Ganglioside Inhibition of Neurite Outgrowth Requires Nogo Receptor Function

IDENTIFICATION OF INTERACTION SITES AND DEVELOPMENT OF NOVEL ANTAGONISTS

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Gangliosides are key players in neuronal inhibition, with antibody-mediated clustering of gangliosides blocking neurite outgrowth in cultures and axonal regeneration post injury. In this study we show that the ganglioside GT1b can form a complex with the Nogo-66 receptor NgR1. The interaction is shown by analytical ultracentrifugation sedimentation and is mediated by the sialic acid moiety on GT1b, with mutations in FRG motifs on NgR1 attenuating the interaction. One FRG motif was developed into a cyclic peptide (N-Ac-CLQKFRGSSC-NH₂) antagonist of GT1b, reversing the GT1b antibody inhibition of cerebellar granule cell neurite outgrowth. Interestingly, the peptide also antagonizes neurite outgrowth inhibition mediated by soluble forms of the myelin-associated glycoprotein (MAG). Structure function analysis of the peptide point to the conserved FRG triplet being the minimal functional motif, and mutations within this motif inhibit NgR1 binding to both GT1b and MAG. Finally, using gene ablation, we show that the cerebellar neuron response to GT1b antibodies and soluble MAG is indeed dependent on NgR1 function. The results suggest that gangliosides inhibit neurite outgrowth by interacting with FRG motifs in the NgR1 and that this interaction can also facilitate the binding of MAG to the NgR1. Furthermore, the results point to a rational strategy for developing novel ganglioside antagonists.

Antibodies that bind to the ganglioside GT1b inhibit neurite outgrowth (1–3). Furthermore, passive immunization with anti-ganglioside antibodies directly inhibits axonal regeneration after injury in mice (4). A considerable body of evidence also suggests that autoimmune anti-ganglioside antibodies might contribute to the poor prognosis of some patients with Guillain–Barre syndrome (5) and other peripheral neuropathies (6). A better understanding of the mechanisms whereby ganglioside antibodies inhibits neurite outgrowth and the development of agents to circumvent this might lead to novel therapeutic opportunities for some peripheral neuropathies.

The myelin-associated glycoprotein (MAG) can inhibit neurite outgrowth (7, 8). The response to soluble MAG and GT1b antibodies are not obviously different when compared side by side (1). MAG can bind directly to gangliosides including GT1b (9), and the presence of complex gangliosides in neurons is required for MAG function (10). This suggests that in some circumstances MAG inhibits neurite outgrowth by binding to and activating a GT1b receptor complex in an antibody-like manner. In support, the inhibitory response to soluble MAG and ganglioside antibodies requires activation of RhoA (Ras homolog gene family A) (1, 3). It follows that insights into how anti-ganglioside antibodies inhibit neurite outgrowth might be gleaned from understanding how MAG inhibits neurite outgrowth and vice versa.

MAG can bind to a receptor complex in neurons that contain the Nogo-66 receptor NgR1 (11–14), the p75 neurotrophin receptor (p75NTR) (15, 16), and Lingo-1 (17). The p75NTR receptor is the key signaling component of the complex, and GT1b also appears to be associated with the complex as antibodies to GT1b can immunoprecipitate p75NTR from neurons (3, 18). These data suggest that GT1b could facilitate the interaction between MAG and the NgR1, and indeed enzymes that remove sialic acid from complex gangliosides inhibit soluble MAG binding to NgR1 and NgR2 in cells (19) and inhibit MAG function (20). Recent studies using independently generated lines of mice that lack the NgR1 have clearly shown that the ability of soluble MAG to induce growth cone collapse from dorsal root ganglion neurons is dependent on this receptor (21, 22). However, the later study also provided conclusive evidence that the NgR1 is not required for the function of substrate bound MAG.

In the present study we have used analytical ultracentrifugation sedimentation to demonstrate that GT1b can form higher order complexes with the NgR1. The binding required the presence of sialic acid on the ganglioside and was inhibited when any one of three independent FRG motifs in the NgR1 was mutated. One NgR1 sequence that contains an FRG motif (LQKFRGSS) lends itself well to the design of a cyclic peptide mimetic (N-Ac-CLQKFRGSSC-NH₂). This mimetic peptide prevented GT1b antibodies from inhibiting neurite outgrowth. These data suggest that the inhibitory activity of anti-GT1b
antibodies is dependent on NgR1 function. In support we show that GT1b antibodies do not inhibit neurite outgrowth from neurons isolated from mice that have the NgR1 gene genetically ablated from the germline.

The same NgR1 peptide that inhibited the GT1b antibody response also antagonized the response stimulated by soluble MAG, with alanine scanning identifying the FRG sequence as the functional motif within the peptide. These data suggest that in some circumstances soluble MAG can inhibit neurite outgrowth through the ganglioside/NgR1 pathway. In support, mutations of the FRG motif that inhibit GT1b binding to the NgR1 also inhibit MAG binding to the receptor, and the inhibitory activity of soluble MAG was significant attenuated in neurons that do not express the NgR1. However, it is also clear that substrate-bound MAG can inhibit neurite outgrowth in the absence of NgR1 function (22) and, in accord the NgR1 derived inhibitory peptides identified in this study, are not expected to, and indeed do not, inhibit the function of substrate-bound MAG.

