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Myelin-Associated Glycoprotein, a Cell Adhesion Molecule of Oligodendrocytes, Is Phosphorylated in Brain

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Myelin-associated glycoprotein (MAG) has been implicated in the mediation of interactions between oligodendrocytes and neurons during the development of the myelin sheath. Here we show that MAG is phosphorylated in intact myelinating mouse brain primarily at serine residues and to a lesser extent at threonine and tyrosine residues. In vivo, only the larger of the two developmentally regulated MAG isoforms is phosphorylated. MAG can be phosphorylated at tyrosine by the v-*fps* and v-*src* protein-tyrosine kinases in vitro and by a kinase endogenous to myelin membrane preparations. MAG phosphorylated in myelin membranes in vitro also contains phosphoserine and phosphothreonine. These observations suggest that phosphorylation of MAG is physiologically significant in regulating oligodendrocyte-neuron interactions.

Glycoproteins related to the immunoglobulin superfamily mediate cell surface adhesion among neural cell types and are thought to play a key role in the development of the nervous system (7). The myelin-associated glycoprotein (MAG) is a recently recognized member of this group (19) that is expressed on the surface of oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. MAG contains an extracellular domain homologous with the external domains of neural cell adhesion molecule, a transmembrane segment, and two distinct cytoplasmic domains generated by developmentally regulated alternative splicing of a primary transcript (21). Like the neural cell adhesion molecule and other cell adhesion molecules, MAG has potential sites for serine and threonine phosphorylation (1, 15, 21, 23). In addition, one of the MAG isoforms is distinguished by a tyrosine residue in a cytoplasmic sequence that shares marked homology with the major autophosphorylation site of the epidermal growth factor receptor (21). To examine the role of this additional domain, we studied the phosphorylation status of the MAG molecule in vivo and in vitro.

To test whether MAG is phosphorylated in vivo, we isolated MAG from rodent brain proteins that were labeled by intracerebral injection of rats (19 days old) or mice (15 days old) with 1 mCi of $^{32}P_i$ in 150 mM NaCl (16, 24). Briefly, brains were removed and directly homogenized into ice-cold buffer A (0.25 M sucrose, 10 mM sodium fluoride, 10 mM EDTA, 10 mM sodium PP_i, 100 μM ammonium vanadate, 1 mM sodium phosphate [pH 7.4], 0.5 mM phenylmethylsulfonyl fluoride, 10 U of aprotinin per ml). Particulate matter was removed from the homogenate by sedimentation at 3,000 × *g* for 5 min. The supernatant was subjected to centrifugation at 75,000 × *g* for 30 min to produce a membrane-enriched pellet which was subsequently washed in buffer A (minus sucrose) and extracted with chloroform-methanol (2:1, vol/vol). The pellet was dissolved overnight in 1% sodium deoxycholate–0.1% sodium dodecyl sulfate (SDS) and then diluted with 1 volume of 2% Triton X-100–1 M NaCl–1 mM $MgCl_2$–2 mM dithiothreitol–10 mM Tris hydrochloride (pH 7.4) to make buffer B. The following day, insoluble material was removed by centrifugation at 13,000 × *g* for 15 min. The supernatant was mixed with concanavalin A-Sepharose for 2 h at room temperature and washed with buffer B, and finally, bound glycoproteins were specifically eluted with buffer B containing 1 M methyl mannoside and 10 mM EDTA. MAG was immunoprecipitated from these radiolabeled glycoproteins. Phosphorylated MAG was immunoprecipitated with either a pool of anti-MAG monoclonal antibodies (Fig. 1A, lanes 4 and 5) or an anti-MAG polyclonal antiserum (Fig. 1A, lane 3). In control experiments with mock-injected brains and [γ-^{32}P]ATP in the homogenization buffers, we detected no phosphorylation in vitro of brain proteins during the fractionation procedures, indicating that the observed phosphorylation occurred in the live animal.

