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Vertebrate Slit, a Secreted Ligand for the Transmembrane Protein Roundabout, Is a Repellent for Olfactory Bulb Axons

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Summary

The olfactory bulb plays a central role in olfactory information processing through its connections with both peripheral and cortical structures. Axons projecting from the olfactory bulb to the telencephalon are guided by a repulsive activity in the septum. The molecular nature of the repellent is not known. We report here the isolation of vertebrate homologs of the *Drosophila slit* gene and show that Slit protein binds to the transmembrane protein Roundabout (Robo). *Slit* is expressed in the septum whereas *Robo* is expressed in the olfactory bulb. Functionally, Slit acts as a chemorepellent for olfactory bulb axons. These results establish a ligand–receptor relationship between two molecules important for neural development, suggest a role for Slit in olfactory bulb axon guidance, and reveal the existence of a new family of axon guidance molecules.

Introduction

Olfactory information is relayed successively from the olfactory epithelium to the olfactory bulb and then to the olfactory cortex (Price, 1987; Farbman, 1991; Greer, 1991). Odorants are sensed by specific receptors of olfactory neurons in the olfactory epithelium of the nasal cavity (Buck and Axel, 1991; Reed, 1992; Strotmann et al., 1992, 1994; Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Dulac and Axel, 1995; Reed, 1996). Olfactory receptor neurons send the information through their projections to the olfactory bulb (Lancet et al., 1982; Kauer et al., 1987; Imamura et al., 1992; Mori et al., 1992; Katoh et al., 1993; Ressler et al., 1994; Vassar et al., 1994). Axons from the main olfactory epithelium form synapses in the glomeruli with the mitral and tufted cells in the olfactory bulb. These secondary neurons project axons to olfactory cortical areas in the telencephalon including the anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, the anterior cortical nucleus of the amygdala, the periamygdaloid cortex (also known as the posterolateral nucleus of the amygdala), and the lateral entorhinal cortex (Hinds, 1972; Hinds and Ruffett, 1973; Schwob and Price, 1984; Brunjes and Frazier, 1986; Saucier and Astic, 1986; Shepherd and Greer, 1990; Schoenfeld et al., 1994; Shipley et al., 1995; Mombaerts et al., 1996).

Although it is clear that the processing of olfactory information relies on precise connectivity among different parts of the olfactory system, our understanding of mechanisms underlying axon guidance in the olfactory system is quite limited. Recent studies have suggested roles for olfactory receptors and cell adhesion molecules in controlling axons projecting from the olfactory epithelium to the olfactory bulb (Wang et al., 1998; Yoshihara et al., 1997). By contrast, little is known at the molecular level about mechanisms guiding axons from the olfactory bulb to the cortex. Morphological studies have shown that axons of the olfactory bulb turn away from the midline, forming the lateral olfactory tract (LOT), and grow toward the olfactory cortex (Schwob and Price, 1984; Brunjes and Frazier, 1986; Shipley et al., 1995). In vitro explant studies have revealed that the septum at the midline of the telencephalon secretes a diffusible factor(s), which repels the projection axons of the olfactory bulb (Pini, 1993). The molecular nature of repulsive factor(s) in the septum has so far remained unknown.

The ventral midline of the neural tube can provide either attractive or repulsive guidance cues for axons, depending on the type and developmental history of the responding axons. Extensive studies have shown that the floor plate in the spinal cord is initially attractive to axons from commissural interneurons located in the dorsal spinal cord (Oppenheim et al., 1988; Tessier-Lavigne et al., 1988; Yaginuma et al., 1991; Stoeckli and Landmesser, 1995; reviewed in Colamarino and Tessier-Lavigne, 1995a). After crossing the floor plate, commissural axons do not respond to the attractant in the floor plate (Shirasaki et al., 1998), and there is evidence for a repellent activity in the floor plate for commissural, sensory, and motor axons (Guthrie and Pini, 1995; Stoeckli et al., 1997; Keynes et al., 1997).

Molecular mechanisms underlying axon guidance at the midline appear to be conserved between vertebrates and invertebrates. Netrin, a protein secreted by the floor plate, is attractive to commissural axons in vertebrates, *Drosophila*, and *C. elegans* (Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Netrin can also act as a repellent for a specific subset of axons in *C. elegans* and vertebrates (Hedgecock et al., 1990; Colamarino and Tessier-Lavigne, 1995b). Recent studies in *Drosophila* have revealed that a transmembrane receptor encoded by the *roundabout* (*robo*) gene play an important role in ensuring that commissural axons which have already crossed the midline do not recross

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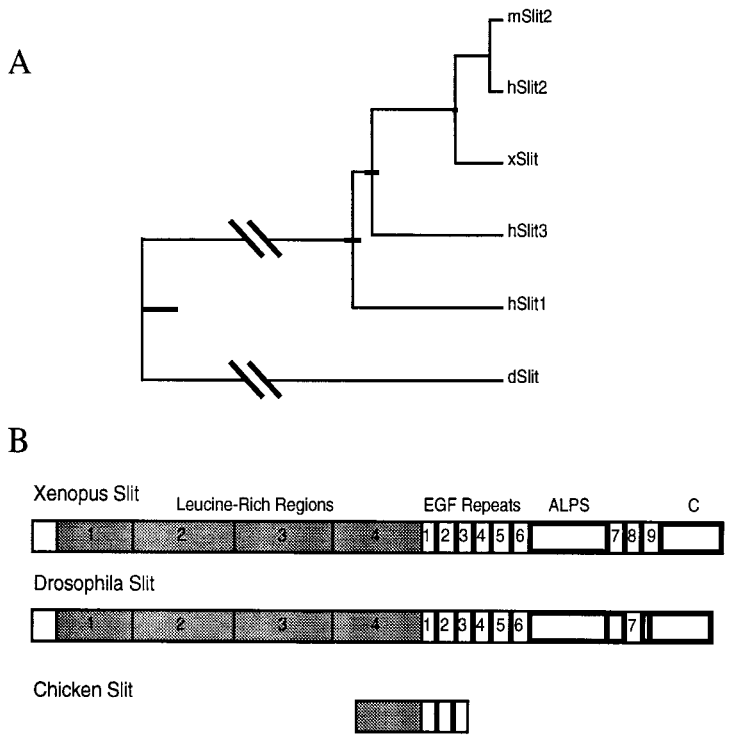

Figure 1. Comparison of the Primary Sequences of Vertebrate and *Drosophila* Slit Proteins

(A) A phylogenetic tree showing sequence similarities among *Drosophila*, *Xenopus*, mouse, and human Slit proteins. Amino acid sequences were aligned and evolutionary distance was calculated using the UPGMA algorithm. The length of the lines is proportional to the evolutionary distance between branchpoints. Shaded bars indicate standard errors at each branchpoint. The *Xenopus Slit* is an ortholog of the mouse and human *Slit2* genes. (B) Diagrammatic illustration of *Xenopus*, *Drosophila*, and chick Slit proteins.

the midline and that other axons which stay ipsilaterally do not cross the midline (Seeger et al., 1993; Kidd et al., 1998a; Zallen et al., 1998). The phenotype of *robo* mutants and the predicted molecular features of the Robo protein suggest the existence of a ligand for Robo at the midline.

