were sacrificed, and spleen cell suspensions were prepared and washed with PBS. Spleen cells were counted and mixed with SP 2/0 myeloma cells (ATCC No. CRL8-006, Rockville, MD) that are incapable of secreting either heavy or light immunoglobulin chains (Kearney et al., 1979) at a spleen:myeloma cell ratio of 2:1. Cells were fused with polyethylene glycol 1450 (ATCC) in 12 96-well tissue culture plates in HAT selection medium according to standard procedures (Kohler and Milstein, 1975).

Screening and Expansion of Hybridomas

Between 10 and 21 days after fusion, hybridoma colonies became visible, and culture supernatants were harvested and screened by ELISA. High-protein-binding 96-well EIA plates (Costar, Cambridge, MA) were coated with 50 μl /well of a 5 $\mu\text{g}/\text{ml}$ solution (0.1 $\mu\text{g}/\text{well}$) of PLP or synthetic PLP peptides and incubated overnight at 4°C. The excess solution was aspirated, and the plates were washed with PBS (three times) and blocked with 1% bovine serum albumin (BSA) solution for 1 hr at room temperature (RT) to inhibit nonspecific binding. The BSA solution was removed, and 50 μl /well of hybridoma supernatant from each fusion plate were added. The plates were then incubated for 45 min at 37°C and washed three times with PBS. Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (Zymed, South San Francisco, CA) diluted 1:4,000 in 1% BSA/PBS was added to each well, and the plates were incubated for 45 min at 37°C. After washing, 50 μl /well of ABTS (Zymed) were added. The intensity of the green color of positive wells at 405 nm was assessed on a Vmax microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). All hybridoma wells that gave a positive response were then expanded to 24-well cultures, subcloned by limiting dilution, and analyzed by ELISA. The three best producing subclones were expanded further. Supernatants were isotyped with an Isostrip kit (Boehringer Mannheim Corp., Indianapolis, IN).

Antibody Purification

The antibodies were purified using Millipore Prosep Protein A resin. They were diafiltered into PBS and concentrated with an Amicon Ultra 4 (30-kDa cutoff) device according to the manufacturer's protocol.

Epitope Identification by ELISA

The antibodies selected from the fusions were screened by ELISA to determine the epitope or region of PLP that they recognized. Each antibody supernatant was screened against a panel of overlapping PLP peptides (1–19, 10–29, 20–39, 30–49, 50–69, 60–79, 80–99, 100–119, 110–123, 110–129, 120–139, 130–149, 139–151, 140–159, 150–169, 170–189, 178–191, 180–199, 190–209, 200–219, 215–232, 220–239, 240–259, 250–269, 260–276, and 269–276), human and bovine PLP, and bovine MBP. The peptides were dissolved in 0.1 M bicarbonate buffer, pH 9.0, and bound overnight to EIA plates with 50 μl /well of a 5 $\mu\text{g}/\text{ml}$ solution (0.25 $\mu\text{g}/\text{well}$) and then processed as described above.

Immunoblots

Fifty micrograms of human PLP were mixed with sample buffer containing 0.7% dithiothreitol, 1 mM EDTA, 10 mM Tris-HCl, 5% sodium dodecyl sulfate (SDS), 13% glycerol, and 0.007% bromophenol blue and electrophoresed on a 12% SDS-PAGE under reducing conditions. The proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and treated with blocking buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween, 5% dry milk solids) for 1 hr at RT. The membranes were probed overnight with the purified PLP-specific Ab at a concentration of 200 ng/ml in buffer containing 5% BSA and 0.025% sodium azide. After washing, the membranes were incubated with HRP-labeled goat anti-mouse Ab (0.4 mg/ml; Jackson ImmunoResearch, West Grove, PA) in the above-mentioned buffer for 1 hr. The membranes were then washed and autoradiographed by using an ECL Western blotting detection kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations.

Immunofluorescent Staining of Mouse Oligodendrocytes

Oligodendrocyte-enriched shake-off cultures were prepared from 2-day-old (SJL \times B10)F₁ newborn mice by using a modified version of the method of McCarthy and de Vellis (1980) as described by Dyer et al. (1995). Live cultures were treated with undiluted hybridoma supernatant, followed by addition of appropriate fluorescein isothiocyanate (FITC; Boehringer Mannheim Corp.) or TRITC (Jackson ImmunoResearch) goat anti-mouse IgG or IgM secondary antibody diluted 1:40. Each step was carried out for 15 min at 37°C in 5% CO₂. To detect internal epitopes, the cells were fixed in 4% paraformaldehyde for 5 min and permeabilized with 0.05% saponin for 15 min prior to staining with the supernatant or primary mAb. The O4 hybridoma was grown and purified as described elsewhere (Dyer et al., 1995). SMI-99 mouse mAb reactive with myelin basic protein (MBP) was purchased from Sternberger Monoclonals (Baltimore, MD). Images were captured with a 35-mm camera system on a Leitz DMR fluorescent microscope with Tmax ASA 400 film.

Tissue Samples

Archival samples of routinely fixed, paraffin-embedded CNS tissue from our previous studies and additional samples of vertebrate CNS tissues were obtained from other investigators. Six-micrometer-thick serial sections from each paraffin block were stained with Luxol fast blue-H&E stain and with the mAbs by immunohistochemistry. A human CNS tissue block contained at least three levels of normal spinal cord from an autopsy of a 24-year-old male. The nonhuman samples consisted of a cow brainstem (N = 3 blocks), two rabbit brains (N = 2 blocks), three Lewis rat brains (N = 3 blocks), the brains and spinal cords of SJL/J and C57/Bl6 mice (N = 2 blocks each), the brains and spinal cords of three *Rana pipiens* (N = 3 blocks), two *Xenopus* brains (N = 2 blocks), and one block each from the brains of a barn owl, a Malawi cichlid, and a leopard shark. All vertebrate species had Luxol fast blue-positive myelin.

TABLE I. pgf Proteins

Vertebrate group	Myelin pgf proteins	Accession number	Abbreviation ^a	Neuronal pgf proteins	Accession number ^b	Abbreviation ^a
Mammals	Bovine PLP	P04116	bPLP	Human M6a	P51674	hM6a
	Rabbit PLP	P47789	rbPLP	Human M6b	Q13491	hM6b
	Rat PLP	P60203	rtPLP	Rat M6a	Q812E9	rtM6a
	Mouse PLP	P60202	mPLP	Rhombex-29	Q9JJK1	Rh-29
				Mouse M6a	P35802	mM6a
				Mouse M6b	P35803	mM6b
Aves	Zebra finch PLP	P47790	ZF PLP	Chicken GPM6a	Q5EES3	Ch M6a
	Chicken PLP	P23289	Ch PLP	Chicken GPM6b	Q5EES2	Ch M6b
Amphibians	<i>Xenopus</i> PLP1	P35801	Xe PLP1	<i>Xenopus</i> DMβ	Q98ST4	Xe DMβ
	<i>Xenopus</i> PLP2	P23290	Xe PLP2			
	<i>Xenopus</i> DMγ1	Q98ST3	Xe DMγ1			
	<i>Xenopus</i> DMγ2/GPM6b	Q98ST2	Xe DMγ2/M6b ^c			
Teleosts	Rainbow trout DM20	P79826	RTr DM20	Zebrafish M6a	Q8UUT4	Zfsh M6a
	Zebrafish DMα1	Q8UUT6	Zfsh DMα1	Zebrafish Gpm6ab	Q6D117	Zfsh M6b
	Zebrafish DMα2a	Q8UUT5	Zfsh DMα2a	Zebrafish DMβ2a	Q8UUS9	ZFsh DM
	Zebrafish DMα2b	Q8UUT3	Zfsh DMα2b	Zebrafish DMβ2b	Q8UUT1	ZFsh DMβ2b
	Zebrafish DMα2c	Q8UUT2	Zfsh DMα2c			
	Zebrafish DMγ1	Q8UUT0	Zfsh DMγ1			
	Zebrafish DMγ2	Q8UUS8	Zfsh DMγ2			
Elasmobranchs	Squalus DMα ^c	P36963	Sq DMα			
	Squalus DMβ ^c	P36964	Sq DMβ			
	Squalus DMγ ^c	P36965	Sq DMγ			

^aThe sequences of the pgf proteins listed were compared to with the human PLP sequences. The abbreviations are used in Supplemental Tables A–C.

^bUniProtKB/Swiss-prot or/TrEMBL entry.

^cThe *Xenopus* DMγ2/GPM6b protein sequence and the squalus DM protein sequences were used for analyses of both myelin and neuronal staining.

Tissue Immunohistochemistry

Six anti-PLP mAbs generated in the present study (two to 50–69 and one each to 100–123, 178–191, 200–219, and 264–276) were used for the immunohistochemical analyses. This panel was supplemented with the previously described mouse mAbs to 139–151 (1D5 and 1C5, both IgG₁; Greer et al., 1996b) and a rat mAb to 264–276 (AA3, IgG_{2b}; Yamamura et al., 1991). Reactivities of the nine purified mAb supernatants in the tissue sections were titrated for optimal staining using immunoperoxidase staining as described elsewhere (Sobel, 2005). Controls included substitution of mAb supernatants with PBS.

