THE ADHESION MOLECULE FASCICLIN II IN NEURONAL DEVELOPMENT

by
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CERTIFICATE OF APPROVAL

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Abstract

Receptor-mediated cellular adhesion is a key feature of cell activity. Not only does adhesion provide stability for a cell, but adhesion molecules serve a tremendous array of functions, including roles as guidance molecules for migrating cells, as regulators of differentiated states, and mediators of signal transduction cascades that flow from the environment to the cytoplasm and in the reverse direction, from the cytoplasm to the extracellular domain. Cell adhesion molecules (CAMs) have been studied extensively in the nervous system, where appropriate adhesion to specific substrates is crucial for the correct wiring of a developing nervous system.

We utilize an embryonic model system that has proven useful in studying aspects of nervous system development and cell migration. The work presented here identifies a particular CAM, fasciclin II, in the moth embryo *Manduca sexta*, and demonstrates its requirement for several aspects of neuronal development, including the promotion of motility, adhesion, and guidance. *Manduca* fasciclin II (MFas II) was originally identified as the antigen recognized by a monoclonal antibody (TN-1), which labels major nerve tracts of the central, peripheral and enteric nervous systems. Peptide sequence from purified antigen was used to design primers to screen a cDNA library. The full length protein sequence was predicted from cDNAs isolated from this screen.

Antibodies that target the extracellular domain of *Manduca* fasciclin II (MFas II) and synthetic peptides which bind the extracellular domain of MFas

II interfered with nervous system development, consistent with a requirement for MFas II-mediated adhesion for neuronal guidance and motility.

We generated isoform-specific riboprobes and antibodies to characterize the expression and function of the two isoforms of MFas II, a transmembrane form and a GPI-anchored form. We found that the transmembrane isoform was expressed by neurons and primarily localized to motile axons, whereas the GPI-linked isoform was found on glia and some premigratory neurons. Finally, using antisense oligonucleotides and enzymatic ablation of GPI-anchored MFas II, we examined the specific role of each isoform in nervous system development. We found that the GPI-linked isoform is required for adhesion of non-motile cells, while the transmembrane form is necessary for neuronal motility. Our observations of the expression and role of the GPI-anchored isoforms are entirely novel and could suggest roles for homologous CAMs in other invertebrate and vertebrate species.

Introduction

Cells are the building blocks of life, and we study them to learn how they survive as autonomous organisms and also how they interact with their environment in a multicellular context. Eukaryotic cells were first seen in the 1660's, yet an understanding of their significance, structure, and function was not immediately forthcoming. Nearly two centuries passed before a general paradigm, the cell theory, was codified. This established that cells are the fundamental units of life, that all life is comprised of cells, that cells arise only from other cells, and that the biology of an organism can be understood through sufficient understanding of its cellular activities. In the 1950's electron microscopy offered high resolution (0.1 nm) snapshots of cellular structures, and more recent advances in other fields have made possible the investigation of living cell dynamics. Indeed, we have the ability to edit the genetic library of cells, to induce or suppress gene expression, to introduce directed mutations, and even to transplant gene expression into foreign cell types. These technologies are illuminating the underpinnings of cell biology, allowing us to investigate just how cells carry out their activities at the level of protein-protein interactions. We can also examine how these processes are regulated and ask the question: what cellular events lead to proliferation or differentiation or death?

Cellular activities fall into a broad range of types and include much more than simply promoting the survival of a given cell. Cells comprising metazoan organisms are highly specialized to serve integrative functions and are unable, for the most part, to survive outside their native contexts (although advances in cell culture techniques allow us to maintain a variety

of cell types under optimized conditions). Instead, these cells are highly dependent on interactions with their environment for survival and to support functions that manifest at the level of the whole organism.

Cellular interactions with other cells and the non-cellular environment are achieved through a number of mechanisms, but typically cells insert receptors through the plasma membrane to contact the environment. Any given receptor has a very narrow range of chemical motifs that it will bind with high affinity, conferring high specificity of receptor-ligand complexes. A diverse array of receptor types can give cells a detailed resolution of the environment and myriad ways to respond to environmental cues. Many of these functional responses are governed by receptors that were initially defined on the basis of their ability to promote adhesion to cellular or non-cellular substrates, and are thus termed cell adhesion molecules (CAMs). These molecules participate in homophilic (like-to-like) interactions as well as heterophilic interactions and are discussed below. A common theme that has rapidly emerged, though, is that it is inappropriate to separate adhesion from other cellular functions. It has been found that many classic cell adhesion molecules participate in intracellular signalling events, stimulate cellular motility, function as guidance molecules, and modulate signal cascades associated with other cell surface receptors (e.g. Brümmendorf and Rathjen, 1996, Seeger et al., 1999, Steinberg and McNutt, 1999).

The first technical evidence that CAMs promote specific or preferential cell-cell associations came from experiments in developmental biology (e.g. Townes and Holtfreter, 1955). These showed coordinated cell sorting following chemical dissociation of early embryos (i.e. cells derived from like tissue layers would aggregate, excluding cells from different tissue layers),

thus suggesting homophilic interactions between cells. As our technology has advanced, a wide variety of homophilic and heterophilic CAMs have been identified and it is clear that their biological function is complicated. Many of these molecules bind components of the extracellular matrix (ECM) as well as other cells. The majority of cell adhesion events are mediated by four families of CAMs: integrins, cadherins, selectins, and the immunoglobulin superfamily (IgSF) of receptors.

CAM families

Integrins are a family of heterodimeric CAMs that have major roles in the formation of focal adhesions. These are sites of contact rich in dynamic cytoskeletal activity, containing characteristic groups of proteins, including actin, actin-binding proteins that link receptors to the cytoskeleton, and regulatory proteins such as small GTPases and tyrosine kinases. Integrins mediate adhesion to the ECM, and both the α and ß chains participate in this interaction. The α chain confers calcium dependence and ligand specificity, promoting interactions between diverse matrix proteins (reviewed in Aplin et al., 1998), while the ß-subunit mediates receptor clustering and is responsible for the recruitment of intracellular molecules such as the focal adhesion kinase (FAK), talin, α-actinin, and vinculin (Priddle et al., 1998, Lafrenie and Yamada, 1996). The primary role for integrins is thought to be the integration of the ECM with intracellular activity, with the resulting ability to generate cytoskeletal tension that influences proliferation and differentiation (González-Amaro and Sánchez-Madrid, 1999). Not only are integrins capable of transducing signals from the environment to the cytoplasm, they also demonstrate the fascinating ability to convey "inside-out" signalling, due to their capacity to respond to intracellular events that

mediate integrin clustering and adherence (O'Toole et al., 1994). Thus, integrin-mediated adhesion is not merely a static anchoring mechanism. Integrins are involved in two-way signalling, and the structural elements of integrin-associated foci are critically associated with fundamental aspects of cell function and survival.

Cadherins are also calcium-dependent CAMs, but these receptors are homophilic, and therefore cadherins on neighboring cells act as their ligands. These proteins are major regulators of tissue formation and participate in morphological changes in populations of cells during development (Alpin et al., 1998). They require intracellular binding to catenins in order to effectively participate in extracellular adhesion. The intracellular domain of the cadherin binds ß-catenin, which in turn binds α-catenin. As a result, α-catenin can bind directly to actin or the actin-binding protein α-actinin. A subgroup of cadherins are found in desmosomes and attach to intermediate filaments instead of the actin cytoskeleton. Cadherin/catenin adhesion is necessary for the survival of an organism, and mutations in cadherins are implicated in tumor formation (Berx et al., 1998). ß-catenin is also a multifunctional molecule. It is a target for multiple tyrosine kinases and its phophorylation state regulates cadherin adhesion (Roura et al., 1999). It also participates in diverse signalling pathways associated with myogenesis in the *Xenopus* embryo, cell cycle regulation, and programmed cell death (Vallerosi et al., 2000, Tian et al., 1999, Miller et al., 1999). So like integrins, cadherin function is not suitably defined as just adhesion.

Selectins encompass a small family of receptors that moderate heterophilic interactions through binding to the carbohydrate motifs of glycosylated proteins on other cells or in the ECM (González-Amaro and Sánchez-Madrid, 1999). They function primarily in the adherence of

leukocytes to vessel walls, promoting the rolling of these cells along these substrates prior to the recruitment of integrins, which promote firmer adhesion. Only recently have experiments suggested that selectins participate in signal transduction events independent of those modulated by integrins or other CAMS (Lorenzon et al., 1998).

IgSF adhesion receptors have classically been associated with neuronal guidance, but they are found in all tissue types. These proteins contain Ig-like domains, as well as fibronectin type III repeats. They participate in homophilic adhesion with a high degree of specificity. Some IgSF adhesion molecules have also been shown to associate with intracellular kinase activity, growth factor receptors, integrins, cadherins, and other IgSF family members (Walsh and Doherty, 1997, Olive et al., 1994). In addition, some members of this family, like integrins, have been shown to transduce outside-in as well as inside-out signalling (Hortsch et al., 1998). A more focused treatment of a limited number of IgSF receptors follows, but it should be noted that IgSF molecules are like other CAMs in that simple adhesion to extracellular substrates is only one aspect of their biological function.

The main point of the preceding discussion is that the major CAM families are not involved in static adhesion between cells or between cells and the ECM. Instead, the process of adhesion is intimately associated with other major cellular activities, and these CAMs, while promoting adhesion, also participate in a tremendous array of signal transduction events. The range of activity of these molecules has only recently become accessible to our investigation. A number of different CAMs on a given cell function in parallel and interactive modes, and the cell's response to the environment is the sum result of these interactions. This must be remembered when attempting to dissect the function of a single CAM either *in vivo* or *in vitro*.

Our work focuses on one such molecule, and some of our findings are best understood in this context: the role of one adhesion molecule in a cast of many.

The fasciclin II/NCAM/apCAM group of receptors

The topic of this thesis is the role of the insect IgSF receptor fasciclin II in the development of the moth nervous system. The relevance of these investigations lies in the dilemma of how a complex nervous system can arise in higher metazoan animals. A central mystery in nervous system development is how neurons find their correct target cells. Both the distance between a given neuron and its target and the recognition between neuron and target (a needle-in-a-haystack problem) are conceptually significant obstacles to neuronal development. In addition, a tremendous number of neurons make a large number of connections to other cells in more evolved nervous systems, giving rise to an integrated system wherein the sum activity of individual cells facilitates the response of an entire organism to environmental cues. To put this daunting phenomenon in perspective, the human brain alone contains over a trillion neurons, each one of these making an average of 1000 connections to other cells, and precise wiring is required for appropriate functions to occur (Tessier-Lavigne and Goodman, 1996).

The ability of individual neurons to migrate and extend processes to their targets is absolutely dependent on precise adhesive interactions and proper response to guidance cues, which are two overlapping topics. We have selected the embryonic moth as a model system due to its relative simplicity and accessibility to study. It is the complexity of other systems which often makes them intractable for analysis with available techniques. I

will conclude this introduction with a very brief discussion of some salient features regarding fasciclin II and our model system.

Fasciclin II contains five stereotypic extracellular Ig motifs and two fibronectin type III domains and was first identified and biochemically characterized in grasshopper as a neuronal cell surface antigen (Bastiani et al., 1987, Snow et al., 1988). Subsequent analysis in *Drosophila* demonstrated that fasciclin II mediates the formation of axons into bundles (fascicles) in the embryonic CNS and PNS (Lin et al., 1994, Lin and Goodman, 1994) and demonstrated its role as a CAM. The first chapter of this thesis describes the identification of fasciclin II in the moth. *Manduca* fasciclin II (MFas II) was first postulated to be the antigen recognized by the monoclonal antibody TN-1, based on similarities in expression pattern to fasciclin II in other insect nervous systems (Carr and Taghert, 1988). Homologs to fasciclin II can be found in the *Aplysia* apCAM and the vertebrate neuronal NCAM. While IgSF adhesion molecules share considerable domain homology and sequence identity, the IgSF proteins can be sorted on the basis of whether or not they cross the plasma membrane. IgSF adhesion molecules can be expressed as proteins with single transmembrane motifs, or as extracellular proteins anchored to the plasma membrane via a glycosylphosphatidyl-inositol (GPI) linkage. The transmembrane isoform provides contact between the extracellular domain and the cytoplasm, while the GPI-anchored isoform tethers the receptor to the outer leaflet of the plasma membrane with no cytoplasmic contact. Most IgSF proteins in the nervous system are expressed as either one or the other of these general isoforms. Fasciclin II, NCAM, and apCAM, however, are expressed in both forms as the result of alternative splicing of mRNAs transcribed from single genes (Lin et al., 1994, Mayford et al., 1992, Nguyen et al., 1986).

This thesis investigates the potential for specific and distinct roles for each fasciclin II isoform. We have chosen to examine this because precedence exists for IgSF receptors to function in signal transduction events and because the profound differences in domain structure between isoforms suggests disparate functional capabilities of each isoform. The established interactions of other IgSF molecules are complex and include both intracellular and extracellular associations with other CAMs and signalling molecules.

The structure of the Ig domains has been shown for some IgSF molecules to mediate homophilic interactions in *trans* orientation (Zhou et al., 1993, Ranheim et al., 1996, Wikstrom et al., 1996) and heterophilic interactions with other IgSF family members in *cis* (reviewed in Brümmendorf and Rathjen, 1996). In addition, some IgSF molecules have been shown to bind ECM components (Hortsch, 1996, Brümmendorf et al., 1993). Structural similarities among IgSF family members suggest that MFas II might have heterophilic binding targets *in vivo* (Engel, 1996), although none have yet been identified. The ability to bind a variety of extracellular molecules is not proof that IgSF adhesion molecules are capable of signal transduction or the promotion of motility; however, some IgSF proteins are also capable of interacting with cytoplasmic proteins, which does lend support to their participation in these roles.