Our previous studies on the developmental origin of the eyes in the forebrain indicate that midline signaling is essential for segregating an initially equipotential morphogenetic field into two retina primordia (Li et al., 1997). We have therefore been searching for novel signaling molecules at the vertebrate midline, which led us to pursue vertebrate homologs of the *Drosophila slit* gene. *slit* mutations were originally obtained in a saturation mutagenesis for mutations affecting larval cuticular patterning (Nüsslein-Volhard et al., 1984). *slit* cDNA was isolated when Rothberg et al. (1988) screened for genes encoding EGF repeats that hybridized to a probe made from *Notch*, a gene involved in cell fate determination. *slit* mRNA is expressed in midline glial cells, whereas its secreted protein product was found in the midline cells and on axons traversing the midline cells (Rothberg et al., 1988, 1990). Loss of *slit* function was thought to cause defects in the differentiation of midline cells and the separation of longitudinal axonal tracts (Rothberg et al., 1988, 1990). Vertebrate *Slit* genes have recently been identified from rats and humans (Nakayama et al., 1998; Itoh et al., 1998). We have isolated cDNAs for *Slit* genes from *Xenopus*, chicken, and mouse and determined patterns of *Slit* expression in the embryos. Biochemical and cellular studies indicate that vertebrate Slit protein is a ligand for Robo. Functional studies demonstrate that Slit is a chemorepellent for olfactory bulb axons. Thus, Slit is the first chemorepellent implicated in guiding axon pathfinding between the olfactory bulb and the cortex.

Results

Identification of Vertebrate *Slit* Genes

Based on the *Drosophila slit* sequence, we designed several sets of primers to amplify vertebrate *slit* homologs by the polymerase chain reaction (PCR). One pair of primers allowed us to isolate *slit* homologs from *Xenopus* and chick embryos. A probe made from the PCR fragment of *Xenopus Slit* was used to screen a *Xenopus* embryonic cDNA library, and cDNA clones encoding a full-length *Xenopus* Slit protein were isolated (Figure 1). By low-stringency hybridization, cDNAs for three *Slit* genes were isolated from the mouse. Sequence comparison shows that the *Xenopus Slit* gene is an ortholog of the *Slit2* genes of mice (*mSlit2*) and humans (Figure 1A).

The predicted full-length *Xenopus* and mouse Slit proteins share all the features of the *Drosophila* Slit protein. At the amino (N) terminus, there is a putative signal peptide characteristic of secreted proteins. There are four leucine-rich repeats (LRR), each surrounded by an N-terminal and a carboxyl (C)-terminal flanking region. In *Xenopus* Slit and mouse Slit2, there are nine epidermal growth factor (EGF) repeats, whereas there are seven EGF repeats in *Drosophila* Slit (Rothberg et al., 1990; Rothberg and Artavanis-Tsakonas, 1992). Near the C terminus, there is a laminin G domain with similarities to agrin, laminin, and perlecan (also known as the ALPS domain), followed by a cysteine-rich carboxyl-terminal region (Rothberg et al., 1990; Rothberg and Artavanis-Tsakonas, 1992). Although the chicken *Slit* sequence is

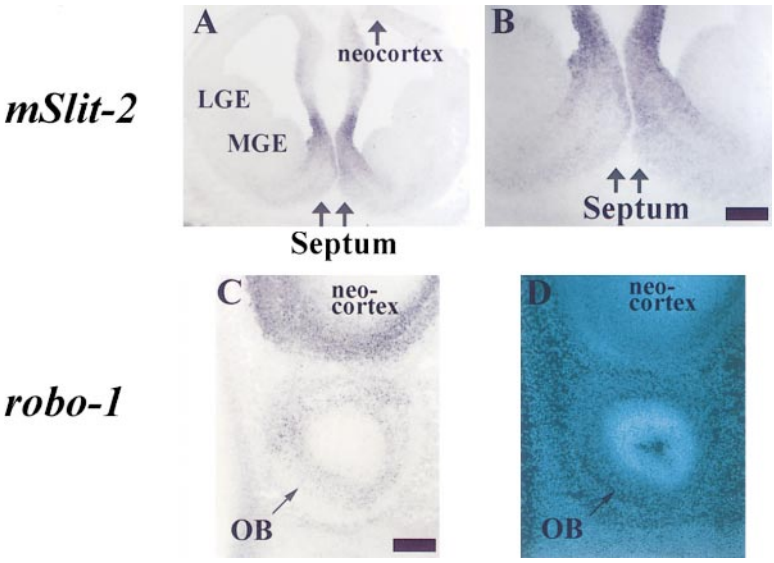

Figure 2. Expression of Mouse *Slit2* and *Robo1* Genes in the Olfactory System

Results of in situ hybridization are shown here. Bars are 100 μm in length.

(A) A coronal section of an E13.5 mouse embryo showing expression of *mSlit2*, the ortholog of the *Xenopus Slit* gene, in the septum, but not in the lateral or medial ganglionic eminence (LGE and MGE), or the neocortex.

(B) A higher magnification view of the same section as shown in (A), showing *mSlit2* in the septum.

(C) A coronal section of E14.5 mouse embryo showing expression of *Robo1* in the olfactory bulb (OB) and the neocortex. Dorsal is up and ventral is down.

(D) A fluorescent view of the same section as that in (D), showing distribution of nuclei revealed by Hoechst dye staining. OB indicates the olfactory bulb.

partial, it contains both LRR and EGF repeats and appears to be an ortholog of the *Xenopus Slit* gene (Figure 1B).

Expression of *Slit* and *Robo1*

We have determined the expression patterns of the *Slit* genes in vertebrate embryos by in situ hybridization. Because the development of the olfactory system is best described in rodents, we show here the expression of the mouse *Slit* and *Robo1* genes in regions relevant to the guidance of axon projections from the olfactory bulb. Both *mSlit1* and *mSlit2* are expressed in the septum (the latter shown in Figures 2A and 2B), the region previously implicated in guiding axons from the olfactory bulb (Pini, 1993). *Robo1*, on the other hand, is expressed in the olfactory bulb (Figure 2C). *Robo1* is also abundantly expressed in the neocortex (Figure 2C).

In chick embryos, *Slit* expression is detectable first in Hensen's node at stage 4 (Figure 3A). From stage 5 to stage 8, it is expressed in Hensen's node, the notochord, the prechordal plate, and the paraxial mesoderm (Figures 3B–3E). At stage 6, *Slit* expression is in the ventral midline of the neural tube including the floor plate (Figure 3E). *Slit* expression in the roof plate proceeds spatiotemporally in a rostral to caudal order: *Slit* is expressed in the roof plate at the prechordal level in a stage 10 embryo (Figure 3F), but not yet at the spinal cord level in the same embryo (Figure 2G). *Slit* expression extends to the most rostral ends of both the ventral and dorsal midlines of the neural tube (see Figures 3J–3M for dorsal, anterior, ventral, and lateral views of a stage 15 embryo). *Slit* expression in the motoneuron columns can be detected in stage 17 embryos (shown in Figure 3H is a stage 18 embryo). *Slit* disappears from the mesodermal midline so that, by stage 21, strong expression at the spinal cord level can be detected in the floor plate, the motor neurons, and the roof plate, but not in the notochord (Figure 3I). *Slit* expression is not only in the neural tube, but also in the somites (the dermomyotome as shown in Figure 3H), the amacrine cells in the retina (Figures 3M and 3N), and the limb bud (Figure 3O). These results suggest that *Slit* may function in many regions of the embryo.

Biochemical Characterization of Slit and Robo Interaction

The ligand for the transmembrane receptor Robo is previously unknown, but is expected to be expressed at the ventral midline. The expression pattern of *Slit* in the midline of vertebrate neural tube suggests the possibility that Slit can be a ligand for Robo, which is further supported by the complementary expression of *Slit* in the septum and that of *Robo1* in the olfactory bulb. This possibility is also consistent with phenotypic similarities between *slit* and *robo* mutants in both the central and peripheral nervous systems of *Drosophila* embryos and the apparently complementary patterns of *slit* and *robo* expression in *Drosophila* embryos (Rothberg et al., 1988, 1990; Seeger et al., 1993; Kolodziej et al., 1995; Kidd et al., 1998a).

