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Neuropilin Is a Semaphorin III Receptor

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Summary

The semaphorin family contains a large number of phylogenetically conserved proteins and includes several members that have been shown to function in repulsive axon guidance. Semaphorin III (Sema III) is a secreted protein that in vitro causes neuronal growth cone collapse and chemorepulsion of neurites, and in vivo is required for correct sensory afferent innervation and other aspects of development. The mechanism of Sema III function, however, is unknown. Here, we report that neuropilin, a type I transmembrane protein implicated in aspects of neurodevelopment, is a Sema III receptor. We also describe the identification of neuropilin-2, a related neuropilin family member, and show that neuropilin and neuropilin-2 are expressed in overlapping, yet distinct, populations of neurons in the rat embryonic nervous system.

Introduction

The complex wiring of the adult nervous system is dependent upon the occurrence during neurodevelopment of an ordered series of axon guidance decisions that ultimately lead to the establishment of precise connections between neurons and their appropriate targets. These guidance events can act over long or short distances, and they can be either attractive or repulsive in nature (Tessier-Lavigne and Goodman, 1996). An important first step in elucidating the mechanisms by which long-distance chemotropic cues mediate axon guidance is identification of the receptors that bind these cues. Identification of two phylogenetically conserved gene families, the semaphorins and the netrins, has advanced our understanding of the cellular and molecular basis of long-range influences on axon guidance. Semaphorins and netrins function as chemotropic cues for specific populations of neurons during development (Keynes and Cook, 1995). The netrins have been implicated in long-range attractive and repulsive guidance events in Caenorhabditis elegans (UNC-6), vertebrates (netrin-1 and netrin-2), and Drosophila (netrin-A and netrin-B). Genetic studies in both invertebrates and vertebrates, and biochemical studies in vertebrates, show that two immunoglobulin (Ig) superfamily subgroups, one including the Deleted in Colorectal Cancer (DCC), UNC-40, and Frazzled proteins, and the other including the UNC-5, UNC-5H1, UNC-5H2, and RCM proteins, contain netrin receptors involved in mediating attractive and repulsive netrin functions (Ackerman et al., 1997; Fazeli et al., 1997; Leonardo et al., 1997; and see references in Tessier-Lavigne and Goodman, 1996). At present, however, semaphorin receptors have not been identified.

The semaphorins comprise a large family of both transmembrane and secreted glycoproteins, suggesting that some semaphorins act at a distance while others act locally (Kolodkin, 1996; Puschel, 1996). Semaphorins are defined by a well conserved extracellular semaphorin (sema) domain of approximately 500 amino acids. Secreted semaphorins contain an Ig domain that is C-terminal to the sema domain, while transmembrane semaphorins can have an Ig domain, type 1 thrombospondin repeat, or no obvious domain motif N-terminal to their transmembrane domain. Semaphorins are present in a variety of neuronal and nonneuronal tissues. Their function in neuronal growth cone guidance, however, has been addressed most extensively.

Two secreted semaphorins, vertebrate collapsin-1/Sema III/Sem D (species homologs) and Drosophila semaphorin II (D-sema II) (Matthes et al., 1995), have been shown to function selectively in repulsive growth cone guidance during development. Collapsin-1 (Coll-1) was identified in a search for growth cone collapsing factors from the membranes of adult chick brain tissue (Luo et al., 1993). Acute application of recombinant Coll-1 induces the collapse of a subset of dorsal-root-ganglia (DRG) neuron growth cones at subnanomolar concentrations, but has no effect on chicken retinal ganglion cell growth cones. Brain-derived membrane extracts enriched for Coll-1 and immobilized to beads, however, provided sensory neurons in culture with a localized repulsive cue capable of steering growth cones away from beads rather than causing complete growth cone collapse (Fan and Raper, 1995).

Genes encoding human, rat, and mouse Sema III/Sem D (referred to below as Sema III) were identified based on their similarity to other semaphorins (Kolodkin et al., 1993; Messersmith et al., 1995; Puschel et al., 1995; Giger et al., 1996). Sema III can act as a chemorepellent for NGF-dependent embryonic (E14) DRG sensory neurons. It has little effect, however, on neurotrophin-3 (NT-3)-responsive E14 DRG sensory afferents. The E14 ventral spinal cord secretes a chemorepellent activity selective for NGF-, not NT-3-, dependent E14 DRG sensory afferents (Fitzgerald et al., 1993; Messersmith et al., 1995; Puschel et al., 1996). This correlates well with the expression pattern of *sema III* in the ventral cord during the time of sensory afferent innervation, and the segregation of NT-3- and NGF-dependent sensory afferents, respectively, into ventral and dorsal targets in the spinal cord (Messersmith et al., 1995).

Indeed, antibody perturbation of Coll-1 at analogous stages in chick neurodevelopment supports the idea that Coll-1 is the ventral cord repellent (Shepherd et al., 1997). This is further supported by the observation that mice with a targeted deletion of the *sema III* gene exhibit defects in the trajectories of certain NGF-responsive sensory afferents (Behar et al., 1996). In addition, functional studies show that Sema III can act as a chemorepellent for spinal motor neurons and a subset of cranial

motor neurons (Varela-Echavarria et al., 1997). Coupled with extensive analysis of *sema III* and *Coll-1* expression (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996), all of these data suggest that specific populations of embryonic and adult neurons require Sema III for establishment, and possibly maintenance, of their appropriate patterns of connections. The rapid response of DRG growth cones in culture to Coll-1 and Sema III, and the low concentrations of these factors needed to elicit a response, strongly suggest that a receptor-mediated signal transduction mechanism underlies the action of these proteins on the cytoskeletal reorganization events that ultimately influence growth cone guidance.

In the present study, we report the identification of a high affinity Sema III receptor and show it to be neuropilin, an axonal glycoprotein extensively characterized by Fujisawa and colleagues (see Discussion). In a parallel study, Z. He and M. Tessier-Lavigne have also identified neuropilin as a Sema III receptor (He and Tessier-Lavigne [1997, this issue of *Cell*]). Further, we have found that neuropilin is one of a family of proteins that is expressed differentially in the mammalian nervous system during development.

Results

Neuropilin Is a Sema III–Binding Protein

To identify cell surface receptors for Sema III, we used a COS cell expression cloning strategy that employed a Sema III–secreted placental alkaline phosphatase fusion protein (Sema–AP) (Flanagan and Leder, 1990; see Experimental Procedures). A COS cell cDNA expression library was constructed using mRNA obtained from rat E14 spinal cord and DRG. cDNAs generated from these mRNAs should encode functional Sema III receptors since Sema III as well as Sema–AP induce collapse of growth cones from NGF-responsive DRG neurons (data not shown). The cDNA expression library was divided into 140 pools, each containing approximately 750 clones, and cDNA from each pool was transfected into separate wells of COS cells. Two days after transfection, COS cells were fixed, incubated with a solution containing Sema–AP, washed, and then stained for alkaline phosphatase (AP) activity. One positive pool was identified by the presence of a single COS cell with Sema–AP binding activity. This positive pool of cDNAs was subdivided and rescreened several times until a single cDNA was obtained that conferred Sema–AP binding when expressed in COS cells (Figure 1A).

