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Slit Branches Out: A Secreted Protein Mediates Both Attractive and Repulsive Axon Guidance

Minireview

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Slit is a large, modular extracellular matrix protein containing four arrays of leucine-rich repeat (LRR) sequences, followed by a string of epidermal growth factor (EGF)–like repeats (Rothberg et al., 1990). *slit* mutations were first identified in the famous Nüsslein-Volhard/Wieschaus patterning screen because they affect external midline structures in the *Drosophila* embryo (Nüsslein-Volhard et al., 1984). *Drosophila* and *C. elegans* have a single *slit* gene, while humans and rats have three (Holmes et al., 1998; Itoh et al., 1998; Nakayama et al., 1998; Brose et al., 1999; Li et al., 1999).

Slit is expressed by midline glia in the fly embryo; and in *slit* mutants these glia are ventrally displaced and the ladder-like axon scaffold of the central nervous system (CNS) collapses down to a single tract at the midline (Figure 1B). Mutations that delete all midline glia produce similar phenotypes, so Slit was thought to be primarily involved in the control of midline cell fates. The collapse of the axon ladder was assumed to be a secondary consequence of these cell fate changes. A series of recent papers in *Cell* and *Neuron* (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Wang et al., 1999; Nguyen Ba-Charvet et al., 1999) and a paper in press in *Development* (Battye et al., 1999), however, now show that Slit's major functions are likely to be in the direct control of axon guidance decisions. Remarkably, Slit has been shown to have at least two distinct guidance activities, discovered through complementary genetic and biochemical approaches.

Analysis of mutant phenotypes in *Drosophila* embryos showed that Slit is likely to represent a postulated activity at the midline that repels growth cones (Kidd et al., 1999). Vertebrate Slit proteins were shown to be capable of repulsion of axons in explant cultures (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). The biochemical experiments that identified Slit were based on a different premise. Many vertebrate neurons extend collateral branches from their axon shafts after the primary growth cone has already advanced far ahead. In some cases the main axon is later retracted, and the collateral branches become the connections to the major target area. In other cases both the collaterals and the primary axon are maintained, allowing the neuron to simultaneously communicate to multiple target areas. The factors that induce collateral branching far from the primary axon's target have not been molecularly characterized to date. Accordingly, an assay was devised to detect activities in brain extracts that could promote branch formation from axons of dissociated rat dorsal root ganglion (DRG) neurons. The purified branch-promoting activity turned out to be the N-terminal portion of the Slit protein (Wang et al., 1999).

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Slit Is Required for Repulsion of Axons from the Midline in Drosophila

In the fly CNS and the vertebrate spinal cord, axons grow to the midline because attractive molecules such as netrins are expressed there. Midline repulsive activities may also be necessary, however, to prevent longitudinal axons that express attractive netrin receptors from crossing the midline. Furthermore, repulsion is required to allow the growth cones of commissural neurons to leave the midline as they travel across the CNS, and to keep them from later returning to the midline. The transmembrane protein Roundabout (Robo), which is expressed on neuronal growth cones and axons, is a receptor for this midline repulsive signal in *Drosophila*. In *robo* mutants, some longitudinal axons fail to be repelled from the midline and cross over to the contralateral side of the CNS, while commissural axons follow looping paths around the midline, crossing it multiple times (Kidd et al. 1998a; Figure 1B).

Robo function is controlled by the Commissureless (Comm) protein. Comm is also a transmembrane protein, but it is expressed on midline glia and is transferred to commissural axons by an unknown mechanism (Tear et al., 1996). Comm causes degradation or downregulation of Robo in the commissures. After commissural axons

