Interaction of Lipid-Bound Myelin Basic Protein with Actin Filaments and Calmodulin†

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Received February 2, 2000; Revised Manuscript Received April 27, 2000

ABSTRACT: Myelin basic protein (MBP) binds to negatively charged lipids on the cytosolic surface of oligodendrocytes (OLs) and is believed to be responsible for adhesion of these surfaces in the multilayered myelin sheath. MBP in solution has been shown by others to bind to both G- and F-actin, to bundle F-actin filaments, and to induce polymerization of G-actin. Here we show that MBP bound to acidic lipids can also bind to both G- and F-actin and cause their sedimentation together with MBP−lipid vesicles. Thus it can simultaneously utilize some of its basic residues to bind to the lipid bilayer and some to bind to actin. The amount of actin bound to the MBP−lipid vesicles decreased with increasing net negative surface charge of the lipid vesicles. It was also less for vesicles containing the lipid composition predicted for the cytosolic surface of myelin than for PC vesicles containing a similar amount of an acidic lipid. Calmodulin caused dissociation of actin from MBP and of the MBP−actin complex from the vesicles. However, it did not cause dissociation of bundles of actin filaments once these had formed as long as some MBP was still present. These results suggest that MBP could be a membrane actin-binding protein in OLs/myelin and its actin binding can be regulated by calmodulin and by the lipid composition of the membrane. Actin binding to MBP decreased the labeling of MBP by the hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), indicating that it decreased the hydrophobic interactions of MBP with the bilayer. This change in interaction of MBP with the bilayer could then create a cytosol to membrane signal caused by changes in interaction of the cytoskeleton with the membrane.

Myelin basic protein (MBP) is 30% of the total protein of myelin. It is bound to the cytosolic side of the oligodendrocyte (OL) membrane, primarily through electrostatic interactions with acidic lipids (reviewed in ref *1*). It is probably involved in adhesion of the cytosolic surfaces of the multilayered myelin sheath (*2, 3*). Another function of MBP may be to interact with the cytoskeleton in oligodendrocytes, in cytosolic inclusions in myelin, and even in compact myelin, where MBP, actin, and tubulin occur in the radial component, a series of tight junctions that pass through many layers of myelin (*4*−*6*). MBP in solution binds to F-actin in a 1:1 mole ratio and induces the formation of ordered bundles of F-actin filaments (*7*). It also binds to G-actin in solution at a MBP/actin mole ratio of 1:2 and causes its polymerization into filaments under nonpolymerizing low ionic strength conditions (*8, 9*). MBP also binds Ca^{2+}-calmodulin (*10, 11*) and this results in dissociation of MBP from actin bundles (*7*) and in depolymerization of actin filaments bound to MBP in solution (*8*). The binding to calmodulin has been shown to be Ca^{2+}-dependent (*7, 8, 10, 11*). Association of a protein in a detergent-insoluble complex usually suggests interaction with the cytoskeleton or glycosphingolipids or both (*12*−*14*). The Triton X-100 insoluble fraction of OLs/myelin contains MBP and actin in addition to tubulin, some other myelin proteins, and glycosphingolipids (*4*−*6, 15*).

Although MBP can interact with actin in solution, it is not known whether it can also interact with actin when MBP is bound to the membrane surface. MBP (p*I* ∼ 10) interacts with both anionic lipids and anionic actin monomers through electrostatic interactions. Interaction with both at once may affect the affinity for one or both constituents and may affect the conformation or type of interaction of MBP with the bilayer. To understand the potential of MBP to anchor actin to the membrane surface and/or transmit signals to or from the cytoskeleton, it is necessary to determine whether membrane-bound MBP can also interact with actin. Calmodulin is also anionic and often binds to basic domains on proteins such as the actin-binding proteins synapsin (*16*) and MARCKS (*17*). Thus it may bind to MBP through electrostatic interactions, and binding of MBP to an acidic lipid bilayer may then prevent its interaction with calmodulin. In

† This work was supported by the Multiple Sclerosis Society of Canada and the Medical Research Council of Canada

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[1] Abbreviations: Cyt-LUVs, large unilamellar vesicles with the lipid composition characteristic of the cytosolic side of myelin; LUVs, large unilamellar vesicles; MBP, myelin basic protein; MLVs, multilamellar vesicles; OLs, oligodendrocytes; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; chol, cholesterol; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; [^{14}C]DPPC, [^{14}C]dipalmitoylphosphatidylcholine.

10.1021/bi0002129 CCC: $19.00
Published on Web 06/09/2000

the present study we show that MBP can still interact with actin filaments when it is bound to acidic lipid vesicles and that calmodulin can still regulate binding of actin filaments under these conditions. We also determined the effect of interaction of actin with membrane-bound MBP on the degree of hydrophobic interaction of MBP with the bilayer, using the hydrophobic photolabel TID (*18*) to label MBP.

