**Caenorhabditis elegans** PlexinA, PLX-1, interacts with transmembrane semaphorins and regulates epidermal morphogenesis

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**SUMMARY**

The plexin family transmembrane proteins are putative receptors for semaphorins, which are implicated in the morphogenesis of animal embryos, including axonal guidance. We have generated and characterized putative null mutants of the *C. elegans* plexinA gene, *plx-1*. *plx-1* mutants exhibited morphological defects: displacement of ray 1 and discontinuous alae. The epidermal precursors for the affected organs were aberrantly arranged in the mutants, and a *plx-1::gfp* transgene was expressed in these epidermal precursor cells as they underwent dynamic morphological changes. Suppression of *C. elegans* transmembrane semaphorins, *Ce-Sema-1a* and *Ce-Sema-1b*, by RNA interference caused a displacement of ray 1 similar to that of *plx-1* mutants, whereas mutants for the *Ce-Sema-2a/mab-20* gene, which encodes a secreted-type semaphorin, exhibited phenotypes distinct from those of *plx-1* mutants. A heterologous expression system showed that *Ce-Sema-2a* and PLX-1 both play roles in the regulation of cellular morphology during epidermal morphogenesis, they function rather independently.

Key words: *C. elegans*, Plexin, Semaphorin, Epidermis, Cell arrangements

**INTRODUCTION**

Morphogenesis in animals involves specific changes in cell shape and position. Several external signals have been identified to modulate the morphology and motility of cells by regulating various cellular properties such as adhesion and cytoskeletal organization. Members of the semaphorin protein family have been implicated as extrinsic guidance cues during the development of the nervous systems (Raper, 2000). The semaphorin family comprises a large number of secreted and transmembrane proteins classified into seven classes; class 1 and 2 in invertebrates and class 3 to 7 in vertebrates, all characterized by the signature sema domain of 500 amino acid residues (The Semaphorin Nomenclature Committee, 1999). Vertebrate sema3A/collapsin, a founding member of the semaphorin family, was first identified as a potent chemorepellant for growing axons in vitro (Luo et al., 1993). Sema3A collapses growth cones of a subset of neurons by reorganizing their cytoskeleton (Fan et al., 1993; Fan and Raper, 1995). Analyses in *Drosophila* (Kolodkin et al., 1992; Kolodkin et al., 1993; Mattes et al., 1995; Yu et al., 1998) and vertebrates (Tanguchi et al., 1997; Shoji et al., 1998) have proved that members of the semaphorin family play important roles in the formation of neural circuits in vivo. While many members of the semaphorins are known to have repulsive activity, some are suggested to function as attractive cues for growing axons (Wong et al., 1997; Wong et al., 1999; Bagnard et al., 1998; de Castro et al., 1999). It has also been revealed that some semaphorins mediate biological functions outside of the nervous system (Hall et al., 1996), though their roles are less understood.

Recently, receptors for semaphorins have been identified (Nakamura et al., 2000). Transmembrane protein neuropilins were shown to bind to class 3 secreted-type semaphorins and to be necessary for mediating growth cone collapse (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Kitsukawa et al., 1997; Fujisawa and Kitsukawa, 1998). The intracellular region of neuropilins, however, was shown to be unnecessary for semaphorin signaling (Nakamura et al., 1998; Giger et al., 1998). Moreover, some invertebrate species, such as *Drosophila* and *C. elegans*, have no neuropilin gene in the genome, indicating that other receptors or intracellular proteins, which may interact with neuropilins, must be involved in semaphorin signaling. Quite recently, the plexins, a family of transmembrane proteins (Ohta et al., 1992; Ohta et al., 1995; Kameyama et al., 1996a; Kameyama et al., 1996b; Maestrini et al., 1996; Fujisawa et al., 1997), were found to serve as receptors for semaphorins. A viral semaphorin, Vaccinia A39R, was found to bind to Plexin C in the vertebrate immune system (Comeau et al., 1998), and biochemical and genetic interactions between class 1 semaphorins and plexinA were shown in *Drosophila* (Winberg et al., 1998). In
vertebrates, several transmembrane-type semaphorins were shown to bind to plexins directly (Tamagnone et al., 1999), and plexins were shown to form a functional receptor complex with neuropilins for class 3 semaphorins in cultured cells (Takahashi et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000; Takahashi and Strittmatter, 2001). More recently, CD72, a member of the C-type lectin superfamily was shown to be a receptor for CD100/Sema4D (Kumanogoh et al., 2000).

