Characterization of Myelin Ligand Complexes with Neuronal Nogo-66 Receptor Family Members*§

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Nogo, MAG, and OMgp are myelin-associated proteins that bind to a neuronal Nogo-66 receptor (NgR/NgR1) to limit axonal regeneration after central nervous system injury. Within Nogo-A, two separate domains are known interact with NgR1. NgR1 is the founding member of the three-member NgR family, whereas Nogo-A (RTN4A) belongs to a four-member reticulon family. Here, we systematically mapped the interactions between these superfamilies, demonstrating novel nanomolar interactions of RTN2 and RTN3 with NgR1. Because RTN3 is expressed in spinal cord white matter, it may have a role in myelin inhibition of axonal growth. Further analysis of the Nogo-A and NgR1 interactions revealed a novel third interaction site between the proteins, suggesting a trivalent Nogo-A interaction with NgR1. We also confirmed here that MAG binds to NgR2, but not to NgR3. Unexpectedly, we found that OMgp interacts with MAG with a higher affinity compared with NgR1. To better define how these multiple structurally distinct ligands bind to NgR1, we examined a series of Ala-substituted NgR1 mutants for ligand binding activity. We found that the core of the binding domain is centered in the middle of the concave surface of the NgR1 leucine-rich repeat domain and surrounded by differentially utilized residues. This detailed knowledge of the molecular interactions between NgR1 and its ligands is imperative when assessing options for development of NgR1-based therapeutics for central nervous system injuries.

When nerve fibers of the brain and spinal cord in adult mammals are severed, little to no regrowth occurs. Astroglial scarring and central nervous system myelin pose extrinsic barriers to regeneration (1, 2). From central nervous system myelin, at least three proteins capable of inhibiting axonal growth in vitro are recognized: Nogo-A, MAG, and OMgp (1, 2). Nogo-A has several domains that participate in inhibiting axonal growth. The hydrophilic Nogo-66 domain flanked by two hydrophobic segments is detectable on the oligodendrocyte surface (3, 4). Together, these three segments form a reticulon (RTN) homology domain (RHD) of ~200 amino acids, characteristic of reticulin family members (5).

Nogo-66 binding provided the basis for the identification of a Nogo-A receptor (NgR/NgR1) (6). Remarkably, MAG and OMgp also bind to NgR1 to inhibit axonal growth in vitro (7–9). NgR1 is a leucine-rich repeat (LRR)-containing glycosylphosphatidylinositol-anchored neuronal protein; the structure of its LRR domain has been determined (10, 11).

Perturbation of Nogo function by antibodies (12–14), peptide, or the soluble NgR1 ectodomain (15–19) leads to enhanced axonal growth, plasticity, and functional recovery after spinal injury or stroke. Genetic studies of Nogo-A (20–22) and NgR1 (23, 24) have, however, found less clear-cut evidence of their role in axonal regeneration. It is plausible that adaptive compensation for chronic genetic loss of NgR1 or Nogo-A may explain this observation in part.

Alternatively, the less pronounced genetic versus pharmacological phenotype might relate to redundancy among the myelin inhibitory proteins and their signaling pathways. NgR1 is the founding member of the three-member NgR family (11, 25, 26); Nogo-A belongs to a four-member RTN family (5). MAG and OMgp have no known paralogs. A recent report demonstrated that, in vitro, MAG can bind and exert its inhibitory function via NgR2 as well as NgR1 (27). The ability of OMgp to bind other NgR family members has not been assessed. The functions of other RTNs are largely enigmatic (28). As other RTNs are also present in the central nervous system and contain homologous RHDs, we hypothesized previously that they could interact with NgR family members (25). Here, we have mapped the NgR family binding properties of all RTNs, MAG, and OMgp. We also demonstrated that RTN3, which we found to bind to NgR1, is expressed in spinal cord white matter.

Several topologies of Nogo-A relative to the lipid bilayer have been supported experimentally. The extended length, 35 and 36 amino acids (3), of the hydrophobic segments flanking the Nogo-66 segment suggests that these segments might not be single-pass transmembrane regions. A recent report supports the existence of a conformation in which all three of the hydrophobic segments of the RTNs are on the same side of the lipid bilayer (29). This raises the possibility that C-terminal amino acids...
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FIGURE 1. Analysis of interactions between RTN family and LRR-containing protein superfamily members shows that RTN2-66 and RTN3-66 interact specifically with NgR1. RTN1-66 at either 6 nm (A) or 3 nm (A') did not bind to NgR1, whereas 6 nm RTN2-66 (B), RTN3-66 (C), and RTN4-66/Nogo-66 (D) showed strong binding. Similarly, binding of 3 nm RTN2-66 (B'), RTN3-66 (C'), and RTN4-66 (D') was clearly detectable. No binding of RTN1-4-66 at 50 nm to NgR2 (E–H, respectively) or to NgR3 (I–L, respectively) was detectable. None of the RTNs bound at 50 nm to other members of the LRR-containing protein superfamily tested: Lingo-1 (M–P, respectively) and TLR4 (Q–T, respectively). The dissociation constants for RTN1-4-66 interactions with NgR1 were determined (U). A summary of the $K_d$ values for RTN1-4-66/NgR1 interactions (means ± S.E. from five experiments) is presented (V), as is a sequence alignment of the mouse RTN1-4-66-amino acid loop regions (W). mm, Mus musculus. Identical amino acids are indicated with asterisks; high physicochemical similarity of amino acids is indicated with colors and similarity with periods. In RTN4, the amino-terminal half (underlined) is indispensable for receptor binding. Non-conserved amino acids are highlighted in yellow, and putative residues in RTN1-66 responsible for the lost NgR1 affinity are highlighted in red. Scale bars = 100 μm (D and T).

