Dual function of Slit2 in repulsion and enhanced migration of trunk, but not vagal, neural crest cells

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Dual function of Slit2 in repulsion and enhanced migration of trunk, but not vagal, neural crest cells

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Neural crest precursors to the autonomic nervous system form different derivatives depending upon their axial level of origin; for example, vagal, but not trunk, neural crest cells form the enteric ganglia of the gut. Here, we show that Slit2 is expressed at the entrance of the gut, which is selectively invaded by vagal, but not trunk, neural crest. Accordingly, only trunk neural crest cells express Robo receptors. In vivo and in vitro experiments demonstrate that trunk, not vagal, crest cells avoid cells or cell membranes expressing Slit2, thereby contributing to the differential ability of neural crest populations to invade and innervate the gut. Conversely, exposure to soluble Slit2 significantly increases the distance traversed by trunk neural crest cells. These results suggest that Slit2 can act bifunctionally, both repulsing and stimulating the motility of trunk neural crest cells.

Introduction

Neural crest cells emerge from the dorsal neural tube and migrate along pathways that are characteristic of their axial level of origin, with cells from different axial levels populating different derivatives (Le Douarin, 1986; Bronner-Fraser et al., 1991; Le Douarin et al., 1992). For example, vagal neural crest cells emerge from the caudal hindbrain and migrate into and along the rostrocaudal extent of the gut where they form the enteric ganglia. In contrast, trunk neural crest cells never enter the gut, even if transplanted to vagal levels (Le Douarin and Teillet, 1973, 1974; Erickson and Goins, 2000). Little is known about the molecular mechanisms underlying these differences in the migratory pathways taken by trunk and vagal neural crest cells.

Several inhibitory molecules have been shown to play important roles in neural crest migration. For example, ephrinB family members prevent neural crest entry into the caudal portion of each somite, resulting in segmental migration in the trunk region (Krull et al., 1997; Wang and Anderson, 1997). Other molecules implicated in neural crest cell guidance include chondroitin sulfate proteoglycans (Oakley et al., 1994) and Semaphorin3A and 3C (Eickholt et al., 1999; Brown et al., 2001; Feiner et al., 2001). However, none of these explain differences between the ability of vagal and trunk neural crest populations to enter the gut.

Slit proteins play important roles in axonal guidance in both vertebrates and invertebrates (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). These glycoproteins are known to be potent chemorepellents for midline axons in Drosophila as well as olfactory, forebrain, and dentate gyrus axons in mammals (Brode et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Bagri et al., 2002). Slits function as repulsive factors during migration of neurons and glia (Hu, 1999; Wu et al., 1999; Zhu et al., 1999; Kinrade et al., 2001) and also can regulate axon elongation/branching in mammals (Wang et al., 1999).

In this study, we examine the potential role of Slit in neural crest migration. Slits are expressed in places where they could influence migrating neural crest cells (in the dorsal neural tube from which neural crest cells emigrate and near the entrance to the gut, which is selectively invaded by vagal, but not trunk, neural crest cells). Consistent with this, we find that only trunk neural crest cells possess Robo receptors for Slit. We tested the possible functional role of Slit on neural crest migration in vitro and in vivo by confronting neural crest cells with Slit2-producing cells or soluble Slit2. The results reveal for the first time a dual role for Slit2 in a migratory cell type, both inhibiting movement when in direct confrontation and enhancing motility when in solution. Our results at least partially account for the differential ability of vagal, but not trunk, neural crest cells to invade and innervate the gut.
Results

Slit and Robo receptor expression during development

We examined the distribution pattern of vertebrate Slits and their Robo receptors at the time of active neural crest migration in chick embryos at embryonic days 2 and 3. Both whole mount and section in situ hybridization were performed for transcripts encoding Slits 1, 2, and 3 as well as Robo1 and Robo2.

We found that the patterns of expression for Slt2 (Fig. 1, a, d, and e), Slt1 (Fig. 1, b and c), and Slt3 (Fig. 1 f) largely overlapped. All were expressed prominently in the splanchnic mesoderm that marks the entrance to the gut. In addition, there was marked expression in the ventral neural tube, notochord, and dorsomedial dermomyotome. Slt1 and Slt2 were expressed strongly in the roof plate, whereas Slt3 had little or no expression in this site. Interestingly, Slt1 and Slt2 were expressed in both the floor plate and in forming motor neuron pools. In contrast, Slt3 was expressed in motor neurons but had low expression in the floor plate. These expression patterns are similar to those noted previously in the mouse (Yuan et al., 1999).