NCAM has been implicated in signal transduction involving the MAP kinase pathway (Schmid et al. 1999) and focal adhesion kinase activity (Beggs et al., 1997). Also, the *src*-family tyrosine kinase fyn is apparently required for NCAM-stimulated NCAM-mediated neurite outgrowth (Beggs et al., 1994). Other IgSF molecules, in the nervous system and elsewhere, are biochemically associated with a variety of intracellular tyrosine kinases (Olive et al. 1995, Zisch et al. 1995). The cytoplasmic C-terminus of *Drosophila*

fasciclin II contains a PDZ-binding sequence, and is localised to synapses with the Shaker potassium channel through binding to a PDZ domain found on Discs-large, a membrane associated guanylate kinase (Zito et al., 1997). Cytoskeletal attachments are also indicated for several IgSF molecules, directly or indirectly. For example, NrCAM exhibits actin-dependent mobility (Faivre-Sarrailh et al., 1999), and growth cone orientation mediated by apCAM requires the cytoplasmic domain of the receptor, suggesting a cytoskeletal association (Suter et al., 1998).

Several recent studies on NCAM and apCAM isoforms show that the transmembrane isoforms have certain biological activities that the GPI-linked isoforms do not. These investigations characterize GPI-linked NCAM as a dominant negative inhibitor of transmembrane NCAM (Saffell et al., 1995) and suggest that GPI-linked apCAM is a nonfunctional isoform with regards to the activities examined (Bailey et al., 1997, Suter et al., 1998). However, even though GPI-anchored molecules have no direct contact with the cytosol, mounting evidence shows that many GPI-linked IgSF receptors are also associated with intracellular *src*-family tyrosine kinases. In some instances, this association might be through *cis* interactions betweeen the GPI-linked receptor and transmembrane molecules in the same plasma membrane (Olive et al., 1995), but in other cases the mechanism of signal transduction is unclear. Interestingly, some IgSF molecules found in leukocytes are expressed as both transmembrane and GPI-linked isoforms, and some of these GPI-linked isoforms can activate *src*-family kinases while the transmembrane isoforms cannot (Shenoy-Scaria et al., 1993). Such evidence raises the possibility that GPI-linked fasciclin II might have some functional activity beyond simple adhesion, as opposed to the nonfunctional or dominant negative roles proposed for GPI-linked NCAM and apCAM. The results

described in this thesis question whether the role of GPI-MFas II is to act as a dominant negative receptor.

The model system

In order to study aspects of cell adhesion and response to guidance cues in a developing animal, we have selected the embryo of the moth, *Manduca sexta*. This model allows us to maintain cells in a relatively complete biological context. Three major features are especially advantageous in this system: first, the developing nervous system is relatively simple in comparison to more complex vertebrate models. The enteric nervous system has only ~300 neurons, while the central and peripheral nervous systems have relatively few primary neuronal tracts per body segment. Second, this model is very accessible to pharmacological manipulation during the most important phases of nervous system development (i.e. when neuronal migration and process extension are occurring). This has allowed us to introduce experimental compounds in a highly controlled fashion. Third, the developmental time course of the events we examine occur within about a day. Key periods of neuronal motility in the enteric nervous system occur between about 40% and 75% of development (about 35 hours at 27°C), while the major motor neuron tracts extend from the CNS to the periphery between 18% and 45% of development. This brief time frame not only allows us to perform experiments relatively rapidly, but also reduces the potential effects of bacterial contamination seen in longer culture systems. This system has recently been used to charactize the role of a number of factors in the directed migration of enteric neurons, including heterotrimeric G-proteins (Horgan et al., 1998), cGMP (Wright et al., 1998), and tyrosine kinase activity (unpublished observations).

While considerable divergence has occurred between *Manduca* and vertebrate species, many aspects of nervous system development are conserved between these groups (Reichert and Boyan, 1997). Therefore our findings should be applicable to other preparations and might predict the role of IgSF molecules in more complex systems

Chapter 1

A role for fasciclin II in the guidance of neuronal migration

This chapter describes the cloning and sequencing of the TN-1 antigen and identifies it as the moth homolog to *Drosophila* and grasshopper fasciclin II. Manipulations in culture demonstrate a role for *Manduca* fasciclin II (MFas II) in the migration and process extension of enteric neurons. This is shown via the introduction of antibodies and peptide fragments that perturb MFas II homophilic adhesion between the migrating neurons and their substrate. Also demonstrated is that PI-PLC, which cleaves GPI-anchored MFas II (GPI-MFas II), inhibits neuronal migration. This result is further addressed in Chapter 2.

Chapter 2

Different isoforms of fasciclin II play distinct roles in the guidance of neuronal migration during insect embryogenesis

Here we extend our analysis of MFas II in the developing enteric nervous system (ENS) by examining the pattern of expression of each MFas II isoform using specific antibodies and riboprobes. We present dramatic distinctions between the patterns of expression of each isoform. Transmembrane MFas II (TM-MFas II) is expressed exclusively by neurons, while GPI-MFas II is expressed by glia and neurons. An interesting result is that the neuronal expression of GPI-MFas II ceases just prior to migration, at which time the neurons express only TM-MFas II. Culture experiments show that GPI-MFas II is required to keep pre-migratory neurons in close apposition, but only TM-MFas II is required for actual migration. We suggest that the inhibition of migration caused by PI-PLC in Chapter 1 might be due to the cleavage of other GPI-anchored molecules, or could be an artifact caused

by the high concentration of PI-PLC used in culture. In this chapter we show that far lower concentrations of PI-PLC affect migration, although indirectly. Treatment of EP cells with PI-PLC prior to the switch in expression of MFas II isoforms from the GPI to the transmembrane isoform blocks later migration. We propose that the cleavage of GPI-MFas II prevents adequate adhesion and this affects migration either by causing displacement of the neurons beyond the range of permissive contact, or else by blocking the ability of the neurons to extend processes due to altered surface tension induced by cell rounding.

Chapter 3

Fasciclin II isoforms in the central and peripheral nervous systems

Here we use the isoform specific antibodies and riboprobes developed in Chapter 2 to investigate the expression of MFas II isoforms in the central and peripheral nervous system. We show that TM-MFas II is expressed solely by neurons and GPI-MFas II is expressed solely by glia. We introduce a modified culture system and investigate the role of GPI-MFas II in supporting the guidance of TM-MFas II-positive axons. We find that GPI-MFas II is required for correct guidance of some axons, but suggest this is not due to GPI-MFas II-TM-MFas II-dependent adhesion. Instead we suggest that GPI-MFas II is necessary to maintain some glial populations in tight apposition until specific developmental stages are reached.

A ROLE FOR FASCICLIN II IN THE GUIDANCE OF NEURONAL MIGRATION

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SUMMARY

The insect cell adhesion receptor fasciclin II is expressed by specific subsets of neural and non-neural cells during embryogenesis and has been shown to control growth cone motility and axonal fasciculation. Here we demonstrate a role for fasciclin II in the guidance of migratory neurons. In the developing enteric nervous system of the moth *Manduca sexta*, an identified set of neurons (the EP cells) undergoes a stereotyped sequence of migration along the visceral muscle bands of the midgut prior to their differentiation. Probes specific for *Manduca* fasciclin II show that while the EP cells express fasciclin II throughout embryogenesis, their muscle band pathways express fasciclin II only during the migratory period. Manipulations of fasciclin II in embryonic culture, both with blocking antibodies and enzymatic removal of glycosyl phosphatidylinositol -linked fasciclin II, produced concentration-dependent reductions in the extent of EP cell migration. These results support a novel role for fasciclin II, indicating that this homophilic adhesion molecule is required for the promotion or guidance of neuronal migration.

INTRODUCTION

The directed migration of neurons or their precursors along specific pathways is essential to the formation of both the central and peripheral nervous system and can affect the expression of mature phenotypes by the post-migratory cells. While the phenomenon of neuronal migration was first characterized in vertebrates, it is now clear that this same process is also critical to the formation of invertebrate nervous systems, where many cells can be uniquely identified throughout development (Hedgecock et al., 1987; Klambt et al., 1991). A particularly dramatic example of directed migration has been documented within the developing enteric nervous system (ENS) of the moth, *Manduca sexta* . During the formation of the ENS, a population of ~300 post-mitotic cells (the EP cells) delaminates from a neurogenic placode to form a discrete packet of undifferentiated neurons at the foregut-midgut boundary (Copenhaver and Taghert, 1991). The completion of their development is delayed, however, until a new set of migratory pathways differentiates on the adjacent midgut, where a set of visceral muscle bands coalesce at eight specific locations around the midgut surface. Once these pathways have formed, subsets of EP cells then migrate rapidly onto each muscle band, traveling several hundred microns over the course of 7-10 hours (Copenhaver and Taghert, 1989b). Only after this migratory phase is complete do the neurons form mature synaptic contacts (Wright et al., 1998) and express transmitter phenotypes (Copenhaver and Taghert, 1989a), a developmental sequence that is regulated in part by the migratory process (Copenhaver et al., 1996).

Exploiting the relative simplicity and accessibility of the ENS in *Manduca*, we have examined the cellular mechanisms regulating neuronal migration within the developing embryo. Intracellular injections of

individual EP cells in vivo have revealed a typical migratory profile: each cell extends a leading process enriched with dynamic filopodial extensions that contact both the muscle bands and the non-supportive interband musculature as the neurons migrate (Horgan and Copenhaver, 1998). Manipulations of the developing ENS in embryonic culture have demonstrated that contact with a muscle band pathway is both necessary and sufficient for EP cell migration, whereas muscles in the interband regions (which are eventually innervated by these neurons) are strongly inhibitory for migration (Copenhaver et al., 1996). These and other studies have indicated that one or more molecular components associated with the muscle bands (but not the interband musculature) are essential for the support of neuronal migration in this system.

One candidate molecule that may function as a guidance cue for EP cell migration is the adhesion receptor fasciclin II. Originally identified by a monoclonal antibody screen of the grasshopper nervous system (Bastiani et al., 1987), cDNA clones encoding fasciclin II were subsequently isolated from both grasshopper and *Drosophila* (Grenningloh et al., 1991; Harrelson and Goodman, 1988; Snow et al., 1988). Fasciclin II is a member of the immunoglobulin (Ig)-related superfamily of cell adhesion receptors (Brummendorf and Rathjen, 1993) with structural similarity to the vertebrate receptor NCAM (Grenningloh et al., 1990). Like NCAM, fasciclin II is a membrane glycoprotein that has an extracellular domain containing five Ig-like C2 domains and two fibronectin (FN)-type III domains (Brummendorf and Rathjen, 1993). Also like NCAM, fasciclin II is expressed in multiple isoforms, including one or more transmembrane forms and a membrane-associated form that is attached via a glycosyl phosphatidylinositol (GPI) linkage to the outer leaflet of the plasma membrane (Grenningloh et al., 1991;

Lin and Goodman, 1994). Extensive studies on the function of fasciclin II in the developing insect nervous system have demonstrated that this molecule acts primarily as a homophilic cell adhesion receptor (Grenningloh et al., 1991) and participates in a number of different aspects of neuronal differentiation, including the regulation of axonal fasciculation (Lin et al., 1994; Lin and Goodman, 1994), synaptic stabilization and growth (Schuster et al., 1996), and the control of proneural gene expression and neurogenesis during metamorphosis (Garcia-Alonso et al., 1995; Whitlock, 1993). However, unlike NCAM, which has been implicated in the guidance of several different classes of migratory neurons (Cremer et al., 1994; Ono et al., 1994), a role for fasciclin II in the control of neuronal migration has not been explored.

In this paper, we report the cloning and characterization of fasciclin II from *Manduca* (hereafter designated MFas II), and we describe the developmental patterns of fasciclin II expression with respect to EP cell migration. We have also shown that during the migratory period, both the neurons and their muscle band pathways express MFas II, whereas the non-supportive interband musculature does not. Lastly, using an embryonic culture preparation, we have tested the functional role of MFas II with respect to the guidance of EP cells along their normal migratory pathways. Our results indicate that MFas II plays a key role in the migration of these identified neurons, serving to promote or guide their motile behavior in a tightly restricted manner within the developing ENS.

MATERIALS AND METHODS

Tissue preparation and immunoanalysis

Synchronized egg collections were obtained from a colony of *Manduca sexta* and maintained at 25°C, at which temperature embryogenesis is complete in 100 hours (1% of development = 1 hour). Embryo staging and whole-mount immunohistochemistry were performed as previously described (Copenhaver and Taghert, 1989a; Wright et al., 1998). For immunoblot analysis, tissues were collected on dry ice and rapidly homogenized in sample buffer (1% SDS, 10% glycerol, 50 mM Tris, pH 6.7) at 100°C. Approximately 100 µg of protein from each sample was separated on a 10% SDS polyacrylamide gel under non-reducing conditions, transferred to nitrocellulose, and reacted with antisera (Horgan et al., 1994). For detecting MFas II, we used either the monoclonal antibody TN-1 at 1:20,000 (gift of Dr. Paul Taghert; Carr and Taghert, 1988) or a mouse polyclonal antiserum at 1:2000 (gift of Dr. James Nardi). Also used in this study were antibodies against *Drosophila* fasciclin I, fasciclin II, and semaphorin Ia (each at 1:5 and 1:20; gifts of Drs. Cory Goodman and Alex Kolodkin), fasciclin III (at 1:200; gift of Dr. Peter Snow); and *Manduca* neuroglian (at 1:2000; gift of Dr. James Nardi). Concentrated aliquots of several of these antibodies were also used for the in vivo blocking experiments described below.