**EXPERIMENTAL PROCEDURES**

**Neurite Outgrowth Assays—**Cerebellar granule neurons isolated from post-natal day 2–3 rats or from day 3–5 mice were cultured over monolayers of 3T3 cells essentially as previously described (23). Monolayers were established by seeding ~80,000 3T3 cells into individual chambers of an 8-chamber tissue culture slide coated with poly-L-lysine and fibronectin. In general, co-cultures were established by removing the media from the monolayers and seeding ~6000 dissociated cerebellar neurons into each well in SATO medium (Dulbecco’s modified Eagle’s medium supplemented with 0.062 mg/liter progesterone, 16.1 mg/liter putrescine, 0.4 mg/liter thyroxine, 0.039 mg/liter triiodothyronine, 10 mg/liter insulin (bovine pancreas), 100 mg/liter transferrin (human)) supplemented with 2% fetal calf serum. However, in some experiments the co-cultures were maintained in neurobasal medium + B27 + 1% L-glutamate + 1% penicillin/streptomycin + 25 mM KCl. Monolayers were established for 24 h before the addition of the neurons, and the cultures were maintained for ~23–27 h before fixation with 4% paraformaldehyde. In general, the neurons were stained with a GAP-43 antibody, and the mean length of the longest neurite per cell was measured for ~120–150 neurons, again as previously described (23). However, in some experiments neurons were labeled using TUJ1 (anti-βIII tubulin) followed by anti-mouse IgG-Alexa488, and nuclei were labeled with Hoechst. Mean total neurite length was calculated using the Neuronal Profiling bioapplication on a Cellomics ArrayScan. Similar results were obtained using both methods.

For neurite outgrowth on substrate-bound MAG, 96-well plates were coated with a thin layer of nitrocellulose before incubating with 1 μg/ml MAG(d1–5) at 4 °C overnight. Wells were subsequently coated with 17 μg/ml of poly-D-lysine (Sigma) followed by incubation in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Rat cerebellar granule neurons were dissociated and seeded at a density of 1 × 10⁴ cells per well. Cells were cultured for 18–20 h before being fixed with 4% paraformaldehyde and stained with a neuronal specific anti-βIII-tubulin antibody. The average of total neurite lengths from each neuron was measured automatically by the MetaXpress Neurite Outgrowth module (Molecular Devices) from at least 200 neurons per well, in triplicate wells per experiment. Results were repeated independently more than three times.

**Immunoprecipitations and Western Blots—**Chinese hamster ovary (CHO) K1 cells (100 mm dishes) were transfected with p75NTR and various mutants of the NgR1. The cells were harvested after 24 h and lysed in 1 ml of radioimmune precipitation assay buffer (Sigma) supplemented with Complete protease inhibitor mixture (Roche Applied Science). After centrifugation at 14,000 × g for 15 min, the supernatants were collected, and a protein assay (Bio-Rad) was performed. Protein lysates (0.5 mg) were preincubated with protein G-Sepharose beads (GE Healthcare) at 4 °C for 1 h, then incubated with 2 μg of goat anti-human NgR antibody (R&D Systems) plus protein G-Sepharose at 4 °C overnight. The beads were washed three times with radioimmune precipitation assay buffer and boiled in Laemmli sample buffer (Bio-Rad). Supernatants were subjected to 4–12% NuPAGE (Invitrogen), transferred onto nitrocellulose membrane (Bio-Rad), and probed with antibodies to the NgR and p75NTR (Promega). Western blot images were analyzed on a Storm gel imaging system using ImageQuant software (GE Healthcare).

Whole brains from adult 129/lex, NgR1 knock-out, and NgR2 knock-out mice were homogenized and sonicated in radioimmune precipitation assay buffer (Sigma). Supernatants were collected after centrifugation, and a protein assay (Bio-Rad) was performed. Protein lysates were subjected to 4–12% NuPAGE (Invitrogen), transferred onto nitrocellulose membrane (Bio-Rad), and probed with antibodies to NgR1 or NgR2 (R&D Systems). Western blot images (see supplemental Fig. 1b) were scanned and analyzed by Odyssey infrared imaging system (Li-Cor).

**Construction of NgR1 Mutants—**Human NgR1 point mutants were constructed using the QuikChange XL site-directed mutagenesis kit (Stratagene) following the manufacturer’s recommended protocol. The wild type human NgR1 cDNA (IMAGE:21210453) in a mammalian expression vector was used as a template to construct all the described mutants.

