

Fasciclin IV: Sequence, Expression, and Function during Growth Cone Guidance in the Grasshopper Embryo

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Summary

Monoclonal antibody 6F8 was used to characterize and clone fasciclin IV, a new axonal glycoprotein in the grasshopper, and to study its function during growth cone guidance. Fasciclin IV is dynamically expressed on a subset of axon pathways in the developing CNS and on circumferential bands of epithelial cells in developing limb buds. One of these bands corresponds to the location where the growth cones of the Ti1 pioneer neurons make a characteristic turn while extending toward the CNS. Embryos cultured in the 6F8 antibody or Fab exhibit aberrant formation of this axon pathway. cDNA sequence analysis suggests that fasciclin IV has a signal sequence; long extracellular, transmembrane, and short cytoplasmic domains; and shows no homology with any protein in the available data bases. Thus, fasciclin IV appears to be a novel integral membrane protein that functions in growth cone guidance.

Introduction

Neuronal growth cones use a variety of cell surface, extracellular matrix, and diffusible cues for their guidance. These cues can function as either attractive or repulsive signals. One approach to the study of growth cone guidance has been to turn to simple model systems, such as insect embryos. The advantages of these organisms range from the relative simplicity and accessibility of neurons in the grasshopper embryo, to the opportunity for genetic analysis in *Drosophila*. Fortunately, from what has been learned thus far, the mechanisms of growth cone guidance appear remarkably similar in both insects and mammals (e.g., Harrelson and Goodman, 1988; Dodd and Jessell, 1988; Klose and Bentley, 1989; McConnell et al., 1989).

The growth cones that pioneer sensory and motor pathways in insects use a variety of cues for their guidance, including other neurons, glia, epithelial cells, and mesodermal cells. For example, in the grasshopper embryo, the growth cones of the pair of pioneer neurons in the developing limb bud recognize guid-

ance cues such as the limb bud epithelium, including particular regions of epithelium near segment borders, and another pair of neurons (e.g., Condic and Bentley, 1989a, 1989b; O'Connor et al., 1990). Guidance along an epithelium and its secreted extracellular matrix has also been observed in the developing wing imaginal disc of *Drosophila* (e.g., Blair et al., 1987). Nonneuronal cues are also important for the establishment of axon pathways in the developing insect CNS (Jacobs and Goodman, 1989; Klämbt et al., 1991). For example, glia provide guidance cues for the growth cones that pioneer one of the peripheral nerve roots in the grasshopper embryo (e.g., Bastiani and Goodman, 1986).

In contrast to the initial pioneers, most later growth cones in the developing insect CNS are followers, and they find themselves in an environment surrounded by the axons of earlier differentiating neurons. Experimental analysis reveals their ability to distinguish one bundle of axons, or fascicle, from another (called selective fasciculation), leading to the labeled pathway hypothesis (e.g., Raper et al., 1984; Bastiani et al., 1984, 1986; du Lac et al., 1986).

In an attempt to identify molecules involved in selective fasciculation, a series of monoclonal antibody (MAb) screens were conducted in both grasshopper and *Drosophila* to identify surface glycoproteins that are differentially expressed on subsets of axon pathways. Four surface glycoproteins were initially characterized (fasciclin I, fasciclin II, fasciclin III, and neuroglial; Bastiani et al., 1987; Patel et al., 1987; Bieber et al., 1989). With the exception of neuroglial, which is more widely distributed, the other three proteins are dynamically expressed on restricted subsets of axon pathways during development. The genes encoding these four proteins were cloned in grasshopper and *Drosophila* (Patel et al., 1987; Zinn et al., 1988; Harrelson and Goodman, 1988; Snow et al., 1989; Bieber et al., 1989; Grenningloh et al., 1991), and their cDNAs were used for *in vitro* transfection assays to show that each can function as a homophilic cell adhesion molecule (Snow et al., 1989; Elkins et al., 1990b; Grenningloh et al., 1990). Genetic analysis shows that two can function during growth cone guidance (Elkins et al., 1990a; Grenningloh et al., 1991; Fetter et al., unpublished data).

In the present study we used the MAb 6F8 to characterize and clone a new axonal glycoprotein in grasshopper, fasciclin IV, and to study its function during growth cone guidance. Fasciclin IV was initially identified because of its dynamic expression on a subset of axon pathways in the developing CNS. Thus, it is a prime candidate to play a role in selective fasciculation. However, additional analysis revealed that fasciclin IV is also expressed on circumferential bands of epithelial cells in developing limb buds, one of which corresponds to the location where the growth cones

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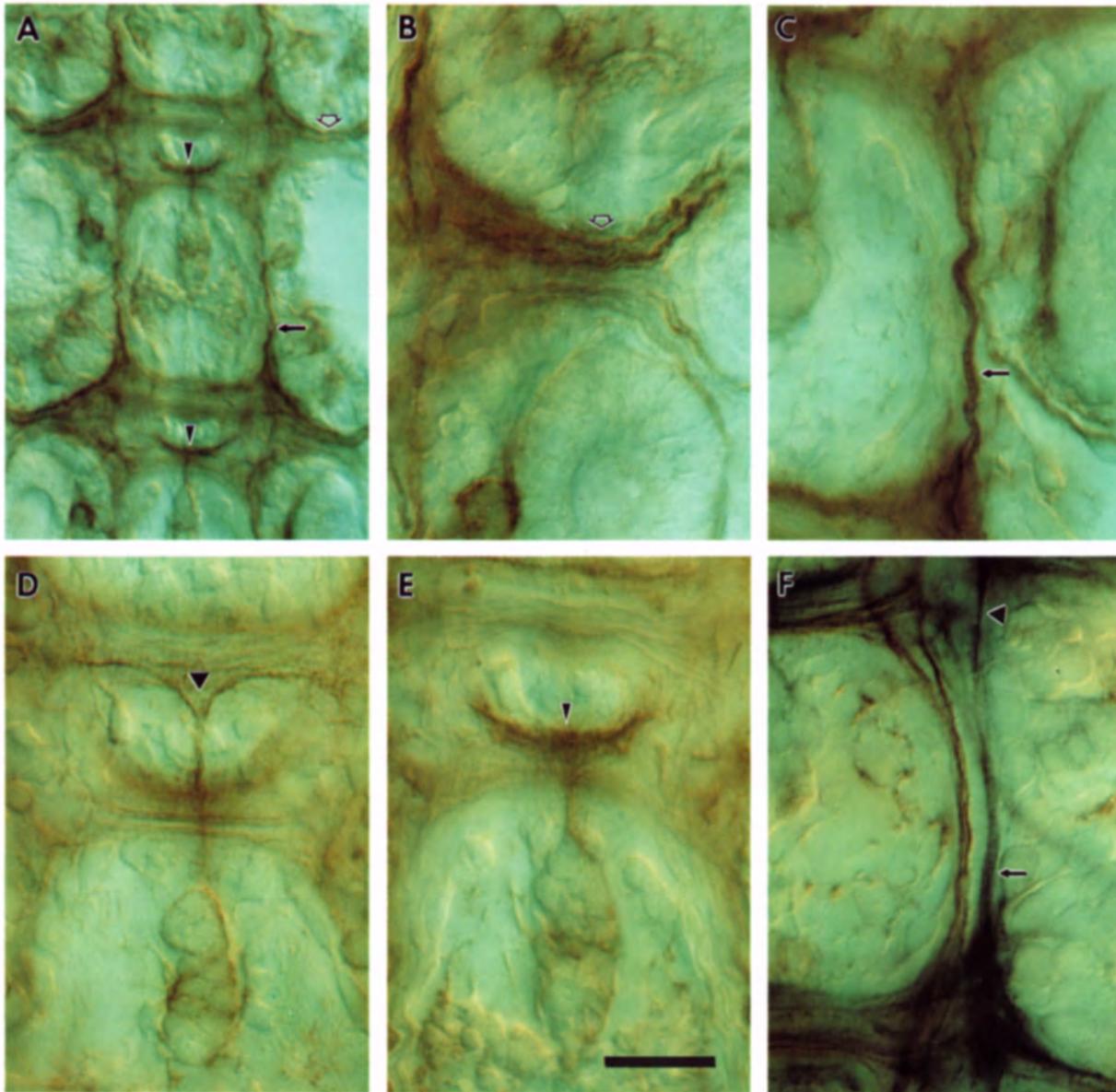


Figure 1. Fasciclin IV Is Expressed on a Subset of Axon Pathways in the Developing CNS

Photographs showing fasciclin IV expression in the developing CNS of dissected grasshopper embryos at 40% of development, viewed with Nomarski optics from the dorsal (inner) surface of the neuroepithelium. (A–E) Brown staining is fasciclin IV expression as visualized using MAb 6F8 and HRP immunohistochemistry. (F) Black staining is fasciclin IV (MAb 6F8) and brown is fasciclin II (MAb 8C6), as visualized using NiCl₂-enhanced HRP immunohistochemistry and standard HRP immunocytochemistry, respectively.

