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PlexinA1 Autoinhibition by the Plexin Sema Domain

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Summary

Semaphorin 3A (Sema3A) binds to neuropilin-1 (NP1) and activates the transmembrane Plexin to transduce a repulsive axon guidance signal. Here, we show that Sema3 signals are transduced equally effectively by PlexinA1 or PlexinA2, but not by PlexinA3. Deletion analysis of the PlexinA1 ectodomain demonstrates that the sema domain prevents PlexinA1 activation in the basal state. Sema-deleted PlexinA1 is constitutively active, producing cell contraction, growth cone collapse, and inhibition of neurite outgrowth. The sema domain of PlexinA1 physically associates with the remainder of the PlexinA1 ectodomain and can reverse constitutive activation. Both the sema portion and the remainder of the ectodomain of PlexinA1 associate with NP1 in a Sema3A-independent fashion. Plexin A1 is autoinhibited by its sema domain, and Sema3A/NP1 releases this inhibition.

Introduction

Accurate axonal path finding during development is critical for the establishment of nervous system structure and function. Developing axons are guided by both attractive and repulsive cues (Tessier-Lavigne and Goodman, 1996). The semaphorin family of proteins has been characterized primarily as repulsive cues (Luo et al., 1993; Kolodkin, 1996). All semaphorins possess a conserved 500 aa sema domain (Kolodkin, 1996). Over 30 semaphorins have been identified and are classified into eight groups based on their primary sequence and source (Semaphorin Nomenclature Committee, 1999). Class 3 semaphorins (Sema3A–Sema3F) are secreted semaphorins with a high-potency growth cone–collapsing activity for certain sets of neurons (Luo et al., 1993; Koppel et al., 1997; Takahashi et al., 1998). Gene-targeting studies demonstrate an in vivo function for Sema3A as an axon-repulsive molecule (Behar et al., 1996; Taniguchi et al., 1997).

Neuropilin-1 (NP1) and neuropilin-2 (NP2) have been isolated as receptors for class 3 semaphorins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Sema3A binds to only NP1, whereas Sema3B, Sema3C, and Sema3F bind to NP1 and NP2 (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takahashi et al., 1998). Antibody perturbation, virus-mediated gene transfer, and gene-targeting experiments demonstrate that NP1 is required for Sema3A action and that NP2 is essential for Sema3B, Sema3C, and Sema3F activity (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Chen et al., 1998; Giger et al., 1998; Takahashi et al., 1998; Chen et al., 2000; Giger et al., 2000). Interestingly, Sema3B and Sema3C compete with Sema3A for binding to NP1 and can function as antagonists of NP1 receptors (Takahashi et al., 1998).

Plexins (Plex) are known to be the primary binding sites for some semaphorins that do not bind to NP (Comeau et al., 1998; Winberg et al., 1998; Tamagnone et al., 1999). Members of the plexin family have been classified into four groups: A–D (Tamagnone et al., 1999). A member of the PlexC family, VESPR (PlexC1), was isolated as a receptor for the viral Sema VA (Comeau et al., 1998). By virtue of genetic studies of *Drosophila melanogaster*, fly PlexinA is recognized as a functional receptor for Sema1A/B (Winberg et al., 1998). In vertebrates, PlexB1 and PlexC1 are binding sites for Sema4D and Sema7A, respectively (Tamagnone et al., 1999). Plexin is a single membrane-spanning protein that possesses a sema domain near the amino terminus, followed by a cysteine-rich motif and several glycine-proline-rich motifs (Ohta et al., 1995; Winberg et al., 1998.)

Deletion studies of NP1 revealed that the amino-terminal CUB domain is crucial for semaphorin binding (Giger et al., 1998; Nakamura et al., 1998). Sema3A signal transduction also requires the extracellular, juxtamembrane MAM domain of NP1 (Nakamura et al., 1998). Strikingly, the transmembrane and cytoplasmic regions of NP1 are not necessary for Sema3A action (Nakamura et al., 1998). These findings imply that another NP-interacting transmembrane protein transduces Sema3A signals to the cytoplasm. Recently, plexins were found to be NP-interacting, signal-transducing proteins (Takahashi et al., 1999; Tamagnone et al., 1999). NP1 and PlexA1 form a complex with an enhanced binding affinity for Sema3A, as compared with NP1 alone (Takahashi et al., 1999). NP1 plus PlexA1, but not NP1 or PlexA1 alone, mediates contraction of the COS-7 cell perimeter in response to Sema3A (Takahashi et al., 1999). Overexpression of a mutant PlexA1 lacking the cytoplamsic domain blocks Sema3A action in dorsal root ganglion (DRG) growth cones (Takahashi et al., 1999) and *Xenopus* spinal axons (Tamagnone et al., 1999). These findings strongly support the idea that a Plex is the signal-transducing protein for Sema3-NP complexes. The cytoplasmic mediators of Sema3 signaling are poorly understood, and the intracellular domain of Plex has little significant sequence similarity with known signaling proteins. Rac1 (Jin and Strittmatter, 1997; Fournier et al., 2000) and collapsin response mediator protein (CRMP) (Goshima et al., 1995) are downstream cytoplasmic mediators of Sema3A signaling, but their molecular relationship to Plex is not defined. Intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) levels modulate Sema3A effects on the growth cone (Song et al., 1998).

In situ hybridization and immunohistochemistry experiments demonstrate the expression of PlexA1, PlexA2, and PlexA3 in Sema3-responsive DRG and sympathetic neurons (Takahashi et al., 1999; Tamagnone

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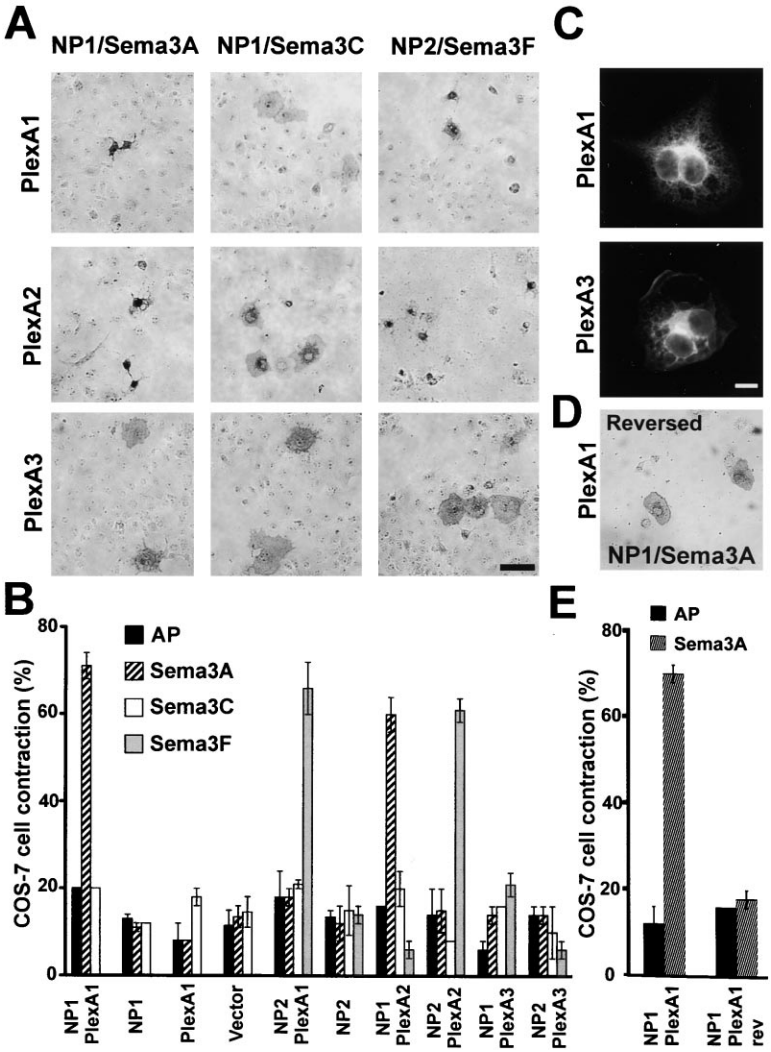

Figure 1. PlexA1 and PlexA2 but Not PlexA3 Mediate Sema3/NP-Induced COS-7 Cell Contraction

(A) AP-Sema3 staining of COS-7 cells expressing NP/Plex. Sema3A but not Sema3C induces the contraction of COS-7 cells transfected with NP1/PlexA1 or NP1/PlexA2. COS-7 cells expressing NP2/PlexA1 or NP2/PlexA2 are small in the presence of Sema3F. Neither Sema3A, Sema3C, nor Sema3F induces the contraction of COS-7 cells transfected with NP/PlexA3. Scale bar, 70 μm.

