Nr-CAM Promotes Neurite Outgrowth from Peripheral Ganglia by a Mechanism Involving Axonin-1 as a Neuronal Receptor

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Nr-CAM is a neuronal cell adhesion molecule (CAM) belonging to the immunoglobulin superfamily that has been implicated as a ligand for another CAM, axonin-1, in guidance of commissural axons across the floor plate in the spinal cord. Nr-CAM also serves as a neuronal receptor for several other cell surface molecules, but its role as a ligand in neurite outgrowth is poorly understood. We studied this problem using a chimeric Fc-fusion protein of the extracellular region of Nr-CAM (Nr-Fc) and investigated potential neuronal receptors in the developing peripheral nervous system. A recombinant Nr-CAM-Fc fusion protein, containing all six Ig domains and the first two fibronectin type III repeats of the extracellular region of Nr-CAM, retains cellular and molecular binding activities of the native protein. Injection of Nr-Fc into the central canal of the developing chick spinal cord in ovo resulted in guidance errors for commissural axons in the vicinity of the floor plate. This effect is similar to that resulting from treatment with antibodies against axonin-1, confirming that axonin-1/Nr-CAM interactions are important for guidance of commissural axons through a spatially and temporally restricted Nr-CAM positive domain in the ventral spinal cord. When tested as a substrate, Nr-Fc induced robust neurite outgrowth from dorsal root ganglion and sympathetic ganglion neurons, but it was not effective for tectal and forebrain neurons. The peripheral but not the central neurons expressed high levels of axonin-1 both in vitro and in vivo. Moreover, antibodies against axonin-1 inhibited Nr-Fc-induced neurite outgrowth, indicating that axonin-1 is a neuronal receptor for Nr-CAM on these peripheral ganglion neurons. The results demonstrate a role for Nr-CAM as a ligand in axon growth by a mechanism involving axonin-1 as a neuronal receptor and suggest that dynamic changes in Nr-CAM expression can modulate axonal growth and guidance during development.

Key Words: Nr-CAM; axonin-1; cell adhesion molecule; neurite outgrowth; axon guidance.

INTRODUCTION

During nervous system development, positive and negative cues along the axon pathway act in concert to route axons through a complex environment to their target fields (Tessier-Lavigne and Goodman, 1996). Neurons express specific receptors on their growth cones to sense various secreted, extracellular matrix-bound, as well as cell surface ligands and thereby modulate axonal growth and guidance. Axon surface glycoproteins belonging to the immunoglobulin (Ig) superfamily are believed to play roles in neuronal migration, axon elongation, and pathfinding (Brummendorf and Rathjen, 1995). Most of these molecules promote neurite outgrowth and can direct growth along spatially restricted trajectories, while others have repulsive properties (Tessier-Lavigne and Goodman, 1996). Interestingly, there is increasing evidence that Ig superfamily CAMs can mediate biological functions as both ligands and receptors.

Nr-CAM (also known as Bravo) belongs to the L1 subfamily of Ig CAMs which includes L1, Ng-CAM (the L1 homologue in chicken), CHL1, and neurofascin (Brummendorf and Rathjen, 1995; Grumet, 1997). All these proteins consist of six Ig-like domains, four to five fibronectin type III (Fn III) repeats, a single transmembrane domain, and a cytoplasmic region that can bind to the cytoskeletal protein ankyrin (Davis and Bennett, 1994). Multiple splicing isoforms of Nr-CAM have been reported, but functional differences between individual isoforms are still unclear (Davis et al., 1996; Grumet et al., 1991; Kayyem et al., 1992; Lane et al., 1996; Wang et al., 1998). The extracellular region of Nr-CAM appears to be cleaved at a furin-like cleavage site.
in the third Fn III repeat and the cleaved amino-terminal fragment of ~140 kDa lacking the transmembrane region still associates with the membrane (Grumet et al., 1991; Kayyem et al., 1992), but the function of cleavage is unknown. Nr-CAM is expressed on neurons and glial cells including Schwann cells and Muller cells (Grumet et al., 1991; Kayyem et al., 1992; Krushel et al., 1993; Suter et al., 1995). It is expressed in a restricted manner during development and its expression persists at elevated levels in adult, in contrast to L1 and Ng-CAM that are downregulated in the adult (Davis and Bennett, 1994; Krushel et al., 1993). During chick development, Nr-CAM shows a more restricted expression pattern than Ng-CAM and also shows unique focal expression such as in the floor plate of the spinal cord (Denburg et al., 1995; Krushel et al., 1993; Stoeckli and Landmesser, 1995). Its expression has also been shown recently outside the nervous system, i.e., in human pancreas and adrenal gland (Wang et al., 1998).

