Antibody-induced demyelination is an important component of pathology in Multiple Sclerosis. In particular, antibodies to myelin oligodendrocyte glycoprotein (MOG) are elevated in Multiple Sclerosis patients and they have been implicated as mediators of demyelination. We have shown previously that antibody cross-linking of MOG in oligodendrocytes results in the repartitioning of MOG into glycosphingolipid-cholesterol membrane microdomains (“lipid rafts”), followed by changes in the phosphorylation of specific proteins, including dephosphorylation of β-tubulin and the β subunit of the trimeric G protein, and culminating in rapid and dramatic morphological alterations. In order to further elucidate the mechanism of anti-MOG mediated demyelination, we have carried out a proteomic analysis to identify the set of proteins for which the phosphorylation states or expression levels are altered upon anti-MOG treatment. We demonstrate that treatment of oligodendrocytes with anti-MOG alone leads to an increase in calcium influx and activation of the MAPK/Akt pathways that is independent of MOG repartitioning. However, further cross-linking of anti-MOG/MOG complexes with a secondary anti-IgG results in the lipid raft dependent phosphorylation of specific proteins related to cellular stress response and cytoskeletal stability. Oligodendrocyte survival is not compromised by these treatments. We discuss the possible significance of the anti-MOG induced signaling cascade in relation to the initial steps of MOG-mediated demyelination.

The investigation of the transfer of information between myelin and axons is a forefront of myelin biology and demyelinating disease (1-3). Loss or damage of myelin, such as occurs in the human demyelinating disease Multiple Sclerosis, results in axonal degeneration with severe, generally irreversible, functional consequences (4). Multiple Sclerosis-related demyelination is in part induced by antibodies directed against surface antigens of myelin and oligodendrocytes (OLs), the myelin producing cells of the central nervous system (5). In particular, antibodies to myelin oligodendrocyte glycoprotein (MOG), a highly encephalitogenic glycoprotein concentrated in the outer lamella of the myelin sheath and thus exposed to the environment (6), have been implicated as mediators of demyelination (7-10).

Upon ligand or antibody cross-linking, a variety of plasma membrane receptors undergo enhanced partitioning into glycosphingolipid-cholesterol membrane microdomains (‘lipid rafts’) as an obligatory first step towards participation in early signal transduction events (11-13). We have previously shown that antibody cross-linking of MOG on the surface of OLs results in the rapid repartitioning of a significant fraction of MOG into lipid rafts, followed by dephosphorylation of β-tubulin and the β subunit of the trimeric G protein (Gβ), and a rapid retraction of OL processes and myelin-like membranes (14). In order to elucidate this mechanism and better consider its relationship to antibody-mediated demyelination in Multiple Sclerosis, we have taken a proteomic approach to identify target proteins for which the phosphorylation states and/or expression levels are altered upon MOG cross-linking. We show that antibody-induced
activation of MOG signaling in its non-lipid raft state triggers the activation of MAPK and Akt pathways, and an increase in calcium influx. Further cross-linking of anti-MOG/MOG complexes by anti-IgG leads to a requisite MOG repartitioning into lipid rafts, followed by the activation of signaling pathways related to stress response and cytoskeletal instability, and finally dramatic changes in cellular morphology. These processes are reversible and, at least in the short run, non-toxic. We propose that the signaling cascade triggered upon antibody-mediated repartitioning of MOG into lipid rafts is pertinent to the initial steps of MOG-mediated demyelination in Multiple Sclerosis.

**EXPERIMENTAL PROCEDURES**

**Antibodies.** Antibodies were obtained as follows: mouse monoclonal anti-MOG (8-18C5; Dr. C. Linington, University of Aberdeen, UK); anti-MAG 513mAb (Dr. R. Quarles, NIH, MD); anti-CRMP-2 (Dr. S. Strittmatter, Yale University, New Haven, CT); anti-EF-2 (Dr. A. Nairn, Yale University, New Haven, CT); anti-β, -HSC70, -Fyn, -enolase and -HSP a/b (Santa Cruz, Santa Cruz, CA); anti-β-actin, -β-tubulin, -acetylated and -tyrosine tubulin, and -heme oxygenase-1 (HSP-32) (Sigma, St. Louis, MI); anti-phosphoserine and - phosphothreonine (Zymed, San Francisco, CA); anti-annexin vi and -phosphotyrosine (4G10), and goat anti-mouse IgG (Transduction Laboratories, San Diego, CA); phospho-Akt, phospho-EF-2 and phospho ErbB2 antibody sampler kit (Cell Signaling Technology, Beverly, MA); anti-insulin growth factor-1 receptor, -Src pY418 and pY529 phospho-specific antibodies (Biosource, Camarillo, CA); anti-HSP-74/Grp75 (Calbiochem, San Diego, CA); anti-active MAPK and -pan Erk1/2 (Promega, Madison, WI); anti-myelin basic protein (MBP) (Sternberger Monoclonals, Baltimore, MD); O4 monoclonal antibody (15) was prepared in our laboratory (16).

**Cell Culture.** Mixed primary cultures and highly enriched populations of maturing OLs were prepared as previously described (17, 18). OL populations were grown in defined medium [modified N2 (mN2) (19, 20)] for 7 days to obtain MOG-expressing OLs.

