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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-1a, but not Ce-Sema-2a, physically bound to PLX-1. Our results indicate that PLX-1 functions as a receptor for transmembrane-type semaphorins, and, though 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 (Taniguchi 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 know 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 (*mab-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 play important roles in neural development, *mab-20* animals exhibited relatively minor defects in the morphology of the nervous system (Roy et al., 2000).

Except for *mab-20*, much of the semaphorin/plexin signaling system in *C. elegans* has remained unexplored. Whether plexins are functional receptors for semaphorins has yet to be confirmed in *C. elegans*, and the specificity of interactions between three semaphorins and the two plexins has not been established. What roles the two transmembrane semaphorins as well as two plexins play in the development of *C. elegans*, in particular in that of the nervous system, remain to be elucidated. We have adopted a reverse genetic approach to analyze the function of one of the *C. elegans* plexins, PLX-1. We now report that *plx-1* mutants exhibit defects in epidermal morphogenesis, which cannot be explained simply by the repulsive action of semaphorin. We also present genetic and biochemical evidence that PLX-1 interacts with Ce-Sema-1a and Ce-Sema-1b, but not with Ce-Sema-2a/MAB-20.

MATERIALS AND METHODS

C. elegans strains, N2, DR466 *him-5(e1490)*, EM67 *mab-20(bx24); him-5(e1490)* and PS3352 (*syIs50*) were obtained from the *C. elegans* Genetic Stock Center, care of T. Stiernagle (The University of Minnesota). NW1074 *mab-20(ev574)* was provided by J. Culotti. SU93 (*jcIs1*) was provided by J. Simske. Basic methods for worm culture and genetics were performed as described by Brenner (Brenner, 1974) and compiled by Wood (Wood, 1988). Standard techniques of molecular biology described by Sambrook et al. (Sambrook et al., 1989) were employed. Primer sequences and the conditions for PCR are available on request.

Cloning and sequencing of cDNAs

The following cDNA clones were isolated by Y. Kohara's group as part of the Kohara cDNA project: *yk535f1* encoding the C terminal region for PLX-1; *yk88a4* and *yk450a4* containing the entire coding region for *Ce-sema-1a* and *Ce-sema-2a*, respectively. The 5′ end of the *plx-1* cDNA was generated by 5′RACE. A full-length cDNA for *Ce-sema-1b* was synthesized by RT-PCR. DNA sequences were determined for both strands.

PCR of the *plx-1* genomic fragment

For transformation rescue of *plx-1* mutants, two PCR fragments corresponding to the *plx-1* genomic DNA fragments, Y55F3AL.3065-18373nt and Y55F3AL. 17542nt- Y55F3AM.4730nt, were generated (TaKaRa LA PCR Kit). The two PCR products (0.05 mg/ml each) were mixed with pRF4 (0.2 mg/ml) containing *rol-6(su1006)* and injected into the gonad of *plx-1(nc37); him-5(e1490)* hermaphrodites (Mello and Fire, 1995). F_1 progeny exhibiting the dominant rolling phenotype induced by *rol-6(su1006)* were transferred individually to establish a transgenic line, *plx-1(nc37); ncEx[plx-1(+), rol-6(su1006)]*.

To examine expression of the *plx-1* gene, a PCR fragment corresponding to the *plx-1* genomic DNA, Y55F3AL.3065-18373nt, was cloned into *Kpn*I-*Bam*HI-digested pFXneEGFP (S. Mitani, personal communication), which resulted in translational fusion of the N-terminal half of PLX-1 with EGFP (Living Colors Fluorescent Proteins, Clontech).

Isolation of insertion 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 Axioplan 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 *jcIs1[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; Podbilewicz and White, 1994). The *jcIs1* 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 *jcIs1; 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 RNeasy 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 *Hin*dII-*Bgl*II 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 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-ΔC-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 5 to 30 minutes.

For quantitative binding assays, cells were incubated with Ce-Sema-1a-ΔC-Fc-AP and lysed with 250 μm of 10 mM Tris-HCl (pH 8.0) containing 1% Triton X-100. The cell lysates were subjected to a colorimetric analysis to measure the AP activity (Flanagan and Leder, 1990).

For immunoblot analysis, protein blots were reacted either with goat anti-human Fc conjugated with AP (Cappel), or with anti-Myc antibody, 9E10 (Evan et al., 1985), and then with goat anti-mouse IgG/M conjugated with AP (Boehringer), and immunoreactivity was detected with the NBT/BCIP system (Boehringer).

