A Neutralizing Anti-Nogo66 Receptor Monoclonal Antibody Reverses Inhibition of Neurite Outgrowth by Central Nervous System Myelin*

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The Nogo66 receptor (NgR1) is a neuronal, leucine-rich repeat (LRR) protein that binds three central nervous system (CNS) myelin proteins, Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein, and mediates their inhibitory effects on neurite growth. Although the LRR domains on NgR1 are necessary for binding to the myelin proteins, the exact epitope(s) involved in ligand binding is unclear. Here we report the generation and detailed characterization of an anti-NgR1 monoclonal antibody, 7E11. The 7E11 monoclonal antibody blocks Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein binding to NgR1 with IC50 values of 120, 14, and 4.5 nM, respectively, and effectively promotes neurite outgrowth of P3 rat dorsal root ganglia neurons cultured on a CNS myelin substrate. Further, we have defined the molecular epitope of 7E11 to be DNAQLR located in the third LRR domain of rat NgR1. Our data demonstrate that anti-NgR1 antibodies recognizing this epitope, such as 7E11, can neutralize CNS myelin-dependent inhibition of neurite outgrowth. Thus, specific anti-NgR1 antibodies may represent a useful therapeutic approach for promoting CNS repair after injury.

Growth-inhibitory molecules present in the CNS1 myelin contribute, at least in part, to the inability of mammalian CNS axons to regenerate upon injury (1). Three inhibitors that have been identified are Nogo (2–5), myelin-associated glycoprotein (MAG, Refs. 6–9), and oligodendrocyte-myelin glycoprotein (OMgp, Ref. 10). Nogo is a member of the Reticulin family and occurs in three forms, NogoA, NogoB, and NogoC, that are generated from alternate splicing (2–4). NogoA, -B, and -C all contain a 66-amino acid extracellular domain (Nogo66) that alone can inhibit neurite outgrowth and induce growth cone collapse (2–5). MAG is a type I membrane protein composed of five extracellular immunoglobulin (Ig)-like domains (6, 7), whereas OMgp is a glycosylphosphatidylinositol-anchored protein that contains 8 LRR domains (10). Nogo66, MAG, and OMgp inhibit neurite outgrowth by interacting with a neuronal protein termed Nogo66 receptor or the Nogo receptor (NgR1, Refs. 8–11). NgR1 is a glycoprotein that contains 8 LRR domains, a LRR cap region in the respective N and C termini, and a stalk region composed of 100 amino acid residues (11). All 8 LRR domains in the NgR1 are necessary for binding to Nogo, MAG, and OMgp; however, the exact epitope(s) on NgR1 that interacts with these myelin proteins is unknown (8–10). Because NgR1 is a glycosylphosphatidylinositol-anchored protein, it is not a signaling molecule (12, 13). The low affinity neurotrophin receptor, p75, has been identified as a co-receptor for NgR1, and transduces a signal upon interaction with the myelin ligands (14–16). The downstream signaling pathway involves RhoA activation (17, 18), which in turn regulates the cytoskeletal protein assembly and mediates inhibitory effects on neurite growth (13, 19, 20).

A rational strategy to promote CNS axonal regeneration and hence functional recovery after CNS injury is to inhibit the interactions of Nogo, MAG, and OMgp with NgR1 (13). Indeed, molecules that modulate the interaction of an individual myelin protein with NgR1 have been evaluated for the ability to promote CNS axonal regeneration. Successful examples include the anti-NogoA antibody, IN-1 (21, 22), and the Nogo66 antagonist peptide, NEP1–40 (23), both of which reversed CNS myelin-dependent inhibition of neurite outgrowth, promoted axonal regeneration, and improved functional recovery in an animal model of spinal cord injury (21, 22, 24). However, these reagents only target a single myelin protein, Nogo, which may not be sufficient to facilitate maximal CNS axonal regeneration because other inhibitors such as MAG and OMgp are present in the CNS myelin environment.

