The divergent C. elegans ephrin EFN-4 functions in embryonic morphogenesis in a pathway independent of the VAB-1 Eph receptor

Ian D. Chin-Sang*,†, Sarah L. Moseley†, Mei Ding, Robert J. Harrington, Sean E. George and Andrew D. Chisholm‡

Department of Molecular, Cell, and Developmental Biology, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA

*Present address: Department of Biology, Queen’s University, Kingston, Ontario K7L 3N6, Canada
†These authors contributed equally to this work
‡Author for correspondence (e-mail: chisholm@biology.ucsc.edu)

Accepted 4 September 2002

SUMMARY

The C. elegans genome encodes a single Eph receptor tyrosine kinase, VAB-1, which functions in neurons to control epidermal morphogenesis. Four members of the ephrin family of ligands for Eph receptors have been identified in C. elegans. Three ephrins (EFN-1/VAB-2, EFN-2 and EFN-3) have been previously shown to function in VAB-1 signaling. We show that mutations in the gene mab-26 affect the fourth C. elegans ephrin, EFN-4. We show that efn-4 also functions in embryonic morphogenesis, and that it is expressed in the developing nervous system. Interestingly, efn-4 mutations display synergistic interactions with mutations in the VAB-1 receptor and in the EFN-1 ephrin, indicating that EFN-4 may function independently of the VAB-1 Eph receptor in morphogenesis. Mutations in the LAR-like receptor tyrosine phosphatase PTP-3 and in the Semaphorin-2A homolog MAB-20 disrupt embryonic neural morphogenesis. efn-4 mutations synergize with ptp-3 mutations, but not with mab-20 mutations, suggesting that EFN-4 and Semaphorin signaling could function in a common pathway or in opposing pathways in C. elegans embryogenesis.

Key words: Morphogenesis, C. elegans, Ephrin, Semaphorin, EFN-4, VAB-1

INTRODUCTION

The epidermis of C. elegans is a simple epithelium that forms the skin of the animal. Morphogenesis of the C. elegans epidermis provides a model for several aspects of epithelial biology, including epithelial cell fate determination, cell polarity, cell motility and cell shape (Chin-Sang and Chisholm, 2000; Michaux et al., 2001; Simske and Hardin, 2001). Epidermal cells arise on the dorsal surface of the embryo, where they form junctions to give rise to an epithelial sheet. The epidermal sheet then spreads laterally and ventrally to close up at the ventral midline, an example of epiboly referred to in C. elegans as epidermal enclosure (Williams-Masson et al., 1997). Epidermal enclosure itself can be divided into two steps: in the first step, two bilateral pairs of epidermal cells extend actin-rich processes over a substrate of neural cells. These processes meet their contralateral counterparts at the ventral midline, leading to the rapid formation of junctions between the two pairs of cells (Raich et al., 1999). In the second step, other epidermal cells enclose the posterior body, possibly by a supracellular actin purse-string mechanism (Simske and Hardin, 2001).

We have previously shown that the C. elegans Eph receptor VAB-1 and its ephrin ligand EFN-1 (previously called VAB-2) function in epidermal enclosure (Chin-Sang et al., 1999; George et al., 1998). During epidermal enclosure, VAB-1 and EFN-1 are predominantly expressed and required in underlying neurons but not in the epidermal cells. Time-lapse analysis of vab-1 and efn-1 mutant embryos revealed specific defects in the organization of neural precursors, manifested as a failure to close the ‘gastrulation cleft’, a depression in the ventral surface of the embryo left after ingression of endodermal and mesodermal precursors. Because epidermal cells migrate over the descendants of these ventral neural precursors, a simple model for the non-autonomous effects of vab-1 or ephrin mutants on epidermal enclosure is that they arise as a consequence of an altered neural substrate. The defects in neural cell organization in C. elegans Eph signaling mutants may resemble cell sorting defects observed in vertebrates when Eph signaling is disrupted (Mellitzer et al., 1999; Xu et al., 2000).

The relative simplicity of the C. elegans Eph signaling network allows a systematic analysis of its roles in a single morphogenetic process. A single Eph receptor (VAB-1) and four ephrins (EFN-1 through EFN-4) have been identified in C. elegans (Wang et al., 1999). efn-1 mutations, obtained from forward genetic screens, cause phenotypes weaker than but otherwise indistinguishable from those of vab-1 mutants;
expression, binding and genetic data indicate that EFN-1 functions as a ligand for VAB-1 in embryonic morphogenesis (Chin-Sang et al., 1999). efn-2 and efn-3 mutants, obtained by reverse genetics, appear essentially normal during embryogenesis; EFN-2 is expressed embryonically in a pattern similar to EFN-1. The efn-1 null phenotype is much weaker than that of vab-1, and an efn-1 efn-2 efn-3 triple mutant resembles a vab-1 knockout, suggesting that efn-2 and efn-3 play subtle roles in embryogenesis that are partly redundant with efn-1 (Wang et al., 1999) (S. L. M. and A. D. C., unpublished) The similarity of the efn-1 efn-2 efn-3 triple mutant phenotype to the vab-1 null phenotype suggests that these three ephrins might account for all signaling functions of the VAB-1 Eph receptor. VAB-1/EFN-1 signaling also appears to function partly redundantly with a pathway involving the LAR-like receptor tyrosine phosphatase PTP-3 (Harrington et al., 2002).