The molecular basis for NgR1 interaction with multiple ligands has not been defined. LRR domains are commonly involved in protein/protein interactions presumably because the non-globular extended surface of the LRR domain provides ample opportunities for high affinity interactions. Here, we show that NgR1 utilizes certain residues to interact with multiple ligands in a central binding region and other surrounding residues to interact with specific ligands. These data helps us to understand the ligand specificity of different NgR family members and contribute to the elucidation of how central nervous system axonal plasticity and regeneration are limited by the interaction between multiple ligands and NgR family members.

acids might contribute to NgR1 binding, a hypothesis we test here.

EXPERIMENTAL PROCEDURES

Recombinant DNA Constructs—The alkaline phosphatase (AP)-Nogo-66, AP-Y4C (human Nogo-A amino acids 950–1018), AP-Nogo-A-24 (human Nogo-A amino acids 995–1018) AP-MAG, AP-OMgp, and AP-Lingo-1 constructs have been described (6, 8, 9, 30, 31). To generate additional AP fusion proteins, DNA fragments encoding 66-amino acid sequences of RTN1, RTN2, and RTN3 (as shown in Fig. 1W) were cloned into pAPtag5 (kindly provided by Dr. J. G. Flanagan, Harvard Medical School) (32) with restriction enzyme XbaI. For the AP-C-terminal Nogo-A construct (AP-Nogo-C39), the DNA encoding the last 39 amino acids of human Nogo-A was cloned into the XbaI site of pAPtag5. An additional MAG-AP construct was generated by cloning the sequence encoding the MAG ectodomain devoid of signal peptide into the HindIII and BglII restriction sites in pAPtag5. Mouse MAG, TLR4, NgR2, and NgR3 expression constructs contain Igκ signal peptide and Myc and His$_6$ tags, followed by respective open reading frames devoid of signal peptides. These inserts were cloned into the XbaI restriction site of a modified pSecTag2a plasmid in which the stop codon was replaced with the XbaI site. pSecTag2a was modified using the QuikChange II site-directed mutagenesis kit (Stratagene). The Myc-NgR1 expression construct has been described (6). Also, an additional AP-Nogo-66 construct based on the pAPtag5 plasmid backbone was used in some experiments. This was created by subcloning an insert from the original pcAP-5-Nogo-66 plasmid (6) into the pAPtag5 vector. All constructs were sequenced to confirm that no unwanted changes had occurred.

NgR1 Mutagenesis—NgR1 mutagenesis was accomplished using the QuikChange II multisite-directed mutagenesis kit (Stratagene). The Myc-NgR1 expression construct was used as a template. All mutant NgR1 constructs were analyzed by sequencing.

Recombinant Proteins—Expression vectors encoding AP fusion proteins were transfected into HEK293T cells, and conditioned media were collected after 5–7 days. In some cases, conditioned media were concentrated using Amicon Ultra centrifugal filtration devices (Millipore Corp.). His$_6$-tag-containing
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AP-MAG, MAG-AP, and AP-OMgp were purified from conditioned media using nickel-nitrilotriacetic acid affinity resin (Qiagen Inc.) according to the manufacturer’s guidelines.

**COS-7 Ligand Binding Assay**—COS-7 binding assays were performed as described (6). Conditioned media containing AP-fused ligands or purified ligands were incubated with COS-7 cells transfected with the indicated constructs for 1–2 h at room temperature before washing and fixation. Bound AP was visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium reaction.

**Immunocytochemistry**—Live cell immunostaining was performed by incubating cells in Hanks’ balanced saline solution with 0.05% bovine serum albumin and AP-conjugated anti-Myc antibody (9E10, Sigma; 1:200 dilution) or anti-NgR1 antibody (6) at room temperature or on ice for 1 h, followed by washing and fixation. The cells were then incubated for 1.5 h in 65 °C. Finally, bound antibodies were visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium reaction.

**In Situ Hybridization**—In situ hybridization was performed as described (25). The RTN probes described previously (25) were designed to recognize the splice variants containing the sequence encoding the 66-amino acid loop region.

**RESULTS**

**NgR1 Is a High Affinity Receptor for Several RTN Family Members**—We prepared N-terminal AP fusion proteins of all RTN 66-amino acid loop regions and analyzed their binding properties for NgR family members (Fig. 1). We found that, in addition to Nogo-66, RTN2-66 and RTN3-66 interact with NgR1, but not with other NgR family members or other related members of the type I transmembrane LRR-containing protein superfamily tested (Fig. 1). The affinities of RTN2-66, RTN3-66, and Nogo-66 for NgR1 appeared to be similar, with $K_d$ values for RTN2-66 and Nogo-66 of ~1 nM and for RTN3-66 of ~4 nM (Fig. 1V). RTN1-66 showed no affinity for NgR1 even at the highest concentration tested (50 nM).