The C. elegans genome contains three semaphorin genes; two for the transmembrane semaphorin 1a and semaphorin 1b (Ce-sema-1a and 1b), and one for the class 2 secreted semaphorin 2a (mba-20/Ce-sema-2a) (Roy et al., 2000). The C. elegans genome also contains two plexin genes, plx-1 and plx-2. Compared with vertebrates, in which more than 20 semaphorins and at least nine plexins are present (Artigiani et al., 1999), this simplicity makes C. elegans an attractive system for the study of the semaphorin/plexin signaling system. Mutations in the gene mab-20/Ce-sema-2a cause various defects including embryonic lethality and abnormal body shape (Roy et al., 2000). These defects are the consequences of aberrant epidermal cell migration and ectopic cell contacts that affect the morphogenetic movement known as ventral enclosure. The arrangement of epidermal cells in mab-20 larvae is also altered, which leads to the fusion of sensory processes in the male tail called rays (Baird et al., 1991).

Interestingly, despite the prevailing notion that semaphorins and at least nine plexins are present (Artigiani et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000; Takahashi and Strittmatter, 2001). More recently, CD72, a member of the C-type lectin superfamily was shown to be a receptor for CD100/Sema4D (Kumanogoh et al., 2000).

Enzyme selection and deletion alleles for plx-1

To generate loss-of-function mutations in the plx-1 gene, we performed Tc1 transposon-mediated deletion mutagenesis using a mutator strain MT3126 according to a protocol described previously (Shibata et al., 2000). The mutants were out-crossed 10 times to N2.

Microscopic observation

For examination of GFP expression, worms were mounted on 4% agarose containing 1 mM levamisol and were examined with a Zeiss Axioskop microscope using Zeiss filter set #10. Images were recorded with a CCD camera (PXL camera system, Photometrix).

The boundary of epidermal cells was visualized by observing GFP expression using an insertion allele jcsI1[jam-1::gfp]. The monoclonal antibody MH27 recognizes an antigen at the adherens junctions (Francis and Waterston, 1991; Priess and Hirsh, 1986; Baird et al., 1991; Podbielwicz and White, 1994). The jcsI1 allele contains all known sequences required to target MH27 to the cellular junction, pRF4 and F35D3(unc-29+DNA) in an N2 background (Mohler et al., 1998). Most observations of mutant phenotypes were made with jcsI1; plx-1(nc37); him-5(e1490) animals. We have observed that plx-1(nc37); him-5(e1490) animals exhibited similar epidermal defects by immunostaining with MH27, confirming that the defects are caused by plx-1(nc37) by itself.

RNA interference

A Ce-sema-1a cDNA fragment (nucleotides 1-566) and a Ce-sema-1b cDNA fragment (nucleotides 1289-1951) subcloned into pBluescript SK (Stratagene) were amplified by PCR with primers CMo24 and CMo422 (Craig Mello, personal communication), and were used as templates for RNA synthesis with T7 RNA polymerase (Boehringer). Double-stranded RNAs (100 μg/ml) purified with an RNAeasy kit (Qiagen) and mixed with FITC dextran, were injected into the gonad of adult him-5 hermaphrodites, and F1 progeny were examined.

Binding analysis

To produce the Ce-sema-1a ectodomain fused to the Fc fragment of human IgG heavy chain (Fc) and the human alkaline phosphatase (AP) (Ce-sema-1a-ΔC-Fc-AP), a cDNA fragment corresponding to amino acids 20-578 of Ce-sema-1a was inserted into pCEP-SYFcAP (a gift from Dr Mizuno) whose HindIII-BglII fragment corresponding to the rat Sema6a cDNA was removed. To produce Ce-sema-2a fused to Fc (Ce-sema-2a-Fc), a cDNA fragment corresponding to amino acids...
acids 20-658 of Ce-Sema-2a of was inserted into pEF-Fc (Nishimura et al., 1987; Mizushima and Nagata, 1990). The plx-1 cDNA was inserted into pCAGGS (Niwa et al., 1991). In all the expression constructs used in the binding assay, a native signal sequence and sequences immediately upstream of the translation initiation codon for C. elegans proteins were replaced with that of the mouse Sema3A (amino acids 1-25) (Puschel et al., 1995) and a vertebrate Kozak consensus sequence (CCACC), respectively (Kozak, 1992). PLX-1 was tagged with a Myc-specific sequence (GEQKLISEEDL) at the N terminus (Evan et al., 1985).

