Myelin-associated Glycoprotein Interacts with Ganglioside GT1b

A MECHANISM FOR NEURITE OUTGROWTH INHIBITION*

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Myelin-associated glycoprotein (MAG) is expressed on myelinating glia and inhibits neurite outgrowth from postnatal neurons. MAG has a sialic acid binding site in its N-terminal domain and binds to specific sialylated glycans and gangliosides present on the surface of neurons, but the significance of these interactions in the effect of MAG on neurite outgrowth is unclear. Here we present evidence to suggest that recognition of sialylated glycans is essential for inhibition of neurite outgrowth by MAG. Arginine 118 on MAG is known to make a key contact with sialic acid. We show that mutation of this residue reduces the potency of MAG inhibitory activity but that residual activity is also a result of carbohydrate recognition. We then go on to investigate gangliosides GT1b and GD1a as candidate MAG receptors. We show that MAG specifically binds both gangliosides and that both are expressed on the surface of MAG-responsive neurons. Furthermore, antibody cross-linking of cell surface GT1b, but not GD1a, mimics the effect of MAG, in that neurite outgrowth is inhibited through activation of Rho kinase. These data strongly suggest that interaction with GT1b on the neuronal cell surface is a potential mechanism for inhibition of neurite outgrowth by MAG.

Expression of myelin-associated glycoprotein (MAG; siglec 4a),¹ is restricted to myelinating glial cells on myelin membrane adjacent to the axon and is required for maintenance of myelin integrity (1–4). In vitro, MAG inhibits outgrowth of postnatal neurons (5–8), involving activation of Rho GTPase, a key signaling step for the inhibitory effect of myelin on regeneration of neurons in vivo (9). MAG is therefore thought to contribute to the inhibitory properties of myelin, which is in part responsible for the lack of regenerative capacity of the central nervous system after injury or disease (10, 11).

Like other siglecs, MAG binds to sialic acid residues at the termini of glycans on opposing cells through a sialic acid binding site located in the N-terminal V-set Ig domain (12–22). MAG binds specifically to terminal sialic acid residues in α2–3 linkage to galactose, which occurs in glycans linked β1–3 to GalNAc or GlcNAc or β1–4 to GlcNAc (12, 23–25). Use of sialic acid analogues has identified specific groups on sialic acid essential for interaction with MAG (26), consistent with interactions seen between sialic acid and conserved amino acids in the siglec 1 crystal structure (21). It is also thought that the core glycan structure on which the terminal sialic acid is presented plays a role in recognition by MAG (23, 26, 27). The ability of MAG to bind specific gangliosides bearing terminal α2–3-linked sialic acid has been well documented. Gangliosides bind to MAG with the relative potencies GT1a > GT1b, GD1a > GD1a, GT1b > GM3, GM4, whereas GM1, GD1b, GD3, and GQ1b do not support adhesion (23, 27, 28).

Although the binding of MAG to sialylated glycans and gangliosides is well characterized, the functional importance of these interactions to the inhibition of neurite outgrowth by MAG is unclear. MAG binding to neurons is dependent on the presence of cell surface sialic acid (7). Furthermore, addition of exogenous sugars or neuraminidase treatment of neurons reduces the effect of MAG on neurite outgrowth (7), suggesting that the interaction between MAG and sialylated cell surface receptors results in inhibition of neurite outgrowth. However, mutation of arginine 118, an amino acid which is predicted to form hydrogen bonds with the carboxylate group of sialic acid (21), failed to inactivate the protein (29). This led to the suggestion that a second, sialic acid-independent, site on MAG interacts with an unknown counter-receptor on neurons, triggering the intracellular signaling cascade leading to inhibition of neurite outgrowth (29).