(A) Photograph of two segments showing fasciclin IV expression including, for example, the U longitudinal pathway (thin arrow), the median fiber tract and its projection into the posterior commissure (arrowheads), and the motor axons exiting in the anterior branch of the segmental nerve (open arrow).

(B) The motor axons exiting in the anterior branch of the segmental nerve (open arrow) express high levels of fasciclin IV, whereas the axons in the posterior branch do not.

(C) In the longitudinal connectives between adjacent segmental neuromeres, fasciclin IV is expressed on the U pathway (thin arrow) but not on other longitudinal pathways; it is also expressed on at least some of the motoneurons that turn laterally and exit in the intersegmental nerve (out of focus).

(D) Fasciclin IV is expressed on the cell bodies of the pairs of MP4, MP5, and MP6 progeny, as well as median neuroblast progeny (the cluster of cell bodies in the lower middle of the photograph) and on their axons in the median fiber tract. This focal plane shows fasciclin IV expression on the MP4 axons extending anteriorly across the posterior commissure and then bifurcating (triangle) in the posterior end of the anterior commissure.

(E) In a different focal plane of the commissures and midline, fasciclin IV expression is seen on the MP6 axons as they extend anteriorly and bifurcate (arrowhead) in the anterior end of the posterior commissure (see also arrowheads in [A]).

(F) In the longitudinal connectives between adjacent neuromeres, fasciclin IV is expressed on the U pathway (black) and fasciclin II is expressed on the MP1 pathway (brown). Within the segmental neuromere (top of photo), fasciclin IV is also expressed on the axons in the A/P fascicle (triangle).

Bar, 100 μ m (A); 50 μ m (B–F).

of the Ti1 pioneer neurons make a characteristic turn while extending toward the CNS. Thus, the expression of fasciclin IV on epidermal cells makes it an excellent candidate for a molecule involved in the guidance of pioneer growth cones. In this paper, we test and confirm this second hypothesis by culturing grasshopper embryos of the appropriate age in either 6F8 MAb or Fab. These experimental embryos exhibit aberrant formation of the Ti1 axon pathway in the developing limb bud.

Fasciclin IV cDNAs were cloned, and the deduced amino acid sequence reveals a signal sequence, a long extracellular domain, a transmembrane domain, and a short cytoplasmic domain. Fasciclin IV shows no homology with any protein in the available data base. Thus, fasciclin IV appears to be a novel membrane protein that functions in the guidance of the pioneer growth cones in the developing grasshopper limb bud, and whose pattern of expression suggests other guidance functions as well, particularly during the selective fasciculation of follower growth cones in the CNS.

Results

To identify cell surface molecules that function in selective fasciculation, a series of MAb screens was conducted. The immunogen used for most of these screens was membrane from the longitudinal connectives (the collection of longitudinal axons) between adjacent segmental ganglia of the nervous system of the larval grasshopper (Bastiani et al., 1987). From these screens, MAbs 3B11 and 8C6 were used to purify and characterize two surface glycoproteins, fasciclin I and fasciclin II (Bastiani et al., 1987); the genes encoding both were subsequently cloned (Snow et al., 1989; Zinn et al., 1988; Harrelson and Goodman, 1988).

Another MAb isolated during these screens, MAb 6F8, was chosen for the present study because, just as with fasciclin I and fasciclin II, the antigen recognized by this MAb is expressed on a different but overlapping subset of axon pathways in the developing CNS. The 6F8 antigen appears to be localized on the outside of cell surfaces, as indicated by MAb binding when incubated both in live preparations and in fixed preparations in which no detergents have been added (data not shown). Because the 6F8 antigen is a surface glycoprotein expressed on a subset of axon fascicles (see below), we call it fasciclin IV.

Fasciclin IV expression begins early in embryonic development, before axonogenesis. At 29% of development (see Bentley et al., 1979, for staging), expression is seen on the surface of the midline mesectodermal cells and around 5–7 neuroblasts and associated ectodermal cells per hemisegment. This expression is reminiscent of the mesectodermal and neuroblast-associated expression observed with both fasciclin I and fasciclin II (Bastiani et al., 1987; Harrelson and Goodman, 1988); however, in each case, the pattern

resolves into a different subset of neuroblasts and associated ectodermal cells.

At 32% of development, shortly after the onset of axonogenesis in the CNS, fasciclin IV expression is seen on the surface of the axons and cell bodies of the three pairs of MP4, MP5, and MP6 midline progeny, on the three U motoneurons, and on several unidentified neurons in close proximity to the U neurons (data not shown). This is in contrast with fasciclin II, which at this stage is expressed on the MP1 and dMP2 neurons, and fasciclin I, which is expressed on the U neurons but not on any midline precursor progeny.

The expression of fasciclin IV on a subset of axon pathways is best observed at around 40% of development, after the establishment of the first longitudinal and commissural axon pathways. At this stage, the protein is expressed on two longitudinal axon fascicles, a subset of commissural axon fascicles, a tract extending anteriorly along the midline, and a subset of fascicles in the segmental nerve (SN) and intersegmental nerve (ISN) roots (Figure 1A).

Specifically, fasciclin IV is expressed on the U fascicle (Figures 1C and 1F), a longitudinal pathway (between adjacent segmental neuromeres) pioneered in part by the U neurons (Bastiani et al., 1986), and on the A/P longitudinal fascicle (in part an extension of the U fascicle within each segmental neuromere) (Figure 1F). In addition, fasciclin IV is expressed on a second narrower, medial, and more ventral longitudinal pathway (data not shown). The U axons turn and exit the CNS as they pioneer the ISN; the U axons and many other axons within the ISN express fasciclin IV. The continuation of the U fascicle posterior to the ISN junction is also fasciclin IV positive. The specificity of fasciclin IV for distinct subsets of longitudinal pathways can be seen by comparing fasciclin IV and fasciclin II expression in the same embryo (Figure 1F); fasciclin IV is expressed on the U and A/P pathways, whereas fasciclin II is expressed on the MP1 pathway.

The axons in the median fiber tract (MFT) also express fasciclin IV (Figures 1A, 1D, and 1E). The MFT is pioneered by the three pairs of progeny of the midline precursors MP4, MP5, and MP6 (Goodman et al., 1981). The MFT actually contains three separate fascicles. The axons of the two MP4 progeny pioneer the dorsal MFT fascicle and then bifurcate at the posterior end of the anterior commissure (Figure 1D); whereas the axons of the two MP6 progeny pioneer the ventral MFT fascicle and then bifurcate at the anterior end of the posterior commissure (Figures 1A and 1E). Fasciclin IV is expressed on the cell bodies of the six MP4, MP5, and MP6 neurons and on their growth cones and axons as they extend anteriorly in the MFT and bifurcate in one of the two commissures (Figures 1D and 1E). However, this expression is regional in that once these axons bifurcate and begin to extend laterally across the longitudinal pathways and toward the peripheral nerve roots, their expression of fasciclin IV greatly decreases. Thus, fasciclin IV is a label for the axons in the MFT and their initial bifurcations in both

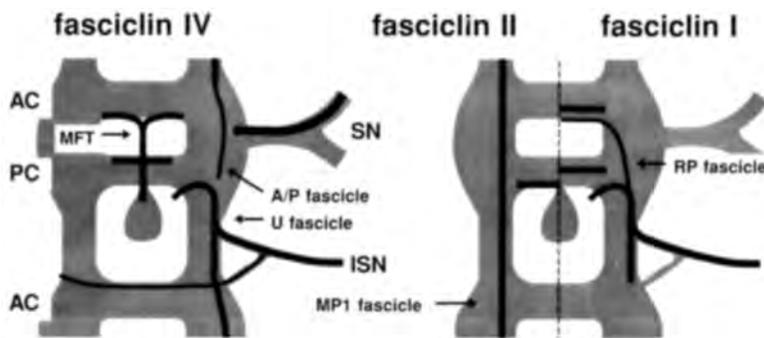


Figure 2. Summary of CNS Expression of Fasciclin IV as Compared with Fasciclin I and Fasciclin II

Schematic diagram showing (from left to right) the expression of fasciclin IV, fasciclin II, and fasciclin I in a pair of adjacent segmental neuromeres in a grasshopper embryo at 40% of development. Note that all three axonal glycoproteins are expressed on different but overlapping subsets of axon pathways at this stage of development. All three patterns of expression are dynamic and transient. See text for further details. AC, anterior commissure; PC, posterior commissure; SN, segmental

nerve root; ISN, intersegmental nerve root; MFT, median fiber tract (note bifurcation of fasciclin IV-positive from this tract in both the AC and the PC); A/P, U, MP1, and RP fascicles, names of well-characterized bundles of axons.

the anterior and posterior commissures. It appears to be expressed on other commissural fascicles as well. However, the commissural expression of fasciclin IV is distinct from the transient expression of fasciclin II along the posterior edge of the posterior commissure, or the expression of fasciclin I on several different commissural axon fascicles in both the anterior and posterior commissure (Figure 2) (Bastiani et al., 1987; Harrelson and Goodman, 1988).