**Antibody Perturbation.** OLs were first incubated at 37 C with monoclonal anti-MOG antibody (155 µg/mL IgG) for various time intervals (5-60 min). Antibody was then washed out by two changes of Dulbecco's modified Eagle's medium (DMEM). In most cases the OLs were then further incubated at 37 C with goat anti-mouse IgG (1:500) for 5-60 min to cross-link the anti-MOG/MOG complexes formed in the first step. In some cases cells were treated prior to antibody incubation with the protein kinase inhibitors (Calbiochem, San Diego, CA) PD098059 (50 µM), LY294002 (50 µM) or Y27632 (15 µM) for 2 h, or with methyl-β-cyclodextrin (MCD, 5 mM, Sigma, St Louis, MI) for 15 min at 37 C. Controls were subjected to the same schedule of washes and incubations.

**Detergent Extraction and Sucrose Gradient Ultracentrifugation.** Cells were scraped into 0.5 mL of a solution of 150 mM NaCl, 5 mM EDTA, 25 mM Tris-Cl buffer (pH 7.5) containing 1 mM PMSF, 10 µg/mL leupeptin/aprotinin, 50 mM NaF, 10 mM NaPO4, 1 mM Na o-Vanadate and Triton X-100 (1% final concentration) as previously described (14). Samples were centrifuged (13,000xg, 15 min) to separate them into a detergent insoluble pellet and a detergent soluble supernatant fraction, and processed for immunoprecipitation and SDS-PAGE. For sucrose gradient ultracentrifugation (14, 21), pellet fractions were mixed with 2 M sucrose, overlaid with 1 M and 0.2 M sucrose, and centrifuged for 16 h at 200,000xg at 4 C. After centrifugation, 0.5 mL fractions were collected at 4 C from the top (fraction 1) to the bottom (fraction 10) of the gradient.

**Immunoprecipitation.** Soluble and insoluble fractions of the TX-100 extracts were solubilized in 500 µL of RIPA buffer (10 mM Tris pH 7.6, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% NP-40, 1% TX-100) and incubated overnight with anti-Fyn (1:50) at 4°C. Protein A Sepharose (0.01 mg/mL) beads (Amersham Biosciences, Piscataway, NJ) were added for 2 h at 4°C. Immunocomplexes were separated by centrifugation, washed 4 times with RIPA buffer, and analyzed by SDS-PAGE/Western blot with anti-pan or -phosphorylated Fyn (anti-pY418 or anti-pY529).
SDS-PAGE and Immunoblotting Analysis. Solubilized samples (above) were loaded onto acrylamide gels (Protein II mini-cell apparatus, BioRad, Richmond, VA). SDS-PAGE was performed as previously described (14). Densitometric analysis of immunoblots was carried out using NIH Image 1.62.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). 2D-PAGE was carried out as previously described (2, 14, 22). Briefly, OL extracts (300 μg of protein) were solubilized in 7 M urea, 2 M thiourea, 2% ASB-14, 0.5% IPG buffer 4-7, 100 mM DTT, 0.001% bromophenol blue. Samples were applied to Immobiline™ Dry Strip pH 4-7 isoelectric focusing gels (Amersham Biosciences, Piscataway, NJ). Proteins were separated in the first dimension (200 V, 1 h; 500 V, 1 h; 1,000 V, 1 h; ramped to 8,000 V, 30 min; held at 8,000 V for 30,000 Vh) at 20°C using an IPGPhor electrophoresis unit (Amersham Biosciences, Piscataway, NJ). For the second dimension, SDS-PAGE was carried out in 10% acrylamide running gels.

Mass Spectrometric Analysis. Coomassie blue-stained spots were excised and digested overnight with trypsin. Tryptic peptides were separated by microcapillary liquid chromatography coupled to a tandem mass spectrometer (LCQ-DECA; Finnigan-MAT, San Jose, CA; Center for Proteomics and Biological Mass Spectrometry at the University of Connecticut Health Center). Peptide fragmentation spectra were analyzed against the OWL database by using the SEQUEST algorithm followed by INTERACT and PEPTIDE PROPHET (2).

Immunofluorescence Microscopy. Mature OLs were stained live with anti-sulfatide [O4 (16)], or fixed with 4% paraformaldehyde and stained with either phospho-specific (1:50) or total EF-2 (1:100) antibodies, or with anti-MBP, as previously described (14). The diameter of randomly chosen cells (100 cells per experiment) was determined by using a calibrated microscopic grid.

Detection of Cell Death. Cell death was detected by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Apoptag kit, Serologicals Corporation, Norcross, GA) according to the manufacturer’s instructions and as described before (23); the total cell number was determined by Hoechst dye staining. Percentages of TUNEL+/total cells were estimated by analysis of at least 30 fields from three independent experiments (100 cells were analyzed in each experiment).

