slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains

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The Drosophila slit locus encodes a protein with four regions containing tandem arrays of a 24-amino-acid leucine-rich repeat (LRR) with conserved flanking sequences (flank-LRR-flank surrounding these arrays), followed by two regions with epidermal growth factor (EGF)-like repeats. Each of these motifs has been implicated in protein-protein interactions as part of an extracellular domain in a variety of other proteins. Analysis of slit cDNA clones reveals that as a consequence of alternative splicing, the locus can code for two distinct protein species differing by 11 amino acids at the carboxyl terminus of the last EGF repeat. The existence of a putative signal sequence and the absence of a transmembrane domain suggest that slit is secreted, an observation supported by an analysis of its expression in tissue culture. Examining the expression pattern of slit in the embryo by antibody staining, enhancer trap detection, and in situ hybridization, we demonstrate that the protein is expressed by a subset of glial cells along the midline of the developing central nervous system. Through immunoelectron microscopy, slit can be seen on the commissural axons traversing the glial cells although it is absent from the cell bodies of these neurons, implying that slit is exported by the glia and distributed along the axons. Finally, we demonstrate that a reduction in slit expression results in a disruption of the developing midline cells and the commissural axon pathways. The embryonic localization, mutant phenotype, and homology of slit to both receptor-binding EGF-like ligands and adhesive glycoproteins suggest that it may be involved in interactions between the midline glial cells, their extracellular environment, and the commissural axons that cross the midline.

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Thus, we became interested in determining what role slit might play in the development of the midline glia and the commissural axon tracts.

Here we report the full structure of the slit protein, as well as its sites of production and distribution. We find that in addition to containing EGF homologous domains, the slit protein also has four regions bearing homology to the leucine-rich repeats [LRRs] found in a family of proteins involved in protein–protein interactions (Titani et al. 1987, Schneider et al. 1988; McFarland et al. 1989; Field et al. 1990, Krantz and Zipursky 1990). In addition, we show that sequences flanking the LRRs of slit exhibit homology to sequences in corresponding positions in some of the other LRR-containing proteins. We demonstrate that slit is necessary for the normal development of the midline of the CNS, including particularly the midline glial cells, and for the concomitant formation of the commissural axon pathways. Furthermore, this process is dependent on the level of slit protein expression. We also present evidence indicating that the slit protein is excreted from the midline glial cells where it is synthesized and is eventually associated with the surfaces of the axons that traverse them. In addition, slit protein is tightly localized to the muscle attachment sites and to the sites of contact between adjacent pairs of cardioblasts as they coalesce to form the lumen of the larval heart. The implications of the structure and distribution of the slit protein in development are discussed.

Results

Molecular characterization of the slit transcript and P-element alleles

The isolation and partial characterization of slit EGF-homologous genomic sequences and corresponding cDNA clones was described previously [Rothberg et al. 1988]. Here we extend our molecular analysis to include the entire slit-coding sequence, its genomic organization, characterization of a splicing variant, and the molecular basis of four P-element-induced mutations. The slit embryonic transcript was estimated to be ~9 kb by Northern analysis. Using both conventional hybridization screening procedures and methods employing the polymerase chain reaction [PCR], we obtained cDNA clones representing 8.3 kb of this sequence [see Materials and methods]. Sequencing of genomic DNA indicates a consensus Drosophila transcriptional initiation sequence [Hultmark et al. 1986] 53 bp upstream of our longest cDNA. Figure 1 shows the slit transcript aligned with a restriction map of the corresponding genomic regions. The known intron/exon boundaries are indicated in Figure 1A and were determined by a comparison of the cDNA sequence with known genomic sequence [Rothberg et al. 1988]. The slit cDNA sequence spans a genomic region of ~20 kb and contains a single 4440-bp open reading frame [ORF]. The nucleotide and deduced amino acid sequences of the ORF are shown in Figure 2. The slit-coding sequence [Codonpreference; Gribskov et al. 1984] starts with a translational start site consistent with the Drosophila consensus [Cavener 1987].

Restriction mapping and sequence analysis of slit cDNA clones revealed two classes of transcripts differing by 33 nucleotides. The location of this sequence variation is shown in Figure 2. The presence of a minor sequence variation prompted a more careful analysis of slit cDNA clones to detect whether other transcript variants existed that might not have been detected by Northern analysis. Utilizing a cDNA screening procedure based on PCR [see Materials and methods], the only detectable size variation was confined to the same region as in the original variant. A comparison of the genomic and cDNA sequences demonstrates that the 33-nucleotide size variation is the result of alternate RNA splicing. The two species of slit cDNA differ in the location of a donor [5'] splice site, whereas the acceptor [3'] site is identical.

Our molecular characterization has been extended to include the determination of the site of P-element insertion in four slit alleles [slitP1, slitP119, slitE158, and slit175], which were recovered during a P-element-based enhancer trap screen [Bellen et al. 1989; Bier et al. 1989].

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Figure 1. Transcription unit and molecular characterization of slit P-element enhancer trap alleles. The slit transcript [A] is shown aligned above the corresponding genomic sequence [B]. Transcription is shown from left to right. Alternating light and dark shading patterns are used to represent the five EcoRI restriction fragments in the cDNA with the numbers above indicating their size in base pairs. Where known precisely, the locations of splice sites are shown by a connecting “V.”