Sequence Comparisons

Percentages of sequence identities to the human PLP mAb epitopes were determined from BLAST searches (<http://au.expasy.org/tools/blast/>) and estimated by aligning sequences of the pgf myelin or neuronal proteins in the same or most closely related species for which the sequences are known (Table I). The pgf proteins listed are at least 30% identical to one or more of the PLP mAb epitopes.

RESULTS

Selection of Antibodies

Approximately 8,000 hybridomas were generated from fusions of spleens from six PLP^{-/-} mice and one BALB/c mouse that had been immunized with human PLP and boosted with synthetic PLP peptides. After ini-

tial screening against human PLP, 25 reactive hybridomas were identified, subcloned, and isotyped. In additional testing, two of these showed consistent reactivity to the region encompassing PLP 50–69, one to PLP 100–123, two to PLP 178–191, five to PLP 200–219, and one to PLP 264–276. Eleven hybridomas initially showed reactivity to PLP 139–151, but this reactivity was not consistent through subcloning; all but one of those hybridomas were determined to be of the IgM subclass. Three hybridomas showed reactivity to whole PLP but did not show consistent reactivity to any of the peptides tested. Thus, their epitope specificities could not be determined.

Epitope Assignments

Table II shows the reactivities to PLP and representative PLP peptides of selected hybridomas. All of these mouse hybridomas recognize human and bovine PLP and not MBP. Each hybridoma shown specifically recognizes a different region of human PLP. Based on these and additional ELISA screening using overlapping peptides, assignments for reactivity of the mAbs used for further studies were made as follows: F4.4C2 and F3.9E9 (PLP 50–69), F4.2D2 (PLP 100–123), P7.6A5 (PLP178–191), F4.8A5 (PLP 200–219), and P5.12A8 (PLP 264–276). Hybridoma P5.12A8 is an IgM; F3.9E9 is an IgG_{2aK}; all of the other hybridomas are IgG_{1K}. All but one of these selected hybridomas were from PLP^{-/-} mice; the anti-PLP 178–191 hybridoma was from the

TABLE II. Epitope Mapping of Representative PLP Antibodies by ELISA

Hybridoma	Epitope assignment	MBP	Human PLP	Bovine PLP	Absorbance (OD/405 nm) ^a					
					PLP peptide tested					
					50–69	120–139	139–151	178–191	200–219	260–276
F4.4C2	50–69	0.078	<i>0.699</i>	<i>1.070</i>	<i>0.433</i>	0.057	0.059	0.107	0.071	0.074
F4.2D2	100–123 ^b	0.060	<i>0.584</i>	<i>1.149</i>	0.047	<i>0.409</i>	0.043	0.050	0.048	0.058
P7.6A5	178–191	0.052	<i>0.487</i>	n.d. ^c	0.094	0.075	0.076	<i>0.656</i>	0.089	0.076
F4.8A5	200–219	0.056	<i>0.379</i>	<i>0.921</i>	0.045	0.043	0.046	0.047	<i>0.653</i>	0.053
P5.12A8	264–276	0.074	<i>0.693</i>	<i>0.250</i>	0.100	0.084	0.090	0.140	0.151	<i>0.268</i>

^aItalicized values indicate positive results.

^bAssignment based on reactivity with PLP 100–119, 110–123, 110–129, and 120–139 but not with peptide 130–149 or 139–151.

^cn.d., Not done.

BALB/c mouse. In additional testing by ELISA of one of the previously reported anti-139–151 mAbs (1C5), the specific reactivity of this mAb to a synthetic peptide encompassing the 139–151 region and lack of reactivity to a peptide encompassing the 100–123 region confirmed its specificity.

Western Blots

A representative Western blot demonstrates that all five of the hybridomas tested recognize a band corresponding to PLP (Fig. 1). All but one mAb also recognize a slightly lower band corresponding to DM-20. Because this mAb recognizes PLP 100–123, it does not recognize DM-20, and there is a single band on the blot. None of the mAbs recognized MBP (not shown).

Immunofluorescent Staining of Cultured Oligodendrocytes

Only the one anti-50–69 mAb (F4.4C2) of the five mAbs tested detected a PLP epitope on the surface of cultured oligodendrocytes. A prominent membrane staining pattern is evident in Figure 2A,B. Figure 2C shows the same cells shown as in Figure 2B stained with the oligodendrocyte marker antibody O4, confirming that the cells are oligodendrocytes. However, the distributions of PLP and antibody O4 are distinct: O4 is uniformly distributed, whereas PLP occurs in a punctate pattern throughout the sheet and is also concentrated along the edges (compare areas indicated by arrows in Fig. 2B,C).

The mAbs recognizing PLP peptides 100–123, 178–191, 200–219, and 264–276 did not stain the surface of live oligodendrocytes. Representative results for the mAb to PLP 264–276 are shown in Figure 2D. However, each of the mAbs to these regions stained fixed and permeabilized oligodendrocytes. Figure 2E shows a representative fixed oligodendrocyte stained for PLP 264–276 with this mAb. PLP 264–276 has been shown to be on the cytoplasmic portion of the membrane (Konola et al., 1992), and staining for this epitope shows a distribution in the cell body, along cytoskeletal veins, and in the membrane sheet (highlighted by arrows). Figure 2F shows the same cell stained for MBP.

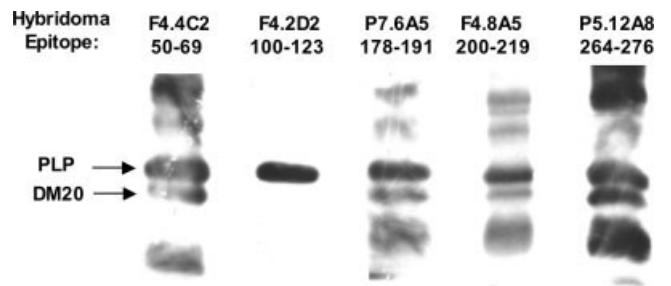


Fig. 1. Representative immunoblot of whole human PLP with selected mAbs. With one exception, i.e., the mAb to 100–123, all of the mAbs tested recognize two bands corresponding to PLP and DM-20. The 100–123 epitope is part of the region absent in DM-20 (residues 116–150). Therefore, the mAb to this region stains only PLP and not DM-20. The higher molecular weight bands represent aggregates of multimeric complexes and the lower bands are presumed degradation products. All but the mAb to 200–219 were run in the same gel. The data are representative of six gels performed for different human PLP samples.

The staining pattern for MBP differs from that of PLP 264–276 in that it appears more diffuse (compare areas indicated by arrows in Fig. 2E,F). In summary, only an anti-50–69 mAb immunostained live oligodendrocytes, confirming that this region is on the extracellular face of the membrane, whereas all of the other mAbs tested stained fixed, permeabilized oligodendrocytes.

Immunostaining of Archival Paraffin Sections

For each of the purified mAbs, optimal staining was observed at dilutions of 1:100–1:500; diffuse and nonspecific patterns were observed when they were more concentrated. The three pairs of mAbs to the same regions (50–69, 139–151, and 264–276) gave essentially identical staining results. Staining controls were negative.

Mammalian and Avian CNS Myelin

In the normal human spinal cord, all anti-PLP mAbs specifically stained CNS myelin, but not PNS myelin or axons in adjacent spinal nerve roots (Fig. 3A–F). In gray matter areas, e.g., the posterior horns, they stained individual myelinated fibers but not the back-

