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Truncated Soluble Nogo Receptor Binds Nogo-66 and Blocks Inhibition of Axon Growth by Myelin

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CNS myelin contains axon outgrowth inhibitors, such as Nogo, that restrict regenerative growth after injury. An understanding of the mechanism of Nogo signaling through its receptor (NgR) is critical to developing strategies for overcoming Nogo-mediated inhibition. Here we analyze the function of NgR domains in outgrowth inhibition. Analysis of alkaline phosphatase (AP)-Nogo binding in COS-7 cells reveals that the leucine-rich repeat domain is necessary and sufficient for Nogo binding and NgR multimerization. Viral infection of embryonic day 7 chick retinal ganglion cells with mutated NgR demonstrates that the NgR C-terminal domain is required for inhibitory signaling but not ligand binding. The NgR glycosylphosphatidylinositol domain is not essential for inhibitory signaling but may facilitate Nogo responses. From this analysis, we have developed a soluble, truncated version of the Nogo receptor that antagonizes outgrowth inhibition on both myelin and Nogo substrates. These data suggest that NgR mediates a significant fraction of myelin inhibition of axon outgrowth.

Key words: Nogo; myelin; axon inhibition; Nogo receptor; CNS; leucine-rich repeat

The inability of injured CNS neurons to spontaneously regenerate is caused in part by the presence of myelin-associated inhibitory molecules at the CNS injury site. Multiple inhibitors have been identified in myelin (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Niederost et al., 1999), and one of the most potent inhibitors may be Nogo (Caroni and Schwab, 1988a; Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). This contention is supported by experiments showing that treatment with the inhibitor neutralization (IN-1) antibody, which recognizes Nogo-A, enhanced neurite outgrowth and functional recovery after CNS spinal cord injuries in rats (Caroni and Schwab, 1988b; Schnell and Schwab, 1990). However, IN-1 recognizes several other proteins in CNS myelin extracts; therefore, experiments with specific Nogo reagents are necessary to determine the relative contribution of Nogo to myelin-based axon inhibition.

Nogo is a member of the reticulon protein family, and its inhibitory activity has been demonstrated in multiple assays in both non-neuronal and neuronal cells (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Fournier et al., 2001). The mechanism of action of Nogo inhibition may be complex, because two different inhibitory domains have been identified in the Nogo protein. Both the N-terminal portion of Nogo-A (Amino-Nogo; residues 1–1024) (Chen et al., 2000; Prinjha et al., 2000) and a 66 amino acid hydrophilic protein segment in the C-terminal region of Nogo (Nogo-66) (GrandPre et al., 2000; Fournier et al., 2001) have inhibitory activity. Although both domains may have important biological activities, Nogo-66 is expressed on the surface of oligodendrocytes (GrandPre et al., 2000) and has specific inhibitory effects on neurons in a soluble form. Because the epitope recognized by IN-1 is not defined, the relative contribution of Amino-Nogo and Nogo-66 to myelin action on axons is poorly defined by published studies.

A receptor for Nogo-66 (NgR) has been identified (Fournier et al., 2001). NgR is a 473 amino acid protein containing a signal sequence, a leucine-rich repeat (LRR)-type N-terminal domain, eight LRR domains, a cysteine-rich LRR-type C-terminal flanking domain, a unique C-terminal region, and a glycosylphosphatidylinositol (GPI) anchorage site. The LRR domains of the NgR share moderate amino acid sequence similarity to many other LRR-containing proteins. Because other LRR proteins serve a wide variety of functions (Buchanan and Gay, 1996), they offer little insight into the mechanism of NgR signaling.

The presence of a GPI anchor in the NgR raises several issues with regard to NgR signaling mechanisms. First, the GPI-linked nature of NgR suggests an interaction with a transmembrane receptor subunit capable of intracellular signal transduction. Second, the GPI domain might play a critical role in lipid raft localization and signal transduction as shown for glial cell line-derived neurotrophic factor (GDNF) family receptors (Tansey et al., 2000). Third, the GPI anchor may provide an NgR cleavage site for the release of soluble NgR from the cell surface. Such cleavage might render the affected cell insensitive to Nogo and/or modulate Nogo signaling on adjacent cells.

In this study, we attempt to clarify the mechanism of NgR action by systematically deleting NgR domains and testing these deletion mutants in both ligand binding and Nogo signaling assays. By studying alkaline phosphatase (AP)-Nogo binding in COS-7 cells, we have determined that all of the NgR LRR domains are required for Nogo binding. We have also identified a domain in the C-terminal portion of NgR that is necessary but not sufficient for NgR signaling. The GPI linkage of NgR is not critical for Nogo signaling but may play a modulatory role in inhibitory signaling. This analysis has led to the identification of

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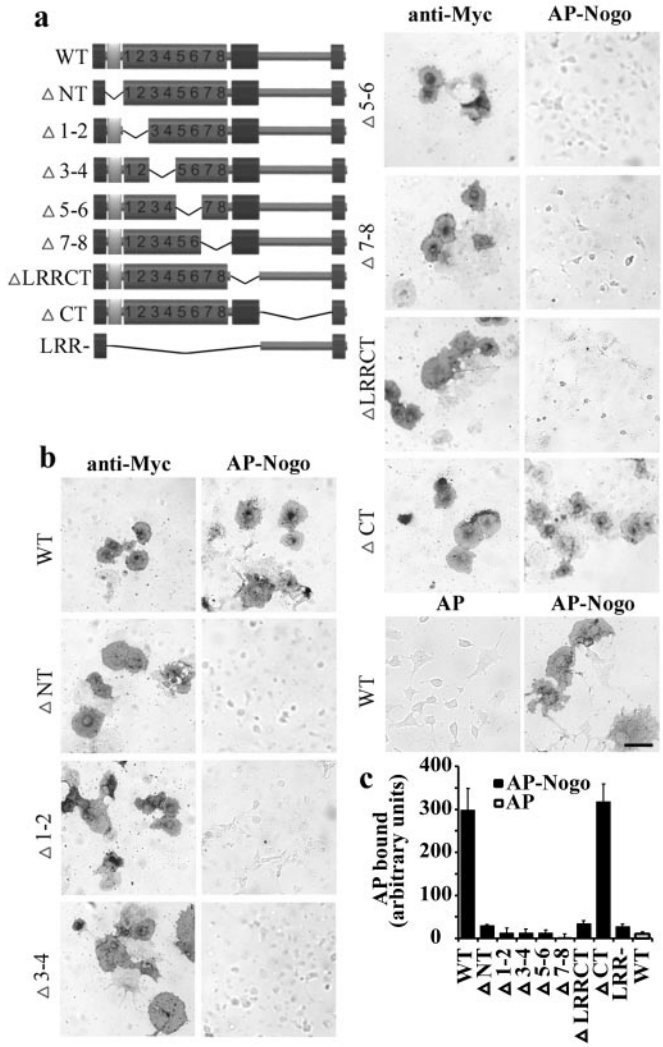

