The leech receptor protein tyrosine phosphatase HmLAR2 is concentrated in growth cones and is involved in process outgrowth

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SUMMARY

Developing neurons extend long processes to specific distal targets using extracellular molecules as guidance cues to navigate through the embryo. Growth cones, specialized structures at the tip of the extending processes, are thought to accomplish this navigation through receptors that recognize guidance cues and modulate growth accordingly. In *Drosophila*, several receptor tyrosine phosphatases (rPTPs), including DLAR, have been shown to participate in directing neurite outgrowth. As yet, however, it is not known how rPTPs act to affect navigation. To gain insight into the mechanisms of rPTP-mediated outgrowth guidance, we have investigated the role of HmLAR2, a *Hirudo medicinalis* homologue of DLAR, in process outgrowth. HmLAR2 is expressed by, among other cells, a transient neuron-like template cell, the Comb cell. Here we present evidence that HmLAR2 protein becomes concentrated within their growth cones at a stage when C cell processes undergo rapid outgrowth. When antibodies raised against the extracellular domain of HmLAR2 were injected into intact embryos, they bound specifically to the C cell surface at growth cones and along processes and caused the partial internalization of HmLAR2 receptors. Moreover, the C cell processes were found to project aberrantly, to deviate from their normally highly regular trajectories and to extend shorter distances in the presence of the antibodies. We propose that HmLAR2 is required by the C cell for guidance and extension and suggest that it functions via its ectodomain to transduce extracellular guidance cues.

Key words: Receptor tyrosine phosphatase, LAR homologue, Growth cone, Neurite, *Hirudo medicinalis*, Embryogenesis

INTRODUCTION

The growth cone of a neurite is like a cruising automobile. Both have at any given moment a limited range of behaviors: stop, go forward, go back, turn and, for growth cones, branch. Both are able to traverse varied and complex paths. Both depend on information obtained en route in order to arrive at the correct destination, and both must discern appropriate directional cues against a noisy background rich in irrelevant, potentially distracting information. An automobile requires a human driver to recognize road signs, to integrate information and to choose when to stop, to go or to turn. The growth cone accomplishes its analogous tasks of interpreting and responding to guidance molecules with an array of membrane receptors and intracellular effector systems.

Many membrane receptors that detect guidance cues are members of the cell adhesion molecule (CAM) family of proteins (Stoeckli and Landmesser, 1995; Zhukareva and Levitt, 1995). CAMs are also found in the extracellular matrix on which neurites grow, and neurites can respond to extracellular CAMs by altering their rate and direction of outgrowth. CAMs on neurites and growth cones are thought to interact with extracellular CAMs either by homophilic or specific heterophilic binding (Chiba and Keshishian, 1996). For example, process outgrowth can be perturbed by interfering with the ability of the neuronal CAMs axonin-1 and Nr-CAM to bind their extracellular CAM substrates by masking the neuronal CAMs with specific antibodies (Stoeckli et al., 1997).

When CAMs or other receptors bind their ligands, the event must be transduced so that the cell can respond appropriately. A great deal remains to be learned about the molecular means by which extracellular positional information is mediated inside the neurite. Intracellular protein tyrosine phosphorylation, however, has been shown to correlate inversely with the rate that neurites extend along specific substrates (Wu and Goldberg, 1993). Furthermore receptor tyrosine kinases, such as the Eph receptors, are known to transduce extracellular cues by phosphorylating proteins and to influence growth cone navigation (Tessier-Lavigne, 1995). Increasing evidence also implicates receptor protein tyrosine phosphatases (rPTP) in directing neurites.

