INTRODUCTION

Understanding how axon and cell migrations are guided is fundamental to our understanding of metazoan development. During directed migrations, the leading edge of growing axons and motile cells must continually establish transient contacts to neighboring cells and the surrounding extracellular matrix (ECM) via membrane extensions. It is through the modulation of the cytoskeletal network within these membrane extensions that molecular cues can guide cells to their target destination (Tanaka and Sabry, 1995; Carlier, 1998).

Simplistically, migratory guidance cues can be classified as those that attract and those that repel. There are also cues that can both attract and repel, depending on the receptor complex receiving the cue (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Chan et al., 1996) and the activity of signaling components downstream of the activated complex (Song et al., 1998). Attractive cues are thought to promote membrane extension towards increasing concentrations of the cue by increasing the affinity of the extension to the substratum (de la Torre et al., 1997). In contrast, repulsive cues can induce localized collapse of membrane extensions through the disassembly of microfilaments within the extensions (Fan and Raper, 1995). However, exactly how these cues regulate the cytoskeletal elements required for membrane extension and cell motility is not clear. Even less is known about the way migrating cells transiently make and break contacts with the ECM and other cells that line their migratory paths. To address these issues, we have begun a genetic analysis of semaphorin function in the nematode Caenorhabditis elegans.

The semaphorins are a family of secreted and transmembrane proteins known to elicit growth cone repulsion and collapse. We made and characterized a putative null mutant of the C. elegans gene semaphorin-2a (Ce-sema-2a). This mutant failed to complement mutants of mab-20 (Baird, S. E., Fitch, D. H., Kassem, I. A. A. and Emmons, S. W. (1991) Development 113, 515-526). In addition to low-frequency axon guidance errors, mab-20 mutants have unexpected defects in epidermal morphogenesis. Errant epidermal cell migrations affect epidermal enclosure of the embryo, body shape and sensory rays of the male tail. These phenotypic traits are explained by the formation of inappropriate contacts between cells of similar type and suggest that Ce-Sema-2a may normally prevent formation or stabilization of ectopic adhesive contacts between these cells.

Key words: Caenorhabditis elegans, Semaphorin, mab-20, Cell contact, Adhesion

mab-20 encodes Semaphorin-2a and is required to prevent ectopic cell contacts during epidermal morphogenesis in Caenorhabditis elegans

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Kitsukawa et al., 1995). In principle, the cardiovascular defects may be explained by the promiscuity of the neuropilin-1 receptor, as it also binds an isofrom of VEGF (Soker at al., 1998). The mechanisms underlying the other non-neuronal defects induced by neuropilin-1 overexpression, and those of the Drosophila and mouse semaphorin mutants remain obscure; however, a recent report suggests that chicken SEMA3A can repel neural crest cells in vitro and possibly in vivo (Eickholt et al., 1999).

To further study semaphorin function in vivo, we used transposon-mediated reverse genetics to obtain a putative null allele of the semaphoran-2a gene in C. elegans (Ce-sema-2a). This allele failed to complement mutations in the previously uncloned mab-20 gene (Baird et al., 1991). The mutant phenotype demonstrates that the predicted gene product Ce-Sema-2a is required for axon guidance and cell migrations. However, a major role of Ce-Sema-2a is to regulate the morphogenesis of the epidermis, historically called the hypodermis in C. elegans. Ce-sema-2a mutants exhibit several inappropriate hypodermal cell contacts during embryonic development. Mutant defects in hypodermal enclosure of the embryo, and in the morphogenesis of the body wall and the sensory rays of the male tail can be explained by these inappropriate contacts. Our results suggest that Ce-Sema-2a may play a key role in tissue morphogenesis by preventing the formation or stabilization of adhesive contacts between cells of a similar type.

MATERIALS AND METHODS

Standard molecular biology methods were used (Sambrook et al., 1989) unless otherwise noted. General procedures used for the culture, maintenance and storage of the nematodes are compiled by Wood (1988). All strains not isolated in our laboratory were obtained from the C. elegans Genetics Center, care of T. Sternagle (The University of Minnesota). Primer sequences are available upon request.

cDNA construction

The λzAP11 (Strategene) cDNA clones yk80 and yk100 were provided by Y. Kohara and excised in vivo. To obtain the 5′ end of the cDNA, the RACE procedure was used (Frohman et al., 1988) on total RNA from a mixed stage population of wild-type nematodes (N2). First-strand cDNA was synthesized using the mab-20-specific primer II.R. A polymerase chain reaction using an oligo(dT) primer and the gene-specific primer II.RI generated a 1.25 kb product from a first-strand cDNA template tailed with dATP, and was then cloned into pBluescript KS (+). A polymerase chain reaction using the splice leader sequence 1 (SL1) 5′ primer (Krause and Hirsh, 1987) and the mab-20-specific primer II.RI generated a 1.55 kb product, which was cloned into pBluescript KS (+). The full-length cDNA was spliced together using the SL1 PCR product’s 5′ end to BamHI, the 5′ RACE product’s BamHI-SmaI fragment, and yk80 3′ from the SmaI restriction site. The cDNA was sequenced in its entirety.

The evCe-sema-2a cDNA was released from its vector using polynucleotyl xbaI and xhoI, and used to probe a northern blot of 5 μg of total RNA from a mixed-stage population of wild-type nematodes (N2), prepared as described in Leung-Hagestein et al. (1992).