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 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 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

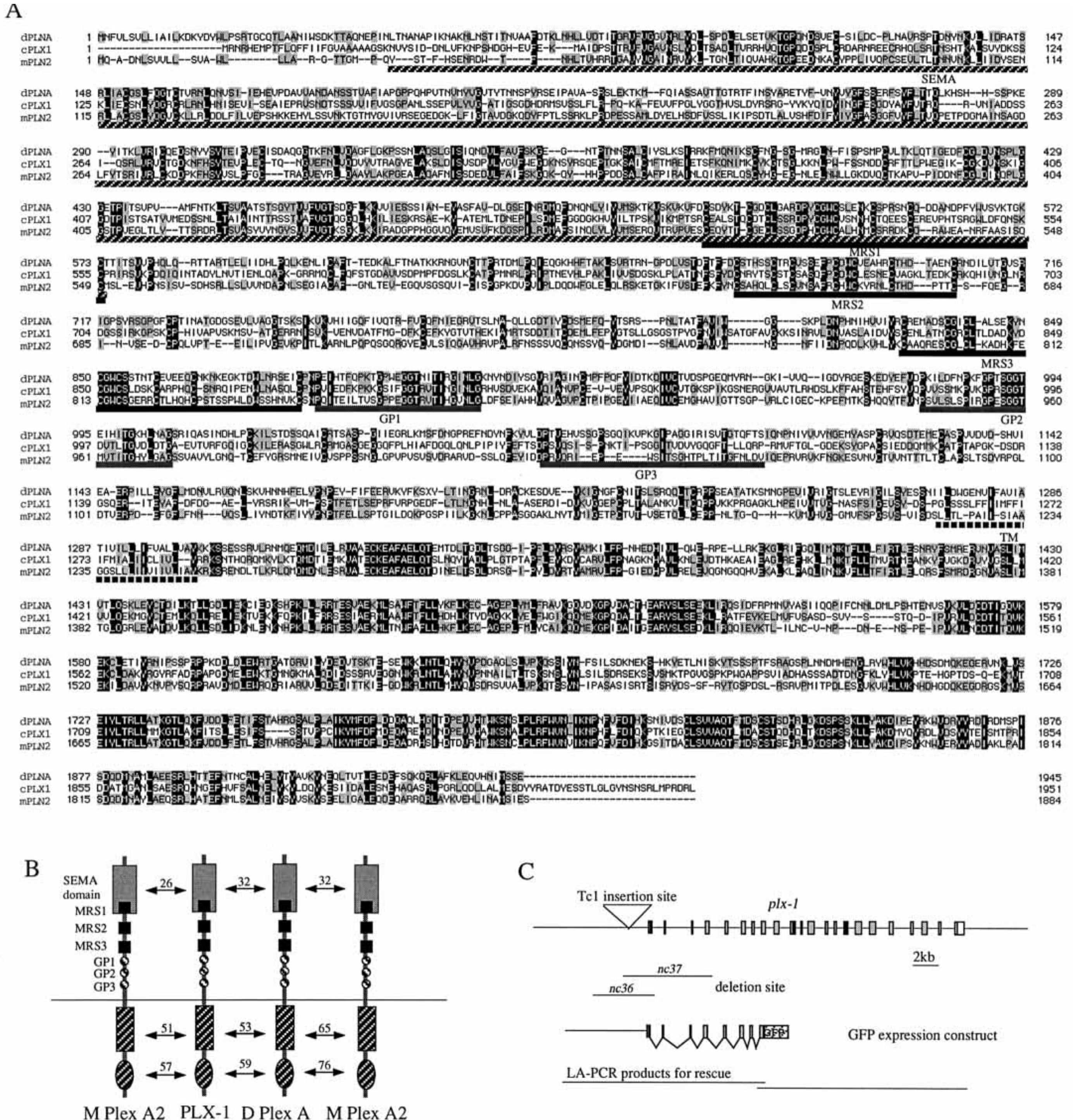

Fig. 1. The structure of the *plx-1* gene and its product. (A) The primary structure of PLX-1 aligned with the *Drosophila* PlexA and the mouse plexin-A2 sequences. (B) The similarity (%) between each region of PLX-1 and that of *Drosophila* PlexA and mouse plexin-A2. (C) A scheme showing the structure of the *plx-1* gene, deletions, the construct *plx-1::egfp* used in the expression analysis and the PCR products used for rescue experiments. DDBJ Accession Number for the *plx-1* cDNA is AB080022.