Microsequence analysis and cloning of MFas II

An affinity-purified fraction of MFas II (generously provided by Dr. James Nardi; see Nardi, 1992) was analyzed at the HHMI Biopolymer Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven). Primary and secondary sequences were obtained from two fragments of this protein and compared with known sequences in the Non-Redundant Protein Sequences database using the NCBI BLAST program (Altschul et al., 1990). Oligonucleotide primers based on this

microsequence data were used in a PCR reaction to amplify a partial clone of MFas II from cDNA prepared from embryonic midgut mRNA. Random primed ^{32}P-labeled probes were prepared from this PCR product and used to screen a cDNA library (Uni-Zap^{tm} XR Custom Library from Stratagene; gift of Dr. James Nardi) generated from mRNA derived from larval CNS. Approximately 6 X 10^5 plaques were screened, resulting in the isolation of 18 positive clones. Nucleic acid sequencing was performed on an automatic sequencer (Model 373 Stretch; ABI) and confirmed by manual sequencing.

Northern blot analysis and in situ hybridization histochemistry

For Northern blots, ^{32}P-labeled probes were prepared from a 3 kb subclone of the extracellular domain of MFas II after restriction digest with either Pst I (sense) or Apa I (antisense) and used at final concentrations of 1 x 10^5 cpm/ml in hybridization buffer (after Horgan et al., 1995). Poly-A$^+$-mRNA was purified from pupal wing using the Oligotek Direct mRNA kit (Qiagen), and 1 µg of each sample was separated on a 1% agarose gel. Samples were then transferred to nylon membranes (Zeta Probe; from Biorad) and hybridization with probe was performed in hybridization buffer at 65°C overnight. Blots were washed repeatedly and exposed to film. For whole-mount *in situ* hybridization histochemistry, both sense and antisense digoxygenin-labeled probes were also prepared from the same inserts described above using digoxigenin-11-UTP (Boeringer) after the methods of Patel and Goodman (1992). Unhydrolyzed probes (~3,000 b) were found to yield a stronger signal in the EP cells than probes that had been hydrolyzed to ~400 b fragments. Embryos were fixed in 5% paraformaldehyde, 0.8% Triton-X-100 in PBS (pH 8.0) for 1 hour and incubated with the probes (1:100 in hybridization buffer; Horgan et al., 1995) overnight at 58°C. Embryos were

washed and reacted with an alkaline phosphatase-conjugated anti-digoxygenin antibody (at 1:2,000; Boehringer). The embryos were then washed in PBS/Triton for 2 hours, and bound antibody was visualized by reaction with the appropriate substrates.

Embryonic culture

Staged embryos were isolated in a modified culture medium (Horgan et al., 1994), restrained in Sylgard-coated chambers, and a small incision was made in the dorsal body wall to expose the developing ENS . Experimental and control solutions were then flushed repeatedly into this opening to ensure complete exposure of EP cells and the muscle bands. Embryos were then allowed to continue to develop at 28°C for 12-20 hours, then fixed in 4% paraformaldehyde and processed for whole-mount immunohistochemistry. For in vivo applications of blocking antibodies, IgG fractions were purified using HiTrap Protein G columns (Pharmacia). Phosphatidylinositol-specific phospholipase C (PI-PLC) and phospholipase B (PLB) were obtained from Boehringer. All other reagents were obtained from Sigma, unless otherwise specified. The distance of EP cell migration and axon outgrowth (measured from the foregut-midgut boundary on each of the four dorsal muscle bands) was then analyzed by photomicroscopy and *camera lucida* techniques. Data from experimental preparations were normalized to matched control groups and subjected to statistical analysis using a 2-tailed Students *t* test. Histograms indicate means $\pm$ SEM.

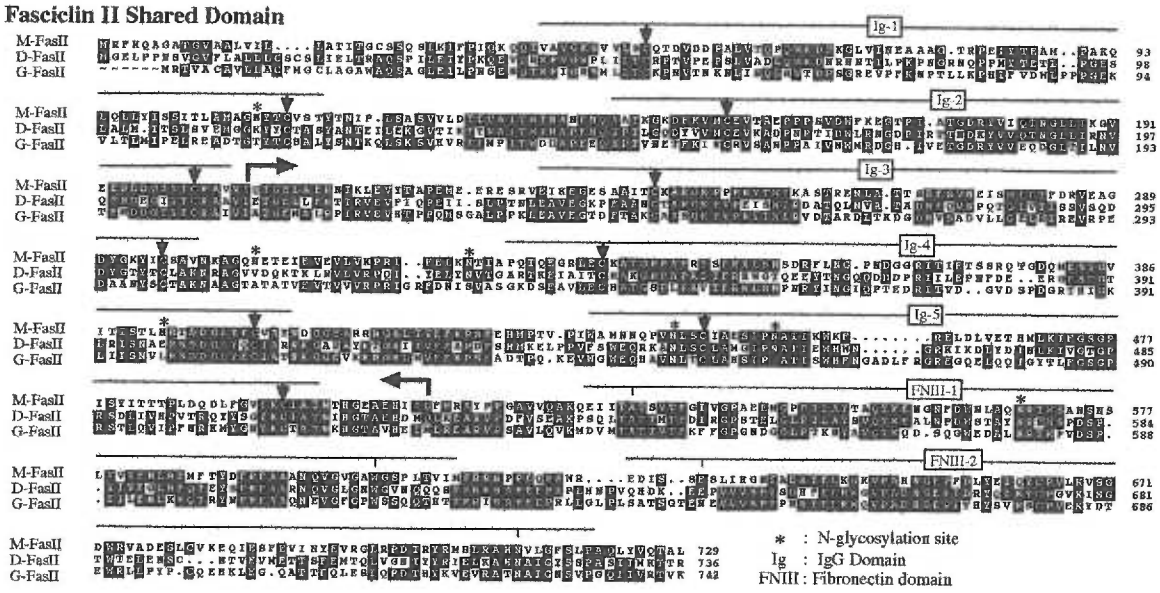
RESULTS

Multiple isoforms of fasciclin II are expressed in the developing ENS

In previous work, Carr and Taghert (1988) showed that the TN-1 antibody produced a staining pattern in the developing CNS of *Manduca* resembling the expression pattern of fasciclin II in grasshopper and *Drosophila* (Bastiani et al., 1987; Grenningloh et al., 1991). Subsequently, Nardi (1990) showed that TN-1 recognized two proteins with apparent molecular weights of 91 kDa and 94 kDa, and that partial amino acid sequences obtained from the 91 kDa protein showed some homology with fasciclin II sequences from other species (Nardi, 1992). Because these sequences were insufficient for the complete characterization of the TN-1 epitope, we obtained two additional microsequences from tryptic fragments of the 91 kDa protein (Glu-Met-Gln-Glu-Arg-Glu-Ser-Arg-Val-Glu-Ile and Val-Phe-Ala-His-Ser-Gly-Glu-Phe-Iso-Asp-Lue-Tyr-Glu-Ile-Gln-Tyr-Cys-Phe-Val-Leu; see methods). These peptides showed 63% and 65% homology with *Drosophila* fasciclin II, respectively, and were used to construct degenerate oligonucleotide primers for a PCR reaction against cDNA obtained from embryonic midgut mRNA. The resultant product was found to have strong sequence similarity with the coding regions for fasciclin II in other species (not shown).

Using probes derived from this PCR product, we isolated eight separate clones from a *Manduca* cDNA library that contained sequences encoding fasciclin II-like proteins. Two of these clones (with inserts of 4.0 and 5.5 kb, respectively) were found to contain full-length coding regions for proteins with significant homology to *Drosophila* and grasshopper fasciclin II (Fig. 1). Each clone contains one long open reading frame encoding proteins with calculated masses (Mr) of ~86.5 kDa and 92.5 kDa, respectively. The two clones share the same 5' untranslated region and signal sequence and are 100% identical over the first 2547 nucleotides. As illustrated in Fig. 1, the

Fasciclin II Shared Domain



GPI-Linked Carboxyl Terminus

Transmembrane Carboxyl Terminus

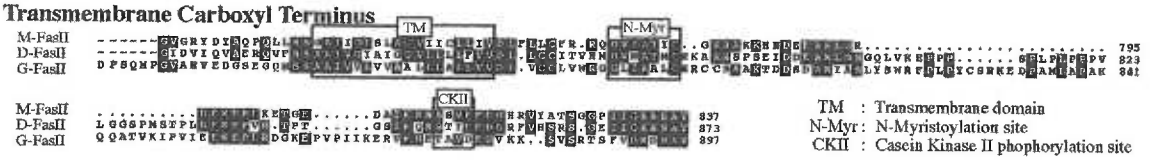


Figure 1. Alignment of the deduced amino acid sequence of *Manduca* fasciclin II (MFas II) with fasciclin II isoforms from *Drosophila* (D-Fas II) and grasshopper (G-Fas II). Conserved amino acid residues are highlighted in black; similar residues are shaded in gray. The five immunoglobulin C2 domains (Ig-1 through Ig-5) and the two fibronectin type III domains (FNIII-1 and FNIII-2) are indicated by the labeled bars above the appropriate sequences. Potential sites for N-glycosylation are indicated by asterisks. The two isoforms of MFas II share a common extracellular domain (residues 1-730) but have divergent C-terminal extensions, as indicated. Characteristic features distinguishing the GPI-linked isoform of MFas II include a putative site for GPI attachment (boxed residues) separated from a C-terminal hydrophobic domain (dotted region) by a short linker region. The transmembrane isoform contains a predicted transmembrane domain (TM) of hydrophobic residues and potential sites for N-myristoylation (N-Myr) and casein kinase II phosphorylation (CKII) within the cytoplasmic domain.

proteins encoded by this region (amino acid residues 1-730) align well with the extracellular domains of fasciclin II from other species, containing five putative Ig-like C2 domains, two FN III domains, and seven potential sites for N-glycosylation (Fig. 1, asterisks). However, the 3' ends of the two clones are divergent. The larger clone contains a predicted transmembrane domain (TM) and a short cytoplasmic tail with potential sites for N-myristoylation and phosphorylation by casein kinase II. The smaller clone encodes a protein with the characteristic features of a 3' GPI attachment site (Udenfriend and Kodukula, 1995), including a triplet of small amino acids that comprise the putative GPI linkage site (boxed region) followed by a short linker region and a C-terminal hydrophobic domain (dotted region). The deduced transmembrane protein shares substantial sequence identity with transmembrane isoforms of fasciclin II in *Drosophila* (44%) and grasshopper (39%), with lower sequence similarity to mouse NCAM (26%). The putative GPI-linked protein shares 42% amino acid identity with the GPI-linked isoform of *Drosophila* fasciclin II. Based on these similarities, we conclude that these two clones encode transmembrane and GPI-linked forms of MFas II.

We next used a combination of Northern blot and protein immunoblot analyses to verify that both isoforms of fasciclin II are expressed in *Manduca*. Riboprobes generated from a portion of the shared 5' region of the two MFas II clones were hybridized with mRNA from developing adult wing (an abundant source of MFas II protein; Nardi, 1990). As shown in Fig. 2A, two strongly hybridizing transcripts of approximately 5 kb and 6.5 kb were consistently detected by these probes, as well as a less prevalent mRNA species at ~11 kb. These results indicate that the native mRNA species encoding MFas II contain additional untranslated sequences that are not present in our cDNA

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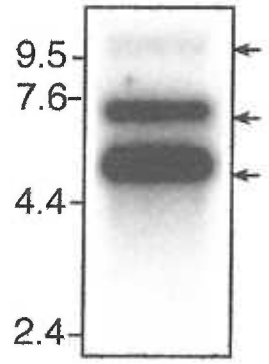

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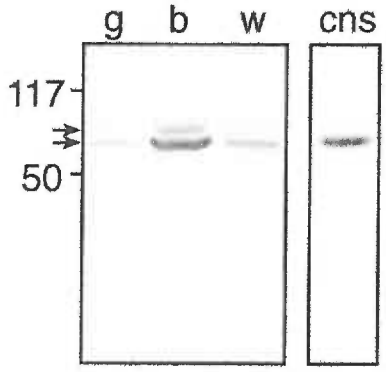

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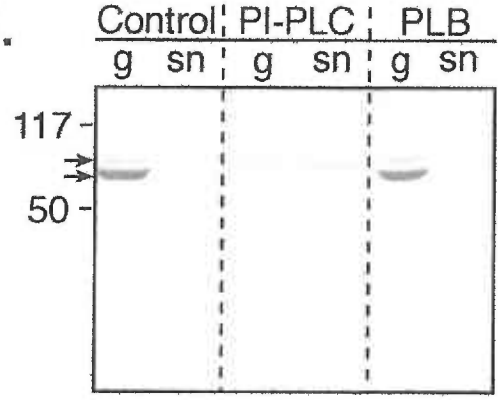

Figure 2. Analysis of MFas II-specific mRNA transcripts and protein isoforms. **A.** Northern blot of poly-A^+-mRNA (purified from pupal wing) reacted with a ^{32}P-labeled probe that was made from a 3 kb subclone of the shared extracellular domain of MFas II. Two transcripts of ~5 kb and 6.5 kb were strongly labeled, while a third transcript (~11 kb) was more faintly labeled. **B.** Immunoblot of proteins extracted from embryonic gut (**g**), larval midgut muscle bands (**b**), developing adult wing (**w**), and larval nervous system (**cns**) that were labeled with an anti-MFas II antibody. Under non-reducing conditions, two prominent bands with apparent molecular weights of ~90 kDa and 95 kDa were consistently seen in tissue extracts containing the EP cells (including embryonic guts and larval muscle bands), as well as in developing adult wing extracts. Only the smaller band was detected in the larval nervous system. **C.** Immunoblots of proteins extracted from embryonic guts (**g**) or from the surrounding supernatant (**sn**) after 2 hours in culture and brief centrifugation. In control medium, both MFas II-specific bands (~90 kDa and 95 kDa) remained associated with the gut tissue, whereas in medium containing PI-PLC, virtually all of the 90 kDa band was liberated into the surrounding medium. Incubation in PLB did not result in a change is distribution of either protein band. For each sample, gut tissue was collected from 10 embryos (60-65% of development) and pooled in culture.

clones. Whether the 11 kb band represents an additional, as yet uncharacterized isoform of MFas II or is simply an unprocessed form of one of the smaller transcripts remains to be determined. Similarly, immunoblots using anti-MFas II antibodies also revealed two strongly labeled bands (Fig. 2B) in protein extracts from embryonic midgut (**g**; containing the migratory EP cells) and from larval midgut muscle bands (**b**; containing the post-migratory EP cells). Both bands were also detected in immunoblots of developing adult wing (**w**), although only the lower band was consistently stained in extracts from larval central nervous system (**cns**). Under the non-reducing conditions used in this study, the apparent molecular weights of the two proteins were ~90 kDa and 95 kDa, consistent with the predicted sizes of the proteins encoded by our cDNA clones after glycosylation.