We report our analysis of a fourth worm ephrin, EFN-4, focusing on its functions in embryonic morphogenesis. We show that EFN-4 is also required for movement of embryonic neural precursors and enclosure of the epidermis, but that its role is distinct from those of VAB-1 or EFN-1. Unexpectedly, efn-4 mutations show strong synergistic interactions with mutations in vab-1 and efn-1, suggesting that EFN-4 may function in a VAB-1-independent pathway. efn-4 mutations also synergize with ptp-3 mutations, suggesting that EFN-4 does not function solely in the PTP-3 pathway. Through a comprehensive analysis of vab-1 efn double mutants we find that only efn-4 mutations show strong synergism with vab-1 receptor mutants. It had been suggested that the Semaphorin-2A homolog MAB-20 might function in the EFN-4 pathway (Harrington et al., 2002), and injected at a concentration of ~70 ng/µl. Injected hermaphrodites were allowed to recover for 12 hours; eggs laid over the ensuing 24 hours were analyzed. RNAi of vab-1 efn-4 double mutants could be propagated very slowly, and displayed >99% lethality (see Fig. 5). Some vab-1; efn-4 double mutants were maintained as homozygous lines balanced by an extrachromosomal array (juEx350) carrying the wild-type copies of vab-1 and efn-4. juEx350 was formed by co-injection of the vab-1(+)/cosmid M03A1 (1 ng/µl), the efn-4(+) cosmid F56A11 (1 ng/µl), the plasmid pRf4 (30 ng/µl), the SUR-5-GFP marker pTG96 (30 ng/µl) and pBluescript (40 ng/µl). ptp-3 efn-4 double mutants and vab-1 ptp-3 efn-4 triple mutants were analyzed from balanced strains of genotype ptp-3 (or vab-1 ptp-3/mnl1 mls14; efn-4).

mab-20 efn-4 double mutants were constructed using dpy-9 as a marker in trans to efn-4; all double mutants were confirmed by multiple complementation tests. We used one-way ANOVA (Statview) to compare differences in penetrance of phenotypes.

**MATERIALS AND METHODS**

**Genetic analysis**

*C. elegans* strains were cultured using standard methods, at 20°C unless stated (Brenner, 1974). Mutations used are described elsewhere (Riddle et al., 1997), in papers indicated or in this paper. Mutations used were:

- LGI, mab-20(e574, e819, bx24);
- LGII, vab-1(dx31, e118, ju8, e699, e856, e1029), ptp-3(op147) (Harrington et al., 2002);
- LGIV, dpy-9(e12), efn-1(e96, jul, ju90) (Chin-Sang et al., 1999);
- LGX, efn-3(e696) (Wang et al., 1999).

Rearrangements used were: *mnl1 mls14* (Edgley and Riddle, 2001); and *mDp1(IV; f)*.

All efn-4 mutations were EMS-induced, with the exception of bxs (diepoxybutane). S. Brenner isolated the efn-4 mutations e36 and e660; J. Hodgkin isolated e1746; X. Huang isolated ju134. All efn-4 alleles failed to complement bxs. efn-4(ju314), isolated independently from e1746, was found to cause similar phenotypes and to result from the same molecular lesion.

*efn-19* is an X-ray-induced mutation isolated by S. Brenner and assigned to *mab-20* by mapping and complementation tests (data not shown). *mab-20(e819)* causes embryonic and larval lethal phenotypes similar in penetrance to those of *mab-20(e574)*, suggesting that it causes a strong loss of function. The weak vab-1 allele e1029 has been found to result from a mis-sense mutation (C122Y) in the extracellular domain of VAB-1. Penetration of lethality and morphogenetic defects was quantitated as described previously (George et al., 1998); all estimates of penetrance were obtained from counts of between 3 and 6 complete broods.

**Double mutant construction and analysis**

*efn-4* double mutants were completely inviable and were maintained as balanced strains. We initially used *dpy-9* as a balancer for *efn-4*; however, such strains were unstable, owing to recombination between *dpy-9* and *efn-4*. We subsequently constructed more stable balanced strains of genotype *vab-1/mln1 mls14; efn-4*. *vab-1 efn-4* double homozygotes were identified as non-GFP-expressing progeny from such heterozygotes. Some *vab-1 efn-4* double mutants constructed using weak *vab-1* alleles were viable; rare viable and fertile non-GFP-expressing progeny were identified as segregants from *mln1 mls14* balanced strains. Such homozygous *vab-1 efn-4* double mutants could be propagated very slowly, and displayed >99% lethality (see Fig. 5). Some *vab-1; efn-4* double mutants were maintained as homozygous lines balanced by an extrachromosomal array (juEx350) carrying the wild-type copies of *vab-1* and *efn-4*. juEx350 was formed by co-injection of the *vab-1(+) cosmid M03A1* (1 ng/µl), the *efn-4(+) cosmid F56A11* (1 ng/µl), the plasmid pRf4 (30 ng/µl), the SUR-5-GFP marker pTG96 (30 ng/µl) and pBluescript (40 ng/µl). ptp-3 efn-4 double mutants and vab-1 ptp-3 efn-4 triple mutants were analyzed from balanced strains of genotype *ptp-3 (or vab-1 ptp-3/mnl1 mls14; efn-4).*

**RNA interference of *efn-4***

For dsRNA-mediated interference of *vab-1* a 1.9 kb *Spr1-PstI* fragment of the *vab-1* cDNA was subcloned into the RNAi vector L4440. RNAs were synthesized and annealed as described (Harrington et al., 2002), and injected at a concentration of ~70 ng/µl. Injected hermaphrodites were allowed to recover for 12 hours; eggs laid over the ensuing 24 hours were analyzed. RNAi of *vab-1* resulted in phenotypes approximately half as penetrant as the null mutant (data not shown).

**Molecular analysis of *efn-4***

*efn-4* corresponds to the predicted gene F56A11.3. cDNAs from this gene had been isolated in a genome-wide cDNA sequencing project (Maeda et al., 2001). The longest cDNA, yk449e2, was sequenced completely; this cDNA lacks the first 11 bases of the predicted coding sequence. The predicted EFN-4 preprotein consists of 348 amino acid residues; an N-terminal signal sequence is predicted to be cleaved near S328. The lesions of the alleles were determined by sequencing genomic DNA from the mutants, as described (George et al., 1998). Sequences of primers are available on request. Using Southern blotting of genomic DNAs and sequencing, we determined that *bxs* is a deletion of 1838 bp, encompassing exon 2 and parts of introns 1 and 2. The breakpoints of *bxs* are: 5’ ggttgtaacaaca[a][bxs][a]acactaatattt 3’.

**Transgenic rescue and reporter constructs**

To generate a genomic clone containing *efn-4*, we subcloned a 16.1 *HindIII-EagI* genomic DNA fragment from cosmid F56A11 into pSL1190, creating pCZ148. This clone contains 5.3 kb genomic sequence 5’ to the EFN-4 ATG. Transgenic lines (juEx150, juEx152, juEx153) were made by co-injection of pCZ148 and the marker plasmid pRF4 (both at ~50 ng/µl) in an *efn-4(bxs)* background and the penetrance of the posterior morphology (Vab) phenotypes quantitated in transgenic (Roller) animals. In a typical transgenic line the penetrance of the Vab phenotype was 0.7% (2/250 Rollers were
EFN-4 functions in morphogenesis 5501

Vab, compared with 27% in control lines bearing pRF4 alone (n=175). To overexpress EFN-4 we also used the transgene juIs350 described above.