Figure 1. Nogo binding to NgR deletion mutants. *a*, Schematic of WT-NgR (*WT*) and the NgR deletion mutants used in this study. NgR mutants include deletions of the N-terminus (Δ*NT*), LRR domains 1 and 2 (Δ*1–2*), 3 and 4 (Δ*3–4*), 5 and 6 (Δ*5–6*), 7 and 8 (Δ*7–8*), the LRR C-terminus (Δ*LRRCT*), the C-terminus (Δ*CT*), and the complete LRR domain (*LRR−*). *b*, COS-7 cells transfected with NgR deletion mutant plasmids were stained for Myc immunoreactivity or tested for 20 nM AP or AP-Nogo binding. All NgR mutant proteins were expressed in COS-7 cells as shown by Myc immunoreactivity. Only WTNgR- and NgRΔCT-transfected COS-7 cells bound to AP-Nogo. Scale bar, 100 μm. *c*, Quantification of AP-Nogo or AP binding to COS-7 cells transfected with NgR deletion mutants.

a soluble, truncated NgR (NgREcto) that antagonizes the neurite outgrowth-inhibitory effects of Nogo. NgREcto also antagonizes myelin-dependent inhibition, suggesting that signaling through the NgR mediates a significant proportion of myelin inhibition.

MATERIALS AND METHODS

Nogo receptor deletion mutants and chimeras. Myc-tagged mouse wild-type NgR (WTNgR) in pSecTag2Hygro (Invitrogen, Burlingame, CA) (Fournier et al., 2001) was used as a template for NgR deletion and chimeric mutants. To generate NgR with the CT region deleted (NgRΔCT), the LRR region (residues 1–310) and GPI region (residues 445–473) were amplified separately, ligated together at a *Not*I site, and then ligated into the *Bam*H1/*Xho*I sites of pSecTag2. LRR deletions were generated using the ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA). Deletions were as follows: NgRΔNT, residues 1–57; NgRΔ1–2, residues 58–105; NgRΔ3–4, residues 106–154; NgRΔ5–6, residues 155–202; NgRΔ7–8, residues 203–250; and NgRΔLRRCT, residues 260–310. NgRCT (residues 310–445) was amplified and cloned into the *Bam*H1/*Eco*R1 site of pGEXTAG2. Cloning of HSVPlexA1 (herpes simplex virus–plexin A1) has been described previously (Takahashi et al., 1999). To construct HSVWTNgR, HSVNgRCT, and HSVNgRΔCT, the corresponding NgR was amplified with the signal sequence from pSecTag2 and ligated into the *Hin*dIII/*Sal*I sites of pHSVPrPUC. The HSV transfer vector for the NgRL1 chimeric protein was constructed by substituting the cDNA encoding amino acids 1–451 of mouse NgR for the neuropilin-1 coding region in the HSV-NP1-L1 vector (Nakamura et al., 1998).

Purified NgREcto protein was a generous gift from Biogen Inc. (Cambridge, MA). To construct NgREcto, cDNA encoding amino acids 1–310 of rat NgR was cloned by PCR into the expression vector PV90, and the resulting plasmid was transfected into CHO cells. Amino acid substitutions were present, resulting in the following sequence: MKRASAGGSR LLAWVLWLQA WRVATPCPGA CVCYNEPKVT TSCPQQGLQA VPTGIPASSQ RIFLHGNRIS YVPAASFQSC RNLTILWLHS NALAGIDAAA FTGLTLLEQL DLSDNAQLRV VDPTTFRGLG HLHTLHLDRC GLQELGPGLF RGLAALQYLY LQDNNLQALP DNTFRDLGNL THLFLHGNRI PSVPEHAFRG LHSLGRLLLH QNHVARVHPH AFRDLGRLMT LYLFANNLSM LPAEVLVPLR SLQYLRLNDN PWVCGCRARP LWAWLQKFRG SSSEVPCNLP QRLAGRDLKR LAASDLQGCA. A clone derived by limiting dilution that was expressing high levels of NgREcto was expanded in serum-free culture medium. Conditioned medium was collected, and NgREcto was purified by cation-exchange chromatography on an SP-Sepharose column. The resultant protein was ~85% pure. N-Terminal sequence analysis of the rat NgR1 (1–310) product verified that the mature protein started with Cys-27, which matched the predicted start site.

Preparation of recombinant proteins. To construct the AP-NgR vector, the NgR coding sequence from residues 27–451 was ligated in frame with the signal sequence-histidine 6 (His6)-AP sequence of pAP-6. To express AP-NgR, plasmid was transfected into HEK293T cells, and conditioned medium was collected after 4 d. Secreted protein was purified by Ni^{2+} affinity chromatography (Nakamura et al., 1998). AP-Nogo or AP-NgR binding in COS-7 cells was assessed as described previously (Takahashi et al., 1998; Fournier et al., 2001).

