The Mouse SLIT Family: Secreted Ligands for ROBO Expressed in Patterns That Suggest a Role in Morphogenesis and Axon Guidance

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The Slit gene encodes a secreted molecule essential for neural development in Drosophila embryos. Here we report the identification of three Slit homologues in the mouse. We demonstrate that the mouse SLIT1 protein can bind ROBO1, a transmembrane receptor implicated in axon guidance. Both whole-mount and section in situ hybridization studies reveal unique and complementary patterns of expression of the three mouse Slit genes and of Robo1, both within the central nervous system and in other developing tissues. The complementary expression patterns of Slit and Robo1 and their in vitro interaction suggest a ligand–receptor relationship. The expression of all three Slit genes in the floor plate suggests that they are likely to share the same functional properties with their Drosophila homologue in midline neural development and axon guidance. The complementary expression of Slit and Robo1 in different subdivisions of the somites suggests their possible function in axon pathfinding and neural crest cell migration. The unique expression pattern in limb and other organs indicates additional potential functions of the Slit gene family. © 1999 Academic Press

INTRODUCTION

Development of the nervous system requires the migration of neural progenitors and the projection of neural growth cones over long distances. The molecules that guide axons and regulate cell migration belong to several groups: diffusible long-range chemotropic factors, nondiffusible extracellular matrix (ECM) molecules, short-range ECM-bound growth factors, cell surface adhesion molecules, and cell surface receptors.

In the central nervous system most axons cross the midline and then project longitudinally along the contralateral midline. Commisural axons are attracted to the midline by netrin and once at the midline mechanisms exist to allow the commissural axons to cross the midline and to prevent recrossing of axons (Kennedy et al., 1994). The guidance of commissural axons is regulated by several genes. In Drosophila, mutations in the commissureless (comm) gene cause axons to fail to cross the midline, whereas in roundabout (robo) mutants, axons cross the midline multiple times. Comm encodes a cell surface protein that may be released from midline cells and taken up by overlying axons (Tear et al., 1996). Kidd et al. (1998b) showed that comm may serve to regulate the expression of robo on commissural axons.

Robo encodes a large transmembrane protein with immunoglobin repeats and fibronectin type III repeats (Kidd et al., 1998a). Robo1 is expressed on longitudinal axons in the Drosophila CNS and may serve as a receptor for repulsive guidance molecules. Robo expression is upregulated in axons once they have crossed the midline and may serve to prevent axons from recrossing the midline a second time (Kidd et al., 1998a).

Homology searches revealed two Robo homologs in both rat and human (Kidd et al., 1998a). In situ hybridization

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showed expression of rat Robo1 in the dorsal spinal cord at embryonic day 13 in a population of cells that comprise commissural and motor neurons. Immunoelectron microscopy localized the highest levels of Robo expression to the growth cone and lower levels of expression in axons (Kidd et al., 1998a). Genetic interaction studies in Drosophila show that Robo does not interact with netrin A or B (Kidd et al., 1998a). Thus it is likely that Robo interacts with an as yet unidentified molecule that functions as a repellent for axons that cross the midline.

SLIT is a secreted protein containing leucine-rich and EGF-like repeats. In Drosophila, the SLIT protein is expressed in midline cells and is thought to be required for the normal development of midline structures (Rothberg et al., 1988). Mutations in the Slit gene result in abnormalities in midline cells or their progeny and the fusion of longitudinal axons. Slit is also expressed on the surface of midline glial cells and immunoelectron microscopy suggests that SLIT is produced and exported from these cells and binds along commissural axons (Rothberg et al., 1988, 1990).

The four leucine-rich repeats (LRR) which are flanked by conserved sequences and seven EGF-like repeats are likely to mediate extracellular protein–protein interactions (Rothberg et al., 1990). Recently two Slit homologues have been identified in the rat and three in human (Itoh et al., 1998; Nakayama et al., 1998). The vertebrate homologues show 28–40% identity with Drosophila slit and contain similar conserved motifs (four LRR, nine instead of seven EGF-like repeats, and a laminin-G domain) which are arranged in a similar order (Itoh et al., 1998).

In this paper we have identified three mouse homologues of the Slit gene family. We show by in situ hybridization that during embryogenesis the three Slit genes and Robo1 are expressed in unique and complementary patterns in the central nervous system and in other tissues. The expression patterns suggest specific roles for SLIT and ROBO in regulating axon pathfinding and/or tissue morphogenesis. In vitro binding studies demonstrated that mouse SLIT1 binds rat ROBO1 and the complementary expression patterns suggest that SLIT may be a physiological ligand for ROBO.