In the typical structure of the rPTP family of proteins, a CAM-like extracellular domain and intracellular tandem protein tyrosine phosphatase domains are brought together. Clearly this structure suggests a mechanism for directing neurite outgrowth in response to extracellular cues. Direct experimental evidence has demonstrated that several *Drosophila* rPTPs, including DLAR, participate in axonal navigation. In DLAR loss-of-function mutants, DLAR-
expressing neurons make pathfinding errors. Specifically, the SNb motor neurons fail to branch off the common motor pathway and fail to arrive at their target (Krueger et al., 1996). The means by which the loss of DLAR activity brings about this effect, however, is not known, although it has been shown that DLAR normally acts in concert with other rPTPs (Desai et al., 1997).

HmLAR2 is a leech homologue of DLAR (Gershon et al., 1998). Like DLAR, HmLAR2 has an ectodomain comprising three amino-terminal immunoglobulin (Ig) repeats followed by nine fibronectin type 3 (FN-III) repeats, a transmembrane region and two intracellular phosphatase domains. In embryos of Hirudo medicinalis, HmLAR2 mRNA is expressed exclusively by a small, fixed number of neurons in the CNS and a neuron-like template cell, the Comb (C) cell (Jellies and Kristan, 1988), in the body wall. Immunological detection of HmLAR2 reveals that the protein is expressed on the processes of the C cell, and particularly on growth cones and filopodial extensions (Gershon et al., 1998). Thus, HmLAR2 is ideally positioned on C cells to affect navigational behavior by directly sensing guidance cues and influencing the mechanisms of outgrowth.

The C cells of the developing leech provide an excellent opportunity to observe process extension and growth cone behavior. Each of the leech’s 21 midbody segments contains a pair of C cells and each C cell has approximately 70 processes, each ending in a large growth cone (Jellies and Kristan, 1988). These processes extend in a highly regular formation in which half project posteromedially and half anterolaterally, forming an oblique lattice around the animal (Fig. 1). This lattice provides the template on which the oblique muscle layer forms (Jellies and Kristan, 1988; reviewed in Jellies, 1990). The comb cell processes extend in a simple, straight trajectory, that is rigidly maintained. Any variation in trajectory caused by experimental perturbation is thus easily detected. Comb cell development proceeds from an initial relative quiescent period, up to around embryonic day 9 (E9), during which they establish their arrays of growth-cone-tipped processes. However, beginning around E10 and continuing up until E14, the C cell processes undergo a stage of rapid growth wherein the processes encircle the developing embryo (Jellies and Kristan, 1988). Importantly, it is during this stage of rapid outgrowth that HmLAR2 protein expression is detected at its highest level on the membrane surfaces of the C cell, especially at the growth cone (Gershon et al., 1998).

In this study we have investigated the role of HmLAR2 in C cell development. To test the hypothesis that HmLAR2 is a receptor for directional guidance cues, we raised antisera against the extracellular domain of the protein. These antisera allowed us to determine that HmLAR2 becomes concentrated at the growth cones of the C cells at the time when rapid C cell process extension normally occurs. Surprisingly, we found that most of the HmLAR2 protein in growth cones is not present on the cell surface, but is internally sequestered. Lastly, we obtained evidence suggesting that interfering with the extracellular domain of the receptor causes the C cells to grow aberrantly. In embryos injected with antibodies raised against the extracellular domain of HmLAR2, the C cell processes were found to deviate from their normally straight, oblique trajectories, were frequently found to collide with each other and often grew shorter distances. Based on these data, we suggest that HmLAR2 protein interacts with the extracellular environment to help control the direction and extent of process extension.

MATERIALS AND METHODS

Antigen preparation
A cDNA fragment encoding the extracellular Ig domain of HmLAR2 was isolated using PCR with pfu polymerase (Stratagene). The HmLAR2 fragment was cloned into pGEX2t and expressed in bacteria as a GST fusion protein according to the methods of Ausubel et al. (1995). The expressed protein was insoluble and purified according to the methods of Harlow and Lane (1988). Briefly, insoluble matter from bacterial cultures was recovered by centrifugation after lysis, and then solubilized with SDS and B-mercaptoethanol. Fractions were run on large polyacrylamide gels and visualized by CuCl2 negative staining. The appropriate band of protein was cut out and electroeluted from the polyacrylamide using an Elutrap device (Schleicher and Schuell) according to the manufacturer’s instructions. Purified protein was then quantitated by the Bradford reagent (Bio-Rad) method.

