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Isoforms Reveals Neuronal–Glial Interactions during Peripheral Nerve Growth

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During the formation of the insect peripheral nervous system (PNS), the cell adhesion receptor fasciclin II has been shown to play a prominent role in axonal fasciculation and synapse formation during motor neuron outgrowth. In the moth *Manduca,* fasciclin II (MFas II) is expressed both as a transmembrane isoform (TM-MFas II) and a glycosyl phosphatidylinositol-linked isoform (GPI-MFas II). By using RNA and antibody probes, we have shown that these two isoforms are expressed in nonoverlapping patterns: TM-MFas II is expressed exclusively by neurons and becomes localized to their most motile regions, while GPI-MFas II is expressed primarily by the glial cells that ensheath the peripheral nerves. This cell-type specificity of expression allowed us to monitor the nature of neuronal–glial interactions during PNS development. The outgrowth of TM-MFas II-positive axons in many regions preceded the arrival of GPI-MFas II-expressing glial processes that enwrapped them. In a few key locations, however, GPI-MFas II-positive glial cells differentiated before the arrival of the first axons and prefigured their subsequent trajectories. Prior inhibition of GPI-MFas II expression disrupted the subsequent outgrowth of axons at these locations but not elsewhere in the PNS. Our results suggest that the two isoforms of MFas II play distinct roles with respect to cellular motility and nerve formation.

Key Words: **growth cone; fasciculation; adhesion; insect; *Manduca sexta.***

INTRODUCTION

The formation of the nervous system involves a complex interplay between neurons and glia that is crucial to the differentiation and survival of both cell types. In the developing central nervous system (CNS) of vertebrates and invertebrates, glial cells prefigure many of the pathways taken by migrating neurons and growing axons (Jacobs and Goodman, 1989a; Klämbt *et al.,* 1991; Rakic, 1971; Silver, 1984), and perturbation of these interactions results in significant pathfinding errors (Granderath and Klämbt, 1999; Hatten, 1999; Hidalgo and Booth, 2000; Mason and Sretavan, 1997). Neuronal-specific proteins have also been shown to induce changes in glial differentiation that are critical to their normal function (Rio *et al.,* 1997; Zheng *et al.,* 1996). In the vertebrate peripheral nervous system (PNS), the roles of neurons and glia are reversed with respect to guidance: growing axons act as scaffolds for migrating Schwann cell precursors and maintain their survival (Bhattacharyya *et al.,* 1994; Mirsky and Jessen, 1999), although later the ensheathing Schwann cells promote neuronal survival, as well as the maturation of the perineurial sheath and stabilization of the neuromuscular junction (Davies, 1998; Jessen and Mirsky, 1999; Sanes and Lichtman, 1999). Guidance interactions between neurons and glial cells in the PNS of insects are more diverse. Motor axons initially orient along identified sets of glial cells when leaving the CNS (Bastiani and Goodman, 1986; Klämbt and Goodman, 1991) but then may be guided by a variety of substrates, including both ectodermal and mesodermal cell types and components of the extracellular matrix (Anderson and Tucker, 1989; Rajan and Denburg, 1997; Younossi-Hartenstein and Hartenstein, 1993). While the pathfinding of peripheral axons is typically thought to be independent of glial activity, in some instances, glial scaffolds prefigure neuronal tracts before the first axons arrive (Carr and Taghert, 1988; Gorczyca *et al.,* 1994).

The mechanisms that govern these interactions are poorly understood but are thought to involve a combination

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of diffusible, matrix, and membrane-associated cues, including a variety of cell-adhesion molecules (CAMs). Among the CAMs identified in the insect nervous system, fasciclin II has been particularly well characterized for its role in mediating neuron–neuron interactions. Fasciclin II was first identified in the grasshopper nervous system as a neuronal glycoprotein capable of promoting intercellular adhesion (Bastiani *et al.*, 1987; Harrelson and Goodman, 1988; Snow *et al.*, 1988). Subsequent analysis in *Drosophila* demonstrated that fasciclin II helps mediate axonal fasciculation and growth cone guidance in the embryonic CNS and PNS (Grenningloh *et al.*, 1991; Lin *et al.*, 1994; Lin and Goodman, 1994). Fasciclin II is a member of the immunoglobulin-related superfamily of CAMs (IgSFs) with structural similarity to the vertebrate receptor NCAM and the *Aplysia* receptor apCAM (Grenningloh *et al.*, 1990; Mayford *et al.*, 1992). Like NCAM and apCAM, the extracellular portion of fasciclin II contains five immunoglobulin domains and two fibronectin type III domains. The NCAM/apCAM/fasciclin II subfamily is also unique in that each of these CAMs can be expressed as alternative isoforms with divergent membrane attachments: whereas most IgSFs occur either as transmembrane proteins or as membrane-associated proteins anchored via a glycosyl phosphatidylinositol (GPI) linkage, fasciclin II, NCAM, and apCAM are expressed in both forms as a result of alternative mRNA splicing (Grenningloh *et al.*, 1991; Mayford *et al.*, 1992; Nguyen *et al.*, 1986). Aside from its role in axon outgrowth, fasciclin II has been shown to participate in the control of synaptic stabilization and growth, activity-dependent plasticity, and proneural gene expression (Davis *et al.*, 1997; Garcia-Alonso *et al.*, 1995; Goodman *et al.*, 1997). However, while considerable work has been performed on the functions of transmembrane isoforms of fasciclin II, the role of the GPI-linked isoform and the relative contributions of the different isoforms to specific developmental processes have remained unexplored.

We recently cloned fasciclin II from the moth, *Manduca sexta*, and demonstrated that it plays an essential role in guiding the migration of neurons and glial cells within the embryonic enteric nervous system (ENS; Wright *et al.*, 1999). Using probes that selectively recognize the transmembrane (TM) and GPI-linked isoforms of *Manduca* fasciclin II (MFas II), we showed that TM-MFas II was expressed exclusively by motile neurons, while GPI-MFas II was primarily expressed by glial cells (Wright and Copenhaver, 2000). In addition to these distinct expression patterns, each isoform of MFas II appears to mediate specific functions. Inhibition of TM-MFas II expression in the ENS demonstrated that it helps promote neuronal migration and axon outgrowth. In contrast, removal of GPI-MFas II had no direct effect on neuronal motility but disrupted intercellular adhesivity among cells expressing this isoform. We have now used a combination of riboprobes and antibodies to investigate whether isoform-specific expression patterns of MFas II occur in the embryonic CNS and PNS. Unexpectedly, we have found that the pattern of MFas II in the CNS (which is similar to the patterns of fasciclin II reported for other insects) is entirely due to the expression of TM-MFas II. In contrast, the expression of GPI-MFas II is largely confined to glial populations associated with the developing peripheral nerves. By selective manipulation of GPI-MFas II expression in embryonic culture, we have investigated the potential role of these peripheral glial cells with respect to axonal guidance in the PNS. Our results indicate that, in some instances, GPI-MFas II expression by specific glial cells is relevant to axonal pathfinding, but, in other instances, it is not.

MATERIALS AND METHODS

Embryos were collected from a stable *Manduca sexta* colony as previously described (Copenhaver and Taghert, 1989a). When maintained at 25°C, embryos complete development in approximately 100 h (1 h is equivalent to 1% of development). Sets of internal and external markers characterized in previous work were used to identify particular stages of development (Copenhaver and Taghert, 1989a; Dorn *et al.*, 1987). For studies employing whole-mount immunoanalysis and *in situ* hybridization histochemistry, embryos were restrained in Sylgard-coated chambers and opened along a lateral anterior–posterior line, flattened, and pinned in place. This dissection permitted visualization of the developing CNS and the complete innervation of the lateral body wall musculature on one side (one intact hemisegment per segment).

For tissue fixation, we used a modified version of our original protocol (Horgan *et al.*, 1994) to enhance signal sensitivity while reducing tissue degradation. PBS-Triton (1.8 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 175 mM NaCl, 0.8% Triton X-100) was diluted 1:1 with stock (37.6%) formaldehyde solution (Mallinckrodt) and adjusted to pH 9.5 with 1 N NaOH. The tissue was fixed for 15 min followed by 3× 5-min washes in PBS-Triton. Immunohistochemical staining was performed as previously described (Horgan *et al.*, 1994). A mouse polyclonal antibody recognizing both MFas II isoforms and two guinea pig antisera raised against unique sequences within each isoform were characterized by immunoblot analysis (Wright and Copenhaver, 2000) and used for whole-mount immunohistochemistry. Because both isoform-specific antibodies were generated in the same host species, we were unable to perform simultaneous double labeling with these two antibodies. Instead, we visualized the TM-MFas II-specific antibody with alkaline phosphatase (to generate a purple reaction product) and the mouse antibody against both isoforms with a horseradish peroxidase reaction (generating a brown reaction product; Horgan *et al.*, 1994). Isoform-specific riboprobes complementary to the mRNA sequences encoding each MFas II isoform were characterized in Northern blots and used for whole-mount *in situ* hybridization histochemistry, as previously described (Wright and Copenhaver, 2000).