Other exonic regions are shown as blocks aligned approximately with corresponding genomic sequence. The location of primers used to confirm the splice variation in the slit transcript and the resulting 33-bp alternate segment [see text] are indicated by opposing horizontal arrows and a vertical bar, respectively. The location of the primer used to detect the P-element inserts is shown by a left-pointing arrow near the S' end of the transcript. [B] A restriction map of the genomic sequence containing the slit transcription unit. Labeled solid triangles indicate the sites of insertion of the enhancer trap construct in the various P-element slit alleles. Their nucleotide positions relative to the consensus transcription initiation site are shown in parentheses. [B] BamHI; [E] EcoRI; [H] HindIII; [S] SalI.

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Genomic DNA from each line was employed in the PCR using primers designed to detect P-element insertions in regions 5' of the slit-coding sequence [Materials and methods]. By direct sequencing of the PCR products, these lines were shown to contain insertions upstream of both the slit consensus transcription initiation sequence and ORF (see Fig. 1B) confirming their initial characterization as slit alleles and suggesting their utility in the characterization of slit expression.

**slit Codes for flank-LRR-flank and EGF domains**

The slit transcripts potentially encode two proteins of 1469 and 1480 amino acids, with molecular masses of ~166 kD. The predicted initiating methionine is followed by an amino acid sequence containing structural regions characteristic of a secretory signal sequence (Fig. 2). However, hydropathy plots do not predict a transmembrane domain [data not shown]. An examination of the slit-coding domain reveals that the majority of the protein is composed of two repeated motifs: the 24 amino acid LRR and the 40-amino acid EGF repeat (Fig. 2). Figure 3A shows schematically the positions of these repeats and indicates a higher level of organization among the LRRs. The LRRs are arranged in four groups, each composed of four or five LRRs [Fig. 3B] surrounded by conserved amino- and carboxy-flanking regions [see key in Fig. 3A]. The presence of both the LRRs and EGF-like repeats within a single protein make slit unusual; this combination is not found in any other proteins in invertebrate proteins involved in protein-protein interactions. Moreover, slit contains seven copies of the EGF motif [Figs. 2 and 3A], which also has been shown to participate in extracellular protein–protein interactions [Rothberg et al. 1988]. The last EGF repeat is of special interest because the alternate mRNA splicing noted earlier potentially results in the insertion or removal of 11 unique amino acids at the carboxyl terminus of this repeat [see Figs. 2 and 3A].

**slit is exported from glial cells and distributed along axon tracts**

We have shown previously that slit transcript and protein could be detected at the highest levels in the midline glial cells [Rothberg et al. 1988]. However, despite the presence of the slit protein on the axons in the embryonic commissural and longitudinal axon pathways, we failed to detect any transcript or protein in the cell bodies of these neurons. This raised the possibility that the slit protein, which is synthesized in and presumably secreted by the midline cells, can become associated with axons. Here we explore this question further in whole-mount embryo preparations by comparing the sites of slit expression, as assayed by in situ hybridization and the detection of β-galactosidase in slit enhancer trap lines, with the subsequent localization of the protein as assayed by antibody staining [summarized in Fig. 4].

All four enhancer trap alleles [slit^581, slit^519, slit^518, and slit^175] express β-galactosidase within the ventral midline to varying levels (see Materials and methods). The location of the P-element constructs 5' of the slit-coding domain, the resulting mutant phenotypes [described below], and especially their expression patterns are all consistent with their being under the transcriptional control of slit regulatory elements. A summary of
Figure 2. (See facing page for legend.)
the embryonic localization of the slit mRNA and protein, and the β-galactosidase expression of slitE158 is shown in Figure 4. The expression of β-galactosidase from the enhancer trap construct in slitE158 shows excellent overall agreement with mRNA localization data at all embryonic stages [Fig. 4, cf. A, D, G, and J with C, F, I, and L]. Each method reveals a nearly identical expression pattern starting at gastrulation [Fig. 4A–C]. At germ-band extension, all of the midline mesectodermal cells [see Crews et al. 1988; Thomas et al. 1988] show the highest level of slit expression [Fig. 4D–F]. During germ-band retraction and nerve cord shortening, expression is most restricted to the six midline glial cells that are derivatives of the midline neuroepithelium [Fig. 4G–I]. Localized expression is also evident in the cardioblasts [Fig. 4J–L] during dorsal closure. Figure 5, A and B, shows that the slit protein is most highly localized to the points of contact between opposing pairs of cardioblasts as they coalesce to form the dorsal vessel (presumptive larval heart). All three methods also reveal expression in the walls of the gut [Fig. 4J–L] and in a segmentally reiterated pattern near the muscle attachment sites in the ectoderm [apodemes; Fig. 4G–I]. Precise protein localization to the sites where the muscles are attached to the apodemes is seen by confocal microscopy [Fig. 5A, C].

