Disruption of pioneer growth cone guidance in vivo by removal of glycosyl-phosphatidylinositol-anchored cell surface proteins

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Summary

Cell surface proteins anchored to membranes via covalently attached glycosyl-phosphatidylinositol (GPI) have been implicated in neuronal adhesion, promotion of neurite outgrowth and directed cell migration. Treatment of grasshopper embryos with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves the GPI anchor, often induced disruptions in the highly stereotyped migrations of peripheral pioneer growth cones and afferent neuron cell bodies. In distal limb regions of embryos treated with PI-PLC at early stages of pioneer axon outgrowth, growth cones lost their proximal orientation toward the central nervous system (CNS) and turned distally. Pioneer growth cones in treated limbs also failed to make a characteristic ventral turn along the trochanter-coxa (Tr-Cx) segment boundary, and instead continued to grow proximally across the boundary. Treatment at an earlier stage of development caused pre-axonogenesis Cx1 neurons to abandon their normal circumferential migration and reorient toward the CNS. None of these abnormal phenotypes were observed in limbs of untreated embryos or embryos exposed to other phospholipases that do not release GPI-anchored proteins. Incubation of embryos with PI-PLC effectively removed immunoreactivity for fasciclin I, a GPI-anchored protein expressed on a subset of neuronal surfaces. These results suggest that cell surface GPI-anchored proteins are involved in pioneer growth cone guidance and in pre-axonogenesis migration of neurons in the grasshopper limb bud in vivo.

Key words: growth cone, axon guidance, adhesion, cell migration, glycosyl-phosphatidylinositol, PI-PLC, *Schistocerca americana*.

Introduction

The guidance of neuronal growth cones to appropriate targets during embryonic development is a requisite event in the formation of functional connections within a nervous system. Growing axons often travel relatively long distances through a changing environment of cell surfaces and extracellular matrices before reaching their correct synaptic partners. Labeling of axonal trajectories in vivo has shown that growth cones can make highly stereotyped turning decisions at certain choice points along their pathway, where they encounter a variety of possible guidance cues (Goodman et al., 1984; Tosney and Landmesser, 1985; Eisen et al., 1986; Caudy and Bentley, 1986b). These observations indicate that neuronal growth cones detect external guidance information and transduce extracellular signals into intracellular events that lead to directed axonal growth.

Potential sources of guidance information include extracellular matrix (ECM) and cell surface adhesion/recognition molecules (Anderson, 1988; Jessell, 1988; Fessler and Fessler, 1989; Sanes, 1989), which may be differentially distributed temporally and/or spatially on substratum cells or in the ECM, thus offering selective labels for recognition by growth cones. A growing number of cell surface molecules that may mediate adhesion or cell recognition have been identified that do not have transmembrane or cytoplasmic domains and are instead anchored to the outer leaflet of the plasma membrane by covalently attached glycosyl-phosphatidylinositol, or GPI (Low and Saltiel, 1988; Ferguson and Williams, 1988; Cross, 1990). GPI-anchored molecules that may be involved in neuronal adhesion and recognition include NCAM-120 (Doherty et al., 1990), F3/F11 (Gennarini et al., 1991), TAG-1 (Furley et al., 1990), T-Cadherin (Ranscht and Dours-Zimmerman, 1991), heparan sulfate proteoglycans (Carey and Stahl, 1990) and alkaline phosphatase (Zackson and Steinberg, 1988) in vertebrates, and chaoptin (Krantz and Zipursky, 1989), fasciclin I (Elkins et al., 1990b; Hortsch and Goodman, 1990), and one form of fasciclin II (Grenningloh et al., 1991) in insects.

Patterns of growth cone migration can be constrained by either attractive or aversive interactions with

extrinsic molecules. Surfaces that are low affinity, non-permissive or actively repulsive have now been described in several systems and may be a normal feature of growth cone guidance (Keynes and Cook, 1990). One of the best characterized examples of substratum preference in vitro is that of growth cones of temporal retinal neurons in the chick for anterior over posterior tectal membranes (Walter et al., 1987). A 33×10^3 M_r GPI-anchored protein located on posterior membranes appears to mediate this preference by promoting growth cone retraction (Stahl et al., 1990). Thus, GPI-anchored proteins are implicated in both attractive and aversive interactions.

In addition to their proposed roles in cell:cell adhesion and recognition, GPI-anchored cell surface proteins may affect transmembrane signalling upon binding extracellular ligands, presumably through interactions with other proteins. Binding of monoclonal antibodies to the GPI-anchored protein Thy-1, results in an influx of extracellular calcium ions in both T-lymphocytes (Krozcek et al., 1986) and sensory neurons (Saleh et al., 1988). Also, the receptor for ciliary neurotrophic factor was recently identified (Davis et al., 1991) and is the first example of a GPI-anchored receptor protein for a soluble ligand. Double mutation analysis in *Drosophila* suggests that fasciclin I, a GPI-anchored protein, may interact with intracellular signal transduction pathways mediated by cytoplasmic tyrosine kinases (Elkins et al., 1990a). Genetic analysis of fasciclin II in *Drosophila* embryos has shown that complete null mutation of this protein, which exists in both GPI-anchored and transmembrane forms, results in disrupted formation of an early fascicle of longitudinal axons in the CNS (Grenningloh et al., 1991).

The peripheral nervous system of embryonic grasshoppers offers a suitable system for testing the involvement of GPI-anchored proteins in growth cone guidance and neuronal migration. In embryonic limb buds, the Ti1 pioneer neurons are the first to initiate axonogenesis (Bate, 1976). Their growth cones migrate along a highly stereotyped pathway through the limb to the central nervous system (Bentley and Keshishian, 1982; Ho and Goodman, 1982; Caudy and Bentley, 1986b; O'Connor et al., 1990). Other later-arising afferent neurons use the pathway established by the Ti1 pioneers as a scaffold for their axonal outgrowth (Keshishian and Bentley, 1983; Klose and Bentley, 1989). As schematically represented in Fig. 1, the growth cones of the Ti1 neurons contact a series of guidance cues during their migration. These include intrasegmental epithelium and basal lamina (Caudy and Bentley, 1986a; Anderson and Tucker, 1988; Condic and Bentley, 1989a), pre-axonogenesis (guidepost) neurons and, at presumptive limb segment boundaries, high-affinity and low-affinity circumferential bands of boundary epithelial cells (Caudy and Bentley, 1987; Condic and Bentley, 1989b). Upon contact with these guidance cues at specific limb locations, the growth cones make a series of discrete alterations in direction termed steering decisions. The pathfinding behavior of the Ti1 pioneer neurons is extremely robust: it persists
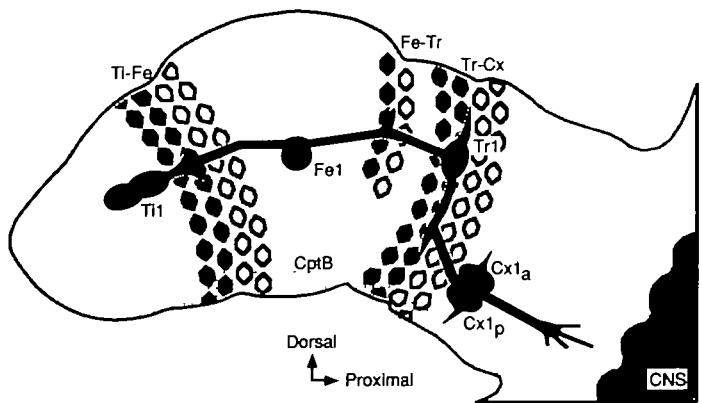

Fig. 1. Schematic diagram of the Ti1 pioneer neuron pathway at 35% of embryonic development. The sibling Ti1 pioneers arise at the limb tip. Their growth cones migrate proximally to the central nervous system (CNS) along a highly stereotyped route which is determined by encounters with a series of guidance cues. These include a set of pre-axonogenesis afferent (guidepost) neurons (Fe1, Tr1, Cx1$_{a,p}$) and presumptive limb segment boundaries (Ti-Fe, Fe-Tr, Tr-Cx). At each developing limb segment boundary, a distal, high-affinity circumferential band (filled hexagons) of epithelial cells is adjacent to a proximal, low-affinity band (unfilled hexagons). Only the Tr-Cx bands are well-developed when first encountered by the Ti1 growth cones. Ti-Fe, tibia-femur; Fe-Tr, femur-trochanter; Tr-Cx, trochanter-coxa.

following isolation of the limb bud from the body, geometrical distortion of the limb epithelium, removal of the mesoderm (Lefcort and Bentley, 1987), removal of the basal lamina, treatment with trypsin, chymotrypsin, elastase, papain, collagenase or ficin (Condic and Bentley, 1989c), intracellular injection of tubulin (Sabry et al., 1991) and UV illumination (O'Connor et al., 1990). Because most experimental manipulations do not alter the formation of the Ti1 pathway, those that do reproducibly perturb pioneer growth cone guidance without adversely affecting cell viability or growth can provide information about specific cues that are involved in normal pathway formation. In the experiments described in this report, we exposed embryos to phosphatidylinositol-specific phospholipase C (PI-PLC) to assay guidance of pioneer growth cones following removal of GPI-anchored cell surface proteins.

