Myelin Basic Protein as a "PI(4,5)P_2-Modulin": A New Biological Function for a Major Central Nervous System Protein[†]

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ABSTRACT: The 18.5 kDa isoform of myelin basic protein (MBP) is multifunctional and has previously been shown to have structural and phenomenological similarities with domains of other membrane- and cytoskeleton-associated proteins such as MARCKS (myristoylated alanine-rich C kinase substrate). Here, we have investigated whether 18.5 kDa MBP can sequester phosphatidylinositol-(4,5)-bis-phosphate (PI(4,5)P_2) in membranes, like MARCKS and other "PIPmodulins" do. Using fluorescence-quenching and electron paramagnetic resonance (EPR) spectroscopy, and model membranes containing BODIPY-FL- or proxyl-labeled PI(4,5)P_2, respectively, we have demonstrated that MBP laterally sequesters PI(4,5)P_2. The MBP-PI(4,5)P_2 interactions are electrostatic, partially cholesterol-dependent, and sensitive to phosphorylation, deimination, and Ca^{2+}-CaM binding. 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. On the basis of these findings as well as the overwhelming convergence of functional properties, modifying enzymes, and interaction partners, we propose that MBP is mechanistically related to GAP-43, MARCKS, and CAP-23. During myelinogenesis, it may mediate calcium and phosphorylation-sensitive plasma membrane availability of PI(4,5)P_2. This regulation of PI(4,5)P_2 availability at the cell cortex may be coupled to the elaboration and outgrowth of the membranous cellular processes by oligodendrocytes.

During central nervous system (CNS[1]) development, oligodendrocytes, the myelinating cells of the CNS, undergo complex morphological changes with restricted gene expression. At the onset of myelination, mature oligodendrocytes extend elaborate cell processes that terminate in membranous lamellipodia with many motile filopodial extensions that anchor onto and enwrap axons (*1, 2*), to form concentric spiral wrappings of myelin membrane necessary for saltatory nerve conduction. The intrinsic factors and the underlying molecular mechanisms by which mature oligodendrocytes elaborate and extend these membranous cellular processes remain unresolved. More specifically, the mechanistic roles that myelin-specific proteins play in myelin formation, if any, are not understood.

In the CNS, myelin basic protein (MBP) constitutes 30% of the total myelin protein by weight, representing the second most abundant protein in CNS myelin, after proteolipid protein (PLP). The biological role of MBP in myelin is traditionally understood to be the maintenance of myelin sheath compaction. Owing to its extreme net positive charge, and ability to organize anionic lipid-containing vesicles into multilamellar structures *in vitro*, MBP is believed to serve as an adhesive molecule in myelin sheaths, bringing together the cytosolic surfaces of the oligodendrocyte membrane and

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[1] Abbreviations: bC1−bC6, bovine MBP charge components, 1 to 6; bC1-PhT, *in vitro* MAPK-phosphorylated natural component bC1 (on threonyl residues); BCA, bicinchoninic acid; BODIPY-FL-PI(4,5)P_2, fluorescent derivative of PI(4,5)P_2; C1−C8, MBP charge isomers, or components, 1 to 8; CaM, calmodulin; CAP-23, cortical/cytoskeleton-associated protein of 23 kDa; CNS, central nervous system; DMSO, dimethylsulfoxide; ED, effector domain; EDTA, ethylenediamine tetraacetic acid; EPR, electron paramagnetic resonance; GAP-43, growth-associated protein of 43 kDa; Golli, genes of oligodendrocyte lineage; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; hPAD2, human peptidylarginine deiminase isoform 2; LUV, large unilamellar vesicle; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C kinase substrate; MS, multiple sclerosis; NAP-22, neural axonal myristoylated protein of 22 kDa; N-WASP, neural Wiskott-Aldrich syndrome protein; PAD, peptidylarginine deiminase, EC 3.5.3.1; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PI(4,5)P_2, phosphatidylinositol-4,5-bis-phosphate; PKC, protein kinase C; PLP, proteolipid protein; proxyl-PI(4,5)P_2, spin-labeled derivative of PI(4,5)P_2; PS, phosphatidylserine; rmMBP, recombinant murine 18.5 kDa classic MBP isoform; rmC1, rmC8, recombinant murine MBP charge isomers, or components, 1 and 8; rmC1-Cit, *in vitro* deiminated (citrullinated) rmC1; rmC1-PhS, *in vitro* PKC-phosphorylated rmC1 (on seryl residues); TPI, triphosphoinositides; Tris, tris(hydroxymethyl)aminomethane; WASP, Wiskott-Aldrich syndrome protein; WAVE, Wiskott-Aldrich syndrome protein family verprolin homologous.

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causing myelin compaction around axons (reviewed in refs *3* and *4*). In addition, a growing body of evidence suggests that MBP may be involved in signaling pathways involved in cellular morphogenesis and membrane organization (*5*).

The expression of classic MBP isoforms by oligodendrocytes, at the terminal differentiation stage, coincides with marked morphological changes. The elaboration and outgrowth of the membranous cellular processes, which form concentric multilamellar myelin sheaths around axons, are intimately linked to the synthesis of MBP in the distal end of the growing myelin. Shiverer mice, which carry recessive MBP gene deletions and lack MBP expression, exhibit severe hypomyelination (reviewed in ref *6*). In cultured shiverer oligodendrocytes, the absence of MBP gene products has been shown to cause abnormal cell process formation, and aberrant organization of the actin and tubulin cytoskeleton (*7*). The involvement of MBP with cytoskeletal assembly is also supported by various *in vitro* and *in vivo* studies (*4, 8, 9*).

Myelin basic protein shares several important functional and physical properties with myristoylated alanine-rich protein kinase C substrate (MARCKS) (*10, 11*), growth-associated protein of 43 kDa (GAP-43) and cortical/cytoskeleton-associated protein of 23 kDa (CAP-23) (*12, 13*), and the CAP-23 human homologue, neural axonal myristoylated protein of 22 kDa (NAP-22) (*14*). These proteins have been recognized as morphogenetic competence determinants in cells that express them, and are similar to MBP in that they are intrinsically disordered, at least in part (*15*). Though they share very little sequence identity, these proteins contain a common basic amino acid rich domain that facilitates much of their known cellular functions. This domain, often referred to as the effector domain (ED), binds calmodulin and actin, and contains seryl residues which are phosphorylated by protein kinase C (PKC) (*16*−*18*). The electrostatic interaction of the basic effector domain with anionic membrane surfaces, and the membrane insertion of an N-terminal hydrophobic acyl moiety, cooperatively allow these proteins to associate with the cell membrane (*19*). Binding of Ca^{2+}-calmodulin, or phosphorylation by protein kinase C, induces the membrane dissociation of the effector domain (*12, 20, 21*). We have previously noted several similarities between the MARCKS-ED and 18.5 kDa MBP in terms of their strong membrane association, high degree of phosphorylation, and calmodulin-binding (*10*). These observations were extended further by noting similarities with other actin-polymerizing proteins, and the unique structuring that MBP induced in phosphoinisotide-containing membranes, especially PI(4,5)P_2 (phosphatidylinositol-4,5-bis-phosphate) (*3, 22*).

Several biophysical and biochemical studies have demonstrated that, when membrane-bound, the basic effector domains of these proteins laterally sequester PI(4,5)P_2 through reversible, nonspecific, electrostatic interactions (*14, 23*−*25*). Cell culture studies have also provided evidence that these proteins can modulate the plasmalemmal spatial distribution of PI(4,5)P_2, and form cholesterol-dependent PI(4,5)P_2 microdomains at the cell surface (*12*). As such, these proteins are collectively designated as intrinsic PI(4,5)P_2-modulins in cells that express them. The formation of PI(4,5)P_2 microdomains has been shown to be dependent on the effector domain in these proteins, and constructs lacking this domain functioned in a dominant negative manner which failed to generate PI(4,5)P_2-clustering in cells (*12*). Plasma membrane PI(4,5)P_2-clustering by these proteins was shown to promote stimulus-induced neurite outgrowth and cell spreading. This activity is correlated with PI(4,5)P_2-microdomain-dependent cell surface actin accumulation, and possible corecruitment of proteins that regulate the dynamics and assembly of cell cortex actin cytoskeleton (*12*).

