Analysis of a Zebrafish Semaphorin Reveals Potential Functions In Vivo

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ABSTRACT The semaphorin/collapsin gene family is a large and diverse family encoding both secreted and transmembrane proteins, some of which are thought to act as repulsive axon guidance molecules. However, the function of most semaphorins is still unknown. We have cloned and characterized several semaphorins in the zebrafish in order to assess their in vivo function. Zebrafish semaZ2 is expressed in a dynamic and restricted pattern during the period of axon outgrowth that indicates potential roles in the guidance of several axon pathways. Analysis of mutant zebrafish with reduced semaZ2 expression reveals axon pathfinding errors that implicate SemaZ2 in normal guidance. Dev Dyn 1999;214:13–25. © 1999 Wiley-Liss, Inc.

Key words: axon guidance; semaphorin; collapsin; zebrafish

INTRODUCTION

As the nervous system develops, axons navigate along specific pathways by following guidance cues located in precise locations of their environment. These guidance cues can be either attractive or repulsive to the axon’s growth cone. Recently, the search for molecules capable of guiding growth cones has led to the identification of several gene families encoding proteins thought to act as axon guidance molecules (reviewed in Culotti and Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996). The semaphorin/collapsin gene family is a large and diverse family encoding both secreted and transmembrane proteins, some of which are repulsive axon guidance molecules. Semaphorins have a characteristic extracellular sema domain of approximately 500 amino acids, and are grouped into six classes based on other structural domains of the proteins they encode (Adams et al., 1996; Kolodkin, 1996; Zhou et al., 1997). To date, numerous semaphorins have been identified in humans (Kolodkin et al., 1993; Hall et al., 1996; Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996), rodents (Inagaki et al., 1995; Püschel et al., 1995; Adams et al., 1996; Furuyama et al., 1996; Giger et al., 1996; Eckhardt et al., 1997; Kikuchi et al., 1997; Zhou et al., 1997; Christensen et al., 1998), chick (Luo et al., 1993, 1995), and insects (Kolodkin et al., 1993). In addition, analysis of the human EST database indicates that the number of semaphorins in the vertebrate genome may exceed 20.

The evidence that semaphorins may act as axon guidance molecules has come from a number of in vitro and in vivo studies. The first vertebrate semaphorin identified, chick Collapsin-1 (Coll-1) (Luo et al., 1993), and its mammalian homologs SemaD/III (Kolodkin et al., 1993; Püschel et al., 1995), can inhibit specifically the outgrowth of particular spinal and cranial sensory and motor axons (Püschel et al., 1995, 1996; Messersmith et al., 1995; Kobayashi et al., 1997; Shepherd et al., 1997; Varela-Echavarria et al., 1997), suggesting it normally acts to guide these axons. In support of this hypothesis, mice lacking the semaI/D gene show defects in the outgrowth and pathfinding of spinal sensory and cranial nerve axons (Behar et al., 1996; Taniguchi et al., 1997). Analyses of the in vivo function of semaphorins in Drosophila and grasshopper have shown that the transmembrane semaphorin Semal may have a dual role in axon guidance, acting as an attractive/permissive cue for some axons, and a repulsive cue for others (Kolodkin et al., 1992; Wong et al., 1997; Yu et al., 1998). Moreover, a role for Drosophila SemaI in directing an axon’s choice of synaptic target has been demonstrated by experiments in which Semal expression levels were perturbed on target muscle fibers (Matthes et al., 1995; Winberg et al., 1998).

Increasing evidence indicates that semaphorins function not only in axon guidance and synapse formation but also in a broad range of developmental processes. One mouse semalII knockout line displays defects in bone and cartilage formation as well as excessive growth of the heart, suggesting that SemalII may function to restrict growth of these tissues during development (Behar et al., 1996). Interestingly, two human semaphorin genes, semaV and semaV, were...
isolated from a chromosomal region that is deleted in several lung cancers, suggesting that these semaphorins may possess tumor suppressant activity and/or may function in normal lung development (Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996). In addition, a role for semaphorins in immune system function has recently been demonstrated. The gene encoding a leukocyte activation antigen, human CD100, was cloned and determined to be a transmembrane member of the semaphorin family (Hall et al., 1996). These studies show that semaphorins may act as signaling molecules with widespread functions including regulation of cell movements, cell differentiation, and tissue modeling. Our understanding of how the combined action of the large number of semaphorins contributes to development is far from complete, as the in vivo function of most family members has yet to be determined.