Nr-CAM shows homophilic binding (Mauro et al., 1992) as well as heterophilic binding to molecules including other Ig superfamily CAMs (contactin, axonin-1, and neurofascin) (Davis et al., 1996; Morales et al., 1993; Suter et al., 1995; Volkmer et al., 1996), proteoglycans (neurocan and phosphacon) (Milev et al., 1996; Sakurai et al., 1997), and the extracellular matrix molecule laminin (Grumet and Sakurai, 1996). Evidence for Nr-CAM as a receptor to promote neurite outgrowth has been obtained from studies using chick tectal cells grown on substrates coated with contactin/F11 and neurofascin (Morales et al., 1993; Volkmer et al., 1996). Nr-CAM also acts as a coreceptor with contactin on tectal neurons to receive neurite outgrowth signals from the short receptor form of RPTPβ (Sakurai et al., 1997).

Nr-CAM can also act as a ligand. In the spinal cord, Nr-CAM is expressed in the floor plate and on commissural fibers, and it has been implicated in guidance of these fibers by a mechanism involving axonin-1, presumably acting as a receptor on commissural axons (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). As a substrate in culture, Nr-CAM has been shown to induce neurite outgrowth from chick tectal cells using contactin as their neuronal receptor (Volkmer et al., 1996). Nr-CAM can bind to dorsal root ganglia (DRG) neurons and this adhesive interaction was found to involve axonin-1 on the neuron (Suter et al., 1995), but the functional relevance of this interaction was not explored. We have addressed this issue here by studying the response of various populations of neurons to Nr-CAM applied as an Fc fusion protein. We found robust neurite outgrowth from two populations of peripheral neurons but only weak response from two major populations of CNS neurons. Moreover, we showed that the responding peripheral neurons express axonin-1 robustly and that axonin-1 serves as a receptor for Nr-CAM-mediated neurite outgrowth. Finally, the results suggest that the amino-terminal region of Nr-CAM that is defined by the furin-like cleavage site is a potent modulator of neurite outgrowth for certain neurons and may play a role in axon guidance in vivo.

FIG. 1.
Model of Nr-CAM and purity of the Nr-Fc fusion protein. (A) Schematic view of Nr-CAM and Nr-Fc fusion protein. Nr-CAM contains six immunoglobulin-like domains (omega) and five fibronectin type III repeats (rectangles). Arrow depicts the furin-like cleavage site in the third fibronectin type III repeat. Nr-Fc contains all six Ig-like domains and the first two fibronectin type III repeats fused to the constant region of human immunoglobulin. Note the disulfide linkages creating a dimer of Nr-Fc chimeras. (B) SDSPAGE profile of purified Nr-Fc fusion protein. Markers are indicated in kilodaltons.

MATERIALS AND METHODS

Construction and purification of chick Nr-Fc fusion proteins. The EcoRI (multicloning site)-KpnI fragment of chick Nr-CAM cDNA (Sakurai et al., 1997) containing nucleotides 1–1961 was cloned into pUC19 using EcoRI and KpnI sites. A cDNA fragment containing nucleotides 1961–2515 of chick Nr-CAM was PCR amplified with a modified 3' end to have a BamHI site enabling cloning into the pUC19-chick Nr-CAM construct using the KpnI and BamHI sites. The cDNA containing nucleotides 1–2515 which encodes six Ig domains and two fibronectin type III repeats was cut out by EcoRI and BamHI and cloned into a plasmid containing the human Fc region cDNA with a BamHI site at the 5' end in pCMN1. As a result, chick Nr-CAM cDNA was fused with the human Fc region cDNA in frame and driven by the CMV promoter. The sequence was confirmed by DNA sequencing. The plasmid was cotransfected with pSV2neo plasmid (5:1 weight ratio) into 293 cells derived from human kidney epithelial cells using lipofectamine reagents and opti-MEM (Gibco BRL), and clones were selected by G418 (Gibco BRL, 0.7 mg/ml, effective concentration) in DMEM containing 10% FCS. High expressers were screened by immunoprecipitation from culture supernatant with protein A antibody conjugated with HRP (Jackson Laboratory). Typical yield of Nr-Fc in culture media is 1–2 μg/ml. The clones were expanded and cultured in DMEM containing 2% FCS. Pooled culture supernatant (~600 ml) was used for the purification of Nr-Fc either by immunoaffinity column conjugated with anti-chick monoclonal antibody C38 (Denburg et al., 1995) or by a new method involving protein A (Sakurai et al., 1998). Purified protein was dialyzed against PBS.