Here, in an attempt to provide insight into the mechanistic role of MBP in myelinogenesis and myelin maintenance, we have investigated whether MBP may be functionally related to these PI(4,5)P_2-sequestering proteins, as we originally hypothesized (*3, 10, 22*). In contrast to these proteins, the basic residues of MBP are distributed throughout its sequence, enabling it to adhere two cytoplasmic membrane leaflets to each other, and it has a shorter N-terminal alkyl chain. Nevertheless, its high net positive charge allows it to bind tightly to acidic lipid surfaces and to sequester other negatively charged lipids, phosphatidylserine (PS) and phosphatidylinositol (PI) (*26, 27*). Using fluorescence-quenching and electron paramagnetic resonance (EPR) spectroscopy, and model membranes containing BODIPY-FL- or proxyl-labeled PI(4,5)P_2, respectively, we have demonstrated that MBP laterally sequesters PI(4,5)P_2 through nonspecific, electrostatic interactions, and in a manner that is partially cholesterol-dependent and sensitive to net charge reduction by protein phosphorylation and deimination, and by Ca^{2+}-CaM binding. Confocal microscopy of cultured oligodendrocytes also revealed patched colocalization of MBP and PI(4,5)P_2 in the plasma membranes of these cells.

MATERIALS AND METHODS

Reagents and Antibodies. Triton X-100 was purchased from Fluka (Buchs, Switerland). Monoclonal anti-MBP (SMI99) was purchased from Sternberger Monoclonals (Lutherville, MD). Monoclonal anti-PI(4,5)P_2 IgM (2C11) was obtained from Abcam (Cambridge, MA). The following AlexaFluor-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR) and were used for immunofluorescence analysis: AlexaFluor 594 goat antirabbit IgG (H+L), AlexaFluor 488 goat antimouse IgM (μ chain), AlexaFluor 488 rabbit antimouse IgG (H+L), and AlexaFluor 594 rabbit antimouse IgG (H+L).

Preparation of Large Unilamellar Vesicles (LUVs). The lipids phosphatidylcholine (PC, brain), phosphatidylserine (PS, brain), PI(4,5)P_2, and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL) and aliquotted as chloroform stocks. The BODIPY-FL-C5,C6-phosphatidylinositol-4,5-diphosphate (BODIPY-FL-PI(4,5)P_2) was obtained from Molecular Probes and dissolved in dimethylsulfoxide (DMSO). The proxyl-PI(4,5)P_2 was a gift from Dr. D. Cafiso (University of Virginia).

Unless otherwise noted, vesicles for fluorescence studies were prepared fresh each day from chloroform stocks of PC and cholesterol, and BODIPY-FL-PI(4,5)P_2 in a 59.9:40:0.1 molar ratio, respectively. Lipid mixtures were first dried under a stream of nitrogen, redissolved in 15 μL of methanol, dispersed by vortexing (30 min at room temperature) in 10 mM HEPES−NaOH, 1 mM EDTA, 140 mM NaCl, pH 7.4 buffer (1.0 mL, final volume), and then extruded through a 100 nm polycarbonate filter (Lipofast, Avestin Inc., Ottawa, ON) to prepare large unilamellar vesicles (LUVs) (*28*). For the LUVs used to study the effects of the presence of other

negatively charged lipids, PS was included in a PC:PS:Chol:BODIPY-FL-PI(4,5)P_2 molar ratio of 39.9:20:40:0.1. For comparison with cholesterol-containing vesicles, vesicles without cholesterol were prepared from PC with 0.1 mol % BODIPY-FL-PI(4,5)P_2. For the experiments in which the effects of Ca^{2+}-calmodulin were studied, vesicles were prepared in 10 mM HEPES−NaOH, 140 mM NaCl, pH 7.4, without EDTA. For the experiments in which the effects of salt concentration were studied, vesicles were initially prepared in 10 mM HEPES−NaOH, 1 mM EDTA, 140 mM NaCl, pH 7.4, but then diluted (>10-fold) into the same HEPES buffer with NaCl replaced by KCl of various concentrations.

For EPR studies, PC-LUVs with or without 3 mol % unlabeled PI(4,5)P_2 were prepared as above at a concentration of 20 mM, and labeled in the outer monolayer with proxyl-PI(4,5)P_2 as described (*29*). Briefly, a chloroform solution of proxyl-PI(4,5)P_2 was evaporated in a thin film in a test tube, and dried in a lyophilizer. The suspension of LUVs was shaken gently in the tube containing the dried proxyl-PI(4,5)P_2 for 45 min at room temperature. The final concentration of proxyl-PI(4,5)P_2 in the LUVs was 0.5 mol % of the lipid. In all experiments, the phospholipid concentration was determined using the Bartlett assay for phosphorus as described (*30*).

Recombinant Protein Expression, Purification, and Modification. Recombinant murine MBP C1 and C8 charge component mimics (denoted as rmC1 and rmC8, respectively) were expressed in *Escherichia coli*, and purified as previously described (*31, 32*). Natural charge components of the 18.5 kDa isoform of bovine MBP (denoted bC1−bC6) were purified from the delipidated white matter of a 2-year-old steer brain as described (*33*), and were a kind gift from Drs. D. D. Wood and M. A. Moscarello (Hospital for Sick Children, Toronto, ON). Calmodulin (CaM, *Xenopus laevis*) was expressed in *E. coli* and purified as described (*34*). The purity of all protein preparations was assessed by SDS−PAGE, and concentrations were determined with the micro-BCA assay (Pierce Biotechnology, Rockford, IL) using either rmC1 or BSA as a standard. The concentrations were also assessed by measuring the absorbance at 280 nm, using the extinction coefficients 0.623 L g^{-1} cm^{-1} (rmC1), and 0.152 L g^{-1} cm^{-1} (CaM). These values (in 6.0 M guanidine hydrochloride, 0.02 M phosphate, pH 6.5), were calculated on the basis of the amino acid sequences using the ProtParam software tool available at the Web site http://www.expasy.ch.

Protein kinase C (PKC) phosphorylation of rmC1was achieved by incubating 300 μg of rmC1, at 30 °C for 4 h, with 1 μg of PKCα (Upstate, Chicago, IL) in the presence of 0.15 mM ATP, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, and PKC lipid activator (Upstate) in 20 mM HEPES−NaOH, pH 7.4, buffer. The products are denoted as rmC1-PhS. The final reaction concentrations of the components of the PKC lipid activator used were 0.06% Triton X-100, 0.2 mg/mL PS, and 0.02 mg/mL diacylglycerol.

Recombinant p42 mitogen-activated protein kinase (MAPK) was purchased from New England Biolabs and stored at −80 °C until use. Component bC1 from bovine brain was phosphorylated with MAPK as described to yield bC1-PhT (*9*). Analysis on an alkaline tube gel indicated that one site was phosphorylated, most likely Thr97 (bovine 18.5 kDa sequence numbering), the dominant MAPK site of MBP.

Phosphorylation of the bovine MBP charge components and the PKC-phosphorylated rmC1 were evaluated using Pro-Q Diamond phosphoprotein gel stain (Invitrogen, Molecular Probes, Mississauga, ON) as per the manufacturers' instructions. The Pro-Q phosphoprotein stained gels were imaged with Typhoon 94000 (Amersham Biosciences, Piscataway, NJ) with excitation at 532 nm and a 560 nm long-pass emission filter. These gels were subsequently stained with SYPRO-Ruby protein gel stain for total protein detection. The deimination state of the natural bovine MBP charge components was also assessed using the modified citrulline detection kit (Upstate) as per the manufacturer's instructions.

