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Structural Polymorphism and Multifunctionality of Myelin Basic Protein[†]

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ABSTRACT: Central nervous system myelin is a dynamic entity arising from membrane processes extended from oligodendrocytes, which form a tightly wrapped multilamellar structure around neurons enabling rapid and efficient signal propagation. The gene of oligodendrocyte lineage (golli) gives rise to a variety of developmentally regulated splice isoforms of myelin basic protein (MBP), denoted golli for early forms and classic for later ones. In mature myelin, the predominant splice isoform of classic MBP is 18.5 kDa; its central role is to maintain the structural integrity of the myelin sheath, by holding together the apposing cytoplasmic leaflets of the oligodendrocyte membrane in a tight, spiral, multilamellar arrangement. This protein's extreme physicochemical properties, net charge of +19 at neutral pH, low proportion of hydrophobic residues, alternating regions of predicted intrinsic disorder and order, induced folding upon association with membranes and other proteins, and diversification via combinatorial post-translational modifications, define not only its role as a molecular Velcro in compact myelin, but as a multifunctional hub that may also bind to a number of other proteins and small molecule ligands in myelinating oligodendrocytes. In particular, MBP may link the underlying cytoskeleton and proteins containing SH3 domains to the membrane, allowing it to transduce transmembrane signals to the cytosol. These associations are facilitated by MBP being an intrinsically disordered protein, creating a large effective protein surface, and by the formation of transient and/or induced ordered secondary structure elements for molecular recognition. These processes can be modulated by a molecular barcode of numerous post-translational modifications and interactions with proteins such as calmodulin. In the human demyelinating disease multiple sclerosis, an aberrant pattern of modifications may contribute to demyelination and confound inherent attempts at repair. The conformational dynamics of the various isoforms and modified variants of MBP and their interactions with other proteins potentially allow them to participate in events coupling extracellular signals to cytoskeletal organization during myelination or remyelination. Various biophysical and cell biological approaches are beginning to elucidate these properties of MBP and are leading to a new understanding of the role of this protein as a linker and/or hub in structural and signaling networks in oligodendrocytes and myelin.

Myelin is the lipid-rich multilamellar assembly of membrane processes extending from oligodendrocytes and Schwann cells, forming an insulating sheath around nerve axons in the central and peripheral nervous systems, respectively (*1*). The myelin sheath is a dynamic system that communicates with both the underlying axon and external environment, and enables rapid transmission of nerve impulses by saltatory conduction. In the central nervous system (CNS[1], brain and spinal cord), the adhesion of the cytoplasmic surfaces of multilamellar internodal compact myelin is maintained by myelin basic protein (MBP), one of the two predominant proteins, the other being proteolipid protein (PLP). The myelin basic protein family is a product of differential splicing of a single mRNA transcript arising from one of three transcription start sites of the gene complex called golli (gene of oligodendrocyte lineage) (*2*). Various golli proteins arising from transcription start site 1 are produced in developing myelin and other cells in the nervous and immune systems. These isoforms can translocate between the nucleus and cellular processes, suggesting multiple roles in events as diverse as T-cell

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[1]Abbreviations: Akt, protein kinase B; Aze, azetidine-2-carboxylic acid; C1, C8, charge components C1 and C8 of myelin basic protein; CAP-23, cortical/cytoskeleton-associated protein of 23 kDa; CNS, central nervous system; CSK, cytoskeleton; Cyt-LUV, cytoplasmic large unilamellar vesicles; DIGS, detergent-insoluble glycosphingolipid and cholesterol-enriched microdomains; DM20, a PLP isoform; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; Fyn, a Src family tyrosine kinase; GalC, galactosylceramide; GAP-43, growth-associated protein of 43 kDa; GFP, green fluorescent protein; golli, gene of oligodendrocyte lineage; MAG, myelin-associated glycoprotein; MARCKS, myristoylated alanine-rich C kinase substrate; MAPK, mitogen-activated protein kinase (p42 and p44, 42, and 44 kDa isoforms, respectively); MBP, myelin basic protein; PI, phosphatidylinositol; PI_3K, phosphoinositide 3-kinase; $PI(4,5)P_2$, phosphatidylinositol-(4,5)-bis-phosphate; PKC, protein kinase C; PLC, phospholipase C; PLP, proteolipid protein; PPII, polyproline type II; PRMT, protein arginine methyltransferase; PTM, post-translational modification; RFP, red fluorescent protein; rmMBP, recombinant murine MBP (rmC1, rmC8 are recombinant murine C1, C8 variants, respectively); SDSL, site-directed spin labeling; SL, methanethiosulfonate spin label; SH3, Src homology domain 3; siRNA, silencing RNA; ssNMR, solid-state NMR; TFE-d_2, perdeuterated trifluoroethanol; VOCC, voltage-operated calcium channels.

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activation, Ca^{2+} influx, gene regulation, and signal transduction pathways in myelination (e.g., ref *3*).

The classic (canonical) MBP isoforms arise primarily from transcription start site 3, and splice variants range in molecular mass from 14 kDa to 21.5 kDa. The major adult classic MBP isoform in humans is 18.5 kDa, which is highly conserved in sequence in mammals (*4*). A 3′ untranslated region targets the mRNA transcript to distal cell processes where the protein is synthesized, becoming peripherally membrane-associated and concentrated in compact myelin (*5*). The classic 17.22 kDa and full-length 21.5 kDa isoforms have an additional 26 amino acids generated by exon-6/II, which causes them to become partially translocated to the nucleus (*6*). Our focus in this current topic is on the most-studied 18.5 kDa MBP isoform, which we shall henceforth refer to simply as MBP, using murine sequence numbering unless otherwise noted (Figure 1). The mutant line of *shiverer* mice lack classic MBP isoforms because of an ablation of that portion of the golli gene; they have only one or two layers of poorly compacted myelin, which can be rescued by MBP-producing cells (*7*). Because of this centrality, MBP has been called the "executive" protein of myelin (*8*) and has been the subject of a number of review articles and a recent book (e.g., refs (*4* and *9*−*13*)).