MATERIALS AND METHODS

Isolation and Sequence Analysis of the Mouse Slit Family

The nomenclature that we have adopted for mouse Slit genes conforms to the published sequences of three human Slit cDNAs (Itoh et al., 1998). Two pairs of PCR primers based on homology to Drosophila slit (Rothberg et al., 1988) and Xenopus slit (Li et al., 1999) were used for RT-PCR from mouse E11.5 head RNA: primer pair 1, 5′-CTCACTG/TAGTACCCGACCAACAACAYC/T/AAAC-3′ (sense) and 5′-AGGCTACAACG/GGCTGG-3′ (antisense), and primer pair 2, 5′-TACCCGACCAACAACAYC/T/AAAC-3′ (sense) and 5′-TTACGGTCGTTATCCACCCAGCCACG/AA-3′ (antisense). The first pair of primers amplified a 1476-bp fragment. The second pair of primers amplified a 750-bp fragment. The two DNA fragments were subcloned and then used to screen a newborn mouse brain cDNA library (Stratagene, No. 936314). The clones identified using the 1476-bp probe encode mSlit1, while the 750-bp probe identified clones encoding mSlit3. Near full-length sequences were obtained by rescoring the library with probes made to the 5′ end of the identified fragments. The 5′ end including the initiation methionine of the mSlit1 cDNA was cloned using the 5′-RACE method (Gibco BRL). The 3′ end was identified by sequencing a mouse EST, clone mJTG011.1, obtained from the WashU-HMM1 Mouse EST project. To verify the sequence of the full-length mSlit1 expression construct, overlapping fragments were amplified by RT-PCR from E11.5 head RNA, subcloned, and then sequenced.

The 5′ end of the mSlit3 cDNA was cloned by two RT-PCR walks using a 3′-specific primer and a 5′ primer based on homology to Drosophila and Xenopus sequences and finally by 5′-RACE (Gibco BRL). During the RT-PCR walk using the primers shown below a PCR fragment with high homology to but distinct from mSlit1 and mSlit3 was obtained and hence named mSlit2. Primers were 5′-CGTGAGTGGGCTGCTTGGGCT-3′ (sense) and 5′-GCCGAGCAAGCAATCTCTTTTC-3′ (antisense). The full-length mSlit2 sequence was then cloned by similar methods. EST m38a09.1 was subsequently identified and corresponds to mSlit2.

Sequences were assembled and analyzed using Geneworks software (IntelliGenetics, Inc.) on a Macintosh computer and using the BLAST algorithm at the NCBI. The phylogenetic tree was constructed using the UPGMA method (Nei et al., 1985). Sequences used were Drosophila (Rothberg et al., 1988), Xenopus (Li et al., 1999), rat (Nakayama et al., 1998), and human (Itoh et al., 1998). Accession numbers for the three mouse slit genes are AF144627, AF144628, and AF144629.

Assembly of the mSlit1 cDNA and Expression Vector

The coding region for full-length mSlit1, obtained by RT-PCR amplification of mouse E11.5 head cDNA and primers with appropriate restriction sites, was cloned into a pGEM T vector (Promega, Inc.). The full-length cDNA was then assembled in frame in the pcDNA2 expression vector (Gibco). The 3′ end was identified by sequencing a mouse EST, clone mJTG011.1, and hence named mSlit2. Primers were 5′-CGTGAGTGGGCTGCTTGGGCT-3′ (sense) and 5′-GCCGAGCAAGCAATCTCTTTTC-3′ (antisense). The full-length mSlit2 sequence was then cloned by similar methods. EST m38a09.1 was subsequently identified and corresponds to mSlit2.

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Cell Culture and Binding Studies

HEK293T cells were maintained in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (Sigma). Cells were allowed to grow to 70% confluence on 6-cm culture dishes and were then transfected with 6 μg of plasmid DNA in the presence of Lipofectamine for 24 h according to the manufacturer's instruction (Gibco). Transfection efficiency was monitored by cotransfecting with a green fluorescent protein expression vector (pGL, Life Technologies, Inc.) and monitoring fluorescent cells under UV illumination.

For coimmunoprecipitation, plasmids encoding mSLIT1-myc or ROBO1-HA (Li et al., 1999) or the pcDNA2 expression vector, which contains six copies of the myc epitope tag (Rupp et al., 1994), using a PCR-generated linker encoding the 3′ end of the mSlit1 open reading frame. The full-length coding regions of mSlit1 and the continuity with the myc epitope tag were verified by sequencing (ABI, Inc.).
EDTA, 50 mM NaF, 1 mM Na3VO₄, 1 mM DTT, 1 mM PMSE, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 150 μg/ml benzamidine. Conditioned medium containing mSLIT1-myc was mixed with lysates from ROBO1-HA or control cells. Immunoprecipitation was carried out as described (Kopan et al., 1996), using anti-myc antibodies (Babco, Inc.) and protein A-Sepharose. Precipitated proteins were detected after Western blotting with anti-HA antibodies (Babco, Inc.) with enhanced chemiluminescence according to the manufacturer’s instructions (Amersham, Inc.).

Whole-Mount in Situ Hybridization

Whole-mount in situ hybridization was carried out as published (Henrique et al., 1995; Nieto et al., 1996) with minor modifications. Probes were labeled according to the manufacturer’s instructions using the DIG RNA labeling mix (Boehringer Mannheim). Probes longer than 1 kb were hydrolyzed by adding 60 μl bicarbonate buffer (60 mM Na2CO3, 40 mM NaHCO3, pH 10) at 65°C for 30 min to obtain ~500-nt fragments. The probes used for in situ hybridization were mSlit1, 1476-bp PCR fragment corresponding to position 556 to 2031 bp of the coding region; mSlit2, 1327-bp PCR fragment corresponding to position 114 to 1440 bp of the coding region; mSlit3, 1935-bp library fragment corresponding to position 556 to 2031 bp of the coding region; and rat Robo1, 4956-bp fragment containing the entire ROBO1 coding region (Kidd et al., 1998; Li et al., 1999).