To demonstrate that one isoform of MFas II is anchored to the external leaflet of the plasma membrane by a GPI-modification, we treated the developing ENS in culture with PI-PLC, an enzyme that has been shown to cleave GPI-linkages and release glypiated molecules into the surrounding medium (Udenfriend and Kodukula, 1995). Sets of embryonic guts containing the EP cells (at 60-65% of development; 10 guts per set) were incubated in culture medium in the presence or absence of PI-PLC for 2 hours. The tissue was then gently pelleted, and detergent-solubilized extracts of both the gut tissue (**g**) and the surrounding supernatant (**sn**) were assayed by immunoblot analysis to determine the distribution of the two MFas II isoforms (Fig. 2C). In control medium without PI-PLC, both isoforms remained associated with the embryonic gut tissue, with no apparent degradation of the proteins (compare with control immunoblot in Fig. 2B). In contrast, incubation with PI-PLC caused virtually all of the ~90 kDa isoform to be released from the gut tissue into the surrounding medium. Phospholipase B (PLB), which does not cleave

GPI linkages, caused no detectable change in the distribution of either isoform. In summary, the results shown in Fig. 2 demonstrate that two isoforms of MFas II are associated with the developing ENS: a transmembrane isoform (~95 kDa), and a GPI-linked isoform (~90 kDa), as predicted by the deduced amino acid sequences of the clones described above.

Fasciclin II is expressed by both the migratory EP cells and their muscle band pathways

In previous work, antibodies against MFas II were found to label the EP cells at various stages of their differentiation (Copenhaver and Taghert, 1989b; Copenhaver and Taghert, 1991). Since fasciclin II has been shown to serve as a neuronal recognition molecule for axon guidance in the developing insect CNS (Harrelson and Goodman, 1988; Lin and Goodman, 1994), we examined the developmental expression of MFas II with respect to EP cell migration. Developmentally matched embryos were labeled either with the TN-1 antibody (which recognizes both MFas II isoforms) or by whole-mount *in situ* hybridization histochemistry, using a digoxygenin-labeled probe against the shared extracellular domain of the two MFas II clones described above (Fig. 3).

Prior to the onset of EP cell migration (50% of development), both MFas II protein and MFas II-specific mRNA were expressed throughout the packet of EP cells at the foregut-midgut boundary, as well as by cells within the developing esophageal nerve on the foregut (**en**; Fig. 3A, D). In contrast, no detectable expression of MFas II was observed within the adjacent muscle cell layers on the foregut and midgut, although a variety of other ectodermal and mesodermal cell types stained positively for MFas II at this time (not shown). During the subsequent period of EP cell migration (55-60% of development), all of the migratory neurons continued to express MFas II as they traveled

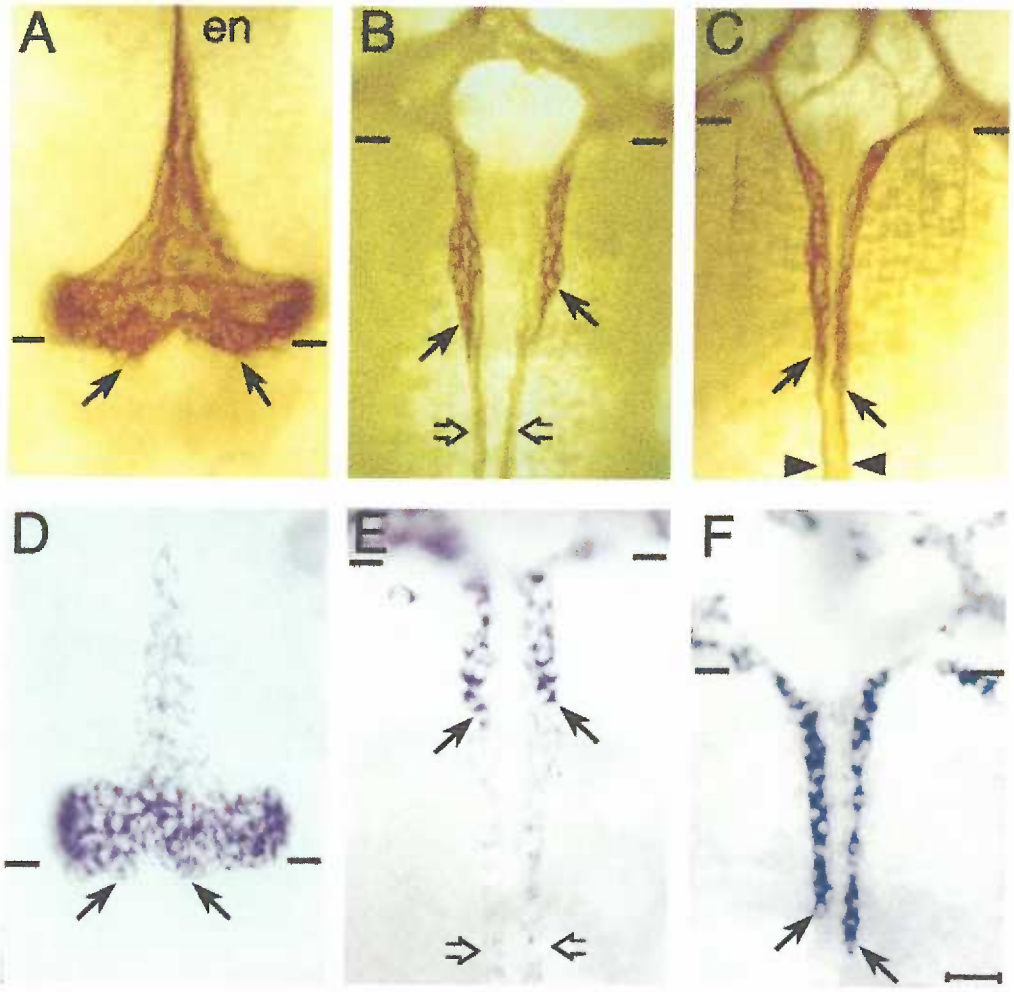
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Figure 3. Developmental expression of MFas II transcripts and protein in the developing ENS. (**A-C**): whole mount immunostaining of the developing ENS with an anti-MFas II antibody; (**D-F**): whole-mount in situ hybridization histochemistry of the ENS with digoxygenin-labeled probes against MFas II-specific mRNA. **A** and **D**: at 50% of development, the premigratory EP cells form a tight packet at the foregut-midgut boundary and show strong levels of MFas II expression. Positive immunostaining can be seen in the short filopodial processes extended by some of the EP cells onto the adjacent midgut epithelium (**A**, black arrows). MFas II expression can also be seen within axon bundles and the presumptive glial cells forming the recurrent nerve (**en**) that joins the EP cells to the more anterior enteric ganglia (out of view). **B** and E: at 58% of development, subsets of EP cells (black arrows) have begun to migrate out of the original packet and continue to exhibit strong levels of MFas II expression. In addition, the muscle cells forming the band pathways also contain detectable levels of MFas II protein and message (open arrows; only the mid-dorsal pair of muscle bands is shown), whereas the adjacent interband muscle cells do not (see also Fig. 4). **C** and **F**: at 65% of development, the EP cells have completed their migration, but they continue to extend axonal processes posteriorly along the muscle band pathways. MFas II expression remains high in the post-migratory EP cells (black arrows), including robust immunostaining within the outgrowing bundles of axons (**C**, arrowheads). In contrast, the underlying muscle bands no longer exhibit detectable levels of either MFas II-related protein or message. Paired black hatchmarks indicate the foregut-midgut boundary; scale bar = 20 μm.

onto the coalescing muscle bands of the midgut (Fig. 3B, E, arrows), including strong immunolabeling of the leading processes of the migratory neurons (visible in Fig. 3B). In addition, however, both MFas II protein and MFas II-specific mRNA could also now be seen within the muscle cells forming the band pathways (open arrows in Fig. 3B, E). In contrast, no detectable MFas II expression was detected within the adjacent interband musculature. By the end of the migratory period (65% of development), this dual pattern of expression had changed: while the post-migratory neurons continued to show strong levels of MFas II expression (Fig. 3C, F), including robust levels of MFas II immunoreactivity within their axonal processes (arrowheads), the muscle bands no longer show any detectable MFas II mRNA or protein.

This transient pattern of fasciclin II expression by the midgut muscle band pathways was unexpected and is better illustrated in Fig. 4. As previously noted, the muscle bands coalesce from subsets of the longitudinal muscle cells at eight specific locations around the midgut circumference, just prior to the onset of EP cell migration (Copenhaver and Taghert, 1989b). Shortly after their formation, MFas II expression could be detected along the entire length of the muscle bands (Fig. 4A and D, open arrows), coincident with the migration of the EP cells onto the anterior ends of the bands at the foregut-midgut boundary (black arrows). Notably, positive staining within the visceral mesoderm was restricted to the component cells of the muscle bands, a distinction that was readily apparent in regions of the midgut posterior to the migratory neurons (Fig. 4B, E). As shown in Fig. 3, by 65% of development, the post-migratory EP cells had begun to extend axons along the muscle bands (Fig. 4C, arrowheads), a process that continues over the next 20% of development before they branch laterally to innervate the interband musculature (Copenhaver and Taghert, 1989a; Wright et al., 1998). However,

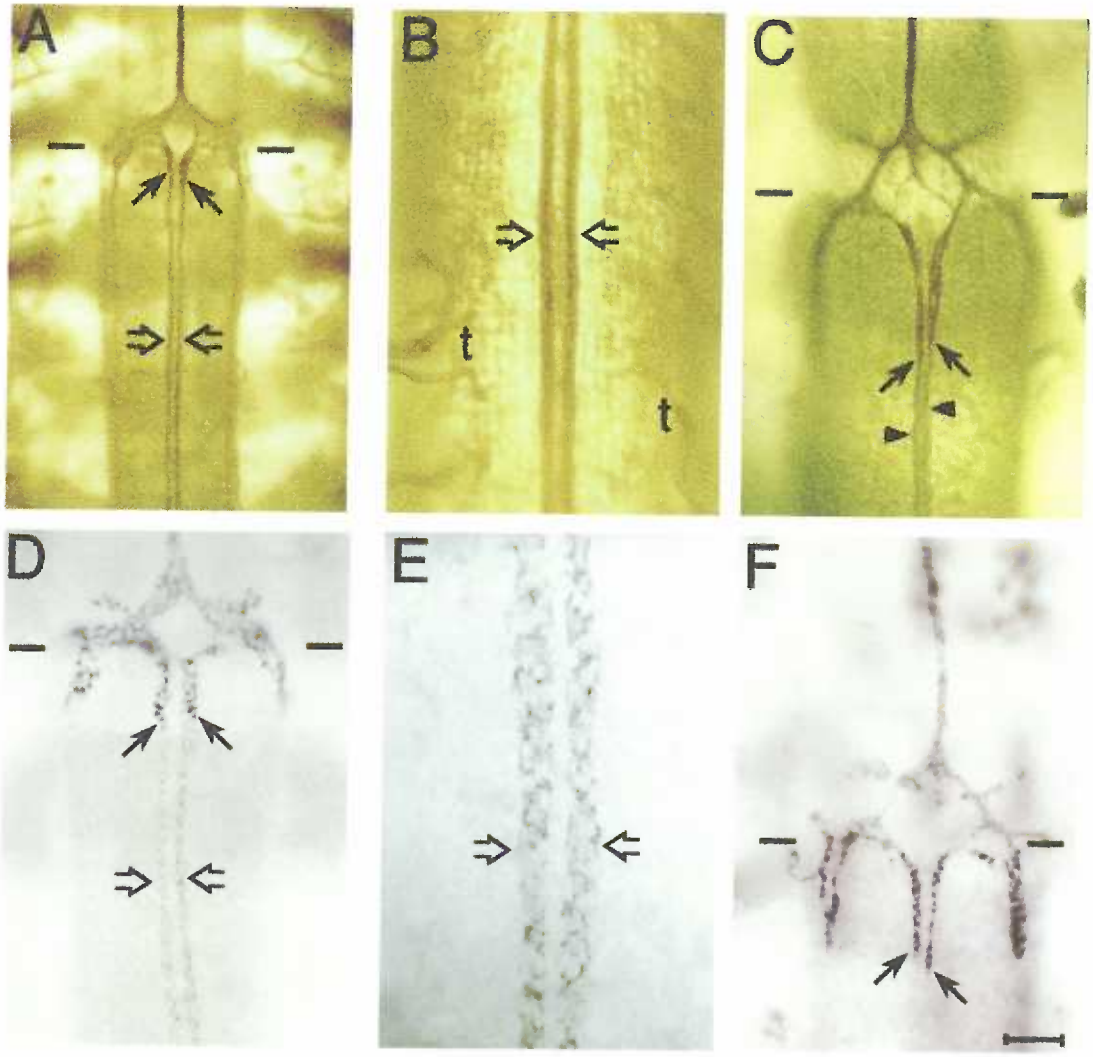
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Figure 4. The muscle band pathways transiently express MFas II along the entire length of the midgut during EP cell migration. (**A-C**): whole mount immunostaining of the developing ENS with an anti-MFas II antibody; (**D-F**): whole-mount in situ hybridization histochemistry of the ENS with digoxygenin-labeled probes against MFas II-specific mRNA. At 58% of development (**A** and **D**), the EP cells have commenced their migration onto the midgut muscle bands (black arrows). In addition, the eight longitudinal muscle bands of the midgut have also begun to express MFas II (open arrows indicate the mid-dorsal pair of bands; the dorsolateral pair of bands can also be seen near the margins of the midgut in **A**). Note that the muscle bands extend the entire length of the midgut (>1 mm), whereas the EP cells will only migrate for approximately 200 -250 μm before stopping (compare with Fig. 3). **B** and **E**: higher magnified view of the mid-dorsal muscle bands at 58% of development on the posterior midgut (distal to the position of the migratory EP cells). Positive expression of both MFas II-related protein and mRNA can clearly be detected within the muscle band cells (open arrows) but not in the surrounding interband musculature. Curving structures labeled "t" are tracheolar branches growing onto the midgut. **C** and **F**: at 65% of development, the post-migratory EP cells (black arrows) have begun to extend axonal processes along the muscle bands (arrowheads; see Fig. 3). By this stage, the muscle bands have ceased to exhibit detectable levels of MFas II. Paired black hatchmarks indicate the foregut-midgut boundary, where appropriate. Scale bar = 50 μm in panels A, C, D, and F and 20 μm in panels B and E.

by the time that migration was complete, MFas II expression could no longer be detected within the muscle band cells at any position along the midgut (Fig. 4C, F). Thus, both the EP cells and their muscle band pathways express MFas II during the migratory period but do so in developmentally distinct patterns: whereas the migratory neurons maintain strong levels of MFas II both before and after migration, the muscle bands express this cell adhesion receptor transiently, exhibiting detectable levels of MFas II only during the specific time during which the neurons are actively migrating on them.