Myelin basic protein isolated from brains shows extensive post-translational modifications (PTMs) including deimination, phosphorylation, deamidation, methylation, and N-terminal acylation (*8*, *14*) (Figure 1C). These varying degrees of modifications give rise to multiple charge variants denoted as C1 to C8, when separated on cation exchange resin. The C1 component represents the least modified and the most cationic component with net charge +19 at neutral pH; it is the most abundant form in healthy adult humans. The remaining components, known as C2−C8, differ by the successive additional loss of one unit of positive charge as a result of combinations of different PTMs. Increased (and irreversible) deimination of MBP is associated both with normal developing and multiple sclerosis adult myelin (*12*, *14*−*16*), whereas (reversible) phosphorylation is implicated in signaling and myelin restructuring (*11*, *17*, *18*).

ELECTROSTATIC AND HYDROPHOBIC INTERACTIONS OF MBP WITH MEMBRANES

In electron micrographs of sectioned myelin sheaths, MBP is localized in the major dense lines, electron-dense lamellae formed by the tight apposition of the cytoplasmic leaflets of the oligodendrocyte membrane. Myelin is unusual compared to other cellular membranes in that it has a very high lipid to protein mass ratio (roughly 75:25 to 80:20), and high proportions of the glycosphingolipids (galactocerebrosides and sulfatides), and of cholesterol (>40 mol %) (*19*, *20*). The *in vitro* reconstitution of MBP with unilamellar vesicles containing anionic lipids results in vesicle aggregation and the formation of multilamellar structures similar to those seen in myelin sheaths (*19*). The most effective lipid vesicle-aggregating charge variant of MBP is the most cationic C1 component, as shown by various studies (*21*−*23*).

The interaction of MBP with lipid monolayers and bilayers (formed by Langmuir, Langmuir−Schaeffer, and Langmuir−Blodgett approaches, or as lipid vesicles) has been studied by differential scanning calorimetry, use of lipid probes, small-angle X-ray and neutron scattering, atomic force microscopy and spectroscopy, and miscellaneous other biophysical techniques (*19*, *24*−*26*). These studies show that MBP interacts with lipid membranes by a combination of electrostatic and hydrophobic interactions, as do many other peripheral membrane proteins such as MARCKS (myristoylated alanine-rich C kinase substrate) (*27*). However, MBP differs from such proteins in having its basic residues distributed over its entire length rather than in a cluster (Figure 1A,B). Thus, it does not have a distinct membrane-interacting domain as in other proteins; this property may be what allows it to cause tight adhesion of two lipid bilayers and also act as a scaffolding protein, binding other proteins to the membrane. Hydrogen bonding with phospholipid headgroups such as inositols may also contribute to the interaction (*28*). In addition to coupling two leaflets of membranes together, MBP can fill defects in them (*24*), which may allow it to bind at the interface between lipid-ordered and lipid-disordered domains.

The MBP−membrane interactions rely on a balance of interactions between the basic residues of MBP and the acidic headgroups of the lipid bilayer, in order to assemble the proper multilamellar structure seen in myelin sheaths (*24*, *26*, *28*). Small changes to this balance could result in significant changes in myelin adhesion or stability and can be achieved by modifying the protein post-translationally and/or the local lipid composition. Generally, maximum adhesion would be expected to occur when each positively charged residue on MBP associates with a phospholipid via simple electrostatic interaction (salt bridge) (*26*, *28*); a depletion or excess of MBP results in membrane repulsion or formation of a hydrated gel by the excluded protein, respectively.

Various other factors may also regulate MBP-mediated adhesion or association with the membrane. Variations in local ionic strength and pH, which are known to occur *in vivo* (*29*, *30*), can modulate MBP's adhesive strength by altering the repulsion between negatively charged membrane leaflets themselves (*28*). Zinc is a physiologically important cofactor; the concentration of zinc in myelin (about 50 μM) is substantially higher than that of any other trace element, and zinc specifically binds to MBP and may stabilize its association to the CNS myelin membrane (*31*). Finally, the classic MBP isoforms are acylated at their amino-termini, although the chain length is heterogeneous and rarely exceeds 10 carbons (*32*). Nevertheless, it affects local conformation (*33*) and may affect membrane association of the N-terminal domain.

The lipid composition of different membrane domains in myelin may also affect association with MBP. In monolayer and bilayer studies, MBP preferentially associates with and penetrates into liquid-expanded (effectively liquid-disordered *vide infra*) domains at low surface pressures and is then squeezed out of the membrane (while remaining bound) as the pressure increases or when the lipid undergoes the transition to the ordered gel phase (*19*, *34*). In mixtures of lipids, MBP causes phase separation of fluid and ordered domains and binds to the fluid domains (*35*). In mixtures of neutral and charged lipids, it binds preferentially to the charged lipids (*19*, *36*).

The general principles of MBP−lipid interactions deduced from *in vitro* studies of reconstituted systems are confirmed by X-ray scattering studies of intact myelin, where experimental variables include the composition and pH of the swelling solution (e.g., ref *20*). One important datum provided by such work is an estimate for the composition of the inner (cytoplasmic) leaflet of the oligodendrocyte membrane: 44% cholesterol, 27% phosphatidylethanolamine, 13% phosphatidylserine, 11% phosphatidylcholine, 2% phosphatidylinositol, and 3%