Embryos were isolated from time-mated Swiss Webster mice counting the morning of the vaginal plug as embryonic day 0.5. More accurate staging was by comparison with atlas-staged embryos (Kaufman, 1995).Embryos were fixed in 4% paraformaldehyde at 4°C overnight. Embryos were dehydrated in a graded ethanol series (50%, 70%, 80%, 95%, 100%), and then incubated in 1 ml NTMT containing 3.4 μl/ml NBT (100 mg/ml in dimethyl formamide) and 3.5 μl/ml BCIP (X-phosphate; 50 mg/ml in 70% DMF) for several hours to 2 days.

For sections of whole-mount in situ hybridization-stained embryos, embryos were embedded in 7.5% low-melting-point agarose. Both whole embryos and sections were digitally imaged under stereomicroscopy (Zeiss).

Section in Situ Hybridization

Digoxigenin-labeled riboprobes were synthesized according to the manufacturer’s instructions (DIG RNA labeling kit; Boehringer Mannheim). Tissues were dissected out, embedded in OCT, frozen in dry ice–ethanol bath, and sectioned to 20 mm thick. Tissue sections were hybridized overnight at 58°C (0.2 μg/ml riboprobe, 50% formamide, 0.3 M NaCl, 20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 40 mM DTT, 50 μg/ml yeast tRNA, and 30 μg/ml denatured herring sperm DNA). Following hybridization, the slides were rinsed in 2× SSC for 15 min at room temperature, treated for 30 min at 37°C with 20 mg/ml RNase A in buffer A (10 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA), and sequentially washed in 2× SSC for 15 min at 37°C, 1× SSC for 15 min at 37°C, 0.1× SSC for 10 min at 37°C twice, 1× PBS (1× PBS with 0.1% Tween 20) for 5 min at room temperature. Following blocking at room temperature for 1 h, the slides were incubated with anti-DIG-AP-conjugated antibody (FAB fragment; Boehringer Mannheim) at 1:2000 dilution for overnight at 4°C. The signal was detected according to the manufacturer’s instructions (Boehringer Mannheim) using the nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate substrate solution.

RESULTS

Cloning and Sequence Analysis of Mouse Slit cDNAs

Two PCR primer pairs were designed according to regions of conserved sequence between Drosophila (Rothenberg et al., 1988) and Xenopus (Li et al., 1999) Slit. Amplification of reverse-transcribed E11.5 mouse head RNA yielded a 1476- and a 750-bp amplification product with the respective primer pair. Sequence analysis of these fragments showed homology to both Drosophila and Xenopus Slit cDNAs. These initial amplification products were used to screen a newborn mouse brain cDNA library. Several clones were identified and sequenced and were shown to encode closely related but nonidentical transcripts representing two Slit homologues, now named mSlit1 and mSlit3. The third Slit gene, mSlit2, was identified during an RT–PCR walk which was used to extend the 5’ end of mSlit3. 5’ sequences were

**FIG. 1.** Amino acid alignments of mouse SLIT proteins. Shaded boxes indicate amino acid identities among the three SLIT proteins. Each SLIT sequence contains a putative signal sequence (bold), four regions containing four to six tandem arrays of a typical 24-amino-acid LRR (underlined with a black bar, with six arrays in each of the first three LRRs and four arrays in the fourth LRR). There are also two groups of EGF-like motif repeats (six in the first group and three in the second, underlined with shaded bars), which are separated by a laminin-G domain (underlined with an open bar). The C-terminus contains a Cys knot structure (underlined with a hatched bar).
obtained by RACE cloning using E11.5 head RNA as the template.

mSlit1 encodes a 1531-aa open reading frame (ORF), mSlit2 encodes a 1521-aa ORF, and mSlit3 encodes a 1523-aa ORF (Fig. 1). Like Drosophila Slit, mSlit ORFs contain a signal peptide and thus are likely to be secreted proteins. Additionally, mouse SLIT proteins contain several conserved motifs such as four leucine-rich repeats; nine epidermal growth factor (EGF)-like sequences; a single laminin-G domain containing agrin, laminin, and perlecan homologies (termed ALPS) (Itoh et al., 1998); and a carboxy-terminal cysteine-rich region. These conserved motifs are likely to modulate potential homophilic interactions of SLIT proteins and heterophilic interactions with other soluble proteins and potential membrane receptor proteins.

Subsequent to our identification of three mouse Slit genes, two rat Slit homologues (Nakayama et al., 1998), two partial mouse Slit cDNAs (Holmes et al., 1998), and three human Slit sequences (Itoh et al., 1998) have become available. Sequence alignment of the predicted proteins showed an overall identity of 94–98% between the corresponding mouse, rat, and human sequences. Phylogenetic analysis using the UPGMA algorithm shows that the single identified Slit gene from Xenopus laevis is most closely related to mammalian Slit2 and that Drosophila Slit is equidistant from all three vertebrate homologues, suggesting a common evolutionary origin for the Slit gene family (Fig. 2).

