Repellent Signaling by Slit Requires the Leucine-Rich Repeats

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Slit is a repellent axon guidance cue produced by the midline glia in *Drosophila* that is required to regulate the formation of contralateral projections and the lateral position of longitudinal tracts. Four sequence motifs comprise the structure of Slit: a leucine-rich repeat (LRR), epidermal growth factor-like (EGF) repeats, a laminin-like globular (G)-domain, and a cysteine domain. Here we demonstrate that the LRR is required for repellent signaling and *in vitro* binding to Robo. Repellent signaling by *slit* is reduced by point mutations that encode single amino acid changes in the LRR domain. By contrast to the EGF

or G-domains, the LRR domain is required in transgenes to affect axon guidance. Finally, we show that the midline repellent receptor, Robo, binds Slit proteins with internal deletions that also retain repellent activity. However, Robo does not bind Slit protein missing the LRR. Taken together, our data demonstrate that Robo binding and repellent signaling by Slit require the LRR region.

Key words: axon guidance; *Drosophila*; midline glia; Slit; repulsion; Roundabout

Growing axons navigate through the developing nervous system by integrating varied adhesive, attractive, and repellent signals, delivered by cell contact or diffusion through the extracellular matrix (ECM) (Tessier-Lavigne and Goodman, 1996). The midline of the CNS is an important source of guidance signals. Most interneurons cross once and avoid the midline thereafter. At least two signals regulate axon guidance at the midline of all nervous systems examined to date. The Netrin pathway communicates an attractive cue from midline cells to enable midline axon crossing (Kennedy et al., 1994). The Slit pathway communicates a repellent cue from midline cells to restrict axon crossing (Battye et al., 1999; Kidd et al., 1999; Yuan et al., 1999; Zhou et al., 2000).

Midline guidance signals Netrin and Slit are both produced by the midline glia (MG) in *Drosophila* (for review, see Jacobs, 2000). Slit, a secreted glycoprotein, is found on the surface of the MG and at low levels on the surface of all CNS axons (Rothberg et al., 1988; Rothberg et al., 1990; Kidd et al., 1999). Slit binds to three Roundabout receptors (Robo, Robo2, and Robo3) on axons and growth cones to inhibit filopodial extension and growth toward the midline and to specify the lateral position of axons in the longitudinal tracts (Brose et al., 1999; Li et al., 1999; Murray and Whittington, 1999; Rajagopalan et al., 2000; Simpson et al., 2000a,b).

The functions of the structural domains of Slit are unknown. Four leucine-rich repeats (LRRs) comprise most of the protein. This motif is found in matrix proteins that bind to collagen or laminin (Matsushima et al., 2000), in transmembrane receptors, and in cell surface adhesion molecules (Gay et al., 1991; Nose et al., 1992; Yamagata et al., 1994). LRRs are thought to be required

for protein recognition and adhesion events (Hocking et al., 1998).

Drosophila Slit also contains seven EGF repeats, similar in sequence to the Notch EGF repeats. These repeats also are implicated in protein recognition and ligand binding events (Lieber et al., 1992). The C-terminal region of Slit contains a laminin-like globular (G)-domain, also termed an ALPS (for agrin, laminin, perlecan, slit) domain (Rothberg and Artavanis-Tsakonas, 1992). The agrin G-domain is required for nerve-to-muscle signaling that triggers formation of the neuromuscular synapse (Cornish et al., 1999).

Slit can be proteolytically cleaved. The amino terminal fragment enhances sensory axon branching *in vitro* (Wang et al., 1999). Repellent activities of either fragment have not been determined.

Our sequence analysis of seven mutations indicates that single amino acid changes in the LRR regions and terminations of translation in the EGF repeats reduce repellent signaling by *slit*. We have also generated three *slit* transgenes, with different internal deletions, to determine which structural domains are required for repellent signaling. An intact LRR domain is required in transgenes to restore midline guidance in *slit* mutants and for Slit binding to the Robo receptor *in vitro*. Expression of transgenes lacking the EGF or G-domain restore *slit* function and encode proteins that bind Robo. These data indicate that the LRR domain is required for repellent signaling by Slit.

MATERIALS AND METHODS

Stocks. Most *slit* and *robo* alleles were isolated on a background deficient for fasciclin III and fasciclin I (Seeger et al., 1993). *slit*² was isolated by Nüsslein-Volhard et al. (1984), *slit*^{E158} was isolated by A. Kolodkin (Johns Hopkins University), and *slit*^{F81} and *slit*^{F119} were isolated by H. Bellen (Rothberg et al., 1990). *robo*¹ was provided by G. Tear (Seeger et al., 1993). All stocks have been restored to a common wild-type background. *Df(2R)WVG* was obtained from the Indiana stock center. Transgene expression was regulated by *P[slit1.0-GAL4]* (Christian Klämbt, Münster), *P[en-GAL4]* (Orihara et al., 1999), and *P[elavC155-GAL4]* (Lin and Goodman, 1994).

