Plexins Are a Large Family of Receptors for Transmembrane, Secreted, and GPI-Anchored Semaphorins in Vertebrates

Luca Tamagnone,[1,6] Stefania Artigiani,[1,7] Hang Chen,[2,7]
Zhigang He,[2,7] Guo-li Ming,[4] Hong-jun Song,[4,8]
Alain Chedotal,[5] Margaret L. Winberg,[3]
Corey S. Goodman,[3] Mu-ming Poo,[4]
Marc Tessier-Lavigne,[2] and Paolo M. Comoglio[1]
[1]Institute for Cancer Research and Treatment
University of Torino
10060 Candiolo
Italy
[2]Howard Hughes Medical Institute
Departments of Anatomy and
   Biochemistry and Biophysics
University of California
San Francisco, California 94143
[3]Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720
[4]Department of Biology
University of California
San Diego, California 92093
[5]INSERM U106
Hôpital de la Salpetrière
75013 Paris
France

Summary

In *Drosophila*, plexin A is a functional receptor for sema-phorin-1a. Here we show that the human plexin gene family comprises at least nine members in four sub-families. Plexin-B1 is a receptor for the transmem-brane semaphorin Sema4D (CD100), and plexin-C1 is a receptor for the GPI-anchored semaphorin Sema7A (Sema-K1). Secreted (class 3) semaphorins do not bind directly to plexins, but rather plexins associate with neuropilins, coreceptors for these semaphorins. Plex-ins are widely expressed: in neurons, the expression of a truncated plexin-A1 protein blocks axon repulsion by Sema3A. The cytoplasmic domain of plexins asso-ciates with a tyrosine kinase activity. Plexins may also act as ligands mediating repulsion in epithelial cells in vitro. We conclude that plexins are receptors for multiple (and perhaps all) classes of semaphorins, ei-ther alone or in combination with neuropilins, and trig-ger a novel signal transduction pathway controlling cell repulsion.

Introduction

We previously identified a novel human gene family named after the prototype member SEX (Maestrini et

[6] To whom correspondence should be addressed (e-mail: ltamagnone @ircc.unito.it).
[7] These authors contributed equally to this work.
[8] Present address: Molecular Neurobiology Laboratory and Howard Hughes Medical Institute at the Salk Institute, La Jolla, California 92037.

al., 1996). Murine and amphibian members of this family were independently cloned and named plexins (Ohta et al., 1995; Kameyama et al., 1996a, 1996b). We refer here to this family as plexins. Plexins encode large transmem-brane proteins whose cysteine-rich extracellular do-mains share regions of homology with the scatter factor receptors (encoded by the Met gene family). The extra-cellular domains of plexins also contain ~500 amino acid semaphorin domains (see below). The highly con-served cytoplasmic moieties of plexins (~600 amino acids), however, have no homology with the Met tyrosine kinase domain nor with any other known protein. Met-like receptors and their ligands the scatter factors medi-ate a complex biological program including dissociation of cell–cell contacts, motility, and invasion (for a review, see Tamagnone and Comoglio, 1997). During embryo-genesis, scatter factor-1 and Met promote the dissocia-tion of cell layers in the somites and drive the migration of myogenic cells to their appropriate location (Bladt et al., 1995; Maina et al., 1996). Met and scatter factor-1 are also involved in controlling neurite outgrowth and axonal guidance (Ebens et al., 1996; Maina et al., 1997, 1998).

The first clue regarding a possible function for plexins came from the finding that a novel plexin, VESPR, inter-acts with the viral semaphorin A39R (Comeau et al., 1998). Semaphorins are a large family of secreted and membrane-bound molecules that are characterized by an extracellular domain containing an ~500 amino acid semaphorin domain (Kolodkin et al., 1993). As noted above, plexins contain a more divergent but neverthe-less conserved semaphorin domain.

Semaphorins were originally characterized in the ner-vous system, where they have been implicated in repul-sive axon guidance (Kolodkin et al., 1993; Luo et al., 1993; Tessier-Lavigne and Goodman, 1996). More re-cently, semaphorins have been further implicated in car-diac and skeletal development (Behar et al., 1996), in the immune response (Hall et al., 1996), in the regulation of angiogenesis (Miao et al., 1999), and in tumor growth and metastasis (Christensen et al., 1998).

We recently identified two plexins in *Drosophila mela-nogaster*, showed that both are expressed in the devel-oping nervous system, used biochemical analysis to show that plexin A is a receptor for class 1 semaphorins (Sema-1a and Sema-1b), and used genetic analysis to show that plexin A is a functional semaphorin receptor that controls motor and CNS axon guidance (Winberg et al., 1998).

In vertebrates, almost 20 semaphorins are known, classified into five subfamilies depending on distinctive structural features (see The Semaphorin Nomenclature Committee for review), but little is known about the re-ceptors that mediate the actions of these semaphorins. Members of one class of semaphorins, the class 3 (se-creted) semaphorins, have been shown to interact with transmembrane molecules called neuropilins (NP-1 and NP-2; Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). However, neuropilins have very
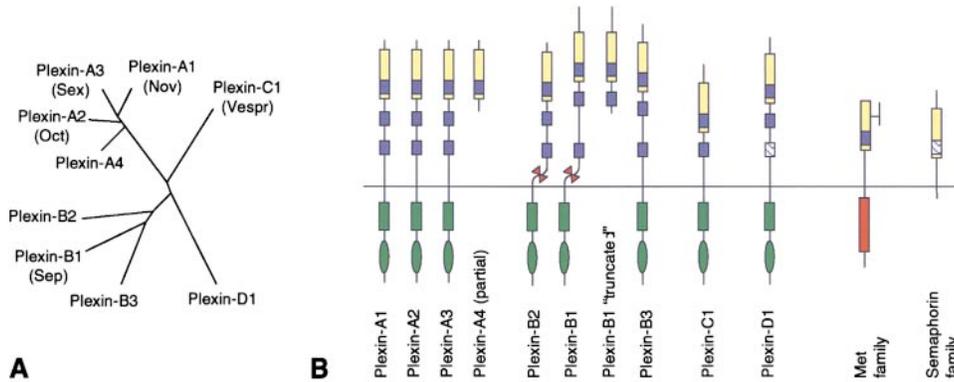
Figure 1. Human Plexin Family and Related Proteins

(A) Phylogenetic tree of human plexins. Known family members cluster in two major groups: plexin-A and plexin-B subfamilies (see text).
(B) Structural features of plexins, Met-like receptors, and semaphorins. In the extracellular moieties, yellow boxes indicate "*sema*" domains and blue boxes mark cysteine-rich MRS motifs, some of which are stippled to mark their atypical sequence. Sequence identity among *sema domains* ranges from 15%–50%, as previously described (see Winberg et al., 1998). Potential furin-like proteolytic sites are marked by red ribbons. Plexin-B1 "truncated" is the product of a splicing variant (see text). Plexin-D1 and plexin-C1 (VESPR) are more distant family members, since they include atypical features in their extracellular domains. The highly conserved intracellular domain of plexins (*SP domain*) contains two separate regions of high homology (Maestrini et al., 1996) (green oval and box). Met-like receptors are disulfide-bound heterodimers and include a cytoplasmic tyrosine kinase domain (red box). Mammalian semaphorins can be either secreted or cell surface proteins.

short cytoplasmic tails and are thought to require interaction with as yet unknown transmembrane coreceptor(s) for signal transduction (Nakamura et al., 1998). Moreover, nothing is known about the receptors for the many other classes of vertebrate semaphorins.