(B) The percentage of stained cells with an area less than 1600 μm^2. Cells were transfected and treated with the indicated plasmids and proteins. The means $\pm$ SEM from three to five experiments are presented.

(C) The subcellular distribution of PlexA1 and PlexA3, as detected by anti-Myc immunohistology of transfected COS-7 cells, is similar. Scale bar, 20 μm.

(D) Sema3A-treated NP1/PlexA1-expressing cells appear well-spread 12 hr after a 1-hr exposure to Sema3A (reversed). Scale bar, 70 μm.

(E) Percentage of stained cells with an area less than 1600 μm^2. Cultures were transfected with NP1/PlexA1 plasmids and treated with Sema3A for 1 hr. Cells were either fixed (NP1/PlexA1) or incubated for an additional 12 hr without Sema3A (NP1/PlexA1/rev). The means $\pm$ SEM from three to five experiments are presented.

et al., 1999). However, precisely which Plex mediates signaling by which Sema3/NP complex is not known. Furthermore, the mechanism by which Sema3/NP complexes activate Plex is a mystery. Interestingly, the ectodomain of Plex contains a sema domain related to Sema3 ligands. What is the role of the Plex sema domain? In this study, we show that PlexA1 and PlexA2, but not PlexA3, mediate Sema3/NP signaling. Furthermore, we present evidence that the sema domain of PlexA1 is necessary to maintain PlexA1 in its basal inactive state. Sema3A/NP1 complexes appear to release this autoinhibition.

Results

PlexA1 and PlexA2 but Not PlexA3 Mediate Sema3/NP-Induced COS Cell Contraction

Given that PlexA1, PlexA2, and PlexA3 are all expressed in DRG and sympathetic neurons (Takahashi et al., 1999; Tamagnone et al., 1999), we considered whether PlexA2 and PlexA3 might have physiological roles in determining the specificity of Sema3 signaling in combination with NPs. For this investigation, a cell-based morphologic assay allowing varied protein expression is required. Since this is not possible in primary neurons because of endogenous Plexins and inefficient transfection rates, we have utilized a COS-7 cell contraction assay. This assay is based on our previous observation that class3 semaphorins induce the contraction of COS-7 cells expressing both PlexA1 and NP (Takahashi et al., 1999). Here, we expressed PlexA1, PlexA2, or PlexA3 in COS-7 cells together with NP1 or NP2. Transfectants were incubated with 3 nM Sema3-AP (alkaline phosphatase) at 37°C for 1 hr and visualized by AP staining or epitope-tag immunostaining. We measured the fraction of cells with a reduced cellular area (Takahashi et al., 1999). In such double-transfection assays, greater than 85% of HA-NP-1/Myc-PlexA1-transfected cells express both epitope tags (data not shown). Sema3A, but not Sema3C and Sema3F, induces the contraction of NP1/PlexA1- or NP1/PlexA2-expressing COS-7 cells (Figures 1A and 1B). NP2/PlexA1- or NP2/PlexA2-expressing COS-7 cells are contracted in the presence of Sema3F, but not Sema3A and Sema3C (Figures 1A and 1B). Therefore, the function of PlexA2 appears identical with PlexA1 in terms of Sema3 signaling. On the other hand,

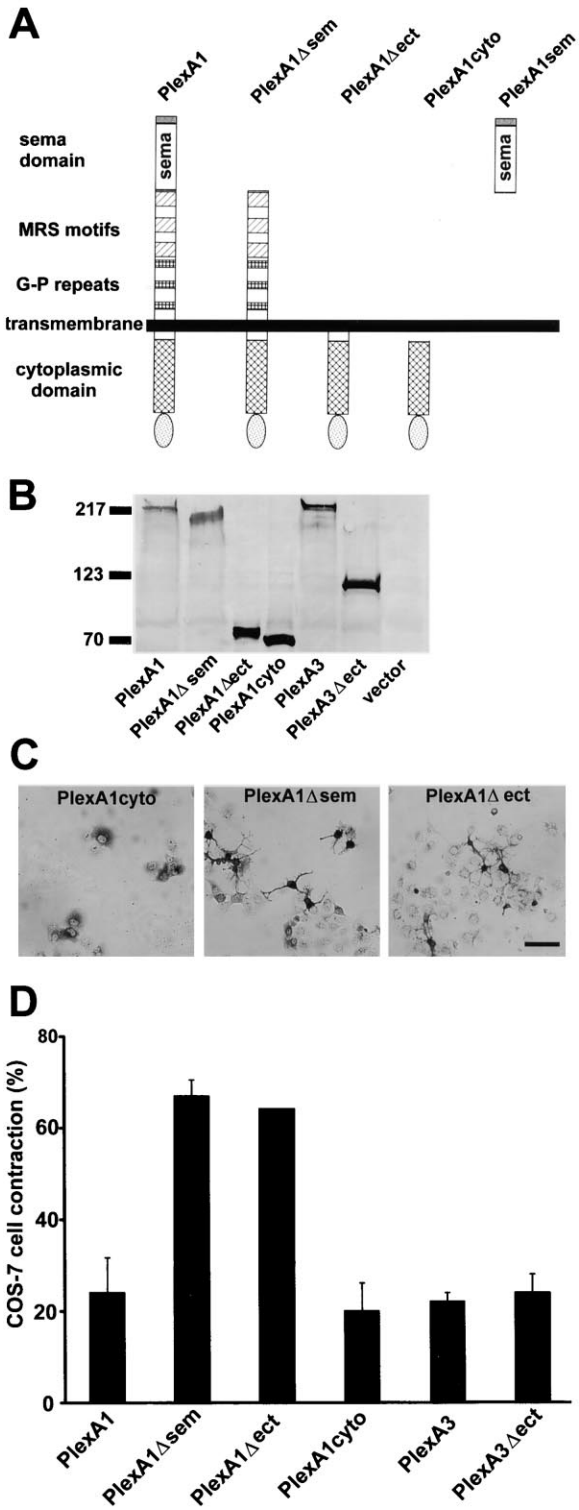

Figure 2. PlexA1Δsem and PlexA1Δect Are Constitutively Active in COS-7 Cells

(A) Schematic diagram of PlexA1 deletion constructs.

(B) Immunoblotting of cell lysates of COS-7 cells expressing PlexA1, PlexA1Δsem, PlexA1Δect, PlexA1cyto, PlexA3, PlexA3Δect, or vector. Migration of molecular size markers (kDa) is at left.

(C) COS-7 cells were transfected with vectors encoding Myc-tagged PlexA1cyto, Myc-tagged PlexA1Δsem, or Myc-tagged PlexA1Δect and stained with anti-Myc antibody 24 hr after transfection. Although PlexA1cyto-expressing COS-7 cells are well spread, COS-7 cells expressing PlexA1Δsem or PlexA1Δect are much smaller. Scale bar, 70 μm.

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

none of these Sema3 proteins induce the contraction of NP/PlexA3-expressing COS-7 cells (Figures 1A and 1B). This can not be attributed to poor PlexA3 expression as immunohistology and immunoblot analyses reveal similar PlexA1 and PlexA3 patterns (Figures 1C and 2B). Furthermore, PlexB1 and PlexB2 were also inactive in this assay (data not shown). Thus, Sema3 signaling requires PlexA1 or PlexA2, but specificity is largely determined by the NP compliment of the cell.