In order to analyze the in vivo function of semaphorins during development of the vertebrate nervous system, we initiated the cloning and analysis of zebrafish semaphorins. The zebrafish has several characteristics advantageous for investigation of semaphorin gene function during development. Embryos are transparent, develop rapidly, and are accessible at all stages of development. The axonal pathways of the nervous system are established by a relatively small number of identified neurons whose individual trajectories are well characterized (Eisen et al., 1986; Mendelson, 1986; Myers et al., 1986; Bernhardt et al., 1990; Chitnis and Kuwada, 1990; Kuwada et al., 1990a,b; Wilson et al., 1990; Ross et al., 1992). A potential zebrafish homolog to Coll-1, SemaZ1a (Marc Roos, Robert Bernhardt, and Melitta Schachner, personal communication), which is another candidate zebrafish homolog, was cloned and determined to be a transmembrane member of the semaphorin family (Hall et al., 1996). These studies show that semaphorins may act as signaling molecules with widespread functions including regulation of cell movements, cell differentiation, and tissue modeling. Our understanding of how the combined action of the large number of semaphorins contributes to development is far from complete, as the in vivo function of most family members has yet to be determined.

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**RESULTS**

**Cloning Of Zebrafish Members Of The Semaphorin Gene Family**

To identify SemaZ2, we screened a zebrafish embryonic cDNA library at reduced stringency with a probe complementary to chick collapsin-1. Weakly hybridizing plaques were purified further by hybridization at reduced stringency to the zebrafish semaphorin, SemaZ1a. One phage insert was a 2.5 kb cDNA with an open reading frame lacking only the sequence 5’ to the sema domain, and showed strong sequence similarity to chick collapsin-2 (Luo et al., 1995). We obtained the entire coding sequence for SemaZ2 by using rapid amplification of cDNA ends (5’-RACE), and ligated the RACE PCR product to the 2.5 kb cDNA to generate a 2.9 kb cDNA containing a complete open reading frame.

The cDNA sequence for SemaZ2 predicts a protein of 764 amino acids with structure similar to other semaphorins (Fig. 1A). The amino-terminal region shows characteristics of a signal sequence with a predicted signal peptide cleavage site at amino acid 42. Because the remainder of the sequence contains no potential transmembrane domain, the protein is likely secreted. The deduced amino acid sequence contains four potential sites for N-linked glycosylation and 19 cysteine residues conserved between SemaZ2 and chick Collapsin-2 (Coll-2), including the 13 cysteines conserved among all vertebrate sema domains. SemaZ2 has the same structural domains as the semaphorins of class III and shares the highest sequence identity with chick Coll-2 (64% amino acid identity over the entire coding region). The sema domain of SemaZ2 shares 71% amino acid identity with that of Coll-2, and is followed by a single immunoglobulin-like (Ig) domain with 62% amino acid identity to Coll-2. SemaZ2 also has a highly basic carboxy terminal region sharing 72% amino acid identity with Coll-2. The degree of amino acid identity between SemaZ2 and Coll-2 is markedly higher than between SemaZ2 and all other semaphorins, including the most closely related zebrafish semaphorin, SemaZ1a, suggesting that SemaZ2 may be a zebrafish homolog to Coll-2 (Table 1).

In parallel experiments, we used a PCR-based approach to search for zebrafish genes with sequence similarity to chick collapsins. We used degenerate oligonucleotide primers to conserved regions of the sema domain to amplify cDNA sequences from total RNA of zebrafish embryos at 24 hr post-fertilization (hpf). We obtained five different PCR products of the expected length (0.8 kb). Sequence analysis revealed that these semaphorin fragments encode partial open reading frames covering the carboxy half of the sema domain of five different zebrafish semaphorins (Fig. 1B). The nucleotide sequence of one of these was identical to the corresponding portion of the semaZ2 cDNA. The other four, however, proved to be novel sequences different from semaZ1a, Z2, Z7, and Z8. One of the four has sequence most similar to Coll-1/SemaD/III (86% amino acid identity) and SemaZ1a (84% identity), and has considerably lower similarity to all other semaphorins (Table 1). This sema fragment is in fact identical in sequence to another recently identified zebrafish semaphorin, SemaZ1b (Marc Roos, Robert Bernhardt, and Melitta Schachner, personal communication), which is another candidate zebrafish homolog to Coll-1/SemaD/III. We have named the remaining three partial semaphorins semaZ9–11 until their complete sequences are obtained and they can be defini-
**A**

Fig. 1. A: Comparison of the sequences of SemaZ2 with related chick collapsins Coll-1 and Coll-2. Alignments of predicted amino acid sequences were generated with the MacVector ClustalW alignment program. Identical amino acids are boxed and similar amino acids are shown in bold. Brackets delineate the sema domain, double underline denotes the Ig domain, and single underline denotes the basic carboxy terminus. Asterisks mark conserved cysteine residues.