MATERIALS AND METHODS

Lipids and Proteins. Egg L-α-phosphatidylcholine (PC) was purchased from Sigma (St. Louis, MO). L-α-Phosphatidylglycerol (PG; prepared from egg PC), bovine brain L-α-phosphatidylserine (PS), bovine brain L-α-phosphatidylethanolamine (PE; mostly plasmalogen form), bovine liver L-α-phosphatidylinositol (PI), and bovine brain sphingomyelin (SM) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (chol) was from Supelco, Inc. (Bellefonte, PA). [^{14}C]Dipalmitoylphosphatidylcholine ([^{14}C]DPPC) (specific activity 110 mCi/mmol) was purchased from Dupont NEN (Boston, MA). Myelin basic protein (MBP) was isolated from bovine brain white matter as described (*19*). The least modified, most highly positively charged isomer, C1, was used, purified as described (*20, 21*). Rabbit muscle acetone powder was prepared and actin was prepared from 10 g of it at a time as described (*22*). It was purified by batch treatment of F-actin with DEAE-cellulose. After two depolymerization−repolymerization steps, it was stored as 2−2.5 mg/mL aliquots as F-actin at 4 °C in 2 mM Tris buffer, pH 8.0, containing 0.2 mM $CaCl_2$, 1 mM Na_2-ATP, 0.1 M KCl, 1 mM $MgCl_2$, and 0.2 mM DTT. Before use it was depolymerized for use as G-actin by dialysis against G buffer (2 mM Tris-HCl, pH 8.0, containing 0.2 mM Na_2ATP, 0.2 mM $CaCl_2$, and 0.2 mM DTT) (*23*). For use as F-actin, F buffer (2 mM Tris-HCl, pH 7.5, containing 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM $CaCl_2$, 0.5 mM Na_2-ATP, and 0.2 mM DTT) was added to it (*23*). It was at a concentration of 1.1 mg/mL. This actin preparation was used for most of the experiments. Under similar conditions, at 4−25 °C, the critical concentration of muscle actin has been reported as 30 μg/mL (*24*). For a few experiments, G-actin was purchased from Cytoskeleton (Denver, CO) and stored lyophilized at 4 °C. It was made up to 10 mg/mL in distilled water and then diluted to 2.4 mg/mL with G buffer and used immediately. Calmodulin was purchased from ICN, Irvine, CA. Na_2ATP (grade 1) was purchased from Sigma (St. Louis, MO). The hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) (specific activity 10 mCi/μmol) was purchased from Amersham (Canada). It was supplied as an ethanolic solution.

Preparation of Multilamellar Vesicles and Large Unilamellar Vesicles. Aliquots of chloroform solutions of the lipids were combined in the desired mole ratio. For LUVs containing the lipid composition estimated to be characteristic of the cytoplasmic surface of myelin (Cyt-LUVs) by Inouye and Kirschner (*25*), chol, PE, PS, PC, SM, and PI were combined in the mole ratio 0.44:0.27:0.13:0.11:0.03:0.02 as described previously (*26*). [^{14}C]DPPC was added to give a specific activity of 100 000 cpm/10 μmol of lipid. The solvent was evaporated under a stream of nitrogen and the lipid film was evacuated in a lyophilizer for 2 h. The dry lipid film was dissolved in 1−2 mL of benzene, frozen, and lyophilized overnight. The lipid (10−20 μmol) was hydrated in 500 μL of modified G or F buffer, with the divalent cations omitted. Multilamellar vesicles (MLVs) were prepared by freeze−thawing five times in a dry ice/actone bath followed by a 40 °C water bath and dispersing the lipid by vigorous vortex mixing. MLVs were used for G-actin or were extruded through 0.1 μm pore filters to give large unilamellar vesicles (LUVs), as described (*27*), for F-actin. LUVs were diluted in modified F buffer to a final concentration of 1.5 mg or 1.95 μmol/100 μL. For TID labeling experiments, Cyt-LUVs and PC/PG LUVs were used at equal total weights of lipids present, 1.5 mg/100 μL.

Determination of Interaction of Actin and MBP with LUVs. MBP was dissolved in modified G buffer at a concentration of 100 μg/100 μL. MBP (100 μg) was added to 100 μL of LUVs in Eppendorf microcentrifuge tubes, followed by an aliquot of G-actin solution containing 240 μg of G-actin. The sample was incubated at room temperature for 30 min to allow interaction of the proteins before addition of F buffer. Then sufficient G or F buffer (containing divalent cations; a minimum of 618 μL) was added to bring the total volume to 1.0 mL. The samples were mixed gently and incubated for an additional 30 min at room temperature. When calmodulin was used, it was dissolved in modified F buffer at a concentration of 92 μg/100 μL and 92−184 μg was added to the samples after they had been incubated with F buffer for 30 min. They were then incubated an additional 30 min at room temperature. The mole ratio of MBP/actin/calmodulin was 1:1:1 or 1:1:2.

The samples were centrifuged at 11800*g* for 30 min at 4 °C in an Eppendorf bench centrifuge. The supernatant was removed and aliquots were taken for counting [^{14}C]DPPC, for protein assay, and for running on gels. Aliquots for gel electrophoresis were lyophilized and taken up in NuPage sample buffer. The pellets were redispersed gently in 1 mL of G or F buffer and placed on a discontinuous sucrose density gradient in order to separate lipid-free actin−MBP complexes from lipid-bound MBP−actin complexes. The gradient was prepared by layering 1 mL each of 40% sucrose, 20% sucrose, 15% sucrose, and 10% sucrose, each made up in G or F buffer in 5 mL Beckman Ultra-Clear ultracentrifuge tubes. The samples were centrifuged at 109000*g* for 3 h at 4 °C in a SW 55Ti rotor in a Beckman Optima L-90K ultracentrifuge. The lipid without protein sedimented in a hazy band on top of the 10% layer while the lipid with protein sedimented as sharp bands that could be clearly seen by eye. The position and appearance of the band was noted, each sucrose layer was collected, and the bands were collected separately. After removal of most of the 40% layer, the bottom of the tube was washed out with 300 mL of G or F buffer to collect any pellet at the bottom. Aliquots of the layers without bands were counted for ^{14}C and for protein assay. The bands were put into 13.5 Nalgene polycarbonate (Ultrabottles) tubes, diluted with F buffer, and centrifuged at 109000*g* for 1.5 h. The supernatant was removed and aliquots were taken for counting and protein assay. The pellet was redispersed in water and aliquots were taken for counting, for protein assay, and for running on gels.

