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Plexin-Neuropilin-1 Complexes Form Functional Semaphorin-3A Receptors

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Summary

Class 1 and 3 semaphorins repulse axons but bind to different cell surface proteins. We find that the two known semaphorin-binding proteins, plexin 1 (Plex 1) and neuropilin-1 (NP-1), form a stable complex. Plex 1 alone does not bind semaphorin-3A (Sema3A), but the NP-1/Plex 1 complex has a higher affinity for Sema3A than does NP-1 alone. While Sema3A binding to NP-1 does not alter nonneuronal cell morphology, Sema3A interaction with NP-1/Plex 1 complexes induces adherent cells to round up. Expression of a dominant-negative Plex 1 in sensory neurons blocks Sema3A-induced growth cone collapse. Sema3A treatment leads to the redistribution of growth cone NP-1 and plexin into clusters. Thus, physiologic Sema3A receptors consist of NP-1/plexin complexes.

Introduction

The semaphorins are a large family of proteins sharing a sema homology domain of approximately 550 amino acid residues (aas; Kolodkin et al., 1993). Based on associated features such as transmembrane carboxyl segments, immunoglobulin domains, thrombospondin repeats, or glycophosphatidylinositol linkage sites, the family has been divided into eight classes (Semaphorin Nomenclature Committee, 1999). It is likely that they exert a number of effects in vivo ranging from axonal guidance to cell migration to vascular morphogenesis to immunologic modulation to neoplastic transformation (Kolodkin et al., 1993; Behar et al., 1996; Tessier-Lavigne and Goodman, 1996; Comeau et al., 1998; Soker et al., 1998; Eickholt et al., 1999). The class 3 semaphorins, exemplified by Sema3A (chick collapsin-1, SemD, Sema III), are secreted proteins with potent axon repulsive activity that can abruptly collapse certain growth cones (Luo et al., 1993). A repulsive role for Sema3A in vivo is supported by the excessive peripheral dorsal root ganglion (DRG) axon growth seen in *Sema3A*$^{-/-}$ mice (Taniguchi et al., 1997).

Neuropilin-1 (NP-1) and neuropilin-2 (NP-2) have been identified as high-affinity binding sites for class 3 semaphorins (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Sema3A binds only to NP-1 (Chen et al., 1997). In contrast, Sema3B, Sema3C, and Sema3F bind to both NP-1 and NP-2 (Chen et al., 1997; Takahashi et al., 1998). They function as agonists at NP-2 sites on sympathetic neurons and as antagonists of NP-1 sites on DRG neurons (Chen et al., 1998; Giger et al., 1998; Takahashi et al., 1998). Antibody perturbation, gene knockout, and gene transfer studies have confirmed that NP-1 is required for Sema3A function (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Takahashi et al., 1998). Structure–function analysis of NP has revealed that the amino-terminal CUB (complement-binding protein homology) domain functions as a Sema-binding site (Chen et al., 1998; Giger et al., 1998; Nakamura et al., 1998). The transmembrane and cytoplasmic domains are not required for sema signaling through NP-1 (Nakamura et al., 1998). Instead the extracellular, juxtamembrane MAM (meprin, A5, μ) domain is crucial, implicating a second transmembrane protein as the transducing protein for Sema3A action (Nakamura et al., 1998). The identity of this transducing protein has not been defined, but downstream intracellular mediators include CRMP and Rac1 (Goshima et al., 1995; Jin and Strittmatter, 1997). In addition, intracellular guanosine 3′, 5-cyclic monophosphate (cGMP) levels influence growth cone steering by Sema3A (Song et al., 1998).

A second semaphorin-binding protein, plexin, has been identified more recently (Comeau et al., 1998; Winberg et al., 1998). Expression screening for the cellular binding sites of a pox virus–derived semaphorin yielded VESPR, a plexin (Comeau et al., 1998). In *Drosophila melanogaster*, genetic data implicate plexin A as a receptor for Sema-1a/b with axon defasciculating activity (Winberg et al., 1998). Furthermore, expression of plexin A in heterologous cells creates high-affinity Sema-1a and -1b binding sites (Winberg et al., 1998). NP is not required for fly Sema-1a/b binding to plexin A. Fly plexin A does not bind vertebrate class 3 or 4 semaphorins (Winberg et al., 1998). Interestingly, plexin shares sequence similarity with the conserved sema domain of semaphorins, suggesting that these families derive from a common evolutionary ancestor with homophilic binding properties (Winberg et al., 1998). The plexin family is comprised of at least nine members in vertebrates (Kameyama et al., 1996; Maestrini et al., 1996; Comeau et al., 1998; Tamagnone et al., 1999 [this issue of *Cell*]).

The molecular analysis suggests two independent semaphorin signaling cascades, one initiated by Sema3A and NP, and the other by Sema1 and plexins. However, several factors suggest that two pathways might not be fully independent. The physiologic actions of NP-binding semaphorins and plexin-binding semaphorins

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are similar. In both cases, axons are repelled either from areas expressing soluble ligand or from adjacent axons (Luo et al., 1993; Taniguchi et al., 1997; Winberg et al., 1998). Furthermore, Sema3A-responsive DRG neurons contain both NP-1 and a plexin (Plex 3; Maestrini et al., 1996). In addition, the sema domain of plexin might be expected to possess affinity for NP directly.

To explore the relationship between these two semaphorin-binding proteins, we sought to characterize any molecular interactions between NP and plexin. We find that NP-1 and Plex 1 form a complex with enhanced affinity for Sema3A. Furthermore, this NP-1/Plex 1 complex, but neither protein alone, can mediate a morphologic response to Sema3A in nonneuronal cells. A mutant of Plex 1 lacking the intracellular domain interacts with NP-1 and disrupts Sema3A signaling in DRG neurons. These data suggest that all semaphorin signaling may occur via plexins. The role of NP may be to enhance binding affinity for soluble Sema3A, much as NP-1 plays a facilitory role in VEGF signaling through some VEGF receptors (Soker et al., 1998).

Results

Plexin 1 and NP-1 Form a Complex

We first considered whether there was a direct interaction between mouse NPs and Plex 1. Transfection of HEK293T cells yields coexpression of HA-tagged NPs and Myc-tagged Plex 1. Plex 1 can be immunoprecipitated with immobilized anti-Myc antibody. Anti-HA immunoblots of Plex 1 immunoprecipitates demonstrate the presence of coexpressed NP-1 or NP-2 (Figure 1). The binding of Sema-1a/b to Plex A is known to require divalent cations (Winberg et al., 1998), but chelation of divalent cations with EDTA plus EGTA does not alter the extent of NP-1/Plex 1 coprecipitation. The specificity of the NP-1/Plex 1 association is demonstrated by total protein stains of material precipitated with antibodies directed against the native proteins (Figure 1B). The stoichiometry of the NP-1/Plex 1 complex is close to 1:1.

Sequence homologies suggest that the sema domain of Plex 1 might bind to the semaphorin-binding site in NP-1. If so, Sema3A might compete with Plex 1 for binding to NP-1. However, the addition of Sema3A does not alter the degree of coprecipitation (Figure 1A), nor does Plex 1 coexpression block Sema3A binding to NP-1 (Figure 2). Furthermore, a NP-1 mutant (0111; Nakamura et al., 1998), lacking the ability to bind the sema domain of Sema3A, still coprecipitates with Plex 1 (data not shown). Thus, the formation of Plex 1/NP-1 complexes cannot be explained solely through a semamimetic activity of Plex 1 at NP-1.