**B**

Alignment of the predicted amino acid sequences of the semaphorin fragments SemaZ1b and SemaZ9-Z11. Alignment was generated with the MacVector ClustalW alignment program. Identical amino acids are boxed and similar amino acids are shown in bold. Asterisks denote conserved cysteine residues.
TABLE 1. Percentage Amino Acid Identity Within the Sema Domain

<table>
<thead>
<tr>
<th>Sema Domain</th>
<th>SemaZ2</th>
<th>SemaZ1b</th>
<th>SemaZ9</th>
<th>SemaZ10</th>
<th>SemaZ11</th>
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<td>SemaZ1a</td>
<td>55.8</td>
<td>84.5</td>
<td>66.7</td>
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<tr>
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<td>67.4</td>
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<tr>
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<td>60.9</td>
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<tr>
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<td>67.1</td>
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<td>64.0</td>
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<tr>
<td>SemaZ7</td>
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<td>38.5</td>
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<tr>
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</table>

* Amino acid identities for SemaZ8 are within its partial sema domain.

** M-SemaF and M-SemaG are those cloned by Inagaki et al. (1995) and Furuyama et al. (1996).

Globally, SemaZ10 shares the greatest sequence similarity to mouse SemA (Pueschel et al., 1995) (60% amino acid identity) and shows much lower similarity to other semaphorins.

**Forebrain/midbrain.** By 16 hpf in the zebrafish, the major brain divisions can be distinguished and the first neurons are beginning to extend axons in the brain. No expression of semaZ2 mRNA was detected in the telencephalon at any age examined. However, beginning at 16 hpf and continuing at least through the second day of development, semaZ2 mRNA is expressed in two regions of the diencephalon (Fig. 2A–F). One expression domain is located in the rostral most diencephalon, spanning the midline at the base of the optic stalks. The second domain covers the width of the neuroepithelium at the caudoventral diencephalon and may extend into the midbrain tegument.

Because semaphorins are thought to influence growing axons, we examined the relationship between these semaZ2 expressing cells and the early developing axonal pathways in the diencephalon. Axons were labeled with an antibody to acetylated tubulin in embryos that had previously been processed for in situ hybridization to semaZ2 (Fig. 2D,E). The axons that form the initial scaffold of tracts in the brain for the most part grow around but not through the domains of semaZ2 expression, suggesting semaZ2 may act as an inhibitory guidance molecule. In particular, axons of the nucleus
of the posterior commissure (nucPC) located dorsally in the caudal diencephalon initially extend ventrally and then turn caudally away from the semaZ2 expressing cells upon reaching the ventral diencephalon. Likewise, the epiphysial neurons, which lie in the dorsal diencephalon, extend their axons ventrally, pioneering the

Figure 2.
Hindbrain/spinal cord. In the developing hindbrain, semaZ2 mRNA exhibits a dynamic pattern of expression during the stages examined. semaZ2 expression can be detected beginning at 12 hpf in a subset of hindbrain rhombomeres (Fig. 2H–K). Double in situ hybridization with probes to semaZ2 and to krox-20, a gene expressed specifically in rhombomeres 3 and 5 (r3 and r5, Oxtoby and Jowett, 1993), shows that the semaZ2 expression is restricted to rhombomeres 3, 4, and 5 (Fig. 2). At 12 hpf, semaZ2 expression is seen only in r3 and r5 (Fig. 2H). By 15 hpf, expression in r3 and r5 is visible in both a dorsal and ventral domain, with lower expression levels detectable in the dorsal most region of r4 (Fig. 2I). By 18 hpf, the expression in r3 and r5 has decreased, while expression in r4 has increased (Fig. 2K). semaZ2 expression in r4 continues until at least 36 hpf.

During the time period when the first axons are beginning to extend in the hindbrain (16–20 hpf), semaZ2 transcripts are detected in an unusual pattern in the floor plate cells at the ventral midline of the CNS. semaZ2 is expressed in the floor plate cells of the mesencephalon and of the caudal hindbrain rhombomeres r4–r7 (Fig. 2I–K). However, no expression is present in the floor plate of the rostral hindbrain rhombomeres r1–r3, or in the floor plate of the spinal cord. By 24 hpf, only a small group of floor plate cells at the region of the caudal hindbrain/rostral spinal cord are still expressing semaZ2 (Fig. 2L). The only cells in the spinal cord that do express semaZ2 mRNA are the single row of roof plate cells at the dorsal midline (Fig. 2L).