Recently, a soluble fragment of NgR1 containing the ligand-binding domain (sNgR310) was shown to block both Nogo66 and MAG-dependent inhibition of neurite outgrowth (9, 25). When an Ig-fusion protein containing this soluble NgR fragment (sNgR310-Fc) was administered intrathecally to rats with spinal cord injury, enhanced axonal growth in the cortical spinal and raphespinal tracts, accompanied by improved functional recovery were observed.2 sNgR310-Fc treatment led to a more significant increase in sprouting complexity and in the number of cortical spinal tract fiber sprouts than NEP1–40, which may be attributed to its ability to neutralize the interaction of all three myelin ligands with NgR1.2

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‡ The abbreviations used are: CNS, central nervous system; MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; Ig, immunoglobulin; LRR, leucine-rich repeat; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; AP, alkaline phosphatase; DRG, dorsal root ganglion; PI-PLC, phosphatidylinositol-specific phospholipase C; aa, amino acid(s).

1 The abbreviations used are: CNS, central nervous system; MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; Ig, immunoglobulin; LRR, leucine-rich repeat; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; AP, alkaline phosphatase; DRG, dorsal root ganglion; PI-PLC, phosphatidylinositol-specific phospholipase C; aa, amino acid(s).

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To better understand the functional properties of NgR1, we have generated a specific monoclonal antibody (mAb). This antibody, 7E11, blocks the interactions of Nogo, MAG, and OMPg with NgR1 and reverses the inhibitory effects of CNS myelin on neurite outgrowth in primary neurons and is the first reported neutralizing anti-NgR1 antibody. Through epitope mapping for this antibody, we have defined a key epitope on NgR1 that is conserved and capable of inducing the functional neutralizing antibody, 7E11 is an invaluable reagent for assessing the role of NgR1 in CNS axonal regeneration. Our data suggest that neutralizing anti-NgR1 antibodies may be a useful therapeutic for treating CNS injuries, such as spinal cord injury, traumatic head injury, stroke, and multiple sclerosis.

**EXPERIMENTAL PROCEDURES**

**Soluble NgR310-Fc (sNgR310-Fc) and sNgR344-Fc—The generation of sNgR310-Fc was described by Li et al.** Soluble NgR344-Fc (sNgR344-Fc) was similarly generated, except that the cDNA encoding amino acid residues 27–344 of rat NgR1 was fused in-frame to the rat IgG hinge and Fc was contained in the expression plasmid vector pVY90 (27). After sequence verification, the plasmid was transfected into CHO-DG44 cells. Secreted sNgR344-Fc in the conditioned medium was purified on Protein A-Sepharose. The purity of the protein obtained was ~95% as analyzed by SDS-PAGE. N-Terminal sequence analysis verified that the secreted sNgR344-Fc protein began with Cys7.

**Recombinant Proteins—The generation of the AP-Nogo66 cDNA construct was described by Fournier et al. (17). AP-Nogo66 was expressed in CHO-DG44 cells, and purified by sequential chromatography steps on TMAE-triacetate and Ni²⁺-nitrilotriacetic acid-agarose. AP-OMpg (10) was a generous gift from Dr. Sha Mi of Biogen Idec. MG-Fc was obtained from R & D Systems (Minneapolis, MN). Rat IgG was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA).

**CNS Myelin—**White matter from bovine brains (Pel-Freeze Biologicals, Inc.) was isolated and homogenized in 0.32 M sucrose including protease inhibitors (Roche). The homogenate was subjected to centrifugation on a sucrose density gradient containing 0–0.85 M sucrose at 32,000 × g for 4 h at 4°C. The white interface was collected and osmotically shocked twice with cold water. The pellet was resuspended in 0.32 M sucrose and again subjected to the sucrose gradient centrifugation protocol. Hybridomas were maintained in 1.1% CM supplemented Dulbecco balanced salt solution containing 0.1% ovalbumin and bound AP-Nogo66 or AP-OMpg were detected by measuring AP activity using p-nitrophenyl phosphate under standard conditions (Pierce). Bound MAG-human Fc was detected using horseradish peroxidase-conjugated goat anti-human Fc antibody (Jackson ImmunoResearch Laboratory) and developed using 3,3’,5,5’-tetramethylbenzidine substrate under standard conditions. 