Sequence analysis revealed that the Sema–AP binding protein was the full-length rat homolog of mouse neuropilin, a protein previously identified and well characterized in mice and other vertebrates (Kawakami et al., 1995; see Discussion). Neuropilin is a type I transmembrane protein that is expressed in a number of populations of neurons, including DRG neurons and spinal motor neurons (Kawakami et al., 1995; Figures 2E and 4E). The neuropilin protein consists of a large extracellular domain, a single transmembrane domain, and a short 39 amino acid intracellular domain (Figure 5). Sema–AP fusion protein bound to neuropilin via its Sema III domain, not the AP domain, because secreted placental alkaline phosphatase (SEAP) alone did not bind to

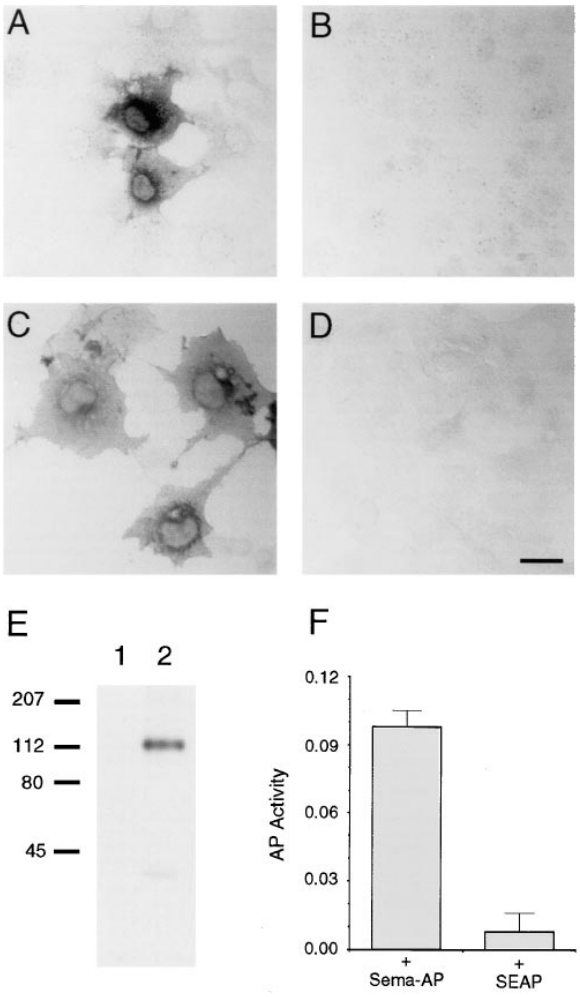

Figure 1. Sema–AP Binds to Neuropilin

(A–D) COS cells were transfected with an expression vector encoding neuropilin (A–C) or the empty vector (D). After two days, cells were incubated with Sema–AP (A) or SEAP (B) and then processed for alkaline phosphatase activity, or cells were fixed and subjected to immunocytochemistry using anti-neuropilin IgG (C and D). No neuropilin immunoreactivity was detected when COS cells expressing neuropilin were incubated with preimmune IgG (data not shown). Scale bar = 25 μm.

(E) Anti-neuropilin immunoblot analysis of whole cell extracts prepared from COS cells that were transfected with the empty expression vector (lane 1) or an expression vector encoding neuropilin (lane 2).

(F) Sema–AP binds directly to the extracellular domain of neuropilin. Either Sema–AP or SEAP was incubated with soluble myc-tagged neuropilin extracellular domain (myc-neuropilin^{ex}). Then, myc-neuropilin^{ex} was immunoprecipitated with an antibody directed against the myc epitope, and alkaline phosphatase activity in the immune complex was measured as described in Experimental Procedures and is reported as OD^{405}/second. Shown are the means ± SEM of three independent experiments.

COS cells expressing neuropilin (Figure 1B). Moreover, Sema–AP binding to COS cells expressing neuropilin was inhibited by myc epitope–tagged Sema III (Sema-myc), and Sema-myc bound directly to COS cells expressing neuropilin but not to untransfected COS cells (data not shown). Lastly, anti-neuropilin antibodies,

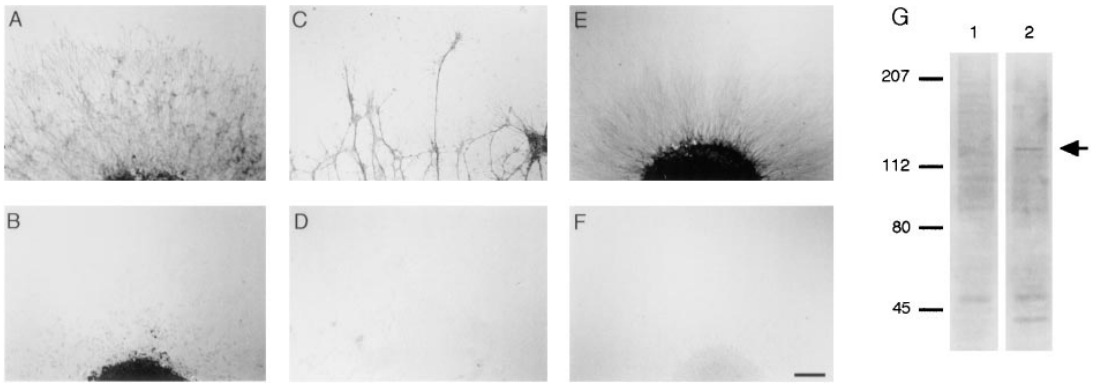

Figure 2. Sema–AP Binding Sites and Neuropilin Are Coexpressed on Growth Cones and Axons of Sema III–Responsive Neurons

DRG explants obtained from E14 rat embryos were grown in tissue culture for two days in the presence of NGF, then processed for in situ Sema–AP binding (A and C), SEAP binding (B and D), or immunocytochemistry with either anti-neuropilin IgG (E) or preimmune IgG (F). Note that both Sema–AP binding activity and anti-neuropilin immunoreactivity are detected on axons and growth cones of DRG neurons. (G) shows extracts from E14 DRG and spinal cord subjected to immunoblotting using either preimmune (1) or immune (2) IgG. A single band of ~130 kDa was detected with immune but not preimmune IgG. The ~40 kDa band is likely to be a neuropilin degredation product. Scale bar = 100 μm in (A), (B), (E), and (F); 25 μm in (C) and (D).

directed against a bacterial fusion protein that included the C-terminal MAM domain of neuropilin, detected neuropilin in COS cells transfected with a neuropilin expression vector, as shown by immunocytochemistry (ICC) and immunoblotting (Figures 1C and 1E). Together, these results demonstrate that Sema III binds to neuropilin that is expressed on the surface of COS cells.