Materials and methods

Embryos

Embryos were obtained from a colony of *Schistocerca americana* maintained at the University of California at Berkeley and were dissected and staged according to Bentley et al. (1979). Culture experiments were performed on embryos staged between 30%-35% of development, during which time the Ti1 pioneer neurons are born and establish their axonal trajectory into the central nervous system. Results from examination of pioneer neurons in 271 limb buds from 60 PI-PLC-treated embryos and 247 limb buds from 53 carrier-treated control embryos in 16 culture experiments are

Table 1. *Frequency of growth cone and cell behaviors after PI-PLC treatment*

	Distal growth[a]	Boundary crossing[b]	Cell body towing[c]	Axon defasc.[d]	Cx1 cell migration[e]
Control	0/216	0/148	0/216	30/216[f]	0/50
	(0%)	(0%)	(0%)	(13.9%)	(0%)
PI-PLC	42/231	27/151	36/231	47/231[f]	19/92
	(18.2%)	(17.9%)	(15.6%)	(20.3%)	(20.7%)

[a]Limbs were scored positive if one or both Ti1 growth cones turned more than 90° from proximal migration along the longitudinal limb axis (except at Tr-Cx boundary).

[b]Limbs were scored positive if one or both Ti1 growth cones which reached the Tr-Cx boundary crossed into the coxa without turning ventrally.

[c]Limbs were scored positive if one or both Ti1 cell bodies were displaced across the Ti-Fe segment boundary into the femur.

[d]Limbs were scored positive if the two Ti1 axons were separated for more than 50 μm.

[e]Individual Cx1 cells in prothoracic limbs were scored positive if they left the circumferential path and migrated toward the CNS.

[f]*t*-test; 10 paired experimental replications; significant at 0.05 level.

presented here and are summarized in Table 1. In addition, limbs from 59 embryos treated with control phospholipase enzymes were examined to determine the ability of these enzymes to affect growth cone guidance, cell migration and release of GPI-anchored proteins in vivo.

Enzyme treatment and embryo culture

Eggs were sterilized in 0.02% benzethonium chloride in 70% ethanol and dissected in sterile grasshopper saline (Bentley et al., 1979) containing 0.1% bovine serum albumin (BSA). The dorsal closures of all embryos were opened to ensure access of reagents to the limb interior. In pilot experiments, a range of concentrations of PI-PLC enzyme (from 0.5 to 5.0 Units ml^{-1}) was tested to determine a minimum dose level that produced a maximal response. In the experiments presented, embryos were treated for 1.5 hours at 30°C with 1.5 Units ml^{-1} PI-PLC from *Bacillus thuringiensis* (ICN, or generously provided by Dr. Martin Low, Columbia University; the latter preparation is highly purified recombinant enzyme, which is essentially free from contaminating activity of other phospholipases), or an equivalent volume of carrier (50% glycerol in 10 mM Tris, pH 7.0), diluted in RPMI 1640 medium supplemented with 130 mM sucrose, 10 mM xylose, 0.2% sodium bicarbonate, 1 mM oxaloacetic acid, 0.45 mM sodium pyruvate, 2 mM L-glutamine, 25 mM D(+) glucose, 50 μg ml^{-1} gentamycin, 4 μM β-ecdysterone, 0.2 Units ml^{-1} insulin, 1 mM N,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate, 0.1 μM retinoic acid, 0.4 mM CaCl$_2$ and 0.4 mM MgSO$_4$, pH 6.9. Culture plates were gently agitated for several seconds every 20-30 minutes during the enzyme incubation. Embryos were then washed for 30 minutes and cultured in supplemented RPMI medium at 30°C in a 5% CO$_2$ incubator for 10-24 hours. Control enzyme treatments were performed using phosphatidylcholine-specific phospholipase C (PC-PLC, Boehringer-Mannheim), phospholipase B (PLB) and Type VII phospholipase D (PLD). For each experiment, a single clutch of eggs was used, and 3-6 embryos were immediately fixed following dissection to determine the locations of the pioneer neuron growth cones at the start of the incubation and culture period. Except where noted, all reagents were purchased from Sigma Chemical Co.

Immunocytochemistry

For indirect immunofluorescence studies, neurons were labeled with serum antibodies against horseradish peroxidase (anti-HRP), which selectively bind to insect neurons (Jan and Jan, 1982; Snow et al., 1987), according to the protocol of Caudy and Bentley (1986a). Briefly, embryos were fixed in 3.7% formaldehyde (Fisher) in grasshopper saline for 2-12

hours, then rinsed in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.5% Triton X-100 and 0.02% sodium azide (PBT) for 1-2 hours. Embryos were incubated with rabbit anti-HRP antisera (Cappel/Organon-Teknika) diluted 1:1000 in PBT for 10-16 hours at 4°C, then washed in PBT containing 1% BSA for 4-6 hours. Rhodamine-conjugated goat anti-rabbit IgG (Jackson) was applied at a dilution of 1:200 in PBT + 1% BSA overnight at 4°C. Embryos were then rinsed in several changes of PBT + 1% BSA and mounted under coverglass with 40 or 60 μm wire spacers in 90% glycerol in PBS, with 0.15% Hanker-Yates reagent added as an antioxidant. Slides were viewed and photographed with Zeiss or Nikon epifluorescence microscopes.

Monoclonal antibodies (mAbs) 3B11 and 8C6 (anti-fasciclin I and anti-fasciclin II, respectively; Bastiani et al., 1987) were kindly provided by N. Patel and C. S. Goodman. The immunocytochemical methods used for these antibodies were essentially the same as those described by Bastiani et al. (1987). Embryos were fixed for 30 minutes in 3.7% formaldehyde in 0.1 M Pipes, 2 mM EGTA, 1 mM MgSO$_4$, pH 6.95, then washed in three changes of PBS, followed by three changes of PBS containing 0.1% saponin and 0.2% BSA (PBSS). Embryos were blocked in 5% normal goat serum (NGS) in PBSS for 30 minutes, then incubated with a 1:1 mix of hybridoma supernatant:PBSS +5% NGS overnight at 4°C. Embryos were then washed in PBSS for 2-4 hours, blocked again for 30 minutes in PBSS + 5% NGS, then incubated in HRP-conjugated goat anti-mouse IgG (Jackson) at 1:500 in PBSS + 5% NGS overnight at 4°C. Following several washes in PBSS, the HRP reaction product was visualized using 0.3 mg ml^{-1} diaminobenzidine in PBS, with 0.015% H$_2$O$_2$ and 0.05% NiCl$_2$. Embryos were washed in PBS and then cleared in stepwise concentrations of glycerol in PBS (50, 70, 90%) before being dissected and mounted in 90% glycerol. Slides were viewed and photographed on a Nikon microscope equipped with Nomarski optics.