The *in vitro* deimination of rmC1 (300 μg) was performed overnight at 37 °C, in the presence of recombinant human peptidylarginine deiminase isoform 2 (hPAD2, 10 μg) in 100 mM Tris-HCl, 5 mM $CaCl_2$, 2 mM DTT, pH 7.5, buffer. The hPAD2 had been purified as previously described (*35*) and was a gift from Dr. R. Raijmakers (Radboud Universiteit Nijmegen, The Netherlands). The degree of global deimination of rmC1, after the overnight reaction, was determined using freshly prepared COLDER citrulline detection solution (11.5% (v/v) H_3PO_4, 21.8% (v/v) H_2SO_4, 1.1 mM NH_4-Fe(SO_4), 20 mM diacetyl monoxime, and 0.5 mM thiosemicarbazide) as described (*36*).

Steady-State Fluorescence Measurements of PI(4,5)P_2-Sequestration. The sequestration of PI(4,5)P_2 by MBP was assayed using BODIPY-FL-labeled PI(4,5)P_2 as previously described (*14, 24*). Unless otherwise noted, a preparation of LUVs (50 μM, in 10 mM HEPES−NaOH, 1 mM EDTA, 140 mM NaCl, pH 7.4 buffer) was titrated with small aliquots of dilute MBP (1 μL at a time). All fluorescence measurements were obtained with a PTI Alphascan-2 spectrofluorometer (Photon Technologies International, South Brunswick, NJ) equipped with thermostatted cell holders. The steady-state fluorescence emission spectra of the BODIPY-FL-PI(4,5)P_2-containing LUVs, in the presence or absence of MBP, were recorded from 500 to 600 nm while exciting at 490 nm. The spectral bandwidths for the excitation and emission wavelengths were set as 2 and 4 nm, respectively. For routine titrations, data were recorded with excitation and emission wavelengths set at 490 and 511 nm, respectively. The fluorescence signals were integrated over a 5 s (5 points/s) time window in order to minimize photobleaching. Vesicle light scattering was minimized using a 495 nm cutoff filter (Oriel Corporation, Rockford, IL) placed in the emission light path. All measurements were made at 25 °C, in ultramicro quartz cuvettes (Hellma, Concord, ON) with 3 mm excitation and emission path lengths. The self-quenching of BODIPY-FL-PI(4,5)P_2 fluorescence, upon addition of MBP to the vesicles, was evaluated by normalizing the emission intensity of the vesicles at each titration point (F) to the initial fluorescence intensity (F_0) of the vesicle solution alone. During titration, the sample was mixed thoroughly and allowed to incubate for 1 min after each addition of MBP, and prior to each measurement. All intensity values were corrected for the small volume change during titration. Measurements were made at least in triplicate.

Electron Paramagnetic Resonance (EPR) Spectroscopic Measurements of PI(4,5)P_2-Sequestration. Solutions of bC1, bC1-PhT, and rmC8 were prepared in distilled water at 300−550 μM. Aliquots of the proxyl-PI(4,5)P_2-labeled LUVs, protein solutions, and buffer (10 mM HEPES−NaOH,

1 mM EDTA, 140 mM NaCl, pH 7.4) were combined to give a final lipid concentration of 7.5 mM, and a final protein concentration of 18.75 μM. This resulted in a protein to lipid molar ratio of 1:400, and a protein to proxyl-$PI(4,5)P_2$ molar ratio of 1:2. Since the net positive charge of bC1 is +19 at neutral pH (the amino terminus is acylated, and there are 31 arginyl and lysyl residues, and 11 glutamic and aspartic residues), the molar ratio of protein positive charges to proxyl-$PI(4,5)P_2$ was roughly 10 for bC1. The samples were incubated for 30 min at room temperature, and taken up in 50 μL capillary tubes which were sealed with Critoseal. The EPR spectra were immediately recorded at room temperature on a Bruker ECS 106 spectrometer (Bruker BioSpin, Billerica, MA).

Oligodendrocyte Cell Culture. Wistar rat pups' (7−8 days old; Charles River Canada, Saint-Constant, QC) spinal cord oligodendrocytes were cultured as described previously (*37*). Cells were seeded at a concentration of 10^6 cells/mL on polylysine-coated coverslips, and grown for 8−9 days.

Immunofluorescent Staining and Confocal Microscopy of Cultured Oligodendrocytes. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed 4× with PBS (phosphate-buffered saline), permeabilized (0.05% saponin), and blocked with 5% normal goat serum and 5% normal donkey serum. Cells were then double immunostained for MBP and $PI(4,5)P_2$, using monoclonal anti-MBP (1:400), and anti-$PI(4,5)P_2$ (1:200) antibodies, respectively, in blocking solution. After extensive washing, appropriate AlexaFluor-conjugated antibodies were incubated (2 h, gentle shaking) with the cells, followed by further washing. Coverslips were then mounted with IMMU-MOUNT medium (Thermo-Electron Corporation, Pittsburgh, PA) and allowed to harden overnight, in the dark, on a flat surface. Slides were viewed in a Zeiss Model LSM-510 confocal laser-scanning microscope controlled by the LSM-510 program, and imaged using the sequential scanning mode.

RESULTS AND DISCUSSION

Sequestration of $PI(4,5)P_2$ by MBP in Model Membranes: Fluorescence-Quenching. The fluorescent probe BODIPY-FL, as BODIPY-TMR, has highly superimposable absorption and emission spectra, and exhibits self-quenching properties (due to static and/or dynamic quenching) when two or more molecules are brought together in close proximity. Previous studies have demonstrated that peptides corresponding to the basic effector domains of NAP-22 and MARCKS result in a "dose-dependent" decrease in fluorescence when titrated into vesicles containing BODIPY-TMR-labeled $PI(4,5)P_2$, due to the lateral clustering of the labeled $PI(4,5)P_2$ (*14, 24*). Following a similar strategy, we have explored the ability of MBP to sequester $PI(4,5)P_2$ laterally in model membranes, using LUVs containing BODIPY-FL-labeled $PI(4,5)P_2$.

Figure 1A shows the fluorescence emission spectra of vesicles containing labeled $PI(4,5)P_2$ as increasing amounts of MBP were added (0−1.3 μM final; from top to bottom). As shown, the addition of MBP aliquots to the vesicles containing BODIPY-FL-labeled $PI(4,5)P_2$, PC, and cholesterol (0.1:59.9:40) resulted in a dramatic, dose-dependent reduction of the vesicle fluorescence (Figure 1A, top trace without MBP, and bottom trace in the presence of a final concentration of 1.3 μM MBP). The inset diagram illustrates the basis of the assay. As MBP is titrated into the vesicle solution, it is expected that two or more of the labeled, acidic $PI(4,5)P_2$ molecules would interact with basic residues on the protein and be brought together, laterally. However, because the concentration of $PI(4,5)P_2$ in these vesicles is extremely low (0.1 mol % of total lipids), and $PI(4,5)P_2$ molecules would be uniformly distributed, binding of MBP to the vesicles in the absence of any clustering effect is not expected to influence their fluorescence. Consequently, the observed dose-dependent fluorescence-quenching caused by MBP illustrates the ability of the protein to cluster/sequester $PI(4,5)P_2$ laterally along the plane of the membrane.

To understand the effects that other anionic lipids or cholesterol may have on MBP-$PI(4,5)P_2$-sequestration, we performed experiments in which cholesterol was removed from the vesicle preparation, or in which the anionic phospholipid phosphatidylserine (PS) was included. Previously, titration of the NAP-22 basic effector domain into fluorescently labeled $PI(4,5)P_2$-containing vesicles caused cholesterol-dependent fluorescence-quenching, and this peptide was shown to form cholesterol-dependent $PI(4,5)P_2$ membrane domains as revealed by total internal reflectance fluorescence microscopy (*14*). In contrast, the basic effector domain of MARCKS is unique among PIPmodulins because it has been shown to sequester $PI(4,5)P_2$ similarly in model membranes with and without cholesterol (*24*). Here, the normalized fluorescence-quenching profiles obtained from PC- (○), PC:cholesterol- (●), and PC:PS:cholesterol- (◆) vesicles containing PC:BODIPY-FL-$PI(4,5)P_2$ are compared in Figure 1B. The addition of cholesterol resulted in a robust fluorescence-quenching, greater than in its absence. These observations indicate that MBP-$PI(4,5)P_2$-sequestration is cholesterol-sensitive.