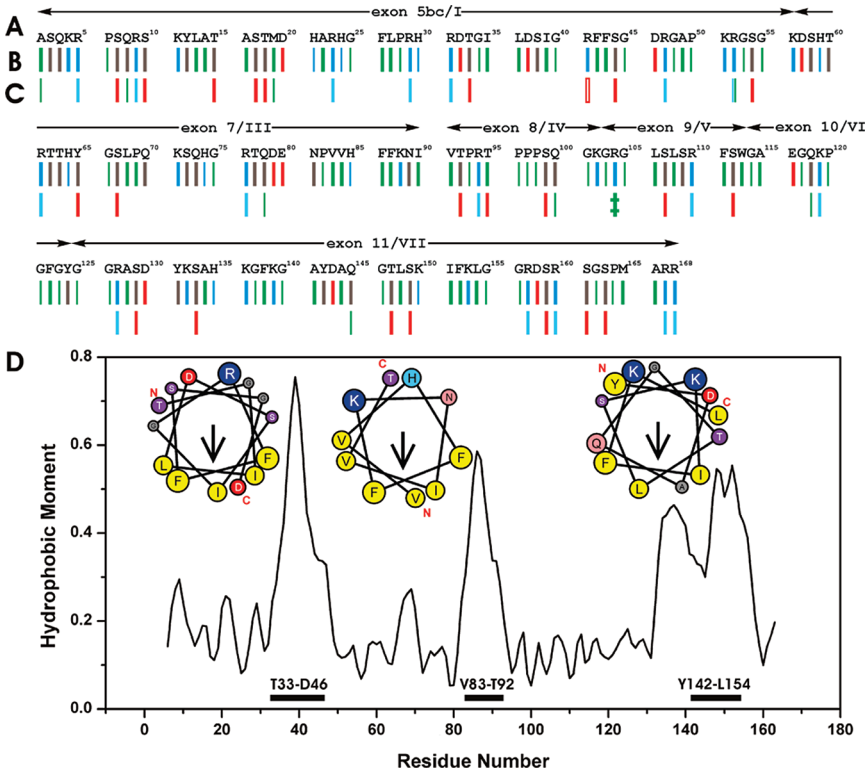

FIGURE 1: Residue roadmap of the murine classic 18.5 kDa MBP isoform. (A) Amino acid sequence indicating the exons (golli numbering in Arabic/Sanskrit numerals, classic numbering in Roman numerals). The recombinant rmC1 form has a C-terminal LEH_6 purification tag (not shown). (B) The molecular barcode defined by the physicochemical properties of the amino acids: blue bar, basic residues (R, K); thin blue bar, H (can be protonated depending on local pH fluctuations); green bar, apolar residues (A, V, F, I, L, W, M); thin green bar, special apolar residues (G, P); dark brown bar, polar residues (S, T, N, Q, Y, C); red bar, acidic residues (D, E). There is no apparent charged or apolar domain structure; all types of residues appear uniformly distributed throughout the sequence. (C) The molecular barcode due to diverse and combinatorial post-translational modifications (*10*, *14*): red bar, phosphorylation (S, T, Y) (murine residues T92 and T95 are MAP-kinase phosphorylation sites, lying in the vicinity of a putative molecular switch); hollow red bar, an unusual case of reported arginyl phosphorylation (*14*); ice blue bar, citrullination (the murine residues R25, R33, K119, R127, R157, and R168 correspond to primary arginine deimination sites in the C8 component of the human protein (*22*) and were used to generate a pseudodeiminated rmC8 variant by glutamine substitution (*38*)); green bar, diverse PTMs (acylation, deamidation, ADP-ribosylation, and sulfoxide oxidation of methionine); crossed green bar, methylation (either ω-$N^{G'}$-monomethylation or symmetric ω-$N^{G},N^{'G}$-dimethylation of R104). The density of PTMs is greatest at both termini. (D) Plot of hydrophobic moment over a moving 11-residue window for 18.5 kDa murine MBP and averaged per residue. Insets show helical wheel representations of selected segments of 18.5 kDa murine MBP, suggested by solution NMR spectroscopy and chemical shift analysis in 30% TFE-d_2 (*42*) to be α-helical, and all shown here to be strongly amphipathic. From left to right, we present segments (T33-D46), (V83-T92), and (Y142-L154). The hydrophobic moment plot was calculated by Mr. Kyrylo Bessonov (University of Guelph), and the helical wheel representations were generated using the HeliQuest program (at the Web site http://heliquest.ipmc.cnrs.fr/) and redrawn.

sphingomyelin (molar percentages) (*20*). The galactocerebrosides and their sulfates are assumed to be localized on the extracellular leaflet. Another parameter provided by these studies is the width of the major dense line in which classic MBP resides, varying from 3 to 4 nm.

MAPPING THE INTERACTION OF 18.5 kDA MBP WITH LIPID MEMBRANES

Electron paramagnetic resonance (EPR) spectroscopy is a powerful tool for probing the association of proteins such as MBP with lipid membranes. When reconstituted with lipids to mimic its natural environment in myelin, MBP forms large, semisolid assemblies that are amenable to this technique, as recently reviewed (*37*). Although MBP is a water-soluble protein without long stretches of hydrophobic amino acids (Figure 1), it was earlier found to have a perturbing effect on lipid bilayers, e.g., affecting the motion of the fatty acid chains even at locations deep within the bilayer, and decreasing the lipid phase transition temperature (reviewed in ref *19*). The two methionines, Met20 and Met167 (corresponding to murine Met19 and Met165), were spin-labeled, and their EPR spectra showed that the mobility of the side chains was sensitive to the degree of lipid order indicating greater penetration of the side chains into the fluid phase and extrusion from the ordered gel phase. Furthermore, the mobility of the spin-labeled side chains was sensitive to the perturbation of the lipid order caused by penetration of the side chains of residues elsewhere in the protein. Penetration of the protein side chains into the bilayer could allow MBP to detect extracellular signals which affect membrane order or membrane domain formation. This could cause a conformational change of MBP and greater perturbation of the lipid, resulting in transduction of these signals to the cytoplasmic side of the membrane (*11*).

More recently, we have used site-directed spin-labeling (SDSL) in combination with EPR spectroscopy to map out which residues of MBP penetrate into the bilayer and to determine the topology and structure of MBP on the bilayer surface (*37*−*40*). The SDSL procedure involves first introducing cysteines (natively absent in the 18.5 kDa isoform) at positions of interest in the protein for labeling with a small sulfhydryl-specific spin label, such as methanethiosulfonate (MTS-SL, or simply SL). The protein topology revealed by this technique

demonstrates which regions of MBP are accessible on the membrane surface for tethering other proteins to the membrane or for modification or cleavage by enzymes. We have compared two recombinant forms of the murine 18.5 kDa protein, which we denote as rmC1 and rmC8, corresponding to the respective natural isoforms (*10*, *38*). The rmC8 variant was generated from unmodified rmC1 by six Arg/Lys → Gln substitutions to mimic citrulline and thus the isoform of reduced net charge found in increased proportion in multiple sclerosis patients. We generated cytoplasmic large unilamellar vesicles (Cyt-LUVs) with the lipid composition described above, mimicking the cytoplasmic leaflet of the myelin sheath. The Ångstrom level penetration of the spin label into the bilayer can be determined from the solvent accessibility of the spin-labeled site using EPR spectroscopy, after distance calibration with spin-labeled lipids of known depth penetration. Regular secondary structure is revealed by the periodicity of the label's accessibility to these reagents (*39*, *40*).