Fasciclin IV is also expressed on a subset of motor axons exiting the CNS in the SN. The SN splits into two major branches, one anterior and the other posterior, as it exits the CNS (Figure 1B). Two large bundles of motoneuron axons in the anterior branch express fasciclin IV at high levels (Figures 1A and 1B); one narrow bundle of motoneuron axons in the posterior branch expresses the protein at much lower levels. Fasciclin IV is also expressed on many of the axons in the ISN.

The CNS and nerve root expression patterns of fasciclin IV, fasciclin I, and fasciclin II at around 40% of embryonic development are summarized in Figure 2. Although there is some overlap in their patterns (e.g., both fasciclin IV and fasciclin I label the U axons), these three surface glycoproteins label distinct subsets of axon pathways in the developing CNS.

Fasciclin IV Is Expressed on Epithelial Bands in the Developing Limb Bud

The developing limb bud in the grasshopper embryo is a model system for studies on the guidance of pioneering growth cones (e.g., Bentley and O'Connor, 1992). The first neurons to extend processes are the two sibling Ti1 neurons located at the distal end of the developing limb bud; they initiate growth cones at 30% of development. Over the next 5% of development (~24 hr), these growth cones encounter a variety of substrates, including epidermal cells, basal lamina, mesoderm, and other newly differentiated neurons. The pathway pioneered by the Ti1 axons is highly stereotyped and consists of a proximal extension toward the CNS that is abruptly interrupted midway down the limb by a ventral circumferential turn. This

first turn is followed by a second, proximal turn and subsequent extension into the CNS. The first turn normally occurs at an axial position coincident with the proximal side of the trochanter (Tr1) guidepost cell; both the Tr1 cell and the Ti1 axons lie within the trochanter close to the axial position where the trochanter-coxa boundary differentiates (Caudy and Bentley, 1987). Sufficient information for this turn appears to be available on the surface of the epithelial cells (Lefcort and Bentley, 1989; Condic and Bentley, 1989a). In contrast, it appears to be the pair of Cx1 neurons that lie just proximal to the trochanter-coxa boundary that are responsible for the second turn that guides the Ti1 growth cones proximally toward the CNS (Bentley and Caudy, 1983; O'Connor et al., 1990).

Observation of fixed and live-labeled Ti1 neurons reveals a dramatic change in growth cone morphology upon contact with the epithelium in the trochanter adjacent to the trochanter-coxa segment boundary (Caudy and Bentley, 1987; O'Connor et al., 1990). Growth cones abruptly discontinue proximal growth and typically extend lengthy filopodia and branches both dorsally and ventrally along this epithelium. Eventually the ventral branch is selected for further growth, and the dorsal branch is retracted. Experiments in which the basal lamina is removed enzymatically reveal that the Ti1 growth cones form a highly adhesive, load-bearing interaction with these epithelial cells, whereas their adhesion with more distal epithelium in the femur is not as strong (Condic and Bentley, 1989b). Thus morphological and adhesive characteristics are profoundly affected by the band of trochanter epithelial cells located just distal to the trochanter-coxa boundary.

Fasciclin IV is expressed on the developing limb bud epithelium in circumferential bands (Figure 3). At 34.5% of development, these bands can be localized with respect to constrictions in the epithelium that mark presumptive segment boundaries. In addition to a band just distal to the trochanter-coxa segment boundary (Figure 3A), bands are found in the tibia, femur, and coxa; later in development, a fifth band is found in the tarsus. Fasciclin IV is also expressed in

the nascent chordotonal organ in the dorsal aspect of the femur. The bands in the tibia, trochanter, and coxa completely encircle the limb. However, the femoral band is incomplete, containing a gap on the anterior epithelia of this segment (Figure 3). This pattern of expression is summarized in Figure 6A.

The position of the Ti1 axon pathway with respect to these bands of fasciclin IV-positive epithelia is shown in Figure 3B (summarized in Figure 6B). Certain aspects of this pattern suggest a potential role for fasciclin IV in guiding the Ti1 growth cones. First, the band of fasciclin IV expression in the trochanter, which is approximately 3 epithelial cell diameters in width when encountered by the Ti1 growth cones, is the axial location where the growth cones reorient from proximal migration to circumferential branch extension (Caudy and Bentley, 1987; O'Connor et al., 1990). The Tr1 cell (Figure 3B; see Figure 6B), which marks the location of the turn (Caudy and Bentley, 1987), lies within this band, usually over the central or the proximal cell tier. Second, although there is a more distal fasciclin IV-expressing band in the femur, where a change in Ti1 growth is not observed, there exists a gap in this band such that fasciclin IV-expressing cells are not traversed by the Ti1 growth cones (Figure 3B; see Figure 6B). The Ti1 axons also may encounter a fasciclin IV-expressing region within the coxa, where interactions between the growth cones, the epithelial cells, and the Cx1 guidepost cells have not yet been investigated.

The initial encounter of the Ti1 growth cones with epithelium expressing fasciclin IV occurs just distal to the trochanter-coxa boundary, which is precisely where the Ti1 growth cones make their first major reorientation, as summarized in Figure 6B. In addition to its expression over the surface of bands of epithelial cells, fasciclin IV, as visualized with MAb 6F8, is found on the basal surface of these cells in a punctate pattern (Figure 3C). This punctate staining is not an artifact of the horseradish peroxidase (HRP) immunocytochemistry, since fluorescence visualization of MAb 6F8 is also punctate (data not shown). The nonneuronal expression of fasciclin IV is not restricted to limb buds. Circumferential epithelial bands of fasciclin IV expression are also seen on subesophageal mandibular structures and on the developing antennae (data not shown).

MAb Directed against Fasciclin IV Can Alter the Formation of the Ti1 Axon Pathway in the Limb Bud

The expression of fasciclin IV on an epithelial band at a key choice point in the formation of the Ti1 axon pathway led us to ask whether this protein is involved in growth cone guidance at this location. To answer this question, we cultured embryos, or epithelial fillets (e. g., O'Connor et al., 1990), during the 5% of development necessary for normal pathway formation either in the presence or absence of MAb 6F8 or 6F8 Fab fragments. Under the culture conditions used

for these experiments, defective Ti1 pathways are observed in 14% of limbs (Chang et al., 1992); this defines the baseline of abnormalities observed using these conditions. For controls we used other MAbs, and their Fab fragments, that either bind to the surfaces of these neurons and epithelial cells (MAb 3B11 against the surface protein fasciclin I; Bastiani et al., 1987) or do not (MAb 4D9 against the nuclear protein engrailed; Patel et al., 1989). To assess the impact of MAb 6F8 on Ti1 pathway formation, we compared the percentage of aberrant pathways observed following treatment with MAb 6F8 with that observed with MAbs 3B11 and 4D9. Our cultures began at 32% of development, when the Ti1 growth cones have not yet reached the epithelium just distal to the trochanter-coxa boundary and therefore have not encountered epithelial cells expressing fasciclin IV. Following approximately 30 hr in culture (~4% of development), embryos were fixed and immunostained with antibodies to HRP in order to visualize the Ti1 axons and other neurons in the limb bud. Criteria for scoring the Ti1 pathway and the definition of "aberrant" are described in detail in Experimental Procedures.

Although MAb 6F8 does not arrest pathway formation, several types of distinctive, abnormal pathways are observed (Figures 4B-4D; see Figures 6C and 6D). These defects generally begin where growth cones first contact the fasciclin IV-expressing cells in the trochanter. Normally, the Ti1 neurons each have a single axon, and the axons of the two cells are fasciculated in that portion of the pathway within the trochanter (Figure 4A). Following treatment with MAb 6F8, multiple, long axon branches are observed within, and proximal to, the trochanter. Two major classes of pathways are taken by these branches; in 36% of aberrant limbs, multiple, long axon branches extend ventrally in the region distal to the Cx1 cells, which contains the band of fasciclin IV-expressing epithelial cells (Figures 4B and 4C; see Figure 6C). In the ventral region of the trochanter, these branches often independently turn proximally to contact the Cx1 cells and thus complete the pathway in this region.

In the second major class of pathway defect, seen in 47% of aberrant limbs, axon branches leave the trochanter at abnormal, dorsal locations and extend proximally across the trochanter-coxa boundary (Figures 4C and 4D; see Figure 6D). These axons then veer ventrally, often contacting the Cx1 neurons. The remaining 17% of defects include defasciculation distal to the trochanter, axon branches that fail to turn proximally in the ventral trochanter and continue into the posterior compartment of the limb, and axon branches that cross the trochanter-coxa boundary and continue to extend proximally without a ventral turn.