Growth cone collapse and neurite outgrowth assays. Preparation of embryonic day 7 (E7) chick retinal explant cultures and recombinant HSV preparations have been described in detail previously (Fournier et al., 2000a). Retinal explants were grown for 12 hr and then incubated for an additional 24 hr with HSVNgR preparations. Explants were treated for 30 min with 0, 50, 250, or 500 nM glutathione *S*-transferase (GST) Nogo-66 (GrandPre et al., 2000), fixed, and stained with phalloidin (Molecular Probes, Eugene, OR). Growth cone collapse was assayed as described previously (Luo et al., 1993). For neurite outgrowth assays on Nogo, myelin, or aggrecan substrates, Permanox chamber slides (Fisher Scientific, Pittsburgh, PA) were coated with 100 μg/ml poly-L-lysine and washed, and then 3 μl drops of PBS containing 0, 50, or 150 ng of GSTNogo-66, myelin, or aggrecan with or without 500 ng of NgREcto were spotted and dried. After three PBS washes, slides were coated with 10 μg/ml laminin. Dissociated E13 chick dorsal root ganglia (DRG) neurons were grown for 4–8 hr, fixed, and stained with phalloidin, and neurite outgrowth lengths were assessed using NIH Image. GSTNogo-66 and myelin were prepared as described previously (Fournier et al., 2000b; GrandPre et al., 2000). Aggrecan was obtained from Sigma (St. Louis, MO).

Analysis of membrane fractions on flotation gradients. HEK293T cells were cultured in 6 cm culture dishes and transfected with HSVWTNgR or HSVNgRL1 plasmids. After 48 hr, cells were rinsed with PBS and then lysed on ice with 375 μl precooled TNE buffer (in mM: 50 Tris-HCl, pH 7.4, 150 NaCl, and 5 EDTA) containing 0.1% Triton X-100, 10 mM NaF, and protease inhibitors (TNEX). Cells were homogenized by passing the ice-cold lysates through a 27 gauge needle 10 times. Extracts were adjusted to 35% OptiPrep (Invitrogen, Gaithersburg, MD) by adding 525 μl of 60% OptiPrep–0.1% Triton X-100, placed in an ultracentrifuge tube, and overlaid with 8.75 ml of 30% OptiPrep in TNEX and 1 ml of TNEX. After centrifugation (4 hr; 200,000 × *g*; 4°C), seven fractions were collected, precipitated in TCA, washed with acetone, air dried, and resuspended in Laemmli's sample buffer. Fractions were analyzed by 8% SDS-PAGE and immunoblotting with the NgR antibody (Fournier et al., 2001). For detection of transferrin receptor, a mouse monoclonal antibody (Zymed, San Francisco, CA) was used.

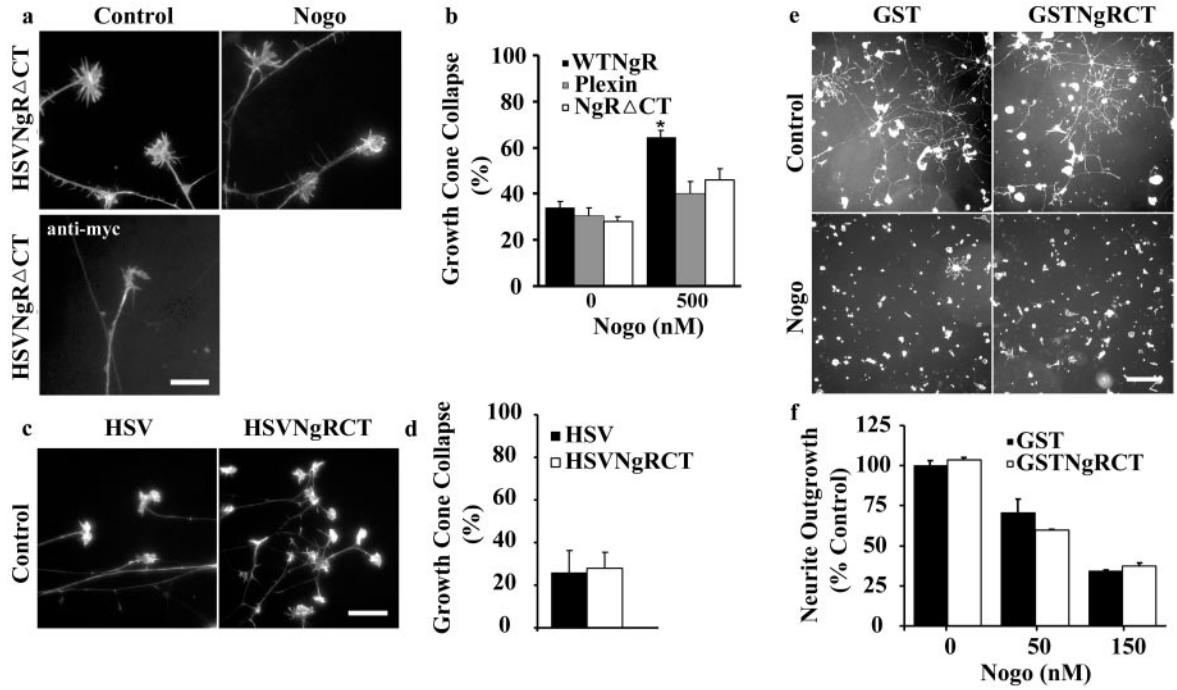

Figure 2. The CT region of NgR is necessary but not sufficient for Nogo inhibition. *a*, E7 retinal explants were infected with a recombinant viral preparation of the NgRΔCT. NgRΔCT-infected RGCs were insensitive to a 40 min treatment with 500 nM GSTNogo-66. Expression of NgRΔCT in RGC neurites and growth cones was verified by Myc immunostaining. Scale bar, 50 μm. *b*, Quantification of the growth cone collapse response of RGCs to GSTNogo-66 after viral infection with NgRΔCT, WTNgR, or a control plexinA1 virus (*Plexin*). Means ± SEM from 4–10 experiments are reported. Student's *t* tests comparing WTNgR or NgRΔCT to control plexinA1 values at the indicated Nogo concentration are reported. $^*p < 0.001$. *c*, E7 RGC explants were infected with recombinant viral preparations of control HSV particles (*HSV*) or HSVNgRCT. NgRCT alone does not cause growth cone collapse. Scale bar, 100 μm. *d*, Quantification of E7 RGC growth cone collapse after control HSV or HSVNgRCT infection. Means ± SEM for three experiments are reported. *e*, Neurite outgrowth of dissociated E13 DRGs plated on control or Nogo substrates and treated with 500 nM soluble GST or GSTNgRCT. GSTNgRCT does not inhibit neurite outgrowth on control spots or modify the response of E13 DRGs to Nogo inhibition. Scale bar, 200 μm. *f*, Nogo dose–response of E13 DRG neurite outgrowth in the presence of GST or GSTNgRCT. Neurite outgrowth is calculated as micrometers of growth per cell. Means ± SEM from three experiments are reported.