Results

Disoriented Ti1 growth cone migration in the distal limb

At the 30% stage of embryogenesis, the Ti1 pioneer neurons arise from a precursor cell at the tip of the limb epithelium. The region of epithelium where they are born will become the proximal portion of the tibia. The pioneers emerge from the epithelium so that they lie between the basal lamina and the basal surface of the

A

B

C

D

E

F
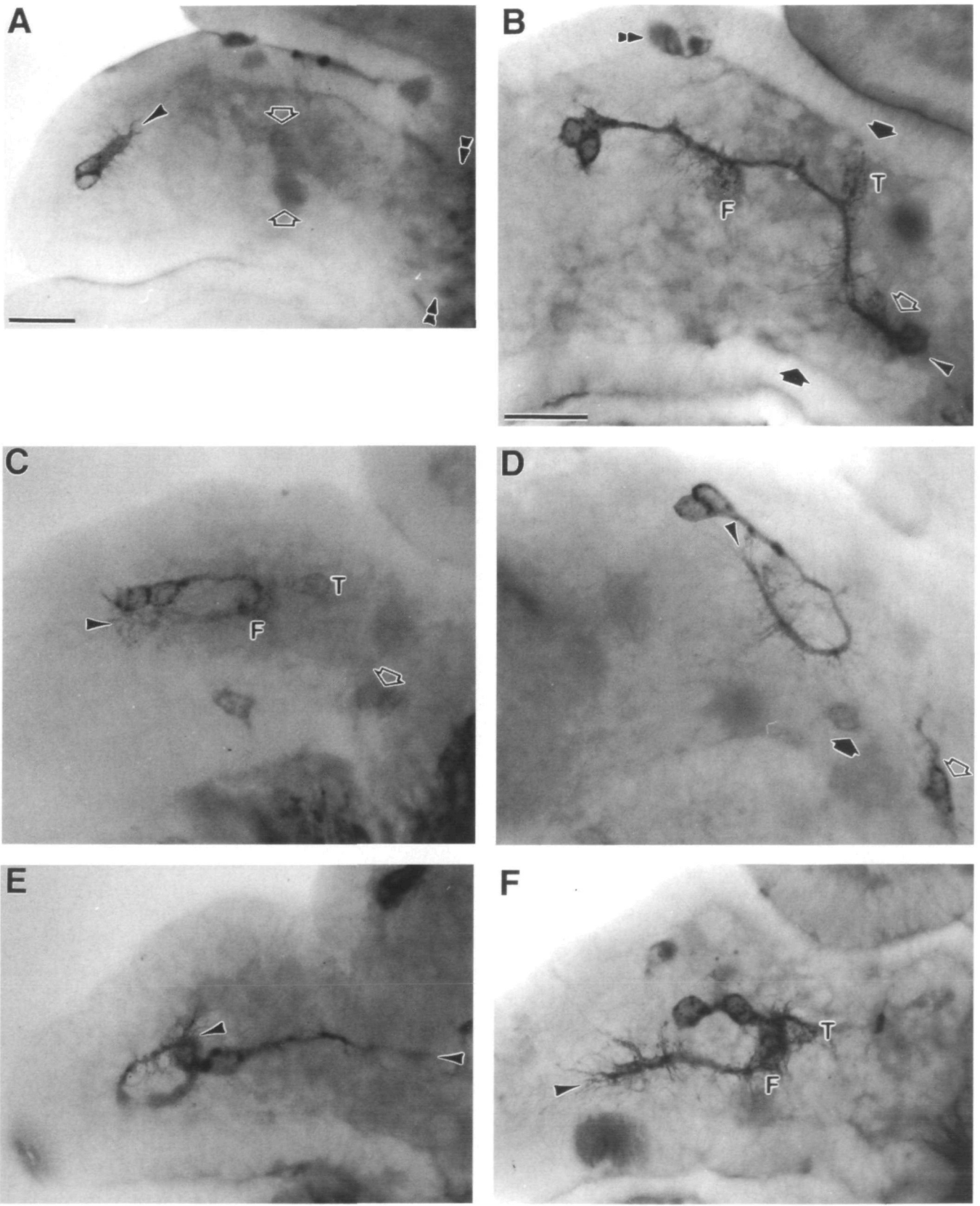
epithelium, and initiate axonogenesis. The growth cones emerge from the proximal poles of the cells and migrate proximally along the longitudinal limb axis

toward the CNS (Figs 1, 2A, 3A). Their migration path through the tibia, the femur, and the trochanter is relatively straight, with small angle deflections resulting

Fig. 2. Disoriented Ti1 growth cone migration in the distal limb following PI-PLC treatment. (A) Control limb showing normal Ti1 outgrowth at the onset of treatment (31.5% stage). The growth cones (arrowhead) migrated proximally into the femur along the longitudinal limb axis. Cx1 guidepost cells (unfilled arrows) are visible out of the focal plane approximately midway between the Ti1 cell bodies and the border of the CNS (double arrowheads). (B) Control growth of Ti1 neurons in an embryo placed in culture at the 31-32% stage and incubated for 24 hours. The growth cones (arrowhead) have migrated along the normal pathway, contacting guidepost cells Fe1 (F) and Tr1 (T) before turning ventrally along the Tr-Cx segment boundary (black arrows), and then proximally to the pair of Cx1 cells (unfilled arrow). Double arrowhead: differentiating cells of the femoral chordotonal organ. (C-F) Ti1 outgrowth in embryos treated with PI-PLC at 31-32% of development and cultured for 20-24 hours. (C) The Ti1 growth cones (arrowhead) turned distally after contacting guidepost cell Fe1 (F) and lie adjacent to the Ti1 cell bodies. Unfilled arrow: Cx1 cells; T: Tr1 cell. (D) The Ti1 growth cones reoriented near the presumptive Tr-Cx boundary, which is marked by the location of cell Tr2 (black arrow), and grew distally toward the Ti1 cell bodies. The Cx1 cells (unfilled arrow) probably have migrated toward the CNS (the trailing process appears to indicate the location of the cell bodies at the time of enzyme treatment; see text and Fig. 6). (E) The growth cone (left arrowhead) of the distal pioneer grew ventrally and distally around the tip of the limb, then migrated proximally in the posterior limb compartment (plane of focus). The growth cone (right arrowhead) of its sibling grew in the normal proximal direction in the anterior limb compartment (out of focal plane). The axons failed to establish their normal fasciculation. (F) Both Ti1 growth cones (arrowhead) turned distally in the mid-femur and migrated into the limb tip. In addition, the Ti1 cell bodies were towed or displaced into the femur near cell Fe1 (F), and cell Tr1 (T) has also been displaced (compare to Fig. 2B). Neurons labeled with anti-HRP antibodies in whole-mounted embryos. Dorsal, up; proximal, to right. Bar: 50 μm (A, D, on A; B, C, E, F, on B).

from contact with guidepost cells Fe1 and Tr1 (Figs 2B, 3B).

When embryos at 31%-32% of development, a stage at which the Ti1 growth cones are growing in a proximal orientation through the femur (Fig. 2A), were exposed to PI-PLC and then maintained in culture for 20-24 hours, disoriented pioneer growth cone migration was often observed. Ti1 growth cones in PI-PLC-treated limbs could migrate abnormally in any direction, including straight distally (Fig. 2C-F). Turns of 180 degrees could occur within short distances, often with a turning radius of less than 20 μm, along the longitudinal limb axis (Fig. 2C, D). In contrast, Ti1 growth cones in control-treated limbs followed their stereotyped pathway of migration in culture, maintaining their proximal growth orientation through the femur and trochanter (Fig. 2B). When growth cones that turned more than 90 degrees from proximal growth were scored, one or both pioneer neurons in 18.2% of enzyme-treated limb buds showed this behavior, as opposed to 0% in limbs of both non-treated, cultured control embryos (Table 1),

and embryos exposed to control phospholipase enzymes. In nine separate experiments in which this phenotype was scored, the mean score per experiment was $22.6 \pm S.E. 5.0\%$ (n=minimum of 12 limbs scored per experiment).

Subdivision of the grasshopper limb into anterior and posterior compartments on the basis of posterior compartment-specific expression of the *engrailed* homeodomain-containing protein has previously been described (Patel et al., 1989; Bentley and Toroian-Raymond, 1989). The Ti1 growth cones normally migrate in the anterior limb compartment. Distally migrating growth cones in PI-PLC-treated embryos could grow around the interior of the limb tip, enter the posterior limb compartment, and extend proximally within that compartment (Fig. 2E).

