Classic 18.5- and 21.5-kDa Myelin Basic Protein Isoforms Associate with Cytoskeletal and SH3-Domain Proteins in the Immortalized N19-Oligodendroglial Cell Line Stimulated by Phorbol Ester and IGF-1

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Abstract

The 18.5-kDa classic myelin basic protein (MBP) is an intrinsically disordered protein arising from the Golli (Genes of Oligodendrocyte Lineage) gene complex and is responsible for compaction of the myelin sheath in the central nervous system. This MBP splice isoform also has a plethora of post-translational modifications including phosphorylation, deimination, methylation, and deamidation, that reduce its overall net charge and alter its protein and lipid associations within oligodendrocytes (OLGs). It was originally thought that MBP was simply a structural component of myelin; however, additional investigations have demonstrated that MBP is multi-functional, having numerous protein-protein interactions with Ca²⁺-calmodulin, actin, tubulin, and proteins with SH3-domains, and it can tether these proteins to a lipid membrane in vitro. Here, we have examined cytoskeletal interactions of classic 18.5-kDa MBP, in vivo, using early developmental N19-OLGs transfected with fluorescently-tagged MBP, actin, tubulin, and zonula occludens 1 (ZO-1). We show that MBP redistributes to distinct ‘membrane-ruffled’ regions of the plasma membrane where it co-localizes with actin and tubulin, and with the SH3-domain-containing proteins cortactin and ZO-1, when stimulated with PMA, a potent activator of the protein kinase C pathway. Moreover, using phospho-specific antibody staining, we show an increase in phosphorylated Thr98 MBP (human sequence numbering) in membrane-ruffled OLGs.

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Previously, Thr98 phosphorylation of MBP has been shown to affect its conformation, interactions with other proteins, and tethering of other proteins to the membrane in vitro. Here, MBP and actin were also co-localized in new focal adhesion contacts induced by IGF-1 stimulation in cells grown on laminin-2. This study supports a role for classic MBP isoforms in cytoskeletal and other protein-protein interactions during membrane and cytoskeletal remodeling in OLGs.

Keywords

Membrane ruffling; Focal adhesion contacts; Phorbol ester; IGF-1; Laminin-2; Cytoskeleton; Myelination; Live-cell imaging; TIRF microscopy; Confocal microscopy

Introduction

Oligodendrocytes (OLGs) are the myelinating cells in the central nervous system (CNS). In nearly all vertebrates, mature OLGs extend membrane processes, which concentrically wrap segments of the axon forming the multilamellar structure of myelin during late foetal and early postnatal stages [1–6]. The CNS myelin, although mostly composed of lipids, possesses many fundamental proteins which are essential for proper structural and functional integrity of the sheath (reviewed by [7]). Among these essential proteins are the myelin basic proteins (MBPs) [8–13], a family composed of developmentally regulated members arising from different transcription start sites of the Golli (Gene of Oligodendrocyte Lineage) complex, with further alternatively-spliced isoforms and combinatorial post-translational modifications [14]. ‘Classic’ MBP isoforms arise from transcription start site 3 and range in size from 14 kDa to the full-length 21.5-kDa transcript. They also undergo combinatorial post-translational modifications including phosphorylation and deimination of arginine, resulting in a number of charge components [11]. They adapt structurally and bind to a variety of different polyanionic proteins such as actin and tubulin, and also have been shown to have other important interactions with calmodulin and SH3-domain proteins such as Fyn kinase, the actin-polymerizing protein cortactin, and PSD-95 (presynaptic density protein 95) [8, 10–12, 15–18]. Although PSD-95, a membrane-associated guanylate kinase (MAGUK), is not known to be present in OLGs, its SH3-domain has 57% similarity to that of the MAGUK ZO-1 (zonula occludens 1) [19], which is present in OLGs and in peripheral myelin [20–22].

Investigations have shown that all MBPs including the 18.5-kDa isoform can polymerize and bundle G-actin and F-actin in solution, and bind actin filaments and bundles to lipid vesicles [17, 23–26]. Moreover, purified recombinant and bovine sources of MBP have been demonstrated to polymerize and bundle tubulin in vitro [27], and to bind them to actin filaments and to lipid vesicles [26]. Classic MBP has also been shown to stabilize microtubules from depolymerizing in the cold in vitro, and in cultured OLGs [28, 29]. In cultured OLGs isolated from the *shiverer* mutant mouse, in which MBP is lacking, the microtubules and actin filaments were abnormal in size and distribution, and production of processes and membrane sheets was abnormal [30]. These studies support an important role for interaction of MBP with the cytoskeleton in OLG development and myelination [31–33]. The 18.5-kDa MBP splice isoform has also been shown to bind the SH3-domain of Fyn to

lipid vesicles [34]. To date, these in vitro biochemical techniques have added to our understanding of MBP's interactions with other proteins in vitro. However, there have been limited investigations performed to examine these protein-protein interactions within living cells and their physiological role, although we have shown recently that MBP transfected into immortalized N19-OLG cells was co-localized with Fyn [35]. The association of MBP with Fyn was dependent on the integrity of the SH3-domain ligand in MBP, as has also been demonstrated for their interaction in vitro by solution NMR spectroscopy [36].

Here, we treat N19-OLG cells with the phorbol ester phorbol-12-myristate 13-acetate (PMA) and with IGF-1 to induce cytoskeletal rearrangement to examine the interactions between MBP and the cytoskeletal proteins actin and tubulin, and the SH3-domain proteins, cortactin and ZO-1, in cells. The N19-OLGs were transfected with fluorescently-tagged recombinant versions of classic murine 18.5- and 21.5-kDa MBP isoforms, and with the pseudo-deiminated form MBP-C8 with six R/K-to-Q substitutions to mimic the deiminated 18.5-kDa C8 charge component [37], and fluorescently-tagged recombinant actin, tubulin, and ZO-1.

Using both fixed and live cells, we have found that the two different recombinant 18.5-kDa charge components (MBP-C1, the unmodified form, and MBP-C8, the pseudo-deiminated form), and the full-length 21.5-kDa form, of classic MBP become enriched in distinct membrane-ruffled regions at the cell cortex and associate with β - and γ -actin, cortactin, α -tubulin, and ZO-1 following PMA-stimulation. The MBP-C1 variant also was co-localized with actin in new focal adhesion contacts induced by IGF-1 in cells grown on the extracellular matrix protein, laminin-2. These active areas of membrane ruffling and focal adhesion are areas of dynamic cytoskeletal assembly and disassembly. These results demonstrate that MBP may participate in and/or mediate cytoskeletal protein-protein interactions during membrane and cytoskeletal remodeling in OLGs [8–10, 12].