**Rtn3 and Nogo-A Are Expressed in the Same Glial Cell Population of Spinal Cord White Matter**—A previous report showed Nogo-A expression in central nervous system oligodendrocytes (4). However, the possible expression of other RTNs in spinal cord white matter has not been analyzed. We analyzed the expression of all RTN mRNAs in adult mouse lumbar spinal cord by in situ hybridization. Consistent with the previous study (25), we found that all RTN mRNAs were expressed in neurons (Fig. 2). Interestingly, we found prominent expression of Rtn3 and Rtn4 mRNA transcripts in the white matter/lateral funiculus (Fig. 2, I and L, arrowheads). Nissl counterstaining of the sections enabled large and weakly stained nuclei (neurons) to be distinguished from small and strongly stained nuclei (glia). We noted that glial cell nuclei of the stained cryosections displayed dichotomy in size distribution and that Nogo-A and Rtn3 mRNAs were expressed by the same cell population characterized by larger and more weakly stained nuclei compared with other glial cells. The probes used in these experiments were designed to cover the conserved RHDs and are relatively homologous: in the most homologous 203-nucleotide sequence stretch, Rtn3 and Nogo-A probes are 73% identical, but contain no identical nucleotide stretches longer than 11 nucleotides. Notably, in the Purkinje cell layer of the cerebellum, strong Nogo-A mRNA expression was observed, but no or low level expression of Rtn3 mRNA was detected, thus confirming the specificity of the hybridization reaction (supplemental Fig. 1). No signal was detected with control sense probes (data not shown).

**Nogo-A Interaction with NgR1 Involves Three Segments of Nogo-A**—We produced an AP fusion protein of the C-terminal 39 residues of Nogo-A (AP-Nogo-C39) and measured its affinity for NgR family members. We found that it interacted with high affinity and specificity with only NgR1 (Fig. 3). The NgR1 binding affinity ($K_d$) of AP-Nogo-C39 was determined to be nearly the same as that of the AP-Nogo-A-24 fragment; however, AP-Nogo-66 showed the highest affinity for NgR1. We also reconsidered the $K_d$ of the Nogo-66/NgR1 interaction. The AP-Nogo-66 protein we used in previous studies was found to have been proteolytically cleaved to a significant degree between the AP moiety and Nogo-66. Re-cloning of the same insert into the pAPlag5 plasmid serendipitously led to formation of a stable recombinant protein (supplemental Fig. 2). This allowed us to determine Nogo-66 affinity for NgR1 more accurately than in previous studies and suggests that partial ligand degradation is likely to have resulted in an underestimation of Nogo-66 affinity for NgR1. We concluded that Nogo-66 binds to NgR1 with a $K_d$ of 1 nM.
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The C-terminal Fragment of Nogo-A Interacts Exclusively with the LRR Domain of NgR1—We mapped the interaction site of Nogo-C39 in NgR1 using a series of deletion mutants lacking pairs of the LRR or other structural elements in NgR1 as described (33). The LRR domain of NgR1 was indispensable for Nogo-C39 binding (Fig. 4).

MAG Binds to NgR1 and NgR2 with Moderate Affinity and Shows No Affinity for NgR3—Venkatesh et al. (27) reported that MAG-Fc interacts with NgR2 and that NgR2 mediates MAG-dependent growth inhibitory signaling other than NgR1-mediated signaling. Previously, we failed to observe AP-MAG fusion protein binding to human NgR2 (11). As Venkatesh et al. also failed to see AP-MAG/NgR2 interaction, they proposed that AP tagging of MAG sterically interferes with the binding to NgR2, but not to NgR1. Since then, we have identified a non-conservative nucleotide variant in the PCR-derived NgR2 expression construct used in our previous study. When using a wild-type NgR2 expression plasmid, we found that AP-MAG bound with similar affinity to NgR2 and NgR1 (Fig. 5). Because of the higher expression level of the transfected Myc-NgR2 construct, we observed higher maximal binding of AP-MAG to NgR2- than to NgR1-expressing cells. However, as analyzed by Scatchard analysis, the dissociation constants for these interactions were essentially identical using these constructs. MAG had no detectable affinity for NgR3. Binding experiments using MAG-AP resulted in similar results (data not shown).

OMgp Binds MAG with Very High Affinity and NgR1 with Moderate Affinity and Does Not Interact with NgR2 or NgR3—OMgp was identified previously as a myelin inhibitory molecule interacting with NgR1 (9). To investigate whether OMgp can also interact with NgR2 or NgR3, we prepared AP-OMgp recombinant protein and assessed its affinity for NgR family members. As reported previously (9), we found that OMgp interacted with NgR1. Unexpectedly, under other conditions tested, we found that OMgp interacted with MAG and that this interaction had significantly higher affinity than the binding affinity of OMgp/NgR1 interaction. The $K_d$ values for OMgp/MAG and OMgp/NgR1 interactions are 3–6 and 10–20 nM, respectively (Fig. 5, J and K) (data not shown). We did not detect OMgp binding to NgR2 or NgR3 (data not shown).