Caveolin was detected with a rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY).

RESULTS

Leucine-rich repeats are required for AP-Nogo binding

To better understand structure–function relationships for the NgR, deletion mutants were generated and tested for Nogo binding. NgR contains a signal sequence, an LRR-type N-terminal region (LRRNT) (Pfam accession number PF01462), eight LRR domains (LRR1–8) (Pfam accession number PF00560), an LRR-type C-terminal domain (LRRCT) (Pfam accession number PF01463), a unique C-terminal domain, and a GPI linkage domain (Fournier et al., 2001). Domains of the NgR were systematically deleted using PCR-based site-directed mutagenesis (Fig. 1*a*). Individual NgR mutants expressed in COS-7 cells exhibit the predicted mobility as verified by Myc immunoblots (data not shown). The ability of individual NgR mutants to bind to Nogo-66 was assessed using an AP-Nogo binding assay (Fournier et al., 2001). COS-7 cells were transfected with individual NgR deletion mutant constructs, treated with AP or AP-Nogo conditioned medium, and assayed for AP binding. AP-Nogo binding was detected in WTNgR or NgRΔCT (Fig. 1*b*). NgR deleted in any pair of the LRRs (NgRΔ1–2, NgRΔ3–4, NgRΔ5–6, NgRΔ7–8, and NgRLRR−) or in the N-terminal or LRR-type C-terminal flanking regions of the LRRs (NgRΔNT and NgRΔLRRCT, respectively) does not support AP-Nogo binding. The AP-Nogo binding pattern suggests that dispersed amino acid residues within the NgR LRR region are required for AP-Nogo binding. Alternatively, mutations in individual LRR regions may disrupt the tertiary structure of the NgR, resulting in a loss of AP-Nogo binding. For other LRR-containing receptors, similar deletions do not disrupt the function of remaining repeats (Song et al., 2001a,b), suggesting that multiple LRRs participate directly in Nogo-66 binding.

NgRCT domain is required but not sufficient for NgR-dependent inhibition

Although the NgRCT domain is not required for Nogo binding, we considered the possibility that it participates in Nogo-dependent inhibition of axon growth. E7 retinal ganglion cells (RGCs) were infected with recombinant HSVNgRΔCT preparations, and growth cone collapse in response to GSTNogo-66 was assessed (Fig. 2*a*,*b*). Under these conditions, wild-type NgR supports Nogo-66-dependent growth cone collapse. RGCs infected with NgRΔCT are not sensitive to Nogo in the growth cone collapse assay. The CT region of NgR is therefore required for effective NgR inhibitory signaling.

One possibility is that the CT domain of NgR binds to a transducing receptor component to initiate an intracellular signaling cascade after ligand binding. This would explain why NgR deleted in the CT region is signaling incompetent. If this were the case, it is also possible that the CT region of NgR is capable of constitutive receptor activity. To test this possibility, recombinant viral preparations expressing GPI-anchored NgRCT were used to

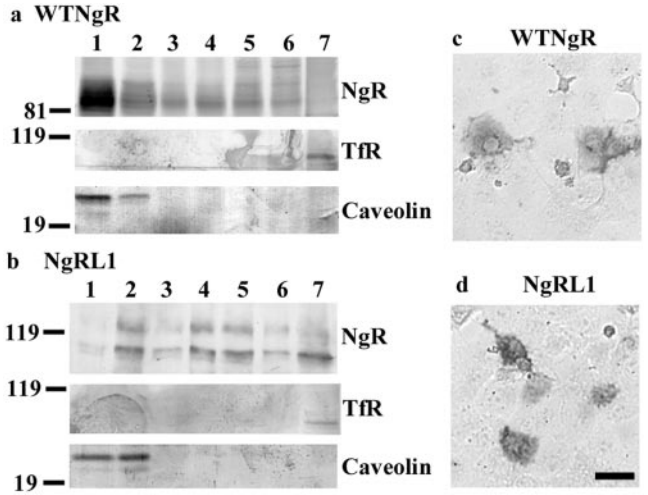

Figure 3. Characterization of NgRL1. *a*, Cell lysates from HEK293T cells transfected with HSVWTNgR or HSVNgRL1 plasmids were fractionated on flotation gradients. WTNgR is found almost exclusively in the caveolin-positive detergent-insoluble fraction. *b*, NgRL1 is in multiple membrane fractions, with a small proportion in the caveolin-positive detergent-insoluble fraction. *c*, COS-7 cells were transfected with WTNgR and tested for 10 nM AP-Nogo binding. *d*, COS-7 cells were transfected with NgRL1 and tested for 10 nM AP-Nogo binding. Cells expressing WTNgR or NgRL1 bind similar amounts of AP-Nogo. Scale bar, 100 μm.