Genomic clones

1.3×10^6 plaques from an Embl3 genomic phage library were screened using a 1.25 kb evCe-sema-2a cDNA fragment released from its vector using the EcoRI cDNA restriction sites. One of ten positive isolates, evpZH13, contained the predicted mab-20 gene in its entirety. A subclone of this phage isolate, evpPR11.2, was demonstrated, by restriction analysis, sequencing and transgenic rescue of the mutant, to contain an entire functional mab-20 gene. The transcriptional fusion reporter evpPR11.67 and evpPR11.75 were constructed by inserting 2.5 kb of DNA 5′ to the predicted initiator methionine of mab-20 into the GFP vectors pPD95.67 and pPD95.75, respectively. The translational fusion reporter evpPR11.14 was constructed by inserting in frame the GFP-coding sequence and introns contained within a 1.1 kb EcoRI fragment of DNA excised from pPD95.85 into the fourth EcoRI site of evpPR11.2. The pPD series of vectors were a gift from A. Fire.

Screening for Tc1 alleles and deletion derivatives

The strategy for the construction of our Tc1 library is an extension and modification of previous work (Zwaal et al., 1993) and is detailed in Roy (1999). Alpha-pool DNA was screened for an insertion allele in mab-20 using nested PCR with gene-specific primers II.A and II.B and Tc1-specific primers. AmpliTaq Gold™ (Perkin-Elmer) was used in all PCR reactions. To obtain a deletion allele of mab-20, a sublibrary was constructed and screened in the same fashion as the original, with the exception of using NW1034 [mab-20(ev573::Tc1); mut-2(r1459); dpy-19(n1347)] as the founding strain. To screen the alpha-pools of the sublibrary, PCR was carried out using mab-20-specific primers II.Y, II.Z, II.9, and II.10. The resulting mab-20(ev574) allele was outcrossed with N2 or him-5(e1490) at least 11 times before being analyzed further.

Genetic linkage and complementation

From ev574+/dpy-5(e61)/+ double heterozygotes, 115 Dpy animals were picked that had no morphological defects like those of ev574 homozygotes. Only 23 of these clones threw ev574 homozygous progeny, showing loose linkage to dpy-5 on chromosome I, as is also true for mab-20 mutants, which have similar phenotypes. mab-20(bx24) males raised at the permissive temperature (16°C) were mated with ev574 hermaphrodites. Male cross progeny raised at 25°C were scored for ray fusions. Since neither mab-20(bx24) nor mab-20(ev574) male homozygotes can successfully mate, the complementation test between these two alleles was done by crossing mab-20(bx24)/+ males to mab-20(ev574) hermaphrodites. All male cross progeny were scored for ray fusions. Since no deficiency uncovers the mab-20 locus, the supposition that ev574 is a genetic null cannot be tested.

Transgenics

Extrachromosomal arrays of Ev[mab-20(+)(evpPRI1.2); rol-6(su1006)] were generated by co-microinjection of the DNAs into N2 oogonia (Mello and Fire, 1995) at a concentration of 27.5 ng/μl and 100 ng/μl, respectively. F1 and F2 transformants were selected based on the dominant rolling phenotype induced by rol-6(su1006). The extrachromosomal arrays were integrated into random double-stranded breaks in the chromosomes generated by 3000 rads from a 137Cs source, resulting in intrachromosomal arrays evl574a, evl574b, evl574c and evl574d. Similar procedures were used to create reporter arrays.

Microscopy

Male tail ray fusions, ventral enclosure defects and DTC migratory defects were scored by mounting 1 mM levamisole-treated animals on 2% agarose pads for observation using DIC optics (Leica DMR microscope). The mec-4: gfp reporter (a gift from M. Driscoll) was used to assess the migrations of QL and QR. The migration of QL was considered aberrant if the PVM was situated near or anterior to the vulva.

For whole-mount immunohistochemistry, animals were permeabilised as described by Finney and Ruvkun (1990), and incubated overnight at room temperature with mAbMH27 or anti-
LIN-26 antibodies. The next day the animals were incubated with FITC-conjugated goat secondary antibody (Sigma™).

Circumferential microfilaments were observed by fixation in 4% paraformaldehyde and 0.5% glutaraldehyde, followed by a brief 5 minute exposure to rhodamine-conjugated phalloidin and 0.2% Triton X-100 (Priess and Hirsh, 1986). Although this procedure is intended for viewing the circumferential microfilaments, sarcomeric microfilaments were sometimes observed and cell outlines were visible.

SEM analysis of embryos was carried out according to the methods of Williams-Masson et al. (1997), except that specimens were coated with 1-2 nm of gold and observed with a JOEL 820 scanning electron microscope.

The numerous short-range migrations and rearrangements of wild-type seam cells and their precursors during the creation of the seam was observed through the use of the demonstration version of the BioCell CD ROM™, freely available from R. Schnabel (Schnabel et al., 1997).