Animal injection
Animals were maintained, injected with antigen and bled by HTI Bioproducts. The HmLAR2 antigen was injected into rats in three doses of 100 µg, 2-3 weeks apart. After 8 weeks animals were exsanguinated.

Affinity purification
Serum was affinity purified according to methods described by Harlow and Lane (1988). Embryonic leech and GST-producing bacterial cell lysates, as well as heterologously expressed, gel-purified HmLAR2 antigens were fixed to 1 ml portions of cyanogen-bromide-
activated (CnBr) sepharose beads (Pharmacia) according to the manufacturer’s instructions. These beads were then loaded into columns and the columns were washed extensively with PBS. Antiseras, filtered to 0.2 μm and diluted 1:10, were passed through first the bacterial and leech columns, then through the HmLAR2 column. Antibodies binding to the HmLAR2 column were eluted successively with acid and base and the concentration of antibodies was roughly determined by the measuring the absorbance of light of 280 nm wavelength. The most concentrated fractions were pooled, dialyzed against PBS and used in subsequent immunohistochemical techniques. Antiserum was stabilized with bovine serum albumin (BSA) to a concentration of 10 mg/ml.

The affinity-purified HmLAR2 antiserum was found to label bacterial HmLAR2-Ig fusion protein as assayed by immunobLOTS (data not shown). Moreover, when whole-mount immunostaining of leech embryonic body wall (see below) with this new antiserum was compared to the immunolabeling of a different polyclonal antibody that recognizes the intracellular phosphatase domain of HmLAR2, an identical pattern of C cell staining was detected (Gershon et al., 1998; Nitabach, 1995). Based upon these results, and the fact that the only peripheral cell that expresses HmLAR2 mRNA is the C cell, we conclude that the antiserum used in the perturbation experiments selectively recognizes the Ig domain of HmLAR2.

Antibody perturbations

For antibody perturbation experiments, antibodies were sterilized by filtration, and then purified and concentrated by diafiltration using microcon 100 devices (Amicon). In one set of experiments, however, antibodies were concentrated by ammonium sulfate precipitation. While the concentration of antibodies was not measured quantitatively, it was approximately 10 times the concentration used to produce good staining results at 1:10 dilution. Fast green (0.1%) was added to the injected solution in order to monitor visually the diffusion of the solution in the embryo. Antiseras were injected into embryos at E7, and the embryos were allowed to develop at 18°C until they reached the equivalent of E14, 1 day before the fusion of the dorsal midline would be expected to take place if the embryos were kept at 23°C. At 18°C development is considerably slowed, such that progress from E7 to the equivalent of E14 takes approximately 10-14 days. During this time embryos were injected every 3-4 days. For each injection, 2 μl of antiserum were loaded into a freshly pulled micropipet that was then broken underwater close to the animals to be injected. The micropipet was then pushed through the germinal plate and the contents were ejected into the fluid-filled space between the yolk and the germinal plate. As controls, BSA, antibodies raised against the intracellular domain of the closely related protein HmLAR1, or goat anti-mouse secondary antibodies were injected in place of antibodies to HmLAR2 ectodomain. At approximately E14, animals were dissected, fixed and processed for HmLAR2 immunohistochemistry and/or Laz 10-1, a muscle-specific monoclonal antibody, as described below.