It is not known whether growth cones can become disoriented at any location along the longitudinal limb axis, or whether locations where disorientation can occur are spatially restricted. Distal to the Tr-Cx segment boundary, it appeared that growth cones could become disoriented at any axial position. Inappropriate distal turns by growth cones were observed immediately upon leaving the cell body (Fig. 2E), when approaching the Tr-Cx boundary (Fig. 2D), and at locations in between these extremes (Fig. 2C, F). Distal turns could occur when growth cones were migrating on epithelial cells (Fig. 2D, E), or when they were growing over guidepost cells (Fig. 2C, F). However, no Ti1 growth cones that had crossed the Tr-Cx boundary, either at the normal ventral location or at an abnormal dorsal location (see below), were observed to turn distally. Therefore, disorientation from proximal migration by Ti1 growth cones following PI-PLC treatment could occur anywhere within the region distal to the Tr-Cx boundary.

Pre-axonogenesis (guidepost) neurons Fe1, Tr1 and the Cx1 pair are located along the Ti1 pathway and constitute high-adhesivity substrata for growth cone migration. The maintenance of growth cone contacts with the Cx1 cells when placed under mechanical loads indicates that this affinity is at least partially based upon adhesivity (Condic and Bentley, 1989b). In PI-PLC-treated limbs, Ti1 growth cones often contacted guidepost cells Fe1 and Tr1 and grew across their surfaces (Figs 2C, F, 3C). In some cases, the trajectory of the nascent Ti1 axons clearly followed the curvature of the guidepost cell (Fig. 3C: cell Tr1). In other cases, guidepost cells appeared to be aligned along the Ti1 axons (Fig. 2F: Fe1, Tr1; Fig. 3C: Fe1). Therefore, it appears that considerable Ti1 growth cone:guidepost cell affinity persisted following PI-PLC treatment.

The Ti1 cell bodies and the guidepost cells lie at specific locations in the limb (Figs 1, 2B, 3B). Following PI-PLC treatment, Ti1 cell bodies were sometimes displaced into the femur (Table 1). In some cases, the Ti1, Fe1, and Tr1 cell bodies were found in a single cluster (Fig. 2F). Therefore, the Ti1 cell bodies may be towed behind the migrating growth cones, or might secondarily be drawn into a new limb location where the growth cones have migrated, following removal of
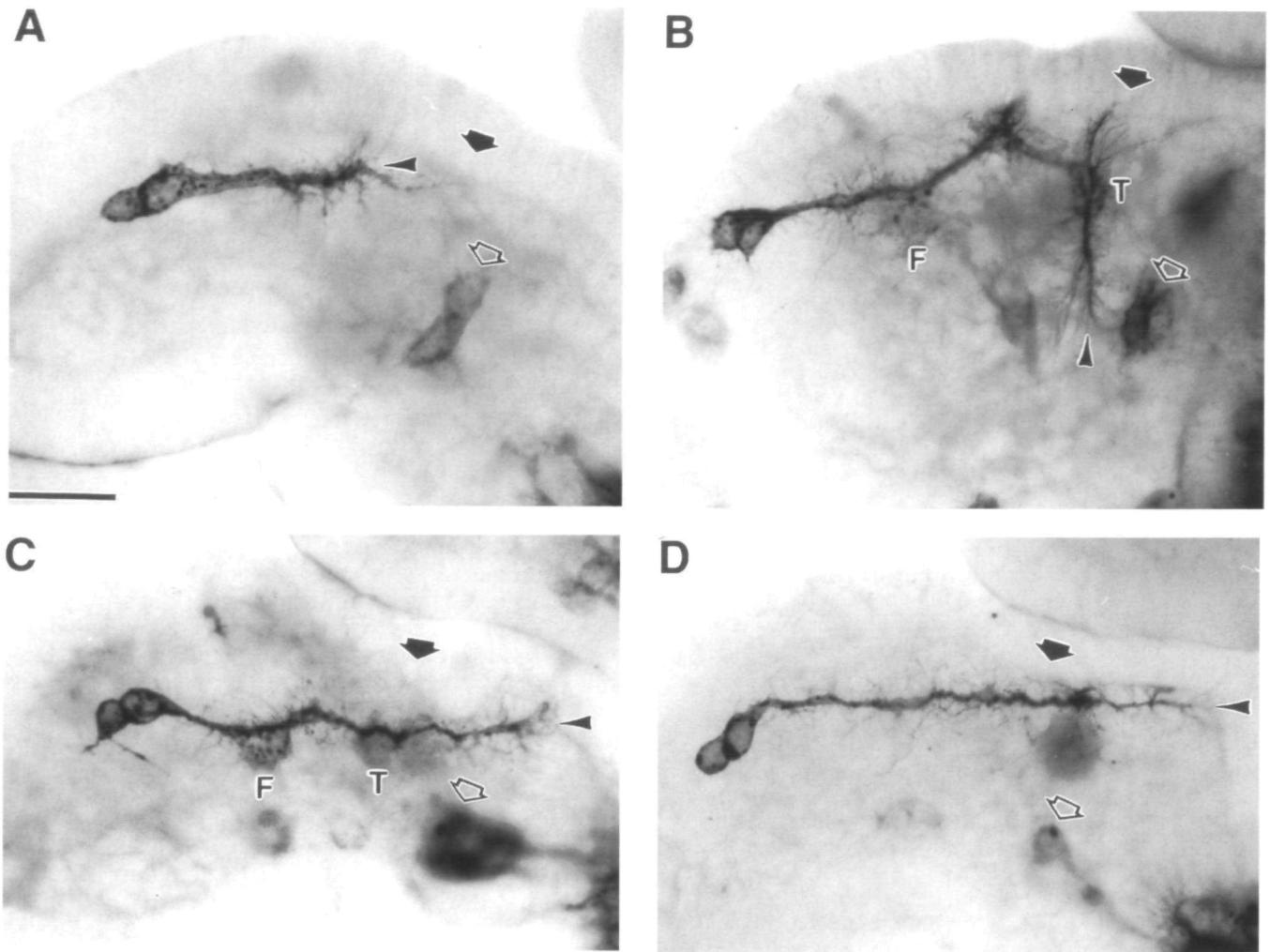
Fig. 3. Failure of pioneer growth cones to reorient ventrally at the trochanter-coxa segment boundary following PI-PLC treatment. (A) Control showing normal Ti1 outgrowth at the onset of treatment (32.5% stage). The growth cones (arrowhead) migrated proximally along the longitudinal limb axis and approached the Tr-Cx segment boundary (black arrow). Unfilled arrow: Cx1 cells. (B) Control growth of Ti1 neurons in an embryo placed in culture at the 32% stage and incubated for 20 hours. The growth cones (arrowhead) contacted cells Fe1 (F) and Tr1 (T), and made a normal sharp ventral turn along the Tr-Cx boundary (black arrow). Unfilled arrow: Cx1 cells. (C-D) Ti1 outgrowth in embryos treated with PI-PLC at 32-33% of development and cultured for 20-24 hours. (C) These Ti1 growth cones (arrowhead) grew toward, and then along the surface of, cell Fe1 (F) and then to cell Tr1 (T). However, at the location of Tr1, they failed to turn ventrally along the Tr-Cx segment boundary (black arrow) and instead continued to migrate proximally though the coxa. Unfilled arrow: Cx1 cells. (D) These Ti1 growth cones (arrowhead) also have grown straight into the coxa with no sign of reorientation or branching at the Tr-Cx segment boundary (black arrow). Neurons labeled with anti-HRP antibodies. Unfilled arrow: Cx1 cells. Dorsal, up; proximal, to right. Bar: 50 μm.

GPI-anchored surface proteins by PI-PLC. These observations suggest that adhesion between the substratum and the afferent neuron cell bodies is reduced following PI-PLC treatment.

Fasciculation between the two Ti1 axons is usually maintained during axonogenesis, although 10-20% of control axons do grow separately for distances of 50-100 μm (Table 1; Jay and Keshishian, 1990). In PI-PLC-treated embryos, the number of limbs with defasciculated axons increased by about 50% (Table 1; Fig. 2E). Therefore, the loss of GPI-anchored proteins might reduce the ability of Ti1 axons to establish or maintain fasciculation.