**Neurite Outgrowth Assay—**Labtek culture slides (4 wells) were coated with 0.1 mg/ml poly-d-lysine in PBS (Sigma). CNS myelin prepared from bovine white matter was confirmed to contain OMPg, MAG, and Nogo by Western analysis as described below. Different concentrations of CNS myelin in PBS, 6, 9, and 15 mg/mm², were spotted as 3 µl drops. Each well contained 3 drops of CNS myelin and 1 drop of PBS control. Fluorescent microscopes (Polysciences, Warrington, PA) were added to the myelin/PBS to facilitate demarcation of the drops (2). Labtek slides were then rinsed and coated with 10 µg/ml laminin (Invitrogen). DRGs from post-natal day 3 (or day 4) Sprague-Dawley rats were dissociated with 1 mg/ml collagenase type I (Worthington Biociences), triturated with fire-polished Pasteur pipettes, and pre-plated for 1 h to enrich for neurons. Cells were plated at 23,000 cells/well in Ham’s F-12 culture medium containing 5% heat inactivated donor horse serum (JRH Bioscience, Logan, UT), 5% heat inactivated fetal bovine serum (JRH Bioscience), and 50 ng/ml murine nerve growth factor (JRH Bioscience), and incubated at 37°C and 5% CO₂ for 6 h. Test reagents were added immediately after plating. At the end of the experiment, cells were fixed for 20 min with 4% paraformaldehyde in PBS containing 20% sucrose, blocked with PBS containing 10% normal goat serum (Vector, Burlingame, CA) and 0.1% Triton-X-100, and stained for the neuronal marker β-III tubulin using an anti-β-III-tubulin antibody (Covance, Princeton, NJ) diluted 1:500 in PBS containing 10% normal goat serum and 0.1% Triton X-100. A secondary goat anti-mouse IgG Fc (γ) specific Alexa-594 antibody (Molecular Probes, Eugene, OR) was used at a dilution of 1:300 (1 µg/ml) in PBS. Slides were washed with PBS and sealed with a coverslip with Gelmount (Biomedea, Foster City, CA). Digital images (5×, 1 image/drop, 6 images/data point) were acquired and the images were analyzed using the Metamorph software (Universal Imaging Co., West Chester, PA) for quantification of neurites. Total neurite outgrowth per field was obtained and normalized to the number of neurons per field.

**Epitope Mapping for 7E11—**The LDLSDNAQLR<sub>V</sub>VDPTDP<sub>T</sub> peptide was suspended in distilled water at 500 µg/ml. For each digestion, 40 µg of the peptide was treated with either 8 µl of immobilized trypsin on glass beads (Sigma) or 0.2 µg of sequencing grade endopeptidase Lys-C (Applied Science) in 1M sodium bicarbonate, pH 9.0, or in 1M Tris-HCl, pH 8.0. After incubation at 37°C for 2 h, the immobilized trypsin was removed by centrifugation. The resulting cleavage products were incubated at 37°C for 18 h with 400 µg of 7E11 Fab. The 7E11 Fab-peptide complex was desalted on a G-25 column to separate the complex from the unbound peptide fragments. The 7E11 Fab-peptide complex was subjected to reverse phase high performance liquid chromatography on a C<sub>4</sub> column that was eluted with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid to separate the 7E11 Fab from the bound peptide. The fragments associated with the 7E11 Fab were collected and subjected to liquid chromatography-mass spectrometry analysis. The peptide sequences were determined by liquid chromatog-
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Fig. 1. Generation of the 7E11 mAb. **A,** the rat NgR1 protein sequence translated from the NgR1 cDNA was subjected to antigenicity analysis using Vector NTi software. The results are plotted against the NgR1 sequence. Positive values represent potentially antigenic residues. Sequences with high antigenicity were next subjected to BLAST searches for identifying homology with other proteins. Only sequences that share <25% identity with other proteins were selected. A peptide comprising amino acids 110–125, LDLSDNAQLRVDPTT, was selected as the antigen based on these criteria. The lower scheme illustrates the key structural features of the NgR1 protein. The peptide selected as the antigen is located in LRR3 of rat NgR1. **B,** 7E11 binds to immobilized LDLSDNAQLRVDPTT. Left, LDLSDNAQLRVDPTT was immobilized on Maxisorp microtiter plate and incubated with 7E11 at concentrations ranging from 0.01 to 10 μg/ml. Bound 7E11 was detected by horseradish peroxidase-conjugated goat anti-mouse antibody. Data are mean values of 3 readings and error bars indicate S.E. Nonlinear regression curve fit and EC_{50} were generated using Prism. **Right,** the binding affinity of 7E11 to sNgR344-Fc was similarly obtained. **C,** 7E11 binds to rat NgR1 expressed on COS-7 cells. COS-7 cells expressing rat NgR1 or parental untransfected COS-7 cells were incubated with 7E11 and subjected to FACS analysis (a, parental untransfected COS-7 cells incubated with 30 μg/ml 7E11; b, transected COS7 cells incubated with 30 μg/ml 7E11).