For culture experiments, embryos were prepared essentially as described (Wright *et al.*, 1998), but, because the stages investigated in these studies were much younger than in our previous work (25–45% of development), the following modifications were introduced: embryos at 25–28% of development were dechorionated in sterile medium using fine forceps, but the inner extraembryonic membranes were kept intact. A small opening was then made in these membranes, permitting the insertion of a glass electrode attached to a Hamilton syringe with polyethylene tubing. Approxi-

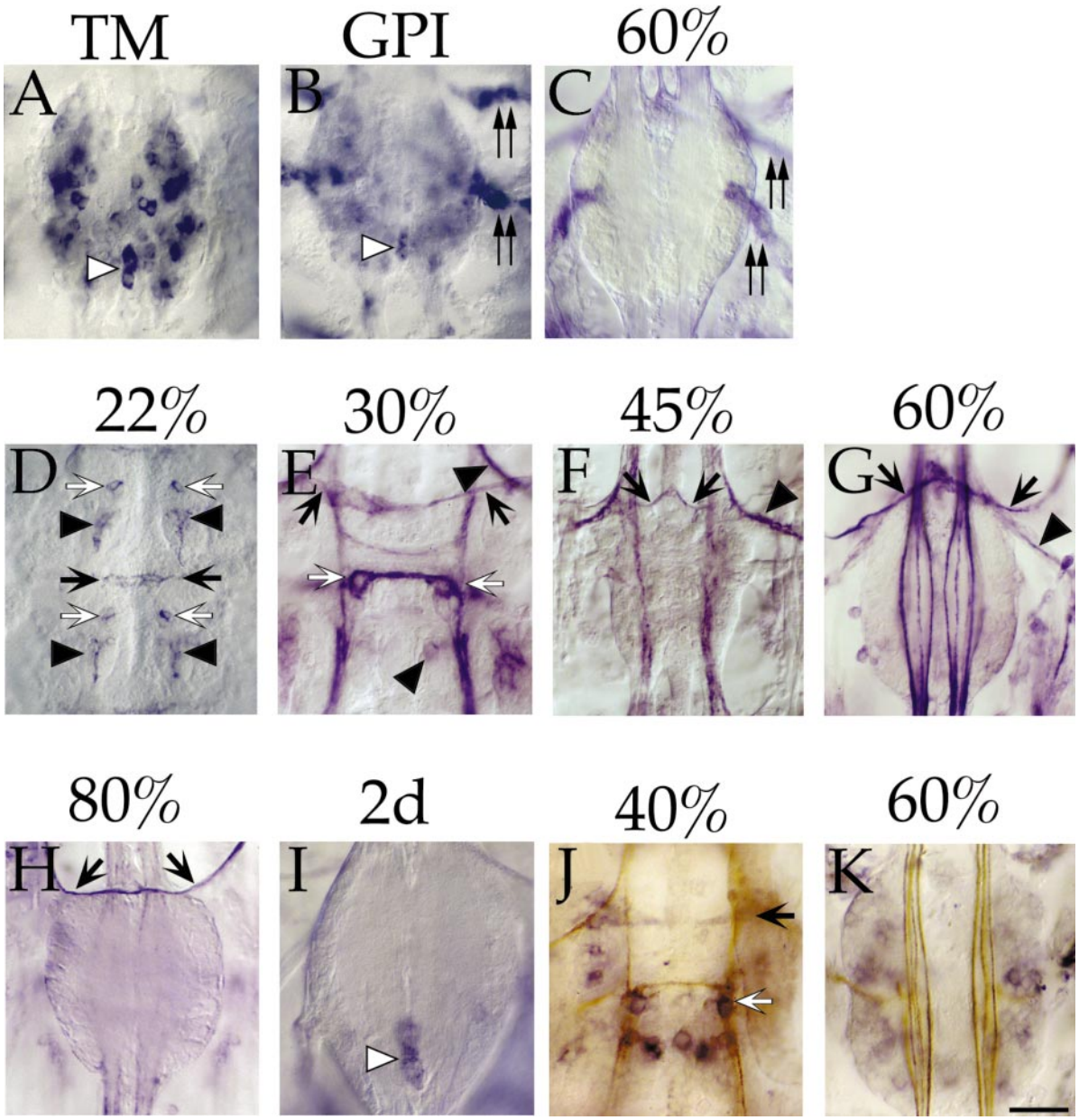

FIG. 1. Developmental expression of MFas II isoforms in the embryonic CNS. Each panel shows a representative abdominal ganglion (anterior is towards the top of the page). (A) *In situ* hybridization histochemistry using a TM-MFas II-specific riboprobe stained a large number of neuronal somata in an abdominal ganglion at 60% of development (white arrowhead indicates a set of UM cells). (B) 60% ganglion reacted with a GPI-MFas II-specific probe produced much weaker staining in CNS neurons but robust staining in glial cells surrounding each of the peripheral nerves leaving the ganglion (black arrows; white arrowhead indicates similar UM cells as in A). (C) Immunohistochemical labeling with a GPI-MFas II-specific antibody produced no detectable staining within the CNS at 60% of development (or any other time during embryogenesis) but did stain the peripheral glial cells (arrows). (D—I) Immunohistochemical staining with a TM-MFas II-specific antibody labeled specific sets of cells and processes in a stage-specific manner. (D) TM-MFas II expression at 22% in the aCC/pCC cell cluster (black arrowheads), in a putative set of RP neurons (white arrows), and in a set of nonneural cells that prefigure the transverse nerve (black arrows). (E) TM-MFas II expression at 30% is still apparent in a few somata (lower arrowhead indicates aCC/pCC cell cluster) but was increasingly localized to their growing axons (upper arrowhead indicates fascicle containing the aCC axon from the next anterior ganglion exiting the dorsal nerve). Strong labeling was also seen in longitudinal fascicles running through the ganglion and in specific commissural tracts (white arrows indicate axons of the putative RP neurons shown in D). Black arrows indicate persistent staining in the non-neural cells forming the transverse nerve. (F) At 45%, persistent TM-MFas II staining was seen in the longitudinal fascicles and axons exiting the dorsal nerve (black arrowhead), but all commissural staining had disappeared. Staining was also visible in the bilateral branches of the spiracular neurons entering the transverse nerve (arrows). (G) At 60%, eight separate TM-MFas II-positive longitudinal fascicles could be distinguished within each hemisegment; axons within the transverse nerve (arrows) and dorsal nerve (arrowhead) also remained immunopositive. (H) By 80%, all TM-MFas II immunoreactivity had disappeared from the CNS except for a bilateral fascicle coursing through the transverse nerve (arrows). (I) By 2 days posthatch, TM-MFas II expression had reappeared in a pair of UM cells (white arrowhead). (J, K) Ganglia at 40 and 60% of development double-stained by *in situ* hybridization histochemistry for TM-MFas II mRNA (purple) and by immunohistochemistry for TM-MFas II protein (brown); arrows in J label the same structures as in E. Scale = 25 μM.

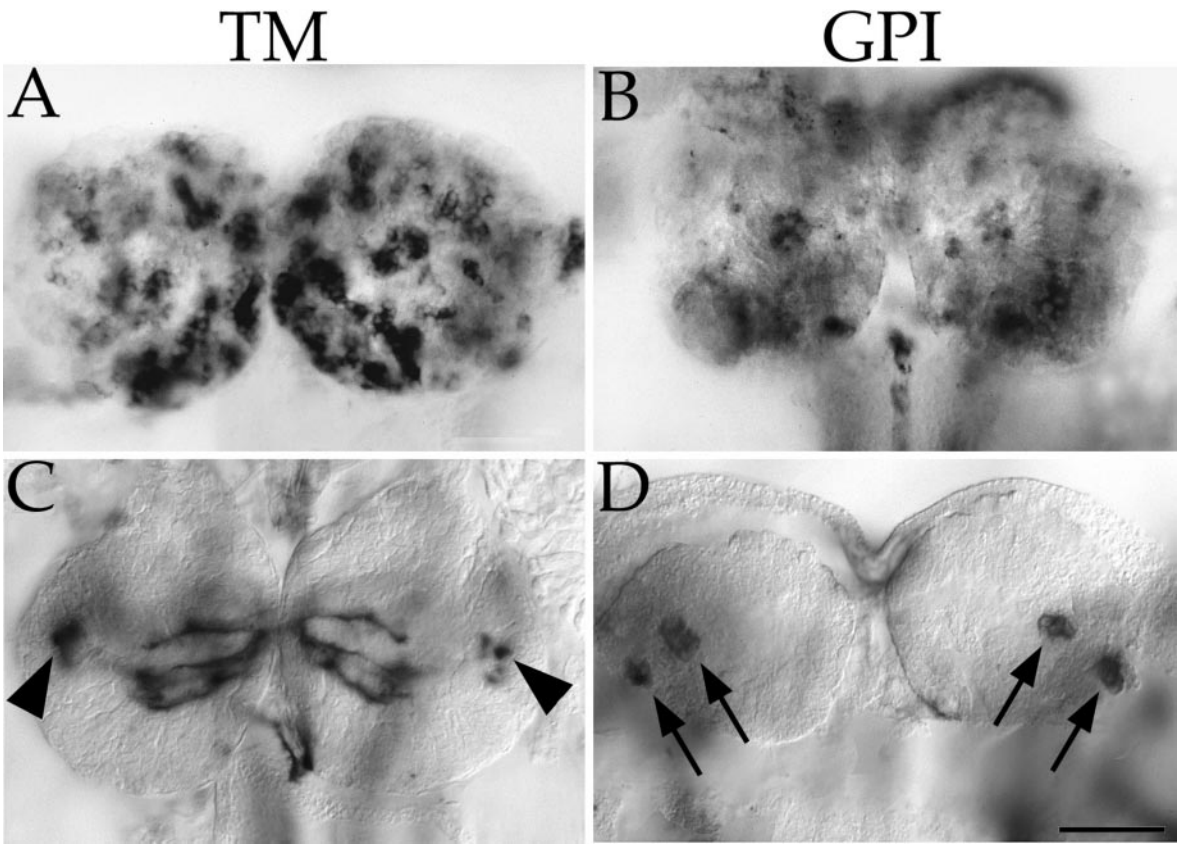

FIG. 2. MFas II isoform expression in the embryonic brain at 60% of development. (A, B) *In situ* hybridization histochemical labeling for TM-MFas II mRNA and GPI-MFas II mRNA, respectively. (C, D) Immunostaining for the same isoforms. Arrowheads in C show labeled fascicles in profile that run between the brain and the subesophageal ganglion (located ventral to the brain). Arrows in D indicate two bilaterally paired clusters of putative glial precursor cells. Scale = 25 μM.

mately 10 μl of either control or experimental solutions were then introduced into the vicinity of the developing nervous system. Embryos were cultured overnight at 37°C prior to fixation and immunohistochemical staining. Phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Boehringer and diluted in sterile medium.

Oligodeoxynucleotides (ODNs) were prepared as phosphorothioate-modified 20-mers (Synthegen, Houston, TX) derived from the mRNA sequence for GPI-MFas II. Three distinct regions within the proximal 3′ untranslated region (UTR) of GPI-MFas II mRNA were selected based on their predicted lack of strong secondary structure (Fenster *et al.*, 1994). Specific 20-mers were then selected from within these regions by using the following criteria: predicted hybridization to only one site within the GPI-MFas II sequence, GC content $<$ 50%, Tm values $>$ 55°C, lack of self-complementarity or poly-base sequences, and absence of significant sequence identity with other known genes (determined using the NCBI BLAST program; Altschul *et al.*, 1990). The following ODNs were constructed: GPI-ODN-1, corresponding to nt 2187–2207; GPI-ODN-2, corresponding to nt 2398–2418; and GPI-ODN-3, corresponding to nt 2490–2510. Sense and scrambled sequence ODNs were used as controls, as previously described (Copenhaver and Wright, 2000). The ODNs were HPLC-purified, filtered on a G25 Sephadex column, and then resuspended in sterile defined saline at 2 μg/μl. Working dilutions (0.1 μg/μl) were prepared with serum-free culture medium and delivered to cultured embryos as described above. After overnight incubation at 37°C, the preparations were fixed and immunostained with a monoclonal antibody recognizing the extracellular domain shared by both isoforms of MFas II. Each experimental manipulation was repeated a minimum of three times by using replicate sets of identically staged embryos. Analysis of axon outgrowth in cultured preparations was performed by using photomicrographic and *camera lucida* techniques.