While our results suggest that neuropilin is a Sema III–binding protein, it remained possible that Sema–AP bound to a complex of neuropilin and an endogenous COS cell protein(s), or that neuropilin induced the expression of an endogenous Sema III binding protein in COS cells. Therefore, we next asked whether Sema–AP binds directly to neuropilin in a coimmunoprecipitation assay. For these experiments, an N-terminal myc-tagged neuropilin protein lacking the neuropilin transmembrane and intracellular domains (myc-neuropilin^{ex}) was used to assess whether the extracellular domain of neuropilin can directly interact with Sema III. myc-neuropilin^{ex} was expressed in COS cells, and the tissue culture medium was then incubated with either Sema–AP or SEAP alone. Upon precipitation of the myc-neuropilin^{ex} with a monoclonal antibody directed against the myc epitope, coprecipitation of Sema–AP was determined by the presence of AP activity in the immune complex (Figure 1F). In contrast, no AP activity above background levels was detected in anti-myc immune complexes collected from samples in which myc-neuropilin^{ex} was incubated with SEAP alone. These results demonstrate that Sema III associates directly with neuropilin.

We next compared the spatial distribution of neuropilin and Sema III–binding sites present on the surface of cultured NGF-dependent DRG neurons. Sema–AP binding sites were detected all over the neurons, including their growth cones (Figures 2A and 2C). Again, SEAP alone did not bind to DRG neurons, demonstrating that Sema–AP binding was dependent on the Sema III domain, not the AP domain, of the fusion protein (Figures 2B and 2D). To assess neuropilin distribution, we first subjected extracts of E14 DRG and spinal cord to immunoblotting using our anti-neuropilin antibodies described above. These antibodies detected a single ~130 kDa band that was not observed with preimmune IgG (Figure 2G). Using these antibodies, neuropilin immunoreactivity was seen on growth cones, axons and cell bodies of cultured DRG neurons (Figure 2E).

Sema III Binds to Neuropilin with High Affinity

Because Coll-1 elicits biological effects at subnanomolar concentrations (Luo et al., 1993), we predicted that a bona fide Sema III receptor should bind to Sema III with high affinity. To determine the affinity of Sema–AP for neuropilin, neuropilin was transiently expressed in COS cells, and whole cell binding analyses were performed two days later (Figure 3A). For comparison, the affinity of Sema–AP for its receptor(s) present on NGF-dependent sensory neurons prepared from dissociated E14 DRG was also determined (Figure 3B). Sema–AP bound to COS cells expressing neuropilin with a high affinity; the calculated equilibrium dissociation constant (K_D) was approximately 1.5 nM. There were approximately 125,000 Sema–AP binding sites per COS cell. Interestingly, Sema–AP bound to DRG neurons with an equivalent affinity, and DRG sensory neurons had approximately 20,000 binding sites per cell. These binding affinities are similar to those recently described for netrins and their receptors (Keino-Masu et al., 1996; Leonardo et al., 1997), and they are consistent with a role for neuropilin in Sema III–mediated growth cone collapse and in repulsive guidance of DRG neurons during neurodevelopment. Taken together, these data suggest that neuropilin is a high affinity Sema III receptor expressed on Sema III–responsive DRG neurons.

Neuropilin Antibodies Inhibit Sema III–Mediated Repulsion of DRG Neuron Growth Cones

If neuropilin is a receptor for Sema III, then it should be possible to block neuropilin function in NGF-dependent DRG neurons and to prevent Sema III from acting as a repulsive cue. To block neuropilin function, we used our anti-neuropilin antibodies described above. In addition to immunoblotting analysis of extracts of E14 DRG and

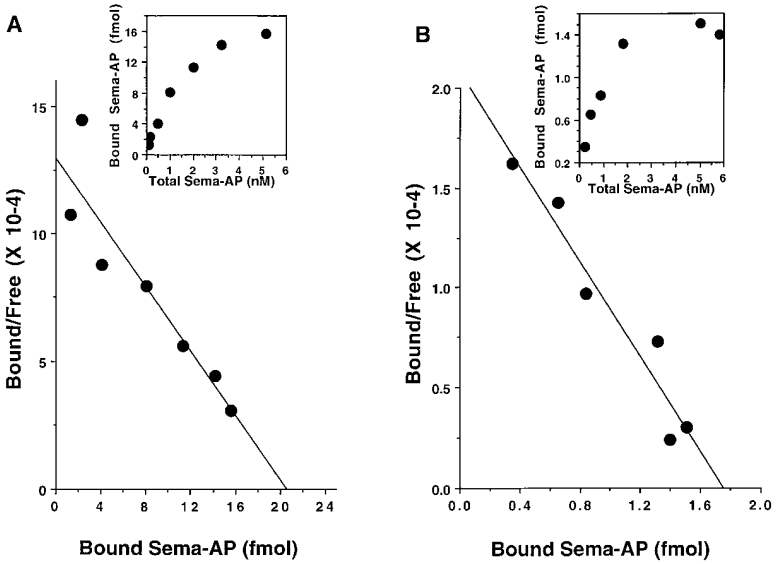

Figure 3. Scatchard Analyses of Sema–AP Bound to COS Cells Expressing Neuropilin and to DRG Sensory Neurons

Sema–AP binding analyses were performed with COS cells that were transfected with a neuropilin expression vector (A) or primary cultures of dissociated rat embryonic DRG neurons (B). Nonspecific Sema–AP binding was less than 10% of total binding as measured by Sema–AP binding to untransfected COS cells. Binding characteristics for the experiments shown were as follows: COS cells expressing neuropilin had approximately 125,000 Sema–AP binding sites per cell, and the $K_D = 1.5 \times 10^{-9}$ M. Dissociated DRG neurons had approximately 20,000 Sema–AP binding sites per cell, and the $K_D = 0.9 \times 10^{-9}$ M. Similar results were seen in at least three independent COS cell and dissociated DRG binding experiments.

spinal cord (Figure 2G), these antibodies were further assessed for specificity by immunostaining sections from E14.5 rat embryos. They specifically reacted with a protein expressed in a subset of neurons including DRG neurons, sympathetic neurons (Figure 4E), and trigeminal sensory neurons (Figure 4G). These populations of neurons also express robust levels of *neuropilin* mRNA as determined by in situ hybridization analysis (Figures 4H and 6B; Kawakami et al., 1995). No immunoreactivity was detected on tissue sections incubated with IgG purified from preimmune serum (Figure 4F). In combination with immunoblot analysis, as well as the expression pattern of the other identified member of the neuropilin family (*neuropilin-2*, see below), these data strongly suggest that these antibodies specifically recognize neuropilin in DRG neurons.