Failure of growth cones to respond to the trochanter-coxa limb segment boundary

During normal embryogenesis, the Ti1 growth cones turn ventrally along the Tr-Cx segment boundary (Figs 1, 3B). The location of the turning response is at the interface between two circumferential bands of epithelial cells, a distal band to which the growth cones strongly adhere (Condic and Bentley, 1989b), and a low-affinity (or non-permissive) proximal band (Caudy and Bentley, 1986a, 1987). Monoclonal antibody studies in the grasshopper have revealed the expression of antigens in very spatially restricted patterns along the high-affinity distal band (A. Kolodkin, D. Matthes and

C. S. Goodman, personal communication) and low-affinity proximal band (Kotrla and Goodman, 1982; Taghert et al., 1982; Singer et al., 1991; Bastiani et al., in press) of epithelial segment boundary cells in the developing limb. Studies in the cockroach embryo have also demonstrated that bands of cells at limb segment boundaries are antigenically distinct, and thus differentiated, from neighboring cells (Norbeck and Denburg, 1990). Upon encountering the interface of cells at the trochanter-coxa boundary, the growth cones cease proximal migration, extend branches both dorsally and ventrally along the distal (high-affinity) epithelial band, and then invariably turn ventrally (O'Connor et al., 1990).

PI-PLC treatment of embryos at the 32%-33% stage, when Ti1 growth cones are approaching the Tr-Cx boundary (Fig. 3A), resulted in an abnormal response to the boundary. Approximately 18% of growth cones in limbs of treated embryos neither ceased proximal growth nor turned ventrally, and instead continued migrating straight into the dorsal region of the coxa (Fig. 3C, D; Table 1). In nine separate experiments in which this phenotype was scored, the mean score per experiment was 17.2 ± S. E. 6.1% (n=minimum of 11 scored limbs per experiment). The failure to turn along the Tr-Cx boundary was not observed in any of the limbs from carrier-treated embryos (Table 1) or control phospholipase-treated animals. There are several notable features of this behavior. First, the growth cones migrated toward the Tr1 guidepost cell, contacted its surface and maintained their normal extensive apposition to this cell (Fig. 3C). Secondly, branches did not extend dorsally and ventrally along the high-adhesivity band of epithelial cells on the distal side of the boundary (Fig. 3C, D). Finally, in the twenty-seven examples of Tr-Cx boundary crossing that we observed, all growth cones remained proximally oriented within the coxa (Fig. 3C, D). Disoriented or distal growth cone turning was never observed in this region of the limb after PI-PLC treatment.

Embryos at the 33%-35% stages, when growth cones have already turned ventrally along the boundary or proximally toward the Cx1 cells (Figs 1, 2B), were also treated with PI-PLC. Exposure to enzyme at these later stages of development did not cause the nascent axons to become detached from the substratum or to alter their course of growth in culture.

Aberrant migration of Cx1 neurons

The failure of the Ti1 growth cones to respond to the Tr-Cx boundary following PI-PLC treatment suggested that GPI-anchored receptors or ligands might be involved in detection or delineation of circumferential guidance cues in the limb. Therefore, we examined a different set of afferent neurons, the Cx1 cells, which normally respond to a different circumferential guidance cue during their pre-axonogenesis migration (Bentley and Toroian-Raymond, 1989). In the prothoracic limb, the two Cx1 cells arise separately from the epithelium, about 150 degrees apart around the limb circumference. They migrate circumferentially, at a

specific position along the longitudinal limb axis, to meet one another near the ventral boundary of the anterior and posterior limb compartments (Fig. 1). In Fig. 4A, the leading edges of the migrating cells have met one another to give the cell pair a dumbell-shaped appearance. After the two cell bodies have made contact, they stop migration, initiate axonogenesis and extend their axons together proximally to the CNS (Fig. 4B).

PI-PLC treatment of 30%-31% stage embryos, when the two Cx1 cells are in the process of migrating on their circumferential route, often resulted in abnormal behavior. Both Cx1 cells could reorient so that their longitudinal axis was aligned with the limb axis rather than the circumferential route, and could then leave the circumferential route and migrate toward the CNS (Fig. 4C). Their former location on the circumferential route was often marked by a patch of anti-HRP antibody labeling material (Fig. 4C). In some limbs, Cx1 cells were evident in the margin of the CNS, and in others appeared to have migrated completely into the CNS. About 21% of Cx1 cells in prothoracic limb buds (the only limb bud where both Cx1 cells label with anti-HRP antibodies at this stage of development) of PI-PLC-treated embryos displayed this atypical behavior, as opposed to 0% in untreated limb buds (Table 1).

PI-PLC removal of fasciclin I in vivo, and additional controls

Fasciclin I is an extracellular GPI-anchored, adhesive glycoprotein expressed on a subset of central and peripheral neurons in grasshopper and *Drosophila* embryos (Bastiani et al., 1987; Zinn et al., 1988; Hortsch and Goodman, 1990). In the grasshopper limb, fasciclin I is expressed on the Ti1 pioneer cell bodies and axons, and on afferent neuron precursors of the femoral chordotonal organ (Fig. 5A; Keshishian and Bentley, 1983; Kutsch, 1989). To determine whether PI-PLC treatment cleaved GPI-anchored proteins from cell surfaces in vivo, we labeled embryos with mAb 3B11, which recognizes grasshopper fasciclin I (Bastiani et al., 1987), following PI-PLC and control incubations.