In situ hybridization [Fig. 4D, G, and J] and the expression from the enhancer trap lines [Fig. 4E, I, and L] both support the observation that initially all of the midline cells, and subsequently primarily the six midline glia, are producing slit while lateral neurons are not. However, antibody labeling is seen strongly in the midline glia [Fig. 4E, H] and on the commissural and longitudinal axon tracts [Fig. 4E, H, and K] while it is absent from lateral neuronal cell bodies, which supply the bulk of the axons to these bundles. These results suggest that the antibody labeling along the commissural and longitudinal axon tracts is due to the distribution of slit protein exported from the midline glial cells. The protein is also absent from the peripheral nerve roots and peripheral axon tracts.

Immunoelectron microscopy was used to determine the subcellular localization of the slit protein in the ventral nerve cord. Dissected embryonic nerve cords demonstrate staining on the midline cells, as well as on the commissural and longitudinal nerve bundles. Light and electron micrographs of a similarly prepared sample are shown in Figure 6. Although all the derivatives of the neuroepithelium initially express slit, this expression becomes restricted to the midline glial cells during nerve cord condensation and axonal outgrowth. The midline glial cells surround the developing commissural axons, and growth cones have been shown to track along their surface [see Jacobs and Goodman 1989a]. Antibody staining can be seen both on the surfaces of the midline glial cells, where they abut growing axons, and on the axons themselves. No detectable variation in the amount of slit staining among subsets of axons or fascicles is detected [see Materials and methods].

We are able to detect slit along the length of the axonal projections in the commissural and longitudinal axon tracts though we are unable to detect any signal above background from the lateral neuronal cell bodies supplying these axons [Fig. 6]. Our immunoelectron microscopy demonstrates the extracellular localization of the slit protein and supports the expression data, indicating that the slit protein on the axon tracts is not produced by the neurons whose axons comprise them. Thus, it appears that the axonally distributed slit protein is first secreted from the midline glial cells and then becomes associated with these axons as they traverse the midline.

To obtain direct biochemical evidence that slit is expressed from the cells in which it is produced, we investigated slit expression in Drosophila tissue culture cell lines. Schneider line S2 was found to normally express the slit protein, and it can be seen on the surface of a subset of the cells by immunofluorescence [data not shown]. Immunoblotting of immunoprecipitated protein extracts from Drosophila embryos and S2 cell lines revealed a single 200-kD band [Fig. 7A, lanes 1 and 2]. This size is consistent with expectations of a glycosylated form of the predicted slit protein. Conditioned Schneider cell media [see Materials and methods] also was found to contain a similar 200-kD species [Fig. 7A, lane 3] in addition to two other species that may represent differences in glycosylation [J.M. Rothberg, unpubl.]. The presence of the slit protein in the culture media was confirmed by immunoprecipitations of the same molecular mass species from media in which 35S metabolically labeled S2 cells had been growing [Fig. 7B]. These experiments further support the suggestion that slit is an ex-
Figure 3. (See facing page for legend.)
Table 1. LRR-containing proteins

| Proteins                                      | Arrangement          | Function                        | Reference               |
|-----------------------------------------------|----------------------|---------------------------------|-------------------------|
| Glycoprotein lbα                              | LRR-flank            | receptor/adhesion               | Titani et al. (1987),   |
|                                               |                      |                                 | Lopez et al. (1987)     |
| Glycoprotein lbβ                              | flank-LRR-flank      | receptor/adhesion               | Lopez et al. (1988)     |
| Glycoprotein IX                               | flank-LRR-flank      | receptor/adhesion               | Hickey et al. (1989)    |
| Lutropin—choriogonadotropin receptor          | LRR                  | receptor                        | McFarland et al. (1989) |
| Collagen-binding 59-kD protein [fibromodulin] | flank-LRR            | ECM binding                     | Oldberg et al. (1989)   |
| Small interstitial proteoglycan PC-S1 [biglycan] | flank-LRR           | ECM binding                     | Fisher et al. (1989)    |
| Small interstitial proteoglycan PC-S2 [decorin, PG-40] | flank-LRR          |                                 |                         |
| Adenylate cyclase*                            |                      |                                 |                         |
| Ribonuclease/angiogenin inhibitor*            | LRR                  | protein—protein                 | Schneider et al. (1988) |
| Chaoptin                                      | LRR                  | homotypic adhesion              | Reinke et al. (1988);   |
|                                               |                      |                                 | Krantz et al. (1990)    |
| Leucine-rich α2-glycoprotein                  | LRR                  | ??                              | Takahashi et al. (1985) |
| Oligodendrocyte—myelin glycoprotein           | flank-LRR            | adhesion?                       | Mikol et al. (1990)     |
| *Toll*                                        | 2 × LRR-flank        | dorsal—ventral polarityb        | Hashimoto et al. (1988) |
| *slit*                                        | 4 × flank-LRR-flank  | morphogenesisb                  | this work               |

*Intracellular proteins; all others are extracellular or cell surface proteins.