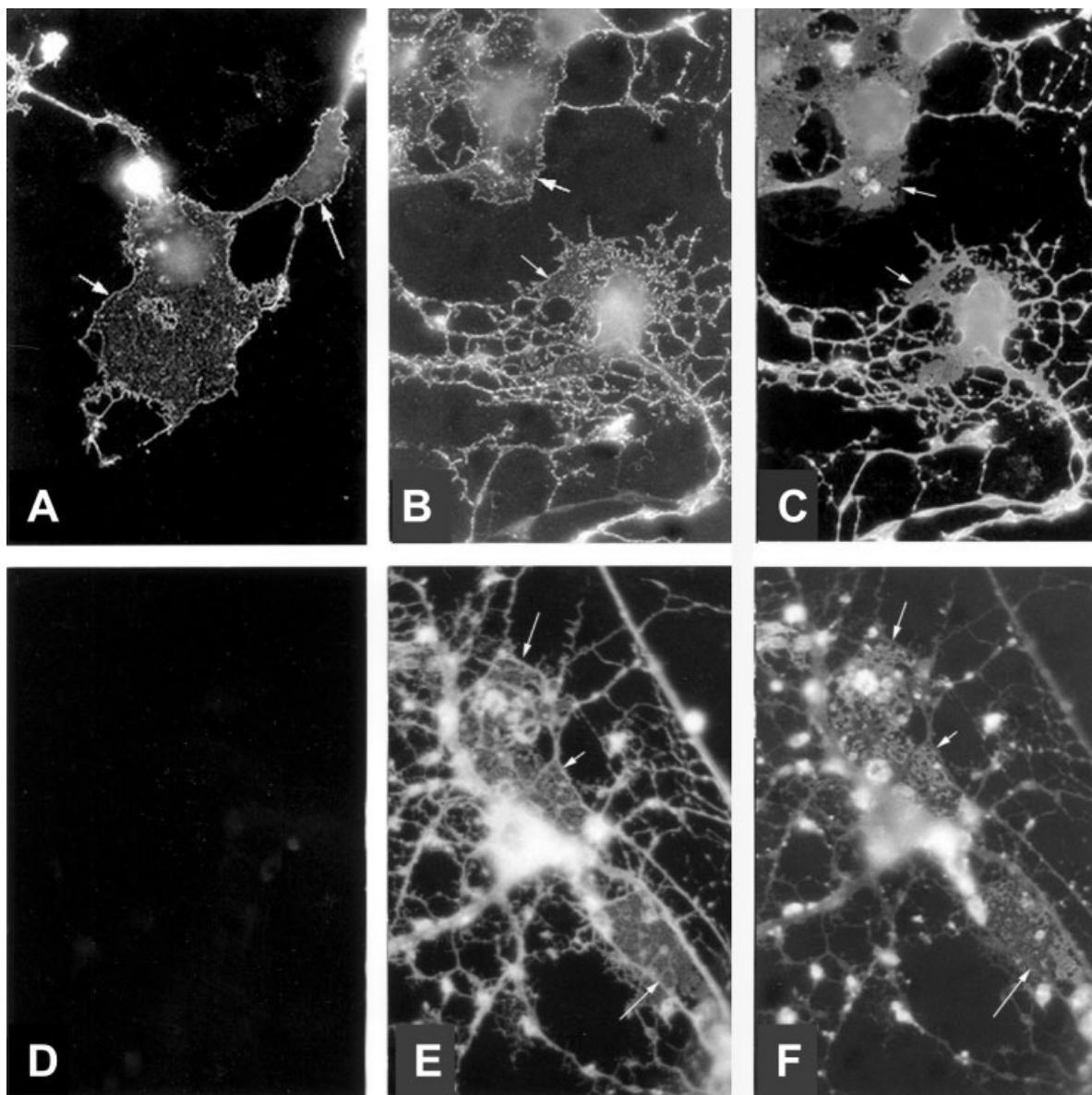


Fig. 2. Surface and internal staining of PLP epitopes in oligodendrocytes. **A,B:** Staining of live cultured oligodendrocytes with an anti-50–69 mAb. Arrows indicate intensely stained perimeter of the membrane. **C:** Same cell as in B stained for O4 showing diffuse staining. Arrows in B and C indicate the same locations on the cell surface, demonstrating the difference between PLP and O4 staining. **D:**

Staining of live oligodendrocyte cultures with anti-264–276 mAb; note lack of stain. **E:** Fixed and permeabilized oligodendrocyte stained with the anti-264–276 mAb shows diffuse PLP staining. **F:** Same cell as in E stained for MBP. Arrows in E and F point to cytoskeletal veins that are stained for the PLP epitope in E but not MBP in F. A–C: $\times 250$; D: $\times 150$; E,F: $\times 350$.

ground neuropil. They did not stain neurons in this sample. There was similar specific staining of myelin bundles and individual fibers in all samples of cow, rabbit, rat, and mouse CNS tissues, consistent with the 95–100% identity with the human sequence of the PLP in these mammals (Greer and Lees, 1992). Figure 4 illustrates this staining pattern in rabbit basal ganglion. All mAbs also stained compact myelin in the barn owl, although the PLP sequence is not known for the barn owl, the PLP sequences of other avian species (chicken

and zebra finch) are also highly conserved in most of the regions recognized by the mAbs (Campagnoni et al., 1994; Supplemental Tables A–C). Occasional staining of oligodendrocytes in gray matter, particularly with the mAbs to the C-terminus region, was also observed in the mammals and barn owl (not shown).

Amphibian CNS Myelin

All of the mAbs stained compact myelin in the *Rana pipiens* brain and spinal cord; the staining intensity

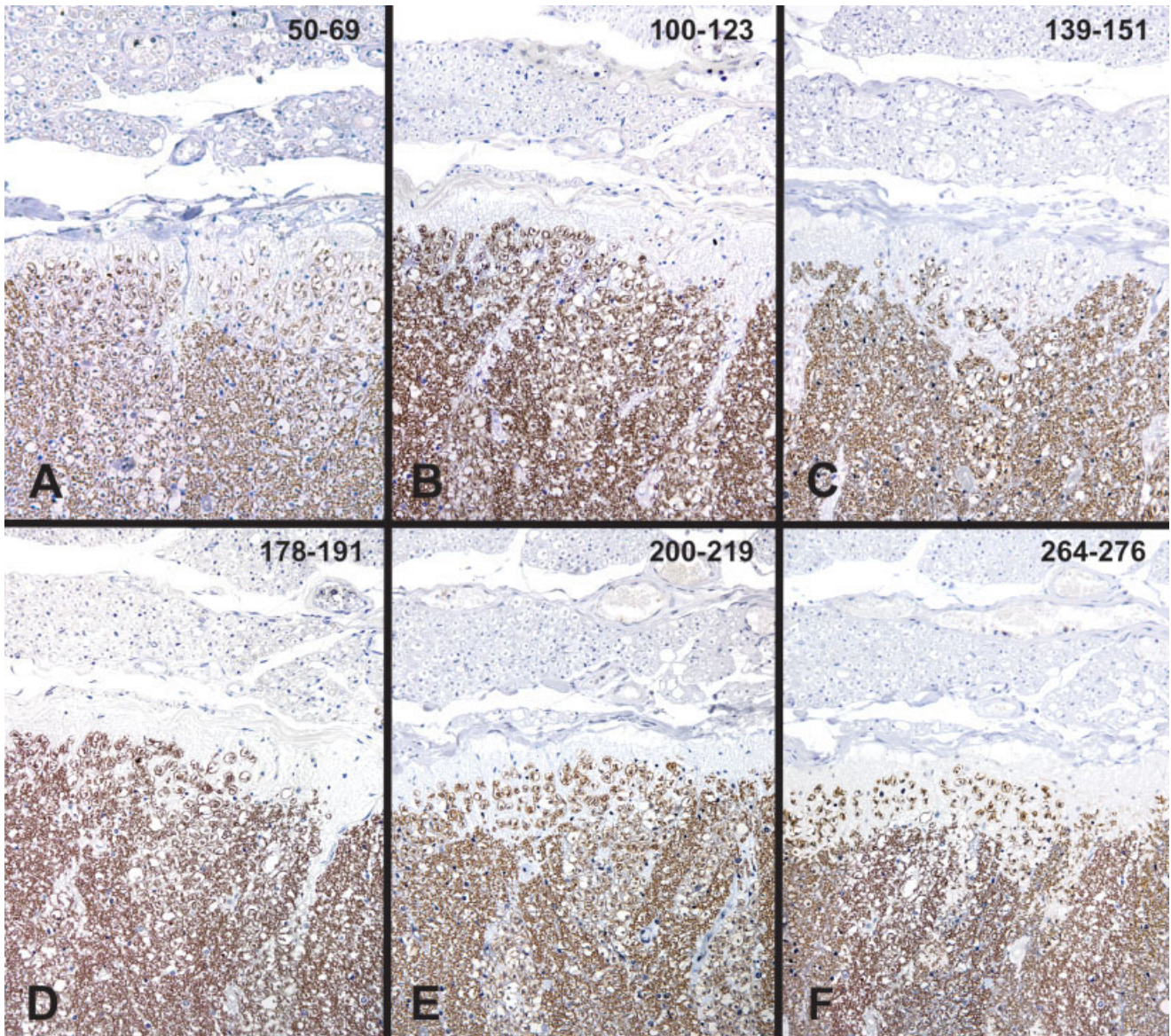


Fig. 3. Immunohistochemistry of a normal human spinal cord sample. Anterior spinal cord with spinal nerve roots (upper portion) and white matter (lower portion) shows specific staining of CNS myelin (brown) with the indicated mAb in each panel. PNS myelin, which lacks PLP, and nerve root axons (blue) are not stained. All are counterstained with hematoxylin. $\times 80$.

was greatest in myelinated fiber tracts of the cord (Figs. 5A–C, 9C). This pattern is consistent with the distribution of PLP in *Xenopus* (Yoshida et al., 1999). To our knowledge, pgf proteins in *Rana pipiens* have not been identified or sequenced. Therefore, staining correlations were made with *Xenopus* protein sequences (Supplemental Tables A–C). The *Xenopus* CNS samples studied were limited to the brain, and only staining of single myelinated fibers could be identified in those sections with all mAbs, except for mAb to 100–123 (Fig. 5D–F). The *Xenopus* PLP sequences are at least 80% identical to the human sequence in all regions analyzed,

except 100–123 and 262–276. *Xenopus* pgf myelin proteins have approximately 50–60% identity with human PLP in the 100–123 and 264–276 regions. In the frog samples, therefore, the minimum sequence identity that resulted in recognition of compact CNS myelin by the anti-PLP mAbs was approximately 60%.