Protein was assayed by the Peterson method (*28*). Samples were run on NuPage Bis-Tris 10% polyacrylamide gels (Novex, San Diego, CA) with known amounts of actin, MBP, and calmodulin standards. Coomassie blue-stained gels were analyzed with a UVP image analyzer and band areas were

related to those of the standards in order to quantitate the amount of each protein in the sample. Samples of 0.2 μg of each protein could be easily detected on the gels. The results of representative experiments are shown in Figure 2B and Table 2, and the results from several experiments were averaged and summarized in Table 1.

Photolabeling Reaction with TID. Samples to be labeled with TID were prepared as above and centrifuged in an Eppendorf centrifuge. The supernatant containing unbound protein was removed and the pellet was resuspended in 1 mL of fresh F buffer. The samples were flushed with nitrogen, 8−10 μL of TID solution containing (1−1.5) × 10^7 cpm was added to 1 mL of vesicle suspension, and the samples were mixed in the dark. They were equilibrated at room temperature in the dark for 20−30 min and then each sample was irradiated for 2 min with a 100 W high-pressure Hg lamp (Photochemical Research Associates, London, ON) with the sample tube immersed in ice water as described (*26*, *29*, *30*). The light beam was cooled by passage through a reservoir of circulating cold water and directed through a filter consisting of a saturated solution of $CuSO_4$.

The labeled MBP was delipidated by extraction with chloroform/methanol (1:1 v/v) containing 5% 0.1 N HCl as described previously, but it was not dialyzed or chromatographed on C18 Sep-Pak columns (*26*, *29*, *30*) because of loss of protein by these procedures. Actin was lost into the organic phase and was not analyzed further. The aqueous phase containing MBP was assayed for protein concentration, lyophilized, and redissolved in sample buffer for gel electrophoresis. Samples (5−10 μg/lane) were loaded on NuPage Bis-Tris 10% polyacrylamide gels and the specific activity of MBP was determined from the area of Coomassie blue-stained bands determined as above and from the radioactivity of the stained band, by cutting the gel into strips and counting the strips in a Beckman Gamma8000 γ counter. The area was converted to weight of protein by comparison with the area of a band from a known amount of MBP standard run on the same gel. The specific activity was compared to that of a control sample in which MBP was added to labeled lipid after irradiation and taken through the same purification and gel electrophoresis procedures. The specific activity of this control was less than 20% of the irradiated MBP sample. This is higher than obtained when the protein is dialyzed or chromatographed on Sep-Pak columns (*26*, *29*, *30*) but indicates that the extraction procedure combined with gel electrophoresis removed most of the noncovalently bound labeled lipid from the protein.

For each experiment, the specific activity of MBP bound to PC/PG LUVs in the absence of actin was set at 100. The relative percent labeling in the presence of actin was calculated as the ratio of specific activities in the presence and absence of actin times 100. The relative percent labeling of MBP bound to Cyt-LUVs relative to PC/PG 8.5:1.5 LUVs in the absence of actin was calculated similarly. The relative percent labeling values for a number of experiments were averaged.

RESULTS

Interaction of Actin with MBP. The interaction of actin with membrane-bound MBP was determined from cosedimentation of actin and MBP with the lipid vesicles. Samples were first centrifuged at low speed (11800*g*), such that F-actin alone did not sediment, to remove unbound, uncomplexed MBP and G- or F-actin. The amount of actin and MBP in the supernatant and pellet were determined by protein assay and by separation of the proteins on gels (Figure 1) and quantitation of each by comparison of the density to that of standard bands. The pellets were then resuspended in G or F buffer and ultracentrifuged at high speed (109000*g*) on a discontinuous sucrose gradient in order to separate an actin−MBP complex unbound to lipid from a lipid-bound MBP−actin complex and MBP−lipid or protein-free lipid vesicles. Bands and different sucrose layers were collected and the amounts of lipid, actin, and MBP in each were determined. For G-actin under nonpolymerizing conditions in low-salt G buffer, PC/PG MLVs were used since they can be sedimented easily when bound to MBP (Figure 2A, set 2), while PC/PG LUVs bound to MBP do not sediment by low-speed centrifugation under these conditions (*21*). However, in the presence of high-salt F buffer, LUVs bound to MBP can be sedimented at low speed (Figure 2B, set 2), so LUVs were used under actin-polymerizing conditions in F buffer.

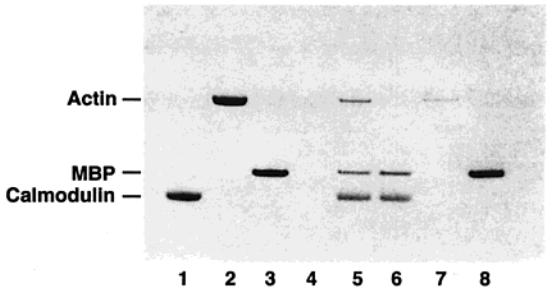

FIGURE 1: Representative Coomassie blue-stained gel of supernatants from PC/PG 9:1 LUVs containing MBP, actin, and calmodulin in F buffer. Lane 1, 2.3 μg of calmodulin standard; lane 2, 2.4 μg of actin standard; lane 3, 2 μg of MBP standard; lane 5, supernatant of LUVs with MBP, actin, and calmodulin; lane 6, supernatant of LUVs with MBP and calmodulin; lane 7, supernatant of LUVs with MBP and actin; lane 8, supernatant of LUVs with MBP. Different percentages of each sample were applied to the gel.

Actin−MBP Complex in the Absence of Lipid. F-Actin and MBP in solution by themselves remain in the supernatant on low-speed centrifugation (Figure 2B, sets 4 and 5, respectively). However, in both G and F buffer, when actin and MBP were added together, all of the actin and MBP sedimented on low-speed centrifugation, indicating that they interacted with each other (Figure 2A,B, set 6). This confirms that MBP induced polymerization of G-actin under nonpolymerizing conditions as reported by Dobrowolski et al. (*8*) and also that it caused larger aggregates of filaments such as the bundles reported by Barylko and Dobrowolski (*7*), since actin filaments alone do not sediment at low speed.