**Receptor Binding Assay—**COS-7 cells were co-transfected with either wild type or mutant NgR1 constructs along with a cytomegalovirus-β-gangliosidase plasmid (pCMVβ, BD Biosciences) as a transfection control. Transfection was performed in six-well plates using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. The next day cells were trypsinized and seeded at 30,000 cells per well in poly-l-lysine-coated 96-well plates (BD Biosciences). At that time, two sister plates were established, one of which was used in the binding assay, and the other was used to correct for transfection efficiency by measuring β-galactosidase activity (see below). Remaining cells were separately plated and assayed for surface expression of the NgR1 proteins by immunocytochemistry and for total NgR1 protein levels by Western blotting. All mutant proteins were expressed on the cell surface and produced in comparable amounts to the wild type protein (data not shown). The next day wells were rinsed once with Hanks’ balanced salt solution containing 0.5 mg/ml bovine serum albumin, 0.1% NaN₃, 20 mM HEPES, pH 7.0, at room temperature followed by incubation with 100 μl of fusion protein MAG-alkaline phosphatase (AP) diluted to a final concentration of 10 μg/ml in 20
mm HEPES, pH 7.0, for 90 min. After this, wells were washed 6 times with gentle shaking in 20 mm HEPES, pH 7.0, at room temperature, 5 min each wash. Cells were then fixed with acetone-formaldehyde (60-3%), in 20 mm HEPES, pH 7.0) for 15 s at room temperature then washed 3 times for 5 min each with Hanks’ balanced salt solution. Binding of AP-tagged ligands was then measured using the Great EscApE SEAP kit (BD Biosciences) following the manufacturer’s recommended protocol. Briefly, after aspirating Hanks’ balanced salt solution, a 60-µl dilution buffer was added to each well, and the plates were sealed and then incubated at 65°C for 90 min. Plates were cooled on ice, and then 60 µl of assay buffer was added per well and incubated at room temperature for 5 min. Sixty microliters of diluted chemiluminescent alkaline phosphatase substrate was then added per well, incubated 10 min at room temperature, and then read on a LMAXII luminometer (Molecular Devices). Absolute binding numbers were corrected by subtracting average binding values obtained from mock-transfected controls. Binding was further corrected for sample-to-sample variations in transfection efficiency by normalizing to β-galactosidase activity. β-Galactosidase activity was measured using the luminescent β-galactosidase detection kit II (BD Biosciences) following the manufacturer’s recommended protocol. Three independent binding experiments were conducted with at least six replicates per experiment. Background was subtracted, and β-galactosidase-corrected binding values were expressed relative to the wild type receptor.

Preparation of AP-tagged Fusion Proteins—A fusion protein (Nogo66-AP) containing an N-terminal human placental AP and a C-terminal Nogo66 domain was constructed by ligating nucleotides encoding amino acids 1055–1120 of human NogoA (reticulon-4, NP_065393) to sequences encoding amino acids 23–511 of AP (NM_001632). This fusion was further modified by changing amino acid 47 of the Nogo66 sequence from cysteine to valine and introducing six consecutive histidine residues at the C terminus. The coding sequence was inserted into a mammalian expression vector and transiently transfected into HEK293GT cells (Invitrogen) using LipoFectamine 2000 (Invitrogen). The next day serum-free medium (Free Style 293, Invitrogen) was added, and cells were incubated a further 48 h before collection of crude conditioned medium. Nogo66-AP concentration was determined by measuring alkaline phosphatase activity and by Western blotting for alkaline phosphatase. A stable CHO cell line expressing a fusion protein containing an N-terminal human myelin-associated glycoprotein (human MAG; NM_002361; amino acids 1–516) and a C-terminal AP domain (amino acids 23–511) bearing six C-terminal histidine residues was created (referred to as MAG-AP). Cells were incubated in serum-free medium for 48 h; the conditioned medium was collected, and the fusion protein was purified using TALON cobalt affinity chromatography (Clontech) following the manufacturer’s protocol. MAG-AP concentration was determined by measuring alkaline phosphatase activity and by Western blotting for alkaline phosphatase and MAG.

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed on a Beckman XLI/XLA analytical ultracentrifuge. NgR1(310)-fc (0.21 or 0.38 µM final) was added to ganglioside at increasing ganglioside concentrations from 0 to 48 µM. Mutant protein used in the sedimentation velocity experiments corresponded to the column fraction of greatest purity based on SDS gel analysis. Wild type or mutant NgR(310)-Fc was added to Tris-buffered saline (TBS) buffer or TBS buffer containing GT1b to a final concentration of 16–30 µg/ml protein and 0 or 22 µM GT1b in a microcentrifuge tube. The solution, 400 µl, was loaded into 2-channel (1.2-cm path length) carbon-Epon centerpieces in an An-50-Ti rotor. Scans were recorded at 20°C with a rotor speed of 35,000 rpm, and the signal was detected at 230 nm with a spacing of 0.006 in the continuous mode. Sedimentation profiles were analyzed by the program Sednterp (25) to obtain the sedimentation coefficient distributions. The solvent density (1.006) and partial specific volume (0.72) were calculated using the program Sednterp (25).

Neuraminidase Treatment—CHO parental cells and NgR1 stable cells were seeded at 30,000 cells per well in 96-well plates the night before the assay. Various concentrations of Vibrio cholerae neuraminidase (Roche Applied Science) in growth medium (Dulbecco’s modified eagle medium containing 10% fetal bovine serum) were incubated with cells for 1 h at 37°C. Medium was replaced with affinity-purified MAG-AP or Nogo66-AP in Hanks’ balanced salt solution supplemented with 1% fetal bovine serum and 20 mm HEPES and incubated at room temperature for 90 min. Cells were then washed four times with supplemented Hanks’ balanced salt solution. AlloPhos (0.6 mg/ml) (Promega) was added to indicate of bound ligands. After a 30-min incubation at room temperature, the plates were read at emission/excitation wavelength of 400 nm/505 nm by FlexStationII384 ( Molecular Devices).