To test directly for a possible ligand–receptor relationship, we expressed Slit and Robo proteins in cultured cells and examined their biochemical interactions. cDNAs expressing the full-length *Xenopus* Slit protein tagged with the myc epitope (Slit-myc) and the full-length rat Robo1 protein tagged with the hemagglutinin (HA) epitope (Robo-HA) were separately transfected into human embryonic kidney (HEK)–derived 293T cells. A monoclonal anti-myc antibody can specifically detect and immunoprecipitate the Slit-myc protein, whereas the Robo-HA protein could be detected by a monoclonal anti-HA antibody.

After incubation of Slit-myc in the medium with Robo-HA in the lysate, Slit-myc was coimmunoprecipitated by the anti-HA antibody (Figure 4A). Similarly, Robo-HA was coimmunoprecipitated with Slit-myc by the anti-myc antibody only if Robo-HA was preincubated with Slit-myc (Figure 4B). These results provide biochemical evidence that the secreted Slit protein can bind to Robo.

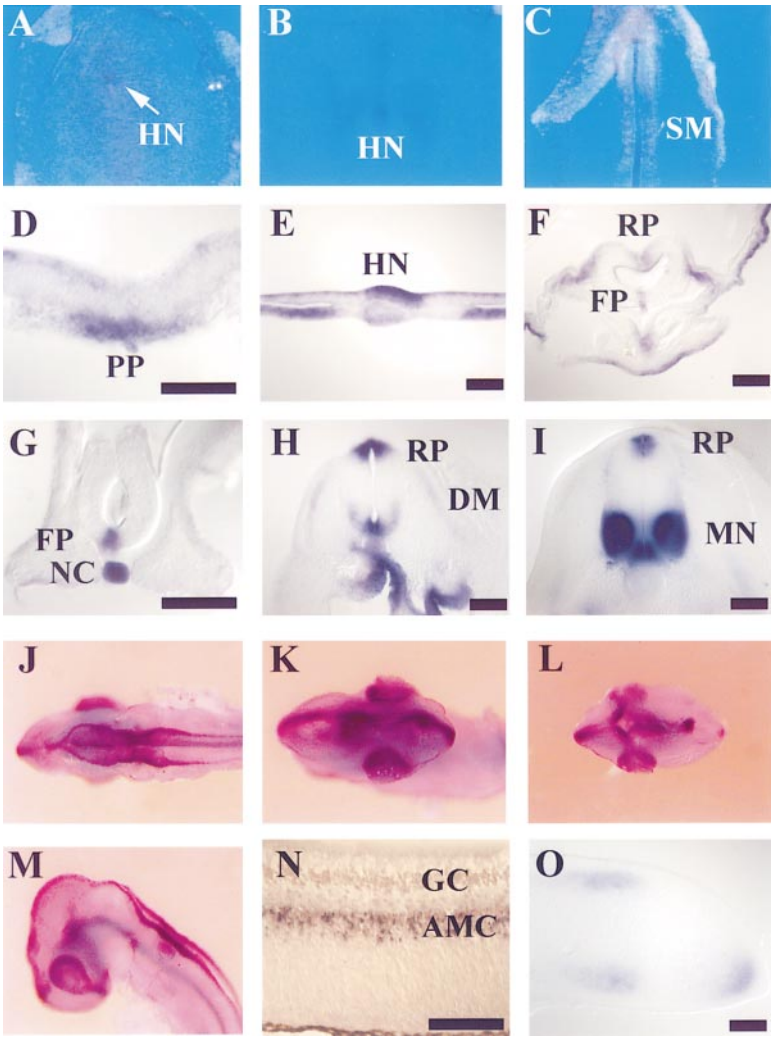

Figure 3. Expression of *Slit* in Chick Embryos

Bars are 100 μm in length.

(A) A dorsal view of a stage 4 embryo showing expression in Hensen's node (HN).

(B) A dorsal view of a stage 5 embryo showing expression in the prechordal plate, Hensen's node (HN) and the paraxial mesoderm.

(C) A dorsal view of a stage 8 embryo showing expression in the prechordal plate, the notochord, and the somites (SM).

(D) A transverse section (at a prechordal level) of a stage 6 embryo showing expression in the prechordal plate (PP).

(E) A transverse section (at the level of Hensen's node) of a stage 6 embryo showing expression in Hensen's node (HN) and the paraxial mesoderm.

(F) A transverse section (at the prechordal level) of a stage 10 embryo showing expression in the prechordal plate, the floor plate (FP), and the roof plate (RP).

(G) A transverse section (at the spinal cord level) of a stage 10 embryo showing expression in the notochord (NC) and the floor plate (FP).

(H) A transverse section (at the spinal cord level) of a stage 18 embryo showing expression in the notochord, the floor plate, the motor neurons, the roof plate (RP), and the dermomyotome (DM) in the somite.

(I) A transverse section (at the spinal cord level) of a stage 21 embryo showing expression in the floor plate, the motor neurons (MN), the roof plate (RP), and the somite.

(J) A dorsal view of a stage 15 embryo with the anterior to the left, showing expression in the neural fold of the hindbrain.

(K) An anterior view of the same stage 15 embryo as shown in (J), noting that the expression goes all the way to the anterior end.

(L) A ventral view of the head of the same stage 15 embryo as shown in (J) and (K).

(M) A lateral view of the same stage 15 embryo as shown in (J)–(L).

(N) A transverse section of a stage 30 retina, showing expression in the amacrine cells (AMC). GC, ganglion cells.

(O) A transverse section of a stage 21 limb bud showing expression in the progress zone underlying the apical ectodermal ridge, and in dorsal and ventral mesenchyme underlying the surface ectoderm. Proximal is to the left and dorsal is up.

Binding of Soluble Slit Protein to Cell Surface Robo Protein

To confirm the binding of Slit to Robo detected by immunoprecipitation, we examined the binding of a secreted Slit protein to the membrane of cells expressing Robo protein.

An alkaline phosphatase (AP)–tagged Slit (Slit-AP) was made by fusing AP to the C terminus of Slit. Slit-AP binding to Robo-HA was first tested by in vitro biochemical binding. Similar to Slit-myc, Slit-AP could bind to Robo in immunoprecipitation experiments (Figure 4C). To test for cell surface binding, control or *Robo*-transfected cells were incubated with Slit-AP and cell surface AP activity was measured (Flanagan and Leder, 1990; Cheng and Flanagan, 1994). Significant Slit-AP binding was observed only after cells were transfected with *Robo* (Figure 4D, column 1). lFng-AP, another AP-tagged fusion made by the fusion of AP to the secreted protein lunatic Fringe (Wu et al., 1996), did not bind to *Robo*-transfected cells (Figure 4D, column 3).

The availability of Slit-AP allowed us to examine the relationship between Slit-AP binding and Robo-HA expression. After detection of Slit-AP binding to cells by AP reaction, anti-HA immunofluorescent staining was used to reveal cells expressing Robo-HA (Figure 5). Slit-AP binding was found to correlate with Robo-HA expression (Figures 5A–5C), providing strong evidence for the binding of soluble Slit protein to Robo on the cell surface.