Solid-phase protein binding assay. Binding analysis of Nr-Fc fusion protein to purified adhesion molecules was performed as described (Sakurai et al., 1997). Contactin-Fc (Peles et al., 1995) and normal human Ig (Sigma) were used as controls.

Fc fusion protein binding to DRG neurons. Cultured E9 chick embryo DRG neurons were established as described (Einheber et al., 1997). Briefly, the ganglia were isolated, trypsinized, and triturated to prepare single cells that were plated on ammoniated rat tail collagen- (Biomedical Technologies Inc.) coated coverslips.
FIG. 2. Molecular and cellular binding activities of Nr-Fc fusion protein. (A) Solid-phase protein binding assay. Purified proteins (50 μg/ml) were coated on plastic petri dishes and blocked with 1% BSA. After incubation with Fc fusion proteins (1 μg/ml), binding of Fc fusion proteins was detected with anti-human Fc antibody conjugated with alkaline phosphatase, followed by BCIP/NBT alkaline phosphatase substrates (Vector). Nr-Fc binds strongly to Nr-CAM (Nr), axonin-1 (Ax-1), and Ng-CAM (Ng), while weakly to laminin (Lm) and not at all.
of Nr-Cam binding by anti-axonin-1 antibodies. Chick DRG neurons were incubated with Nr-Fc in the presence of normal rabbit (top) or normal human Ig (nH-Ig; 20 μg/ml) to fibronectin (Fn). (B) Nr-Fc binding to DRG neurons. Chick DRG neurons were incubated with Nr-Fc or normal human Ig (20 μg/ml) and bound Fc fusion proteins were clustered with anti-human Fc antibody labeled with fluorescence. Bar, 100 μm. (C) Inhibition of Nr-Fc binding by anti-axonin-1 antibodies. Chick DRG neurons were incubated with Nr-Fc in the presence of normal rabbit (top) or anti-axonin-1 (bottom) Fab' fragments (200 μg/ml). Pictures were taken with the same exposure times to compare binding. DIC pictures (right) are shown to identify neurons in the fields.

Axonin-1 Is a Neuronal Receptor for Nr-CAM

FIG. 3. Immunolocalization of Nr-Cam and axonin-1 in developing spinal cord. Both Nr-CAM and axonin-1 are expressed on commissural cell axons in the E4 chick spinal cord. Nr-CAM is still expressed on these axons through E6 when axonin-1 expression is downregulated. Note the increase in expression of Nr-CAM in the fibers coursing through the Nr-CAM-positive floor plate (black arrow). White arrowheads point out the ventral regions of the dorsal funiculus where Nr-CAM expression is enhanced. Bars, 50 μm.

Neurite outgrowth assay. Neurite outgrowth assays were performed with slight modifications to those described (Friedlander et al., 1994). Purified proteins were diluted to 30 μg/ml in filtered PBS and 1 μl was coated onto sterile polystyrene petri dishes for 1.5 h in a humidified chamber. Protein spots were removed and the dishes were washed in PBS before blocking the dish with 1% BSA/PBS. E8–E9 chick embryos were used to isolate peripheral ganglia and E6 embryos were used to isolate totoal and forebrain neurons. Following isolation of single-cell suspensions, neurons were plated on the protein-coated dishes at a concentration of 1 × 10^5 cells/ml in N3 medium (for DRG and SG cells) (Felsenfeld et al., 1994) or in DMEM/F12 (ITS+ for tectum and forebrain) (Sakurai et al., 1997). Cultures were incubated for 16–18 h in 5% (for N3) or 10% (for DMEM/F12 (ITS+) CO_2 atmosphere. In case of antibody inhibition experiments, after 1 h of culture, media were replaced with the same media containing 200 μg/ml antibody Fab’ fragments. Anti-axonin-1 antibodies used were described previously (Shiga et al., 1997). The dishes were gently fixed in warm fresh 3.5% parafomaldehyde in PBS. Coverslips were mounted on glass slides in Citifluor (Ted Pella Inc.), and Fc fusion protein binding was visualized under a Zeiss Axiophot fluorescent microscope. For antibody inhibition of Nr-Fc binding, binding assay was performed as above except L-15-containing anti-axonin-1 Fab’ or normal rabbit Fab’ fragments (200 μg/ml) were used.