Fasciclin II is necessary for EP cell migration

As noted earlier, surgical manipulations of the developing ENS in vivo have shown that the muscle band pathways are both necessary and sufficient for the normal migratory dispersal of the EP cells: manipulations that prevent contact between the EP cells and a muscle band preclude migration, while transplantation of the EP cells onto a muscle band will promote migration (Copenhaver et al., 1996). Because fasciclin II has been shown to act as a homophilic adhesion molecule in other systems (Grenningloh et al., 1990; Lin and Goodman, 1994), the transient upregulation of MFas II on the muscle bands coincident with the onset of EP cell migration (Figs. 3 and 4) suggested a role for MFas II in the guidance of the migratory neurons. To test this hypothesis, we used antibodies against MFas II as a means of inhibiting fasciclin II-mediated interactions in vivo. Embryos were placed in culture at 50-52% of development (prior to migration onset) and minimally dissected to expose the ENS (Fig. 5A). The EP cells were then treated either with control medium or with medium containing affinity-purified antibodies against MFas II, and the preparations were allowed to develop for an additional 12-15 hours (through the completion of the migratory period). In contrast to control

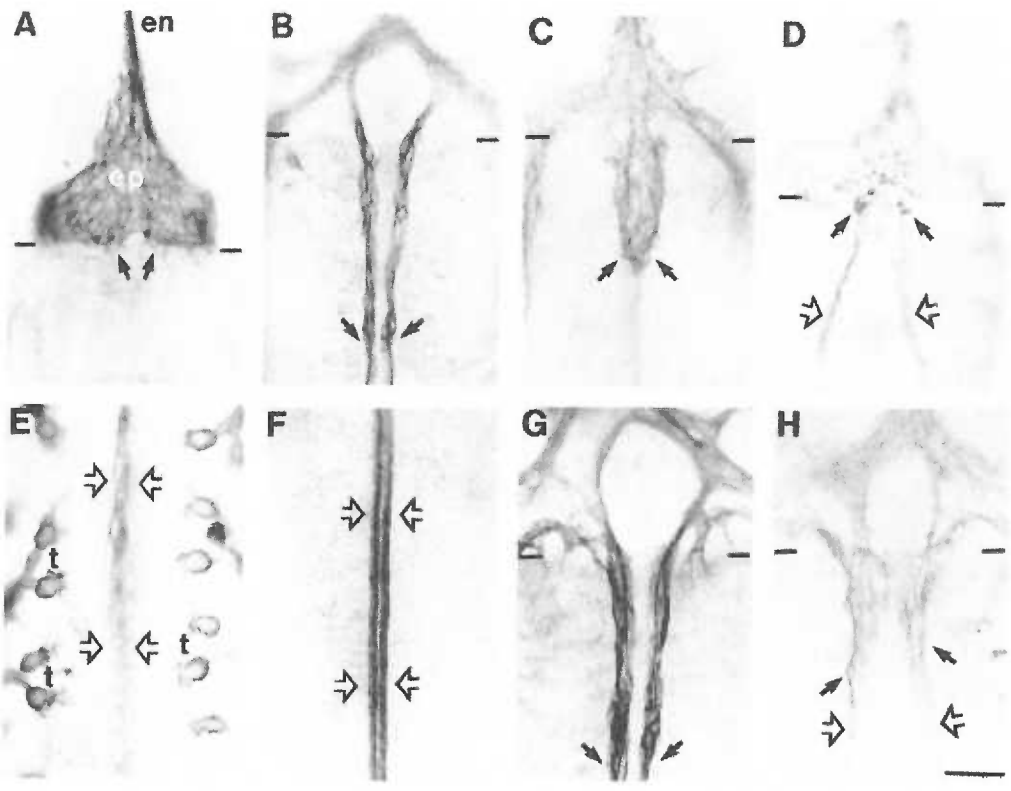
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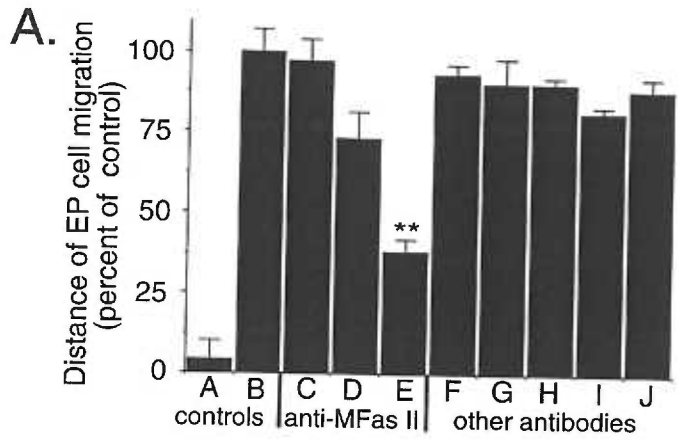
Figure 5. Perturbations of MFas II in embryo culture inhibits EP cell migration. Preparations were fixed and immunostained with anti-MFas II antibodies unless otherwise noted. **A**. Premigratory EP cells (**ep**) in an embryo placed in culture at ~53% of development and then fixed immediately to show the initial positions of the EP cells (black arrows) at the onset of the culture experiments. MFas II expression was also present in the cells and processes of the adjacent recurrent nerve (**en**). **B**. Control embryo that was placed in culture at 53% of development (panel A) and allowed to develop in normal medium for 12 hours before fixation; note that the migration of the EP cells (arrows) along the muscle bands proceeded normally. **C**. Embryo that was cultured in medium containing 20 μg/ml anti-MFas II antibody (affinity-purified IgG). The extent of EP cell migration was reduced, and the neurons exhibited some aberrant clumping on the muscle bands (arrows). **D**. Embryo that was cultured in the presence of 200 μg/ml anti-MFas II IgG. In this preparation, EP cell migration was completely inhibited, with the leading neurons (arrows) remaining at the foregut-midgut boundary. Formation of the mid-dorsal muscle bands (open arrows) was also partially disturbed, possibly do to incomplete gut closure. **E**. Immunohistochemical staining of a 65% embryo with anti-fasciclin III antibodies labeled a stripe of epithelium underneath the mid-dorsal muscle bands (open arrows); the ingrowing tracheolar cells (**t**) were also positively stained. **F**. Immunohistochemical staining of a 65% embryo with anti-neuroglian antibodies also labeled the muscle bands (open arrows). **G**. Embryo that was cultured in the presence of 100 μg/ml anti-neuroglian IgG; the migration of the EP cells and formation of the muscle bands appeared normal (compare with panel **B**). **H**. Embryo that was treated in culture with PI-PLC (1 U for 60 minutes) and then allowed to continue development in

normal medium for 12 hours. Migration of the EP cells (black arrows) was inhibited compared to control preparations and the overall level of MFas II immunoreactivity was reduced. The coalescence of the muscle bands was also partially disrupted (open arrows), although they still appeared to support neuronal migration. Paired black hatchmarks indicate the foregut-midgut boundary, where appropriate. scale bar = 20 μm.

embryos, in which the EP cells migrated normally along their muscle band pathways (Fig. 5B), exposure to anti-MFas II antibodies caused a significant inhibition in neuronal migration (Fig. 5C-D). At relatively low antibody concentrations, the effects on migration were variable, resulting in abnormal clumping of the EP cells that had partially migrated onto the muscle bands (Fig. 5C). At higher antibody concentrations, migration was significantly inhibited: whereas in control embryos, EP cells had migrated an average of 164 μm after 8 hours, in embryos treated with 200 μg/ml anti-MFas II IgG, the average distanced of migration was only 65 μm, and 20% of the preparations, virtually all of the EP cells remained clustered at the foregut-midgut boundary (Fig. 5D). The coalescence of the muscle band pathways also appeared to be perturbed, a finding that is discussed below. In addition, the overall level of MFas II staining was reduced in these preparations, possibly due to antibody-induced receptor internalization; however, we did not observe any evidence of necrosis or cell death within the EP cell packet or the muscle bands.

The effect on migration following treatment with either anti-MFas II antibodies or antibodies against a number of other cell adhesion receptors is also shown in Fig. 6A. As compared with cultured control embryos, antibodies against MFas II caused a concentration-dependent inhibition of EP cell migration along the midgut muscle bands. In contrast, antibodies against *Drosophila* fasciclin I, fasciclin III, and semaphorin 1a (fasciclin IV) had no detectable effect on migration. While not all of these antibodies labeled components of the ENS in *Manduca*, anti-fasciclin III antibodies did stain the midgut epithelium underneath the muscle bands (Fig. 5E, open arrows), as well as the adjacent tracheolar cells growing onto the midgut surface. More significantly, affinity-purified antibodies against *Manduca* neuroglian, another member of the Ig-superfamily of cell adhesion receptors (Chen et al., 1997;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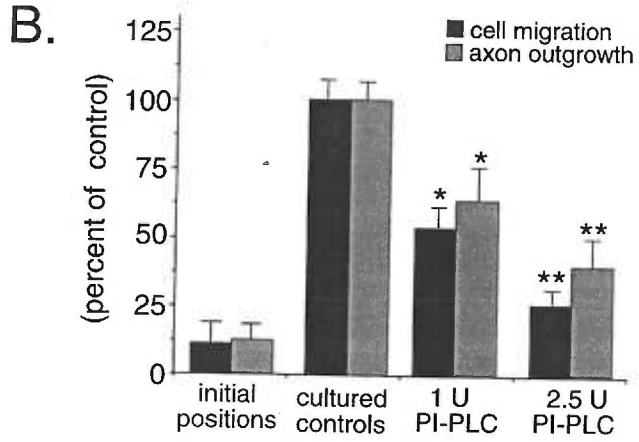
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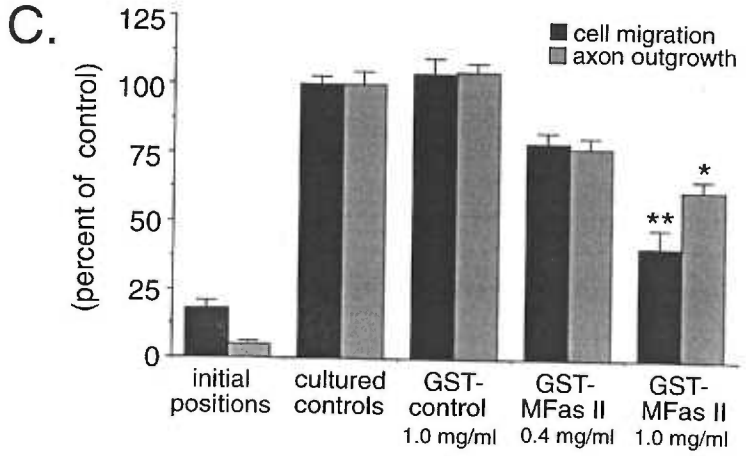
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GST-MFas II 1.0 mg/ml

Figure 6. Inhibition of MFas II-mediated interactions significantly inhibits migration. **A.** The extent of EP cell migration in control embryos and in embryos incubated with antibodies against a variety of cell adhesion receptors. The distance of migration was measured from the foregut-midgut boundary for the leading neuron on each of the four dorsal muscle bands and normalized with respect to control embryos. Columns labeled A and B show the extent of migration in cultured embryos at the onset and completion of the culture period. Columns C-E show the extent of migration in embryos treated with anti-MFas II IgG at 1 μg/ml, 20 μg/ml, and 200 μg/ml, respectively. Treatment with 200 μg/ml anti-MFas II IgG caused a significant inhibition of migration (*$P < 0.001$). In contrast, treatment with a variety of other antibodies produced no detectable effect on migration, including antibodies against fasciclin I (column F), fasciclin III (column G), semaphorin 1a (column H), and neuroglian (100 μg/ml IgG; column I). **B.** Both EP cell migration (black histograms) and axon outgrowth (gray histograms) were reduced by exposure to PI-PLC in a concentration-dependent manner. *$P < 0.01$; **$P < 0.001$. Each histogram represents data collected from at least 10 animals; histograms indicate mean values ± SEM. Data analysis was performed using a 2-tailed Student's *t* test.