There are two isoforms of MAG which differ in their polypeptide backbones (large MAG [L-MAG] and small MAG [S-MAG], 72,000 and 67,000 daltons, respectively) but when fully glycosylated migrate as a single broad band (100,000 daltons) on SDS-polyacrylamide gels. The two MAG isoforms can be resolved electrophoretically when analyzed as nascent polypeptides after in vitro translation of brain mRNA (9) or after deglycosylation of the mature proteins (21). We used the latter approach to determine which of the isoforms is phosphorylated in vivo. In control experiments, we were able to show that both L-MAG and S-MAG could be detected after chemical deglycosylation of adult rat MAG (Fig. 2A) but that S-MAG was barely detectable if preparations from 19-day-old animals were used (data not shown). MAG was isolated from myelinating rat brains labeled in vivo with $^{32}P_i$ and mixed with unlabeled adult MAG before deglycosylation to ensure that detectable amounts of both L-MAG and S-MAG would be present. The two isoforms were resolved by electrophoresis and visualized by immunoblotting with an alkaline phosphatase-conjugated second antibody and then autoradiography of the immunoblot to detect the labeled isoform(s). Only L-MAG was detectably phosphorylated in vivo (Fig. 2B) and in vitro (data not shown). Additionally, comparison of lanes 1 and 4 in Fig. 2B suggests that only the upper region of the broad

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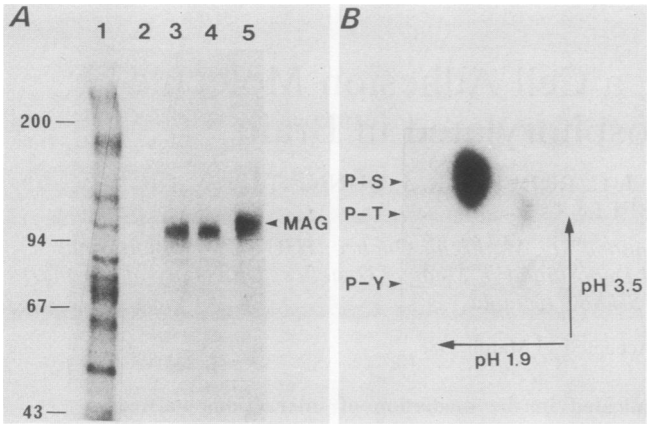

FIG. 1. MAG is phosphorylated in live animals. Purified concanavalin A-binding ^{32}P-labeled brain glycoproteins were incubated with the indicated antibody overnight at 4°C and then with an affinity-purified goat anti-mouse serum (Jackson Laboratory, Bar Harbor, Maine) for 1 h. The immune complexes were precipitated with protein A-agarose, resolved on SDS-7.5% polyacrylamide gels, and detected by autoradiography. (A) Lane 1, Eluate from concanavalin A-Sepharose containing specifically eluted ^{32}P-labeled mouse brain glycoproteins. Lane 2, Eluate immunoprecipitated with rabbit preimmune serum. Lane 3, Eluate immunoprecipitated with a rabbit polyclonal anti-MAG (a gift of H. deF. Webster). Lane 4, Eluate immunoprecipitated with a combination of three anti-MAG monoclonal antibodies (Gen3 S1, Gen3 S3, and Gen3 S5, generously provided by N. Latov). Lane 5, Rat myelin proteins purified as described above and immunoprecipitated with the pooled anti-MAG monoclonal antibodies. The mobilities of the molecular size markers (in kilodaltons) are indicated on the left. (B) Phosphoamino acid analysis of MAG immunoprecipitated from ^{32}P-labeled brains. ^{32}P-MAG was electroeluted from polyacrylamide gels, precipitated with 10% trichloroacetic acid, washed in ice-cold acetone, and hydrolyzed with 6 N double-distilled HCl for 75 min at 100°C. The hydrolysate was lyophilized, mixed with authentic phosphoamino acids, spotted onto MN cel 400 thin-layer cellulose plates, and resolved by two-dimensional electrophoresis as previously described (6). The thin-layer plates were sprayed with ninhydrin to locate the positions of the phosphoamino standards before detection of radioactive phosphoamino acids by autoradiography. The positions of the phosphoamino acid standards are indicated on the left: P-S, phosphoserine; P-T, phosphothreonine; P-Y, phosphotyrosine. Electrophoresis was done sequentially at the pHs indicated; migration toward the anode is indicated by arrows.