When cultured in the presence of MAb 6F8, 43% of limbs exhibited malformed Ti1 pathways ($n = 381$) as compared with 11% with MAb 3B11 ($n = 230$) and 5% with MAb 4D9 ($n = 20$) (Figure 5). These percentages are pooled from treatments with MAbs concentrated from hybridoma supernatant, IgGs isolated from

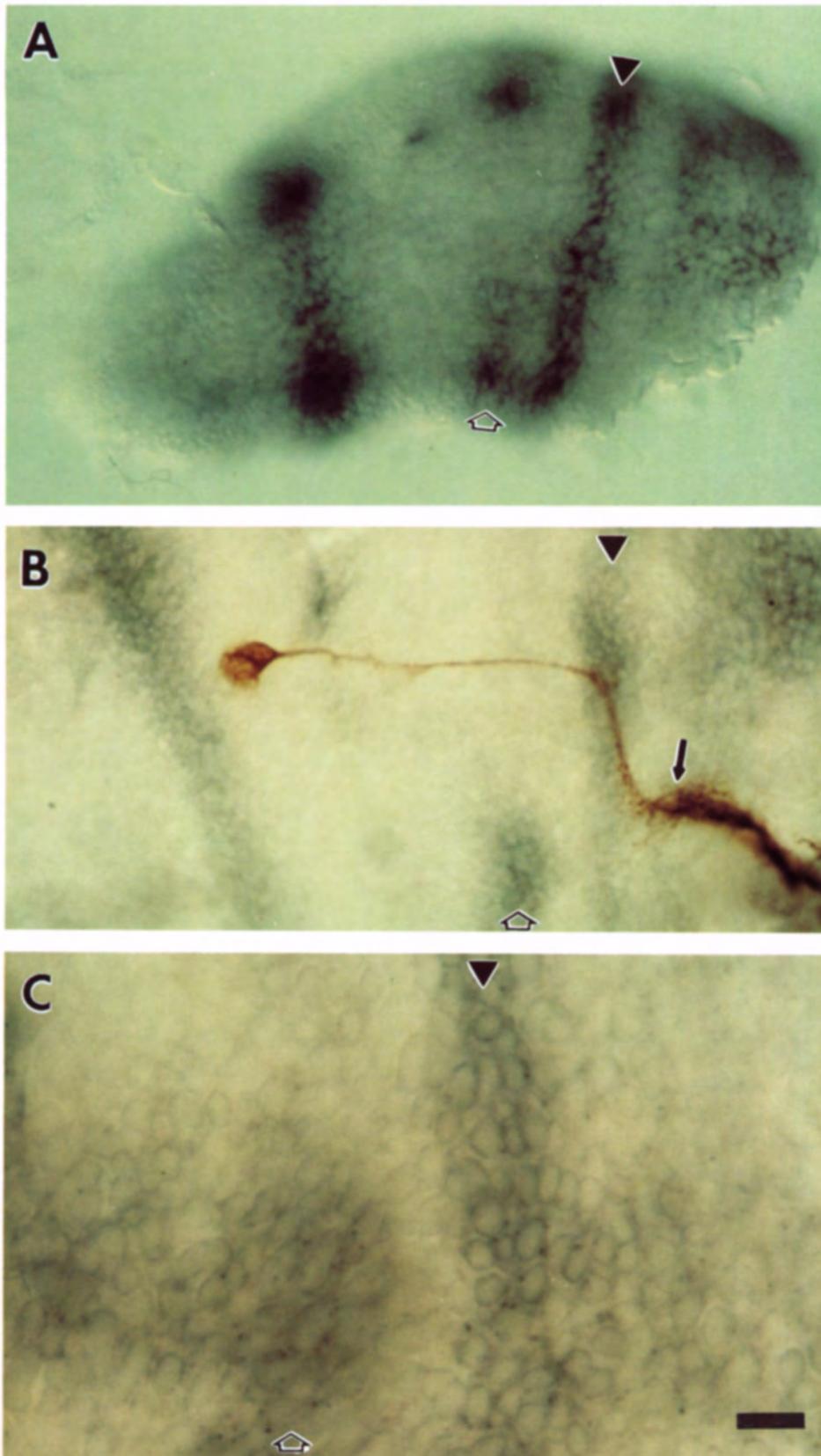


Figure 3. Fasciclin IV Is Expressed in Epithelial Bands in the Developing Limb Bud
Photographs of T3 limb buds in the grasshopper embryo at 35% (A) and 35.5% (B and C) of development showing the epithelial bands:

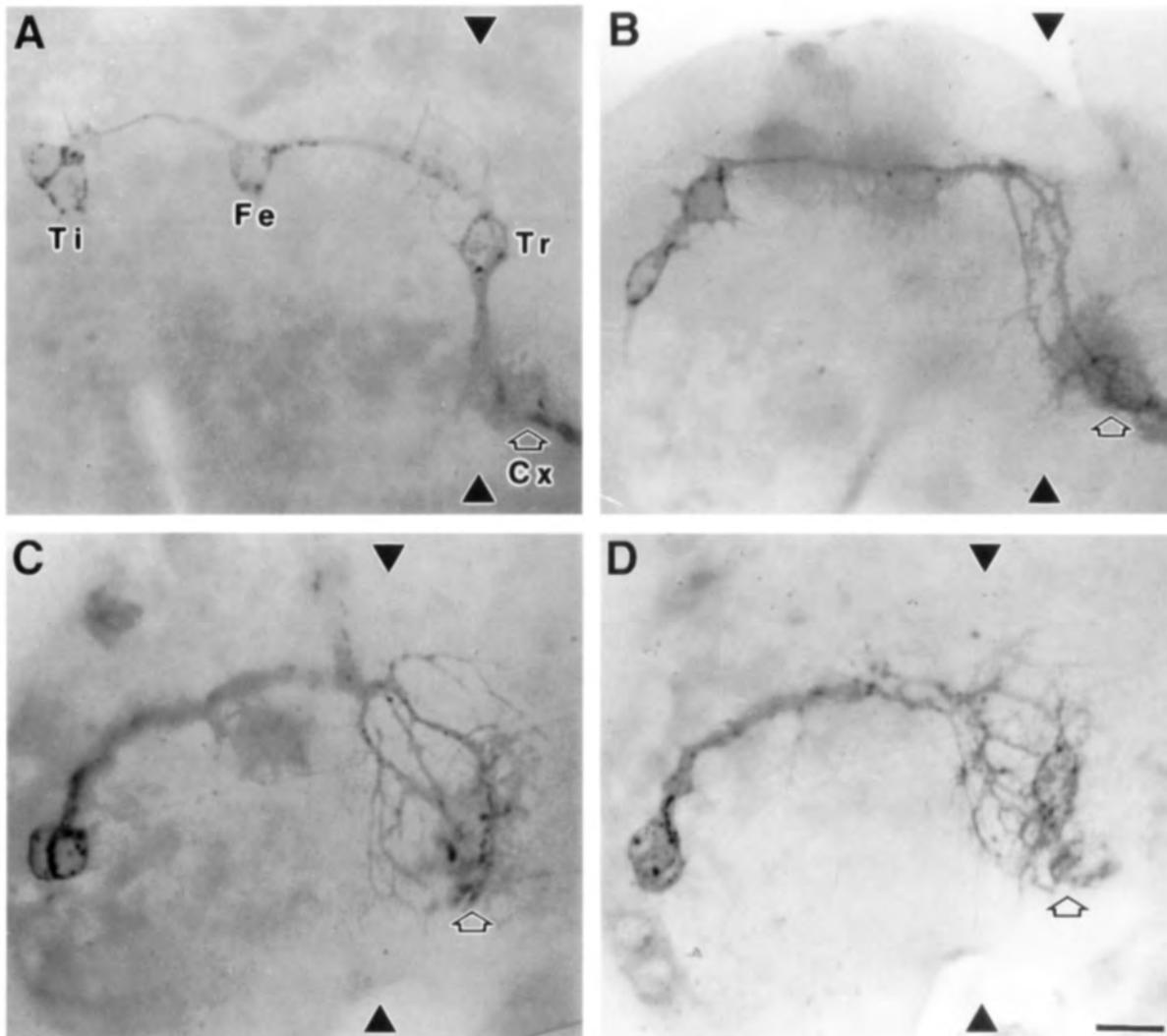


Figure 4. MAb Directed against Fasciclin IV Disrupts Ti Pathway Formation at the Trochanter-Coxa Boundary

Photographs of whole-mount limb buds (32% + 30 hr in culture) labeled with RITC-conjugated anti-HRP to reveal the Ti axon pathway, following culture in either the absence (A) or the presence (B–D) of MAb 6F8 to block fasciclin IV. The three examples of experimental limb buds (B–D) are representative of the range of defects and show examples of multiple branching (B–D) as well as branches crossing the trochanter-coxa boundary (C and D). The triangle marks the location of the epithelial band just distal to the trochanter-coxa boundary, where the Ti1 growth cones normally make their characteristic ventral turn. The open arrow marks the Cx1 neurons. Bar, 30 μ m.

of fasciclin IV expression (black staining) as visualized using MAb 6F8 with NiCl-enhanced HRP immunohistochemistry (dorsal is up; distal is left). The brown staining in (B) reveals the pair of Ti1 pioneer neurons in the limb bud visualized using MAb 8G7 (which recognizes a cell surface epitope on the Ti1 axons; Tiemeyer and Goodman, unpublished data) with standard HRP immunocytochemistry. In all panels, the fasciclin IV band just distal to the trochanter-coxa boundary is marked at the top with a triangle and the partial ventral band in the femur is marked at the bottom with an open arrow. Fasciclin IV is also expressed in a more distal band in the tibia (A and B) and in other bands as well (data not shown).