Slit antibody generation. Slit hybridoma 6D.4 was donated by S. Artavanis-Tsakonas (Rothberg et al., 1988) and reestablished in RPMI Medium (Life Technologies-BRL, Gaithersburg, MD) supplemented

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with 10% fetal calf serum (Life Technologies-BRL) and penicillin streptomycin (Life Technologies-BRL). Medium from subcloned mAb 6D.4 was purified on protein-G Sepharose 4 beads (Pharmacia Biotechnology, Arlington Heights, IL). This antibody binds to a region of *in vitro* translated Slit between EGF-2 and EGF-6.

Immunocytochemistry. *Drosophila* embryo collection, fixation, and immunocytochemistry were adapted from Patel (1994). BP102, mAb 1D4, mAb Slit, mAb Robo, [provided by N. Patel (University of Chicago), C. Goodman (University of California at Berkeley), and G. Tear (King's College), respectively], and anti- β -galactosidase (gal) (Cappel, West Chester, PA) were diluted in PBS containing 0.5% Triton X-100. Incubation in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was followed by incubation in Vector Laboratories Elite ABC. The first label reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Life Technologies-BRL) in the presence of 0.03% nickel/cobalt chloride. Second or single labels were reduced with DAB. Nerve cords were dissected or embryos were mounted whole and visualized on a Zeiss Axiophot microscope. Figures were prepared from scanned slides with Adobe Photoshop.

Viability. Viability of each *slit* mutant was assessed in a *yw* background, using a *CyO[y⁺]* balancer as a late embryonic and larval marker. Embryonic lethality monitored 1000 selected embryos, plated on apple juice agar at 22°C. After 48 hr, unhatched embryos were dissected, and counts were taken from infertile, balanced, and mutant embryos lacking pigmented mouth hooks. Larvae were selected from the same collections, based on absence of the *CyO[y⁺]* balancer. Larvae were aged at 22°C and observed until pupation or death.

Nerve cord length. BP102-labeled homozygous *slit* mutants embryos were collected on the basis of *lacZ* balancer staining and measured under camera Lucida at 25 $\times$ projection.

Fluorescence microscopy. Manually devitelinized embryos were dissected on glass in PBS and fixed for 10 min in 4% paraformaldehyde. The dissections were washed in buffer and incubated for 30 min in rhodamine-labeled phalloidin (Verheyen and Cooley, 1994) (Molecular Probes R415) and mounted in glycerol with *p*-phenylene *D*-amine (Sigma, St. Louis, MO) as an anti-bleaching agent. Projections made from confocal images were collected with a Zeiss 310 microscope.

Genomic analysis. Several cosmids from region 52D were screened by PCR for 5' and 3' domains of the *slit* DNA (5' for 5'-ACTCGAGCGA-CTGGACATCT-3', 5' rev 5'-GTCGTGCGAAAGCTGGAGAAC-3', 3' for 5'-GCACAGCAGGCATACAAGAA-3', and 3' rev 5'-AGCAATTGGGTAGTCGCATC-3'). Three genomic clones (EDGP cosmids 60E2, 11G7E, and 11G10) that contained both 5' and 3' *slit* cDNA were isolated and sequenced and confirmed the revised cDNA sequence (AF126540). PCR primer pairs were designed to flank each of the 19 *slit* exons and tested using cosmid and wild-type genomic DNA and a stock deficient for *slit*, (*Df(2R)WMG*). Mutant genomic DNA was obtained from single homozygous embryos or larva not carrying a *CyO[y⁺]* balancer in a *yw*⁻ genetic background. Individuals were washed in sterile ddH₂O and transferred to a PCR tube containing 83 μ l of PCR-buffered sterile ddH₂O in which they were crushed, and then the remaining PCR reagents were added. Pure single-banded products were purified (Qiagen 28104; Qiagen, Hilden, Germany) for each individual sample and sequenced. At least three independently sequenced products were generated for each primer pair and genetically mutant allele. Sequencing revealed 18 silent polymorphisms and an Ile/Asn (ATC/AAC) dimorphism at nucleotide 2660.

***slit* cDNA.** A new *slit* cDNA was constructed on the basis of fragments of clones provided by Rothberg et al. (1988) and PCR-generated bridges that were based on genomic cosmids (11G10 and 60E2). Primers were used that overlaid known restriction sites and in addition generated a 5' *HindIII* (5'-CATAAAGCTTCCACAATGGCCGCGCC-3') and a 3' *SpeI* (5'-CATCGTGCAGCAAGTGCAGGAAGTACTAGTAACAA-3') restriction site for subcloning into Stratagene pBS KSII+. All products were completely sequenced and verified against the amended *slit* cDNA and genomic sequence.

Transgenic deletion constructs. Three transgenic constructs were created that deleted specific domains of the *slit* cDNA shown in Figure 4. From a complete *slit* construct [*HindIII* (-8 bp) to *SpeI* (4461 bp)], we generated *slit* Δ L1-L4 [Bpu1102I (134) to *Bst*EII(2539) treated with mung bean nuclease (MBN; Life Technologies-BRL)], *slit* Δ E2-E6 [NsiI (2913) to MunI (3463) treated with MBN], and *slit* Δ G-E7 [SspBI (3733) to *SfiI* (4231) treated with T4 DNA polymerase]. Deletion constructs engineered with a C-terminal HA epitope tag were subcloned into

P[UAST], and transformant lines were generated and mapped by standard procedures. The epitope tag was weakly immunodetectable *in vivo*.