Determination of cAMP and Calcium (Ca2+) levels. Changes in cAMP levels upon MOG cross-linking were determined by using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer’s instructions. To estimate changes in Ca2+ levels, mature OLs were labeled with 10 μg/mL Fluo-4 AM (Molecular Probes, Eugene, OR) for 30 min at 37 C. After 30 min of recovery with fresh media, anti-MOG antibody followed by anti-mouse IgG (5 min incubation each antibody at 37 C), anti-MAG antibody followed by anti-mouse IgG (5 min incubation each antibody at 37 C) or anti-mouse IgG alone (5 min incubation at 37 C) was added to the culture. Fluorescence intensity changes (estimating Ca2+ levels) were recorded every 15 sec (starting before the addition of anti-MOG or anti-MAG antibody) by time lapse confocal microscopy (LSM 510 Zeiss) using a thermo-controlled chamber. Image analysis was carried out with NIH Image 1.62. Ionomycin was used as a positive control. A total of 20 cells were analyzed for each condition.

Statistical Analysis. Means and SEMs were calculated, and evaluation of statistical significance between conditions was made using Student’s two-tail t-test.

RESULTS

Identification of Proteins that Change their Phosphorylation State Upon MOG Cross-Linking

We previously have shown (14, 21) that treatment of live OLs with anti-MOG followed by cross-linking secondary antibody (subsequently referred to as MOG cross-linking) causes rapid repartitioning of MOG into a detergent insoluble fraction. At least half of this fraction has key biochemical characteristics of lipid rafts, including detergent solubility at 37 C or at 4 C upon prior cholesterol perturbation, and low density. MOG repartitioning is followed rapidly by changes in the phosphorylation state of specific proteins, and dramatic changes in OL cytoarchitecture. In
contrast, treatment with anti-MOG alone was ineffective at inducing MOG repartitioning or changes in cell morphology (14); these effects were confirmed in this study, even when treatment with anti-MOG alone was extended to 60 min (Fig. 1A).

To identify the proteins undergoing MOG cross-linking-mediated changes in phosphorylation state, we first resolved the total population of cellular proteins by 2D-PAGE, using a method that allows increased resolution of transmembrane and GPI-anchored proteins from lipid-rich membranes such as myelin and myelin-like membranes from OLs (22) (Fig. 1B-D). No changes were observed in the overall pattern of proteins identified by silver staining (Fig. 1B). However, upon further analysis by anti-phosphoaminoacid immunoblots generated from these 2D gels, we found significant differences in the phosphorylation state of specific proteins (Fig. 1C). These phosphoproteins were then identified by mass spectrometry and/or 2D-Western blot with specific antisera (Fig. 1D). As noted previously (14), G\textbeta and \beta-tubulin underwent dephosphorylation on threonine (Fig. 1C, spot 4) or tyrosine (Fig. 1C, spot 5), respectively. In addition, eight other proteins were identified (Fig. 1C-D, 2A) that underwent hyperphosphorylation of either tyrosine [heat shock protein 74 (HSP74, spot 1), annexin vi (spot 2), Fyn (spot 3), mitogen-activated protein kinase (MAPK, spots 6-6'), \alpha-enolase (spot 7)], threonine [collapsin response mediator protein-2 (CRMP-2, spot 8), elongation factor 2 (EF-2, spot 9), and/or serine [\alpha-enolase (spot 7), CRMP-2 (spot 8), Akt (identified by 1D immunoblot with a phosphospecific antibody; Fig. 2A)]. In contrast to MOG, the detergent insolubility of these proteins was not changed upon cross-linking (data not shown). These proteins fall into three general functional categories involving cellular stress response, signal transduction and cytoskeletal stability.

Response of OLs to Anti-MOG Alone Compared to MOG Cross-Linking

ERK members of the MAPK kinase family, in close association with PI-3 kinase (PI3K) and its downstream effector Akt (24), promote OL survival (25, 26). Further investigation showed that in contrast to the other phospho-proteins that required MOG cross-linking (e.g., EF-2, Fig. 2B, and data not shown), treatment with anti-MOG antibody alone (i.e., no secondary cross-linking) led to hyperphosphorylation of both MAPK and Akt (Fig. 2B).

In many cases, an interrelationship has been observed between protein phosphorylation and the intracellular levels of calcium (Ca\textsuperscript{2+}) and/or cyclic AMP (cAMP). For example, G\textbeta down-regulates Ca\textsuperscript{2+} influx by modulating the activity of voltage-gated Ca\textsuperscript{2+} channels (27); increased Ca\textsuperscript{2+} levels lead to the phosphorylation of certain proteins, including MAPK (28-30); calcium-calmodulin kinase III phosphorylates EF-2 in response to both Ca\textsuperscript{2+} and cAMP (31). Therefore, we examined Ca\textsuperscript{2+} and cAMP levels before and after exposure of OLs to antibodies. Calcium levels rapidly increased (≤1 min) upon treatment with anti-MOG alone; they remained elevated without further increase, even after the addition of secondary cross-linking antibody (starting at 300 sec, see arrow; Fig. 3A). As controls, this response was not observed when cross-linking was carried out using anti-myelin associated glycoprotein (anti-MAG) antibody or secondary antibody alone (Fig. 3A). In contrast, the levels of cAMP in OLs were unchanged by treatment with either anti-MOG antibody alone, anti-MOG followed by secondary antibody, or secondary antibody alone (Fig. 3B).

We conclude that treatment with anti-MOG antibody alone is sufficient to cause increased Ca\textsuperscript{2+} levels, and hyperphosphorylation of MAPK and Akt.