(A) Whole mount limb bud viewed from the anterior surface.

(B and C) The anterior surface of filleted limb buds. The Ti1 axons (stained brown in [B]) make a characteristic ventral turn along the band of fasciclin IV expression just distal to the trochanter-coxa boundary. The Tr1 neuron lies within the fasciclin IV band where the Ti1 axons turn. Note the gap in staining of the fasciclin IV band in the femur; the Ti1 axons extend proximally through this gap, thus encountering fasciclin IV-expressing epithelial cells for the first time when they reach the band just distal to the trochanter-coxa boundary. Ventrally, the axons leave the band to cross to the Cx1 guidepost cells (thin arrow). (C) Higher magnification photograph of epithelial cells expressing fasciclin IV to show the punctate expression (small dark spots) in addition to the general cell surface expression.

Bar, 30 μ m (A); 20 μ m (B); 8 μ m (C).

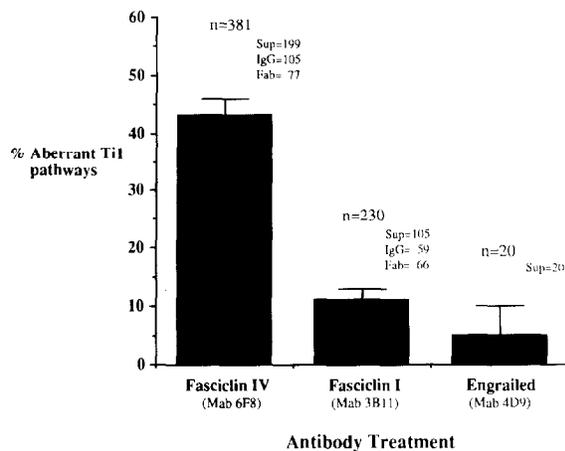


Figure 5. Summary of Aberrant Ti1 Pathways in Antibody Blocking Experiments

Percentage of aberrant Ti1 pathways in the T1–T3 limb buds of the grasshopper embryo following culture in the presence of MABs against fasciclin IV (6F8), fasciclin I (3B11), and engrailed (4D9). Data are pooled from four experiments in which three different preparations of MAB (concentrated hybridoma supernatant, purified IgG, and Fab fragments) were used as blocking reagents. All three types of preparation showed a similar percentage of aberrant pathways and a similar spectrum of pathway phenotypes. The four pooled experiments showed a similar degree of pathway disruption for each reagent used. Raw data are presented in Table 1. n, the total number of limbs scored for each MAB treatment; Sup, number of limbs treated with concentrated hybridoma supernatant; IgG, number of limbs treated with purified IgG; Fab, number of limbs treated with Fab fragments.

these supernatants, and Fab fragments isolated from these IgG preparations (see Experimental Procedures). The frequency of malformed Ti1 pathways and the types of defects observed showed no significant variation regardless of the method of antibody preparation or the type of antibody used. Since Fab fragments showed results similar to those with IgGs, the effects of MAB 6F8 are not due to cross-linking by the bivalent IgG.

In summary, following treatment with MAB 6F8, the Ti1 pathway typically exhibits abnormal morphology beginning just distal to the trochanter and at the site of fasciclin IV expression. The two most common types of Ti1 pathway defects described above occur in 36% of experimental limbs (treated with MAB 6F8), but are seen in only 4% of control limbs (treated with MABs 3B11 and 4D9).

Fasciclin IV cDNAs Encode a Novel Integral Membrane Protein

Grasshopper fasciclin IV was purified by passing crude embryonic grasshopper lysates over a MAB 6F8 column. After affinity purification, the protein was eluted, precipitated, denatured, modified at cysteines, and digested with either trypsin or Lys-C. Individual peptides were resolved by reverse-phase high pressure liquid chromatography and microsequenced using standard methods.

The amino acid sequences derived from these proteolytic fragments were used to generate oligonucleotide probes for polymerase chain reaction (PCR) experiments, resulting in products that were used to isolate cDNA clones from the Zinn embryonic grasshopper cDNA library (Snow et al., 1988). Sequence analysis (Figure 7) of these cDNAs reveals a single open reading frame encoding a protein with two potential hydrophobic stretches of amino acids: an amino-terminal signal sequence of 20 residues (von Heijne, 1983) and (beginning at amino acid 627) a potential transmembrane domain of 25 amino acids (Chou and Fasman, 1974). Thus, the deduced protein has an extracellular domain of 605 amino acids, a transmembrane domain, and a cytoplasmic domain of 78 amino acids. The calculated molecular mass of the mature fasciclin IV protein is 80 kd and is confirmed by Western blot analysis of the affinity-purified and endogenous protein as described below. The extracellular domain of the protein includes 16 cysteine residues that fall into three loose clusters but do not constitute a repeated domain and are not similar to other known motifs with cysteine repeats. There are also six potential sites for N-linked glycosylation in the extracellular domain. Treatment of affinity-purified fasciclin IV with N-glycanase demonstrates that fasciclin IV does indeed contain N-linked oligosaccharides (data not shown). Fasciclin IV shows no sequence similarity when compared with other proteins in the PIR data base using BLASTP (Altschul et al., 1990) and is therefore a novel type I integral membrane protein.

A polyclonal antiserum directed against the cytoplasmic domain of the protein encoded by the fasciclin IV cDNA was used to stain grasshopper embryos at 40% of development. The observed staining pattern was identical to that seen with MAB 6F8 (data not shown). On Western blots, this antiserum recognizes the protein we affinity purified using MAB 6F8 and then subjected to microsequence analysis (Figure 8, lane 1). Additionally, the polyclonal serum recognizes a protein of similar molecular mass from grasshopper embryonic membranes (Figure 8, lane 4). Taken together, these data indicate that the sequence we have obtained is indeed fasciclin IV.

Four other cell surface proteins that label subsets of axon pathways in the insect nervous system (fasciclin I, fasciclin II, fasciclin III, and neuroglian) are capable of mediating homophilic cell adhesion when transfected into S2 cells in vitro (Snow et al., 1989; Elkins et al., 1990b; Grenningloh et al., 1990). To ask whether fasciclin IV can function as a homophilic cell adhesion molecule, the fasciclin IV cDNA with the complete open reading frame was placed under the control of the inducible metallothionein promoter (Bunch et al., 1988), transfected into S2 cells, and assayed for its ability to promote adhesion in normally nonadhesive S2 cells (Snow et al., 1989). Following induction with copper, fasciclin IV was synthesized in these S2 cells, as shown by Western blot analysis (Figure 8, lanes 2

