Microtubule-associated protein 1B: a neuronal binding partner for myelin-associated glycoprotein

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Myelin-associated glycoprotein (MAG) is expressed in periaxonal membranes of myelinating glia where it is believed to function in glia–axon interactions by binding to a component of the axolemma. Experiments involving Western blot overlay and coimmunoprecipitation demonstrated that MAG binds to a phosphorylated neuronal isoform of microtubule-associated protein 1B (MAP1B) expressed in dorsal root ganglion neurons (DRGNs) and axolemma-enriched fractions from myelinated axons of brain, but not to the isoform of MAP1B expressed by glial cells. The expression of some MAP1B as a neuronal plasma membrane glycoprotein (Tanner, S.L., R. Franzen, H. Jaffe, and R.H. Quarles. 2000. J. Neurochem. 75:553–562.), further documented here by its immunostaining without cell permeabilization, is consistent with it being a binding partner for MAG on the axonal surface. Binding sites for a MAG-Fc chimera on DRGNs colocalized with MAP1B on neuronal varicosities, and MAG and MAP1B also colocalized in the periaxonal region of myelinated axons. In addition, expression of the phosphorylated isoform of MAP1B was increased significantly when DRGNs were cocultured with MAG-transfected COS cells. The interaction of MAG with MAP1B is relevant to the known role of MAG in affecting the cytoskeletal structure and stability of myelinated axons.

Introduction

The localization of myelin-associated glycoprotein (MAG)* in periaxonal membranes of myelinating oligodendrocytes and Schwann cells (SCs) suggests that it functions in glia/axon communication by interacting with a component(s) of the axonal surface membrane (for review see Quarles, 1997). Myelin formation is relatively normal during development of MAG-null mice (Li et al., 1994; Montag et al., 1994), but there is degeneration of myelinated axons of peripheral nerve as the mice age (Fruttiger et al., 1995; Yin et al., 1998). The axonal degeneration, which involves decreased caliber and cytoskeletal abnormalities such as reduced phosphorylation and closer packing of neurofilaments (Yin et al., 1998), suggests that the interaction of MAG with a component on the axon is required for normal long term maintenance of myelinated axons. In addition, MAG can promote or inhibit neurite outgrowth in vitro (for review see Cai et al., 2001), also demonstrating that MAG affects neuronal properties. However, little is known about the molecular mechanisms by which MAG affects axonal structure, particularly its neuronal binding partner(s).

MAG has been shown to interact with a variety of neuronal glycoproteins (DeBellard and Filbin, 1999; Strenge et al., 1999) and gangliosides (Collins et al., 1999) based on its lectin specificity for 2,3-linked sialic acid (Kelm et al., 1994). However, there is evidence indicating that sialic acid–binding may reflect a docking mechanism and that a separate peptide-binding site in MAG is crucial for its effects on neurons (Tang et al., 1997). In this report, we describe the binding of MAG to proteins of neuronal membrane preparations from the peripheral and central nervous systems, respectively, that would be expected to contain physiological MAG binding partners, i.e., membranes from dorsal root ganglion neurons (DRGNs) and axolemma-enriched fractions (AEFs) from myelinated axons of brain. In both cases, MAG bound primarily to a high molecular weight protein that was identified as microtubule-associated protein 1B (MAP1B). A previous report from our laboratory (Tanner et al., 2000) and new findings in this paper demonstrate that some MAP1B is expressed as a plasma membrane glycoprotein in neurons, supporting the concept that MAP1B is an axonal binding partner for MAG.
Results and discussion

MAG interacts with neuronal MAP1B

To identify MAG-binding proteins in axonal membranes that would be expected to contain the MAG binding partner, total proteins were solubilized from cultured DRGNs and isolated AEFs. Western blots of the proteins were incubated with affinity-purified native MAG. MAG bound most strongly to a high molecular weight protein above the 250 kD standard in both DRGNs and AEFs (Fig. 1 A). This protein was identified initially as MAP1B by mass spectral analysis of the band excised from SDS gels in a manner similar to that described previously (Tanner et al., 2000). When blots on which MAG binding had been demonstrated were stripped and immunostained for MAP1B, the MAG-binding component corresponded to the MAP1B bands (Fig. 1 A).