RESULTS

Isoform-Specific Expression of MFas II in the Central Nervous System

Previous work by Goodman and colleagues (1997) showed that fasciclin II is dynamically expressed by characteristic subsets of fasciculating axons in the nervous systems of both grasshopper and fly embryos (Bastiani *et al.*, 1987; Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988). To investigate the relative contributions of different fasciclin II isoforms with respect to this expression pattern, we applied isoform-specific probes against TM-MFas II and

GPI-MFas II to the developing CNS of *Manduca* throughout embryogenesis. When embryos at ~60% of development were reacted with a digoxigenin-labeled RNA probe against TM-MFas II, a large number of cells within each segmental ganglion showed strong labeling (Fig. 1A), including a set of unpaired median (UM) cells near the posterior ventral midline (white arrowhead). In contrast, an RNA probe against GPI-MFas II produced only faint levels of staining within the CNS (Fig. 1B), although a few cells consistently stained more strongly, including a similar set of UM cells as indicated in Fig. 1A (white arrowhead). Robust levels of GPI-MFas II mRNA expression were also detected within populations of glial cells distributed along the peripheral nerves of each ganglion, demarcating the course of each nerve from its origin at the margin of the ganglion (Fig. 1B, arrows).

To determine the distribution of MFas II-related proteins in the developing nervous system, we immunostained staged embryos with either an antibody against the shared extracellular domain of MFas II (recognizing both isoforms) or antibodies against peptide sequences unique to each MFas II isoform. Although some minor differences were seen between the pattern of MFas II expression in *Manduca* and fasciclin II staining reported in fly and grasshopper (Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988), the overall pattern of developmental expression was largely conserved, involving characteristic sets of identified neurons and their processes at specific stages of development. Surprisingly, this entire pattern was recapitulated by TM-MFas II immunostaining, whereas no detectable GPI-MFas II expression was detected within the thoracic or abdominal ganglia (Fig. 1C). At 22% of development (shortly after the onset of neurogenesis), TM-MFas II expression could be detected within the aCC/pCC cell clusters (Fig. 1D, arrowheads) and on the aCC axons projecting posteriorly in each segment; these axons subsequently help pioneer the dorsal nerve (equivalent to the intersegmental nerve in other insects) of the next posterior ganglion (upper arrowhead in Fig. 1E; arrowheads in Figs. 1F and 1G). Early TM-MFas II expression was also seen more anteriorly on a bilateral pair of neurons (white arrows) that by their position and projections may correspond to a set of RP neurons (Jacobs and Goodman, 1989b; Sink and Whitington, 1991), and on cells prefiguring the future transverse nerve at the anterior boundary of each ganglion (equivalent to the segment boundary nerve in *Drosophila*; black arrow in Fig. 1D). This latter set of cells are nonneural cells that will later contribute to the "strap" structure that forms before the arrival of axons in the transverse nerve (Carr and Taghert, 1988).

Throughout the remainder of embryogenesis, the pattern of TM-MFas II expression was progressively modified as the ganglia differentiated. At 30% of development, the most robust staining was associated with a subset of longitudinal axon fascicles extending between ganglia and within a smaller number of commissural axons (Fig. 1E). Unlike other insects, the strongest commissural staining in *Manduca* was seen in the processes of a bilateral pair of neurons (the putative RP cells identified in Fig. 1D; white arrows) which extended contralateral processes across the posterior boundary of the anterior commissure. Fainter staining was also seen in another set of axons at the anterior edge of this commissure, while no stained processes were apparent in the posterior commissure. Persistent staining was also seen in the "strap" cells just anterior to each ganglion (Fig. 1E, black arrows). By 45%, TM-MFas II expression was no longer detectable in either these nonneural cells or any of the commissures, although the outgrowing axons of the spiracular motor neurons (originating from the next anterior ganglion; Carr and Taghert, 1988) had reached the developing transverse nerve and were now TM-MFas II-positive (Fig. 1F, arrows). Of note was that TM-MFas II staining appeared in these axons only after they had traversed the median nerve (running longitudinally between the two connectives) and had bifurcated out both sides of the developing transverse nerve.

TM-MFas II expression in the CNS at 60% of development was at its maximal level (Fig. 1G), including strong staining of eight separate fascicles in each hemisegment and in fascicles within the dorsal nerve (arrowhead) and transverse nerve (arrows). By 80%, most of this immunoreactivity in the CNS had declined (Fig. 1H, arrows), but the elimination of TM-MFas II from axons exiting the ganglia occurred in a proximal–distal gradient so that expression persisted within their more peripheral branches (described below). Except for the earliest stages of neurogenesis (Figs. 1D and 1E), TM-MFas II immunoreactivity was excluded from the somata of positively expressing cells and was confined to their growing axons. This localized pattern of expression was particularly evident in preparations double-labeled for TM-MFas II expression by both *in situ* hybridization and immunohistochemistry (Figs. 1J and 1K), in which the somata expressing TM-MFas II mRNA (shown in purple) were often spatially separated from their immunolabeled processes (shown in brown). TM-MFas II immunoreactivity was no longer apparent in either the CNS or PNS by the completion of embryonic development. Within 48 h, however, a new phase of TM-MFas II expression had commenced in a set of UM cell somata (Fig. 1I, white arrowhead), neuromodulatory neurons that continue to express TM-MFas II in their somata and terminal processes throughout postembryonic development (Knittel *et al.*, 2001).

Similar aspects of MFas II expression were seen in the developing brain. A large number of neurons exhibited strong levels of TM-MFas II-specific mRNA (Fig. 2A), while only a few cells expressed much lower levels of GPI-MFas II mRNA (Fig. 2B). As in the ventral ganglia, TM-MFas II protein was primarily localized to specific axonal tracts within the brain (Fig. 2C) and to a set of bilateral fascicles connecting the brain and subesophageal ganglion (arrowheads). Unlike the ganglia, GPI-MFas II protein was also present within a subset of cells in the brain (Fig. 2D, arrows). Each lobe contained two populations of four to eight clustered cells that appeared to be glial precursors,

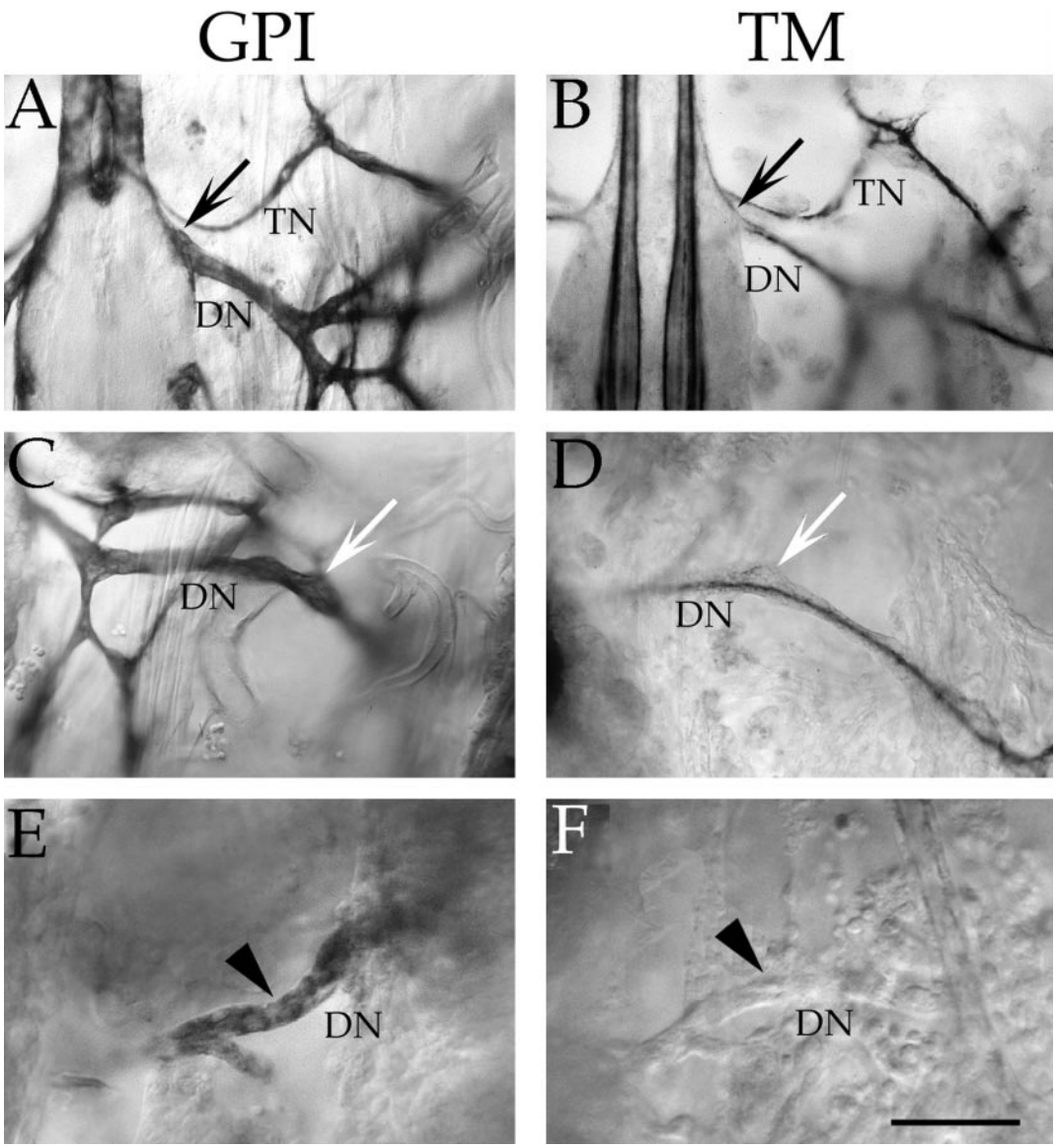

FIG. 3. MFas II isoform expression in the peripheral nervous system at 65% of development. (A) GPI-MFas II immunostaining shows strong labeling of the ensheathing glial cells surrounding all peripheral nerve branches up to their exit point from an abdominal ganglion. (B) TM-MFas II immunostaining of an identically staged ganglion shows strong labeling in the CNS (see Fig. 1) and in a subset of axon fascicles within the dorsal nerve and transverse nerve (arrows in A and B indicate similar regions of the dorsal nerve). (C) Higher magnification of a peripheral region of the dorsal nerve stained with GPI-MFas II; glial cells ensheathing all peripheral nerve branches are strongly immunopositive. (D) Similar region of the dorsal nerve shown in C, immunostained for TM-MFas II. The unstained glial sheath is clearly visible under Nomarski optics (compare position of white arrows in C and D). (E) *In situ* hybridization histochemical labeling for GPI-MFas II mRNA stains the peripheral glia of the dorsal nerve (arrowhead). (F) *In situ* hybridization labeling for TM-MFas II mRNA shows no staining associated with the peripheral nerves. Scale = 25 μM in A–D, 10 μM in E, F.

based on their size and position (P.F.C., unpublished data). GPI-MFas II immunoreactivity was first detectable in these cells at 40% of development and persisted until about 90%, by which time glial differentiation in the embryonic brain appears largely complete.