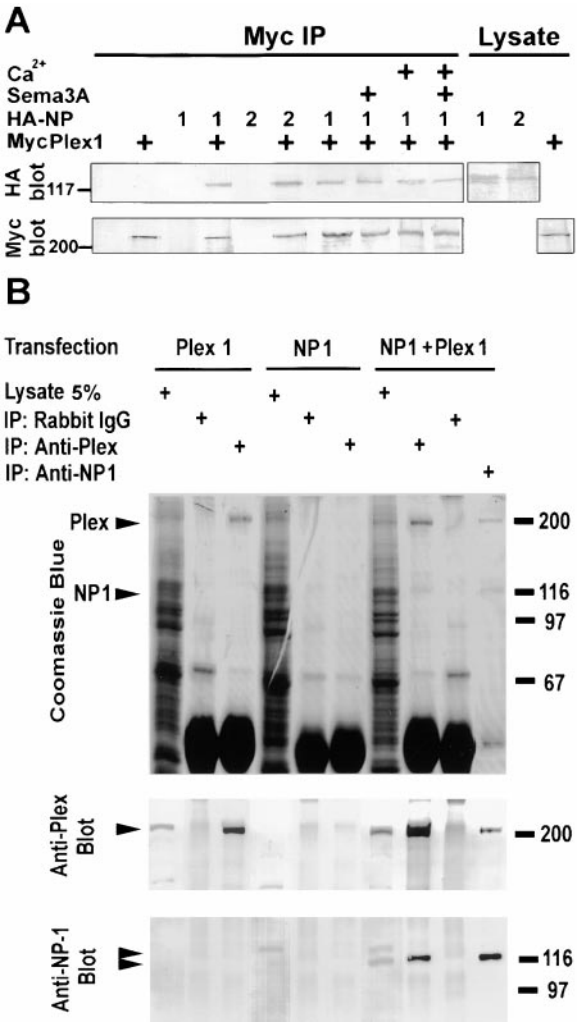

Figure 1. Neuropilins Immunoprecipitate with Plex 1

(A) HEK293T cells were transfected with HA-NP-1, HA-NP-2, or vector together with Myc-Plex 1 or vector. Cell lysates were then immunoprecipitated with anti-Myc antibody and blotted with anti-HA or anti-Myc antibody. Note that HA-NP-1 and HA-NP-2 are coprecipitated with Myc-Plex 1. This association is not influenced by Sema3A or divalent cations. The estimated molecular weights of HA-NP-1, HA-NP-2, and Myc-Plex 1 are 120, 120, and 250 kDa, respectively. Molecular size markers (in kDa) are shown at left.

(B) HEK293T cells were transfected with untagged NP-1 or vector together with myc-Plex 1 or vector. Cell lysates were then immunoprecipitated with anti-NP-1 antibody or anti-Plex 1 antibody and analyzed by Coomassie blue staining or immunoblot. Either 5% of the original lysate or the entire immunoprecipitate was analyzed. The positions of Plex 1 and NP-1 are shown by arrowheads at left. Note that NP-1 and Plex 1 are the major proteins other than Ig in the immunoprecipitates. The NP-1 doublet resolved on these gels is likely to reflect differential glycosylation and has been observed in previous studies (Nakamura et al., 1998). Molecular size markers (in kDa) are shown at right.

Plex 1 Increases NP-1 Affinity for Sema3A

In considering the ability of Sema3A and Plex 1 to compete for binding to NP-1, we noted that NP-1/Plex 1 coexpressing cells possess a 6-fold higher affinity for an alkaline phosphatase (AP) fusion protein of Sema3A than do NP-1-expressing cells (Figure 2). Plex 1 coexpression does not alter the B_{max} for AP-Sema3A binding (Figure 2) or the expression of NP-1 protein by immunoblot analysis (Figure 1). Plex 1 alone exhibits no detectable affinity for AP-Sema3A. The affinity of the NP-1/Plex 1 complex for Sema3A matches more closely than does that of NP-1 alone with the 50 pM EC_{50} of Sema3A-induced growth cone collapse in DRG cultures

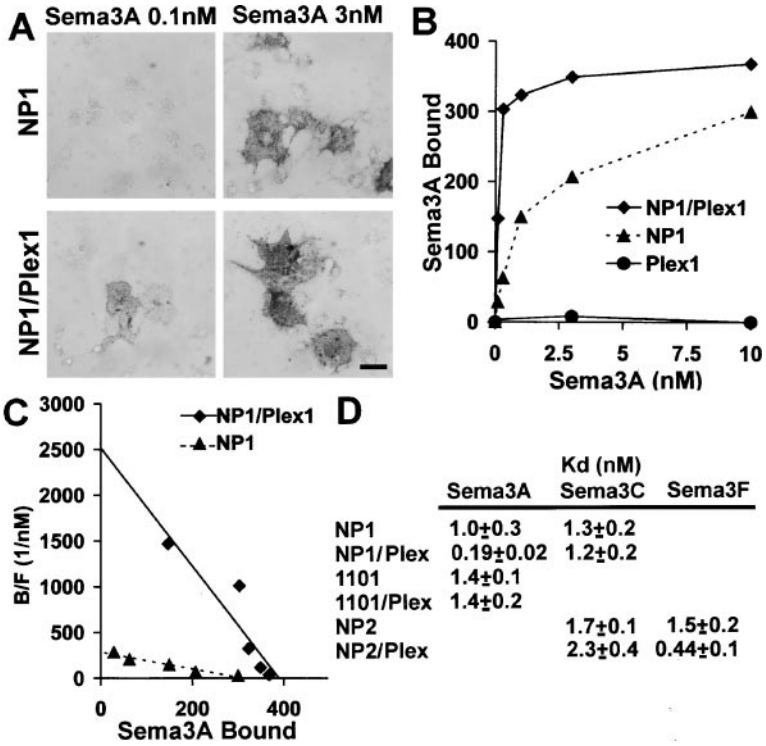

Figure 2. Plex 1 Increases NP-1 Affinity for Sema3A

(A) AP-Sema3A staining of transfected cells. COS-7 cells were transfected with expression vector for NP-1, Plex 1, or both, and then AP-Sema3A binding was visualized histologically. Sema3A binding to NP-1/Plex 1–expressing COS-7 cells is indistinguishable from NP-1-expressing COS-7 cells at saturating concentrations of ligand (3 nM). However, low concentrations of Sema3A (0.1 nM) bind preferentially to NP-1/Plex 1–expressing COS-7 cells. Scale bar, 70 μm.

(B) Saturation of AP-Sema3A binding. NP-1/Plex 1–expressing COS-7 cells exhibit higher binding affinity for Sema3A than do NP-1-expressing COS-7 cells. Plex 1 does not bind to AP-Sema3A. One of five experiments with similar results is shown.

(C) Scatchard analysis of Sema3A binding to COS-7 cells. The K_Ds for Sema3A binding to NP-1/Plex 1- and NP-1-expressing COS-7 cells are 189 ± 18 pM and 1016 ± 287 pM, respectively (mean ± SEM for n = 5). One of five experiments with similar results is shown.

(D) The affinity of semaphorin binding to various NP/Plex 1 complexes. Data from experiments as in (C) are summarized. All data are the means ± SEM for n ≥ 3.

(Takahashi et al., 1998). This suggests that a NP-1/plexin complex, rather than NP-1 alone, reflects physiologically relevant growth cone binding sites for Sema3A.