**Expression of Mouse Slit and Robo1 during Embryonic Development**

**Expression of mouse Slit1.** The expression of mSlit1 was examined between 8.5 and 17.5 days of mouse embryogenesis (E8.5–E17.5). During this period of development, mSlit1 expression was primarily observed in neural tissues. In the developing spinal cord, mSlit1 was detected in both the roof plate and the floor plate between E8.5 and E12.5 (Figs. 3 and 4). Beginning at E13.5 the expression in roof plate became weak. At this stage, relatively weak expression was also observed in the motor columns, dorsal root ganglia (DRG), and commissural neurons (Fig. 3D).

Rostrally, from E8.5 to 9.5, mSlit1 could be detected in the neural fold overlying the fourth ventricle and midbrain (Figs. 4B–4D). By E10.5 to 11.5 intense expression was observed in the rhombic lip and in the midline of the developing tectum (Figs. 5A–5C). In the developing brain from E13.5 to E17.5, mSlit1 was expressed in the cortex and in the medial and lateral ganglionic eminencies (Figs. 8A and 8E). At this stage mSlit1 was also expressed in the ventral diencephalon, dorsal mesencephalon, and ventral metencephalon (data not shown).

In other tissues and organs, between E8.5 and E9.5 mSlit1 mRNA was detected in the primordium of the branchial arches (Figs. 4A–4D). From E9.5 to 10.5, mSlit1 was expressed in a segmented pattern in the posterior dermamyotome (Figs. 5A, 6G, and 6J). By E11.5 the segmented expres-
Expression of mouse Slit genes. mSlit1 was prominently expressed in neural and mesodermally derived tissues. From E8.5 to 9.5, mSlit2 was expressed strongly in the roof plate, floor plate, and notochord (Figs. 3E, 4E, and 4H). Beginning at E10.5, intense expression was also observed in the motor columns (Figs. 3F and 3G). By E13.5, similar to mSlit1, the expression of mSlit2 decreased in the roof plate.
but was still retained in the floor plate and motor columns (Fig. 3H).

In the rostral CNS between E8.5 and E9.5, mSlit2 was expressed intensely in the dorsal neuroepithelium overlying the hindbrain, in the dorsal midline of the midbrain and forebrain, and in the ventral midbrain region (Figs. 4E–4H). By E10.5–11.5, additional intense expression was observed in the rhombic lip and the rostral midline (Figs. 5E–5H). From E13.5 to E17.5, the expression decreased dorsally and continued to be detected in the ventral mesencephalon and diencephalon (data not shown).

Between E8.5 and E9.5, mSlit2 expression was detected in the clefts between the first, the second, and the third branchial arches (Figs. 4F and 4H). From E10.5 to E11.5, expression was detected in the nasal pit, the developing eye, the otic vesicle, and the visceral grooves (Figs. 5E–5H). From E13.5 to E17.5, mSlit2 expression was observed in the developing cochlea (in a pattern consistent with expression in the organ of Corti), in the olfactory epithelium, and, similar to mSlit1, in the inner neuronal layer of the retina and in the optic nerve (Figs. 7B, 8B, and 8F). At this stage, mSlit2 was also expressed in the tongue, in the tooth primordium (Fig. 8B), and in the outer root sheath of the whisker follicle in the layer surrounding the bulb (Fig. 7E).

At E11.5, mSlit2 was intensely expressed in the rostral lateral ridge flanking the forelimb buds and in lateral ridge tissue between the fore- and the hindlimb buds (Fig. 5G). Weak expression was observed in a segmented pattern in the posterior part of the sclerotome (Figs. 6H and 6K). Expression was notably absent in the base of the limb buds and weak expression was observed in the interdigital regions of the distal limb bud beginning at E11.5 (Fig. 6A). By E13.5 intense expression was observed in interdigital mesenchyme (Fig. 6D).

Expression of mSlit3. mSlit3 was detected as early as E8.25 in the ventral neural tube (Fig. 3I). From E9.5 to E11.5, mSlit3 was expressed in the floor plate and motor columns (Figs. 3J and 3K); however, its expression was weaker than that of mSlit2 (Figs. 3F and 3G). This pattern of expression continued until at least E17.5 and remained weak (Fig. 3L and data not shown).

Rostrally, at E8.25 mSlit3 was expressed in the ventral midline of the neural groove and in the neural fold prior to closure of the neural tube (Fig. 4I). Between E8.5 and E9.5, expression was observed in the ventral side of the mesencephalon and metencephalon and in the commissural plate after closure of the neural tube (Figs. 4J and 4K). After E13.5, the expression of mSlit3 was weak in the developing CNS.