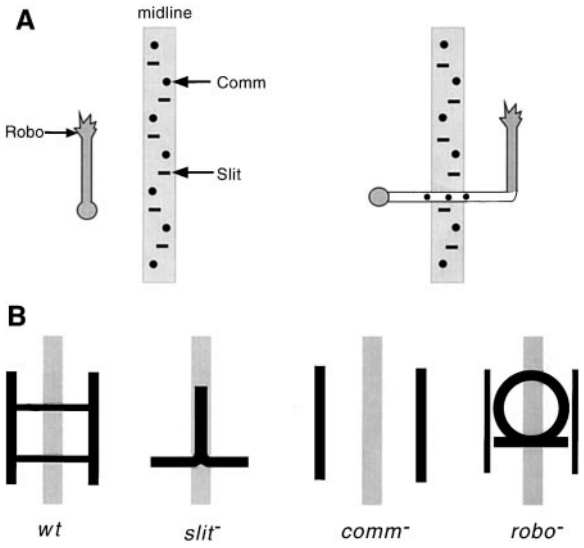

Figure 1. Mutations Affecting Growth Cone Behavior at the Midline

(A) Normal behavior of longitudinal and commissural axons. Comm and Slit are expressed by midline glia, while Robo is on CNS growth cones. Comm is transferred to commissural axons and Robo is downregulated when commissural growth cones contact the midline.

(B) Wild-type and mutant CNS axon arrays. In the wild-type embryo, two longitudinal axon bundles extend along the length of the embryo. In each segment, there are two commissures crossing the midline. The *robo* and *slit* cartoons represent a subset of axons that are strongly affected by the *robo* mutation.

cross the midline, Robo protein inserted into the membrane at the growth cone may escape Comm-mediated downregulation, because the growth cone is no longer in the zone within which it can acquire Comm from the midline glia. Robo on the growth cone would now stimulate its growth, driving it away from the midline repellent. Repulsion can thus facilitate axonal growth as well as reduce it, allowing formation of axon tracts that cross over the repellent source (Figure 1A). In a *comm* mutant, Robo fails to be downregulated, so that all axons are repelled from the midline and no commissures form (Figure 1B; Kidd et al., 1998b).

A clue that secreted Slit might be the midline repellent for the Robo receptor came from experiments in which Comm was expressed on all neurons. Comm expression at moderate levels caused Robo to be downregulated on all axons and therefore generated a *robo*-like phenotype. High-level Comm expression, however, produced a phenotype like that of *slit*, in which all axons converged onto the midline (Kidd et al., 1999). Thus, Comm has additional targets involved in repulsion by the midline, and when all of these are eliminated axons grow to the midline and never leave. One such target might be a second Robo protein, Robo2 (Kidd et al., 1998a).

The potential relationship between Slit as ligand and Robo as receptor suggested by these results was then tested by making double mutants, and it was found that *slit* and *robo* mutations interact in a dosage-sensitive manner (i.e., *slit*/+, *robo*/+, and *robo*/*robo* phenotypes are similar; Kidd et al., 1999). A dosage-sensitive relationship is usually taken as evidence that two mutations affect proteins in the same pathway. Slit was also demonstrated to be a repellent by overexpressing it either at the midline or in stripes across the CNS, resulting in phenotypes in which axons turned away from Slit-expressing regions (Battye et al., 1999).

Slit Binds to Robo

To study the interactions of Slit and Robo in vitro, fly and vertebrate proteins were epitope-tagged and expressed in transfected mammalian cells. Slits bind to Robo-expressing cells, and vice versa. The two proteins can also be coprecipitated from a mixture of Slit and Robo-containing lysates. In cross-species binding experiments, the fly and mammalian Slits and Robos were able to interact with each other. The K_ds for binding of vertebrate Slits to Robos are in the low nanomolar range (Brose et al. 1999; Li et al., 1999).

Slit Repels Motor and Olfactory Bulb Axons

The mRNAs encoding the three mammalian Slits are localized in complex, overlapping patterns that are consistent with the involvement of Slit proteins in multiple guidance pathways. They are expressed, however, at two places and times where repulsion of axons apparently occurs. These are the floor plate in the spinal cord and the septum in the forebrain, at E11–E13 (Holmes et al., 1998; Itoh et al., 1998; Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). Both regions have been shown to be capable of repelling axons in explant cultures (Pini, 1993; Guthrie and Pini, 1995).