In this report, we investigate the roles of sialic acid and ganglioside recognition by MAG in the inhibition of neurite outgrowth. We show that, in the absence of arginine 118, the potency of MAG is significantly reduced, but the residual inhibitory activity also involves carbohydrate recognition. We then show that interaction of MAG with ganglioside GT1b is a potential mechanism for the inhibitory effect of MAG on neurite outgrowth. These results strongly suggest that GT1b represents a potential receptor for MAG, mediating inhibition of neurite outgrowth.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, all reagents were purchased from Sigma Chemical Co. (UK). Tissue culture media and B27 supplement were from Life Technologies (Paisley, UK). BCA protein quantification kit was purchased from Pierce (Chester, UK). Anti-MAG antibody (MAB1567) and isotype control antibody were purchased from Chemicon (Harrow, UK). SDB-polyacylamide gel electrophoresis gels were purchased from Bio-Rad. ECL reagents were from Amersham Pharmacia Biotech (UK). Neu5Ac, 3-sialyllactose, and purified gangliosides were purchased from Sigma. Anti-GT1b (clone GM5R, an IgM) and biotinylated anti-GT1b and anti-GD1a (clone GMR17, an IgM) were purchased from Seikagaku America (Falmouth, MA). Anti-GM1 (an IgG) was from Cambio (Cambridge, UK) and A2B5 (an IgM that recog-
nizes uncharacterized ganglioside(s) (30, 31)) was from Roche Molecular Biochemicals (Mannheim, Germany). TRITC-conjugated anti-mouse immunoglobulin was from Dako (Cambridge, UK) and streptavidin-Texas red was from Amersham Pharmacia Biotech. Y27632 was from Tocris (Bristol, UK).

Recombinant Protein Production—The constructs MAG-Fc/pIg and MAGR118A-Fc/pIg have been described elsewhere (29) and were kindly provided by Prof. M. T. Filbin. The rat SIRP-Fc/pIg construct consisted of the three extracellular N-terminal Ig-like domains fused to human IgG1 and was provided by Dr. L. Vernon-Wilson. Recombinant protein was produced by transient transfection of COS-7 cells as described previously (19).

ELISA—ELISA was carried out using standard methods (19, 29). Briefly, a 96-well plate was coated with 10 μg/ml goat anti-human IgG overnight at 4 °C. After washing, Fc proteins were applied at varying concentrations and incubated at 37 °C for 2 h. Wells were washed and incubated with anti-MAG antibody at 10 μg/ml for 1 h. This was followed by visualization using anti-mouse horseradish peroxidase and O-phenylenediamine substrate.

Primary Neuronal Cell Culture—The hippocampi of gestational day 18 rat embryos were dissected out, incubated in trypsin (0.08%, 30 min at 37 °C), and dissociated mechanically (32). Hippocampal cells were resuspended in neurobasal medium supplemented with B27, anti-oxidants, 1 mM glutamine, and 25 mM pyruvate, and plated at a density of 3000 cells/well into 96-well dishes that had previously been coated with poly-t-lysine followed by 10% FCS. Cerebellar granule neurons were prepared from postnatal day 8 Harlan Sprague-Dawley rat pups. Cerebella were enzyme-digested, triturated, and plated into poly-t-lysine-coated 96-well plates at a density of 20,000 cells/well in Eagle’s basal medium supplemented to contain 25 mM KCl, 10% FCS, and 50 μg/ml gentamicin (32).