Hortsch et al., 1990), showed strong immunolabeling of the midgut muscle bands (Fig. 5F) but had no effect on EP cell migration (Fig. 5G, 6A). All of these experiments were done in complement-free conditions, but the limited amount of available antibody precluded the use of Fab' fragments; it is therefore possible that some of the effects induced by anti-MFas II IgG were due to steric interference. Nevertheless, these results support the hypothesis that the coordinated expression of MFas II by the EP cells and their muscle band pathways serves to promote neuronal migration within the developing ENS.

As noted above, both the transmembrane and GPI-linked isoforms of MFas II appear to be expressed within the developing ENS (Fig. 2). Because PI-PLC has been previously shown to remove GPI-linked adhesion receptors from living cells in other systems (Chang et al., 1992; Doherty et al., 1990), we used our embryonic culture preparation to expose the premigratory EP cells to PI-PLC for 30-60 minutes and then allowed the embryos to continue development in normal medium. As shown in Fig. 5H, treatment with PI-PLC caused a marked reduction in the extent of EP cell migration and also reduced the overall level of MFas II immunoreactivity. The effects of PI-PLC pre-treatment (before migration onset) were both concentration-dependent and statistically significant (Fig. 6B), inhibiting both the migration and axon elongation of the EP cells. In contrast, phospholipase B had no significant effect on migration (not shown). Although PI-PLC treatments should remove other GPI-linked receptors besides MFas II, these treatments did not result in a general disruption of gut development. Rather, the enzymatic removal of GPI-linked MFas II at this stage of development appeared to affect selectively the progression of EP cell migration. Together with the inhibitory effects of blocking antibodies described above, these results indicate that fasciclin II plays

an essential role as a guidance molecule for neuronal migration in the developing ENS.

DISCUSSION

Fasciclin II was originally characterized as a neuronal recognition molecule in the developing insect nervous system, based on its selective pattern of expression by specific subsets of growing axons within both the CNS and PNS (Bastiani et al., 1987; Harrelson and Goodman, 1988). A combination of molecular and genetic manipulations of fasciclin II subsequently demonstrated that it may serve a variety of functions, including the selective fasciculation of growing axons (Lin et al., 1994), synaptic stabilization and plasticity (Schuster et al., 1996; Thomas et al., 1997), and possibly the modulation of growth cone responses to other cell adhesion receptors (Fambrough and Goodman, 1996; Lin and Goodman, 1994). In the present study, we have shown that fasciclin II is also expressed by an identified class of migratory neurons (the EP cells) and their cellular pathways (the midgut muscle bands) during the normal period of migration. Moreover, we have shown that in vivo manipulations designed to perturb fasciclin II-mediated interactions consistently inhibited EP cell migration. These results demonstrate a new role for fasciclin II, indicating that this adhesion receptor participates in the control of multiple forms of neuronal motility.

The overall structure of fasciclin II (five Ig C2 domains plus two FN-III domains, expressed by multiple isoforms with divergent C-terminal sequences) suggests that it shares a common ancestral molecule with the vertebrate adhesion receptor NCAM (Grenningloh et al., 1990). Like fasciclin II, NCAM can be detected on growing axons and motile neurons in many regions of the CNS and PNS (Goridis and Brunet, 1992), although its

distribution is much more widespread than the restricted patterns of cell-specific expression described for fasciclin II (Harrelson and Goodman, 1988). Also like fasciclin II, the specific functions of NCAM appear to be more complex than originally proposed: genetic deletions of both of these receptors produced surprisingly subtle defects (Cremer et al., 1994; Grenningloh et al., 1991; Tomasiewicz et al., 1993), suggesting that their primary role may be to promote axonal adhesion rather than directional guidance *per se* (Lin and Goodman, 1994; Tang and Landmesser, 1993). However, one striking effect of deleting NCAM was a significant reduction in granule cell populations within the olfactory bulb, due to the absence of NCAM-mediated interactions required for the migration of their precursors from the subventricular zone (Hu et al., 1996; Ono et al., 1994). Similarly, our results indicate that fasciclin II-dependent events are essential for the migration of the EP cells along their muscle pathways. Thus, just as the phenomenon of cell migration occurs in all developing nervous systems, the molecular mechanisms underlying this basic process may be evolutionarily conserved, as well.

The deduced amino acid sequences that we obtained for MFas II (Fig. 1) aligned well with sequences for fasciclin II from other species (Grenningloh et al., 1991; Snow et al., 1988), including the transmembrane isoforms of fasciclin II from both *Drosophila* and grasshopper and the GPI-linked isoform from *Drosophila* (curiously, no GPI-linked form has been identified in grasshopper). The proteins from all three species share similar degrees of sequence conservation (between 39-44% amino acid identity) and are similarly divergent from related vertebrate molecules (MFas II shares 26% identity with mouse NCAM). Unlike the transmembrane forms of fasciclin II described for grasshopper and fly, the cytoplasmic region of transmembrane MFas II dose not contain a PEST domain (a motif that may confer instability to proteins

intended for rapid turnover; Rechsteiner, 1988). The existence of a transmembrane isoform of fasciclin II lacking a PEST domain in *Drosophila* has also been mentioned (Lin and Goodman, 1994). Whether a third, PEST+ isoform of MFas II is expressed in *Manduca* remains to be determined. However, we did detect numerous ATTTA motifs in the 3' untranslated regions of both isoforms of MFas II (eight in the GPI-linked form and ten in the transmembrane form; not shown), which have been shown to affect mRNA stability in other systems (Chen and Shyu, 1995). The transient expression of MFas II by the muscle band pathways during the migratory period may therefore be regulated in part by a rapid turnover of MFas II-specific mRNA within these cells.

How does fasciclin II regulate EP cell migration?

The manipulations that we performed in embryo culture to perturb fasciclin II-mediated interactions consistently inhibited EP cell migration: both the application of blocking antibodies against MFas II and removal of GPI-linked MFas II with PI-PLC caused significant reductions in the extent of migration without inducing obvious signs of damage to the EP cells. While both of these experimental approaches must be interpreted with some caution, the fact that a number of other antibodies (including anti-neuroglian, which also labels the muscle bands) and other enzymes (including phospholipase B) had no effect on ENS development supports our conclusion that fasciclin II plays an essential role in promoting EP cell migration. Still unresolved is the specific mechanism by which fasciclin II-mediated interactions affect neuronal motility. The simplest interpretation of our results is that the onset of MFas II expression within the newly formed muscle bands establishes a permissive substrate for EP cell migration onto the midgut, so that MFas II-mediated

homophilic interactions between the neurons and the muscle bands leads to their migration along these pathways. This hypothesis is supported by previous work which showed that the muscle bands are both necessary and sufficient for neuronal migration, in that the EP cells will not migrate onto the midgut musculature unless they are in direct contact with one of the muscle bands (Copenhaver et al., 1996). Moreover, the application of anti-MFas II antibodies did not result in a general dissociation of the premigratory packet of EP cells; rather, this treatment appeared to interfere specifically with the formation of homophilic interactions between the neurons and the newly formed muscle bands. However, our manipulations of MFas II in culture also perturbed the coalescence of the muscle bands themselves (Fig. 5D, H), suggesting that fasciclin II may play a role in the differentiation of the bands from the visceral mesoderm. Although we have observed in culture that the EP cells will migrate onto the muscle band cells even when the bands have not completely coalesced (unpublished data), it is possible that our manipulations of MFas II affected migration indirectly by preventing the normal differentiation of their migratory pathways. We are currently testing whether MFas II expressed in the absence of other muscle band proteins can support EP cell migration in vitro.

Manipulations of fasciclin II by a variety of means have suggested that it may play a number of different roles within the developing nervous system. For example, application of blocking antibodies to cultured grasshopper embryos caused fasciclin II-expressing growth cones to stall and to elaborate filopodia along inappropriate directions (Harrelson and Goodman, 1988). Removal of fasciclin II from peripheral pioneer neurons using chromophore-assisted laser inactivation also inhibited axonogenesis, but only at a restricted stage of neuronal differentiation (Diamond et al., 1993). The results of these

acute treatments were qualitatively similar to the dramatic effects that we observed with respect to neuronal migration, suggesting that fasciclin II plays an important role in the guidance of motile cells and processes. In contrast, genetic deletions of fasciclin II have produced more subtle alterations in the developing nervous system, primarily due to errors in axonal fasciculation (Lin et al., 1994; Lin and Goodman, 1994). It is therefore possible that the true role of fasciclin II in the developing ENS of *Manduca* is simply to maintain the apposition of the EP cells with the muscle bands, while other pathway-specific cues are needed to promote directional migratory behavior. As has been shown in other systems (e.g.Fishman and Hatten, 1993; Newgreen and Minichiello, 1995), it is likely that a combination of positive and negative guidance cues regulate the timing and directionality of migration in the ENS. However, acute inhibition of fasciclin II in culture (such as with blocking antibodies or enzymatic cleavage) may also reveal fasciclin II-dependent processes that might be obscured in a genetic mutation, where other guidance cues with overlapping functions might be able to compensate for the lack of fasciclin II over time. Manipulations of MFas II expression within the EP cells and the muscle band pathways will be needed to determine which aspect of neuronal migration is specifically regulated by this adhesion receptor.

A related question concerns the mechanism by which EP cell migration is first initiated. As noted above (Fig. 3), all of the EP cells express MFas II prior to the onset of migration, at which time the neurons are tightly clustered at the foregut-midgut boundary. Although our data support a role for MFas II in guiding the EP cells along the muscle bands once these pathways have formed, an additional process must stimulate the preferential release of fasciclin II-dependent adhesive interactions within the premigratory cluster in order for migration to occur. By analogy, NCAM is also expressed by a variety of

premigratory neurons in the vertebrate nervous system (e.g.Chuong et al., 1987; Rutishauser et al., 1988). While some cells may down-regulate NCAM as they become motile (Akitaya and Bronner-Fraser, 1992; Hynes and Lander, 1992), the migration of granule cell precursors into the olfactory bulb coincides with the expression of a polysialated NCAM (NCAM-180), an isoform of NCAM that has anti-adhesive properties and promotes neuronal plasticity and growth (Hu et al., 1996; Tang and Landmesser, 1993). Although fasciclin II has not been found to be polysialated, several different isoforms have been identified (Grenningloh et al., 1991; Lin and Goodman, 1994; and this paper) which may play distinct roles in development. Alternatively, modulation of fasciclin II-fasciclin II interactions by other guidance cues associated with the muscle bands may facilitate the exodus of the EP cells from their original packet (cf. Fambrough and Goodman, 1996). An analysis of isoform-specific expression patterns for MFas II should lend insight into the role that these molecules may play with respect to EP cell migration.

As with other examples of neuronal migration, the EP cells remain motile for only a specific time during development; however, following the migratory period, they continue to extend axonal processes along the same muscle band pathways on the midgut. Intriguingly, this behavioral transition from migration to axon outgrowth coincides with the disappearance of MFas II expression from the muscle bands, although MFas II levels within the axons of the EP cells remain high (Fig. 3c; and unpublished observations). It is therefore possible that the termination of fasciclin II expression in the muscle bands precludes further migration by the neurons, while other guidance cues associated with the muscle bands (such as neuroglian; Fig. 5F) continue to support axonal outgrowth. The persistent expression of MFas II in the EP cell axons may simply serve to promote their fasciculation until they branch

laterally to innervate the interband musculature (Wright et al., 1998). Alternatively, fasciclin II may also interact with other adhesion molecules expressed by the muscle bands once migration is complete. Heterophilic interactions of this type have been reported for a number of related adhesion receptors, including NgCAM, F_3/F_{11}, and possibly NCAM (Brummendorf and Rathjen, 1993; Horstkorte et al., 1993), but have not yet been investigated with respect to fasciclin II. Similarly, several related molecules (including NCAM and L1) have been shown to associate with non-receptor tyrosine kinases, a relationship that is required for at least some aspects of their adhesive functions (Beggs et al., 1994; Ignelzi et al., 1994). In preliminary studies, we have shown that changes in tyrosine kinase activity accompany the transition of the EP cells from a premigratory to a migratory state (unpublished data); however, a functional link between fasciclin II and a particular intracellular signaling pathway remains to be determined.

Besides contributing to the formation of the nervous system, the process of cell migration is essential to the formation of many other embryonic tissues, where similar molecular mechanisms may regulate the motility of markedly different cell types (reviewed in Caterina and Devreotes, 1991; Hynes and Lander, 1992). In this regard, it is noteworthy that MFas II is also expressed by subsets of cells within a number of different ectodermal and mesodermal tissues at specific times during embryogenesis (Carr and Taghert, 1989; Grenningloh et al., 1991; Wright and Copenhaver, unpublished observations). Moreover, high levels of MFas II expression have been specifically correlated with periods of cell migration and reorganization in both the embryonic tracheal system (Nardi, 1990) and the developing adult wing (Nardi, 1992). The results that we have obtained concerning the role that fasciclin II plays in controlling neuronal migration may therefore be relevant

to more general aspects of morphogenesis and regulated cell movements within the developing embryo.