Western Blot Analysis—COS-7 cells expressing rat, mouse, or human NgR1, or human NgR2 or NgR3 were either lysed directly in 100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, pH 6.8, or treated with phosphatidylinositol-specific phosphopholipase C (PI-PLC, 1U/10^6 cells, Sigma) at 37 °C for 1 h in Dulbecco’s modified Eagle’s medium supplemented with 1% bovine serum albumin. Cell lysates and the supernatant fractions of PI-PLC treatments were subjected to SDS-PAGE on a 4–20% gradient gel under reducing conditions. Protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 2% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween 20, membranes were stained with antibodies to NgR1 and analyzed by Western Blotting.
Tween 20 (TBST), the polyvinylidene difluoride blots were incubated with 7E11 (5 μg/ml) in TBST containing 1% nonfat dry milk for 3 h. Blots were washed with TBST three times and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000, Bio-Rad). The blots were again washed three times with TBST and developed using the Odyssey system (Li-Cor Biosciences, Lincoln, NK).

CNS myelin (20 μg) were run on SDS-PAGE gels (4–20% Novex Tris glycine gels, Invitrogen) under reducing conditions and transferred to nitrocellulose filter. Blots were reversibly stained and visualized with Memcode Blue (Pierce) followed by blocking with a 10% nonfat dry milk solution solution in TBST at 25 °C for 3.5 h. Blots were then incubated with 5 μg/ml of the following antibodies at 25 °C for 2 h: goat monoclonal anti-MAG antibody (R & D System), rabbit polyclonal anti-NogoA antibody (obtained from Dr. Stephen M. Strittmatter) (31), and rabbit polyclonal anti-OMgp antibody (obtained from Dr. Sha Mi, Biogen Idec). Blots were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:100,000, Jackson Immuno-Research Laboratory) and developed using a Femto Chemiluminescence kit (Pierce).

**RESULTS**

**mAb 7E11 Recognizes Rat and Human NgR1—7E11 mAb was generated using a synthetic peptide derived from amino acid residues 110–125 in the rat NgR1 sequence (Fig. 1A). These residues are located in the LRR3 domain on NgR1. 7E11 bound to this synthetic peptide and sNgR344-Fc immobilized on Maxisorp plates with EC_{50} values of ~0.55 and 0.75 nm, respectively (Fig. 1B). Furthermore, 7E11 recognized rat NgR1 expressed on COS-7 cells by FACS (Fig. 1C). Western blot**
analyses using COS-7 cells expressing rat, mouse, and human NgR, respectively, showed that 7E11 recognized both rat and human NgR1, either in the total cell lysate fraction (Fig. 2A) or the supernatant fraction after PI-PLC treatment (Fig. 2B). 7E11 did not recognize mouse NgR1, human NgR2, or human NgR3 (Fig. 2, A and B). In the rat NgR1 and human NgR1 cell lysate fractions, two stained bands were observed that may represent alternate glycosylation forms of NgR1. A 15-kDa band was specifically found in the human NgR1-PI-PLC fraction, which may be generated by contaminating proteases during the PI-PLC treatment (Fig. 2B). Western blot analyses indicate that 7E11 is a sensitive and highly specific anti-NgR1 antibody.