To evaluate whether recombinant Slit could function as a repellent, Brose et al. (1999) cocultured aggregates of Slit-expressing cells with explants of ventral spinal cord. Spinal motor axons grow profusely out of these explants, and these axons are repelled by floor plate cells (Guthrie and Pini, 1995). It was observed that when an explant was placed adjacent to a Slit-expressing cell aggregate, axonal outgrowth was greatly reduced on the side of the explant that faced the aggregate (Figure 2B). Thus, Slit can repel motor axons. This repulsion might be mediated by Robo, since Robo mRNAs are expressed in the motor columns. Slit had no effect on spinal commissural axons, which by analogy to the fly system might be expected to be repelled by Slit after they cross the floor plate.

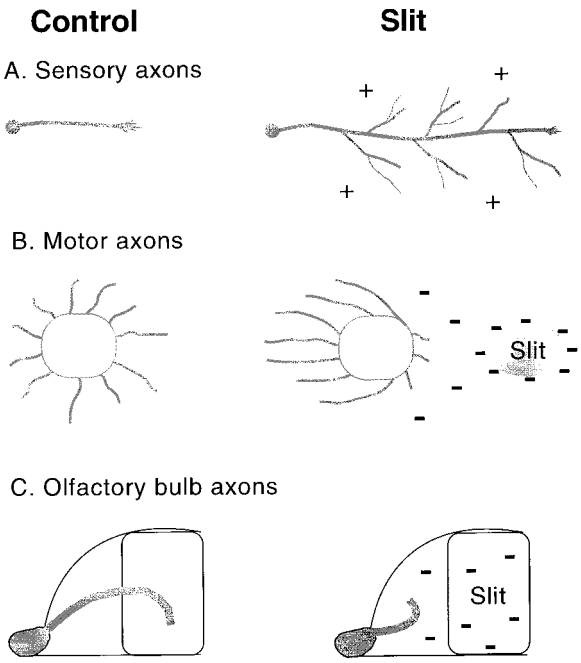

Figure 2. Slit Activities

(A) Slit promotes branching of cultured NGF-responsive DRG axons.
(B) Slit repels motor axons extending from a spinal cord explant.
(C) Olfactory bulb projection axons turn away from Slit-expressing cells covering the telencephalon.

The axons of olfactory bulb projection neurons follow the lateral olfactory tract into the olfactory cortex, avoiding the septum. Septal tissue repels olfactory bulb axons in explant cultures (Pini, 1993). Slit-expressing cells were found to also be capable of repelling these axons when placed adjacent to olfactory bulb explants (Li et al., 1999; Nguyen Ba-Charvet et al., 1999). To examine the effects of Slit on axon outgrowth from the olfactory bulb in its normal context, an intact piece of tissue containing the olfactory bulb and telencephalon was cultured, and the telencephalon was covered with aggregates of Slit-expressing or control cells. Olfactory bulb projection axons turned away from regions covered with Slit cells, showing that Slit is capable of repelling these axons when they are growing along normal telencephalic pathways (Li et al., 1999; Figure 2C). Finally, Slit induced growth cone collapse when added to olfactory bulb cultures (Nguyen Ba-Charvet et al., 1999). Thus, Slits resemble semaphorins, the best-characterized chemorepellents, in that they can both collapse growth cones in short-term assays and inhibit directional axon outgrowth in longer-term cultures.

Slit Promotes Axonal Branching

The identification of factor(s) that promote extension of collateral branches required the development of an assay in which branch formation could be easily quantitated. To do this, Wang et al. (1999) took advantage of certain properties of cultured DRG neurons. In the rat, DRG axons contact the spinal cord at the dorsal root entry zone, bifurcate and extend longitudinally in both rostral and caudal directions, and then branch and send collaterals into the dorsal spinal cord. Nerve growth factor (NGF)–responsive small-diameter DRG neurons begin to extend collateral branches into the spinal cord at E16. When E14 DRG neurons were cultured at low density in a collagen matrix in the presence of NGF, their development was slowed, so that they extended simple axons with few branches during the first four days. Later, however, the axons elaborated more complex branches. This assay provided a way to search for activities that could promote the precocious formation of branches from E14 neurons.