Although the role of these proteins in *Drosophila* development is known, it is not known how their function is mediated.

slit mutants exhibit disruptions in midline cells and commissural axon pathways

An analysis of *slit* null mutant embryos reveals the collapse of the normal scaffold of commissural and longitudinal axons. However, the *slit* protein is detectable in the midline neuroepithelial cells well before the time of axonal outgrowth [Rothberg et al. 1988]. This raised the possibility that the *slit* protein influences the differentiation of midline cells from the neuroepithelium and that the observed collapse of the axonal scaffold is the result of an earlier developmental abnormality. To examine the development of the midline before axon outgrowth, we followed the fate of the MP2 cells [an identified neuronal precursor cell that normally develops in the most medial row of neuroblasts in the lateral neuroepithelium], as well as the midline neuroepithelium and its progeny [see Materials and methods] in both wild-type and mutant embryos.

In wild-type embryos at the germ-band-extended stage, the MP2 cells are separated by the midline neuroepithelium [Fig. 8A], whereas in *slit* embryos these cells appear closer together [Fig. 8B]. In addition, cell autonomous markers [lines 8-7 and 242] for some of the midline neuroepithelial cells and their progeny [Fig. 8C-E, and G] are either absent or ectopically expressed before [Fig. 8D] and during axonal outgrowth [Fig. 8F,H]. For example, in *slit* mutant embryos, some of these cells appear absent and others come to lie in an abnormal position along the ventral surface of the nerve cord [Fig. 8F,H]. These results clearly show a perturbation in the development of the midline neuroepithelial cells as early as the germ-band-extended stage. This disruption further leads to a disruption of their progeny, including the midline glial cells, resulting in a lateral compression.

Figure 3. Conservation of flank-LRR-flank domains in known adhesive proteins. [A] Schematic representation of the *slit* protein. The putative signal sequence and amino- and carboxy-terminal ends of the protein are indicated. The four consecutive flank-LRR-flank regions, the seven EGF-like repeats, and the 11-amino-acid connecting segment—the result of differential splicing at the carboxy-terminal of the seventh EGF repeat—are shown [see key]. Single LRRs have been shown to form β-sheets in solution and, as depicted here, may form antiparallel sheets [Krantz and Zipursky 1990]. Tandem EGF-like repeats in other ECM proteins have been shown to be arranged in a rod-like conformation and are depicted here as such [see Engle et al. 1989], with the individual EGF repeats modeled after the solution structure of human EGF [Cooke et al. 1987]. [B] The amino acid sequence of the 24-amino-acid LRRs comprising the central regions of the flank-LRR-flank structures in *slit* is presented aligned. Residues identical in >50% of the compared LRRs are lightly shaded. Only the asparagine residue [N] [heavily shaded] is found to be invariant. Note the predominance of the aliphatic residues [I, L, V] at the consensus positions. [C] Alignment of the four amino-flanking regions of *slit* and comparisons with similar regions from other LRR-containing proteins. [a] Aliphatic residues. Sequences preceding the LRRs in other proteins, which show some similarity but do not meet our definition for the amino-flanking region, are shown below the consensus. [D] Carboxy-flanking regions from *slit* are aligned with corresponding regions from other LRR-containing genes. Flanking regions truncated by transmembrane domains are indicated [Tm].
of the nerve cord (confirmed by histological analysis; J.R. Jacobs, unpubl.). Given the disruption in the development of the midline of the CNS, the ensuing collapse of the axonal scaffold is not unexpected (see a similar phenotype of the sim mutant; Crews et al. 1988; Thomas et al. 1988).

Mutations caused by the insertion of the enhancer trap P-element allow for a further exploration of the relationship between the level of slit expression and the extent of the nerve cord defect. In the wild-type embryo, as observed with antibodies specific to neuronal membranes [anti-horseradish peroxidase (HRP); Jan and Jan 1982], commissural and longitudinal axon pathways appear to form a regular ladder-like structure (Fig. 9A). A wild-type embryo stained with anti-slit antibodies also shows labeling of the CNS axon pathways, as well as

**Figure 4.** Comparison of in situ, antibody, and enhancer trap staining. The slit message, protein, and promoter activation are visualized at three stages of embryogenesis by in situ hybridization \([A,D,G,J]\), antibody staining \([B,E,H,K]\), and enhancer trap detection \([C,F,I,L]\). The following stages during embryogenesis are shown: gastrulation in a dorsal view \((A-C)\), germ-band-extended stage in a dorsal view \((D-F)\), and nerve cord condensation, from both dorsal \((G-J)\) and sagittal \((J-L)\) views. Staining can be demonstrated by all three methods in the midline neuroepithelium (arrow in \(D-F\)), midline glial cells (bold arrow in \(G-K\)), and cardioblast [open arrow in \(J-L\)], as well as in the walls of the gut and in a segmentally reiterated pattern near the muscle attachment sites (thin arrow in \(G-I\)). Note that although no signal above background is detected from the lateral neuronal cell bodies, antibody staining [red arrow in \(H\)] is visible on the axonal projections from these neurons (see also Figs. 6 and 9B).
prominent staining of the midline glial cells [Fig. 9B]. Embryos homozygous for slit^C107 do not have any detectable slit expression either in the midline cells or on the axonal bundles (Fig. 9D). This null allele is embryonic lethal; mutant embryos exhibit a lateral compression of the nerve cord (Fig. 9D) and a single fused longitudinal axon tract (Fig. 9C).