Many important issues related to plexin function in vertebrates therefore remain unanswered. First, what is the diversity of plexin proteins in vertebrates? Second, are the plexins receptors for vertebrate semaphorins, and if so, are they receptors for all classes of semaphorins? Third, how do they signal? In this paper, we begin to provide answers to these questions. Our results indicate that plexins are receptors for multiple subfamilies of semaphorins in vertebrates and suggest that plexins belong, with the Met receptor family, to a large protein superfamily controlling cell dissociation and repulsion in a variety of tissues.

Results

Sex/Plexins: A Wide Gene Family Encoding Cell Surface Proteins

We identified a novel human gene family (*SEX/plexins*) including four genes initially named *SEX*, *SEP*, *OCT*, and *NOV* (Maestrini et al., 1996). Three homologous murine genes (Kameyama et al., 1996a, 1996b) and another related human gene, *VESPR*, were also described (Comeau et al., 1998). By RT-PCR cloning and searching sequence databases (see Experimental Procedures), we have now identified four novel human plexins. Together with the five previously reported, these proteins fall into four homology groups, which we and our colleagues (M. Spriggs and H. Fujisawa, personal communications) propose to name the plexin-A, -B, -C, and -D subfamilies (Figure 1A). The plexin-A subfamily includes plexin-1/Nov (now plexin-A1), plexin-2/Oct (now plexin-A2), plexin-3/Sex (now plexin-A3), and a novel member, plexin-A4, whose corresponding gene is located on human chromosome 7. The plexin-B subfamily includes the former Sep (now plexin-B1) and two novel members,

plexin-B2 and plexin-B3 (genes located on human chromosomes 22 and X, respectively). *plexin-B3* maps very close to the *plexin-A3* genomic locus on Xq28. The plexin-C subfamily is defined by VESPR (now plexin-C1). A novel and atypical plexin, plexin-D1, is the prototype of the plexin-D subfamily.

We found that human *plexin-B1* is expressed in at least two different mRNA forms deriving from the alternative splicing of 583 nucleotides within the sequence encoding the extracellular domain. The two splice variants are found in comparable amounts in several cell types that we have examined (data not shown). The long mRNA corresponds to the previously reported sequence (Maestrini et al., 1996), while the short mRNA variant includes an early stop codon (due to a shift in the reading frame; see Experimental Procedures). In COS cells, the short *plexin-B1* cDNA is abundantly translated in a glycoprotein of ∼120 kDa ("plexin-B1 truncated" in Figure 1B), bound to the cell surface, and partially secreted (not shown).

Like previously identified plexins, these proteins have in their large extracellular domains regions of homology with two other protein families: (1) scatter factor receptors, encoded by the *MET* oncogene family (Tamagnone and Comoglio, 1997), and (2) semaphorins (Kolodkin et al., 1993; Figure 1B). In particular, plexins and Met-like receptors contain short cysteine-rich motifs, termed "Met-related sequences" (MRS), whose minimal consensus is: C-X(5-6)-C-X(2)-C-X(6-8)-C-X(2)-C-X(3-5)-C (Maestrini et al., 1996; Tamagnone and Comoglio, 1997; blue boxes in Figure 1B). The proteins of the Met family contain a single MRS (in their receptor β chains), whereas in plexin family members, there are three repeated MRS motifs; plexin-C1 is an exception, as its extracellular domain is smaller and includes only two MRS. Furthermore, all plexin family members contain in their extracellular moiety a 500 amino acid region similar to the *sema domain* of semaphorins (Kolodkin et al., 1993; Winberg et al., 1998; yellow boxes in Figure 1B).

Plexin-B proteins are the closest homologs to Met-like receptors. In particular, the extracellular domains

of plexin-B1 and -B2 include potential cleavage sites, satisfying the optimal consensus for furin-like proteases (R-X-K/R-R; indicated by red ribbons in Figure 1B; for a review, see Steiner, 1998). In the Met-like receptors there are similar polybasic sites, which are cleaved during biosynthesis by a furin-like convertase, yielding the mature form of the receptor that consists of two disulfide-linked α and β chains (Giordano et al., 1989). *Drosophila* plexin B (D Plex B) also includes a similar potential cleavage site in the same position as in human plexin-B1 and plexin-B2. We conclude that h-plexin-B1 and -B2, as well as D Plex B, may undergo furin-dependent proteolytic processing. Considering this intriguing observation, we suggest here that the two fly plexins D Plex A and D Plex B may share the same ancestry as the mammalian plexin A and B gene subfamilies, respectively.

The cytoplasmic domain of plexins contains an ∼600 amino acid domain that we term the *SP domain* ("*Sex and Plexins*," marked in green in Figure 1B) that is highly conserved within the family (57%–97% similarity) and in evolution (over 50% similarity between invertebrates and humans). The *SP domain* does not share homology with any known protein. It includes a number of potential tyrosine phosphorylation sites but lacks the typical motifs of catalytic tyrosine kinases. Interestingly, the predicted secondary structure of the *SP domain* includes long, conserved alpha helices typically found in protein–protein interaction modules. Furthermore, there are several dihydrophobic amino acid motifs (such as LL or LI) known to mediate the internalization and downregulation of transmembrane receptors (Sandoval and Bakke, 1994).