Whole-mount immunostaining

Embryos were dissected and fixed in 4% formaldehyde at room temperature for 1 hour, washed for at least 1 hour in wash buffer (PBS with or without 0.5% Triton-X), treated for 30 minutes in blocking buffer (wash buffer containing 2% normal donkey serum) and incubated overnight in primary antiseras diluted in blocking buffer. Preparations were then treated with secondary antibodies conjugated to either 10 nm colloidal gold (Ted Pella; 1:40 dilution) or horseradish peroxidase (HRP; Jackson Laboratories; diluted 1:50) in 0.5% Triton-X PBS. HRP-conjugated antibodies were then visualized either by treating tissue with 0.3% hydrogen peroxide and 0.03% diaminobenzidine in PBS until stained to satisfaction, or with fluorescein-tyramide (NEN) according to manufacturer’s protocol, while gold-conjugated antibodies were visualized using a commercially available silver enhancement kit (Ted Pella) according to the manufacturer’s protocol. Some silver-stained preparations were subsequently labeled with the muscle-specific monoclonal antibody Laz 10-1 (diluted 1:4 in 0.5% Triton-X PBS; generously provided by Dr Birgit Zipser), in order to visualize the circular and oblique muscle layers. Following Laz 10-1 treatment, preparations were washed and treated with fluorescein-conjugated donkey anti-mouse secondary antibodies (Jackson Laboratories), diluted 1:200 in 0.5% Triton-X PBS. After a final wash, preparations were mounted and examined with a Zeiss microscope equipped with Nomarski and fluorescence optics.

RESULTS

By embryonic day 10 (E10), when rapid outgrowth begins, HmLAR2 is concentrated at the growth cones of C cells

Standard immunohistochemistry using Triton-X permeabilization indicated that, from E10 onward, HmLAR2 was more concentrated at growth cones (Fig. 2A,B) than along C cell processes (CCPs), whereas prior to E10 the most intense labeling was found in the somata (Gershon et al., 1998). To verify that the intensity of growth cone labeling with HmLAR2 antibodies resulted from a higher concentration of HmLAR2 protein at the growth cones relative to the CCPs, rather than from the relatively larger volume of the growth cone, we used confocal microscopy to examine optical sections that included both growth cones and attached processes. In such optical sections, the thickness of a growth cone and of its process were equivalent, and the growth cones continued to label more intensely (Fig. 2C). Thus, HmLAR2 protein is not evenly distributed in the CCPs after E10, but rather accumulates at the tips. This increase in HmLAR2 protein in the growth cone from E10 onwards, as well as the general distribution of the protein throughout the C cell, have also been documented using an antiserum raised against the intracellular phosphatase domain of HmLAR2 (Nitabach, 1995), indicating that the staining with ectodomain antibodies reflects the distribution of the ectodomain together with the intracellular catalytic domain.

Most of the HmLAR2 protein at the growth cones is internally sequestered

When embryos were labeled by immunohistochemistry without detergent to permeabilize cell membranes during primary antibody incubation, the distribution pattern of HmLAR2 protein that emerged was different from that observed with permeabilization. In unpermeabilized preparations, HmLAR2 protein appeared to be evenly distributed on the surfaces of growth cones and CCPs (Fig. 2D,E). The same result was obtained using fluorescent labeling and confocal microscopy (Fig. 2F). It follows, therefore, that most of the protein concentrated at the growth cones must be stored internally. As would be expected, the overall intensity of labeling was much greater when preparations were permeabilized.

Antibodies to HmLAR2 ectodomain injected into the developing embryo are internalized by the C cells

Embryos were injected at E7 with affinity-purified antibodies to HmLAR2 ectodomain in order to interfere with the normal binding of HmLAR2 ligands. Control embryos were injected with BSA, or with a variety of antibodies that were not
expected to bind to HmLAR2 (see Materials and Methods). Some of the embryos injected with HmLAR2 antibodies were fixed several days later and stained without additional application of primary antibody in order to determine if the injected antibodies had reached all of the CCPs and growth cones. In every case thus assayed, CCPs and their growth cones were labeled by the injected antibodies. Interestingly, the somata were labeled much more strongly than the rest of the C cells (Fig. 3). This somatic labeling was apparently internal, and did not resemble the clearly external light labeling of somata in unpermeabilized preparations treated with primary antibody after fixation. Control antibodies did not label the C cell somata or CCPs (not shown). The somatic labeling by HmLAR2 antibodies injected into the live embryo demonstrates that these antibodies were taken up by the C cells, presumably upon binding to the HmLAR2 antigen. The site of antibody uptake could be anywhere in the cell, since HmLAR2 ectodomain is present throughout the surface of the C cell (Gershon et al., 1998).