Since biological membranes such as myelin contain other anionic lipids in addition to $PI(4,5)P_2$, we examined the ability of MBP to cluster $PI(4,5)P_2$ in membrane vesicles that contained 20 mol % PS, at a concentration 200-fold higher than that of $PI(4,5)P_2$. When MBP was titrated into vesicles containing PS (Figure 1B, ◆), significant fluorescence-quenching was observed only at higher protein concentrations. In comparison to the LUVs that contained only 0.1 mol % $PI(4,5)P_2$, the presence of 20 mol % PS was expected to enhance MBP's affinity for the now highly anionic membrane surface. In addition, MBP would have, under these conditions, an enhanced ability to organize the unilamellar vesicles into multilamellar structures. Both of these effects can reduce the lateral diffusion of MBP necessary for the clustering of the relatively scarcely populated $PI(4,5)P_2$. Nonetheless, these data indicate that MBP can cluster the more highly charged $PI(4,5)P_2$ even in the competing presence of a physiological excess of a singly charged anionic lipid.

In similar, earlier experiments in which a peptide that corresponds to the basic effector domain of MARCKS was titrated into fluorescently labeled $PI(4,5)P_2$ containing vesicles, the presence of 17−30 mol % PS in the membrane bilayer was also shown to reduce the efficiency of the fluorescence-quenching, when compared to vesicles without PS (*24*). As shown here for MBP, the effector domain of MARCKS did sequester $PI(4,5)P_2$ and caused significant fluorescence-quenching in the presence of PS, albeit requiring a higher

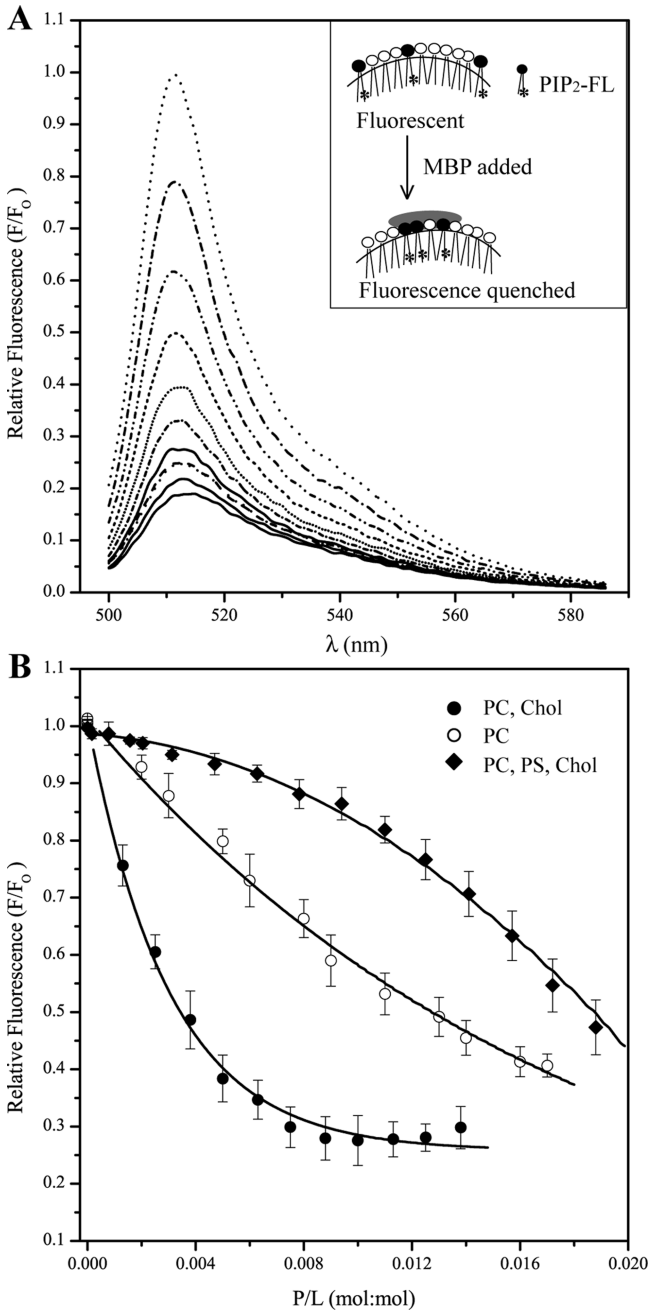

FIGURE 1: MBP sequesters PI(4,5)P_2 in model membrane vesicles. (A) Fluorescence emission spectra of BODIPY-FL-PI(4,5)P_2 containing LUVs titrated with rmC1. The concentration of LUVs was 50 μM, and their composition was 59.9:40:0.1 (mol %) PC:cholesterol:BODIPY-FL-PI(4,5)P_2). The MBP was added sequentially, ~0.15 μM protein per scan, from 0 μM (top trace) to 1.3 μM (bottom) final concentrations. All measurements were made in 10 mM HEPES−NaOH, 1 mM EDTA, 140 mM NaCl, pH 7.4, at 25 °C, and the emission was recorded from 500 to 600 nm while exciting at 490 nm. The spectral bandwidths for the excitation and emission wavelengths were set as 2 and 4 nm, respectively. Inset: Illustration of self-quenching of BODIPY-FL as PI(4,5)P_2 is sequestered together by MBP. (B) Normalized fluorescence emission intensity of BODIPY-FL-PI(4,5)P_2 (0.1 mol %)-containing LUVs (50 μM) in 10 mM HEPES−NaOH, 1 mM EDTA, 140 mM NaCl, pH 7.4, plotted against protein−lipid molar ratio (P/L) as rmC1 was titrated into the vesicle solution. The lipid and cholesterol compositions of the LUVs used were: 99.9 mol % PC (○); 59.9:40 mol % PC:cholesterol (●); or 39.9:20:40 mol % PC:PS:cholesterol (◆). All measurements were made, at 25 °C, with excitation and emission λ set at 490 and 511 nm, respectively, as described in Materials and Methods.

peptide/lipid ratio. Theoretical calculations involving the application of electrostatic theory to atomic models of PS- and PI(4,5)P_2-containing bilayers, with adsorbed MARCKS effector domain peptide, also supported these conclusions (*24, 38*). These calculations showed that the basic residues of the membrane-bound peptide provide a concentrated, positive electrostatic surface potential that can attract and sequester the multivalent, anionic PI(4,5)P_2 even in the presence of an overwhelming amount of monovalent acidic PS (cf. ref *39*).

Sequestration of PI(4,5)P_2 by MBP in Model Membranes: EPR Spectroscopy. A spin-labeled derivative of PI(4,5)P_2, proxyl-PI(4,5)P_2, has also been shown to be a useful probe of sequestration of PI(4,5)P_2 by the effector domain of MARCKS (*24, 29*). Protein-induced clustering of the proxyl-PI(4,5)P_2 in PC vesicles results in spin−spin interactions and line broadening, which reduces the height of the spectrum. Figure 2 shows that component bC1 also causes a decrease in height of the spectrum of PC-LUVs containing 0.5 mol % proxyl-PI(4,5)P_2. Although MBP could cause a decrease in mobility of the lipid, including the spin label, which would also reduce the height of the spectrum, this mechanism was not the cause of the observed reduction in height. This was shown by incorporation of 3 mol % of unlabeled PI(4,5)P_2 in the PC-LUVs to compete with proxyl-PI(4,5)P_2 for component bC1 and decrease clustering of the spin label. In the presence of excess PI(4,5)P_2, bC1 caused little or no reduction in the height of the spectrum, indicating that it did not decrease the lipid mobility (Figure 2B).