Teleost CNS Myelin

The mAbs to 50–69, 200–219, and 264–276 immunostained CNS myelin in the Malawi cichlid brain (Fig. 6A,F,G). In these regions, there are considerable

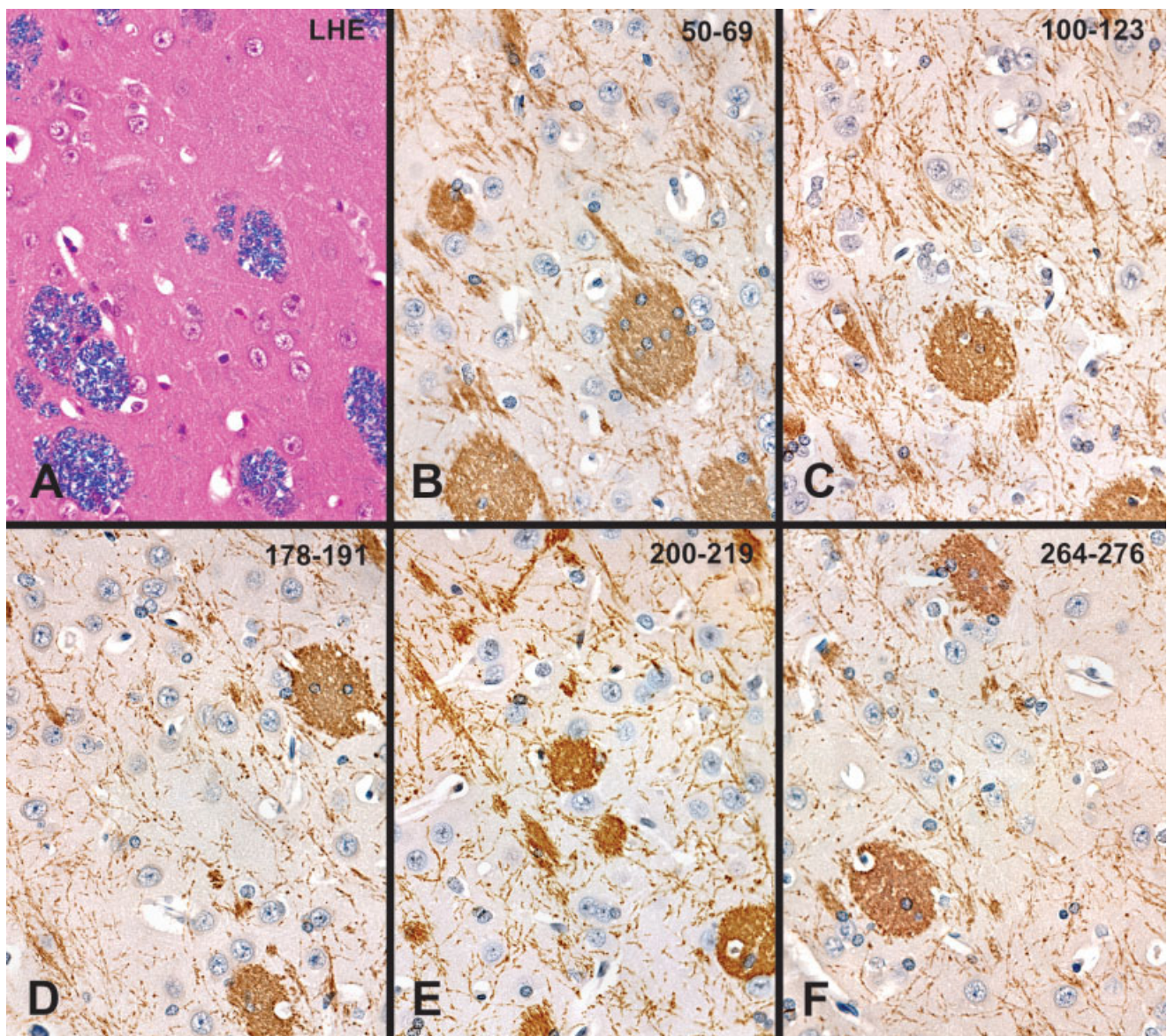


Fig. 4. Myelin staining in rabbit corpus striatum demonstrated by Luxol fast blue-hematoxylin and eosin (A) and by immunostaining for the indicated PLP epitopes (B–F). Dense fiber bundles and individual myelinated fibers but not the background neuropil or neurons are stained. This pattern is representative of the staining observed in all mammals and in the barn owl. $\times 160$.

similarities (approximately 50–75% identity) between the human PLP sequence and sequences of rainbow trout and zebrafish DM proteins (Supplemental Tables A, C). The anti-100–123 mAb did not stain the cichlid myelin (Fig. 6B), consistent with the expected absence of residues 116–150 in the major pgf myelin proteins in teleosts (Supplemental Table A). By contrast, both mAbs to the 139–151 region stained portions of the brain myelin in a distinct pattern, i.e., predominantly on fibers in peripheral portions of deep gray matter tract bundles, leaving larger central portions of the bundles unstained (Fig. 6C,D). The zebrafish DM γ 2 and the rainbow trout

DM-20 proteins have approximately 30–45% identity with 139–151 (Supplemental Table B), but the implications and significance of this distinct partial staining pattern are presently unclear. The anti-178–191 mAb did not stain the cichlid myelin (Fig. 6E), despite the approximately 38% identity of rainbow trout DM-20 and the 54% identity of zebrafish DM γ 2 with the human PLP 178–191 sequence. The species of Malawi cichlid tested and its DM sequences are not known, and pgf myelin protein sequences are more diverse in teleosts than in tetrapods (Geltner et al., 1998). In summary, the mAbs to highly conserved regions, i.e., those in which

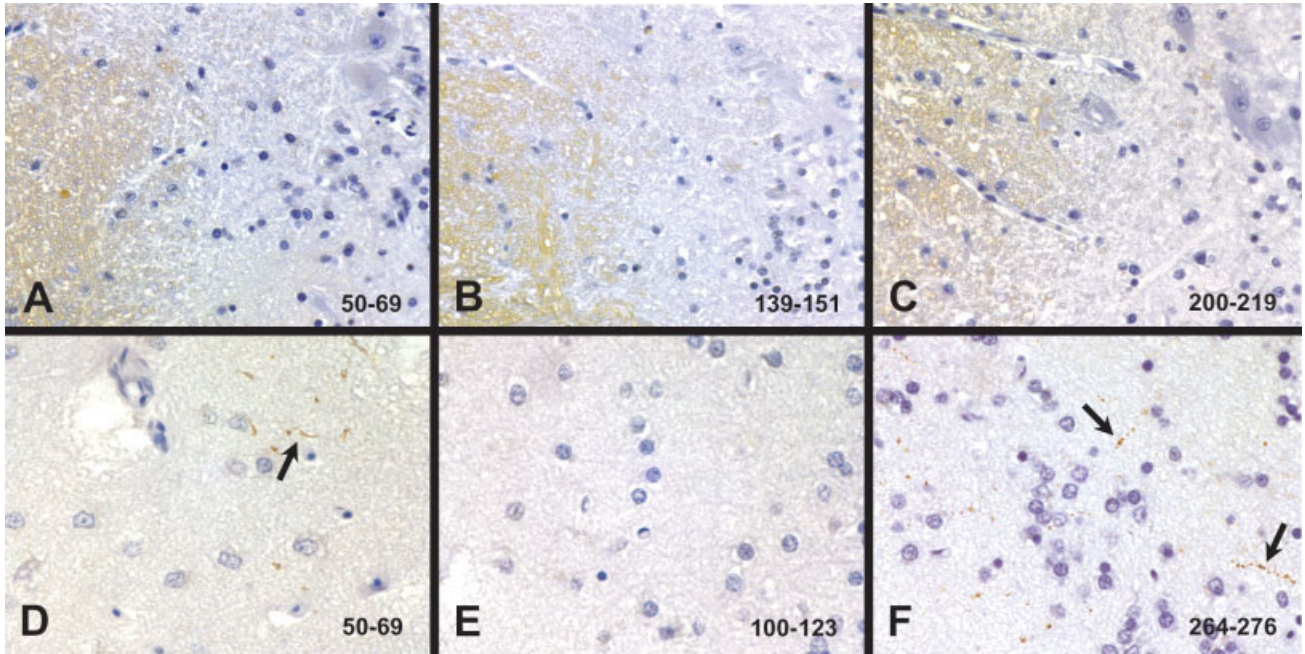


Fig. 5. mAb staining in *Rana pipiens* and *Xenopus*. Myelinated fiber tracts are stained with mAbs to the indicated epitopes in *Rana pipiens* spinal cord (A–C). Large neurons in these fields (upper right corners) are not stained. In *Xenopus* brain sections stained with mAbs to the indicated epitopes (D–F), individual fibers are stained in beaded patterns for 50–69 and 264–276 (arrows in D,F). There is no staining for 100–123 in E. A–C: $\times 160$; D–F: $\times 240$.