An EFN-4::GFP reporter was constructed by insertion of the GFP intron cassette from vector pPD121.89 into the Sac II site of the rescuing genomic clone pCZ148, to create the EFN-4::GFP clone pCZ147. This clone is predicted to encode EFN-4 with GFP inserted in-frame close to the N terminus of the mature protein, after residue 33. Three transgenic lines (juEx151, juEx205 juEx206) were established by injection of pCZ148 (40 ng/μl) with the co-injection marker plasmid pRF4, and two lines (juEx206, juEx210) formed by co-injection of pCZ147 and the lin-15(+) plasmid pLin-15EK into lin-15(n765) animals. All EFN-4::GFP transgenic lines yielded similar expression patterns. Chromosomal integrants of juEx210 (jus109 and jus110) were made by X-ray mutagenesis. The lethality of an efn-4(bx80) strain is suppressed from ~32% to ~5% by the jus109 transgene. Staining with anti-EFN-4 antisera was performed on animals overexpressing EFN-4, either from the EFN-4::GFP array (jus109) or from EFN-4-overexpressing animals (juEx350). To examine co-expression of EFN-4::GFP and VAB-1, we established a VAB-1-overexpressing array (juEx44S) by injection of the vab-1(+) cosmid M03A1 (15 ng/μl) and pRF4 (30 ng/μl) into jus109-carrying animals. Co-expression of EFN-1 and EFN-4 was examined by making animals transgenic for jus109 and the efn-1 transgene jus53.

Anti-EFN-4 antibody production

To express EFN-4 in bacterial cells, we cloned DNA encoding amino acid residues 26-325 (i.e. EFN-4 lacking secretion and GPI-addition signal sequences) into pET21a (Novagen), creating the expression clone pC5S51. The resulting fusion protein is tagged with 6xHis at the C terminus. EFN-4-6His was induced with 0.1 mM IPTG in BL21 (DE3) bacteria and the protein is tagged with 6xHis at the C terminus. EFN-4-6HIS was purified on Ni-NTA beads (Qiagen) under denaturing conditions (8 M urea). Purified EFN-4-6HIS was dialyzed in 1× PBS, lyophilized and injected into rabbits for antibody production (Animal Pharm). Anti-EFN-4-6His antibodies were preabsorbed to total protein acetone powders derived from, E. coli, HEK 293T and efn-4(bx80) protein lysates. Affinity-purified anti-EFN-4 antibodies were kept at a final concentration of 1-5 mg/ml.

Fixation and staining of C. elegans embryos were as described (Finney and Ruvkun, 1990). Chicken anti-GFP antibodies (Chemicon), affinity-purified anti-EFN-4, anti-EFN-1 (Chin-Sang et al., 1999), anti-VAB-1 (S. E. G. and A. D. C., unpublished) and MH27 monoclonals (Francis and Waterston, 1991) were used at 1:200 to 1:500 dilution. All fluorescently conjugated secondary antibodies were used at 1:500 dilution in antibody buffer A. Data were collected on a Leica TCS-NT confocal microscope.

Accession Number

The GenBank Accession Number for the efn-4 cDNA is AF410936.

RESULTS

mab-26 mutations affect EFN-4

EFN-4, encoded by C. elegans gene F56A11.3, is a divergent member of the ephrin family of ligands for Eph receptors (Fig.

Fig. 1. Structure of the efn-4 locus and sequence comparisons. (A) Genetic and physical maps of the efn-4 region. mab-26/efn-4 is located at the left end of linkage group IV. (B) Alignment of the conserved domain of EFN-4 (residues 25-170) with those of other ephrins, showing point mutations; locations of predicted secondary structure elements are based on structures of ephrin B2 (Toth et al., 2001; Himanen et al., 2001). The ephrin with highest similarity to EFN-4 within the conserved domain is EFN-1 (31% identity, 46% similarity). The EFN-4 conserved domain is approximately equally similar in sequence to the ephrin A and ephrin B subclasses of vertebrate ephrins (25-20% identity; sequences shown are human ephrin A1 and ephrin B2). Alignments were made using ClustalW; identities are in black and similarities in gray. Secondary structure elements are aligned according to Himanen et al. (Himanen et al., 2001). The strong allele juIs109 affects a non-conserved threonine residue; the weaker allele efn-4 affects a conserved proline, and the weakest allele, efn-1, affects a non-conserved alanine residue. When aligned according to Toth et al. (Toth et al., 2001) juIs134 affects an exposed residue of β-strand D, which may play a role in ephrin dimerization; e660 affects a residue at the end of β-strand C in the tetramerization interface; and e1746 affects a buried residue between β-strand D and α-helix E. (C) Phylogenetic tree of C. elegans, Drosophila and vertebrate ephrin sequences. Phylogenetic analysis suggests that the C. elegans ephrins diverged after the last common ancestor of vertebrates and nematodes; among the C. elegans ephrins, EFN-4 is the most rapidly evolving. Vertebrate ephrin sequences used are from humans except for ephrin A6 (chick). This tree was generated using a neighbor-joining algorithm (MEGA); bootstrap values are indicated at branches.
Like other C. elegans ephrins, EFN-4 shows similarities to both trans-membrane and GPI-anchored vertebrate ephrins within its receptor-binding domain (Fig. 1B). However, the conserved domain of EFN-4 also contains a 21-residue insertion relative to other ephrins (Fig. 1B); this insert is predicted to affect a loop between $\beta$-strands F and G of the ephrin crystal structure (Toth et al., 2001). Phylogenetic analysis suggests that EFN-4 is a rapidly diverging member of the C. elegans ephrin family (Fig. 1C).