The interaction of MAG with MAP1B was also demonstrated by coimmunoprecipitation (Fig. 1 B). Isolated AEFs contain MAG because of the presence of periaxonal oligodendroglial membranes in addition to the axolemma (DeVries et al., 1983). MAG was immunoprecipitated from the AEF with rabbit anti-MAG antiserum, and the immunoprecipitates were fractionated by SDS-PAGE and immunostained for MAP1B. MAP1B was in the immunoprecipitate obtained with anti-MAG antiserum but not in the one obtained with normal rabbit serum (Fig. 1 B, left). MAP1B is phosphorylated both by proline-directed serine/threonine kinases and casein kinase II (for review see Gordon-Weeks and Fischer, 2000). The broader appearance of the MAP1B band in the whole AEF in the left lane is due to the fact that the AA6 monoclonal antibody (mAb) recognizes all isoforms of MAP1B whether or not they are phosphorylated (Fig. 1 B, left), whereas the MAP1B that coimmunoprecipitated with MAG appeared to correspond to phosphorylated MAP1B with higher apparent molecular weight. This interpretation is supported by reactivity of the coimmunoprecipitated MAP1B with mAb SMI-31 to phosphorylated MAP1B (Fig. 1 B, right).

The presence of MAP1B in AEFs isolated from myelinated axons, which had been described previously (Sapirostein et al., 1992), is consistent with it being a physiological axonal binding partner for MAG. We confirmed the well-known decrease in the total amount of MAP1B in brain during maturation (Gordon-Weeks and Fischer, 2000), but a high concentration of MAP1B is retained in the AEF even from adult brains when it could not be detected in whole brain homogenate (Fig. 1 C). This enrichment of MAP1B in the periaxonal compartment of adult myelinated axons supports the possibility that it has a functional role in glia-axon interactions at this site.

Colocalization of MAP1B and MAG-binding sites on the surface of neurons

When the binding of MAG to MAP1B was first observed, it seemed that MAP1B was unlikely to be a physiological binding partner for MAG on the axonal surface because it is generally thought to be an intracellular cytoskeletal component (Gordon-Weeks and Fischer, 2000). However, MAP1B had been proposed to be a membrane glycoprotein based on a hydrophobic amino acid sequence that could be a transmembrane domain, potential sites for N-linked glycosylation and its role as a substrate for ecto-kinases (Muramoto et al., 1994).

Subsequent studies in our laboratory supported this hypothesis that some MAP1B is expressed as a membrane glycoprotein on the surface of neurons (Tanner et al., 2000). Our results pointed to the expression of MAP1B as a type II transmembrane glycoprotein in which the COOH terminus and glycosylation sites are extracellular and the microtubule binding domains are in the neuronal cytoplasm. Immunostaining of DRGNs showed that MAP1B is concentrated in varicosities distributed along the neurites (Tanner et al., 2000). New evidence for the expression of MAP1B as a surface protein on neurons is shown in Fig. 2 A, demonstrating a similar localization of MAP1B at neuronal varicosities whether or not the neurons were permeabilized before immunostaining. The surface immunostaining was observed both with mAb AA6 (A1) and with the polyclonal antiserum recognizing amino acid sequence 2056–2070 (unpublished data), which is in the extracellular domain of MAP1B, according to its model as a transmembrane glycoprotein (Tanner et al., 2000).

To determine if MAG-binding sites on neurons colocalize with MAP1B, cultured DRGNs were incubated with a MAG-Fc chimera (Fig. 2 B). MAG-Fc–binding sites (B2) were discontinuously distributed along the neurites in a punctate pattern in accordance with the report of Turnley and Bartlett (1999). The binding pattern was similar to the immunostaining for MAP1B (B1), and the overlapping signals in B3 show the colocalization of MAG-binding sites with MAP1B on neuronal varicosities. There was no binding of human-Fc chimera used as a negative control (Fig. 2 C). Also, there was no MAG-Fc binding when MAP1B was blocked previously with polyclonal antiserum to MAP1B (Fig. 2 D), demonstrating the specificity of the interaction.