A recent report described Sema3A-induced cell death of nerve growth factor (NGF)-dependent sensory neurons after a 24 hr exposure to semaphorin (Gagliardini and Fankhauser, 1999). This raises the issue of whether the cell contraction response is part of an irreversible cell death response or whether it is a reversible effect on the cytoskeleton similar to growth cone collapse. NP1/PlexA1-expressing COS-7 cells were treated with Sema3A for 1 hour to induce contraction and were then incubated in Sema3A-free medium for an additional 12 hr (Figures 1D and 1E). The total number of NP1-expressing cells in NP1/PlexA1-transfected cultures is not reduced by Sema3A treatment, during the period 0–12 hr after exposure to Sema3A (108 ± 13% of control cultures at 0 hr after treatment and 98 ± 17% of control at 12 hr after treatment). Furthermore, the majority of cells expressing NP1 resume a spread morphology during the washout phase, indicating that the contraction response is reversible and not secondary to the onset of a cell death process (Figures 1D and 1E). The data are most consistent with COS-7 contraction reflecting a cytoskeletal event similar to growth cone collapse.

Sema Domain Deletion Mutants of PlexA1 Are Constitutively Active

Plexins have a large extracellular portion that includes a sema domain. The conservation of this domain between ligand and receptor raises the questions of its functional role. For Sema3A, it is clear that the sema domain is the primary signal directing axonal repulsion. To explore the role of sema domain of Plex, we have analyzed the activity of various Plex deletion mutants. Because only PlexA1 and PlexA2 mediate Sema3 signaling in COS-7 cells and because they are functionally identical, we have focused on PlexA1. The PlexA1 vectors direct expression of PlexA1 lacking the sema domain (PlexA1Δsem), the entire ectodomain (PlexA1Δect), or both ectodomain and transmembrane region (PlexA1cyto) (Figure 2A). Each of the PlexA1 mutant proteins includes a Myc epitope tag. The morphology of COS-7 cells expressing each of these proteins was assessed after a 24 hr incubation by Myc immunohistology. Although wild-type PlexA1-expressing COS-7 cells are well-spread, PlexA1Δsem- or PlexA1Δect-expressing COS-7 cells are contracted (Figures 2C and 2D). We also examined PlexA3 as a control because PlexA3 does not mediate Sema3/

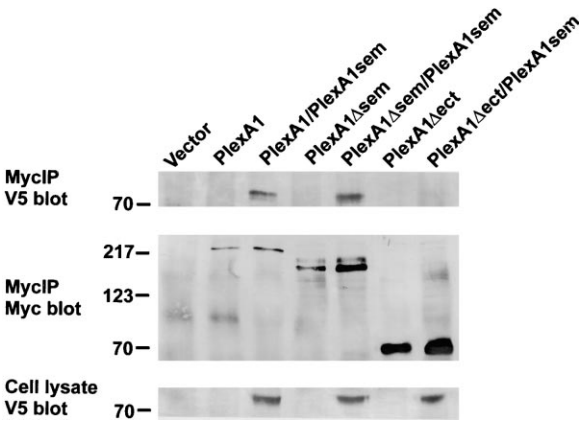

Figure 3. PlexA1sem Associates with PlexA1Δsem

HEK293T cells were transfected with vector, or V5-tagged PlexA1-sem together with Myc-tagged PlexA1, Myc-tagged PlexA1Δsem, or Myc-tagged PlexA1Δect. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-V5 or anti-Myc antibody. PlexA1sem coprecipitates with Myc-PlexA1 or Myc-PlexA1Δ-sem but not with PlexA1Δect. The molecular weights of PlexA1, PlexA1Δsem, PlexA1Δect, and PlexA1sem are 250, 200, 70, and 75 kDa, respectively. Molecular size markers (in kDa) are shown on the right.

NP signaling. In contrast to the case of PlexA1 mutants, expression of a PlexA3 mutant lacking a large portion of ectodomain (PlexA3Δect) does not alter the morphology of COS-7 cells (Figure 2D). All of the Plex mutants are expressed at similar levels in COS-7 cells (Figure 2B).

To examine whether the mutant PlexA1 forms were regulated by physiologic signals, cells were cotransfected with vectors directing the expression of NP and the PlexA1 mutants and were then exposed to semaphorins. The presence of NP1, Sema3A, or both does not change the small cellular area of PlexA1Δsem- and PlexA1Δ ect-expressing cells (data not shown). Therefore, the PlexA1Δsem and PlexA1Δect mutant proteins appear to act as constitutively active receptors.

We also tested whether the transmembrane region of PlexA1 is required for the constitutive activity of PlexA1-Δect. In contrast to PlexA1Δect mutant, expression of PlexA1cyto mutant lacking both the ectodomain and the transmembrane regions does not induce a change in COS-7 cell morphology (Figures 2C and 2D). The transmembrane region is clearly required for the activity of the Plex intracellular domain. Although a specific function might be associated with specific residues in the transmembrane portion, it seems more probable that this domain acts to tether the cytoplasmic domain at the inner surface of the plasma membrane.

PlexA1 Sema Domain Associates with PlexA1Δsem Protein

Based on the fact that PlexA1Δsem is constitutively active, we hypothesized that the PlexA1 sema domain (PlexA1sem) maintains basal inactivity by binding and inhibiting the remainder of PlexA1. As a first step, we examined the association between PlexA1Δsem and PlexA1sem. We cotransfected an expression vector for

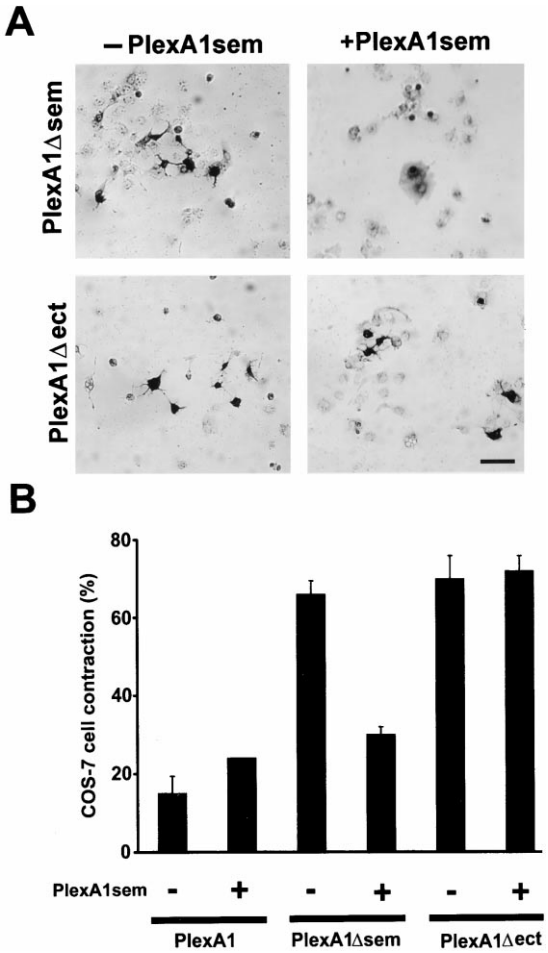

Figure 4. PlexA1sem Reverses the Constitutive Activation of PlexA1Δsem

(A) COS-7 cells were transfected with Myc-tagged PlexA1Δsem or Myc-tagged PlexA1Δect with or without V5-tagged PlexA1sem. One day later, cells were fixed and stained with anti-Myc antibody. Although COS-7 cells transfected only with PlexA1Δsem are contracted, COS-7 cells expressing both PlexA1Δsem and PlexA1sem are much larger. The decreased spreading of PlexA1Δect-expressing cells is not reversed by co-expression of PlexA1sem. Scale bar, 70 μm.

(B) The percentage of stained cells with an area less than 1600 μm^2. Cells were transfected with the indicated plasmids. The means ± SEM from three to five experiments are presented.

V5-tagged PlexA1sem together with Myc-tagged wild-type PlexA1, PlexA1Δsem, or PlexA1Δect into HEK293T cells. Wild-type PlexA1 and mutant proteins were immunoprecipitated with immobilized anti-Myc antibody. Immunoprecipitates were immunoblotted with anti-Myc antibody or anti-V5 antibody. Immunoblotting with anti-Myc antibody reveals an equal expression level for wild-type PlexA1, PlexA1Δsem, and PlexA1Δect (Figure 3). Anti-V5 immunoblot demonstrates that PlexA1sem physically associates both with PlexA1Δsem and wild-type PlexA1, but not with PlexA1Δect. Anti-V5 antibody blotting of cell lysates reveals an equal amount of PlexA1 sem expression among these preparations. These results demonstrate that the sema domain of PlexA1 binds to the carboxyl half of the PlexA1 ectodomain.