C cells exposed to HmLAR2 antibodies extend processes aberrantly

In embryos that were injected with antibodies to HmLAR2 and allowed to develop for 7-10 days (see Materials and Methods), the normal growth of the CCPs was clearly perturbed. CCPs failed to maintain their normally straight trajectories, they diverged from their normally oblique orientation and, failing

Fig. 2. HmLAR2 is concentrated within C cell growth cones after E10. (A-C) Preparations were labeled by immunohistochemistry with Triton-X permeabilization; (D-F) preparations were processed without Triton-X until the application of secondary antibodies. (A) E11 embryo. The soma (CC) of a C cell, as well as the posteriorly directed processes and growth cones of this C cell and its contralateral homologue, are labeled by the HmLAR2 antibody. Note the intense labeling of growth cones compared to processes. The segmental ganglion and connective nerves (CNS) are out-of-focus. (B) HmLAR2 protein concentrated at the distal portion of C cell growth cones at E14, visualized by HRP-DAB immunolabeling. HmLAR2 was also present on filopodia and on the surface of the processes’ shafts. (C) Thin optical section through C cell processes and growth cones of an E13 embryo, generated by confocal microscopy. This section was chosen so as to cut approximately through the center of the growth cones. Although the spatial resolution of the fluorescence technique is not as good as that of the HRP-DAB technique (cf. with B), it does help demonstrate that labeling is much more intense within growth cones than on shafts. (D) In an embryo roughly at the same stage as the one in A, HmLAR2 protein appears to be evenly distributed on the surface of C cell processes and growth cones. (E) In an embryo at roughly the same stage as that shown in B, HmLAR2 protein was evenly distributed on the outside of processes and on growth cones. (F) In an optical section comparable to the one shown in C, HmLAR2 was detected in equal distribution on the surface of processes and growth cones when detergent was omitted from the early steps in the procedure. HmLAR2 was visualized by immunolabeling with colloidal gold-silver (A,D,E), with HRP-DAB (B), or with HRP-fluorescein (C,F; see Materials and Methods). In all panels, anterior is up, black arrowheads point to CCPs, red arrowheads to growth cones and bars represent 10 μm.

Fig. 3. HmLAR2 ectodomain antibodies are internalized by living C cells and concentrated in the perinuclear region. Antibodies to the HmLAR2 ectodomain, injected into live embryos 1 day prior to fixation, labeled C cell bodies (arrows), process shafts (black arrowheads) and growth cones (red arrowheads); the posteriorly directed process of the C cell body is indicated by the white arrowhead. This preparation was treated after fixation with secondary antibodies, but its only exposure to primary antibodies occurred while the animal was alive and intact. Much of the primary antibody is localized inside the soma, indicating that the antibody molecules were internalized by the C cells. The soma, processes and growth cones of the C cell on the right are mostly not in the plane of focus of this photomicrograph. Anterior is up and the bar represents 10 μm.
to maintain a regular distance between each other, they frequently intersected (Fig. 4A-C). In addition, while it has been estimated that about 1 in 1000 processes normally makes a 90° turn and grows in an inappropriate oblique orientation (Jellies and Johansen, 1995), in experimental animals, processes made turns relatively frequently and, when they did so, were not constrained to grow in an opposite oblique course (e.g., see outlined CCPs in Fig. 4A).