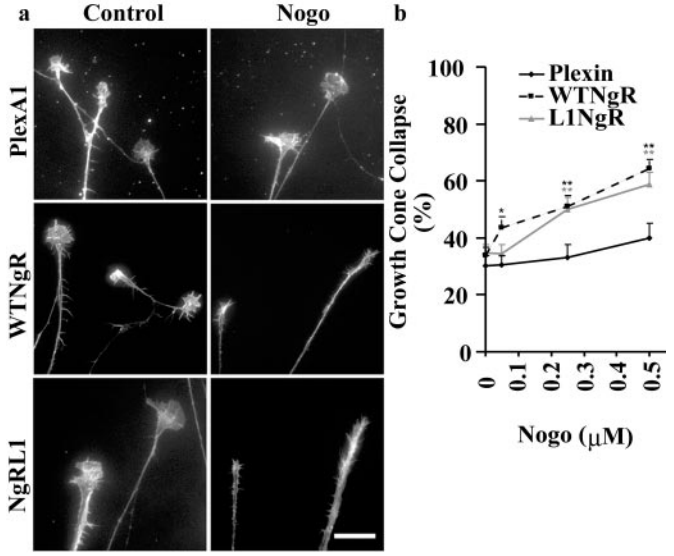

Figure 4. The GPI linkage region of NgR is not required for Nogo-mediated inhibition. *a*, E7 retinal explants were infected with recombinant viral preparations of PlexA1, WTNgR, or NgRL1. Explants were treated with 500 nM GSTNogo-66 for 40 min, fixed, and stained with rhodamine–phalloidin. RGCs infected with PlexA1 control virus are insensitive to Nogo, whereas those infected with WTNgR or NgRL1 collapse in response to Nogo. Scale bar, 50 μm. *b*, Dose–response of RGCs to GSTNogo-66 after infection with NgR viral preparations. Student's *t* tests comparing WTNgR or NgRL1 to PlexA1 at the indicated Nogo concentration are reported. $^{*}p = 0.01$; $^{**}p < 0.01$. Significance indicators (*) are coded with the appropriate infection. Means ± SEM for 6–10 experiments are reported.

infect E7 RGCs (Fig. 2*c*,*d*). Expression of NgRCT does not cause growth cone collapse in infected RGCs. In a second assay, NgRCT was purified as a soluble GST fusion protein (GST-NgRCT) and tested for its ability to disrupt signaling in a dominant negative manner. E13 chick DRGs were dissociated and plated in the presence or absence of 500 nM soluble GST or GSTNgRCT. In this assay, GSTNogo-66 inhibits neurite outgrowth (Fournier et al., 2001). Soluble GSTNgRCT does not alter neurite outgrowth lengths on control substrates, nor does it attenuate or enhance the response of dissociated E13 DRGs on Nogo substrates (Fig. 2*e*,*f*). Together, these experiments indicate that the CT region of NgR is necessary but not sufficient for NgR-dependent inhibition.

NgR GPI domain is not required for NgR signaling

In some cases, GPI anchors are critical for receptor function. The GPI anchor of GDNF receptor-α1 (GFRα1) plays a critical role in localizing receptor components to lipid rafts and permitting receptor tyrosine kinase (RET) activation (Tansey et al., 2000). To assess the role of the GPI anchor in mediating inhibitory Nogo signals, a chimeric NgR was generated by exchanging the NgR GPI domain with the transmembrane domain of the L1 cell adhesion molecule (Nieke and Schachner, 1985) in a pHSVPrPUC vector (HSVNgRL1). GPI-linked proteins localize to detergent-insoluble sphingolipid and cholesterol-rich lipid microdomains that exist as phase-separated "lipid rafts" in the plasma membrane (Simons and Ikonen, 1997; Brown and London, 1998). Sphingolipids and cholesterol in cell membranes are resistant to solubilization with nonionic detergents at 4°C, allowing lipid rafts to be isolated as detergent-resistant membrane fractions (Brown and Rose, 1992).

HEK293T cells were transfected with HSVWTNgR or HSVNgRL1, and membrane fractionation on flotation gradients was performed (Fig. 3). As expected for a GPI-anchored protein, WTNgR localizes primarily to the caveolin-positive lipid raft fractions. In contrast, the vast majority of chimeric NgRL1 localizes to the caveolin-negative fractions, and the small proportion cosedimenting with caveolin is likely to reflect incomplete separation rather than any raft localization. The ability of NgRL1 to bind to Nogo with an affinity similar to that of the WTNgR was verified by assaying AP-Nogo binding in transfected COS-7 cells (Fig. 3*c*,*d*). To test the signaling capability of NgRL1, recombinant HSVNgRL1 preparations were produced and used to infect E7 RGCs. Infected RGCs were treated with GSTNogo-66, and growth cone collapse was assessed (Fig. 4). At high concentrations of Nogo, NgRL1 transduces Nogo signals as efficiently as WTNgR. However, a 50 nM Nogo dose collapses RGC growth cones infected with WTNgR, whereas NgRL1-infected RGCs are unresponsive. Therefore, NgRL1 mediates Nogo signaling less efficiently than WTNgR. The GPI anchorage site may play a modulatory role in Nogo signaling.

NgR binds NgR

One hypothesis for the diminished signaling efficiency of NgRL1 is that NgRL1 fails to concentrate in lipid rafts, and the consequent loss of receptor clustering leads to inefficient Nogo signaling. To consider this possibility, we determined whether NgR was capable of interacting with itself. COS-7 cells were transfected with WTNgR or NgR deletion mutant plasmids and stained with AP-NgR conditioned medium (Fig. 5). Clearly, the extracellular domain of NgR has significant affinity for surface-bound NgR. Binding saturation was difficult to achieve reliably; however, the K_d of this interaction is estimated to be $\geq$50 nM. Considering that endogenous NgR molecules are likely to interact in *cis* within lipid rafts, this affinity is consistent with physiological relevance. Analysis of AP-NgR binding to NgR deletion mutants reveals