As judged by antibody staining intensity in whole-mount embryo preparations, all four enhancer trap slit alleles show reduced levels of slit expression in the homozygous state at 18°C and exhibit an intermediate phenotype. Because the P-element construct resides upstream of slit-coding sequences, it is reasonable to assume that it is not the disruption of the slit protein per se that is responsible for the observed mutant phenotypes but, rather, a reduction in the level of slit expres-
Figure 5. Confocal localization of the slit protein to cardioblasts and muscle attachment sites. [A] An optical, horizontal section of an embryo undergoing dorsal closure stained with anti-slit antibodies shows the slit protein to be localized on the surface of cardioblasts (apposing arrows) and at the muscle attachment sites to the body wall (long arrow). [B] A higher magnification view of the cardioblasts shows that the highest concentration of slit protein is localized to the regions of contact (long arrow) between apposing pairs of cardioblasts (apposing arrows) as they come together to form the lumen of the larval heart. [C] A sagittal view (dorsal side up) shows the slit protein to be localized to the sites of muscle attached to the ectoderm (long arrows). Autofluorescence from gut is also visible.
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morphogenetic events accompanying the formation of early structures have been shown to be dependent on the properties of the molecules that form their extracellular environment [see Jessell 1988]. In vitro and in vivo studies suggest that growth cone guidance and axonal pathway selection are influenced by adhesive interactions between axons and ECM molecules [see Sanes 1989]. Furthermore, specific constituents of the extracellular environment have been shown to affect neurite outgrowth in vitro and have been detected in vivo in the developing central and peripheral nervous systems [see Rutishauser 1989].

In this paper we show that the slit locus, whose mutant phenotypes indicate that it plays a major role in the development of the specialized midline glial cells and the commissural axon tracts that traverse them, encodes a unique extracellular protein containing two structural motifs associated with adhesive interactions. The slit protein has four regions containing tandem arrays of a 24-amino-acid leucine-rich repeat with conserved flanking sequences [flank-LRR-flank] and two regions with EGF-like repeats. Although the LRR and EGF motifs are not found together in any other proteins in the NBRF data bank, each has been found in conjunction with other sequence motifs, often forming a distinct region of a larger protein involved in protein–protein interactions. As part of larger proteins, each of these

Discussion

It has long been thought that the extracellular environment influences the regulation of gene expression and the morphogenesis of cells during embryonic development [see McDonald 1989]. In the nervous system, the

![Figure 6](Image)

Figure 6. Immunoelectron microscopic localization of slit in the embryonic CNS to midline cells and axonal tracts. Staining with anti-slit antibody in a frontal section through the plane of the longitudinal and commissural axonal tracts, detected by silver intensification of an HRP-conjugated secondary antibody. At the electron microscopic level, labeling is both on the axons comprising the longitudinal connectives [lc], anterior [ac] and posterior [pc] commissures, and on the cells lying between them including the processes of the midline glial cells [arrows]. A light level frontal view of a similarly prepared dissected nerve cord shows strong axonal labeling with respect to the midline cells (insert). No signal above background is seen on lateral neuronal cell bodies [N], either at the light or electron microscopic level. Bar, 5 μm.

![Figure 7](Image)

Figure 7. Secretion of slit from cultured cells. [A] An immunoblot with anti-slit antibodies of slit protein immunoprecipitated from embryos [lane 1] and S2 culture cells [lane 2] shows a common protein species of ~200 kD [arrow]. This species is also immunoprecipitated from S2 cell line-conditioned media [lane 3], indicating that the slit protein can be exported from the cells in which it is produced [see text for discussion]. [Lane 4] By immunoblotting, the 200-kD slit protein species can also be detected in the matrix materials deposited by the S2 cells in culture [see Materials and methods]. The predominant band seen in immunoprecipitations is immunoglobulin heavy chain [H]. [B] The media in which 35S metabolically labeled S2 cells had been cultured were immunoprecipitated with anti-slit antibodies, separated by SDS-PAGE, and detected by autoradiography. Consistent with the immunoblotting results, a major 200-kD species is detected [arrow]. Tick marks indicate position of 100- and 220-kD size standards.
Figure 8. (See facing page for legend.)
motifs has been shown to contribute directly to these interactions.

The LRRs in slit are similar to those that were first identified in human leucine-rich α2-glycoprotein and later in a variety of vertebrate and invertebrate proteins involved in protein-protein interactions, both inside and outside the cell (Table 1). In the extracellular environment, the LRRs have been found in conjunction with a variety of conserved protein motifs [McFarland et al. 1989, Mikol et al. 1990]. Of greatest interest to us, however, is the fact that the LRRs in extracellular proteins are often found accompanied by either one or both of the conserved amino- and carboxy-flanking regions identified in the slit protein (see Table 1). In all of the cases where the LRRs are accompanied by these flanking regions, the proteins have either been shown, or are believed, to participate in extracellular adhesive interactions. Although we do not yet know the significance of the individual flanking regions in these interactions, a functional role for at least the carboxy-flanking sequence has been demonstrated in vivo: Mutations in the cysteines of this region in the Drosophila Toll protein confer a dominant phenotype [K. Anderson, pers. comm.].