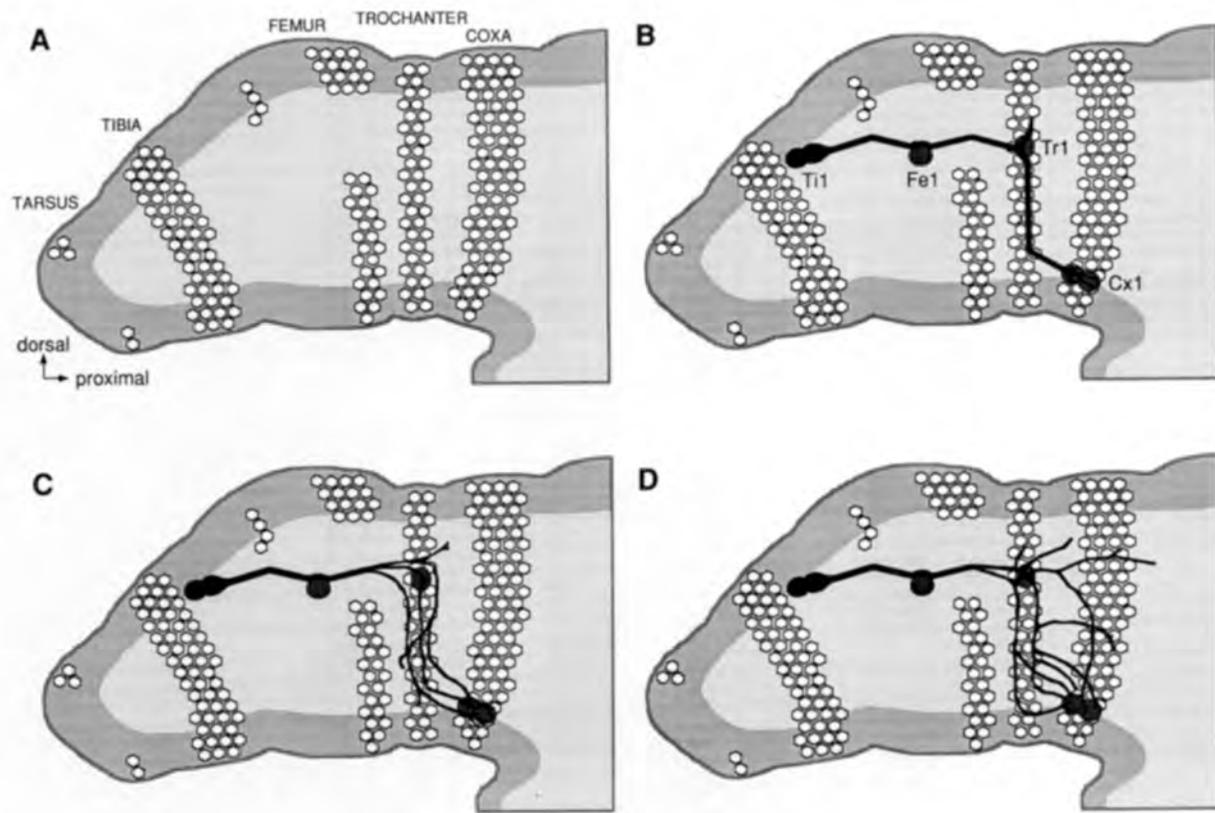


Figure 6. Relationship of the Ti1 Pathway and Fasciclin IV Expression in Control and Antibody Blocking Experiments
Schematic diagrams of limb buds showing the epithelial bands of expression of fasciclin IV (open hexagonal epithelial cells) in relationship to the locations of neuronal cell bodies and the trajectory of the Ti1 axons (dark circles and black lines in [B]–[D]) in the grasshopper embryo at 34.5% of development. (A and B) Normal limb buds. (C and D) Representative examples of Ti1 axon branching and pathways in experimental embryos incubated in MAb 6F8. These two examples illustrate the range of abnormal Ti1 pathways. Note the multi-branch phenotype (C and D) and the extension of processes across the trochanter–coxa boundary (D).

and 3) and cell surface staining of induced S2 cells with the polyclonal antiserum described above (data not shown).

We observed no evidence for aggregation upon induction of fasciclin IV expression, thus suggesting that, in contrast with the other four proteins, fasciclin IV does not function as a homophilic cell adhesion molecule. Alternatively, fasciclin IV-mediated aggregation might require some further posttranslational modification or cofactor not supplied by the S2 cells, but clearly this protein acts differently in the S2 cell assay than the other four axonal glycoproteins previously tested. This is consistent with the pattern of fasciclin IV expression in the embryonic limb, since only the epithelial cells and not the Ti1 growth cones express fasciclin IV and yet antibody blocking experiments indicate that fasciclin IV functions in the epithelial guidance of these growth cones. Such results suggest that fasciclin IV functions in a heterophilic adhesion or signaling system.

Discussion

Neuronal growth cones are guided by a variety of

neuronal and nonneuronal substrates. For example, in the grasshopper embryo, pioneer growth cones in the limb buds navigate in part using cues in the epithelium, whereas follower growth cones in the CNS navigate in part using cues on the surfaces of other axons. The present study began with a MAb screen to identify molecules of the second type, namely, those involved in axon–axon interactions in the developing CNS. Yet what was ultimately discovered was a novel integral membrane protein, fasciclin IV, that is expressed not only on a subset of axon pathways in the developing CNS, but also on bands of epithelial cells in developing limb buds, one of which coincides precisely with where the limb bud pioneer growth cones make a characteristic turn in their trajectory. Antibody blocking experiments suggest that fasciclin IV does indeed function in the epithelial guidance of the Ti1 growth cones that pioneer the first axon pathway in the developing limb bud.

Fasciclin IV is expressed on groups of axons that fasciculate in the CNS, suggesting that, much like other insect axonal glycoproteins, it might function as a homophilic cell adhesion molecule binding these axons together. Yet, in the limb bud, fasciclin IV is

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001  MRAALVAVAA LLWVALHAAA WVNDVSPKMY VQFGEERVQR FLGNESHKDH
051  FKLLEKDHNS LLVGARNIVY NISLRDLTEF TEQRIEWHSS GAHRELQYK
101  GKSEDDGONY IRVLAKIDDD RVLIGTNAY KPLGRHYALK DGDYVVEKEY
151  EGRGLQPFDP DHNSTAIYSE GQLYSATVAD FSGTDPLIYR GPLRTERS DL
201  KQLNAPNFVN TMEYNDFIFF FFRETAVEYI NGGKAIYSRV ARVQKHDKGG
251  PHQFGDRWTS FLKSRNLGSV PGDYPFYFNE IQSTSDIIEG NYGGQVEKLI
301  YGVFTTPVNS IGGSAVQAFS MKSILESFDG PFKEQETMNS NWLAVPSLKV
351  PEPRPGQGVN DSRTLDPDVSV NFKSHTLMD EAVPAFFTRP ILIRISLQYR
401  FTKIAVDQV RTPDGKAYDV LFIGTDDGKV IKALNSASF DSSDVTDSVVI
451  EELQVLPQGV PVKNLYVVRM DGDSDKLVVV SDDEILA IKL HRQGS DKITN
501  QREQVSLQDP YQAWDNVELK QTAVGSPDWS AGKRRFIQNI SLGEHKAQGG
551  RPQTEIVASP VPTQPTTKSS GDPVHSIHQA EFEPEIDNEI VIGVDDSNVI
601  PNTLAEINHA GSKLPSSEK LPIYTAETLT IAIVTSCLGA LVVGFJSGFL
651  FSRRCRGEDY TDMFPDQRH QLNRLTEAGL NADSPYLPPC ANNKAAINLV
701  LNVPPKNANG KNANSSAENK PIQKVKKTYI

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Figure 7. Deduced Amino Acid Sequence of Fasciclin IV

Predicted amino acid sequence of the fasciclin IV protein based on cDNA sequence analysis (nucleotide sequence not shown but entered in data base). The putative signal sequence at the amino terminus is underlined. The transmembrane domain near the carboxyl terminus is double underlined. Potential N-linked glycosylation sites are noted with arrows. All 16 extracellular cysteines are highlighted.

expressed on a band of epithelium but not on the growth cones that reorient along this band, suggesting a heterophilic function. This notion that fasciclin IV functions in a heterophilic rather than homophilic fashion is supported by the lack of homophilic adhesion in S2 cell aggregation assays. In contrast, fasciclin I, fasciclin II, fasciclin III, and neuroglian all can function as homophilic cell adhesion molecules (Snow et al., 1989; Elkins et al., 1990b; Grenningloh et al., 1990).

cDNA sequence analysis indicates that fasciclin IV is an integral membrane protein with a novel sequence not related to any protein in the present data base. Thus, fasciclin IV represents a new type of protein that functions in the epithelial guidance of pioneer growth cones in the developing limb bud. Given its expression on a subset of axon pathways in the developing CNS, fasciclin IV is likely to function in the guidance of CNS growth cones as well. Experiments are in progress to determine the role of fasciclin IV in axon guidance in the CNS.

The results from the MAb blocking experiments illuminate several issues in Ti1 growth cone guidance and axon morphogenesis in the limb. First, the most striking change in growth cone behavior in the limb is the cessation of proximal growth and initiation of circumferential extension of processes upon encountering the trochanter-coxa boundary region (Bentley and Caudy, 1983; Caudy and Bentley, 1987). This could be because the band of epithelial cells within the trochanter promotes circumferential growth, or because the cells constituting the trochanter-coxa boundary and the region just proximal to it are nonpermissive or aversive for growth cone migration, or both. The extension of many axon branches across the trochan-

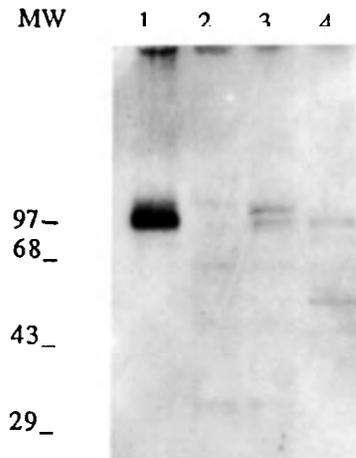


Figure 8. Western Blot Analysis of Fasciclin IV Protein

Western blot incubated with a polyclonal antibody directed against a fasciclin IV bacterial fusion protein using the carboxy-terminal portion of fasciclin IV (see Experimental Procedures). Lane 1, fasciclin IV affinity purified from grasshopper membrane lysates and used for microsequencing. Lane 2, control S2 cell membranes. Lane 3, S2 cell membranes from a line transfected with the fasciclin IV cDNA under the control of the metallothionein promoter and induced with copper ions. The presence of two fasciclin IV bands in lane 3 may reflect differential modifications that occur in S2 cells. Lane 4, grasshopper embryonic membranes (40%-60% of development). The lower molecular weight bands in this lane are most likely the result of proteolysis.

ter-coxa boundary following treatment with MAb 6F8 suggests that the trochanter-coxa boundary cells, which do not express fasciclin IV, are not aversive or nonpermissive. Thus the change in behavior at the boundary appears to be due to the ability of fasciclin IV-expressing epithelial cells to promote circumferential extension of processes from the Ti1 growth cones.