Coculturing E14 DRG and Sema III–expressing COS cells in a collagen matrix provides a robust assay for the chemorepulsive activity of Sema III on these neurons (Messersmith et al., 1995). As seen previously, NGF-dependent DRG neurons were repelled from COS cells that expressed Sema III (Figure 4A). Including anti-neuropilin antibodies, however, resulted in an inhibition of the repulsive activity of Sema III (Figure 4B). The amount of axon outgrowth on the side of the DRG adjacent to the Sema III–expressing COS cells was more than 2-fold greater in the presence of anti-neuropilin antibodies as compared to cocultures grown in the absence of added antibodies or equal amounts of preimmune IgG fraction (Figures 4C and 4D). The results of these antibody perturbation experiments indicate that neuropilin activity is required to mediate the repulsive effects of Sema III on NGF-dependent E14 DRG neurons. Since we have shown that neuropilin is a Sema III–binding protein, and since neuropilin is expressed on the axons and growth cones of these neurons, these results demonstrate that neuropilin is an endogenous receptor for Sema III.

Neuropilin Is the First Member of the Neuropilin Gene Family

The great diversity within the semaphorin family of proteins, both with respect to primary amino acid sequence and tissue distribution, led us to investigate the possibility that neuropilin defines a family of conserved semaphorin-binding proteins. A search of the dbEST database identified several human expressed sequence tags that encode proteins either identical to or related to neuropilin. Sequence information from one of these sequence tags was used for the amplification from E14 rat spinal cord/DRG cDNA of a 400 base pair PCR product that was found to encode a portion of a *neuropilin*-related gene (referred to below as *neuropilin-2*). This amplification product was used to screen an E14 rat spinal cord/DRG cDNA library. Several cDNAs containing the *neuropilin-2* open reading frame were isolated, one of which was sequenced over the entire *neuropilin-2* open reading frame (ORF) (see Experimental Procedures).

Conceptual translation of the *neuropilin-2* ORF revealed that it encodes a protein that has the same overall extracellular and intracellular organization as neuropilin (Figures 5A and 5B). Like neuropilin (Takagi et al., 1991; Kawakami et al., 1995), neuropilin-2 has (N-terminal to C-terminal) a signal sequence, an a1/a2 domain similar to the noncatalytic regions of the complement components C1r and C1s (CUB domain; Bork and Beckman, 1993), a b1/b2 domain similar to the C1 and C2 domains of coagulation factors V and VIII, a c region that contains a MAM domain, a transmembrane domain, and a short cytoplasmic domain unique to neuropilins. The length and spacing of these domains in neuropilin and neuropilin-2 are very similar. Neuropilin and neuropilin-2 share 44% amino acid identity over their entire length; however, different domains have different degrees of conservation. For example, the a1/a2 and b1/b2 domains are 55% and 44% identical, respectively, whereas the MAM portions of domain c are only 37% identical. Further, the putative transmembrane domains are 71% identical, and the cytoplasmic domains are 53% identical and of the same length. These features clearly show that *neuropilin* and *neuropilin-2* are members of a gene family encoding related proteins and have implications for their distinct roles in semaphorin signaling.

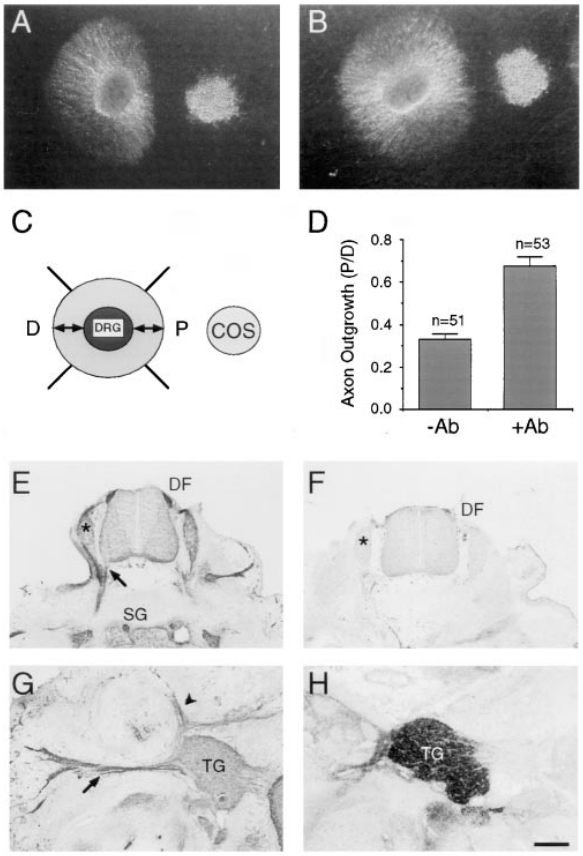

Figure 4. Neuropilin Antibodies Inhibit Sema III–Mediated Repulsion of NGF-Dependent DRG Neurons

(A and B) DRG explants were cocultured with COS cells expressing myc-Sema III and grown for 40 hr in the absence (A) or presence (B) of anti-neuropilin antibodies (100 μg/ml IgG fraction).

(C) schematic diagram depicting DRG neurons, COS cells, and parameters measured in experiments presented in (D). (P) = proximal; (D) = distal.

(D) Quantitation of the effects of anti-neuropilin antibodies on the repulsive activity of Sema III. Shown are the means ± SEM of axon outgrowth (proximal length/distal length) of DRG neurons in the coculture assay grown in the absence (−Ab) or presence (+Ab) of anti-neuropilin IgG fraction. The degree of axon outgrowth was determined in three separate experiments. Anti-neuropilin antibodies significantly inhibited the repulsive activity of Sema III as determined by a Students t test ($P < 0.0001$). The average amount of axon outgrowth on lateral sides of the explants as well as the average distance between the explants and the COS cell clumps were not different between the various groups. Although the cocultures for these experiments were grown in the presence of anti-neuropilin or in the absence of rabbit antibodies, additional experiments showed that pre-immune IgG (100 mg/ml) had no effect on the repulsive activity of Sema III ($P<0.001$; $n=21$, pre-immune IgG fraction and $n=26$, immune IgG fraction).