Lipid-Bound F-Actin−MBP Complex. In the presence of F buffer, about half of the total actin cosedimented with all of the MBP and PC/PG 4:1 lipid vesicles on low-speed centrifugation (Table 1, line 1; see Figure 2B, set 3, for a representative experiment). In the absence of MBP, actin does not sediment with the lipid vesicles in G or F buffer (Figure 2A,B, set 1). Ultracentrifugation of the pellet on a sucrose density gradient showed that the actin−MBP complex was bound to the lipid vesicles. Protein-free LUVS sedimented as a hazy band on top of the 10% sucrose layer.

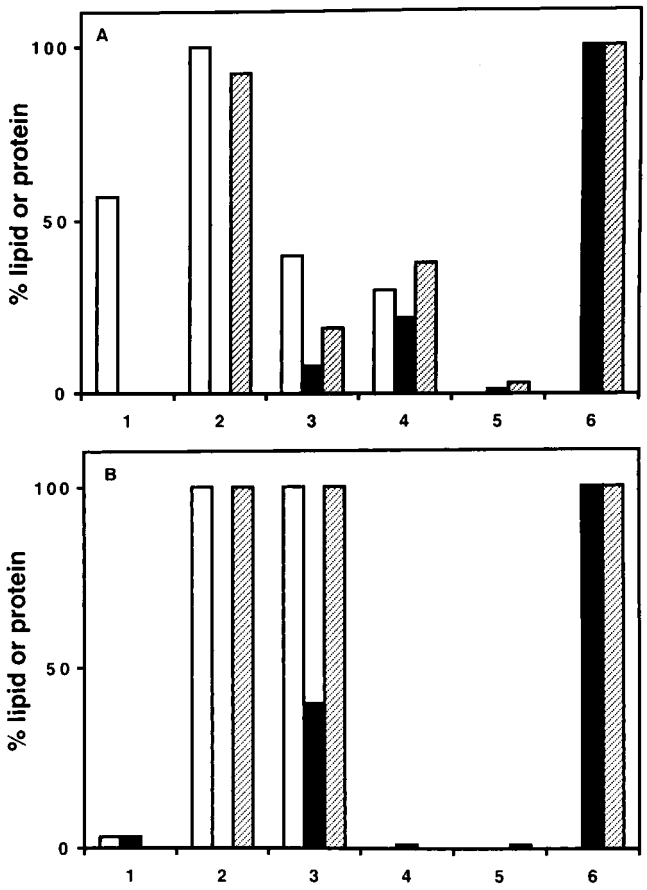

FIGURE 2: Percent added lipid (open bars), actin (solid bars), and MBP (hatched bars) in lipid−protein or protein samples. (A) PC/PG 9:1 MLVs in G buffer; lipid−protein or protein pellets after low-speed centrifugation or lipid−protein bands from sucrose density gradient. Actin and MBP in sets 3 and 4 were quantitated from gels. Set 1, MLVs + actin; set 2, MLVs + MBP; set 3, MLVs + actin + MBP, upper band from sucrose gradient; set 4, MLVs + actin + MBP, lower band from sucrose gradient; set 5, MLVs + actin + MBP, pellet from bottom of sucrose gradient; set 6, actin + MBP, pellet after low speed centrifugation. (B) PC/PG 4:1 LUVs in F buffer; lipid−protein or protein pellets after low-speed centrifugation. Data from a representative experiment are shown. Set 1, LUVs + actin; set 2, LUVs + MBP; set 3, LUVs + MBP + actin; set 4, actin only; set 5, MBP only; set 6, actin + MBP.

The actin−MBP complex in the absence of LUVs sedimented to the bottom of the tube and could be seen as a pellet; 100% of the added protein was recovered in this pellet (Table 2). F-Actin alone was found throughout the gradient (Table 2), but because of the initial low-speed centrifugation of all lipid−protein or MBP−actin samples, any F-actin unbound to MBP or lipid would have been removed from the sample. MBP−LUVs sedimented as a sharp band just into the top of the 10% layer, and MBP−actin−LUVs sedimented as a sharp band about $^3/_4$ of the way into the 10% layer. Assay of other sucrose layers and the bottom of the tube for lipid and protein for all samples revealed that except for F-actin alone there was very little lipid or protein in other parts of the gradient outside of the bands and pellets referred to above (Table 2). It is particularly important to note that there was no protein at the bottom of the tube for the MBP−actin−LUVs. Thus the MBP and actin found in the pellet on low speed centrifugation were associated with the lipid vesicles, indicating that actin filaments could associate with membrane-bound MBP.

Since gel electrophoresis showed that the supernatant from the low-speed centrifugation contained only actin and no MBP and since sucrose density gradient centrifugation showed that all actin and MBP in the pellet was associated with the lipid, the amount of MBP and actin associated with the lipid vesicles in the pellet could be quantitated from the protein assay of the supernatant. This was more accurate than quantitation of both proteins in the pellets or bands from the sucrose density gradient on gels. Protein assay of the supernatant indicated a mean mole ratio of actin to MBP bound to lipid vesicles of 0.52 (Table 1, line 1). For most samples, the mole ratio of actin to MBP in the band recovered from the sucrose density gradient was also determined by quantitation of the proteins on gels. In most cases this determination agreed with that based on analysis of the supernatant from low-speed centrifugation (Table 1). However, in the case of the actin−MBP−PC/PG 4:1 vesicles recovered from the sucrose gradient, the mean ratio of actin to MBP determined on gels was 0.83. This may reflect some intrinsic error in this assay. Regardless of the method of determination, the mole ratio of actin to MBP associated with the PC/PG 4:1 lipid vesicles was lower than the starting ratio (Table 1, line 1), in contrast to the actin−MBP complex in the absence of lipid, for which the ratio was similar to the starting ratio (Table 1, line 7). Thus less actin binds to MBP−PC/PG 4:1 LUVs than to MBP in solution.