Reagents—Synthetic peptides were all obtained from a commercial supplier (Multiple Peptide Systems). All peptides were purified to the highest grade by reverse-phase high performance liquid chromatography and obtained at the highest level of purity (>97%). With all peptides there was no indication of higher molecular weight species. Where peptide sequences are underlined, this denotes a peptide that has been cyclized via a disulphide bond between the given cysteine residues. All peptides were acetylated and amide-blocked. Recombinant MAG-Fc chimera was obtained from R&D Systems and used at a final concentration of 5–25 µg/ml. The monoclonal antibody to GT1b (clone GM5R) was obtained from Seikagaku America and was used at a final concentration of 20 µg/ml. All reagents were diluted into the co-culture media and in general added to the cultures just before plating of the neurons. GT1b and GM1 were a kind gift of Dr. Gino Toffano. Asialo-GM1 was obtained from Sigma. The recombinant NgR1(310)-Fc chimera and the extracellular portion of MAG(d1–5) were expressed and purified in-house. Pharmacological reagents were obtained from Calbiochem and/or Sigma.

Generation and Characterization of NgR1 and NgR2 Knock-out Mice—Targeting vectors (see supplemental Fig. 1a) were introduced into mouse embryonic stem cells of the 129SvBrd background via electroporation. Homologously targeted integrants were selected in G418 (0.4 g/liter) and identified by Southern blotting using probes external to the targeting vector on both the 5’ and 3’ sides. Retention of the 5’-most loxP site in the targeted cells was determined by PCR of genomic DNA using the following primers: for NgR1 allele, 5’-GGTCTAGG-
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![Identification of putative functional motifs on the NgR1.](image)

**FIGURE 1.** Identification of putative functional motifs on the NgR1. The protein binding site of the NgR1 is shown in a. The concave face of NgR1 is shown in space-filled mode, and the residues critical for protein binding (MAG, OMGP, Nogo66) are shown in red. Two putative small ligand binding cavities are shown on the convex face of NgR1 in b. Clusters of energy minima for a simple 3.5 Å diameter van der Waals probe define two small pockets (in proximity to the red and yellow spheres in b). The three occurrences of the FRG motif are also highlighted in (b) (red, green, and yellow patches), with the $^{190}$FRG$^{200}$ and $^{278}$FRG$^{280}$ peptides seen to be neighboring the predicted small molecule binding pockets. In c, a ribbon diagram of the NgR is shown to highlight those parts of the receptor (with corresponding sequences) that are obviously amenable to a cyclic peptide mimetic approach for determining functional significance of the highlighted loops. The figures were generated with DS Viewer from Accelrys.

GATGCATCTCAG and 5'-ACATCTGAAGGCCTTCTGG; for the NgR2 allele, 5'-GGTCTAGGGATG-CATCTCAG and 5'-GGCCCTGCCCCTCCTAC. Embryonic stem cells were used to establish mouse lines and animals bearing the NgR1 and NgR2 alleles were crossed to Prm-cre transgenic mice (26). The Prm-cre transgene directs expression of cre recombinase in the male germline. Therefore, males harboring the transgene along with the NgR1 or NgR2 allele transmit the non-functional NgR1 or NgR2 alleles, respectively, to their offspring. Cre-mediated recombination of the floxed (f) alleles to the deleted (d) alleles was detected by PCR of genomic DNA using the following primers: for NgR1, 5'-GTGCTAGGGATGCATCTCAG and 5'-GTGGCTGTGTGCTGTCCGTGC; for NgR2, 5'-GGTGGTGGGTTCTTCAGG and 5'-GGGCGCCTAGCAGC. All alleles were maintained on the 129SvBrd background.

**RESULTS**

**Binding Motifs on the NgR1**—There are two published crystal structures of the NgR1, Protein Data Bank accessions 1OZN (27) and 1P8T (28), but currently no ligand-receptor complex structure has been solved. Detailed mutagenesis studies have recently mapped the residues critical for the binding of MAG to the receptor (29), and these are illustrated in Fig. 1a. Small ligand binding sites show up as cavities and can be revealed by the clustering of a small probe under the influence of a van der Waals potential. In Fig. 1b we show the two lowest energy clusters for a probe with a van der Waals radius of 3.5 Å. The potential binding pockets lie on the convex side of the protein, and interestingly, both neighbor FRG triplet motifs that can be found in the other NgRs (discussed in detail below). Sialic acid residues on gangliosides and possibly other glycoconjugates bind directly to an FRG motif in MAG itself (30). These observations have led us to develop the hypothesis that gangliosides can interact with FRG motifs in the NgR and that this interaction might facilitate MAG binding to the receptor.