Neurite Outgrowth Assays—One hour after plating primary neuronal cells, Fc proteins or anti-ganglioside antibodies at various concentrations were added in equal volumes of PBS to triplicate wells. All antibodies used in these assays were previously dialedyzed against cell culture medium. For preincubation experiments, 10 μg/ml anti-MAG or control antibody or various concentrations of sugars were incubated with MAG-Fc or SIRP-Fc for 1 h at room temperature prior to addition to cells. For ganglioside preincubation experiments, gangliosides were reconstituted at 25 mg/ml in chloroform:methanol (1:1) and stored at −20 °C. Fresh stock solutions of 250 μg/ml ganglioside in 1% fatty acid-free bovine serum albumin in PBS in glass tubes were prepared by vigorous vortexing immediately before each experiment. Gangliosides were diluted to 2 μg/ml into Fc protein solution in sterile glass tubes and incubated at 1 h at room temperature before being added to cultures as above. After 24 h (cerebellar granule neurons) or 48 h (hippocampal cells), cells were fixed with 4% paraformaldehyde for 1 h on ice, washed with PBS, and stained using Coomassie Blue (11). Briefly, 50 μl of 0.1% Coomassie Blue R-250 (in 40% methanol, 10% acetic acid) were added per well and incubated for 30 s. Stain was tipped off and the wells were washed three times with PBS. Assays were quantified using a KS300 image analysis system (Imaging Associates, UK). For each cell measured, the length from the edge of the cell to the end of the longest neurite was measured for 100 cells/well for each treatment in triplicate. Results are expressed as a percentage of the length of neurites of cells treated with control-treated with PBS alone. The length of neurites from control-treated neurons varied between experiments (between 35 and 50 μm for hippocampal neurons after 48 h of culture and 20–40 μm for cerebellar neurons cultured for 24 h). Therefore control-treated cells were included on every 96-well plate and results for each treatment in an experiment were expressed as a percentage of the length of control-treated cells. This allowed data from three independent experiments to be pooled. Data points therefore represent mean and S.E. of data pooled from three independent experiments.

Immunocytochemistry—Primary neuronal cells were plated onto 8-well chamber slides coated as described above. After 24 h (cerebellar granule neurons) or 48 h (hippocampal neurons), cells were fixed as above and stained by standard immunocytochemistry techniques. 10 μg/ml primary antibody (or isotype control) or biotinylated cholera toxin in PBS/10% FCS was incubated overnight at 4 °C. Secondary reagents were then incubated at room temperature for 1 h as follows: for biotinylated anti-CTb and cholera toxin, 1:200 dilution of streptavidin-Texas Red; for anti-GD1a and A2B5, 1:30 dilution of anti-mouse-TRITC.

RESULTS

Arginine 118 Is Required for Optimal Inhibition of Neurite Outgrowth by MAG—To assess their structural integrity, MAG-Fc and MAGR118A-Fc recombinant proteins were tested in an ELISA assay using the anti-MAG monoclonal antibody, which recognizes a conformation-dependent epitope. MAG-Fc and MAGR118A-Fc reacted identically in a dose-dependent manner (Fig. 1a) indicating that both proteins were correctly folded.

To investigate the role of arginine 118 in inhibition of neurite growth by MAG, neurite outgrowth experiments were carried out in primary cultured hippocampal neurons. Consistent with previous reports, MAG-Fc potently inhibited neuronal outgrowth (Fig. 1b). SIRP-Fc (consisting of the three extracellular N-terminal Ig-like domains of rat SIRP (33) fused to the Fc region of human IgG1) had no effect on neurite outgrowth from hippocampal neurons (Fig. 1b), consistent with previous observations using cerebellar granule neurons (34), therefore showing that inhibition of outgrowth by MAG-Fc was specific for MAG and not a result of the presence of the Fc region. MAGR118A-Fc also inhibited neurite outgrowth but with lower potency compared with the unmutated form (Fig. 1b). This inhibition was specific when compared with negative control Fc protein SIRP-Fc, and was reversed with the anti-MAG antibody mAb 513 but not mouse IgG1 isotype control. A similar effect was seen for neurite outgrowth from cerebellar granule neurons (data not shown).

Neurite Outgrowth Inhibition by MAGR118A-Fc Is Blocked by Preincubation with Mono- and Trisaccharides—To investigate the basis for the residual activity of MAGR118A-Fc, we carried
out neurite outgrowth assays using \( \text{MAG}^{R118A}\text{-Fc} \) in the presence of mono- and trisaccharides. The sugars tested did not affect neurite length when added to concentrations up to 5 mM in the absence of \( \text{MAG}^{R118A}\text{-Fc} \) (data not shown). Inhibition of neurite outgrowth by \( \text{MAG}^{R118A}\text{-Fc} \) was blocked by preincubation with \( \alpha \)-methyl sialic acid (Neu5Ac) and the sialylated trisaccharide 3'-sialyllactose (Neu5Aca2-3Galb1-4Glc) (Fig. 2); however, incubation with 0.1 mM 6'-sialyllactose did not significantly reverse inhibition (data not shown). This suggests that \( \text{MAG}^{R118A}\text{-Fc} \) inhibits neurite outgrowth via recognition of sialylated glycans on the surface of neurons.