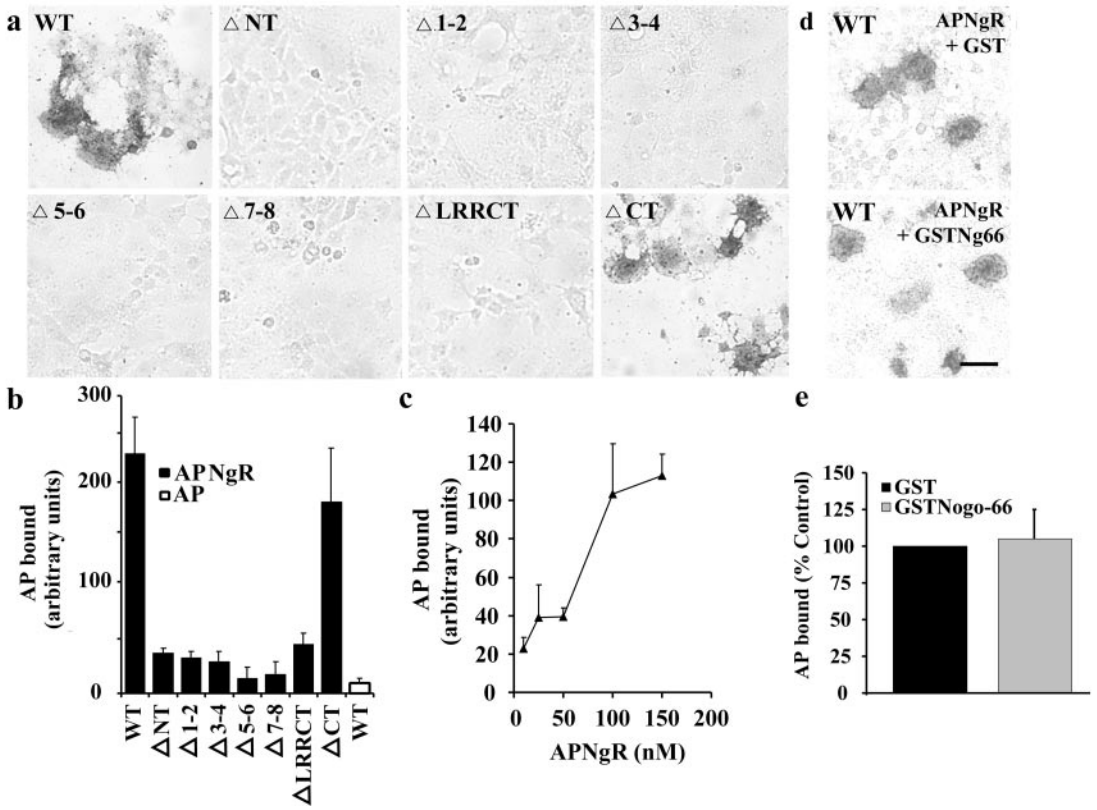

Figure 5. NgR interacts with itself. *a*, COS-7 cells were transfected with WTNgR or NgR deletion mutant plasmids and tested for AP or AP-NgR binding. WTNgR- and NgRΔCT-transfected COS-7 cells bind to AP-NgR. *b*, Quantification of AP or AP-NgR binding to COS-7 cells transfected with NgR deletion mutants. *c*, AP-NgR binding to COS-7 cells transfected with NgR as a function of AP-NgR concentration. Mean ± SEM for three experiments. *d*, COS-7 cells were transfected with WTNgR and treated with AP-NgR in the presence of 25 nM GST or GSTNogo-66 (*GSTNg66*). AP-NgR interaction with WTNgR is not modified by the presence of GST or GSTNogo-66. Scale bar, 100 μm. *e*, Quantification of AP-NgR binding to WTNgR-transfected COS-7 cells in the presence of 25 nM GST or GSTNogo-66.

that the receptor multimerization domain, like the Nogo-66 binding site (Fig. 1), is localized to the LRR domains.

Receptor multimerization does not appear to be regulated by ligand. First, the presence of Nogo has little, if any, effect on AP-NgR binding to NgR-transfected COS-7 cells (Fig. 5*b*). Second, when transfected HEK293T cells were treated with Nogo, the membrane fractionation profile of WTNgR and NgRL1 remained unchanged (data not shown). This suggests that Nogo does not modulate NgR localization to lipid raft compartments in HEK293T cells. It should be noted that both of these assays were performed in non-neuronal cell lines. It is possible that Nogo affects NgR multimerization in neurons that may possess additional components required for Nogo signaling. It is also possible that ligand binding modifies the localization of additional unidentified signaling components within the lipid raft, as is the case for ephrins (Davy et al., 1999). Because the components of the NgR intracellular signaling cascade have not been identified, the effect of Nogo on the recruitment of signaling components to lipid rafts remains an open question.

Truncated NgR antagonizes Nogo and myelin-dependent inhibition

On the basis of the structure–function analysis of NgR, a truncated soluble NgR (NgREcto) was assayed for antagonism of Nogo-66 signaling. NgREcto consists of residues 1 through 310, which includes the entire binding region for Nogo-66, but lacks the NgRCT region that is required for NgR signaling and the GPI linkage region of the receptor. NgREcto protein was purified from the conditioned medium of stably transfected CHO cells. The ability of NgREcto to antagonize Nogo–NgR interactions was tested by treating WTNgR-expressing COS-7 cells with 7 nM Nogo-AP in the presence or absence of 70 nM NgREcto (Fig. 6*a*,*b*). NgREcto significantly reduces Nogo-AP binding to transfected cells. To test the effect of NgREcto on signaling through the NgR, E13 dissociated DRGs were plated on mixed NgREcto–Nogo-66 substrates (Fig. 6*c*,*d*). Neurite outgrowth from E13 chick DRGs plated on GSTNogo-66 substrates without NgREcto is strongly inhibited. NgREcto to a great extent reverses this neurite outgrowth inhibition by Nogo. NgREcto is unable to overcome the inhibitory activity of the chondroitin sulfate proteoglycan aggrecan (Seidenbecher et al., 1998), suggesting that the NgREcto reagent is acting specifically on the NgR pathway. Because bound NgREcto might alter the surface properties of the laminin–Nogo substrate, purified NgREcto was also applied as a soluble protein to test for its ability to antagonize Nogo inhibition (Fig. 6*f*). The inhibitory effect of Nogo-66 is significantly diminished in the presence of 2 μM soluble NgREcto protein.