We reasoned that mutations in efn-4 might cause defects in epidermal morphogenesis similar to those caused by mutations in the VAB-1 Eph receptor or the EFN-1 ephrin. The mab-26(bx80) mutation, identified on the basis of male tail morphogenetic defects (Chow and Emmons, 1994), causes variable defects in epidermal morphology throughout the body, and maps to the same genomic region as efn-4 (Fig. 1A). We found that a 16.1 kb genomic DNA clone (pCZ148) containing the efn-4 locus completely rescued all mab-26(bx80) phenotypes in transgenic lines (see Materials and Methods).

The bx80 mutation is a 1.8 kb deletion in efn-4, and four additional mab-26 alleles result from point mutations in this gene, confirming that efn-4 corresponds to mab-26; we subsequently refer to this gene as efn-4. All five efn-4 mutations are recessive and cause similar morphogenetic defects in larval epidermis. We determined the sequence of the four point mutations, and the deletion of the bx80 allele, and used this information to map the location of each lesion.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Embryonic arrest (%)</th>
<th>Larval arrest (%)</th>
<th>Adult, Vab (%)</th>
<th>Adult, non-Vab (%)</th>
<th>Wild-type sequence</th>
<th>Mutant sequence</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>bx80</td>
<td>14.1</td>
<td>18.8</td>
<td>12.6</td>
<td>54.4</td>
<td>ACC</td>
<td>ATC</td>
<td>1838 bp deletion of exon 2</td>
</tr>
<tr>
<td>ju134</td>
<td>15.2</td>
<td>10.0</td>
<td>20.2</td>
<td>54.4</td>
<td>TAT</td>
<td>TAA</td>
<td>Y960che</td>
</tr>
<tr>
<td>e36</td>
<td>11.1</td>
<td>10.5</td>
<td>17.7</td>
<td>60.6</td>
<td>TAT</td>
<td>TAA</td>
<td>P54L</td>
</tr>
<tr>
<td>e660</td>
<td>7.0</td>
<td>5.2</td>
<td>21.4</td>
<td>66.4</td>
<td>CGG</td>
<td>CGG</td>
<td>A75V</td>
</tr>
<tr>
<td>e1746</td>
<td>3.6</td>
<td>5.7</td>
<td>18.6</td>
<td>72.0</td>
<td>GCG</td>
<td>GCG</td>
<td>A75V</td>
</tr>
</tbody>
</table>

Fig. 2. efn-4 embryonic and larval phenotypes. Frames from 4D Nomarski DIC movies of individual embryos are shown; all embryos are shown as ventral views; times are relative to first cleavage and are the same for all genotypes. Scale bar: 20 $\mu$m in A-C; 50 $\mu$m in D-G.

(A) Embryogenesis of wild type (N2), ventral views. Note complete closure of the ventral gastrulation cleft (outlined) by 280 minutes. (B,C) Frames from movies of two representative efn-4(bx80) embryos, showing enlarged ventral gastrulation clefts. A total of 58 efn-4(bx80) and 36 efn-4(e36) embryos were recorded for 4D analysis; the range and penetrance of phenotypes was similar in both mutants and the data are considered together below. Some phenotypes of efn-4 embryos could be classified using the same phenotypic classes as used to classify vab-1 and efn-1 phenotypes (George et al., 1998; Chin-Sang et al., 1999). Twenty percent (19/94) of efn-4 animals displayed the Class I phenotype shown in B, in which the gastrulation cleft was enlarged and persistent (dotted outline, 230-280 minutes), the epidermis failed to enclose and internal cells ruptured during early enclosure (330 minutes); this phenotype is seen in 19% of vab-1(0) animals and 12% of efn-1 embryos.

Three out of 94 animals exhibited a slightly weaker phenotype resembling the Class II phenotype (enlarged gastrulation cleft; epidermis encloses, embryo turns then ruptures). One out of 94 efn-4 embryos displayed normal gastrulation clefs and arrested during elongation (vab-1 Class III phenotype), compared with 38% of vab-1 or 16% efn-1 embryos. Sixty-one out of 94 efn-4 embryos displayed the phenotype shown in C, in which the gastrulation cleft was transiently enlarged (230-280 minutes) and later closed; epidermal enclosure and elongation occurred with normal timing, and the embryo hatched with deformations in the posterior epidermis. Ten out of 94 embryos displayed wild-type development. (D-G) L1 larvae, showing wild type (D), efn-4(e36) mutant tail morphology (arrow, E,F) and efn-1(ju1) Notch head morphology (arrow, G).
defects (Table 1; Fig. 2). The three strongest alleles (bx80, e36, and ju134) probably abolish efn-4 function.

When expressed in human 293T cells, EFN-4 is localized to the cell surface, as detected with anti-EFN-4 antibodies (data not shown). In experiments using VAB-1-AP fusion proteins to determine the affinity of VAB-1/EFN-4 interactions, as previously described (Chin-Sang et al., 1999), we were unable to detect binding of VAB-1-AP (10 nM) to EFN-4-expressing cells. These data are consistent with previous evidence that the VAB-1/EFN-4 interaction may be weak (Wang et al., 1999) relative to other VAB-1/EFN interactions.

**EFN-4 regulates embryonic neuroblast movements and epidermal morphogenesis**

*efn-4(bx80)* mutants display defects in the morphogenesis of post-embryonic male tail sensilla (Chow and Emmons, 1994; Hahn and Emmons, 2002). *efn-4* mutants also have widespread defects in embryonic morphogenesis of the posterior body: about 20-30% of *efn-4* larvae and adults have blunted or mis-shapen posteriors (Fig. 2E,F), although the head morphology defects (‘Notched head’; Fig. 2G) of *vab-1* or *efn-1* mutants (George et al., 1998; Chin-Sang et al., 1999) are rarely seen in *efn-4* mutants. In general the post-embryonic phenotypes of *efn-4* mutants partly overlap those of *vab-1* or *efn-1* mutants.

The posterior morphology defects of *efn-4* mutants arise during embryogenesis. We analyzed the development of *efn-4* embryos using four-dimensional (4D) time-lapse microscopy and found that *efn-4* mutants displayed defects in the same embryonic morphogenetic processes as are affected in *vab-1*: neuroblast movements during closure of the ventral gastrulation cleft, and enclosure of the epidermis (George et al., 1998; Chin-Sang et al., 1999). Although some embryonic defects of some *efn-4* mutants could be classified according to the criteria used previously in our analyses of *vab-1* and *efn-1*, we also observed additional phenotypic classes, detailed below.