Fig. 2 E shows double immunostaining of a section of myelinated peripheral nerve for MAP1B and MAG. MAP1B...
was enriched at the periphery of axons in the region of the axolemma, and MAG appeared as rings corresponding to the periaxonal SC membrane. Colocalization of MAP1B and MAG, revealed by the overlapping yellow signal, is consistent with an interaction of MAG and MAB1B at the SC-axon junction in myelinated nerve.

Lack of binding of MAG to glial MAP1B
Because MAP1B is also expressed by SCs (Ma et al., 1999) and oligodendrocytes (Vouyiouklis and Brophy, 1993), a possibility was that the MAG binding to MAP1B represents a cis interaction within myelinating glia. Fig. 3 A shows that primary SCs and immortalized S16 SCs express MAP1B, but it is of lower molecular weight than the primary neuronal isoform and does not react with the SMI-31 antibody to phosphorylated epitopes in neuronal MAP1B. Ma et al. (1999) also reported that phosphorylation of MAP1B in SCs is different from that in neurons. Furthermore, blot overlay and coimmunoprecipitation experiments with SCs and oligodendrocytes gave no evidence for an interaction of MAG with glial MAP1B. For example, Fig. 3 B shows an overlay experiment in which binding of MAG to the higher molecular weight form of MAP1B in DRGNs was clearly demonstrated, but there was no binding to MAP1B of S16 SCs. This appears to be due to a difference in the isoforms expressed by neurons and SCs, respectively, because the MAG binding corresponds to the higher molecular weight phosphorylated MAP1B in neurons, which is not expressed in SCs (Fig. 3 A, second panel, and B, third panel). Coimmunoprecipitation experiments also demonstrated the specificity of the interaction of MAG with neuronal MAP1B (Fig. 3 C). MAG was in the immunoprecipitate when MAP1B was immunoprecipitated from the AEF with anti-MAP1B antiserum. This is the inverse of the experiment.

Figure 2. Colocalization of MAG-binding sites and MAP1B on the surface of neurons in vitro and of MAG and MAP1B in myelinated nerves. (A) Similar immunofluorescent staining of MAP1B at neuronal varicosities of cultured DRGNs by mAb AA6 was observed without (A1) or with (A2) prior permeabilization with Triton X-100. (B) Double immunostaining of cultured embryonic DRGSCs that had been maintained for 3 h in the presence of MAG-Fc chimera. Immunostaining for MAG-Fc (FITC) shows MAG-binding sites on the neurons (B2), and B1 shows the same neuron immunostained for MAP1B (Rhodamine). Overlapping red and green images result in yellow signals (B3), demonstrating the colocalization of MAG-Fc-binding sites with MAP1B on neuronal varicosities (arrows). (C) Negative control for panels B1 and B2. The MAP1B-positive varicosities (C1) did not bind human Fc (C2). (D) Antibody blocking. MAG-Fc binding to DRGNs (D1) was inhibited when the culture was pretreated for 2 h with antiserum to MAP1B sequence 2056–2070 before adding MAG-Fc (D2) but not when the pretreatment was with an antibody to synaptophysin (unpublished data), a plasma membrane protein similarly distributed at varicosities of DRGSCs (Tanner et al., 2000). (E) Double immunostaining of MAP1B and MAG in rat sciatic nerve. These localizations of MAP1B (FITC) and MAG (Rhodamine) are similar to those shown previously in ventral roots (Tanner et al., 2000), and here we demonstrate their colocalization at the glia-axon junction by the overlapping yellow signal (arrows). Schmidt-Lanterman incisure was strongly reactive for MAG (arrowhead). Bars: (A) 5 μm; (B) 3.3 μm; (C) 10 μm; (D) 10 μm; (E) 8 μm.
shown in Fig. 1 B in which coimmunoprecipitation was with anti-MAG antiserum and further documents the interaction between MAG and neuronal MAP1B. Although the immortalized S16 SC line is differentiated toward myelination and expresses a substantial amount of MAG (Toda et al., 1994), there was no MAG coimmunoprecipitated with MAP1B from S16 SC extracts by the same antiserum.