In particular, the segment (Val83-Thr92) in rmC1, which has a large hydrophobic moment, was shown to form an amphipathic α-helix *in situ* (see Figure 1D) (*39*). The α-helix was embedded into the lipid bilayer and tilted slightly (roughly 9° with respect to the membrane surface) (Figure 2) as a result of the larger hydrophobic potential profile around its C-terminal end. The constituent hydrophobic side chains of unmodified rmC1 (Val83-Thr92) penetrated at a depth of up to 12 Å into the bilayer, thus explaining the ability of MBP to perturb lipid packing. The Phe86-Phe87 pair was important for anchoring this region to the bilayer. The deepest penetrating spin label on the polar face of the helix was Lys88Cys-SL (5.5 Å into the bilayer). This residue was in an ideal position for snorkeling, i.e., positioning the positively charged group of the amino acid in the polar region, whereas the aliphatic part was in the hydrophobic portion of the bilayer (Figure 2). In peripheral membrane proteins, snorkeling is thought to allow the long and bendable side chain of lysine to place the charged amino group in the more polar interface region, while keeping the hydrocarbon part of the side chain inside the hydrophobic part of the membrane, resulting in stronger binding. This study provided the first experimental evidence of specific, local secondary structure in MBP when bound to a lipid bilayer, including site-specific hydrophobic and electrostatic interactions.

Moreover, this segment of the protein is a B-cell epitope and an important T-cell recognition site in human MBP. This primary immunodominant epitope formed a more highly surface-exposed and shorter amphipathic α-helix in rmC8 than in rmC1 (Figure 2), explaining why rmC8 was more readily cleaved by cathepsin D at the Phe-Phe pairs than rmC1 (*40*). This important observation is consistent with earlier findings which showed the unusual accessibility of this epitope, *in vivo*, in degenerating myelin of multiple sclerosis lesions in close proximity to activated microglia and myelin-laden macrophages. Proteolytic processing of such a surface-exposed epitope by myelin-associated proteases, and/or phagocytosis of the damaged myelin by immune-derived cells, could result in the release of this encephalitogenic epitope from the central nervous system and its processing for antigen presentation to the peripheral immune system. Deimination of MBP may thus, in addition to reducing the degree of myelin compaction, also participate in the autoimmune pathogenesis of the disease by revealing otherwise inaccessible antigens of myelin to the surveillance of immune cells.

MBP contains two other regions with a large hydrophobic moment, in addition to residues (Val83-Thr92) (Figure 1D). It is also important to determine their topologies with respect to the membrane and how they are affected by protein deimination. That of the C-terminal region (Figure 1D), is presently under investigation. Besides the region (Val83-Thr92), as noted above, there was some notable divergence between rmC1 and rmC8 in terms of depth of penetration of other sites into the Cyt-LUV membrane (*38*). In particular, two spin-labeled residues of rmC8 in the C-terminal half were significantly more exposed than in rmC1, consistent with the fact that four of the six Arg/Lys → Gln substitutions were in this region (*38*). This greater accessibility of the C-terminal half of deiminated MBP in multiple sclerosis would potentially facilitate its interactions with other proteins, such as proteases or calmodulin (*41*−*43*), which may be involved in myelinogenesis during development, but may result in deleterious effects on myelin in adults with multiple sclerosis (*12*, *16*).

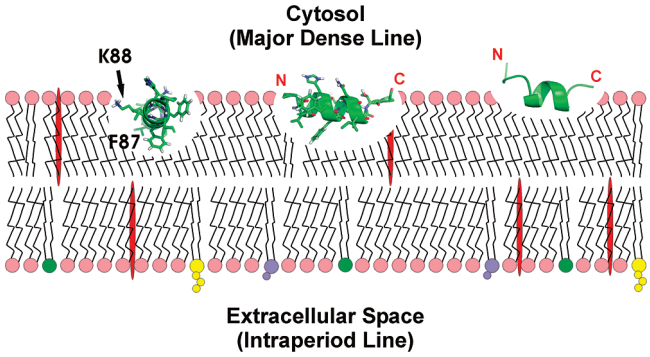

FIGURE 2: Disposition of an amphipathic α-helix (immunodominant epitope comprising residues V83-T92) of rmC1 (leftmost and middle peptides) and rmC8 (rightmost peptide) on a lipid membrane. The region from Val83 to Thr92 was studied by Cys-substitution and spin-labeling of each residue (within the intact protein molecule), followed by EPR spectroscopy of each protein species in turn (*39*). The periodicity of the penetration depth was fitted to a sine function. For rmC1, this analysis revealed a periodicity of 3.6 residues, and amplitude of 10 Å, indicative of an amphipathic α-helix. Moreover, the helix was slightly tilted with respect to the plane of the bilayer, but not as great as fusogenic peptides (leftmost and center peptide). The most exposed residue is His85, and the deepest penetrating residue Phe87 is indicated. Here, Lys88 is shown in a snorkeling orientation interacting with the negatively charged phosphate group of a phospholipid. Upon pseudodeimination of the protein, the α-helix in rmC8 is disrupted at Val83 and Val84, is less deeply embedded in the membrane (rightmost peptide), and the protein molecule is more exposed to proteases resulting in increased release of this peptide (*40*). The rest of the MBP molecule at the N-and C-terminal ends of this peptide is not depicted.

MEMBRANE MICRODOMAINS IN OLIGODENDROCYTES AND MYELIN

Biological membranes in all cells, including myelin-associated ones, comprise a dynamic, hierarchical organization of discrete structures, viz., microdomains of distinct lipid and protein composition, concentrated spatially to perform a specific task. These microstructures, often referred to as lipid rafts, are isolated as DIGs (detergent-insoluble glycosphingolipid and cholesterol-enriched microdomains). Myelin microdomains *in vivo* are developmentally regulated, diverse, and dynamic entities to which many myelin-specific proteins, including MBP, are localized (*44*−*48*). The association of MBP with myelin DIGs and by implication its participation in myelin signaling, appears, in part, to be due to its modification by kinases.

The mitogen-activated protein kinase (MAPK) family is important in oligodendrocyte proliferation, cell survival,

differentiation, and apoptosis (*49*). The p42/p44-MAPK in myelin is active and phosphorylates exogenous MBP (*44*) and endogenous MBP, CNP (myelin 2′,3′-cyclic nucleotide 3′-phosphodiesterase), and tubulin (Gong, Y., Min, W., and Boggs, J. M., unpublished data). The phosphorylation of murine Thr95 (human Thr98) within the segment Thr92-Pro93-Arg94-Thr95-Pro96 (discussed further below) by MAPKs is regulated by action potential generation in axons (*18*). We have characterized DIGs of mature and developing bovine myelin (*50*). In mature myelin, the DIGs were enriched in the MAPK-phosphorylated form of MBP, but the citrullinated and methylated forms of MBP were excluded. Moreover, nonphosphorylated MBP is part of a nonraft domain composed of phospholipids and sulfatide, also containing PLP (but not DM20, a smaller PLP isoform), S-MAG (but not L-MAG, the two isoforms of myelin-associated glycoprotein), and p42-MAPK (but not p44-MAPK) (*51*). In addition to partial nuclear localization, the exon-6/II-containing isoforms have been found to be localized to the tight junction/radial component (*52*). This differential targeting of phosphorylated and splice variants of MBP may be important in myelination and in maintaining myelin homeostasis. Furthermore, it has been postulated that MBP may form a specific microdomain that acts as a diffusion barrier between compact myelin and the paranodal loop region (*53*).