Lipid-Bound actin−MBP Complex in G Buffer. In the presence of lipid vesicles in G buffer, 76% of the added actin and all of the MBP sedimented with the MBP−lipid vesicles on low-speed centrifugation (not shown). Centrifugation of this pellet on a sucrose density gradient showed that the vesicles were heterogeneous in composition, giving two distinct lipid−protein bands, one about a quarter way into the 10% sucrose layer and one on top of the 20% layer. MBP−MLVs alone sediment on top of the 15% layer. Each of the lipid−protein bands contained both actin and MBP. The lighter band contained less protein than the heavier one, but the actin/MBP mole ratios were relatively similar, 0.42 and 0.58, respectively (Figure 2A, sets 3 and 4, respectively). Almost all of the added actin and MBP were associated with the lipid vesicles since only a small amount of actin and MBP was found elsewhere in the gradient; this was all at the bottom of the tube, indicating a small amount of actin−MBP complex unbound to lipid (Figure 2A, set 5).

Effect of Lipid Composition on Actin−MBP Binding to Lipid Vesicles in F Buffer. The ratio of PC to PG and the ratio of PG to MBP affects the surface charge of the vesicles (*26*). To determine the effect of the surface charge of the vesicles on F-actin binding to MBP−vesicles, the PC/PG ratio was varied. For the PC/PG 4:1 vesicles, the mole ratio of PG to MBP was 74. If the LUVs are single-layered and MBP binds only to the outside, the ratio on the outer monolayer would be 37. Since MBP has a net positive charge of 20 at neutral pH, the MBP−LUVs would have a significant excess negative charge of 17 per molecule of MBP, which may inhibit binding of negatively charged actin to the membrane-bound MBP. Therefore, the PG content was reduced to 10 mol % so that MBP would be able to completely neutralize the negative charge of these vesicles. These PC/PG 9:1 vesicles bound only 62% of the added MBP in the absence of actin, but in the presence of actin in F buffer, all of the added MBP and virtually all of the added

Table 1: Actin and MBP Associated with Lipid Vesicles or with Each Other in Absence of Lipid, and Effect of Calmodulin

line	sample	calmodulin[a]	% added MBP bound to lipid or in complex[b]		% added actin in complex[b]	actin/MBP (mol/mol) with lipid or in complex	
			no actin	with actin		*b*	*c*
1	PC/PG 4:1	0	95 ± 5	100 ± 0	52 ± 10	0.52 ± 0.1	0.83 ± 0.04
2	PC/PG 4:1	1×	56	52	34	0.65	0.66
3	PC/PG 8.5:1.5	0	100 ± 0	100 ± 0	90 ± 8	0.90 ± 0.08	nd
4	PC/PG 9:1	0	62 ± 10	100 ± 0	97 ± 1	0.97 ± 0.01	1.1 ± 0.2
5a	PC/PG 9:1	2×	*d*	*d*	*d*		*d*
5b				20[e]	75[e]		3.75[e]
6	Cyt − LUVs	0	90 ± 15	100 ± 0	58 ± 10	0.58 ± 0.1	0.37
7	actin + MBP[e]	0		90	100	1.11	1.03
8	actin + MBP[e]	1×		57	83	1.46	1.5
9	actin + MBP[e]	2×		0	13		

[a] Initial mole ratio of MBP/actin/calmodulin for PC/PG 4:1 LUVs was 1:1:1 (1×) and for PC/PG 9:1 LUVs it was 1:1:2 (2×). In the absence of lipid it was 1:1:1 (1×) and 1:1:2 (2×). Zero indicates no calmodulin was added. [b] Percent added MBP or actin found in lipid−protein complex (lines 1−5a and 6), or in protein complex for samples in the absence of lipid (lines 7−9) and for the lipid sample in the presence of 2× calmodulin (line 5b). Calculated amount and mole ratios (mol/mol) from each protein found in the supernatant, after low-speed centrifugation, are shown, assuming the remainder is associated in the complex with lipid and MBP or in the MBP−actin pellet in the absence of lipid. If more than one protein was present in the supernatant, the ratios were quantitated from gels. There was no actin or MBP−actin pellet at the bottom of the sucrose density gradient for any of the lipid samples except in the presence of 2× calmodulin. [c] Mole ratios (mol/mol) calculated from the amounts of the two proteins in the lipid−protein bands from the sucrose gradient or MBP−actin pellet, detected by gel electrophoresis. This was not determined (nd) for every sample represented in the table. [d] No lipid−protein pellet was found for this sample, suggesting that most of the MBP and actin in the supernatant was not bound to the lipid. MBP and actin were in the lipid-free pellet at the bottom of the sucrose gradient as indicated in line 5b. [e] In lipid-free MBP−actin pellet from low-speed centrifugation or at bottom of sucrose gradient after high-speed centrifugation.