MFas II Expression in the Peripheral Nervous System

The distinction between TM-MFas II and GPI-MFas II expression in the developing peripheral nervous system was

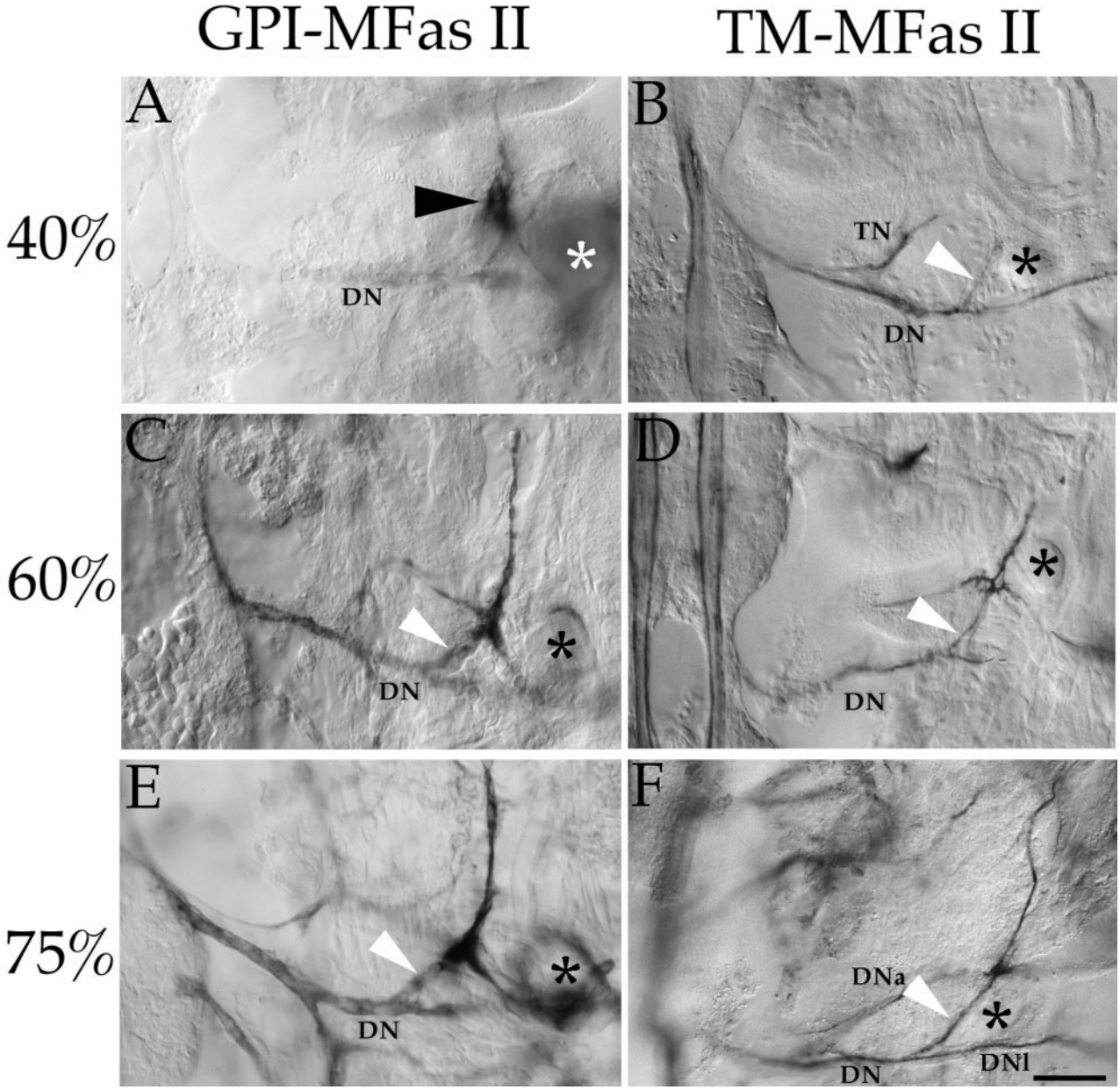

FIG. 4. GPI-MFas II-positive glial cells prefigure the trajectory of one branch of the dorsal nerve. (A) At 40% of development, a cluster of peripheral glial cells have coalesced to form a glial "bridge" (black arrowhead) anterior to the dorsal nerve and proximal to the spiracle (asterisk). (B) The first growth cones pioneering the anterior branch of the dorsal nerve (DNa branch; white arrowhead) have just begun to defasciculate from the dorsal nerve. (C) By 60%, the glial bridge has assumed its four-armed "star"-like morphology (Carr and Taghert, 1988) that serves as a scaffold for several peripheral nerves. (D) The DNa axons have reached the bridge and have grown along its anterior arm. (E, F) By 75%, GPI-MFas II-positive glial cells have ensheathed all of the branches of the dorsal nerve, including the TM-MFas II-positive axon fascicles that have grown anteriorly (the DNa branch) and those that have grown laterally (the DNl branch). White arrowheads in B–F indicate the position of the DNa branch. Scale = 20 μM.

particularly striking. GPI-MFas II immunoreactivity was absent from the central ganglia (as noted above) but prominent within the glial cells that surrounded the peripheral nerves exiting the CNS (Fig. 3A; arrow indicates origin of the dorsal nerve). In contrast, TM-MFas II immunostaining was localized to specific axon fascicles within the dorsal and transverse nerves and their peripheral branches (Fig. 3B). More distally, GPI-MFas II-positive glial cells could be

seen lining the entire surface of the dorsal nerve (Fig. 3C), while TM-MFas II expression was confined to fasciculated axons within the nerve (Fig. 3D). The unlabeled glial cells surrounding the TM-MFas II-positive axons were easily visible in Fig. 3D by using Nomarski optics (white arrow). *In situ* hybridization labeling verified that these peripheral glial cells expressed substantial levels of mRNA specific for GPI-MFas II (Fig. 3E). No detectable levels of TM-MFas II mRNA were detected in the dorsal nerve (Fig. 3F), confirming that all of the axonal TM-MFas II protein in this nerve originated from centrally located somata. These results show that, unlike the CNS, the pattern of MFas II mRNA isoform expression and protein synthesis in the PNS precisely coincide.

Since the expression of MFas II isoforms was clearly cell-type specific, we used our antibodies against TM-MFas II and GPI-MFas II to examine neuronal–glial dynamics within the developing PNS. These studies revealed two distinct modes of interaction that contributed to the differentiation of peripheral nerves. The first of these modes was best illustrated during the formation of an anterior branch of the dorsal nerve (DNa) commencing at about 40% of development (Fig. 4). A subset of TM-MFas II-positive growth cones (Fig. 4B, white arrowhead) was seen to leave the dorsal nerve and grow anteriorly to contact a cluster of nonneuronal cells that had already begun to express GPI-MFas II (Fig. 4A, black arrowhead). These cells subsequently differentiated into a glial "bridge" (Fig. 4C) that prefigured the routes taken by several peripheral nerve branches, as previously shown by Carr and Taghert (1988). Axons forming the DNa nerve branch had grown through the bridge by 60% of development (Fig. 4D) and had begun to extend towards muscles anterior to the spiracle (asterisk). By 75%, the DNa axons had reached their targets and the bridge cells had differentiated into an enveloping glial sheath (Figs. 4E and 4F). Thus, during the establishment of the DNa nerve branch, glial cells expressing GPI-MFas II formed a presumptive scaffold prior to the arrival of TM-MFas II-positive axons.

In contrast, a second mode of interaction between growing axons and peripheral glia was seen during the formation of the lateral branch of the dorsal nerve (DNl; Fig. 4F), which extended posterior to the spiracle to innervate dorsal musculature (Fig. 5). By 40% of development, TM-MFas II-positive growth cones pioneering the DNl had grown beyond the spiracle into the differentiating body wall mesoderm (Fig. 5B). Unlike the formation of the DNa branch, there was no evidence of glial cells prefiguring this pathway, either by GPI-MFas II immunolabeling or when viewed with Nomarski optics. Instead, faintly labeled glial precursors (GPI-MFas II-positive) were seen advancing along the more proximal portion of the DNl that had already formed (Fig. 5A). The somatic mesoderm had begun to differentiate into organized muscle fibers by 60% of development, and TM-MFas II-positive terminal branches of the DNl had begun to extend along these fibers (Fig. 5D). Glial cells had also grown along the DNl into the vicinity of the developing muscles but had not yet extended to the tips of the leading neuronal processes (Fig. 5C). However, by 75% of development (when initial innervation of the skeletal muscle was largely complete), glial processes had not only elaborated over all of the TM-MFas II-positive processes (thereby delineating the neuromuscular junctions), but, in several locations, had extended well beyond them, ramifying over additional regions of the peripheral muscle (compare Fig. 5E with 5F). Consequently, the resulting network of GPI-MFas II-positive glial extensions was considerably more elaborate at this stage than the TM-MFas II-positive motor neuron terminals (compare Fig. 5E with 5F).