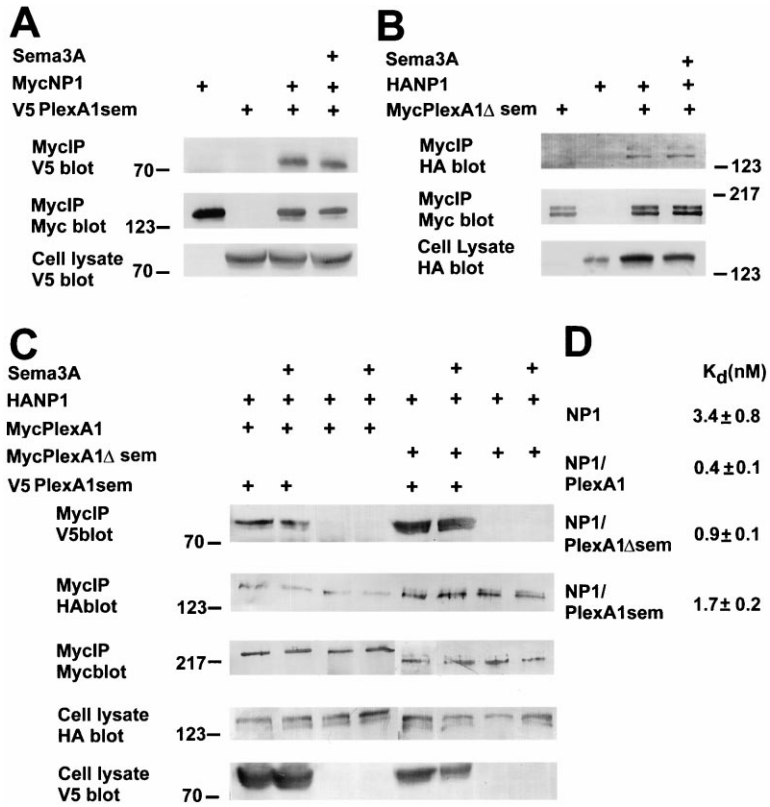

Figure 5. NP1 Associates Both with PlexA1-sem and PlexA1Δsem

(A) HEK293T cells were transfected with vector or Myc-tagged NP1 together with vector or V5-tagged PlexA1sem. One day later, cultures were treated with or without 3–5 nM Sema3A for 1 hr at 37°C. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-V5 antibody or anti-Myc antibody. Note that PlexA1sem coprecipitates with NP1. Molecular size markers (in kDa) are shown in the left.

(B) HEK293T cells were transfected with vector or Myc-tagged PlexA1Δsem together with vector or HA-tagged NP1. Twenty-four hours after transfection, cells were treated with 3–5 nM Sema3A as in A. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-HA or anti-Myc antibody. Note that NP1 coprecipitates with PlexA1Δ-sem. Molecular size markers (in kDa) are shown in the right.

(C) HEK293T cells were transfected with vector, Myc-tagged PlexA1, or Myc-tagged PlexA1Δsem with vector and HA-tagged NP1 or V5-tagged PlexA1sem and HA-tagged NP1. Twenty-four hours later, cells were incubated with 3–5 nM Sema3A as described in this article. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-HA antibody, anti-Myc antibody, or anti-V5 antibody. Molecular size markers (kDa) are shown in the left. The molecular weights of NP1, PlexA1Δsem, PlexA1, and PlexA1sem are 125, 200, 250, and 75 kDa, respectively.

(D) Kd (nM) of Sema3A-AP binding to COS-7 cells expressing NP1, NP1/PlexA1, NP1/PlexA1Δsem, or NP1/PlexA1sem. The means ± SEM from three to five experiments are presented.

Addition of PlexA1sem Inhibits the Constitutive Activity of PlexA1Δsem

If intramolecular sema domain binding to the remainder of the ectodomain is required to avoid the constitutively active state, then the PlexA1sem domain should reverse constitutive activity of PlexA1Δsem. We examined the morphology of COS-7 cells expressing V5-tagged PlexA1 sem plus Myc-tagged PlexA1, Myc-tagged PlexA1Δ-sem, or Myc-tagged PlexA1Δect. PlexA1-expressing cells were detected and analyzed by anti-Myc antibody. As described, expression of PlexA1Δsem or PlexA1Δect, but not PlexA1, induces the contraction of COS-7 cells (Figure 2). On the other hand, COS-7 cells expressing both PlexA1sem and PlexA1Δsem spread as completely as do PlexA1 or PlexA1sem/PlexA1-expressing COS-7 cells (Figure 4). Because the immunoprecipitation experiments show that PlexA1sem does not interact with PlexA1Δect, expression of PlexA1sem should not block the constitutive activity of PlexA1Δect. Indeed, COS-7 cells expressing PlexA1sem/PlexA1Δect exhibit no increased cellular area as compared with PlexA1-Δect-expressing COS-7 cells (Figure 4). This confirms the specificity of PlexA1sem morphologic effects for PlexA1Δsem-expressing cells. The simplest interpretation is that PlexA1sem inhibits the constitutive activity of PlexA1Δsem by binding to the remainder of the PlexA1 ectodomain.

PlexA1sem Associates with NP1

How might PlexA1sem mediate autoinhibition of PlexA1 participate in Sema3A signaling through a NP1/PlexA1 complex? To begin a molecular examination, we tested the interaction of PlexA1sem with NP1. Extracts from HEK 293T cells expressing Myc-tagged NP1 and V5-tagged PlexA1sem were immunoprecipitated with anti-Myc antibody-conjugated agarose. Anti-V5 immunoblotting of the immunoprecipitates reveals PlexA1sem association with NP1 (Figure 5A). This interaction is not significantly affected by the presence of excess Sema3A, indicating that the PlexA1sem binding site on NP1 is different from that for Sema3A. This is consistent with our previous finding that PlexA1 does not compete with Sema3A for binding to NP1 but actually enhances Sema3A binding to NP1 (Takahashi et al., 1999). Indeed, cotransfection of PlexA1sem with NP1 slightly enhances the binding of Sema3A to NP1 (Figure 5D).

We also examined the interaction between NP1 and PlexA1Δsem in this coimmunoprecipitation assay. HEK 293T cells were transfected with HA-tagged NP1 and Myc-tagged PlexA1Δsem. Anti-HA antibody blotting of anti-Myc immunoprecipitates reveals an association between NP1 and PlexA1Δsem (Figure 5B). The addition of Sema3A does not have a detectable effect on this association. Furthermore, Sema3A binding affinity to NP1/PlexA1Δsem-expressing COS-7 cells is more avid

than to NP1-expressing COS-7 cells (Figure 5D). Thus, both the PlexA1 sema domain and the remainder of the PlexA1 ectodomain interact with NP1 at sites distinct from the Sema3A binding site of NP1, and the two Plex domains cooperate to enhance Sema3A binding to NP1.

During Sema3A stimulation of a NP1/PlexA1 complex, PlexA1sem autoinhibition of PlexA1Δsem appears to be released. This might be correlated with a physical dissociation of the PlexA1Sem domain from a Sema3A/NP-1/PlexA1Δsem complex. Alternatively, although PlexA1sem may no longer repress PlexA1Δsem, it may remain physically associated with Sema3A/NP1. To distinguish these possibilities, we transfected Myc-tagged PlexA1 or Myc-tagged PlexA1Δsem together with HA-tagged NP1 and V5-tagged PlexA1sem into HEK293T cells and immunoprecipitated with anti-Myc antibody following a 1 hr exposure to 5 nM Sema3A-AP or AP. Anti-V5 immunoblots reveal that the amount of coprecipitated PlexA1sem from Sema3A-treated cells is almost identical to that from AP-treated cells. Therefore, PlexA1sem remains associated with a Sema3A/NP1/PlexA1Δsem complex, and the effect of ligand must be on the conformation rather than the composition of the complex.