At E9.5, mSlit3 was detected in the otic vesicle and in the clefts between the first and the second branchial arches (Fig. 4L). This expression pattern was similar to that of mSlit2 at this stage of development. Between E10.5 and E11.5, mSlit3 was prominently expressed in the otic vesicle but decreased in the branchial clefts (Figs. 5I and 5J). From E13.5 to E17.5, mSlit3 expression was observed in the cochlea, in the pigment layer of the retina, and in the olfactory epithelium (Figs. 7C, 8C, and 8G). This pattern resembled that of mSlit2. At E13.5 mSlit3 expression was observed in the whisker follicle surrounding the bulb and shaft (Fig. 7F).

In the developing limb, mSlit3 was first detected at E10.5 in distal limb bud mesenchyme (Fig. 5I). At this stage, mSlit3 was also observed in lateral ridge tissue flanking the limb bud (Fig. 5I). This pattern persisted through E11.5 but unlike with mSlit2, expression was not observed in the inter-limb bud lateral ridge tissue (Fig. 5J). At E11.5, expression of mSlit3 was first detected in both the fore- and the hindlimb was most intense in the distal anterior mesenchyme and in the proximal posterior cleft between the limb bud and the lateral ridge (Figs. 5J and 5B). At E13.5, mSlit3 could be detected in the wrist and weakly in palm and proximal part of the digits excluding the tips of the digits (Fig. 6E).

Expression of Robo1. The three Slit genes and Robo1 exhibited somewhat complementary patterns of expression throughout development. In contrast to the expression pattern of the three Slit genes in the spinal cord, Robo1 was never expressed in either the floor plate or the roof plate at any stage of development (Figs. 3M–3P and data not shown). However, beginning at E10.5, Robo1 expression was observed in the spinal cord in a population of cells that comprise commissural and motor neurons (Figs. 3O and 3P). This pattern of expression was similar to but more widespread than that observed by Kidd et al. (1998a).

At E8.75 Robo1 was expressed in the midbrain in the ventricular zone (Figs. 4M and 4N). By E9.5 Robo1 expression was also detected in the ventricular zone of the telencephalon (Fig. 4P). Between E10.5 and E11.5, weak expression was still observed in the midbrain and in the groove between the nasal process and the telencephalon (Figs. 5K and 5L). At E15.5, Robo1 was prominently expressed in whole embryos between E8.5 and E9.5. (A–D) mSlit1 mRNA was detected in the roof plate (rp), neural fold (nf), and floor plate (fp) of the spinal cord and in the primordium of the branchial arches. (E–H) mSlit2 mRNA was detected in the dorsal neuroepithelium overlying the hindbrain (neural fold), the ventral midbrain (vm), the tectum (tc), and the commissural plate (cp). mSlit2 was also expressed in the optic eminence (ey in H) and in the clefts between the first (I), the second (II), and the third branchial arches (F, H). (I–L) mSlit3 mRNA was detected in the ventral midline of the neural groove (floor plate) and the commissural plate before and after closure of the neural tube (I–K). At E9.5 mSlit3 expression was observed in the otic vesicle (ov) and in the clefts between the first and the second branchial arches (L). (M–P) Robo1 mRNA was detected in the midbrain ventricular zone (m), in the otic vesicle, and in the distal tip of the first branchial arch (M, N). By E9.5 Robo1 formed a ring outlining the otic vesicle and was prominently expressed in the first branchial arch and ventricular zone (ov) of the telencephalon (O, P).
FIG. 5. Expression of Slit and Robo1 in whole embryos between E10.5 and E11.5. (A–D) mSlit1 mRNA was detected in the roof plate (rp), in the tectum (tc), and in a segmented pattern in the posterior somites. Weak expression was also observed in the nasal pit at E11.5 (D). (E–H) mSlit2 mRNA was detected in the roof plate and floor plate (fp) of the spinal cord, the rhombic lip (rl), the tectum, the commissural plate, the nasal pit (np), the eye (ey), the otic vesicle (ov), and the visceral grooves. At E11.5, intense expression was observed in lateral ridge tissue (lr) flanking the limb buds and in lateral ridge tissue between the fore- and hindlimb buds (G). Weaker expression was observed in a segmented pattern in somites (G). (I, J) mSlit3 mRNA was detected in distal limb bud (lb) mesenchyme and in lateral ridge tissue flanking the limb bud. At E11.5 (J), expression of mSlit3 in both the fore- and the hindlimb was most intense in the distal anterior mesenchyme and...
pressed in the outer layers of the cerebral cortex and the intermediate zone of the ganglionic eminence (Fig. 8D).

At E8.75, Robo1 was expressed in the otic vesicle and in the distal tip of the first branchial arch (Figs. 4M and 4N). This expression pattern persisted and by E9.5 Robo1 formed a ring outlining the otic vesicle and was prominently expressed within the first branchial arch (Figs. 4O and 4P). By E10.5 expression was prominent in both the mandibular and the maxillary components of the first branchial arch and in tissue surrounding the nasal process (Fig. 5K). By E11.5 expression was prominent in the branchial arch (Fig. 5L). From E13.5 to E17.5, Robo1 was also expressed in the trigeminal ganglia, in the olfactory epithelium, and in the inner nuclear layer of the retina (Figs. 7D, 8D, and 8H).