Trunk, but not vagal, neural crest cells express both Robo1 and Robo2 receptors (Figs. 2 and 3). Whole mount in situ hybridization reveals Robo receptors on neural crest cells as they migrate through the trunk somites (Fig. 2, a–c, e, and g). However, neural crest cells at vagal levels, which can be visualized by their expression of the HNK-1 epitope (Fig. 2 f), lack detectable Robo receptors. Robo1 expression was similar to that of Robo2 but initiated at later stages. By section in situ hybridization, which gives better cellular resolution, we noted that Robo1 (Fig. 3, a and b) and Robo2 (Fig. 3, c and d) were expressed on migrating trunk neural crest cells. In contrast, vagal neural crest cells lacked Robo receptor expression above background (Fig. 3, e–h). Not all HNK-1 positive cellsFUNCTIONAL IMAGING OF MULTIPLE HUMAN GASTROINTESTINAL TUMORS

By employing functional imaging techniques, we were able to detect and characterize multiple lesions within the gastrointestinal tract of patients with known gastrointestinal malignancies. The images obtained provided valuable information about the extent of disease and helped guide treatment decisions.

In conclusion, our study highlights the potential of functional imaging in the multimodal approach to the management of gastrointestinal malignancies. Further research is needed to validate these findings and to explore the role of functional imaging in other diseases of the gastrointestinal tract.
Expression of Slits during neural crest migration. In situ hybridization of Slit2, 1, and 3 demonstrates that Slits are expressed in the mesenchyme at the entry to the gut as well as in the neural tube and somites. (a–c) Whole mounts of stage 17 chicken embryos. (a) Whole mount in situ hybridization with a Slit2 probe reveals that it is expressed in the dorsal neural tube (black arrow), ventral neural tube (white arrow), and gut mesenchyme (black arrowhead). (b and c) Whole mount in situ hybridization with a Slit1 probe reveals that it is expressed in the dorsal neural tube, dorsomedial dermomyotome (red arrow), and gut mesenchyme. (b) A higher magnification of c. (d) Transverse section in situ hybridization through the lumbar level of a stage 17 chick embryo shows Slit2 in the floor plate and developing motor neurons (white arrow), the roof plate of the neural tube (black arrow), and the mesenchyme immediately dorsal to the gut (black arrowhead); the Slit1 pattern (not depicted) looked identical. (e) A similar expression pattern for Slit2 and Slit1 (not depicted) was observed at the hindlimb level of a stage 19 embryo. (f) Slit3 in a stage 16 embryo appeared similar to the other Slits, except that staining was reduced or absent in the floor plate and dorsal neural tube.

Expression of Robos during neural crest migration. Whole mount in situ hybridization with Robo1 and Robo2 probe reveals that the receptor is expressed in the trunk neural tube, on migrating trunk neural crest cells within the somites, and in the dermomyotome, but is not expressed by vagal neural crest cells. (a) In a stage 15 chicken embryo, there is no staining for Robo2 at vagal levels (bracket) though there is staining at truncal levels. (b and c) At stage 18 during the peak of neural crest migration, Robo1 (b) and Robo2 (c) are strongly expressed in neural crest cells at the trunk levels (black arrows) but not in the vagal region (brackets). (d) Robo2 labeling in the head of a stage 15 embryo shows staining on the dorsal half of the otic vesicle (ov), trigeminal placode, and dorsal neurons in the mesencephalon; the posterior part of the second/hyoid branchial arch is also positive for Robo2 (arrowhead). (e–g) At stage 20, when neural crest cells are condensing to form the dorsal root ganglia (arrows), Robo1 (e) and Robo2 (g) are strongly expressed in trunk, but not vagal (brackets), regions. (f) The same stage embryo labeled with HNK-1 shows neural crest cells migrating and beginning to condense into dorsal root ganglia at both vagal and trunk levels.
In addition to Slit2, we also tested on trunk neural crest cells the effects of exposure to Slit1-expressing cells using the same in vitro paradigm as described above for Slit2. These results were identical (not depicted), suggesting that Slit1 is also a repellent for trunk neural crest cells.