Second, treatment with MAb 6F8 results in frequent defasciculation of the axons of the two Ti1 neurons and in formation of abnormal multiple axon branches within the trochanter over fasciclin IV-expressing epithelial cells. Previous studies have shown that treatment with antibodies against ligands expressed on nonneural substrates (Landmesser et al., 1988), or putative competitive inhibitors of substrate ligands (Wang and Denburg, 1992) can promote defasciculation and increased axon branching. Our results suggest that Ti1 axon-axon fasciculation and axon branching also are strongly influenced by interactions with substrate ligands and that fasciclin IV appears to be a component of this interaction within the trochanter.

Third, despite the effects of MAb 6F8 on axon branching, and on crossing the trochanter-coxa boundary, there remains a pronounced tendency for branches to grow ventrally both within the trochanter and within the distal region of the coxa. Consequently, all signals that can promote ventral migration of the growth cones have not been blocked by MAb 6F8

treatment. Antibody treatment may have a threshold effect in which ventral growth-directing properties of fasciclin IV are more robust and less incapacitated by treatment than other features. Alternatively, guidance information promoting ventral migration may be independent of fasciclin IV. Resolving some of these issues will require future time-lapse video experiments to determine how the abnormal pathways we observe actually form.

These results suggest that fasciclin IV functions as a guidance cue for the Ti1 growth cones just distal to the trochanter-coxa boundary and is required for these growth cones to stop proximal growth and spread circumferentially. It appears as if a ventralizing tendency is not blocked in these experiments. The simplest model to explain the function of fasciclin IV in Ti1 pathway formation is that interactions between an unidentified receptor-ligand on the Ti1 growth cones and fasciclin IV on the surface of the band of epithelial cells results in changes in growth cone morphology and subsequent reorientation. Fasciclin IV could elicit this change in growth cone morphology and orientation via regulation of adhesion, by a signal transduction function, or by a combination of the two.

Fasciclin IV is not the only molecule to be expressed in circumferential bands in the grasshopper limb bud. Annulin (initially termed Epi-1; Taghert et al., 1982) is a membrane-associated cytoplasmic protein that is expressed in several bands in the developing limb bud (Bastiani et al., 1992; Singer et al., 1992). One of these bands comprises the distal cells of the coxa. When the morphological epithelial invagination that demarcates the trochanter-coxa boundary becomes evident at about the 35% stage of development (about 2% after the Ti1 axons reach the boundary region), the epithelial cells underlying the invagination are seen to be the distal-most tier of annulin-expressing cells (Singer et al., 1992). These cells appear to correspond to the coxal band of DSS-8 antigen-expressing cells in the cockroach embryo (Norbeck and Denburg, 1990, 1991). The DSS-8 antigen, a 164 kd membrane protein, is also expressed on a subset of axon pathways in the embryonic cockroach CNS that is different from the subset of pathways in the grasshopper embryonic CNS that express fasciclin IV (Wang et al., 1992). A third set of epithelial bands, which extend across limb segment boundaries and which express glycosyl-phosphatidylinositol-anchored alkaline phosphatase, also have been identified in the grasshopper embryo (Chang, Zachow, and Bentley, unpublished data).

Previous biochemical and molecular manipulations of the insect limb bud in culture have also resulted in the formation of aberrant Ti1 pathways, but none of these experiments displays the kind of spatial specificity described here. Treatment with phosphatidylinositol-specific phospholipase C results in a variety of pathway defects in the grasshopper embryo that include distal axon extension, trochanter-coxa boundary crossing by one or both Ti1 axons, towing of the

Ti1 cell bodies across the tibia-femur boundary, and defasciculation of the Ti1 axons (Chang et al., 1992). This treatment results in distinctive guidance phenotypes at the trochanter-coxa boundary and more distally in the limb. Presumably, these multiple phenotypes reflect the removal of several glycosyl-phosphatidylinositol-anchored cell surface molecules that are important for pathway formation.

Addition of glycosaminoglycans also results in the perturbation of the Ti1 pathway in the cockroach limb bud (Wang and Denburg, 1992); the most common defects observed in these experiments are defasciculation and disorientation of the axons in this pathway. Endogenous glycosaminoglycans are distributed throughout the basal lamina of the limb bud. Taken together, these results suggest a role for glycosaminoglycans in pathway maintenance and/or formation.

Evidence that fasciclin I is involved in the fasciculation of the Ti1 axons in the limb bud was reported by Jay and Keshishian (1990), who used a novel method referred to as chromophore-assisted laser inactivation (CALI). However, it is difficult to reconcile the CALI-induced defasciculation of sensory axons in the grasshopper embryo with the observation that a protein null mutation in the fasciclin I gene in *Drosophila* on its own does not display gross defects in axon fasciculation in the developing PNS (Elkins et al., 1990b). Perhaps fasciclin I plays more of a redundant role in axon fasciculation in *Drosophila* as compared with the grasshopper PNS. Alternatively, however, CALI might alter the function of several proteins complexed together or, by altering the extracellular domain of a molecule, lead to the activation of its cytoplasmic function.

What is unique about the results presented in this paper is that, using antibodies to block a single molecule, defects are observed in the Ti1 pathway, and most important, these defects are selective for a specific portion of the pathway that correlates with the normal expression of that molecule. The correlation of the location of the epithelial band of fasciclin IV expression and the defect in the Ti1 pathway in this region encourages us that these experimental results are revealing an important guidance function for this molecule.

Antibody blocking experiments have a potential problem in that, instead of simply blocking a molecule's function, the antibodies used might actually trigger some form of signal transduction (Schuch et al., 1989). This seems less likely in the fasciclin IV experiments described here, simply because fasciclin IV is not itself expressed on the surface of the growth cones whose behavior is altered by the treatment. Nevertheless, it is formally possible that the antibodies against this molecule might in some way alter the function of another epithelial molecule that itself actually guides the Ti1 growth cones. This sort of scenario is generic to most such antibody blocking experiments. This might best be resolved in the future by genetic analysis of the fasciclin IV homolog in *Dro-*

sophila, if in fact one exists. For example, in the case of fasciclin II, the basic result of the antibody blocking experiments in grasshopper (Harrelson and Goodman, 1988) have been confirmed by genetic analysis in *Drosophila* (Grenningloh et al., 1991; Fetter et al., unpublished data). Given this caveat, the experiments described here provide the most compelling evidence to date in this model system for an identified molecule (fasciclin IV) being involved in a specific aspect of the guidance of the pioneering growth cones.

Experimental Procedures

Immunocytochemistry

Grasshopper embryos were obtained from a colony maintained at the University of California, Berkeley, and staged by percentage of total embryonic development (Bentley et al., 1979). Embryos were dissected in phosphate-buffered saline (PBS), fixed for 40 min in PEM-FA (0.1 M PIPES [pH 6.95], 2.0 mM EGTA, 1.0 mM MgSO₄, 3.7% formaldehyde), washed for 1 hr with three changes in PBT (1 × PBS, 0.5% Triton X-100, 0.2% bovine serum albumin), blocked for 30 min in PBT with 5% normal goat serum, and incubated overnight at 4°C in primary antibody. PBSap (1 × PBS, 0.1% saponin, 0.2% bovine serum albumin) was used in place of PBT with MAb 8C7. Antibody dilutions were as follows: MAb 6F8, 1:1; polyclonal antisera directed against a fasciclin IV bacterial fusion protein (#98-3), 1:400; MAb 8C7, 1:4; MAb 8C6, 1:1. The embryos were washed for 1 hr in PBT with three changes, blocked for 30 min, and incubated in secondary antibody for at least 2 hr at room temperature. The secondary antibodies were HRP-conjugated goat anti-mouse and anti-rat IgG (Jackson Immunoresearch Lab), and were diluted 1:300. Embryos were washed in PBT for 1 hr with three changes and then reacted in 0.5% diaminobenzidine in PBT. The reaction was stopped with several washes in PBS, and the embryos were cleared in a glycerol series (50%, 70%, 90%), mounted, and viewed under Nomarski or bright-field optics. For double-labeled preparations, the first HRP reaction was done in PBT containing 0.06% NiCl₂, followed by washing, blocking, and incubation overnight in the second primary antibody. The second antibody was visualized with a diaminobenzidine reaction as described above. Embryos cultured in the presence of MABs were fixed and incubated overnight in goat anti-HRP (Jackson Immunoresearch Labs) conjugated to RITC (Molecular Probes), washed for 1 hr in PBT with three changes, mounted in 90% glycerol, 2.5% DABCO (Polysciences), and viewed under epifluorescence. S2 cells were stained with polyclonal sera #98-3 diluted 1:400 and processed as described previously (Snow et al., 1989).