By 36 hpf, when the initial axon scaffolding has been established and many later growing axon tracts are still forming, semaZ2 displays a segmental expression pattern that reflects the hindbrain rhombomeres (Fig. 2M–O). Bilateral clusters of semaZ2 expressing cells are present dorsally at each rhombomere boundary region. The identity of these cells is not known; they lie in a position medial to the clusters of commissural neurons also present in rhombomere boundary regions (Trevarrow et al., 1990). Expression of semaZ2 in r4 has persisted, although it is now present only in the ventral aspect of the rhombomere, spanning the ventral midline.

semaZ2 mRNA is Expressed in Neural Crest, Pharyngeal Arches, and Placodal Derivatives

In the zebrafish, the cranial neural crest arises as distinct masses of cells lying lateral to the neural keel at the level of the midbrain and hindbrain (Schilling and Kimmel, 1994). These cells begin migrating at 15–16 hpf, moving ventrally to populate the pharyngeal arches, which are the primordia of the jaw structures and gills. semaZ2 is expressed in a subset of cranial neural crest from the onset of migration. At 14 hpf, a cluster of semaZ2 expressing cells can be seen lateral to rhombomeres 3 and 4 (Fig. 3A). Cell lineage experiments have shown that neural crest cells from this region populate predominantly the second (hyoid) pharyngeal arch, although they can contribute to the first (mandibular) arch as well (Schilling and Kimmel, 1994). By 16–18 hpf, this mass of cells is larger and has migrated rostrally and ventrally (Fig. 3B). Neural crest cells lying caudal to the otic vesicle, which populate the gill arches, begin expressing semaZ2 by about 20 hpf. Between 20–24 hpf, three clusters of semaZ2 expressing cells are present in the region of the pharyngeal arch primordia (Fig. 3C). Later, as the arches develop further (36–72 hpf), the expression of semaZ2 is localized to a subset of cells in each arch mesenchyme (Fig. 3D–F). At 72 hpf, the cartilage and muscle cells of the pharyngeal arches can be recognized by their characteristic morphologies: the muscle striations are visible, and cartilage cells show a distinct cobblestone-like appearance. At this stage, semaZ2 expression is present in a small group of distal arch cells that do not appear to be either differentiated cartilage or muscle based on these morphologies (Fig. 3F). In addition to the semaZ2 expression in cranial neural crest, transcripts are also detected in small clusters of cells along the medial surface of each somite. These cells are in the position of trunk neural crest migrating along their medial migration pathway (Fig. 3G).

semaZ2 expression is also detectable in several placodal structures: the nasal placode, the otic placode, and the migrating primordium of the lateral line. Between 22 to 36 hpf, semaZ2 expressing cells are present at the medial aspect of the nasal pit, at its interface with the telencephalon (Fig. 3H). In the otic vesicle, two small spots of semaZ2 expression can be seen at 2 days of development (Fig. 3E). These expressing cells lie in the positions of the developing sensory maculae, and may correspond to these sensory patches. The zebrafish posterior lateral line sensory neuromasts develop in part from a placoidly derived migrating primordium that moves caudally along the embryo, depositing cells that will differentiate into neuromasts. At 20 hpf, semaZ2 is expressed in the region caudal to the otic vesicle where the placode initially forms, and later by
the cells in the leading half (caudal half) of the migrating primordium as it progresses along the trunk (Fig. 3I).

**semaZ2 Is Expressed in the Notochord, Pectoral Fin Bud, and Tail Bud**

semaZ2 mRNA is expressed in the notochord from its earliest stages of development. Initially, from 12–18 hpf, expression is seen throughout the notochord (Fig. 2H,I). Between 18 to 24 hpf, expression is downregulated in a rostral to caudal wave such that only the rostral most notochord is still expressing semaZ2 by 24 hpf (Fig. 3L). At this stage semaZ2 transcripts are also detectable in the underlying hypocard.

The pectoral fin bud initiates as an area of condensing mesenchyme lateral to somites 2–3 at 26 hpf. Weak semaZ2 expression is present from early fin bud formation. By 36–50 hpf, semaZ2 mRNA is strongly expressed throughout the developing cartilage of the fin bud (Fig. 3J). After 72 hpf, when the fin has developed a broadened fin blade with cartilaginous actinotrichia, semaZ2 expression continues, although it is now restricted to the most distal differentiating cartilage cells (Fig. 3K). Expression of semaZ2 in the fin bud occurs during the stages that motor axons are extending toward and innervating the fin bud, suggesting Semaz2 could play a role in defining domains of motor innervation into the fin, perhaps preventing axons from innervating cartilage.

Expression of semaZ2 mRNA is also detected in the developing tail bud throughout the embryonic stages examined (Fig. 3L).