**7E11 Blocks NgR1 Binding to MAG, OMgp, and Nogo66**—The biochemical effects of 7E11 on the interactions of MAG,
OMgp, and Nogo with NgR1 were tested, using protein-based ELISA binding assays. For these studies, ELISA plates were coated with sNgR344-Fc or control rat Ig, and the binding of MAG-Fc, AP-OMgp, and AP-Nogo66 in the presence or absence of 7E11 was studied in detail. 7E11 inhibited the binding of all three myelin proteins, MAG-Fc, AP-OMgp, and AP-Nogo66, to immobilized sNgR344-Fc in a dose-dependent manner, with IC₅₀ values of 14, 4.5, and 120 nM, respectively (Fig. 3). 7E11 had no effect on the binding of MAG, OMgp, and Nogo66 to the control rat Ig protein. The efficacy of sNgR310-Fc protein in promoting CNS axonal regeneration and behavioral motor function recovery in an animal model of spinal cord injury has been demonstrated previously.² We next compared the potency of 7E11 relative to the sNgR310-Fc in the same series of binding studies with MAG-Fc, AP-OMgp, and AP-Nogo66 and obtained the respective IC₅₀ values of 74, 60, and 8600 nM. In these assay systems, 7E11 is a more potent inhibitor than sNgR310-Fc with at least 15-fold differences in the relative IC₅₀ values for each myelin inhibitor (Table I). Thus, 7E11 represents the first specific anti-NgR1 antibody that inhibits NgR1 binding to Nogo, MAG, and OMgp.

7E11 Promotes DRG Neurite Outgrowth on CNS Myelin—The functional ability of 7E11 to prevent CNS myelin-dependent inhibition of neurite outgrowth of DRG neurons was next evaluated. In the absence of CNS myelin, DRG neurons sent out long neurites after 6 h in culture (Fig. 4A). In contrast, concentration-dependent inhibition of neurite outgrowth was observed when the neurons were cultured on a dried-down CNS myelin substrate. Half-maximal inhibition was observed with ~9 ng/mm² CNS myelin (Fig. 4B). When 7E11 was added to the cultures at the time of plating, it significantly attenuated the inhibitory properties of CNS myelin on neurite outgrowth. 7E11 (100 nM) effectively antagonized the effects of coated CNS myelin up to 15 ng/mm², resulting in more neurite outgrowth (Fig. 4, C and D). When sNgR310-Fc was tested in the same assay, ~1 µM sNgR310-Fc was required to produce similar effects (data not shown). Thus, the effects of 7E11 and sNgR310-Fc on neurite outgrowth correlate well with the IC₅₀ values obtained in the binding studies. Similarly, 7E11 reversed the inhibition of MAG-Fc on neurite outgrowth in a dose-dependent manner. Almost complete reversal of the MAG-Fc inhibition was observed with high doses of 7E11 (Fig. 4G). 7E11 is the first neutralizing anti-NgR1 monoclonal antibody that can promote neurite outgrowth against CNS myelin inhibition in vitro.

Defining the Molecular Epitope of 7E11—To test if 7E11 recognizes other sequence epitopes on rat NgR1, fragments of rat NgR1 were generated and tested for 7E11 binding (Fig. 5). A recombinant fragment of the rat NgR1 (sNgR310) that contains all 8 LRR domains and the N- and C-terminal caps was treated with either acid or cyanogen bromide (CNBr). Gel electrophoresis showed that the apparent molecular mass of untreated sNgR310 is about 42 kDa (Fig. 5, left, lane 1). Acid treatment of sNgR310 produced two major cleavage products of 15 (aa 27–122) and 30 kDa (aa 123–310) (Fig. 5, left, lane 2). CNBr treatment generated three fragments, a 33/35-kDa doublet (aa 27–229), which may represent fragments with heterogeneous glycosylation, a 10-kDa product (aa 241–310), and an 11-amino acid fragment (aa 230–240), which was not retained on the gel (Fig. 5, left, lane 3). These cleavage products were subjected to Western blotting analysis using 7E11 (Fig. 5, right). As expected, 7E11 recognized intact rat NgR1 (aa 27–310, lane 4), the 15-kDa fragment (aa 27–122, lane 5), and the 35-kDa fragment (aa 27–229, lane 6) that both contained the LDLSDNAQLRVDPTT but not the 30-kDa acid cleavage fragment (aa 123–310) (lane 5) or the 10-kDa CNBr fragment (aa 241–310) (lane 6). This result is consistent with 7E11 binding to the LDLSDNAQLRVDPTT sequence on NgR1.