The Physical Nature of MBP-PI(4,5)P_2-Sequestration. We have examined the physical nature of MBP-PI(4,5)P_2-sequestration, and whether a physiologically relevant salt concentration could interfere with this interaction. High salt concentrations have been shown to decrease the PI(4,5)P_2-sequestration ability of the effector domains of NAP-22 (*14*) and MARCKS (*24*). Figure 3 shows the fluorescence-quenching isotherm of LUVs incubated with a saturating level of MBP (0.12 protein:lipid molar ratio) in the presence of increasing concentrations of KCl (0−1 M). As expected, the MBP-PI(4,5)P_2-sequestration is largely electrostatic in nature. Increasing the salt concentration of the assay buffer reduced the fluorescence-quenching with a sigmoidal dose−response. When the data were fitted to Boltzmann's sigmoidal function, the estimated IC_{50} for KCl (the concentration of KCl for 50% reduction in fluorescence-quenching) was calculated to be ~250 mM. This value is well above the salt concentration inside the cell, indicating that MBP-PI(4,5)P_2-sequestration could occur *in vivo*, at physiologically relevant salt concentrations.

Putative Regulation of MBP-PI(4,5)P_2-Sequestration by Protein Phosphorylation. Phosphorylation of the basic effector domains of PI(4,5)P_2-modulin proteins, in response to cellular stimuli that include the activation of PKC, has been proposed to be a possible mechanism of reversing their spatial PI(4,5)P_2-sequestration and allowing the local release of PI(4,5)P_2 (*40*). The phosphorylation by PKC of the basic effector domains of these proteins at several sites diminishes the electrostatic binding to anionic lipids. Phosphorylation of MARCKS by PKC, *in vivo*, has been shown to result in its translocation from the plasma membrane to the cytoplasm (*41*). Similar results have also been obtained *in vitro* using membrane vesicles, for peptides corresponding to the basic

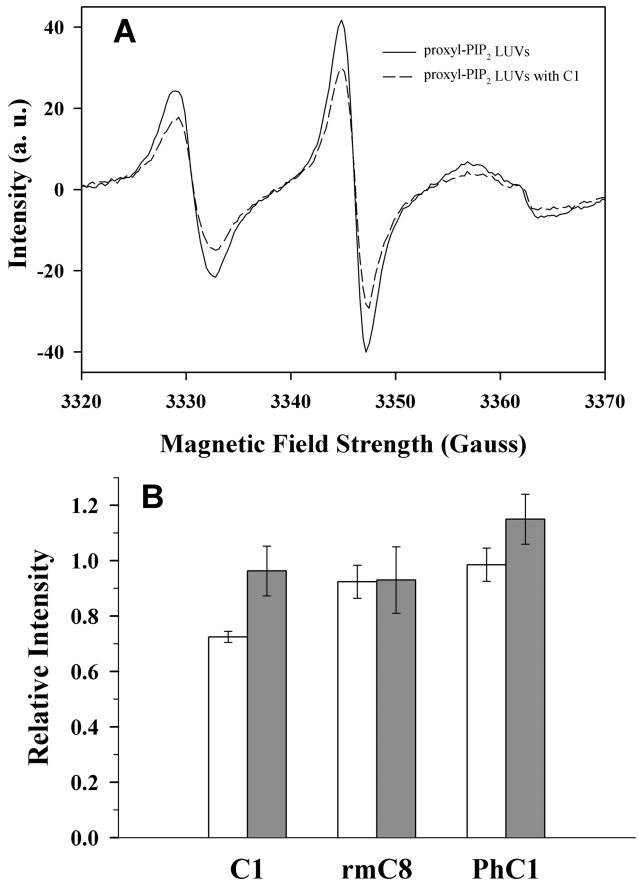

FIGURE 2: Component bC1 of MBP causes clustering of proxyl-PI(4,5)P_2 in PC-LUVs. (A) The EPR spectra of PC-LUVs containing 0.5 mol % proxyl-PI(4,5)P_2 in the absence (solid line) or presence of bC1 (dashed line). The molar ratio of bC1 to lipid was 0.0025, and that of bC1 to proxyl-PI(4,5)P_2 was 0.5. Spectral intensity is in arbitrary units (a.u.). (B) Effect of bC1, rmC8, or bC1-PhT (MAPK-phosphorylated bC1) on the relative intensity of the center line of the EPR spectrum of PC-LUVs containing 0.5 mol % proxyl-PI(4,5)P_2 without (white bars) or with 3 mol % unlabeled PI(4,5)P_2 (gray bars). The relative intensity is determined from the intensity of the center line of the EPR spectrum of proxyl-PI(4,5)P_2-containing LUVs in the presence of MBP divided by that in its absence. Values represent the mean plus or minus standard deviation of three experiments for bC1, and the mean plus or minus range of two experiments for rmC8 and bC1-PhT. The value for bC1 in the absence of unlabeled PI(4,5)P_2 was significantly ($p < 0.05$) different from the value in the presence of unlabeled PI(4,5)P_2.

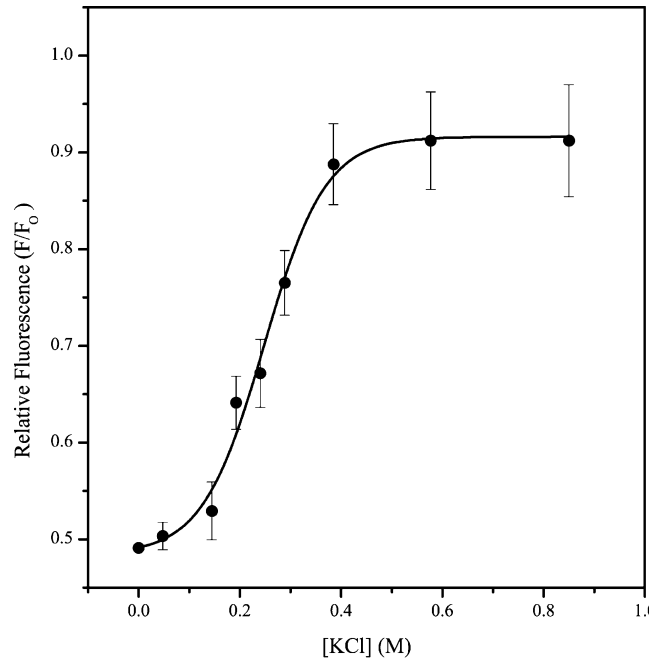

FIGURE 3: The sequestration of PI(4,5)P_2 by MBP is electrostatic in nature. Normalized fluorescence intensity of PC:cholesterol: BODIPY-FL-PI(4,5)P_2 (59.9:40:0.1 mol %) LUVs incubated with rmC1 (at 0.12 mol:mol P/L) as a function of KCl concentration (0−0.9 M) in 10 mM HEPES−KOH, 1 mM EDTA, pH 7.4 buffer (with a residual 14 mM NaCl; see Materials and Methods). Data were fitted to Boltzmann's sigmoidal function ($r = 0.997$), and the IC_{50} for KCl was determined to be 247.6 ± 0.01 mM.

effector domains of MARCKS and GAP-43, as well as intact MARCKS (*20, 42*).

Myelin basic protein is a substrate both *in vivo* and *in vitro* for diverse families of protein kinases such as PKC, MAPK, Ca^{2+}-calmodulin-dependent protein kinase, and cyclic AMP-dependent protein kinase (*43*−*45*). Phosphorylation of MBP reduces the membrane affinity of this protein and modulates its interactions with cytoskeletal proteins (*8, 9*). Figure 4A shows a comparison of vesicle fluorescence-quenching caused by *in vitro* PKC-phosphorylated rmC1 (rmC1-PhS) and unmodified rmC1. Figure 4B compares the vesicle fluorescence-quenching of several *in vivo* phosphorylated natural charge components of MBP purified from bovine brain (bC4−bC6), of decreasing net positive charge as a consequence of various post-translational modifications, including phosphorylation (*46*). The most abundant, and most highly positively charged, natural bC1 component is not phosphorylated. We confirmed that the C4−C6 charge components, and rmC1-PhS, were phosphorylated using the Pro-Q Diamond phosphoprotein gel stain (Figure 4C).