**Altered semaZ2 Expression in Zebrafish Mutants Correlates with Axon Pathfinding Errors**

We analyzed mutant lines of zebrafish to search for alterations in semaZ2 expression and to determine whether the alterations could be correlated with errors in axon pathfinding. In the mutant cyclops (cyc), ventral midline structures of the nervous system at all axial levels are missing (Hatta et al., 1991). The two domains of semaZ2 expression in the diencephalon are completely absent in cyc embryos (Fig. 4A,B), as is the expression in the floor plate of the midbrain and hindbrain (Fig. 4C,D). In contrast, other aspects of semaZ2 expression are normal. Previous work has demonstrated errors in axon pathfinding in cyc that could arise from the absence of an inhibitory guidance cue in these regions of normal semaZ2 expression. For example, epiphysial axons initially extend normally along their ventral trajectory in cyc; however they make pathfinding errors at the ventral diencephalon (Patel et al., 1994). In each case, the growth cones failed to turn rostrally, rather they turned caudally or continued ventrally to cross the other side. The pathways followed by the epiphysial growth cones in cyc embryos are consistent with the loss of a signal that normally renders the caudal direction unattractive. In the hindbrain of cyc embryos, axons also make errors at the ventral midline. Normally, axons of some hindbrain reticulospinal neurons extend toward the ventral midline in the region of semaZ2 floor plate expression, and then turn and descend ipsilaterally, without crossing the midline. In cyc, these axons sometimes inappropriately cross the midline, consistent with the loss of an inhibitory signal in the floor plate (Hatta et al., 1992).

Another zebrafish mutant, whitetail (wit), also shows altered semaZ2 expression and corresponding axon pathfinding errors. In wit, neurogenesis is affected with embryos displaying increased numbers of early differentiating neurons (Jiang et al., 1996). The expression of semaZ2 in the diencephalon of wit embryos is dramatically reduced (Fig. 4E,F). In cyc, this reduction in semaZ2 expression is correlated with errors in epiphysial axon navigation. We found epiphysial axons in wit embryos that diverge from the normal DVDT pathway and extend inappropriately in the caudal direction (Fig. 4F). This type of error was found in 74% of wit embryos (n=23), while only 4% of wild-type embryos (n=23) displayed such errors. In addition to reduced expression in the diencephalon, semaZ2 expression is completely absent from the spinal cord roof plate of wit embryos (Fig. 4G,H). Interestingly, this lack of semaZ2 expression is again associated with aberrant axon trajectories. The peripheral axons of the Rohon-Beard (RB) sensory neurons extend from cell bodies in the dorsal spinal cord and arborize in the skin. These axons normally innervate only one side of the embryo and do not cross the dorsal midline (Fig. 4I). In wit embryos, RB peripheral axons freely cross the dorsal midline and extend over the skin on both sides of the embryo (Fig. 4J).

**DISCUSSION**

We have identified several new zebrafish members of the semaphorin family. One of the sema fragments we isolated is identical in sequence to semaZ1b, a recently cloned potential zebrafish homolog to Coll-1/SemaD/Ili (Marc Roos, Robert Bernhardt, and Melitta Schachner, personal communication). The other three sema fragments, semaZ9–11, do not have strikingly high sequence identity to other vertebrate semaphorins. Until their complete sequence and domain structure is obtained, it is not possible to determine whether these represent novel semaphorins or are homologs to those previously described. SemaZ2 is potentially a zebrafish homolog to chick Coll-2. These two genes share some common aspects of their expression patterns; both are expressed in the notochord, fin/limb buds, and optic tectum (Luo et al., 1995). However, there are also significant differences in their expression, most notably in the spinal cord, where Coll-2 is expressed in the floor plate, motor neuron pools, and in much of the dorsal spinal cord with the exception of the roof plate. In contrast, semaZ2 is expressed only in the spinal cord roof plate. Because the functions of Coll-2 and semaZ2 are not yet known, it is not clear whether these two genes share a common function. It is possible that the function of Coll-2 is served by more than one gene in the
Fig. 3. Whole mount in situ hybridization showing developmental expression pattern of semaZ2 in the neural crest, pharyngeal arches, and placodal derivatives. A: Dorsal view of a 14 hpf hindbrain. Clusters of semaZ2 expressing cells in the position of neural crest cells are indicated with arrowheads. Expression of semaZ2 is also present in r3 and r5 at this age. B: Dorsal view of 18 hpf hindbrain. Clusters of semaZ2 expressing neural crest (arrowheads) have increased in size and migrated further rostral and ventral. Rhombomeres 3 and 5 are labeled in red with a probe for krox20, and semaZ2 expression in blue is apparent in r4. C: Dorsal view of 24 hpf embryo showing three clusters of semaZ2 expressing cells (arrowheads) in the region of the pharyngeal arch primordia. D: Lateral view of pharyngeal arches at 36 hpf. Arrowheads denote semaZ2 expressing cells in the arches. Expression is also strong in the developing fin bud (fb). E: Lateral view of pharyngeal arches at 48 hpf. Arrowheads denote semaZ2 expression in the arches, and arrows denote semaZ2 expression in the otocyst (oto). F: Ventral view of pharyngeal arches at 72 hpf. Arrowheads denote semaZ2 expression. At this stage, semaZ2 does not appear to be expressed in differentiated muscle (m) or cartilage (c). G: Lateral view of rostral trunk at 24 hpf showing small streams of semaZ2 expressing cells medial to each somite in the position of migrating neural crest (arrowheads). H: Dorsal view of 27 hpf head showing semaZ2 expression (arrowheads) at the medial surface of the nasal placodes (np). I: Lateral view of rostral trunk at 24 hpf. Migrating primordium of the posterior lateral line (mp) is outlined with dotted line. semaZ2 is expressed in cells at the leading (caudal) half of the migrating primordium. J: Dorsal view of pectoral fin bud (fb) at 50 hpf showing strong semaZ2 expression in the developing cartilage. K: Dorsal view of pectoral fin bud at 72 hpf showing more restricted semaZ2 expression in distal cartilage. L: Lateral view of 24 hpf tail. semaZ2 expression in notochord (noto) is limited to the caudal region at this age, and expression is also present in the tail bud (tb). Scale bars = 50 µm (A, E–K) and 100 µm (B–D, L).
zebrafish. Indeed, additional members of several gene families have been isolated in zebrafish and are hypothesized to be the result of chromosomal duplications during zebrafish evolution (Ekker et al., 1992; Akinmenko et al., 1995; Prince et al., 1998).