While HmLAR2 antibody injection made the array of CCPs more irregular and caused individual processes to follow various abnormal routes, it also produced effects that were universal across the array. Normally, the CCPs grow at close to a 45° angle with respect to the orthogonal lateral and longitudinal axes of the embryo (Figs 1, 5A,B). In experimental animals, however, processes grew in formation along curved trajectories, at varying angles with respect to these axes. In an area around the ventral midline, both anteriorly directed and posteriorly directed processes grew at angles abnormally close to the transverse axis (Fig. 5C,D), thus closer to the orientation of the circular muscle layer (Fig. 6).

By comparison, when processes grew through more lateral regions, they gradually turned towards the anteroposterior axis, thus orienting at angles progressively closer to the longitudinal muscles (Fig. 5C,D). However, since in the ventral region the perturbed CCPs were misdirected towards the lateral
axis, the turning towards longitudinal brought the CCPs into orientations within the range observed for normal CCPs. Thus, the longitudinal turning of the CCPs may represent an attempt to return to normal trajectories.

The result of these opposite effects was that anteriorly directed processes, which do not cross the ventral midline, followed crescent-shaped trajectories (Fig. 5D), while posteriorly directed processes followed ‘S’-shaped trajectories (Fig. 5C). Individual CCPs did not all turn to the same degree, however, and where processes turned more sharply than their neighbors, collisions and intersections occurred (Fig. 4B,C). Lastly, CCPs in experimental animals often did not extend as far as CCPs in matched control animals. While both anteriorly and posteriorly directed processes from antibody-treated animals demonstrated a pronounced decrease in total CCP length, this was particularly true for posteriorly directed CCPs, which were 40-60% shorter than those from control C cells (cf. Fig. 5B,D, red arrowheads; Table 1).

The extent of perturbation varied among injected animals, and not all were noticeably affected. However, as shown in Table 1, half of all animals receiving the antibody injections displayed curved CCP trajectories. In contrast, no control animals (those receiving either non-HmLAR2 antibodies or BSA) displayed curved C cell trajectories. The strongest effects were observed in embryos injected at or before E8, re-injected every 3-4 days thereafter, and allowed to develop at 18°C until a stage approximately equivalent to E14. Embryos injected at E10 and left for 2 days at 18°C were also affected, but less strongly. While examples of irregular CCP outgrowth were found in these embryos, including turning and process intersection, they were less obvious and less frequent than in the most strongly affected animals. These animals are not included in the table or figures.

Aberrant C cells continued to direct oblique muscle cell development, producing abnormal oblique muscles

In even the most strongly affected experimental animals, muscle cells grew along the CCPs. These muscle cells were easily visualized with the muscle-specific monoclonal antibody Laz 10-1, and generally conformed to the trajectories of the CCPs. In Fig. 6A,B, fluorescent micrographs of regions near the ventral midline of Laz 10-1-labeled control (A) and experimental (B) embryos at several focal planes were scanned into a computer and the fibers of the circular, longitudinal and oblique muscle layers were traced. In the experimental embryo, the oblique muscle fibers, like the CCPs (Figs 4A-C, 5C,D) followed curved trajectories.

Perturbation by HmLAR2 antibodies does not cause a loss of growth cones or filopodia

Stoekli et al. (1996) found that application of antibodies to axonin-1 disturbs pathfinding and causes growth cones to collapse. In experimental embryos treated with HmLAR2 antibodies, however, C cell growth cones appeared to have a normal morphologies. Immunohistochemical labeling with HmLAR2 antisera revealed robust growth cones with many filopodia, the same as seen in normal animals (Fig. 7A,B). Growth cones in both normal and affected animals were extremely variable in shape, however, making it difficult to establish a canonical shape for detailed comparison. HmLAR2 antibody labeling, furthermore, does not permit the visualization of growth cone morphology as effectively as intracellular dye injection. While it is thus impossible to discount the possibility that subtle changes in growth cone morphology occur, it is clear that the injection of HmLAR2 antibodies did not cause a significant permanent loss of filopodia, or a loss of HmLAR2 protein on these structures, though transient effects could have occurred and not been detected by our methods.