Almost all *efn-4* mutant embryos displayed an enlarged and persistent gastrulation cleft (Fig. 2B,C, 230 and 280 minutes); by contrast, *vab-1* or *efn-2* mutants display a much lower penetrance of enlarged clefts. In wild-type development, the gastrulation cleft is 1-2 μm deep and remains visible as a gap in the ventral neuroblast sheet for ~30 minutes to 1 hour (Fig. 2A, 230 min). By contrast, the gastrulation cleft in *efn-4* mutants varies between 2 μm and 10 μm in depth (*n*=94), and persists for longer than in the wild type. About 20% of *efn-4* embryos displayed an enlarged gastrulation cleft that remained open until the time of epidermal leading cell migration, resulting in a failure of epidermal enclosure and rupture at the ventral midline, as shown in Fig. 2B; this phenotype resembles the Class I phenotype of *vab-1* or *efn-1* mutants. In contrast to *vab-1* or *efn-1* mutants, which frequently rupture during epidermal elongation (*vab-1* class IV), less than 1% of *efn-4* embryos arrested in later embryogenesis. Most *efn-4* embryos (65%) displayed a novel phenotype in which the gastrulation cleft was enlarged and delayed in closing, yet epidermal enclosure and elongation proceeded normally; such animals hatch with normal morphology or defective posterior morphology (Fig. 2C). Six percent of *efn-1* animals display similar transient abnormalities in the gastrulation cleft that do not result in later epidermal rupture (Chin-Sang et al., 1999). In summary, although there is some overlap, the spectrum of embryonic defects in *efn-4* mutants is different from that seen in *vab-1* or *efn-1* mutants.

**EFN-4 is expressed in the developing nervous system**

To determine the expression pattern of EFN-4 we generated anti-EFN-4 antisera; these antisera were able to detect EFN-4 in animals overexpressing EFN-4 although not in wild-type animals. We also examined the expression of a functional EFN-4::GFP reporter construct (see Materials and Methods). We observed indistinguishable expression patterns using both methods.

We first detected EFN-4 at about the 100-cell stage in a large number of ventral surface cells, identified as neural or epidermal precursors (Fig. 3E). During and after enclosure of the epidermis, EFN-4 was expressed in many neurons and not in epidermal cells (Fig. 3A-C). In later embryos, larvae and adults, EFN-4-GFP was expressed in several head neurons, pharyngeal cells and a small number of lateral and tail neurons, within which EFN-4 was localized throughout the neuronal cell body and in axonal processes (Fig. 3M-O). When compared with the expression pattern of EFN-1, EFN-4 expression is relatively widespread (Fig. 3D-I) and only overlapping in part.

If EFN-4 is a cell-surface ligand for VAB-1 then it should be expressed at least in part in cells that contact VAB-1-expressing cells. Our data show that this is the case. During epidermal enclosure, EFN-4- and VAB-1-expressing cells are widely distributed in the ectoderm, allowing many possible points of contact (Fig. 3J-L). We conclude that EFN-4 expression in embryos is consistent with the possibility that it interacts with VAB-1; interestingly, some cells appear to express both VAB-1 and EFN-4 (e.g. circled cell in Fig. 3J-L).

**efn-4 mutations enhance vab-1 (Eph receptor) null phenotypes and are synthetic-lethal with weak vab-1 mutations**

We reasoned that if *efn-4* functions only in the same pathway as the VAB-1 Eph receptor, then the phenotype of a VAB-1 receptor null mutant [vab-1(0)] should not be enhanced by loss of EFN-4. Alternatively, if EFN-4 has functions that are independent of the VAB-1 receptor, then a vab-1(0); *efn-4* null double mutant might be enhanced relative to a *vab-1(0)* mutant. We constructed strains doubly mutant for *vab-1* and *efn-4*, and found that *efn-4* mutations enhanced *vab-1* null alleles. All *vab-1*; *efn-4* double mutants displayed dramatically enhanced lethality relative to the single mutants, and were maintained as balanced strains for phenotypic analysis (see Materials and Methods). Most *vab-1(0); efn-4(0)* double mutant animals showed the embryonic-lethal phenotype shown in Fig.4A, which is more severe than observed in either *vab-1* or *efn-4* single mutants, in that the gastrulation cleft is deeper (average ~10 μm, *n*=19) and the entire embryo appears mildly disorganized.

VAB-1 has both kinase-dependent and kinase-independent functions (George et al., 1998). To determine if *efn-4* mutations synergized with either or both functions, we compared the phenotypes of *vab-1(0)* *efn-4* double mutants with those of *vab-1(0); efn-4(0)* double mutants. We found that *vab-1(0); efn-4(0)* double mutants displayed strong synergistic phenotypes that were nevertheless weaker than those of *vab-1(0)*; *efn-4(0)* double mutants (compare Fig. 4A...
Fig. 3. Expression pattern of EFN-4. (A-C) EFN-4 expression during and after epidermal enclosure, detected using the EFN-4-GFP transgene jul1s109 or in animals overexpressing EFN-4 from the transgene juxEx530. Genotype in A is lin-15(n765); jul1s109[EFN-4::GFP, lin-15(+)], stained with anti-GFP (green) and the monoclonal antibody MH27 to illustrate epidermal adherens junctions (red); ventral view. (B,C) EFN-4 overexpressing embryos, stained with anti-EFN-4 (green) and MH27 (red). B is a confocal midline section at about the same stage as A; C shows a lateral view confocal projection at comma stage, after enclosure. Note the broad distribution of EFN-4-positive cells in the head. Genotype for D-I is lin-15(n765); EFN-4-GFP(jul1s109); EFN-1(jul1s53), stained with anti-GFP, anti-EFN-1 and MH27. (D-F) In early embryo (~100-cell stage; ventral views). Both EFN-4 and EFN-1 are broadly expressed. The expression of EFN-4 and EFN-1 appears slightly complementary: some cells expressing high levels of EFN-4 (circled) express lower levels of EFN-4, and vice versa. (G-I) Expression of EFN-1 and EFN-4 during late epidermal enclosure, subventral views. EFN-4 expression (green) partly overlaps (yellow) that of EFN-1 (blue). (J-L) Double staining (midline confocal section) of EFN-4-GFP (green) and VAB-1 (purple) during epidermal enclosure. Circle indicates pharyngeal cell expressing EFN-4-GFP and VAB-1. Genotype is n765; jul1s109; juxEx445, stained with MH27 antibodies. (M) EFN-4-GFP in L1 stage larva. Double staining with anti-GFP and MH27. (N,O) EFN-4-GFP expression in late stage larva. Note localization of EFN-4-GFP to nerve ring (arrowhead, N) and ventral cord (arrowhead, O). Genotype for M-O is jul1s109. Scale bar: 10 μm (A-L); 20 μm (M); 50 μm (N,O).