**Slit2 repulsive effect on trunk neural crest is contact mediated**

Although Slit2 is soluble and can be secreted, it is most commonly found in membrane extracts and tightly associated with the cell surface (Brose et al., 1999; Nguyen Ba-Charvet et al., 2001a). To determine if the effects of Slit2 on trunk neural crest cells were caused by soluble Slit2 present in the media (Wu et al., 1999) or by cell membrane–associated Slit2 (Hu, 2001; Nguyen-Ba-Charvet et al., 2001b), we repeated the in vitro assay above with cells that were dried and dead but retained membrane-bound Slit2 (not depicted). Under these conditions (Figs. 5, e–h), trunk, but not vagal, neural crest cells avoided Slit2 membrane ghosts, confirming that the effects of Slit2 are cell surface dependent. These results are summarized in Table I.

**Soluble Robo reverses Slit2 repulsion of trunk neural crest**

Slit2 is a known ligand for the Robo1 and Robo2 transmembrane receptors (Brose et al., 1999). We tested the ability of a soluble form of the receptor to neutralize the effects of exogenous Slit2. RoboN consists of the extracellular portion of Robo1 (Wu et al., 1999). When trunk neural crest cells were grown in media conditioned by the RoboN-secreting cells, the repulsive effect caused by Slit2 was reversed (Table II), suggesting that the effect was indeed Slit mediated. In contrast, RoboN media had no significant effect on trunk neural crest cells when encountering control HEK cells. Similarly, trunk neural crest cells showed no altered behavior when encountering RoboN-secreting cells alone (not depicted).

**Soluble Slit2 affects trunk, not vagal, neural crest migratory properties**

The observation that the membrane-bound form of Slit2 appears to be responsible for its chemorepulsive effects on neural crest cells does not rule out a possible function of the soluble
form of the molecule. In fact, we noted that trunk neural crest cells in the vicinity of Slit2-expressing cells in vivo and in vitro appeared more elongated than in the presence of control cells, raising the possibility that cytoskeletal and/or motility changes may occur in the presence of soluble Slit2.

To examine the effects of soluble Slit2, migrating neural crest cells were placed on fibronectin-coated substrates transfilter to Slit2-expressing cells, control cells, Slit2-conditioned medium (CM),* or control CM (Wu et al., 2001). This assures that crest cells under experimental conditions would be exposed only to the soluble form of Slit2. With either transfilter Slit2 cells or Slit2 CM, the maximum distance traveled by neural crest cells was an average of 21% more than that observed under control conditions (n > 100 neural tubes in a total of 12 separate experiments; Table III; Fig. 6). In contrast, vagal neural crest cells failed to exhibit enhanced migration under identical conditions (n = 12 neural tubes in a total of three separate experiments; Table III).

*Abbreviations used in this paper: CM, conditioned medium.

Table I. Trunk, but not vagal, neural crest cells avoid Slit2-expressing cells

<table>
<thead>
<tr>
<th></th>
<th>In vivo</th>
<th>In vitro</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Slit2</td>
</tr>
<tr>
<td>Live cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk</td>
<td>1/10</td>
<td>12/15</td>
</tr>
<tr>
<td>Vagal</td>
<td>0/6</td>
<td>1/8</td>
</tr>
<tr>
<td>Dried cell ghosts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk</td>
<td>0/5</td>
<td>5/7</td>
</tr>
<tr>
<td>Vagal</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Numbers correspond to individual embryos (in vivo) or neural tubes with migrating neural crest (in vitro) that showed avoidance when encountering either the control or Slit2-expressing cells.

In a second assay for Slit2’s effects on migrating neural crest cells, we examined the rapidity with which neural crest cells closed an injury-induced gap within their population (Fig. 7 a). In the presence of Slit2 CM, >20% of cultures had completely sealed the gap within 2 h, compared with <10% of cultures exposed to control medium (Fig. 7 b; Table IV). Moreover, when cells were “primed” with CM before the wound assay, >40% of cultures had sealed the wound within 2 h, compared with <20% of controls (P < 0.005; Table IV). The increased migratory response occurred rapidly, within ~1 h of exposure to Slit2. Given this short time interval, an increase in the number of migratory cells is unlikely (Mason et al., 2001). Consistent with this, no increase in cell division was observed by antiphosphohistone3 labeling, when neural crest cells were exposed to Slit2 (unpublished data). Furthermore, we noted no alteration in cell death, as assayed by pyknotic nuclei identified by DAPI staining (unpublished data). In contrast to trunk neural crest cells, vagal neural crest cells were unaffected by the presence of Slit2 in the medium.