These data raise the possibility that Plex 1 might be the previously unidentified signaling molecule downstream of Sema3A/NP-1 complexes. For G protein–coupled receptors, it has been shown that the affinity of agonists, but not antagonists, is altered by the presence and activation state of downstream heterotrimeric G proteins (Gilman, 1987). We considered the relative effect of Plex 1 on the affinity of NP-1 for an antagonist. Previous studies demonstrated that Sema3C binds as an antagonist to NP-1 and does not mediate growth cone collapse in DRG neurons (Takahashi et al., 1998). The calculated K_D for Sema3C binding to NP-1-expressing COS-7 cells is indistinguishable from that of NP-1/Plex 1–expressing COS-7 cells (Figure 2). The specificity of the Plex 1–induced affinity shift of NP-1 for the agonist Sema3A is consistent with Plex 1 serving as the transmembrane signal–transducing protein for Sema3A/NP-1 complexes.

To provide further evidence that the Sema3A affinity shift induced by Plex 1 reflects a conformational change relevant to physiologic signaling, we examined a nonsignaling form of NP-1. The NP-1 mutant lacking a MAM domain (1101) binds Sema3A tightly but cannot couple to downstream signal transduction systems in chick retinal ganglion cell growth cones (Nakamura et al., 1998). For this form of NP-1, coexpression of Plex 1 produces no alteration in Sema3A affinity (Figure 2D), extending the correlation of an affinity shift with an agonist conformation.

Previous experiments have suggested that Sema3C is an agonist for NP-1/2 heterodimers but not for either NP-1 or NP-2 homodimers, while Sema3F is an agonist for NP-2 homodimers (Chen et al., 1998; Giger et al., 1998; Takahashi et al., 1998). To consider the correlation of Plex-induced affinity shifts with agonist/receptor complexes across the Sema3 family, we assessed the effect of Plex 1 coexpression on Sema3C and Sema 3F binding to NP-2 (Figure 2D). Sema3C binds with equal affinity to NP-2- and NP-2/Plex 1–expressing cells, while Sema3F has increased affinity for NP-2/Plex 1–expressing cells. Overall, the Plex 1–induced Sema3 affinity shift is very well correlated with current knowledge of the molecular determinants of biologically active Sema3/NP complexes.

Plex 1/NP-1 Complexes Are Sufficient for Sema3A-Induced Morphological Changes

Sema3A binding to NP-1 in DRG growth cones produces a dramatic morphologic effect, growth cone collapse, but results in no detectable morphologic change in non-neuronal COS-7 cells. Presumably, COS-7 cells lack necessary signal transduction components. Given the data suggesting that NP-1/Plex 1 complexes form physiologic Sema3A-binding sites, we considered the possibility that this complex might be adequate to mediate a morphologic response in COS-7 cells. Cells expressing NP-1, NP-1 plus Plex 1, or Plex 1 were incubated with 3 nM Sema3A-AP or AP for 1 hr at 37°C or 4°C. Transfected COS-7 cells were visualized by AP staining or epitope tag immunostaining. NP-1/Plex 1 coexpressing cells exhibit a dramatically reduced cell area after incubation with Sema3A at 37°C (Figure 3). To simplify the quantitation of these changes, cells were classified as possessing cell areas greater than or less than 1600 μm^2. In contrast to NP-1/Plex 1–expressing cells, NP-1-expressing, Plex 1–expressing, and control cells show

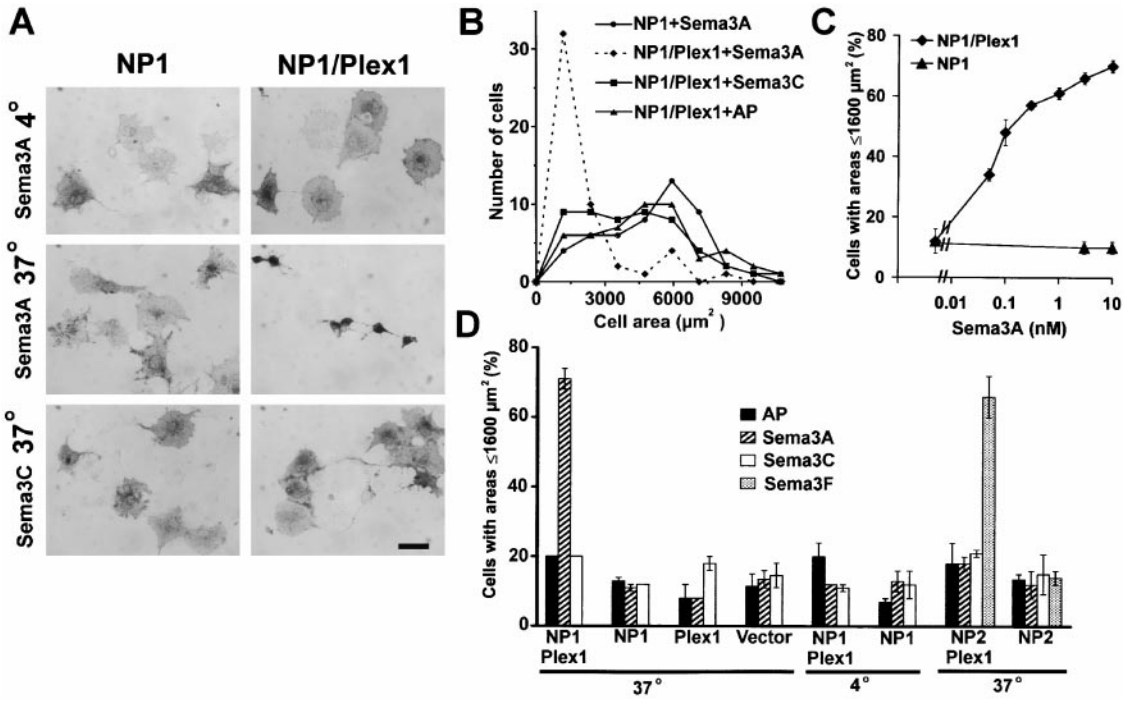

Figure 3. Plex 1/NP-1 Complexes Are Sufficient for Sema3A-Induced Morphological Change

(A) AP-Sema3A staining of transfected COS-7 cells. Cells transfected with NP-1 or NP-1/Plex 1 were incubated with the 3 nM Sema3A-AP or Sema3C-AP for 1 hr at 37°C or 4°C. Sema3A treatment at 37°C reduces the size of cells expressing NP-1/Plex 1 but not NP-1 only, as visualized by AP staining. This morphological change is not observed after incubation at 4°C. Sema3C does not induce the morphological change. Scale bar, 70 μm.

(B) The distribution of the cellular areas. COS-7 cells were transfected and treated as in (A) using the indicated plasmids and proteins. The area for 50 consecutive NP-expressing cells is plotted. There is an increase in the proportion of small cells in the Sema3A-treated NP-1/Plex 1–expressing cultures as compared to Sema3A-treated NP-1-expressing COS-7 cells and to Sema3C- or AP-treated NP-1/Plex 1 transfectants. On the basis of this distribution, 1600 μm^2 was utilized as a definition of contracted cells.

(C) Sema3A dose–response curve for the morphological change of NP-1/Plex 1 or NP-1 transfectants at 37°C. The means ± SEM from five experiments are presented.