Plexins Are Specific Receptors for Cell Surface Semaphorins in Vertebrates

Plexin-C1 (VESPR) has been shown to bind the soluble viral semaphorins SemaVA (A39R) and SemaVB (AHV) (Comeau et al., 1998), and we recently found that D Plex A interacts with transmembrane Sema-1a (Winberg et al., 1998). We therefore examined in vertebrates whether the extracellular domain of several different cellular semaphorins—fused to alkaline phosphatase—could bind members of the human plexin-A, -B, and -C subfamilies. Multiple secreted semaphorins of class 3 (Sema3A, Sema3C, or Sema3F; see below) did not interact with plexins-A1, -A2, -A3, -B1, B2, or -C1 (data not shown). In contrast, plexin-C1 (VESPR) specifically bound Sema7A (Sema-K1) (Figure 2A), a GPI membrane-linked semaphorin (class 7). This result is not entirely unexpected, since Sema7A may represent the cellular counterpart of viral semaphorin SemaVB, previously shown to interact with this plexin (Comeau et al., 1998). More interestingly, the class 4 transmembrane semaphorin Sema4D (CD100) did interact strongly and specifically with plexin-B1 (Figure 2A). Thus, the prototypes of two distinct plexin families are the receptors for members of two distinct semaphorin subclasses. We also found that Sema7A and Sema4D do not bind to neuropilin-1 or -2 alone, nor did cotransfection of either neuropilin with plexin-B1 significantly modify its binding efficiency (not shown). Neuropilins thus seem so far to function as receptors only for vertebrate semaphorins of class 3.
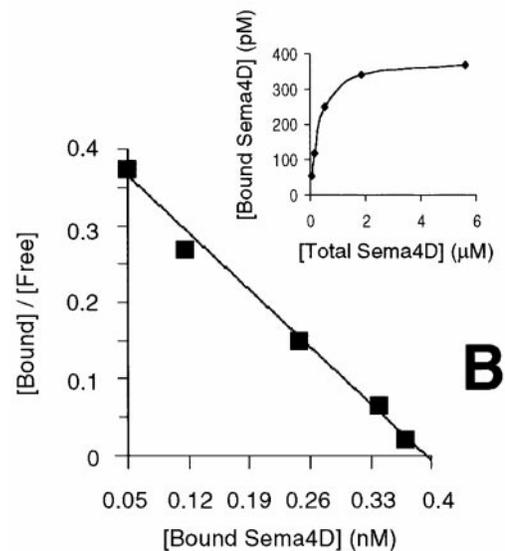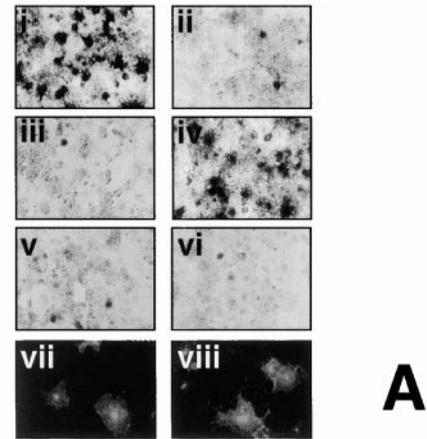
Figure 2. Cell Surface Semaphorins Specifically Bind Human Plexins

(A) Micrographs of the binding assays (see Experimental Procedures) that tested (i) the extracellular domain of Sema4D (CD100) fused to alkaline phosphatase (Sema4D-AP) on COS cells transfected with *plexin-B1* cDNA; (ii) control AP on plexin-B1; (iii) Sema4D-AP on plexin-B2; (iv) Sema4D-AP on the entire extracellular domain of plexin-B1; (v) Sema4D-AP on isolated "plexin-B1 truncated" (including *sema domain* and 1° and 2° MRS); (vi) Sema4D-AP on a "plexin-B1-Δsema" (including 2° and 3° MRS; see Experimental Procedures for details); (vii) extracellular domain of SemaVA (A39R) fused to AP, on plexin-C1 (VESPR); (viii) Sema7A-AP on plexin-C1. The final detection of the binding was done either using alkaline phosphatase substrates (i–vi) or by immunofluorescence (vii and viii).

(B) Scatchard analysis and binding curve of Sema4D-AP to plexin-B1 ($K_D = 0.9$ nM $\pm$ 0.15); see Experimental Procedures.

The affinity constant of Sema4D for plexin-B1 was estimated by Scatchard plot to be in the subnanomolar range ($K_D = 0.9$ nM, Figure 2B; the estimated K_D of Sema7A for plexin-C1 is 2.1 nM, not shown). These values are consistent with those observed for semaphorin–neuropilin and fly semaphorin 1–plexin A interactions (He and Tessier-Lavigne, 1997; Winberg et al., 1998).

We used two deletion constructs of plexin-B1 to explore the semaphorin-binding sites of plexins. As shown

in Figure 2A, neither the N'-terminal half of plexin-B1 extracellular domain ("plexin-B1 truncated"; see previous section) nor its C'-terminal half ("plexin-B1-Δsema"; see legend) was sufficient alone to bind Sema4D, suggesting that the binding of Sema4D depends on multiple structural determinants in the extracellular domain of plexin-B1.

Plexins Associate with Class 3 Semaphorin Receptors, Neuropilins

As outlined above, secreted semaphorins of subclass 3 are known to bind neuropilins (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). However, the short cytoplasmic tail of neuropilins seems to be dispensable for their biological activity (Nakamura et al., 1998), indicating the requirement of an associated coreceptor for signal transduction. Interestingly, in *Drosophila* (where neuropilins have not been identified to date), plexin A is sufficient to mediate the biological response to semaphorin 1 in axon guidance (Winberg et al., 1998).

In an initial set of experiments, we could not observe binding of the class 3 semaphorins Sema3A (Sema III), Sema3C (Sema E), or Sema3F (Sema IV) to plexin-A1, -A2, -A3, -B1, -B2, or -C1 (not shown). To test whether plexins might be coreceptors with neuropilins for class 3 semaphorins, we set up coprecipitation experiments in COS cells to test whether neuropilins may interact with plexins. Three tested plexins (plexin-A1, -A3, and -B1) associated both with neuropilin-2 (NP-2, shown in Figure 3) and neuropilin-1 (not shown). The binding was specific, inasmuch as neither neuropilin nor any plexin coimmunoprecipitated with the netrin receptor DCC under conditions where DCC coimmunoprecipitated with the other netrin receptor, UNC5H2 (Figure 3 and data not shown). We observed finally that the plexin-neuropilin association is mediated by the *sema domain* of plexins, as demonstrated using either the "plexin-B1 truncated" splice variant (Figure 3) or an even shorter form of the extracellular domain ("plexin-B1-sema"; see Experimental Procedures; not shown).

To further support the idea of a plexin-neuropilin multimeric receptor complex for semaphorins, we show here that *plexin-A3* (for example) is expressed in a large number of neuronal classes, including sensory, sympathetic, motor, and olfactory bulb neurons (Figure 4 and data not shown), which are known to respond to class 3 semaphorins and express either neuropilin-1 or neuropilin-2, or both (Chen et al., 1997; Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Thus, plexin-A3 is a candidate for a physiological coreceptor involved in mediating class 3 semaphorin effects on these axons. Other plexins may also have a role as neuropilin coreceptors in specific cell populations, such as *plexin-A2*, which is expressed in a subset of sensory neurons and in dorsal horn cells, and *plexin-A1*, which is expressed at low levels and broadly in the spinal cord (Figure 4).