We repeated the wound assay but preexposed the cells to either control or Slit2 CM mixed with RoboN CM (1:1) to see if this reversed the effects of Slit2. RoboN CM reduced (P < 0.02) the percentage of cells closing the gap

Table II. Slit2 repulsion is neutralized by RoboN

<table>
<thead>
<tr>
<th>Explants with inhibition out of total number</th>
<th>− RoboN</th>
<th>+ RoboN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3/23</td>
<td>1/22</td>
</tr>
<tr>
<td>Slit2</td>
<td>16/27</td>
<td>3/22*</td>
</tr>
</tbody>
</table>

Neural tubes were cultured overnight on fibronectin-coated slides containing control medium or Slit2 with or without RoboN. Inhibition is defined by the presence of a clear separation between neural crest cells and the Slit2 or control cell lines. Control, untransfected HEK293; Slit2, combined results from two different Slit2-expressing cell lines.

*P < 0.00001, Chi-squared test.
within 2 h by half, a level similar to that observed under control conditions (Fig. 7c; Table IV).

Slit2 increases trunk neural crest cell motility

The results suggest that soluble Slit2 enhances neural crest migration, as the distance traveled by the cells as a function of time increases significantly. To view this at higher resolution, we tracked individual cell movement by imaging neural crest cells in the presence or absence of soluble Slit2 by time-lapse video microscopy. To aid visualization, trunk neural tubes were electroporated with a GFP expression vector in vivo (Megason and McMahon, 2002), and then the tubes were explanted in vitro. Cultures were primed with Slit2 or control CM and filmed under a confocal microscope. The resultant movies were analyzed to examine the total and net distance navigated, speed, directionality, and persistence of movement of individual, GFP-labeled neural crest cells.

The results confirmed static pictures showing that trunk neural crest cells moved more dynamically and for greater distances in Slit2 than in control medium (Fig. 8; see Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200301041/DC1). With or without Slit2, neural crest cells moved in a somewhat erratic manner, with frequent changes in direction (Kulesa and Fraser, 1998, 2000). The cells appeared to collide more frequently in the presence of Slit2, raising the possibility that cell–cell collision may effect their degree of movement.

Fig. 9a, which illustrates by groups the total path length of all cells normalized to a total of 2.5 h, shows that the Slit2-exposed neural crest cells tend to move further than control-treated cells, with twice as many within the range of 100–200 μm from the neural tube (74 experimental and 65 control cells analyzed from five separate experiments). We also noted a significant difference in both the total distance traveled (i.e., including the various turns made by the cells) and the net distance away from the neural tube of neural crest cells exposed to soluble Slit2 compared with control CM (Fig. 9b). Accordingly, the net path length was 36% longer in the Slit2-exposed cells compared with control-treated length (38 ± 3 μm in Slit2 compared with 28 ± 3 μm in control medium, P < 0.02, Mann-Whitney test), as was the total path length (139 ± 15 μm for control and 167 ± 14 μm for Slit2, P < 0.005, Mann-Whitney test; Fig. 9b). We analyzed the speed of movement in groups of cells assayed for similar lengths of time and found an average

| Table III. Slit2 enhances migration of trunk, not vagal, neural crest cells |
|-----------------------------|------------------|
|                             | Control         | Slit2            |
| Trunk                       | 133 ± 3.8       | 161 ± 4.2        | 21%^a |
| Vagal                       | 132 ± 7         | 125 ± 6          | -6%  |

Neural tubes were cultured overnight on fibronectin. Values correspond to the average distance traveled by the neural crest cells away from the neural tube explant ± SEM. For trunk, there were a total of >100 neural tubes per condition in 12 separate experiments. For vagal, there were 12 neural tubes per condition in three separate experiments.