In addition to Toll and the OMgp, two distinct families of adhesive proteins have slit homology extending to the LRR-flanking sequences. The first includes a set of functionally related interstitial proteoglycans known to bind directly to ECM proteins: biglycan, fibromodulin and decorin. Biglycan binds laminin and fibronectin [A. Skubitz, pers. comm.] while fibromodulin, and decorin bind collagen and fibronectin and have a regulatory effect on collagen fibril formation [Vogel et al. 1984; Schmidt et al. 1987; Hedbom and Heinegärd 1989; Oldberg et al. 1989]. The second set comprises the proteins of the glycoprotein Ib-IX [GPIb-IX] complex, which together function as a receptor for the von Willebrand factor [vWF] and thrombin and are responsible for vWF-dependent platelet to blood vessel adhesion. In this complex, the LRR-containing region of the GPIbα chain binds one of a set of three repeated 200-amino-acid sequences termed A domains in vWF [Mohri et al. 1988; Titani et al. 1987]. In addition to demonstrating the role of the LRR motif in protein-protein interactions, this homology also raises the possibility that similar regions in slit might bind to proteins containing repeats homologous to the A domains of vWF. In vertebrates, these proteins include both ECM molecules and integrins [see Larson et al. 1989].

The conservation of the amino-terminal sequences flanking a LRR region in a family of proteins that participate in direct adhesion to ECM components suggests that this structure may play a similar role in slit. Alternatively, the conservation of the entire flank-LRR-flank motif in slit and the GPIb-IX complex offers the intriguing possibility that the interactions of slit with the ECM, like that of the vWF and thrombin receptor, could be mediated by additional factors.

In comparing the various proteins known to contain the EGF-like motif, it is clear that this sequence is always found in an extracellular environment; and in many instances, these sequences have either been implicated or shown to function directly in protein-protein interactions [Appella et al. 1988]. In addition, these repeats are found in conjunction with a variety of other structural and catalytic domains in molecules involved in blood coagulation [see Furie and Furie 1988] and in adhesive ECM glycoproteins [see Engel 1989]. Tandem arrays of EGF-like repeats comprise the majority of the extracellular domains of the cell-surface proteins Notch [Wharton et al. 1985] and Delta [Vassil et al. 1987; Kopczynski et al. 1988] and have been implicated in Ca²⁺-dependent heterotypic adhesive interactions between the two proteins as well as in homotypic interactions in the Delta protein [Fehon et al. 1990].

The EGF-like repeats in slit are arranged in two groups in a fashion similar to the arrangement found in cell-surface and extracellular adhesive proteins and in EGF-like ligands, respectively [see Appella et al. 1988; Lander 1989]. An additional similarity between the EGF-like repeats in slit, Delta, and Notch is a conserved recognition site for a post-translational modification involved in Ca²⁺ binding [Rees et al. 1988] and a consensus sequence implicated in Ca²⁺-dependent protein-protein interactions [Handford et al. 1990]. By these criteria, the third and fifth EGF-like repeats of slit are potential candidates for β-hydroxylation and may participate in Ca²⁺-dependent interactions. The seventh and last EGF domain in slit is separated from the tandemly arranged EGF-like repeats by 202 amino acids. It will be of interest to determine whether the alternate splicing variant seen in slit, which results in a change of coding capacity at the

Figure 8. Null mutant embryos exhibit disruptions in midline cells. The pattern of expression of β-galactosidase in MP2 cells [A and B] and the midline neuroepithelium and its progeny [C–H] is compared in wild-type and null mutant embryos (see Materials and methods). Anterior is toward the left. (A and B) A dorsal view shows the MP2 cells [arrows] well separated by cells of the midline neuroepithelium at the extended germ-band stage in wild-type embryos [A] but closer together in a slit mutant background [B], indicating an early disruption along the midline. (C and D) The midline neuroepithelium at the germ-band-extended stage [arrow in C] and its midline progeny [E and G] are clearly labeled in wild-type embryos. In comparison, following germ-band extension in slit mutant embryos there is either no midline neuroepithelial labeling or low levels of labeling slightly later [arrow in D]. (E and F) A sagittal view during nerve cord condensation shows the bulk of the midline cells of each neuromere clearly expressing β-galactosidase in the wild-type embryo [arrow in E]. However, in slit mutant embryos, the expressing cells are reduced in number and displaced to the ventral edge of the nerve cord [arrow in F]. (G and H) A dorsal view of a similarly staged wild-type [G] and slit mutant [H] embryo. In the wild type the midline cells can be seen in the space separating adjacent neuromeres within a segment. In slit mutant embryos, expressing cells can be seen to lie irregularly shifted laterally, as well as ventrally [arrow].