with 4B) suggesting that EFN-4 acts redundantly with both kinase-dependent and kinase-independent functions of vab-1. In addition, we constructed strains containing vab-1 extracellular domain mis-sense mutations (e699, e856, e1029 or jul8) with either of the hypomorphic efn-4 mis-sense alleles (e660 or e1746), and found that all eight such double mutant combinations showed synergistic lethality (data not shown). Because the synthetic-lethal interaction of vab-1 and efn-4 is allele nonspecific we conclude that vab-1 and efn-4 mutations affect parallel, partly redundant pathways.

The synergistic interaction of efn-4 mutations with mutations in the VAB-1 Eph receptor stands in contrast to efn-1 mutations, which do not enhance vab-1(0) embryonic phenotypes (Chin-Sang et al., 1998) (data shown for comparison in Fig. 5). We therefore asked if other C. elegans ephrins showed similar interactions with vab-1 that might be suggestive of vab-1-independent functions. We found that loss of function in efn-2, but not in efn-1, slightly but significantly enhanced the embryonic and larval lethal phenotypes of a vab-1 null mutant (Fig. 5). efn-2 mutants and vab-1 efn-1 double mutants also displayed a reduced brood size (average=158 progeny) compared with vab-1 null mutants [average=281 for vab-1(DS141)] or other efn mutants (data not shown); we have not investigated the basis for the reduced fertility of efn-2 strains. We further tested whether efn-1, efn-2 and efn-3 might have redundant functions in VAB-1-independent pathways using vab-1 dsRNAi (see Materials and Methods) in an efn-1 efn-2 efn-3 triple mutant background. Such vab-1(RNAi) efn-1,2,3 quadruple-mutant embryos displayed morphogenetic defects similar in range and penetrance to the efn-1,2,3 triple mutant (data not shown). Although this experiment is subject to the caveat that RNAi does not fully abolish vab-1 function, we provisionally conclude that efn-1, efn-2 and efn-3 are unlikely to function redundantly in VAB-1-independent processes in morphogenesis.

efn-4 mutations are synthetic-lethal with ptp-3 mutations

Mutations in the LAR-like receptor tyrosine phosphatase ptp-3 cause low-penetrance defects in neural and epidermal morphogenesis and also show synergistic interactions with vab-1 and efn-1 mutations (Harrington et al., 2002) that are, in general, weaker than those of efn-4 mutations. The similarity of the ptp-3 and efn-4 phenotypes and genetic interactions led us to test whether ptp-3 might function in the same pathway as efn-4. We found that ptp-3; efn-4 double mutants displayed a fully penetrant synergistic embryonic lethality, inconsistent with PTP-3 functioning in a linear pathway with EFN-4 (Fig. 4C). vab-1(0) ptp-3; efn-4 triple mutant embryos displayed abnormal gastrulation clefts that were larger than in vab-1(0) efn-4 or ptp-3 efn-4 double mutants (compare outlines in Fig. 4D with 4A and 4C; see legend to Fig. 4 for quantitation of gastrulation cleft morphology). Such triple mutant embryos appeared qualitatively more abnormal than the double mutants,
EFN-4 functions in morphogenesis

in that cells were more mobile and less tightly packed in the embryo. Because vab-1 ptp-3 efn-4 triple mutants appeared more severely affected than vab-1 ptp-3, vab-1 efn-4 or ptp-3 efn-4 double mutants, these data suggest that VAB-1, PTP-3 and EFN-4 may function in distinct yet partly redundant pathways in embryonic morphogenesis.

EFN-4 and MAB-20 Semaphorin-2A may function in common or opposing pathways in early embryonic morphogenesis

Loss of function of the C. elegans secreted Semaphorin-2A homolog MAB-20 causes defects in male tail morphogenesis (Baird et al., 1991) similar to those of efn-4 mutants. It has been suggested that these two genes might affect a common pathway in male tail development (Chow and Emmons, 1994; Roy et al., 2000). To ask whether mab-20 and efn-4 might function in the same pathway in embryogenesis, we examined the embryonic morphogenetic defects of mab-20 mutants using 4D analysis. We found that mab-20 mutants were frequently defective in closure of the gastrulation cleft (Fig. 6A, arrow at 230 minutes); in general, the defects resembled those seen in efn-4 mutants. Thus, in addition to its role in enclosure of the posterior epidermis, semaphorin signaling is also involved in the earlier process of neuroblast migration.

We examined the consequences of reducing both MAB-20 signaling and ephrin signaling on embryonic morphogenesis. We made several mab-20 efn-4 double mutant strains using either null or weak alleles of either gene. In contrast to vab-1, efn-1 or ptp-3 mutations, mab-20 mutations did not show synthetic-lethal interactions with efn-4 mutations. In all mab-20 efn-4 double mutant strains the penetrance of...
Embryonic lethality was not significantly different from that of the corresponding mab-20 single mutants (Fig. 7A). Using 4D microscopy, we confirmed that most mab-20 efn-4 double mutant embryos showed morphogenetic defects that were less severe than expected from additivity of the single mutants. In particular, the gastrulation cleft defects of mab-20 efn-4 double mutants were sometimes less severe than in either single mutant (Fig. 6C,D). The less than additive effect of mab-20 and efn-4 in embryonic morphogenesis suggests that these two genes might either have antagonistic roles or that they might operate in a common pathway.