HEK293T cells were transfected with the plx-1-expression constructs, or with Ce-Sema-1a- or Ce-Sema-2a-expression constructs, using Trans Fast Transfection Reagent (Promega) and the calcium phosphate co-precipitation technique, respectively. Two days after transfection, culture medium containing Ce-Sema-1a-AC-Fc-AP or Ce-Sema-2a-Fc was collected, concentrated by ultrafiltration (Ultrafree-15 centrifugal Filter Device, Millipore) and added to transfectants expressing PLX-1. After incubation at 37°C for 60 minutes, the cultures were washed with fresh culture medium, fixed with 4% paraformaldehyde overnight at 4°C, and rinsed with TBST [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Tween 20]. Then the cultures were reacted with goat anti-human Ig-Fc conjugated with AP (20 g/ml, Cappel) in TBST containing skim milk (50 mg/ml) at room temperature for 1 hour. After a wash with TBST, the cultures were stained in NBT/BCIP solution (Boehringer) at room temperature for 1 hour. Then, two deletion alleles, nc36 and nc37, were isolated. nc36 deleted 5443-9668 including the entire exon1, which contained the sequences corresponding to 53 amino acid residues at the N terminus. nc37 deleted 6963-14576 including exon 1-exon 4, which contained the sequences corresponding to 235 amino acid residues at the N-terminus. We have not detected gross morphological defects in the nervous system. Instead, close examination revealed that the mutants have defects in epidermal morphogenesis, including the formation of rays embedded within a cuticular spade-shaped fan. In adult males, the anterior-most ray was displaced anteriorly with high penetrance (Fig. 2A). NC36 deleted 5443-9668 including the entire exon1, which contained the sequences corresponding to 53 amino acid residues at the N terminus. nc37 deleted 6963-14576 including exon 1-exon 4, which contained the sequences corresponding to 235 amino acid residues at the N-terminus. We have not been able to examine the genetic nature of the mutations by using chromosomal deficiencies deleting the relevant region of LGIV, as such deficiencies are currently not available. However, lacking the predicted initiator methionine, the signal sequence as well as most of the sema domain, plx-1(nc37) is likely to be null molecularly and was mainly used in the subsequent phenotypic analysis.

Animals homozygous for both deletion mutations are healthy and viable. They are fertile, but their brood size is slightly reduced [N2: 338±8; plx-1 (nc36): 270±19; plx-1 (nc37): 244±11 (n=10)]. We have not detected gross morphological defects in the nervous system. Instead, close examination revealed that the mutants have defects in epidermal morphogenesis, including the formation of rays, seam cells and vulvae. In this paper, we will focus on the defects of rays and seam cells.

Ray 1 is displaced in plx-1 mutants

The adult male tail is a specialized structure used for copulation, and contains a set of nine male-specific genital sensilla (simple sense organ) known as rays embedded within a cuticular spade-shaped fan. In adult plx-1 males, the anterior-most ray was displaced anteriorly with high penetrance (Fig. 3B,D; Table 1). The displaced ray was thin and had an opening on the dorsal surface of a fan, which are morphological

**RESULTS**

**Structure of the plx-1 gene and the plx-1 cDNA**

A BLAST search (Altschul et al., 1994) of C. elegans genome sequences provided by the C. elegans Sequencing Consortium uncovered 2 genes, Y55F3AL.1 and K04B12.1, with high homology to the mouse PlexinA2 cDNA (Kameyama et al., 1996a). We and our colleague (J. Culotti) propose to name the genes plx-1 and plx-2, respectively. The plx-1 gene on the YAC clone Y55F3 was mapped to the left arm of LGIV, and consisted of 25 exons including a splicing leader SL1 (Fig. 1C).

We reconstructed a full-length plx-1 cDNA by combining a cDNA clone yk535f1 encoding the C terminal region of PLX-1 with the 5’ RACE products. The plx-1 cDNA has a splicing leader sequence 1, SL1, and contains an open reading frame of 5853 bp encoding a polypeptide of 1951 amino acid residues, which conserves the authentic features of Plexin A (Fig. 1A,B). PLX-1 has a sema domain (27-555), three MET-related sequence (MRS) repeats (503-555, 651-709, 830-881), three glycine-proline-rich (G-P) repeats (884-911, 980-1007, 1062-1089) and an intracellular domain, which is also well conserved (about 50% identical to mouse PlexA2) (Fig. 1B).

**plx-1::gfp is expressed in a subset of epidermal cells and neurons**

First, we examined the expression of plx-1 using a reporter transgene. A PCR fragment, spanning from –6kb upstream of the putative translation site to the eighth exon of the plx-1 gene, was fused in-frame to EGFP cDNA (Fig. 1C), and a transgenic line, him-5; ncEx[plx-1::egfp, rol-6(su1006)], was generated. EGFP expression was first observed at the lima bean stage in P and V epidermal cells and intestinal cells (data not shown). In larvae, EGFP was expressed intensely in motoneurons in the ventral nerve cord and several neurons in the nerve ring and in the tail. The seam cells showed moderate EGFP expression throughout development (Fig. 2E). In hermaphrodites, vulval precursor cells and their descendants expressed EGFP intensely throughout development. In the male tail, R(n) cells (Fig. 2A) and their descendants (Fig. 2C) all expressed EGFP intensely. Another transgenic line independently established with the same construct also showed the similar patterns of EGFP expression. The expression patterns of EGFP correlated well with the epidermal phenotype of plx-1 mutants. Whether the expression of the reporter gene faithfully represents the actual expression of the plx-1 gene, however, should be confirmed by other means, such as antibody staining or in situ hybridization, in future analyses.