Midline rescue and overexpression. Transformant lines generated from the *slit* deletion constructs were crossed into a *slit*² background and mated with *P[slit1.0-GAL4];slit²/CyO[lacZ]* flies. Embryos were assayed by staining with mAbs BP102 and 6D.4 and then selected by screening of the marked balancer using α - β -gal. Overexpression was produced by crosses generating *P[slit1.0-GAL4];P[UAS-slit]* and similarly using *P[en-GAL4]* and *P[elavC155-GAL4]* lines. Embryos were immunolabeled with mAb BP102, and *slit* overexpression was monitored in embryos expressing transgenes with the Slit epitope to mAb 6D.4 or with antibody to the HA epitope.

In vitro binding. To determine properties of Slit and Robo binding *in vitro*, 20 μ l of biotin-translated protein from each of the *slit* constructs (Biotin *In Vitro* translation kit, Roche) was incubated with 20 μ l of unlabeled Robo, rotated at 4°C for 2 hr. After incubation, 25 μ l of avidin Agarose (Roche) was added to each tube and incubated for 15–20 hr rotating at 4°C. After washing, the biotin bound to the avidin complex was eluted by rotating the Agarose with 50 μ l of elution buffer (2 mM D-Biotin-B-Hydroxysuccinimidester (Roche), 0.1 M PB, 0.15 M NaCl, pH 7.2) for 1 hr after which 10 μ l of 5 $\times$ sample buffer was added, and each mixture was heated to 80°C for 5 min. For each construct, 12 μ l of supernatant was loaded onto each of three 4% gels for SDS-PAGE alongside a wide range color maker and then blotted onto a polyvinylidene difluoride membrane. Each membrane was washed and blocked before being separated for incubation with mAb Slit, mAb Robo, or avidin-HRP. After washing, the Slit and Robo membranes were incubated with α M HRP, and then all membranes were washed and then reacted with 2 ml of ECL (Amersham Pharmacia Biotech) chemoluminescence for detection of HRP-bound primary antibody or biotinylated proteins on Kodak X-OMAT AR Film.

RESULTS

Midline axon guidance in embryos mutant for hypomorphic alleles of *slit*

To identify the structural requirements for repellent signaling by *slit*, we have examined the midline guidance phenotype of 13 alleles of *slit* generated by P-element insertions and ethylmethyl sulfonate (EMS) mutagenesis (Rothberg et al., 1990; Seeger et al., 1993). These alleles present a series of reduced midline repellent phenotypes, as assessed by the frequency of midline crossing of longitudinal tract axons labeled by fasciclin II. For illustrative purposes, we have grouped the alleles on the basis of midline guidance phenotype into phenotypic severe, moderate, and mild alleles (Table 1).

Wild-type late embryonic nerve cords have a bilateral set of three fasciclin II-expressing longitudinal fascicles that maintain a constant distance from the midline (Fig. 1A). Midline separation is maintained in embryos with a single copy of the *slit* gene (Fig. 1B). Complete midline fusion and a loss of all intersegmental longitudinal axons are seen in embryos homozygous for a deficiency uncovering *slit* (Fig. 1C). All of the mutant alleles that we examined generate *slit* transcript, assessed with a probe complementary to the G-domain. However, *slit* alleles 2, *GA20*, and *1912* generate no detectable Slit protein, using a monoclonal antibody raised to the EGF and G-domains of Slit (Rothberg et al., 1988). These severe mutants have a similar midline fusion of axons and retain few intersegmental axon projections (Fig. 1D,E, arrows). Nerve cords with fewer intersegmental axons had less nerve cord condensation (shortening) during development. The wild-type nerve cord was 73% of embryo length at stage 16, whereas a severe *slit* nerve cord was 90% of embryo length (Table 1).

The remaining five EMS and three insertion alleles produce immunodetectable protein. Three P-element insertion alleles (*slit*^{F81}, *slit*^{F119}, and *slit*^{E158}) label less intensely for mRNA than the EMS alleles (data not shown). These alleles generate less complete midline fusion of axons. In moderate alleles, all longitudinal

Table 1. *slit* phenotypes and *robo/slits* interactions

Allele	First larval instar: percentage of expected ^a	Latest observed stage	Nerve cord length	Slit protein detected	Midline guidance phenotype ^b	<i>robo</i> ¹ / <i>slit</i> : percentage of segments with crossovers ^c
<i>yw</i> [−]	98.0 (429)	Adult	0.73 ± 0.04	Y	Normal	0 (250)
EMS alleles						
<i>slit</i> ²	1.0 (978)	L1	0.88 ± 0.06	N	Severe	45 (227)
<i>slit</i> ^{GA20}	1.9 (956)	L1	0.90 ± 0.02	N	Severe	60 (219)
<i>slit</i> ^{GA178}	13 (914)	L1	0.91 ± 0.03	Y	Severe	61 (227)
<i>slit</i> ^{GA945}	2.3 (475)	L1	0.88 ± 0.02	N	Severe	46 (215)
<i>slit</i> ³¹⁴⁹	5.1 (473)	L2	0.94 ± 0.02	Y	Severe	63 (318)
<i>slit</i> ¹⁹¹²	7.4 (484)	Pupa	0.92 ± 0.02	N	Moderate	60 (231)
<i>slit</i> ⁵⁵⁰	0.5 (924)	L1	0.89 ± 0.03	Y	Moderate	48 (283)
<i>slit</i> ⁵³²	7.1 (971)	Adult	0.76 ± 0.05	Y	Moderate	46 (281)
<i>slit</i> ²⁹⁹⁰	2.0 (438)	Adult	0.85 ± 0.04	Y	Mild	45 (267)
P-element insertions						
<i>slit</i> ^{F81}	11 (970)	Adult	0.82 ± 0.06	Y	Mild	41 (232)
<i>slit</i> ^{F119}	17 (943)	Adult	0.80 ± 0.07	Y	Mild	37 (229)
<i>slit</i> ^{E158}	3.1 (953)	Pupa	0.82 ± 0.04	Y	Mild	35 (222)