To examine the interactions between the growing axons and glial processes in better detail, we double-labeled preparations with the anti-TM-MFas II antibody (using an alkaline phosphatase-conjugated secondary) followed by the anti-MFas II polyclonal antibody recognizing both isoforms (using an HRP-based detection system; see Materials and Methods). This strategy resulted in a compact purple labeling of the TM-MFas II-positive axon fascicles that could readily be distinguished from the more diffuse brown labeling of the GPI-MFas II-positive glial processes (Fig. 6). Unexpectedly, these preparations revealed that the neuronal and glial processes contributing to a developing nerve overlapped only partially during their initial outgrowth. At 60% of development, the more proximal segments of the dorsal nerve were entirely ensheathed by the surrounding glial cells (Fig. 6A; proximal is to the left). In the vicinity of the target muscles, however, the glial trajectories became largely independent of the neuronal trajectories. Although, at this stage, there were still numerous neuronal branches extending beyond the glial cells, the leading glial processes were often found to be growing parallel to but separate from their neighboring axons. In Fig. 6A (which is a montage of four focal planes), the black arrows indicate regions where the axons and glial processes appeared to be in direct contact, while the white and black arrowheads indicate regions where the axons and glial cells were maximally separated in the z-axis. All TM-MFas II-positive processes subsequently became ensheathed by GPI-MFas II-positive glial cells (at 75%; Fig. 6B, solid arrow). However, as indicated in Fig. 5, adjacent glial branches that were independent of TM-MFas II-expressing axons were also found on the target muscles (Fig. 6B, open arrow), apparently enwrapping an additional set of unlabeled neuronal processes.

Thus, unlike the formation of the DNa branch, in which a glial scaffold prefigured the trajectory of growing axons, the formation of the DNl involved the initial extension of TM-MFas II-positive axons into the periphery prior to the differentiation of any glial support. The subsequent outgrowth of glial cells that eventually ensheathed the DNl axons also proceeded in a manner that was partially independent of glial–neuronal contact. Whether the more elaborate glial arbor seen in mature muscles (Figs. 5E and 6B) reflects the selective down-regulation of TM-MFas II by a

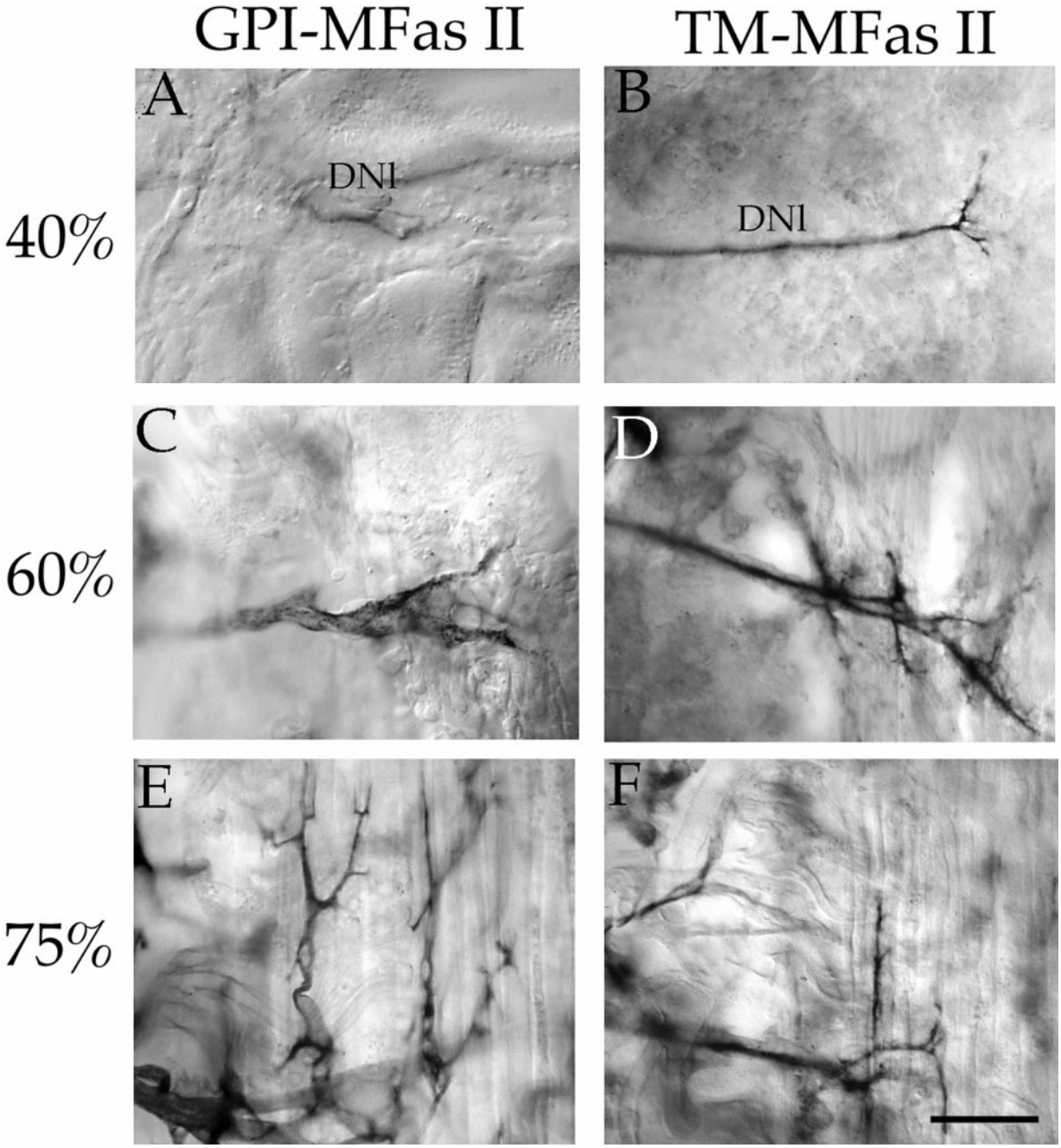

FIG. 5. TM-MFas II-positive growth cones pioneer the lateral branch of the dorsal nerve prior to the arrival of GPI-MFas II-positive glia. (A) At 40% of development, GPI-MFas II immunoreactivity is only faintly detectable in the most distal cluster of glial cells on the DNl branch (the spiracle is just out of the field of view to the left). (B) TM-MFas II-positive processes pioneering the DNl branch have already extended significantly beyond the most distal glial cells that can be detected by immunolabeling or Nomarski optics (compare with Fig. 3D). (C, D) By 60%, GPI-MFas II-positive glial cells have spread laterally along the DNl branch but still have not encompassed the most distal terminals of the TM-MFas II-positive axons that have extended onto the differentiating skeletal muscle. (E, F) By 75%, GPI-MFas II-positive glial processes now extend over the entire DNl and significantly beyond the most distal TM-MFas II-positive nerve terminals. Scale = 20 μM.

subset of motor axons or is due to glial ensheathment of other MFas II-negative cell types is discussed below.

Guidance of TM-MFas II-Positive Axons by GPI-MFas II-Positive Glia

Our observations that growing axons expressing TM-MFas II colocalize with GPI-MFas II-positive glial cells in a variety of circumstances suggested that neuronal–glial interactions might play a role in axonal pathfinding. In particular, the formation of the GPI-MFas II-positive glial bridge prior to the outgrowth of the TM-MFas II-positive DNa axons (Fig. 4) raised the possibility that these glia form a requisite scaffold that guides these axons towards their correct targets, and that this neuronal–glial interaction might be fasciclin II-dependent. We therefore treated cultured embryos with low doses of PI-PLC at 35% of development (shortly before DNa outgrowth) and allowed them to develop overnight at 37°C. In previous studies, we showed that PI-PLC treatment (which selectively cleaves GPI linkages) was an effective means of removing GPI-MFas II, but not TM-MFas II, from cell membranes both *in vitro* and *in vivo* (Wright *et al.*, 1999; Wright and Copenhaver, 2000). In cultured control embryos, the GPI-MFas II-positive bridge glia formed normally (not shown), and the branches of both the DNa and DNl nerves grew along their appropriate trajectories (Fig. 7A; white and black arrowheads, respectively). The extension of the DNl into the periphery was similarly unperturbed in PI-PLC-treated embryos (Figs. 7B–7D, black arrows): both the overall length of the DNl (averaging ~200 μm) and its innervation of target muscles in enzymatically treated embryos were indistinguishable from controls (not shown).

In contrast, the trajectory of the DNa branch was markedly perturbed in 92% of treated embryos, which exhibited a variety of abnormal phenotypes (Figs. 7B–7D, white arrowheads). One-third of treated embryos exhibited a complete failure in the defasciculation of the DNa from the DNl (Fig. 7C). The divergence of the DNa from the DNl proceeded normally in the remaining animals (compare Fig. 7A with 7B and 7D), indicating that GPI-MFas II-positive bridge cells were not essential for the initial guidance of the DNa growth cones away from the DNl. The subsequent trajectories taken by these processes was often abnormal, however. In 16% of these embryos, the DNa axons stalled approximately at the position of the bridge and extended abnormal branches in several inappropriate directions (Fig. 7B). More frequently, the DNa branches emerged at variable locations along the DNl and exhibited a "switchback" phenotype (Fig. 7D), indicating the presence of additional guidance cues capable of misdirecting the DNa growth cones when GPI-coupled guidance cues associated with the bridge were disrupted.

To determine whether the formation of the bridge itself was disrupted in these preparations, we also immunostained embryos with a variety of antisera that label glial cells in other insect species; unfortunately, none of these reagents provided a convincing independent marker for *Manduca* embryonic glia. Moreover, while PI-PLC treatments selectively affected the outgrowth of the DNa but not the DNl branch, these results might be due in part to the removal of additional GPI-linked molecules besides GPI-MFas II. We therefore designed a panel of antisense phosphorothioate ODNs against unique sequences within the 3′ UTR of GPI-MFas II as a means of selectively inhibiting the expression of this isoform. Several published studies have previously demonstrated that antisense ODNs directed against the 3′ UTR regions of specific mRNAs can have potent inhibitory effects on protein expression (Chiang *et al.*, 1991; Lipp *et al.*, 1995; Ronnov-Jessen and Petersen, 1996), and we have successfully used this strategy to inhibit TM-MFas II expression in migratory neurons (Wright and Copenhaver, 2000). Each ODN was applied to cultured embryos at 28–30% of development, after the initial formation of the dorsal nerve (Fig. 1E) but prior to the differentiation of the glial bridge and outgrowth of the DNa branch (see Figs. 4A and 4B). After application of the ODNs, the preparations were allowed to develop for an additional 12–16 h, then fixed and immunostained with an antibody that recognized the conserved extracellular domain of the two MFas II isoforms. This protocol allowed us to monitor both the trajectories of TM-MFas II-positive axons and residual levels of GPI-MFas II expression in the adjacent glial cells in these culture preparations.