Expression of phosphorylated MAP1B in neurons is increased in the presence of MAG

When DRGNs were cocultured with MAG-transfected COS cells, total MAP1B and phosphorylated MAP1B were increased in comparison to DRGNs cocultured with mock-transfected COS cells (Fig. 4 A). Densitometry of Western blots from three separate experiments revealed that total MAP1B was increased 2.1 \pm 0.12-fold \((p < 0.01)\) and total phosphorylated MAP1B was increased 1.8 \pm 0.12-fold \((p < 0.05)\) in the presence of MAG. Since COS cells do not express MAP1B (Kirkpatrick and Brady, 1994), we hypothesize that a MAG–MAP1B interaction could provide a structural link between the periaxonal membrane of MAG causes increased expression of its phosphorylated MAP1B binding partner.

**General comments**

Studies summarized in the Introduction on MAG-null mice and on the effects of MAG on neurite outgrowth have pointed to MAG acting as a ligand for an axonal receptor that affects the cytoskeletal structure of neurons. Here, several different methods were used to demonstrate that MAP1B is a neuronal binding partner for MAG including blot overlay, coimmunoprecipitation, and colocalization.

MAP1B appears to play roles in neuronal differentiation (Gordon-Weeks and Fischer, 2000) and axonal formation (Gonzalez-Billault et al., 2001) by regulating cytoskeletal organization, but its exact functions remain unclear. Disruption of MAP1B expression in vivo by gene targeting has generally supported such roles (Edelmann et al., 1996; Takei et al., 1997; Meixner et al., 2000). Of particular interest, with regard to the potential role of MAP1B as a neuronal binding partner for MAG, is the impaired myelination reported for two of these mutants (Takei et al., 1997; Meixner et al., 2000).

It is well established that the cytoskeletal structure of axons is modulated by surrounding myelin sheaths, based on studies of dysmyelinating mutants and comparisons of myelinated and nonmyelinated regions of the same axon (Kirkpatrick and Brady, 1999). For example, the neurofilaments in peripheral nerves of Trembler mutants exhibit decreased phosphorylation and increased density in comparison to normally myelinated nerves. The similar changes observed in MAG-null mice (Yin et al., 1998) suggest that MAG may be involved in the molecular mechanism by which myelin affects the axonal cytoskeleton. Furthermore, the stability of microtubules and the phosphorylation of several MAPs, including MAP1B, are decreased in the absence of normal myelin in Trembler mice (Kirkpatrick and Brady, 1994). Here, we demonstrate that the expression and phosphorylation of MAP1B are increased in DRGNs cocultured with MAG-expressing cells. These findings are all consistent with an effect of MAG on the phosphorylation and structure of cytoskeletal elements in the axon, including MAP1B. Based on the findings reported here, we hypothesize that a MAG–MAP1B interaction could provide a structural link between the periaxonal membrane of...
the myelin-forming cell and the axonal cytoskeleton, thereby contributing to the known capacity of myelin to affect the structure and stability of myelinated axons.

Materials and methods

Cell culture and subcellular fractionation

DRGcs from 16-d-old fetal rats were cultured as described previously (Tanner et al., 2000). 5 d later, neurons were solubilized in PBS containing complete protease inhibitor cocktail (Roche Molecular Biochemicals), phosphatase inhibitors (50 mM NaF and 100 μM Na2VO4), and 1% Triton X-100 (PPI buffer). The proteins of primary SC cultures and immortalized S16 SCs (Toda et al., 1994) were solubilized in the same way. The heavy AEF was isolated from myelinated axons of rat brain (DeVries, 1981).

Antibodies. Mouse mAbs to MAP1B were AABs that reacts with all isoforms (Sigma-Aldrich) and SMI-31 that is specific for mode I phosphorylated epitopes (Sternberger). Rabbit polyclonal antisera to synthetic peptides correspond to MAP1B sequences included one to the NH2 terminus from Dr. P. Brophy (University of Edinburgh, Edinburgh, Scotland) and another to amino acids 2056–2070 raised by Zymed Laboratories. Antibodies to MAG included an mAb S13 to native MAG (Poltorak et al., 1987) and an mAb (B11F7) and rabbit polyclonal antisera raised in our laboratory.