DISCUSSION

HmLAR2 and the C cells

The observed perturbations following antibody injection are consistent with HmLAR2 playing a critical role in the establishment of the highly regular lattice of CCPs. The binding of antibodies to HmLAR2 ectodomain caused
individual processes to grow aberrantly and caused generalized deviation from the normal pattern of CCP outgrowth. Individual processes were found to depart from the common direction of growth, to bunch together and to cross over each other. Entire formations of CCPs curved away from their normal, oblique trajectories. Finally, treated embryos displayed consistently displayed CCP that were shorter in length, particularly those directed towards the posterior of the animal. Despite these changes in growth and trajectory, however, CCPs did not extend in random directions but maintained ordered, if less regular, patterns. The order that was seen to persist in experimental animals might be interpreted as evidence (1) that the effect of injected antibodies was to block only incompletely normal HmLAR2 function, or (2) that, in the absence of normal HmLAR2 function, other guidance molecules continue to affect the direction of CCP outgrowth and their effects give rise to the pattern of outgrowth observed.

These interpretations of our observations presume that HmLAR2 antibodies affect CCP outgrowth by acting directly on the C cells. It must be considered, however, that HmLAR2 antibodies may affect CCP outgrowth by acting on other cells, thus altering the environment in which the C cells grow. For example, the observed large-scale effects might be attributable to abnormal circumferential growth of the embryo, or to a disruption of body wall muscles secondary to the antibodies disrupting their innervation, possibly by affecting HmLAR2-expressing central neurons. These possibilities seem unlikely for a number of reasons. Firstly, injected animals appeared normal with regard to their relative proportions. Secondly, experimental and control animals both varied over approximately the same range in their size. Finally, other aspects of body wall development appeared normal, including the formation of the longitudinal and circular muscle layers (Fig. 6A,B) and the FMRFaminergic innervation of the lateral heart tubes by central motor neurons (data not shown). While these markers of normal development were observed in both experimental and control animals, only the C cells in the body wall of experimental animals internalized HmLAR2 antibodies, demonstrating that the antibodies had acted selectively on these cells. Furthermore, since the C cells are the only peripheral cells that express HmLAR2 mRNA, and since identical and selective labeling of C cells and their processes was observed when using two different antisera raised against either the intracellular or extracellular domains of HmLAR2 (Gershon et al., 1998; Nitabach, 1995), it seems reasonable to conclude that the effects reported herein are specific and selective to a perturbation of HmLAR2 function alone.

Exactly how antibodies to HmLAR2 ectodomain domain act when bound to the receptor is unclear. The antibodies might act by blocking the normal binding of the ligand of HmLAR2, or by actually mimicking the effect of ligand binding. Because antibodies were introduced as whole, bivalent immunoglobulins rather than as Fab fragments, they might, theoretically, bring about receptor dimerization. Such dimerization is an essential aspect of the activation of many rPTPs. The presence of relatively high levels of HmLAR2 antibodies within C cell somata only in those preparations that were exposed for a prolonged period to the antibody in vivo indicates that endocytosis, and possibly retrograde transport, of HmLAR2 protein bound to antibodies takes place. One effect of antibody binding, therefore, might be to deplete the levels of HmLAR2 on the surface of the cell and thereby decrease the phosphatase activity of HmLAR2. Finally, it might be argued as well that the disrupted CCPs were a secondary result of this translocation process, perhaps due to the co-internalization of another, more crucial membrane-bound molecule. While such a possibility cannot be excluded at this time, we consider it unlikely given the involvement of LAR-like rPTPs in neuronal navigation in Drosophila (Krueger et al., 1996; Desai et al., 1997). Uncertainty about the exact action of the antibody on the living C cell is mirrored by uncertainty about how rPTPs transduce signals. So little is known about rPTPs that it is not clear whether the binding of ligand by the extracellular domain regulates the activity of the intracellular domain, its subcellular localization, or even if regulation is positive or negative. Interpretation of the perturbation data must therefore proceed by assuming that HmLAR2 might be either masked or inappropriately activated by antibodies, with the potential results of increased or decreased phosphatase activity. Whichever is the case, these data are consistent with the hypothesis that HmLAR2 is a receptor for guidance cues, and that it participates in the normal navigation of the C cell growth cones. These conclusions, therefore, complement observations in Drosophila that show that neurons that normally express DLAR make pathfinding errors in dlar mutants (Krueger et al., 1996; Desai et al., 1997), and implicate the extracellular