Table 2: Percent Lipid and Protein Added That Was Found in Supernatant and Fractions from Sucrose Gradient[a]

			sucrose gradient fractions									
	supernatant[b]		buffer[c]		10% sucrose		20% sucrose		30% sucrose		40% sucrose	
sample	% lipid	% protein	% lipid	% protein	% lipid	% protein	% lipid	% protein	% lipid	% protein	% lipid	% protein
LUVs only	92		73		7		4		0.4		0.5	
LUVs + MBP	0	8.4	3	3	80	73	0	5	0	8	0	4
LUVs + MBP + actin	5	27	0	0	76	73	2	0	0	0	0	0
MBP + actin		0		0		2		0		0		98
actin		99		10		18		12		23		36
MBP		99		39		39		0		7		15

[a] Pellet from low-speed centrifugation was recentrifuged at high speed on sucrose gradient. For samples where there was no low-speed pellet, the supernatant was centrifuged on the sucrose gradient. Lipid vesicles were PC/PG 4:1 LUVs. From a representative experiment. [b] Supernatant from low-speed centrifugation. [c] Buffer layer after high-speed centrifugation from application of sample to top of sucrose gradient.

actin were found in the pellet on low-speed centrifugation (Table 1, line 4). On sucrose density gradient ultracentrifugation, MBP−PC/PG 9:1 LUVs sedimented on top of the 10% layer while the MBP−actin−LUVs sedimented all the way through the 10% layer to the top of the 15% sucrose layer (not shown), indicating that more protein was bound than for the 4:1 vesicles. No protein was found at the bottom of the tube (not shown). The actin to MBP ratio in the recovered band was close to the starting ratio (Table 1, line 4). Since more MBP bound to the lipid−protein complex than in the absence of actin, the additional MBP in the complex may have been bound to the actin complexed to the lipid−MBP vesicles rather than directly to the lipid. PC/PG 8.5:1.5 LUVs bound all of the MBP and 90% of the actin (Table 1, line 3) and thus were intermediate in actin-binding capacity between the 4:1 and 9:1 LUVs.

The interaction of MBP and actin with Cyt-LUVs (*26*) having the composition estimated to be that of the cytosolic surface of myelin (*25*), with 15 mol % acidic lipid (mostly PS and some PI), was also determined. These LUVs bound 90% of the added MBP in the absence of actin, and all of the MBP in the presence of actin (Table 1, line 6). However, only 58% of the added actin was found in the pellet on low-speed centrifugation. On ultracentrifugation of the pellet on a sucrose density gradient, MBP−Cyt-LUVs sedimented just into the 10% sucrose layer and the MBP−actin−Cyt-LUVs sedimented all the way through the 10% layer to the top of the 15% layer. There was no protein at the bottom of the tube or elsewhere on the gradient outside of the lipid−protein band. The mole ratio of actin to MBP bound to the Cyt-LUVs determined from analysis of the supernatant from low-speed centrifugation was 0.58, and that determined for the recovered band from the sucrose density gradient by quantitation of the proteins on gels was 0.37, considerably less than the starting ratio and less than that for PC/PG LUVs with 15 mol % PG (Table 1).

Thus the dependence of actin binding on the PC/PG ratio shows that an excess of negative surface charge inhibits actin binding to membrane-bound MBP for PC/PG LUVs. However, there is an additional loss of actin binding to MBP−Cyt-LUVs since the cytosolic lipid composition results in less actin binding than PC/PG 8.5:1.5, with a similar percentage of acidic lipid. This may be due to differences in the interaction of MBP with the two types of vesicles, as we reported earlier (*26*).

Effect of Calmodulin. Calmodulin was added to MBP−actin−LUVs using PC/PG 4:1 LUVs in the presence of F buffer (which contained 0.2 mM Ca^{2+}) at a 1:1 mole ratio of calmodulin to MBP. Calmodulin did not bind to the protein-free LUVs nor to the MBP−LUVs (Figure 3A, sets