As shown in Figure 4A, PKC-phosphorylation of MBP diminished this protein's ability to sequester PI(4,5)P_2 (○), as evident from the reduced fluorescence-quenching in comparison to unmodified rmC1 (●). Component bC1 that had been phosphorylated *in vitro* at Thr97 by MAPK (bC1-PhT) also had diminished ability to sequester proxyl-PI(4,5)P_2 (Figure 2B). The bC1-PhT-induced increase in height of the spin label in PC-LUVs containing 3 mol % unlabeled PI(4,5)P_2 may be due to an increase in mobility of the lipid caused by bC1-PhT. Similarly, the bC4−bC6 charge isomers showed significantly reduced fluorescence-quenching ability in comparison to the unmodified bC1 component or the recombinant rmC1 equivalent; the latter two proteins showed a comparable degree of fluorescence-quenching. Taken together, these observations suggest that phosphorylation of MBP by PKC and MAPK, in response to cellular stimuli, may impede this protein's ability to sequester PI(4,5)P_2 and allow its local release.

Putative Regulation of MBP-PI(4,5)P_2-Sequestration by Calmodulin-Binding. Another mechanism by which PI(4,5)P_2-sequestering proteins can release PI(4,5)P_2, and make it available for its multifaceted functions in cells, is through the binding of Ca^{2+}-calmodulin (CaM) to their effector domains (*40*). This interaction is known to dissociate the effector domain from the membrane surface. For example, binding of Ca^{2+}-CaM to the membrane-bound basic effector domain of MARCKS has been shown to increase the rate of dissociation of this domain from the membrane (*19*). Equally, Ca^{2+}-CaM markedly reduced the binding of MARCKS to acidic membrane vesicles (*20*). The 18.5 kDa isoform of

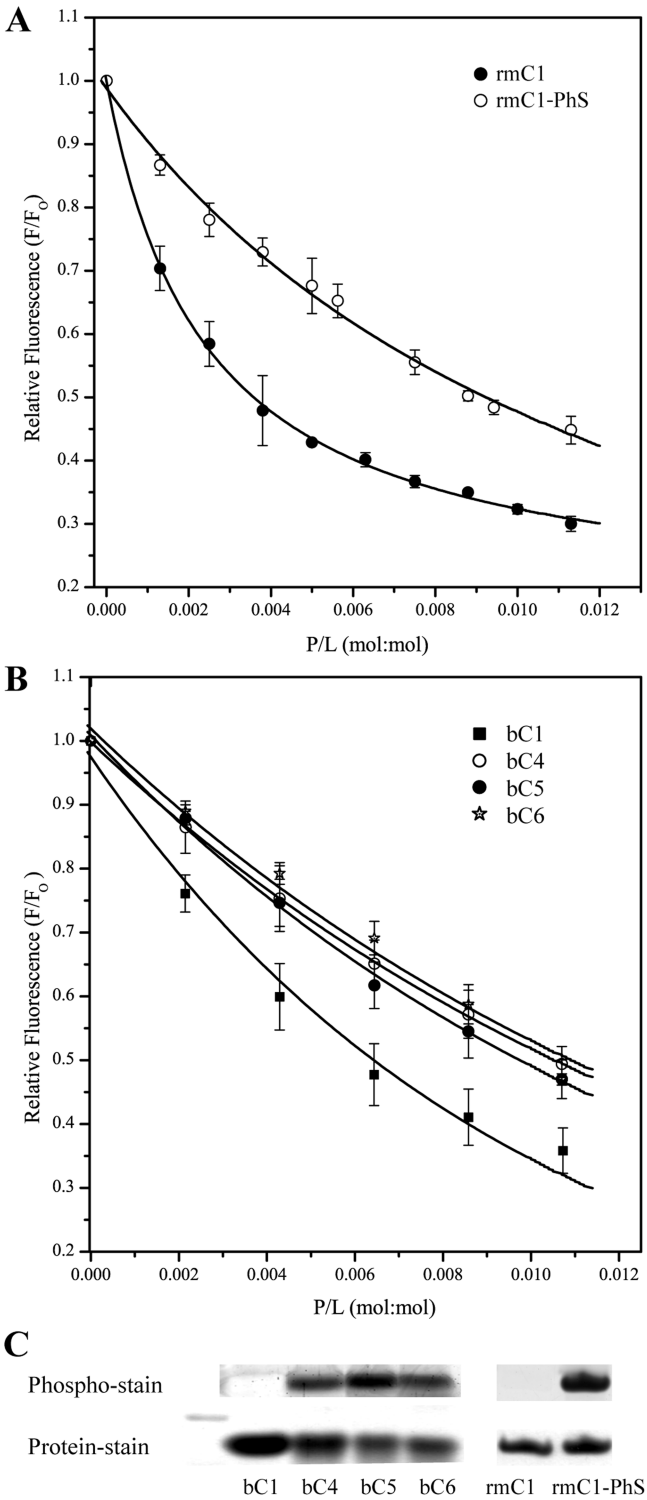

FIGURE 4: Phosphorylation of MBP impedes PI(4,5)P_2-sequestration. (A) Normalized fluorescence emission intensity of BODIPY-FL-PI(4,5)P_2 (0.1 mol %)-containing LUVs (50 μM) in 10 mM HEPES−NaOH, pH 7.4, 1 mM EDTA, 140 mM NaCl, plotted against the protein−lipid molar ratio (P/L) of unmodified rmC1 (closed circles, ●) and PKC-phosphorylated rmC1 (rmC1-PhS, open circles, ○). (B) Normalized fluorescence emission intensity of BODIPY-FL-PI(4,5)P_2 (0.1 mol %)-containing LUVs, as above, plotted against the protein−lipid molar ratio (P/L) of purified bovine MBP charge isomers (bC1, bC4, bC5, bC6). These isomers differ in net positive charge due, in large part, to various Ser/Thr phosphorylations. (C) Global phosphorylation state of the charge isomers of bovine MBP and PKC-phosphorylated rmC1 (rmC1-PhS) as determined from samples separated on SDS-PAGE and stained with Pro-Q Diamond phosphoprotein gel stain, followed by SYPRO protein gel stain.

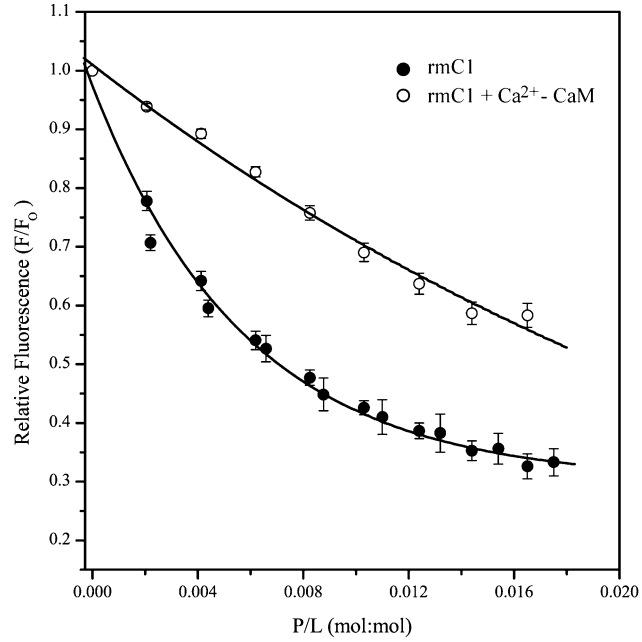

FIGURE 5: Calcium-calmodulin (Ca^{2+}-CaM) diminishes MBP-PI(4,5)P_2-sequestration. Normalized fluorescence emission intensity of BODIPY-FL-PI(4,5)P_2 (0.1 mol %)-containing LUVs (50 μM) in 10 mM HEPES−NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4, plotted against the protein−lipid (P/L) ratio of added rmC1-MBP in the presence and absence of Ca^{2+}-CaM. Either the CaM was added to the vesicles in a 2-fold excess of the final concentration of MBP at saturation, or CaM and MBP were premixed (2:1 molar ratio, respectively), and the mixture titrated to the LUVs solution. Both procedures gave the same result; only one is shown.

