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Diversity and Specificity of Actions of Slit2 Proteolytic Fragments in Axon Guidance

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The Slits are secreted proteins that bind to Robo receptors and play a role in axon guidance and neuronal migration. In vertebrates, Slit2 is a major chemorepellent for developing axons and is involved in the control of midline crossing. *In vivo*, Slit2 is cleaved into 140 kDa N-terminal (Slit2-N) and 55–60 kDa C-terminal (Slit2-C) fragments, although the uncleaved/full-length form can also be isolated from brain extract. We explored the functional activities of Slit2 fragments by engineering mutant and truncated versions of Slit2 representing the N-, C-, and full/uncleavable (Slit2-U) fragments. Only Slit2-N and Slit2-U bind the Robo proteins. We found that in collagen gel, olfactory bulb (OB) but not dorsal root ganglia (DRG) axons are repelled by Slit2-N and Slit2-U. Moreover, only Slit2-N membranes or purified protein-induced OB growth cones collapse. Finally, we found that only recombinant Slit2-N could induce branching of DRG axons and that this effect was antagonized by Slit2-U. Therefore, different axons have distinct responses to Slit2 fragments, and these proteins have different growth-promoting capacities.

Key words: repulsion; guidance; collapse; Robo; Slit2; branching

Developing axons are guided by cues that can be either diffusible or bound to the extracellular matrix (ECM) or the cell membrane. The Slits have been characterized recently as extracellular ligands for the *roundabout* transmembrane receptors (Kidd et al., 1999). Three distinct slit genes, named *slit1*, *slit2*, and *slit3*, have been cloned in mammals (Holmes et al., 1998; Itoh et al., 1998; Brose et al., 1999). In tissue culture, Slit1 and Slit2 proteins have been shown to function as chemorepellents and collapsing factors for olfactory, motor, hippocampal, and retinal axons (Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). Slit1 and Slit2 can also repel tangentially migrating interneurons in the mouse telencephalon (Hu, 1999; Zhu et al., 1999), and *Drosophila* Slit (dSlit) repels muscle precursors in the fly embryo (Kidd et al., 1999). However, there is also some positive effect associated with Slits, because Slit2 stimulates the formation of axon collateral branches by NGF-responsive neurons of the dorsal root ganglia (DRG) (Wang et al., 1999).

Slits are large ECM glycoproteins of ~200 kDa (Fig. 1*A*), comprising, from their N terminus to their C terminus, a long stretch of four leucine reach repeats, seven to nine EGF repeats, and a domain, named ALPS [for "agrin, laminin, perlecan, slit" (Rothberg and Artavanis-Tsakonas, 1992)], LNS [for "laminin, neurexin, slit" (Rudenko et al., 1999)], or laminin G-like (LG) module (Hohenester et al., 1999). Full-length hSlit2 is proteolytically processed into 140 kDa N-terminal and 55–60 kDa C-terminal fragments in cell culture and *in vivo*. *Drosophila* Slit appears to be similarly processed *in vitro* and *in vivo*, suggesting conserved mechanisms for Slit proteolytic processing across species (Brose et al., 1999; Wang et al., 1999). There is some evidence that the different Slit2 fragments may have different functional activities *in vivo*. The purification of a DRG axon elongation- and branch-promoting activity suggested that the N-terminal fragment of Slit2, but not the full-length protein, is capable of stimulating elongation and branching (Wang et al., 1999). However, the purification of a repellent activity for migrating subventricular zone olfactory precursor interneurons and retinal axons revealed that these activities also correspond to the full-length Slit2 protein (Hu, 1999; Niclou et al., 2000). It is not clear which fragment or fragments are responsible for the other Slit2-associated activities, including the repellent effects of Slit2 on axons and cells in vertebrates and *Drosophila* (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wu et al., 1999; Zhu et al., 1999). It is also not known whether proteolytic processing is required for the repulsive activity of Slit2.

In this paper, we have begun to explore these questions by engineering mutant versions of human Slit2 (hSlit2) representing the two cleavage fragments, N- and C-, and the full/uncleavable molecule and examining the activities of these mutants in binding and functional assays. We show that different fragments have different activities but also that the response of an axon to Slit2 is not absolute but rather depends on the type of axon.

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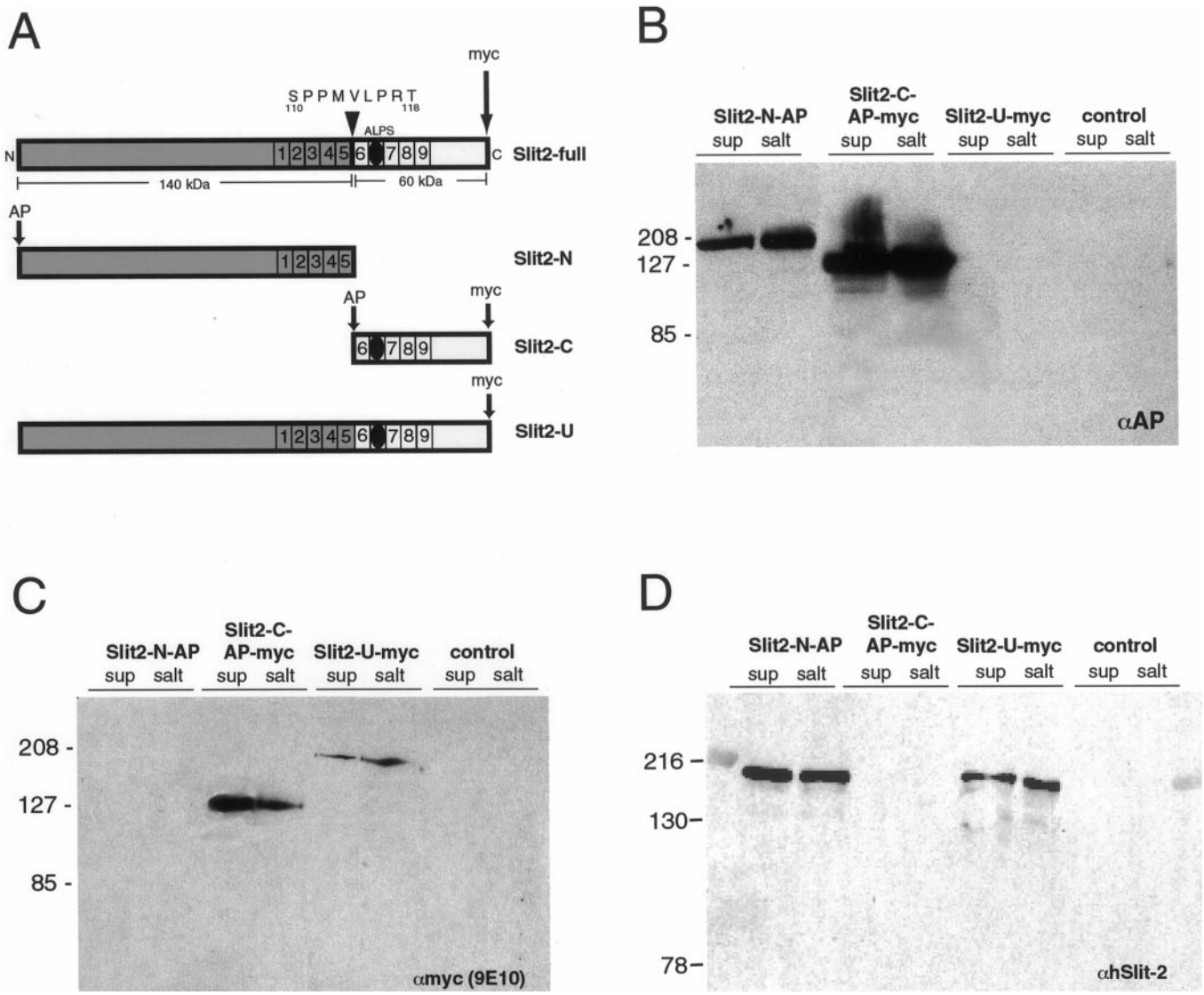