To directly test the possible involvement of plexins in class 3 semaphorin signal transduction, we studied the repulsive responses of *Xenopus laevis* spinal neurons to Sema3A, which is mediated by a receptor mechanism involving neuropilin-1 (Song et al., 1998). We asked whether these responses could be altered by expression
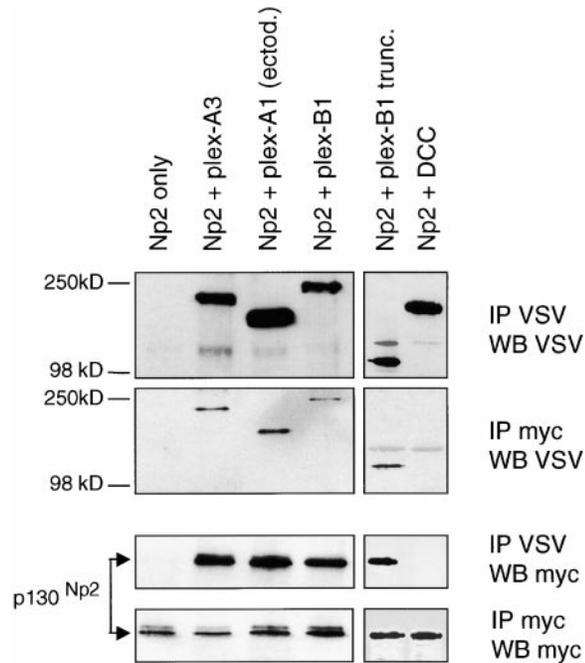
Figure 3. Plexins Associate with Neuropilins

Western blots of immunoprecipitated samples from cells coexpressing neuropilins and plexins. Specific MoAbs were used, directed against the VSV tag included in plexins and in DCC, or the Myc tag included in neuropilin-2 (NP-2, 130 kDa). NP-2 coimmunoprecipitates with plexins, such as plexin-A3 (220 kDa), the extracellular domain of plexin-A1 (~160 kDa), and plexin-B1 (250 kDa), but not with the unrelated cell surface receptor DCC (170 kDa). NP-2 can also associate a truncated form of the extracellular moiety of plexin-B1 ("plex-B1 trunc.," ~110 kDa) containing the *sema domain*.

of a presumed dominant-negative plexin-A1 construct lacking the cytoplasmic domain of the protein. Transmembrane proteins can be reliably expressed in these neurons by injecting the encoding mRNA at the developmental two-cell stage, allowing the embryos to grow to tadpole stage, and then removing the spinal cord and culturing the neurons (Hong et al., 1999). We therefore injected the mRNA encoding the truncated plexin-A1 construct together with mRNA encoding GFP (as a reporter) and then studied the responses of spinal neurons expressing GFP that were derived from these embryos. Whereas control spinal neurons are repelled by Sema3A (Figures 5A and 5B; Song et al., 1998), neurons from embryos injected with mRNA for truncated plexin-A1 did not respond with either repulsion or attraction to Sema3A (Figures 5C and 5D). This blocking effect appeared to be specific, since expression of a different heterologous receptor, UNC5H2, did not impair repulsion by Sema3A (Hong et al., 1999) and since expression of the truncated plexin construct did not block attractive responses to netrin-1 (Figures 5E and 5F). Figures 5G and 5H quantify these effects. As can be seen, the effect of Sema3A is completely abolished by the truncated plexin; although there is a slight apparent decrease in the attractive effect of netrin-1, the effect is not statistically significant.

Although we have used a truncated plexin-A1 construct, this construct may be expected to interfere with the function of various plexins, since all the plexins

Plexin-A1   Plexin-A2   Plexin-A3
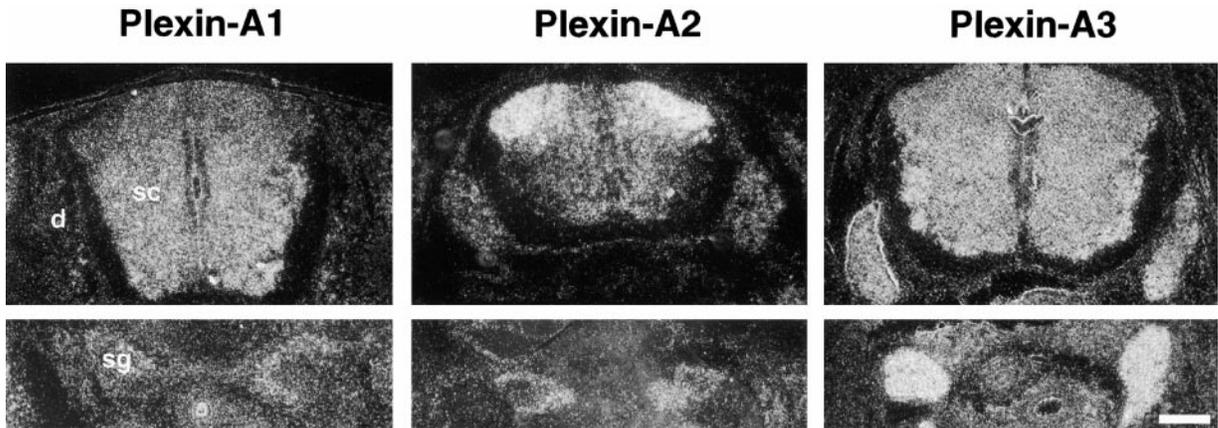
Figure 4. Plexin Expression in the Spinal Cord, Dorsal Root Ganglia, and Sympathetic Ganglia

Expression of mRNAs for plexin-A1, -A2, and -A3 in the spinal cord (sc), dorsal root ganglia (d), and sympathetic ganglia (sg) of E13.5 mouse embryos. Expression of the mRNAs was detected by RNA in situ hybridization. Scale bar, 1 μm.

tested (A1, A3, and B1) associated with neuropilin-1. These results do not, therefore, give any information on the complement of plexins expressed by the *Xenopus* spinal neurons; they do, however, support a role for one or more plexins in mediating the repulsive Sema3A signal in these neurons.