RESULTS

Cloning of the C. elegans semaphorin-2a gene

A C. elegans gene (corresponding to yae Y54E5B.1 on linkage group I) predicted to encode a semaphorin-1 homolog (Ce-Sema-1a) was initially cloned using degenerate oligonucleotides and sequenced (Roy, 1999). This sequence was used to search the C. elegans genome database using the BLAST program (Altschul et al., 1994). Two additional semaphorin genes (D1037.2 and Y71G12A_205.G) also on linkage group I were identified. D1037.2 encodes another semaphorin-1 homolog (Ce-Sema-1b) while Y71G12A_205.G encodes a semaphorin-2 homolog (Ce-Sema-2a). Here we focus on the mutagenesis and characterization of the semaphorin-2a gene as it produces more severe defects than mutations in the semaphorin-1 related genes (P. J. R., V. Ginzburg and J. G. C., unpublished results).

A 2.4 kb cDNA (Fig. 1) was isolated and used to probe a northern blot of mRNA purified from a mixed-stage population of wild-type (N2) worms. This reveals a single RNA species of approximately 2.4 kb (data not shown). The evCe-sema-2a cDNA is predicted to encode a 657 amino acid protein, consisting of an amino-terminal hydrophobic signal sequence, followed by a semaphorin domain, a C2-type immunoglobulin domain, and a non-conserved carboxy-terminal region (Fig. 1). This modular configuration is the same as that predicted for Drosophila Sema-2a (Kolodkin et al., 1993) which is 32% identical and 41% similar to Ce-Sema-2a and conserves 17 of the 19 cysteines of Ce-Sema-2a. The semaphorin domain of Ce-Sema-2a has 13 of the 14 conserved cysteines that define this domain.

The evCe-sema-2a cDNA was used to isolate a 15.4 kb genomic lambda phage clone. A 14.6 kb subclone of this phage, evpPRII.2 (Fig. 2), rescues a putative null Ce-sema-2a mutation (ev574), suggesting that the subclone contains all elements required for proper functioning of the Ce-sema-2a gene (Table 1).

The isolation and molecular characterization of a C. elegans sema-2a mutant

A Ce-sema-2a silent transposon-insertion mutant, strain NW1034 [Ce-sema-2a(ev573::Tc1)I, mut-2(r459)I; dpy-19(n1347)III], was obtained by PCR screening of a frozen library of worms containing active Tc1 transposons. NW1034 was propagated and screened for a deletion derivative of ev573::Tc1, resulting in the isolation of NW1074 [Ce-sema-2a(ev574)]. PCR, Southern analysis and sequencing show that ev574 contains a deletion of 1479 bp that includes part of the first intron, the entire first exon, and 641 bp 5’ to the predicted initiator methionine, thereby eliminating the predicted signal sequence (Fig. 2). As no other methionine is present until residue 120, ev574 is predicted to be a molecular null allele of Ce-sema-2a.

NW1074 worms are deformed (see Fig. 4), a trait that maps to linkage group I (see Materials and Methods). Concurrent with mapping studies, phenotypic analysis of ev574 homozygotes revealed that the normally distinct sensory rays of the adult male tail are fused, a phenotypic trait referred to as Mab, or male abnormal (see Fig. 5). The fusion pattern is similar to that observed in mutants of mab-20 on linkage group.
I (map position -10.4; Baird et al., 1991). Genetic crosses revealed that ev574 does not complement mab-20(bx24) or mab-20(bx61) (Table 1), suggesting that mab-20 encodes Ce-Sema-2a.

SSCP, PCR, Southern and DNA sequence analyses revealed that bx24 is an internal, out-of-frame, tandem duplication of a 2.2 kb sequence normally found immediately 5' to the predicted stop codon of Ce-sema-2a (Fig. 2). The temperaturesensitive allele bx61 is a missense mutation that changes the 188th codon from a proline to a leucine. The nature of the three mutations is consistent with the relative penetrance of all of the phenotypic traits of the three mab-20 alleles examined, which...
can be placed into a simple allelic series (Table 1). The Ce- 
sema-2a gene is hereafter called mab-20, and the predicted 
protein product Ce-Sema-2a or simply Sema-2a.

mab-20 mutants have ventral enclosure defects

Initial observations of mab-20 mutants suggested that 
hermaphrodites have a low fecundity, a defect that is rescued 
zygotically upon the introduction of mab-20(+ either by 
matings or as a transgene (data not shown). In a given 
population, 84% (n=452) of mab-20(ev574); him-5(e1490) 
embryos die, apparently from the extrusion of internal contents 
from the ventral side during the elongation phase of 
development (Fig. 3A,B). The embryonic lethality of ev574 is 
rescued by a mab-20(+) transgene array; only 28% (n=256) 
of mab-20(ev574); evls74d (mab-20(+ ) (evpRII.2); rol- 
6(su1006)); him-5(e1490) embryos die. Incomplete rescue is 
attributed to the lethality conferred by the integrated evIn74d 
array; 40% (n=235) of evls74d; him-5(e1490) embryos die, 
most from the extrusion of internal contents. In addition, 15% 
(n=223) of him-5(e1490) control embryos die for unknown 
reasons, a result that has been repeated with several isolates of 
him-5(e1490). Interestingly, mab-20 mutant embryos burst at 
the ventral midline in a manner similar to animals mutant for 
the VAB-1 Eph receptor tyrosine kinase (Fig. 3C). vab-1 
mutants are thought to burst because of a failure to properly 
enclose the ventral surface with hypodermis (George et al., 
1998). These observations prompted an investigation of ventral 
enclosure of mab-20(ev574) mutant embryos. The process of 
ventral enclosure is reviewed below.