Changes in Stress Response-Related Proteins Upon MOG Cross-Linking

The cellular stress response (e.g., heat shock) is a protective mechanism that enhances cell survival in times of environmental stress by down-regulating the level of protein synthesis, thereby limiting the accumulation of misfolded proteins (32). The MOG cross-linking dependent hyperphosphorylations of EF-2, HSP-74 and \alpha-enolase [another heat shock protein (29)] are consistent with a cellular stress response. In addition, the level of HSC-70, a member of the HSP-70 family previously implicated in Multiple Sclerosis (33), was also significantly increased upon MOG cross-linking, whereas the total levels of two other heat shock proteins, HSP-90 and HSP-32, were unaffected (Fig. 4A). Since EF-2 is inactivated by phosphorylation at residue Thr56,
thereby preventing the elongation step of protein synthesis (31), we used an antibody directed against phospho-EF-2/Thr56 to further study this protein in OLs. We found that EF-2 did in fact undergo hyperphosphorylation at Thr56 upon MOG cross-linking, and that this occurred in the TX-100 soluble fraction; as shown also in Fig. 1B, the total level of EF-2 was unmodified (Fig. 4A). Immunocytochemistry further confirmed the cross-linking dependent hyperphosphorylation of EF-2 in OLs, accompanied by the predicted retraction of OL processes (Fig. 4Bd).

The changes in stress responding proteins and OL morphology could in principle be an indication of cell death. However, the number of TUNEL+ cells was unchanged even when cells were cross-linked for either up to 2 h and analyzed immediately, or for 30 min and then analyzed 2 to 14 h after antibody removal (supplementary Fig. 1). Similar results were obtained when cell death was evaluated by propidium iodine staining (not shown). We conclude that antibody cross-linking of OLs in culture does not lead to cell death over the time periods studied, and in fact previous studies have shown that the physiological changes induced by MOG cross-linking are reversible (14).

Role of Cytoskeletal-Related Proteins in MOG-Mediated Events

The cytoskeleton is a critical mediator of cellular physiology. Several proteins that change their phosphorylation state upon MOG cross-linking (Fig. 1) regulate cytoskeletal dynamics. CRMP-2 activation is related to depolymerization of both actin microfilaments and microtubules (34, 35); changes in β-tubulin and Fyn phosphorylation affect microtubule integrity (36-38); and annexin vi phosphorylation increases its calcium binding affinity (39), leading to an inhibition of F-actin polymerization (30).

Noting that the phosphorylation of CRMP-2 by active Rho serine/threonine kinase leads to actin depolymerization during growth cone collapse (35), we studied the effect of exposing OLs to a Rho kinase inhibitor (Y27632) (40) prior to MOG cross-linking (Fig. 5). As expected, this treatment prevented the increased phosphorylation of CRMP-2 upon cross-linking (Fig. 5A), and in addition, significantly attenuated, but did not completely eliminate, the MOG cross-linking-mediated retraction of OL processes (Fig. 5Bb,f, 5Cb,f). The Rho kinase inhibitor did not prevent changes in either the phosphorylation state of MAPK or Akt (Fig. 5D) or of the other proteins whose phosphorylation state was altered by MOG cross-linking (data not shown). These data indicate that although Rho kinase is clearly an important mediator of the MOG cross-linking-mediated changes in OL morphology, presumably operating through CRMP-2, other molecules unaffected by the Rho kinase inhibitor also must contribute to this process. Further, although pre-treatment of OLs with inhibitors of either MAPK (PD098059) or PI3K (LY294002) prior to MOG cross-linking eliminated the increased phosphorylation of MAPK and Akt, respectively (Fig. 5D), it did not prevent changes in OL morphology (Fig. 5Bc-d, g-h, 5Cc-d,g-h).

Tyrosine phosphorylation of β-tubulin is stimulated by activation (i.e., phosphorylation) of insulin growth factor receptor (IGF-R), EGF receptor (a member of Erb family of receptor tyrosine kinases), and by src kinases such as Fyn (37). Although the expression of EGF receptor is down-regulated in OLs upon terminal differentiation, in mature OLs high levels of ErbB2 tyrosine kinase are maintained (41), and ErbB2 is necessary for normal OL differentiation and survival; further, the astrocytic expression of ErbB2 is reduced in Multiple Sclerosis lesions (42). However, the examination of the effect of MOG cross-linking on these receptors showed that neither IGF-R nor ErbB2 phosphorylation was altered (Fig. 6A).

Although Fyn became hyperphosphorylated upon MOG cross-linking (Fig. 1B), its reactivity with anti-PY418 (indicating kinase activity) was reduced in the detergent insoluble fraction (Fig. 6A). We hypothesized that the overall hyperphosphorylation of Fyn could be due to Csk-mediated phosphorylation of the carboxy-terminus of Fyn (PY529), a reaction that blocks Fyn kinase activity (43). Consistent with this hypothesis, we found that upon MOG cross-linking, there is an increased reactivity in the detergent insoluble fraction against PY529 on Fyn (Fig. 6A). These data suggest that since Fyn kinase can phosphorylate β-tubulin, inactivation of Fyn by MOG cross-linking could lead to β-tubulin dephosphorylation, in turn affecting the integrity of microtubules.

Microtubule depolymerization is also
triggered by the addition of a carboxy-terminal tyrosine or deacetylation of α-tubulin (37). However, the levels of either acetylated or tyrosinatable α-tubulin were not changed upon MOG cross-linking (Fig. 6B).