Following PI-PLC treatment, mAb 3B11 binding was absent or reduced to very low levels in the limb (Fig. 5B). Neither the Ti1 neurons nor the femoral chordotonal organ cells appeared to be labeled. The absence of fasciclin I immunoreactivity suggests that the exposure to PI-PLC effectively released this GPI-anchored protein from cell surfaces in the limb. Furthermore, fasciclin I was not significantly re-expressed on peripheral neurons during the time course of the culture experiments (Fig. 5B). Embryos that were incubated with PI-PLC enzyme and cultured for 18-24 hours showed barely detectable expression of fasciclin I in the limbs, most of which was localized to the presumptive femoral chordotonal organ. In contrast, embryos that were treated with equivalent doses of carrier alone or other phospholipases which do not cleave the GPI anchor, displayed a normal distribution of fasciclin I immunoreactivity. Thus, for at least one characterized GPI-anchored cell surface molecule expressed on the
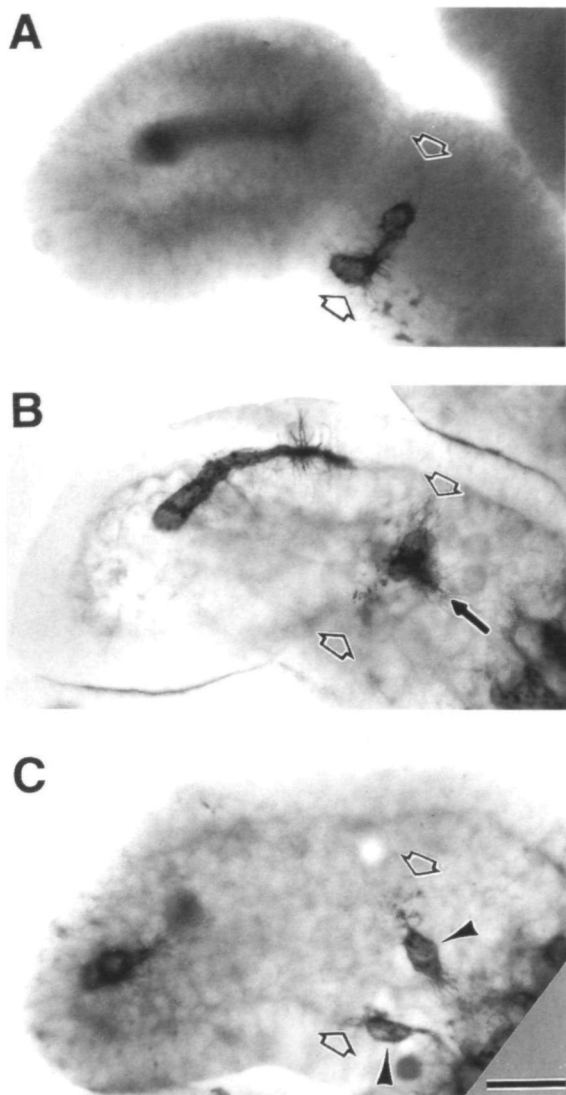
Fig. 4. Abnormal proximal migration of the Cx1 guidepost neurons in prothoracic limb buds following PI-PLC treatment. (A) Control limb showing the normal path of migration of the Cx1 cells. Cell Cx1$_a$ (upper unfilled arrow) arises in the anterior limb compartment, and cell Cx1$_p$ (lower unfilled arrow) arises in the posterior limb compartment. Each migrates ventrally along a circumferential route just proximal to the Tr-Cx segment boundary (see Fig. 6A). In this limb, the leading edges of the migrating cells have just made contact. (B) In a control limb at a slightly later stage, the two Cx1 cells have met one another near the anterior-posterior compartment boundary. The cell bodies remain at this axial position (unfilled arrows), and extend axons (black arrow) toward the CNS. (C) In an embryo treated with PI-PLC at the 30% stage of development and cultured for 22 hours, the circumferential migration of Cx1 cells was disrupted. Both Cx1 cells (arrowheads) left the normal circumferential path (unfilled arrows) and migrated proximally towards the CNS. Trailing processes, and characteristic amorphous patches of anti-HRP labeling material (unfilled arrows) mark the path of migration. Neurons labeled with anti-HRP antibodies. Dorsal, up; proximal, to right. Bar: 50 μm.
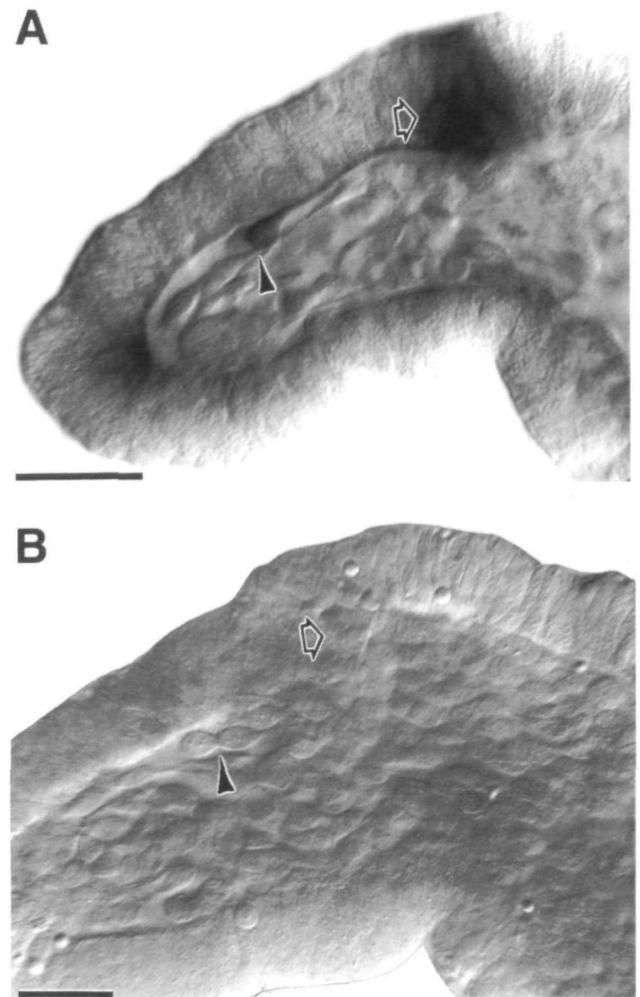
Fig. 5. Removal of fasciclin I, a GPI-anchored cell-surface protein, from Ti1 neurons by in vivo treatment with PI-PLC. Embryos were stained with mAb 3B11 (anti-fasciclin I) and viewed as whole-mounts with Nomarski optics. (A) A 32% stage limb from a control-treated embryo showing the normal distribution of fasciclin I immunoreactivity on the Ti1 pioneer neuron cell bodies (arrowhead) and nascent axons, and on cells of the presumptive femoral chordotonal organ (arrow; see also Fig. 2B). (B) A limb from an embryo that was treated with PI-PLC at the 34% stage, then maintained in culture for 19 hours. No fasciclin I immunoreactivity is detectable on either the Ti1 cell bodies (arrowhead) or the femoral chordotonal organ (arrow). This indicates that the protein was released from cell surfaces by PI-PLC treatment in vivo, and that it was not re-expressed substantially during the culture period. Dorsal, up; proximal, to right. Bars: 50 μm.

pioneer neurons, the majority of the protein was removed by PI-PLC and did not appear to be significantly re-expressed during the course of embryo culture experiments.

To test the specificity of the effects of PI-PLC, embryos were treated with equivalent doses (Units ml^{-1}) of phosphatidylcholine-specific phospholipase C (PC-PLC), phospholipase B (PLB), and Type VII

phospholipase D (PLD), enzymes that have varying degrees of specificity for membrane phospholipids but do not release GPI-anchored proteins. Exposure of embryos (n=minimum of 30 Ti1 and Cx1 pairs examined per enzyme) to these enzymes did not produce any of the growth cone guidance or cell migration defects described for PI-PLC, nor did any of these enzymes reduce or eliminate fasciclin I immuno-reactivity. These observations strongly suggest that the abnormal phenotypes observed were specific to re-moval of GPI-anchored proteins by PI-PLC and were unlikely to be caused by contaminating phospholipase activity in the enzyme preparations or non-specific damage to membrane integrity. This assertion is also supported by the observation that membranes of neurons and epithelial cells in PI-PLC-treated embryos appeared morphologically intact, with no signs of microvesiculation or blebbing. Furthermore, Ti1 pio-neer axons in PI-PLC-treated limbs often grew a comparable distance to axons in untreated control limbs (compare Fig. 3C and 3D with Fig. 3B), suggesting that exposure to PI-PLC did not impair the ability of neurons to assemble and extend axons.

We also tested the specificity of the enzyme treatment by determining whether incubation with PI-PLC removed a cell surface protein that has a transmem-brane domain. For these experiments, we used mAb 8C6, which recognizes grasshopper fasciclin II, a glycoprotein expressed on a subset of axons in the CNS (Bastiani et al., 1987; Harrelson and Goodman, 1988). Although it is possible, as in *Drosophila* embryos, that there are GPI-anchored as well as transmembrane forms of fasciclin II (Grenningloh et al., 1991), the only form of this molecule characterized to date in grass-hopper has a transmembrane domain. When embryos were incubated with PI-PLC, and then stained with mAb 8C6, the intensity and distribution of staining in both the limb and the CNS were not significantly different from control-treated embryos (data not shown). Therefore, the observation that a substantial amount of an identified transmembrane molecule survived the PI-PLC treatment in vivo supports the conclusion that the enzyme treatment conditions used in these experiments selectively removed GPI-anchored proteins from cell surfaces without inducing non-specific damage to membranes or other membrane proteins.

The several aberrant phenotypes were not clustered in a small percentage of limbs or embryos. Of all limbs treated with PI-PLC, 46.8% contained neurons which displayed at least one of these phenotypes (11.3% displayed two phenotypes; 4.8% displayed three; 0.4% displayed four). Defasciculation of the Ti1 axons was the behavior most frequently correlated with other phenotypes; 61.7% of limbs with defasciculated pioneer axons also exhibited other phenotypes.