Immunostaining. Embryos were removed from the egg and staged (Hamburger and Hamilton, 1951) before fixation in 4% paraformaldehyde overnight. Embryos were cryoprotected in 30% sucrose, mounted in Lipshaw (Shandon) on dry ice, and cryosectioned (15–20 μm) on a HackerBright cryostat. Sections mounted on gelatin-coated slides were immunostained as described (Shiga et al., 1997). Staining of cultured cells was performed essentially as for the tissue sections, with the exception that primary antibodies were diluted an additional twofold.

Fc fusion protein injection into chick embryo in ovo. In ovo injections were performed essentially as described by Dr. E. Stoeckli (Stoeckli and Landmesser, 1995). Briefly, E3 chick embryos (stage 18) were accessed through a window in the egg shell. A total of four to five injections (0.1 μl each during the period of E3 to E5) of antibody or purified protein were made in the central canal of the embryo. Fab’ antibody injections were performed using Fab’ fragments concentrated to 20 mg/ml in Millipore Ultratree-CL centrifugal filters (Sigma). Similarly, Fc fusion proteins and control normal Ig were concentrated to 2 mg/ml. At E5 (stage 25) the embryos were removed from the egg and the lumbar spinal cord was isolated. The roof plate of the spinal cord was cut open with fine forceps and pinned on Sylgard-(Dow/Corning) coated dishes to create the open-book spinal cord preparation for Dil labeling. Dil was allowed to diffuse for 1–2 weeks in order to label the commissural cell axons. To quantitate crossing errors, fluorescence levels of pre- and postcommissural turning fibers were compared by eye, and, where resolution allowed, fibers were counted. When fibers could not be counted, if only one or two fibers were seen turning precommissurally, it was considered <1%, while if the Dil-labeled precommissural turning tract was as densely labeled as the postcommissural turning fibers, it was counted as >20% (Stoeckli and Landmesser, 1995).

Neurite outgrowth assay. Neurite outgrowth assays were performed with slight modifications to those described (Friedlander et al., 1994). Purified proteins were diluted to 30 μg/ml in filtered PBS and 1 μl was coated onto sterile polystyrene petri dishes for 1.5 h in a humidified chamber. Protein spots were removed and the dishes were washed in PBS before blocking the dish with 1% BSA/PBS. E8–E9 chick embryos were used to isolate peripheral ganglia and E6 embryos were used to isolate totoal and forebrain neurons. Following isolation of single-cell suspensions, neurons were plated on the protein-coated dishes at a concentration of 1 × 10^5 cells/ml in N3 medium (for DRG and SG cells) (Felsenfeld et al., 1994) or in DMEM/F12 (ITS+ for tectum and forebrain) (Sakurai et al., 1997). Cultures were incubated for 16–18 h in 5% (for N3) or 10% (for DMEM/F12 (ITS+) CO_2 atmosphere. In case of antibody inhibition experiments, after 1 h of culture, media were replaced with the same media containing 200 μg/ml antibody Fab’ fragments. Anti-axonin-1 antibodies used were described previously (Shiga et al., 1997). The dishes were gently fixed in warm fresh 3.5% parafomaldehyde, 2% sucrose, 0.1% glutaraldehyde in Hanks’ buffered saline solution (Gibco BRL) for 2 h and stored in PBS. Images of neurons grown on the protein spots were digitized using a Scion AG-5 image-capture card linked to a Nikon Diaphot inverted microscope. Digitized images were set to scale and single neurite lengths for each cell body were marked by hand in NIH-Image and measured. Data were analyzed to obtain the neuron length plot profile and graphed in Microsoft Excel as described (Sakurai et al., 1997).
RESULTS

To study Nr-CAM as a ligand, we prepared a recombinant Fc fusion protein consisting of the six Ig domains and first two Fn III repeats of Nr-CAM coupled to the constant region of human Ig (Fig. 1A). Fc fusion proteins have been shown to be powerful tools for analyzing protein functions (Ashkenazi and Chamow, 1997) and the amino-terminal region of Nr-CAM was chosen because it represents most of the major 140-kDa fragment generated by cleavage at the furin site (Grumet, 1997). We purified the fusion protein from culture supernatants of 293 cells stably transfected to produce Nr-Fc, and SDS–PAGE analysis (Fig. 1B) showed one major protein corresponding to Nr-Fc migrating at $\sim 180$ kDa. Using this preparation, we sought to analyze the function of the extracellular region of Nr-CAM as a ligand.