PlexA1sem Inhibits Sema3A-Induced Contraction of NP1/PlexA1-Expressing COS-7 Cells

One mechanism for Sema3A activation of PlexA1 in a NP1/PlexA1 complex is reduced autoinhibitory PlexA1sem binding to the remainder of the PlexA1 ectodomain. If this mechanism is operable, then excess PlexA1sem might be predicted to substitute for endogenous PlexA1 sem and shift an equilibrium toward PlexA1 signaling inactivity. To explore this prediction, COS-7 cells expressing NP1/PlexA1 with or without PlexA1sem were incubated with 3 nM of AP-Sema3A for 1 hr at 37°C. Alkaline phosphatase activity was utilized to visualize the Sema3A binding cells. Although Sema3A induces the contraction of NP1/PlexA1-expressing cells in the absence of excess PlexA1sem, COS-7 cells expressing NP1/PlexA1/PlexA1sem do not respond to Sema3A and remain spread (Figure 6). Therefore, overexpression of PlexA1sem blocks Sema3A signaling in COS-7 cells. The effect may be attributable to secreted PlexA1sem throughout the culture medium or to locally higher concentrations of PlexA1sem near the surface of transfected cells.

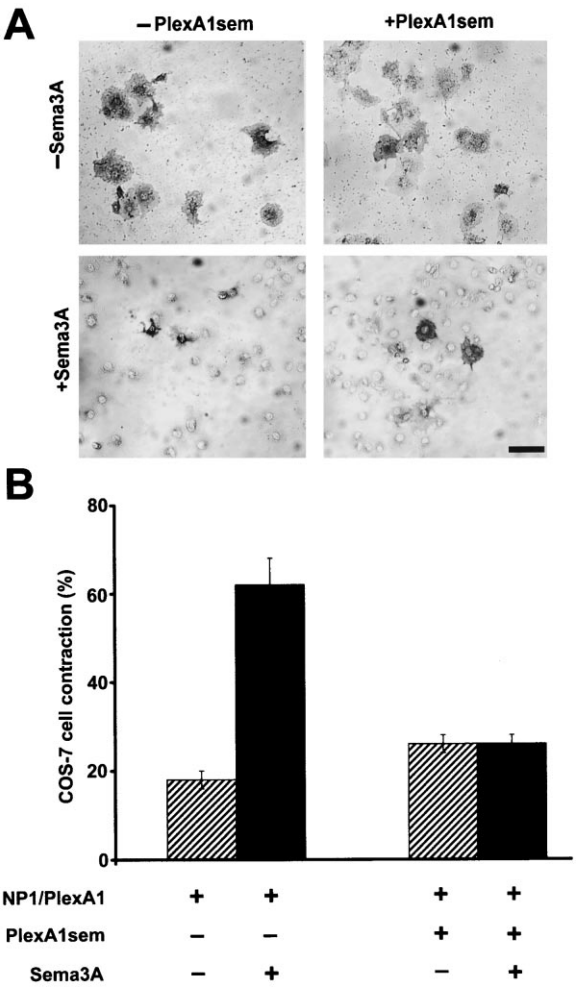

Figure 6. PlexA1sem Inhibits Sema3A-Induced Contraction of COS-7 Cells Expressing NP1/PlexA1

(A) COS-7 cells expressing NP1/PlexA1 with or without PlexA1sem were treated with 3 nM Sema3A or AP. Cells treated with AP-Sema3A were visualized via AP histochemistry. Cells treated with AP were visualized by anti-HA antibody staining. Although Sema3A induced the contraction of NP1/PlexA1-expressing cells in the absence of PlexA1sem, COS-7 cells transfected with NP1/PlexA1/PlexA1sem are spread after Sema3A treatment. Scale bar, 70 μm.

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

PlexA1Δsem and PlexA1Δect Are Constitutively Active in DRG Neurons

To confirm that PlexA1Δsem and PlexA1Δect mutants are consitutively active in neuronal cells as well as COS-7 cells, we introduced these mutant genes into dissociated E7 chick DRG neurons by infection with recombinant herpes simplex virus (HSV). One day later, growth cone morphology was examined. Growth cones of uninfected, wild-type PlexA1, or PlexA3Δect-expressing DRG neurons exhibited similarly broad lamelipodia. In contrast, the majority of PlexA1Δsem and PlexA1Δect-expressing DRG growth cones were collapsed and were indistinguishable from Sema3A-treated DRG growth cones (Figures 7A and 7B). Thus, both PlexA1Δsem and PlexA1Δect appear constitutively active in neuronal growth cones. If the collapsing effect of PlexA1Δsem is due to the activation of specific signaling cascade rather than a nonspecific toxicity, then it should be reversed by excess PlexA1sem, as observed in COS-7 cells (Figure 4). Indeed, coinfection of PlexA1sem virus reverses PlexA1Δsem-induced growth cone collapse, but not PlexA1Δect-induced collapse (Figure 7C). Thus, there is an intramolecular plexA1 activation pathway controlling DRG growth cone morphology.

Growth cone collapse by Sema3A is associated with reduced neurite outgrowth. To the extent that the truncated PlexA1 mutants function in a similar fashion to Sema3A-activated receptors, a reduction in neurite out-

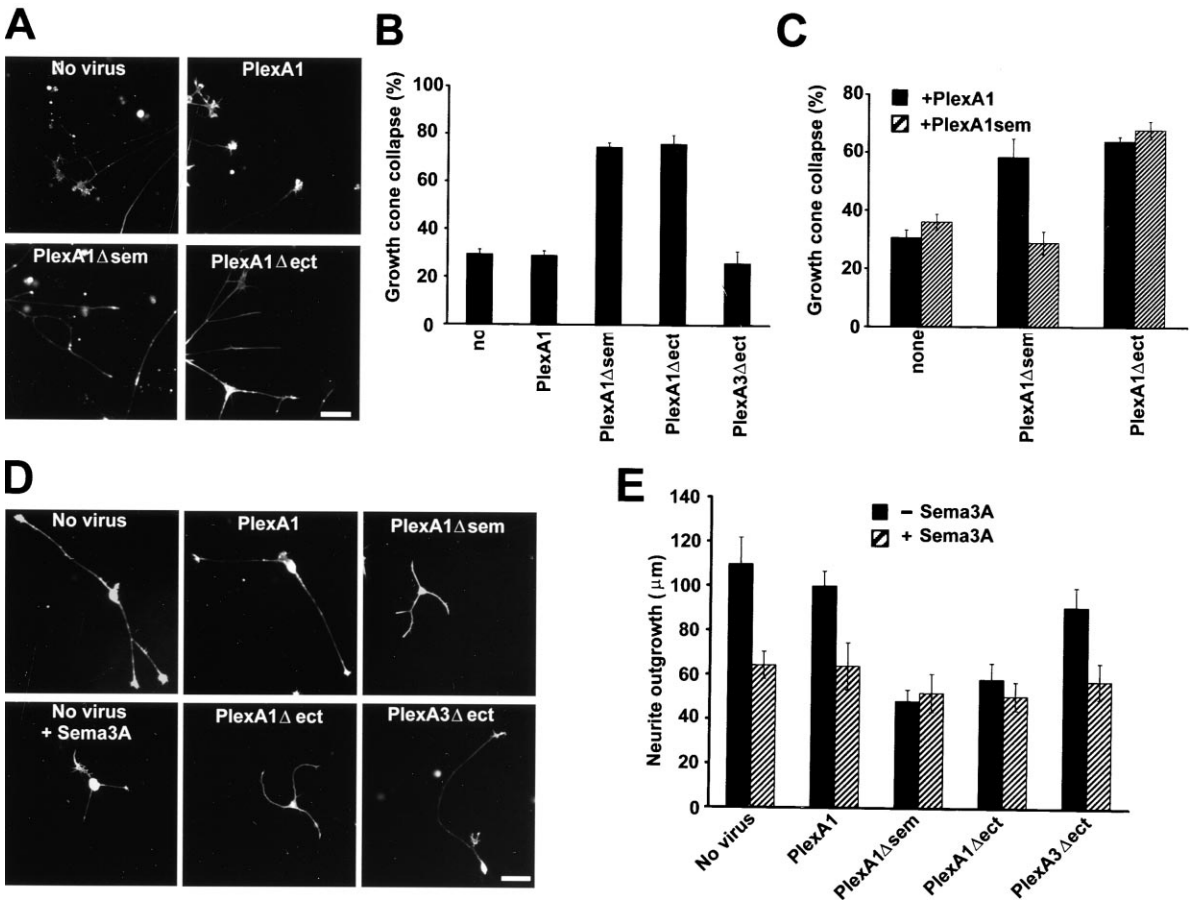

Figure 7. PlexA1Δsem and PlexA1Δect Are Constitutively Active in DRG Neurons

(A) Dissociated chick E7 DRG cultures were exposed to no virus or to PlexA1, PlexA1Δsem, PlexA1Δect, or PlexA3Δect recombinant HSV. One to two days later, cultures were fixed and stained with rhodamine-phalloidin to visualize F-actin in the growth cones. Note that nonvirus-infected, PlexA1 virus-infected growth cones have typical well-spread lamelipodia. In contrast, a majority of PlexA1Δsem virus or PlexA1Δect virus-treated growth cones are collapsed. Scale bar, 20 μm.