The isolation and molecular characterization of plx-1 mutants

We have generated deletion mutations of the plx-1 gene by transposon-mediated mutagenesis. First, nc38::Tc1 was isolated in which a transposon, Tc1, was inserted at 7659–7660 of Y55F3AL, which is ~1.5kb upstream to the putative translation initiation site (9132) of the plx-1 gene (Fig. 1C). Animals homozygous for nc38::Tc1 had apparently no phenotype. Then, two deletion sites, plx-1(nc36) and plx-1(nc37), were isolated. nc36 deleted 5443-9668 including the entire exon1, which contained the sequences corresponding to 53 amino acid residues at the N terminus. nc37 deleted 6963-14576 including exon 1-exon 4, which contained the sequences corresponding to 235 amino acid residues at the N-terminus. We have not been able to examine the genetic nature of the mutations by using chromosomal deficiencies deleting the relevant region of LGIV, as such deficiencies are currently not available. However, lacking the predicted initiator methionine, the signal sequence as well as most of the sema domain, plx-1(nc37) is likely to be null molecularly and was mainly used in the subsequent phenotypic analysis.

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characteristics of normal ray 1, indicating that it is a displaced ray 1. Often, ray 1 was located outside of a fan, and the tip of the ray was located laterally, rather than ventrally, on the body wall (class I defect) (Fig. 3B). The displaced ray was usually short and a small fan-like structure formed around it (Fig. 2). In some cases, ray 1 remained in a fan, but was no longer associated with neighboring ray 2 (class II defect) (Fig. 3B). The other rays appeared normal. The phenotypic traits were rescued by a *plx-1*(*+*) transgene (Table 1). Animals heterozygous for *plx-1*(nc37) showed weak ray 1 displacement defects, indicating that *plx-1* may be haplo-insufficient. As the positions of adult rays are determined by the site of...
attachment of ray precursor clusters to the surface in larvae (Baird et al., 1991), we next examined ray precursor cells in plx-1 mutants. The epidermal development of the male tail has been studied extensively by Sulston et al. (Sulston et al., 1980) and Emmons and his colleagues (reviewed by Emmons and Sternberg, 1997), and will be briefly summarized here. Nine ray precursor cells, R(n) cells, are generated by specialized epidermal cells (seam cells) on each side of the posterior body of larval males. Each ray precursor cell, after several divisions, gives rise to a ray precursor cluster comprising three cells, which later differentiate into two neurons and one support cell of a mature ray. Each ray precursor cell also produces R(n), which fuses later with other Rn.p.s to form a multi-nucleated cell called the tail seam. During these processes, cells change their shape and position dynamically, suggesting that they are arranged actively through specific ray cell-epidermal cell interactions (Baird et al., 1991; Emmons and Sternberg, 1997).

When the positions of cells during morphogenesis for the male tail were examined by visualizing the adherence junctions of epidermis with a jam-1::gfp transgene (jcIs1), the processes of ray precursor cluster 1 were often found to be located anteriorly in plx-1 animals, sometimes just posterior to the junction of the body seam and the tail seam, at the middle L4 or later stages when R1.p and R2.p had already fused (Fig. 4D). Before the fusion of Rn.p.s, the processes of a ray precursor cluster are localized to the site associated with the junction of three or more epidermal cells, Rn.p.s and hyp7, which surround the ray precursor cluster as described by Baird et al. (Baird et al., 1991) (Fig. 4A). As this raises the possibility that the position of the junctional site between R1.p, R2.p and hyp7 might be affected in plx-1 mutants, we examined the precursors at the stage when R1.p and R2.p had yet to fuse or were just in the process of fusing. We found that R1.p was abnormally small, and the boundary between R1.p and R2.p shifted anteriorly in plx-1 mutants [plx-1(nc37): 88%, n=104, N2: 0% (e1490)]. Thus, at least in some cases, an abnormality in R1.p shape may lead to the aberrant positioning of ray precursor cluster 1. We failed to detect any abnormality in the position and morphology of ray 1 precursors when the processes of ray precursors were still thick.