(E–H) Neuropilin immunoreactivity was specifically detected in neurons previously shown to express neuropilin mRNA (Kawakami et al., 1995; Figure 6). (E) shows a cross-section of an E14.5 rat spinal cord. Strong neuropilin immunoreactivity was found in DRG (asterisk) and their central and peripheral projections. The dorsal funiculus (DF) and motoraxons that leave the ventral horn (arrow; data not shown) display strong neuropilin immunoreactivity. The sympathetic chain ganglion (SG) was stained. In (F), no labeling was detected on parallel sections processed with the preimmune IgG. In (G), parasagittal sections of the head show strong neuropilin immunoreactivity in the sensory trigeminal ganglion (TG), including the ophthalmic (arrowhead) and maxillary (arrow) branches. A corresponding section stained for *neuropilin* mRNA by in situ hybridization (H) revealed very strong staining in cell bodies of the trigeminal ganglion.

Scale bar = 400 μm in (A) and (B); 300 μm in (E) and (F); 180 μm in (G) and (H).

Neuropilin and Neuropilin-2 Are Expressed in Distinct Populations of Neurons in the Developing Rat Spinal Cord

The existence of neuropilin-2 is consistent with the hypothesis that there are multiple, structurally related semaphorin receptors. Because neuropilin is present in discrete populations of neurons (Kawakami et al., 1995), and because individual semaphorins have distinct neuronal expression patterns (Luo et al., 1995; Puschel et al., 1995; Wright et al., 1995; Adams et al., 1996; Giger et al., 1996; Shepherd et al., 1996; Zhou et al., 1997), we compared the patterns of expression of *neuropilin* and *neuropilin-2* by in situ hybridization. Cross sections of E14.5 rat embryos stained for the presence of *neuropilin* mRNA displayed discrete labeling in the spinal cord and a subset of DRG neurons (Figure 6B), consistent with previous observations of *neuropilin* expression (Kawakami et al., 1995). For comparison, *sema III* spinal cord expression is shown in Figure 6A. In the ventral spinal cord, strong *neuropilin* expression was observed in motor pools and in a thin stripe of cells in the intermediolateral column. Weaker *neuropilin* expression was detected in the dorsal horn. *neuropilin* expression was not seen in the spinal neuroepithelium. In contrast, a very different expression pattern was observed for *neuropilin-2* (Figure 6C). Unlike *neuropilin*, which is strongly expressed in DRG, *neuropilin-2* expression was not detected in neurons within the DRG. Moreover, staining in the spinal cord was largely confined to the ventral horn, the intermediate grey, and a thin dorsally extending stripe of cells at the border of the neuroepithelium. Robust *neuropilin-2* expression was seen in the lateral motor pools of the ventral cord and lateral part of the basal plate neuroepithelium. The roof plate and the floor plate also showed moderate *neuropilin-2* expression. Examination of other CNS structures revealed that *neuropilin* and *neuropilin-2* are expressed in overlapping, but distinct, populations of neurons. For example, expression of *neuropilin*, but not *neuropilin-2*, was detected in the trigeminal ganglion (Figure 4, data not shown). However, expression of *neuropilin-2*, but not *neuropilin*, was observed in the accessory olfactory bulb (data not shown). Lastly, as has been observed for *neuropilin*, *neuropilin-2* expression was not restricted to the nervous system. Strong nonneuronal expression of *neuropilin-2* was detected in several tissues, including the mesenchymal tissue lining the ribs (data not shown). Together, these results demonstrate that *neuropilin* and *neuropilin-2* are expressed in overlapping yet distinct populations of neurons in the CNS and that both genes are expressed in neuronal as well as nonneuronal cells.

Discussion

We have identified the transmembrane glycoprotein neuropilin as a receptor for Sema III, a secreted chemo-

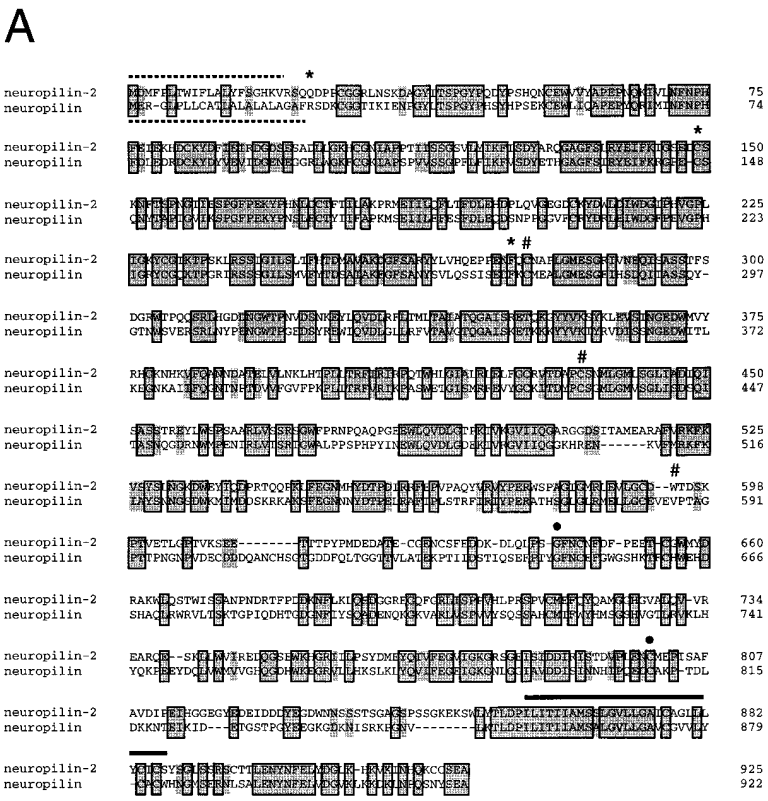

Figure 5. Comparison of the Deduced Amino Acid Sequences of Rat Neuropilin and Npn-2

(A) Putative signal sequence (dashed line), the two complement binding domains (CUB domains; between the asterisks), the two coagulation factor domains (between the number symbols), the single MAM domains (between the closed circles), and the putative transmembrane domains (solid lines) of neuropilin and neuropilin-2 are indicated.