Of the three GPI-MFas II-specific ODNs employed, two of them (GPI-1 and GPI-3) produced no significant changes in dorsal nerve formation: both the levels of GPI-MFas II expression by the bridge glia and the trajectory of the DNa nerve branch in embryos treated with these ODNs were indistinguishable from cultured control preparations (Figs. 8A and 9A). In contrast, exposure to ODN GPI-2 frequently resulted in aberrant glial bridge formation and disrupted DNa outgrowth (Figs. 8 and 9). Although somewhat variable, the level of GPI-MFas II expression by the glial cells in these embryos was often reduced when compared with control cultures, and the overall organization of the bridge was noticeably disrupted (Figs. 8B–8F). In contrast, the level of TM-MFas II expression in the outgrowing axons of the dorsal nerve appeared unchanged, demonstrating that the effect of ODN GPI-2 on fasciclin II expression was isoform-specific. Concurrent with these effects on bridge formation, ODN GPI-2 also induced a range of defects in the formation of the DNa branch that were similar to the abnormalities seen in PI-PLC-treated embryos. In some preparations, the DNa was completely absent (Figs. 8B and 9B). More frequently, axons could be seen to have left the dorsal nerve at approximately the normal position of the DNa but then stalled or branched inappropriately around the disorganized bridge glia (Figs. 8C, 8D, 9C, and 9D). In a small number of segments, the DNa exhibited a "switchback" phenotype (Figs. 8E and 9E) similar to that shown in Fig. 7D, in which axons that would normally extend through the bridge to more lateral muscle targets instead projected back towards the CNS. Preparations treated with sense and scrambled

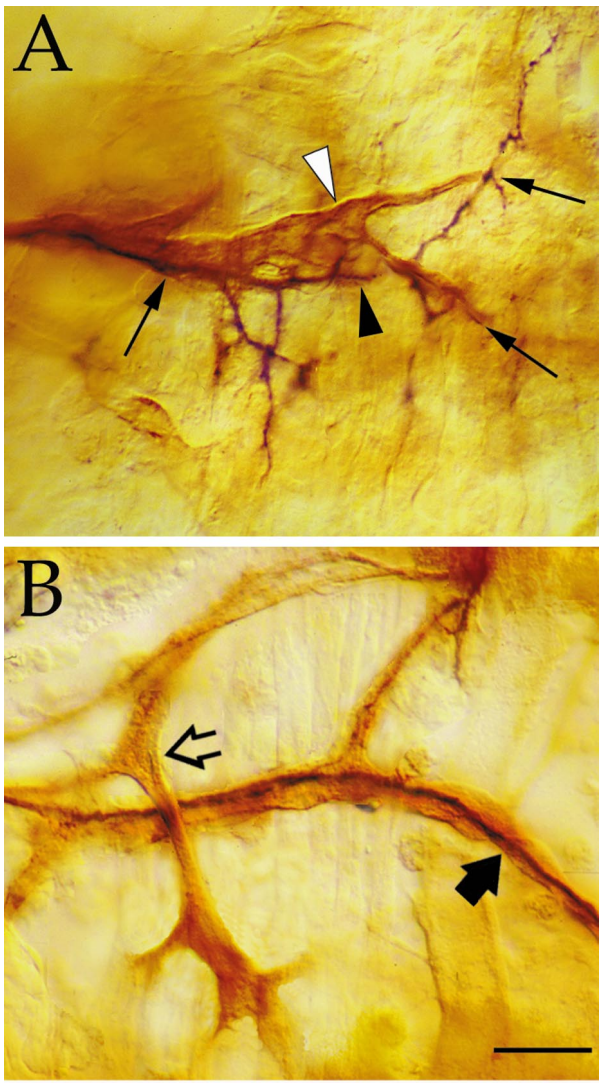

FIG. 6. TM-MFas II-positive axons and GPI-MFas II-positive glial processes follow partially independent trajectories. (A) Peripheral region of the DNl in a preparation immunostained to show TM-MFas II-positive axons (purple) and GPI-MFas II-positive glial processes (brown; this figure is a montage of four focal planes). Although the neuronal processes extend substantially beyond the glial cells at this stage, the trailing glial cells remain closely apposed to the axons at only some positions (arrows). Elsewhere, the glial processes follow distinct trajectories (white and black arrowheads indicate a region where neuronal and glial processes are completely separate in the z-axis). (B) By 75% of development, all of the TM-MFas II-positive axons are completely ensheathed by GPI-MFas II-positive glial cells (black arrow). However, additional glial branches (GPI-MFas II-positive) extend onto adjacent regions of the target muscles devoid of any TM-MFas II-positive axons (open arrow). Scale = 20 μM.

sequence control ODNs (containing the same combination of nucleotide residues as ODN-GPI-2) were indistinguishable from other control preparations (not shown).

These results substantiate our data obtained using PI-PLC treatments, demonstrating a functional role for neuronal–glial interactions in the formation of the DNa nerve. Whether GPI-MFas II serves as a *bona fide* axonal guidance cue or is simply needed for the differentiation of the glial bridge (which subsequently provides other guidance cues required for DNa outgrowth) remains to be determined. Nevertheless, our results indicate that the formation of some nerve pathways in the PNS requires axonal guidance by preformed glial assemblies, while, in other instances, the outgrowth of neuronal and glial processes appears to proceed in a partially independent manner.

DISCUSSION

Isoform-Specific Expression Patterns of MFas II during Neural Development

The data presented in this paper demonstrate compelling differences between the distribution of MFas II isoforms in the developing nervous system, both in terms of cell type-specific expression and subcellular localization. TM-MFas II was expressed exclusively by neurons and was localized to regions of active motility (elongating axons and growth cones). In contrast, GPI-MFas II protein was found almost exclusively on peripheral glial cells and was distributed uniformly across their surface membranes. The overall pattern of TM-MFas II in the embryonic CNS recapitulated the patterns of fasciclin II described in other insects (Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988), although there were some notable differences with respect to the initial sequence of fasciclin II expression by identified neurons and specific axon fascicles. However, this is the first demonstration that all neuronal fasciclin II expression in the CNS consists of the transmembrane isoform. We also found that TM-MFas II protein was rapidly localized to the growing regions of developing neurons, so that their somata were only briefly immunopositive at the time of initial outgrowth (Figs. 1D and 1E). The progressive redistribution of TM-MFas II protein from the neuronal somata into their distal processes was particularly evident in preparations that were double-labeled to show TM-MFas II mRNA and protein expression (Figs. 1J and 1K). These observations are consistent with the localized distributions of fasciclin II reported in grasshopper and fly (Bastiani *et al.*, 1987; Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988).

The striking lack of GPI-MFas II protein in neurons within the CNS was unexpected, both because of the extensive analyses of fasciclin II in other systems and because of the presence of low but detectable levels of GPI-MFas II-specific mRNA in the ventral ganglia (Fig. 1B) and brain (Fig. 2B). The expression of mRNA encoding both isoforms in CNS neurons might reflect "leaky" transcriptional regulation of the two splice variants, although no evidence for this phenomenon was seen within the GPI-MFas II-positive glial cells in the peripheral nerves (Fig. 3).

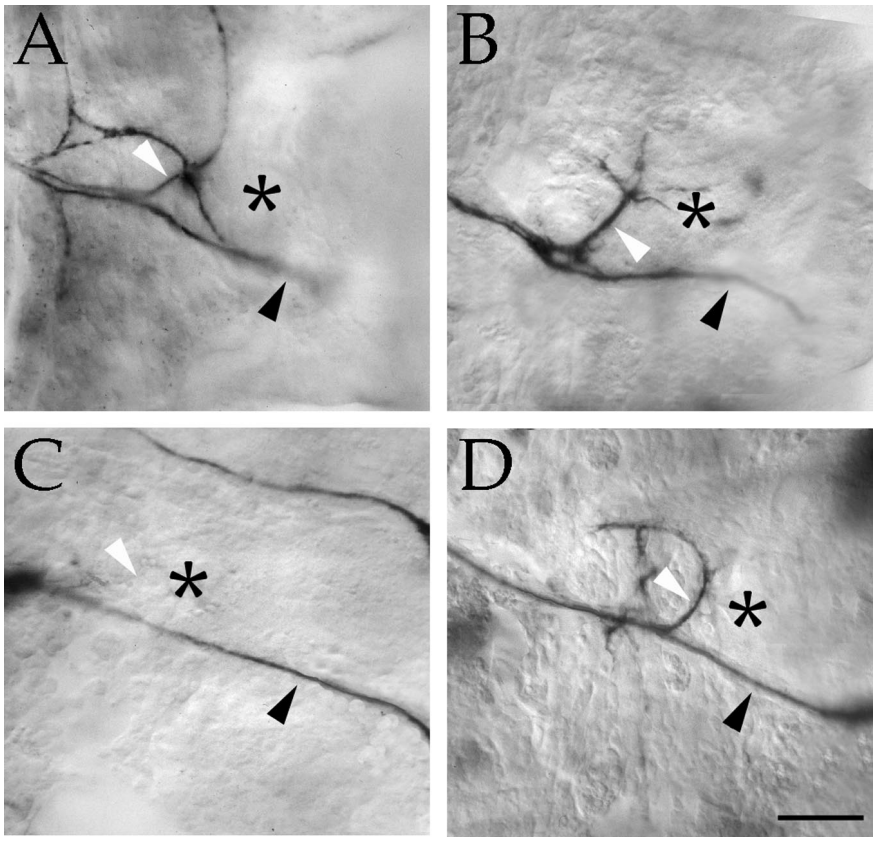

FIG. 7. Enzymatic removal of GPI-MFas II from the bridge glial cells disrupts the formation of the DNa branch but not the DNl branch. All preparations were immunostained for TM-MFas II. (A) Control embryo maintained in normal culture medium showed normal outgrowth of both the DNa (white arrowhead) and the DNl branch (black arrowhead; asterisk indicates the spiracle). (B—D) In embryos treated with PI-PLC prior to the outgrowth of the DNa, a variety of abnormalities were subsequently detected in the DNa. (B) Example of a preparation in which the DNa defasciculated from the dorsal nerve correctly and grew into the normal vicinity of the glial bridge but then stalled and sent short processes in several inappropriate directions. (C) Preparation in which the DNa branch completely failed to defasciculate from the dorsal nerve. (D) Preparation in which the DNa branch defasciculated correctly but then exhibited a "switchback" phenotype, curving proximally back towards the CNS. In all of these preparations, the outgrowth of the DNl branch was unaffected (black arrowheads). Scale = 20 μM.

Previous work on the ENS also showed no evidence of mRNA expression for a particular isoform in the absence of detectable protein (Wright and Copenhaver, 2000). It is possible that low levels of GPI-MFas II protein in the CNS were not detectable by immunohistochemical staining, but the sensitivity of the antibodies used in this study argues against this possibility. Whether mRNA encoding GPI-MFas II is simply never translated in CNS neurons or whether there is a rapid degradation of GPI-MFas II in these cells is unknown, although we have shown that both MFas II isoforms can be rapidly down-regulated in other contexts (Wright and Copenhaver, 2000). In general, however, our data show that developing neurons express only the transmembrane isoform of fasciclin II, while the GPI-linked isoform is primarily confined to peripheral glia.