Materials and Methods

Plasmid Construction

We constructed plasmids coding for RFP-tagged versions of classic MBP variants possessing a 3' UTR (untranslated region) namely, pERFP-C1-rmMBPC1-UTR, pERFP-C1-rmMBPC8-UTR, and pERFP-C1-rmMBP21.5-UTR, which were used throughout these investigations, and which have been previously described in detail [38]. (*n.b.*, The "C1" of the RFP vector designation is not to be confused with the "C1" charge component of MBP, the latter which will be the primary usage throughout this paper).

The pAcGFP1- α -tubulin vector was purchased from Clontech (Mountain View, CA), whereas GFP-tagged β - and γ -actin were constructed using recombinant DNA techniques. The pAcUW51 expression vector containing full-length templates for β - and γ -actin were a kind gift from Dr. John Dawson (University of Guelph), and restriction enzymes and reagents were purchased from New England Biolabs (Mississauga, ON), Fisher Scientific (Nepean, ON, Canada), and Stratagene (La Jolla, CA). Full-length β - and γ -actin DNA was amplified using a common reverse primer ActRp 5' - ATATGGATCCTCAGAAGCACTTGCGG-3' and the primers β -ActFp 5' -

ATATCTCGAGATGGATG ACGATATC-3' and γ -ActFp 5'-ATATCTCGAGATGGAA GAAGAAATCG-3' for β - and γ -actin DNA, respectively. Polymerase chain reaction (PCR) amplifications were performed using a BioRad thermal cycler PCR system using *Pfu* Ultra Polymerase (Stratagene, CA) with the following cycling parameters: initial denaturing temperature of 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, followed by a final 4°C hold. These primers introduced *Xho*I and *Bam*HI restriction sites to the 5' and 3' ends of both amplified products, which were subsequently digested with corresponding enzymes and ligated into the pEGFP-C3 vector to produce pEGFP-C3- β -actin and pEGFP-C3- γ -actin.

For small-scale plasmid DNA extractions, the Roche High Pure Plasmid isolation kit (Roche Diagnostics, IN) was used, and positive clones were confirmed by restriction digests and sequencing (Laboratory Services, Guelph, ON). For transfection experiments requiring larger quantities of DNA, the PureLink HiPure Plasmid Purification kit (Invitrogen Life Technologies, Burlington, ON) was used. Other remaining reagents used for these studies were purchased from Sigma-Aldrich unless otherwise stated.

The pGEX2T-ZO-1-SH3 plasmid, coding for amino acids 496–579 which encompass the SH3-domain of ZO-1 linked to GST, was a kind gift from Dr. Maria Balda (University College London, UK) [19]. The pGFP-ZO-1 plasmid encoding full-length ZO-1 fused to GFP was a kind gift from Dr. Heidi Wunderli-Allenspach (Institute of Pharmaceutical Sciences, ETH-Zürich) [39].

GST Purifications and Binding Assays

The pGEX2T-ZO-1-SH3 plasmid (encoding amino acids 496–579 encompassing the SH3-domain of ZO-1 linked to GST) and pGEX2T-empty plasmid vectors were transformed and were expressed in *E. coli* BL21-Codon-Plus(DE3)-RP cells (Stratagene, La Jolla, CA) containing appropriate antibiotics. A 10 mL overnight culture grown at 37°C was back-diluted the next day into 1 L of 2xYT (yeast-tryptone) media containing appropriate antibiotics. These new cultures were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at an $A_{600\text{nm}}$ ~0.7–0.8, and were grown for an additional 3 h. Cells were harvested by centrifugation, and the pellet was frozen at +20°C, and was subsequently resuspended in 25 mL lysis buffer (300 mM NaCl, 50 mM TRIS base, 5% glycerol, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine), containing 0.3% v/v sodium lauroyl sarcosinate. The lysis buffer containing the cell pellet was homogenized and stirred at 4°C for 1 h, and was subsequently centrifuged at 15,000 $\times$ g prior to loading on a pre-equilibrated 4 mL bead volume column packed with glutathione-Sepharose beads (GE Healthcare Life Sciences, Montréal, QC). Crude lysate was loaded onto the column and was washed with 45 mL of lysis buffer (without sodium lauroyl sarcosinate), and was then eluted in 7 mL of 40 mM glutathione resuspended in ddH₂O. Eluted purified protein was dialyzed twice against a 2 l volume of ddH₂O at 4°C using tubing with a M_r cutoff of 6,000–8,000 Da. The final product was filtered through a 0.45 μ m membrane (Pall Life Sciences, Mississauga, ON), frozen, and was lyophilized prior to experimentation.

The hexahistidine-tagged recombinant murine 18.5 kDa isomer representing the unmodified C1 charge component (rmMBP-C1) was expressed and purified by nickel-chelation chromatography as described before, with minor modifications [40].

For GST-pull-down assays, both GST and GST-ZO-1-SH3 were resuspended in phosphate-buffered saline (PBS) at similar concentrations, and 0.7 mg and 0.1 mg of each protein, respectively, were added to a slurry (100 μ l) of pre-equilibrated glutathione-Sepharose beads, respectively. A 7-fold decreased amount of GST-ZO-1-SH3 was used compared to GST, to illustrate further the higher binding affinity of 18.5-kDa MBP (viz., rmMBP-C1) to ZO-1-SH3. This mixture was incubated with 100 μ g of MBP for 2 h at 4°C, and was then washed 5 times with 1 mL PBS. The protein complexes were eluted from the beads using 35 μ l of 40 mM glutathione resuspended in ddH₂O. Equal volumes (17.5 μ l) from each reaction were analyzed using SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and Coomassie blue staining.