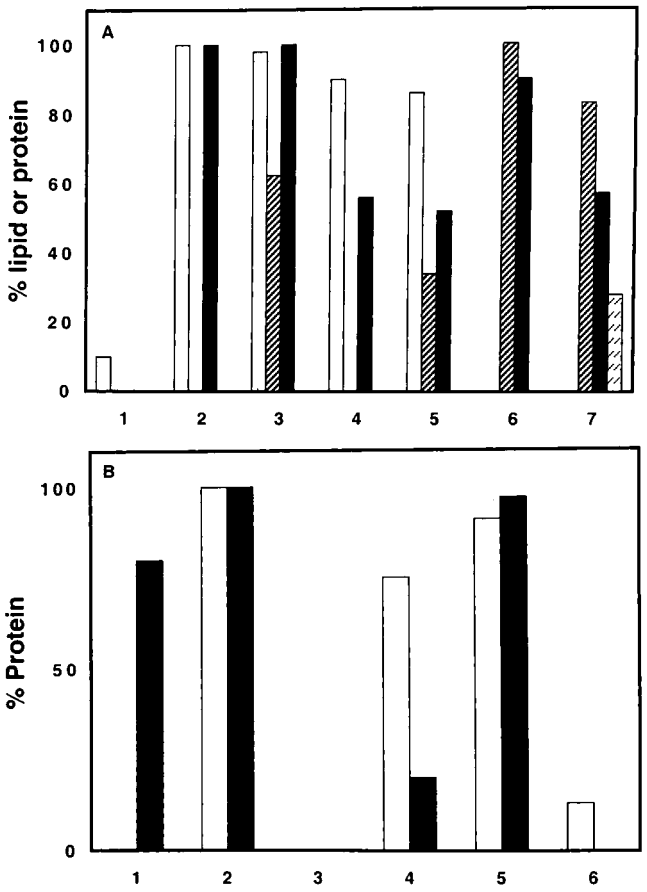

FIGURE 3: (A) Percent added lipid (open bars), actin (heavily hatched bars), MBP (solid bars), and calmodulin (lightly hatched bar) in lipid−protein bands from sucrose gradient or in protein pellets after low-speed centrifugation of PC/PG 4:1 LUVs in F buffer. Actin/MBP/calmodulin mole ratio was 1:1:1. Set 1, LUVs + calmodulin; set 2, LUVs + MBP; set 3, LUVs + MBP + actin; set 4, LUVs + MBP + calmodulin; set 5, LUVs + MBP + actin + calmodulin; set 6, MBP + actin; set 7, MBP + actin + calmodulin. Value for calmodulin in pellets is 0 for sets 1, 4, and 5. (B) Percent added actin (open bars) and MBP (solid bars) in lipid−protein bands from sucrose gradient or from protein pellets after low-speed centrifugation of PC/PG 9:1 LUVs in F buffer. Actin/MBP/calmodulin mole ratio was 1:1:2. Set 1, LUVs + MBP; set 2, LUVs + MBP + actin; set 3, LUVs + MBP + calmodulin (no pellet was obtained, thus value for MBP in pellet is 0); set 4, LUVs + MBP + actin + calmodulin, (values for MBP and actin in pellet at bottom of tube; no lipid−protein band was found); set 5, MBP + actin; set 6, MBP + actin + calmodulin (value for MBP is 0).

1 and 4, respectively). On ultracentrifugation on a sucrose gradient, the MBP−actin−LUVs just entered the 10% sucrose layer in the presence of calmodulin, while they went to the bottom of this layer in the absence of calmodulin. There was no protein at the bottom of the tube or elsewhere on the gradient other than the lipid−protein band. Calmodulin caused dissociation of 44% of the MBP from the MBP−LUVs (Figure 3A, compare sets 2 and 4) and a similar amount from the MBP−actin−LUVs (Figure 3A, compare sets 3 and 5). It also resulted in significantly less binding of actin to the MBP−LUVs. Only 34% of the added actin was present with the MBP−LUVs, in contrast to 62.5% in the absence of calmodulin (for the same experiment, Figure 3A). The recovered lipid−protein band contained actin and MBP in a mole ratio of 0.66 (Table 1, line 2), while that in the absence of calmodulin was 0.87 (determined similarly and in the same experiment). Calmodulin also decreased binding of actin to MBP in solution (Figure 3A, compare sets 6 and 7), causing 17% of the actin and 43% of the MBP to remain in the supernatant after low-speed centrifugation (Table 1, line 8). The pelleted actin−MBP complex in the presence of calmodulin contained actin and MBP in a mole ratio of 1.5, indicating that once polymerized and bundled by MBP, the assembled structures did not require a 1:1 mole ratio of MBP to actin in order to remain assembled.

In a second experiment, a 2:1 mole ratio of calmodulin to MBP was used for PC/PG 9:1 LUVs. At this calmodulin to MBP ratio, the lipid and MBP remained entirely in the supernatant for MBP−LUVs after centrifugation (Figure 3B, compare sets 1 and 3). For MBP−actin−LUVs, all of the lipid, 80% of the MBP, and 25% of the actin remained in the supernatant. No lipid−protein bands were observed on the sucrose density gradient on high-speed centrifugation (Table 1, line 5a). This indicates that calmodulin caused partial and probably complete dissociation of MBP and of the MBP−actin complex from the LUVs, since protein binding to the LUVs would have caused them to sediment on centrifugation. The dissociated MBP−actin complex was found at the bottom of the sucrose gradient and contained 75% of the added actin and only 20% of the added MBP (Table 1, line 5b; Figure 3B, set 4). Thus calmodulin also caused dissociation of most of the MBP from the bundled actin filaments but did not dissociate the actin bundles once formed. However, in the absence of lipid, a 2:1 mole ratio of calmodulin to MBP dissociated all of the MBP from the actin and greatly reduced the amount of actin in the actin bundles at the bottom of the tube (Table 1, line 9; Figure 3B, compare sets 5 and 6).

Effect of Interaction of Membrane-Bound MBP with Actin on TID Binding to MBP. MBP bound to lipid can be labeled by the hydrophobic photolabel TID and by lipid photolabels with the photolabel group at the end of an acyl chain (*30*). This indicates that hydrophobic side chains of MBP lying on the bilayer surface dip into the bilayer. The effect of actin binding to membrane-bound MBP on its accessibility to TID labeling was thus determined as a measure of the effect of actin on the degree of hydrophobic interaction of MBP with the bilayer. In the presence of actin, MBP bound to PC/PG LUVs was labeled about 30% less than in its absence (Figure 4, sets 1−3). This indicates that actin binding caused a decrease in the degree of hydrophobic interaction of MBP with the lipid. MBP was labeled more when bound to Cyt-LUVs (Figure 4, set 4) than PC/PG 8.5:1.5 LUVs with the same concentration of acidic lipid, suggesting greater hydrophobic or hydrogen-bonding interactions with the former, as reported previously (*26*). The TID labeling of MBP bound to Cyt-LUVs was also decreased by actin but the difference was not significant. Actin binding had no significant effect on TID labeling of MBP in solution in the presence of PC vesicles (Figure 4, set 5). Note that MBP does not bind to PC vesicles. Thus labeling of MBP in the presence of PC vesicles is due to labeling of MBP in solution and is at least 50% less than that of MBP bound to lipid (*26, 30*).