MBP has previously been demonstrated to interact with calmodulin (CaM) in a Ca^{2+}-dependent manner (i.e., specifically) *in vitro*, at a 1:1 (MBP:CaM) ratio under near-physiological conditions with a dissociation constant of 144 ± 76 nM (*47*). Although this association in solution appears relatively weak compared to MARCKS, for example, it is sufficiently strong that the addition of Ca^{2+}-CaM causes the dissociation of MBP and MBP−actin complexes from model membrane vesicles (*48*). Subsequent proteomics analyses have revealed MBP to be one of many CaM-binding proteins in the brain (*49*).

Here, we monitored the fluorescence-quenching caused by MBP in the presence (Figure 5, ○) and absence (Figure 5, ●) of a saturating level of Ca^{2+}-CaM. Either the Ca^{2+}-CaM was premixed with MBP at a 2:1 molar ratio and the mixture titrated into the vesicle solution, or Ca^{2+}-CaM was added to the LUVs in excess and MBP titrated into this solution. Both of these approaches produced comparable results, although the latter experiments were easier to reproduce. As shown in Figure 5, Ca^{2+}-CaM binding markedly reduced the efficiency of vesicle fluorescence-quenching caused by MBP. Titration of Ca^{2+}-CaM or apoCaM (in the absence of Ca^{2+}) into the vesicle solution alone caused no changes in vesicle fluorescence. Equally, titration of MBP into the vesicle solution in the presence of an excess amount of apoCaM caused no appreciable reduction in fluorescence-quenching. This reduction in MBP-PI(4,5)P_2-sequestration caused by Ca^{2+}-CaM may be attributed to either the reduced membrane affinity of Ca^{2+}-CaM-bound MBP, as shown previously (*48*), or a shielding, upon Ca^{2+}-CaM binding, of the positive charges of the protein that are required for PI(4,5)P_2-sequestration, as proposed for other PI(4,5)P_2-sequestering

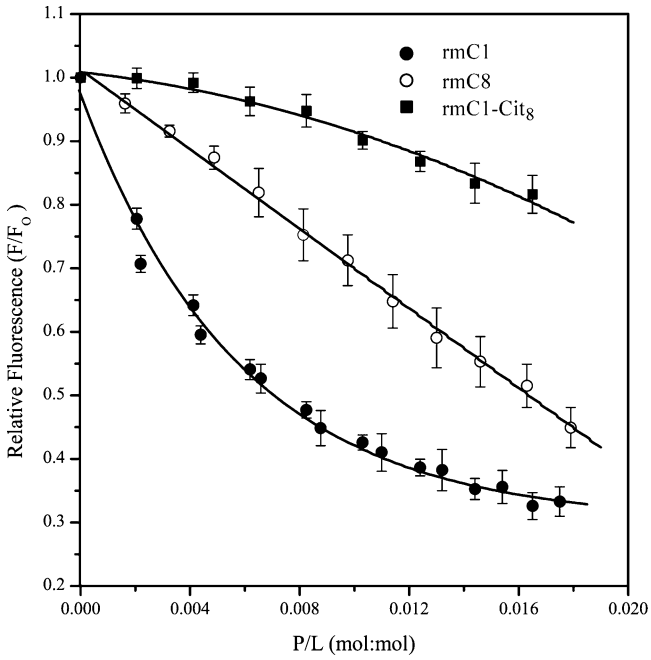

FIGURE 6: Developmental and disease-associated reduction in net positive charge of MBP (deimination) eliminates MBP-PI(4,5)P_2-sequestration. Normalized fluorescence emission intensity of BODIPY-FL-PI(4,5)P_2 (0.1 mol %)-containing LUVs (50 μM) in 10 mM HEPES−NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4, plotted against the protein−lipid (P/L) ratio of added rmC1, rmC8, and deiminated rmC1-Cit_8 (on average) MBP variants.

proteins (*40*). Recent solution NMR spectroscopic studies of rmC1−Ca^{2+}-CaM interactions show that all residues of 18.5 kDa MBP appear to interact with Ca^{2+}-CaM, even though the C-terminus appears to be the primary binding site (*50*), thereby supporting this conjecture.

Effects of MBP Deimination on Its PI(4,5)P_2-Sequestering Ability. Another post-translational modification to MBP, which reduces its net positive charge, is conversion of its arginyl residues to the neutral amino acid citrulline by deimination. This post-translational modification is catalyzed *in vivo* by the calcium-dependent peptidylarginine deiminase (PAD) family of enzymes (*51*). Deimination of MBP reduces its ability to form multilamellar structures, and is aberrantly increased in the human demyelinating disease multiple sclerosis, as reviewed in ref *52*. Here, we examined the PI(4,5)P_2-sequestration abilities of enzymatically deiminated MBP (rmC1-Cit), and the recombinant deimination-mimetic MBP (rmC8, in which 6 Arg residues had been converted to Gln).

Figure 6 shows a comparison of the vesicle fluorescence-quenching caused by unmodified recombinant MBP (rmC1, ●), deimination-mimetic recombinant MBP (rmC8, ○), and enzymatically deiminated rmC1 (rmC1-Cit, ■). Compared to the fluorescence-quenching profile of rmC1, both rmC8 and rmC1-Cit showed a substantial reduction in fluorescence-quenching ability. The quasi-deiminated recombinant variant rmC8 also had diminished ability to cause clustering of proxyl-PI(4,5)P_2 (Figure 2B). Care was taken to ensure that the observed differences were not due to differences in protein concentration. Surprisingly, the decrease in fluorescence-quenching effects of the recombinant rmC8 was moderate in comparison to the enzymatically modified rmC1-Cit. To understand the nature of this difference in fluorescence-quenching between these two proteins, we quantified the degree of citrullination of the overnight enzymatically modified rmC1. The rmC1-Cit was estimated to contain, on average, 8 citrullinyl residues per molecule (results not shown), representing a further reduction in net positive charge of the protein by only 2 compared to rmC8. However, given that enzymatic modification is a continuous one-step process, the rmC1-Cit population is likely to be heterogeneous, with some protein molecules containing more than 8 citrullinyl residues. Consequently, it is likely that the differences in vesicle fluorescence-quenching between the recombinant rmC8 and enzymatically modified rmC1-Cit may be due to differences in net positive charge of a significant portion of the latter preparation.