By 250 minutes after first cleavage in wild-type animals, two 
dorsal, two lateral and two ventral rows of hypodermal cells are 
aligned longitudinally and sit on the embryo in a dorsal-
posterior locale, leaving the ventral blast cells exposed (Podbielwicz and White, 1994). The ventrally directed 
migration of the hypodermis is led by two anterior pairs of 
ventral hypodermal cells on either side of the embryo called 
leader cells. Of all ventral hypodermal cells, only the leader 
cells have been reported to extend substantial actin-filled 
membrane extensions toward the ventral midline (Fig. 3D; 
Williams-Mason et al., 1997). After the contralateral leader 
cells meet and form junctions at the ventral midline, the 
trailing posterior ventral hypodermal cells, called pocket cells, leave a 
ventral pocket of neuroblasts uncovered (see Fig. 3G). A 
microfilament purse-string mechanism is thought to draw the 
pocket cells together and complete hypodermal enclosure of the 
embryo (Williams-Mason et al., 1997). Twelve of the seventeen 
pocket cells are P neuroectoblasts (P cells) and are arranged in 
two longitudinal rows of six cells each (Podbielwicz and White, 
1994). After enclosure, all ventral hypodermal cells fuse into 
either the hyp6 or hyp7 hypodermal syncytium except for the 
six pairs of P cells. The dorsal side of each of the twelve 
rhomboid P cells contacts two lateral hypodermal cells, called
Fig. 3. Ventral enclosure defects of mab-20 mutants are associated with ectopic pocket cell contacts. (A-C) DIC photomicrographs of control (A), a mab-20(ev574) (B), and a vab-1(e2027) (C) embryo at approximately the threefold stage of development. In mab-20(ev574) and vab-1(e2027) animals, internal contents (red arrows) have extruded from the ventral midline. Where visible, heads and tails are indicated with long or short white arrows, respectively. (D-J) Animals from a ventral-lateral perspective. Anterior is to the left. (D-F) Deconvolved images of phalloidin-stained embryos with ventral hypodermal leader cells (large asterisks) and pocket cells (small asterisks) migrating toward the ventral midline (down). The leading edges of these migrating cells are actin-rich relative to other parts of the submembranous cytoskeleton. (D) A control embryo extending an actin-rich process (arrow) from a leader cell toward the ventral midline. (E,F) mab-20(ev574) embryos in which the pocket cells also have substantial actin-rich processes, but unlike leader cell processes do not extend toward the midline (red arrows). Note that, in F, the pocket cell that extends many processes also ectopically contacts a non-neighboring anterior pocket cell. The leader cell extensions are not affected in the mutant. (G,H) Scanning electron micrographs of a control (G) and a mab-20(ev574) (H) embryo during ventral enclosure showing leader cells (large asterisks) and pocket cells (small asterisks). Ectopic contacts between pocket cells (red arrowhead) are clearly visible in the mutant and may result in the obvious kink of the embryo. In both panels, neuroblasts fill the pocket ventral to the migrating leading edge hypodermal cells. (I,J) Control (I) and mab-20(ev574) (J) hatchlings stained for hypodermal cell boundaries with the MH27 antibody. The six P neuroectoblasts on the left side of the wild-type animal (P1/P2 etc.) are surrounded by the hypodermal syncytium, hyp7. The mab-20(ev574) animal exhibits a starburst pattern of P cells with numerous ectopic contacts to non-neighboring cells at the midline. Only the P7/P8 cell is labeled for reference in J. All animals shown carry the him-5(e1490) mutation. Scale bars are 10 μm.

The abnormal body shape of mab-20 mutants may be caused by the misalignment of circumferential microfilaments during embryonic elongation

The most obvious phenotype of mab-20(ev574) homozygotes is the severe deformities in body size and shape (Fig. 4A,B). Newly hatched animals are shorter than the wild type and frequently have bulges and constrictions randomly about the body and head. This phenotypic trait is first observed after embryonic elongation begins. Approximately 88% of larvae have obvious morphological defects, compared to none for wild-type and mab-20(+) rescued lines. Many of the larvae contact non-neighboring P cells (n=100), as determined by immunostaining with mAbMH27, which recognizes a component of adherens junctions of epithelia (Francis and Waterston, 1991). The ectopic contacts often result in a starburst pattern of cell junctions (Fig. 3I,J). No wild-type animals have ectopic P cell contacts (n=100), while only one vab-1(e2027) mutant was observed to have an ectopic P cell contact (n=100). Although both vab-1 and mab-20 mutant embryos burst from the ventral side during elongation, the underlying cellular defects responsible for this trait are apparently different in the two mutants.

The SEM analysis predicts that those embryos that survive enclosure and elongate might exhibit vestigial ectopic pocket cell contacts. 78% of the mutant embryos have P cells that contact non-neighboring P cells (n=100), as determined by immunostaining with mAbMH27, which recognizes a component of adherens junctions of epithelia (Francis and Waterston, 1991). The ectopic contacts often result in a starburst pattern of cell junctions (Fig. 3I,J). No wild-type animals have ectopic P cell contacts (n=100), while only one vab-1(e2027) mutant was observed to have an ectopic P cell contact (n=100). Although both vab-1 and mab-20 mutant embryos burst from the ventral side during elongation, the underlying cellular defects responsible for this trait are apparently different in the two mutants.