Figure 1. *A*, Schematic representation of the various Slit2 constructs. *Slit2-full* represents the native full-length Slit2. The nine EGF repeats and the ALPS domain are represented, and the nine amino acids sequence deleted to generate Slit2-U is indicated (*arrowhead*). All proteins except Slit2-N are also myc tagged at their C termini, and Slit2-N and Slit2-C are tagged by the alkaline phosphatase at their N termini. *B–D*, Western blots using anti-alkaline phosphatase (*B*), 9E10 anti-myc (*C*), and anti-hSlit2 (*D*) antibodies. All Slit2 fragments can be detected in conditioned medium (*sup*) and after extraction in 1 M NaCl (*salt*). Slit2-NAP is recognized by anti-hSlit2 and anti-alkaline phosphatase (*B*, *D*), whereas Slit2-U is detected by anti-myc and anti-hSlit2 (*C*, *D*). Slit2-C can be detected by the anti-alkaline phosphatase and the anti-myc antibodies (*B*, *C*). All protein fragments migrate at the expected molecular weight, and Slit2-U migrates as a single band and therefore is not cleaved.

MATERIALS AND METHODS

Generation of Slit2 fragment expression constructs. The N-terminal cleavage construct (Slit2-N) was made by cloning the N terminus of human Slit2, consisting of sequences encoding amino acids QACPAQ to FSPPMY, upstream of the putative cleavage site into the *Bam*HI–*Xba*I sites of pSectagB vector (Invitrogen, San Diego, CA) and thus contains both myc and His epitope tags at its C terminus. A version of this construct bearing an N-terminal alkaline phosphatase tag (Slit2-NAP) was constructed by inserting coding sequences for human alkaline phosphatase into the *Hin*dIII site of Slit2-N.

The C-terminal cleavage construct (Slit2-C) was made by cloning the C terminus of human Slit2, consisting of sequences encoding amino acids immediately downstream of the putative cleavage site beginning at TSPCD to the stop into the *Xho*I–*Xba*I sites of pSectagB. A version of this construct bearing an N-terminal alkaline phosphatase tag (Slit2-N-AP) was constructed by inserting coding sequences for human alkaline phosphatase into the *Hin*dIII site of Slit2-C.

Sequences encoding the amino acids SPPMVLPRT were deleted from human Slit2 using PCR mutagenesis. The deletion construct was also cloned into the pSectagB. This mutation abolishes the cleavage of Slit2. The resulting protein was called Slit2-U (for uncleavable).

Antibodies and Western analyses. To examine processing of hSlit2, conditioned media and high salt (1 M NaCl) extracts from cells transfected with truncation constructs consisting of the N-terminal (tagged with AP, and detectable with the anti-hSlit2 and the anti-alkaline phosphatase antibodies), the C-terminal (tagged with AP and myc) or the uncleavable (myc tagged, and detectable with the anti-Slit2 antibody) fragments were collected. These extracts were TCA precipitated, solubilized in SDS-PAGE sample buffer, run out on an SDS-PAGE (7.5%) gel, and Western blotted by standard methods with a monoclonal 9E10 anti-myc antibody (gift of J. M. Bishop, Stanford University), an anti-alkaline phosphatase antibody (Dako, Glostrup, Denmark), or an anti-Slit2 antibody (see below).

Purification of Slit2-U, Slit2-N, and Slit2-C fragments. Slit2-N and Slit2-U proteins were purified on the basis of the association of the N terminus with wheat germ agglutinin (WGA). Salt extracts of cells expressing either Slit2-U or Slit2-N were prepared by incubating cells with 1 M NaCl/10 mM HEPES, pH 7.5, for 15 min at room temperature;

the procedure was repeated twice. Salt concentration was adjusted to 1 M NaCl/10 mM HEPES, $CaCl_2$ to 1 mM, and $MnCl_2$ to 1 mM. Protease inhibitors were present at all times, and extracts were kept at 4°C. Extracts were centrifuged for 10 min at 12,000 × *g* to remove cellular debris. Cleared extract was added to prewashed (with 1 M NaCl/10 mM HEPES/1 mM $CaCl_2$/1 mM $MnCl_2$) WGA-Agarose (Vector Laboratories, Burlingame, CA) and allowed to batch bind overnight, rocking at 4°C. A column was packed with bound beads, and media was loaded onto the column. Flow-through was reapplied to the column at least once to maximize binding efficiency. The column was washed with 10 column volumes of 1 M NaCl/10 mM HEPES/1 mM $CaCl_2$/1 mM $MnCl_2$ and eluted stepwise with 5 M urea/0.5 M *n*-acetylglucosamine/1 mM NaCl/10 mM HEPES, pH 7.5/1 mM $CaCl_2$/1 mM $MnCl_2$. The eluted protein was dialyzed overnight against F12.