Seam cells are separated by gaps in plx-1 mutants

Another phenotype of plx-1 mutants is missing seam cells. We found that the alae, cuticular structures running longitudinally along the lateral surface of the body wall, are often discontinuous in plx-1 mutant adults (Fig. 5C). As seam cells underlying the cuticle secrete the alae (White, 1988), we

Table 1. Ray 1 displacement in plx-1, Ce-sema-1a(RNAi) and Ce-sema-1b(RNAi) animals

<table>
<thead>
<tr>
<th>Animal</th>
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<tr>
<td></td>
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<td>8</td>
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<td>mab-20(bx24)</td>
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<td>mab-20(bx24); plex-1(nc37)</td>
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<tr>
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<td>Ce-sema-1a(RNAi); Ce-sema-1b(RNAi)*</td>
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<td>36</td>
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</table>

The percentage of sides of male tails with a displaced ray 1 is shown. The defects are divided into class I (strong) and class II (mild), according to the extent of displacement (see text for detail). All strains contained him-5 (e1490).

*All the progeny laid later than 1 day after RNA injection were scored.

† Only progeny that were fluorescent with FITC-dextran at the L1 stage were collected and scored later. We observed many fluorescent embryos that failed to hatch. Similar defects were observed when using other RNAs, indicating the nonspecific toxicity caused by a large amount of exogenous RNA.
examined seam cells by visualizing their boundaries with surrounding hypodermal cells in the jcIs1 background. The boundaries, which formed continuous parallel lines in wild-type animals (Fig. 5B), had a gap in plx-1 mutants (Fig. 5D) where the alae were missing, indicating that seam cells were missing in that region. Some 18% (n = 100) of the sides of the plx-1(nc37); him-5 adult males had a gap in the seam cell queue, compared with 0% (n = 100) of control him-5 adult males. We also have observed an extra cell boundary within a seam cell (Fig. 5F), where the alae made an abnormal bifurcation (Fig. 5E).

Next, we examined larval seam cells, which are precursors of adult seam cells and produce epidermis forming the lateral body wall of larvae (Sulston and Horvitz, 1977; White, 1988). Seam cells lose contact with each other as they go through cell division at the beginning of each larval stage. While the non-stem daughters fuse with the surrounding hyp7 syncytium, the seam stem cells elongate longitudinally to reach both anterior and posterior cells, and regenerate a continuous row of seam cells (Austin and Kenyon, 1994; Podbilewicz and White, 1994). Visualization with the jam-1::gfp transgene revealed that larval seam cells formed a continuous chain of cells arranged anteroposteriorly along the body wall in wild-type animals (Fig. 5G). However, in plx-1 mutants, the arrangement of seam cells was often disrupted (Fig. 5H). Cells sometimes formed dorsoventral contacts with neighboring cells. While this led to extended contact areas on one side of the cell, in some cases, cells concomitantly failed to make contact with neighbors on the other side, resulting in a gap in a continuous row of cells; 80% of sides of plx-1(nc37) jcIs1; him-5 (n = 100) had gaps while 5% of those of jcIs1; him-5 (n = 100) had gaps at the late L2 stage. Similar defects were detected by using strains with syIs50, which expresses GFP in seam cells under the promoter of the cdh-3 gene and visualizes the entire profile of the cells (Pettitt et al., 1996). The frequency of sides with gaps in a row of the seam cells at the L4 stage was 41% (n = 126) in syIs50; plx-1 (nc37), 19% (n = 104) in syIs50, and 16% (n = 100) in syIs50; plx-1 (nc37); ncEx[plx-1(+), rol-6(su1006)].

**Fig. 3.** Male tail defects of plx-1 mutant, mab-20 mutant and Ce-sema-1 (RNAi) animals. All animals contain him-5. Anterior is towards the left. Arrows indicate ray 1 and arrowheads indicate ray 2. (A,B) DIC photomicrographs of ventral views of (A) a control and (B) a plx-1(nc37) mutant adult tail. In B, ray 1 on both sides show displacement defects but to different extents; the right ray 1 (arrow) is located outside of a fan (class I defect) and the left ray 1 remains in a fan (class II defect). (C-G) DIC photomicrographs of lateral views of a control (C), plx-1(nc37) (D), mab-20(bx24) (E), Ce-sema-1a, Ce-sema-1b (RNAi) (F) and mab-20(bx24); plx-1(nc37) (G) animal. In the mab-20(bx24) animal (E), ray 1 fused to ray 2, and rays 3-5 fused together. The Ce-sema-1a, Ce-sema-1b (RNAi) animal (F) shows displacement of ray 1 similar to the plx-1(nc37) animal (D). In the mab-20(bx24); plx-1(nc37) animal (G), ray 1 is displaced anteriorly and ray 3 fuses to ray 4. Scale bars: 20 μm.