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

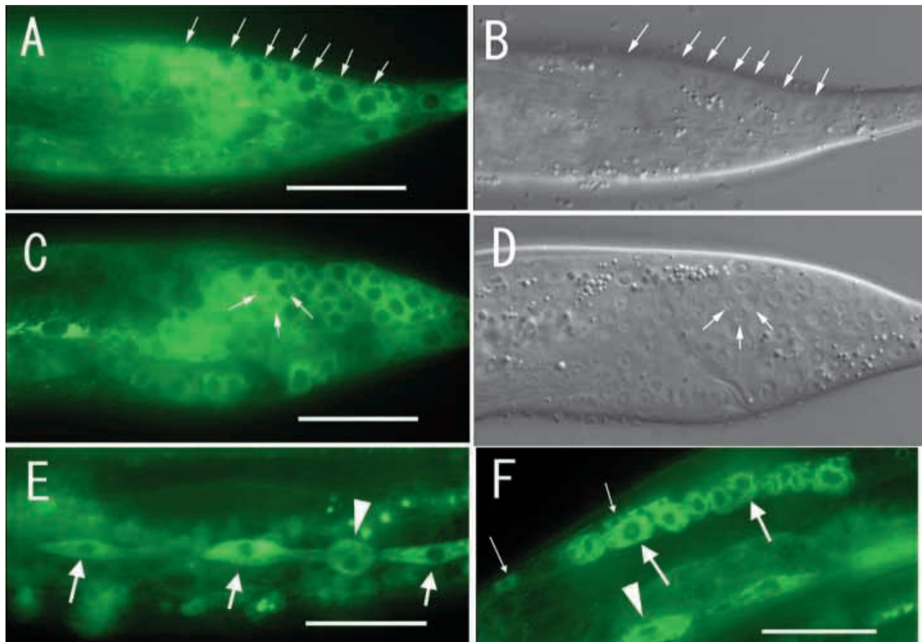

Fig. 2. Expression of *plx-1::egfp* in *him-5; ncEx[plx-1::egfp, rol-6(su1006)]*. The animals are shown with anterior towards the left. (A) A lateral view of the left side of a third larval-stage male tail at about 30 hours after hatching. GFP expression is observed in R(n) cells (arrows). (C) An early to mid-L4 stage male at about 35 hours after hatching. The ray precursor clusters (arrows) express GFP. (B,D) The corresponding DIC images shown in A,C, respectively. (E) A lateral view of seam cells at L4 stage. Both parental seam cells (arrows) and a daughter cell (arrowhead) express GFP. (F) A ventral view at early L3 stage. GFP expression is observed in ventral cord motoneurons (small arrows) and seam cells (an arrowhead). All vulval precursor cells (some are indicated with arrows) aligned along the ventral midline also express GFP. Scale bars: 20 μm.

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

	Ray 1 displacement		
Animal	Class I (%)	Class II (%)	*n*
Control	0	0	313
plx-1(nc37)	62	30	100
plx-1(nc36)	44	29	100
plx-1(nc37)/+	0	15	137
plx-1(nc37); Ex[plx-1, rol-6(su1006)]	11	8	100
mab-20(ev574)	0	0	100
mab-20(bx24)	0	0	100
mab-20(ev574); plx-1(nc37)	15	10	100
mab-20(bx24); plx-1(nc37)	22	22	105
*Ce-sema-1a(RNAi)**	1	7.7	614
*Ce-sema-1b(RNAi)**	0.4	5.4	1057
*Ce-sema-1a(RNAi); Ce-sema-1b(RNAi)**	1.4	27.1	280
Ce-sema-1a(RNAi); Ce-sema-1b(RNAi)†	15	36	53

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.

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 Rn.p, which fuses later with other Rn.ps 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.ps, the processes of a ray precursor cluster are localized to the site associated with the junction of three or more epidermal cells, Rn.ps 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%. *n*=100] (Fig. 4B). 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