Robo1 was also expressed in the whisker follicle although, in contrast to mSlit2 and mSlit3, the expression was within the bulb, rather than surrounding it and also weakly in dermal papilla and in the hair root (Fig. 7G).

In the limb bud, Robo1 expression was prominent in proximal mesenchyme at E11.5 and appeared complementary to the expression pattern of mSlit2 and mSlit3 in the distal limb bud (Figs. 6D–6F) and to that of mSlit2 and mSlit3 in lateral ridge tissue (compare expression in Figs. 5G, 5J, and 5L). In the somites, Robo1 expression included the entire dermamyotome except within the intersomite boundary from E9.5 to E11.5 (Figs. 3M, 3N, 5K, and 6L).

However, by E11.5 Robo1 was expressed in a segmented pattern in the anterior sclerotome (Figs. 5L, 6I, and 6L), which is complementary to the expression pattern of mSlit2 in the posterior sclerotome (compare Figs. 6H and 6I).

Interaction between mSlit1 and Robo1
Recent work demonstrated that Xenopus Slit (ortholog of mSlit2) interacts with ROBO1 (Li et al., 1999). Furthermore, the expression patterns and phenotype of Drosophila Slit and Robo mutants suggest that these molecules may interact (Kidd et al., 1998a; Rothberg et al., 1990). The complementary expression patterns (see below) of the three mouse Slit genes and of ROBO1 are also suggestive of a signaling pathway between these molecules. To test whether mSLIT1 can interact with ROBO1, both molecules were engineered to contain unique epitope tags and were assayed for interaction by immunoprecipitation and Western blotting. Six copies of the myc epitope were fused to the C-terminus of mSLIT1 and the HA epitope was fused to the C-terminus of rat ROBO1. Following transient expression in HEK293 cells, mSLIT1-MYC protein could be recovered and detected when coimmunoprecipitated in the presence of ROBO1-HA and anti HA antibodies (Fig. 9, lane 3). Controls in which either ROBO1-HA was not present (Fig. 9, lane 2) or the HA antibody was left out (Fig. 9, lanes 4 and 5) failed to coimmunoprecipitate mSLIT1-MYC. These data demonstrate that mSLIT1 and ROBO1 can directly interact with each other in vitro.

SLIT Proteins Interact with ROBO1
The interaction between mSLIT1 and ROBO1 in vitro suggests a ligand-receptor relationship between these molecules. This relationship is supported by similar phenotypes in Drosophila Slit and Robo mutants and by the complementary expression pattern of Slit and Robo1 during mouse development. Subsequent to the submission of this paper, recent studies have demonstrated the ligand-receptor relationship between ROBO and SLIT (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999).

Binding studies show that both SLIT1 and SLIT2 can bind ROBO1, suggesting that multiple SLIT molecules are capable of interacting with a single ROBO receptor (Brose et al., 1999; Li et al., 1999, our data). In mammals two Robo genes have been identified (Kidd et al., 1998a), allowing for possible combinatorial interactions of varying specificity.
and the potential for functional redundancy. The overlapping expression patterns of the three Slit genes in specific tissues are remarkable (see, for example, expression in the spinal cord, somite, eye, and septum) and imply redundancy of function at these sites. In an in vitro explant culture experiment, we found that mSLIT1-expressing cells, like

![Image of expression patterns in the forelimb bud and somites](A-D) mSLIT2 expression in the interdigital region of the limb bud at E11.5 (arrow in A) and E13.5 (D). mSLIT2 was also expressed in lateral ridge tissue at E11.5 (arrowhead in A). Cross section showing mSLIT2 within the mesodermal compartment of the interdigital region at E13.5 (arrow in D inset). (B) mSLIT3 expression in the anterior (left) tip of the limb bud at E11.5. At E13.5, mSLIT3 was weakly expressed in the wrist and superficial layers proximal to the wrist (E). (C) Robo1 expression in the proximal part of the limb bud at E11.5. By E13.5, Robo1 is expressed at the tip of the digits (F), shown in cross section in inset (arrow). Large arrowheads in D and F indicate the plane of section shown in insets. In A–F anterior is on the left. (G–I) Sagittal cross sections through somites. (G, J) mSLIT1 expression in a segmented pattern in the posterior part of the dermamyotome at E10.5. (H, K) mSLIT2 expression in the posterior part of the sclerotome at E11.5 (arrowhead in K). (L) Robo1 expression in dermamyotome (open arrowhead) and medial and lateral sclerotome (filled arrowheads). (I) Sagittal section localizing Robo1 expression in the anterior somite. sc, spinal cord; a, anterior; p, posterior.

*FIG. 6.* Expression of Slit and Robo1 in the forelimb bud and somites. (A, B) mSLIT1 and mSLIT2 expression in the neural layer (especially the inner nuclear layer of the retina) and weakly in the lens epithelium. (B) Also shown is mSLIT2 expression in the optic nerve (arrow). (C) mSLIT3 expression in the pigment layer of the retina and lens epithelium. (D) Robo1 expression in the inner nuclear layer of the retina and weakly in the optic nerve (arrow). (E-G) Expression in the whisker follicle. (E, F) mSLIT2 and mSLIT3 expression in the outer root sheath along the shaft and the layer surrounding the bulb. (G) Robo1 expression in the bulb and weakly in the dermal papilla and in the hair root.