Nr-Fc Binds to Purified CAMS and to Cultured DRG Neurons

We first tested the binding of Nr-Fc to various purified CAMs to clarify whether the recombinant protein retains the same binding activities as the native Nr-CAM molecule (Grumet, 1997). Using a solid-phase binding assay, we detected binding of the Fc fusion protein to several adhesion molecules spotted on plastic dishes. Nr-Fc bound strongly to axonin-1 as well as to Nr-CAM itself while less strongly to Ng-CAM (Fig. 2A) and contactin (data not shown), indicating that Nr-Fc has similar binding activities as reported for the native Nr-CAM (Grumet, 1997).

A previous report suggested a role for Nr-CAM in neuron–Schwann cell interactions and raised the possibility that Nr-CAM could bind to neurons via axonin-1 (Suter et al., 1995). Therefore, we analyzed the ability of Nr-Fc to bind to

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein injected</th>
<th>No. injected</th>
<th>&lt;5% errors</th>
<th>&gt;20% errors</th>
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<tr>
<td>Anti-axonin-1</td>
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<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Nr-Fc</td>
<td>13</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Normal human Ig</td>
<td>8</td>
<td>8</td>
<td>0</td>
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Note: Number of errors (see Fig. 3) seen in treated and control-injected embryos in spinal cord open-book preparations. Quantitation of ipsilateral turning errors was performed as described (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997).

**FIG. 4.** Effect of Nr-Fc injection on axon guidance in chick spinal cord in ovo. Open-book preparations (A) from chick embryos injected with proteins in ovo. E5 chick embryo spinal cords were prepared in an open-book preparation (Stoeckli and Landmesser, 1995) and the commissural cell bodies were injected with Dil (10 mg/ml); axons were labeled anterogradely for 2 weeks by incubation at room temperature. The normal pathway of the commissural cell axons' guidance was observed (B) following injection of normal human Ig (nH-Ig). Anti-axonin-1- (C) and Nr-Fc- (D and E) treated embryos have commissural cell axons turning prematurely into the ipsilateral ventral fiber tracts (arrowheads). Floor plate (FP) was visualized by phase-contrast microscopy. Bar, 100 $\mu$m.
live DRG neurons cultured in vitro. Anti-human Fc antibodies were used to cluster bound Fc fusion proteins as well as to visualize their binding to live cells in culture. Sequential treatment with Nr-Fc and anti-human Fc conjugated with fluorescein yielded clusters that were detected on the neuronal cell surface (Fig. 2B, left). Neurons incubated with control Fc fusion proteins or normal human Ig (Fig. 2B, right) did not show clusters detected with the anti-human Fc antibodies. The binding of Nr-Fc to DRG neurons was inhibited by preincubation with Fab fragments of anti-axonin-1 antibodies (Fig. 2C, bottom), indicating that axonin-1 serves as a neuronal receptor for Nr-CAM (Fig. 2C). We also concluded that recombinant Nr-Fc (consisting of six Ig-like domains and two Fn III repeats) retained binding activities similar to native Nr-CAM in both molecular interactions and cellular interactions and could be used for further analysis of Nr-CAM function.

**Spinal Cord Commissural Axon Guidance Is Perturbed by Injection of Nr-Fc**

In the developing spinal cord, commissural axons express axonin-1 transiently (from E3 to E5) as they project axons ventrally toward the floor plate, while Nr-CAM expression on these cells and in the floor plate persists even until E6 (Fig. 3 and Dodd et al., 1988; Shiga and Oppenheim, 1991; Sakurai et al., 1994). Nr-CAM has been proposed to act as a ligand in the floor plate for the guidance of commissural axons based on perturbation experiments with anti-axonin-1, anti-Nr-CAM, and axonin-1 protein in the developing chick spinal cord (Stoeckli and Landmesser, 1995). Since our Nr-Fc fusion protein mimics binding activities of Nr-CAM, we tested its ability to interfere with the Nr-CAM–axonin-1 interaction in vivo in this paradigm. We injected Nr-Fc into chick embryos from E3 to E5 and analyzed commissural axons following DiI tracing in open-book preparations (Fig. 4). We observed dramatic axon guidance errors in Nr-Fc-injected embryos (Figs. 4D and 4E). Premature turning errors in the commissural axons were seen where fibers failed to cross the midline and instead joined the ipsilateral fascicle. Similar effects were observed following injection of anti-axonin-1 antibody (Fig. 4C) as shown previously (Stoeckli and Landmesser, 1995). As a control, injection of a human Ig (Fig. 4A) or normal rabbit
Fab' fragments (not shown) did not show guidance errors. Quantitation of the frequency of errors showed that all embryos injected with either Nr-Fc or anti-axonin-1 antibodies had a high percentage of guidance errors, while few or no errors were observed in controls (Table 1). This result demonstrated that Nr-Fc is a potent biological agent that can modulate molecular interactions involving Nr-CAM in vivo and confirmed the importance of Nr-CAM in guidance of commissural axons in the spinal cord.