These results suggest that a rearrangement and depolymerization of the cytoskeleton is an early event following MOG cross-linking, consistent with the observed morphological alterations.

**Lipid Rafts are Mediators of the Signaling Response to MOG Cross-Linking**

We previously showed that upon MOG cross-linking, the detergent insoluble MOG is distributed between a low density lipid raft fraction and a higher density fraction that is likely to be based on protein-protein interactions (14). In order to further assess the importance of the low density fraction in the response of OLs to MOG cross-linking, we disrupted lipid raft integrity by treatment with methyl-β-cyclodextrin (MCD), an oligomer that depletes cholesterol from the plasma membrane (11). Under these conditions, GM1 ganglioside (a widely used marker for lipid rafts) was solubilized (1% TX-100, 4 °C), the low density fraction of MOG was eliminated, and the detergent insoluble MOG was associated entirely with the high density fraction (Fig. 7A). Further, this pre-treatment with MCD prevented the MOG cross-linking induced changes in the phosphorylation states of not only β-tubulin, Gpβ and Fyn, proteins that reside within lipid rafts (14, 44-46), but also of the non lipid raft-associated proteins EF-2, annexin vi, HSP-74, α-enolase, and of the expression level of HSC-70 (Fig. 7B). In addition, the MOG cross-linking-mediated retraction of OL processes was prevented by MDC pre-treatment; i.e., the cell diameters (estimated by MBP immunofluorescent microscopy; mean ± SEM in arbitrary units) of MOG cross-linked OLs after MCD treatment was significantly larger (5.29 ± 0.22) than those without MCD pre-treatment (2.56 ± 0.12) and similar to untreated (5.56 ± 0.23) (Fig. 7Ca-d). Offering a convenient control, we found that the anti-MOG induced increase in phosphorylation of MAPK and Akt (MOG repartitioning independent) were unaffected by treatment with MCD (Fig. 7B). We conclude that two signal transduction pathways are activated by the binding of anti-MOG to its ligand, one acting through MOG in its non-raft state (i.e., MAPK and Akt), and another that requires MOG repartitioning into lipid rafts and that induces morphological alterations in OLs.

**DISCUSSION**

An important aspect of the pathogenesis of demyelination in Multiple Sclerosis involves brain inflammation mediated by T cells, leading to local activation of microglia and impairment of the integrity of the blood brain barrier. In addition, there is increasing evidence that anti-MOG antibodies play an important role by entering the central nervous system through a compromised blood brain barrier and destroying myelin (8-10). Although there is still some controversy on the specificity of the antibody response in Multiple Sclerosis patients, the role of anti-MOG in those patients with pattern II demyelination has been clearly demonstrated (5). A similar pathology with severe demyelination is found in an experimental allergic encephalomyelitis in both rodents and primates upon the presentation of either purified MOG or anti-MOG antibody (8, 47-49). In addition, demyelination has been produced in aggregating brain cell cultures by anti-MOG, whereas antibodies against other myelin proteins had no effect (50).

In spite of the evidence that MOG/anti-MOG interactions could be mediators of demyelination in EAE and Multiple Sclerosis, the mechanism is unclear. We have proposed a model in which antibody binding to MOG on the surface of OLs leads to altered signal transduction mediated by glycopshingolipid-cholesterol microdomains (lipid rafts) (14). Specifically, we showed that complement-independent antibody cross-linking of MOG on the surface of OLs results in the rapid repartitioning of MOG into lipid rafts, leading to specific changes in the pattern of protein phosphorylation, and culminating in a rapid retraction of OL processes and myelin-like membranes (14). It is noteworthy that these changes are specific for anti-MOG; for example, although cross-linking Myelin Associated Glycoprotein (MAG) also instigates signaling modifications, these are distinct from those observed with MOG and do not result in morphological alterations (3). Further, whereas...
both anti-MOG and anti-MAG antibodies are detected among the abundance of autoantibody responses in Multiple Sclerosis, only anti-MOG antibodies appear to be pathogenic in this disease (5, 51, 52).

In the present study, we have obtained important insights into the anti-MOG/MOG-mediated mechanism by identifying a specific set of proteins that change their phosphorylation state upon MOG cross-linking and placing them in a functional context. These data are accompanied by analyses of signal transduction pathways that are likely to be involved, and the demonstration that OL cell death does not occur.

On the basis of these results we propose the following model (Fig. 8). Upon OL treatment with anti-MOG antibody alone, MAPK and Akt pathways are activated and Ca²⁺ levels increase. These signaling modifications appear to be lipid raft independent since neither MOG, MAPK or Akt are found in these microdomains under these conditions. Further, since the Akt, and probably MAPK, pathways promote survival of OLs (25, 26), these changes may have implications in the normal physiology of the cell, in which stimulation of MOG with a hypothetical ligand may support survival of OLs. Upon further cross-linking the MOG/anti-MOG complexes by anti-IgG antibodies, MOG becomes repartitioned into detergent insoluble complexes, at least half of which exhibits characteristics of lipid rafts. According to current models, lipid rafts provide a specialized environment for novel molecular interactions that can activate signal transduction pathways (11). Here we demonstrate the lipid raft-dependent activation of two physiological events. One, a stress-related pathway is implicated by virtue of the phosphorylation of α-enolase, HSP-74 and EF-2, and increased levels of HSC-70. Two, a reduction of cytoskeletal integrity is indicated by inactivation of Fyn that can promote dephosphorylation of β-tubulin, and the phosphorylation of CRMP-2 and annexin vi, leading to retraction of cell processes and loss of membranes. In addition, the observed inactivation of Gβ may alter the βγ-mediated regulation of calcium channels (27) and survival pathways (53).