MAB Blocking Experiments

To test for functional blocking, MAB reagents were prepared as follows. Hybridoma supernatant was brought to 20% with H₂O-saturated NH₄SO₄, incubated in ice for 1 hr, and spun at 15,000 × g at 4°C for 20 min. The supernatant was brought to 56% with H₂O-saturated NH₄SO₄, incubated overnight at 4°C, and spun as above. The pellet was resuspended in PBS using approximately 1/40 volume of the original hybridoma supernatant (often remaining a slurry) and dialyzed against 1 × PBS overnight at 4°C with two changes. This reagent is referred to as "concentrated hybridoma supernatant." Purified IgG was obtained by using the ImmunoPure Plus Immobilized Protein A IgG Purification Kit (Pierce) to isolate IgG from the concentrated hybridoma supernatant. Fab fragments were obtained using the ImmunoPure Fab Preparation Kit (Pierce) from the previously isolated IgGs. For blocking experiments, each reagent was diluted into freshly made supplemented RPMI culture media (O'Connor et al., 1990) and dialyzed overnight at 4°C against 10 vol of the same culture media. Dilutions were as follows: concentrated hybridoma supernatant, 1:4; purified IgG, 150 µg/ml; Fab, 75 µg/ml.

Embryos for culture experiments were carefully staged to be-

Table 1. Summary of Experimental Observations of Ti1 Pioneer Pathways after Culturing in the Presence of MAB 6F8, 3B11, or 4D9

Experiment	Antibody	Total Observations	Abnormal Pathways	% Abnormal
1	6F8-Sup	92	41	45
	3B11-Sup	39	4	10
	4D9-Sup	20	1	5
2	6F8-Sup	45	21	47
	3B11-Sup	26	1	4
3	6F8-Sup	62	26	42
	3B11-Sup	40	6	15
4	6F8-IgG	105	33	31
	6F8-Fab	77	41	53
	3B11-IgG	59	5	9
	3B11-Fab	66	9	14

MAB 6F8 is directed against fasciclin IV; MAB 3B11, against fasciclin I; MAB 4D9, against the engrailed protein. Sup, concentrated hybridoma supernatant.

tween 31% and 32% of development. As embryos in each clutch typically differ by less than 1% of embryonic development from each other, the growth cones of the Ti1 neurons at the beginning of the culture period were located approximately in the mid-femur, well distal to the trochanter-coxa segment boundary. From each clutch, at least two limbs were filleted and the Ti1 neurons were labeled with the lipophilic dye Dil (Molecular Probes) as described (O'Connor et al., 1990) in order to confirm the precise location of the Ti1 growth cones. Prior to culturing, embryos were sterilized and dissected (Chang et al., 1992). The entire amnion and dorsal membrane were removed from the embryo to ensure access of the reagents during culturing. Embryos were randomly divided into groups and cultured in one of the blocking reagents described above. Cultures were incubated with occasional agitation at 30°C for 30 hr. At the end of the culture period, embryos were fixed and processed for analysis as described above in immunocytochemistry.

For each culture experiment, the scoring of the Ti1 pathway in each limb was confirmed independently by a second observer. There was no statistically significant variation between the two observers. Limbs from MAB cultured embryos were compared with representative normal limbs from non-MAB cultured embryos and were scored as abnormal if any major deviation from the normal Ti1 pathway was observed. The Ti1 pathway was scored as abnormal for one or more of the following observed characteristics: defasciculation for a minimum distance of approximately 25 µm anywhere along the pathway; multiple axon branches that extended ventrally within the trochanter; the presence of one or more axon branches that crossed the trochanter-coxa boundary dorsal to the Cx1 cells, but then turned ventrally in the coxa and contacted the Cx1 cells; the presence of axon branches that crossed the trochanter-coxa segment boundary, did not turn ventrally, but continued proximally toward the CNS; and failure of ventrally extended axons within the trochanter to contact and reorient proximally to the Cx1 cells. For each MAB tested, the data are presented as a percentage of the abnormal Ti1 pathways observed. The raw data are presented in Table 1.

Protein Affinity Purification and Microsequencing

Grasshopper fasciclin IV was purified by passing crude embryonic grasshopper lysate (Bastiani et al., 1987) over an Affi-Gel 15 column (Bio-Rad) conjugated with the MAB 6F8. Protein was eluted with 50 mM DEAE (pH 11.5), 0.1% lauryldimethylamine oxide (Calbiochem), 1 mM EDTA. Protein was then precipitated, denatured, modified at cysteines, and digested with either trypsin or Lys-C (Boehringer Mannheim). Individual peptides were resolved by reverse-phase high pressure liquid chromatography

and microsequenced (Applied Biosystems 4771 Microsequencer) using standard chemistry.

PCR Methods

cDNA to poly(A)⁺ RNA from grasshopper embryos at 40%–50% of development was prepared (Sambrook et al., 1989). PCR was performed using Perkin Elmer Taq polymerase, (Saiki et al., 1988) and partially degenerate (based on grasshopper codon bias) oligonucleotides in both orientations corresponding to a portion of the protein sequence of several fasciclin IV peptides as determined by microsequencing. These oligonucleotides were designed so as not to include all of the peptide-derived DNA sequence, leaving a remaining 9–12 bp that could be used to confirm the correct identity of amplified products. All possible combinations of these sequences were tried. Forty cycles were performed; the parameters of each cycle are as follows: 96°C for 1 min; a sequentially decreasing annealing temperature (2°C per cycle, starting at 65°C and ending at 55°C for the remaining 35 cycles) for 1 min; and 72°C for 1 min. Reaction products were cloned into the SmaI site of M13 mp10 and sequenced. Two products, 1074 bp and 288 bp in length, contained DNA 3' to the oligonucleotide sequences that encoded the additional amino acid sequence of the fasciclin IV peptide from which the oligonucleotides were derived. These two fragments have one end in common, and the oligonucleotides used to amplify them correspond to the amino acid sequences MYVQFGEE and MDEAVPAF (fasciclin IV residues 29–386) and HTLMDEA and KNYVVRMDG (fasciclin IV residues 376–472).

cDNA Isolation and Sequence Analysis

Both PCR products were used to screen 1×10^6 clones from a grasshopper embryonic cDNA library (Snow et al., 1988). Twenty-one clones that hybridized to both fragments were recovered, and a single 2600 bp clone was sequenced using the dideoxy chain termination method (Sanger et al., 1977) and Sequenase (US Biochemical Corp.). Templates were made from M13 mp10 vectors containing inserts generated by sonication of plasmid clones. One cDNA was completely sequenced on both strands using oligonucleotides and double strand sequencing of plasmid DNA (Sambrook et al., 1989) to fill gaps. Two additional cDNAs were analyzed by double strand sequencing to obtain the 3' 402 bp of the transcript. All 3 cDNAs were used to construct a plasmid containing the entire transcript. The complete transcript sequence is 2860 bp in length with 452 bp of 5' and 217 bp of 3' untranslated sequences containing stop codons in all reading frames. The predicted protein sequence was analyzed using the FASTDB and BLASTP programs (Intelligenetics). The fasciclin IV open reading frame unambiguously contains 10 of the 11 peptide sequences determined by microsequencing the fasciclin IV trypsin and Lys-C peptides.

Generation of Polyclonal Antibodies from Bacterial Fusion Proteins

Bacterial trpE fusion proteins were constructed using pATH (Koerner et al., 1991) vectors, three restriction fragments encoding extracellular sequences, and one fragment (770 bp HindIII-EcoRI, which includes amino acids 476–730) encoding both extracellular and intracellular sequences (designated #98-3). Fusion proteins were isolated by making an extract of purified inclusion bodies (Spindler et al., 1984), and rats were immunized with ~70 µg of protein emulsified in RIBI adjuvant (Immunochem Research). Rats were injected at 2 week intervals, and serum was collected 7 days following each injection. Sera were tested histologically on grasshopper embryos at 45% of development. Construct #98-3 showed a strong response and exhibited a staining pattern identical to that of MAb 6F8. Two of the extracellular constructs responded weakly but also showed the fasciclin IV staining pattern. All preimmune sera failed to stain grasshopper embryos.

S2 Cell Transfections, Aggregation Assays, and Western Analysis

A restriction fragment containing the full-length fasciclin IV

cDNA was cloned into pRmHa-3 (Bunch et al., 1988) and cotransformed into *Drosophila* S2 cells (Schneider, 1972) with the plasmid pPC4 (Jokerst et al., 1989), which confers α-amanitin resistance. S2 cells were transformed using Lipofectin Reagent and the recommended protocol (Bethesda Research Laboratories) with minor modifications. All other S2 cell manipulations are essentially as described (Snow et al., 1989), including adhesion assays. Fasciclin IV expression in transformed cell lines was induced for adhesion assays and histology by adding CuSO₄ to 0.7 mM and incubating for at least 48 hr. Northern analysis confirmed transcription of fasciclin IV, and surface-associated staining of the S2 cells with polyclonal serum #98-3 strongly suggests that fasciclin IV is being transported to the cell surface. Preparation of membranes from S2 cells and from grasshopper embryos, polyacrylamide gel electrophoresis, and Western blot analysis were performed as previously described (Elkins et al., 1990b), except that signal was detected using the enhanced chemiluminescence immunodetection system kit (Amersham). The amount of protein per lane in each sample loaded was as follows: fasciclin IV, ~5 ng; S2 cell membranes, 40 µg; grasshopper membranes, 80 µg. The amounts of protein loaded were verified by Ponceau S staining of the blot prior to incubation with the antibody.

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