One particular type of microdomain is that enriched in phosphatidylinositol-(4,5)-bis-phosphate ($PI(4,5)P_2$). Using fluorescence-quenching and EPR spectroscopy, we have recently demonstrated that MBP laterally sequesters phosphatidylinositol-(4,5)-bis-phosphate ($PI(4,5)P_2$) in model membranes through nonspecific electrostatic interactions and in a manner that is partially cholesterol-dependent and sensitive to phosphorylation, deimination, and Ca^{2+}-calmodulin binding (*54*). Confocal microscopy of cultured oligodendrocytes also revealed patched colocalization of MBP and $PI(4,5)P_2$, indicating the spatial clustering of $PI(4,5)P_2$ in the plasma membrane of these cells (Figure 3). We further demonstrated colocalization of MBP and MARCKS in primary rat oligodendrocytes and codistribution of MBP, MARCKS, and GAP-43 (growth-associated protein of 43 kDa) in DIGS recovered from Triton X-100 detergent-extracted isolated myelin and brain homogenates (*55*). In an independent study, Nawaz et al. (*56*) demonstrated that $PI(4,5)P_2$ is essential for the stable association of MBP to the oligodendrocyte membrane. The 14 kDa MBP isoform was preferentially targeted to the plasma membrane when transfected into oligodendroglial precursor cells (interestingly, regardless of whether the 3′ untranslated region was present). This targeting was found to require $PI(4,5)P_2$ and was destabilized by ionomycin-triggered Ca^{2+}-influx (which would activate phospholipase C) and ATP-depletion. Thus, MBP may behave similarly to GAP-43, MARCKS, and CAP-23 (cortical/cytoskeleton-associated protein of 23 kDa), a class of proteins known collectively as PIPmodulins, and that mediate calcium and phosphorylation-sensitive plasma membrane availability of $PI(4,5)P_2$. The basic nature of MBP may also serve a strong charge-neutralizing function.

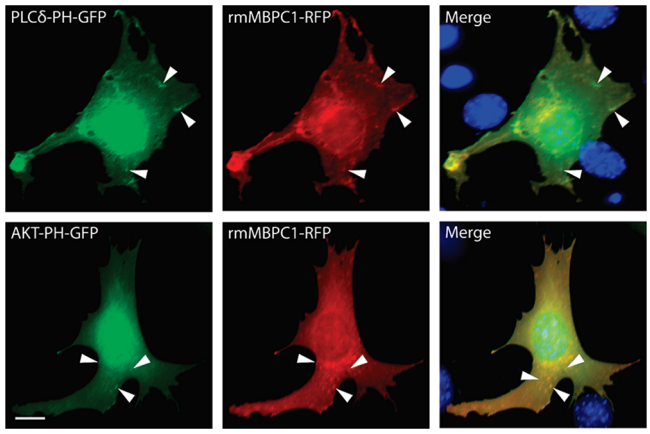

FIGURE 3: The 18.5 kDa murine MBP isoform is a PI(4,5)P2-modulin. Colocalization analysis of RFP-tagged rmMBP (rmC1 variant) with specific $PI(4,5)P_2$ and $PI(3,4,5)P_3$ markers (PLCδ-PH-GFP and Akt-PH-GFP, respectively) in N19 oligodendrocyte cells (PLC, phospholipase C; Akt, protein kinase B; RFP, red fluorescent protein; GFP, green fluorescent protein). Cells were transiently transfected with 0.75 μg of each plasmid DNA coding for the corresponding protein or marker and cultured for an additional 48 h. Following transfection, cells were fixed using formaldehyde and were washed twice before mounting for microscopy. Slides were directly counterstained using DAPI (4′-6-diamidino-2-phenylindole) (blue) and were imaged using epifluorescence microscopy. The areas of colocalization with rmC1-RFP are shown in the merged image (white arrows) and agree with our results using anti-$PI(4,5)P_2$ antibody (*54*) and the independent study of Nawaz et al. (*56*). Scale bar = 15 μM. Unpublished micrographs provided by Mr. Graham Smith (University of Guelph).

BEYOND LIPIDS: BINDING PARTNERS AND FUNCTION AS A SCAFFOLDING PROTEIN

Cytoskeletal Organization and Tethering to the Myelin Membrane. The sole function of MBP has long been thought to be adhesion of the cytosolic surfaces of the oligodendrocyte membranes of CNS myelin. However, MBP is known to bind to a number of proteins, including actin and tubulin, and can cause their polymerization and bundling *in vitro* (*11*, *57*, *58*), similar to that in MARCKS (*59*). It can also tether actin filaments and bundles, and microtubules to a membrane surface *in vitro* (*57*, *60*−*62*). These interactions can be regulated by physiological PTMs of MBP (primarily Ser/Thr phosphorylation and Arg deimination to citrulline), by Ca^{2+}-calmodulin binding to MBP and by a physiological change in membrane surface potential (Figure 4). The interaction of MBP with calmodulin *in vitro* is calcium-dependent and specific, with moderate (submicromolar) binding affinity, with the C-terminus of MBP being the primary binding target but not excluding other binding sites (*41*−*43*, *63*). The preponderance of interactions of highly positively charged MBP with other ligands is electrostatic. Deimination of MBP affects its interactions with calmodulin (*41*), and phosphorylation would also be expected to do so (*63*). Phosphorylation, a reversible modification, reduces the net charge of the protein by 2 and alters the local electrostatic properties and interactions of proteins significantly (*64*).