Cell line Culture and Transfection

Tissue culture reagents were purchased from Gibco/Invitrogen (Invitrogen Life Technologies, Burlington, ON). The FuGene HD transfection reagent was purchased from Roche (Roche Diagnostics, IN). The N19 immortalized oligodendroglial cell line was grown in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (foetal bovine serum) and 1% penicillin/streptomycin, and cultured in 10 cm plates at 34°C/5% CO₂. At 70–80% confluency (4–7 days), cells were detached using 0.25% trypsin for 5 min, and were seeded onto 2 cm plates containing a glass coverslip. Cells were grown overnight to a confluency of 15% prior to transfection using 100 μ l serum-free media, 0.75–3.0 μ g of plasmid DNA, and 4 μ l of FuGene HD (Roche Diagnostics, IN). The DNA was allowed to complex for 5 min at room temperature, and was directly added to cells following incubation. Cells were cultured for an additional 48 h at 34°C prior to treatment, fixation, or immuno processing.

Immunofluorescence, Epifluorescence, and Confocal Microscopy and Image Analyses

Following protein expression and treatment with reagents, cells were directly fixed using a 4% formaldehyde solution in PBS for 15 min with gentle rocking, or used for live-cell imaging as described below. For induction of membrane ruffles, PMA—an agonist of protein kinase C (PKC)—was used. The PMA was added to a final concentration of 250 nM, and cells were incubated for 5 min with gentle rocking. Following incubation, cells were fixed and mounted as previously described [38]. Samples requiring immunoprocessing were permeabilized using 0.1% v/v Triton X-100 for 20 min, and were subsequently washed once with 1 mL of PBS. Slides were blocked for 1 h using 10% normal goat serum (NGS) and, following this incubation, the primary antibody was added and incubated for an additional hour. The slides were then washed three times with 1 mL PBS, and the secondary antibody (1:400 dilution) was applied for 20 min. Once again, the slides were washed four times with 1 mL of PBS, and were mounted using ProLong Gold AntiFade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Slides were viewed using either a Leica epifluorescence microscope (DMRA2), or a multiphoton scanning confocal microscope (Leica DM6000 TCS SP5). Images were processed and analyzed using ImageJ software

(National Institutes of Health—NIH, <http://rsb.info.nih.gov/ij/>), and were compiled using Adobe Photoshop CS3. The following antibodies were obtained from commercial sources: rabbit polyclonal anti-cortactin (1:10 dilution) (Cell Signaling technology Cat#3502), mouse monoclonal anti-phospho-Thr97 (1:400 dilution) (Millipore). For three-dimensional digital reconstructions, image sets of 0.3 μ m optical serial sections of individual cells expressing both MBP and other transfected proteins were acquired using a Leica DM6000 TCS SP5 microscope and digital reconstruction for three-dimensional images was performed using NIH ImageJ software [41, 42].

Live-cell Imaging

For imaging live samples, similar procedures for cell culture and transfection were employed as previously described, with the following modifications. Standard glass coverslips were substituted with #1.5–25 mm glass coverslips (Warner Instruments, CT) and were transferred to a Chambridge CMB magnetic culture chamber (Quorum Technologies, Guelph, ON). Cultures were grown in phenol-free DMEM high-glucose media, supplemented with 10% FBS and 1% penicillin/streptomycin at 34°C/5% CO₂. Cells were imaged using a Quorum WaveFX spinning disc microscope, and PMA-induced membrane ruffling was stimulated after establishing a steady baseline. Images were acquired at 10 s intervals and, following acquisition, images were processed and analyzed using standard routines written for NIH ImageJ software. Images and movies demonstrating co-localization for the red and green channels show co-localization as yellow or white false-colored pixels.

For TIRF microscopy, cells were grown on round glass coverslips (#1.5–18 mm, Warner Instruments). Transfected cells were treated with 250 nM PMA as above. For treatment with IGF-1, the coverslips were acid-washed and coated with poly-L-Lysine (30–70 kDa, Sigma) by overnight incubation in a 0.10 mg/mL solution of poly-L-Lysine HBr in PBS at room temperature. They were then rinsed with sterile water and air-dried. The coverslips were further treated with laminin-2 by incubation in a 10 μ g/mL laminin-2 solution for 3–4 h in an incubator set at 37°C with 5% CO₂ [43]. The laminin-2 (derived from human placenta, Sigma) was supplied as a concentrated solution in 50 mM TRIS buffer at pH 7.4, and was diluted to the required concentration in PBS. Following treatment with laminin-2, the coverslips were rinsed with sterile water and were then seeded with N19 cells. Cells were co-transfected 24 h later with RFP-MBP and β -actin-GFP plasmids, respectively, as described above.

Transfected cells were imaged by TIRF microscopy before and after addition of 250 nM PMA or 50 ng/mL IGF-1 to the cells on the microscope stage at 34°C. The IGF-1 solution was prepared by initially dissolving the protein desiccate in water to a concentration of 1 mg/mL, and then diluting it to a working concentration of 10 μ g/mL (in 50 mM TRIS buffer, pH 8, containing 0.1% bovine serum albumin (BSA)). The TIRF microscope images of live cells were acquired with an Olympus IX81 inverted microscope equipped with an objective-based motorized cell^{TIRF} illuminator (Olympus) coupled with 491 nm and 568 nm lasers. The motorized cell^{TIRF} illuminator allows a smooth transition from epifluorescence to TIRF mode, and enables control over parameters used for obtaining TIRF illumination. The critical angle required for TIRF was achieved by off-the-axis illumination of the lasers at the

back focal plane of a high numerical aperture (1.49) 60 × objective (Olympus). The cells were illuminated at an angle (66.86°) exceeding the critical angle (63.86°) for the coverslip-cell interface used. Images were recorded as 16-bit grayscale, with no binning and no averaging, and were of dimensions 512 × 512 pixels. The images were acquired with a cooled high-quantum-efficiency EMCCD (Quant EM:512SC) camera using the image acquisition software Metamorph (Version 7.7).

Time-lapse TIRF images with exposure times less than 50 ms were recorded prior to, immediately after, and at 5 min intervals up to 20 min after addition of 250 nM PMA, or 50 ng/mL IGF-1, while maintaining the cells at 34°C (ambient temperature for the cells). The acquired images were analyzed with ImageJ software. Correlation analysis was done by comparison of the intensity values due to RFP-MBP and β -actin-GFP along a line drawn across a region where changes in their distribution occurred after PMA or IGF-1 treatment.