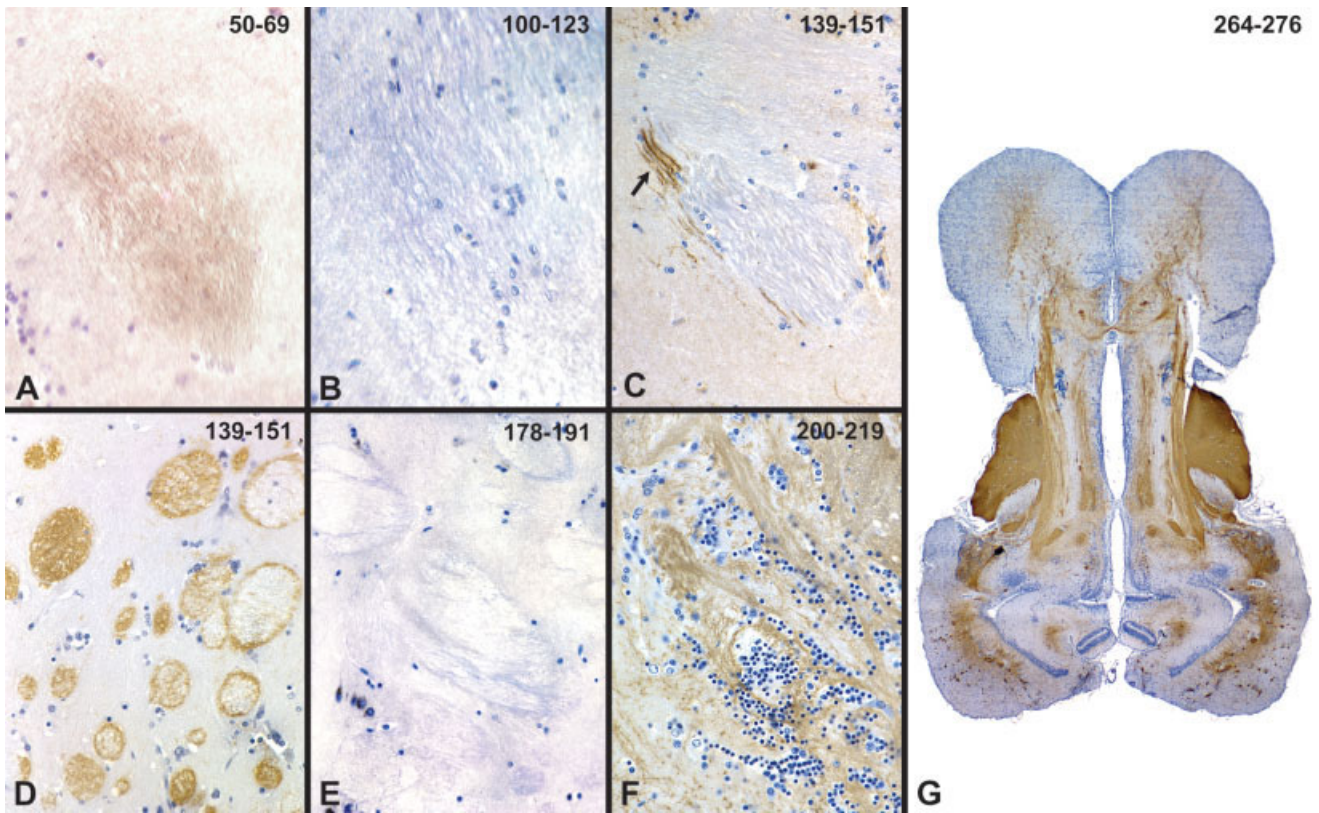


Fig. 6. mAb staining in Malawi cichlid brain. Compact myelin is stained with mAbs to 50–69 (A), 200–219 (F), and 264–276 (G, whole-brain section). No myelin is stained with mAbs to 100–123 (B) or 178–191 (E). Partial staining with two different anti-139–151 mAbs of peripheral portions of myelinated fiber bundles (arrow in C) with central portions of the bundles remaining unstained is shown in C and D. A–F: $\times 160$; G: $\times 4$.

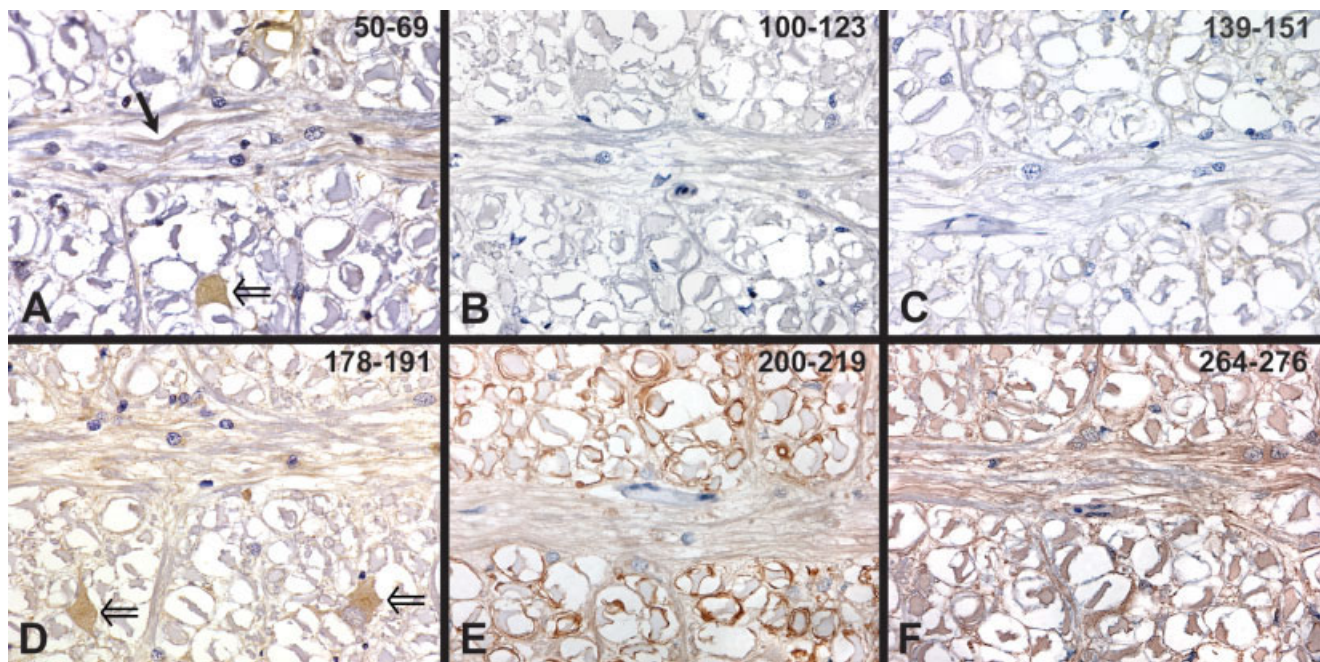


Fig. 7. mAb staining in leopard shark brainstem. Compact myelin is stained with mAbs to 50–69 (solid arrow in **A**), 178–191 (**D**), 200–219 (**E**), and 264–276 (**F**). There is no staining with the mAb to 100–123 (**B**), and the staining is minimal with an mAb to 139–151 (**C**). Additional neuron cell bodies are stained in **A** and **D** (open arrows). $\times 160$.

there is approximately 50% or greater identity of zebra-fish and rainbow trout pgf proteins and PLP epitopes recognized at least some compact CNS myelin in the cichlid brain, whereas the mAbs to less well conserved regions were recognized to a lesser extent.

Elasmobranch CNS Myelin

The mAbs to the 50–69, 200–219, and 264–276 regions also immunostained compact myelin in the leopard shark brain (Fig. 7A,E,F). The sequences of squalus DM α and DM γ are greater than 60% identical to the human PLP sequences in these regions (Supplemental Tables A, C). Myelin staining with the anti-139–151 (Fig. 7C) and -178–191 (Fig. 7D) mAbs was positive although less uniform than the staining of the other mAbs; the squalus DM γ sequences are approximately 46% and 61% identical to the human PLP 139–151 and 178–191 sequences, respectively (Supplemental Table B). The mAb to 100–123 did not stain the compact myelin (Fig. 7B), consistent with the absence of the PLP 116–150 sequence in elasmobranchs (Supplemental Table B).