Discussion

We treated grasshopper embryos with bacterial PI-PLC

at different stages of embryogenesis to assess the role of GPI-anchored cell surface proteins in axon guidance and cell migration in the developing peripheral nervous system. In whole embryo culture following removal of GPI-anchored proteins, a significant number of axons of pioneer neurons in the limb bud became disoriented and deviated from an extremely stereotyped pattern of growth (Fig. 6B, C). The aberrations in axonal growth and cell migration occurred with moderate frequency (about 20%; Table 1), but were reproducible from experiment to experiment and were completely absent in control embryos. A variety of factors may account for the proportion of neurons exhibiting effects of PI-PLC treatment. These include uncertainty regarding the exact location of growth cones at the time of enzyme treatment, variation in the degree to which all cell surfaces were exposed to the enzyme, incomplete removal of all GPI-anchored proteins by the enzyme (Low et al., 1988), and redundancy in adhesion and guidance systems (Bixby et al., 1987). The axonal phenotypes that we did observe include loss of proximal orientation, as evidenced by inappropriate turning and growth in the distal direction, and failure of growth cones to reorient along a circumferential guidance cue located at a developing segment boundary. The circum-ferential migration of pre-axonogenesis afferent neur-ons was also reproducibly disrupted by incubating embryos with PI-PLC (Fig. 6A). The enzyme treatment was sufficient to release fasciclin I, a GPI-anchored neuronal glycoprotein (Zinn et al., 1988; Hortsch and Goodman, 1990), from cell membranes within the limb bud, and did not have obvious detrimental effects on cell viability or growth. The removal of fasciclin I suggests that limited access of the enzyme to the limb interior was not a major factor in the percentage of aberrant phenotypes. Identical results were obtained using either a commercially available or a highly purified recombinant preparation of PI-PLC enzyme. Parallel treatments with other phospholipases that do not cleave the GPI anchor neither removed fasciclin I immunoreactivity nor affected growth cone and cell migrations. From these observations, we propose that normal guidance of pioneer neuron growth cones, and migration of a pair of pre-axonogenesis neurons, involves one or more GPI-anchored proteins present on neuronal and/or epithelial cell surfaces.

GPI-anchored proteins and cell adhesion

Our results suggest that GPI-anchored proteins are involved in at least two aspects of cell adhesion during generation of the Ti1 pathway. First, the cell bodies of Ti1 pioneer neurons and other guidepost neurons normally adhere to the basal surfaces of epithelial cells, and may also adhere to the basal lamina (Anderson and Tucker, 1988; Condic and Bentley, 1989b). The fre-quent displacement of Ti1 and guidepost cell bodies following PI-PLC treatment indicates that GPI-anchored proteins are probably involved in this ad-hesion. Secondly, the two Ti1 axons usually fasciculate during outgrowth. Following PI-PLC treatment there was about a 50% increase in the frequency of
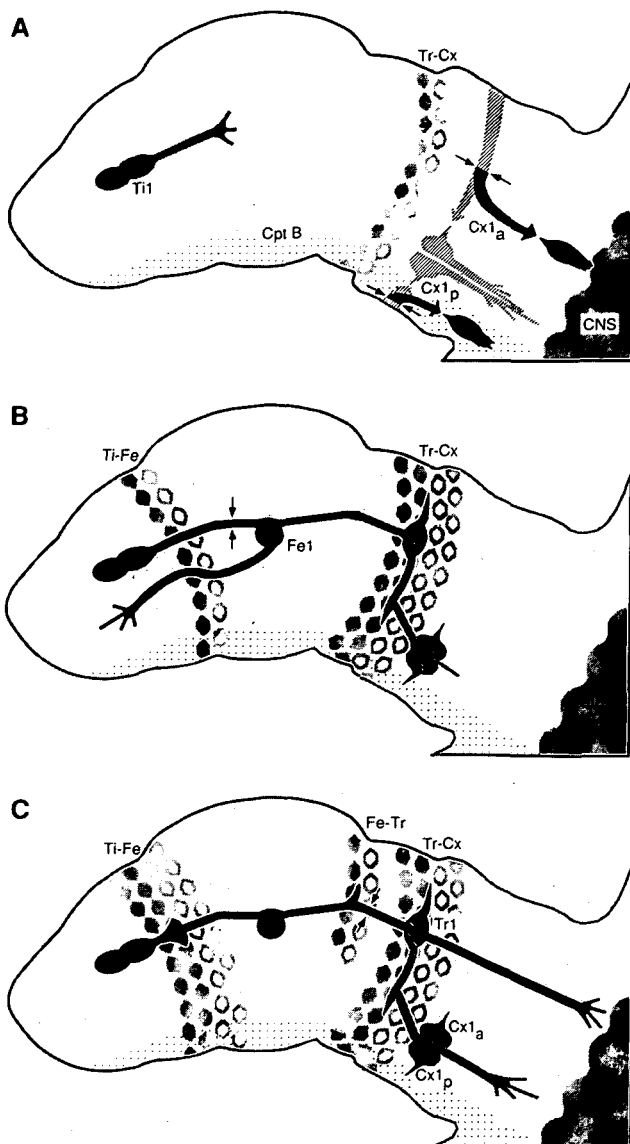
A

B

C

defasciculation. This suggests, but does not provide unequivocal evidence for, involvement of one or more GPI-anchored proteins in fasciculation.

A GPI-anchored protein which is expressed by Ti1 neurons and was released by the in vivo PI-PLC treatment is fasciclin I (Bastiani et al., 1987; Zinn et al., 1988; Hortsch and Goodman, 1990). *Drosophila* fasciclin I has been shown to function as a homophilic adhesion molecule that can mediate selective sorting of S2 (Schneider) cells transfected with fasciclin I cDNA (Elkins et al., 1990b). Using chromophore-assisted laser inactivation (CALI), Jay and Keshishian (1990) reported that fasciculation of the two Ti1 pioneer axons in vivo could be disrupted by inactivation of fasciclin I. Pathfinding events by Ti1 growth cones, such as maintenance of proximal growth orientation in the femur and ventral turning along the Tr-Cx segment boundary, were not affected by CALI of fasciclin I. Since the epithelial substratum under the Ti1 cell bodies expresses little, if any, fasciclin I, homophilic adhesion involving this molecule is unlikely to contribute to cell

Fig. 6. Schematic diagrams of alterations in Ti1 pioneer growth cone migration and Cx1 cell migration following release of GPI-anchored proteins in vivo. (A) Cx1 migration (31.5% stage limb): the Cx1 cells arise from the epithelium of the anterior (Cx1$_a$) and posterior (Cx1$_p$) compartments of the limb. They migrate ventrally around the limb along a circumferential route (cross-hatched arrows) just proximal to the Tr-Cx boundary. After meeting near the boundary (CptB) between the posterior (stippled) and anterior limb compartments, they extend axons (cross-hatched) toward the central nervous system (CNS). Following PI-PLC treatment, the Cx1 cells leave the circumferential path and migrate (stippled arrows) toward the CNS. (B) Ti1 growth cone disorientation (34% stage limb): if GPI-anchored proteins are removed when the Ti1 growth cones are migrating within the femur (black arrows), the growth cones may lose their proximal orientation and migrate in an abnormal, distal direction. Cross-hatching indicates the normal pathway. (C) Failure of Ti1 growth cones to turn (35% stage limb): if GPI-anchored proteins are removed as the Ti1 growth cones are approaching (black arrows) the Tr-Cx segment boundary, the growth cones may cross the boundary into the coxa without making their normal ventral turn (shown by cross-hatching). However, the growth cones appear to continue to respond to other guidance cues since they maintain proximally oriented growth along the longitudinal limb axis toward the CNS. Segment boundaries: tibia-femur (Ti-Fe), femur-trochanter (Fe-Tr), trochanter-coxa (Tr-Cx). Guidepost cells: Fe1, Tr1, Cx1$_a$, Cx1$_p$. Filled hexagons: distal, high-affinity epithelial segment boundary cells. Unfilled hexagons: proximal, low-affinity epithelial segment boundary cells. Dorsal, up; proximal, to right.

body: substratum adhesion. Fasciclin I, therefore, may contribute to axon fasciculation during outgrowth by homophilic adhesion, but is not yet implicated in guidance or in neuron:substratum adhesion.