*FIG. 7.* Expression of Slit and Robo1 in the eye and whisker follicle. (A–D) Expression in the developing eye. (A, B) mSLIT1 and mSLIT2 expression in the neural layer (especially the inner nuclear layer of the retina) and weakly in the lens epithelium. (B) Also shown is mSLIT2 expression in the optic nerve (arrow). (C) mSLIT3 expression in the pigment layer of the retina and lens epithelium. (D) Robo1 expression in the inner nuclear layer of the retina and weakly in the optic nerve (arrow). (E-G) Expression in the whisker follicle. (E, F) mSLIT2 and mSLIT3 expression in the outer root sheath along the shaft and the layer surrounding the bulb. (G) Robo1 expression in the bulb and weakly in the dermal papilla and in the hair root.
FIG. 8. Expression of Slit and Robo1 in the head between E14.5 or 15.5 (A–D) and at E17.5 (E and F). (A, E) mSlit1 expression in the cortical plate (cp), medial and lateral ganglionic eminence (lge), neural layer (especially inner nuclear layer) of retina (nl), and trigeminal ganglia (tg) and weakly in the olfactory epithelium (oe). (B, F) mSlit2 expression in neural layer of the retina, tongue (tn), olfactory epithelium, tooth...
Xenopus SLIT-expressing cells, can also repel olfactory axons (data not shown). This suggests that the functional redundancy is consistent with the ability of multiple SLIT ligands to interact with a single ROBO receptor.

In contrast to vertebrates only one Slit gene has been identified in Drosophila, yet two Robo genes have been found (although the function of ROBO2 has not been genetically characterized) (Kidd et al., 1998a). If this is the extent of the family in Drosophila, it may explain the observation that the Drosophila Slit mutant has a more severe phenotype than the Drosophila Robo1 mutant (Kidd et al., 1998a) and predicts that Drosophila SLIT should be able to interact with either ROBO molecule to regulate diverse biological processes. Future studies will be required to determine the relative binding affinities and specificities of vertebrate SLIT molecules for ROBO1 and ROBO2 and the significance of the specificities in different tissues and at different embryonic stages.

The Slit Family May Function in Midline Development and as a Midline Axon Repellent

Phylogenetic analysis shows that the Slit gene family is highly conserved between Drosophila and vertebrates. In many cases the conservation in structure is consistent with the maintenance of function throughout evolution. Interestingly, hSlit3 was found to colocalize with the disease arthrogryposis multiplex congenita neurogenic type (AMCN) on chromosome 5q35 (Nakayama et al., 1998). Severely affected newborns show defects in neuronal migrations in the spinal cord and in the cerebral cortex (Brodkorb et al., 1994). Addressing a role for SLIT in neuronal migration will help to determine whether mutations in Slit3 cause AMCN.

Bilateral symmetry is a conserved property from insects to vertebrates. To initiate and maintain bilateral symmetry, it is necessary to define the midline and coordinate development with respect to the midline axis. In Drosophila, the Slit mutation affects midline development and results in embryonic lethality (Kidd et al., 1998a,b; Rothberg et al., 1988, 1990). However, it is still controversial whether SLIT functions in midline development. Kidd et al. suggest that SLIT does not control midline cell fate and differentiation but functions only in axon guidance (Kidd et al., 1999). In a similar genetic study in Drosophila, Battye et al. suggest dual functions for SLIT: in axon guidance and in establishing midline cytoarchitecture (Battye et al., 1999). The three mouse Slit homologues are expressed consistently in the floor plate of the neural tube, which corresponds to the midline in Drosophila. These expression patterns and recently published data on SLIT activity as a neuronal repellent (Brose et al., 1999; Li et al., 1999) suggest that mouse SLIT proteins are likely to share some functional properties with their Drosophila homologue in midline neural development.

Possible Roles of Slit and Robo in Axon Pathfinding and Neural Crest Cell Migration

mSlit2, mSlit3, and Robo1 are expressed in the motor neuron columns between E10.5 and E13.5, while mSlit1 is expressed weakly in the dorsal root ganglia and throughout the spinal cord. These expression patterns suggest possible roles in midline development and axon guidance. Future studies will be required to determine the relative binding affinities and specificities of vertebrate SLIT molecules for ROBO1 and ROBO2 and the significance of the specificities in different tissues and at different embryonic stages.
roles for SLIT and ROBO in the development and maintenance of motor and sensory neurons.

The subdivision of sclerome into anterior and posterior components is well known. Morphologically, it is further reflected by the presence of a visible boundary (von Ebner's fissure) (Christ and Ordahl, 1995). The posterior sclerome inhibits axon passage and neural crest cell migration, while the anterior sclerome is permissive. This is due largely to an inhibitory activity that renders the posterior half of the sclerome inhospitable to axon growth (Tannahill et al., 1997). SLIT proteins may act as repulsive molecules in the guidance of motor neurons because Slit and Robo are expressed in complementary patterns in the sclerome: mSlit2 is expressed in the posterior sclerome while Robo1 is expressed in the anterior sclerome.