^aP < 0.0001, t test.
Slit2 inhibits trunk neural crest migration | De Bellard et al. 275

Table IV. Slit2 enhances wound closure by trunk, not vagal, neural crest cells

<table>
<thead>
<tr>
<th></th>
<th>Nonprimed</th>
<th>Primed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trunk</td>
<td>Trunk + RoboN</td>
</tr>
<tr>
<td>Control</td>
<td>9 ± 4</td>
<td>13.8 ± 5.5</td>
</tr>
<tr>
<td>Slit2</td>
<td>22 ± 5</td>
<td>41.6 ± 5.6*</td>
</tr>
</tbody>
</table>

Trunk neural tubes were cultured overnight on fibronectin. After one day, media was changed to one conditioned by control or Slit2-secreting cells. A wound of one to two cells width was made with a fine pipette. After 2 h, the percent of wounds with cells crossing and sealing the gap was determined. For trunk, there were a total of >100 neural tubes per condition in five or seven separate experiments (nonprimed and primed, respectively). For vagal, there were >70 neural tubes per condition in three separate experiments.

*P < 0.005, t test, in trunk exposed to control versus Slit2 media.

P < 0.02, t test, in trunk exposed to Slit2 versus Slit2 + RoboN media.

Discussion

Neural crest cells arising from the vagal axial level form the enteric ganglia that innervate the gut. They invade the foregut and migrate long distances from rostral to caudal regions of the gut to populate its entire length. In contrast, trunk neural crest cells fail to invade gut tissue immediately below their migratory pathways. Even if transplanted to vagal levels, trunk neural crest cells do not enter the gut (Le Douarin and Teillet, 1973; Erickson and Goins, 2000). Several inhibitory molecules have recently been shown to play important roles in the general migratory process of neural crest cells, e.g., ephrinB family members, Semaphorin3A, and chondroitin sulfate proteoglycans (Oakley et al., 1994; Krull et al., 1997; Eicholtz et al., 1999). Here, we show that substrate-bound Slit2 is a chemorepellent for migratory trunk, but not vagal, neural crest cells. The differential effects of Slit2 provide the first molecular explanation for the differences in the migratory properties of these two populations.

The expression of Slits in the mesentery is uniform throughout the rostrocaudal extent of the gut mesenchyme. Thus, the selective entry of vagal neural crest cells lies not in the distribution of the ligand but rather with expression of Slit receptors, Robo1 and Robo2, in different neural crest populations (Li et al., 1999; Holmes and Niswander, 2001; Vargesson et al., 2001). Migratory and condensing trunk neural crest cells express both Robo receptors, whereas neither receptor was detected on vagal neural crest. The absence of Robo receptors on vagal neural crest cells is consistent with their lack of response to Slit2. In previous publications that have examined the embryonic distribution of Robos, no expression on migratory neural crest cells was noted. By using higher resolution in situ hybridization and, in particular, in situ performed on tissue sections, we detected expression of both Robo1 and Robo2 on trunk neural crest cells. Not all of the HNK-1–positive cells expressed detectable levels of Robo receptors; it is possible that some of the migrating cells expressed levels of receptors that were below the sensitivity of this method. Consistent increase in speed of 52% in Slit2-containing medium relative to the control (P < 0.009, t test). Interestingly, the persistence and directionality were not significantly different between control and Slit2 exposed. Thus, the cells change directions with the same frequency but move further in the same time in the presence of Slit2.

Figure 7. Slit2 enhances trunk neural crest motility in a wound assay. Trunk neural tubes were cultured overnight on fibronectin. After one day, media was changed to one conditioned by control or Slit2-secreting cells. A wound of one to two cells width was made with a fine pipette. After 2 h, the percent of wounds with cells crossing and sealing the gap was determined. (a, unsealed) Image of a neural crest culture fixed immediately after wounding and stained with the HNK-1 antibody. (a, sealed) Image of a similar culture fixed 4 h after wounding showing that many neural crest cells have sealed the gap with a fine pipette. After 2 h, percent of wounds with cells crossing and sealing the gap was determined. (b) Percentages of HNK-1–positive cells in the wound. Data correspond to one representative experiment out of eight. (c) Slit2 enhances wound healing of trunk, not vagal, neural crest, and this effect can be reversed by soluble Robo. The enhanced migration of trunk neural crest by Slit2 was significantly reduced by the presence of RoboN in the media. Neural crest cultures were primed for 2 h before performing the wound with media conditioned for 5 d by control HEK cells or Slit2-secreting cells. Nonprimed corresponds to neural crest cells that were not preexposed to Slit2 in the media before the wound. Data correspond to one representative experiment out of six.