**MAG and MAG\( ^{R118A} \) Interact with Gangliosides GT1b and GD1a—** Specific gangliosides are known to bind MAG via their carbohydrate epitopes with relative potencies GT1b \( \sim \) GT1a \( \sim \) GD1a \( \sim \) GM1. The recent availability of anti-ganglioside antibodies (35, 36) has provided the opportunity to investigate the functional significance of these interactions. Although MAG binds to the \( \alpha \)-series gangliosides with higher affinity, their expression in brain is very low (0.5, 0.9, and 0.3 mg/kg for GT1b, GT1a, and GD1a, respectively) compared with that of GD1a and GT1b (abundance of GD1a is 1200 mg/kg), which are among two of the four major brain gangliosides (27). If gangliosides are involved in neurite outgrowth inhibition in response to MAG, GD1a and GT1b would be predicted to be involved, because MAG affects all neuronal types tested.

To examine whether GD1a and GT1b could compete with endogenous neuronal MAG receptors for MAG binding, we preincubated Fc protein with gangliosides prior to addition into neurite outgrowth assays (37). Preincubation of MAG-Fc with a mixture of brain gangliosides, purified GT1b or GD1a but not asialo GM1 or GM1, blocked the inhibitory action of MAG (Fig. 3a). The inhibitory activity of \( \text{MAG}^{R118A}\text{-Fc} \) was also partially reversed by GT1b or GD1a preincubation (Fig. 3b).

**GT1b and GD1a Are Expressed on the Surface of Primary Neurons That Respond to MAG—** Immunocytochemistry using anti-ganglioside antibodies confirmed the expression of GT1b and GD1a on the surface of primary cerebellar and hippocampal neurons (Fig. 4). Both these antibodies showed most intense staining associated with cell bodies. Weaker staining was observed on some neurites. Gangliosides GM1 and the A2B5 antigen (an uncharacterized epitope carried by several gangliosides) were also found to be expressed on the surface of these cells (Fig. 4). A lack of fluorescence for cells stained with secondary antibody alone confirmed specificity (data not shown).

**GT1b Mediates Inhibition of Neurite Outgrowth—** The effect of anti-ganglioside antibodies on neurite outgrowth was then assessed. If MAG inhibits neurite outgrowth by binding to either GD1a or GT1b on the surface of neurons, antibodies recognizing these gangliosides may be expected to mimic the effect of MAG on neurite outgrowth. Anti-GT1b antibody, but not isotype control or antibodies recognizing GD1a, GM1, or the A2B5 antigen inhibited neurite outgrowth in a dose-dependent…

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**Fig. 2.** Neurite outgrowth activity of MAG\( ^{R118A}\text{-Fc} \) is blocked by small sugars. MAG\( ^{R118A}\text{-Fc} \) (300 nM) was preincubated with increasing concentrations of \( \alpha \)-methyl sialic acid 3'-sialyllactose prior to addition to cultured hippocampal neurons and outgrowth assays were carried out as described for Fig. 1.

**Fig. 3.** Neurite outgrowth activity of MAG and MAG\( ^{R118A} \) is blocked by preincubation with purified gangliosides GT1b and GD1a. MAG-Fc (60 nM, a) or MAG\( ^{R118A}\text{-Fc} \) (300 nM, b) were added to hippocampal neurons alone (Cont) or following preincubation with 2 \( \mu \)g/ml of a mixture of gangliosides (Mix) or individual purified gangliosides. Neurite outgrowth assays were carried out as described for Fig. 1. aGM1, asialo GM1.