Slit2-C proteins were purified by nickel-affinity chromatography based on the C-terminal His tag. Salt extracts of transfected cells were prepared as described above. Conditioned media and salt extracts were combined, supplemented with protease inhibitors, and centrifuged as described above. Cleared extracts were added to prewashed (with 1 M NaCl/10 mM HEPES) nickel Agarose beads (Qiagen, Hilden, Germany) overnight, rocking at 4°C. A column was packed with bound beads, and media was loaded onto the column. The column was washed first with 10 column volumes of 1 M NaCl/10 mM HEPES, pH 7.5, then washed with 10 column volumes of 10 mM imidazole/1 M NaCl/10 mM HEPES and eluted with 250 mM imidazole/1 M NaCl/10 mM HEPES. Eluted protein was dialyzed overnight against F12.

Generation of antibodies against hSlit2. Sequences coding for EGF repeats 1–5 of hSlit2 were cloned into pGEX-4T vector (Amersham Pharmacia Biotech, Les Ulis, France). Bacteria transformed with this construct were used to produce GST fusion proteins. To generate optimal quantities of purifiable protein, expression of fusion proteins was induced at room temperature for 3 hr. Fusion proteins were purified under native conditions following the manufacturer's protocol and injected into two rabbits to raise antibodies (Babco, Richmond, CA). Western blots of recombinant Slit1, -2, and -3 revealed that this antiserum was specific to Slit2.

Collagen gel assay. OBs from embryonic day (E) 14–15 rats were dissected out and cultured as described previously (de Castro et al., 1999). DRG from E15 rats were dissected as described elsewhere (Messersmith et al., 1995). All explants were cocultured with aggregates of untransfected COS cells or COS cells transfected with secreted alkaline phosphatase (AP), using the AP-Tag-4 vector (gift of Dr. J. Flanagan, Harvard Medical School), or *slit2-U*, *slit2-N*, *slit2-C*. Heparin (100 ng/ml) was added to the culture medium because this treatment appears to release some of the Slit2 that is normally associated with the plasma membrane (Brose et al., 1999).

Explants were fixed in ice-cold 4% paraformaldehyde and immunolabeled as described in Nguyen Ba-Charvet et al. (1999). Quantification of the explants was performed as described previously (Chédotal et al., 1998).

Collapse assay. DRG or OB explants from E14–E15 Sprague Dawley rat embryos were cultured on glass coverslips coated with 0.2 mg/ml poly-L-lysine (Sigma, St. Louis, MO) in borate buffer and 20 μg/ml laminin in PBS. Collapse assays with membranes were performed as described previously (Nguyen Ba-Charvet et al., 1999). For protein collapse assays, the purified recombinant proteins were gently mixed into the culture medium. The cultures were incubated at 37°C, 5% CO_2 for 1 hr and then fixed with 4% paraformaldehyde containing 10% sucrose. The proteins were tested at a final concentration ranging from 0.5 to 10 μg/ml.

Branching and elongation assay of sensory axons. Dissociated DRG neurons from E14.5 rat embryos were prepared and cultured in collagen gels as described previously (Wang et al., 1999). They were incubated in the medium with 25 ng/ml NGF for the first day, and the medium was replaced with a fresh one without NGF but with different Slit2 protein fragments on the second day. Cells were fixed at ~48 hr, and neurites were visualized with an antibody against neurofilament-M (gift of Dr. Virginia Lee, University of Pennsylvania) (Wang et al., 1999). The neurite length and branch numbers were measured from at least four random images of each sample.

Binding. Binding and equilibrium binding of individual Slit2 fragments were performed and analyzed as described previously (Brose et al., 1999).

RESULTS

Characterization of Slit2 protein fragments

To examine the activities of various cleavage fragments of Slit2, we generated cDNA constructs encoding truncated proteins corresponding to just the N-terminal and the C-terminal cleavage fragments of human Slit2 (with one ending and the other starting at Thr 1118, the putative cleavage site, located at the start of EGF repeat 6) (Fig. 1*A*). We also engineered a cDNA construct encoding a presumptive uncleavable form of Slit2. For this, the DNA sequence encoding the nine amino acids preceding Thr 1118 (SPPMVLPRT) was removed from the human Slit2 cDNA, on the assumption that cleavage of the full-length protein would require some or all of that sequence. The three mutant proteins were expressed at high levels in COS cells and are found in both media conditioned by these cells and in high salt (1 M NaCl) extracts of these cells, as assessed by Western blot using antibodies against AP (Fig. 1*B*) or the myc epitope (Fig. 1*C*). The Slit2-N-AP protein runs on gel at approximately the same level as Slit2-U (Fig. 1, compare *B*, *C*), because it was fused to the alkaline phosphatase (see Materials and Methods), which has a molecular weight of 67 kDa. When the cDNA deprived of the 27 nucleotides was transfected in COS or 293 cells, the expressed protein was of the approximate size of full-length Slit2 but was not cleaved (Fig. 1*C*). We name this mutant protein Slit2-U. Finally, a polyclonal antiserum raised against EGF repeats 1–5 of hSlit2 recognizes both Slit2-N and Slit2-U, but not Slit2-C (Fig. 1*D*) (see also Hu, 1999; Nguyen Ba-Charvet et al., 1999). This antibody is specific for hSlit2 and does not recognize recombinant hSlit1 or hSlit3 (data not shown).

Binding to Robo receptors is mediated by amino-terminal Slit2 sequences

The binding properties of various Slit2 protein fragments with Robo receptors were tested in cell overlay assays. We first tested the ability of soluble Slit2-N, Slit2-C, and Slit2-U (in conditioned media from transfected COS cells) to bind to transfected COS cells expressing Robo1 or Robo2. Both Slit2-N (Fig. 2*A*) and Slit2-U (Fig. 2*B*) bound cells expressing Robo1 proteins, whereas Slit2-C did not (Fig. 2*C*). The same results were obtained with cells expressing Robo2 proteins (data not shown). No staining was observed using supernatant from mock-transfected COS cells (Fig. 2*D*), and none of the proteins bound control (mock-transfected) COS cells (Fig. 2*E*) or COS cells expressing several other Ig superfamily members (DCC, TAG-1, or L1; data not shown). The binding affinities for the interactions between Robo and Slit2 protein fragments were estimated in equilibrium binding experiments in which Robo-Fc fusion proteins were applied to COS cells expressing Slit2-N, Slit2-C, or Slit2-U or to control COS cells (Fig. 2*F–I*). These experiments took advantage of the fact that although all three Slit2 fragments are secreted and diffusible, a significant fraction of the proteins remain associated with cell surfaces (as detected by Western blot analysis and immunohistochemistry). We found that Robo1-Fc and Robo2-Fc bound cells expressing Slit2-N and Slit2-U, but there was no detectable binding to cells expressing Slit2-C (data not shown). Specific binding of Robo1-Fc and Robo2-Fc to Slit2-N- and Slit2-U-expressing cells showed saturation, and binding curves were fitted to the Hill equation (Fig. 2*F–I*). The estimated dissociation constants (K_d) for Robo1-Fc binding to Slit2-N and Slit2-U were 8.6 and 10.1 nM, respectively. Those for Robo2-Fc binding to Slit2-N and Slit2-U were 5.9 and 7.3 nM. The binding interactions observed in each of the four pairwise combinations