A Library of NgR1 Mutants Is Expressed in a Similar Fashion Compared with Wild-type NgR1—NgR1 has the capacity to bind Nogo-66, MAG, OMgp, Lingo-1, Nogo-A-24 and Nogo-C39 (6–9, 31). To better define how multiple ligands with so wide a structural diversity bind to NgR1, we examined a series of Ala-substituted NgR1 mutants for ligand binding activity. An Ala substitution was generated for each of the charged residues predicted to be solvent-accessible at the surface of the ligand-binding LRR domain of NgR1 (10, 11). We generated mutants in which 1–8 surface residues localized within 5 Å of one another were Ala-substituted. Because of the coiling nature of the LRR structure, residues juxtaposed on the protein surface are separated by ~25 residues in the primary structure. In addition to mutations in specific charged surface patches, other mutations were targeted to glycosylation sites (Asn82 and Asn179) and to regions predicted to be involved in ligand binding based on the NgR1 structure (10, 11). A variant corresponding to a human polymorphism present in the GenBank Data Bank was also examined (D259N). None of the mutations altered the Leu residues critical for the tertiary LRR structure or the Cys residues involved in disulfide bond formation in the N- and C-terminal capping domains. The vast majority of such surface Ala-substituted mutants were expressed as immunoreactive polypeptides with molecular masses and expression levels indistinguishable from those wild-type NgR1 (Fig. 6A) (data not shown). Those that where not expressed were excluded from further analysis. Moreover, all of the NgR1 mutants that
were analyzed for ligand binding exhibited a cellular distribution in transfected COS-7 identical to that of the wild-type protein (Fig. 6B) (data not shown). Notably, those mutations that removed both glycosylation sites in the LRR domain (amino acids 82 and 179) did not alter expression levels or surface localization, although the molecular masses were reduced as determined by immunoblot analysis (data not shown).

Myelin Ligand Binding to NgR1 Requires Overlapping but Separate Residues—We used the library of 74 NgR1 mutants to test for their AP-Nogo-66, AP-Nogo-Y4C, AP-Nogo-C39, AP-MAG, AP-OMgp, and AP-Lingo-1 binding abilities. The properties of the NgR1 mutants fall into one of three major categories (Fig. 7B and Table 1). A number of Ala-substituted NgR1 variants bound all of the ligands at wild-type levels. We concluded that the corresponding residues do not play an essential role in ligand interactions. Many of these residues are situated on the convex side of the NgR1 structure, indicating that this surface is not a primary site for these interactions. In addition, a significant extent of the concave surface is dispensable for ligand binding. Glycosylation at residue 179 is not essential for ligand binding. The D259N polymorphic variant exhibited normal ligand binding properties (Table 1).

A second group of mutants exhibited weak or no binding to each of the ligands (Fig. 7B and Table 1). One interpretation is that these residues are required for NgR1 folding, so their substitution with Ala results in misfolded protein with no ligand binding. However, there are several reasons to favor the alternative hypothesis that many of these residues contribute to the binding of multiple NgR1 ligands in a common binding pocket. Critically, the NgR1 expression levels and subcellular distribution were not altered for these mutants (data not shown). In contrast, misfolded proteins might be expected to be unstable and mislocalized. Notably, the majority of those residues that could not be mutated to Ala without losing affinity for all ligands are clustered near one another. Thus, we concluded that the NgR1 surface created by residues 67/68, 111/133, 133/136, 158/160, 163, 182/186, and 232/234 constitutes a primary binding site for these ligands. Mouse and human NgR1 are identical at all 13 of these positions, supporting a conserved functional role for these residues. Human and mouse NgR1 differ from human and mouse NgR2 at four of these positions (Arg/Leu at position 68, Ser/Lys at position 113, His/Gln at position 133, and Asp/Glu at position 163), whereas human and mouse NgR1 differ from human and mouse NgR3 at three of these positions (Ser/Gly at position 113, His/Tyr at position 136, and Tyr/Phe at position 232). The non-conservative changes at these sites may account for the inability of NgR2 and NgR3 to bind several ligands specific for NgR1. Removal of both NgR1 N-linked glycosylation sites (residues 82 and 179) abrogated binding to all ligands. Because the 82/179 mutant was expressed at the cell surface, the lack of binding indicates that glycosylation contributes to either protein folding or ligand binding directly.