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DIFFERENT ISOFORMS OF FASCICLIN II PLAY DISTINCT ROLES IN THE GUIDANCE OF NEURONAL MIGRATION DURING INSECT EMBRYOGENESIS

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ABSTRACT

During the formation of the enteric nervous system (ENS) of the moth *Manduca sexta*, identified populations of neurons and glial cells participate in precisely timed waves of migration. The cell adhesion receptor fasciclin II is expressed in the developing ENS and is required for normal migration. Previously, we identified two isoforms of *Manduca* fasciclin II (MFas II), a glycosyl phosphatidylinositol-linked isoform (GPI-MFas II) and a transmembrane isoform (TM-MFas II). Using RNA and antibody probes, we found that these two isoforms were expressed in cell type-specific patterns: GPI-MFas II was expressed by glial cells and newly generated neurons, while TM-MFas II was confined to differentiating neurons. The expression of each isoform also corresponded to the motile state of the different cell types: GPI-MFas II was detected on tightly adherent or slowly spreading cells, while TM-MFas II was expressed by actively migrating neurons and was localized to their most motile regions. Manipulations of each isoform in embryo culture showed that they played distinct roles: whereas GPI-MFas II acted strictly as an adhesion molecule, TM-MFas II promoted the motility of the EP cells as well as maintaining fasciculation with their pathways. These results indicate that precisely regulated patterns of isoform expression govern the functions of fasciclin II within the developing nervous system.

INTRODUCTION

The formation of the nervous system requires the directed migration of both neurons and glial cells through a complex landscape of potential guidance cues. Within the nervous systems of both vertebrates and invertebrates, the developmental origins of nascent neuronal and glial populations are intimately linked (Bossing *et al.*, 1996; Bronner-Fraser, 1994; Hartenstein *et al.*, 1998; Wetts and Fraser, 1988), and reciprocal interactions between adjacent neurons and glial cells have been shown to regulate the migration and subsequent differentiation of both cell types (Ebens *et al.*, 1993; Hardy and Reynolds, 1993; Hatten, 1999; Klämbt *et al.*, 1991). Within particular regions of the nervous system, similar guidance cues have often been found to regulate the motility of neurons and glial cells (e.g. Orentas and Miller, 1996; Zerlin *et al.*, 1995), including components of the extracellular matrix, soluble factors, and a variety of cell adhesion receptors (Delannet *et al.*, 1994; Fishman and Hatten, 1993; Kiss, 1998; Wehrle-Haller and Chiquet, 1993). Although the effects of many potential guidance cues on cell migration have been extensively characterized *in vitro*, the molecular mechanisms by which these cues regulate motility in the intact nervous system remain unclear (Lumsden and Gulisano, 1997).

To address this issue, we have established the enteric nervous system (ENS) of the moth, *Manduca sexta* as a preparation in which neuronal and glial migration can be examined *in vivo*. During embryogenesis, a population of ~300 post-mitotic neurons (the **EP cells**) delaminates from a neurogenic placode in the posterior foregut (Copenhaver and Taghert, 1990); concurrently, a second population of glial precursor cells emerges from an adjacent proliferative zone associated with the developing esophageal nerve (formerly named the recurrent nerve; Copenhaver, 1993). These two cell

groups become distributed across the foregut and midgut via a stereotyped sequence of motile behaviors, giving rise to the enteric plexus that spans the foregut-midgut boundary (see Fig. 1). The EP cells first migrate and then extend axons along specific sets of visceral muscles on both the foregut and midgut (Copenhaver and Taghert, 1989b), and then the glial cells spread along these same pathways, ensheathing the post-migratory neurons (Copenhaver, 1993). Notably, the migrating cells and their pathways remain accessible to manipulation throughout embryogenesis, permitting an *in vivo* analysis of the mechanisms controlling this migratory sequence (e.g. Copenhaver *et al.*, 1996; Horgan and Copenhaver, 1998; Wright *et al.*, 1998).

Recently, we showed that the cell adhesion receptor fasciclin II is required for the normal migration of the EP cells (Wright *et al.*, 1999). Fasciclin II is a member of the immunoglobulin (Ig)-related superfamily of cell adhesion receptors. Like the vertebrate receptor NCAM, fasciclin II has an extracellular domain containing five Ig-like C2 domains and two fibronectin (FN)-type III domains (Grenningloh *et al.*, 1990) and is expressed in multiple isoforms via alternative splicing from a single gene (Lin and Goodman, 1994; Snow *et al.*, 1988). In *Drosophila*, two transmembrane isoforms of fasciclin II have been reported, as well as a third isoform that is attached to the outer leaflet of the plasma membrane via a glycosyl phosphatidylinositol (GPI) linkage (Goodman *et al.*, 1997). Immunohistochemical studies (directed primarily against the transmembrane isoforms) have shown that fasciclin II is expressed in a dynamic fashion on subsets of cells and axons within both the central and peripheral nervous system (Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988). Although originally characterized as a homophilic adhesion molecule that regulates axonal fasciculation (Lin *et al.*, 1994), fasciclin II has also been shown to participate in the control of proneural gene

expression (Garcia-Alonso *et al.*, 1995; Whitlock, 1993), synaptic patterning and stabilization (Davis *et al.*, 1997; Schuster *et al.*, 1996b), and activity-dependent plasticity (reviewed in Goodman *et al.*, 1997). The transmembrane isoforms also share a PDZ-binding motif that has been shown to promote interactions between fasciclin II and several proteins localized at the neuromuscular junction, including the membrane-associated guanylate kinase Discs-large and the voltage-sensitive Shaker potassium channel (Thomas *et al.*, 1997; Zito *et al.*, 1997). In contrast, a specific role for the GPI-anchored form of fasciclin II has not been investigated.

Multiple forms of *Manduca* fasciclin II (hereafter designated MFas II) have also been identified, including a single transmembrane isoform (TM-Mas II) and a GPI-linked isoform (GPI-MFas II; Wright *et al.*, 1999). Previously, we showed that both the neurons and glial cells of the developing ENS express MFas II throughout embryogenesis while their muscle band pathways express MFas II only transiently, coincident with the active period of cell migration (Copenhaver and Taghert, 1989b; Wright *et al.*, 1999). We also demonstrated that perturbation of MFas II-dependent adhesion disrupted both the migration and outgrowth of the EP cells. However, whether these effects were mediated by one or both isoforms of MFas II was not determined. We have now used specific riboprobes and antibodies to show that the two isoforms of MFas II are expressed in distinct and dynamic patterns within the developing ENS at different phases of neuronal and glial differentiation. We also present evidence that the two isoforms subserve distinct functions: whereas TM-MFas II plays an essential role in neuronal motility (including EP cell migration and axon outgrowth), GPI-MFas II is associated primarily with maintaining intercellular adhesivity.

MATERIALS AND METHODS

Immunoanalysis

Synchronized embryos were collected from a colony of *Manduca sexta* and maintained at 25°C; at this temperature, 1 hour is equivalent to 1% of development. Embryo staging and whole-mount immunohistochemistry were performed as previously described (Copenhaver and Taghert, 1989b; Wright *et al.*, 1998). Immunoblot analyses of protein extracted from staged embryos or developing adult wings were performed as described in Wright *et al.* (1999). For detecting both isoforms of MFas II, we used a mouse polyclonal antiserum (1:500) that was generated against gel-purified MFas II protein (provided by Dr. James Nardi). For detecting individual isoforms, guinea pig polyclonal antisera (Pocono Rabbit Farm & Laboratory; Canadensis, PA) were generated against peptide conjugates (Macromolecular Resources; Fort Collins, CO) corresponding to unique sequences contained in the two MFas II isoforms. For TM-MFas II, the sequence (C)TGEDAIKRNSSVEFDGHRV was used, corresponding to a region within the cytoplasmic tail near the C-terminus. For GPI-MFas II, the sequence (C)GEYNSESNEVPRQPGFYDV was used, corresponding to the unique extracellular domain located adjacent to the GPI attachment site (Wright *et al.*, 1999; see fig. 2). Antisera were used for immunohistochemistry at 1:2,000-1:10,000 and detected with anti-guinea pig secondary antibodies conjugated either to alkaline phosphatase or rhodamine (KPL, Gaithersburg, MD). An antiserum against ELAV (generously provided by Drs. Kalpana White and Steven Robinow) was used at 1:500 and detected with antibodies conjugated with horseradish peroxidase or fluorescein.

Detection of Mas II-specific mRNA

Northern blots of poly A+ mRNA and whole-mount *in situ* hybridization histochemistry were performed essentially as described in Wright *et al.* (1999). For Northern blots, ^{32}P-labeled probes were generated from templates derived from the 3'-UTR of each MFas II isoform or from a restriction fragment of the shared extracellular domain. For *in situ* hybridization histochemistry, the same templates were used to generate digoxigenin (dig)-labeled probes after the methods of Patel and Goodman (1992). As previously noted (Wright *et al.*, 1999), an improved signal was obtained using unhydrolyzed probes with intact lengths of 400 (TM-MFas II), 1,400 (GPI-MFas II), and 3,000 bases (shared extracellular domain), respectively. Bound probes were detected using an alkaline phosphatase-conjugated anti-dig antibody (Boehringer) followed by reaction with the appropriate substrates. Sense probes were used as controls to confirm specificity.

Manipulations of MFas II expression in embryonic culture

Staged embryos were isolated and restrained in Sylgard-coated chambers using either a modified culture medium or defined saline (Horgan and Copenhaver, 1998). For young cultures (starting at 40% of development), the eggshell and outer membranes were carefully removed and a small incision was made in the vitelline membrane perpendicular to the developing embryo. Experimental reagents (dissolved in culture medium) were then introduced into the open body cavity of the embryo via microinjection using a glass electrode attached to a Hamilton syringe (10 μl) by polyethylene tubing. Embryos were then allowed to develop for 12-24 hr in culture at 37°C. For older cultures (starting at 50-53% of development), a small incision in the dorsal body wall was made to expose the developing

ENS on the gut surface, permitting the EP cells and enteric glia to be treated directly with bath-applied compounds (Horgan *et al.*, 1994). Subsequently, the embryos were allowed to develop an additional 24-48 hr at 28°C. Dissected control embryos were routinely used with each manipulation. At the completion of each culture experiment, embryos were fixed and immunostained with anti-MFas II antibodies. The extent of migration and outgrowth exhibited by the treated cells was analyzed by photomicroscopy and *camera lucida* techniques (Horgan and Copenhaver, 1998). The data from each experimental group were normalized to matched control groups and analyzed using a 2-tailed Student's *t* test. Phosphatidylinositol-specific phospholipase C (PI-PLC; Boehringer) was used at 0.05-0.1 U/ml.

Oligodeoxynucleotides (ODNs) were prepared as phosphorothioate-modified 20-mers (Synthegen; Houston, TX) derived from the mRNA sequence for MFas II. Four distinct regions within the proximal 3' untranslated region (UTR) of TM-MFas II mRNA were initially chosen based on their predicted lack of strong secondary structure (Fenster *et al.*, 1994) using the *mfold* program by Zuker and Turner (Mathews *et al.*, 1999; Zuker *et al.*, 1999). Specific 20-mers were then selected from within these regions by the following criteria: predicted hybridization to only one site within the TM-MFas II sequence, GC content < 50%, Tm values > 55°C, and lack of self-complementarity or poly-base sequences. ODNs were also examined using the NCBI BLAST program (Altschul *et al.*, 1990) to verify that the selected sequences did not share significant identity with any known genes other than MFas II. Four antisense ODNs were constructed corresponding to nt # 2821-2840 (ODN 1); nt # 2876-2895 (ODN-2); nt # 3007-3026 (ODN-3); and nt # 3685-3704 (ODN-4). The region spanning the predicted translation initiation codon of MFas II (shared by both isoforms) was similarly analyzed for the

construction of a fifth ODN (nt # 336-355; ODN-Start) containing a biotin molecule attached with 12-atom linker to the 5' end. The sequence for ODN-2 (GAATCGGCTACATCACTACT) was also used to construct a sense control (ODN-Sense; AGTAGTGATGTAGCCGATTC) and a scrambled sequence control (ODN-Scramb; ACGATTCACGTACTCTACGT). All ODNs were HPLC-purified and filtered on a G25 Sephadex column.

For embryonic culture experiments, lyophilized ODNs were resuspended in sterile defined saline (Horgan and Copenhaver, 1998) at 2 μg/μl and stored at -20°C; working dilutions (0.03 μg/μl-0.3 μg/μl) were prepared with culture medium lacking serum and antibiotics. Solutions containing the ODNs were then delivered directly to the EP cells in cultured embryos at 52-54% of development. Immediately following ODN exposure, the incision in the dorsal body wall of each embryo was closed using glass electrodes, and serum plus antibiotics was added to the surrounding culture medium. In the course of these experiments, a variety of compounds known to enhance cell transfections *in vitro* were also tested, including Lipofectamine and Lipofectamine 2000 (Gibco/BRL; Grand Island, NY); Suprafect (Qiagen; Valencia, CA), and ExGen 500 cationic polymer (MBI Fermentas; Amherst, NY) according to the manufacturers' instructions. However, inclusion of these reagents never enhanced (and in some cases reduced) the effects of the ODNs on EP cell development, and they were subsequently omitted. At the completion of each experiment, the preparations were immunostained and analyzed as described above. To confirm ODN penetration, preparations treated with ODN-Start (containing the biotin linker) were first incubated with avidin-HRP and visualized with diaminobenzidine (DAB)-H_2O_2 in the presence of NiCl (to produce a black reaction product), followed by immunostaining with anti-MFas II antibodies

in the absence of NiCl (to produce a brown reaction product; Copenhaver *et al.*, 1996).