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Figure 9. Levels of slit expression correlate with disruptions of midline cells and axon pathways. The major axonal pathways are labeled with anti-HRP antibodies (A, C, and E) [Jan and Jan 1982] and compared to the staining pattern seen with antibodies against the slit protein (B, D, and F). In these horizontal views, anterior is toward the left. (A and B) In wild-type embryos the ladder-like arrangement formed by the commissural and longitudinal axonal tracts is visible. Staining with antibodies against the slit protein (B) shows labeling of the midline glial cells (black arrow), as well as axonal staining (red arrow). (C and D) Anti-HRP stained null mutant embryos (C) exhibit a single centrally located longitudinal nerve bundle along the length of the CNS. No detectable slit staining is seen (D). The lateral neuronal bodies are shifted inward toward the center, filling the space normally occupied by the midline cells. An overall reduction in the width of the nerve cord is also observed (double-ended arrow). (E and F) slit^{E188} mutants exhibit an intermediate phenotype characterized by a partial collapse of the axonal scaffold. Relatively weak slit staining is visible along the length of the axonal bundles (F). Segments with the highest levels of slit staining (black arrow), have more midline cells and a less severe collapse of the longitudinal connectives (short red arrow) in comparison to segments with lower expression levels (long red arrow). Segments with reduced levels of slit expression exhibit nerve cord compression and a concomitant fusion of the axon tracts (long red arrow).

carboxyl terminus of this EGF-like repeat, provides for a unique functional constituent of the protein with altered binding specificity.

Export and cell binding

Using both whole-mount in situ hybridization and slit enhancer trap alleles, we are able to demonstrate that slit is produced in the developing midline neuroepithelium, as well as in its progeny midline glial cells along the dorsal midline of the CNS but not in the neuronal cell bodies whose axons form the major commissural and longitudinal axon tracts in the CNS. Light and immunoelectron microscopy indicate that slit is exported from the midline glial cells and is associated with the axons that traverse them. If, as is suggested by these
data, the *slit* gene product is not produced in the neurons of the axons on which it resides, we expect that it is secreted from the midline cells and “picked up” by passing axons. This, in turn, raises the possibility that the axons that carry *slit* on their surface may be expressing specific receptors capable of interacting with *slit* in a direct or indirect manner. An analysis of *slit* expression in *Drosophila* cell culture demonstrates that *slit* can be localized to the surface of individual cells. Additional biochemical support for the extracellular, secreted nature of the protein was provided by demonstrating that tissue culture cells producing *slit* are secreting the protein into the media. Moreover, consistent with the hypothesis that *slit* functions as an ECM molecule, we found the protein to be accumulated in the matrix materials deposited by these cells.

**Morphogenetic regulation of the neuroepithelium**

A model for *slit* function wherein it regulates the morphological differentiation of a cell by attaching to both the ECM and cell-surface receptors is consistent with its predicted structure, expression pattern, and phenotype. Like the other ECM glycoproteins, *slit* is composed of repetitive structural motifs and lacks the hydrophilic regions characteristic of membrane-spanning cell-surface adhesion molecules. ECM glycoproteins play a diverse role in development, acting as signals for cell differentiation, growth, and migration. Furthermore, the *slit*-homologous proteoglycan decorin is involved in the control of cell proliferation and has the ability to convert transformed cells to morphological regularity (Yamaguchi and Ruoslahti 1988).

Here we demonstrate the involvement of *slit* in the development and differentiation of the midline neuroepithelium and the subsequent formation of commissural axon pathways. In a *slit* mutant background the midline cells do not undergo proper differentiation or morphological movements; instead of filling the midline of each neuromere as they do in the wild-type embryo, they appear at the base of the nerve cord and are fewer in number. This is followed by the complete collapse of the axonal scaffold. The in vivo effects of reductions in *slit* expression further indicate that the morphogenesis of the midline cells and the subsequent axonal pathway formation are dependent on the concentration of *slit* protein. Using P-element-induced *slit* alleles, we are able to demonstrate that a reduction in *slit* expression is coincident with the lack of development of the midline cells of an individual segment and specifically, with the development of the midline glial cells. We show further that the variability in the extent of collapse of the midline of the nerve cord is mirrored by the extent of collapse of the commissural and longitudinal axon pathways.

We note with interest that the extent of disruption in the ventral nerve cord in *slit* alleles corresponds to the range of phenotypes exhibited by mutations of the *Drosophila* EGF-like receptor homolog (DER). Given the homology between *slit* and EGF-receptor ligands, the colocalization of the DER and *slit* proteins to the midline glial cells and the muscle attachment sites (Zak et al. 1990) raises the possibility that *slit* functions as a DER ligand. This speculation is particularly attractive, as the activation of a receptor tyrosine kinase by the *slit* protein would offer a mechanistic explanation for the influence of *slit* on either the development or maintenance of the midline cells and provide for a direct molecular link between the ECM and genes involved in cellular proliferation and differentiation (see Yarden and Ullrich 1988).

**Implications of *slit* expression**

The three major regions of *slit* expression are the midline neuroepithelium of the CNS, the attachment sites of muscle to epidermis, and the cardioblasts of the dorsal tube. The expression of *slit* in the cardioblasts as they meet and form the lumen of the dorsal tube may be of general interest given that in vertebrate tissue culture, the ECM has been shown to be involved in endothelial cell alignment and the induction of capillary tube formation (see Ingber and Folkman 1989). This process is one of the best characterized morphogenetic processes in vitro and has allowed for an analysis of the molecular mechanisms by which ECM molecules, specifically collagen, laminin, and fibronectin, are able to control capillary morphogenesis (Grant et al. 1989).