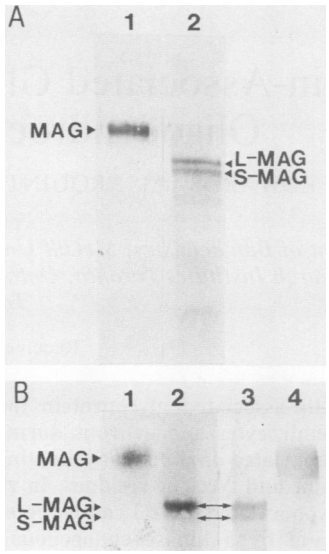

FIG. 2. Identification of phosphorylated MAG species. Adult rat myelin (1 mg) was resolved on 7% polyacrylamide gels. Partially pure MAG was obtained by electroelution. MAG was precipitated with 12% trichloroacetic acid, and the resulting pellet was washed twice with acetone. Half of the precipitate was solubilized in gel electrophoresis sample buffer, and the remaining MAG was deglycosylated by a modification (22) of the procedure of Edge et al. (8). The deglycosylated MAG was washed twice with acetone and was then solubilized into gel electrophoresis sample buffer. (A) Immunoblot analysis of adult rat MAG and deglycosylated adult rat MAG. Adult rat MAG (lane 1) and deglycosylated adult rat MAG (lane 2) were resolved by gel electrophoresis, transferred to nitrocellulose paper, probed with a pool of three monoclonal antibodies directed toward MAG (generously provided by N. Latov), and visualized with an alkaline phosphatase-coupled second antibody. The mobilities of the fully glycosylated MAGs and the deglycosylated forms are indicated on the left- and right-hand side, respectively. (B) Deglycosylation of MAG labeled in vivo with ^{32}P. MAG was immunoprecipitated from ^{32}P-labeled rat brains (19 days old) and was further purified by SDS-polyacrylamide gel electrophoresis and electroelution. One-third of the electroeluted MAG was combined with purified unlabeled adult MAG, precipitated with trichloroacetic acid, and dissolved into SDS sample buffer. Two-thirds of the ^{32}P-MAG was also combined with unlabeled adult MAG, deglycosylated, and then solubilized for gel electrophoresis. The samples were analyzed by autoradiography and immunoblotting. Lanes: 1, autoradiography of 19-day-old rat MAG labeled in vivo; 2, autoradiography of deglycosylated 19-day-old rat MAG labeled in vivo; 3, immunoblot of deglycosylated MAG; 4, immunoblot of MAG.

fully glycosylated MAG band contains phosphorylated residues. From these results, we conclude that in myelinating rat brain only the L-MAG isoform undergoes phosphorylation.

The phosphoamino acids present in immunoprecipitated MAG labeled in vivo were identified by partial acid hydrolysis and two-dimensional thin-layer electrophoresis (Fig. 1B). We detected phosphoserine, phosphothreonine, and phosphotyrosine residues in the ratio shown in Fig. 1B. Clearly, the major residue phosphorylated during in vivo labeling experiments is phosphoserine, though we consistently observed phosphothreonine and phosphotyrosine at low levels.

To further characterize the tyrosine phosphorylation of MAG, we incubated partially purified MAG preparations (18, 20) in vitro with the tyrosine kinases encoded by the v-*fps* and v-*src* oncogenes. The P130$^{gag\text{-}fps}$ and p60$^{v\text{-}src}$ tyrosine kinases were immunoprecipitated from v-*fps*- or v-*src*-transformed rat-2 cells (26). Briefly, 10^7 cells were washed twice with ice-cold Tris-saline and lysed in 2 ml of ice-cold lysis buffer (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1 mM sodium orthovanadate), and the lysate was clarified by centrifugation at 12,000 × *g* for 15 min. The cell lysate was incubated with mouse monoclonal antibodies specific for either P130$^{gag\text{-}fps}$ (R254E anti-p19^{gag} [13]) or p60^{src} (antibody 327 [17]) at a 1:100 dilution at 4°C for 45 min. *Staphylococcus aureus* coated with rabbit anti-mouse immunoglobulin was added for a further 45 min. The *S. aureus*-associated immune complexes were pelleted by centrifugation at 12,000 × *g* for 15 s and extensively washed with lysis and reaction (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], 10 mM $MnCl_2$, 0.1 mM vanadate) buffers. Finally, immune complexes were resuspended in 40 μl of reaction buffer in the presence or absence of partially purified MAG (5 μg); 25 μCi of [γ-^{32}P]ATP was added, and phosphorylation was allowed to occur at 20°C for 15 min. MAG was