The consistent expression of GPI-MFas II the peripheral glial cells provided an unambiguous means of visualizing the distribution of these cells throughout most of embryogenesis (Figs. 3, 4, and 6). Although the lack of additional antibodies that unambiguously labeled *Manduca* glial cells limited some aspects of our analysis, we were nevertheless able to monitor the interactions between growing axons and their glial partners in specific locations. Still unresolved is the source of these peripheral glial populations. In *Drosophila*, an identified population of exit glia originates from stem cells in the CNS and delineates the positions at which the peripheral nerves leave the CNS (Jacobs and Goodman, 1989b). Subsequent work has indicated that the progeny of these cells continue to migrate along the growing axons in a manner reminiscent of Schwann cell migration in the vertebrate CNS (Edwards *et al.*, 1993; Sepp *et al.*, 2000). In other regions, a mesodermal origin has been indicated for at least some of the peripheral glia that form scaffolds for nerve growth in *Drosophila* (Gorczyca *et al.*,

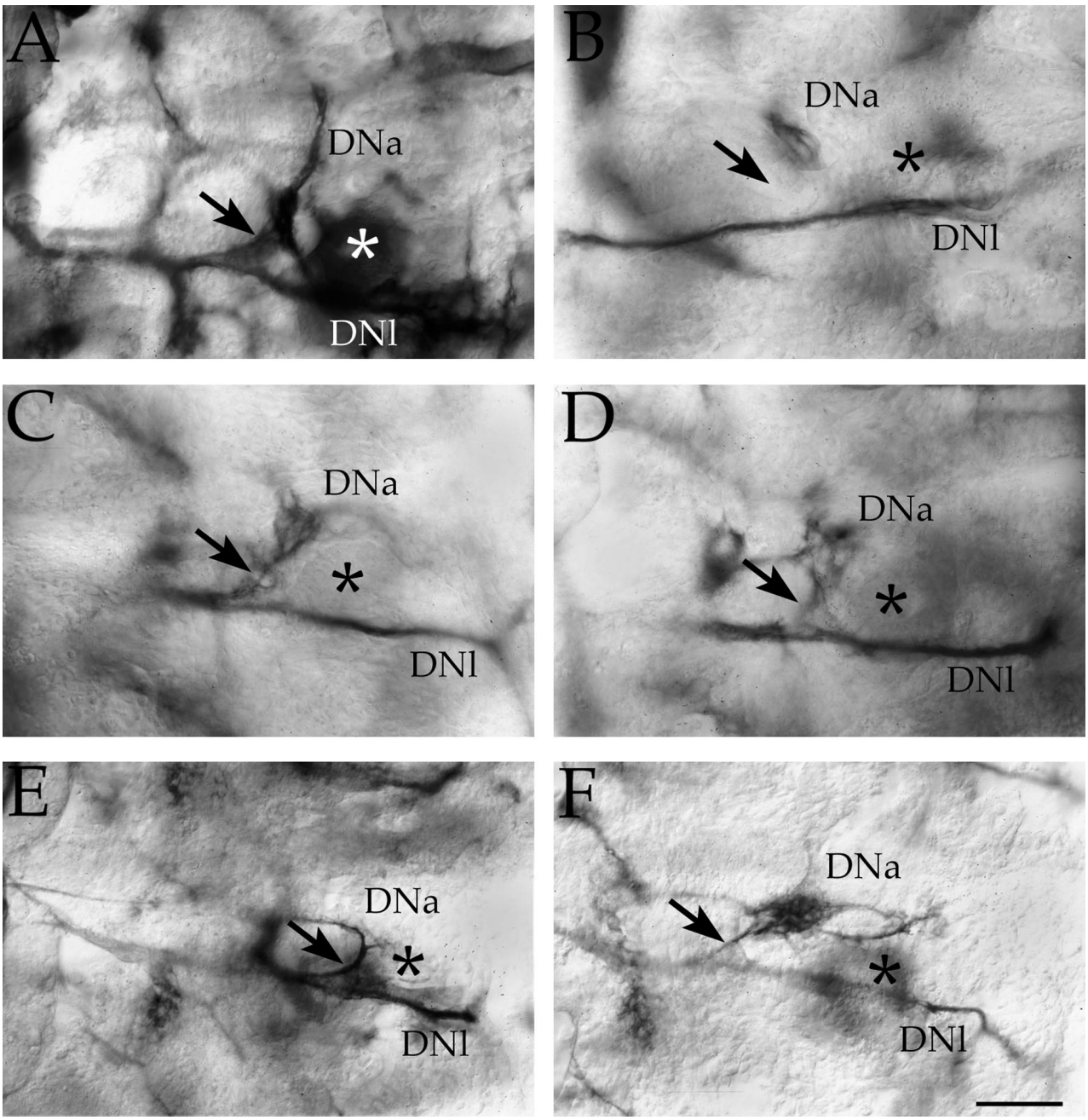

FIG. 8. Inhibition of GPI-MFas II expression with an antisense ODN reduces GPI-MFas II expression in peripheral glia and disrupts the formation of both the glial bridge and the DNa nerve. All preparations were immunostained with an antibody against the shared extracellular domain of MFas II. (A) Control embryo grown in normal culture medium exhibited normal bridge formation and DNa outgrowth from the dorsal nerve (DN). The peripheral glia lining the branches of the dorsal nerve and forming the glial bridge showed robust levels of GPI-MFas II expression. (B–F) Embryos treated with ODN GPI-2 showed a variety of defects in bridge formation and DNa outgrowth. (B) Example of a peparation in which the DNa failed to fasciculate from the dorsal nerve; the glia surrounding the branches of the dorsal nerve also showed substantially reduced levels of GPI-MFas II expression, and the glial cells that normally form the bridge had not coalesced correctly. (C, D) Two examples of preparations in which the DNa axons emerged from the dorsal nerve at approximately the normal position but then stalled and send short processes in several inappropriate directions. The glial bridge cells showed reduced levels of GPI-MFas II expression and appeared disorganized. (E) Preparation in which the DNa branch exhibited a "switchback" phenotype after reaching the disorganized glial bridge. (F) Preparation in which the bridge glia expressed more robust levels of GPI-MFas II than seen in B–E, but the bridge still appeared malformed and the DNa axons grew in several inappropriate directions. DNl indicates the lateral branch of the dorsal nerve, which was unaffected. Asterisks indicate the position of the spiracle. Scale = 20 μM.

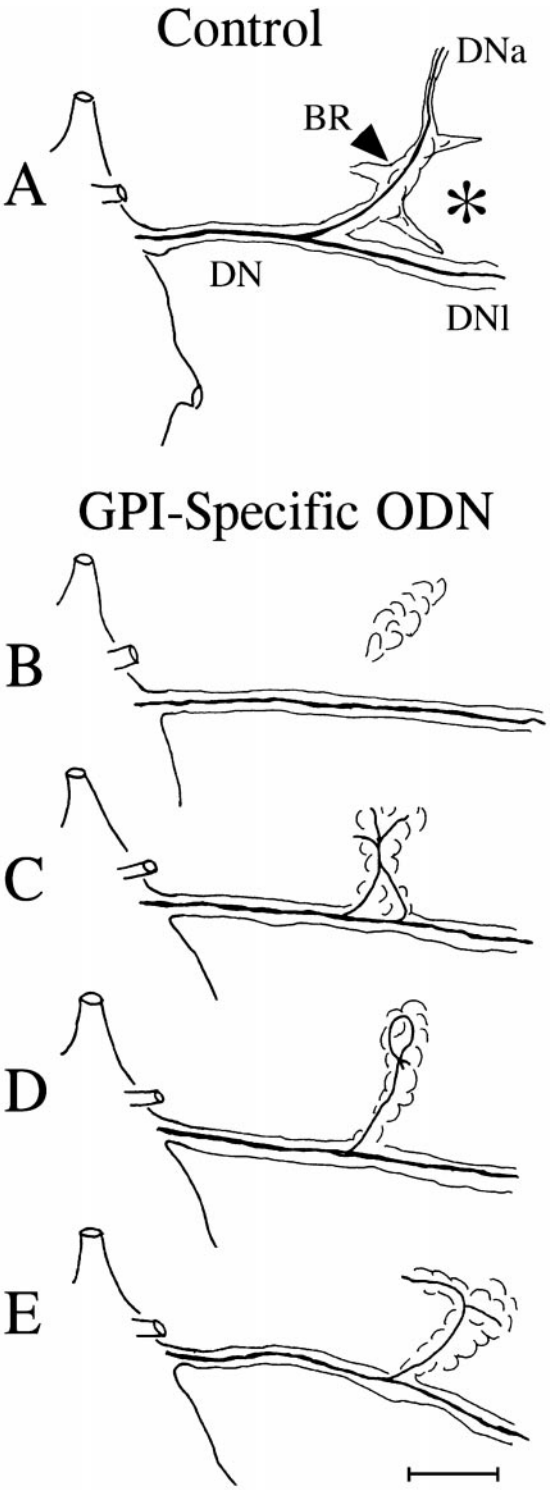

FIG. 9. Effects of ODN GPI-2 on bridge formation and DNa nerve outgrowth (redrawn from *camera lucida* images). (A) Cultured control preparation showing the normal morphology of the glial bridge (BR) and the trajectory of the DNa branch from off of the dorsal nerve (DN). Asterisk indicates the position of the spiracle; other nerves that extend through the bridge are not shown. (B–E) Examples of cultured preparations that were treated with ODN-2 prior to bridge formation and DNa outgrowth (compare with Fig. 8). (B) Preparation in which the DNa failed to defasciculate from the dorsal nerve. (C, D) Two preparations in which the DNa axons emerged from the dorsal nerve but then stalled in the vicinity of the bridge and extended short processes in a variety of inappropriate directions. (E) Example of the "switchback" phenotype in which some of the DNa axons projected back towards the CNS. Note that the organization of the glial cells that normally form the bridge is substantially less coherent than in the control preparation. DNl, lateral branch of the dorsal nerve. Scale = 20 μM.

1994), and several identified glial structures in *Manduca* (including the glial bridge that guides the DNa nerve) also appear to arise within surrounding mesodermal tissues prior to the arrival of any peripheral growth cones (Fig. 4; Carr and Taghert, 1988). Glial phenotypes have been shown to be induced in mesodermal cells by glial-specific transcription factors (Bernardoni *et al.*, 1998), suggesting that a combination of ectodermal and mesodermal precursors may contribute to peripheral glial populations in insects. A complete analysis of the different glial populations described in this study will require additional markers that can be used to trace their origins in better detail.