**Binding of MAG, but Not Nogo66, to NgR1 Is Partially Sensitive to Neuraminidase**—In neurons soluble MAG binds to the NgR1 and NgR2 in a sialic acid-dependent manner (19). In the present study we confirmed the neuraminidase sensitivity of MAG binding to the NgR1 expressed in CHO cells. The data show that over a wide range of concentrations (2.5–20 μg/ml) the specific binding of the MAG-AP fusion protein to NgR1-expressing cells is partially inhibited (55%) by treating the CHO cells with neuraminidase. The effect was dependent upon the concentration of neuraminidase, and even at the highest concentration Nogo66-AP binding remained completely unaffected (Fig. 2). These data suggest that MAG binding to the NgR1 is dependent at least in part on sialic acid binding to the receptor.

**Effects of Loop 2 and Additional FRG Mutations on GT1b Binding to the NgR1**—GT1b is a sialic acid-containing ganglioside that has previously been reported to be a key component of the MAG receptor complex (18, 31). We have tested whether GT1b can bind directly to the ectodomain of the NgR1 using analytical ultracentrifugation. In the absence of GT1b, the dimeric NgR1(310)-Fc migrates with a sedimentation coefficient of ~6.5 S (Fig. 3). In the presence of low μM concentrations of GT1b, the 6.5 S species decreases, and additional peaks with higher sedimentation coefficients appear in a dose-dependent manner (Fig. 3a). In this assay, GM1 can also interact with NgR1 (Fig. 3b), which suggests that the interaction might be dependent on sialic acid. In support, no change in sedimentation coefficient of the NgR1(310)-Fc is observed in the presence of asialo-GM1. No effect was observed upon the addition of 22 μM GT1b to anti-hNgR AF 1208 antibody from R&D, which provides additional evidence that the interaction of GT1b with NgR1 is specific (not shown).

We next determined if the binding of GT1b to the NgR1 was sensitive to mutation of the FRG motifs. Importantly, based on the relative ratios of the ~6.7- and ~11-S peaks, it can be estimated that mutation of the arginine 279 to an aspartic acid reduced binding to ~56% that of wild type NgR, suggesting this site plays a role in mediating the interaction (Fig. 3d). Mutation of arginine 151 (Fig. 3e) or arginine 199 (Fig. 3f) also reduced GT1b binding to 49 and 33% that of wild type, respectively. These data suggest that all three FRG sites might be important...
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in facilitating GT1b binding to NgR1. In all three instances the sedimentation coefficient curves can be seen to be qualitatively different to the curve seen with the wild type NgR1 construct. Whereas a higher migrating species (11 S) becomes the dominant species in the presence of GT1b with the wild type receptor, lower migrating species remain dominant with all three mutated receptors (Fig. 3, d–f).

A FRG-containing Mimetic of an NgR1 Loop Inhibits the Function of a GT1b Antibody—In general antibodies that bind to neurons do not inhibit neurite outgrowth (including antibodies to neural cell adhesion molecule, N-cadherin, L1, and the fibroblast growth factor receptor, and THY-1, e.g. see Refs. 32 and 33). However, antibodies that cluster GT1b inhibit neurite outgrowth, most likely by clustering GT1b with consequent clustering and activation of an inhibitory molecule complex (1–3). One of the FRG motifs implicated in GT1b binding to the NgR1 is part of an exposed loop that lends itself well to the design of a cyclic peptide mimetic (see Fig. 1c). In the present study post-natal day 2/3 cerebellar neurons were cultured over monolayers of 3T3 fibroblasts for ~23 h in the presence and absence of a GT1b antibody. As previously reported, the antibody inhibits neurite outgrowth in a dose-dependent manner with a robust inhibition seen at 40 μg/ml (Fig. 4). When the antibody was added in the presence of the 100 μg/ml cyclic peptide (N-Ac-CLQKFRGSSC-NH2) that mimicked the FRG motif-containing loop (the NRL2 peptide), it failed to inhibit neurite outgrowth when tested at up to 40 μg/ml (Fig. 4). By showing that a NgR1-derived peptide can inhibit the GT1b antibody response, the data further substantiate the hypothesis that the GT1b antibody response might rely on GT1b binding to the FRG motifs in the NgR1 (see also effect of NgR1 ablation; see Fig. 8).

Effects of the NRL2 Peptide on MAG Inhibition of Neurite Outgrowth—A wide range of Fc chimeras that bind to neurons do not inhibit neurite outgrowth (32, 34, 35). In contrast, a soluble MAG-Fc chimera inhibits neurite outgrowth in a manner that can depend upon both ganglioside and NgR function (see the Introduction). In the present study the MAG-Fc inhibited neurite outgrowth from post-natal day 2/3 cerebellar neurons in a dose-dependent manner (not shown) with robust inhibition seen at 25 μg/ml (Fig. 5a). The NRL2 peptide again had no effect on basal neurite outgrowth, but it was striking that the MAG-Fc fails to substantially inhibit neurite outgrowth when this peptide is present in the growth media (Fig. 5a). As a control we tested cyclic versions of the three other exposed NgR loops (see Fig. 1c for details) for their effects on neurite outgrowth. These peptides were coded NRL1 (N-Ac-CYNEP-KVTC-NH2), NRL3 (N-Ac-CSLPQRLAC-NH2), and NRL4 (N-Ac-CAGRDLKRC-NH2). When tested at 100 μg/ml, these peptides had no effect on basal neurite outgrowth or on the suppressed neurite outgrowth seen in the presence of the MAG-Fc. We next looked at the dose-response curve for the NRL2 peptide. It can be seen to have no significant effect on neurite outgrowth in control media when tested at up to 200 μg/ml. In contrast, the peptide promotes neurite outgrowth in a dose-dependent manner in the presence of the MAG-Fc with the response reaching a plateau at around 50 μg/ml (~45 μM) (Fig. 5b).