The 7E11 binding site was further analyzed by trypsin peptide mapping. High performance liquid chromatography analyses of the tryptic digests of sNgR310 yielded multiple peaks, consistent with the presence of 24 trypsin-sensitive lysine and arginine residues in the NgR1 sequence (Fig. 6A, upper panel). Only a single trypsin digest peptide bound to 7E11 (Fig. 6A, lower panel). Subsequent MS and sequence analyses identified the bound peptide to be AAAFGTLLEQLDLSDNAQLR, thus providing additional evidence that 7E11 binds to a single epitope on NgR1.

To define the molecular epitope for 7E11, the LDLSDNAQLRVDPTT peptide was subjected to further mapping analysis. First, the peptide was digested with trypsin to yield two major fragments, LDLSDNAQLR and VVDPTT, and these fragments were tested for their ability to bind 7E11 (Fig. 6B). MS analysis of the bound fraction revealed that peptide LDLSDNAQLR was recognized by the antibody, and therefore contains the binding epitope for 7E11. Within this peptide fraction, detailed MS analysis identified several scrambled peptides that also bound 7E11, including peptides with deamination at Asn¹¹⁵ and Gln¹¹⁷, addition of alanine at amino acid position 112 or 113, or addition of serine at amino acid position 114 (Fig. 6B). These indicate that many amino acid residues located within this LDLSDNAQLR peptide fragment may not be critical for 7E11 binding. Second, the peptide was digested with endoprotease Asp-N and analyzed for 7E11 binding. Endoprotease Asp-N cleaved the peptide into 3 peptide fragments, L, DLS, and DNAQLRVDPTT. Of these products, the DNAQLRVDPTT peptide was recognized by 7E11 (Fig. 6B). Taken together, the trypsin and endoprotease Asp-N cleavage data further localize the 7E11 binding epitope to the DNAQLR sequence. To verify that this sequence is the 7E11 binding epitope, peptides containing DNAQLR, a scrambled version of the peptide, and two variant sequences were synthesized and tested for binding to 7E11. The results showed that 7E11 bound to DNAQLR while weaker binding to the other peptides occurred (Fig. 6C). Although 7E11 binds to DNAQLR, this was a weaker interaction when compared with 7E11 binding to the NgR1 protein, suggesting that additional flanking amino acid residues and/or protein structure may contribute to optimal binding. Interestingly, both DNAQLR and LDLSDNAQLR, either in solution or when coupled to bovine serum albumin, did not affect Nogo66, MAG, and OMgp binding to NgR1 (data not shown).

Additional data that helped to identify critical residues in...
Fig. 6. Localization of the 7E11 binding epitope by mapping with trypsin and endoprotease Asp-N. A, sNgR310 was digested with trypsin. The cleavage products were incubated with the 7E11 Fab. Unbound peptides were removed through desalting. The single bound peptide was analyzed by MS and subjected to sequence analysis (indicated by arrow). B, top, the synthetic peptide LDLSDNAQLRVDVPTT was digested with either trypsin or endoprotease Asp-N. Arrows indicate the cleavage sites. The cleavage products were incubated with the 7E11 Fab. Peptides LDLSDNAQLR and DNAQLRVDVPTT bound 7E11, and the overlap sequence in the bound peptides are underlined. B, top, when LDLSDNAQLRVDVPTT was digested with trypsin, MS analysis showed that several scrambled peptides also bound 7E11 (B, bottom). C, synthetic peptides containing DNAQLR sequence were synthesized and tested for binding to 7E11 in an ELISA format. Only DNAQLR shows significant binding to 7E11. Error bars indicate S.E.
the 7E11 binding epitope were obtained by analyzing the amino acid sequences of NgR1, NgR2, and NgR3 from various species because Western blotting and FACS analysis (data not shown) had revealed that the 7E11 mAb recognized rat and human NgR1 but not mouse NgR1, human NgR2 or NgR3. Sequence alignments revealed that the rat and human NgR1 sequences corresponding to amino acids 110–125 in rat NgR1 are identical and that the mouse NgR1 differs only by one amino acid at position 119 (Arg119 in rat and human NgR1, and His119 in mouse NgR1; Table II). Because 7E11 binds well to rat and human NgR1 but poorly to mouse NgR1, Arg119 on NgR1 must contribute to 7E11 binding. Similarly, we can infer from the NgR3 binding data that Ala116 is involved in the epitope, because within the DNAQLR sequence NgR3 only differs from NgR1 by an arginine at the corresponding sequence. Within the DNAQLR sequence, 4 of 6 of the residues in NgR2 are identical to rat NgR1. Ala116 and Gln117 are replaced with arginine and histidine, respectively. Whereas we can infer that Ala116 is an important amino acid residue contributing to 7E11 binding, from sequence alignment we cannot exclude the involvement of Gln117.