Among the phosphorylation changes affecting cytoskeletal integrity, the activation of CRMP-2 appears to be of particular importance since a substantial attenuation of the OL morphological damage is achieved by preventing Rho kinase-mediated CRMP-2 phosphorylation. These results agree with previous observations showing that Rho kinase activation inhibits process extension of OLs (54). Moreover, Fyn kinase down-regulates Rho kinase activity during OL differentiation (55), suggesting that the activation of CRMP-2 through Rho kinase shown in our model may be a downstream effect of Fyn inactivation. We conclude that the rearrangement of the OL cytoskeleton is a key event following MOG cross-linking, leading to severe morphological, and thus functional, implications. In principle, a reversion of harmful effects of MOG cross-linking could be effected by preventing these cytoskeletal changes, suggesting a potential target for therapeutic intervention in Multiple Sclerosis.

Since our model proposes that a secondary cross-linking antibody is required in order to activate demyelinating signaling, the question arises as to the identity of a binding partner for anti-MOG that could be playing that role in vivo. For example, macrophages and complement are logical candidates for mimicking the effect of the secondary cross-linking antibody by virtue of their ability to bind IgG molecules (56). Alternatively, MOG binds the complement component C1q (57), although recent structural data on MOG suggests that the proposed C1q site is oriented toward the membrane surface, adopting a conformation that would prevent the binding of C1q in vivo (58). Nevertheless, in our model, once anti-MOG antibody binds to the OL surface, C1q could bind to the Fe region of anti-MOG and possibly mimic the effects that we observe using a cross-linking secondary antibody. A similar effect could be triggered upon binding of Fe receptors from macrophages. In addition, the simultaneous presence of polyclonal anti-MOG antibodies that recognize different epitopes of MOG in Multiple Sclerosis patients could be sufficient to cross-link MOG and initiate a lipid-raft mediated demyelinating signaling, in this case eliminating the requirement for a secondary cross-linking molecule. The further elucidation of both physiologic and pathological-induced MOG/anti-MOG binding partners offers exciting potential for novel therapies for Multiple Sclerosis.
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**FOOTNOTES**

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Abbreviations: MOG (myelin oligodendrocyte glycoprotein); OLs (oligodendrocytes); Gβ (β subunit of the trimeric G protein); MBP (myelin basic protein); mN2 (modified N2); DMEM (Dulbecco's modified Eagle's medium); MCD (methyl-β-cyclodextrin); 2D-PAGE (two dimensional polyacrylamide gel electrophoresis); MAPK (mitogen-activated protein kinase); CRMP-2 (collapsin response mediator...
protein-2); EF-2 (elongation factor 2); PI3K (PI-3 kinase); MAG (myelin associated glycoprotein); IGF-R (insulin growth factor receptor).

FIGURE LEGENDS

Figure 1: MOG cross-linking induces repartitioning of MOG into detergent insoluble fractions and changes the phosphorylation of specific proteins. (A) MOG is solubilized (S) by 1% TX-100 at 4 °C from untreated OLs, or from OLs treated with anti-MOG antibody for 5 to 60 min, or with secondary antibody alone. Treatment with anti-MOG (15 min) plus secondary cross-linking antibody (15 min, “MOG Cross-linked”) results in the repartitioning of MOG to the TX-100 insoluble fraction (P). (B-D) Identification by 2D PAGE electrophoresis of proteins that change their phosphorylation state upon MOG cross-linking (pI range 4-7). (B) Silver staining. (C) Immunoblots with anti-phosphotyrosine, anti-phosphothreonine and anti-phosphoserine antibodies. (D) Immunoblots with specific antibodies (the positions of these proteins in B and C are indicated): (1) HSP-74, (2) annexin vi, (3) Fyn, (4) Gβ, (5) β-tubulin, (6-6’) MAPK, (7) α-enolase, (8) CRMP-2 and (9) EF-2. Note that the number of spots in D is higher than in C due to the existence of multiple protein isoforms, not all of which are phosphorylated. Asterisks (*) indicate phosphospots. α-Enolase is phosphorylated on both tyrosine and serine, and CRMP-2 on both serine and threonine residues. Typical results out of three independent experiments are shown.

Figure 2: Anti-MOG alone or MOG cross-linking leads to differential signaling responses. (A) Immunoblot analyses of TX-100 (1%, 4 °C) soluble (S) and insoluble (P) fractions upon MOG cross-linking (anti-MOG 15 min, followed by secondary antibody 15 min, at 37 °C; “anti-MOG+2 Ab”) using phosphoprotein-specific antibodies show the hyperphosphorylation of MAPK (levels of total MAPK are unchanged) and Akt (levels of actin are used as loading controls). The graph shows the ratios in arbitrary units (mean ± SEM) of p-MAPK/pan MAPK and p-Akt/actin of control (c) vs. cross-linked (t) (n = 3, p<0.0001). (B) Treatment with anti-MOG alone (5-60 min) also increases the phosphorylation of MAPK and Akt, but not of EF-2. Typical results out of three independent experiments are shown.