CNS Myelin Staining Correlations

The left column in Table III summarizes the myelin staining and correlates positive staining results in each species tested with the percentage identities of the PLP mAb epitope and the most similar known pgf myelin

TABLE III. Summary of Myelin and Neuron Staining Correlations*

<i>Identity of mAb epitope and most similar pgf myelin or neuronal protein (%)</i>	<i>Myelin staining (No. positive/No. in group)</i>	<i>Neuronal staining (No. positive/No. in group)</i>
0–29	0/1	0/4
30–39	0/1	10/18
40–49	3/4	9/16
50–59	4/5	4/5
≥ 60	43/43	3/11
Total positive/total sequence comparisons	50/54	26/54

*Correlations of myelin and neuronal staining by the mAbs to six PLP regions in nine vertebrate species (54 correlations for each) with the extent of sequence identity of the epitopes with pgf proteins. The data are grouped according to the percentage identity of the mAb epitope and the most similar sequence in the known pgf myelin or neuronal proteins in each or the most closely related species (Table I). See Supplemental Tables A–C for specific correlations.

and neuronal proteins. Because there is a high degree of conservation of pgf myelin proteins, all 43 samples in which a pgf myelin protein is at least 60% identical to the human PLP epitope showed myelin staining with the mAbs. Staining was also very often observed when there was 40–60% identity of the mAb epitope and the corresponding sequence in the pgf myelin proteins of the species tested or the most closely related species. Staining was not observed when there was less than 40%

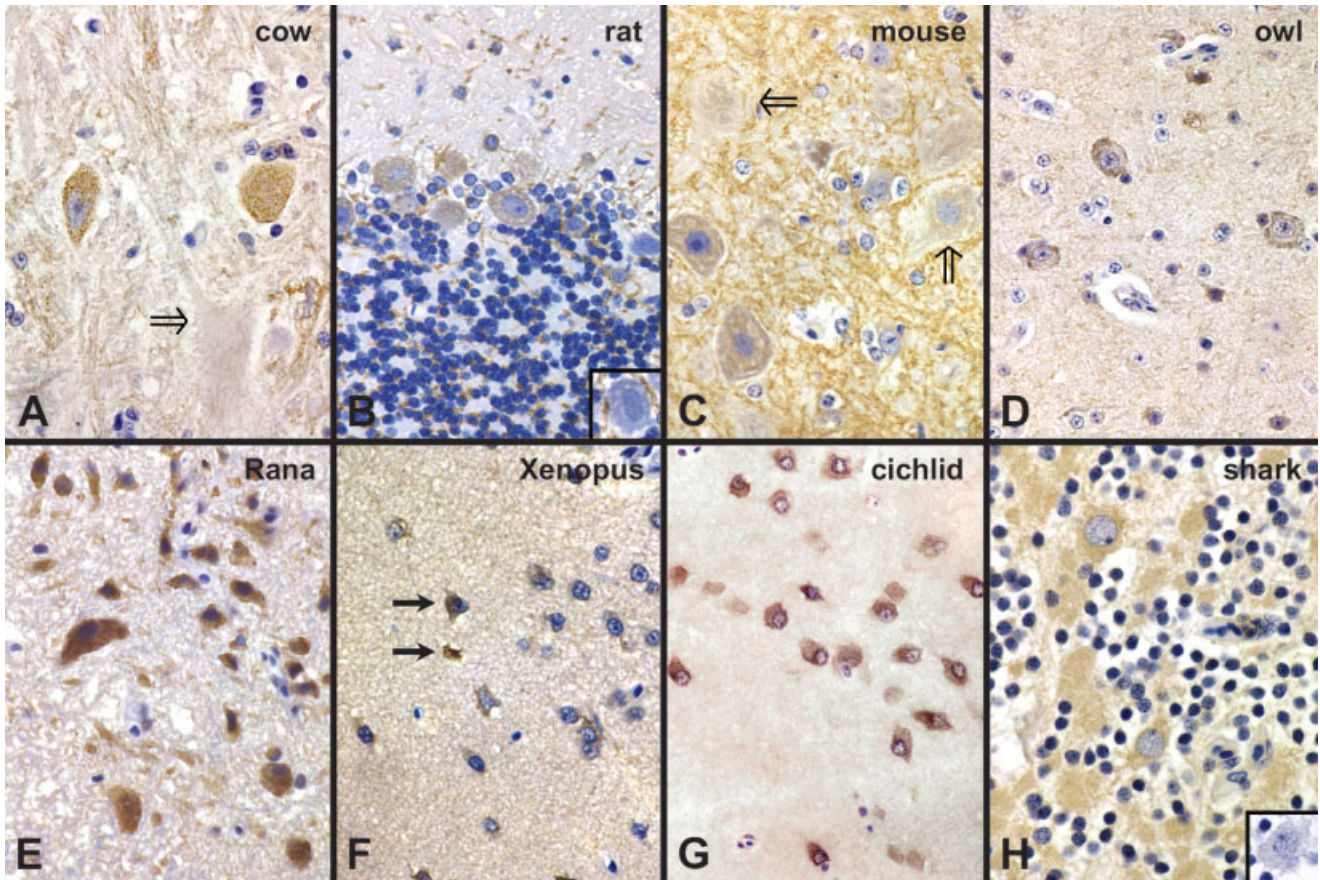


Fig. 8. Neuron subpopulations stained with the anti-178–191 mAb. **A:** Cow brainstem with adjacent cell in field (open arrow) unstained. **B:** Rat Purkinje cells are stained; granular layer neurons are not stained. **Inset:** Unstained Purkinje cell from adjacent section stained for 50–69 indicates epitope specificity. There is staining of individual myelinated fibers in both. **C:** Mouse ventral horn cells with adjacent cells in field (open arrows) showing little or no staining. **D:** Many large neurons in the barn owl forebrain show peripheral soma stain-

ing. **E:** Large neurons in the *Rana pipiens* spinal cord are diffusely stained. **F:** Neurons in *Xenopus* brain (arrows) are stained. Unstained cells are seen in the same field. **G:** All neurons in this field from the cichlid brain are stained. **H:** Large Golgi-like neurons in the leopard shark cerebellar granular layer are stained, and there is diffuse staining of neuropil fibers. **Inset:** Unstained large Golgi-like neuron with an anti-139–151 mAb indicates epitope specificity of the anti-178–191 mAb staining. $\times 240$.

sequence identity or when the epitope was absent in the known pgf proteins.

CNS Neuron Staining by the Anti-178–191 mAb in All Vertebrate Species

In addition to CNS myelin, the anti-178–191 mAb unexpectedly immunostained neuron subpopulations in all species tested. This positive staining included but was not limited to rare large neurons in the bovine brainstem (Fig. 8A) temporal cortical neuron subpopulations in the rabbit, rat, and mouse; Purkinje cells in the rat (Fig. 8B); ventral horn cell subpopulations in mouse spinal cord (Fig. 8C); many large gray matter neurons in the barn owl (Fig. 8D); large neurons in the spinal cord and brains of the frogs (Fig. 8E,F); and many neurons and neuropil in the cichlid and shark brains (Fig. 7D, 8G,H). The specificity of this staining

was indicated by identification of unstained similar-appearing neurons in the same microscopic fields (e.g., Fig. 8A–C,F), and the absence of staining of the same neuron subpopulations with the other mAbs in adjacent serial tissue sections (e.g., insets in Fig. 8B,H) and in negatively staining controls.

The staining patterns of individual neurons were variable in different regions, but individual subpopulations tended to have uniform staining patterns in the same anatomic areas across species. For example, in most neurons in the mammals and barn owl, the immunoperoxidase reaction product appeared to be localized on soma membranes, with less staining around nuclei (Fig. 8B–D). In other species and anatomic regions, neuron cell bodies were more often diffusely stained (Fig. 8A,E–H). Axonal staining could generally not be assessed because of intense myelin staining. However, nonmyelinated, i.e., Luxol fast blue-negative, neuropil also showed diffuse staining, e.g.,