Identification of Key Functional Amino Acids in the NRL2 Sequence—Structural analysis of the NgR1 shows that the most conspicuous amino acids within the loop corresponding to the NRL2 peptide are the positively charged lysine and arginine; both are highly solvent-exposed, with their side chains clearly available for binding (not shown). Of the surrounding amino acids, the phenylalanine is buried in the structure but might play a role in stabilizing the local region. The glycine and serine are partially solvent-exposed but look less likely to mediate a binding interaction. Based on this analysis, we designed two small peptides that both have the exposed lysine and arginine within them. These were the NRL2a (N-Ac-CKFRGSC-NH2) and NRL2b (N-Ac-CQKFRGC-NH2) peptides, and it should be noted that they contain a common four-amino acid motif (KFRG). Both peptides had no effect on neurite outgrowth in control media (not shown), with their ability to promote
growth in the presence of the MAG-Fc shown in Fig. 6a. Within the inhibitory environment, both peptides promote neurite outgrowth with significant effects seen at 25 μg/ml (30 μM) and maximal effects seen at 50 μg/ml (60 μM). At this higher concentration the inhibitory activity of the MAG-Fc was effectively abolished. This suggests that the functional activity within the NRL2 peptide sequence resides within the KFRG motif. To identify key amino acids within this short region, we designed four peptides (A1–A4) with individual alanine substitutions within the KFRG sequence of the NRL2 peptide. When tested at 100 μg/ml, peptides with alanine substitutions in positions 1 and 2 were as effective as the parental peptide in inhibiting the MAG response (Fig. 6b). However, when tested over a range of concentrations, substitution at position 1 had no obvious effect on the efficacy of the peptide, whereas substitution at position 2 is seen to reduce efficacy by a factor of ~2 (Fig. 6c and d). In contrast, substitutions at position 3 or 4 rendered the peptides ineffective at inhibiting the MAG response when tested at 100 μg/ml (Fig. 6b). Also, a linear version of the QKFRG peptide did not inhibit the MAG response (LNRL2b, Fig. 6b). These data demonstrate that to be functional, the QKFRG motif needs to be constrained by a disulfide bond and that single mutations to any amino acid within the FRG motif compromises activity of the peptide.

To determine whether a relatively metabolically stable peptide would retain biological activity, we cyclized the NgR1 sequence via a stable peptide bond (homodetic cyclization), and we replaced the amino acids by their chiral partners. Specifically, we replaced the L-type amino acids of the original peptide by non-native D-type amino acids. The peptide sequence is reversed to ensure that the side chain orientations are preserved. Such peptides are referred to as retro-inverso peptides. Explicitly, the sequence of the homodetic retro-inverso peptide (hriNRL2) is c[sGrfkq], where c[ ] refers to homodetic cyclization, and the lowercase letters refer to D-type amino acids. Note that glycine has no chirality because it has no side chain. When tested in the MAG-Fc assay, this peptide can be seen to retain full efficacy in inhibiting the MAG response (Fig. 6e).

Neuraminidase inhibits the function of soluble but not substrate-bound MAG (20, 36). We interpret this as suggesting that soluble MAG requires a sialic acid containing co-receptor for maximal efficacy. Interestingly, we have been unable to inhibit the function of substrate-bound MAG with any of the NRL2 peptides, and this is shown for the hriNRL2 peptide in Fig. 6f. In this example the hriNRL2 peptide has no significant effect on neurite outgrowth when tested at up to 200 μg/ml on the suppressed growth that is seen on the MAG substrate. The NRL2 peptides do not promote growth over substrate-bound myelin (data not shown), confirming that they do not have nonspecific effects on neurite outgrowth.

Effects of Loop 2 Mutations on Ligand Binding to the NgR1—The above data suggest that the 277KFRG280 motif in loop 2 in the NgR1 plays an important role in the context of soluble but not substrate-bound MAG function. Given that the lysine 277 and arginine 279 are positively charged and highly solvent-exposed, the effects of mutating both residues to negatively charged aspartic acids or neutral alanines was determined. In both instances the mutations had no obvious effect on the level of expression of the NgR1 (Fig. 7a), and based on co-immunoprecipitation, a normal interaction between the mutated NgR1 constructs and p75NTR, presumably in the cell membrane, was apparent (Fig. 7a).
p75NTR did not co-immunoprecipitate with a control antibody (not shown). When soluble MAG was tested in binding assays, a significant reduction in binding (~60%) was seen to the mutated NgR1s irrespective of whether the exposed lysine and arginine were substituted with aspartic acids or alanines (Fig. 7b). When these positively charged amino acids were individually mutated to alanines, the data clearly suggests that arginine 279 is more important for MAG binding than lysine 277 (Fig. 7b) with a 36% reduction in binding seen after this single point mutation.