(B) Domain alignment and amino acid identity between rat neuropilin and rat neuropilin-2. (ss) denotes the putative signal sequence; (a1) and (a2), the complement binding domains; (b1) and (b2), the coagulation factor domains; (TM), the transmembrane domain; (cy), the cytoplasmic domain.

repellent that functions in inhibitory growth cone guidance during neurodevelopment. Similar results have been obtained by Z. He and M. Tessier-Lavigne (He and Tessier-Lavigne, 1997). Sema III binds directly to neuropilin with high affinity, and the affinity of Sema III for neuropilin expressed on the surface of COS cells is equivalent to the affinity between Sema III and its binding site(s) present on Sema III–responsive DRG sensory neurons. Moreover, neuropilin is present on growth cones and axons of these neurons. Finally, antibodies directed against neuropilin inhibit the chemorepulsive activity of Sema III on NGF-dependent DRG neurons in culture. Therefore, neuropilin is a Sema III receptor and is likely to be critical for the chemorepulsive activity of Sema III in vivo. In addition, we have identified and characterized neuropilin-2, a protein related to neuropilin, showing that the neuropilins are members of a gene family encoding proteins that are differentially expressed in the developing nervous system.

Neuropilin-1 Is Expressed and Functions in Sema III–Responsive Tissues

Neuropilin (previously known as A5) was first identified as a membrane-associated glycoprotein expressed in the tectum of Xenopus laevis (Takagi et al., 1987). More recent analyses have demonstrated that mammalian, Xenopus, and avian neuropilin are present in a number of discrete neuronal populations (Kawakami et al., 1995; Satoda et al., 1995; Takagi et al., 1995). Importantly, neuropilin distribution patterns in the developing mouse nervous system support our conclusion that it is a Sema III receptor. During mouse development, neuropilin is present in several populations of neurons known to be responsive to Sema III, including DRG sensory neurons,

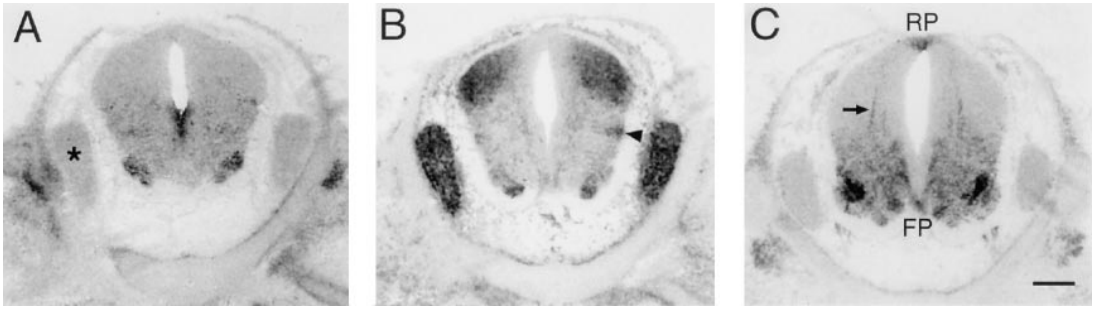

Figure 6. *neuropilin* and *neuropilin-2* Are Expressed in Distinct Populations of Cells within the Spinal Cord and DRG

In situ hybridization of cross-sections of E14.5 rat spinal cord with DIG-labeled cRNA probes specific for *semaphorin III* (A), *neuropilin* (B), and *neuropilin-2* (C).

(A) Expression of *semaphorin III* was restricted to the ventral spinal cord, including the basal plate neuroepithelium.

(B) Strong expression of *neuropilin* was observed in DRG (asterisk), motor pools in the ventral horn, the intermedolateral column (arrowhead), and the dorsal horn.

(C) *neuropilin-2* expression was detected in motor pools, the ventral horn, intermediate zone, and two dorsally extending stripes at the lateral border of the ventricular zone (small arrow). Roof plate (RP) and floor plate (FP) displayed moderate *neuropilin-2* expression. Scale bar = 150 μm.

postganglionic sympathetic neurons, trigeminal motor neurons, and spinal motor neurons (Takagi et al., 1987, 1991, 1995; Kawakami et al., 1995; Messersmith et al., 1995; Puschel et al., 1995, 1996; Shepherd et al., 1996; Varela-Echavarria et al., 1997). Neuropilin is also expressed in many other populations of developing neurons whose ability to respond to Sema III has yet to be determined. These include several cranial nerve sensory ganglia, primary olfactory neurons, and neurons within the hippocampus and neocortex.

In addition to biochemical evidence and expression patterns, genetic evidence also suggests that neuropilin is a receptor for Sema III in vivo. Transgenic mice that overexpress *neuropilin* and mutant mice with a targeted deletion of the *sema III* gene have remarkably similar phenotypes, indicating that both *neuropilin* and *sema III* are likely to contribute to the morphogenesis of a similar, if not identical, set of tissues (Kitsukawa et al., 1995; Behar et al., 1996). In the nervous system, loss of *sema III* function and overexpression of neuropilin produce defects in DRG sensory afferent projections in the spinal cord. In addition, neuropilin overexpression results in defasciculation and ectopic sprouting of spinal motor nerves in regions where nonneuronal Sema III is likely to function as a guidance cue (Wright et al., 1995; Giger et al., 1996). Further, *sema III* mutant and neuropilin overexpressing mice have similar cardiovascular and bone defects. These phenotypes, coupled with the expression of *neuropilin* and *sema III* in these tissues (Kawakami et al., 1995; Kitsukawa et al., 1995; Wright et al., 1995; Giger et al., 1996), suggest that both gene products function in a common signaling pathway. In addition to providing indirect, yet compelling, support for our conclusion that neuropilin is a receptor for Sema III in vivo, these data show that Sema III and neuropilin influence development of both neuronal and nonneuronal cells.

Neuropilins and Semaphorin Signaling

Secreted and transmembrane semaphorins are likely to affect neurodevelopment, at least in part, through their influence on repulsive growth cone steering decisions (secreted semaphorins: as described above; transmembrane semaphorins: H.-H. Yu., H. Araj, S. Ralls, and A. K., unpublished data). In vitro, application of Coll-1 to NGF-dependent DRG neurons induces collapse of their growth cones, and this event is mediated by changes associated with the actin cytoskeleton within the growth cone. Growth cones exposed to Coll-1-enriched membrane extracts undergo a loss of F actin at their leading edges relative to their centers that is not accompanied by alterations in intracellular Ca^{2+} levels (Ivains et al., 1991; Fan et al., 1993). Coll-1–induced growth cone collapse is pertussis toxin (PTX)–sensitive, though at present it is unclear whether this effect is directly mediated by ADP ribosylation of G proteins (Goshima et al., 1995; Kindt and Lander, 1995). Our finding that neuropilin is a Sema III receptor suggests that Sema III intracellular signaling does not proceed directly through a G protein–coupled mechanism. Recently, a gene encoding collapsin response mediator protein (CRMP-62) was cloned and found to be necessary both for mediation of Coll-1–induced inward ion currents in oocytes and for the activity of Coll-1 on DRG neurons (Goshima et al., 1995). CRMP-62 is a member of a family of related intracellular proteins that includes four members variously expressed in the developing and adult rat nervous system, and also the C. elegans protein UNC-33 protein, which is required for axonal elongation and fasciculation (McIntire et al., 1992; Wang and Strittmatter, 1996). The mechanism by which CRMP-62 mediates Coll-1 or Sema III effects on DRG growth cones is unknown.