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either outgrow the phenotype or fail to survive, leaving only 55% of adults with body deformities.

We investigated the arrangement of hypodermal cells during embryonic elongation in detail since the defects in body shape first appear during this phase. Immediately preceding elongation, bundles of parallel microfilaments align along the entire circumference of the body wall (Priess and Hirsh, 1986), which will be referred to here as the circumferential contour. The bundled microfilaments connect opposite sides of each hypodermal cell and are situated near the apical surface. Following ventral enclosure, a threefold circumferential constriction of the microfilaments results in a fourfold elongation of the embryo with no increase in cell number or volume. The force generated by the microfilaments during elongation is evenly distributed across the surface by both the embryonic sheath and hypodermal microtubules, without which animals develop bulges and constrictions (Priess and Hirsh, 1986). In principle, an altered arrangement of the hypodermal microfilaments could affect the distribution of force on the body wall and create similar bulges and constrictions.

In addition to the ectopic contacts between ventral pocket cells described above, mAbMH27 staining of embryos revealed that the lateral hypodermal seam cells also make ectopic contacts (Fig. 4C-F). 56% of mab-20(ev574) mutant embryo sides exhibit a disorganized row of seam cells in which at least one cell makes contacts to more than two neighboring seam cells (n=208), compared to 2% for wild-type sides (n=200). The ectopic contacts are apparent prior to elongation and persist thereafter. Intriguingly, the pattern of seam cell clusters in mab-20 mutants is strikingly similar to earlier stages of wild-type development when the 10 seam cells on each side of the embryo are forming a lateral line. During this process, the seam cells and/or their precursors undergo numerous short-range migrations and rearrangements (see Materials and Methods; Schnabel et al., 1997). The conformation of the mab-20(ev574) seam cell clusters is consistent with an early arrest in the migrations of the seam cells as they form the lateral line.

We postulated that the altered P cell and seam cell hypodermal arrangements in mab-20 mutants might be associated with alterations in microfilament alignment. To address this hypothesis, embryos were stained with rhodamine-conjugated phalloidin (Fig. 4G-J). Surprisingly, the alignment of microfilaments within pocket cells in mab-20(ev574) embryos deviates little from the wild type (Fig. 4H). The most striking defect is microfilament misalignment within clustered seam cells. Several extreme examples were observed in which microfilament arrays were U-shaped within the clustered seam cells, extending back toward the same side of the animal as the side of their origin (Fig. 4I). Bulges along the embryos were observed only in areas where microfilaments within neighboring seam cells were aligned obliquely with respect to the circumference. Bulges were not observed in animals that had normal seam cell arrangements.
cells of a similar type is observed in mab-20 mutant male tails. The adult male tail is a specialized structure used for copulation. It is bilaterally symmetric and each side has nine sensory rays that project laterally and are embedded within a cuticular spade-shaped fan (Fig. 5A). The ectodermal development of the male tail has been described in detail by Sulston et al. (1980) and is summarized here. Differences in the male and hermaphrodite hypoderimal lineage manifest early in the third larval stage (Sulston and Horvitz, 1977). Extra rounds of division in the posterior seam cells results in 9 R(n) cells on each side, each of which gives rise to five or six cells and one programmed cell death. Two or three of the cells within each group fuse to either the surrounding hyp7, or the tail seam. The remaining three cells become a ray precursor cluster (see Fig. 5E) that develops into two neurons and a structural-support cell that en sheathes the dendrites of the two neurons. During the anterior retraction of the entire tail at the end of the fourth larval stage, the nine ray clusters remain attached to the cuticle and become surrounded by hyp7, forming the finger-like rays of the adult.

mab-20(ev574) mutant male tails have fusions of sensory rays 1 through 4, 4 with 6, and 7 with 9, a phenotypic trait that is completely rescued by mab-20(+); mab-20(bx24) and mab-20(bx61) mutants. Consistent with the ray fusions of mab-20(ev574) adults, aggregations of ray precursor clusters 1 through 4, 4 with 6, and 7 with 9 were observed by staining with mAbMH27. Clusters 5 and 8 develop in isolated locales relative to other ray clusters in the wild type and rarely fuse to other clusters in mab-20 mutants.

A model proposed to explain why rays fuse is that each ray expresses a particular identity (Baird et al., 1991; Chow and Emmons, 1994) and that, in some mutants with fusions, ray identities equivocate. In a wild-type genetic background, the unc-129p::GFP reporter is highly expressed in one neuron in each of rays 1, 5, and 7 in the control (B) and mutant (D) animals. Ray 8, anterior to the fusion of 7 and 9, is not labeled. (E,F) Control and mutant mab-20(ev574) larval male tails, respectively, stained for hypodermal cell boundaries with mAbMH27. Each control ray precursor cluster is distinct and appropriately labeled (E), but in the mutant, clusters 1, 2, 3, 4 and 6, as well as clusters 7 and 9 appear as indistinct aggregates (F). For reference, one R(n)p cell, R1.p is labeled in the control (E). The hypodermal syncytium (hyp7), the seam, and the phasmid socket (Ph) are indicated. All animals contain him-5(e1490). Scale bars are 10 μm.