Cellular Colocalization of MBP and PI(4,5)P_2. In order to elucidate whether MBP is colocalized with PI(4,5)P_2 at the cell surface of oligodendrocytes, as previously was shown for GAP-43, MARCKS, and CAP-23 in COS cells and PC12B cells (*12*), we have examined the codistribution of MBP and PI(4,5)P_2 in paraformaldehyde-fixed and saponin-permeabilised cultured oligodendrocytes using a commercially available anti-PI(4,5)P_2 IgM antibody (2C11) that has been previously used to detect endogenous PI(4,5)P_2 in the plasma membrane (*53*). Figure 7 shows a representative confocal image of cultured oligodendrocytes labeled with anti-PI(4,5)P_2 2C11 (green) and anti-MBP (red) antibodies. As seen, some MBP colocalized with PI(4,5)P_2 in the plasma membrane. The immunoreactivities of MBP and PI(4,5)P_2 show both diffuse and patched colocalization (Figure 7, arrowheads), suggesting that some of the membrane-bound MBP and PI(4,5)P_2 are clustered together in the plasma membrane of oligodendrocytes. The patched MBP-PI(4,5)P_2 domains are similar to those observed earlier for other PI(4,5)P_2-sequestering proteins (*12*). Control experiments, in which cells were not permeabilized with saponin, failed to show any staining for either PI(4,5)P_2 or MBP, highlighting the expected absence of both MBP and PI(4,5)P_2 on the outer cell surfaces. Similarly, no cross-reactivity of the respective secondary antibodies was observed when cells that had been incubated with either anti-MBP IgG antibody or anti-PI(4,5)P_2 IgM antibody were probed with AlexaFluor 488 goat antimouse IgM (μ chain) and AlexaFluor 594 rabbit antimouse IgG (H+L), respectively. While further investigation of the factors that influence these PI(4,5)P_2 domains is needed, particularly in living (not fixed) cells, the observed colocalization of MBP and PI(4,5)P_2 in this work suggests that MBP may be an additional element responsible for sequestering PI(4,5)P_2 into lateral domains in the internal leaflet of the plasma membrane of intact oligodendrocytes.

Biological Significance. Morphological cell processes, such as membrane ruffling and lamellipodial extension, are known to require regulated dynamic remodeling of actin-based cytoskeleton at the cell cortex. In this context, actin-based cytoskeleton networks, along with key regulators of actin cytoskeleton assembly such as Arp2/3, N-WASP, WAVE proteins, and the members of the Rho family of small GTPases, Rac, and Cdc42, have all been shown to accumulate at the leading edge of oligodendrocytes (*54*−*56*). How actin and many of the above signaling proteins are recruited here is not well understood. Accumulation of microdomains of PI(4,5)P_2 at the peripheral edges of cells, either through local synthesis and/or protein-driven retention

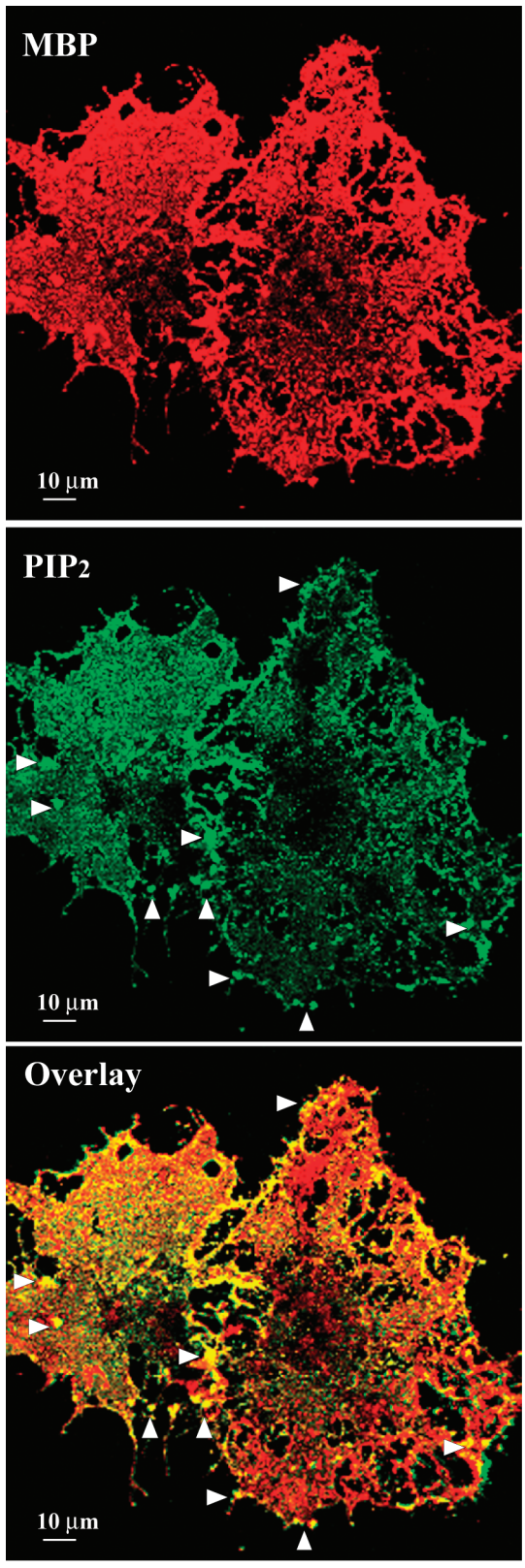

FIGURE 7: MBP colocalizes and accumulates with $PI(4,5)P_2$ at the peripheral plasma membrane of cultured rat oligodendrocytes. Immunofluorescence reactivity of MBP (red) and $PI(4,5)P_2$ (green) detected with affinity purified monoclonal anti-MBP IgG and anti-$PI(4,5)P_2$ IgM antibodies, followed by AlexaFluor 594 rabbit antimouse IgG (H+L) and AlexaFluor 488 goat antimouse IgM (μ chain) secondary antibodies, respectively.

and clustering, has been shown to promote recruitment and dynamic remodeling of actin cytoskeletons that give rise to changes in cell shape and motility (*12, 40, 57, 58*).

Central nervous system myelin is formed by the extension of membranous cellular processes from oligodendrocytes. The movement of the leading edge of these processes is driven first by actin polymerization (*59*), with subsequent stabilization by microtubule infiltration (*60*). It has been reported that an early developmental Golli-MBP isoform is associated with actin and involved in the formation of dorsal ruffles, cleavage furrows, and processes (*61*). The classic MBP isoforms studied here are prominent proteins in late developing and mature myelin, and have been shown to interact with cytoskeletal proteins under Ca^{2+}-bound calmodulin regulation, and in a manner modulated by post-translational modifications (e.g. refs *4*, *8*, *9*, *62*, and *63*). During myelinogenesis, a cytoskeleton-mediated event, MBP may function as an effector of process extension by stabilizing microfilaments.

But the classic 18.5 kDa MBP isoform is also membrane-associated and, in addition to linking actin (and potentially tubulin) to the oligodendrocyte membrane (*9, 48*), interacts so strongly with negatively charged lipids like phosphoinositides that it corrugates and rolls bilayers containing them (*22*). Phosphoinositides, in general, are an integral component of myelin metabolism, and undergo significant turnover (*64*). We have previously noted the strong similarities between MBP and other membrane-associated and actin-assembling proteins such as MARCKS (*10, 22*), and have here established a more specific link to $PI(4,5)P_2$ sequestration.

Although the amount of MBP or ratio to $PI(4,5)P_2$ in oligodendrocytes has not been quantified, MBP appears to be very abundant in these cells. It coats the cytosolic surface of the large membrane sheets of mature oligodendrocytes. In compact myelin, where MBP is 10% and PI is 0.56% of the dry weight (*65*), diphosphoinositides and triphosphoinositides (TPI) have been reported to be present at 36% and 49%, respectively, of the amount of PI (*66*). This allows calculation of a molar ratio of MBP to TPI in myelin of 1.5. The ratio of PI to PS, the major acidic lipid in oligodendrocytes, is higher in oligodendrocytes than in myelin (*67, 68*); thus, the amount of TPI in oligodendrocytes also may be higher than in myelin. However, MBP has a net positive charge of +19 at neutral pH, and so can bind several molecules of TPI. Thus, the amount of MBP in myelin, and probably also in oligodendrocytes, is sufficient to bind to virtually all of the $PI(4,5)P_2$ present, if it is not sequestered by other proteins. Therefore, it is probable that oligodendrocytes employ this protein as an additional modulator of the availability of plasmalemmal $PI(4,5)P_2$. This ability of MBP to sequester $PI(4,5)P_2$ may be linked to cellular functions outside its classically accepted role in myelin, particularly in cellular morphogenesis of oligodendrocytes during maturation, differentiation, and myelinogenesis in health and disease.

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