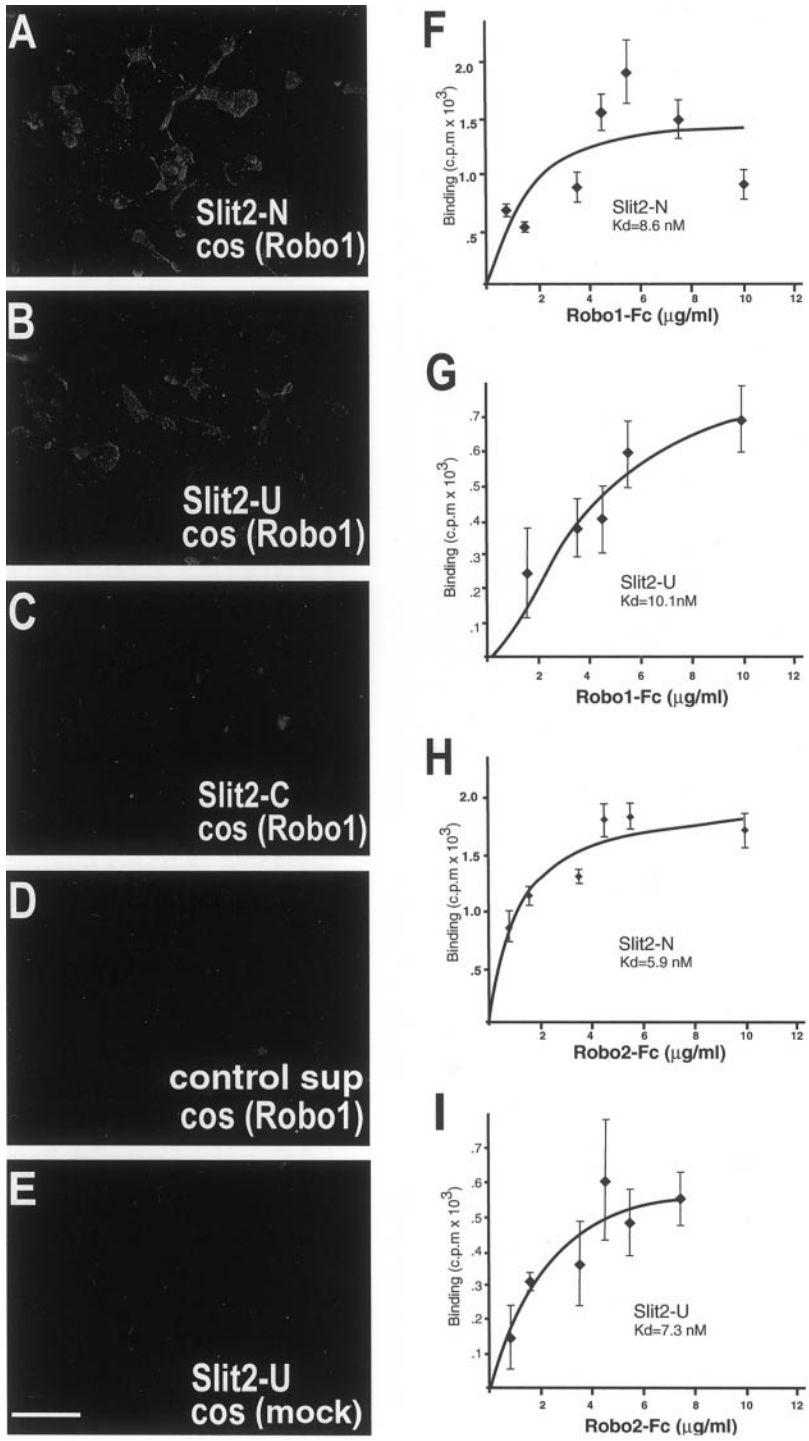

Figure 2. Slit2-N and Slit2-U, but not Slit2-C, bind Robo. Supernatants from cells expressing hSlit2-N (*A*), hSlit2-U (*B*), and hSlit2-C (*C*), or from mock-transfected cells (*D*) were incubated with cells expressing Robo1 proteins. Binding was detected using an antibody against myc epitope (9E10) and corresponding Cy3-conjugated secondary antibodies. No binding is observed when Slit2-U is applied to mock-transfected COS cells (*E*). Scale bar, 100 μm. Equilibrium binding curves for interactions of rRobo1-Fc (*F*, *G*) and rRobo2-Fc (*H*, *I*) to Slit2-N (*F*, *H*) and Slit2-U (*G*, *I*). COS cells transfected with either Slit2-N or Slit2-U expression constructs, or control vector alone, were incubated with indicated concentrations of purified rRobo1-Fc or rRobo-2Fc in PBS/1% BSA for 4 hr, washed, incubated with ^{125}I-labeled anti-human IgG, and washed again. Total binding was determined by measuring radioactivity associated with cells after the final wash. Specific binding was defined as the difference between binding to Slit2-expressing cells and mock-transfected cells. Specific binding curves were fitted using the Hill equation.

(Slit2-N or Slit2-U with either Robo1-Fc or Robo2-Fc) were thus of similar affinity in each case (compare the specific K_d values).