The binding affinity of Slit to Robo was determined with an assay similar to that used to measure the binding affinities of other receptors and ligands including axon guidance molecules (Flanagan and Leder, 1990; Cheng and Flanagan, 1994; Keino-Masu et al., 1996; Leonardo et al., 1997). Robo-expressing cells were incubated with Slit-AP and their binding was determined by measuring AP activities bound to the cells. An apparent dissociation constant (K_D) of 2.75 nM was estimated from the binding curve (Figure 5G), which is comparable to other ligand–receptor interactions (Flanagan and Leder, 1990; Cheng and Flanagan, 1994; Keino-Masu et al., 1996;

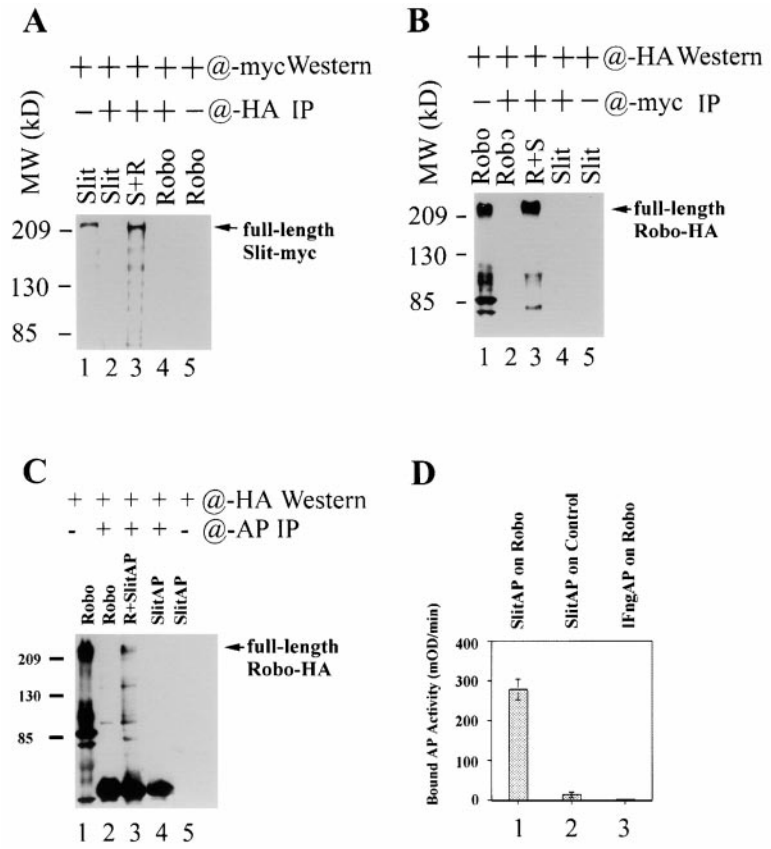

Figure 4. Binding of Slit and Robo Proteins Determined by Immunoprecipitation

Shown here are results from Western blots.

(A) Slit-myc was detected by the anti-myc antibody (lane 1), and Slit-myc alone could not be immunoprecipitated by the anti-HA antibody (lane 2). Slit-myc was coimmunoprecipitated by the anti-HA antibody only if Slit-myc was preincubated with Robo-HA (lane 3). No Slit-myc band was detected if anti-myc was used to stain anti-HA precipitates of Robo-HA lysate (lane 4) or to stain Robo-HA lysate (lane 5). Multiple bands of Slit were observed in addition to the full-length Slit protein.

(B) Robo-HA was coimmunoprecipitated by the anti-myc antibody only if Robo-HA was preincubated with Slit-myc (lane 3), but not by itself (lane 2). No Robo-HA band was detectable if anti-HA was used to stain anti-myc precipitates of Slit-myc medium (lane 4) or to stain Slit-myc medium (lane 5). Multiple bands of Robo were observed in addition to the full-length Robo protein.

(C) Binding of Slit-AP to Robo. Robo-HA could be coimmunoprecipitated by the anti-AP antibody only if Robo-HA was preincubated with Slit-AP (lane 3), but not by itself (lane 2). Lane 1 shows the migration of Robo bands (without immunoprecipitation), while lanes 4 and 5 show that Slit-AP could not be recognized by the anti-HA antibody.

(D) Binding detected by cell surface AP activity. HEK293 cells stably expressing Robo-HA or control cells transfected with the vector were seeded in 24-well dishes and then incubated with Slit-AP or IFng-AP containing media with equivalent AP activities (530 OD/ml/hr). Bound AP activities were measured as described in Experimental Procedures. Each binding test was performed with triplicate wells. Slit-AP showed significant binding to Robo cells (column 1), but little binding to the control cells (column 2). IFng-AP did not bind to Robo cells (column 3).

Leonardo et al., 1997). These results indicated that soluble Slit proteins could bind with high affinity to Robo protein on the cell surface.

Effect of Slit on Axon Outgrowth from Olfactory Bulb Explants

The projection axons of the olfactory bulb do not cross the septum but instead grow laterally in the LOT into the primary olfactory cortex (Schwob and Price, 1984; Brunjes and Frazier, 1986; Shipley et al., 1995). A chemorepellent activity at the septum was found to turn the olfactory bulb axons away from the midline in rat and chicken embryos (Pini, 1993; Keynes et al., 1997). The expression of *Slit* in the septum (Figure 2) suggests Slit as a possible candidate guidance molecule for olfactory bulb axons.

To test this possibility, we used an explant assay in collagen gel matrices previously established by Pini (1993) in the rat, which was later used in chicken embryos for studying olfactory bulb axon guidance (Keynes et al., 1997). Olfactory bulb explants were isolated from chick embryos and cocultured with either HEK cells stably transfected with *Slit-myc* or control HEK cells transfected with the vector plasmid. When cocultured with control cells, axons from the olfactory bulb explants grew symmetrically (n = 85) (Figures 6A and 6E). When cocultured with *Slit*-transfected cells, olfactory bulb explants sent out axons asymmetrically with more axons growing on the side distal to Slit-expressing cells than those on the side proximal to the Slit-expressing cells (n = 94) (Figures 6B and 6E).

Another axon guidance molecule, Netrin, is known to be expressed at the midline. It has been shown previously that Netrin is not only a chemoattractant for commissural axons, but also a repellent for other axons (Hedgecock et al., 1990; Colamarino and Tessier-Lavigne, 1995b). We therefore tested whether Netrin could be a repellent for olfactory bulb axons. In the same assay as that used for examining Slit function, Netrin was not found to repel these axons (n = 30) (Figures 6C and 6E). This was not due to the inactivity of Netrin under our culture conditions because the same Netrin-expressing cell line was effective in attracting commissural axons of the spinal cord (date not shown). Semaphorin III (Sema III) is a member of the Sema family of axon repellents (Kolodkin et al., 1993; Luo et al., 1993, 1995; Messersmith et al., 1995; Kolodkin, 1996; Püschel, 1996). We have tested the effect of Sema III on olfactory bulb axons and found that it was not repulsive to these axons (n = 10) (Figures 6D and 6E). Thus, olfactory bulb axons do not respond to all axon guidance molecules made at the midline, and the effect of Slit appears to be specific.