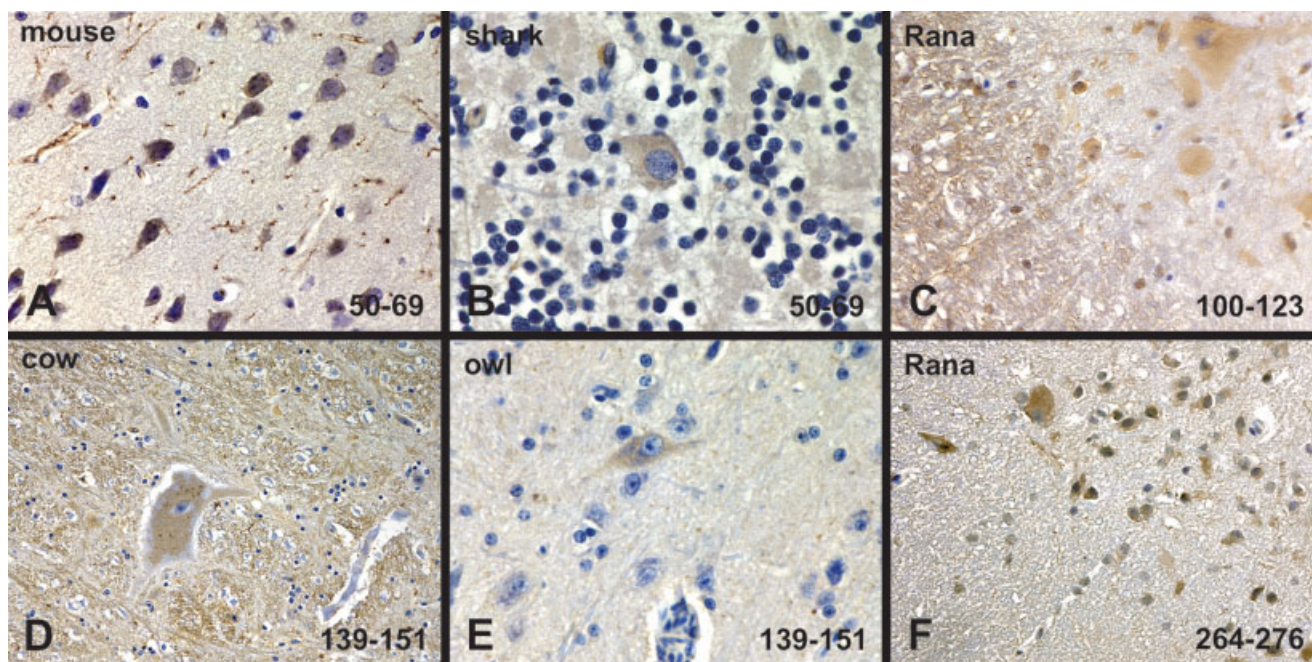


Fig. 9. CNS neuron staining with mAbs to epitopes other than 178–191. **A:** Mouse temporal cortex with many neurons stained for 50–69. **B:** A large Golgi-like neuron in the leopard shark cerebellar granular layer is stained with an anti-50–59 mAb. Absence of neuropil staining indicates specific neuronal soma staining and contrasts with Figure 8H. **C:** Neurons and myelin are stained for 100–123 in

Rana pipiens spinal cord (note contrast to Fig. 5B,C). **D:** Large neuron in the cow brainstem stains for 139–151. **E:** A single neuron stains for 139–151 in the barn owl ventral brainstem. **F:** Numerous neurons are stained for 264–276 in the *Rana pipiens* spinal cord. A,B,E: $\times 240$; C,F: $\times 160$; D: $\times 80$.

in the shark cerebellum (Fig. 8H), suggesting that the antigen recognized was present in some cell processes.

To understand the possible basis for neuronal recognition, the human PLP 178–191 sequence was compared with known sequences of major pgf neuronal proteins (Table I). In human, rat, mouse, chicken, and zebrafish M6a and M6b; zebrafish DM β 2; *Xenopus* DM β ; and squalus DM β and $-\gamma$ proteins, there are regions that are from approximately 30% to 60% identical to the human 178–191 sequence. Most of these sequence identities are in the 35–46% range (Supplemental Table B). Recognition of pgf neuronal proteins with this extent of sequence identity might, therefore, account for the neuronal recognition by the anti-178–191 mAb in the different species.

CNS Neuron Staining by Other mAbs

In addition to staining CNS myelin, the mAbs to regions 50–69, 100–123, 139–151, and 264–276 stained distinct neuron subpopulations in the different species tested. For each of the mAbs, there were variations in staining patterns of neuronal cell bodies and membranes similar to those observed with the anti-178–191 mAb.

The anti-50–69 mAbs stained a subset of mouse temporal cortical neurons (Fig. 9A), many neurons in *Rana pipiens* brain and spinal cord, scattered neurons in the cichlid brain, and scattered neurons in the shark

brain (see, e.g., Fig. 7A) and cerebellum (Fig. 9B). This PLP region has approximately 30–60% identity with sequences in mouse M6b, *Xenopus* DM γ 1 and DM γ 2/GPM6b, zebrafish M6a, and squalus DM proteins (Supplemental Table A).

The anti-100–123 mAb stained ventral horn cells in the *Rana pipiens* spinal cord (Fig. 9C) and faintly stained some neurons in the leopard shark brain but did not stain neurons in any other species. The 100–123 region has approximately 30–40% identity with *Xenopus* DM β and DM γ 2/M6b and squalus DM β (Supplemental Table A).

One of the anti-139–151 mAbs (1C5) immunostained a subset of neurons in the brainstems of the rabbit, rat, and cow (Fig. 9D); both of the anti-139–151 mAbs stained similar neurons in the barn owl ventral brainstem (Fig. 9E). The 139–151 region has a high degree of identity (46%) with human, mouse, and chicken M6b sequences and with the sequence of Rhombex-29, a pgf neuronal protein that has been identified in the rat ventral medulla and is involved in H⁺ channels and the regulation of sensitivity to acidosis (Shimokawa and Miura, 2000; Shimokawa et al., 2005; Supplemental Table B). Neurons of the other species were not stained with the anti-139–151 mAbs.

The mouse anti-264–276 mAb stained rare neurons in the bovine and barn owl brainstem and a subset of ventral horn cells in mouse spinal cord. The rat anti-

262–276 mAb stained these populations and additionally stained neurons in the *Rana pipiens* spinal cord (Fig. 9F) and *Xenopus* and shark brains. Staining of rat caudal brainstem neurons with this mAb has also previously been demonstrated (Miller et al., 2003). This PLP region has from approximately 33% to 61% identity with most of the known M6 and DM sequences and with the Rhombex-29 sequence (Supplemental Table C).

CNS Neuron Staining Correlations

The right column of Table III summarizes the results of neuronal staining and groups them according to the percentage identities of the PLP mAb epitope and the most similar known pgf neuron protein(s). For example, there was from 40% to 49% identity between a PLP mAb epitope and the most similar known pgf neuronal protein in 16 samples. An anti-PLP mAb stained neurons in nine of these. The mAbs to the six PLP regions recognized neurons in nearly half (26 of 54) of the samples tested. Neuron staining was observed frequently in samples in which there is greater than 30% sequence identity of the PLP mAb epitope and the corresponding sequence in the pgf neuron proteins of the species tested or the most closely related species. However, despite high degrees of sequence similarity between PLP sequences and corresponding neuronal proteins, cross-reactivity was not invariably observed. In particular, the PLP 200–219 sequence and most species M6 and DM proteins are 60–75% identical but the mAb to this region did not react with neurons of any species (Supplemental Table C). Thus, a minimal sequence identity of approximately 30% appears to be *necessary* but it may not be *sufficient* for neuronal epitope recognition by an anti-PLP Ab.

DISCUSSION

Generation of Anti-PLP mAbs

We have used a novel strategy to generate a panel of mAbs recognizing different regions of PLP and have demonstrated their usefulness in elucidating patterns of anti-PLP antibody recognition of myelin and neurons in vertebrate CNS tissues. Immunization of PLP^{-/-} mice circumvented immunologic tolerance arising from the identity of human and murine PLP and its expression in lymphoid tissues (Voskuhl, 1998; Klein et al., 2000; Anderson and Kuchroo, 2003). Indeed, most of the PLP-reactive hybridomas were obtained from fusions using PLP^{-/-} mice. Immunization of the mice with whole PLP initiated the immune response, and boosting them with synthetic peptides induced expansion of B-cell clones reactive with specific regions of the protein. Therefore, this strategy facilitated production of mAbs that recognize PLP regions to which mAbs have been difficult to obtain.

We and others have previously generated both polyclonal and mAbs to PLP. Potter and Lees (1988) immunized rabbits with a series of synthetic PLP peptides and obtained polyclonal sera reactive with specific regions of PLP (residues 48–59, 97–105, 183–193, 192–

200, and 264–276) on immunoblots. That study suggested a conformational dependence of antibody recognition of PLP. Gunn et al. (1990) identified the carboxy terminus of PLP as the immunodominant B-cell epitope and raised polyclonal rat antibodies to this region. Yamamura et al. (1991) immunized Lewis rats with PLP and generated mAbs specific for PLP 209–217 and PLP 264–276. Konola et al. (1992) showed that one of these mAbs to the C-terminus, mAb AB3, recognized the cytoplasmic face of myelin. These mAbs were useful for localizing PLP-expressing cells in the CNS. Greer et al. (1996b) immunized different strains of mice with synthetic PLP peptides known to be encephalitogenic in each strain and generated mAbs to four regions of PLP (residues 40–59, 139–151, 178–191, and 215–232). Immunofluorescent staining with these mAbs helped to verify the PLP orientation by identifying regions of PLP that are expressed on the outer or inner surface of the cell membrane. None of the mAbs generated in that study proved useful for immunoblotting, and several of them have been lost.