As a first step toward understanding semaZ2 function in vivo, we analyzed its developmental expression pattern. There are several regions of the developing zebrafish nervous system in which semaZ2 is in a position both temporally and spatially to influence developing axon pathways. For example, semaZ2 is expressed between the pathways of the epiphysial axons and the nucPC axons and could guide their growth cones to turn in opposite directions at the ventral diencephalon (Fig. 5A).

Analysis of axon trajectories in the mutants cyc and wit, where semaZ2 expression in the diencephalon is either completely missing or greatly reduced, supports the hypothesis that Semaz2 normally contributes to the guidance of the epiphysial axons. The nucPC axons, however, do make their normal caudal turn in these mutants, and thus may be guided by additional cues. The increase in the incidence of caudally directed growth of the epiphysial axons in cyc and wit suggests the absence of a signal that normally prevents epiphysial growth cones from extending caudally (Fig. 5B). In addition, cyc embryos have an abnormal morphological crease in the diencephalon rostral to the epiphysial axons that may prevent them from making their normal rostral turn (Patel et al., 1994). However a mechanical barrier cannot account for the epiphysial pathfinding errors seen in wit embryos, because no crease or other abnormal structure is present in the diencephalon of wit. The cyc and wit mutations could also affect other potential guidance cues that may contribute to the observed axon trajectory errors. It is intriguing, however, that the correlation between reduced semaZ2 expression and epiphysial axon errors is present in two separate mutants.

The expression of semaZ2 by cells of the hindbrain floor plate may also reflect a role in guiding axons, and in particular guiding an axon’s decision whether to cross the ventral midline. Numerous studies have
of semaZ2 expression (stippled) and early axon tracts in wild-type (A) and cyc or wit (B). Black ovals represent clusters of cell bodies and black lines indicate axon pathways. Dotted arrows in (B) indicate epiphysial axon pathfinding errors seen in cyc and wit mutants. AC, anterior commissure; POC, post optic commissure; TPOC, tract of the post optic commissure; DVDT, dorsal ventral diencephalic tract; Ep, epiphysial neurons; nuc PC, nucleus of the posterior commissure; VPC, ventral nucleus of the posterior commissure; nucMLF, nucleus of the medial longitudinal fasciculus.

**Fig. 5.** Schematic representations of relationship between semaphorin expressing cells and developing axon pathways in wild type and mutant embryos. A,B: Schematics of lateral view of brain showing regions of semaZ2 expression (stippled) and early axon tracts in wild type (A) and cyc or wit (B). Black ovals represent clusters of cell bodies and black lines indicate axon pathways. Dotted arrows in (B) indicate epiphysial axon pathfinding errors seen in cyc and wit mutants. AC, anterior commissure; POC, post optic commissure; TPOC, tract of the post optic commissure; DVDT, dorsal ventral diencephalic tract; Ep, epiphysial neurons; nuc PC, nucleus of the posterior commissure; VPC, ventral nucleus of the posterior commissure; nucMLF, nucleus of the medial longitudinal fasciculus. C,D: Schematics of dorsal view of hindbrain in wild type (C) and cyc (D) embryos. Stippling indicates regions of the floor plate (FP) expressing semaZ2. Reticulospinal neurons are shown on one side only; however the same neurons are present on both sides of the hindbrain in vivo. Black circles indicate ipsilaterally projecting reticulospinal neurons, and open circles represent contralaterally projecting reticulospinal neurons (there are two contralaterally projecting neurons in rhombomere 6 in vivo). Dotted arc in (D) indicates reticulospinal pathfinding errors seen in cyc. oto, otocyst; r1–r6, rhombomeres 1–6.