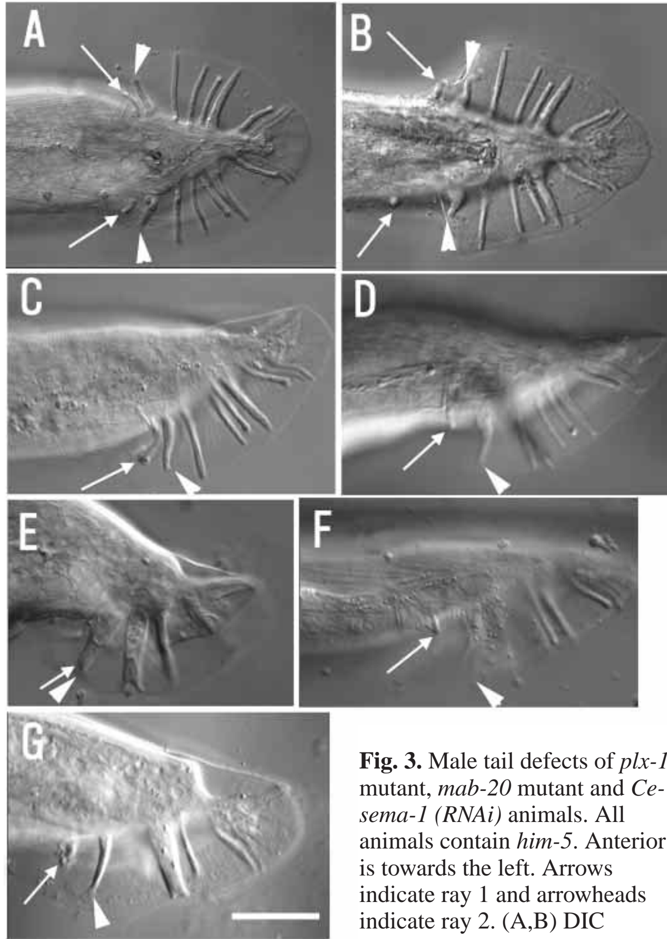

Fig. 3. Male tail defects of *plx-1* mutant, *mab-20* mutant and *Ce-sema-1 (RNAi)* animals. All animals contain *him-5*. Anterior is towards the left. Arrows indicate ray 1 and arrowheads indicate ray 2. (A,B) DIC photomicrographs of ventral views of (A) a control and (B) a *plx-1(nc37)* mutant adult 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.

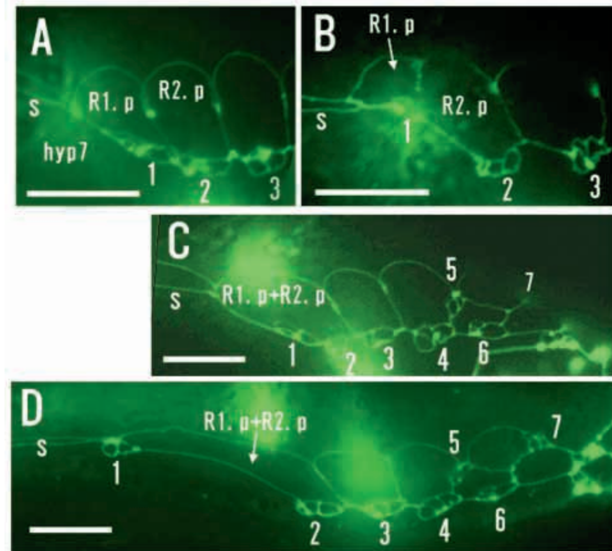

Fig. 4. Displacement of ray precursors in *plx-1* mutants. Cell boundaries of ray precursor clusters were visualized in wild-type (A,C) and *plx-1(nc37)* (B,D) animals with *jam-1*::GFP. All animals contain *him-5*. (B) At the early L4 stage, ray 1 precursor cluster, processes are localized to the junction 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 anteriodorsally 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 syncythium (hyp7) are labeled. Scale bars: 20 μm.

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)]*.