^aTwenty-five percent of total embryos (*n*) were expected to be *slit* mutant; the percentage of *slit* mutant embryos hatching as L1 was recorded.

^bAlleles of *slit* were classified into three groups on the basis of their phenotype, as described in Results.

^cThe percentage of total segments scored (*n*) containing midline crossovers in embryos heterozygous for both *robo* and respective alleles of *slit*.

tracts wander close to the midline (Fig. 1*F,G*), whereas alleles with a mild midline guidance phenotype have midline deviations in the most medial fasciculus II fascicles and retain an intact lateral fascicle (Fig. 1*H,I*).

Very few severe or moderate *slit* mutant embryos hatch; however, up to 17% of embryos mutant for a mild allele of *slit* emerge as larvae (Table 1). These larvae are sluggish and uncoordinated and very slow to grow. Most are unable to feed and die in the first larval instar; a very low number pupate or produce adults (Table 1). Mutant larvae have a midline guidance phenotype comparable to the mild embryonic phenotype, characterized by repeated midline crossing of medial fascicles and an unaffected lateral fascicle (Fig. 1, compare *K* with *J*, wild type).

Our further characterization of *slit* mutations focuses on representative alleles for severe (*slit*²) (Fig. 2*A*) moderate (*slit*¹⁹¹²) (Fig. 2*B*), and mild (*slit*²⁹⁹⁰) (Fig. 2*C*) perturbation of midline guidance. Commissure morphology was assessed with BP102 antibody, which labels most CNS axons. Embryos mutant for severe *slit* alleles have complete medial collapse of the commissures (Fig. 2*A*; compare with wild type in Fig. 6*A*). Most axons in embryos mutant for a moderate allele approach the midline, but medial axons are not compressed into a medial fascicle (Fig. 2*B*). Separation of anterior and posterior commissures is seen in embryos mutant for a weak allele of *slit* (Fig. 2*C*).

Interaction with *robo*

slit has a dosage-dependent interaction with *robo* (Kidd et al., 1999). Embryos with a single copy of each gene make occasional midline guidance errors. If the midline guidance phenotype of *slit* hypomorphs results from reduced signaling to Robo, then we would expect a strong correlation between the severity of the allele of *slit* and the degree of genetic interaction with the null allele *robo*¹. We found that embryos doubly heterozygous for *robo*¹ and a mild allele of *slit* generally had fewer segments with midline guidance errors (Table 1). Nevertheless, more axons can deviate toward the midline in a *robo* heterozygote with a moderate or mild *slit* allele (Fig. 2*E,F*) than with a severe allele (Fig. 2*D*). A moderate allele with a strong interaction with *robo*¹, such

as *slit*¹⁹¹², may generate Slit protein, which binds to but is a poor activator of the Robo receptor.

Midline guidance of ventral muscles

slit function also contributes to the positioning of ventral muscle insertions (Battye et al., 1999; Kidd et al., 1999). Normally the ventral oblique muscles are repelled from the midline above the nervous system and insert into the ectoderm underneath the lateral edges of the nerve cord. We found a consistent relationship between the numbers of ventral oblique muscles remaining on the dorsal surface of the nervous system and the class of *slit* mutant allele. In embryos homozygous for the severe allele *slit*², an average of 6.8 muscle fibers per segment overlie the nerve cord (*n* = 11 segments). In embryos homozygous for a moderate allele, *slit*¹⁹¹², 2.9 fibers cross the midline (*n* = 9), whereas 0.6 (*n* = 10) were found in the hypomorph *slit*⁵³².

Coding changes in *slit* mutations

Nine alleles of *slit* characterized here were generated by EMS mutagenesis and likely represent point mutations in the *slit* locus. A sequence change in a region of the Slit protein required for repellent signaling should generate a strong midline guidance phenotype. We generated a map of the intron–exon boundaries of genomic *slit* based on PCR amplification of gDNA using primers generated from the cDNA sequence. Our map of 19 exons was in agreement with the subsequently released genomic sequence from the Berkeley Drosophila Genome Project (AC005556).