The third group of Ala-substituted NgR1 mutants exhibited selective loss of binding for some ligands, but not others (Tables 1 and 2). The preservation of binding affinity for at least one ligand by each member of this class demonstrates that the Ala replacements do not prevent NgR1 folding and surface expression. Most of the NgR1 residues responsible for differential ligand binding are situated at the perimeter of the primary binding site described above. Many of these substitutions reduced or eliminated MAG, OMgp, and Lingo-1 binding without diminishing binding by Nogo-66, Nogo-Y4C, or Nogo-C39. The simplest interpretation of this topographic relationship is that MAG, OMgp, and Lingo-1 require not only a central ligand-binding domain that is partially shared with multiple
NgR1-interacting Nogo-A fragments, but also an adjacent group of residues for high affinity binding. This adjacent region includes amino acids 78/81, 87/89, 89/90, 95/97, 108, 119/120, 139, 210, and 256/259. Mouse and human NgR1 are identical at 11 and similar at 13 of these 14 residues. Human NgR2 exhibits less conservation at these 14 positions, with 8 identical and 6 non-identical amino acids compared with human NgR1 (Arg/Gly at position 78, Arg/Ser at position 81, His/Phe at position 89, Arg/Thr at position 95, Asp/Tyr at position 97, and Asp/Ala at position 259). One of these changes is conservative (similar/ non-identical amino acids; His/Phe at position 89), and five are non-conservative (dissimilar amino acids). For human NgR3, there are 7 identical and 7 non-identical amino acids compared with human NgR1 (Arg/Ser at position 78, Arg/Pro at position 81, His/Tyr at position 89, Arg/Tyr at position 95, Asp/His at position 97, Ser/Thr at position 120, and Asp/Gly at position 259). Two of these changes are conservative (His/Tyr at position 89 and Ser/Thr at position 120); two are moderately conservative (Asp/His at position 97 and Asp/Gly at position 259); and three are non-conservative. The lack of amino acid conservation at these sites may account for the inability of NgR2 to bind OMgp and of NgR3 to bind MAG and OMgp.

Of special interest is the glycosylation site at residue 82. Mutating this glycosylation site to Ala reduced MAG, OMgp, and Lingo-1 binding. Interestingly, NgR2, which binds MAG, has a potential glycosylation site at Asn82, whereas in NgR3, this has been naturally replaced with Ala, and concomitantly, affinity for MAG has been lost. This suggests that sugar moieties attached to Asn82 contribute to NgR1 interaction with these particular ligands. Consistent with this model, MAG interaction with neurons expressing NgR1 and NgR2 has been shown to be at least partially sialic acid-dependent, and both receptors have been shown to likely be highly sialylated glycoproteins (27).

All three Nogo-A fragments were found to interact with residues located on the central portion of the concave side of the LRR domain. We did not identify mutants that differentially displayed reduced affinity for a certain Nogo-A segment. However, it is possible that higher resolution mapping of NgR1 residues involved in ligand binding could reveal differences in their binding sizes.

**DISCUSSION**

This study has extended our understanding of how myelin inhibitors interact with NgR family members: NgR1 binds three linear segments of Nogo-A as well as MAG and OMgp; mutagenesis defined overlapping NgR1-binding sites for different ligands; RTN2 and RTN3 also bind NgR1 with high affinity; NgR2 binds MAG, but not RTNs; and finally, NgR3 binds none of the known NgR family ligands.

As all RTNs are also present in the central nervous system and as they all contain homologous 66-amino acid loop regions, we hypothesized previously that they could interact with
NgR family members (25). Here, we found that RTN2 and RTN3 interact with NgR1. Because of the high sequence similarity between RTN-66 regions, they very likely interact with the same site in NgR1. We localized this binding site to the center of the concave side of the LRR domain by systematic mutagenesis. The several amino acid changes between NgR1 and NgR2 and NgR3 in this core binding region are likely to explain the specificity of this interaction. Previously, we showed that the amino-terminal half (underlined in Fig. 1W) of Nogo-66 are critical for receptor binding (15). Analysis of different RTN-66 regions suggested that the amino acid changes in RTN1 (shown in red in Fig. 1W) could account for the loss of its affinity for NgR1. Amino acids 36–41 in RTN-66 regions show considerable sequence diversity. This C-terminal part of Nogo-66 is instrumental for activating NgR1 downstream signaling (15). As our previous results showed that glutathione S-transferase-fused RTN1-66 and RTN3-66 recombinant proteins do not cause growth cone collapse (4), other RTNs could thus function as NgR1 antagonists, blocking Nogo-66-induced NgR1 activation. However, as ~95% of these glutathione S-transferase proteins are misfolded and in inclusion bodies, it is plausible that the remaining protein fraction might be inactive as well. Although no Rtn1 or Rtn3 mRNA expression has been detected in the optic nerve (4), expression of other RTNs in spinal cord white matter had not been previously analyzed. We found that the Rtn3 mRNA transcript containing the 66-amino acid loop region is expressed in spinal cord white matter at levels similar to those of Nogo-A. Previous studies showed that different splice forms of endogenous RTN4 interact with each other (34) and that Nogo-B interacts with RTN3 (35). As the Nogo-B/RTN3 interaction is mediated by the RHD (35), Nogo-A and RTN3 may also form complexes in glial cells. The stoichiometry of RTN3/RTN4 complexes might determine their function.