Neuropilin binds Sema III in the extracellular environment of Sema III–responsive growth cones, and it participates, possibly directly, in propagation of the Sema III signal to the intracellular components that influence actin-based changes in growth cone morphology. The extracellular portions of neuropilin and neuropilin-2 consist of three motifs found in other transmembrane proteins: the complement binding domains (CUB), the coagulation factor domains, and the MAM domains. One or all of these domains may be important for semaphorin binding or for other neuropilin functions (Hirata et al., 1993; Takagi et al., 1995). Our finding that anti-neuropilin

antibodies generated against the neuropilin MAM domain inhibited the repulsive activity of Sema III on NGF-dependent DRG neurons suggests that this domain participates in Sema III binding. The MAM domain is found in a diverse group of proteins that includes metalloendopeptidases, receptor protein tyrosine phosphatases, a class C macrophage–specific scavenger receptor, as well as the neuropilins (Beckmann and Bork, 1993; Pearson et al., 1995).

The mechanism by which neuropilin transmits the Sema III signal to the interior of the growth cone remains unknown. The intracellular domain of neuropilin is short and contains no motifs with obvious catalytic function nor any domains that offer clues regarding the mechanism of Sema III signal transduction. However, because the intracellular domains of neuropilin and neuropilin-2 are similar with respect to both primary sequence and length, it is likely that they share a common signaling mechanism. Whether neuropilin functions alone or as part of a receptor complex to propagate the Sema III signal remains to be determined. Future studies will provide insight into the biochemical interactions between neuropilin, CRMP-62, and other signaling molecules that influence cytoskeletal dynamics of growth cones upon encountering Sema III.

Neuropilin and Neuropilin-2 Define a Gene Family

Sema III is one member of a large family of phylogenetically conserved proteins with diverse patterns of neuronal and nonneuronal expression (Kolodkin, 1996; Puschel, 1996). It is likely that these proteins participate in many aspects of development. Therefore, it is important to identify receptors for all of the semaphorins to begin to determine their mechanisms of action in target cells. Our identification of neuropilin-2 (and unpublished data from H. Chen and M. Tessier-Lavigne, personal communication) provides evidence for the existence of a family of neuropilin receptors and for a model in which at least two neuropilin receptors mediate the cellular responses of semaphorin family ligands.

Neuropilin and neuropilin-2 are closely related proteins that share a common domain structure and a significant degree of amino acid identity throughout their entire length. Although we have not shown that neuropilin-2 can bind to any semaphorin family member, the conserved structure and its embryonic expression pattern strongly support the idea that neuropilin-2 is a semaphorin receptor. Ongoing studies on ligand specificity for neuropilin-2 and the requirement for neuropilin-2 during neurodevelopment will address this issue.

In summary, we have identified the transmembrane glycoprotein neuropilin as a cell surface receptor for the secreted semaphorin family member, Sema III. This observation is corroborated by neuropilin and *sema III* expression data as well as the results of recent genetic experiments. In addition, we have identified a neuropilin family member, neuropilin-2, supporting a model in which multiple, distinct, semaphorin receptors mediate the diverse cues provided by semaphorins. It will be important to identify physiological combinations of ligand–receptor interactions between semaphorins and the neuropilins, and also to characterize the mechanism by which neuropilins propagate the semaphorin signal to the intracellular machinery that influences steering decisions of advancing growth cones and axonal projections.

Experimental Procedures

Generation of Sema–AP, Myc-Neuropilin^{ex}

To generate the H-Sema III–alkaline phosphatase fusion protein (Sema–AP) expression vector, the human Sema III coding sequences (Kolodkin et al., 1993) were inserted into the HindIII and BglII sites of pAPtag-1 (Flanagan and Leder, 1990) to generate a Sema–AP fusion. Then, the entire Sema–AP sequence was excised from the pAPtag-1 vector and inserted into the HindIII and XhoI sites of pCEP4, an expression vector designed to provide high level expression in the EBNA subclone of 293 cells (Invitrogen). Myc epitope–tagged, secreted neuropilin (myc-neuropilin^{ex}) expression construct was generated as follows: A 2.5 kb fragment of *neuropilin* lacking the coding determinants of the transmembrane and intracellular domains was obtained by PCR using the entire *neuropilin* ORF sequence in pcDNA3 (Invitrogen) as a template. The PCR fragment was digested with EcoRI and XbaI and subcloned into a pBluescript vector containing a Kozak consensus sequence, myc epitope tag, and signal sequence originating from peptidylglycine α–amidating monooxygenase (PAM) (a gift of Richard Mains and Ruth Marx). This plasmid was digested with NotI, SalI, and ScaI, and a fragment encoding the Kozak consensus sequence, PAM signal sequence, myc epitope tag, and the entire extracellular domain of neuropilin was isolated. This fragment was then cloned into the NotI and SalI sites of the pCIneo mammalian expression vector (Promega).

Expression Library Construction and Screening

Polyadenylated RNA isolated from embryonic day 14 rat spinal cord and associated dorsal root ganglia was used to generate cDNA (ZAP-cDNA Synthesis Kit; Stratagene). Subsequently, the cDNA was size-fractionated, and cDNA within fractions containing the largest fragments was ligated into the pMT21 COS cell expression vector (a modified version of pMT2 [Sambrook et al., 1989; Serafini et al., 1994]). The ligation products were transformed into E. Coli (ElectroMAX DH10B; GIBCO-BRL), and approximately 750 bacterial colonies were grown on 140 separate plates and harvested to generate 140 pools of cDNAs. Plasmid DNA was isolated from each pool using the Wizard DNA purification system (Promega), and then, each cDNA pool was independently transfected into COS cells (1×10^5 cells per 35 mm well of cells) using a Lipofectamine-mediated DNA transfection procedure (GIBCO-BRL). Two days after transfection, cells were incubated with Sema–AP, and bound Sema–AP was visualized following an alkaline phosphatase assay done essentially as described (Flanagan and Leder, 1990). Several pools containing one or more plasmids capable of conferring Sema III–AP binding activity were identified, and these pools were used to generate successively smaller plasmid pools followed by transfection and Sema III–AP binding assays. Ultimately, transfection of a single cDNA clone conferred Sema–AP binding activity in transfected COS cells. This clone, encoding rat neuropilin, was sequenced on both strands using the fluorescent di-deoxy terminator method of cycle sequencing on a Perkin Elmer Applied Biosystems Division 373a automated DNA sequencer.