**Fig. 4.** Displacement of ray precursors in plx-1 mutants. Cell boundaries of ray precursor clusters were visualized in wild-type (A,C) and plx-1(nc37) (B,D) animals with jam-1::GFP. All animals contain him-5. (B) At the early L4 stage, ray 1 precursor cluster, processes are localized to the junction site between R1.p, R2.p and the surrounding hypodermal syncytium (hyp7). (A,B) R1.p of a plx-1(nc37) animal is abnormally small (B) compared with that of the control animal (A). In the plx-1 mutant at the mid L4 stage (D), the position of the ray 1 precursor cluster (1) is shifted anterodorsally from the ray 2 precursor cluster (2) to the body seam cell (s), while it is associated with the ray 2 precursor cluster in the wild-type animal (C). For reference, each ray precursor cluster, R1.p, R2.p, the seam cell (s) and the hypodermal syncytium (hyp7) are labeled. Scale bars: 20 μm.

**plx-1 and mab-20 mutants have distinct phenotypes**

Previous studies showed that mutations in the mab-20 gene,
which encodes a secreted semaphorin, semaphorin 2a, also affect several epidermal morphogenetic processes, including the spatial arrangement of male tail rays (Baird et al., 1991; Roy et al., 2000). However, phenotypes of plx-1 and mab-20 mutants are distinct; mab-20 mutants have ventral enclosure defects, which result in a low fecundity, whereas Roy et al., (2000). However, phenotypes of the spatial arrangement of male tail rays (Baird et al., 1991; mab-20 mutants are distinct; mab-20 mutants have ventral enclosure defects, which result in a low fecundity, whereas Roy et al., 2000). However, phenotypes of mab-20 (Roy et al., 2000) (Table 1), whereas plx-1 mutants seldom exhibited ray-fusion. Non-overlapping phenotypes suggest that the ray-fusion phenotype of mab-20 mutants is not dependent on the presence of the wild-type plx-1 gene. Exceptions were ray 6, and rays 7 and 9, which showed enhancement and reduction of fusion, respectively. Although we did not detect any abnormality in these rays in plx-1 mutants, we observed the expression of plx-1::egfp in all the ray precursors, and it may be that the positions of these precursors are subtly affected in plx-1 mutants.

RNAi of transmembrane-type semaphorins causes displacement of ray 1

C. elegans has three semaphorin genes; mab-20, Ce-sema-1a and Ce-sema-1b. As mab-20 and plx-1 mutants exhibited distinct phenotypes, the candidate ligands for PLX-1 are two transmembrane semaphorins, Ce-Sema-1a and Ce-Sema-1b. We examined the functions of these genes in the morphogenesis of the male tail by RNA interference (RNAi) experiments. A displacement of ray 1 similar to that in plx-1 mutants was observed among the progeny of worms that were subjected to injection of double-stranded RNAs corresponding to either the Ce-sema-1a or Ce-sema-1b gene (Fig. 3F).

RNAi for Ce-sema-1a had a relatively stronger effect than that for Ce-sema-1b. Whereas injection of single RNA species resulted in a very mild phenotype, simultaneous suppression of both genes by injection of mixed RNAs affected nearly 40% of animals, and some exhibited displacement comparable with that of severely affected plx-1 mutants. The results suggest that Ce-Sema-1a and Ce-Sema-1b function redundantly as ligands for PLX-1 in the morphogenesis of the male tail (Table 1).

Table 2. Ray fusion in plx-1 and mab-20 animals

<table>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>mab-20(ev574)</td>
<td>90</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>3</td>
<td>65</td>
<td>95</td>
<td>3</td>
<td>95</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>mab-20(bx24)</td>
<td>73</td>
<td>77</td>
<td>98</td>
<td>98</td>
<td>4</td>
<td>37</td>
<td>58</td>
<td>0</td>
<td>58</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>mab-20(ev574); plx-1(nc37)</td>
<td>68</td>
<td>92</td>
<td>96</td>
<td>100</td>
<td>7</td>
<td>82</td>
<td>58</td>
<td>2</td>
<td>58</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>mab-20(bx24); plx-1(nc37)</td>
<td>27</td>
<td>28</td>
<td>74</td>
<td>81</td>
<td>5</td>
<td>71</td>
<td>21</td>
<td>1</td>
<td>21</td>
<td>62</td>
<td>105</td>
</tr>
</tbody>
</table>

The table gives the percentage of ray fusion to a neighbor within one side of a male tail. >2R refers to the percentage of male tail sides that had more than two rays within a single fusion. All strains contained him-5(e1490).
While the defects caused by the RNAi experiments were much milder than those of plex-1 mutants and many worms showed no abnormalities, this appears to reflect a low efficiency of suppression of genes at late larval stages with the RNAi procedure employed, rather than low expressivity of the phenotype caused by suppression of their functions. When we scored selectively the progeny retaining co-injected dye, which we presume to retain injected RNAs abundantly, the frequency of the defects increased significantly and many worms exhibited class I defects (Table 1).