**Nr-Fc Induces Neurite Outgrowth from Peripheral Ganglion Neurons**

In contrast to the dramatic downregulation of axonin-1 on commissural axons, it persists at high levels in the E6 dorsal funiculus and the DRG in an overlapping pattern with Nr-CAM (Fig. 3). This suggests potential interactions between the CAMs in these sites in the dorsal funiculus and the DRG. Considering that Nr-Fc can bind to DRG neurons, we asked whether Nr-Fc itself could serve as a substrate for neurite outgrowth for DRG neurons in culture. As shown in Fig. 5, Nr-Fc induced neurite outgrowth from chick DRG neurons. Both Nr-Fc and Nr-CAM induced long neurites from dissociated DRG neurons when tested at 30 μg/ml (average length = 145 ± 1 μm for Nr-Fc, 84 ± 9 μm for Nr-CAM). At this concentration, Nr-CAM-Fc was more potent than native Nr-CAM, and the superior activity of Nr-Fc may be due to increased avidity of the molecule as a dimer. The response of DRG neurons to Nr-CAM was roughly comparable to that seen with Ng-CAM (average length = 111 ± 9 μm) which is a potent promoter of neurite growth.

Given that both Nr-CAM and Ng-CAM are structurally related CAMs that promote robust neurite outgrowth for DRG neurons, it was of interest to determine whether other types of neurons responded to these CAMs. When cells from DRG, optic tectum, forebrain, and sympathetic ganglia were compared, we found that only some of these neurons had a robust response to Nr-Fc (Fig. 6). DRG and sympathetic ganglion cells extended long neurites on Nr-Fc, while dissociated tectal and forebrain cells had little neurite outgrowth. On the other hand, Ng-CAM induced robust neurite outgrowth from these cells.
neurite outgrowth from all cells tested, suggesting that restricted neurite outgrowth is dependent on differential responsiveness to Nr-Fc and not on neuronal preparations. Therefore, we conclude that Nr-Fc can induce robust neurite outgrowth from only certain neuronal populations and that Ng-CAM and Nr-CAM are likely to use distinct mechanisms to achieve this effect.

Neurite Outgrowth Promoted by Nr-Fc Is Inhibited by Anti-Axonin-1 Antibodies

Given that Nr-CAM can bind to axonin-1, it is interesting that neurons responsive to Nr-Fc are found in tissues that are strongly axonin-1 positive (Figs. 3 and 7). Axonin-1 is expressed at high levels in certain sensory neurons when they are extending axons. Developing DRG neurons expressed axonin-1 (Figs. 3 and 7), and at E8 (Fig. 7) sympathetic ganglia immunostained intensely for axonin-1 (Hafter et al., 1994). Nr-CAM expression is observed along a subset of pathways of these axonin-1-positive neurons, for example, in the ventral region of the dorsal funiculus where DRG neurons enter the spinal cord (Fig. 7). Moreover, Nr-CAM is coexpressed with axonin-1 at high levels in the sympathetic chain (Fig. 7). In contrast, tectal neuronal cell bodies are not axonin-1 positive and the only axonin-1-positive region in the optic tectum is the stratum opticum which contains retinal afferents (Sakurai et al., 1994; Yamagata et al., 1995). To confirm that the expression of axonin-1 is indeed on neuronal processes, we immunostained cultured neurons from DRG, SG, tectum, and forebrain (Fig. 8). DRG and SG neurons expressed large amounts of axonin-1 while little to no expression was seen on tectum and forebrain neurons in culture. In contrast, all these neuronal processes stained for Ng-CAM and Nr-CAM. These results suggest that neurons expressing axonin-1 can extend neurites in response to Nr-CAM, while neurons deficient in axonin-1 have little or no response to Nr-CAM.

Neurite outgrowth from all cells tested, suggesting that restricted neurite outgrowth is dependent on differential responsiveness to Nr-Fc and not on neuronal preparations. Therefore, we conclude that Nr-Fc can induce robust neurite outgrowth from only certain neuronal populations and that Ng-CAM and Nr-CAM are likely to use distinct mechanisms to achieve this effect.