(B) The percentage of collapsed growth cones for each condition is indicated. The means ± SEM from three to five experiments are presented.

(C) DRG cultures were infected with HSV preparations encoding PlexA1Δsem or PlexA1Δect together with a 3-fold MOI excess of HSV preparations encoding PlexA1 or PlexA1sem. Growth cone morphology measurements from three independent experiments are shown.

(D) Dissociated chick E7 DRG cultures were exposed to no virus or to PlexA1, PlexA1Δsem, PlexA1Δect, or PlexA3Δect recombinant HSV. Infected neurons were cultured for 12 hr in suspension and then replated on laminin-coated dishes with or without 10 nM Sema3A for 6 hr. Note that neurite outgrowth from PlexA1Δsem- and PlexA1Δect-expressing neurons is reduced compared with that form noninfected, PlexA1-, and PlexA3Δect-expressing neurons. Scale bar, 20 μm.

(E) Average neurite outgrowth after viral infection. The means ± SEM from three to five experiments are presented.

growth should result from their expression. To explore this prediction, dissociated E7 chick DRG neurons were infected with recombinant HSV preparations for 12 hr in suspension culture to allow for protein expression, and then outgrowth was assessed during a 6-hr period on laminin-coated surfaces. Neurites of noninfected, PlexA1, or PlexA3Δect-expressing DRG neurons are significantly longer than PlexA1Δsem- or PlexA1Δect-expressing neurons (Figures 7D and 7E). Furthermore, 10 nM of Sema3A decreases neurite outgrowth in control cultures to the same level observed in PlexA1Δsem- or PlexA1Δect-expressing neurons. Consistent with constitutive PlexA1 activation, 10 nM Sema3A had no further inhibitory effect on neurite outgrowth in PlexA1Δsem- or PlexA1Δect-expressing neurons (Figures 7D and 7E). Thus, the truncated PlexA1 mutants fully activate the Sema3A signaling pathway in DRG neurons.

PlexA1sem Blocks Sema3A-Induced Growth Cone Collapse in DRG Neurons

Because PlexA1sem attenuates Sema3A action in nonneuronal COS-7 cells, we tested whether PlexA1sem might block Sema3A-induced growth cone collapse in E7 chick DRG neurons. We purified PlexA1sem by virtue of its His tag from the medium of PlexA1sem-expressing HEK293T cells. Purified PlexA1sem (10 nM) was incubated with chick E7 DRG neurons for 30 min prior to Sema3A exposure. This concentration of PlexA1sem reduces the potency of Sema3A as a growth cone–collapsing agent by a factor of four (Figures 8A and 8B).

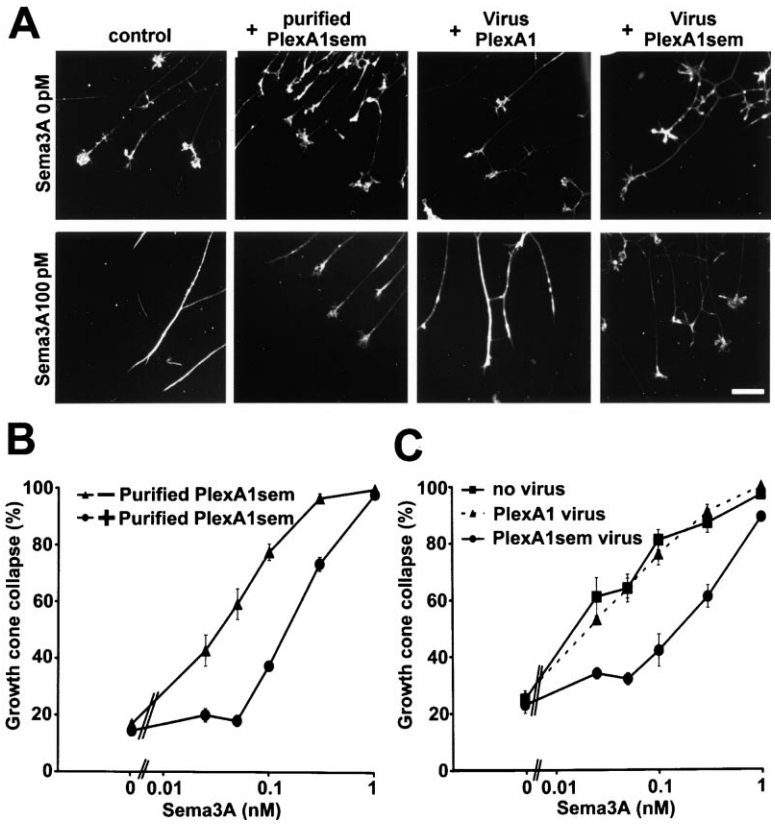

Figure 8. PlexA1sem Inhibition of Sema3A Action in Neurons

(A) Explants of E7 chick DRG were treated with or without 10 nM purified PlexA1sem or PlexA1sem virus. Rhodamine-phalloidin staining of DRG growth cones is presented. Either purified PlexA1sem or PlexA1sem virus infection reduces 100 pM Sema3A-induced growth cone collapse. Scale bar, 20 μm.

(B and C) Sema3A dose–response curve for DRG growth cone collapse. (B) DRG cultures were treated with purified PlexA1sem or buffer for 30 min in DRG medium, and then growth cone collapse assays were performed with the indicated concentrations of Sema3A.

(C) Growth cones were treated with or without PlexA1 virus or PlexA1sem virus for 24–48 hr, and growth cone collapse was performed with various concentrations of Sema3A. The means ± SEM from three to five experiments are presented.

As an alternative method to provide more complete receptor silencing, PlexA1sem was coexpressed with endogenous Sema3A receptor complexes via HSV infection of E7 chick DRG neurons. Overexpression of full-length PlexA1 does not change the responsiveness of DRG growth cones to Sema3A. On the other hand, expression of PlexA1sem shifts the dose response curve for Sema3A to the right by a factor of 10 (Figures 8A and 8C). These results demonstrate that the sema domain of PlexA1 can significantly reduce Sema3A-induced growth cone collapse in DRG neurons.

Discussion

Mechanism of Plexin Activation

The major finding of the present study is that a mutant PlexA1 lacking the sema domain exhibits constitutive activation. This mutant reduces COS-7 cell area, collapses DRG growth cones, and inhibits neurite outgrowth from DRG neurons. These data imply that the sema domain of PlexA1 restricts PlexA1 to a basal inactive state by binding to the remainder of the ectodomain. In support of this interpretation, we show that PlexA1sem physically associates with PlexA1Δsem and that PlexA1sem coexpression attenuates the constitutive activity of PlexA1Δsem. The constitutive activity of PlexA1Δsem and PlexA1Δect does not appear to be due to a nonphysiologic conformational change induced by large-scale deletion because (1) PlexA1sem reverses the constitutive activity of PlexA1Δsem, (2) PlexA3Δect is inactive, and (3) exposure to excess Sema3A does not attenuate neurite outgrowth from PlexA1Δsem- or PlexA1Δect-expressing DRG neurons.