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with this possibility, older embryos during later stages of migration had more prominent Robo expression on migrating trunk neural crest cells, suggesting that receptor levels increase with developmental age.

Our data support the idea that Slits can function as direct chemorepellents for trunk neural crest cells. In vivo, we cannot rule out the possibility that Slits might also influence neural crest cells via indirect interactions with other tissues adjacent to neural crest migratory pathways, as the neural tube and dermomyotome also express Robo receptors. Our in vitro data, however, show that exogenous Slit2 and Slit1 can repel trunk neural crest cells in the absence of neighboring cell types. Furthermore, addition of RoboN specifically reverses the inhibitory effects of Slit2, suggesting a direct interaction.

Slit2 is likely not the only chemorepellent for trunk neural crest cells. Although Slit2 was used for most of the functional experiments performed here, we observed that multiple Slit...
isoforms, Slit1 and Slit3, have similar expression patterns in mesoderm at the entrance to the gut and that Slit1 can function similarly to Slit2. Furthermore, all three Slits share the highly conserved four leucine-rich repeats that have been shown to be responsible for Slit repellent activity (Battye et al., 2001; Chen et al., 2001; Nguyen Ba-Charvet et al., 2001a). Such redundancy has also been shown for olfactory neurons; these neurons that are repelled by endogenous Slit2 in the subventricular zone are also repelled by Slit1 (Wu et al., 1999). However, we cannot exclude the possibility that there are subtle functional differences in the different Slit proteins (Bagri et al., 2002; Plump et al., 2002).

The membrane-bound form appears to be responsible for Slit2’s chemorepulsive effects on trunk neural crest cells. The 200-kD Slit2 glycoprotein is proteolytically processed into a 140-kD NH2-terminal fragment and an ∼60-kD COOH-terminal fragment in cell culture and in vivo (Brose et al., 1999; Nguyen-Ba-Charvet et al., 2001b). The NH2 terminus is more tightly bound to the membrane and has been shown to mediate repellent activities and stimulation of axon elongation (Brose et al., 1999; Wang et al., 1999; Niclou et al., 2000; Battye et al., 2001; Nguyen-Ba-Charvet et al., 2001b). As the COOH terminus does not appear to interact with Robo receptors (Nguyen-Ba-Charvet et al., 2001a), the repulsion we observed on neural crest cells almost certainly involves only the NH2 terminus of the protein. In support of this, the leucine-rich repeats of the NH2 terminus appear to be sufficient to achieve trunk neural crest repulsion (Chen et al., 2001; unpublished data). Moreover, trunk neural crest cells exposed to diffusible Slit2 either in chemotaxis chambers or collagen gels showed no apparent repulsion (unpublished data).

In addition to being a chemorepellent, our data suggest that soluble Slit2 selectively enhances the distance migrated by trunk neural crest cells. Such dual function for Slit2 concurs with observations on axon guidance where Slit family members have been shown to play multiple roles, causing both faster axon growth and induction of branching (Wang et al., 1999; Oztun and Erzurumlu, 2002). For the case of trunk neural crest cells, Slit2 appears to have opposite effects of inhibition and promotion of motility in the same cell type. Our results are consistent with previous studies showing that Slit2 can affect cell motility independent of its repellusive activity (Mason et al., 2001).

Although many trunk neural crest cells exhibited enhanced migration in the presence of soluble Slit2, a subpopulation of cells in explant cultures moved relatively short distances away from the center of the explant, as assayed both by static visualization and by time-lapse video microscopy. One possibility is that there are two separate populations of cells in our cultures. The majority (∼90%) of cells was observed on neural crest cells almost certainly involves only the NH2 terminus of the protein. In support of this, the leucine-rich repeats of the NH2 terminus appear to be sufficient to achieve trunk neural crest repulsion (Chen et al., 2001; unpublished data). Moreover, trunk neural crest cells exposed to diffusible Slit2 either in chemotaxis chambers or collagen gels showed no apparent repulsion (unpublished data).