Figure 3: Levels of second messengers upon MOG cross-linking. (A) Changes in calcium levels were monitored every 15 sec upon treatment with anti-MOG alone (0-300 sec) followed by the subsequent addition of secondary antibody (300-600 sec). Arrow indicates time of addition of secondary antibody. Similar treatment was carried out with anti-MAG followed by cross-linking with secondary antibody, or with secondary antibody alone. Levels of calcium increase upon treatment with anti-MOG alone, but not further after the addition of secondary antibody. MAG cross-linking or treatment with secondary antibody alone does not modify calcium compared to basal levels (1.00). The graph shows the means of the ratio changes (magnitude of fluorescence increase in arbitrary units) of four independent experiments (mean ± SEM). (B) Similar levels of cAMP are found in OLs treated with secondary antibody (5 min) alone, anti-MOG antibody (5 min) alone, or anti-MOG (5 min) followed by secondary antibody (5 min). The graph shows the results of three independent experiments (mean ± SEM).

Figure 4: MOG cross-linking induces changes in stress response-related proteins. (A) Immunoblot analyses of TX-100 (1%, 4 °C) soluble (S) and insoluble (P) fractions upon MOG cross-linking (anti-MOG 15 min, followed by secondary antibody 15 min, at 37 °C; “anti-MOG+2 Ab”) using a phosphoprotein-specific antibody show the hyperphosphorylation of EF-2 (levels of total EF-2 are not modified), and increased total levels of HSC-70 but not of HSP 90 or HSP-32 (levels of actin are used as loading controls). The graph shows ratios in arbitrary units (mean ± SEM) of HSC-70/actin = 0.43 ± 0.01 (control, Pc) vs. 0.59 ± 0.01 (cross-linked, Pt) (n = 3, p<0.001) from the TX-100 insoluble fraction, or 0.59 ± 0.01 (control, Sc) vs. 0.75 ± 0.01 (cross-linked, St) (n = 3, p<0.001) from the TX-100 soluble fraction; and phospho-EF-2/pan EF-2 = 0.29 ± 0.01 (control, Sc) vs. 0.51 ± 0.04 (cross-linked, St) (n = 3,
p<0.001) from the TX-100 soluble fraction. (B) Immunocytochemical analyses of untreated control (a,c) and MOG cross-linked (b,d) cells show that the level of EF-2 is unchanged (a,b), but that the level of phospho-EF-2 is increased upon cross-linking (compare c vs. d). Note that as expected, MOG cross-linked cells show a severe process retraction (one cell in a,b and c, and three cells in d). Photomicrographs were taken with the same exposure time. Bar = 5 μm. Typical results out of three independent experiments are shown.

**Figure 5**: Alterations in OL morphology are dependent on Rho kinase, but not on MAPK or PI3K signaling. (A) 2D-Immunoblot analysis of phospho-CRMP-2 (phosphothreonine immunoblot) from control (c) and MOG cross-linked (t) OLs with or without pre-treatment with Rho kinase inhibitor (RhoK inh) shows that the increased phosphorylation of CRMP-2 upon cross-linking is prevented by inhibition of Rho kinase. (B) Immunostaining with O4 antibody shows OL morphology before (a-d) and after (e-h) MOG cross-linking in the absence (a,e) or presence of inhibitors of Rho (b,f), MAPK (c,g) or PI3K (d,h) kinases. Bar = 5 μm. (C) Analysis of the diameter (arbitrary units) of randomly chosen cells (B) shows that Rho kinase inhibitor attenuates the MOG cross-linking induced reduction in cell diameter (f) (p<0.0001, ***). The results of three independent experiments (mean ± SEM) are shown. (D) Immunoblots from TX-100 (1%, 4 °C) soluble (S) and insoluble (P) fractions from control (c) and MOG cross-linked (t) OLs show that Rho kinase inhibitor does not affect the increased phosphorylation of MAPK and Akt upon MOG cross-linking. However, MAPK and PI3K inhibitors prevent the increased phosphorylation of MAPK and Akt, respectively. Typical results out of three independent experiments are shown.

**Figure 6**: MOG cross-linking induces changes in potential regulators of microtubular stability. Immunoblot analysis of TX-100 (1%, 4 °C) soluble (S) and insoluble (P) fractions from control (c) and MOG cross-linked (t) OLs show (A) no changes in the levels of phosphorylated IGF or ErbB2 receptors upon cross-linking. Following immunoprecipitation with pan-Fyn antibody, the reactivity in the pellet fractions with anti-pY418 (activating site of src family kinase) is reduced, but with anti-pY529 (inactivating site of src family kinase) is increased (total levels of Fyn are unaffected). The graph shows the ratios in arbitrary units (mean ± SEM) of PY418/pan Fyn of control (c) vs. cross-linked (t) (n = 3, p<0.0001), and of PY529/pan Fyn of control (c) vs. cross-linked (t) (n = 3, p<0.05) from the TX-100 insoluble fraction. (B) The levels of acetylated or tyrosine α-tubulin are not modified upon MOG cross-linking. A typical result out of three independent experiments is shown.