Interactions of Slit2 protein fragments with different axonal populations

To permit a fine-grained analysis of the effects of different Slit2 protein fragments on different axonal populations, we first compared the effects of the three recombinant Slit2 protein fragments (-N, -C and -U) on axons of OB neurons (in particular mitral cells) from E14–15 rat embryos and on axons of NGF-responsive sensory neurons from dorsal root ganglia of E14–15 rat embryos ("DRG axons"). We chose to focus on these neurons because both populations express *Robo2* but not *Robo1* mRNA (Nguyen Ba-Charvet et al., 1999; Wang et al., 1999) and because they showed dramatically different responses to Slit2, with olfactory axons being repelled (Nguyen Ba-Charvet et al., 1999) and DRG axons stimulated to elongate and branch (Wang et al., 1999).

The functional properties of the Slit2 proteins were tested in several assays in which they were presented in different ways to axons emanating from tissue explants. First, we used a three-dimensional collagen gel assay to test the ability of the proteins (secreted from COS cells) to function as diffusible chemorepellents (or chemoattractants) for the axons ("diffusion assay").

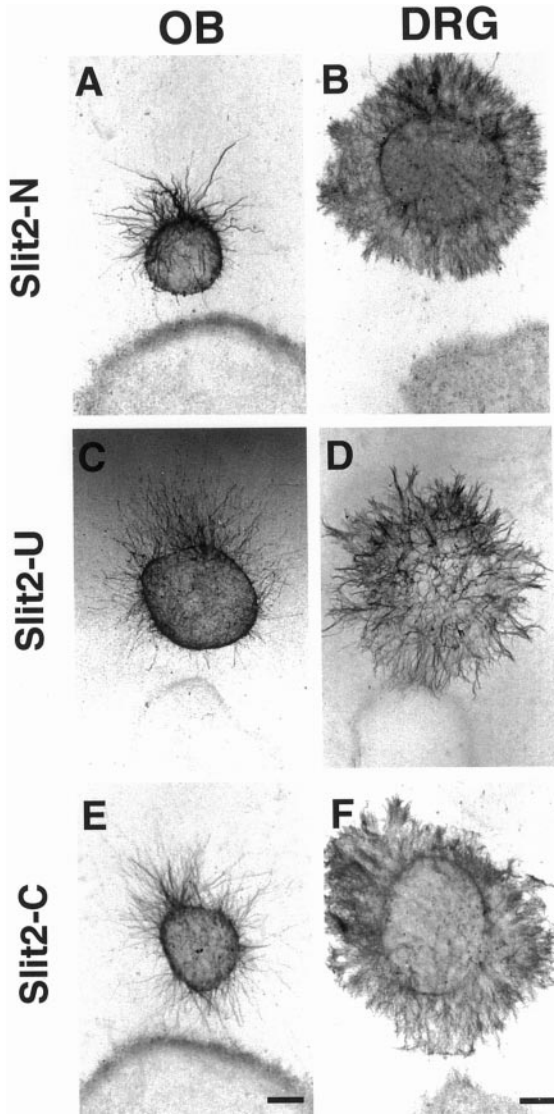

Figure 3. OB axons are repelled by Slit2 but not DRG axons. E15 rat OB and DRG explants were cocultured next to COS cells transfected with Slit2-N (*A*, *B*), Slit2-U (*C*, *D*), and Slit2-C (*E*, *F*) and stained with anti-β-tubulin antibody. OB axons confronted with Slit2-C grow symmetrically (*E*), whereas they grow away from COS cells expressing Slit2-N (*A*) or Slit2-U (*C*). In contrast, DRG axons grow radially when confronted with Slit2N (*B*), Slit2U (*D*), or Slit2C (*F*). Scale bars, 100 μm.

Second, we examined the ability of the proteins, applied acutely, to induce the collapse of growth cones of axons growing on a two-dimensional laminin-coated substrate ("collapse assay"). The results can best be understood by describing first the responses of OB axons in all the assays before turning to DRG axons. Nonetheless, to facilitate comparison, the progression of the Figures is different, with the responses of both OB and DRG axons shown in succession for each assay.

Olfactory bulb axons: requirement of N-terminal sequences for chemorepulsion, and dissociation of chemorepulsion and collapsing activities

In the diffusion assay, OB axons were repelled by cells expressing either Slit2-N or Slit2-U, whereas cells secreting Slit2-C had no effect (Fig. 3*A*,*C*,*E*, Table 1). Thus, the ability of Slit2 protein fragments to function as chemorepellents for these axons correlates with their ability to bind Robo receptors: both properties require amino-terminal sequences of Slit2.

Table 1. Semiquantitative evaluation of axonal outgrowth in different combinations of OB and DRG explants cocultured collagen gels

	Number of explants	Axonal outgrowth			
		+	=	−	−−
OB					
Slit2-N	22			2	20
Slit2-U	15			6	9
Slit2-C	23		21	2	
CTL	22		21	1	
DRG					
Slit2-N	14		14		
Slit2-U	16		16		
Slit2-C	12		12		
CTL	14		14		

Cocultures were classified as follows: +, moderate attraction of axons (axons are two- to threefold longer in the proximal than in the distal quadrant); =, radial axonal growth (axons in the proximal and distal quadrants differed by less than twofold in length); −, moderate axonal repulsion (axons are two- to threefold longer in the distal than in the proximal quadrant); −−, strong axonal repulsion (axons are more than threefold longer in the distal than in the proximal quadrant).

Because Slit2-N and Slit2-U function as diffusible chemorepellents, we expected that both would also induce growth cone collapse because it occurred with extracts containing both native full-length Slit2 and native Slit2-N (Nguyen Ba-Charvet et al., 1999). To our surprise, when comparing the proportion of OB growth cones that collapsed in response to membrane extracts of COS cells expressing recombinant Slit2-N or Slit2-U, we found that only Slit2-N-containing membranes induced collapse (Fig. 4*A*, *E*, Table 2). This result was confirmed by testing purified Slit2-N and Slit2-U and showing that up to 60% of growth cones collapsed in response to Slit2-N (0.5–10 μg/ml), whereas no significant collapse was observed in response to Slit2-U (Fig. 4*B*,*E*, Table 3). Slit2-C could be presented only as pure protein because the low quantity that normally binds to membranes is detached during the preparation of the membrane vesicles (Nguyen Ba-Charvet et al., 1999). We found that purified Slit2-C protein, did not induce OB growth cone collapse (Fig. 4*B*,*E*, Table 3). These results reveal a clear dissociation between the ability of proteins to function as diffusible chemorepellents and their ability to induce growth cone collapse.