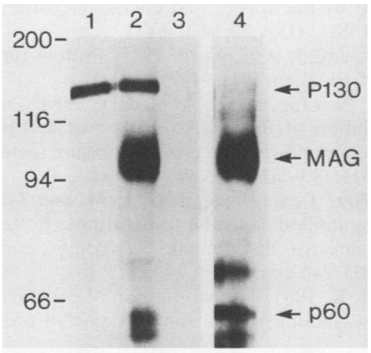

FIG. 3. Phosphorylation of MAG in vitro by the v-*fps* and v-*src* tyrosine kinases. Products from the in vitro kinase reaction as described in the text were solubilized in SDS sample buffer, separated by electrophoresis in an SDS-7.5%-polyacrylamide gel, and detected by autoradiography. Lanes: 1, $P130^{gag\text{-}fps}$ tyrosine kinase alone; 2, $P130^{gag\text{-}fps}$ with MAG (5 μg); 3, MAG alone (5 μg); 4, $p60^{v\text{-}src}$ tyrosine kinase with MAG (5 μg). The mobilities of MAG (100 kilodaltons) and autophosphorylated $P130^{gag\text{-}fps}$ and $p60^{v\text{-}src}$ are indicated.

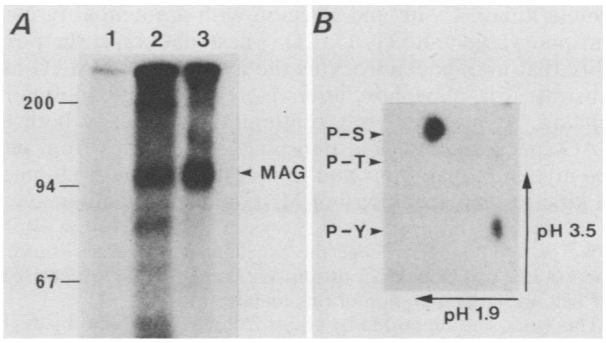

FIG. 4. MAG is phosphorylated by a tyrosine kinase present in myelin membranes. (A) Mouse brain myelin (150 μg of protein) (18) was incubated with 50 μCi of [γ-^{32}P]ATP in a buffer containing 0.05% Triton X-100, 50 mM Tris hydrochloride (pH 7.4), 10 mM NaF, 5 μM EDTA, 2 mM dithiothreitol, and 10 mM $MgCl_2$ at 30°C for 5 min. Ammonium vanadate (200 μm) was included where indicated. The reaction was stopped by the addition of SDS to a final concentration of 1%, and this solution was diluted 10-fold with ice-cold buffer C (1% Triton X-100, 0.15 M NaCl, 10 mM EDTA, 10 mM NaF, 10 mM sodium PP_i, 100 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 U of aprotinin per ml). All further steps were performed at 4°C. MAG was then immunoprecipitated, resolved on SDS-7.5% polyacrylamide gels, and detected by autoradiography. The mobilities of the molecular size markers (in kilodaltons) are indicated on the left. Lanes: 1, MAG immunoprecipitated from ^{32}P-labeled myelin proteins phosphorylated in vitro; 2, MAG immunoprecipitated from ^{32}P-labeled myelin proteins phosphorylated in vitro in the presence of vanadate; 3, MAG immunoprecipitated from mouse brains labeled in vivo. (B) Phosphoamino acid analysis of MAG in lane 2. Details are explained in the legend to Fig. 1B.

strongly phosphorylated by both the $P130^{gag\text{-}fps}$ and $p60^{v\text{-}src}$ viral protein-tyrosine kinases (Fig. 3, lanes 2 and 4). Phosphoamino acid analysis of in vitro-phosphorylated MAG revealed that tyrosine was the only amino acid labeled in these reactions (data not shown). After phosphorylation by $p60^{v\text{-}src}$ or $P130^{gag\text{-}fps}$, the 100 kilodalton polypeptide could be immunoprecipitated by two anti-MAG monoclonal antibodies (Gen3 S1 and Gen3 S3, gift of N. Latov) and an anti-MAG polyclonal antiserum (R3B10, gift of H. deF. Webster), confirming the identity of the tyrosine kinase substrate as MAG (data not shown).