**Figure 7**: MOG cross-linking signaling and alterations in OL morphology depend on lipid raft integrity. Immunoblot analyses are shown of (A) sucrose gradient fractions (fraction 1 lowest density, fraction 10 highest density) of TX-100 insoluble (1%, 4 °C) or soluble (S) material from MOG cross-linked OLs, or (B) from TX-100 soluble (S) and insoluble (P) fractions from control (c) and MOG cross-linked (t) OLs with or without methyl-β-cyclodextrin (MCD) pre-treatment. (A-B) Depletion of cholesterol by MCD to disrupt lipid rafts precludes (A) the association of GM1 and MOG with low density fractions (3-5; density = 1.3540-1.3705 mg/mL; note that although MOG is mainly recovered in fractions 3 and 4, GM1 is concentrated in fraction 3, a residual amount is detected also in 4 in an overexposed film), and (B) changes in the phosphorylation states of phospho-β-tubulin (phosphothreonine immunoblot), phospho-Gβ (phosphothreonine immunoblot), active Fyn (pY418), phospho-EF-2 or the level of HSC-70. In contrast, the increased phosphorylation of MAPK and Akt are unaffected by this treatment. (C) Immunostaining with anti-MBP antibody shows OL morphology before (a,c) and after (b,d) MOG cross-linking in the absence (a,b) or presence of MCD (c,d). MCD pre-treatment prevented the MOG cross-linking induced changes in OL morphology (retraction of OL processes). Bar = 5 μm. Typical results out of three independent experiments are shown.
**Figure 8:** Proposed model for events triggered upon MOG cross-linking in OLs. (A) Under control (untreated) conditions MOG is not significantly associated with lipid rafts. (B) Upon OL treatment with *anti-MOG antibody alone*, MAPK and Akt survival pathways are activated, and calcium levels increase, independently of lipid raft signaling since MOG remains in its non-raft state. (C) Upon *MOG cross-linking with secondary antibodies* (anti-MOG+2 Ab) MOG repartitions into lipid rafts, leading to dephosphorylation of Gβ, alteration of cytoskeletal integrity (inactivation of Fyn, dephosphorylation of β-tubulin, phosphorylation of CRMP-2 and annexin vi), and activation of stress-related pathways (phosphorylation of EF-2, HSP-74 and α-enolase, and increased levels of HSC-70).

**Supplementary Figure 1:** MOG cross-linking does not affect OL survival. TUNEL assay. Control (a) or MOG cross-linked OLs for 5 (b), 15 (c), 30 (d), or 60 (e) min (i.e., anti-MOG followed by secondary antibody, each for the times indicated). In some cases, OLs were first treated as in c, whereupon the antibody-containing medium was removed and the cells are grown further in fresh medium for 2 (f) or 14 (g) h. The numbers of TUNEL+/total cells are not significantly different upon MOG cross-linking (t-test with 90% of confidence). The results are expressed as means + SEM of three independent experiments.
Figure 2

A

Control anti-MOG+2°Ab

p-MAPK
MAPK
p-Akt
actin

P S P S

p-MAPK
p-Akt

Ratio

0 0.5 1.0 1.5

c t c t

***

B

Control 2ndAb anti-MOG alone

p-MAPK
p-Akt
p-EF2

P S P S P S P S P S

5' 15' 30' 60'
Figure 3

A

**Ca\(^{2+}\) levels**

- Diamond: anti-MOG + 2\(^{nd}\) Ab
- Triangle: anti-MAG + 2\(^{nd}\) Ab
- Circle: 2\(^{nd}\) Ab alone

B

**cAMP levels**

- Control
- 2\(^{nd}\) Ab
- Anti-MOG + 2\(^{nd}\) Ab
Figure 4

A

| Control | anti-MOG+2°Ab |
|---------|--------------|
| HSC-70  |              |
| HSP-90  |              |
| HSP-32  |              |
| actin   |              |
| p-EF2   |              |
| EF2     |              |

P | S | P | S

![Graph showing ratios of HSC-70 and p-EF2 with significant differences marked with asterisks.]

B

![Images showing different cell structures labeled as a, b, c, and d.]

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Figure 5

A

- RhoK inh + RhoK inh

p-CRMP-2

c  t  c  t

B

a  b  h  c
e  f  g  h

C

Cell Diameter

a  b  c  d  e  f  g  h

D

| RhoK inh | MAPK inh | PI3K inh |
|---------|---------|---------|
| p-MAPK  |         |         |
| p-Akt   |         |         |

Pc  Sc  Pt  St  Pc  Sc  Pt  St  Pc  Sc  Pt  St
Figure 8

A. Untreated

B. Anti-MOG Alone

C. Anti-MOG+2°Ab

Cytoskeletal Rearrangements

Stress

\[ \begin{align*}
\downarrow \text{pTubulin} & \quad \uparrow \text{pEF-2} \\
\uparrow \text{pAnnexin vi} & \quad \uparrow \text{pHSP74} \\
\uparrow \text{pCRMP-2} & \quad \uparrow \text{p\alpha\text{-enolase}} \\
\end{align*} \]
Signaling cascades activated upon antibody cross-linking of myelin oligodendrocyte glycoprotein. Potential implications for multiple sclerosis
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