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

Previous studies showed that mutations in the *mab-20* gene,

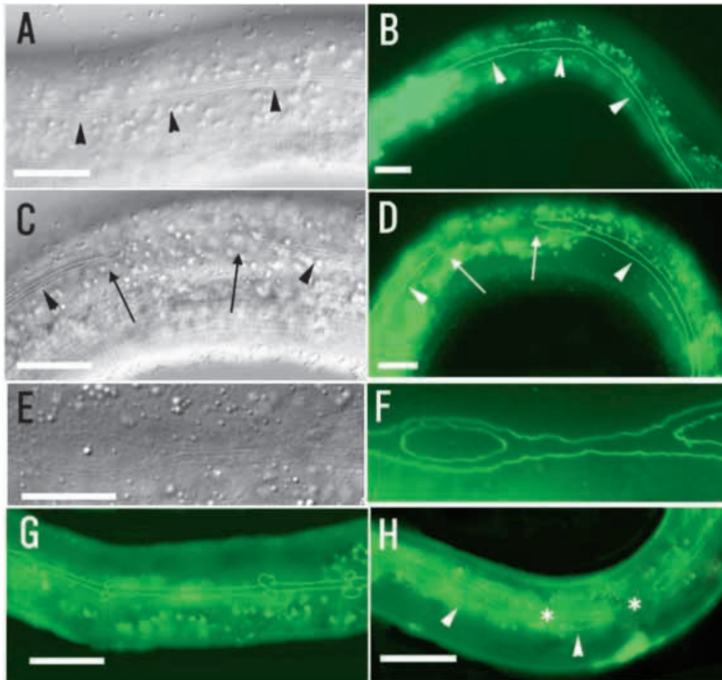

Fig. 5. Seam cell defects of *plx-1(nc37)* hermaphrodites. Lateral views with anterior towards the left. All animals contain *jcIs1; him-5*. (A,C,E) DIC images of adult alae (arrowheads) in a control (A) and *plx-1* mutant (C,E) hermaphrodites. In *plx-1* animals, alae were discontinuous (arrows, C) or bifurcated (E). (B,D,F) Fluorescent images of the *jam-1::gfp* transgene expression in a control animal (B) and *plx-1* mutant animals (D,F) shown in A,C,E. (D) In a *plx-1* animal, the seam cell boundaries closed midway (arrows). The position of the gap without seam cells corresponds with that of the gap of the alae. (F) In a *plx-1* animal, extra cell boundaries formed within a seam cell where the alae made bifurcations. (G,H) Fluorescent images of the *jam-1::gfp* transgene expression in a control (G) and a *plx-1* L4 (H) hermaphrodite. In the *plx-1* animal, the arrangement of seam cells is disrupted (arrowheads). Some seam cells were separated by gaps (asterisks). Scale bars: 10 μm.

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 *plx-1* embryos hatch normally (data not shown). Ray 1 of *mab-20* mutants often fused with posterior rays (Fig. 3E; Table 2), but was never located anteriorly (Roy et al., 2000) (Table 1), whereas *plx-1* mutants seldom exhibited ray-fusion. Non-overlapping phenotypes suggest that the *plx-1* gene and the *mab-20* gene function independently.

To examine genetic relationships between the two genes further, we constructed *mab-20; plx-1* double mutants. *plx-1(nc37)* was combined with either a putative null allele of *mab-20*, *ev574* (Roy et al., 2000), or a hypomorphic allele, *bx24*, and both combinations showed an essentially similar ray phenotype. Although some animals showed ray fusions involving ray 1 similar to those of *mab-20* mutants, some exhibited a ray 1 displacement defect in addition to the fusion of other rays (Fig. 3G, Table 1). The frequency of fusion among the other rays in the double mutants was altered little from that in the *mab-20* mutants (Table 2), indicating 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 ray1 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

Animal	Ray fusion (%)										
	R1	R2	R3	R4	R5	R6	R7	R8	R9	>2R	*n*
Control	0	0	0	0	0	0	0	0	0	0	313
plx-1(nc37)	0	0	0	0	0	0	0	0	0	0	100
mab-20(ev574)	90	98	100	100	3	65	95	3	95	90	100
mab-20(bx24)	73	77	98	98	4	37	58	0	58	43	100
mab-20(ev574); plx-1(nc37)	68	92	96	100	7	82	58	2	58	83	100
mab-20(bx24); plx-1(nc37)	27	28	74	81	5	71	21	1	21	62	105

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)*.