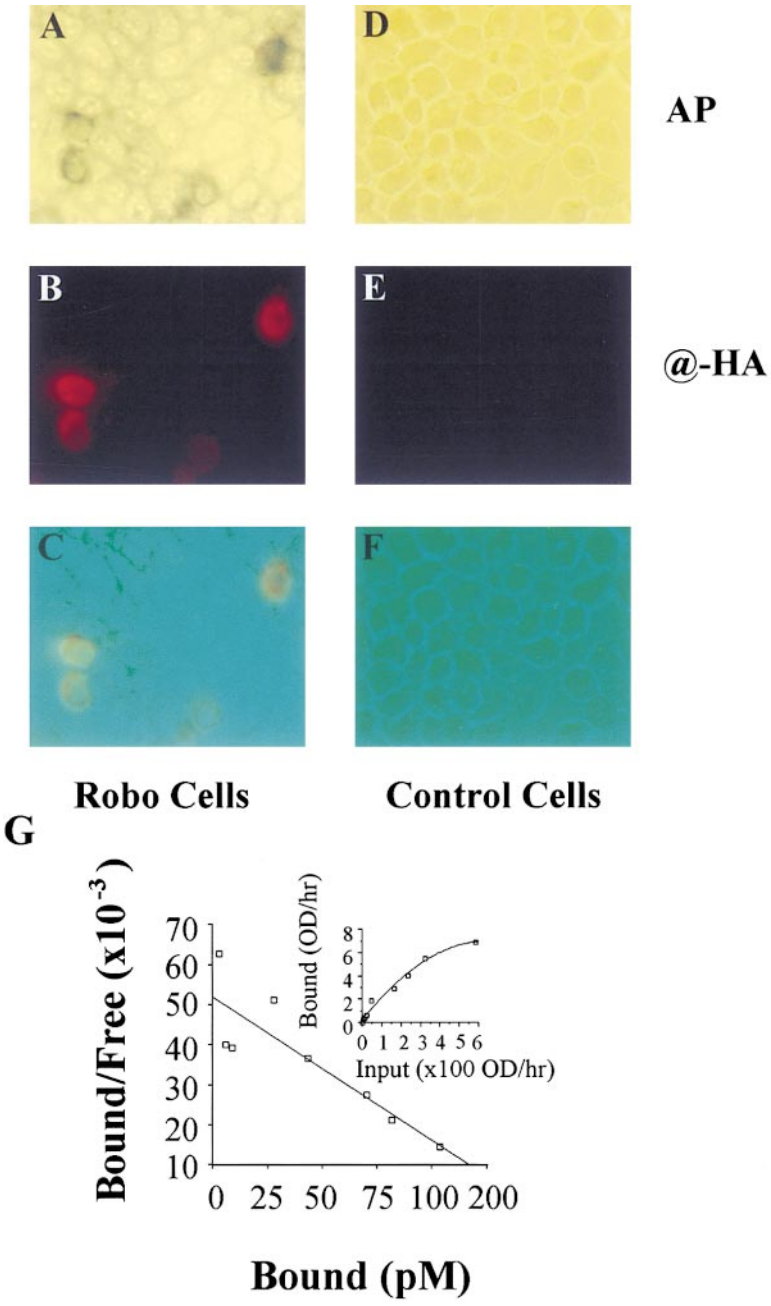

Figure 5. Binding of Slit-AP to Robo-Expressing Cells

HEK293 cells were transfected with either the vector or Robo-HA-expressing plasmids. The transfected cells were incubated with Slit-AP containing media. Slit-AP bound to the cell surface was detected by AP color reaction (A and D). Robo-HA expression was visualized by immunofluorescent staining using the anti-HA antibody and Cy3 conjugated anti-mouse secondary antibody (B and E).

(A) Results of AP staining on cells transfected with *Robo-HA*, showing the presence of Slit-AP on the cell surface. These cells are the same as those shown in (B) and (C).

(B) The same cells as those shown in (A), but viewed under a rhodamine fluorescence filter to reveal Robo-HA expression.

(C) This shows the super-imposition of (A) and (B). Correlation of Slit-AP and Robo-HA was observed.

(D) Results of AP staining on control cells, showing the absence of Slit-AP on the cell surface. These cells are the same as those shown in (E) and (F).

(E) Results of anti-HA staining of the control cells in D, showing the absence of Robo-HA.

(F) This shows the superimposition of (D) and (E).

(G) Slit-AP-containing medium was diluted to different concentrations and incubated with either Robo-expressing cells or control cells. Free and bound AP activities were determined. The numbers shown are those of specific binding after the subtraction of Slit-AP binding to control cells from its binding to Robo cells). The estimated apparent K_D was 2.75 nM.

These results indicate that Slit can act as a repellent for axons from olfactory bulb explants.

Effect of Slit on the Projection of Olfactory Bulb Axons into the Telencephalon

During normal development, olfactory bulb axons grow into the telencephalon, forming the LOT (Schwob and Price, 1984; Brunjes and Frazier, 1986; Shipley et al., 1995). Results from our experiments with olfactory bulb explants, although suggestive of a repulsive activity for Slit, did not address whether Slit could work on olfactory bulb axons when the axons grow in their normal environment, i.e., in the telencephalon.

To study the effect of Slit on axons projecting from the olfactory bulb into the telencephalon, we adopted a whole-mount preparation by which the olfactory bulb together with telencephalon was isolated and cultured (Sugisaki et al., 1996). The telencephalon region was covered with aggregates of control HEK cells or Slit-expressing HEK cells prelabeled with the lipophilic dye DiO. After about 2 days in culture, a crystal of another lipophilic dye, DiI, was inserted into the olfactory bulb to reveal its projection axons. It was therefore possible to visualize both the transfected cells (in green, Figures 7C and 7F) and the olfactory bulb axons (in red, Figures 7B and 7E). It is clear that, while the axons could grow