Plexins Signal via a Novel Type of Tyrosine-Phosphorylated Cytoplasmic Domain

The sequences of plexin cytoplasmic domains are highly conserved among plexins but do not match any known sequences and hence do not provide any clues as to
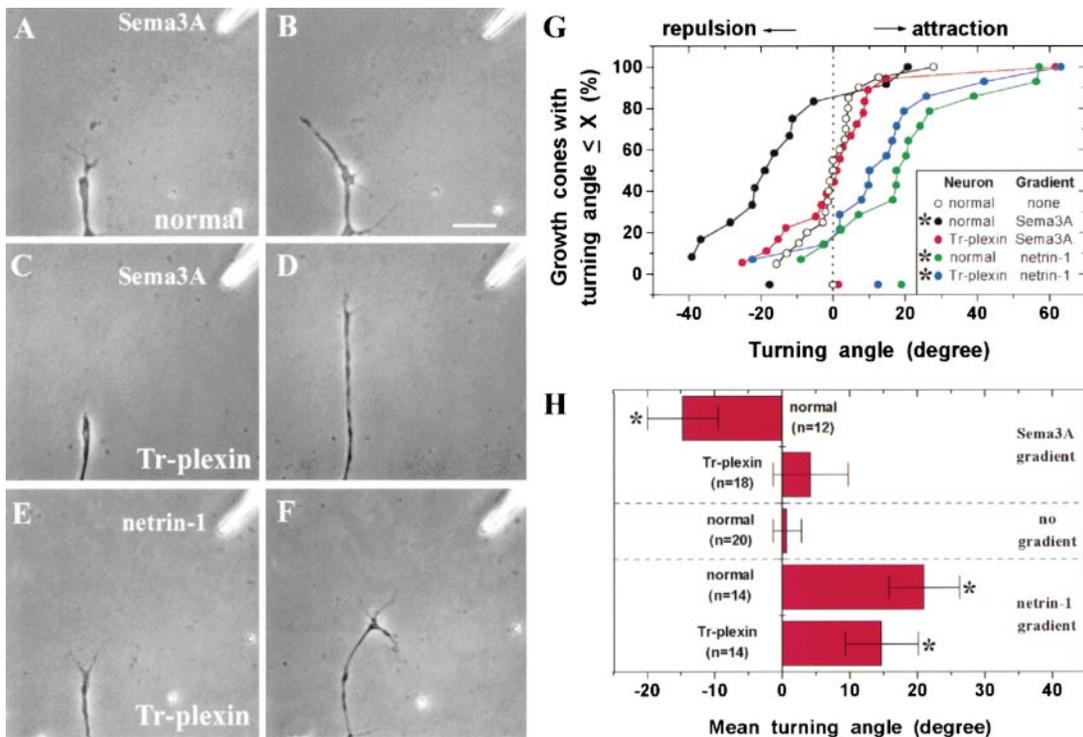
Figure 5. A Truncated Plexin-A1 Protein Blocks Axon Repulsion to Sema3A

(A–F) A control spinal neuron exposed to a gradient of Sema3A emanating from a pipette (A) is repelled away over a period of 1 hr (B). In contrast, a GFP-expressing spinal neuron from an embryo injected with mRNA for the truncated plexin-A1 construct (C) is not affected by Sema3A (D). A similar neuron (E) shows a normal attractive response to netrin-1 (F).

(G) Cumulative distribution plot of turning angles for all the neurons studied. Curves show the percent of neurons with turning angles less than the angle indicated on the abscissa, under different conditions (open circles, control neurons; black and blue circles, control neurons responding to Sema3A or netrin-1, respectively; red and green circles, responses of neurons expressing the truncated plexin-A1 construct to Sema3A and netrin-1, respectively). Open squares, significantly different from the data from neurons in the absence of any gradient (p < 0.05; Kolmogorov-Smirnov test).

(H) Mean turning angle under all the conditions just mentioned.
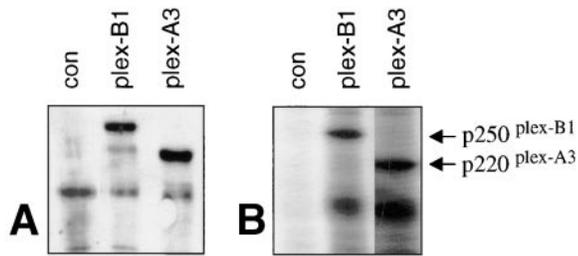
Figure 6. Tyrosine Phosphorylation of Plexins

(A) Western blotting of immunoprecipitated p220$^{plex-A3}$ and p250$^{plex-B1}$ proteins, followed by detection with anti-phosphotyrosine antibody. (B) The same immunopurified samples underwent in vitro kinase assay in the presence of [γ-^{32}P]ATP, Mg^{2+}, and Mn^{2+} ions; the SDS-PAGE was treated with alkali in conditions known to remove phosphate labeling from serine and threonine but not tyrosine residues (see Experimental Procedures).

the mechanism through which plexins signal. However, we found that the plexin-A3 and plexin-B1 proteins are phosphorylated on tyrosine residues when overexpressed in human kidney cells (BOSC-23), as demonstrated using anti-phosphotyrosine antibodies (Figure 6A). Furthermore, after immunoprecipitation and in vitro kinase assays, plexin-A3 and plexin-B1 became phosphorylated (Figure 6B). Resistance to an alkali treatment (see Experimental Procedures) confirmed the specific phosphorylation of tyrosine residues.

The cytoplasmic domains of several receptors, including Met proteins, become tyrosine phosphorylated owing to an intrinsic kinase activity (Ullrich and Schlessinger, 1990). Since the cytoplasmic domain of plexins is not similar to any bona fide or atypical tyrosine kinase, this suggests that a distinct tyrosine kinase coimmunoprecipitates in association with plexins and is responsible for their tyrosine phosphorylation. Although some additional phosphorylated proteins can be found specifically with plexin-A3 and -B1, we have not yet identified this associated kinase. A number of endogenously expressed tyrosine kinases, namely Met, Ron, Abl, and Src, were not found associated with plexin-A3 by immunoprecipitation and Western blotting (not shown). Since tyrosine-phosphorylated residues often function as docking sites for intracellular signal transducers (Cantley et al., 1991), the fact that the cytoplasmic domains of plexins are tyrosine phosphorylated further suggests that they are part of signaling complexes.

Plexin-A3-Expressing Cells Induce Repulsion of Cocultured Cells

Stable transfectants expressing recombinant human plexin-A3 were successfully obtained in four different cell lines: IMR32 and AF8 (human neuroblasts) and BOSC-23 and MDCK (human and canine kidney cells, respectively). We observed modest phenotypic changes in the transfected cells, which generally become flatter and larger in size. The growth rate of plexin-A3-overexpressing cells was comparable to parental lines, and we did not observe differences in the ability to adhere on different substrates (see Experimental Procedures; data not shown).

In keeping with a previous report on the related plexin

of *Xenopus* (Ohta et al., 1995), we observed a modest increase in calcium-dependent homotypic cell aggregation of plexin-A3 transfectants (not shown). Surprisingly, we found that epithelial MDCK cells overexpressing plexin-A3 mediate strong repelling cues for adjacent cells. This was observed by coculturing mock-transfected and plexin-A3-overexpressing MDCK cells together with several nonepithelial cell lines (such as NIH3T3, Kj29, and D17; Figure 7A). Mock MDCKs grew alongside mesenchymal cells until confluency, when both cell types stopped proliferating. In contrast, when plexin-A3-overexpressing epithelial cells were grown in the same conditions, the adjacent mesenchymal cells withdrew from them and ultimately detached from the plate.