In vivo, both actin and tubulin are isolated together with MBP in a low density, detergent-insoluble, glycosphingolipid-enriched fraction of myelin, which also contains caveolin and kinases, and thus may be a membrane signaling domain (*44*, *65*−*67*). Some MBP is colocalized with actin *in vivo* at the membrane edges of cultured oligodendrocytes and with cytoskeletal veins of microfilaments and microtubules (*17*, *58*, *61*, *68*−*70*). Pull-down assays have also revealed a potential interaction of MBP with β-tubulin and the cytoplasmic loop of the gap junction protein connexin43, perhaps indicating an involvement in cell−cell communication (*71*). In oligodendrocytes from the *shiverer* mutant mouse, which lacks MBP, actin microfilaments do not form bundles and are not colocalized with microtubules (*17*, *72*, *73*). Moreover, the microtubule-based structures are abnormal in size, and the cell

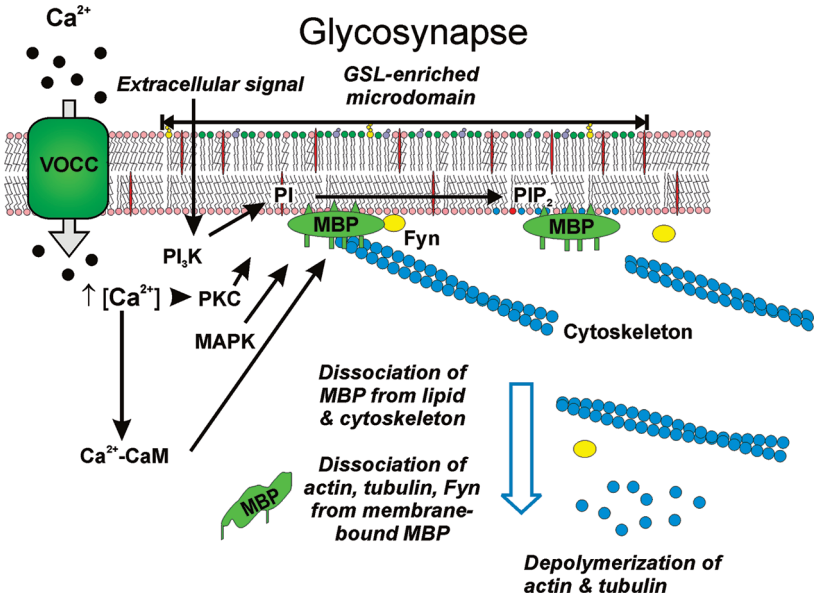

FIGURE 4: Schematic of mechanisms of (1) functions of the classic 18.5 kDa MBP isoform as a membrane scaffolding protein; (2) regulation of binding of actin filaments, microtubules, and SH3 domain-containing proteins to MBP in oligodendrocytes; and (3) transmission of extracellular signals to the cytoskeleton via MBP. The lipids are depicted as follows: $PI(4,5)P_2$ (blue headgroup), other phospholipids (pink headgroup), GalC (green headgroup), sulfatide (purple headgroup), gangliosides (yellow headgroup), and cholesterol (red rod). MBP bound to negatively charged lipids (phosphatidylserine and phosphatidylinositol (PI)) on the cytosolic leaflet tethers actin filaments and microtubules to the membrane. Extracellular signals that impinge on GSL-enriched lipid rafts or membrane signaling domains in the oligodendrocyte membrane, such as those imparted by anti-GalC/sulfatide antibodies or by GalC/sulfatide-containing liposomes, as discussed in the text, cause Ca^{2+}-entry (through VOCC or other Ca^{2+} channels (*3*)) and/or activate kinases such as PI_3K, and result in depolymerization of the cytoskeleton. The binding of Ca^{2+} to calmodulin causes dissociation of actin from MBP and of MBP from the membrane, as well as dissociation of microtubules from MBP. The activation of protein kinase C (PKC) by Ca^{2+} causes phosphorylation of MBP, which may decrease interactions of MBP with actin filaments, as has been shown for phosphorylation of MBP with MAPK. The kinase PI_3K causes phosphorylation of PI and increased amounts of $PI(4,5)P_2$, which are clustered by MBP, a PIPmodulin. The increased PIP_2 increases the membrane negative surface potential causing dissociation of negatively charged actin filaments from the membrane-bound MBP. The free actin filaments may then depolymerize, also causing depolymerization of microtubules, to which they may be bound, mediated by a number of cytoskeleton-binding proteins, and possibly also by MBP. The increased negative surface potential also causes the release of SH3 domain-containing proteins from MBP, thus regulating their activity and local signaling events.

processes are usually smaller than normal, with a larger cell body. These observations and changes in *shiverer* oligodendrocytes suggest that interactions of MBP with actin microfilaments and microtubules occur also *in vivo* in oligodendrocytes and that these interactions are important for cell function, membrane process formation, and myelination.

Interaction of MBP with SH3 Domain-Containing Proteins. Downstream from the membrane-bound amphipathic helix is a TPRTPPPS (Thr92-Ser99, Figure 1A) motif that represents a classic binding motif for SH3 domain (Src homology 3)-containing proteins, such as nonreceptor tyrosine kinases (*8*). Spin-labeled Ser99Cys-SL was found by SDSL/EPR to be exposed to the aqueous phase and thus accessible to modifying enzymes and other proteins, in both membrane-associated rmC1 and rmC8 (*38*). Other studies have shown that tryptic sites near this Ser99Cys-SL position, viz., Arg94-Thr95 and Arg104-Gly105, were cleaved when MBP was bound to a lipid membrane, whereas two other tryptic sites closer to the N-terminus, Arg22-His23 and Arg62-Thr63, were protected (*74*). Thus, one purpose of the preceding surface-associated amphipathic α-helical segment may be to present this proline-rich region appropriately for recognition (by SH3 domain-containing proteins) and/or modification (Figures 1 and 4).

Circular dichroic spectroscopy has indicated that the latter may form a polyproline type II (PPII) structure, and both free and membrane-associated rmC1 and rmC8 have been shown to bind various SH3 domains (*4*, *75*, *76*). Molecular docking simulations of the interaction of the putative SH3-ligand of classic MBP with the human Fyn-SH3 domain indicated that lysyl and arginyl residues in the peptide canonically interact via salt bridges and cation$-\pi$ interactions with negatively charged and aromatic residues in the SH3 domain-binding site (*75*). Both threonines within this motif are MAPK targets (Figure 1C). Post-translational modifications (phosphorylation or methylation) of the ligand *in silico* caused noticeable shifts in the conformation of the flexible peptide and its side chains but did not predict any major inhibition of the binding beyond somewhat less favorable interactions for peptides with phosphorylated seryl or threonyl residues.