Results

Classic 18.5- and 21.5-kDa MBP Isoforms Localize to Membrane-ruffled Regions of N19-OLGs and Co-localize with β - and γ -Actin Following Stimulation with PMA and IGF-1

The conditionally-immortalized N19 oligodendroglial line was chosen as a relevant stage-specific cell line to investigate MBP-cytoskeletal interactions in tissue culture [44]. This cell line stains positive for both the NG2 and A2B5 antibodies, which are markers for oligodendroglial progenitor cells (OPCs), and also lacks expression of classic MBP and proteolipid protein mRNAs [45]. This cell line has previously been used to examine the effects of apotransferrin over-expression on OLG differentiation, elaboration of neurite cell processes in co-culture with neurons, and more recently, the effects of golgi and classic MBP over-expression on calcium homeostasis and cell migration [7, 38, 46–48]. Another interaction of classic MBP that has been studied in N19 cells is that with SH3-domain-containing proteins such as Fyn, where we have demonstrated that disruption and/or pseudo-phosphorylation of the SH3-ligand in MBP has significant effects on cell morphology, and on the trafficking of the protein [35]. We first co-expressed and examined RFP-MBPs, and GFP-tagged versions of β - and γ -actin, to assess their localizations in N19-OLGs under basal conditions, with no stimulation. All MBP constructs had a 3'UTR to facilitate proper intracellular targeting [38, 49, 50]. The classic MBP variants that were compared here were those representing the unmodified 18.5-kDa charge component C1, the pseudo-deiminated 18.5-kDa charge component C8, and the full-length 21.5 kDa transcript containing exonII (classic exon numbering), that we have previously used [35, 38].

Under basal conditions following 48 h of expression, 18.5-kDa MBP-C1 and MBP-C8 variants show some areas of co-localization with actin along the extending membrane processes, and within clustered regions of vesicle-like structures throughout the peri-nuclear region and cell soma (Fig. 1a). The 21.5-kDa MBP isoform is predominantly in the nucleus, as observed previously [38, 51, 52]. Overall, the morphology and phenotype of the N19-OLGs appeared to be similar to those of the non-transfected neighboring cells, indicating that the co-expression of both tagged-proteins was not cytotoxic and did not cause changes in cell phenotype.

Next, we treated the cells with PMA, an agonist of protein kinase C (PKC), to stimulate reorganization of the cytoskeleton in N19-OLGs expressing RFP-tagged variants (charge components and splice isoforms) of MBP. Such PMA-induced membrane ruffling has been employed in other investigations, which have demonstrated its ability to change the properties and appearance of the plasma membrane and rearrangement of the actin cytoskeleton in OLGs and other cells [53–55]. In this study, membranous protrusions or ruffles were seen immediately on introducing PMA into the cell medium, and often but not always disappeared within a few minutes (see below). Here, when N19-OLGs were exposed to a 5 min treatment of PMA at a final concentration of 250 nM, we found that MBPs were enriched in the dorsal and cortical membrane ruffles of the N19-OLG plasma membranes (Fig. 2a). The PMA-treatment induced redistribution of some of the 21.5-kDa MBP from the nucleus to the cytosol where it also was enriched in membrane ruffles (Figs. 1b, 2a). Previously, we have observed that the full-length classic 21.5-kDa MBP is predominantly nuclear-localized (*cf.*, [35, 38]), so its re-translocation to the cytosol observed here is a newly-observed phenomenon.

The PMA-stimulation of PKC in primary OLGs has been shown to result in activation of mitogen-activated protein kinase (MAPK) [55]. Using a phospho-specific antibody toward Thr98 (human 18.5-kDa sequence; bovine Thr97; murine Thr95) of MBP, an identified MAPK [56] and GSK-3 β [57] phosphorylation site, we found that there was an overall increase in Thr98-phosphorylated MBP-C1 in PMA-stimulated N19-OLGs, including at ruffling sites (Fig. 2b).

The PMA treatment induced a significant increase in co-localization of all MBP variants with β - and γ -actin in both cortical and dorsal membrane ruffles in N19-OLGs co-expressing MBP and β - or γ -actin (Fig. 1b, Figures S1, S2). We also observed a reduced number of actin stress fibers in N19-OLGs following PMA treatment due to cytoskeletal reorganization. These results are supported by time lapse live-cell imaging over a 12 min time course following treatment with PMA (Fig. 3). The cells responded rapidly following treatment, and membrane processes and ruffles enriched in MBP and actin extended and retracted, suggesting dynamic changes in polymerization and depolymerization within membrane-ruffled regions at the periphery of the cell and throughout the cell body (Supplementary Information—Videos 1 and 2). Further time course analyses for each dataset, using routines written for NIH ImageJ software, show MBP and β -actin co-localized within newly forming ruffles in Fig. 3a. Significant regions of co-localization are highlighted in Fig. 3b.

We also examined the interaction of MBP-21.5-kDa with β -actin following treatment with PMA. The MBP-21.5-kDa isoform also significantly co-localized with actin in membrane-ruffled regions, and also within areas of the cell undergoing rapid actin polymerization and depolymerization following treatment with PMA (Fig. 1b; Supplementary Information—Video 3).

Here, TIRF microscopy of live cells was used in addition to confocal microscopy to look for coordinated changes in the distribution of β -actin-GFP and 18.5-kDa RFP-MBP-C1-UTR at the cell membrane after exposing cells to 250 nM PMA to induce membrane ruffling or

process formation, or 50 ng/mL IGF-1 to induce formation of focal adhesion contacts to laminin-2 [58, 59]. The advantage of using TIRF microscopy is its superior optical sectioning capability (of the order of 100 nm) as compared to that afforded by a confocal microscope (~600 nm). For this reason, this technique has found widespread use in the study of membrane and cytoskeletal dynamics, protein trafficking, and endo- and exocytosis [60–63]. In TIRF microscopy, a very thin section of the cell membrane closest to the glass coverslip is excited with a weak evanescent beam generated by critical angle illumination of a laser beam with respect to the interface between the glass coverslip and the cell. This evanescent beam penetrates a short distance beyond the interface (theoretically 70–300 nm) and decays exponentially with distance from it. This property allows selective excitation of fluorophores in the region close to the interface and is, therefore, useful for studying cytoskeletal changes occurring at the adherent cell membrane.