We have sequenced each exon of each EMS allele of *slit* amplified from gDNA. We identified 19 consistent polymorphisms, one of which results in an alternate amino acid (see Materials and Methods). Allele-specific coding changes were identified for seven EMS alleles (Fig. 3). Four coding changes were identified in the leucine-rich regions and three in the EGF domain. Severe and moderate mutations include a premature stop codon in the last LRR (*slit*^{GA20}) and amino acid changes in the first and second LRR (*slit*^{GA178}, *GA945*, *532*). Three premature stops in the EGF repeats resulted in either severe (*slit*^{2,1912}) or

Figure 1. Midline guidance phenotypes of *slit* nulls and hypomorphs. In wild-type embryos (*A*), Fasciclin II labels a bilateral set of three fascicles that run symmetrically along the length of the nerve cord but do not cross the midline or each other. A nerve cord heterozygous for *slit*² (*B*) has slight deviations in each longitudinal fascicle, but individual fascicles remain separated. Fasciclin II labeling axons fuse at the midline in embryos deficient for *slit* (*C*) [*Df(2R) WMG*]. *Slit* alleles with severe (*D*, *E*), moderate (*F*, *G*), and mild (*H*, *I*) phenotypes are shown. Alleles that do not make immunodetectable Slit, *slit*² (*D*), and *slit*^{GA945} (*E*) cause a complete fusion of longitudinal axons at the midline. Embryos of moderate alleles of *slit* that generate detectable protein have longitudinal fascicles that crisscross the midline (*arrowhead*) and have thicker intersegmental connections [*slit*³¹⁴⁹ (*F*), *slit*¹⁹¹² (*G*)]. Longitudinal axons in the moderate phenotypes still regularly fuse with adjacent fascicles. *Slit* alleles that produced mild phenotypes included *slit*⁵⁵⁰ (*H*) and *slit*^{F119} (*I*). Mild *slit* phenotypes have a larger separation between axon fascicles, and contralateral projections across the midline are restricted to the two most medial fascicles. Anterior is at *top* in these frontal views of stage 16 nerve cords. Numerous Fasciclin II-labeling longitudinal fascicles are found in a wild-type third instar larval nerve cord (*J*). A second instar-

Figure 2. Commissure structure and the genetic interaction of *slit* alleles with *robo*¹. Embryos homozygous for a severe allele of *slit* (*slit*²) lack all commissure structure (*A*, *arrow*). For comparison, a wild-type axon tract scaffold labeled with BP102 has a ladder-like morphology (Fig. 5*A*). Less midline compression of axons and more intersegmental axon projections are seen with moderate [*slit*¹⁹¹² (*B*)] and mild [*slit*²⁹⁹⁰ (*C*)] alleles. Separation of commissures (*arrow*) seen in mild *slit* mutants is accompanied by greater widening of the ventral nerve cord, resembling a *robo*-like phenotype. The phenotypic interaction with *robo*¹ is contrasted with the same alleles. In embryos heterozygous for *robo*¹ and *slit*², deviations are observed in the most medial longitudinal fascicles within a subset of segments (*D*, *arrowhead*). The remaining fascicles are typical of embryos heterozygous for either *slit*² or *robo*¹. The interactions between *robo*¹ and moderate *slit* alleles [*slit*¹⁹¹² (*E*)] have prominent crossovers (*arrowhead*). More lateral fascicles are less organized (*arrow*). A similar pattern is seen in the *slit*²⁹⁹⁰/*robo*¹ transheterozygotes (*F*), including the fusion of lateral fascicles (*arrow*).

moderate (*slit*⁵⁵⁰) phenotypes. No sequence changes were revealed in two alleles of *slit* (*slit*³¹⁴⁹, *2990*), both of which make immunodetectable protein. Noncoding changes may have altered levels of transcription or the regulation of splicing.

Structural requirements for repellent signaling by *slit*

Characterized *slit* mutations alter the sequence of the LRR, truncate the protein, or in the instance of insertional alleles, lower the levels of immunodetected protein (Rothberg et al., 1990). These data do not reveal a contribution to repellent signaling by the EGF, G-, or cysteine domains of Slit. To explore the function of these domains, we sought to identify the repellent signaling potential of *slit* transgenes that encode forms of Slit with internal deletions.

Drosophila were transformed with three *slit* transgenes with different internal deletions (Fig. 4), regulated with the binary GAL4-UAS system (Brand and Perrimon, 1993). Using the *slit* promoter as the GAL4 driver in a homozygous *slit*² mutant background, we assessed the potential of each transgene to rescue the midline axon guidance phenotype. A complete *slit* transgene partially rescues a *slit* mutant, restoring commissural separation,

*slit*⁵³² hypomorph of the same age has disorganized medial fascicles that re-cross the midline (*K*, *arrowhead*). The most lateral tract is normal (*K*, *arrow*). In this and successive Figures, *arrows* identify intersegmental segments of the longitudinal fascicles, and *arrowheads* indicate midline guidance errors.

Figure 3. Coding changes of seven *slit* alleles. Unique changes in the nucleotide and amino acid sequence of *slit* alleles (right) are mapped to the structural domain of the Slit protein (left) following the protein motif conventions introduced by Rothberg et al. (1990).

although some interruptions in the intersegmental connections persist (Fig. 5B).