NgREcto is a specific Nogo-66 antagonist; therefore, the relative importance of Nogo-66 in inhibition of axon outgrowth by CNS myelin can be assessed. When the same protocol is used as for GSTNogo-66, myelin strongly inhibits chick E13 DRG neurite outgrowth. NgREcto blocks a significant proportion of this inhib-

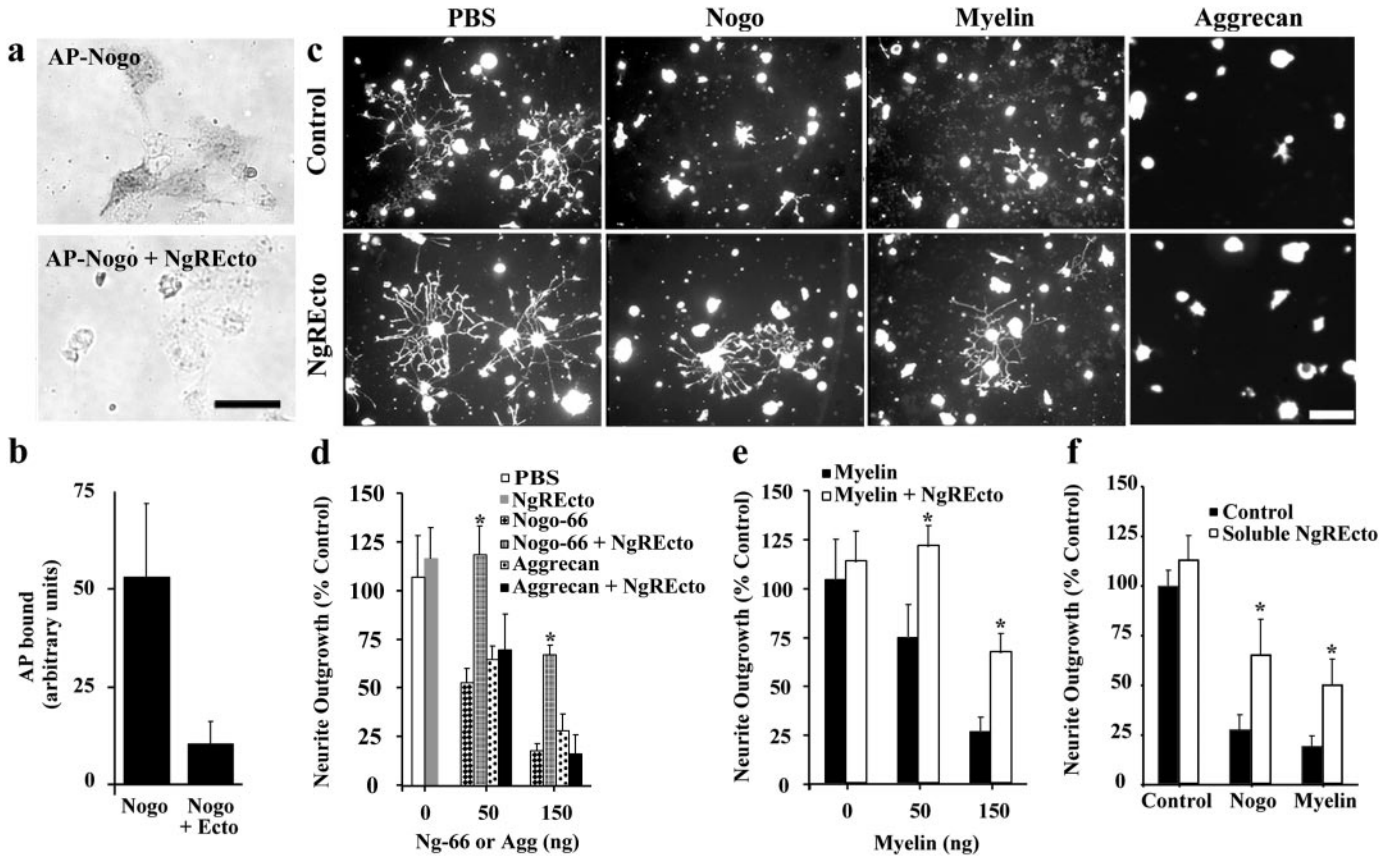

Figure 6. Soluble NgREcto antagonizes neurite outgrowth inhibition on Nogo and myelin substrates. *a*, AP-Nogo stain of COS-7 cells transfected with WTNgR. Cells were stained with 7 nM AP-Nogo in the presence or absence of 70 nM NgREcto. NgREcto blocks AP-Nogo binding to WTNgR. *b*, Quantification of AP-Nogo (7 nM) binding to WTNgR-transfected COS-7 cells in the presence or absence of 70 nM NgREcto. *c*, Dissociated E13 DRGs were plated on spots of PBS or 500 ng of NgREcto mixed with Nogo, myelin, or aggrecan. Nogo and myelin inhibition is partially reversed by the addition of NgREcto, whereas aggrecan inhibition is not. *d*, *e*, Dose–response of E13 DRG outgrowth on spots of PBS or 500 ng of NgREcto mixed with Nogo, myelin, or aggrecan. *f*, E13 DRG neurite outgrowth on Nogo or myelin substrates in the presence of 2 μM soluble purified NgREcto (*Soluble NgREcto*) or PBS. Neurite outgrowth is expressed as micrometers of growth per cell. Means ± SEM for four to six experiments are reported. Student's *t* tests comparing PBS to NgREcto at the indicated Nogo concentration are reported. $^*p < 0.01$. Scale bar, 200 μm.

itory activity (Fig. 6*c,e,f*), consistent with the notion that NgR plays a primary role in mediating myelin action.

DISCUSSION

Previous studies have identified NgR as a highly potent, biologically active receptor for Nogo-66 (Fournier et al., 2001). By generating NgR deletion mutants and chimeric receptors, we demonstrate that the entire LRR region of NgR is required for Nogo binding to NgR and that the CT region of NgR is necessary but not sufficient for inhibitory NgR signaling (Fig. 7). Furthermore, the GPI linkage is not critical for NgR signaling but may modulate the efficacy of NgR-dependent inhibition. We have also identified a soluble, truncated form of NgR that can antagonize the inhibitory effects of Nogo or myelin on E13 chick DRG outgrowth. This supports a central role for NgR in myelin inhibition of axon growth.