NgR1 Function Is Required for Inhibitory Responses to Soluble MAG and GT1b Antibodies—The above results all suggest that soluble MAG and the anti-GT1b antibodies inhibit growth through a common NgR1-dependent manner. It has recently been shown in two independent lines of knock-out mice that the collapse of dorsal root ganglion growth cones induced by soluble MAG requires NgR1 function (21, 22). However, it remains unclear as to whether the NgR1 is required in cerebellar neurons for the inhibition of neurite outgrowth induced by soluble MAG, and this raises an issue about the importance of the receptor for the GT1b antibody response. To directly test this we have studied cerebellar neurons from mice that have the NgR1 or NgR2 genes ablated within the germline (see “Experimental Procedures” for details).

In a series of independent experiments, cerebellar neurons from wild type mice or from NgR1 knock-out mice were cultured in control media (40 μg/ml IgM) or in media supplemented with anti-GT1b antibodies (40 μg/ml). Whereas the GT1b antibodies significantly inhibited neurite outgrowth from the control neurons, neurons isolated from mice that do not express the NgR1 failed to show any response (Fig. 8a). We also tested the effects of the GT1b antibodies on cerebellar neurons isolated from a NgR2 knock-out mouse. These neurons responded as normal to the antibodies, presumably because they do not normally express the NgR2, and retain expression of the NgR1. The above data clearly show that the response to GT1b antibodies is dependent on NgR1 function. We conducted a similar set of experiments using MAG-Fc (25 μg/ml)
to inhibit neurite outgrowth. Again the results clearly demonstrate that a significant component of soluble MAG function is also dependent on NgR1 function in these neurons. In this context the inhibition of neurite outgrowth induced by soluble MAG was significantly \( (p < 0.05) \) greater in neurons isolated from wild type and NgR2 knock-out mice as compared with neurons from the NgR1 knock-out mice (Fig. 8b).

**GT1b Antibodies and Soluble MAG Activate the Same Signaling Cascade**—The above data suggest that GT1b antibodies and soluble MAG inhibit neurite outgrowth by activating the same receptor complex in cerebellar granule cells. A number of drugs have been identified that can inhibit the response to MAG; these include a PKC inhibitor (Go6976), two secretase inhibitors (inhibitor X and inhibitor IX) that act at the level of the receptor complex by inhibiting the intramembrane proteolysis of p75NTR, or drugs that act further downstream including the Rho kinase inhibitor Y27632 and the epidermal growth factor receptor inhibitor AG1478 (for review, see Ref. 37). If the GT1b antibody is activating the MAG receptor complex in cerebellar neurons, then the above drugs should inhibit the response. We find that all of the drugs discussed above that have been reported to inhibit MAG responses also inhibit the GT1b antibody response (Fig. 9).

**DISCUSSION**

In trying to understand how antibodies to gangliosides might inhibit neurite outgrowth in culture and axonal regeneration in vivo, we were guided by studies on ganglioside interactions with MAG. In this context, arginine 118 is part of an FRG motif in MAG that recognizes sialic acid residues on gangliosides (1, 36). This begs the question as to whether gangliosides might be able to interact with FRG motifs in other molecules. Interestingly, the NgR1, a key component of a receptor complex that inhibits neurite outgrowth, contains no less than three conserved FRG motifs.

Using sedimentation assays, we have been able to obtain evidence that GT1b can indeed interact with the NgR1, albeit at low \( \mu \)M concentrations. At these concentrations GT1b forms micelles that migrate with a sedi-
mentation coefficient of ~4.5 corresponding to ~10–12 molecules per micelle (38). This would account for the relatively large shift in the sedimentation coefficient of the NgR1 that is induced, in a dose-dependent manner, by GT1b. This would appear to be a sialic acid-dependent binding as the same shift can be induced by the much simpler GM1 ganglioside. The demonstration that asialo-GM1 does not induce a shift is consistent with the binding being mediated directly by sialic acid. It is perhaps worth noting that gangliosides are present in neuronal membranes at high concentrations (39), and productive interactions with neuronal receptors in the same membrane need not necessarily involve high affinity interactions. However, additional evidence, perhaps including co-crystals of GT1b with the NgR1, will be required for a full understanding of this potential interaction. It is perhaps also worth noting that in this binding assay there are no spatial restrictions in place and no competing molecular interactions. One or both of these might play a role in promoting and/or restricting the interaction of individual gangliosides with the NgR in neuronal membranes. This might explain why GT1b antibodies can inhibit neurite outgrowth in an NgR-dependent manner, whereas GM1 antibodies do not (1).