Most of Nogo-A is localized to the endoplasmic reticulum, where it serves essential functions in a wide variety of cells (29). Accumulating evidence shows that at least a fraction of oligodendrocyte plasma membrane Nogo-A is in a conformation in which the N terminus of the protein faces the extracellular milieu (3, 34). Recently, we demonstrated that a second Nogo-A-specific

FIGURE 6. Examples of NgR1 mutants that show differential binding to myelin ligands. A, to analyze the expression of mutant NgR1 constructs, COS-7 cells were transfected with plasmids encoding the indicated constructs; lysates were subjected to SDS-PAGE; and Western blotting was performed with anti-NgR1 antibody. B, the indicated mutant NgR1 proteins were detected at the surface of transfected COS-7 cells by immunocytochemistry. Live COS-7 cells were incubated at 4 °C with anti-NgR1 antibody prior to fixation and incubation with labeled secondary antibodies, vect, vector. C, shown is the binding of AP or AP-NgR1 ligands to COS-7 cells expressing different NgR1 mutants as indicated. Ng33, AP fusion protein of the N-terminal 33 residues of Nogo-66 (Ng66). The concentrations of ligands applied were as follows: AP, 30 nM; AP-Nogo-66, 5 nM; AP-Nogo-33, 10 nM; AP-Y4C, 10 nM; AP-Y4C66, 0.5 nM; AP-Lingo-1, 10 nM; AP-OMgp, 10 nM; and AP-MAG, 30 nM. These concentrations are close to the binding Kd of these proteins for NgR1, so any decrease in Kd is reflected in staining intensity. D, AP ligand binding to NgR1 mutants was quantitated and is expressed as a percentage of binding to wild-type (WT) NgR1.
domain (Nogo-A-24, human Nogo-A amino acids 995–1018) immediately N-terminal to the first hydrophobic segment has separate high affinity for NgR1 (31). Here, we have reported that the C terminus following the second C-terminal hydrophobic region in the RHD also interacts with NgR1 with high affinity. It is possible that, like several other proteins, Nogo-A could adapt multiple conformations to encompass different functions or to target the protein into different cellular compartments (for review, see Ref. 36). The C-terminal end of Nogo-A possesses an endoplasmic reticulum-targeting sequence, -KXXKK. This pentapeptide has to be cytosolic to be recognized. Thus, it is possible that the population of Nogo-A protein molecules that present their C termini extracellularly or on the luminal side of the endoplasmic reticulum might be enriched on the plasma membrane.

As the three Nogo-A segments that bind to NgR1 are closely connected to each other in a single polypeptide chain, it is plausible that they cannot interact with several distant sites in a single NgR1 molecule. Interestingly, we noted that the binding sites of Nogo-66, Nogo-Y4C, and Nogo-C39 on NgR1 overlap. It is feasible that these fragments might not interact simultaneously with one NgR1 monomer, but that tripartite NgR1 ligand would engage NgR1 clustering. The extracellular domain of NgR1 has significant affinity for surface-bound NgR1, and given the presumably high local concentration of glycosylphosphatidylinositol-anchored NgR1 in lipid rafts, receptor clustering could be facilitated by this basal level homophilic adhesion (33). Consistent with the receptor clustering model, clustering of MAG and Nogo-66 has been shown to increase their potency to activate NgR1 signaling and downstream RhoA activation (37). Previously, we noticed that fusion of Nogo-A-24 (which is able to bind but not activate NgR1) to the NEP-(1–32) antagonist peptide creates a potent agonist peptide (31). This result also raised the possibility that bivalent or multivalent interactions of ligands with NgR1 are critical for its activation.

The observed very high affinity interaction between OMPgp and MAG suggests a model in which a ternary complex consisting of OMPgp, MAG, and NgR1 could regulate...
specific aspects of oligodendrocyte/neuron interactions. At least in some cases, OMgp could also serve as a high affinity neuronal ligand for MAG. This is supported by a report that OMgp is expressed at low levels in oligodendrocytes, whereas neuronal expression of OMgp, as detected by immunohistochemistry and in situ hybridization, is prominent (38). Interestingly, the reported neuronal expression pattern of OMgp overlaps (e.g. in layer V of the cerebral cortex and pyramidal cells of the hippocampus) with that of NgR1. Thus, OMgp could contribute to MAG binding in these cells, and farther downstream signaling might depend on the formation of a ternary complex of MAG, OMgp, and NgR1. Neuronal signaling triggered by OMgp/MAG interaction may also be independent of NgR1.

**TABLE 1**

Summary of the ligand binding properties of the NgR1 mutants

Ala-substituted NgR1 mutants were tested for their binding to AP-Nogo-66, AP-Y4C, AP-Y4C66, AP-Nogo-C39, AP-Lingo-1, AP-OMgp, and AP-MAG, and they fall into three categories: 1) mutants that lose binding to all NgR1 ligands, 2) mutants that still maintain binding to all NgR1 ligands, and 3) differential binding mutants that bind some ligands but lose binding to other ligands. The D259N mutant is a asparagine substitution to mimic a human polymorphism.