Purification of native MAG and binding to proteins on Western blots

Purified native MAG was purified from adult rat brain myelin by immunoprecipitation with the 53 mAb by a procedure similar to that described by Poltorak et al. (1987) using the Immunopure Protein G IgG Orientation Kit from Pierce Chemical Co. MAG binding to the antibody beads was that was 4°C for 1 h in PBS and it was eluted with a pH 11.3 solution of 50 mM ethylene diamine and 5 mM β-octyl glucoside. Proteins in membrane fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated overnight at 4°C in a blocking reagent (from the DIG Glycan Differentiation Kit; Roche) and washed twice in TBS (pH 7.5). After rinsing once in TBS containing 1 mM MgCl2, 1 mM MnCl2, and 1 mM CaCl2, blotS were incubated for 3 h at room temperature with 10 μg per ml of purified MAG in the same buffer. After washing with TBS, bound MAG was detected by immunostaining (overnight at 4°C) with the 53 mAb followed by peroxidase labeled anti–mouse IgG (ICN Biomedicals) and ECL (NEN Life Science Products).

Coimmunoprecipitation

MAG was immunoprecipitated from the proteins of AEFs (50 μg solubilized in PPI buffer) with polyclonal anti-MAG antisera overnight at 4°C followed by protein A Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. MAP1B was immunoprecipitated similarly from the AEF and S16 SC protein extracts with the polyclonal antibody to its NH2 terminus. Immunoprecipitates were washed three times in PPI buffer, solubilized, and fractionated by SDS-PAGE. After transfer to blots and blocking, coimmunoprecipitated proteins were detected by immunostaining with ECL.

Immunostaining and localization of MAG-binding sites

Cultured DRGcs fixed with 2% paraformaldehyde and 0.1% glutaraldehyde were immunostained for MAP1B either without permeabilization or with permeabilization by exposure to 1% Triton X-100 in PBS for 10 min. After blocking for 30 min in 10% normal goat serum (NGS) in PBS, cells were incubated for 1 h with primary antibodies to MAP1B, rinsed with PBS, and incubated with appropriate Texas red–coupled secondary antibodies (Jackson ImmunoResearch Laboratories). Sections of rat sciatic nerve were immunostained for MAG and MAP1B as described previously (Tanner et al., 2000).

For localization of MAG-binding sites on cultured DRGcs, fixed was with 1% paraformaldehyde in PBS. After blocking in 2% PBS and 2% NGS in PBS, the cells were incubated either with a MAG-Fc chimera protein (5 μg/ml; R&D Systems) or with human-Fc protein (5 μg/ml; ICN Biomedicals) for 3 h at 4°C. After three PBS washes, FITC–coupled anti-human Fc antibody was applied for 1 h. The cells were blocked again as above either with or without prior treatment with ice-cold methanol and then incubated with polyclonal antibody to MAP1B followed by rhodamine–coupled anti–rabbit IgG.

Protein analysis of DRGcs cocultured with MAG-expressing COS cells

COS-7 cells were stably transfected by lipofectamine reagent ( Gibco-BRL) with either 2 μg L-MAGpcDNA3.1 (provided by M. Filbin, Hunter Col-

le, New York, NV) or 2 μg pcDNA3.1 (Invitrogen) without the L-MAG insert (mock transfected). E17 DRGcs were plated on 6-well collagen-coated plates at a density of 5 × 104 cells/well and maintained in defined medium (DMEM + N2 + 2% FBS + 100 ng/ml NFG) for 5 d. DRGcs were then seeded onto 100 COS-7 cells for 4 d. Cell lysates were fractionated by SDS-PAGE, bloted, and immunostained for MAP1B with ECL. Densitometry was with NIH Image software (version 1.6), and statistical comparisons were by a two-tailed paired t test.

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