Sensory axon responses of Slit2 proteolytic fragments

Because we had found previously that Slit2-N has a positive action on the axons of NGF-responsive DRG axons, stimulating them to elongate and branch (Wang et al., 1999), we did not expect to see negative (repulsive) effects on these axons. Indeed, in the chemorepulsion assay, in which DRG explants cultured with NGF (to elicit radial axon outgrowth) were confronted with aggregates of control or transfected COS cells, we did not observe any repulsive effect of any of the Slit2 proteins (-N, -U, or -C proteins) (Fig. 3*B*,*D*,*F*, Table 1). Similarly, the growth cones of DRG axons grown on a two-dimensional laminin substrate did not show collapse in response either to membrane fragments from COS cells expressing wild-type Slit2 or to different Slit2 protein fragments (N or U), or to purified Slit2-N, Slit2-U, or Slit2-C tested over a range of concentrations (0.5–10 μg/ml) [Fig. 4*C*–*E*, Tables 4, 5 (and data not shown)] (see also Niclou et al., 2000).

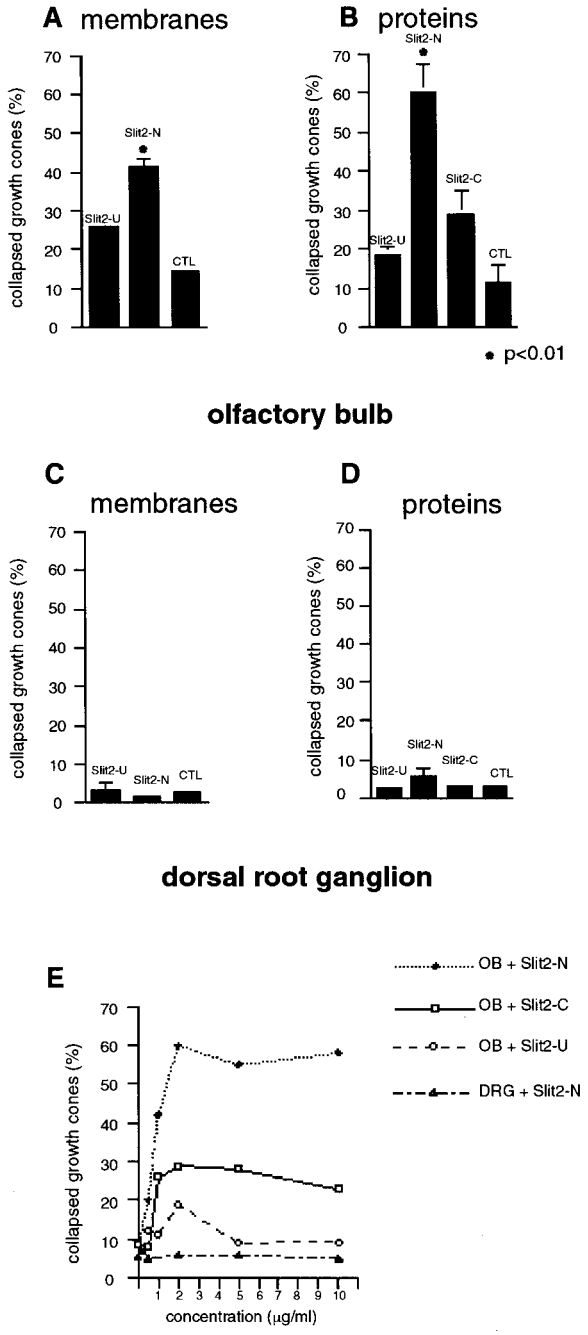

Figure 4. Slit2N collapses only OB axons. Collapse assay of E15 olfactory bulb (*A*, *B*) and DRG (*C*, *D*) growth cones. *A*, Histogram showing that Slit2-N-expressing membranes significantly induced growth cone collapse compared with control membranes or Slit2-U-expressing membranes. *B*, Slit2-N proteins (2 μg/ml) significantly induced growth cone collapse ($p = 0.003$) compared with control (*CTL*), Slit2-U, or Slit2-C. *C*, Neither of the different Slit2-fragment-expressing membranes significantly induces DRG growth cone collapse. *D*, The level of DRG growth cone collapse is insignificant with the different Slit2 fragments tested at 2 μg/ml. *E*, Dose–response curve of the collapsing activity of the various Slit2 fragments on OB and DRG growth cones.

As a positive control for the bioactivity of the Slit2 protein fragments, we examined the ability of recombinant Slit2-N to induce branching and extension of NGF-sensitive sensory axons in the dissociated cell DRG branching assay. Recombinant Slit2-N showed a similar activity in stimulating branching and extension as we reported previously for native Slit2-N purified from cells expressing wild-type full-length Slit2 (Fig. 5*A*) (cf. Wang et al., 1999). We had predicted previously that full-length Slit2 might be an antagonist of Slit2-N (Wang et al., 1999). The availability of purified Slit2-U enabled us to test this prediction. As expected, not only did Slit2-U show no activity in the elongation and branching assay, but it actually functioned as an antagonist of purified recombinant Slit2-N (Fig. 5*B*). Slit2-C did not present any activity in this assay, nor did it function as an antagonist (Fig. 5).

Table 2. Percentage of collapsed growth cone of OB axons with Slit2-containing membranes

Membranes	Collapsed growth cones (%)	*n*
Slit2-N	42 ± 9	328
Slit2-U	26 ± 4	276
CTL	15 ± 1	332

n, Total number of growth cones.

Table 3. Percentage of collapsed growth cone of OB axons with 2 μg/ml of purified Slit2 fragments

Proteins	Collapsed growth cones (%)	*n*
Slit2-N	60 ± 7	339
Slit2-U	19 ± 2	387
Slit2-C	29 ± 6	279
CTL	11 ± 5	440

n, Total number of growth cones.

Table 4. Percentage of collapsed growth cone of DRG axons with Slit2-containing membranes

Membranes	Collapsed growth cones (%)	*n*
Slit2-N	1.6 ± 1	454
Slit2-U	3.5 ± 2	390
CTL	2.8 ± 1	621

n, Total number of growth cones.

Table 5. Percentage of collapsed growth cone of DRG axons with 2 μg/ml of purified Slit2 fragments

Proteins	Collapsed growth cones (%)	*n*
Slit2-N	5.8 ± 2	289
Slit2-U	2.7 ± 1	233
Slit2-C	2.8 ± 1	301
CTL	3 ± 1	376

n, Total number of growth cones.