Since molecular modeling showed that the Fyn-SH3 domain has a negative surface charge density even after binding the MBP ligand, we further investigated the influence of negative membrane surface charge and the effects of post-translational modifications to MBP on the interaction of the Fyn-SH3 domain with membrane-associated MBP (*76*). Using a sedimentation assay with multilamellar vesicles consisting of neutral phosphatidylcholine (PC) and negatively charged phosphatidylinositol (PI), we demonstrated that increasing the negative surface charge of the membrane by increasing the proportion of PI reduces the amount of the Fyn-SH3 domain that binds to membrane-associated MBP because of electrostatic repulsion. When one of the phosphoinositides PI(3)P or $PI(4,5)P_2$ was substituted for PI in equal proportion, none of the Fyn-SH3 domain bound to MBP under the conditions used. Post-translational modifications of MBP which reduced its net positive charge, viz., phosphorylation or arginine deimination, increased the repulsion of

Fyn-SH3 from the membrane surface, an effect further modulated by the lipid charge. This study suggested that changes in membrane negative surface charge due to protein or lipid modifications, which could occur during cell signaling, could regulate the binding of Fyn-SH3 to membrane-associated MBP, as found for actin filaments (*60*, *61*), and thus could regulate the activity of Fyn at the oligodendrocyte membrane surface (Figure 4).

We have already described that MAPK-phosphorylation of MBP targets it to different microdomains, but this modification may have further specific roles. Whereas Thr92 is a MAPK modification site in the membrane-anchoring α-helical segment, the residue Thr95 is a second MAPK target within the putative SH3-ligand. In *in vitro* studies, MAPK phosphorylation of MBP at Thr92 and Thr95 affects its ability to assemble actin and tubulin and tether them to a membrane (*57*, *61*, *62*), and to tether the Fyn-SH3 domain to a membrane (*76*). We thus consider these two residues to be key post-translationally modified sites that can affect local structure, protein−protein interactions, and microdomain targeting and stability, thereby operating as a molecular switch and modulating the functions of classic MBP isoforms (*4*, *11*). Phosphorylation of these threonines may concomitantly result in a localized destabilization of the preceding amphipathic α-helix and/or dissociation of the C-terminus of the central amphipathic α-helix from the phospholipid membrane, a phenomenon similar to what has been observed for the deiminated protein (*40*). Recently, it has been hypothesized that misincorporation of the proline homologue azetidine-2-carboxylic acid (Aze) into MBP during myelin development (which lasts through adolescence in humans) may result in inherent myelin instability and the eventual development of MS (*77*). This unusual amino acid entered European diets in the early nineteenth century with the advent of intensive sugar beet cultivation; roughly two decades later, MS started to become prominent. Aze remains part of our (primarily Western) diet today.

INDUCED FOLDING AND CONFORMATIONAL POLYMORPHISM OF MBP

The myriad interactions of MBP with surfaces and other proteins suggest that it must have great conformational adaptability. The protein has regions predicted to be alternately structured and unstructured (*42*) but can be classified as an intrinsically disordered protein overall, with an extended and flexible conformation that adapts to interactions with its numerous binding partners and surfaces (*78*). The protein alone has little or no ordered secondary structure in aqueous solution (reviewed in ref *10*). Our EPR spectroscopic studies of lipid-vesicle reconstituted MBP (*vide supra*) have provided direct experimental evidence of specific, induced, local secondary structure in the protein, an amphipathic, membrane surface-associated α-helix formed by (Val83-Thr92) (*39*, *40*). This particular result was in agreement with our solution NMR studies of a peptide containing this segment bound to dodecylphosphocholine micelles (*79*). Subsequent solution NMR spectroscopic studies of the full-length protein in aqueous solution and organic solvent (30% TFE-d_2, perdeuterated trifluoroethanol) defined three specific regions of MBP with strong α-helical propensity: (Thr33-Asp46), (Val83-Thr92) (the immunodominant epitope), and (Tyr142-Leu154) (Figure 1D) (*4*, *42*). These investigations also demonstrated that the C-terminal segment of MBP was the primary binding site for calmodulin.

We are beginning to obtain site-specific atomic detail on MBP's association with membranes by solid-state NMR (ssNMR) spectroscopy. In our initial ssNMR spectroscopic study of full-length rmC1 reconstituted with artificial membranes (*80*), we focused first on the highly mobile fragments, which we found to be in almost contiguous runs: (Thr15-Arg23), (Asp46-Ser67), (Pro120-Leu148), and (Leu154-Ser163). Moreover, these mobile segments were mostly disordered and exposed to solvent, likely being located outside the lipid bilayer or in its hydrophilic portion. The extents of the first two of these regions were in good agreement with results from SDSL/EPR spectroscopy (*38*) and correlated well with disordered regions observed by solution NMR spectroscopy of the protein (*42*). It is unclear as to why the segments (Pro120-Leu148) and (Leu154-Ser163) were mobile in this system; they overlap the long C-terminal portion of MBP with a relatively strong α-helical hydrophobic moment (spanning residues (Ser133-Asp160) in Figure 1D), particularly segment (Tyr142-Leu154), which was shown to have a strong α-helical propensity in 30% TFE-d_2 (*42*). Moreover, a C-terminal residue Ser159Cys-SL examined by SDSL/EPR was found to be buried in the bilayer (*38*). The differences between these various studies may be due to the different environments and lipid compositions, which were dictated in large part by experimental requirements. However, a relatively weaker membrane association of the C-terminal region of MBP may make it accessible as a Ca^{2+}-calmodulin target (*41*−*43*, *63*).

Because of the methodology employed in our ssNMR studies of these semisolid reconstituted systems, several of the immobilized fragments of MBP remained undetected. They corresponded to the other two regions with large hydrophobic moments which were predicted to be ordered (and amphipathic) α-helical segments (Figure 1D), and shown to be α-helical in organic solvent (30% TFE-d_2) by solution NMR spectroscopy (*42*), viz., fragments (Ser38-Ala49) and (Pro82-Pro93). These same regions correlated with the membrane-associated regions identified by SDSL/EPR spectroscopy and which contain the Phe42-Phe43 and Phe86-Phe87 pairs (*38*). The whole region (Leu68-Lys102) was completely invisible in these ssNMR experiments, suggesting that it was significantly immobilized. It follows that the other, mobile portions of MBP would thus be available to bind, for example, cytoskeletal and SH3 domain-containing proteins, to the membrane.