mab-20 mutants have defects in axon guidance and cell migration

The DA and DB motor neurons were examined for axon guidance defects in the mab-20 mutants using an unc-129 neural-specific (unc-129p::GFP) reporter (Colavita and Culotti, 1998; Colavita et al., 1998). The cell bodies of these neurons reside in the ventral cord and extend dendrites longitudinally along the ventral nerve cord and axons circumferentially to and then along the dorsal nerve cord (Fig. 6). The axons and dendrites are normally tightly fasciculated within the cords and rarely, if ever, have errant circumferential pioneer guidance defects in control animals (Table 2). 17% of mab-20(ev574) mutants, however, have fasciculation defects and an additional 4% have pioneer guidance defects of at least one of the DA or DB motor axons (Table 2; Fig. 6).
Ce-Sema-2a prevents ectopic cell contacts

In addition to the axon guidance defects, mab-20 mutants have ectodermal and mesodermal cell migration errors. Occasionally, the left Q neuroblast (QL) and the distal tip cells (DTCs) fail to stop migrating at typical positions in mab-20(ev574) mutants. QL normally undergoes a short posterior-oriented migration then divides. Its posterior daughter normally stops migrating and gives rise to the PVM touch receptor neuron (reviewed in Hedgecock et al., 1987). The total migratory distance of QL is less than 1 epithelial cell diameter, leaving PVM in the posterior quarter of the animal. PVM was found near or anterior to the mid-body in 27% of mab-20 animals (n=184) compared to 0% for wild-type controls (n=200), indicating that aberrant anteriorly directed migration of QL occurs frequently in the absence of Sema-2a function (Fig. 6F-H).

The DTCs of late larval stage hermaphrodites migrate on the basal surface of the hypodermis in a series of sequential steps that shape the adult gonad arms. The shape of mutant hermaphrodite gonad arms indicates that the migrating DTCs, which normally turn twice in the wild type, undergo additional turns in 9% (n=131) of mab-20(ev574); him-5 control hermaphrodites. There is no correlation between the body shape abnormalities and either the QL or DTC migratory defects. These results suggest that Ce-Sema-2a may be required to prevent the migration of both the QL and the DTCs beyond their normal stopping points.

Two different types of mab-20 reporter constructs were made.

Table 2. Axon guidance defects of mab-20 mutants and strains expressing ectopic mab-20(+).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% misguided DA/DB axons</th>
<th>% defasciculated nerve cords</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>mab-20(ev574)*</td>
<td>4</td>
<td>17</td>
<td>106</td>
</tr>
<tr>
<td>evIs74[mab-20(+); rol-6(su1006)]</td>
<td>3</td>
<td>8</td>
<td>100</td>
</tr>
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</table>

Axon guidance and nerve cord fascication defects are reported as percentages of animals that contain them. Strains carried the transgene array evIs82b[evpAC12 (unc-129ns::gfp); dpy-20(+)] in order to visualize axons and nerve cords. All strains studied also contained him-5(e1490) except (*).
evpPRII.67 and evpPRII.75 are transcriptional fusions to gfp, while evpPRII.14 encodes a GFP-tagged version of Ce-Sema-2a (Fig. 2). Like the evIs74 series of mab-20(+) transgene arrays (Table 1), animals transgenic for evpPRII.14, but not evpPRII.67 and evpPRII.75, phenocopy mab-20 mutants (data not shown).

Except for an earlier onset of GFP expression from both evpPRII.67 and evpPRII.75, all reporters displayed very similar expression patterns. Embryonic GFP expression reported by evpPRII.14 is first visible at approximately 240 minutes of development and is ubiquitous (Fig. 7), a pattern that persists until hatching. At the beginning of each larval stage, dividing seam cells express mab-20::GFP, as do the ray precursor clusters (arrows in H and I point to a single cluster), which are the R(n).p descendents and are situated in a deeper focal plane. Scale bars are 10 μm.

DISCUSSION

The fundamental molecular mechanisms involved in cell shape changes, cell movements, and the spatial rearrangements of cells that generate form are only beginning to be understood. One of the first examples of a protein that regulates epithelial morphogenesis is the product of the Drosophila decapentaplegic (dpp) gene. Dpp is a member of the TGF-β superfamily of secreted ligands that is required for concerted shape changes in epithelial cells that enclose the embryo through a process known as dorsal closure (Riesgo-Escovar and Hafen, 1997). The row of epithelial cells that constitute the dorsal edge of the epithelium activates a JNK pathway that results in the expression of dpp. Exactly how dpp expression in the leader cells orchestrates a concerted elongation of the lateral epithelial cells in a dorsoventral (DV) direction is unknown. Somewhat analogous to Drosophila dorsal closure is ventral enclosure in C. elegans but, instead of relying on concerted changes in the shape of lateral hypodermal cells, ventral enclosure primarily depends on an active migration of cells on the ventral edge of the hypodermis called leader cells (Williams-Masson et al., 1997). This migration is assisted by VAB-1, which putatively acts in ventral neuroblasts to guide overtaking leader cell extensions to the ventral midline (George et al., 1998). Once membrane extensions from contralateral leader cells have met at the midline, a catenin-cadherin
complex encoded by *hmp-1*, *hmp-2* and *hmr-1* is thought to fortify the contacts and enable the completion of leader cell migration (Costa et al., 1998).