**DISCUSSION**

The plexins constitute an evolutionarily conserved family of proteins in the animal kingdom and have been presumed to play important roles in the development of the nervous system. *C. elegans* has been shown to possess plexin genes (Winberg et al., 1998), but none had been characterized. In this study we generated mutations for one of the *C. elegans* plexin genes, plex-1, and analyzed its function in vivo. We revealed that plex-1 is crucial for epidermal development in *C. elegans*. We also confirmed that plexin is a binding partner for semaphorins in *C. elegans*, and determined the specificity of interactions between members of the *C. elegans* semaphorin family (Ce-Sema-1a, Ce-Sema-1b, Ce-Sema-2a) and PLX-1.

**PLX-1 interacts with Ce-Sema-1a, b but not with Ce-Sema-2a**

We have shown that suppression of *Ce-sema-1a* and *Ce-sema-1b* causes defects in the male tail similar to those caused by plex-1 mutations. We have also shown that Ce-Sema-1a binds to PLX-1. Although binding partners for Ce-Sema-1b remained to be determined biochemically, the present results indicate that PLX-1 is the receptor for the transmembrane-type semaphorins, Ce-Sema-1a and Ce-sema-1b, in *C. elegans*. PLX-1 is a Type A plexin, and in *Drosophila*, a Type A plexin, DplexA, has been also shown to be a receptor for semaphorin I (Winberg et al., 1998). It would be interesting to see whether the class-specific interaction of plexinA and semaphorin I applies to other invertebrate species.
We have shown that *plx-1* and *mab-20*/*Ce-sema-2a* mutants display distinct defects: *plx-1* mutants do not exhibit ventral enclosure defects or Vab phenotypes. Although they both exhibit defects in the male tail, their phenotypes do not overlap. Our preliminary analysis shows that *plx-1* mutants exhibit vulval defects, whereas the vulvae of *mab-20(bx24)* mutants are relatively normal (S. T., unpublished). Together with our finding that Ce-Sema-2a does not bind to PLX-1, the results indicate that, in principle, *plx-1* and *mab-20* function independently. This raises the possibility that the receptor for Ce-Sema-2a is PLX-2, which we are currently examining genetically and biochemically.

**PLX-1 regulates epidermal morphogenesis**

We have shown that *plx-1* mutants have defects in the formation of one ray and seam cells. Three common properties can be pointed out for their epidermal precursors. First, the cells undergo dynamic changes in shape and position, sometimes associated with cell fusion. Second, cells usually form clusters or make contact with each other, and their relationships with neighboring cells changed during morphogenetic movements. Third, *plx-1* appears to be expressed in the cells that are affected in the mutants. Therefore, *plx-1* is likely to function cell autonomously to regulate either cell shape, cell position or cell contact when epidermal cells undergo dynamic morphological changes.

**Rays**

We have revealed an anterior displacement of ray 1 in *plx-1* mutants and *Ce-sema-1a, b* (RNAi) animals. The phenotype is rather subtle and distinct from those of previously isolated mutations affecting the ray morphology, which results in fused rays or missing rays. Many of the previous mutations are presumed to alter the identity or affect the differentiation of rays (Chow et al., 1994; Chow et al., 1995; Ferreira et al., 1999; Sutherlin and Emmons, 1994; Zhang and Emmons, 1995; Lints and Emmons, 1999). In *mab-20* mutants, however, it has been shown that the identities of the rays are not altered. Similar to this, although we have not examined the identity of rays using specific molecular markers, the misplaced ray 1 in *plx-1* mutants retains the morphological characteristics of a normal ray 1, indicating that its identity is not altered.

Our analysis using a *jam-1::gfp* transgene has shown that the displacement of adult ray 1 is a consequence of the mispositioning of the ray 1 precursor cluster in *plx-1* larvae. The mechanisms underlying the allocation of ray precursor clusters are little understood, but our finding that the shape of R1.p is sometimes distorted in *plx-1* mutants indicates that *plx-1* is involved through the regulation of cell morphology.

The present study and a previous report (Roy et al., 2000) showed that both *plx-1* and *mab-20* mutations affect ray positions, and PLX-1 and Ce-Sema-2a/MAB-20 are expressed in the same ray precursor clusters. These observations might imply that Ce-Sema-1s/PLX-1 and Ce-Sema-2a act on identical cells simultaneously and suggest possible crosstalk between the two signaling systems. The effects of *plx-1* and *mab-20* mutations on the position of ray 1 are apparently opposite: the *mab-20* ray 1 precursor cluster makes ectopic contact with the ray 2 precursor cluster, while the *plx-1* ray 1 precursor cluster is apparently repelled by the ray 2 precursor cluster. One possibility is that a normal function of one of the genes would be to suppress signals mediated by the other. Our analysis of animals doubly mutant for the genes, however, did not reveal clear genetic interactions between them. Therefore, two semaphorin-mediated signals appear to function rather independently in ray morphogenesis. An interesting precedence has been shown in the grasshopper limb bud where Sem1 and Sem2a provide functionally distinct guidance information to the same growth cones (Isbister et al., 1999).