To verify these contact points, several peptides that contain point mutations within the LDLSDNAQLR sequence were generated and tested for 7E11 binding. The mutants were immobilized on Maxisorp plate and serial dilutions of the 7E11 mAb were applied. The resulting EC50 values are summarized as in Table III. 7E11 bound to mutants L110A and D111A with similar EC50 values as to the original peptide. When Q117A was tested, the EC50 increased 30-fold and when R119H was tested, the EC50 increased 25-fold. The most significant change in EC50 was observed when Arg119 was mutated to alanine.

The crystal structure of sNgR310 provided a unique opportunity to study the 7E11 binding epitope at the molecular level. We attempted to identify the location of the 7E11 epitope in the NgR1 structure. As expected, the structure shows that the 7E11 epitope is exposed on the surface of the molecule. The side chains of residues Arg119, Gln117, Ala116, and Asp115 protrude outward from the structure, whereas the side chains of Leu118 and Asn116 are pointed inward. The epitope falls on top of an acidic patch within the concave surface of the structure and a basic surface that faces on one of the sides (Fig. 7).

**DISCUSSION**

The CNS myelin proteins Nogo, MAG, and OMgp all bind to NgR1 and recruit co-receptor molecule(s) to mediate their inhibitory effects on neurite outgrowth. We have generated a neutralizing anti-NgR1 antibody, 7E11, to test the hypothesis that reagents targeting NgR1 would antagonize the inhibitory effects of CNS myelin. This mAb specifically binds to a motif comprising six amino acid residues in the LRR3 domain of NgR1. 7E11 recognizes rat and human NgR1, but not NgR2, NgR3, or mouse NgR1, even though they share extensive overall sequence homology with NgR1. Synthetic peptide binding experiments further suggested that the 7E11 epitope contains and requires the sequence DNAQLR, but flanking amino acid residues and/or NgR protein structure may also contribute to optimal 7E11 binding. BLAST analyses of the vertebrate protein databases indicated that several unrelated proteins contain homologous sequences to DNAQLR, however, by FACS analysis of cells lacking NgR1 and Western analyses of cell and brain lysates, cross-reactivity to unrelated proteins was not detected. Thus, 7E11 may have limited cross-species reactivity with other NgRs or proteins. The 7E11 antibody blocks the binding of Nogo66, MAG, and OMgp to NgR1, attenuates the inhibitory effects of CNS myelin on neurite outgrowth of rat DRG neurons, and behaves as a NgR1 antagonist.

The binding sites on NgR1 for Nogo, MAG, and OMgp have not been defined. Even whether these myelin proteins cross-compete with each other for binding to NgR1 remains controversial. For example, it was reported that MAG competed with Nogo66 for binding to NgR1 (8), whereas Liu et al. (9) and Ref. 32 showed that MAG did not compete with Nogo66 for binding to NgR1. These studies are complicated because each group used different fragments of the inhibitors and reagents from different species, which may have an impact on the result. For these reasons, it is intriguing that a single monoclonal anti-NgR1 antibody, such as 7E11, could block the binding of all three myelin proteins to NgR1. This finding clearly implies that the 7E11 epitope may affect ligand binding. Recent crystallographic studies have revealed the structure of the NgR1 ectodomain (29, 33). Other than a relatively “flat banana-shaped surface” similar to that observed in all LRR protein crystal structures, an acidic cavity was noted and postulated to contribute to ligand binding. Interestingly, amino acid residues that constitute this acidic cavity are also contained within the 7E11 epitope and therefore support the notion that this region of the NgR1 protein could be important for ligand binding. Whether 7E11 blocks solely by sterically interfering with ligand binding or inducing a conformational change on NgR1 that discourages ligand/NgR1 interactions remains to be stud-