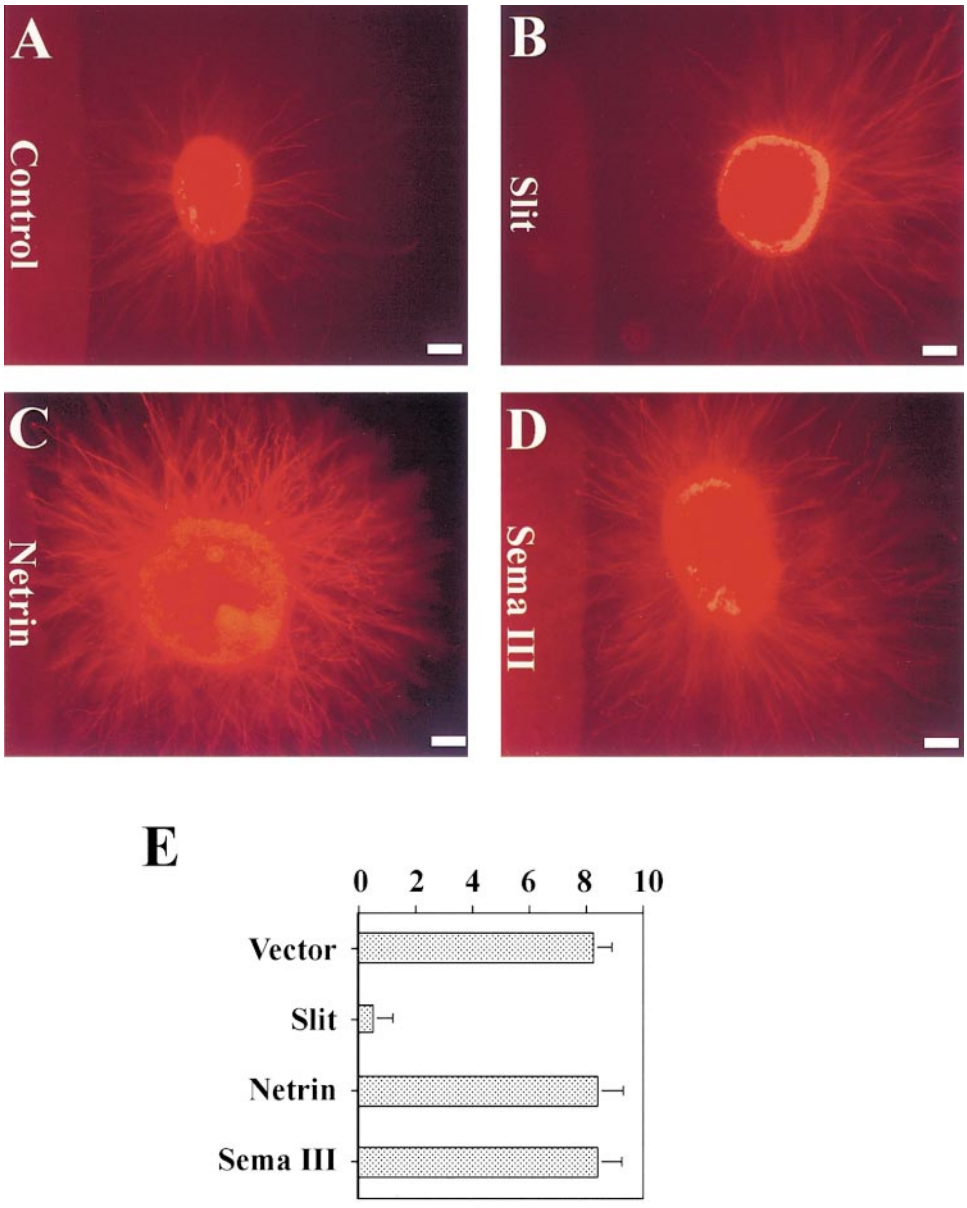

Figure 6. Effect of Slit on Axons Growing from Olfactory Bulb Explants

Olfactory bulb explants were cocultured with control, Slit, Netrin, or Sema III expressing cells. They were fixed 24 hr later and stained with the TuJ1 antibody against neuronal specific β-tubulin. The bar is 100 μm. Differences in the size of the explants in the range shown did not significantly affect their responses to attractants or repellents.

(A) Axons grew symmetrically from the olfactory bulb explant when cocultured with vector-transfected control cells.

(B) There were few axons growing from the olfactory bulb explant in the quadrant proximal to the Slit cells, whereas there were numerous axons growing in the quadrant distal to the explant.

(C) Netrin had neither attractive nor repulsive effect on olfactory bulb axons.

(D) Sema III had neither attractive nor repulsive effect on olfactory bulb axons.

(E) Quantification of axon growth according to the scheme of Keynes et al. (1997). In this scheme, the fewer the number of axons in the proximal quadrant than that in the distal quadrant, the closer the score is to 0. The presence of chemorepellent activity would thus result in a small score. A brief explanation of the quantitation scheme can be found in Experimental Procedure. Each histogram here shows the mean ± SEM.

into the telencephalon covered with control cells (n = 8) (Figures 7A–7C), they turned away from the part of the telencephalon covered with Slit cells (n = 12) (Figures 7D–7F). These results indicated that Slit acted as a repellent for olfactory bulb axons in the natural setting of the telencephalon.

Discussion

Our results have provided evidence suggesting that Slit is a secreted ligand for the transmembrane receptor Robo, and that Slit functions as a chemorepellent for olfactory bulb axons in vertebrates. Together with those

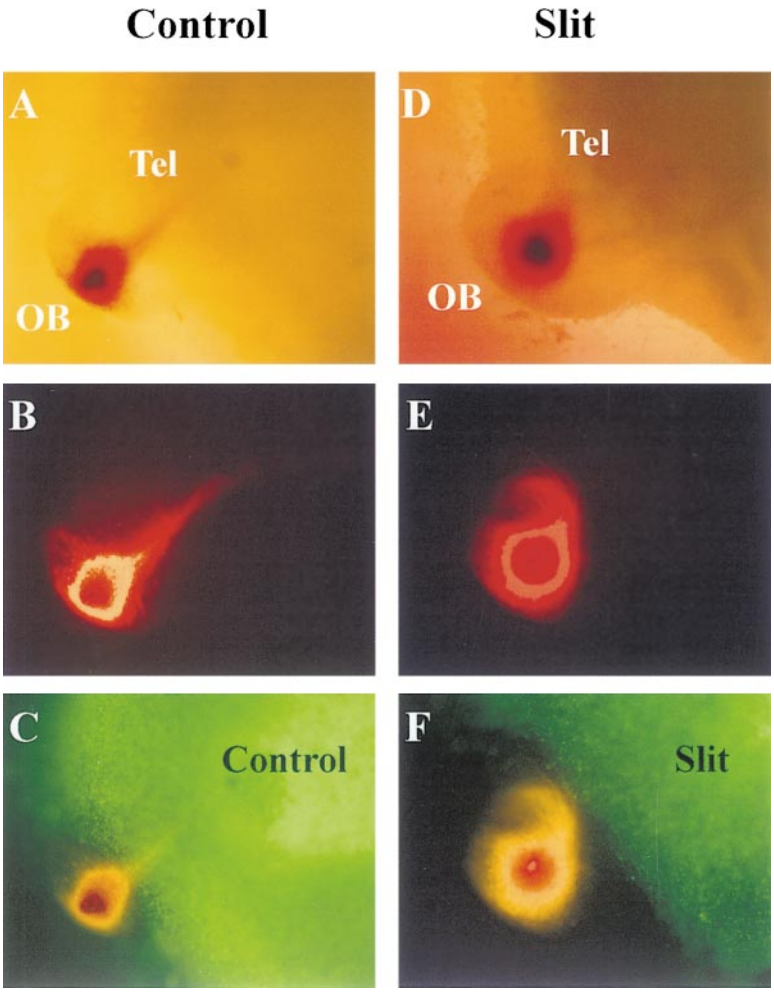

Figure 7. Effect of Slit on Olfactory Bulb Axon Guidance in the Telencephalon

(A), (B), and (C) show different views of the same coculture with control HEK cells laid on top of the telencephalon. (D), (E), and (F) show different views of the same coculture with Slit expressing cells laid on top of the telencephalon.

(A) A bright-field view of the olfactory bulb–telencephalon coculture with DiO-labeled control cells laid on top of the telencephalon and DiI inserted into the olfactory bulb.

(B) A fluorescent view under the rhodamine filter of the same coculture as that shown in (A); note the projection of axons from the olfactory bulb into the telencephalon.

(C) A fluorescent view under the FITC filter of the same coculture as that shown in (A) and (B); note the projection of axons from the olfactory bulb into the telencephalon covered with DiO-labeled (green) Slit cells.

(D) A bright-field view of the olfactory bulb–telencephalon coculture with DiO-labeled Slit cells laid on top of the telencephalon and DiI inserted into the olfactory bulb.

(E) A fluorescent view under the rhodamine filter of the same coculture as that shown in (D); note that olfactory bulb axons turned away from the telencephalon.

(F) A fluorescent view under the FITC filter of the same coculture as that shown in (D) and (E); note that olfactory bulb axons turned away from the region covered with DiO-labeled (green) Slit cells.

presented in the accompanying papers (Brose et al., 1999; Kidd et al., 1999; Wang et al., 1999), these results lead to the conclusion that Slit represents a novel family of signaling molecules of general importance in invertebrate and vertebrate axon guidance.

Slit as a Ligand for Robo

The ligand–receptor relationship between Slit and Robo is supported by several lines of evidence. First, both Slit-myc and Slit-AP proteins bind to Robo in vitro in biochemical assays, indicating that Slit and Robo bind to each other directly. Second, soluble Slit-AP protein binds to the surface of cells expressing Robo protein, indicating that the biochemical binding detected between Slit and Robo in vitro is relevant to binding on the cell surface. Third, the affinity of Slit and Robo binding is within the range of other physiologically important axon guidance molecules. Finally, the activity of Slit in repelling axons is consistent with the activity of Robo in axon guidance (Kidd et al., 1998a).