(D) The percentage of cells with an area less than 1600 μm^2. Cells were transfected and treated with the indicated plasmids and proteins as in (A). The means ± SEM from three to five experiments are presented.

no Sema3A-induced morphologic change. The Sema3A-induced reduction in the size of NP-1/Plex 1–expressing cells is temperature dependent, not occurring during incubation at 0°C. The contraction of the cell perimeter occurs at physiologic concentrations of Sema3A, with an EC_{50} of 100 pM. The morphologic change proceeds over the first 5–30 min after Sema3A addition (data not shown). During the 1 hr incubation, the vast majority of expressing cells remains attached to the substratum, with at most a 5%–25% reduction of total cell number in any group (data not shown). It is clear from these experiments that the NP-1/Plex 1 complex can mediate a morphologic response in nonneuronal COS-7 cells. Many attributes of Sema3A-induced contraction of these cells resemble Sema3A-induced growth cone collapse. This provides further support for the notion that Plex 1 is the transmembrane protein through which Sema3A/NP-1 complexes signal to the neuronal growth cone cytoplasm.

This assay exhibits a high degree of specificity. The NP-1 antagonist Sema3C does not produce this reduction in the cellular area of NP-1/Plex 1–expressing cells (Figure 3D). As suggested above by the affinity shift experiments (Figure 2D), NP-2/Plex 1 expression provides a functional Sema3F receptor but not a functional Sema3C receptor in COS-7 cells (Figure 3D).

NP-1 MAM Domain Is Required for Sema3A-Induced Cell Contraction

If the morphologic changes in NP-1/Plex 1–expressing COS cells are reflective of physiologic mechanisms in axonal growth cones, then similar regions of NP-1 should be required for both responses. In COS-7 cells, as in chick retinal ganglion cells, the GPI-anchored NP-1 mutant that lacks transmembrane/cytoplasmic domains (111G mutant) is as active as the full-length NP-1 (Figures 4A and 4B). In contrast, the NP-1 mutant lacking the MAM domain (1101) does not support Sema3A-induced COS cell contraction when coexpressed with Plex 1 (Figures 4A and 4B). Since the MAM domain of NP-1 contributes to NP oligomerization as well as neuronal signal transduction, we considered the possibility that the inability of 1101 to support Sema3A-induced contraction of Plex 1–expressing COS cells is a result of a failure to complex with Plex 1. After cotransfection of HEK293T cells, HA-tagged 1101 coimmunoprecipitates

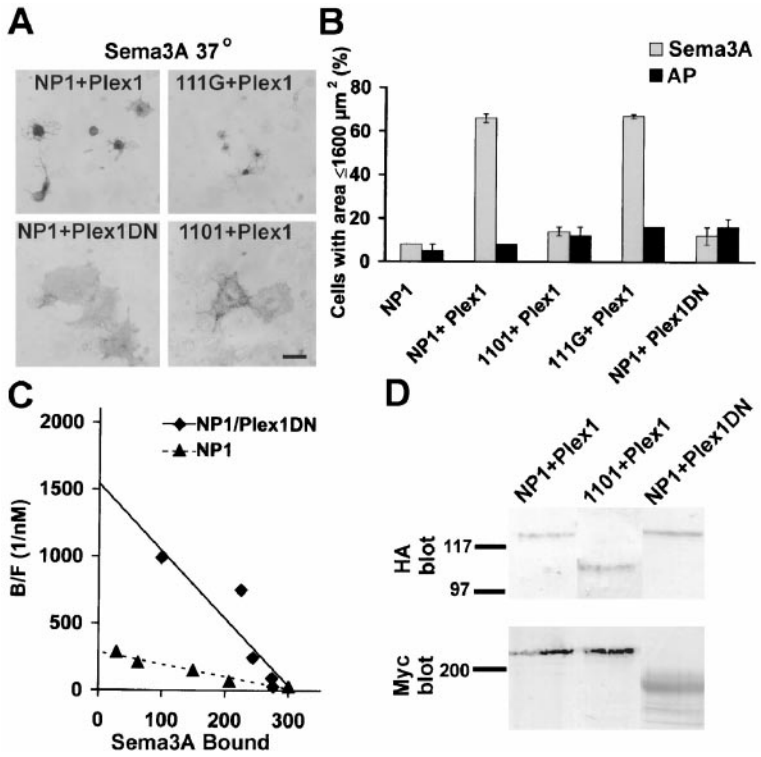

Figure 4. NP-1 and Plex 1 Domains Responsible for Sema3A-Induced Morphological Change

(A) Histology of AP-Sema3A-treated COS-7 cells expressing NP-1/Plex 1, NP-1/Plex 1 DN, 111G/Plex 1, or 1101/Plex 1. The GPI-anchored NP-1 mutant 111G lacks an intracellular domain but mediates Sema3A-induced cell size reduction in the presence of Plex 1. The NP-1 mutant lacking the MAM domain (1101) cannot mediate this reduction in cell area. The Plex 1 mutant lacking most of the intracellular domain (Plex 1 DN) does not mediate Sema3A-induced morphological change. Scale bar, 70 μm.

(B) The percentage of NP-expressing cells with an area less than 1600 μm^2. The fraction of cells with a reduced area is quantitated for the indicated transfection and incubation conditions. The means ± SEM from five experiments are shown.

(C) Scatchard analysis of AP-Sema3A binding to NP-1- or NP-1/Plex 1 DN–expressing COS-7 cells. K_Ds for Sema3A binding to NP-1 and NP-1/Plex 1 DN cells are 1285 ± 185 pM and 235 ± 59 pM, respectively, for n = 5. Results from one of the five experiments are presented.

(D) Ability of 1101 and Plex 1 DN proteins to form NP-1/Plex 1 complexes. HEK293T cells were transfected with HA-NP-1 or HA-1101 together with Myc-Plex 1 or Myc-Plex 1 DN. Lysates prepared from transfected cells were immunoprecipitated with anti-Myc antibody and then analyzed by anti-Myc or anti-HA immunoblot, as in Figure 1A. Immunoblots with anti-HA antibody demonstrate that 1101 binds to Plex 1 and that Plex 1 DN interacts with NP-1.

with Myc-tagged Plex 1 (Figure 4D). Since the 1101 mutant can physically interact with Plex 1, its inactivity in the Plex 1–expressing COS-7 contraction assay suggests that the MAM domain contributes to the transduction of a Sema3A/NP-1 signal to Plex 1 and then to the cell interior. Thus, the NP-1 domains required for high-affinity Sema3A binding by NP-1/Plex 1 complexes, for Sema3A-induced Plex 1–expressing COS-7 contraction, and for Sema3A-induced growth cone collapse are indistinguishable.

Plex 1 Intracellular Domain Mediates Sema3A-NP-1 Signaling in COS-7 Cells

If Plex 1 serves a transmembrane signal transducer for Sema3A/NP-1 complexes, then the cytoplasmic domain of Plex 1, as opposed to that of NP-1, should be necessary for activity. A Plex 1 mutant lacking nearly the entire cytoplasmic domain (Plex 1 DN) was analyzed in the coimmunoprecipitation assay, in the Sema3A/NP-1 affinity shift assay, and in the Sema3A-induced contraction assay. Plex 1 DN is expressed at levels indistinguishable from Plex 1 itself. Plex 1 DN forms a complex with NP-1 and increases the affinity of NP-1 for Sema3A as efficiently as does Plex 1 (Figure 4). However, the Plex 1 DN protein does not support Sema3A-induced contraction of COS-7 cells coexpressing NP-1. These data are consistent with the hypothesis that the extracellular domain of Plex 1 interacts with NP-1 and transduces a Sema3A signal directly to cytoplasm via the intracellular domain of Plex 1.