What is the role of the PlexA1 sema domain in the NP/PlexA1 complex? We demonstrate that PlexA1sem associates with both NP1 and the remainder of PlexA1. Because coprecipitation is not modified by the presence of Sema3A, the PlexA1 sema domain must bind to NP1 at a site different from the Sema3A binding site of NP1. Indeed, coexpression of PlexA1sem with NP1 enhances Sema3A avidity for NP1. PlexA1Δsem also associates with NP1 in a Sema3A-independent fashion and coexpression of PlexA1Δsem increases Sema3A affinity for NP1. This implies that NP1 interacts with three partners at three distinct sites. PlexA1sem and the remainder of PlexA1 ectodomain cooperate to increase the Sema3A affinity for NP1. In the NP1/PlexA1 complex, PlexA1 sema domain interactions with both NP1 and the remainder of the PlexA1ectodomain may keep PlexA1 silent. Presumably, Sema3A binding to NP1 results in a dramatic conformational change in this complex. The simplest model is that this conformational change physically separates and functionally reduces PlexA1sem effects on the remainder of the PlexA1 ectodomain (Figure 9). In support of such a conformational dissociation of the two PlexA1 domains, excess PlexA1sem can reverse constitutive activation or Sema3A-induced PlexA1 activation. Although the two PlexA1 domains are functionally dissociated by Sema3A binding to NP-1, they remain bound in a NP1-dependent complex of altered conformation. The NP1 antagonists, Sema3C, Sema3B, and Sema3F, are expected to occupy the Sema3A site but

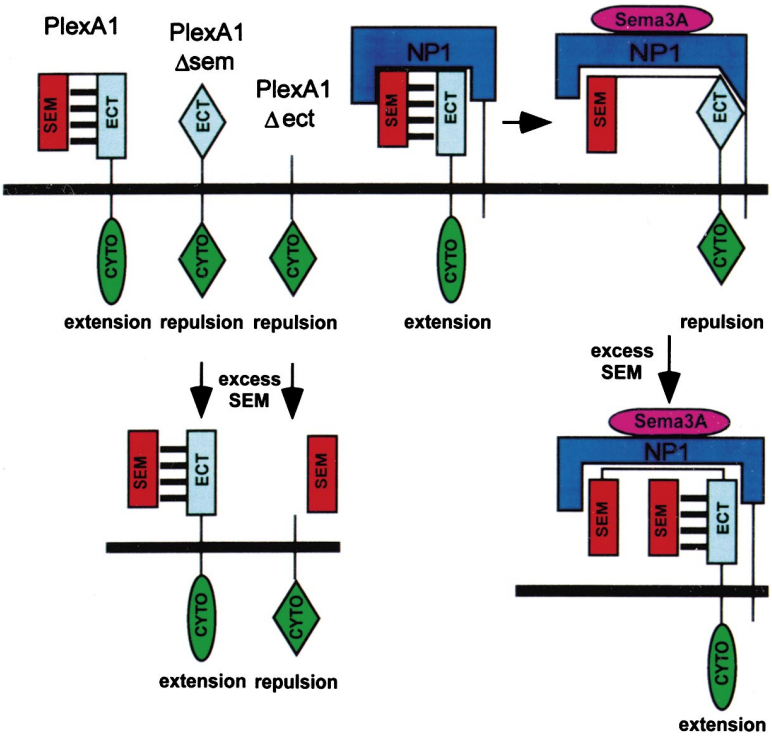

Figure 9. A Model for Plexin Activation

For full-length PlexA1, PlexA1sem binds to the remainder of PlexA1 ectodomain and maintains activity at a low basal level. PlexA1Δsem and PlexA1Δect are constitutively active because of the lack of inhibition by PlexA1sem. The constitutive activity of PlexA1Δsem is reversed by PlexA1sem binding to the remainder of PlexA1 ectodomain. In the NP1/PlexA1 complex, PlexA1sem binds both to NP1 and the remainder of PlexA1 ectodomain and inhibits the activation of PlexA1. Sema3A binding to NP1 induces a conformational change in the complex and dissociates PlexA1sem from the remainder of PlexA1 ectodomain. This leads to the activation of PlexA1 signaling. Excess PlexA1sem can bind to the "empty" PlexA1 ectodomain and inhibit the PlexA1 signaling.

not result in this conformational shift and the release of PlexA1sem from the remainder of the PlexA1 ectodomain.

After conformational changes occur in the extracellular domains of PlexA1, they may be transmitted intramolecularly across the lipid bilayer. Alternatively, macromolecular aggregation state may play a role in transducing conformational changes to the cell interior, as for tyrosine kinase receptors (Yarden and Schlessinger, 1987). Previously, we have shown that Sema3A induces the aggregation of Plex on DRG growth cones and speculated that the formation of Plex aggregates might be crucial for the activation of signaling (Takahashi et al., 1999). It is conceivable that PlexA1 sema domain binding to the remainder of PlexA1 ectodomain prevents the formation of higher order aggregates. Because PlexA1-Δect is constitutively active, this model would imply that the transmembrane + cytoplasmic domain is inherently capable of aggregate formation. It remains unclear whether aggregate formation is the sine qua non of activation or whether it is a secondary consequence of signal transduction. It remains undetermined whether induction of full-length PlexA1 aggregation is sufficient to activate signaling.

A number of semaphorins outside of class 3 can bind directly to Plexins (Comeau et al., 1998; Winberg et al., 1998; Tamagnone et al., 1999). Presumably, these semaphorins disrupt the *cis* binding of the Plex sema domain and activate signaling. They might do so binding to the Plex sema domain as a sema dimer by competing with the Plex sema domain for interaction with the remainder of the Plexin protein or by a combination of these mechanisms.

Specificity of Semaphorin Signaling

Clearly, the signaling specificity within the large semaphorin family is crucial to an understanding of their role in biology. Previously, we found that PlexA1 associates with NP and mediates Sema3A signaling (Takahashi et al., 1999). Here, we demonstrate that PlexA1 and PlexA2 are equally active in transducing Sema3 signals. On the other hand, PlexA3 is inactive in these assays as a mediator of Sema3/NP signaling. The biological function of PlexA3 in DRG and sympathetic neurons is not clear. Because PlexA3 can form a complex with NPs (Tamagnone et al., 1999), it might function as an inactive, buffering component. Further work is required to determine whether multiple PlexAs are associated in a single complex with Sema3 dimers and NP multimers.

At least in part, the inactivity of PlexA3 is determined by the intracellular domain, as the PlexA3Δect protein is not constitutively active in either nonneuronal or neuronal cells. Given that the PlexA1, PlexA2, and PlexA3 intracellular domains are quite similar, the current findings may simplify a search for those residues that determine signaling competency. Although PlexB1 and PlexB2 are inactive as mediators of NP/Sema3 signals, we have not examined whether the PlexB1Δect and PlexB2Δect proteins might be constitutively active. Because the PlexB family possesses furin protease recognition sites in the juxtamembrane region of ectodomain (Tamagnone et al., 1999), proteolysis might regulate the activity of PlexB1 and PlexB2.

With regard to Sema3C, no combination of one Plex and one NP was sufficient for a cell morphology response in COS-7 cells. Although it remains possible that the appropriate plexin has not yet been tested, it seems more likely that both NP1 and NP2 are required for

Sema3C activity. This is supported by the finding that either anti-NP1 antibody or anti-NP2 antibody can block the Sema3C activity in sympathetic neurons (Chen et al., 1998).

Signaling Molecules Downstream of Plexin

CRMP (Goshima et al., 1995), rac1 (Jin and Strittmatter, 1997; Fournier et al., 2000), cGMP (Song et al., 1998), and tyrosine kinase activity (Tamagnone et al., 1999) have all been associated with Sema3A signaling and/or the Plex cytoplasmic domain. However, the exact pathway from cytoplasmic Plex to axon repulsion remains poorly defined. Because dominant negative rac1 blocks Sema3A-induced contraction of NP1/PlexA1-expressing COS-7 cells (T. Takahashi and S. M. Strittmatter, unpublished data), as well as Sema3A-induced growth cone collapse in DRG neurons (Jin and Strittmatter, 1997), similar signaling pathways are likely to operate in both cell types. The current identification of constitutively active Plexins should greatly facilitate further analysis of downstream signaling events. Of note, the cytoplasmic domain of PlexA1 requires its membrane-tethering transmembrane domain for constitutive activity. In this light, it is interesting to note that significant proportions of CRMP and rac1 are membrane-associated (Goshima et al., 1995; Michaely et al., 1999). Furthermore, the activity of several GTPase-regulating proteins is regulated by membrane association (Hasegawa et al., 1996).