Nearly all of the LRR of *slit* was deleted in *Slit*[Δ L1–L4]. This transgene had no effect on the midline axon fusion phenotype of *slit*² (Fig. 5C). Transgenes with smaller internal deletions in the LRR generated identical results (data not shown). In contrast, removal of the first six EGF repeats (Fig. 5D) or the G-domain and seventh EGF repeat (Fig. 5E) resulted in restoration of the midline guidance phenotype. This pattern was consistent for three or more independent insertions of each rescue transposon. The deletion construct removing the EGF motifs was not detectable with Slit antibody; however, all transgene products were detectable, at similar levels of immunolabeling intensity, with anti-HA antibody (data not shown). Viability was not restored in any of our “rescue” experiments, suggesting that *slit* expression elsewhere in the embryo is required for survival.

Ectopic expression of truncated *slit* transgenes

Misexpression of P[UAS-*slit*] in embryos disrupts axon tract establishment (Battye et al., 1999; Kidd et al., 1999). To determine

Figure 4. *slit* transgenes with internal deletions. The regions of *slit* deleted in three transgenes are lightly shaded. Letters at right indicate repellent activity. R, Repellent; NE, no effect.

Figure 5. Restoration of *slit* function using *slit* transgenes with internal deletions. Possible restoration of axon tract architecture was assessed in *Drosophila* mutant for *slit*², also carrying UAS-*slit* constructs (Fig. 4) expressed under *slit1.0-GAL4* regulation. Wild-type expression of Slit in the ventral nerve cord (black) is shown in a BP102-labeled nerve cord (A). The expression of P[UAS-*slit* complete] was sufficient to partially rescue the *slit*² phenotype, demonstrated by greater separation of axons from the midline (B, arrowhead), although intersegmental connections are not restored (B, arrow). A *slit* transgene lacking the leucine domains of *slit* (C) [P[UAS-*slit*ΔL1–L4]] did not rescue the *slit*² phenotype. Midline fusion was not suppressed (arrowhead). A *slit* transgene lacking five EGF repeats, P[UAS-*slit*ΔE2–E6], was able to restore the midline scaffold to near normal conditions, including significant restoration of longitudinal tracts (D). Removal of only the G-domain and final EGF, P[UAS-*slit*ΔG–E7] (E), rescued the *slit*² phenotype to a degree similar to *slit* lacking five EGF repeats (P[UAS-*slit*ΔE2–E6]) (D).

which forms of Slit with internal deletions retain the capacity to disrupt axon tract establishment, we misexpressed the *slit* transgenes in a wild-type pattern (with P[*slit1.0-GAL4*]), in some neurons and the ectoderm at the segment boundary (with P[*engrailed-GAL4*]), and in all neurons (with P[*elav-GAL4*]). The patterns of misexpression are shown in Figure 6, A, F, and K, respectively.

Expression of full-length *slit* resulted in disruption of intersegmental connections and displacement of axon tracts away from the source of Slit (Fig. 6B,G,L). Expression of *slit* lacking the LRR domain had no effect on axon tract organization (Fig. 6C,H,M). However, expression of *slit* lacking the EGF motifs (Fig. 6D,I,N) or lacking the G-domain (Fig. 6E,J,O) resulted in significant disruption of axon tract organization. Longitudinal tracts had numerous breaks and lacked uniform bundling. Commissures were lacking when transgenes were expressed in the midline (Fig. 6D,E). Axon tract perturbations were similar for all functional transgenes when expressed in all neurons (Fig. 6L,N,O).

Figure 6. Ectopic expression of *slit* transgenes with internal deletions. *slit* transgenes (Fig. 4) were expressed in three different patterns using specific P[GAL4] drivers in a wild-type background. P[*slit1.0-GAL4*] (A–E) drives expression in the MG, P[*eng-GAL4*] (F–J) drives ectopic expression at the segmental boundary, and P[*elav-GAL4*] (K–O) directs expression in all neurons. The pattern of ectopic expression is shown in A, F, and K, where each P[GAL4] driver was crossed to P[UAS-*tau-LacZ*] and embryos were labeled with BP102 (black) and α - β -gal (brown). In the remaining panels, BP102 (brown) and Slit immunolabeling (black) are visualized. Expression of the complete *slit* cDNA (B, G, L) disrupted both longitudinal (arrows) and commissural tracts. Some axons misproject laterally (B, asterisk). Ectopic expression of P[UAS-*slit* complete] in all neurons (L) displaced axon tracts toward the midline. Expression of a *slit* transgene that lacks the leucine domains (C, H, M) did not significantly alter axon tract organization. Expression of a *slit* transgene lacking EGF repeats 2 through 6 (P[UAS-*slit* Δ E2-E6]) significantly altered axon tract organization (D, I, N). Intersegmental expression of P[UAS-*slit* Δ E2-E6] resulted in a displacement of axons toward the midline (I, arrowhead) and breaks in the longitudinal tracts (arrow). Expression of P[UAS-*slit* Δ E2-E6] in all neurons also eliminates most intersegmental axons (N, arrow). This construct was not detected by Slit antibody, and only native Slit labeling is seen. Expression of a *slit* transgene lacking the G-domain and the seventh EGF repeat (E, J, O) results in effects similar to expression of the entire construct. Intersegmental expression of P[UAS-*slit* Δ G-E7] resulted in a number of breaks in longitudinal fascicles (J, arrow) and poorly defined anterior and posterior commissures (J, arrowhead). Expression of P[UAS-*slit* Δ G-E7] in all neurons (O) has displaced axons toward the midline (arrowhead).