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The dual functions of Slit2 in both repulsion and stimulation of migration in the same type of cell are not necessarily contradictory; an optimal chemorepellent might logically be expected to stimulate rapid movement away from its source. Previous studies have shown that Slit2 repels axons from motor, olfactory, dentate gyrus, and retinal neurons (Brose et al., 1999; Li et al., 1999; Erskine et al., 2000; Ringstedt et al., 2000), while it inhibits migration of olfactory bulb and cerebral cortical neurons as well as leukocytes (Hu, 1999; Wu et al., 1999, 2001). In addition to repellus effects on axonal growth and cell migration, Slit2 is a potent stimulator of axonal elongation and dendrite branching (Wang et al., 1999; Oztun and Erzurumlu, 2002; Whitford et al., 2002). In the last few years, several axonal pathwayfinding and axonal regeneration inhibitors have been shown to induce both chemorepulsion or chemoattraction, depending on the type or age of the cell (Lohof et al., 1992; Song et al., 1998; Cai et al., 2001; Liu et al., 2002). These opposing effects appear to be due to the internal levels of cyclic nucleotides in the cells, leading to promotion or inhibition of axonal growth (Cai et al., 1999; Hopker et al., 1999).

In summary, we have shown that Slit2 is a true bifunctional molecule: it can both repel neural crest cells and increase their rate of migration. The presence of Slit family members at the entrance of the gut mesenchyme coupled with the presence of Robo receptors on trunk and absence from vagal neural crest provides the first molecular explanation for the differences in migratory behavior of these two subpopulations via Slit chemorepulsion. The results suggest that neural crest cells may not only avoid, but also rapidly move away from, sources of chemorepellents like Slit.

Materials and methods

In situ hybridization

Embryos were removed from the eggs, stripped of membranes, and fixed in 4% paraformaldehyde overnight before being stored in 0.1 M PBS. Patterns of gene expression were determined by whole mount in situ hybridization using DIG-labeled RNA antisense probes as described by Henrique et al. (1995). The 1.7-kb c-slit1, 3.5-kb c-slit2, 1.9-kb cslit3, 1.9-kb c-robo1, and 2.1-kb c-robo2 (provided by E. Lauffer, Columbia University, New York, NY) probes used are described in Vargesson et al. (2001).

In situ sections were performed as follows. Embryos were fixed in modified Carnoy’s and then dehydrated in 70%, 90%, 2 PBS, and then in two changes of 2× SSC. Hybridization was done by diluting 1.5 ng/ml of slit2 and robo1 and 2 probes (Vargesson et al., 2001), incubated at 65°C overnight in 50% formamide, 2× SSC–humidiﬁed chamber. Slides were washed for 15 min in 50% formamide, 1× SSC, 0.1% Tween-20 at 65°C and then in MABT. The slides were then blocked for 1.5 h in MABT with 2% Boehringer blocking reagent and 20% any goat serum. Anti–DIG-AP was diluted 1:2,000 in the above solution and applied overnight at room temperature. The next day, slides were rinsed in MABT in aged bath, 30 min each, and then equilibrated in NTMT (0.1 M NaCl, 0.1 M Tris, pH 9.5, 5 mM MgCl2, 0.1% Tween-20) before BM purple color reaction.

In vivo avoidance assay

Fertilized eggs were incubated at 38°C for 28–56 h, until embryos reached HH10–12 (for vagal neural crest) and HH12–16 (for trunk neural crest). Occasionally, stage 12 embryos received injections at both vagal and trunk levels. Eggs were windowed and visualized by a sub-blastodermic injection of India ink (diluted 1:10 in PBS). Slit2-expressing or control HEK293 cells, labeled with Dil, were injected in the somites at trunk or vagal levels. The eggs were closed with Scotch tape and reincubated for an additional 24 h. Embryos were removed from the eggs, stripped of the membranes, and fixed in 4% paraformaldehyde overnight before being stored in PBS. Embryos were thoroughly washed in PBS and then blocked overnight with PBS containing 1% Triton X-100 and 10% FBS at 4°C. After 3 h at room temperature in washing buffer (PBS with 1% Triton-X100, 1%
fsa, embryos were incubated with 1:300 HNK-1 supernatant in PBS overnight at 4°C. The next day, embryos were extensively washed and incubated with an anti-mouse IgM-specific Alexa 488-conjugated antibody (Molecular Probes). The next day, the embryos were washed extensively, Z stained with 4′,6-diamidino-2-phenylindole (DAPI) and imaged using a Zeiss LSM microscopes. The images were analyzed using MetaMorph and ImageJ software.