Brain protein kinases capable of phosphorylating MAG copurify with myelin membranes. This was demonstrated by incubating myelin membranes prepared from actively myelinating mouse brains with [γ-^{32}P]ATP in the presence or absence of vanadate, a phosphatase inhibitor (14). In the absence of the phosphatase inhibitor, MAG was phosphorylated to very low levels (Fig. 4A, lane 1). The inclusion of vanadate in the reaction mixture markedly increased MAG phosphorylation (lane 2); phosphoamino acid analysis revealed the presence of phosphotyrosine, as well as phosphoserine and low levels of phosphothreonine (Fig. 4B). These experiments suggest that MAG is a substrate for protein kinases as well as phosphatases in purified myelin membranes. Of particular interest is the finding that myelin membranes contain a tyrosine-specific protein kinase capable of phosphorylating MAG in a cell-free kinase reaction.

The activities and ligand-binding properties of a number of cell surface receptors are apparently regulated by phosphorylation of their cytoplasmic domains. Examples include phosphorylation of growth factor receptors at tyrosine and threonine (11), phosphorylation of the fibronectin receptor at tyrosine (10), and phosphorylation of rhodospin (4) and the β-adrenergic receptor at serine and threonine (2). For the fibronectin receptor, tyrosine phosphorylation in v-*src*- and v-*fps*-transformed cells apparently affects both extracellular binding of fibronectin and intracellular binding of talin (P. Tapley and L. Rohrschneider, personal communication). We report here that MAG undergoes multiple phosphorylation events in vivo at serine, threonine, and tyrosine residues, suggesting that several classes of kinases and phosphatases regulate the activity of this transmembrane protein. The brain has proved to be a rich source of both protein-tyrosine kinase and phosphatase activities. Protein-tyrosine kinases such as $p60^{c\text{-}src}$ are thought to be involved in developmental processes in the central nervous system, although no physiologically relevant brain substrates have been identified to account for these suggested properties (5). The phosphorylation of tyrosine residues on MAG in live animals may be especially significant. During myelination, continuous adjustments must be made to oligodendrocyte-neuron interactions. It is possible that phosphorylation-dephosphorylation of MAG at tyrosine residues modulates these interactions. Similarly, protein kinase C, a serine-threonine kinase which can phosphorylate transmembrane proteins in vivo such as the insulin and epidermal growth factor receptors, may play some role in the regulation of MAG activity (3, 12). Indeed, it is interesting that protein kinase C in oligodendrocytes is activated by a cell-substratum interaction, an event that may involve cell adhesion molecules (25).

Two forms of MAG are expressed in rodent brain as a result of developmentally regulated alternative splicing of the same primary transcript (9, 15), suggesting that MAGs play more than one role in the development and maintenance of myelin. These two MAG isoforms share identical extracellular and transmembrane domains, but differ in their cytoplasmic C-terminal regions. The MAG isoforms can only be resolved electrophoretically following extensive deglycosylation of the mature polypeptide (21). Using this approach we demonstrated that only the larger form of MAG is detectably phosphorylated in vivo during myelinogenesis. This MAG isoform is the predominant MAG species expressed when de novo myelination is most rapid and contains all the potential phosphorylation sites including a

protein kinase C site and a region with a potential tyrosine phosphorylation site (1, 15, 21). These data raise the possibility that in oligodendrocytes the availability of MAG as a substrate for phosphorylation is regulated by alternative splicing. At present, we are attempting to purify both the MAG-specific kinase and phosphatase activities from purified myelin preparations and to investigate the developmental kinetics and specificity of MAG phosphorylation.

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