To analyze the dynamics of this repulsion process, we monitored for 36 hr, by time-lapse videomicroscopy, mixed cultures of transfected MDCK cells and fibroblasts in a number of independent experiments. At low cell density, fibroblasts showed intrinsic motility, exploring the surface of the plate with long lamellipodia and filopodia, and thus coming in contact with a high number of stationary MDCK islets. The time length of the contacts between fibroblasts and control MDCK cells varied from 30 min to several hours, lasting mostly over 100 min. However, when fibroblasts were cultured with MDCK cells overexpressing plexin-A3, transient contacts were observed, often lasting less than 30 min (see Figure 7C). At higher cell density, fibroblasts stopped and clustered alongside the islands of control MDCKs, whereas they kept moving in a hectic fashion between the islands of plexin-A3-transfected cells (data not shown).

This cell-repelling effect is not due to the release of soluble factors, since exchanging conditioned media between mixed cultures was without effect (not shown). Moreover, the two different cell populations grew normally until they came into contact, indicating that the repelling effect requires cell–cell interaction. To rule out the possibility that plexin-A3-expressing cells generate an apoptotic signal for fibroblasts, we monitored cell viability and apoptosis by TUNEL staining. As shown in Figure 7B, the clusters of repelled fibroblasts did not include apoptotic cells; furthermore, the detaching cells still excluded Trypan blue stain and were able to spread again on a new culture plate (not shown).

Taken together, these results strongly suggest that, in our experimental system, plexin-A3 mediates cell-repelling cues, presumably by interacting with surface-bound ligands on opposing cells. We could not identify—so far—the specific ligand for plexin-A3; however, it is tempting to speculate that this may be a transmembrane semaphorin. It should be noted that the intracellular domains of transmembrane semaphorins, such as Sema4D, also include tyrosine residues, which may themselves become phosphorylated and associate with cytoplasmic signal transducer molecules, a property shown for ligands of the ephrin family (Holland et al., 1996).

Discussion

In this paper we show that plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. We divide plexins
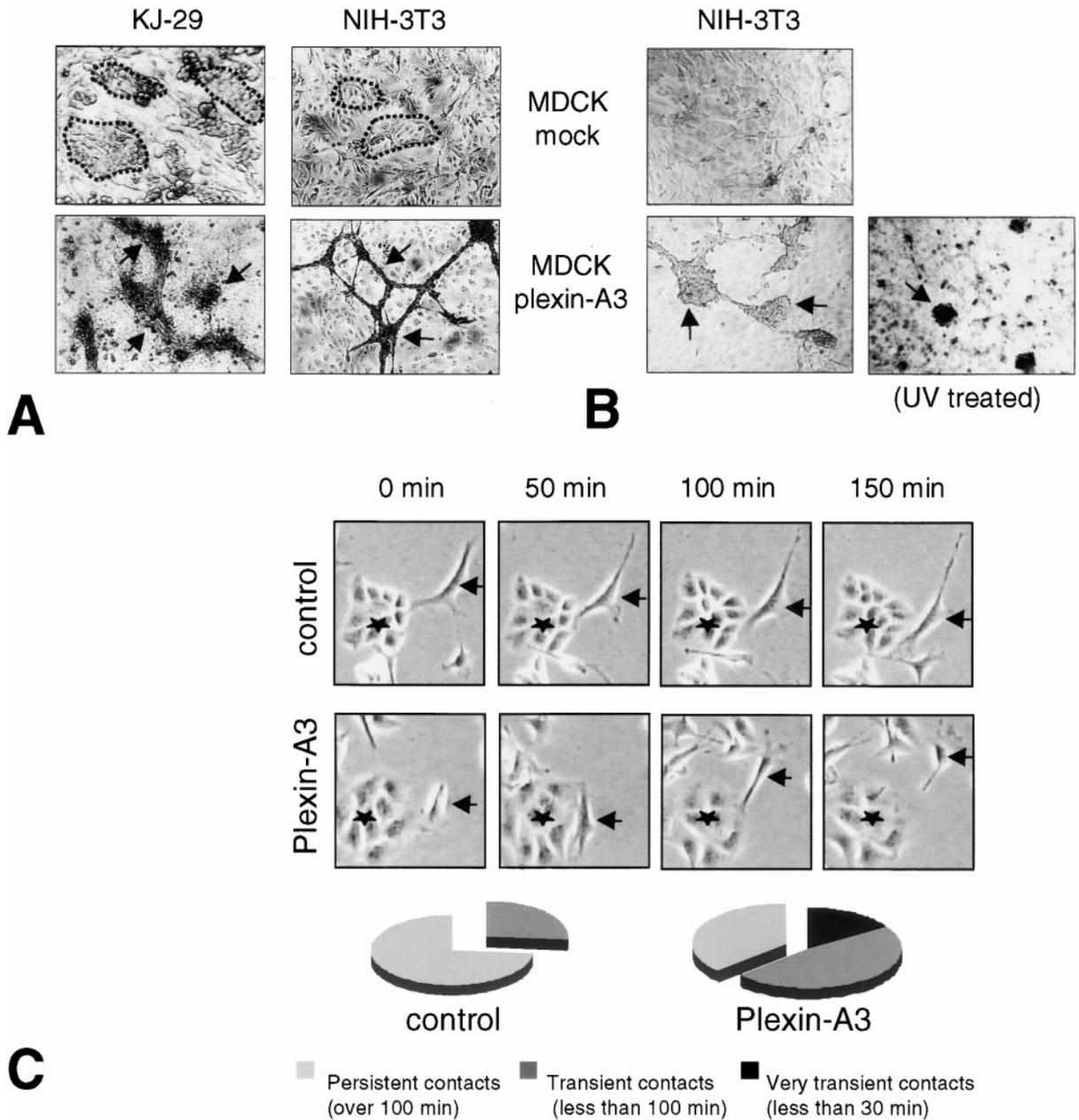
Figure 7. Plexin-A3 Overexpression Mediates Cell-Repelling Cues

(A) Epithelial kidney MDCK cells transfected to overexpress plexin-A3 (or mock transfected) were cocultured with mesenchymal KJ-29 or NIH-3T3 cells. After 16–30 hr, mixed cultures of control cells (upper panels) reached confluency and stopped growing: typically, the epithelial cells formed islets (circled) surrounded by a fibroblast lawn. In contrast, MDCKs overexpressing plexin-A3 (lower panels) overwhelmed the adjacent mesenchymal cells. The latter withdrew and selectively detached from the culture dish (dying cell clusters are indicated by arrowheads), and eventually only epithelial cells survived. To allow an easier detection, mesenchymal cells were previously labeled with DiI (see Experimental Procedures).

(B) Plexin-A3-expressing cells do not induce apoptotic signal on repelled fibroblasts. Mixed cultures of NIH 3T3 and control or plexin-A3-overexpressing MDCKs were tested for the presence of TUNEL-positive cells. Apoptotic cells were not present in clusters of repelled cells (indicated by arrows). The right panel shows a positive control where apoptosis was induced in the same cells by UV treatment.