DISCUSSION

Cleavage of Slit2 proteins

Slit2 is known to be cleaved *in vitro* and *in vivo* into two fragments, Slit2-N and Slit2-C (Brose et al., 1999; Wang et al., 1999), although the cleavage appears only partial because full-length Slit2 is also detected both *in vitro* and in brain extracts (Brose et al., 1999; Hu, 1999; Niclou et al., 2000). We found that transient

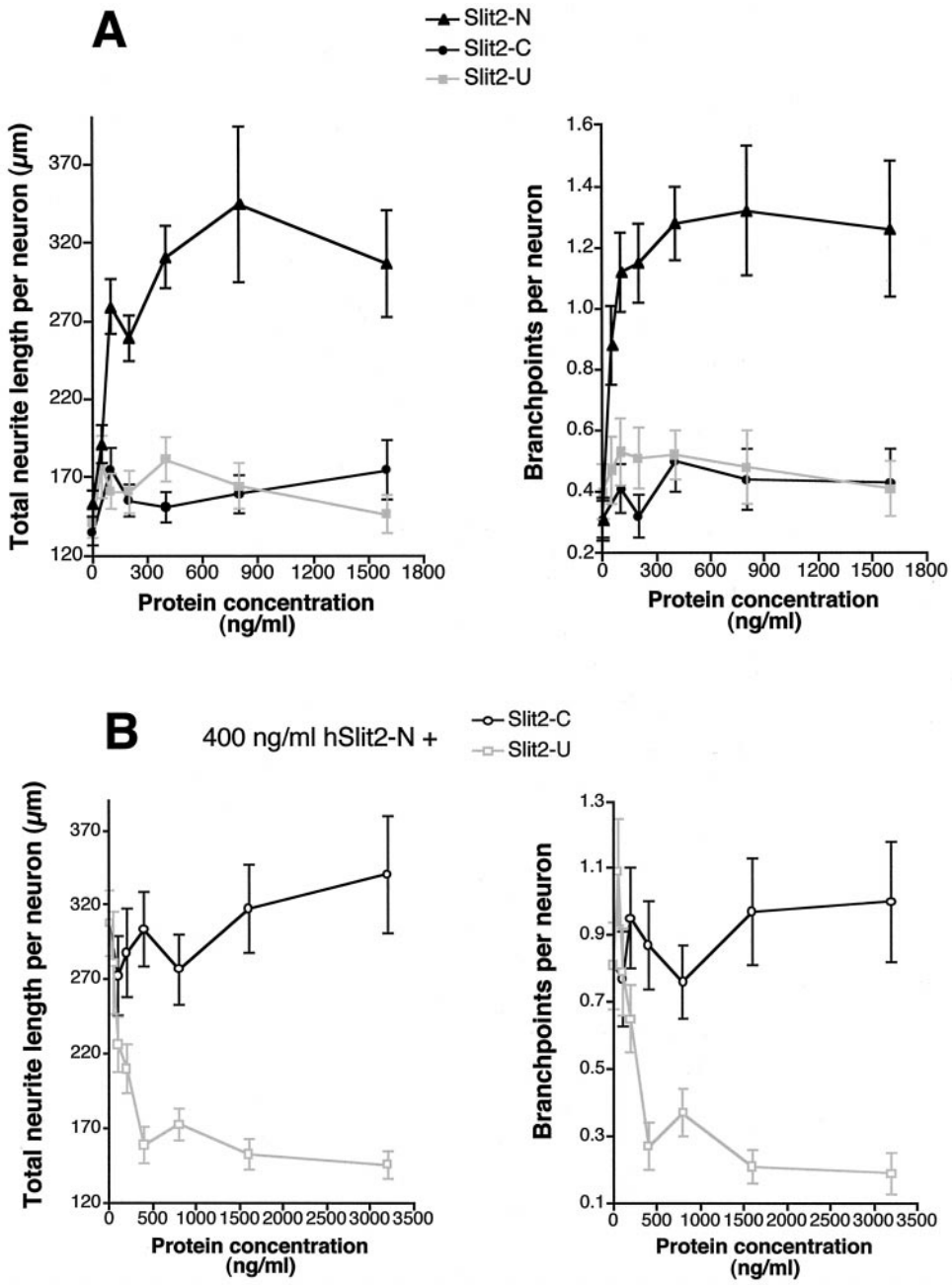

Figure 5. Branching and elongation activities of different Slit2 protein fragments. *A*, The total neurite length (*left panel*) or the branchpoint number (*right panel*) per neuron (mean ± SE; $n > 33$) is plotted against the concentration of different Slit2 protein fragments used in the E14.5 DRG culture. Slit2-N (▲), Slit2-C (●), and Slit2-U (■). *B*, Slit2-U, but not Slit2-C, could block the activity of Slit2-N. The total neurite length or the branchpoint number is plotted against the concentration of either Slit2-U (□) or Slit2-C (○), which was added to the DRG culture stimulated by 400 ng/ml Slit2-N.

transfection of COS cells with an expression construct bearing a deletion of nine amino acids immediately upstream of Thr 1118 resulted in the absence of cleavage in COS cells and the expression of a single protein (Slit2-U) of 190 kDa. We have also exploited the identification of this putative cleavage site to generate recombinant N-terminal and C-terminal Slit2 fragments (ending or starting at Thr 1118, respectively). Recombinant Slit2-N might have a few additional amino acids at its C terminus compared with native Slit2-N if the true cleavage site is amino terminal to Thr 1118; even if this is the case, however, the functional analysis suggests that recombinant Slit2-N and native Slit2-N have similar or identical properties. Similarly, although the deletion of nine amino acids in Slit2-U might in principle alter the functional properties of the full-length protein, the results of functional analysis suggest that its properties are similar or identical to those of native full-length Slit2 (see below).

The availability of Slit2-N, Slit2-C and Slit2-U has enabled us to extend previous studies by examining systematically the functional properties of different Slit2 cleavage products. In the following discussion, the terms Slit2-N and Slit2-C refer to the recombinant forms of the proteins.