An unexplored question concerning ventral enclosure and morphogenesis in general is how concerted cell movements, shape changes and spatial rearrangements of cells, all of which involve the formation and breaking of transient contacts between cells or with the ECM, are regulated. A detailed study of the phenotype of *mab-20* mutants has revealed that *C. elegans* Semaphorin-2a regulates the formation or stabilization of contacts between epithelial cells during epithelial morphogenesis. To put the morphogenetic defects of *mab-20* mutants into context, a brief review of the major morphogenetic events of *C. elegans* development is presented.

*C. elegans* hypodermal morphogenesis can be divided into several steps. First, several short-range migrations and rearrangements of ectoblasts produce six longitudinal rows of hypodermal cells that sit on the embryo in a dorsal-posterior locale, leaving the ventral neuroblasts uncovered (Sulston et al., 1983). Second, while the two dorsal rows of hypodermal cells intercalate, the leader cells of the ventral row of hypodermal cells initiate a migration towards the ventral midline to cover the rest of the body of the embryo in hypodermis in a process called ventral enclosure (Williams-Masson et al., 1998, 1997). It is not known how the head becomes covered with hypodermis. Third, microfilament bundles within the hypodermis align in parallel along the circumferential contour after ventral enclosure. The fourfold elongation of the embryo is mediated by the threefold contraction of these circumferential microfilaments (Priess and Hirsh, 1986). Fourth, six P cells become vulva precursor cells, some of which undergo several divisions, movements and fusions, and then evert to form a vulva by adulthood (Greenwald, 1997). Fifth, the three posterior seam cells on both sides of males undergo extra rounds of division in the third and fourth larval stages to generate the ray precursor cells (Sulston et al., 1980). Finally, an anterior-directed retraction of the entire male tail at the end of the fourth larval stage results in the formation of male tail sensory rays embedded within a cuticular fan. Except for dorsal hypodermal intercalation, head and vulva morphogenesis, and the anterior retraction of the male tail, Ce-Sema-2a is involved in each of these processes.

The earliest phenotypic trait of *mab-20* mutants is the clustering of lateral hypodermal seam cells with each other. During the longitudinal alignment of the seam cell rows, numerous migrations and rearrangements of seam cells and their precursors occur. A comparison of the seam cell positions in *mab-20* mutant embryos to the wild type suggests that migrating seam cells fail to reach their normal anteroposterior (AP) positions, resulting in the observed mutant seam cell clusters.

The next defect observed in *mab-20* mutants is the formation of ectopic contacts between non-neighboring ipsilateral ventral hypodermal cells as they move ventrally (Fig. 3H). These early ectopic contacts likely persist and are later observed as ectopic contacts between P cells after the completion of ventral enclosure (Fig. 3J). The ventral hypodermal cell defects do not appear to depend on prior defects in seam cell arrangements, since they can occur even when all of the seam cells have migrated and intercalated properly.

There are at least two classes of ventral hypodermal cells that exhibit different behaviors during enclosure. On each side there are two leader cells that extend long actin-rich membrane extensions, while the more posterior hypodermal pocket cells fail to extend substantial actin-rich processes (our observations; Williams-Masson et al., 1997). Observation of live embryos by Raich and Hardin (personal communication) suggest that pocket cells exhibit membrane extensions, but these are transient, much shorter than their leader cell counterparts, and cannot be observed by phalloidin staining. It is tempting to speculate that Ce-Sema-2a acts to limit membrane extensions from pocket cells. For example, the actin-rich extensions that emanate from the pocket cells of *mab-20* mutant embryos could represent exploratory processes that would otherwise collapse in the presence of Ce-Sema-2a (Fig. 3E-F). Instead, in the absence of Ce-Sema-2a, these exploratory processes may be free to extend and establish stable ectopic contacts to non-neighboring pocket cells. Leader cells may be unresponsive to the presence of Ce-Sema-2a and therefore extend processes in its presence or absence.

The third defect observed in *mab-20* embryos is the extrusion of internal contents from the ventral surface during embryonic elongation. Contraction of the circumferential hypodermal microfilaments presumably forces internal contents through the incompletely enclosed ventral surface of *mab-20(ev574) embryos in a manner similar to *vab-1(e2027)* mutants. However, our results indicate that enclosure fails in these two mutants for different reasons. It is thought that VAB-1 either directly or indirectly regulates ventral neuroblast interaction with the overlying ventral hypodermis to guide the extensions of contralateral leader cells towards each other (George et al., 1998). In *vab-1(e2027)* mutants, the contralateral ventral hypodermal cells frequently fail to come together at the ventral midline, resulting in embryos that burst during elongation. Our observations show that these *vab-1(e2027)* mutants that escape lethality have no ectopic pocket cell contacts, suggesting that a disruption of neuroblast-hypodermal interaction does not necessarily result in ectopic hypodermal cell contacts. In contrast to *vab-1* mutants, the data presented here suggest that Ce-Sema-2a prevents ectopic contacts between ventral hypodermal cells during enclosure. Thus, *mab-20* mutants most likely burst from the ventral side during elongation because abnormal pocket cell contacts either sterically hinder pocket closure or weaken the contacts formed between the contralateral pocket cells at the ventral midline.