(C) Plexin-A3-overexpressing cells form very transient contacts with fibroblasts. Time-lapse videomicroscopy of control and plexin-A3-overexpressing MDCK cells grown in presence of fibroblasts. Top, snapshot images from the movie, taken every 50 min (real time). In the upper row is shown the persistent contact of a fibroblast (marked by an arrow) with an islet of control MDCK cells (marked by a star). In the lower row, another fibroblast instead forms a transient contact with an islet of plexin-A3-transfected cells, which also in turn reshapes (see Discussion). At the bottom, the diagrams show the relative frequency of persistent, transient, or very transient contacts between fibroblasts and MDCK cells.

into four subfamilies. We show that human plexins of B and C subfamilies bind membrane-bound semaphorins of classes 4 and 7, respectively, while plexins of A and D subfamilies may be predicted to be receptors for other semaphorin subclasses. Interestingly, the ligand of plexin-B1 is Sema4D (CD100), a transmembrane semaphorin mostly expressed in the nervous system and in lymphocytes, where it was shown to mediate interactions between T and B cells (Hall et al., 1996). We found that its plexin receptor, plexin-B1, is widely expressed in human neural, hematopoietic, and endothelial cells (L. T. et al., unpublished data), suggesting that plexin–semaphorin interactions may be crucial for cell–cell signaling events in multiple tissues. Secreted semaphorins of class 3 are potent mediators of growth cone collapse and axon repulsion. Thus far, they have been shown to interact with neuropilins (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Yet neuropilins have only a very short cytoplasmic domain and do not even require their cytoplasmic domain to function as semaphorin receptor components (Nakamura et al., 1998). Thus, another transmembrane subunit is implicated as a neuropilin partner in mediating the function of class 3 semaphorins. In this paper, we demonstrate that plexins can form complexes with neuropilins, implying that the receptors for secreted semaphorins (class 3) may in fact be heterooligomers of plexins and neuropilins. The formation of a receptor complex between NP-1 and plexin-A1 is also recorded independently by Takahashi et al. (1999 [this issue of *Cell*]).

As demonstrated by in situ mRNA expression analysis, plexins and neuropilins are in fact simultaneously expressed in several neuronal populations during embryonic development. In our experimental conditions, the plexin–neuropilin complex seems to predate ligand binding, since the association is not influenced by the presence of class 3 semaphorins. However, since endogenous plexins are expressed at low levels, other factors might regulate complex formation in normal conditions. That the observed plexin–neuropilin complexes are formed in *cis* is furthermore suggested by the experimental conditions used (cotransfection of isolated cells with the two constructs). An interaction in *trans* might also be envisioned (considering that plexins and semaphorins share similar *sema domains*); however, by analyzing mixed cultures of cells separately transfected with plexins and neuropilins, we did not isolate associated complexes (data not shown).

We observed that the main semaphorin-binding domain of neuropilins (CUB domain; Chen et al., 1998; Giger et al., 1998; Nakamura et al., 1998) is not required for the interaction with plexins, as indicated by the association of the relevant neuropilin-2 deletion construct with plexin-B1 (not shown). We thus envision the existence of a ternary complex, where neuropilins use two distinct protein modules to form a bridge between the *sema domain* of semaphorins and the *sema domain* of plexins. Taken together, these findings raise the possibility that plexins are the long-sought functional partners of neuropilins required for transducing signals mediated by class 3 semaphorins. Notably, in flies, which lack both neuropilins and class 3 semaphorins, D Plex A appears sufficient as a functional receptor for Sema-1a, a transmembrane class 1 semaphorin (Winberg et al.,

1998). To further support our hypothesis that plexins are functional coreceptors for secreted semaphorins, we showed that a truncated plexin-A1 construct expressed in *Xenopus* spinal neurons abolishes repulsive responses to Sema3A without markedly affecting attractive responses to netrin-1. Similarly, expression of a dominant-negative plexin-A1 in sensory neurons blocked Sema3A-induced growth cone collapse, as reported independently by Takahashi et al. (1999). These results are consistent with the involvement of plexins, although they do not establish which plexin(s) function in these spinal neurons, nor do they exclude the possible involvement of yet other signaling components.

The intracellular signals transduced by plexins are still largely obscure. The cytoplasmic domain of plexins is large and highly conserved within and across species. Although it still lacks an assigned catalytic function, it contains stretches of alpha helices, which are putative protein–protein interaction domains, and could thus mediate the association with cytosolic partners. Moreover, we show that the cytoplasmic domain of plexins can be tyrosine phosphorylated, suggesting that, like other receptors devoid of intrinsic catalytic activity, plexins may signal by associating a tyrosine kinase (Stahl and Yancopoulos, 1993; Glass et al., 1996).

Finally, in this work we show that expression of plexin-A3 mediates cell-repelling cues. We excluded that the observed effects may be explained by the ability of plexin-A3-expressing cells to primarily transfer an apoptotic signal or by their acquisition of a proliferative advantage or different adhesive properties. Rather, by time-lapse videomicroscopy we observed a true repelling effect on fibroblasts. Intriguingly, we observed that—upon interaction with fibroblasts—the islets of plexin-A3 MDCKs also at times reshaped. This may be explained by the existence of intraepithelial repelling cues balanced by the attractive forces exerted by epithelial cell junctions.

In the nervous system of *Drosophila*, we showed that defasciculating motor axons coexpress both plexin A and one of its interacting partners, the transmembrane semaphorin Sema-1a (Winberg et al., 1998). This indicates the intriguing possibility that plexins may act in vivo either as receptors or ligands for cell surface semaphorins, which may in turn transduce intracellular signals, as reported for ephrins (Holland et al., 1996). Based on the data presented here, we suggest that semaphorins, besides being pivotal in axon guidance, may have a general role in other physiological (morphogenesis) or pathological phenomena (cancer invasion and metastasis) by mediating cell-repelling cues via their interactions with plexins.

Experimental Procedures

Identification and cDNA Cloning of Novel *plexins*
Since the coding sequences of human *plexin-B1 (SEP)*, *plexin-A2 (OCT)*, and *plexin-A1 (NOV)* were incomplete, we obtained the missing cDNA by RT-PCR; primers were designed by homology to orthologous murine sequences and corresponding ESTs. Updated database entries are X87904, X87831, and X87832, respectively. Partial cDNA of *plexin-A4* includes six overlapping human ESTs (HGI THC Report: THC203425; clone 7B19F10) identified in chromosome 7–specific cDNA pools. *plexin-B2* cDNA was amplified by RT-PCR

starting from partial cDNA sequences of clones *MM1* (Shinoura et al., 1995) and KIAA0315 (GenBank database); the genomic locus of *plexin-B2* was identified due to its 100% sequence identity with clone C22_311. *plexin-B3* coding sequence was identified in the genomic sequence of ALD locus (chr. Xq28), using the algorithms HEXON and GENIE. *plexin-D1* was similarly found in the genomic sequence of chr. 3 (pac pDJ70i11). The genomic sequence of *plexin-B1(SEP)*, in the region of the alternative splicing of the extracellular domain, was obtained using the following primers: sense 5′GCAGCACCTGT GCACCCACAAGGC3′ and antisense 5′TGCAGGCTGGACGGGAG GATGAGG3′. The common donor site is CCATCAG/gtgattgt (position 2028 from ATG); alternative splice acceptor sites are: (1) cccccttcag/AGCCC, leading to the canonical plexin-B1 sequence and (2) ctcctctcag/GTGAT, leading to "plexin-B1 truncated" variant (GenBank number AJ011414). The new sequences were analyzed using the algorithms BLAST2, NETPHOS (phosphorylation prediction sites, by Nicolaj Blom), and PH-PREDICT. The phylogenetic tree was generated using AllAll algorithm of the Darwin sequence analysis system (at CBRG).