We analyzed later embryonic and larval phenotypes of mab-20 and mab-20 efn-4 double mutants to explore further whether mab-20 and efn-4 might function in common or opposing pathways later in development. mab-20 mutants display defects in cell-cell associations in the ventral epidermis, manifested as ectopic contacts between P cells (Roy et al., 2000). efn-4 mutants displayed normal contacts between ventral P cells during embryogenesis; furthermore, efn-4 mutations did not suppress the ectopic P cell contacts due to mab-20 mutations (62/78 mab-20(ev574) efn-4(e660) embryos displayed ectopic P cell contacts, compared with 53/75 mab-20(ev574) embryos; P=0.7, Fisher's exact test). Overall, mab-20 efn-4 double mutants showed an increased penetrance of larval lethality consistent with additivity of mutant phenotypes (Fig. 7B). We provisionally conclude that mab-20 and efn-4 act independently in later development.

**DISCUSSION**

**EFN-4 is a divergent member of the ephrin family**

In this paper, we describe the genetic and molecular analysis of the fourth *C. elegans* member of the ephrin family. Although the primary sequence of EFN-4 shows only modest similarity to those of other ephrins, EFN-4 shares all hallmarks of the ephrin family, including a conserved region containing several invariant cysteine and proline residues, and a predicted C-terminal GPI addition signal sequence. The major divergent feature of the EFN-4 sequence is an insertion of 21 residues (relative to vertebrate ephrin A proteins; 24 residues relative to other *C. elegans* ephrins) in the conserved domain.

By reference to the structures of mouse ephrin B2 (Toth et al., 2001) and of the ephrin-B2/EphB2 receptor co-crystal (Himanen et al., 2001), the 21-residue insertion in EFN-4 appears to affect the loop between β-strands F and G. In ephrin B2 this ‘F-G loop’ contributes to the ligand/receptor binding interface. The F-G loop comprises eight residues in vertebrate ephrin A proteins and five residues in vertebrate ephrin B proteins and other *C. elegans* ephrins, suggesting that an enlarged F-G loop could affect the binding specificity of EFN-4. Residues in β-strand G have also been implicated in binding of vertebrate ephrin-A2 to ADAM metallopeptases (Hattori et al., 2000), suggesting that the EFN-4 insert could affect such an interaction; however, the sequence of the metallopeptase binding site itself is poorly conserved in all worm ephrins. Finally, the adjacent G-H domain [Area II by Toth et al. (Toth et al., 2001)] forms the interface between ephrin monomers in the dimeric crystal structure. An intriguing possibility is that a longer F-G loop might affect ephrin homo- or heterodimerization and thus could allow EFN-4 to act as an inhibitory ephrin. Genetic analysis of ephrin double and triple mutants has not uncovered any evidence for such an antagonistic interaction between *C. elegans* ephrins (S. L. M and A. D. C., unpublished). An alternative possibility, suggested by the apparent receptor-independent nature of EFN-4 function (see below), is that the insert sequence in EFN-4 promotes dimerization in the absence of receptor.

**Fig. 5.** Quantitation of embryonic (A) and larval (B) lethality in vab-1 efn double mutants. vab-1; efn double mutants were constructed and lethality quantitated as described in the Materials and Methods. All vab-1; efn-4 double mutants (black bars) showed significantly enhanced lethality compared with the relevant single mutants (white bars). vab-1/0; efn-4 double mutants were fully lethal and maintained as balanced strains of genotype vab-1(mls14); efn-4, vab-1(e2); efn-4 and vab-1(e699); efn-4 double mutants were >96% lethal, with rare escapers; among these escapers, 30-50% were fertile with average brood sizes of 24 and 51, respectively. Data for vab-1; efn-1 double mutants, included for comparison, are taken from Chin-Sang et al. (Chin-Sang et al., 1998). efn-2 mutations significantly increase the embryonic lethality and significantly decrease the larval lethality of vab-1(0) mutants. efn-2 mutations also significantly enhanced the embryonic and larval lethal phenotypes of vab-1(1kinase) mutations, a result that contrasts with the lack of enhancement of vab-1(1kinase) male tail phenotypes by efn-2 (Wang et al., 1999). vab-1; efn-3 double mutants were not significantly different from vab-1 controls. Error bars indicate the s.e.m. Differences in penetrance between vab-1 and vab-1; efn strains were compared using ANOVA; *, P<0.05; **, P<0.01.
Evidence that EFN-4 function is not VAB-1-dependent

Several lines of evidence suggest that EFN-4 can function independently of the VAB-1 Eph receptor. First, the efn-4 mutant phenotype is only partly overlapping with the vab-1/EphR null phenotype: in particular, the penetrance of gastrulation cleft defects is significantly higher than seen in vab-1 strains. Thus, unlike the phenotypes of efn-1 mutants, which are weaker but otherwise indistinguishable from those of vab-1, the efn-4 phenotypes are distinctly different from those caused by loss of receptor function.

A second line of evidence is the strong synergistic interaction between vab-1 and efn-4 mutations. Again, this result contrasts with the other three ephrins: loss of function in efn-1, efn-2, or efn-3 does not exacerbate the phenotypes of vab-1 null mutants, with one exception. The fully penetrant lethality of a vab-1(0); efn-4(0) double mutant could reflect additivity (~80% lethality of a vab-1 null combined with ~30% lethality of an efn-4 null), and thus does not by itself imply redundancy of vab-1 and efn-4. However, double mutants made with the weakest alleles of each gene (each resulting in <10% lethality alone) were >99% inviable, suggesting that loss of efn-4 function strongly sensitizes the animal to loss of vab-1 function, and vice versa. We propose that efn-4 and vab-1 function in parallel pathways that perform related and partly redundant functions in morphogenesis. In support of this, a clear synergistic interaction was also seen in double mutants between efn-4 and efn-1; null mutations in either gene cause ~30% lethality, whereas a double mutant using null alleles was 100% inviable (S. L. M. and A. D. C., unpublished).

A third line of evidence that EFN-4 may not solely signal via VAB-1 is the apparently low affinity of EFN-4/VAB-1 interactions (Wang et al., 1999). The affinity of the VAB-1-EFN-4 interaction appears to be much lower than that of the VAB-1-EFN-1 interaction (K_d ~5 nM) (Chin-Sang et al., 1999). Even if EFN-4 does signal partly via VAB-1, this would not invalidate the conclusion from genetic analysis that EFN-4 also functions in a VAB-1-independent pathway. Genetic and phenotypic analysis suggests that EFN-2 also may have functions independent of VAB-1. Thus, at least two of the four C. elegans ephrins appear to have functions that do not require the only known C. elegans Eph receptor.
How might EFN-4 signal in morphogenesis?