We have also recently used ssNMR and Fourier transform infrared (FTIR) spectroscopy to study the conformation of 18.5 kDa MBP in association with actin microfilaments and bundles (*81*). We have demonstrated induced folding of both protein partners, viz., some increase in β-sheet content in actin, and increases in both α-helix and β-sheet content in MBP, particularly in the terminal fragments and the central immunodominant epitope. The protein was still structurally heterogeneous, however, fitting into the paradigm of fuzzy complexes (*82*). All in all, the recent SDLS/EPR, FTIR, and NMR spectroscopic studies described here support the thesis that MBP is primarily a peripheral membrane protein, with some fragments and side chains embedded in the membrane and immobilized by the strong interactions with lipids. In effect, it can be described as a native molten globule *in situ* (*83*).

The overall conformational polymorphism and adaptability of MBP is consistent with its postulated roles as both a scaffold and linker (membranes and cytoskeleton), and as a potential signaling hub.

ROLE OF MBP IN SIGNALING IN OLIGODENDROCYTES

So far, we have established that MBP can adhere to phospholipid membranes, assemble cytoskeletal proteins, and probably tether the underlying cytoskeleton and SH3 domain-containing signaling proteins to the oligodendrocyte membrane, which may allow it to play a role in signaling. An intriguing series of studies showed that antigalactosylceramide (GalC) antibody added to oligodendrocytes mediates signals which cause Ca^{2+} entry, GalC and sulfatide redistribution in the membrane, phosphorylation changes of MBP, depolymerization of microtubules, and other signal transduction events in cultured oligodendrocytes (*17*, *69*, *70*, *72*, *84*, *85*) (Figure 4). Antisulfatide antibodies also have effects on oligodendrocyte differentiation in culture (*86*). These antibody interactions may be mimicking the effect of natural extracellular ligands impinging on the plasma membrane of oligodendrocytes. Galactosylceramide and sulfatide can bind to each other by Ca^{2+}-mediated *trans* carbohydrate−carbohydrate interactions across apposed membranes and may be extracellular ligands for each other, which cause similar signaling effects (*66*, *67*). *Trans* interactions between other glycosphingolipids in membrane signaling domains in apposed cells trigger signaling and have been proposed to result in the formation of a glycosynapse between the cells (*87*). When GalC/sulfatide-containing liposomes or a multivalent form of galactose-conjugated albumin are added to cultured oligodendrocytes, they interact with GalC and sulfatide in the oligodendrocyte membrane by these *trans* carbohydrate−carbohydrate interactions, with subsequent effects similar to those of anti-GalC antibody, i.e. lipid redistribution, depolymerization of the cytoskeleton, and changes in protein (including MBP) distribution. These results suggest that glycosynapses could also form between apposed oligodendrocyte membranes or between extracellular surfaces in compact myelin through contact between GalC and sulfatide in signaling domains.

This extracellular signaling via GalC/sulfatide to the cytoskeleton may be a signal for compaction of the MBP-containing domains to form myelin since depolymerization of the cytoskeleton is necessary for compaction (*67*). Stabilization of the actin cytoskeleton with jasplakinolide prevented all effects of the GalC/sulfatide-containing liposomes, including GalC and MBP redistribution and microtubule depolymerization, suggesting that the stability of microtubules in oligodendrocytes depends on the integrity of the actin cytoskeleton (*67*). The MBP redistribution following depolymerization of the actin cytoskeleton may result from linkage of actin filaments to MBP in oligodendrocytes, as suggested by our *in vitro* studies. In support of this idea, MBP was present in a Triton X-100 insoluble fraction from oligodendrocytes, which resembled an intact cytoskeleton and which contained actin and tubulin (*65*, *68*). The central involvement of MBP in transduction of the signal is suggested by studies showing that these effects do not occur in *shiverer* oligodendrocytes and are decreased in oligodendrocytes in which MBP expression is inhibited with MBP siRNA (*17*, *67*, *72*). It has also been demonstrated microscopically that association with neurons induced changes in lipid organization, in particular clustering of GalC in oligodendrocyte membranes, a phenomenon not observed in *shiverer* oligodendrocytes, and thus unequivocally requiring MBP (*88*). This MBP-dependent neuron-induced GalC clustering thus represents another extracellular signal which is transduced through MBP.

CONCLUSIONS

We have reviewed a number of *in vitro* studies which show that MBP can interact with different cytosolic proteins and tether them to membranes, suggesting that it can perform as a linker and scaffolding protein. Both *in vivo* studies and studies with cultured oligodendrocytes indicate further that it may be involved in signaling. Oligodendrocytes and myelin also contain more specialized scaffolding proteins. However, the ability of MBP to perform these functions *in vitro* and its great abundance in myelinating oligodendrocytes and myelin strongly suggest that it should be able to perform these functions *in vivo* also. However, it is not clear at what stage of myelination this could occur. *In vivo*, oligodendrocytes do not produce the extensive MBP-containing membrane sheets observed in culture since the oligodendrocyte processes do not produce large amounts of membrane until after contact with axons, during axonal ensheathment. Myelin basic protein is observed only in oligodendrocytes that have migrated into axonal pathways, and it is produced just before the commencement of axonal ensheathment (*89*, *90*). At this stage, the oligodendrocytes produce multiple filopodia which associate with numerous axons. Some may function as sensors to locate mature target axons for myelination, while others perform ensheathment of the axon and myelination (*91*). In mature myelinating oligodendrocytes, MBP redistributes from the soma and primary processes into the myelin sheaths, reflecting a change in the site of MBP mRNA expression (*5*). Cytosol is present in the first few layers of ensheathment before compaction and appearance of the major dense line occur. These early ensheathments actively elongate and undergo extensive remodeling. Varicosities are also observed in immature myelin sheaths indicating the presence of cytosol (*92*). Myelin basic protein must exert many of its putative diverse scaffolding and signaling functions when cytosol and cytosolic constituents are still present, i.e., during the initial association of processes with axons, axonal ensheathment and remodeling, and early stages of myelination. When compaction occurs, its role in adhesion may predominate. However, actin, tubulin, and signaling proteins are present in compact myelin also, possibly associated with the tight junctions of the radial component (*52*, *65*). It is not known how these proteins are organized or associated in compact myelin. Further structural and cell biological studies are necessary to understand the diverse possible roles of MBP in myelination and in the function of compact myelin.

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