**Seam cells**

We have revealed that *plx-1* mutations affect the arrangements of seam cells. During larval development, the seam cells undergo cycles of loss and reformation of cell-cell contacts. Previous studies have shown that the formation of cell contacts between seam cells is a highly active process. Seam cells can extend a cell process laterally to form new cell contacts even after ablation of its normal neighbors, although there appears a limit to the extent of cell extension (Austin and Kenyon, 1994). The gaps in a row of *plx-1* seam cells appear to be caused by failures to reconstitute cell-cell contacts, suggesting that extension of cell processes is affected in the mutants. Seam cells usually make contact with neighboring seam cells on the anterior and posterior sides, and it was suggested that the formation of cell contacts generates a signal that results in a cessation of extension of cellular processes (Austin and Kenyon, 1994). Aberrant contacts along the dorsoventral sides of *plx-1* seam cells indicate that the cells failed to cease lateral growth after making the initial contact with neighboring cells, suggesting that the presumed 'stop' signal is also affected in the *plx-1* mutants.

A previous study has shown that *mab-20* embryos have defects in P cells and V cells, which are embryonic seam cells (Roy et al., 2000). The altered arrangement of *plx-1* larval seam cells revealed in this study is reminiscent of ectopic cell contacts between *mab-20* V cells (Roy et al., 2000), and suggests that these two genes regulate similar cellular events. It has been noted, however, that *plx-1* mutants apparently have no defects in embryonic hypodermal cells, suggesting that these two genes function independently with different temporal patterns.

**How does PLX-1 regulate cell behavior during epidermal development?**

Our finding that transmembrane semaphorins are ligands for PLX-1 indicates that PLX-1 is involved in cell contact-mediated regulation of cell behavior. Although the effects of semaphorins on cell migration have been documented (Eickholt et al., 1999), ray precursor cluster cells and seam cells, which are affected by *plx-1* mutations, do not seem to migrate. Our preliminary analysis shows that the short-range migration of vulval primordial cells is affected in *plx-1* mutants. We have, however, noted that arrangements of vulval primordial cells are also sometimes affected before the onset of cell migration (S. T., unpublished). It seems that a major function of the Ce-Sema-1s/PLX-1 signal in the *C. elegans* epidermal system is the regulation of cell arrangements.

An attractive as well as repulsive action on growth cones has been reported for insect semaphorin 1 proteins (Wong et al., 1997; Wong et al., 1999; Yu et al., 1998; Winberg et al., 1998). While our finding that *plx-1* seam cells fail to reconstitute cell-cell contacts may be explained by an attractive action of Ce-
Sema-1s/PLX-1, the displacement of ray 1 does not appear to be explained simply by mutual attraction or repulsion between ray clusters. It may be that the action of Ce-Sema-1s/PLX-1 is localized within a single epidermal cell, and PLX-1 plays a role as a local modulator of cell morphology by mediating an attractive force in some parts of a cell and/or a repulsive force in others. The effects of semaphorins on axon growth cones are thought to be mediated by cytoskeletal alteration: the localized application of sema3A/collapsin alters the frequency of lamellipodia extensions, and thus the morphology of growth cones (Fan and Raper, 1995). The same mechanisms could underlie changes in epidermal cell morphology. In order to understand the mechanisms by which Ce-Sema-1s/PLX-1 regulates the behavior of epidermal cells, the distribution of Ce-Sema-1s and PLX-1, as well as co-factors or downstream signaling components should be examined at the subcellular level in future studies.

We failed to detect gross morphological defects in the nervous system of plx-1 mutants. Recently identified non-neuronal semaphorins indicate that the semaphorin/plexin signaling system has roles other than in the regulation of neuronal or cellular migrations. In vertebrates, semaphorin is likely to be involved in cartridge formation, immunogenic modulation and vascular morphogenesis (Behar et al., 1996; Hall et al., 1996; Comeau et al., 1998; Miao et al., 1999). Together with a previous study on mab-20 (Roy et al., 2000), our results indicate that the semaphorin/plexin signaling system plays important roles in epidermal morphogenesis in C. elegans. Some vertebrateplexins are expressed in mammalian epithelial cells (H. F., unpublished), and the present results may provide a clue as to their roles. Plexins are likely to have diverse developmental and physiological roles in animals.

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