Cells were imaged prior to introducing 250 nM PMA (Fig. 4a, b, j, k), immediately after (Fig. 4d, e, m, n), and 5 min after (Fig. 4g, h, p, q). Membranous protrusions or ruffles were seen immediately on introducing PMA into the cell medium (Fig. 4d, e, m, n). These membranous protrusions were transient in most cases, disappearing within 5 min of introducing PMA (Fig. 4g, h). In some cases, however, they persisted (Fig. 4p, q). Since all major changes occurred within 5 min of introducing PMA, images acquired beyond 5 min are not shown in Fig. 4. Since ruffling is associated with reorganization of the actin cytoskeleton, the acquired images were analyzed to determine changes in the distribution of β -actin and 18.5-kDa MBP-C1. Correlation analysis of the images was carried out using ImageJ software, focusing on the regions showing changes in the distribution of MBP-GFP and β -actin-RFP. In cell #1 (Fig. 4a–i), the transient appearance of membrane ruffles was associated with the immediate appearance of a peak in the intensity traces corresponding to both β -actin and 18.5-kDa MBP-C1 (Fig. 4f). In cell #2 (Fig. 4j–r), production of ruffles was marked by the immediate appearance of two peaks in the intensity trace (Fig. 4o) corresponding to both actin and MBP-C1, whose localization in the ruffles was strongly correlated following PMA treatment. These peaks persisted for at least 5 min (Fig. 4r).

Insulin-like growth factor 1 (IGF-1) is a neurotrophic factor in the CNS that acts through both autocrine and paracrine signaling [43]. IGF-1 increases cell motility, membrane ruffling, and focal adhesion contacts to extracellular matrix proteins through rapid reorganization of the cytoskeleton in neuronal and glial cells [58, 59]. It has been shown to enhance differentiation of primary OLGs when the cells were grown on a laminin-2 substrate via laminin-dystroglycan interactions [43]. Signaling changes were maximal within 20 min of IGF-1 treatment (*ibid.*).

To stimulate focal adhesion contacts and cytoskeletal reorganization at the adherent membrane surface, N19 cells co-expressing β -actin-GFP and 18.5-kDa RFP-MBP-C1 were grown on laminin-2-coated coverslips and stimulated with 50 ng/mL IGF-1. Here, Fig. 5 shows TIRF microscope images for two cells acquired before (Fig. 5a, b, j, k) and after treating cells with IGF-1 (Fig. 5d, e, m, n after 5 min; Fig. 5g, h, p, q after 20 min). The cells on exposure to IGF-1 showed changes in the distribution of β -actin-GFP and 18.5-kDa RFP-MBP-C1 as revealed by new peaks of β -actin-GFP and RFP-MBP-C1 intensity at certain locations in the cells (Fig. 5f, o, compared to Fig. 5c, l, respectively). The changes occurred

immediately on introducing IGF-1 (Fig. 5d, e, m, n) and many persisted to the end of the measurements, viz., 20 min (Fig. 5g, h, p, q). Correlation analysis of the images by ImageJ software showed that the new peaks of β -actin-GFP and RFP-MBP-C1 intensity that appeared and disappeared with time were strongly correlated with each other after IGF-1 treatment (Fig. 5f, i, o, r).

These increases in co-localization of 18.5-kDa MBP-C1 with actin during cytoskeletal rearrangement suggest that classic MBP isoforms may have direct or indirect interactions with actin in OLGs, supporting conclusions derived from in vitro studies. They indicate that MBP is actively recruited and enriched within membrane-ruffled and focal adhesion contact regions where it is likely to participate along with actin during membrane remodeling.

MBPs Co-localize With the Actin-binding Protein Cortactin in PMA-Stimulated N19-OLGs

The 18.5-kDa isoform of MBP binds to the SH3-domain of the actin-binding protein cortactin [16]. Here, we immunostained PMA-stimulated N19-OLGs for cortactin, to examine whether it is complexed in the membrane-ruffled structures along with MBP and actin. In control cells untransfected with MBP constructs, cortactin had a heterogeneous, punctate distribution in unstimulated cells, whereas it showed increased fluorescence in ruffling regions of PMA-stimulated N19-OLGs (Fig. 6a, Figures S1, S2). Cortactin is known to play a role in regulation of actin dynamics in cell lamellipodia and ruffles [64]. Here, N19 cells over-expressing classic MBP variants, and stimulated with PMA, showed significant increases in co-localization of MBP with cortactin within membrane-ruffled regions around the cortex of the plasma membrane (Fig. 6b, Figures S1, S2). The minimally sufficient region of MBP for actin polymerization is contained in a short peptide region, amino acids (A24-K58) (human 18.5-kDa sequence numbering, murine A22-K56), within its N-terminus [17, 18]. The co-localization of classic MBP with cortactin observed here suggests that, in addition to interactions of MBP with actin via its N-terminal region, its SH3-binding domain may mediate further protein-protein interactions with actin remodeling proteins, such as cortactin, that may contribute towards cytoskeletal arrangement during OLG development and process extension.

Classic MBP Isoforms Co-localize with α -Tubulin in Membrane Ruffles of PMA-Stimulated N19-OLGs

The GFP- α -tubulin was expressed in N19-OLGs, and showed some enrichment in the periphery of the cell processes following PKC activation using PMA (Fig. 7, Figures S1, S2). Following 48 h incubation, unstimulated cells co-expressing 18.5-kDa MBP-C1 and MBP-C8, but not the full-length splice variant MBP-21.5, show some areas of co-localization along the extending membrane processes and throughout the cell body. Upon PMA-induced PKC stimulation, we found that there was an increase of all three MBP variants and α -tubulin in ruffles, and increased co-localization of MBP and microtubules in membrane processes of N19-OLGs (Fig. 7, Figures S1, S2). These increases were also shown by live-cell imaging (Fig. 8; Supplementary Information—Videos 4 and 5). These data suggest that MBP-tubulin interactions may also play a role in cytoskeletal rearrangement when the PKC pathway is activated.

Taken as a whole, these data support the conclusion that classic MBP isoforms are associated with the OLG cytoskeleton, and could play a dynamic role in cytoskeletal modification in PMA-activated N19-OLGs. They further suggest that through its interactions with cytoskeletal proteins, MBP bound to sites on the cytosolic leaflet of the plasma membrane may be involved in process extension or retraction during myelination.