E17 rat spinal cord extracts were found to stimulate axon outgrowth and increase branch number. Similar activities were found in extracts from calf brain membranes, providing an abundant source of material for purification. Calf brain extract was able to increase the number of branchpoints per axon by up to 5-fold (from about 0.5 to 2.5), while also increasing axonal length by up to 2.5-fold (Figure 2A). By fractionating the extract through several columns, it was determined that the presence of a 140 kDa band correlated with activity. The sequences of tryptic peptides from the purified band identified it as bovine Slit2.

Slit1 and *Slit2* mRNAs are expressed in the dorsal spinal cord at the time when DRG neurons extend collateral branches (Wang et al., 1999), so Slits are in the right places to promote collateral formation in vivo. Some or all of the branch-promoting activity found in E17 rat spinal cord extracts is likely to be due to Slit proteins, since Slits are expressed at high levels in spinal cord at this time. We do not know, however, whether Slit can induce collateral branch formation in a system that more closely resembles the environment of the dorsal root entry zone in which DRG neurons branch into the spinal cord during embryogenesis. Thus, although Slit can promote branching in dissociated cultures, there is no evidence yet that it actually does this in vivo.

Interestingly, all three Slit mRNAs are also present in the DRG itself, suggesting the possibility that the elaboration of axonal branches that occurs in the DRG cultures after several days is due to an autocrine effect of Slit produced by DRG neurons. It has not been determined whether Robo proteins, which are likely to be the receptors for the negative repulsive activities of vertebrate Slits, also mediate Slit's positive elongation and branch-promoting activities.

Proteolytic Processing of Slit

The 140 kDa protein that correlated with branching activity was smaller than full-length Slit, indicating that Slit is processed (Wang et al., 1999). When Slit2 was expressed in mammalian cells, it was found to be cleaved into a 140 kDa N-terminal fragment, Slit2-N, and a smaller C-terminal fragment, Slit2-C. Slit2-N is tightly associated with the cell surface, while Slit2-C partitions equally between the cell surface and the medium. *Drosophila* Slit is processed in a similar manner in vivo (Brose et al., 1999). The molecular mass of the N-terminal Slit fragment suggested that the bovine branch-promoting protein might be Slit2-N, and recombinant human Slit2-N was then found to be active in the branching and elongation assay. Full-length Slit2 was inactive, however, and may actually inhibit the activity of Slit2-N (Wang et al., 1999).

Slit Expression and 3D Axon Guidance

The complex geometry of Slit-expressing zones in the brain and spinal cord may be capable of sculpting the trajectories of many axon pathways in three dimensions. This is especially true since these zones may function as repellents for some axons and as attractants for others. For example, as proposed by Li et al. (1999), spinal commissural axons that have crossed the midline might be driven away from it by repulsion from Slit in the floor plate, then forced to turn longitudinally by avoidance of Slit in the motor columns. The detailed geometries of the Slit- and Robo-expressing regions in the hippocampus (Nguyen Ba-Charvet et al., 1999) may be important in shaping its characteristic synaptic pathways and in determining its inputs from and outputs to other cortical regions.

Slit as an Organizer of Guidance Molecules

Slit2 was also found to bind to netrin and laminin, and its affinity for netrin is similar to that for Robo (Brose et al., 1999). This result suggests that Slit, which is a large modular protein with many different conserved binding domains, might be an extracellular "organizer" which could simultaneously bind several different axon outgrowth and guidance factors and deliver them to Slit-responding neurons. The properties of Slit in directing guidance might thus vary depending on what other Slit-binding molecules are present in its vicinity. In this respect, Slit might be like an extracellular version of the large cytoplasmic insulin receptor substrate (IRS) proteins, which contain many distinct tyrosine motifs that are phosphorylated by different kinases and bind to different signaling adaptors. IRS proteins organize different collections of signaling molecules (and thus stimulate or block specific transduction pathways) depending on which tyrosine kinases have been activated and which phosphotyrosine-binding adaptors are available (White, 1998). Slit could perform conceptually similar functions in the extracellular milieu. In this regard, we note that since neither group has evaluated binding between purified proteins, it is not yet known whether Slits and Robos directly interact or if instead they form a "sandwich" complex with another protein expressed in the transfected cells that can bind to both Slit and Robo.