plexin cDNA Expression Constructs and Protein Analysis

Cell transfections were carried out by calcium phosphate and DEAE-dextran methods, using 5–10 μg of each cDNA (1–2 μg each in case of cotransfections). For transient transfections in COS and BOSC-23 cells, the cDNA was cloned in pCDNA3 or derived expression plasmids (Invitrogen). MDCK stable transfectants for *plexin-A3* were obtained using pCEP4 expression plasmid (Invitrogen), and plexin-A3-positive clones were isolated from two independent transfections and showed identical biological properties. Plexin and neuropilin expression constructs included a VSV or a Myc tag at the N′ and C′ protein termini, respectively, detected by monoclonal antibodies anti-VSV-G (cat. V-5507, Sigma) and anti-cMyc-tag (cat. OP10-100UG, Calbiochem).

"Plexin-B1 truncated" splice variant was expressed from a cDNA fragment obtained by RT-PCR and VSV tagged at the N′ terminus: the encoded amino acid sequence spans up to amino acid 676 (including the *sema domain* and two MRS motifs). "Plexin-B1-sema" derives from a further deletion of the plexin-B1 extracellular domain and exclusively includes the *sema domain*. "Plexin-B1-Δsema" protein mutant includes only the C′-terminal half of plexin-B1 extracellular domain, starting from amino acid 606, that is, excluding *sema domain* and first MRS but including second and third MRS, transmembrane, and intracellular domains.

For immunoprecipitations, cells were lysed with EB buffer (20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) in the presence of protease inhibitors and 1 mM Na-orthovanadate. After immunoprecipitations with the appropriate antibodies, high-stringency washes were performed (EB plus 1 M LiCl).

For in vitro kinase assays, immunopurified proteins were incubated with kinase buffer (50 mM HEPES, 100 μM DTT, 5 mM MnCl$_2$, 5 mM MgCl$_2$) in the presence of redivue 5 μCi [γ-^{32}P]ATP (Amersham) for 10 min at 4°C in agitation. Samples were then submitted to SDS-PAGE and autoradiography or analyzed using a Phosphor-Imager system (Molecular Dynamics). Alkali treatment of the polyacrilamide gels was performed with 1 M KOH for 2 hr at 55°C.

Western blots were performed according to standard methods. Specific detection of phosphotyrosines was done with PY20 MoAb (Trasduction Laboratories). Final detection was done with ECL system (Amersham).

Semaphorin–Secreted Alkaline Phosphatase Binding Assays

Soluble forms of semaphorin extracellular domains were expressed as fusion proteins with placental secreted alkaline phosphatase (SEAP) and harvested from the conditioned media of transiently transfected COS cells. Serum-free media were concentrated over 100 times using Centricon Plus-20 filters (Millipore; MW cutoff of 100 kDa). The AP activity of these media was assessed as described (Flanagan and Leder, 1990); the specific activity of fusion proteins was ~1000 U/mg. Concentrated semaphorin-SEAP was diluted as appropriate in a HEPES-buffered saline, additioned with 0.2% BSA, 0.1% NaN$_3$, 5 mM CaCl$_2$, and 1 mM MgCl$_2$ (HBSBA). For binding assays, COS cells transiently transfected with plexins were seeded on multiwell plates and incubated with semaphorin-SEAP fusion proteins (~1–5 nM) for 90 min at room temperature. The binding was detected as described (Flanagan and Leder, 1990). Binding experiments with plexin-C1/VESPR were as described (He and Tessier-Lavigne, 1997; Comeau et al., 1998).

For in vitro binding assays, plexin-B1 was purified from cell extracts by immunoprecipitation with anti-VSV antibody. Extracts of mock-transfected cells were used as control samples. After washing, the immunocomplexes were incubated with serial dilutions of Sema4D-SEAP (prepared as above) for 2 hr at 4°C in continuous agitation. Samples were then washed three times with HBSBA, and the bound alkaline phosphatase activity was measured at A$_{405}$ as described (Flanagan and Leder, 1990). Scatchard analysis was done using Equilibrate (by GertJan C. Veenstra).

In Situ Hybridization Analysis

RNA in situ hybridization was performed essentially as described (He and Tessier-Lavigne, 1997). Briefly, cDNA fragments of plexin-A1, -A2, and -A3 were used to generate ^{35}S-labeled antisense and sense RNA probes, which were used for in situ hybridization histochemistry of cryostat sections of rat embryos.

Xenopus Turning Assay

The methods for injecting mRNA encoding various constructs and for studying the turning responses of the neurons are exactly as described previously (Song et al., 1998; Hong et al., 1999).

Mixed-Culture Assays and Time-Lapse Videomicroscopy

Mock-transfected and plexin-A3-overexpressing MDCK cells were seeded with mesenchymal cells (NIH 3T3, KJ29, D17, among others) in multiwell culture plates by 1:4 or 1:1 ratio. Mesenchymal cells were sometimes labeled by addition of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Fluka) in the culture medium 4 hr before harvesting for the assay; clusters of cells marked with this dye appear blue (in light microscopy) and emit red epifluorecence (TRITC filter). The repelling effect was observed 16–30 hr after confluency by contrast phase microscopy using Leica DM IL. The progress of the assay was also monitored by time-lapse videomicroscopy (320 min recording was converted into 1 min play). To determine the time length of cell contacts, randomly chosen fibroblasts were followed during several hours and the duration of each contact with MDCK cells was measured. Substrate adhesion of plexin-A3-overexpressing MDCKs was analyzed by counting attached cells after 30 min from seeding on microwells coated with fibronectin, collagen, or polylysin in the absence of calf serum: no differences versus control cells were observed.

Apoptosis Detection

TUNEL reaction (Boehringer detection kit) was performed on mixed cultures of MDCK and NIH3T3 cells 24 hr after seeding in a multiwell culture plate. The labeling was converted into a colorimetric signal for analysis by light microscopy using the TUNEL-AP detection kit (Boehringer). As a positive control for the induction of apoptosis, the same cells were treated with UV-C (50 μJ/cm^2).

Acknowledgments

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