MBP Binds ZO-1-SH3 In Vitro, and Co-localizes In Vivo with ZO-1 at the Plasma Membrane in N19-OLGs

Previously, MBP was predicted to bind to ZO-1 based on its binding to PSD-95 on an SH3-domain array [16]. Here we show that ZO-1-SH3 and 18.5-kDa MBP (specifically, rmMBP-C1) bind in vitro using a protein pull-down assay (Fig. 9a). Although the GST-ZO-1-SH3 was purified from a glutathione-Sepharose column with over 95% purity (not shown), little GST-ZO-1-SH3 was eluted from the column with glutathione in the presence of rmMBP-C1. However, more rmMBP-C1 was eluted from the GST-ZO-1-SH3 column than from glutathione-Sepharose in the presence or absence of GST only, indicating that rmMBP-C1 bound to the GST-ZO-1 on the column. Using confocal imaging, it can be seen that, under resting conditions, 18.5-kDa MBP-C1 and ZO-1 are partially co-localized in N19-OLG membrane processes. Moreover, stimulation with PMA results in increased co-localization of MBP and ZO-1 at the membrane surface and in ruffles (Fig. 9b, Figures S1, S2). This was also shown by live cell imaging (Fig. 9c; Video 6, Supplementary Information). This is the first direct evidence that MBP binds to the SH3-domain of ZO-1, and that it is associated with ZO-1 in N19-OLGs.

Discussion

Myelin basic protein appears to be a multi-functional protein which may serve, *inter alia*, as a scaffolding structure that tethers the cytoskeleton to the cytoplasmic surface of the plasma membrane [8, 10, 12]. As in neurons, movement of the leading edge of OLG membrane processes is driven by actin polymerization, with the microtubules located proximal to the actin network. The actin filaments precede the microtubules into the leading edge, and provide tracks for the microtubules to invade regions of new growth [65]. Associated microtubule-associated proteins (MAPs) such as tau link them [31–33, 66, 67].

Oligodendrocytes in culture produce membrane sheets containing major veins and a lacy network of cytoskeletal proteins, where actin filaments and microtubules are co-localized [68]. MBP is co-localized with these cytoskeletal veins of actin filaments and microtubules, and also with cortical actin filaments in cultured OLGs. MBP has also been shown to be important for formation of the cytoskeleton and for stabilizing microtubules in the cold in primary OLGs [29, 30]. In OLGs from the *shiverer* mutant mouse lacking MBP, the microtubules and actin filaments were abnormal in size and distribution, and production of processes and membrane sheets was abnormal, indicating a role for MBP-cytoskeletal interactions for myelination in vivo [30].

Numerous previous studies have shown in vitro that MBP can polymerize both actin and tubulin, and can crosslink actin filaments and microtubules to each other and tether them to a membrane surface [23, 24, 26, 27, 69, 70]. Post-translational modifications (deimination,

deamidation, phosphorylation, etc.) resulting in net charge reduction modulate these diverse interactions. Classic MBP isoforms also have a PXXP motif which is a ligand for SH3-domains, and have been shown to bind to several proteins with SH3-domains and tether the SH3-domain of Fyn to a membrane surface in vitro [16, 34–36]. The phosphorylated form of MBP is enriched along with cytoskeletal proteins and kinases including MAPK, Fyn, and Lyn within insoluble Triton-X100 membrane domains when extracted from myelin [67, 69, 70]. These detergent-insoluble membrane domains from myelin also contain the radial component consisting of a series of tight junctions between myelin layers ([71, 72], see also [73]).

In the present study, we have used immortalized N19 cells transfected with fluorescently-tagged MBP, actin, tubulin, and ZO-1, combined with immunostaining of cortactin, to show that MBP and cytoskeletal proteins and the SH3-domain proteins, cortactin and ZO-1, are enriched in the ruffles and processes induced in these cells by stimulation with PMA. We also showed earlier that Fyn was enriched in these ruffles [35]. In our experience, transfection of primary OLG cultures with various classic MBP constructs such as those described here results invariably in considerable cell death and inconsistent targeting of the protein. In contrast, the N19-OLG cultures have proved to be robust to such manipulation, and have provided evidence for the interaction of classic MBP variants with specific protein partners. We acknowledge that there could be other proteins expressed by more developmentally-mature OLGs (and not in the N19-OLG cell line) that may associate with classic MBP isoforms, and that are worthy of subsequent investigation.

Here, dynamic changes in co-localization of classic 18.5- and 21.5-kDa MBP forms with actin, tubulin, and with ZO-1 were observed to occur during membrane ruffling, with enrichment of these proteins in the membrane ruffles, using time-lapse microscopy of live cells. We also showed that changes in organization of actin induced by IGF-1 in focal adhesion contacts were associated with redistribution of 18.5-kDa MBP to the same regions. These studies support our conclusion that the interactions of MBP with these proteins observed previously in vitro also occur in N19-OLGs. In a separate study, we have shown that mutation of the PXXP ligand in MBP for SH3-domains inhibited binding to SH3-domain proteins and increased the length of membrane processes and branching complexity in N19-OLG cultures [35]. Thus, interactions of classic MBP isoforms with these other SH3-domain-containing proteins may play a physiological role in OLGs and myelin.

The PMA-stimulation also induced phosphorylation of MBP at Thr98, the site phosphorylated by both MAPK and GSK-3 β [56, 57]. (This threonyl residue in the human 18.5-kDa MBP sequence corresponds to residues Thr95/Thr97 in the murine/bovine 18.5-kDa MBP sequences, respectively [11, 15, 35, 74]). The PMA-stimulation activates PKC, which is upstream from MAPK [55]. The phosphorylated form was also enriched in membrane ruffles; the importance of phosphorylation of this threonyl residue is reviewed in references [8, 10–12, 15, 74, 75]. This site is modified during propagation of action potentials [76], and is correlated with healthy myelin, being decreased in myelin from multiple sclerosis patients [77]. This phosphorylated form is associated with non-compact myelin and detergent-insoluble membrane microdomains (“lipid rafts”) [69, 73, 78, 79]. Phosphorylation protects the protein from proteases such as trypsin [80], and has potential

structure-stabilizing effects [74, 81, 82]. The MAPK phosphorylation of MBP decreases its ability to bundle actin filaments and to bind actin filaments, microtubules, and the SH3-domain of Fyn to a lipid membrane [26, 34, 83]. Phosphorylation at these sites may induce a conformational change of this segment of the protein and its disposition with respect to the membrane surface [74, 75]. It also reduces the ability of MBP to neutralize the net negative charge of actin filaments, microtubules, and SH3-domain proteins.