Experimental Procedures

PlexA1, PlexA2, and PlexA3 Expression Vectors

The plasmids containing PlexA1-pcDNA3.1-Myc-His, PlexA1-pHSVPrPUC, HA-tagged NP1-pcDNA1, and Myc-tagged NP1-pcDNA1 were described previously (Nakamura et al., 1998; Takahashi et al., 1998; Takahashi et al., 1999). To obtain PlexA1Δsem-pSecTAG2, a cDNA fragment of PlexA1 (aa 542–1874) was amplified by polymerase chain reaction (PCR) and ligated into the BamHI and EcoRV sites of pSecTag2A. To express PlexA1Δect, a PCR-amplified fragment of PlexA1 (aa 1218–1874) was ligated into the BamHI and EcoRV sites of pSecTag2A. To obtain PlexA1sem-pcDNA3.1-V5-His, the mouse PlexA1 cDNA (Kameyama et al., 1996) was digested with EcoRI and HpaI and then ligated into the EcoRI and EcoRV sites of pcDNA3.1-V5-HisC. A fragment of PlexA1 encoding the intracellular domain (aa 1266–1874) was PCR amplified and ligated into pcDNA3.1-Myc-HisC. Human PlexA2 cDNA (Kazusa, DNA Research Institute; cDNA clone KIAA0463) was digested with KpnI and NotI, and the DNA fragment (aa 1–1885) was ligated into pcDNA3.1-Myc-HisA. For expression of PlexA3Δect, a DNA fragment (aa 1072–1872) was amplified by PCR from E15 mouse cDNA (Quick-Clone; Clontech, Palo Alto, CA) and then digested with BamHI and XhoI. This fragment was ligated into pSecTag2A. To obtain full-length PlexA3, a DNA fragment (aa 21–1071) was amplified by PCR from the E15 mouse cDNA described previously in this article and digested with HindIII and BamHI. This fragment was then ligated into PlexA3Δect-pSecTag2A.

Coimmunoprecipitation

HEK293T cells (1×10^6) were transfected with the expression vector for Myc-PlexA1 (1μg), Myc-PlexA1Δsem, or Myc-PlexA1Δect together with V5-PlexA1sem by the FuGENE-6 method. After 24 hr, cells were lysed with ice-cold immunoprecipitation buffer as described previously (Takahashi et al., 1998; Takahashi et al., 1999). Protein from cell extracts was immunoprecipitated with anti-Myc (9E10) antibody-conjugated agarose beads as described (Takahashi et al., 1998; Takahashi et al., 1999). Bound protein was analyzed by immunoblot with either anti-V5 antibody or anti-Myc antibody (9E10). In other experiments, Myc-NP1 and V5-PlexA1sem, Myc-PlexA1Δsem and HA-NP1, Myc-PlexA1 and HA-NP1 together with V5-PlexA1sem, or Myc-PlexA1Δsem and HA-NP1 together with V5-PlexA1sem were expressed. After a 1-hr incubation with or without Sema3A (5 nM), cells were lysed as described (Takahashi et al., 1999). Cell extracts were immunoprecipitated with anti-Myc antibody-conjugated agarose beads as described (Takahashi et al., 1999). Immunoprecipitates were analyzed with anti-V5 antibody, anti-HA antibody, or anti-Myc antibody as described (Takahashi et al., 1999).

COS-7 Cell Morphology and Binding Assay

COS-7 cells (1×10^5) were transfected with 1 μg of PlexA1-pcDNA3.1-MycHis, PlexA1Δsem-pSecTag2, PlexA1Δect-pSecTag2, PlexA1cyto-pSecTag2 together with 1 μg of pcDNA3.1-MycHis or PlexA1sem-pcDNA3.1-V5His. After 24 hr, these cells were fixed with 3.7% formaldehyde in phosphate-buffered saline. Cells were incubated with anti-Myc antibody (9E10) and fixed with 3.7% formaldehyde again. After 2 hr at 65°C to inactivate endogenous alkaline phosphatase, cells were incubated with alkaline-phosphatase-conjugated anti-mouse IgG. Cells were visualized by the deposition of insoluble AP reaction product from nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The size of stained cells was analyzed as described (Takahashi et al., 1999). In the other experiments, COS-7 cells were transfected with PlexA1-pcDNA-MycHis (1 μg) and HA-NP1-pcDNA1 (0.25 μg) together with PlexA1-sem-pcDNA3.1-V5His (1 μg). After 24 hr, cells were incubated with or without 3 nM of AP-Sema3A at 37°C for 1 hr as described previously (Takahashi et al., 1999). Cells were then washed and fixed. After heat inactivation, Sema3A binding was visualized by NBT/BCIP. Cells not treated with Sema3A were incubated with AP for 1 hr at 37°C. The morphology of such cells was monitored by anti-HA immunohistology.

For Sema3A binding assays, COS-7 cells were transfected with NP1, NP1/PlexA1, NP1/PlexA1Δsem, or NP1/PlexA1sem. Twenty-four hours after transfection, cells were incubated with various concentrations of Sema3A-AP for 2 hr as described (Takahashi et al., 1999). After washing, cells were fixed and heat inactivated as described previously in this article. Binding of Sema3A-AP to COS-7 cells was quantitated as described (Takahashi et al., 1998; Takahashi et al., 1999).

Recombinant HSV Preparations

The coding regions of PlexA1Δsem, PlexA1Δect, and PlexA3Δect (including the pSecTag2 derived signal peptide) were PCR amplified and ligated to pHSVPrPUC vector. For PlexA1sem virus, the coding region of PlexA1sem was excised from pcDNA3.1-MycHis vector and ligated to pHSVPrPUC vector. The resulting plasmids (2μg) were transfected into 2–2 cells (3×10^5) with lipofectamine or FUGENE and then superinfected with 5dl1.2 HSV helper virus 1 day later as described (Nakamura et al., 1998; Takahashi et al., 1998; Takahashi et al., 1999). Recombinant virus was amplified through three passages and stored at −80°C.

Purification of PlexA1sem Protein

HEK293T cells were transfected with PlexA1sem-pcDNA3.1-MycHis by the FuGENE-6 method. Three days after, 200 ml of medium were collected and supplemented with Imidazole to 25 mM and with NaCl to 200 mM. The medium was passed through a 0.45 μm filter and applied to a 0.75 ml Ni-containing resin. Protein was eluted with 200 mM Imidazole, 50 mM NaCl, pH 7.3.

DRG Growth Cone and Neurite Outgrowth Analysis

The culture and HSV infection of chick E7 DRG explants has been described previously (Nakamura et al., 1998; Takahashi et al., 1998; Takahashi et al., 1999). Growth cone collapse assays were performed as described (Igarashi et al., 1993; Goshima et al., 1995; Takahashi et al., 1997). For experiments with purified PlexA1sem, we preincubated cultures with 10 nM of PlexA1sem for 30 min and then performed standard growth cone collapse assays. In tests of the constitutive activity of PlexA1 mutants, E7 DRG neurons were infected with HSV preparations for PlexA1, PlexA1Δsem, PlexA1-Δect, or PlexA3Δect . After 24–48 hr, DRG neurons were fixed with

ice-cold 3.7% formaldehyde/20% sucrose in phosphate-buffered saline. Growth cones were visualized by rhodamine-phalloidin staining. For the analysis of neurite outgrowth, dissociated E7 chick DRG neurons were preplated on uncoated plastic dishes in the presence of HSV preparations encoding PlexA1, PlexA1Δsem, PlexA1Δect, or PlexA3Δect. After 12 hr, neurons were collected and replated on laminin-coated dishes with or without 10 nM Sema3A. Six hours later, cells were fixed stained, and neurite outgrowth was quantitated (Goshima et al., 1995; Jin and Strittmatter, 1997). Neuronal survival was indistinguishable between cultures expressing PlexA1, PlexA1Δsem, PlexA1Δect, and PlexA3Δect.

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