DISCUSSION

This study demonstrates that the amino-terminal six Ig domains and the first two fibronectin repeats of Nr-CAM contain all the known binding activities of the intact protein. In this vein, we show for the first time that Nr-CAM can act as a ligand for neurite outgrowth by a mechanism involving axonin-1 as the neuronal receptor. DRG and sympathetic ganglia were found to express high levels of these proteins in developmental patterns that are consistent with this mechanism. In the CNS, a subpopulation of axonin-1-positive spinal neurons, the commissural cells, are guided across the Nr-CAM-positive floor plate by a mechanism involving axonin-1 and Nr-CAM. The combined results suggest that the restricted Nr-CAM expression in other locations may be critical in guiding axonin-1-positive axons and this process is regulated by dynamic changes in Nr-CAM expression.

Nr-Fc Binding and Bioactivity of Six Extracellular Ig Domains Plus Two Fibronectin Repeats

The Nr-Fc studied here represents the naturally occurring major cleavage product of Nr-CAM except for half of the third fibronectin repeat. It is therefore not surprising that its activity is comparable to that of native Nr-CAM, except for the fact that it was somewhat more active, possibly because it is an Fc dimer that may bind with higher avidity. Moreover, the results suggest that key functions of Nr-CAM as a ligand can be attributed to its extracellular 140-kDa component.
The potency of Nr-Fc was demonstrated in culture and by its ability to induce large numbers of errors in axon guidance of commissural cells growing in the spinal cord in vivo. Previous studies indicated the involvement of axonin-1 and Nr-CAM in this process based on the use of antibodies and purified axonin-1 protein (Stoeckli and Landmesser, 1995), and our study confirms this conclusion by providing additional evidence using the Nr-Fc protein. Stoeckli et al. suggested that the floor plate possesses growth cone-collapsing factors as well as growth-promoting factors (Stoeckli et al., 1997), and the action of the latter, in particular Nr-CAM, is critical for growth across the floor plate by interacting with axonin-1 as a growth cone receptor. A simple interpretation of the perturbations produced by anti-axonin-1 and anti-Nr-CAM antibodies or soluble proteins is that they block interactions between axonin-1 on commissural axons and Nr-CAM in the floor plate. It is also possible that Nr-CAM sends signals to the commissural axon that influence turning after crossing the floor plate, and addition of exogenous Nr-Fc can signal it prematurely. However, there is no evidence for this premature activation insofar as there was no difference between the effects generated by Nr-Fc and the other perturbants.

It is interesting that Nr-CAM becomes selectively enriched in the ventral commissure during the period that axons cross the floor plate. The commissural fibers are weakly Nr-CAM positive before they reach the Nr-CAM-positive floor plate, and their interaction with the floor plate may induce Nr-CAM accumulation in this location. Confocal microscopy showed high levels of Nr-CAM along these fibers, but it was not clear whether Nr-CAM was also enriched in the ventral region of the floor plate cells (unpublished observations). It is possible that enhanced Nr-CAM expression on fibers in the floor plate promotes growth of the later fibers as the commissure expands.
**Nr-CAM Can Act as a Ligand to Promote Neurite Outgrowth**

DRG neurons and sympathetic ganglion neurons respond robustly to Nr-Fc, suggesting that Nr-CAM may induce axonal growth of these neurons in vivo. Nr-CAM is expressed in DRG and the dorsal root entry zone where DRG neurons enter the spinal cord in the dorsal funiculus, and it may support axonal elongation in these locations (Denburg et al., 1995; Krushel et al., 1993; Shiga et al., 1997). At E8, Nr-CAM is restricted to the ventral region of the dorsal funiculus, whereas axonin-1 is expressed throughout the entire region (Shiga et al., 1997; Figs. 3 and 7). The ventral region is composed of sensory afferents from the DRG that bifurcate at right angles in the dorsal funiculus taking "T"-shaped projections (Denburg et al., 1995). The more dorsal regions, which are Nr-CAM negative, are composed of afferents that pass through the Nr-CAM-enriched region and then bifurcate less acutely into "Y"-shaped fibers (Denburg et al., 1995; M. Lustig, unpublished observations).

It is unclear whether these two different trajectories are related to the function of axonin-1 or whether they reflect histotypic differences between these two populations of axons. Nr-CAM and axonin-1 are also prevalent in sympathetic ganglia and along the sympathetic chain during development (Krushel et al., 1993; Fig. 7), suggesting that they may play a role in axonal outgrowth and/or fasciculation in this network.