demonstrated a role for the vertebrate floor plate in guiding axons at the ventral midline of the brain and spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Bovolenta and Dodd, 1991; Bernhardt et al., 1992a,b; Hatta, 1992; Shirasaki et al., 1995, 1996; Tamada et al., 1995). The floor plate has been shown to contain both attractive cues that signal axons to grow ventrally or to cross the midline (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Shirasaki et al., 1995; Tamada et al., 1995), and repulsive cues that direct other axons away from the midline (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Tamada et al., 1995; Shirasaki et al., 1996). In some cases, the guidance molecules Netrin-1 or SemaD/III have been shown to mimic the attractive or repellant activity of floor plate (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). netrin-1 is expressed in the floor plate at all axial levels, suggesting it has a general effect on axons throughout the neuraxis (Kennedy et al., 1994; Serafini et al., 1994; Launderdale et al., 1997; Strähle et al., 1997). Indeed, floor plate from either brain or spinal cord has similar guidance effects on axons from explants of mesencephalon or metencephalon (Tamada et al., 1995). The semaZ2 floor plate expression is particularly interesting because it is present in only a subset of floor plate cells in the hindbrain and midbrain for a limited developmental time period, and is never present in the spinal cord floor plate. It thus may have a highly specific effect on a subset of growing axons, perhaps modifying or fine tuning the effects of more generally expressed guidance cues. The hindbrain reticulospinal neurons, for instance, can be divided into seven classes based on whether their axons cross the midline and which longitudinal pathway they choose (Metcalfe et al., 1986). Each rhombomere contains a unique composition of these classes and may therefore require a unique combination of guidance cues to define their axonal pathways.

The expression pattern of semaZ2 suggests it may also play a role in regulating axon pathway formation in the spinal cord. Although semaZ2 is not expressed in the spinal cord floor plate, it is expressed in the notochord at the time when the first spinal axons are extending toward the ventral midline, and thus could potentially affect their pathfinding decisions at the ventral midline. In fact, the early spinal commissural growth cones cross the midline apposed to the basal lamina that lies between the spinal cord and notochord (Bernhardt et al., 1992a), and could therefore be affected by any cues secreted by the notochord into that basal lamina. When the notochord is laser ablated or absent as a result of the zebrafish notail mutation, growth cones of the spinal commissural axons make pathfinding errors at the ventral midline (Greenspoon et al., 1995), suggesting the notochord provides important cues signaling these growth cones to follow their appropriate pathways. It is possible that SemaZ2 secreted from the notochord may prevent particular classes of ipsilaterally projecting interneurons from crossing the ventral midline. For example, the ventral longitudinal descending (VeLD) axons are potentially responsive axons (Bernhardt et al., 1990; Kuwada et al., 1990a,b). These neurons lie in the ventral spinal cord and extend axons ventrally toward the floor plate. Upon reaching the midline region, the axons turn away from the floor plate and descend ipsilaterally, suggesting they may be repelled by a signal located at the midline.

SemaZ2 secreted from the spinal cord roof plate may play a role in guiding particular spinal axons to a ventral trajectory. A number of studies have indicated
that the attractive guidance molecule Netrin-1, expressed in the ventral spinal cord, functions to direct commissural axons toward the ventral midline in rodent and chick spinal cord (Kennedy et al., 1994; Serafini et al., 1994; 1996), as well as in zebrafish spinal cord (Lauderdale et al., 1997). Spinal commissural axons in netrin-1 deficient mice begin their ventral trajectory normally, suggesting that Netrin-1 is not responsible for the growth cone’s initial extension in the ventral direction (Serafini et al., 1996). Commissural axons may in fact be guided by a combination of repulsion away from the dorsal cord together with attraction toward the ventral cord. Experiments in which rat spinal cord roof plate is co-cultured with dorsal spinal cord explants containing commissural neurons show that a diffusible factor secreted from the roof plate inhibits commissural axon growth (Augsburger et al., 1996). SemaZ2 secreted from the roof plate may initially direct commissural axons to a ventral trajectory, like the role proposed for its potential homolog, chick Coll-2, which is expressed in the chick dorsal spinal cord (Luo et al., 1995). In zebrafish, SemaZ2 could act in concert with SemaZ7, a transmembrane semaphorin expressed in the dorsal spinal cord (Halloran et al., 1998). Because the expression of semaZ2 in the spinal cord roof plate and of semaZ7 in the dorsal cord appears after the very first commissural axons begin extending in the cord, these molecules are likely to affect only later extending axons, such as those of the secondary commissural ascending neurons or the commissural bifurcating neurons (Bernhardt et al., 1996).