We envisage several possibilities for how EFN-4 (and possibly EFN-2) might signal in pathways that do not require the VAB-1 receptor. First, a trivial possibility is that EFN-4 could signal via a component of the MAB-20 pathway (see below). Vertebrate ephrins can bind ADAM family metalloproteases (Hattori et al., 2000); EFN-4 could signal via one of the ADAM family metalloproteases. Mutations in one such metalloprotease, the kuzbanian ortholog sup-17 cause complex phenotypes that reflect reduction of lin-12 and glp-1 function (Wen et al., 1997); we are currently examining whether sup-17 plays additional roles in C. elegans ephrin signaling. The receptor tyrosine phosphatase PTP-3 also functions in parallel to VAB-1 in morphogenesis (Harrington et al., 2002). However, the Ptp-3 and Efn-4 phenotypes are distinct, and ptp-3 mutations display strong synergistic interactions with efn-4 mutations, inconsistent with PTP-3 being the sole EFN-4 receptor. Because vab-1 ptp-3 efn-4 triple mutant embryos appear only slightly more defective than any corresponding double mutants, our data do not rule out a model in which EFN-4 signals redundantly via VAB-1 or PTP-3. However, in the absence of evidence that EFN-4 directly interacts with either receptor, it is equally possible that VAB-1, EFN-4 and PTP-3 define three partly redundant pathways in morphogenesis.

Third, ephrins form dimers and higher-order multimers (Davis et al., 1994; Toth et al., 2001). Cell culture experiments suggest that co-expression of EFN-4 with EFN-1 does not modulate the ability of EFN-1 to bind VAB-1 (I. D. C.-S. and A. D. C., unpublished), although it could alter the specificity of other ephrins so that a heterodimer could interact with an unknown receptor. Additionally, if VAB-1 does bind EFN-4, the co-expression of VAB-1 and EFN-4 in some cells could alter the responsiveness of VAB-1 to other ligands, as demonstrated for co-expressed ephrins and Eph receptors in vertebrate retinal neurons (Dutting et al., 1999; McLaughlin and O’Leary, 1999).

Finally, EFN-4 might not interact directly with any receptor. Recent data from several groups have suggested that GPI-linked ephrins, like their transmembrane counterparts, can also function as bidirectional signaling proteins (Knoll and Drescher, 2002). Vertebrate Ephrin A proteins may function in reverse signaling as a consequence of their localization to membrane microdomains (Bruckner et al., 1999; Davy et al., 1999; Davy and Robbins, 2000), and C. elegans EFN-1 has functions independent of the VAB-1 kinase (Chin-Sang et al., 1999). We speculate that EFN-4 might have evolved to be able to trigger ‘reverse signaling’ pathways in the absence of receptor binding. For example, the F-G loop insert in EFN-4 might allow it to dimerize more easily than other ephrins, leading to activation of pathways in the absence of the VAB-1 receptor. Further analysis of EFN-4 could provide insights into how GPI-linked ephrins function in bidirectional signaling.

Eph signaling, semaphorin signaling and cell adhesion in the early C. elegans embryo

mab-26 (efn-4) was proposed to function in the same genetic pathway as the Semaphorin 2A MAB-20, based on the similarity and non-additivity of mab-20 and mab-26 mutant phenotypes in male tail morphogenesis (Roy et al., 2000). Consistent with the previous analysis of male tail phenotypes we find that the efn-4 and mab-20 embryonic phenotypes are overlapping and show less than additive effects in double mutants. However, our analysis has not conclusively established whether efn-4 and mab-20 act in a common pathway in embryogenesis. The apparent epistasis of mab-20 efn-4 double mutants is also compatible with a model in which mab-20 and efn-4 play opposing roles. MAB-20 is ubiquitously expressed in embryos during morphogenesis stages (Roy et al., 2000), rendering possible several kinds of interaction with EFN-4 pathways. Although ephrin and semaphorin pathways both can function in contact-dependent repulsion of axonal growth cones, the consequence of reducing both pathways simultaneously has not been explicitly addressed, and many other combinatorial interactions have been observed between axon guidance pathways (Yu and Bargmann, 2001). Further analysis of genetic interactions between Eph signaling and
semaphorin signaling will be necessary to elucidate their in vivo functional relationships.

Most functions of Eph signaling involve modulation of cell-cell adhesion. Indeed, ephrins were first identified as contact-dependent axon repellents in the vertebrate central nervous system (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996). Numerous studies have confirmed and extended this anti-adhesive role of ephrin signaling, both in the nervous system and in non-neuronal cells (Wilkinson, 2001). However, it is clear that in some situations ephrin/Eph signaling can promote cell adhesion (Klein, 2001). The cellular basis for the early embryonic defects of C. elegans Eph signaling mutants remains poorly understood, but probably reflects alterations in cell adhesion among neural precursors. The phenotype of vab-1 ptp-3 efn-4 triple mutants, in which cells appear more mobile than in the wild type, hints that in the C. elegans embryo these proteins may function as parts of a redundant cell-adhesion network. However, it remains unresolved whether ephrin signaling directly mediates cell adhesion in the C. elegans embryo, or whether it modulates the function of other cell adhesion molecules, such as LAD-1 (Chen et al., 2001).

We thank Jonathan Hodgkin for information regarding unpublished morphological mutants in the Cambridge strain collection, and Xun Huang and Chris Suh for isolating efn-4 alleles. We thank Yuji Kohara, Andy Fire and the C. elegans genome project for clones, vectors and sequence information; Andrew Hahn and Scott Emmons for sharing unpublished results on efn-4; Martin Hudson for quantitation of juls109 rescue; and Grant Pozgoy for help with the phylogenetic analysis. We thank David Feldheim, Yishi Jin and members of the Jin and Chisholm laboratories for comments on this manuscript. This work was supported by grants to A. D. C. from the NIH (GM54657), the Alfred P. Sloan Foundation and an NSF Instrumentation Award; by a University of California Biotechnology Program postdoctoral fellowship (I. C.-S.)