In *Drosophila*, the larval heart or dorsal vessel is derived from two longitudinal rows of mesodermal cells termed cardioblasts. When these cells meet following dorsal closure along the midline, only their dorsomedial and ventromedial surfaces contact, with the space between forming the lumen of the dorsal vessel (Poulson 1950; Hartenstein and Campos-Ortega 1985). *slit* is expressed in the developing cardioblasts during the time they come together. Confocal microscopic imaging clearly shows the *slit* protein to be concentrated at the point of contact between the cardioblasts as they come together and form the lumen of the larval heart. Given the unique structural characteristics of *slit*, its homology to ECM-binding proteins, and the role of these ECM proteins in vessel formation, an analysis of the role of *slit* in developing cardioblasts and its possible interactions with other proteins expressed in these tissues during larval heart formation may serve as a useful in vivo model for the study of the angiogenic process.

Confocal microscopy shows the *slit* protein to be tightly localized to the points of muscle attachment to the epidermis. This localization is consistent with *slit* functioning as an ECM molecule and suggests its involvement in adhesive events. The muscle attachment sites are known sites of ECM deposition (Newman and Wright 1981), and the position-specific integrins have been shown to be localized here (Leptin et al. 1989). Hence, a role for *slit* in adhesive-mediated events such as muscle attachment and axonal outgrowth is supported both by its structure and its expression pattern. The potential for two variants of the *slit* protein raises the possibility that these roles are mediated by functionally distinct forms of the protein.
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Tissue culture studies have demonstrated that growth cones adhere to and extend neurites onto ECM molecules such as laminin and fibronectin (see Sanes 1989) and that the direction and rate of axonal growth are dependent on these axon–matrix interactions (see Rutishauser and Jessell 1988). Given the homology of slit to the laminin-binding protein biglycan, we note with interest that laminin is expressed on glial surfaces and along the pathways axons follow in the establishment of the commissural and longitudinal axonal tracts in Drosophila (Montell and Goodman 1989).

The possibility that slit binds to matrix materials suggests that its presence on growing axons could influence their interactions with ECM proteins. The ability of axons to fasciculate on one another in all slit mutants indicates that slit is not necessary for axon–axon fasciculation. However, the combination of flank-LRR-flank, tandem EGF, and single EGF motifs in proteins with the unique embryonic distribution of slit could allow for the formation of a “molecular bridge” between axonally associated receptors and ECM molecules. Prompted by the information on the structure of slit, its expression in glial cells, and its presence on axons that extend along these cells, we propose a testable, hypothetical mechanism whereby glial cells can influence the future behavior of an axon: (1) Glial cells secrete multifunctional molecules which have the ability to attach to specific axonal receptors, as well as to specific ECM components into the endoneural basal lamina; (2) passing axons carrying receptors for these proteins pick them up from the glial cell surroundings; and (3) depending on the proteins associated with them, axons are able to respond to cues and interact with molecules in the ECM.

Materials and methods

Drosophila stocks and genetics

slit^B219 and slit^F119 were created by germ-line transformation with the enhancer trap construct P-lacW (Bier et al. 1989) and slit^B52-2 was made using P-lArB (gifts of A. Kolodkin; Bellen et al. 1989). Other slit alleles are as described in Rothberg et al. (1988). slit^B52-157 exhibits some ectopic β-galactosidase expression, whereas slit^B52-1 and slit^F119 (likely the result of the same insertion event) have levels of midline expression lower than levels in slit^B52-2. Lines 8-7 and 242 function as cell autonomous slit alleles and are as described in Rothberg et al. (1988). Line 5704 expresses p-galactosidase from the ftz enhancer trap construct that uses the P-element and 5′ ftz promoter (Philippe et al. 1991).

Whole-mount in situ hybridizations, enhancer trap detection, and antibody labeling

Whole-mount in situ hybridizations were conducted using digoxigenin-derivatized DNA probes from cDNA B52-5 (Tautz and Pfeiffle 1989). Immunocytochemistry was done essentially as described in Rothberg et al. (1988). Anti-β-galactosidase antibody (Promega) was used to detect the signal from the enhancer trap constructs and detected with a HRP-conjugated antimouse antibody (Jackson Immunological Laboratories). Signal from whole-mount in situ hybridizations is cytoplasmic (Tautz and Pfeiffle 1989), enhancer trap signal is localized to the nucleus (Bellen et al. 1989), and antibody staining shows both cytoplasmic and cell-surface staining.

Immunoelectron and confocal microscopy

All preparations were made by dissecting embryos in Schneider medium to expose the nerve cord. Samples were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde for 20 min, followed by primary and secondary antibody labeling without detergent. Primary electron microscopy fixation was performed using 2% glutaraldehyde and 2% paraformaldehyde prior to
silver enhancement of signal from the HRP-conjugated secondary [Amersham]. The silver enhancement procedure prevents accurate distinctions from being made concerning the relative levels of antigen present among subsets of axons. Samples were treated with 1% OsO4 and counterstained with uranyl acetate. Sections were prepared on a Reichert ultramicrotome and visualized on a Jeol electron microscope. Confocal images were made using a Bio-Rad MRC 500 system and a Zeiss Axiovert compound microscope.

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