Plexin Expression in DRG Neurons

The COS-7 cell experiments suggest that a NP-1/plexin complex mediates Sema3A repulsion of DRG axons in vivo. This hypothesis requires plexin expression within these neurons. An antibody directed against the intracellular domain of Plex 1 detects a 250 kDa protein in rat E15 DRG samples (Figure 5A). This antibody is directed against a highly conserved domain of Plex 1 and cross-reacts with Plex 2 and Plex 3 (Figure 5B). Therefore, Plex 1, Plex 2, and Plex 3 may contribute to DRG plexin immunoreactivity and are potential partners with NP-1 in a functional Sema3A receptor. In situ hybridization of E13.5 DRG and spinal cord demonstrates that Plex 3 is expressed strongly in both DRG and spinal cord at this stage (Figure 5C). There is a lower but detectable level of Plex 1 and Plex 2 expression in DRGs. Plex 2 is expressed selectively in the dorsal half of the spinal cord. The expression of Plex 1 in DRG neurons, combined with the COS-7 cell assays (Figures 2 and 3), suggests that Plex 1 contributes to DRG responses to Sema3A. The expression studies are consistent with Plex 2 and Plex 3 participating as well.

Plexin and NP-1 Cocluster during Sema3A-Induced DRG Growth Cone Collapse

Transduction of Sema3A/NP-1 signals by plexins predicts that NP-1 and plexins are colocalized in axonal growth cones. To examine the in situ physical coincidence of NP-1 and plexin, an immunohistologic method was employed. In untreated DRG cultures, both Myc-NP-1 and plexin immunoreactivity are diffusely localized

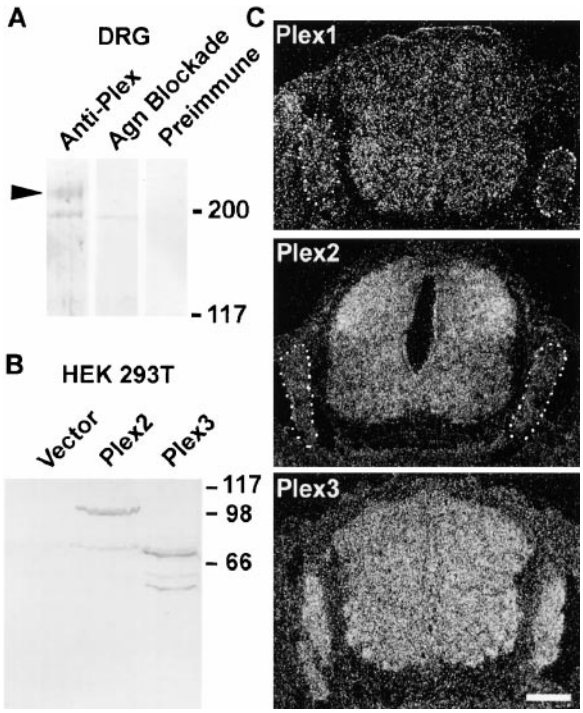

Figure 5. Plexin Expression in Mouse DRG

(A) Plex 1 immunoblot analysis of rat E15 DRG. Specific staining of a 250 kDa species (arrowhead) in transfected cells and in DRG samples is blocked by the addition of excess antigen (Agn blockade). Preimmune serum does not react with the 250 kDa protein. The migration of molecular weight standards of 200 and 117 kDa is shown at the right.

(B) Immunoreactivity of Plex 2 and Plex 3. Fragments of Plex 2 and Plex 3 containing the transmembrane and cytoplasmic domains were expressed in HEK293T cells and immunoblotted with the anti-Plex 1 antiserum. An immunoreactive band with the predicted size of 100 kDa is detected in the Plex 2–expressing cell lysates, and the predicted 75 kDa species is seen in the Plex 3–expressing samples. The migration of molecular weight standards of 117, 98, and 66 kDa is shown at the right.

(C) In situ hybridization for plexins in mouse E13.5 transverse sections. Hybridization of antisense *Plex 1*, *Plex 2*, and *Plex 3* probes is illustrated. Sense probes yielded no signal. DRG location is outlined with white dots. Bar, 200 μm.

throughout the growth cone (Figure 6). Greater than 95% of DRG growth cones cultured under these conditions exhibit plexin immunoreactivity. Shortly after Sema3A addition, but before growth cone collapse occurs, NP-1 and plexin shift to codistributed patches of high concentration. Cell surface glycoproteins containing N-acetyl-β-D-glucosaminyl residues can be detected by wheat germ agglutinin binding and do not localize to these patches (Figure 6). The specific coaggregation of neuronal NP-1 and plexin is consistent with a direct physical interaction between NP-1 and plexins in growth cones and also suggests that receptor aggregation may mediate Sema3A signaling.

Plexins Participate in Sema3A-Induced Collapse of DRG Growth Cones

We sought to provide direct evidence that a plexin transduces Sema3A repulsive signals in DRG growth cones. Since the Plex 1 DN protein can interact with NP-1 but does not induce COS cell contraction, it may be expected to exhibit dominant-negative function in DRG neurons. Plex 1 DN interaction with NP-1 should reduce access of all endogenous plexin family members to the Sema3A/NP-1 complex. We expressed Plex 1 DN or Plex 1 itself in DRG neurons using herpes simplex virus gene transfer, as described previously for NPs (Nakamura et al., 1998; Takahashi et al., 1998). Growth cone collapse assays demonstrate that expression of Plex 1 DN reduces the EC_{50} of Sema3A for growth cone collapse by a factor of 10 (Figure 7). In contrast, HSV-mediated expression of full-length Plex 1 does not alter the collapse potency of Sema3A for DRG growth cones (Figure 7). Since Plex 1 and Plex 1 DN have similar effects in Sema3A binding assays of NP-1-expressing COS cells, this cannot be explained on the basis of reduced affinity of Sema3A-binding sites in the DRG neurons. The Plex 1 DN–expressing cultures exhibit incomplete blockade at higher Sema3A levels and increased cooperativity of Sema3A action. These effects could be due to alterations in the complex kinetics of competitive Plex 1 DN, Plex 1, Plex 2, and Plex 3 interactions with NP-1 in Sema3A-induced multivalent receptor clusters. The data clearly indicate that Plex 1 DN possesses dominant-negative properties for plexin molecules normally playing an essential transducing role in Sema3A-induced growth cone collapse.

Discussion

Plexin Transduces NP-1/Sema3A Signals

The major finding of the present study is that a plexin can serve as signal transducer for the NP-1/Sema3A complex. Previous work had indicated that while NP-1 is necessary and sufficient for growth cone binding of Sema3A, NP-1 does not itself transmit a signal to the cytoplasmic domain of the growth cone (Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Nakamura et al., 1998; Takahashi et al., 1998). Therefore, the interaction of Sema3A/NP-1 complexes with a transmembrane, signal-transducing protein was postulated. Here, we provide several lines of evidence that this transducing protein is a plexin. First, NP-1 and Plex 1 form an immunoprecipitable complex. Second, the complex exhibits an enhanced affinity for Sema3A close to the Sema3A affinity seen in growth cones. Third, the affinity enhancement of the complex requires the signal-transducing MAM domain of NP-1 and is agonist selective. Fourth, the NP-1/Plex 1 complex is sufficient to mediate morphologic responses to Sema3A in nonneuronal cells. Fifth, the NP-1 structural requirements for Plex 1–mediated changes in nonneuronal cell morphology are the same as those for growth cone collapse. Sixth, the cytoplasmic domain of Plex 1 is essential for these Sema3A-induced morphologic changes. Seventh, plexin and NP-1 cocluster during Sema3A treatment of DRG growth cones. Eighth, a dominant-negative form of Plex 1 dramatically reduces Sema3A-induced growth cone collapse. Taken together, the data strongly support the hypothesis that a plexin mediates the actions of Sema3A/NP-1 complexes.