Classic MBP is also modified in vivo by enzymatic deimination of several arginine residues to citrulline, an uncharged amino acid [84]. The deiminated 18.5-kDa C8 form is increased in myelin from children and also from adult multiple sclerosis patients [85], suggesting that it could have both physiological developmental and pathological roles. Although the deiminated form does not bind actin filaments, microtubules, and the SH3-domain of Fyn to a membrane surface as well as the unmodified 18.5-kDa C1 form [23, 34, 86], here it co-localized similarly with other proteins in PMA-stimulated N19-OLGs as the unmodified form did. The full-length classic 21.5-kDa isoform redistributed to the cytosol in PMA-stimulated N19-OLGs and also co-localized with actin, tubulin, and cortactin in membrane ruffles. Although it has been shown previously that PMA inhibited the translocation of 21.5-kDa MBP into the nucleus of HeLa cells [51], this is the first demonstration that it can induce redistribution of this MBP isoform (which contains a 26-residue insertion expressed by classic exonII) from the nucleus to membrane ruffles of oligodendroglial cells. This relocation may account for its presence in myelin, where the content of exonII-containing isoforms in the radial component is higher than that of isoforms lacking exonII [87].

Many myelination events, such as OL process extension, membrane sheet formation, and ensheathment of the axon depend on dynamic changes in the cytoskeleton [31–33, 65, 88, 89] and classic MBP-cytoskeleton interactions may help regulate its dynamics (reviewed in [8, 10–12]). We suggest that the MBP-mediated tethering of the cytoskeleton to the OLG membrane may be involved in regulation of process extension and axonal ensheathment.

Finally, ZO-1 is a scaffold protein associated with integral and adaptor proteins of tight junctions and gap junctions, and with numerous signaling proteins and the cytoskeleton. It is present in OLGs associated with the connexins of OLG gap junctions [20, 21] and in non-compact regions of peripheral nerve myelin [22], and may be part of the tight junctions forming the radial component of CNS myelin [20]. Tight junctions are important selective barriers that regulate the paracellular pathway for the movement of ions and solutes between cells [90, 91]. Gap junctions form between OLGs, and between OLGs and astrocytes, and allow electrical coupling between the cells [92]. Interactions of these junctions with the cytoskeleton may regulate cytoskeletal dynamics, and conversely, the cytoskeleton may regulate junctional activity [93]. Interactions of classic MBP isoforms with the cytoskeleton, ZO-1, and other SH3-domain proteins such as Fyn may allow it to participate in regulation of junctional activity. These results support a multi-functional role for classic MBP in OLGs [8, 10, 12].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
GFP	Green fluorescent protein
Golli	Gene of oligodendrocyte lineage
IGF-1	Insulin-like growth factor-1
MBP	Myelin basic protein
MAGUK	Membrane-associated guanylate kinase
NGS	Normal goat serum
OLG	Oligodendrocyte
OPC	Oligodendroglial progenitor cell
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PNS	Peripheral nervous system
PSD-95	Post-synaptic density protein of 95 kDa
RFP	Red fluorescent protein
TIRF	Total internal reflection fluorescence
UTR	Untranslated region
ZO-1	Zona occludens 1

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Fig. 1.

Fluorescence micrographs of cultured N19-OLGs 2 days post-transfection expressing different classic RFP-tagged MBP variants (*red*), and GFP-tagged versions of β - and γ -actin with nuclei counterstained with DAPI (*blue*). **a** As a control, untreated cells were fixed and viewed prior to experiments with PMA to assess that co-expression of these proteins did not have any effects on cell morphology. Moreover, the transfected N19-OLGs appeared to have similar morphology to that of neighboring, untransfected cells when viewed by phase contrast microscopy (not shown). **b** The N19-OLGs 2 days post-transfection expressing different variants of classic RFP-MBP isoforms (*red*) along with either GFP- β -actin or GFP- γ -actin, following a 5 min stimulation using PMA, with nuclei counterstained with DAPI (*blue*). The merged images of stimulated N19-OLG cells expressing classic RFP-MBP

variants, and GFP- β -actin or GFP- γ -actin isoforms, show areas of co-localization within membrane-ruffled regions around the cell cortex, and on the dorsal surfaces of the N19-OLGs (insets). *Scale bar* = 20 μ m (Color figure online)

Fig. 2.

Fluorescence micrographs of cultured N19-OLGs 2 days post-transfection expressing different variants of RFP-MBP isoforms (*red*), following a 5 min stimulation using PMA, with nuclei counterstained with DAPI (*blue*). Differential interference images are also provided for each fluorescent micrograph shown in (a). **a** The N19-OLGs display membrane-ruffled regions at the cell cortex (*arrowheads*) as well as dorsal membrane regions that are enriched in all MBP variants. **b** The N19-OLGs immunostained using a phosphoThr98-specific antibody, and an AlexaFluor488-conjugated secondary antibody (*green*), show an increased quantity of phosphoThr98 MBP-C1 with enrichment at ruffles. Similar results were observed for the RFP-MBP-C8 and RFP-MBP-21.5 variants (data not shown). *Scale bar* = 20 μ m (Color figure online)

Fig. 3.

a Fluorescence micrographs of cultured N19-OLGs expressing RFP-MBP-C1 (*red*) and GFP- β -actin (*green*), following PKC stimulation by PMA. Images shown are still frames from a time-lapse series for every 30 s following treatment. *Arrowheads* in each channel show areas of co-localization of RFP-MBP-C1 and β -actin. **b** Merged data obtained from time-lapse following PKC stimulation by PMA. Time-course images were acquired at 30 s intervals following treatment; highlighted pixels (*white arrowheads*) show areas of co-localization of RFP-MBP-C1 and β -actin. *Scale bar* = 5 μ m (Color figure online)

Fig. 4.

Live-cell TIRF images at 34°C of N19 cells co-expressing β -actin-GFP (**a, d, g, j, m, p**) and 18.5-kDa RFP-MBP-C1 (**b, e, h, k, n, q**) before and after addition of 250 nM PMA. **a–i**, and **j–r**, represent images of two different cells. Cells were imaged before and after addition of 250 nM PMA. Images were analyzed using ImageJ software to detect localization of β -actin-GFP and RFP-MBP-C1 in membranous protrusions or ruffles following PMA treatment. **a, b, j, k** correspond to images acquired before addition of PMA; **d, e, m, n** correspond to images of the same cells obtained immediately after addition of PMA; **g, h, p, q** were acquired 5 min after addition of PMA. **c, f, i, l, o, r** show intensities due to 18.5-kDa RFP-MBP-C1 (red) and β -actin-GFP (green) measured along a line traced across the region of interest in each cell. Normalized intensities were used in **l, o, r** to overcome the marked difference in signal intensities of the two expressed proteins in this cell. Scale bar = 30 μ m (Color figure online)

Fig. 5.