In vitro avoidance assay
Quail neural tubes from HH10–12 (for vagal crest) and HH13–16 (for trunk, the posterior parts of the tube) were dissociated in 1.5 mg/ml of dispase and cultured in Leibovitz-15 medium. The neural tubes were cultured in small pieces (size of two to three somites) and pipetted in the center of wells coated with fibronectin (10 μg/ml) already surrounded by a monolayer of live, or dried Slit2 or control HEK293 cells (Li et al., 1999). Neural tubes were cultured in DME, 10% FBS, and 100 μg/ml and 100 U of penicillin and streptomycin, respectively, for 18 h, after which they were fixed in 4% paraformaldehyde for 30 min and subsequently blocked for 30 min with PBS, 1% Triton X-100, 10% FBS. Primary antibody was HNK-1 for visualizing neural crest cells, followed by an anti–mouse IgM–Alexa 488 secondary (Molecular Probes). At the end of the experiment, slides were incubated with DAPI in PBS to visualize cell nuclei.

Cell nuclear response was determined as follows. If both types of cells (neural crest and HEK) either mixed or overlapped, it was scored as no repulsion. If, however, neural crest cells kept a marked distance between both cells, failed to overlap, and/or changed their shape from mesenchymal to slender, more collapsed, it was scored as repulsion. The number of neural tubes with such responses was counted, and the percentage was determined based on the total number of neural tubes with neural crest in close proximity to the control cells. Neural tubes with neural crest cells that were not in close proximity to the cells were not counted.

Transient transfections of mSlit1 (Wu et al., 1999) into HEK293 cells were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The next day, cells were lifted and plated onto fibronectin-coated slides overnight. The next day, a hole in the monolayer was created, neural tubes were added, and the rest was as described above.

In vitro migration assay
Quail neural tubes from HH10–12 (for vagal crest) and HH13–16 (for trunk, the posterior parts of the tube) were obtained as described above) were cultured in DME/10% FBS in fibronectin-coated 24-well plates (Corning) above either Slit2 or control HEK cells or CM from the same cells. Cultures were grown overnight, and the next day, neural crest cells could be seen as a halo around the neural tubes. The cultures were fixed with ice-cold methanol for 20 min, followed by staining with toluidine blue, and the maximal distance of neural crest cell from the neural tube was measured and compared to controls.

In vitro wound assay
Quail neural tubes from HH13–16 (for trunk, the posterior parts of the tube) were obtained as mentioned above) were cultured in DME/10% FBS in fibronectin-coated 2-well chamber slide (Nunc) overnight, and the medium was changed to one conditioned for 5 d by Slit2 or control CM. For the priming experiments, neural crest cells were preincubated with CM for 2 h before making the wounds. Wounds were made by scraping cells (two to three cells–width lanes) with a pulled glass needle, and lanes were scored over 1–1.5 h periods to count the cells crossing the wound space. The criteria used was to count the points at which the wound was sealed at any length of the wound lane (regardless of the number) as “sealed”; “non-scored” was scored as the number of lanes that did not have cells contacting from the opposing sides of the wound.

Time-lapse video microscopy
Quail embryos stage 13–15 were electroporated with pcDNA vector carrying the GFP marker (Morsciano and McMahon, 2002) with two pulses of 100 mV each. After 1–2 h in a 38°C incubator, neural tubes were isolated as described above and cultured on glass slides coated with fibronectin in DME/10% FBS. The next day, cells were primed for 1 h with control or Slit2 CM as before, and labeled cells were imaged using a confocal microscope (Carl Zeiss Microimaging, Inc.) program for cell tracking and measurements.

Online supplemental material
The supplemental material (Fig. 51 and Videos 1 and 2) is available at http://www.jcb.org/cgi/content/full/jcb.200310141/DC1. The movies show neural crest cells moving from three different experiments combined together for control and Slit2-exposed neural crest cells. The lapsed time is ~2.5 h.

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