Another potential role for SemaZ2 in the spinal cord roof plate is in guiding the peripheral axons of the sensory Rohon-Beard neurons. RB axons normally do not cross the dorsal midline, suggesting the presence of a guidance cue at the dorsal midline inhibitory to RB growth cones. In fact, semaZ2 is expressed in the spinal cord roof plate during the time the RB axon abors are forming. Because the skin directly overlies the roof plate, SemaZ2 secreted from the roof plate is in a position to affect axons growing in the skin. In wit embryos, where semaZ2 expression in the roof plate is undetectable, the RB axons are no longer restricted to the ipsilateral side. Instead, they readily grow over the dorsal midline, suggesting that SemaZ2 may normally act to prevent their growth across the dorsal midline.

**Experimental Procedures**

**Zebrafish**

Zebrafish (D. rerio) were maintained in a laboratory breeding colony at 28.5°C on a 14 hr light/10 hr dark cycle. Embryos collected from the breeding fish were allowed to develop at 28.5°C and were staged as described by Kimmel et al. (1995). Embryo age is defined as hours post fertilization (hpf). The cyclops mutation (Hatta et al., 1991) is a γ-ray induced mutation, and whitetail52b (Jiang et al., 1996) is an ENU induced mutation.

**Isolation of cDNA clones**

To isolate semaZ2, a 16–20 hpf zebrafish λgt10 cDNA library was initially screened with a chick collapsin-1 probe under low stringency conditions (55°C, 6×SSC). Weakly hybridizing plaques then were rescreened and purified using a semaZ1a fragment as a probe (55°C, 2×SSC). A 1.8 kb cDNA was isolated and subcloned into pBluescript SK-. Both strands of the cDNA insert were sequenced with T7 DNA polymerase (Sequenase 2.0 sequencing kit, Amersham, Arlington Heights, IL). This cDNA encoded a partial semaphorin lacking the 5’ region of the gene. To search for a full length semaZ2 cDNA, the λgt10 library was rescreened under high stringency conditions (65°C, 0.1×SSC) by using a portion of the semaZ2 sense domain as a probe. This screen yielded a longer semaZ2 cDNA that was still lacking the translation initiation site. Rapid amplification of cDNA ends (5’ RACE, Gibco-BRL, Gaithersburg, MD) was then used to synthesize the 5’ end. RACE products from three independent PCR reactions were subcloned into pBluescript SK- and sequenced. A complete semaZ2 cDNA was generated by ligating the RACE cDNA fragment to the cDNA obtained from the λgt10 library screen.

Sequence analyses of cDNAs was carried out with the MacVector software package (Kodak Scientific Imaging Systems, Rochester, NY), and the Genetics Computer Group (GCC) Wisconsin package Version 9.1. Comparisons of the amino acid identity between semaphorins was carried out with the GCG FASTA and GAP programs. The predicted signal peptide cleavage site was determined based on the rules of von Heijne (1986).

**PCR**

PCR was performed on cDNA reverse transcribed from 24 hpf zebrafish total RNA. Primers used were fully degenerate and encoded the first 23 bases of the sequence KWFTFLKA and DPYCAWD. The primers were annealed to the template at 45°C for 1 min and extended at 74°C for 1 min, for 35 cycles. PCR products were subcloned into the pCR-II vector with the TA cloning kit (Invitrogen, La Jolla, CA), and the resulting inserts sequenced.

**Whole Mount In Situ Hybridization and Immunohistochemistry**

Riboprobes labeled with digoxigenin-UTP were synthesized by in vitro transcription and hydrolyzed to an average length of 200-500 bases by limited alkaline hydrolysis (Cox et al., 1994). Whole mount in situ hybridization was performed as described by Schulte-Merker et al. (1992), except for the acetic anhydride/triethanolamine treatment was omitted, and hybridizations were carried out at 65°C. For the two color in situ hybridization with semaZ2 and krox20, FITC-labeled antisense riboprobes to krox20 were synthesized, and hybridization with both probes was carried out simultaneously. After completion of the coloration reaction for
sema2Z2, the alkaline phosphatase was heat inactivated (65°C, 1 hr) and embryos placed in anti-FITC antibody conjugated to alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany) overnight. The krox20 color was developed with Fast Red TR/Naphthol AS-MX (Sigma Chemical Company, St. Louis, MO, F4648). Embryos were mounted in 70% glycerol.

For double-labeling with in situ hybridization and anti-tubulin, the monoclonal antibody for acetylated α-tubulin (Sigma, T6793) was added to the antidigoxigenin antibody during the in situ hybridization. After the in situ color reaction was complete, embryos were fixed in 4% paraformaldehyde overnight. Anti-tubulin labeling was then completed with the Vectastain Mouse IgG ABC immunoperoxidase labeling kit (Vector Labs, Burlingame, CA).

Sections were made with a cryostat after whole mount in situ hybridization. Embryos were equilibrated in 30% sucrose, embedded in OCT compound (Miles, Elkhart, IN), and cut into 16 µm sections on a cryostat. Sections were mounted in 70% glycerol.

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