| Residues | No binding | Binding to all ligands | Differential binding |
|----------|------------|------------------------|---------------------|
| 163      | 61         | 82                     |                     |
| 82, 179  | 92         | 108                    |                     |
| 133, 136 | 122        | 139                    |                     |
| 158, 160 | 127        | 210                    |                     |
| 182, 186 | 131        | 78, 81                 |                     |
| 232, 234 | 138        | 87, 89                 |                     |
| 67, 68, 71 | 151     | 89, 90                 |                     |
| 111, 113, 114 | 176      | 95, 97                 |                     |
| 114, 117, 163 | 179    | 108, 131               |                     |
| 182, 186, 210 | 227   | 256, 259               |                     |
| 210, 232, 234 | 250   | 36, 38, 61             |                     |
| 67, 68, 95, 97 | D259N | 61, 108, 131           |                     |
| 87, 89, 133, 136 | 36, 38 | 95, 97, 122            |                     |
| 182, 186, 158, 160 | 63, 65 | 114, 117, 139          |                     |
| 111, 113, 114, 138 | 114, 117 | 117, 119, 120          |                     |
| 117, 119, 120, 139 | 127, 151 | 216, 218, 220          |                     |
| 95, 97, 188, 189, 191, 192 | 127, 176 | 220, 223, 224          |                     |
| 202, 205, 227, 250, 277, 279 | 143, 144 | 237, 256, 259          |                     |
| 95, 97, 117, 119, 120, 188, 189 | 189, 191, 199 | 256, 259, 284          |                     |
| 196, 199, 200, 223, 224 | 211, 213, 237, 256, 259, 284 | 189, 191, 211, 213, 237, 256, 259, 284 |
NgR Family/Ligand Interactions

Because the NgR1 structure is now defined (10, 11), we probed its surface for ligand-binding sites using Ala substitutions. There appears to be a binding domain located in the central region on the concave side of the NgR1 LRR domain required by Nogo-A-24, Nogo-66, Nogo-C39, MAG, and OMgp ligands. In addition, different ligands require particular residues surrounding this central site. Because all ligands require surface residues centered on the midportion of the concave face of NgR1, their mechanism for activating NgR1 signaling may be similar. Similar to the case with internalin-E-cadherin (39) and glycoprotein Ib von Willebrand factor (40) complexes, the concave side of the receptor serves as a ligand-docking site. Previous work had been divided as to whether binding sites for Nogo-66 and MAG are separate or overlapping. Using the NEP-(1–40) antagonist of Nogo-66, we did not observe inhibition of MAG interactions with NgR1 (8). With a sterically encumbered AP-Nogo-66 ligand, some competition with MAG-Fc binding to NgR1 was detected. Our findings are consistent with partial competition between ligands.

Because NgR1 is considered a target for the development of axonal regeneration therapeutics (41), the definition of this central binding domain shared by multiple ligands may facilitate the design and development of small molecule therapeutics blocking all NgR1 ligands. In contrast, if each ligand had been dated, so future studies can characterize them in greater functional detail. The identification of a central ligand-binding domain holds the promise that general NgR1 antagonists may be created to possibly promote axonal regeneration after central nervous system injury.