<table>
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<th>Experiment number</th>
<th>No. of injected animals*</th>
<th>No. of affected animals†</th>
<th>Distance between growth cones of contralateral anteriorly growing CCPs/ width of embryo (mean)‡</th>
<th>Distance between growth cones of contralateral posteriorly growing CCPs/ width of embryo (mean)‡</th>
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*Number in parenthesis is the number of controls, including BSA injected and non-HmLAR2 antibody injected.
†Animals were judged affected if CCPs curved rather than grew straight.
‡Distances from the line of CCP growth cones on one side of the animal to the line of growth cones on the other side were divided by the distance between the edges of the germinal plate. To maintain consistency as much as possible, all measurements were made at the 10th midbody segment.
¶All animals in this experiment were grown at 23°C rather than 18°C, possibly affecting the outcome.
§All animals were from same cocoon and thus as closely age-matched as possible; experimentals and controls separated in two lines in order to show last two columns.

Table 1. Penetrance and magnitude of effects of HmLAR2 antibody perturbation
domain of LAR-like receptor tyrosine phosphatases as being important in their normal receptor functioning.

**Disruption of HmLAR2 frees the CCPs from constrained growth, revealing separate lateral and longitudinal growth tendencies that coincide with the orientation of the surrounding muscle layers**

The regularity seen in normal CCPs strongly suggests that they grow in a highly constraining environment. Jellies and Johansen (1995) hypothesize that the body wall presents the CCPs with 2 sets of permissive paths that help define the normal, oblique CCP trajectories. In normal embryos, even aberrant CCPs seem able to grow only along these trajectories, while CCPs growing in rotated grafts grow in paths oblique to the longitudinal axis of the donor (Jellies and Johansen, 1995). In embryos injected with antibodies to HmLAR2, however, CCPs were no longer constrained to follow straight, parallel, oblique courses. Individual CCPs turned in many different directions, while the average trajectory of all the processes tended to curve. The loss of constraint may represent the acquired ability to grow over cues that normally repel CCPs from non-oblique paths, or the lost ability to sense and to grow toward strongly attractive oblique cues.

Antibody perturbation caused the CCPs to turn in two opposing ways. Near the ventral midline, processes turned more laterally, more closely following the orientation of the circular muscle cells. Away from the ventral midline, processes turned toward the orientation of the longitudinal muscle cells. As this longitudinal turning brought the CCPs closer to normal trajectories, however, it may be argued that it results from appropriate pathfinding; the CCPs might turn toward the longitudinal axis to resume oblique trajectories. Thus while HmLAR2 antibodies caused two different changes of direction, it may be argued based on available evidence, that the antibodies were the direct cause of only the initial, lateral, change in the ventral region of the animal.

Whatever the underlying causes of the lateral and longitudinal turns, the turns themselves suggest that normal CCP navigation proceeds through the integration of perpendicular forces. Although normal CCPs can only grow obliquely, in the experimental animals laterally and longitudinally directed outgrowth could occur separately. The separate growth tendencies observed in experimental animals, if combined appropriately might account for the oblique paths of the CCPs in normal animals. Interestingly, there is evidence that the *Drosophila* rPTPs DLAR and 99A can exert opposing effects on growing processes (Desai et al., 1997). Perhaps HmLAR2 and another leech rPTP normally exert opposing effects on the CCPs.

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