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**TABLE II**

| Rat, human NgR1 | LDLSDNAQLR |
|----------------|------------|
| Mouse NgR1     | LDLSDNAQLH |
| Rat, human NgR2| LDLSDNAQLR |
| Rat, human NgR3| LDLSDNAQLR |

**TABLE III**

| Mutant peptides | Sequences                  | EC50 (nM) |
|-----------------|----------------------------|-----------|
| Original peptide| LDLSDNAQLRVDPTT            | 0.55      |
| L110A           | LDLSDNAQLRVDPTT            | 0.62      |
| D111A           | LDLSDNAQLRVDPTT            | 0.31      |
| Q117A           | LDLSDNAQLRVDPTT            | 16        |
| R119H           | LDLSDNAQLRVDPTT            | 12        |
| R119A           | LDLSDNAQLRVDPTT            | 88        |

**FIG. 7.** Mapping the epitope for 7E11 on the sNgR310 crystal structure. A space filling model for the sNgR310 crystal structure shows the distribution of charged residues (red, negatively charged; blue, positively charged). An enlargement of the binding epitope with the orientation of the side chain is shown.
ied. Because the IC_{50} values of 7E11 for Nogo66 differs from that of MAG and OMgp by >10-fold, it is likely that the contact surface for Nogo66 may be distinct from that of MAG and OMgp (9, 32).

The fact that Nogo66, MAG, and OMgp all bind to NgR1 (8–10) may explain why axonal regeneration was inefficient in vivo when reagents that blocked a single inhibitory myelin protein were employed. For example, little axonal regeneration after CNS injury in MAG-deficient mice was noted, which may be attributed to the inherent presence of NogoA and OMgp (34, 35). Similarly, in vitro application of anti-NogoA antibody, IN-1, after spinal cord injury only resulted in a small percentage of regenerating axons (21, 22). Likewise, the Nogo66-derived antagonistic peptide, NEP1–40, only blocked the inhibitory effect of Nogo66 on neurite growth but not that of MAG (9). These reports suggest that blocking a single myelin ligand may not be sufficient to facilitate maximal CNS axonal regeneration. Reagents that block all three ligands may result in better efficiencies. In fact, the administration of sNgR310-FC that blocks the binding of Nogo66, MAG, and OMgp to NgR1 led to increased sprouting complexity and a larger number of cortical spinal tract fibers in an animal model of spinal cord injury, when compared with NEP1–40 treatment.\(^2\) In the current study, we directly compared the in vitro blocking activities of sNgR310-FC and 7E11 in competition binding assays and in the neurite outgrowth assay against CNS myelin, and demonstrated that 7E11 is a more potent inhibitor than sNgR310-FC in vitro. Further experiments in relevant animal models may prove the relevance of anti-NgR1 antibodies in promoting CNS axonal regeneration. Thus, targeting the NgR1 pathway with a neutralizing anti-NgR1 antibody is a new strategy to promote axonal regeneration for repairing CNS lesions.

Cells expressing domain-deletion mutant NgR1 variants have been used to evaluate the contributions of individual LRR domains of NgR1 to ligand interactions. One mutant with only the LRR domains but lacking LRRCT and the stalk region bound Nogo66, MAG, and OMgp (9, 10, 25), whereas another mutant with only LRRCT and the stalk region could not bind MAG or OMgp (9, 10); but in one study bound Nogo (10). The studies on 7E11 presented here clearly identify a specific region within LRR3 on NgR1 that is important for ligand binding. Whether other binding sites for Nogo66, MAG, and OMgp co-exist remains to be tested. Future site-directed mutagenesis analysis of NgR1 or crystallography with ligand-receptor complexes may shed light on the exact binding site(s) for these three myelin ligands. Our characterization of a key epitope within LRR3 on NgR1 that affects ligand binding should facilitate rational drug design for finding potent and specific NgR1 antagonists that promote CNS axonal regeneration.

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