Cell Surface Binding Analysis

COS cells were transfected with 2 μg of pMT21-neuropilin, an expression vector encoding neuropilin, or either no DNA or the empty pMT21 expression vector using Lipofectamine (BRL), recovered in growth media, and then grown for 48 hr prior to binding analysis. Dissociated DRG neurons were cultured from E14 DRGs. Briefly, DRG neurons were dissociated in a solution containing trypsin (0.05%), and the dissociated neurons were washed to remove trypsin and then plated on collagen-coated tissue culture plates (400,000 cells/35 mm plate). Cells were grown in DRG growth medium (88% MEM, 10% FBS, 0.2% glucose, glutamine [2 mM] and NGF [30 ng/ml]) and subjected to binding analysis four days after

plating. Quantitiative cell surface binding was done essentially as described (Flanagan and Leder, 1990).

Coprecipitation of Sema–AP and the Secreted, Extracellular Domain of Neuropilin

COS cell supernatant containing myc-neuropilin^{ex}, 293 EBNA cell supernatant containing SEAP, or 293 EBNA cell supernatant containing Sema–AP was filter-sterilized and concentrated. Samples containing equal amounts of control supernatants or supernatants containing myc-neuropilin^{ex} were mixed with samples containing either Sema–AP or SEAP (equal amounts of AP activity). These mixtures were incubated at room temperature for 2 hr. Then, an equal volume of an immunoprecipitation buffer (20 mM Tris [pH 8.0], 140 mM NaCl, 0.5 mM EDTA, and 2% NP-40) was added to each mixture, and the samples were centrifuged at 15,000 × g for 15 minutes at 4°C. Supernatants were recovered, and 4 μl of anti-myc antibody (antibody 9E10 ascites fluid) was added to each, and samples were incubated with mixing at 4°C for 2 hr. Then, 50 μl of protein G–Sepharose was added to each tube, and immune complexes were collected after 1 hr. Immune complexes were washed three times with immunoprecipitation buffer, once with PBS, and then the immune complexes were resuspended in PBS. Liquid alkaline phosphatase assays were performed as described above. Background was defined as the amount of AP activity detected in samples in which myc-tagged myc-neuropilin^{ex} was omitted from the sample incubations, and this value was subtracted from all other measurements.

In Situ Hybridizations

Nonradioactive, digoxigenin (DIG-11-UTP)-labeled cRNA probes with either sense or antisense orientation were synthesized by run-off in vitro transcription using T3 and T7 RNA polymerases (Boehringer Mannheim). Probes were generated from three different cDNA templates, rat *sema III* cDNA (entire coding region), the extracellular domain of *neuropilin* (nucleotides 181–2755 of the coding sequence), and a 2.5 kb fragment of *neuropilin-2* (downstream of nucleotide 1866). Cryosections (20 μm) of E14.5 rat embryos (plug day was E1) were cut at −15°C in a Reichert-Jung cryostat and processed for in situ hybridization essentially as described (Giger et al., 1996).

Neuropilin Antisera Production and Immunoblot Analysis

Anti-neuropilin antibodies were produced by immunizing rabbits with a 6-histidine–tagged neuropilin protein that was produced in E. coli. The bacterial expression construct was made by PCR amplification of a fragment encoding amino acids 583–856 of rat neuropilin and inserted into the EcoRI and HindIII sites of pTrcHisA (Invitrogen). Expressed protein was purified by immobilized nickel-chelate affinity chromatography. Rabbits were immunized with 375 μg of protein in complete Freunds adjuvant and boosted every 2–3 weeks with 250 μg of protein in incomplete Freunds adjuvant. Serum was collected and the IgG fraction was purified by protein A–Sepharose chromatography. Immunoblot analysis was performed as described (Ginty et al., 1994), using extracts of neuropilin-transfected and -untransfected COS cells, and E14 DRG and spinal cord.

Immunohistochemistry

E14.5 rat embryos were fixed for 4 hr in ice cold PBS containing 4% paraformaldehyde and cryoprotected overnight in the same solution containing 15% sucrose. Immunocytochemistry of cryosections (20 μm) using either immune or preimmune rabbit anti-neuropilin, IgG fraction (0.7 μg/ml) was done as described (Kawakami et al., 1995).

***Neuropilin-2* Identification and Molecular Analysis**

A search of the dbEST data base of human expressed sequence tags identified two overlapping clones, GenBank accession numbers AA057388 and AA057680, that displayed sequence similarity to the 3′ end of the *neuropilin* ORF. Degenerate 5′ (TTC/TGAA/GGGIGAA/GATA/C/TGGNAAA/GGG; corresponding to the amino acid residues FEGEIGKG) and 3′ (NAGT/CTCG/AAAG/ATTG/AATG/ATTT/CTC; corresponding to amino acid residues ENYNFEL) oligonucleotides were used for PCR amplification, using 10 ng of E14 rat spinal cord/DRG cDNA and employing 45 amplification cycles (96°C for 1 min; 50°C for 1 min; 72°C for 1 min). Amplification products were cloned into pCRII (Invitrogen) and sequenced. One 400 base pair (bp) amplification product encoded a *neuropilin*-related sequence and was used to screen a rat E14 DRG/spinal cord Lambda Zap II (Stratagene) cDNA library. Several positive clones were isolated, and one 6 kb clone was found to contain the entire *neuropilin-2* ORF. Then, 3371 bp of this clone, including the *neuropilin* ORF, were sequenced on both strands. Alignment of neuropilin and neuropilin-2 sequences was performed using Gene Works (Intelligenetics).

Explant Cocultures and Inhibition of Sema III Activity

E14 DRG and Sema III–expressing COS cells were cocultured for 40 hr as described (Messersmith et al., 1995), except that the culture media was 25% F12 media, 69% OPT-MEM media, 0.04 M glucose, 2 mM glutamine, 0.5% heat inactivated fetal calf serum, and NGF (15 ng/ml). Media was supplemented with either anti-neuropilin or preimmune IgG (100 μg/ml). DRG explants and *H-sema III*–expressing COS cell aggregates were placed ~700 μm apart. For quantitation, the region of neurite growth was divided into four quadrants, as diagrammed in Figure 4C. Neurite outgrowth into the collagen gel was measured from the outer border of each DRG to the perimeter of the bulk of neurites as described (Messersmith et al., 1995). Fixed cocultures were visualized under phase contrast optics on a Zeiss Axiovert 100 inverted microscope and scored blindly by three independent observers. DRG explants with less than 200 μm of outgrowth on the distal side were not scored. Statistical analysis was performed using a Student's t test.

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GenBank Accession Numbers

The accession numbers for rat *neuropilin* and rat *neuropilin-2* are AF016296 and AF016297, respectively.