Concluding Remarks

The identification of Slit as a multifunctional axon guidance factor will undoubtedly soon lead to new findings concerning Slit signaling pathways, receptors for attractive Slit signals, and the phenotypes of *slit* knockout mice. Beyond these obvious experiments, many exciting problems for the future are suggested by the results presented in these papers. This work might eventually have clinical relevance if Slits can stimulate outgrowth and branch formation by regenerating spinal cord axons. Furthermore, activity-dependent collateral branch

formation by cortical neurons might be mediated by Slits, since they are expressed in cortex after birth (Nguyen Ba-Charvet et al., 1999; Wang et al., 1999). For example, visual cortex neurons in an orientation column have secondary collateral branches that selectively form connections within nearby columns with the same orientation specificity. These long-range horizontal connections are thought to be involved in perceiving the continuity of objects (Gilbert, 1992). Perhaps Slit function can be regulated by activity in order to promote formation of appropriate cortical connections such as these. Both positive and negative activities of Slit could be involved in plasticity, since branch formation would lead to the creation of new synaptic connections and repulsion could prevent inappropriate connections from forming. In summary, these papers identify Slit as a central player in repulsive and attractive axon guidance. Further work should clarify how and under what conditions Slit regulates formation and rearrangement of specific connections in the nervous system.

Selected Reading

Battye, R., Stevens, A., and Jacobs, J.R. (1999). Development, in press.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. (1999). Cell *96*, 795–806.

Gilbert, C.D. (1992). Neuron *9*, 1–13.

Guthrie, S., and Pini, A. (1995). Neuron *14*, 1117–1130.

Holmes, G.P., Negus, K., Burridge, L., Raman, S., Algar, E., Yamada, T., and Little, M.H. (1998). Mech. Dev. *79*, 57–72.

Itoh, A., Miyabayashi, T., Ohno, M., and Sakano, S. (1998). Mol. Brain Res. *62*, 175–186.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., Goodman, C.S., and Tear, G. (1998a). Cell *92*, 205–215.

Kidd, T., Russell, C., Goodman, C.S., and Tear, G. (1998b). Neuron *20*, 25–33.

Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Cell *96*, 785–794.

Li, H.-s., Chen, J.-h., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z.-h., Nash, W., Gick, C., Ornitz, D.M., Wu, J.Y., and Rao, Y. (1999). Cell *96*, 807–818.

Nakayama, M., Nakajima, D., Nagase, T., Nomura, N., Seki, N., and Ohara, O. (1998). Genomics *51*, 27–34.

Nguyen Ba-Charvet, K.T., Brose, K., Marillat, V., Kidd, T., Goodman, C.S., Tessier-Lavigne, M., Sotelo, C., and Chedotal, A. (1999). Neuron *22*, 463–473.

Nusslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Roux's Arch. Dev. Biol. *193*, 267–282.

Pini, A. (1993). Science *261*, 95–98.

Rothberg, J.M., Jacobs, J.R., Goodman, C.S., and Artavanis-Tsakonas, S. (1990). Genes Dev. *4*, 2169–2187.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C.S., and Seeger, M.A. (1996). Neuron *16*, 501–514.

Wang, K.H., Brose, K., Arnott, D., Kidd, T., Goodman, C.S., Henzel, C., and Tessier-Lavigne, M. (1999). Cell *96*, 771–784.

White, M.F. (1998). Mol. Cell. Biochem. *182*, 3–11.