Functions and pharmacology of Slit2-N and Slit2-U in repulsion and branching

Slit2-N and Slit2-U were found to have similar activities in repelling OB axons in the collagen gel repulsion assay. The finding that Slit2-U mediates repulsion is consistent with the reports that native full-length Slit2 repels migrating neurons from the subventricular zone (Hu, 1999) and retinal axons (Niclou et al., 2000). The fact that both Slit2-N and Slit2-U function in repulsion is interesting in light of our finding that both also bind to Robo1 and Robo2 with similar affinity (whereas Slit2-C does not). This

observation is consistent with the possibility that repulsion by these Slit proteins is mediated by Robo receptors, a possibility that has been suggested but not yet proven in vertebrates (Brose et al., 1999) by analogy with the fact that Robo is a repulsive receptor for Slit in *Drosophila* (Kidd et al., 1999).

Although Slit2-N and Slit2-U both function in repulsion, we have found that only Slit2-N, not Slit2-U, causes collapse of OB growth cones. This finding was surprising, because it is generally expected that factors that can repel also cause collapse. It also provides the first clear demonstration of a dissociation between the two types of activity. This dissociation may be cell type specific, because Niclou et al. (2000) showed that native full-length Slit2 has both activities for retinal axons in culture. These results cannot be explained by an inability of DRG axons to collapse, because several molecules, including collapsin-1/Sema3A, can collapse these growth cones (Niclou et al., 2000).

Similarly, Slit2-U and Slit2-N have divergent actions in the sensory neuron branching assay. As observed for the native proteins (Wang et al., 1999), we found that recombinant Slit2-N but not Slit2-U stimulates elongation and branching of DRG axons in this assay (and, as expected, neither protein can repel or cause collapse of DRG growth cones). Furthermore, we found that Slit2-U functions as an antagonist of Slit2-N, which is coherent with the observation that native Slit2-N was active only when purified away from full-length Slit2 (Wang et al., 1999).

These results indicate some difference in the receptor mechanisms mediating repulsion and branching. There is no information currently regarding the receptor(s) that mediates branching. However, it is worth noting that DRG and OB neurons express mRNA for Robo2 at the relevant developmental stages, suggesting but not proving the involvement of this receptor in both branching and repulsion.

Does Slit2-C have a role in axon guidance?

There are several well characterized examples of proteins with cleavage that is important to activation but in which only one of the products has bioactivity. For example, hedgehog (Hh) proteins are cleaved into two fragments by autoproteolysis. The remaining C-terminal fragment does not appear to possess any bioactivity of its own, and cleavage is essential because an uncleavable Hh does not possess bioactivity (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1996). Semaphorins provide another example, because class 3 Semaphorins have been reported to be synthesized as inactive proproteins that become chemorepulsive only when their C-terminal end is cleaved (Adams et al., 1997). A parallel may be drawn between such cleavages and Slit2 proteolysis, which is essential to generate Slit2-N, a fragment with branching and collapsing activities.

Although the sole purpose of the cleavage could be to generate bioactive Slit2-N, there are nonetheless reasons for thinking that Slit2-C, on the basis of its structure, might also be bioactive. Slit2-C possesses several EGF-like repeats, protein motifs implicated in cell signaling. It also possesses a domain called ALPS, LNS, or LG-like module (based on the name of the related domain in laminin) (Rothberg and Artavanis-Tsakonas, 1992; Hohenester et al., 1999; Rudenko et al., 1999); this motif is found in the names of the proteins from which these acronyms were derived, and in some of these proteins it has been shown to bind the extracellular proteins neuroligin and dystroglycan (Hohenester et al., 1999; Rudenko et al., 1999).

By analogy, one might expect the Slit2-C moiety to possess a signaling function as well, either in isolation or within the context of the full-length protein. However, in collagen gel we could not observe any effects of Slit2C on DRG, OB, or motor axons (our unpublished observation), although the latter are also repelled by Slit2N and full-length Slit2 (Brose et al., 1999) (data not shown). Nevertheless, we have obtained preliminary evidence indicating that COS cells secreting Slit2-C enhance the outgrowth of motor axons in a collagen/matrigel mixture (our unpublished observation). We have also been able to show that substrate-bound Slit2-C can guide DRG and OB axons in the stripe assay (K. Nguyen Ba-Charvet, unpublished observations). At present, the functional relevance of these effects is unclear, and future studies will aim to determine the *in vivo* role of Slit2-C and to establish whether the LNS domain of Slit2-C is subject to alternative splicing that modulates its activity. The identity of a receptor mediating a possible function of Slit2-C is also unclear because Slit2-C is unable to bind Robo receptors. However, it had been shown that in the brain, Slit-2 is a ligand for the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan glypican-1 (Liang et al., 1999). More recently, it was determined that Slit2 binding to glypican-1 is mediated by the C-terminal portion of Slit2 and most likely by the ALPS domain (Ronca et al., 2000). Moreover, in the rat embryo, glypican-1 is highly expressed in several types of neurons, including motor neurons (Karthikeyan et al., 1994), suggesting that Slit2-C could influence growing axons via this proteoglycan.

Possible function of Slit2 proteolytic fragments *in vivo*

During embryonic development, growth cones often pause and reorient (Godement et al., 1994; Halloran and Kalil, 1994). These changes of direction have been associated with modifications of the expression of some receptors at the surface of the growth cone (Dodd et al., 1988; Kidd et al., 1998) and with abrupt switches of the expression of some axon guidance molecules in the microenvironment of the growth cone (Kolodkin et al., 1992; Nguyen Ba-Charvet et al., 1998; Stoeckli and Landmesser, 1998). Moreover, extracellular proteases, which are known to be important for axonal elongation (Monard, 1988; Seeds et al., 1997), could also be involved in this process by regulating receptor conformation and extracellular matrix composition locally (Galko and Tessier-Lavigne, 2000; Hattori et al., 2000). The identity of the protease involved in Slit2 cleavage is still unknown, but it is likely that one function of this cleavage could be to modify Slit2 function. In the case of DRG axons, Slit2-U has no activity on branching whereas Slit2-N stimulates it. Therefore the activation of Slit2 cleavage locally, for instance on arrival in their target territory in the spinal cord, could stimulate branching. In addition, Slit2-U can antagonize Slit2-N branching activity, which further suggests that the regulation of Slit2 cleavage could regulate *in vivo* axonal elongation and pathfinding. It will be important to determine the mechanism of Slit2 proteolysis and to study *in situ* the expression of Slit2-U and Slit2-N proteins, to understand more precisely Slit2 function *in vivo*, and a possible correlation with orientation choices made by growth cones.

Note added in proof. While this paper was being revised, another paper has appeared (Chen et al., 2001) that showed that the leucine-rich repeats of Slit are sufficient to mediate Slit's repulsive effects.

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