Those *mab-20* mutant embryos that escape lethality frequently develop severe bulges and constrictions during embryonic elongation. Body wall bulges are always coincident with circumferentially misaligned microfilaments contained within the clustered seam cells of *mab-20* mutants. This observation raises two obvious and related questions. First, do misaligned microfilaments cause the bulge or do body wall bulges disrupt the alignment of microfilaments? Several lines of evidence support the former hypothesis. (1) Microfilament alignment is a prerequisite to elongation (Priess and Hirsh, 1986), and the bulges are only observed in mutants well into elongation and past the twofold stage. (2) Single seam cells containing misaligned microfilaments are observed without a corresponding bulge in the body wall (Roy, 1999), but bulges are never observed without a corresponding cluster of seam cells containing misaligned microfilaments. (3) Microfilament arrays are often U-shaped within the clustered seam cells,
extending back toward the same side of the animal as the side of their origin. It is easy to imagine how the contraction of these U-shaped filaments results in the associated bulge. Together, these results strongly suggest that misaligned microfilaments cause body wall bulging and not vice versa.

A second question is whether microfilaments are misaligned because of the ectopic contacts of the seam cells in which they are contained, or because their alignment depends on a global mechanism or cue that is disrupted in mab-20 mutants. The latter hypothesis is not as likely since (1) there are no obvious alterations in the axes of mab-20 mutants and (2) microfilament alignment is never aberrant in dorsal hypodermis and rarely deviates from normal in the ventral hypodermis. Instead, we hypothesize that as a secondary consequence of not properly intercalating within the seam cell queue, the DV and AP axes of clustered seam cells can become skewed. This in turn skews unknown determinants of microfilament polarity and results in a corresponding misalignment of microfilaments prior to elongation.

The last observed morphogenetic defect in mab-20 mutants is the fusion of normally distinct sensory rays of the male tail. In all mab-20 alleles, an exact correlation is found between those ray precursor clusters that make ectopic contacts and those rays that later fuse, suggesting a causal relationship (our observations; Baird et al., 1991; Chow and Emmons, 1994). Intervening hyp7 prevents lasting contacts between neighboring ray precursor clusters, a process that fails in mab-20 mutants. A subtle question that arises is whether the focus of Sema-2a in the male tail is the regulation of contact formation between hyp7 and the R(n)ps or between neighboring clusters. Since hyp7 contacts the dorsal and ventral sides of the R(n)s before they give rise to the ray precursors, it is difficult to imagine anything but a passive role for hyp7 in maintaining distinct ray precursor clusters as they are born. Thus, it is more likely that Sema-2a regulates contact formation between neighboring precursor clusters, possibly by preventing membrane extension between clusters that would otherwise result in stable contacts between clusters and consequent ray fusions.

The role of Ce-Sema-2a in axon guidance
The errors in nerve cord fasciculation and circumferential pioneer axon guidance in mab-20 mutants could be secondary defects that result from the aberrant positioning of cues from a disorganized hypodermis. Alternatively, these cells may require Ce-Sema-2a directly as a guidance cue, an expectation that is based on one known function of the vertebrate semaphorins. The lack of highly penetrant axon guidance and cell migration defects in mab-20 mutants may reflect a function in axon guidance that is redundant with the other two semaphorin genes present in the C. elegans genome (D1037.2 and Y54E5B.1). Our recent recovery of mutations in these genes (P. J. R., V. Ginzburg and J. G. C., unpublished results) should allow us to test this possibility. The characterization of mutants defective in putative signaling components downstream of Sema-2a, such as a Ce-Sema-2a receptor, may also enable us to test whether Ce-Sema-2a directly guides migrations (R. Ikegami and J. G. C., unpublished results).

A model for Ce-Sema-2a activity
We show here for the first time that a member of the semaphorin family regulates both epithelial morphogenesis and the migrations of cells, including epidermal cells, the QL neuroblast and the distal tip cells. In addition, Ce-Sema-2a plays a role in guiding DA and DB pioneer axons. As each of these cells and axons migrate towards their target destination, they must transiently establish contacts to neighboring cells and the ECM via membrane extensions. It is through the modulation of the cytoskeletal network within these membrane extensions that cues such as SEMA3A can guide migrations (Fan and Raper, 1995). One simple model that explains the ectopic hypodermal cell contacts and the errors in cell and axon guidance in mab-20 mutants is that, akin to SEMA3A, Ce-Sema-2a functions to prevent or repel cellular extensions of actin-rich exploratory processes. In the absence of functional Ce-Sema-2a, the QL neuroblast and the distal tip cells migrate beyond their normal stopping points, the DA and DB axons fail to restrict exploration, and epithelial pocket cells, seam cells and ray precursor clusters fail to restrict inappropriate adhesive contacts with each other during morphogenesis.

mab-20 reporters are expressed nearly ubiquitously during mid-embryogenesis and male tail morphogenesis, yet many migrations and cell rearrangements in mab-20 mutants are normal. The ability of a cell to respond to Sema-2a must therefore be controlled by the expression of a Sema-2a receptor, a co-factor or downstream signaling components, rather than by the tight spatiotemporal control of Ce-Sema-2a production. MAB-26 is a candidate for a regulated element of the Ce-Sema-2a pathway since mab-26 mutants share many of the phenotypic traits of mab-20 mutants and do not appear to significantly enhance the mab-20 null allele (Roy, 1999). Sema-2a may be a global regulator of cell-cell interactions and any cell with the ability to receive and transduce the Sema-2a signal may have access to such regulation.

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REFERENCES