**Fig. 4.** Ganglioside expression on the surface of primary neuronal cells. Hippocampal neurons (a) or cerebellar neurons (b) were fixed and stained using antibodies against GT1b or GD1a, the A2B5 antigen, or biotinylated cholera toxin (CTOX), which recognizes GM1.
suggest that MAG inhibits neuronal outgrowth by recognition of MAG and other neuronal cell surface molecules, they strongly
Although these findings do not rule out interactions between ligands and that these sugars and gangliosides, when added
mutated form of MAG retains the ability to bind carbohydrate specific gangliosides shown in this study suggests that this
of sialylated glycans on the neuronal cell surface.
From the crystal structure of siglec 1 (sialoadhesin) complexed with 3'-sialyllactose, it is evident that amino acids other than arginine 118 are involved in binding sialic acid (21). These are conserved in MAG and include tryptophan 2, a key amino acid for sialic acid recognition (21). Furthermore, components of the core glycan on which sialic acid is presented are able to increase the binding affinity of MAG for sialic acid either through direct contacts or more favorable presentations of sialic acid, increasing the affinity of MAG for sialic acid itself (23, 26). The reduced potency of inhibition of MAGR118A-Fc compared with the unmutated protein is therefore consistent with a reduced affinity of binding due to the loss of arginine 118.
Inhibitors of neurite outgrowth in myelin may prevent aberrant sprouting under steady-state conditions (38). The activity of these molecules may require regulation to prevent unwanted neuronal retraction. Linking carbohydrate recognition by MAG to biological function raises the possibility that the effect of MAG on neurons during steady-state or disease conditions could be regulated by changes in lectin activity. Two mechanisms of regulation of lectin activity for other siglecs have been identified. First, "cis" interactions between siglecs (including MAG) and sialylated glycans on the same cell surface (and perhaps on siglecs themselves) have been shown to block binding to opposing cells (13, 14, 29, 39, 40). In the case of siglec 2 (CD22), blocking of lectin activity by cis interactions on resting B-lymphocytes can be "unmasked" on pharmacological or physiological activation, demonstrating the potential for this type of regulation in physiological processes (39). Second, the ability of siglecs to bind sialic acid may be regulated intracellularly. In the case of siglecs containing immunoreceptor tyrosine-based
GT1b Is a Potential MAG Receptor

Gangliosides as Neuronal Receptors for MAG—The sialic acid binding site on MAG creates the potential to bind to many molecules (protein and lipid) bearing the correctly presented terminal sialic acid (23, 27, 28, 42, 43). Extensive studies on the ability of MAG to bind the carbohydrate domains of gangliosides exists in the literature, and if sialic acid recognition by MAG is essential for inhibition of neurite outgrowth by MAG, then gangliosides are candidate MAG receptors. A receptor for MAG that mediates inhibition of neurite outgrowth would be expected to (a) directly interact with MAG, (b) be expressed on the surface of cells that respond to MAG, and (c) be able to trigger the appropriate signaling cascade that results in neurite outgrowth. In this paper, we have shown that the ganglioside GT1b fulfills all of these criteria, and therefore, MAG-GT1b interaction is a possible mechanism for inhibition of neurite outgrowth. The anti-GT1b antibody was raised against purified ganglioside and specifically binds to GT1b but not to other gangliosides tested (Fig. 5 and Seikagaku America product information). However, it is possible that GT1b-like carbohydrate epitopes exist on carrier molecules other than gangliosides. Therefore, the possibility remains that both MAG and the anti-GT1b mAb recognize molecules other than gangliosides.

A MAG receptor that mediates inhibition of neurite outgrowth may also be expected to be present in neurites and growth cones. The immunocytochemistry presented in this paper suggests that GT1b is present on the surface of cells that respond to MAG, although the more intense staining is localized to the cell bodies with much weaker staining in neurites. However, the presence of GT1b has been demonstrated biochemically on purified growth cone membranes (24.4% of the sialic acid at the growth cone is carried by GT1b) (44). Therefore, the absence of strong staining on neurites and growth cones is likely to reflect the use of this particular IgM for staining. A similar result was recently reported for an anti-GD1a IgG that showed more robust GD1a localization than the previously available IgM (45).