In the peripheral nervous system of *Drosophila* embryos, phenotypic similarities between *slit* and *robo* mutants in the orientation and fasciculation of sensory neuron axons is also consistent with their ligand–receptor relationship (Kolodziej et al., 1995). In the central nervous system (CNS) of *Drosophila*, while previous work has clearly shown a role for Robo in guiding commissural axons (Kidd et al., 1998a), the function of Slit in *Drosophila* neural development appeared to be more complicated (Rothberg et al., 1988, 1990). Loss-of-function *slit* mutations were thought to cause two phenotypes in the CNS of *Drosophila* embryos: failure of the midline cells to differentiate as assayed by markers for the midline cells and fusion of longitudinal axon tracts from the two sides of the embryo (Rothberg et al., 1988, 1990). One explanation for the apparent phenotypic differences between *slit* and *robo* mutants could be that Slit binds to multiple Robo receptors in *Drosophila* so that the phenotype of *slit* mutants can be more severe and pleiotropic than that of *robo* mutants. This explanation is plausible because of the existence of *robo2* in *Drosophila* (Kidd et al., 1998a). In vertebrates, it is also clear that there are multiple Slit and Robo proteins.

Function of Slit in Axon Guidance in the Olfactory System

The pattern of *Slit* expression in vertebrate embryos suggests that Slit may guide several major axon pathways (Figures 2 and 3). Results shown here indicate that Slit can guide olfactory bulb axons. Previous studies have revealed that the septum contains a repulsive activity for the olfactory bulb axons (Pini, 1993; Keynes et

al., 1997). Our finding of the expression of *Slit1* and *Slit2* in the septum and the repulsive activity of Slit suggest that one of the Slit proteins can be the repellent in the septum. The expression of *Robo1* in the olfactory bulb suggests that the Robo1 protein can be a receptor in the projection axons. At present, it is not clear whether other molecules, either of the Slit family or of other families, can function as repellents for olfactory bulb axons. It also remains to be investigated whether Robo1 can function alone as a receptor for Slit secreted from the septum, or that it requires other components.

In addition to diffusible activities from the septum, other cues in the LOT and in the olfactory cortex are involved in guiding olfactory bulb axons (Sugisaki et al., 1996; Hirata and Fujisawa, 1997; Sato et al., 1998). Our observation of the effect of Slit on axons projecting to the telencephalon indicates that Slit can override the local LOT cue. One possibility is that Slit may serve as a guidance cue globally in the rostral end of the telencephalon, which determines the distribution of the local cues, such as that in the LOT (Sugisaki et al., 1996; Sato et al., 1998). On the other hand, guidance cues in olfactory cortical areas may function at later steps to target olfactory bulb axons more specifically to distinct regions in the cortex (Hirata and Fujisawa, 1997). In any case, it will be interesting to investigate the functional relationship of Slit with the LOT and cortical cues.

Possible Function of Slit in Controlling Midline Recrossing and Longitudinal Turning of Commissural Axons in the Spinal Cord

In addition to the olfactory bulb axons, another system in which Slit functioning is related to the midline is the projection of commissural axons in the spinal cord. The expression of *Slit* in the floor plate and its binding to Robo support a role for Slit as a repellent at the floor plate for commissural axons. One function of Slit in the spinal cord would be to prevent commissural axons that have already crossed the floor plate from recrossing it. The expression level of Robo protein determines the response of commissural axons to Slit (Kidd et al., 1998a, 1998b), so that commissural axons would respond to Slit after, but not before, the axons have crossed the floor plate.

The expression of *Slit* in motoneurons suggests another possible function: this is to turn the circumferentially growing commissural axons into the longitudinal direction. Although longitudinal turning is a well-known phenomenon, its molecular mechanism is not clear. It is tempting to speculate that the presence of Slit in the floor plate and in the motor column would work together to force commissural axons that have crossed the midline to turn longitudinally. Thus, the expression pattern and repellent activity of Slit suggest that it is possible for a single molecule to play two important roles: to prevent commissural axons from recrossing the floor plate and to turn these axons longitudinally.

The mechanism for rostral–caudal guidance of the longitudinal axons remains unknown at the present. It can not be readily explained by Slit functioning alone because no rostrocaudal difference of *Slit* mRNA distribution in the floor plate and the motor neurons has been detected by in situ hybridization in vertebrate embryos, suggesting possible involvement of other molecules for rostrocaudal guidance. Expression of *Slit* outside the spinal cord, such as the retina and the limb bud, suggests that Slit functioning is not limited to the midline of the central nervous system.

Chemorepulsion and Chemoattraction in Axon Guidance

So far, Slit proteins have only been shown to act as chemorepellents. Repulsive activities have been detected in the ventral and dorsal midlines of the neural tube for axons other than the commissural axons. The ventral spinal cord and the somites are repulsive to sensory and motor axons (Fitzgerald et al., 1993; Guthrie and Pini, 1995; Keynes et al., 1997). Although some of these activities have been attributed to other repellents such as the Semaphorins, the expression of *Slit* in these regions makes it interesting to investigate whether Slit contributes to any of these activities.

Because it is known that the same molecule can be attractive to some axons but repulsive to others (Hedgecock et al., 1990; Colamarino and Tessier-Lavigne, 1995b), and that a molecule can be either attractive or repulsive to the same axons by changing intracellular cAMP and cGMP levels (Song et al., 1997; Ming et al., 1997), it is conceivable that Slit may also act as an attractant under certain circumstances.

Experimental Procedures

Isolation of Vertebrate *Slit* cDNAs

Degenerate primers corresponding to NPFNCNC and CETNIDDC of *Drosophila* Slit protein (amino acid residues 652–658 and 981–988, respectively) were used to clone fragments of *Xenopus* and chicken *Slit* cDNAs by PCR. A cDNA library was constructed using BKCMV vector (Strategene) with mRNAs extracted from stage 17 *Xenopus* embryos. The PCR fragment of *Xenopus Slit* was used to screen the cDNA library to obtain full-length *Xenopus Slit* cDNA clones. The full-length *Xenopus* and partial chicken *Slit* cDNAs were sequenced using an ABI 373A automatic sequencer.

In Situ Hybridization

In situ hybridization was performed as described previously (Li et al., 1997) with some modifications. Briefly, mouse embryos were isolated and fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C, rinsed twice in DEPC-treated PBS, submerged in 30% sucrose in DEPC-treated PBS until the embryos sunk to the bottom of the tube. Embryos were transferred to O.C.T. compound and embedded in O.C.T. for 1–4 hr at 4°C, and then sectioned. The sections were processed as described by Wright and Snyder (1995) with the following two modifications: first, sections were digested in 1 μg/ml proteinase K at room temperature for 5 min, fixed in 4% PFA in PBS for 30 min, washed twice in PBS, and treated with 0.25% acetic anhydride in 0.1 M TEA for 30 min; second, anti-dig-AP conjugated antibody (FAB fragment, Boehringer-Mannheim) incubation was carried out at 4°C overnight. The color reaction was carried out in 34 μg/ml NBT and 340 μg/ml BCIP.

Plasmid Construction

The coding region for full-length *Xenopus* Slit was inserted in-frame into pCS2$^+$ vector containing a six-myc epitope tag or containing the secreted alkaline phosphatase to obtain Slit-myc or Slit-AP, respectively. In both cases, Slit is at the N terminal portion of the fusion proteins. To express Robo as an epitope-tagged protein, the rat Robo1 coding region was obtained by PCR using rat spinal cord cDNA and was then inserted into pCS2$^+$ vector containing a hemmagglutinin (HA) epitope. The HA epitope was at the C terminus

of Robo1. The full-length coding regions of Slit and Robo1 in the plasmids were verified by sequencing. Before being used in transfection experiments, individual plasmids were also tested for expression of the corresponding full-length proteins by coupled in vitro transcription–translation (Promega).