Several other GPI-anchored proteins expressed on neuronal surfaces have been implicated in adhesive interactions. *Drosophila* chaoptin, like fasciclin I, mediates aggregation of transfected S2 cells through homotypic adhesion (Krantz and Zipursky, 1989). Chinese hamster ovary (CHO) cells transfected with cDNA for F3, a mouse neuronal glycoprotein, show increased aggregation over non-expressing controls, and surfaces of these cells promote outgrowth of sensory neurites (Gennarini et al., 1991). Contactin/F11, a chicken homolog of F3, also appears to mediate neural adhesion and neurite extension (Gennarini et al., 1991). TAG-I, a GPI-linked glycoprotein expressed in the developing rat CNS, is released from cell surfaces in vitro and is a strong promoter of neurite outgrowth (Furley et al., 1990). NCAM-120, the GPI-anchored form of NCAM, promotes outgrowth of rat CNS neurons (Doherty et al., 1990), and also has been shown to be required for adhesion and fusion of cultured chick myoblasts (Knudsen et al., 1990).

A variety of CNS proteoglycans in vertebrates also are GPI anchored (Herndon and Lander, 1990). GPI-anchored Schwann cell proteoglycans appear to bind extracellular matrix ligands, particularly laminin (Carey and Stahl, 1990). Since the grasshopper Ti1 cell bodies

are readily displaced following enzymatic removal of the basal lamina (Condic and Bentley, 1989b), as well as by removal of GPI-anchored proteins, similar interactions may occur in the grasshopper system.

Proximally oriented growth cone guidance along the longitudinal limb axis

Pioneer growth cone guidance during the initial phase of outgrowth, proximal migration along the longitudinal limb axis, remains problematical. In theory, outgrowth could be initiated in the appropriate direction and not corrected during migration, or could be oriented by intermittent guidance cues, such as guidepost cells and segment boundaries, or could be continuously guided by external information. Information adequate for normal guidance of Ti1 growth cones is available in limb buds isolated from the embryo, and after removal of mesodermal cells (Lefcort and Bentley, 1987) and basal lamina (Condic and Bentley, 1989c). Thus the limb epithelial cells, and neurons derived from them, appear to provide sufficient guidance for pioneer growth cone migration. Since growth cones emerge from the proximal poles of the nascent neurons at a position highly correlated with the internal cytoskeletal organization of the cell, intrinsic information may be important in the site and orientation of initial axonogenesis (Lefcort and Bentley, 1989). However, rapid disorientation of growth cones when filopodial exploration is suppressed by cytochalasin treatment (Bentley and Toroian-Raymond, 1986) supports the view that extrinsic information is required for maintaining proximal guidance. Although a theory of intermittent guidance by contact with segment boundaries and guidepost cells is plausible, proximal guidance through the femur is not disturbed in limbs where differentiation of the Fe1 cell is delayed (Caudy and Bentley, 1986a). Since filopodia do not extend the length of the femur (O'Connor et al., 1990), guidance information derived from the epithelium within the femur appears to be required. The density of growth cone branches and the degree of membrane apposition to the substratum suggest that the epithelium at the proximal end of the femur, the direction of migration, has higher affinity for the growth cone than the substratum at the distal end of the femur (Caudy and Bentley, 1986a). Transplantation of afferent neurons in *Drosophila* wing imaginal discs (Blair et al., 1987), and transplantation or reorientation of patches of epithelium (Nardi, 1983) also suggest that the epithelium provides guidance information for proximal migration of afferent growth cones. A gradient of substratum affinity would constitute one possible type of orienting information. Alternatives include polarized substratum-associated information not in the form of a gradient (Vinson et al., 1989), or a gradient of a diffusible chemoattractant.

The effect of PI-PLC treatment on proximal migration of Ti1 pioneers suggests that guidance in the distal limb depends upon distributed extrinsic information. The short-radius disoriented turns (Figs 2C-F,

6B) that growth cones can make following removal of GPI-anchored proteins suggest that they may not maintain their correct orientation for more than a few tens of microns in the absence of guidance information. That such turns can occur anywhere from the Ti1 cell bodies to the Tr-Cx segment boundary indicates that this information is required throughout the distal limb region.

A surprising result is that growth cones proximal to the Tr-Cx boundary never became disoriented following PI-PLC treatment (Figs 3C, D, 6C). This suggests that there is a significant difference in guidance information between the limb regions distal and proximal to the Tr-Cx boundary. Whether this difference is quantitative or qualitative is unresolved.

The disruption of proximal guidance by PI-PLC treatment indicates that GPI-anchored proteins are important in the transmission of this guidance information. Whether the relevant molecules are located on the growth cone membrane, on the substratum, or on both surfaces, is unknown. The possible involvement of GPI-anchored proteins in cell migration during embryogenesis has previously been reported (Zackson and Steinberg, 1988, 1989). Migration of pronephric duct and transplanted cranial neural crest cells in the axolotl embryo is inhibited by exposure to PI-PLC (Zackson and Steinberg, 1989). Alkaline phosphatase, a GPI-anchored cell surface protein, is localized in a graded pattern that closely resembles the pathway taken by the migrating cells (Zackson and Steinberg, 1988), and might generate or participate in a molecular gradient which guides the cells. Thus, in at least one other system, stereotyped migration of cells during development appears to be dependent upon interactions with cell surface molecules attached to the membrane by GPI.

Circumferential guidance cues for growth cones and for cell migration

A striking outcome of PI-PLC treatment is the growth of pioneer axons across the Tr-Cx segment boundary (Figs 3C, D, 6C). Normally, the transition from proximal to circumferential growth at this boundary is one of most stereotyped behaviors exhibited by Ti1 pioneer growth cones (Caudy and Bentley, 1987). This behavior could be caused by high growth cone adhesivity of the distal epithelial cell band at the boundary, and/or by relatively low affinity, or even avoidance, of the proximal epithelial band. The adhesivity of the distal band is indicated by preferential extension of branches at that location. More direct evidence is provided by the ability of growth cones that have established contacts with these cells to retain them under the stress of axon shortening and cell body translocation which follow enzymatic removal of the basal lamina (Condic and Bentley, 1989b). Therefore, a possible explanation for the observed segment boundary crossing is that GPI-anchored proteins may function as adhesive ligands on the distal epithelial cells, or growth cone receptors for such ligands. This model is

supported by the failure of growth cones crossing the Tr-Cx boundary to extend branches along the distal cells (Fig. 3C, D).

The low-affinity or non-permissive nature of the proximal epithelial cell band at the boundary is suggested by the failure of filopodia that palpate these surfaces to establish branches (O'Connor et al., 1990), and by the cessation of proximal growth when this, or other differentiated boundaries (Klose and Bentley, 1989), are encountered. Also, when the tibia-femur boundary differentiates, after the Ti1 growth cones have grown across, there is a sharp transition in the amount of adhesion of nascent axon membrane and side-branches to the epithelial surfaces on either side of the boundary (Caudy and Bentley, 1986b; Condic and Bentley, 1989b). Failure to cross boundaries, or to adhere to the proximal cells, could be mediated by a GPI-anchored ligand on the epithelial substratum which counteracts adhesion or induces avoidance, or by a GPI-anchored growth cone receptor for such an inhibitory ligand. Constraint of growth cone migration by negative interactions with substrata has been documented in several systems (Keynes and Cook, 1990). When confronted with a choice between substrata made from anterior or posterior tectal membranes, chick temporal retinal growth cones confine their migration to the anterior-derived substratum (Walter et al., 1987). This preference results from a growth cone repelling activity on the posterior-derived membranes, which appears to be mediated by a 33 × 10^3 M_r GPI-anchored protein (Stahl et al., 1990). A GPI-anchored protein of this type could contribute to pioneer growth cone avoidance of the proximal epithelial band at the Tr-Cx boundary.

This circumferential guidance information, whatever its basis, may be repeated at multiple locations in the limb. Like the Ti1 growth cones, the Cx1 neurons migrate ventrally at a specific circumferential location (Bentley and Toroian-Raymond, 1989). Following PI-PLC treatment, this migration can be abandoned in favor of proximal migration along the limb axis (Figs 4C, 6A). The Cx1 migration route also could be determined by the presence of an adhesive ligand on the path, and/or by a non-permissive ligand adjacent to the path. Either ligands or receptors (or both) could be GPI-anchored. Whichever systems are involved, the failure of both Ti1 growth cones and Cx1 neurons to observe the circumferential guidance information while still responding to the proximal orienting cue indicates that distinct, spatially restricted sets of GPI-anchored guidance molecules or receptors participate in these stereotyped migrations.

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