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REFERENCES

1. Yiu, G., and He, Z. (2006) *Nat. Rev. Neurosci.* 7, 617–627
2. Liu, B. P., Cafferty, W. B., Budel, S. O., and Strittmatter, S. M. (2006) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 1593–1610
3. Oertle, T., van der Haar, M. E., Bandtlow, C. E., Robeva, A., Burfeind, P., Buss, A., Huber, A. B., Simonen, M., Schnell, L., Brossame, C., Kaufmann, K., Vallon, R., and Schwab, M. E. (2003) *J. Neurosci.* 23, 5393–5406
4. GrandPre, T., Nakamura, F., Vartanian, T., and Strittmatter, S. M. (2000) *Nature* 403, 439–444
5. Oertle, T., Klinger, M., Stuermer, C. A., and Schwab, M. E. (2003) *FASEB J.* 17, 1238–1247
6. Fournier, A. E., GrandPre, T., and Strittmatter, S. M. (2001) *Nature* 409, 341–346
7. Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K., Nikulina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., He, Z., and Filbin, M. (2002) *Neuron* 35, 283–290
8. Liu, B. P., Fournier, A., GrandPre, T., and Strittmatter, S. M. (2002) *Science* 297, 1190–1193
9. Wang, K. C., Koprivica, V., Kim, J. A., Sivasankaran, R., Guo, Y., Neve, R. L., and He, Z. (2002) *Nature* 417, 941–944
10. He, X. L., Bazan, J. F., McDermott, G., Park, J. B., Wang, K., Tessier-Lavigne, M., He, Z., and Garcia, K. C. (2003) *Neuron* 38, 177–185
11. Barton, W. A., Liu, B. P., Tzvetkova, D., Jeffrey, P. D., Fournier, A. E., Sah, D., Cate, R., Strittmatter, S. M., and Nikolov, D. B. (2003) *EMBO J.* 22, 3291–3302
12. Schnell, L., and Schwab, M. E. (1990) *Nature* 343, 269–272
13. Bregman, B. S., Kunkel-Bagden, E., Schnell, L., Dai, H. N., Gao, D., and Schwab, M. E. (1995) *Nature* 378, 498–501
14. Wiessner, C., Bareyre, F. M., Allegreini, P. R., Mir, A. K., Frenzler, S., Zurini, M., Schnell, L., Oertle, T., and Schwab, M. E. (2003) *J. Cereb. Blood Flow Metab.* 23, 154–165
15. GrandPre, T., Li, S., and Strittmatter, S. M. (2002) *Nature* 417, 547–551
16. Lee, J. K., Kim, J. E., Sivula, M., and Strittmatter, S. M. (2004) *J. Neurosci.* 24, 6209–6217
17. Li, S., and Strittmatter, S. M. (2003) *J. Neurosci.* 23, 4219–4227
18. Wang, X., Baughman, K. W., Basso, D. M., and Strittmatter, S. M. (2006) *Ann. Neurol.* 60, 540–549
19. Li, S., Liu, B. P., Budel, S., Li, M., Li, B., Walus, L., Li, W., Jirik, A., Rabacchi, S., Choi, E., Worley, D., Sah, D. W., Pepinsky, B., Lee, D., Relton, J., and Strittmatter, S. M. (2004) *J. Neurosci.* 24, 10511–10520
20. Simonen, M., Pedersen, V., Weimann, O., Schnell, L., Buss, A., Ledermann, B., Christ, F., Sansig, G., van der Putten, H., and Schwab, M. E. (2003) *Neuron* 38, 201–211
21. Zheng, B., Ho, C., Li, S., Keirstead, H., Stewart, O., and Tessier-Lavigne, M. (2003) *Neuron* 38, 213–224
22. Kim, J. E., Li, S., GrandPre, T., Qiu, D., and Strittmatter, S. M. (2003) *Neuron* 38, 187–199
23. Zheng, B., Atwal, J., Ho, C., Case, L., He, X. L., Garcia, K. C., Steward, O., and Tessier-Lavigne, M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 1205–1210
24. Kim, J. E., Liu, B. P., Park, J. H., and Strittmatter, S. M. (2004) *Neuron* 44, 439–451
25. Laurén, J., Airaksinen, M. S., Saarma, M., and Timmusk, T. (2003) *Mol. Cell. Neurosci.* 24, 581–594
26. Pignot, V., Hein, A. E., Barske, C., Wiessner, C., Walmsley, A. R., Kaufmann, K., Mayeur, H., Sommer, B., Mir, A. K., and Frenzel, S. (2003) *J. Neurochem.* 85, 717–728
27. Venkatesh, K., Chivatakarn, O., Lee, H., Joshi, P. S., Kantor, D. B., Newman, B. A., Mage, R., Rader, C., and Giger, R. J. (2005) *J. Neurosci.* 25, 808–822
28. Yan, R., Shi, Q., Hu, X., and Zhou, X. (2006) *Cell. Mol. Life Sci.* 63, 877–889
29. Voelz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M., and Rapoport, T. A. (2006) *Cell* 124, 573–586
30. Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., Levesque, M., Allaire, N., Perrin, S., Sands, B., Crowell, T., Cate, R. L., McCoy, J. M., and Pepinsky, R. B. (2004) *Nat. Neurol.* 11, 221–228
31. Hu, F., Liu, B. P., Budel, S., Liao, J., Chin, J., Fournier, A., and Strittmatter, S. M. (2005) *J. Neurosci.* 25, 5298–5304
32. Flanagan, J. G., Cheng, H. J., Feldheim, D. A., Hattoni, M., Lu, Q., and Vanderhaegen, P. (2000) *Methods Enzymol.* 327, 19–35
33. Fournier, A. E., Gould, G. C., Liu, B. P., and Strittmatter, S. M. (2002) *J. Neurosci.* 22, 8876–8883
34. Dodd, D. A., Niederoest, B., Bloechlinger, S., Dupuis, L., Loeffler, J. P., and Schwab, M. E. (2005) *J. Biol. Chem.* 280, 12494–12502
35. Qi, B., Qi, Y., Watari, A., Yohioka, N., Inoue, H., Minemoto, Y., Yamashita, K., Sasagawa, T., and Yutud, M. (2003) *J. Cell. Physiol.* 196, 257–264
312–318
36. Levy, D. (1996) *Essays Biochem.* **31**, 49–60
37. Niederost, B., Oertle, T., Fritsche, J., McKinney, R. A., and Bandtlow, C. E. (2002) *J. Neurosci.* **22**, 10368–10376
38. Habib, A. A., Marton, L. S., Allwardt, B., Gulcher, J. R., Mikol, D. D., Hognason, T., Chattopadhyay, N., and Stefansson, K. (1998) *J. Neurochem.* **70**, 1704–1711
39. Schubert, W. D., Urbanke, C., Ziehm, T., Beier, V., Machner, M. P., Domann, E., Wehland, J., Chakraborty, T., and Heinz, D. W. (2002) *Cell* **111**, 825–836
40. Huizinga, E. G., Tsuji, S., Romijn, R. A., Schiphorst, M. E., de Groot, P. G., Sixma, J. J., and Gros, P. (2002) *Science* **297**, 1176–1179
41. Lee, D. H., Strittmatter, S. M., and Sah, D. W. (2003) *Nat. Rev. Drug Discov.* **2**, 872–878