Role of NgR LRR and CT domains

It is clear that the LRR domains of NgR are required for binding to Nogo. Because the NgRCT region is not sufficient to induce inhibition, it is likely that the LRR domains contribute to additional aspects of inhibitory signaling. The LRR region can also bind to full-length NgR; therefore, this domain may regulate receptor oligomerization and/or bind to an unidentified signal-transducing receptor subunit. The greatest sequence similarity in the NgR LRR region exists with Slit 1–3 and the acid-labile subunit of the insulin-like growth factor-binding protein complex. Slits are a family of extracellular matrix proteins that are expressed at the developing CNS midline and repel axons via receptors of the Roundabout (Robo) family (Brose et al., 1999; Zinn and Sun, 1999). The Slit LRRs have been shown recently to mediate binding to Robo and repellent signaling (Battye et al., 2001). Thus, the Slit–Robo interaction may provide a model for NgR interaction with a signal-transducing protein.

The unique CT domain of the NgR is required for NgR-dependent inhibition. The most plausible model is that this domain participates directly in the activation of a transmembrane signal-transducing component of the NgR. However, its inability to act in a constitutively active manner raises the possibility that the CT domain may facilitate NgR conformational changes that lead to axon inhibition by the LRR domain.

Role of the NgR GPI anchor

The GPI anchor is not absolutely required for NgR inhibitory signaling, because chimeric NgRL1 mediates Nogo-66-induced growth cone collapse when expressed in RGCs. The GPI anchor site could modulate the efficacy of NgR signaling by concentrating

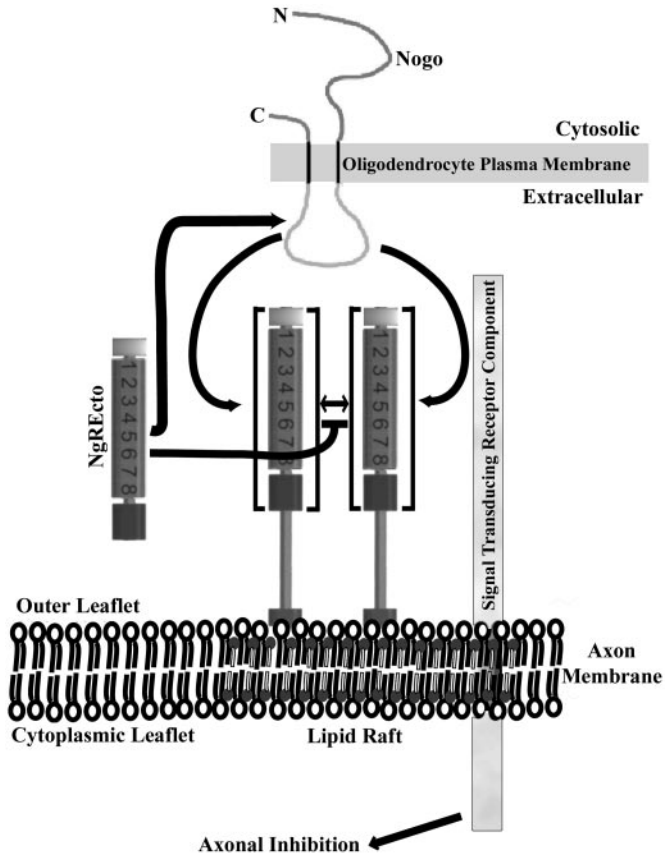

Figure 7. Model of Nogo receptor-mediated signaling. This schematic illustrates the proposed role each Nogo receptor domain plays in Nogo-signal transduction. See Discussion.

NgR in lipid rafts. The importance of raft localization of receptors is clear for the GDNF receptors GFRα1–GFRα4. GFRα1–GFRα4 are GPI-anchored receptors that are responsible for providing specific high-affinity binding sites for individual GDNF family ligands (GFL). All GFRs interact with a common receptor tyrosine kinase, RET (Tansey et al., 2000), that signals intracellularly via its cytoplasmic kinase domain. The GPI anchor plays a critical role in the GFL signaling pathway by restricting GFRα1 protein to lipid rafts, where RET is recruited after GDNF family ligand binding. GFRα1-mediated RET recruitment to the lipid raft is critical for efficient GFL signaling. Lipid rafts are thought to represent specialized signaling compartments within the plasma membrane because of the enrichment of Src family kinases and other signaling proteins that localize to the intracellular leaflet of lipid rafts (Anderson, 1998). The GPI anchor of the NgR could regulate the efficiency of NgR signaling by restricting its localization within the plasma membrane. This restricted distribution might enhance NgR multimerization and access to downstream signaling molecules.

It is also plausible that the role of the NgR GPI linkage is to provide an NgR cleavage site. Although there is as yet no evidence that truncated soluble NgR exists *in vivo*, it is clear that NgREcto is capable of antagonizing myelin inhibition. Released NgR might modulate Nogo signaling by loss of surface NgR from one axon and by diffusible blockade of NgR action.

NgREcto reverses Nogo signaling

The NgREcto protein contains a Nogo-66 binding site and blocks Nogo-66 action. Soluble NgREcto may bind to Nogo and prevent its binding to full-length active Nogo receptors on the neuronal cell surface. Alternatively, NgREcto might interact with surface-bound axonal NgR and prevent receptor oligomerization or NgR interaction with a signal-transducing receptor subunit (Fig. 7). The affinity of AP-Nogo is fivefold to 10-fold higher than AP-NgR for surface-bound NgR. Therefore, it is likely that NgREcto acts primarily by disrupting ligand-receptor interactions.

NgR mediates myelin inhibition

Previous work had not clarified the relative role of myelin-derived inhibitors on axon growth or the role of different Nogo domains. The ability of NgREcto to reverse a majority of myelin-dependent inhibition of axon growth demonstrates that the Nogo-66 receptor is a primary mediator of myelin action. Furthermore, the NgREcto protein is a potential therapeutic agent to promote axon regeneration in the injured adult CNS.

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