Structural requirements for Robo binding

Sequence data from *slit* mutants and the domain requirements for transgene rescue of *slit* mutations both suggest that repellent signaling requires the LRR. If repellent signals are transduced by Robo receptors, then we would anticipate that the LRR is re-

quired to bind to Robo. We translated the *slit* transgenes *in vitro*, incorporating a biotin label, and then bound them to an avidin Agarose column. The column was then incubated with *in vitro* translated *robo* (vector provided by K. Bland and C. Goodman). All *slit* transgenes generated proteins of the predicted size, de-

Figure 7. *In vitro* binding of truncated Slit to Robo. *Slit* transgenes, both complete and containing internal deletions (Fig. 4), were *in vitro* translated incorporating biotin-labeled lysine and then bound to avidin Agarose columns. *In vitro* translated Robo incubated with each column, washed, and eluted with n-biotin. Thirty percent of the extraction from each column was immunoblotted as indicated. Avidin-HRP visualized with chemoluminescence shows that each of the Slit constructs bound and was then eluted from the avidin columns. The Slit antibody detects all constructs except those that delete the EGF2–EGF6 region, to which the antibody binds. The immunoblot for Robo indicates that Slit complete, SlitΔE2-E6, and SlitΔG are capable of binding Robo; however, Slit protein with internal deletions of any or all of the LRR fail to demonstrate any detectable *robo* antigen.

tected with either avidin-HRP or antibody to Slit. Robo was retained only with full-length Slit and Slit lacking either the first six EGF repeats or the G-domain and the last EGF repeat (Fig. 7).

DISCUSSION

We have combined an analysis of mutations of *slit* with a functional assay of *slit* transgenes to determine the structural requirements for repellent signaling by *slit*. Sequence changes that reduce repellent signaling either shorten the protein or modify the structure of the LRR. Similarly, *slit* transgenes containing a complete LRR restore axon repellent signals in *slit* mutants, whereas transgenes that lack a complete LRR generated no repellent signals. Finally, we demonstrated that *slit* transgenes that retain the re-

pellent function produce proteins that bind to Robo, and conversely, Robo did not bind truncated forms of Slit that lack repellent effects.

Dosage-dependent signaling by *slit* mutants

The 13 alleles of *slit* examined here cause a number of midline guidance errors in axons and ventral muscles. Muscle phenotypes range from midline crossovers of all ventral oblique muscles to midline crossing of a few muscle cells per embryo. Axon phenotypes vary from complete midline fusion of all CNS axons to midline crossings of only the most medial axons. Lateral axon tracts are relatively unaffected in mild EMS alleles and P-element insertion alleles of *slit*. All three P-element insertions have been mapped 10–100 bp upstream of the transcription initiation site and apparently reduce the level of Slit protein produced (Rothberg et al., 1990). It is possible that medial axons, being closer to the midline source of secreted Slit, have a higher threshold to respond to Slit and are thus more sensitive to reduced protein levels in mild alleles. Guidance and lateral positioning of the more lateral axon tracts are regulated by the Robo2 and Robo3 receptors, which may respond at a lower threshold to Slit (Rajagopalan et al., 2000; Simpson et al., 2000a).

Sequence changes in *slit* mutants

Three mutations that result in single amino acid changes map to the LRR domain. Point mutations in the β -sheet of LRR 1 and 2 (*slit*^{GA178}, *slit*^{GA945}) generate a severe phenotype. The sensitivity of *slit* phenotype to these conservative coding changes suggests a critical requirement of the LRR domain in repellent signaling. All other sequence changes result in truncated proteins with variable portions of the EGF domain preserved. Truncated proteins lacked the epitope recognized by our antibody; therefore, it was not possible to assess their stability or distribution.

Drosophila Slit is likely proteolytically cleaved at the beginning of the sixth EGF repeat (Brose et al., 1999). Slit synthesized in *slit*⁵⁵⁰ mutants has an incomplete sixth EGF repeat and an altered C-terminal sequence. If the altered C-terminal sequence does not destabilize the protein, we would anticipate that *slit*⁵⁵⁰ could act as a hypermorph, signaling in a manner comparable to the N-terminal portion of endogenously cleaved Slit. *slit*⁵⁵⁰ had the most variable penetrance of all the alleles examined. Nevertheless, the axon guidance phenotype, viability, and *robo* interaction suggest that *slit*⁵⁵⁰ is a hypomorph.

Although the G- and cysteine domains of *slit* represent one-fourth of the coding region, no sequence changes map to this region. It is possible that many point mutations in this region are not lethal and would not have been isolated by mutagenesis.

The leucine-rich repeat is required for repellent signaling

To learn more about the structural requirements for repellent signaling by Slit, we attempted rescue of *slit* mutants with midline expression of *slit* transgenes lacking internal sequences. A *slit* transgene lacking the LRR failed to restore midline guidance and failed to generate effects after ectopic expression. Furthermore, *in vitro* translated Slit, which lacks a full LRR, did not bind to Robo, the repellent receptor. Point mutations encoding single amino acid changes in the LRR also greatly reduced repellent signaling. These data indicate that the LRR of Slit is required for receptor binding and repellent signaling.