GD1a has been shown to bind MAG with equal potency as GT1b (23); however, our results show that cross-linking of cell surface GD1a does not affect neurite outgrowth. This result may reflect the precise epitope recognized by the anti-GD1a antibody. An alternative explanation may be that regulated expression of different gangliosides on the cell surface may in turn regulate the activity of MAG. The α-series gangliosides have been shown to bind MAG with considerably greater potencies than GD1a and GT1b (27) but have an extremely low relative abundance in brain. It is not known whether the interaction of MAG with these gangliosides also results in inhibition of neurite outgrowth or whether they are expressed at critical times to regulate the activity of MAG.

Studies on knockout mice support the theory that interaction between MAG and gangliosides mediates the effects of MAG on neurons. The phenotype of MAG-deficient mice closely resembles that of mice lacking complex gangliosides. This includes decreased central myelination, axonal degeneration in the central and peripheral systems, and demyelination of peripheral nerves (1–4, 46).

Potential Mechanisms for Neurite Outgrowth Inhibition Mediated by GT1b—Gangliosides have been implicated in the modulation of many neuronal functions (47). Glycosphingolipids (including gangliosides) are known to exist in domains (known as lipid rafts, detergent-insoluble glycosphingolipid-enriched domains, caveoli, or caveoli-like domains) on the cell surface (48). Also enriched within these domains are GPI-linked molecules on the outer leaflet of the membrane and signal transducing molecules on the intracellular side (48, 49). These domains are known to exist on the surface of neuronal cells and have been implicated in processes, including signal transduction, cell adhesion, and lipid/protein sorting (50–52). On neurons, ~60% of cell surface gangliosides are found in sphingolipid-enriched domains (53), and the correct structure of these domains and glycosylation of gangliosides within them is essential for neuritogenesis (54, 55).

Within glycosphingolipid-enriched domains, glycosphingolipids are known to make several types of interaction. Glycolipids segregate into domains through hydrogen bonding between ceramide domains and carbohydrate-carbohydrate interactions (49). Furthermore, gangliosides have been shown to interact with proteins within lipid-enriched domains (growth factor receptors, GPI-linked molecules, and signal transducers) and several of these types of interactions have been shown to occur on neurons (49, 56). GM1 interacts directly with the BDNF receptor TrkB, and the quantity of GM1 at the cell surface directly modulates TrkB activity (57–59). GM3 is localized to domains enriched in c-Src, RhoA, and FAK and forms a close association with c-Src (54). GD3 interacts directly with the GPI-linked protein TAG-1 and with the src-family kinase Lyn in cerebellar granule neurons (55, 60). Interaction of gangliosides with extracellular molecules or ganglioside-specific antibodies can trigger intracellular signaling and cellular response, due to modulation of their endogenous interactions. In neurons, anti-GM3 antibody activates c-Src and inhibits melanoma cell growth (61, 62). In neuronal cells, anti-GD3 antibody treatment leads to Lyn activation and phosphorylation of an 80-kDa protein (60), an event that mimics cross-linking of TAG-1 (55).

Specific gangliosides have also been shown to be associated with integrins at focal adhesions (63–64) and to modulate integrin-mediated adhesion (65, 66). In neuronal cells, binding to disialogangliosides by tenasin-C, tenasin-R, or an anti-GD2 antibody causes inhibition of protein kinase C and prevention of integrin-dependent adhesion to fibronectin, resulting in inhibition of neurite outgrowth (37, 67).

It is clear that gangliosides play an important role in modulating intracellular signaling within lipid-enriched domains and at focal adhesions and that interactions between gangliosides and extracellular molecules can modulate cellular responses in a specific manner. GT1b has been shown to interact with proteins (68). MAG binding to GT1b on the surface of neurons may therefore modulate GT1b interactions within the neuronal plasma membrane, resulting in inhibition of neurite outgrowth.

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