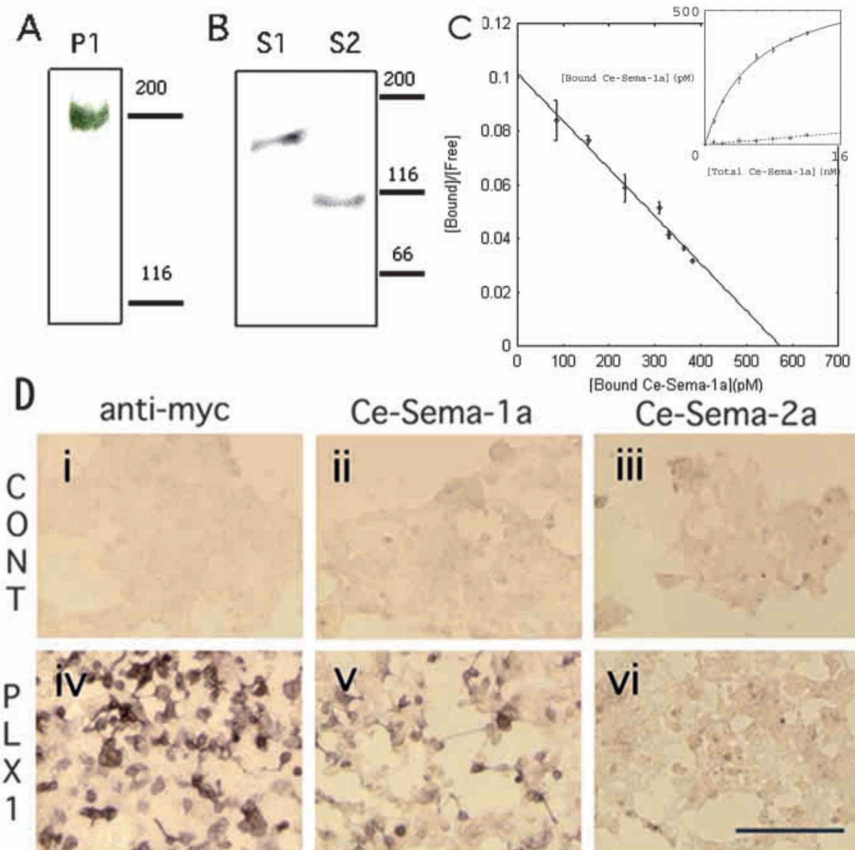

Fig. 6. Binding of *C. elegans* semaphorins to PLX-1 expressed in the cultured cells. (A) A western blot of PLX-1 expressed in HEK293T cells. An immunoreactive band the size of 220 kDa was detected for Myc-PLX-1 with anti-Myc antibody. The predicted size of the peptide is 210 kDa. (B) A western blot of *C. elegans* semaphorins secreted in the culture medium of HEK293T cells. An immunoreactive band the size of 143 kDa (lane 1) and 110 kDa (lane 2) for Ce-Sema-1a-ΔC-Fc-AP and Ce-Sema-2a-Fc was detected, respectively, with anti-Fc antibody. The predicted size of each peptide is 139 kDa and 94 kDa, respectively. (C) Scatchard analysis of the binding of Ce-Sema-1a-ΔC-Fc-AP to PLX-1. The inset shows the binding curves of Ce-Sema-1a-ΔC-Fc-AP to PLX-1 expressed on HEK293T cells (the upper line) and to HEK293T cells transfected with pCAGGS as a control (the lower broken line). (D) Binding of Ce-Sema-1a-ΔC-Fc-AP to PLX-1. (i,ii,iii) Untransfected HEK293T cells. (iv,v,vi) HEK293T cells expressing PLX-1. (i,iv) Cells were reacted with the anti-Myc antibody. (ii,v) Cells were reacted with Ce-Sema-1a-ΔC-Fc-AP. (iii,vi) Cells were reacted with Ce-Sema-2a-Fc. Scale bar: 100 μm.

While the defects caused by the RNAi experiments were much milder than those of *plx-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).

A transmembrane-type semaphorin binds to PLX-1

To confirm further that transmembrane-type semaphorins are ligands for PLX-1, we examined the physical interactions of *C. elegans* semaphorins with PLX-1 in vitro using cultured mammalian cells. The ectodomain of Ce-Sema-1a tagged with an Fc region of human IgG and an alkaline phosphatase (Ce-Sema-1a-ΔC-Fc-AP) or Ce-Sema-2a tagged with an Fc region of human IgG (Ce-Sema-2a-Fc) were expressed in HEK293T cells in culture, and culture supernatants were added to a culture of HEK293T cells transfected with the plx-1 cDNA. Ce-Sema-1a-ΔC-Fc-AP bound to HEK293T cells expressing PLX-1 (Fig. 6C, part v). However, Ce-Sema-2a-Fc failed to bind to PLX-1 expressed on HEK293T cell membrane (Fig. 6C, part vi). The dissociation constant (K_D) value for the interaction of Ce-Sema-1a-ΔC-Fc-AP with PLX-1 estimated by Scatchard analysis was 5.7±0.3 nM (Fig. 6D), which is comparable with that of *Drosophila* semaphorin I with DplexA (Winberg et al., 1999).

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, *plx-1*, and analyzed its function in vivo. We revealed that *plx-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 *plx-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 displaced 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 Sema1 and Sema2a 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 vertebrate plexins 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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