It is striking that, in previous attempts and in the present study, it has not been possible to obtain antibodies to the N-terminal region. A possible explanation may be that the primary structure of PLP precludes immune recognition of this portion of the molecule. This region contains six half-cystines within the first 36 amino acids, and these might shield reactions or limit the flexibility of the native protein (Oteiza et al., 1987; Potter and Lees, 1988).

mAb Characterization

The mAbs generated in the present study and analyzed in detail recognize human and bovine PLP by ELISA, and each recognizes a different region of PLP. Unlike the mAbs previously generated, all of these mAbs also recognize whole PLP on immunoblots, and all but one of them recognize DM-20. Since the anti-100–123 mAb F4.2D2 recognizes an epitope within the 116–150 region found in PLP but absent in DM-20, it distinguishes PLP from DM-20.

The mAb to PLP 50–69 tested recognized PLP on the surface of live, unfixed oligodendrocytes, thereby confirming the previous results of Greer et al. (1996b) showing that this region is on the external face of the membrane. However, the anti-178–191 mAb generated in that study also stained live oligodendrocytes, whereas the anti-178–191 mAb generated in the present study did not. One possible explanation is that those two anti-178–191 mAbs do not recognize the same conformation. Additionally, the 178–191 region of PLP is relatively hydrophobic and may be partially buried in the cell membrane, thus preventing the binding to the cell surface epitope necessary for live staining.

In archival paraffin sections of a normal human spinal cord, the anti-PLP mAbs generated both in the present study and in previous studies showed specific immunostaining of human CNS but not PNS myelin

(Fig. 3). These results are consistent not only with the immunoblot and *in vitro* oligodendrocyte staining data but also indicate that routine tissue processing does not preclude PLP epitope recognition by the mAbs. Consequently, a phylogenetic analysis of *in situ* PLP epitope recognition was feasible.

mAb Recognition of CNS Myelin

The mAbs stained compact CNS myelin in all mammalian species and the barn owl. Examples of this staining in the rabbit corpus striatum are shown in Figure 4. In the other species, highly conserved epitopes, *i.e.*, those with greater than 40% identity with PLP, were also recognized, whereas less well conserved epitopes and those known to be absent from CNS myelin were not (Figs. 5–7, Table III, Supplemental Tables A–C). In particular, the anti-100–123 mAb did not stain the cichlid or leopard shark myelin, which is consistent with the absence of the 116–150 region in the major myelin DM proteins of teleosts and elasmobranchs. These observations further confirm the specificity of the PLP epitope recognition *in situ* by the individual mAbs. Moreover, they indicate that, although most of the PLP sequences are highly conserved across species, as little as 40% sequence identity between an mAb epitope and the known pgf major myelin proteins in each species appeared to be sufficient for myelin staining. This suggests a high degree of evolutionary conservation of pgf myelin epitopes *in situ* despite considerable sequence diversity among pgf myelin proteins in nonmammalian species (Campagnoni et al., 1994; Geltner et al., 1998). It is possible, however, that there are additional pgf or other classes of proteins that have not yet been identified in the various species that have greater sequence identity with the human mAb epitopes. The present data provide information only on the apparent minimal degree of sequence identity of the human PLP epitopes and currently known proteins that may account for the immunostaining results. It should also be emphasized that sequence identities are only surrogate assessments of the similarities among the mAb epitopes. The actual conformation-dependent binding interactions *in situ* cannot be assessed by current techniques.

mAb Recognition of Neurons

In addition to staining CNS myelin, the mAb to PLP 178–191 (P7.6A5) and to a lesser extent the other mAbs stained various CNS neuron subpopulations in all species. Neuronal recognition generally correlated with sequence identity of the anti-PLP mAb epitopes with known neuronal pgf proteins, such as the M6a and -b and DM β proteins and Rhombex-29 (Supplemental Table B). We have also observed that several of these mAbs also immunostain embryonal rat hippocampal neurons grown *in vitro* (unpublished observations). These data imply that the staining might be due to recognition of conserved epitopes in pgf neuronal proteins. In view of the large number of pgf transcripts in oligodendro-

cytes and neurons identified to date (Bongarzone et al., 1999; Werner et al., 2001), however, multiple potential epitopes could be recognized. For example, the anti-139–151 (Fig. 9D,E) and anti-264–276 mAbs stained small numbers of neurons in the ventral brainstem of mammals and the barn owl. There is a relatively high degree of sequence identity with rat Rhombex-29 in these PLP regions, suggesting that the stained neurons might be a specific chemosensitive Rhombex-29-expressing population (Miller et al., 2003; Shimokawa et al., 2005). On the other hand, M6a proteins, particularly M6a, are abundant in mature neurons in the cerebellar granular layer (Yan et al., 1993, 1996; Roussel et al., 1998), and we did not observe widespread staining in that anatomic region with any of the mAbs, suggesting that they might not recognize M6a protein epitopes. Moreover, the staining patterns of the neurons were variable, further indicating that different epitopes in different subpopulations may be expressed and recognized by the mAbs. The present study was not designed as a detailed comparison of specific populations, and it was not possible to compare staining patterns in neurons systematically among the different species. Furthermore, the CNS tissue samples available from each species were limited to certain anatomic regions, *e.g.*, the bovine brainstem, and the extent of antibody recognition of neuron populations in each species may be greater than the present results indicate. For all of these reasons, therefore, although the neuron staining by the various mAbs is specific to distinct subpopulations and likely involves recognition of conserved pgf epitopes, at this time there is considerable uncertainty regarding the identity of the neuronal proteins recognized.

Shared antigens among nonmammalian and mammalian neural cell populations are increasingly being identified (Gould et al., 1995; Morris et al., 2004). These observations imply evolutionary acquisition of new protein functions (Aharoni et al., 2005), which appears to have been the evolutionary progression of the pgf proteins among marine and terrestrial vertebrates (Kitagawa et al., 1993). James et al. (2003) have suggested that conformational diversity, *i.e.*, one sequence adopting multiple structures and functions, as in the conserved sequences among pgf proteins, can increase the numbers of potential antibody targets, thereby enhancing the likelihood of developing cross-reactivity, which can result in autoimmunity (Cohn, 2005). Our present results may also be consistent with that proposition.

Implications for MS

Widespread immunohistochemical staining of neuronal subpopulations by anti-PLP epitope mAbs suggests possible pathogenetic roles for anti-PLP antibodies that are found in humans with demyelinating diseases. Anti-myelin component antibodies generated under pathological conditions might have either adverse or beneficial effects (van der Veen et al., 1986, 1989; Endoh et al., 1986; Sadler et al., 1991; Potter and Stephens, 1994;

Genain et al., 1995; Laman et al., 2001; Morris-Downes et al., 2002; Mitsunaga et al., 2002; Schwab, 2004). Moreover, potential pathogenetic roles for antimyelin antibodies, including anti-PLP antibodies, have been suggested in MS (Sun et al., 1991; Warren et al., 1994; Sellebjerg et al., 1995, 2000; Archelos et al., 2000; Carvalho et al., 2003; Berger et al., 2003; Qin et al., 2003; Kanter et al., 2005; Zhang et al., 2005a). Furthermore, recent studies implicate an autoantibody to nonmyelinating cell populations in the pathogenesis of the MS variant neuromyelitis optica (Lennon et al., 2005) and specific antibody localization on axons in MS plaques (Zhang et al., 2005b). Our present results imply that anti-PLP antibodies that may arise in association with epitope spreading following myelin damage could simultaneously cross-react with neurons *in vivo*. The possible functional consequences of cross-reactivity of antibodies to pgf epitopes are suggested by the demonstration that an anti-M6 mAb inhibits neurite extension of mouse brain cells (Lagenaur et al., 1992) and suppresses neuronal differentiation of M6-transfected PC12 cells (Mukobata et al., 2002). We have also found that some of the anti-PLP mAbs generated in the present study inhibit neurite outgrowth *in vitro* (unpublished observations). Neuronal recognition by cross-reactive anti-PLP epitope antibodies might, therefore, similarly contribute to neuronal injury, altered function, or the failure of axon regeneration in MS patients. Thus, our results suggest a self-molecular-mimicry mechanism (Oldstone, 1998) that might contribute to the axonal injury and loss that are now considered to be major determinants of clinical expression and disease progression in MS (Trapp et al., 1999; Sobel, 2005). Whether there are autoantibody responses to PLP epitopes that simultaneously recognize other pgf proteins and have functional effects on neurons *in vivo* remains to be established.

In summary, we report the generation and characterization of novel anti-PLP epitope mAbs and have proved their usefulness as probes for demonstrating the remarkable evolutionary conservation of pgf protein epitopes in myelin and neurons. The present results also raise many new questions regarding the potential pathogenetic significance of anti-PLP antibodies that cross-react with neurons and may promote neuronal injury or prevent neuronal regeneration in human demyelinating diseases.

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