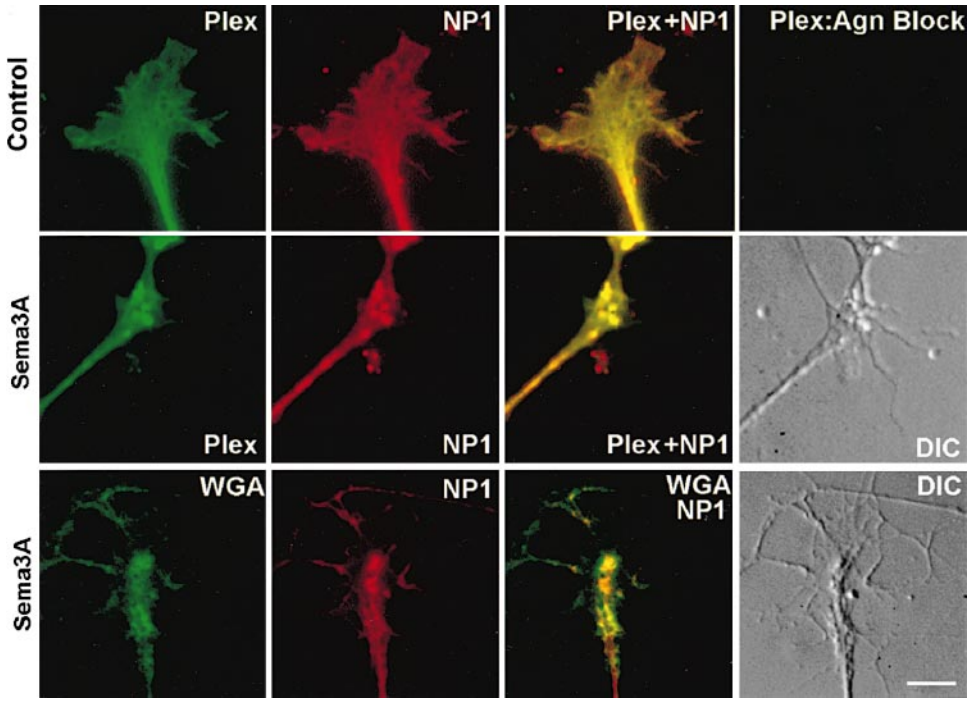

Figure 6. Sema3A Induces Plexin and NP-1 Coclustering in DRG Growth Cones

Chick E7 DRG neurons were infected with Myc-NP-1-HSV preparations and then stained with anti-Myc antibody (red) or anti-Plex antibody (green). Growth cones were treated with or without 1 nM Sema3A for 5 min prior to fixation and staining. Note that the localization of both proteins is diffuse in untreated growth cones but that the proteins cocluster shortly after Sema3A treatment. To demonstrate the specificity of the anti-Plex staining, a separate culture was stained with anti-Plex antibody plus 100 μg/ml of antigen (top right panel). Wheat germ agglutinin (WGA) staining of Sema3A-treated, anti-NP-1-stained growth cones is shown at the bottom. Note that the majority of glycoproteins recognized by WGA do not colocalize with NP-1. Differential interference contrast (DIC) images of immunostained growth cones are at the right in the second and third rows. Scale bar, 12 μm.

Such a model implies a unified scheme for the mechanism of semaphorin action. All semaphorin signals may be mediated via plexins. Soluble class 3 semaphorins utilize NPs as high-affinity binding intermediates in order to access a more general plexin transduction cascade. For class 1 and viral semaphorins, there is convincing evidence that plexins directly bind semaphorin ligands and mediate cellular effects (Comeau et al., 1998; Winberg et al., 1998). Other semaphorins may utilize plexin isoforms directly (Tamagnone et al., 1999), or additional

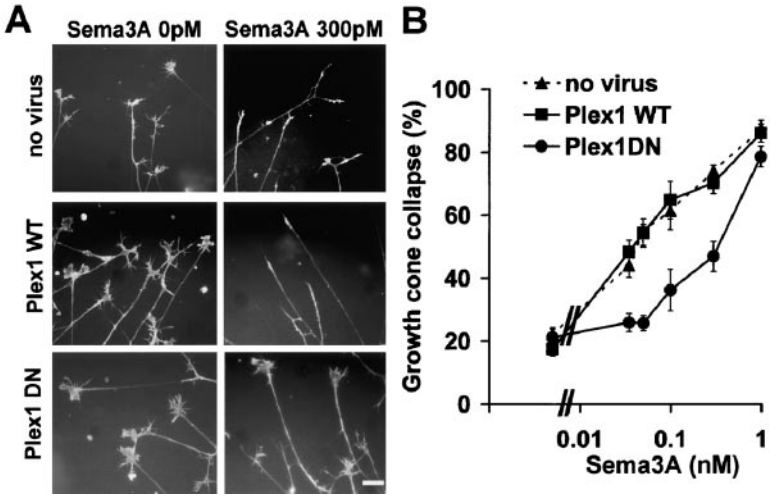

Figure 7. Overexpression of Plex 1 DN Blocks Sema3A-Induced Growth Cone Collapse in DRG Neurons

(A) Response of Plex 1 and Plex 1 DN–expressing DRG neurons to Sema3A. Chick E7 DRG neurons were infected with recombinant HSV preparations directing the expression of full-length Plex 1 (Plex 1 WT) or Plex 1 DN. Expression of full-length Plex 1 does not alter Sema3A-induced growth cone collapse, while expression of Plex 1 DN blocks Sema3A-induced growth cone collapse. Rhodamine-phalloidin staining of actin filaments in DRG growth cones is illustrated. Scale bar, 20 μm.

(B) Sema3A dose–response curve for DRG growth cone collapse. Growth cone collapse was determined after infection with the indicated virus preparations and treatment with the indicated concentrations of Sema3A. The EC_{50} of Sema3A in noninfected cultures and in Plex 1 WT virus-infected cultures is approximately 40 pM. The EC_{50} of Sema3A in the Plex 1 DN virus-infected culture is about 400 pM. Data are means ± SEM for n = 5.

non-NP accessory components may exist. In this model, the role of NPs in Sema3A signaling is quite similar to its role in VEGF-165 signaling through a tyrosine kinase receptor, Flt-1 (Soker et al., 1998). A minor difference is that Flt-1 possesses moderate affinity for VEGF-165 directly (Soker et al., 1998), while Plex 1 has no detectable affinity for Sema3A. It is tempting to speculate that class 3 soluble semaphorins require NPs to enhance binding affinities, whereas membrane-associated semaphorins are confined to the plane of the lipid bilayer and require a lower binding affinity to achieve biological specificity, and this lower affinity is provided by plexins directly. This model also implies that plexins are bifunctional: they can stand alone as Sema-1 receptors and also serve as transducing subunits for Sema3A/NP-1 complexes. A similar conclusion has been drawn from the studies of Tamagnone et al. (1999).