Other studies showed that purified Nr-CAM from adult chick brain could induce neurite outgrowth from chick tectal neurons involving contactin as a neuronal receptor (Volkmer et al., 1996). However, we did not obtain robust neurite outgrowth from chick E6 or E9 tectal cells on native Nr-CAM purified from chick E14 membranes or on chick Nr-Fc. This discrepancy might be explained by differences in splicing isoforms of Nr-CAM and/or differences in substrate-coating methods.

As a ligand, Nr-CAM may also work together with other molecules. In retina, as well as optic nerve, Nr-CAM and axonin-1 are highly expressed (de la Rosa et al., 1990; Grumet et al., 1991; Ruegg et al., 1989; Sakurai et al., 1994), but we and others have not been able to obtain robust retinal neurite outgrowth using purified Nr-CAM (Morales et al., 1996).

**FIG. 9.** Effect of anti-axonin-1 antibody on neurite outgrowth induced by Nr-Fc. DRG and sympathetic ganglion neurons were cultured as described under Materials and Methods, in the presence of normal rabbit Fab' or anti-axonin-1 Fab' fragments (200 μg/ml). A and B show pictures of DRG neurons on Nr-Fc-treated dishes with normal rabbit (A) or anti-axonin-1 (B) antibodies. Bar, 100 μm. Quantification of neurite lengths indicated specific inhibition by anti-axonin-1 antibodies of Nr-Fc-mediated DRG (C, top) and sympathetic ganglion (D) neurite outgrowth. DRG neurons grown on Ng-CAM (C, bottom) were unaffected by anti-axonin-1 as shown previously (Kuhn et al., 1991).
et al., 1996; our unpublished observation). However, NRCAM can moderately potentiate the ability of Ng-CAM to promote retinal neurite outgrowth (Morales et al., 1996) and Ng-CAM may modulate the function of NRCAM depending on their levels of expression.

Axonin-1 Serves as a Neuronal Receptor for NRCAM-Mediated Neurite Outgrowth

In contrast to Ng-CAM, which is widely expressed on neurons and promotes robust neurite outgrowth for many different types of neurons, not all neurons respond to NRCAM. DRG and SG neurons responded robustly while tectal and forebrain neurons did not. NRCAM-responsive neurons express high levels of axonin-1, and anti-axonin-1 antibodies inhibited NRCAM-mediated neurite outgrowth using DRG and SG neurons suggesting that their response is mediated by the axonin-1 receptor. The lack of axonin-1 expression on various other neurons is consistent with their weak axonal outgrowth responses to NRCAM and would imply that modulation of axonin-1 levels on specific neurons regulates their guidance by NRCAM in vivo.

Considering evidence that axonal growth involves signaling in neurons (Kunz et al., 1996), it is likely that as a GPI-anchored molecule, axonin-1 associates with transmembrane coreceptors in order to create a receptor complex to propagate NRCAM signals. Possible candidates for axonin-1 coreceptors include Ng-CAM, neurofascin, and NRCAM. Interestingly, antibodies to Ng-CAM did not affect the growth of DRG (Fig. 8) or SG neurons (M. Lustig, unpublished observations) on NRCAM, indicating that it is not likely to act as a coreceptor with axonin-1 for NRCAM binding, although there is evidence for axonin-1 acting as a coreceptor with Ng-CAM (Buchstaller et al., 1996). Considering that NRCAM can act as a ligand for different receptors (Grumet, 1997), there may be an assortment of receptor complexes that respond to NRCAM in different situations. In addition, axonin-1's glycolipid anchor may be used to signal cytoplasmic proteins associated with the membrane, such as fyn (Kunz et al., 1996).

In summary, we have used a recombinant NRFc fusion protein to analyze the functions of the extracellular region of NRCAM and showed that it can bind to certain neurons and stimulate neurite outgrowth in vitro by a mechanism involving axonin-1 as a neuronal receptor. The restricted expression of NRCAM and axonin-1 during development suggests several potential locations for their interaction in vivo (e.g., on DRG and SG neurons). In addition to the ventral midline, the visual system, and the peripheral ganglia, the early accumulation of NRCAM at developing nodes of Ranvier (Lambert et al., 1997) suggests a role for the molecule in this location. The potency of NRFc as a bioactive agent opens new possibilities for the study of NRCAM functions as both a ligand and receptor in different locations using various bioassays in culture and in vivo.

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