DISCUSSION

The results show that membrane-bound MBP, like MBP in solution (*7, 9*), can still bind to F-actin. Thus it must utilize

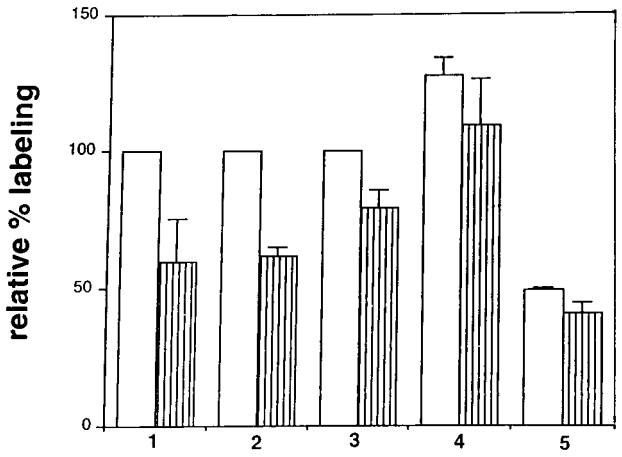

FIGURE 4: TID labeling of MBP in LUVs of different lipid composition in absence (open bars) or presence of actin (striped bars). Relative percent labeling, the percent TID bound to MBP in the presence of actin relative to MBP−PC/PG LUVs without actin, is shown. Values for Cyt-LUVs and PC LUVs with or without actin are also relative to PC/PG samples in the absence of actin, labeled in the same experiment. Set 1, PC/PG 4:1 LUVs; set 2, PC/PG 8.5:1.5 LUVs; set 3, PC/PG 9:1 LUVs; set 4, Cyt-LUVs relative to PC/PG 8.5:1.5 LUVs; set 5, PC LUVs. Labeling of MBP in the presence of PC vesicles is due to labeling of MBP in solution. The mean ± SD of 3−4 experiments is shown.

some of its positively charged residues to bind to the negatively charged lipid and some to bind to actin. The amount of bound actin increased as the excess net negative surface charge of the lipid vesicles decreased, thus decreasing charge repulsion between the bilayer surface and negatively charged actin. Membrane-bound MBP could also bind to G-actin although we cannot tell from these results alone if it caused its polymerization as MBP in solution does (*8*). The actin-binding proteins talin and myosin I have also been shown to bind actin when bound to lipid bilayers (*23, 31*).

Other polycationic proteins such as histone and polyamines can also bind actin and induce its polymerization (*32, 33*). Thus it is not known if the interaction of MBP with actin is a nonspecific electrostatic interaction or if it has a physiological role. However, the interaction of membrane-bound MBP with F-actin suggests that MBP might be able to bind actin to the cytosolic surface of the oligodendrocyte or myelin plasma membrane. Colocalization studies by immunohistochemistry in immature cultured oligodendrocytes indicate that MBP is closely associated with microtubules and actin microfilaments (*34*), although no evidence has been reported to indicate that MBP at the surface of the plasma membrane colocalizes with actin microfilaments. MBP might also bind actin to microtubules. In *Shiverer* mouse OLs, which lack MBP, actin microfilaments are not colocalized with microtubular structures as they are in the wild-type OLs (*35*). It is not known if other actin-binding proteins that mediate interactions between actin and the plasma membrane of many cells, such as vinculin, talin, and α-actinin (*12*), are also present in oligodendrocytes. The radial component in compact myelin is Triton X-100 insoluble and contains tubulin, actin, MBP, CNPase, and glycosphingolipids (*4*−*6*).

Many actin-binding proteins insert into the lipid bilayer, allowing them to anchor actin filaments to the membrane and possibly to also sense mechanical signals transmitted through the membrane. This insertion into the lipid bilayer has been detected by labeling with hydrophobic photolabels (*36*), which react only with groups in the acyl chain region of the bilayer. MBP binds to the lipid bilayer primarily by electrostatic interactions but it can also be labeled by these hydrophobic photolabels when bound to lipid, indicating that it also interacts by hydrophobic interactions, probably due to penetration of hydrophobic amino acid side chains into the bilayer interface region (*26, 29, 30*). It has recently been shown for the basic domain of the MARCKS protein that large hydrophobic amino acids such as Phe cause deeper penetration of the peptide into the bilayer interface by 10−15 Å relative to a similar peptide containing Phe to Ala substitutions (*37*). Like many actin-binding proteins that are myristoylated at the N-terminus (*38,39*), MBP is also acylated at the N-terminus, although in MBP the chain length is heterogeneous and the maximum length is only 10 carbons, with 4 and 6 carbons predominating (*40*). These hydrophobic interactions of MBP with the membrane might allow transmission of signals between other membrane constituents and the cytoskeleton via MBP. Indeed, the reduced labeling of MBP by TID when F-actin is bound indicates that actin binding alters the interaction of MBP with the bilayer. This altered interaction could provide a signal that could be transmitted to other membrane constituents.

The greater labeling of MBP by TID when bound to Cyt-LUVs suggests a deeper penetration of the amino acid side chains of MBP into the bilayer. This has been attributed to a higher affinity binding of MBP to Cyt-LUVs due to hydrogen-bonding interactions with PE and chol in the Cyt-LUVs, in addition to electrostatic interactions with the acidic lipids present. This closer association with the bilayer may result in the lower actin binding found for MBP bound to Cyt-LUVs compared to PC/PG LUVs with a similar amount of acidic lipid.

Calmodulin caused dissociation of some of the actin from the membrane-bound MBP and some of the MBP from the lipid. We did not determine if this effect was Ca^{2+}-dependent. However, it has been shown that the binding of calmodulin to MBP in solution and depolymerization of actin filaments bound to MBP is Ca^{2+}-dependent (*7, 8, 10, 11*). Both apocalmodulin and Ca^{2+}-calmodulin bind to protein domains that contain both positively charged residues and hydrophobic residues (*42, 43*). Thus calmodulin may inhibit both the electrostatic and hydrophobic interactions of MBP with the lipid and electrostatic interactions with actin. This effect of calmodulin indicates that it might be able to regulate attachment of the cytoskeleton to MBP on the cytosolic surface. Such a regulatory mechanism has been proposed for many actin-binding proteins isolated from brain and secretory tissue (*41*). Thus actin binding to MBP may be regulated by both the lipid composition of the membrane and calmodulin.

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BI0002129