Plex 1 and NP-1 can be coimmunoprecipitated from HEK293T cells. The molecular sites of interaction are only partially defined by the current experiments. The intracellular domain of either NP-1 or Plex 1 can be deleted without a reduction in coprecipitation, indicating that the primary site of interaction is outside the cell membrane. The sequence similarity between Plex 1 and the sema domains of semaphorins raises the possibility that the sema domain of Plex 1 might interact with the sema-binding CUB domain of NP-1. We could not detect a competitive effect of Plex 1 on Sema3A/NP-1 interactions or Sema3A on NP-1/Plex 1 interactions. Thus, it seems unlikely that the CUB domain is the sole or major site of Plex 1 binding within NP-1. The study of deletion mutants of NP-1 indicates that Plex 1 binding function is distributed at multiple sites throughout NP-1, with no single domain deletion preventing coprecipitation (T. T. and S. M. S., unpublished data). For Plex 1, no analysis of extracellular domains required for NP-1 interaction has yet been undertaken.

Sema3A-Induced Morphologic Changes in Nonneuronal Cells

Sema3A induces a striking morphologic change in COS-7 cells expressing both Plex 1 and NP-1. Neither of these proteins alone can mediate the morphologic response. At the least, the changes in COS-7 cells appear to reflect activation of physiologically relevant receptors. Thus, this assay is likely to be extremely useful for further dissection of the protein domains required for signaling and for determining which semaphorins interact productively with which NPs and plexins. It remains possible that downstream events are dramatically different in the two cell types. However, it is reasonable to suggest that the reduction in cell area reflects a cellular signaling event akin to DRG growth cone collapse and axon repulsion. Recently, the ability of Sema3A to inhibit neural crest cell migration has been correlated with Sema3A-induced neural crest cell rounding in tissue culture assays (Eickholt et al., 1999). Thus, in growth cones, in neural crest cells, and in NP-1/Plex 1–expressing COS-7 cells, Sema3A initiates lamellipodial retraction. Several molecular attributes are common to both growth cone collapse and the COS-7 cell morphologic change. For both events, the EC_{50} for Sema3A is about 50–100 pM, the MAM domain but not the cytoplasmic domain of NP-1 is required, and Sema3C is inactive for NP-1/Plex-expressing cells. To the extent that downstream signaling events are shared in the two cell types, the NP-1/Plex 1 COS-7 cell contraction assay should also facilitate analysis of subsequent events in the growth cone collapse cascade.

Mechanism of Plexin Signaling

The unified model of semaphorin signaling through plexins presented above emphasizes the paucity of information concerning how the intracellular domain of plexin might transduce a signal. It is noteworthy that the large intracellular domain is highly conserved across the plexin family but does not share strong sequence similarity to another protein, which might suggest an obvious hypothesis for signaling function. There is weak similarity of Plex 1 residues 1667–1825 with a group of R-ras GTPase-activating proteins (GAPs). However, a model for semaphorin signaling based on this similarity is not obvious.

It is known that the NPs and some semaphorins self-associate. Therefore, their association with Plex 1 might create higher-order ligand–receptor complexes. Perhaps receptor aggregation activates a signaling function of the plexin intracellular domain. While Sema3A does not regulate the extent of coprecipitation of NP-1 with NP-1 or NP-1 with Plex 1, the immunohistologic data suggest that Sema3A induces higher-order receptor structures that in turn lead to the activation of signaling cascades and morphologic changes. In support of such a model, we have found that Sema3A-induced NP-1/plexin clusters are associated with Rac1 aggregation, F-actin nucleation, membrane ruffles, and endocytosis (A. F., F. N., R. G. K., and S. M. S., unpublished results).

Semaphorin Specificity

The semaphorin family appears to be comprised of at least 20 members, with a wide range of biological effects and structural features ancillary to the sema domain (Kolodkin et al., 1993; Semaphorin Nomenclature Committee, 1999). Clearly, the basis of specificity in semaphorin signaling lies at the crux of understanding the physiological roles of semaphorins. Previous studies demonstrated that there is a strict specificity in NP-mediated semaphorin signaling (Chen et al., 1997, 1998; Giger et al., 1998; Takahashi et al., 1998). Although Sema3A, -B, -C, and -F all bind to NP-1, only Sema3A functions as an agonist for NP-1 (Chen et al., 1997, 1998; Giger et al., 1998; Takahashi et al., 1998). Sema3C inactivity was observed here for the Plex 1–induced NP-1 affinity shifts and for the Sema-induced contraction of NP-1/Plex 1–expressing COS-7 cells. For Sema3C, both NP-1 and NP-2 may be required for a functional receptor complex. Our studies of NP-1/Plex 1–expressing and NP-2/Plex 1–expressing COS-7 cells support the notion that neither NP-1 nor NP-2 alone is sufficient for Sema3C action. Coexpression of NP-2 with Plex 1 appears to provide a functional receptor for Sema3F in COS-7 cells.

We show that two other rodent plexins, Plex 2 and Plex 3 (Kameyama et al., 1996; Maestrini et al., 1996), are expressed in DRG neurons at levels equal to or higher than Plex 1. Whether coupling with NPs is specific

to Plex 1 or extends to other family members should be explored in future studies.

Insect class 1 semaphorins and a viral semaphorin bind directly to plexins (Comeau et al., 1998; Winberg et al., 1998). Undoubtedly, ligand binding studies will reveal whether other classes of semaphorins bind directly to specific plexins. However, binding studies alone cannot determine whether a ligand is an antagonist or an agonist. Observation of morphologic effects in plexin-transfected nonneuronal cells may be a means for screening the biological activity of various semaphorin classes. If some plexins can interact both with NPs and directly with semaphorins, then the issues of competition, cross-desensitization, and cross-tolerance must be examined to fully understand semaphorin action. Clearly, the interaction of NPs and plexins greatly increases receptor complexity to a level matching that of the semaphorin ligand family.

Signaling Molecules Downstream of Plexin

The intracellular mechanisms underlying Sema3A-induced growth cone collapse are not fully understood. A collapsin response mediator protein (CRMP) is implicated, but its mechanism of action is ill defined (Goshima et al., 1995; Wang and Strittmatter, 1996, 1997). Since no CRMP is present in COS-7 cells, the contraction of the cell perimeter induced by Sema3A in NP-1/Plex 1–expressing cells utilizes a CRMP-independent pathway. Either CRMP plays a facilitory but nonessential role in growth cone collapse or the mechanisms utilized for Sema3A responsiveness in COS-7 cells are distinct from those in neurons. Preliminary experiments suggest that at lower NP-1/Plex 1 expression levels, the COS-7 response to Sema3A is incomplete unless CRMP is coexpressed (T. T. and S. M. S., unpublished data). This argues for the former alternative.

The monomeric GTP-binding protein Rac1 is also implicated in Sema3A-induced growth cone collapse (Jin and Strittmatter, 1997). A dominant-negative form of Rac1 blocks the Sema3A-induced DRG and motoneuron growth cone collapse (Jin and Strittmatter, 1997; Kuhn et al., 1999). In addition, we have recently observed that Rac1 redistributes with NP-1 to sites of actin filament concentration and endocytosis during Sema3A-induced growth cone collapse (A. F., F. N., R. G. K., and S. M. S., unpublished results). This suggests that a Rac1-specific guanine nucleotide exchange factor (Rac-GEF) might link a Sema3A/NP-1/plexin clustered complex to Rac1.

The current data clearly demonstrate that plexins play a central role in class 3 as well as class 1 semaphorin signaling. Plexins serve as signal transducers for Sema3A/NP-1 complexes. Further work will be required to delineate semaphorin specificities for different plexin-based receptor complexes and to reveal the mechanisms by which the intracellular domain of plexin alters axonal morphology.