Live-cell TIRF images at 34°C of N19-OLGs co-expressing β -actin-GFP (**a, d, g, j, m, p**) and 18.5-kDa RFP-MBP-C1 (**b, e, h, k, n, q**) grown on a laminin-2 substrate. **a–i**, and **j–r**, represent images of two different cells. Cells were treated with IGF-1 to measure localization of the two expressed proteins in membranous extensions. The images were analyzed with ImageJ software. **a, b, j, k** correspond to images obtained before addition of 50 ng/mL IGF-1; **d, e, m, n** were measured immediately after introducing IGF-1; **g, h, p, q** were obtained 20 min after introducing IGF-1. **c, f, i, l, o, r** depict intensities due to 18.5-kDa RFP-MBP-C1 (red) and β -actin-GFP (green) measured along a line traced across the region of interest in each cell. Scale bar = 30 μ m (Color figure online)

Fig. 6.

a Untransfected control N19-OLGs immunoprocessed employing a cortactin-specific antibody, and detected using a conjugated AlexaFluor488 secondary antibody (*green*), along with nuclei counterstained with DAPI (*blue*). In untreated N19-OLGs, cortactin appeared to have a heterogeneous punctate distribution, compared to PMA-stimulated cells that showed increased fluorescence intensity within membrane-ruffled regions. Images are provided at different magnifications to illustrate the global changes for multiple cells in culture (*upper panels*, 20+), as well as at higher magnification to visualize individual cells (*lower panels*, 40+). **b** The N19-OLGs following membrane ruffling, showing different classic RFP-MBP variants (*red*) and cortactin (*green*) following a 5 min stimulation using PMA, with nuclei

counterstained with DAPI (*blue*). The N19-OLGs immunoprocessed for cortactin showed significant co-localization of cortactin with RFP-MBP-C1, RFP-MBP-C8, and RFP-MBP-21.5 variants within membrane-ruffled regions, specifically at the leading edge of membrane processes (inset). *Scale bar* = 20 μm (Color figure online)

Fig. 7.

Fluorescence micrographs of cultured N19-OLGs 2 days post-transfection, expressing different variants of RFP-MBP (*red*) and GFP- α -tubulin (*green*), with nuclei counterstained with DAPI (*blue*). As a control (*top three panels*), cells were fixed and viewed prior to performing ruffling treatment to demonstrate that co-expression of these proteins did not have any effects on cell morphology. The transfected N19-OLGs appeared to have a similar morphology to that of untransfected neighboring cells in all control samples. The *lower three panels* labeled “PMA” show N19-OLGs following a 5 min stimulation using PMA, displaying RFP-MBP variants (*red*) and GFP- α -tubulin (*green*), with nuclei counterstained with DAPI (*blue*). The N19-OLGs immunoprocessed for GFP- α -tubulin (*green*) showed co-localization with classic 18.5-kDa RFP-MBP-C1, 18.5-kDa RFP-MBP-C8, and RFP-MBP-21.5 variants within membrane-ruffled regions, specifically at the leading edge of membrane processes (insets). *Scale bar* = 20 μ m (Color figure online)

Fig. 8.

a Fluorescence micrographs of cultured N19-OLGs expressing 18.5-kDa RFP-MBP-C1 (*red*) and GFP- α -tubulin following PKC stimulation by PMA. *Images* shown are still frames from a time-lapse series for every 30 s following treatment; *arrowheads* in each channel show areas of co-localization of RFP-MBP-C1 and α -tubulin. **b** Merged data obtained from time-lapse following PKC stimulation by PMA. Time-course images were acquired at 30 s intervals following treatment; highlighted pixels (*white*) show areas of co-localization of MBP and α -tubulin. *Scale bar* = 5 μ m (Color figure online)

Fig. 9.

a GST pull-down of 18.5-kDa recombinant murine MBP and the ZO-1-SH3 domain: M, molecular mass markers; *Lane 1*, GST alone; *Lane 2*, rmMBP-C1; *Lane 3*, rmMBP-C1 and GST; *Lane 4*, rmMBP-C1 and GST-ZO-1. *Lane 1*, the eluate of glutathione-Sepharose beads incubated with GST (positive control); *Lane 2*, the eluate of glutathione-Sepharose beads incubated with rmMBP-C1. Unmodified 18.5-kDa rmMBP-C1 has a high net charge of +19 at neutral pH, and thus bound independently with moderate affinity to the glutathione-Sepharose beads. *Lane 3*, the eluate of glutathione-Sepharose beads incubated with GST and 18.5-kDa rmMBP-C1. This reaction demonstrated that the highly positively-charged MBP bound non-specifically to both the glutathione-Sepharose beads and also GST. The result was an increased proportion of GST in the final eluate. *Lane 4*, the eluate of glutathione-Sepharose beads incubated with GST-ZO-1-SH3 fusion protein and rmMBP-C1. Significantly less GST-ZO-1-SH3 than GST was incubated with MBP to accentuate the effects of MBP's interaction with GST-ZO-1's SH3-domain. More MBP is eluted from the GST-ZO-1-SH3 column than in the absence of GST-ZO-1-SH3 (*lanes 2 and 3*), which demonstrates an increase in binding of MBP to GST-ZO-1-SH3 compared to glutathione-Sepharose or GST alone. The GST-ZO-1-SH3 domain prior to incubation was greater than 95% in purity. **b** Fluorescence micrographs of cultured resting N19-OLGs (control) and stimulated cells (PMA) expressing RFP-MBP-C1-UTR (red) and ZO-1-GFP (green) and treated with 250 nM PMA at 48 h post-transfection. Nuclei are counterstained with DAPI (blue). Here, RFP-MBP-C1 and ZO-1 are partially co-localized within membrane processes of N19-OLGs (white arrowheads). **c** Time-lapse series of RFP-MBP-C1-UTR (red) and

ZO-1-GFP (*green*) following PKC stimulation by PMA. Time-course of acquired raw images (merged) for every 60 s following treatment; highlighted pixels (*white*) show areas of clear enrichment and co-localization of MBP and ZO-1 within membrane ruffles. *Scale bar* = 50 μm (Color figure online)