Cell Culture, Transfection, and Immunoprecipitation

HEK 293 cells (from American Tissue Culture Center) or 293T cells were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO). Cells were grown to 70% confluence on 10 cm tissue culture dishes and transfected with approximately 25 μg plasmid DNA per plate using calcium phosphate for 16 to 24 hr. GFP-pGL, a plasmid expressing the green fluorescent protein (GFP) (Life Technology), was used in most of the transfection experiments to monitor transfection efficiency.

For coimmunoprecipitation, plasmids encoding Slit-myc, Slit-AP, or Robo-HA or control vector plasmids were transfected into HEK or 293T cells. Conditioned media containing Slit-myc or Slit-AP proteins from the transfected cells were collected 72 to 96 hr after transfection and concentrated using a Boimax-100K ultrafree-15 filter (Millipore). Robo-HA containing cell lysates or control lysates were prepared with lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 150 μg/ml benzamidine). Conditioned media containing Slit-myc or Slit-AP were mixed with lysates from Robo-HA or control cells. Immunoprecipitation was carried out as described in Kopan et al. (1996) using anti-myc (Babco) or anti-AP (Sigma). Precipitated proteins were then detected after Western blotting by anti-HA (Babco) with enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

cDNA fragments encoding Slit-myc and Robo-HA were inserted into modified pIRESneo (Clontech) and pCEP4 (Invitrogen) to make constructs for stable expression of Slit-myc and Robo-HA. Linearized plasmids and their corresponding vector controls were transfected into HEK cells. Antibiotics were added 36 to 48 hr after transfection and selection was carried out for three weeks with the media changed every three days. 300 μg/ml of G418 (Life Technology) was used to select for Slit-myc stable lines, and 200 μg/ml of hygromycin B (Sigma) was used to select for Robo-HA stable lines. Stable cell lines expressing Slit-myc and Robo-HA were obtained after isolating individual colonies and testing for protein expression by both Western blots and immunocytochemical staining (with anti-myc or anti-HA antibodies).

Cell Surface Binding and Immunocytochemistry

HEK293 cells grown in 10 cm dishes were transfected with Robo-HA or vector plasmids. Approximately 30 hr after transfection, cells were suspended by pipetting up and down several times and then seeded onto 6-well or 24-well dishes to 50% confluence. Cells were grown for another 12 to 18 hr before incubation with Slit-AP or lFng-AP conditioned media containing similar amounts of AP activity (approximately 530 OD/ml/hr). After 1 hr of incubation with the conditioned media followed by three to four washes in HBHA buffer (Hank's balanced salt solution, 0.5 mg/ml BSA, 20 mM HEPES [pH 7.0]), cells were fixed for 30 s in acetone-formaldehyde fixative (60% acetone, 3% formaldehyde, 20 mM HEPES [pH 7.0]). Cells were then washed three times in HBS (150 mM NaCl, 20 mM HEPES, [pH 7.0]) and incubated at 65°C for 10 min to inactivate the endogenous cellular phosphatase activity. AP staining buffer (100 mM Tris [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 0.17 mg/ml BCIP, 0.33 mg/ml NBT) was used to detect Slit-AP or lFng-AP bound at the cell surface. Following three washes, mouse anti-HA and anti-mouse conjugated to Cy3 were used to visualize Robo expression. GFP expression indicated similar transfection efficiencies in vector and Robo-HA transfected cells.

Assay for AP Activity after Slit-AP Binding to Robo-Expressing Cells

Stable cell lines expressing Robo-HA or the vector were seeded in 24-well culture dishes precoated with 100 μg/ml polylysine. Cells were grown to 95% confluence and then incubated at 37°C for 1 hr with 200 μl/well concentrated condition media containing different concentrations of Slit-AP with the highest level at approximately 3000 OD/ml/hr of AP activity. Media were collected to assay for the AP activity as free Slit-AP. Cells were washed 4 times with the culture medium without Slit-AP. Cells were then lysed using a lysis buffer containing 50 mM Tris (pH 8), 1% Triton X-100. Cell lysates were cleared by centrifugation for 10 min. Samples were heated at 65°C for 10 min to inactivate the endogenous cellular phosphatase activity. AP activity was assayed by adding equal volumes of 2× AP buffer (2 M diethanolamine [pH 9.8], 1 mM $MgCl_2$, 20 mM homoarginine, 12 mM p-nitro-phenyl phosphate) to cell lysates in 96-well flat bottom microtiter plates. Incubation was carried out at room temperature and AP activity was determined at 405 nm. The concentration of Slit-AP fusion protein in the conditioned media was estimated by comparison with the purified human placenta alkaline phosphatase (HuPAP) (Sigma) both in AP activity assays and in Western blots using anti-AP (Genzyme).

Olfactory Bulb Axon Guidance Assays

Preparation of rat tail collagen and explant culture in collagen gel matrices were carried out according to the protocol described in Guthrie and Lumsden (1994). Cell aggregates were prepared by the hanging-drop method (Fan and Tessier-Lavigne, 1994).

The olfactory bulb explant assay was performed according to Pini (1993; Keynes et al., 1997). Briefly, stage 33 and 34 chick embryos were dissected out in Tyrode's solution. Olfactory bulbs were removed and stored in L15 medium with 5% horse serum (GIBCO) on ice for at least 30 min. Olfactory bulb explants were trimmed to 200–300 μm. Olfactory bulb explants and cell aggregates were transferred onto a collagen pad and covered with collagen. The distance between the olfactory bulb explants and the cell aggregates ranges from 100 to 400 μm. After collagen gel matrices solidified, DMEM with 10% FCS and 100 μg/ml of penicillin and streptomycin was added. Explants and cells were cocultured at 37°C with 5% CO_2 and the effects of Slit on olfactory bulb axons were visible 10 hr after culturing. Explants were fixed after about 12 or 24 hr of coculture. TuJ1 antibody was used in immunocytochemistry to visualize neuronal processes.

Quantification of the axon projections from the olfactory bulb explants was carried out according to a detailed diagram of the scheme Figure 1B of Keynes et al. (1997). A score of 0 is given if there are no or very few axons growing in the proximal quadrant; a score of 2, if there were few axons in the proximal quadrant with strong asymmetry when compared with the distal quadrant; a score of 4, if there was greater outgrowth in the proximal quadrant, with axons in the proximal quadrant still more than 50 μm from the cell aggregates, and strong asymmetry between the distal and proximal quadrants; a score of 6, if axons in the proximal quadrant growing within less than 50 μm from the cell aggregates, still with asymmetry between the distal and proximal quadrants; a score of 8, if axons contacting the cell aggregates, but still with detectable asymmetry; and a score of 10, if axons had grown over the cell aggregates and there was not asymmetry between the proximal and distal quadrants.

Whole-mount preparations of olfactory bulb-telencephalon coculture were carried out with a protocol similar to that described in Sugisaki et al. (1996). Briefly, The telencephalic hemisphere together with the olfactory bulb were dissected out from E12.5 mice, freed from the pia mater, and placed on a collagen gel. Cells transfected with either vector alone or with *Slit-myc* cDNA were previously labeled with DiO (3,3′dioctadecyloxacarbocyanine, Molecular Probes). Aggregates of these cells were put on top of the telencephalon, but not the olfactory bulb. Whole-mount preparations were cultured with DMEM containing 10% FBS at 37°C with 5% CO_2. Forty hours later, small crystals of lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine per-chlorate (DiI; Molecular Probes) were inserted into the olfactory bulbs. Eight hours later, the specimens were fixed with 4% PFA in 10 mM PBS and kept at 4°C before examination under a fluorescent microscope.

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