Experimental Procedures

NP and Plex 1 Expression Vectors

The plasmids encoding HA-tagged NP-1 and Myc-tagged 1101 and 111G NP-1 mutants in pcDNA1 were described previously (Nakamura et al., 1998; Takahashi et al., 1998). To obtain HA-1101 and HA-111G, a HindIII–BglII fragment from HA-NP-1 was transferred to the homologous sites of Myc-1101 and Myc-111G. To express Plex 1, the mouse *Plex 1* cDNA (Kameyama et al., 1996) was digested with EcoRI and BstBI and then treated with the Klenow fragment of DNA polymerase I. This *Plex 1* fragment encoding aas 1–1879 was ligated to EcoRV-digested pcDNA3.1-Myc-His to generate an in-frame epitope tag (Invitrogen). A deletion of the intracellular domain of *Plex 1* (Plex 1 DN-pcDNA3.1, lacking aas 1294–1894) was generated by XhoI digestion and self-ligation of Plex 1-pcDNA3.1-Myc-His. Expression vectors for the carboxyl portions of human Plex 2 (aas 1073–1895) and mouse Plex 3 (aas 1174–1827) were derived from cDNA clones (*Plex 2*, Kazusa DNA Research Institute cDNA clone KIAA0463; *Plex 3*, Kameyama et al., 1996) and utilized the translational start site and signal sequence of pSecTag2 (Invitrogen).

Coimmunoprecipitation

HEK293T cells (1×10^6) were transfected with the expression vector for Myc–Plex 1 (1 μg) together with an expression vector for HA-NPs (1 μg) by the lipofectamine method. After 2 days, cells were incubated with or without Sema3A (5 nM)-containing medium for 1 hr. Cells were then lysed with ice-cold immunoprecipitation buffer (IP buffer: 20 mM HEPES-NaOH, 150 mM NaCl, 10 mM NaF, 2 mM PMSF, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100 [pH 7.0]) or IP buffer containing divalent cations (IP buffer without EDTA and EGTA, with 5 mM $CaCl_2$ and 1 mM $MgCl_2$), with or without 5 nM Sema3A. Protein from cell extracts was immunoprecipitated with anti-Myc-antibody (9E10)-conjugated agarose beads as described in Takahashi et al. (1998) in the presence or absence of 5 nM Sema3A. Bound protein was analyzed by immunoblot with either anti-HA antibody (Y11) or anti-Myc (9E10) antibody.

In other experiments, Plex 1 and untagged NP-1 proteins were expressed and extracted by similar methods and then incubated with anti-plexin or anti-NP-1 antisera. Antibody–antigen complexes were isolated by binding to protein G–Sepharose, washed with IP buffer containing 0.1% SDS, and analyzed by SDS-PAGE.

Anti-Plex 1 Antibody

The predicted intracellular domain of Plex 1 (aas 1266–1894) was expressed in *Escherichia coli* with a hexa-His tag at the amino terminus using the pET system (Novagen). Recombinant protein of 75 kDa was purified by Ni affinity chromatography and utilized for rabbit immunization. Antiserum was used for immunoblot at 1:1000 dilution and for immunohistology at 1:500 dilution. The specificity of staining was verified by the lack of staining after preabsorption of the antiserum with 0.5 mg/ml of recombinant protein and after substitution of preimmune serum for immune serum.

AP-Sema Binding to COS-7 Cells

COS-7 cells (5×10^5) were transfected with 2 μg of pcDNA3.1-MycHis vector, Plex 1-pcDNA3.1-MycHis, or Plex 1 DN-pcDNA3.1MycHis together with 0.5 μg of pcDNA1 vector or various NP-pcDNA1 plasmids by the lipofectamine method. These transfected cells were stained with AP-Sema fusion proteins as described (Takahashi et al., 1997, 1998; Nakamura et al., 1998). The AP-Sema3F (Sema IV) expression vector was a generous gift of M. Tessier-Lavigne (Chen et al., 1997). A microdensitometric method for the quantitation of binding was employed (Takahashi et al., 1998).

Semaphorin-Induced Morphological Effects on COS-7 Cells

COS-7 cells were transfected with 2 μg of pcDNA3.1-MycHis vector, Plex 1-pcDNA3.1MycHis, or Plex 1 DN-pcDNA3.1MycHis together with 0.5 μg of pcDNA1 vector or various HA-NP-pcDNA1 plasmids by the lipofectamine method. After 2 days, cells were incubated with 0–10 nM AP-Sema3A, AP-Sema3C, AP-Sema3F, or AP in standard culture medium at 37°C or 4°C for 1 hr. Cells were washed with Hanks balanced salt buffer containing 0.05% (w/v) BSA six times on ice and fixed with 3.7% formaldehyde in PBS. After fixation, cells were washed once with Hanks balanced salt buffer and incubated at 65°C for 2 hr to inactivate endogenous AP. For most cultures, cells expressing NP and binding AP-Sema were detected histologically by the deposition of insoluble AP reaction product from 5-bromo-4-chloro-3-indolyl phosphate. To visualize NP-expressing AP-treated cells, samples were incubated with anti-HA antibody

followed by peroxidase-conjugated anti-rabbit IgG and diaminobenzidine plus hydrogen peroxide. To visualize cells expressing Plex 1 but not NP, samples were exposed to the anti-Myc antibody and then peroxidase-conjugated anti-mouse IgG followed by peroxidase substrates. The size of stained cells was determined for 50–100 consecutive AP-positive or peroxidase-positive cells from digital images acquired at 10× magnification using an image analysis program (OlymPix version 3.0; Olympus).

Plexin In Situ Hybridization

In situ hybridization was performed as described (Kawakami et al., 1996). ^{35}S-labeled antisense and sense cRNA probes were transcribed from cDNA fragments corresponding to base pairs 564–2732, 2233–2988, and 1296–2389 of the mouse *Plex 1*, *Plex 2*, and *Plex 3* cDNAs, respectively (Kameyama et al., 1996).

Recombinant HSV Preparation

The coding regions of myc-Plex 1 or myc-Plex 1 DN were excised from the pcDNA3.1 vectors and ligated to the pHSVPrPUC vector (Takahashi et al., 1998). The resulting plasmids (2 μg) were transfected into 2-2 cells (3×10^5) with lipofectamine and then superinfected with 5dl5 HSV helper virus 1 day later. Recombinant virus was amplified through three passages and stored at −80°C.

DRG Growth Cone Analysis

The culture and HSV infection of chick E7 DRG explants has been described previously (Goshima et al., 1995; Takahashi et al., 1998). Growth cone collapse assays were performed with Sema3A as previously described (Goshima et al., 1995; Takahashi et al., 1998). For Plex-NP-1 colocalization experiments, DRG explants were infected with HSV-Myc-NP-1 as described (Takahashi et al., 1998). Twenty-four hours after infection, explants were treated with 1 nM of Sema3A for 5 min to induce partial growth cone collapse and fixed in 4% paraformaldehyde, 20% sucrose, 0.1 M Na phosphate (pH 7.5). Explants were double immunostained with fluorescent-conjugated secondary antibodies following an overnight incubation with anti-Myc monoclonal antibody and anti-Plex 1 antibody. For analysis of other growth cone proteins, uninfected cultures were stained with fluorescein-conjugated wheat germ agglutinin and anti-NP-1 and rhodamine-conjugated anti-rabbit IgG.

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