Rostrocaudal segmental subdivision is not only restricted to the sclerome but also extends into the dermamytome. Interestingly, two cholinesterases are differentially expressed in rostral and caudal dermamytome. This differential expression is hypothesized to be important in motor axon guidance (Layer et al., 1988). In this study we observed mSlit1 expression specifically in the posterior dermamytome, suggesting that it may function as a diffusible repellant in this tissue.

Neural crest cells appear to migrate without the guidance of long-range cues, but respond to permissive or nonpermissive substrates in the extracellular matrix (Henderson and Copp, 1997). In mice, trunk neural crest cells initially appear between E8.5 and E11 (Erickson and Weston, 1983; Sendtner et al., 1990; Serbedzija et al., 1994; Wehrle-Haller et al., 1996). Neural crest cell migration follows three possible pathways: ventral medial and ventral lateral, through the anterior sclerome and dorsal lateral, lateral to the dermamytome (Henderson and Copp, 1997). The expression of mSlit2 in posterior sclerome and Robo1 in anterior sclerome at the time of neural crest cell migration suggests that these molecules may also mediate the migration of neural crest cells.

Motoneurons are born on embryonic days 9.5–11 in mice (Ornnes and Cary, 1978) and extend their axons into the periphery as early as E10 (White et al., 1996). Interestingly, mSlit1 and mSlit2 are expressed at this time in the posterior somites. Furthermore, recent studies demonstrate the ability of SLIT proteins to repel motor neuron axons in vitro (Brose et al., 1999). Other molecules are also expressed in the posterior sclerome. These include the 48K and 55K peanut lectin binding proteins, T-cadherin, collagen IX, versican, F-spondin, ephrins, and semaphorins (Tannahill et al., 1997; Debby-Brafman et al., 1999). Ephrins and semaphorins have also been implicated as guidance cues in the somites (Adams et al., 1996; Kikuchi et al., 1997; Krull et al., 1997). The existence of multiple repulsive cues may therefore be required for the accurate guidance of different types of axons and neural crest cells.

The expressions of mSlit2, mSlit3, and Robo1 in the limb suggest that these molecules may guide axons that innervate the limb. Embryonic tissue manipulations demonstrate that the somites and the pelvic girdle precursor are critical for the navigation of axons as they approach the limb (Tosney, 1991). mSlit2 is expressed in lateral ridge tissue (also known as the prospective pelvic girdle), which is a known barrier for axon penetration. The pelvic girdle tissue is discontinuous and has two holes (containing plexus mesenchyme) that allow axons to enter the limb and form nerve trunks (Tosney, 1991). The expression of mSlit2 in the lateral ridge tissue flanking the limb bud suggests that SLIT may also serve as a repellant in this tissue.

**Slit and Robo in Limb Bud Development**

mSlit2, mSlit3, and Robo1 are expressed in distinct and dynamic patterns in the developing limb. The vertebrate limb originates from the lateral plate and somitic mesoderm (Johnson and Tabin, 1997). Unlike FGF8, which is expressed in the most distal ectoderm before limb bud initiation (Crossley et al., 1996), expression of Slit2, Slit3, and Robo1 begins after the emergence of the limb bud. Therefore, it is unlikely for SLIT to be involved in the early initiation process.

An important feature in limb development is the establishment of the three limb axes. Sonic hedgehog (SHH), which is expressed in the zone of polarizing activity (at the posterior distal margin of the limb bud), regulates anterior/posterior patterning (Riddle et al., 1993). Interestingly, mSlit3 is initially (E10.5) expressed in the distal tip of the limb bud and then by E11.5 becomes localized to anterior mesenchyme. This refinement of expression pattern of mSlit3 suggests that mSlit3 transcription may be regulated by A–P acting molecules such as SHH.

In addition to the expression of mSlit3 in the anterior region of the limb bud at E11.5, mSlit2 begins to be expressed in the interdigit region at this stage and remains intensely expressed through E13.5. These regions of SLIT expression are coincident with the anterior necrotic zone and the interdigit necrotic zone, regions that are known to undergo programmed cell death during limb development (no SLIT expression was observed in the posterior necrotic zone). The expression of Slit genes in these regions could prevent axon innervation in these zones and thus guide axons to the digits. Alternatively, SLIT may participate with bone morphogenetic proteins in the regulation of programmed cell death in these regions. Further study of the relationship between SLIT, BMPs, and SHH will help to address this issue. Notably, the complementary pattern of Robo1 expression in the tip of the digits at E13.5 is consistent with the former hypothesis.

Slit genes and Robo1 are also expressed in a variety of tissues and organs including the CNS and the developing sensory organs, such as the eye, ear, nose, and whisker follicles. Slit expression was also observed in some nonsensory organs such as the thymus, and some ESTs encoding mSlit3 were obtained from mammary gland libraries (e.g., ve37e06.r1 and ve77f03.r1). The expression of Slit genes in
these nonneuronal tissues suggests that SLIT may have other functions in addition to axon guidance.

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