Unlike *Drosophila*, in which motor terminals have been reported to remain incompletely ensheathed (Sepp *et al.*, 2000), all of the efferent branches and motor terminals in *Manduca* appeared to become wrapped by glial processes during embryogenesis, a relationship that is maintained throughout postembryonic development (Knittel *et al.*, 2001). In this regard, the neuromuscular junction of *Manduca* is more similar to that of vertebrates than has been described in flies (Atwood *et al.*, 1993; Rheuben, 1992). However, the behavior of the outgrowing glial cells was notable in that they clearly followed additional guidance cues besides the TM-MFas II-positive motor axons: the leading glial processes were often seen extending in parallel but independent of the motor axons (Fig. 6), only subsequently making contact with the adjacent axons and enwrapping them. Moreover, we also found that some GPI-MFas II-positive glial processes grew substantially beyond the TM-MFas II-positive motor terminals on mature muscles (Figs. 5 and 6). It is possible that a subset of motor neurons (or specific motor terminal branches) do not express TM-MFas II but are nevertheless ensheathed by GPI-MFas II-positive glial cells, although in *Drosophila*, all motor neurons have been reported to express fasciclin II during embryogenesis (Schuster *et al.*, 1996a; Van Vactor *et al.*, 1993). A recent examination of *Manduca* neuromuscular junctions in postembryonic animals has also shown that all motor terminals remain ensheathed by GPI-MFas II-expressing glial cells throughout subsequent life stages (Knittel *et al.*, 2001). Alternatively, this additional glial elaboration may reflect the ensheathment of unstained sensory afferent fibers *en route* to the CNS (c.f. Sepp *et al.*,

2000). Simultaneous labeling of sensory neurons and the glial sheath in developing embryos will be needed to address this issue.

Interactions between Axons and Glia Expressing MFas II

The results of this paper indicate that at least two distinct types of interaction occurred between TM-MFas II-positive axons and GPI-MFas II-positive glial cells. The first type, illustrated by the extension of the DNl branch (Fig. 5), occurred when axons expressing TM-MFas II navigated to their targets in advance of glial cells. Axons pioneering the DNl clearly did not rely on GPI-MFas II-expressing glia as a substrate for guidance; rather, the DNl formed in a manner similar to vertebrate peripheral nerves, in which axons extend into target regions prior to the arrival of glial cells (Mirsky and Jessen, 1999). The TM-MFas II-expressing axons subsequently provided one of the substrates followed by the ensheathing glia during their outgrowth, similar to the guidance of Schwann cells by axons during vertebrate development. Based on these results, it is not surprising that manipulations targeting the GPI-linked isoform had no effect on the outgrowth of this nerve (Fig. 7, black arrowheads; Fig. 8), even when the level of GPI-MFas II expression in the glial cells surrounding the DNl were substantially reduced (Fig. 8). Although we did not examine synapse formation in these preparations in any detail, the overall appearance of the terminal branches of the DNl was normal, consistent with the arrival of the axons at their muscle targets prior to ensheathment by the glial processes. Therefore, in the case of the DNl, the axons conduct their initial pathfinding independent of glial interactions and provide support for subsequent glial differentiation.

In contrast, the other class of interaction (illustrated by the formation of the DNa nerve) suggests that GPI-MFas II-positive glial cells play an essential role during axonal guidance in some specific instances. The data shown in Fig. 4 indicated that the glial cells forming the bridge structure were already present and expressing GPI-MFas II prior to the arrival of the DNa pioneer growth cones, and that the morphology of this transient structure prefigured the trajectory subsequently taken by the DNa nerve. Moreover, removal of GPI-MFas II (with PI-PLC) or inhibition of its expression (using antisense ODNs) dramatically altered the formation of this nerve (Figs. 7–9). Both of these manipulations perturbed the trajectory of the DNa axons to some degree in over 90% of the treated preparations, the most dramatic of which involved a complete failure of the DNa to defasciculate from the DNl (Figs. 7C and 8B). More often, the DNa axons initially extended into the area normally occupied by the bridge glia but then either stalled or grew in a variety of inappropriate directions, including back towards the CNS. Treatment with PI-PLC typically resulted in a complete loss of GPI-MFas II expression and tended to produce more pronounced errors in DNa formation. While the relatively low concentration of PI-PLC used in these studies did not cause any obvious defects in the DNl branch (which grows in advance of GPI-MFas II-expressing substrates), this enzyme should also cleave other GPI-linked molecules that might be expressed by the bridge glia. The effects of this treatment on DNa branch formation might therefore reflect the function of additional receptors besides GPI-MFas II. However, our successful application of antisense ODNs designed to inhibit GPI-MFas II expression also produced a similar range of defects in DNa formation, most often resulting in a stalled phenotype in which the axons failed to advance normally beyond the malformed glial bridge. These results indicate that in the case of the DNa branch, axonal pathways are prefigured by a defined glial structure. This phenomenon is reminiscent of the "Blueprint" hypothesis (as noted by Carr and Taghert, 1988), suggesting that some axon pathways may be prespecified by glial or other nonneural structures (Singer *et al.*, 1979; see Silver, 1993).

What is the role of GPI-MFas II with respect to the formation of the DNa branch? Given previous studies demonstrating that fasciclin II can act as a homophilic binding protein (reviewed in Goodman *et al.*, 1997), our initial assumption was that TM-MFas II-positive axons would specifically adhere to GPI-MFas II glial cells either before or after their initial outgrowth. This model would predict that interactions between TM-MFas II receptors on the growth cones and GPI-MFas II on the glial cells normally mediate the guidance of the axons across the glial bridge, and that removal of GPI-MFas II is sufficient to disrupt this process. An alternative possibility is that GPI-MFas II simply acts as an adhesion molecule holding the glial cells in a cohesive group as the bridge structure differentiates, whereupon other molecular components associated with the bridge provide the actual guidance cues for the DNa axons. Contrary to our expectations, we observed instances where TM-MFas II-positive axons grew independent of GPI-MFas II-positive glial cells (Fig. 5), and outgrowth of GPI-MFas II-positive glial processes occurred partially independent of TM-MFas II-expressing axons (Figs. 3 and 6). Although homophilic adhesion between the identical extracellular domains of different MFas II isoforms is formally possible, whether the transmembrane and GPI-linked isoforms directly interact *in vivo* remains to be determined.

Functional Differences between MFas II Isoforms

An important issue with respect to the IgSF receptors in general concerns the potential functions of distinct isoforms. While many of these molecules have been shown to be able to promote cell–cell contact via homophilic interactions (including fasciclin II; Grenningloh *et al.*, 1990), ample precedent has been established that some IgSF receptors mediate functions beyond simple adhesion. In the case of transmembrane IgSFs, both NCAM 140 and L1 have been shown to interact with a variety of intracellular signaling pathways (e.g., Ignelzi *et al.*, 1994; Beggs *et al.*, 1997;

Schmid *et al.*, 1999) which may lead to localized changes in actin assembly and neuronal motility. Similarly, a PDZ-binding domain within the cytoplasmic tail of transmembrane fasciclin II has been shown to promote interactions with both the Shaker potassium channel and the membrane-associated guanylate kinase Discs-large (Thomas *et al.*, 1997; Zito *et al.*, 1997), which may in turn mediate synaptic growth and plasticity. The role of the GPI-linked IgSFs is by contrast less well understood. Although a number of GPI-linked cell adhesion receptors have been shown to interact with nonreceptor tyrosine kinases (Brown, 1993; Casey, 1995; Olive *et al.*, 1995; Zisch *et al.*, 1995), no such association has been demonstrated for GPI-linked isoforms of the NCAM/apCAM/fasciclin II family. Experiments performed in cell culture have shown that GPI-linked receptors (such NCAM-120) can act as substrate molecules that promote neuronal motility *in vitro* (Doherty *et al.*, 1990), although other studies performed *in vivo* have suggested that GPI-linked isoforms of this family serve a strictly adhesive function (Martin and Kandel, 1996; Schuster *et al.*, 1996b).

In the case of MFas II, our studies using cultured embryos support a model in which the two isoforms serve distinct functions with respect to cellular motility. For example, we previously showed that an identified set of newly generated enteric neurons (the EP cells) initially expressed only GPI-MFas II, and that this isoform maintained strong adhesive contact between adjacent neurons but did not contribute to their motility (Wright and Copenhaver, 2000). The EP cells then switched isoforms to express only TM-MFas II shortly before commencing a period of rapid migration and outgrowth, events that were inhibited when TM-MFas II expression was perturbed. Subsequent expression of GPI-MFas II in the ENS was entirely confined to the enteric glial cells that, like the glial cells of the PNS, gradually spread along the pathways formed by the neurons and their processes. We also found that the membrane distributions of the two isoforms were similar in the ENS as reported here: GPI-MFas II protein appeared to be uniformly distributed over the entire surface of cells expressing this isoform, while TM-MFas II was localized to the leading processes of migrating neurons or their growth cones. The consistent localization of TM-MFas II to the most motile regions of a neuron suggests that it may help regulate the cytoskeletal dynamics associated with migration and outgrowth, as proposed for other transmembrane isoforms of this receptor family. In contrast, our data argue that GPI-MFas II may act primarily as a simple adhesion molecule, maintaining strong intercellular contacts without directly promoting intracellular signaling events that might affect cellular motility. Whether the two isoforms directly interact (one providing a substrate for cells expressing the other isoform) or whether either isoform may interact heterophilically with other receptor types encountered *in vivo* remain to be explored.

In summary, we have shown that the two isoforms of fasciclin II in *Manduca* are expressed in dynamic and distinct patterns within the developing nervous system. Notably, we have shown that the well-characterized pattern of fasciclin II in the insect CNS is entirely due to neuronal expression of the transmembrane isoform. In contrast, the GPI-linked isoform of MFas II is almost completely confined to the glial populations that ensheath the peripheral nerves. Our data support a model whereby the localized expression of TM-MFas II in the axons and growth cones of developing neurons may promote their motility, while GPI-MFas II acts as a simple adhesion molecule that may also serve as a substrate for axonal outgrowth in some instances. Finally, we have exploited the isoform-specific patterns of MFas II expression in the PNS to show that at some locations, glial cells ensheath peripheral nerves only after axonal outgrowth, while, in other regions, glial structures form in advance of the first growth cones and may provide necessary substrates for guiding their subsequent trajectories.

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