Nonetheless, several lines of evidence speak to the specificity of the GT1b/NgR1 interaction. In this context, asialo-GM1 did not interact with the NgR1, and this is to be expected for an
interaction that was predicted to be sialic acid-dependent. However, perhaps more importantly, single point mutation within the three FRG motifs in the NgR1 each substantially reduced the interaction. In each case Arg→Glu mutations resulted in less binding determined at close to saturation concentrations of GT1b. This suggests that GT1b can interact with at least three spatially distinct sites on the NgR1. Whereas complex formation was reduced by ~50% when Arg-151 or R279 were mutated, it was reduced by ~70% when Arg-199 was mutated. Of the three NgR1 FRG motifs, two are conserved in NgR2 (Arg-151 and Arg-199), and two are conserved in NgR3 (Arg-199 and Arg-279). The conservation of the site around Arg-199 in all three receptors might argue for a more important function for this motif, and indeed, mutation at this site had the most dramatic effect on GT1b binding. Arg-199 also has three neighboring arginines (196, 223, and 175) arranged in a cluster that may play a key role in forming the site of a binding pocket in the receptor. In this context it is clear that MAG binding to the NgR1 is partly dependent on sialic acid binding to the receptor. However, a protein-protein interaction site that can mediate a direct interaction has also clearly been mapped on the NgR1. Thus, we have not only identified an NgR1-derived peptide that can inhibit the ganglioside response, but we have obtained direct evidence that the NgR1 is required for the response.

Having made clear headway in understanding how antibodies to gangliosides inhibit neurite outgrowth, the question remains as to what extent antibodies to gangliosides and soluble MAG activate common or independent receptor complexes in cerebellar neurons. The data in the present study point to a considerable overlap in that we have shown that both responses require NgR1 function, and both responses are dependent on activation of a common signaling cascade as judged by inhibition with a common set of pharmacological reagents. There are many models that could account for this, with perhaps the key question being whether MAG binding to the NgR1 is dependent or not upon ganglioside interactions with the receptor. In this context it is clear that MAG binding to the NgR1 is partly dependent on sialic acid binding to the receptor; however, a protein-protein interaction site that can mediate a direct interaction has also clearly been mapped on the receptor (Fig. 1a).

A number of observations suggest that the ability of soluble MAG to elicit the inhibitory response is facilitated by a ganglioside/NgR interaction. This includes the fact that soluble MAG was shown to be ineffective in reducing neurite length in the absence of a NgR1. The above data clearly implicate NgR1 FRG motifs as candidate binding sites for the sialic acid moiety on gangliosides. However, some residual GT1b-NgR1 complex formation (~30%) could still be seen after mutating Arg-199, with a similar level seen after mutation of the arginines in all three FRG motifs (data not shown). This suggests that the residual GT1b binding might involve additional sites. Nonetheless, it is also possible that residual binding might reflect a lower affinity interaction with one or more of the mutated FRG sites. The precedence for this comes from studies on MAG itself where mutation of arginine 118 (within an FRG motif) has been interpreted as reducing the affinity, rather than abolishing the binding, of sialic acid to the site (1).
does not inhibit neurite outgrowth in the presence of an agent that blocks the synthesis of complex gangliosides (10). In addition, in the present study we have identified ganglioside binding sites on the NgR1 and shown that a peptide mimetic of one of these not only inhibits the GT1b antibody response but also inhibits the soluble MAG response. The use of short peptides and alanine scanning identified the FRG triplet as the minimal functional motif within the peptide. Moreover, we have shown that mutation of the ganglioside binding site (which is spatially distinct from the direct binding site for MAG) substantially (~60%) inhibits MAG binding to the NgR1 in cells. As a control we have shown that the mutations have no obvious effect on the interaction between the NgR1 with itself or with p75NTR. Also, the same mutations had no significant effect on the binding of soluble Nogo66-AP to the receptor.3

MAG binding to the exposed FRG motif-containing loop could be direct and/or indirect. A number of observations suggest that it is likely to be indirect. First, mutations within the site reduced rather than completely inhibited MAG binding. Second, a much more extensive mutagenesis study has mapped a direct MAG binding site to a different face on the receptor (see Fig. 1a). Finally, we have demonstrated in a direct binding assay that gangliosides can bind to the same FRG region of the receptor, and gangliosides are already well established as being required for MAG binding to cells (see Introduction).

Gangliosides can bind to FRG motifs in MAG itself and to FRG motifs in the NgR1. Because gangliosides such as GT1b have two branches with terminal sialic acids, one possibility is that they facilitate soluble MAG binding to the NgR by cross-linking both molecules via their shared FRG motifs, and in some circumstances this might be sufficient for activation of the NgR1 complex by MAG. Alternatively, a MAG/ganglioside interaction might facilitate a direct protein-protein interaction between MAG and the NgR1. Irrespective of the mechanism, it might be of some value that small FRG motif-containing peptides can disrupt the interaction, possibly by interfering with the ganglioside/NgR interaction or the MAG/ganglioside interaction or both. However, it is also clear MAG and at least two other myelin molecules (OMgp and Nogo-66) can inhibit neurite outgrowth in a NgR-independent manner, particularly when these molecules are substrate-bound (22). Based on this fact, we would not expect the NgR1-derived peptides to inhibit responses to substrate-bound MAG, and this was indeed the case. For these reasons the FRG peptides are unlikely to offer therapeutic opportunities in circumstances where myelin inhibits regeneration. However, a recent study showed that passive immunization with anti-ganglioside antibodies directly inhibits axonal regeneration after axonal injury in mice (4). A considerable body of evidence also exists suggesting that autoimmune, anti-ganglioside antibodies might contribute to the poor prognosis of some patients with peripheral neuropathies (6). The results obtained in the present study might be of value in considering therapeutic opportunities for peripheral neuropathies where antibodies to gangliosides might play a pathologic role.

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