Slit is the first protein for which receptor binding and signaling have demonstrated a requirement for the LRR. The LRR defines a superfamily of proteoglycans of the ECM having tandem re-

peats of xxI/V/LxxxxF/P/LxxL/PxxLxxL/IxLxxNxI/L, where x is any amino acid (for review, see Matsushima et al., 2000). The best-characterized members are decorin and biglycan (for review, see Hocking et al., 1998). A *Drosophila* cell surface receptor (Toll), a GPI-linked proteoglycan (Connectin), and transmembrane Choptin also contain LRR (Rothberg and Artavanis-Tsakonas, 1992). LRR-containing proteins of the ECM are implicated in binding of collagen [fibromodulin, decorin, lumican, and biglycan (Hedbom and Heinegard, 1993), laminin (biglycan), and fibronectin [decorin and biglycan (Hocking et al., 1998)]. Biglycan, decorin, and fibromodulin bind TGF- β (Hildebrand et al., 1994) and may act as a tumor suppressor (Santra et al., 1997).

Fly LRR-containing proteins are involved in cell adhesion (Keith and Gay, 1990). Connectin is required for homophilic adhesion during motoneuron pathfinding and target recognition in *Drosophila* (Nose et al., 1997). Toll promotes homophilic adhesion (Keith and Gay, 1990) but also appears to inhibit formation of neuromuscular junctions on Toll-expressing muscle (Rose et al., 1997). Choptin is required for fasciculation of photoreceptor axons (Van Vactor et al., 1988). Repellent signaling by Toll and Connectin has been suggested and later discounted (Nose et al., 1994; Rose et al., 1997). The possibility of common functions of LRRs deserves reexamination.

Other structural domains of *slit*

It has been suggested that full-length Slit associates with the cell surface and that proteolytic cleavage at the start of the sixth EGF repeat generates two fragments (Brose et al., 1999). Three independent lines of evidence considered here identify a requirement for the LRR, in the N-terminal proteolytic fragment, for Robo binding and repellent signaling. Deletion of the EGF or G-domain did not reduce repellent signaling. These transgenes were more potent than full-length Slit in restoring midline axon guidance in *slit* mutants. This may be because these truncated proteins were more stable or were expressed at higher levels. Our epitope tag labeling of the transgene products verified the presence of all transgene products but could not resolve relative levels of expression. An alternative interpretation is that the EGF or G-domain may have a regulatory influence on repellent signaling by the LRR, perhaps in establishing the gradient of repellent signal by retention on the MG cell surface. Functions of the EGF and G-domains deserve further investigation.

Robo binding did not require an intact EGF, G-, or cysteine domain. Internal deletion of the EGF domains also removed the putative cleavage site in EGF repeat 6; nevertheless, this protein still signals as a repellent. Therefore, it is likely that uncleaved Slit has repellent signaling function.

Protein interactions of the EGF, G-, and cysteine domains await analysis. They may play a role in binding laminin and Netrin, which bind vertebrate Slit (Brose et al., 1999). The EGF repeats of Slit are very similar to the non-calcium-binding repeats of Notch and Delta. Notch, Delta, Slit, laminin, and sea urchin fibropellin share a PGYTG motif within the EGF region. The EGF domains are implicated in specific protein recognition events, for instance, the recognition of Notch by Delta (Lieber et al., 1992). The laminin EGF domain can promote neurite extension and modulate attractive signaling by netrin (Hopker et al., 1999).

The G-domain of laminin (also termed the ALPS motif) (Rothberg et al., 1990) is also found in Slit, neurexin, agrin, and perlecan, juxtaposed with EGF repeats. All members of this family participate in morphogenetic activities in the ECM. Lami-

nin, neurexin, and agrin are also implicated in cell signaling during cell differentiation. Laminin binding to syndecan and integrin requires the G-domain (Hoffman et al., 1998; Kadoya et al., 1998). The G-domains of agrin promote postsynaptic differentiation of the neuromuscular synapse. Removal of the adjacent EGF repeats enhances this activity (Cornish et al., 1999). Our study did not reveal an essential role for the G-domain in repellent signaling by Slit. The role of this domain in Slit may be revealed when its binding partner is identified.

The C terminus of vertebrate Slit contains a cysteine knot (Brose et al., 1999), also found in growth factors that dimerize (McDonald and Hendrickson, 1993). The C terminal of *Drosophila* Slit, also cysteine rich, does not have appropriately spaced cysteines to form looped intermolecular disulfate bonds. Our internal deletion constructs that retained repellent effects did not remove the cysteine domain; therefore, we cannot exclude a contribution from this domain in Slit signaling.

In contrast to the other domains of the Slit protein, only the LRR domain is required in *slit* transgenes to restore midline guidance. Furthermore, translated Slit that lacks a full LRR did not bind to Robo, the repellent receptor. These data and the mutant sequence data indicate that LRR of Slit is required for, although not necessarily sufficient for, receptor binding and repellent signaling. This is the first demonstration that the LRR motif can function as a ligand for signal transduction.

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