RESULTS

Neuronal and glial migration in the developing ENS

As previously described (Copenhaver, 1993; Copenhaver and Taghert, 1989b), the formation of the ENS in *Manduca* involves three discrete phases of migration. Neurogenesis in the ENS is complete by 40% of development, at which time the EP cells have formed a condensed packet of post-mitotic neurons adjacent to the foregut-midgut boundary (Fig. 1, blue cells). Concurrently, a smaller number of large glial progenitor cells have been generated that lie at the anterior margin of this packet (red cells). Over the next 15% of development, the EP cells commence a slow, circumferential phase of migration, during which the packet of neurons spreads bilaterally around the foregut along the foregut-midgut boundary (Copenhaver and Taghert, 1989b). Throughout this first phase of migration, the leading neurons extend short filopodial processes onto the adjacent epithelial surfaces, but the cells remain tightly adherent within the packet. By 55% of development, the EP cells have encircled the foregut, and groups of neurons align with eight longitudinal muscle bands that have coalesced on the midgut surface. These muscle bands form requisite pathways for the next phase of migration (55-65% of development), during which subsets of EP cells disperse from the original packet and travel rapidly along the bands onto the midgut (Copenhaver *et al.*, 1996). Additional sets of neurons also migrate anteriorly and laterally along radial muscles that form on the foregut (Fig. 1, 58-65%; foregut muscles not shown). In contrast, the glial precursors adjacent to the EP cells continue to divide but undergo only minor changes in position during these first two migratory phases. However, towards the end of EP cell migration, proliferating glial progeny commence a third phase of migration, during which they spread down along the same pathways established by the

EP cells to form the glial sheath of the enteric plexus (Copenhaver, 1993). Unlike the rapid, transient migration of the EP cells, the extension of this glial sheath continues more gradually, so that the post-migratory neurons are not completely enwrapped until 80-85% of development. Notably, the enteric glia ensheath the branches of the enteric plexus only to the extent that the neurons have migrated but do not extend along the more distal axons and terminal branches elaborated by the EP cells on the adjacent gut musculature (Fig. 1, 100%).

Isoform-specific expression of MFas II transcripts by distinct cell types

To investigate whether the two isoforms of MFas II are differentially expressed in the developing ENS, riboprobes were generated against unique sequences within the 3' UTR regions of each isoform. As previously shown (Wright *et al.*, 1999), Northern blot analysis of embryonic mRNA using a ^{32}P-labeled riboprobe against the shared 5' extracellular domain of MFas II revealed two prominent bands of approximately 5 kb and 6.5 kb, respectively (Fig. 2A). In contrast, riboprobes against TM-MFas II recognized only the 6.5 kb band and probes against GPI-MFas II recognized only the 5 kb band (Fig. 2A), confirming that these probes were specific for the targeted isoform. Digoxigenin-labeled riboprobes were then used for whole-mount *in situ* hybridization histochemistry to determine the pattern of MFas II isoform expression within the developing ENS. In previous work, we demonstrated that both the EP cells and associated glia express MFas II throughout their migratory phases of development (Copenhaver, 1993; Copenhaver and Taghert, 1989b; Wright *et al.*, 1999). As shown in figure 3 (top row), preparations reacted with the riboprobes against the shared extracellular

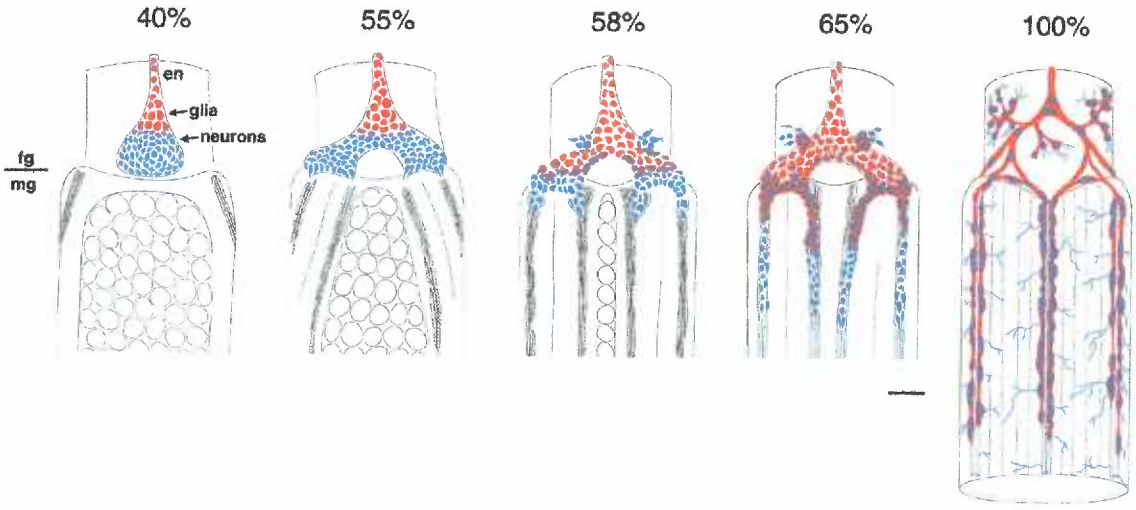
40%
55%
58%
65%
100%
en
glia
neurons
fg
mg

Figure 1. Sequence of neuronal and glial migration that forms the enteric plexus in the developing ENS. By 40% of development, the post-mitotic neurons (EP cells; blue) have delaminated from the foregut adjacent to a population of mitotically active glial precursors (red). Between 40-55%, the EP cells undergo a slow spreading phase of migration around the foregut but remain tightly adherent at the foregut-midgut boundary. Between 55-65%, the EP cells undergo a fast, dispersive phase of migration, during which subsets of neurons travel posteriorly along eight pre-formed muscle bands on the midgut (only the four dorsal bands are shown) and anteriorly along radial muscle fibers on the foregut (radial muscles not shown). Subsequently, the EP cells continue to extend axons along the muscle bands and then branch laterally to innervate the gut musculature. Between 58-80%, glial progeny spread along the pathways pioneered by the EP cells, thereby ensheathing the branches of the enteric plexus. **en** = esophageal nerve of the foregut; scale bar = 50 μM.

domain ("MFas II") showed strong labeling in both the EP cells (solid arrows) and glial precursors (open arrows) during all phases of migration.

Surprisingly, when we applied the isoform-specific probes to identically staged embryos, we found that GPI-MFas II and TM-MFas II were expressed in complementary patterns within the ENS. At 53% of development, GPI-MFas II transcripts were confined to cells at the anterior margin of the EP cell packet (open arrow), an area that is populated by glial precursors (Copenhaver, 1993; Fig. 1). In contrast, TM-MFas II expression at 53% was localized to the adjacent population of EP cells that had spread around the foregut-midgut boundary (solid arrows). This distinction persisted throughout subsequent periods of development. During the fast phase of migration (58% of development), GPI-MFas II mRNA expression remained confined to the foregut regions occupied by the proliferating glial cells (open arrows), while TM-MFas II mRNA was localized to the actively migrating neurons (solid arrows; EP cells that have migrated anteriorly onto the foregut musculature are out of focus). Even after neuronal migration was complete (75% of development), the dispersed EP cells on both the foregut and midgut continued to express only TM-MFas II transcripts (solid arrows). In contrast, GPI-MFas II transcripts now appeared within a trailing population of cells that had begun to spread along the pathways established by the neurons and their processes (75%, open arrows). These distinct patterns of expression for TM-MFas II and GPI-MFas II mRNA match the dynamic changes exhibited by the neuronal and glial populations of the ENS, respectively (see Fig. 1), suggesting that transcripts encoding the two isoforms of MFas II are expressed in a cell type-specific manner during this period of development.

MFas II isoforms are dynamically expressed during ENS development

To examine the distribution of MFas II proteins within the developing ENS, polyclonal antisera were generated against unique peptide sequences within the cytoplasmic tail of TM-MFas II or to an extracellular region of GPI-MFas II adjacent to the GPI attachment site (Fig. 2B, lower figure). When tested by protein immunoblot analysis, anti-TM-MFas II antisera selectively recognized the larger transmembrane isoform (running at ~95 kDa), while anti-GPI-MFas II antisera selectively recognized the smaller glypiated isoform (running at ~90 kDa). In contrast, antibodies against the shared extracellular domain bound to both isoforms, as expected (Fig. 2B, middle lane; Wright *et al.*, 1999). For each antiserum, all immunostaining was eliminated when it was pre-adsorbed with the peptide against which it was generated but not when pre-adsorbed with peptides derived from the complementary isoform (not shown). Having established the specificity of these antisera, we used them to investigate how each MFas II isoform is expressed at the protein level during the three phases of cell migration in the ENS.

By 40% of development, both the newly formed packet of EP cells and the adjacent cluster of glial precursors have emerged onto the foregut surface (see Fig. 1) and are MFas II-positive (Copenhaver and Taghert, 1990). Unexpectedly, when we immunostained preparations at this age with the isoform-specific antibodies, we found that all of the cells (both neurons and glial precursors) expressed GPI-MFas II but not TM-MFas II (Fig. 4). Uniform expression of mRNA specific for GPI-MFas II but not TM-MFas II was similarly seen at this age (not shown). Over the next 10% of development (from 40-50%), during the slow, circumferential migration of the EP cells around the foregut, both the neurons and glial precursors continued to show robust levels of GPI-MFas II immunoreactivity. The distribution of GPI-MFas II appeared uniform over the entire plasma membrane of these cells,

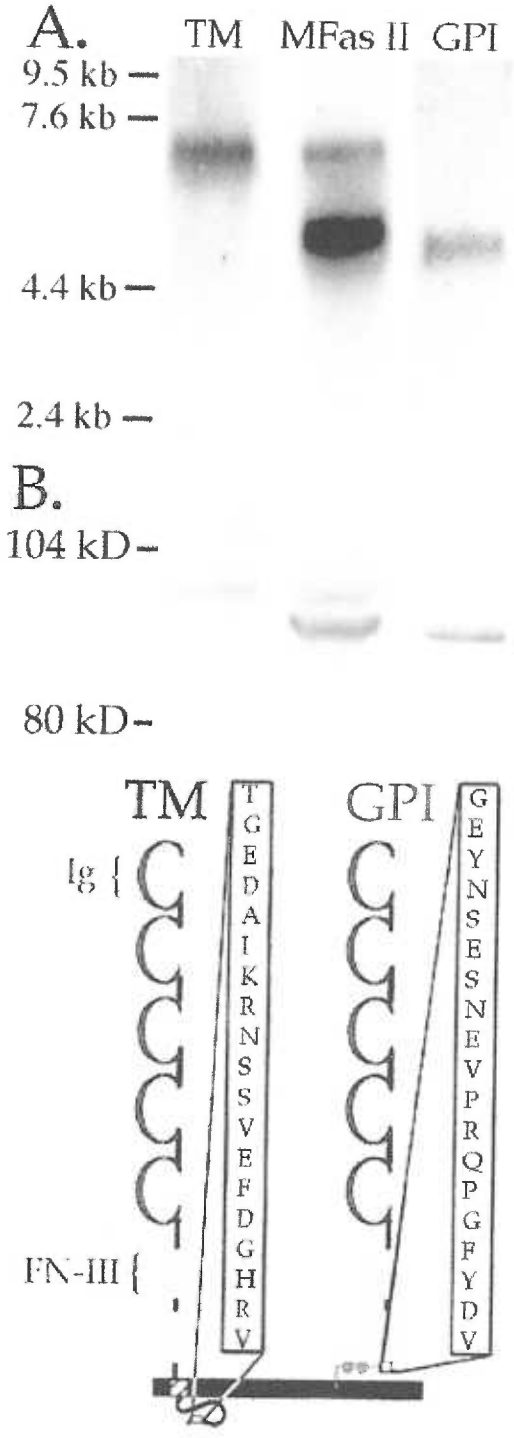
A.
TM
MFas II
GPI
9.5 kb
7.6 kb
4.4 kb
2.4 kb
B.
104 kD
80 kD
TM
GPI
Ig
FN-III
TGEDAIKRNSSVEFDGHRV
GEYNSESNEVPRQPGFYDV

Figure 2. Isoform-specific probes distinguish the two isoforms of MFas II. **A**. Northern blots of mRNA extracted from developing adult wing reacted with ^{32}P-labeled probes specific for the 3'-UTR of TM-MFas II (left) and GPI-MFas II (right); middle lane was reacted with a probe against a 5'-region shared by both isoforms. **B**. Immunoblots reacted with antibodies generated against peptides derived from isoform-specific regions (shown in schematic): for TM-MFas II, a 19-AA sequence was chosen from the cytoplasmic tail; for GPI-MFas II, a 19-AA sequence was chosen adjacent to the GPI-attachment site. Center lane of the immunoblot was reacted with a mouse polyclonal antiserum recognizing the shared extracellular domain of both isoforms. Ig = immunoglobulin domain; FN-III = fibronectin type III domain; solid black line represents the plasma membrane.

revealing numerous small filopodia that extended from the leading EP cells onto the adjacent epithelial surfaces (arrowheads). In contrast, expression of TM-MFas II commenced only gradually during this first migratory phase, appearing in the spreading packet of EP cells but not within the more anterior glial precursors (Fig. 4). By 50% of development, much of the TM-MFas II immunoreactivity appeared to be concentrated in the growing processes of these neurons, producing a reticular pattern of staining within the EP cell packet and strongly labeling the fasciculated axons that had grown into the esophageal nerve (arrowhead).

To confirm the apparent distinction between neuronal and glial cell distributions at this stage of development, we immunostained identically staged embryos with an antibody against ELAV, an RNA-binding protein that is expressed exclusively in insect neurons but not glial cells (Robinow and White, 1991). Previously, we used this antibody to distinguish the distributions of the EP cells and enteric glia at the end of migration (Copenhaver, 1993). As shown in figure 4 (bottom panel), application of anti-ELAV antibodies at 50% of development resulted in a pattern of staining that closely matched the distribution of cells expressing TM-MFas II. These results indicate that the neuronal and glial populations of the ENS exhibit distinct patterns of MFas II expression during the first phase of migration: whereas both cell types show persistent expression of GPI-MFas II throughout this period, only the EP cells commence the expression of TM-MFas II as they spread bilaterally around the foregut.

The expression of both isoforms by the EP cells during this initial phase of migration was unexpected, given the restricted patterns of mRNA expression detected during later stages of development (Fig. 3). However, by 55% of development (just prior to the onset of fast migration), the pattern of

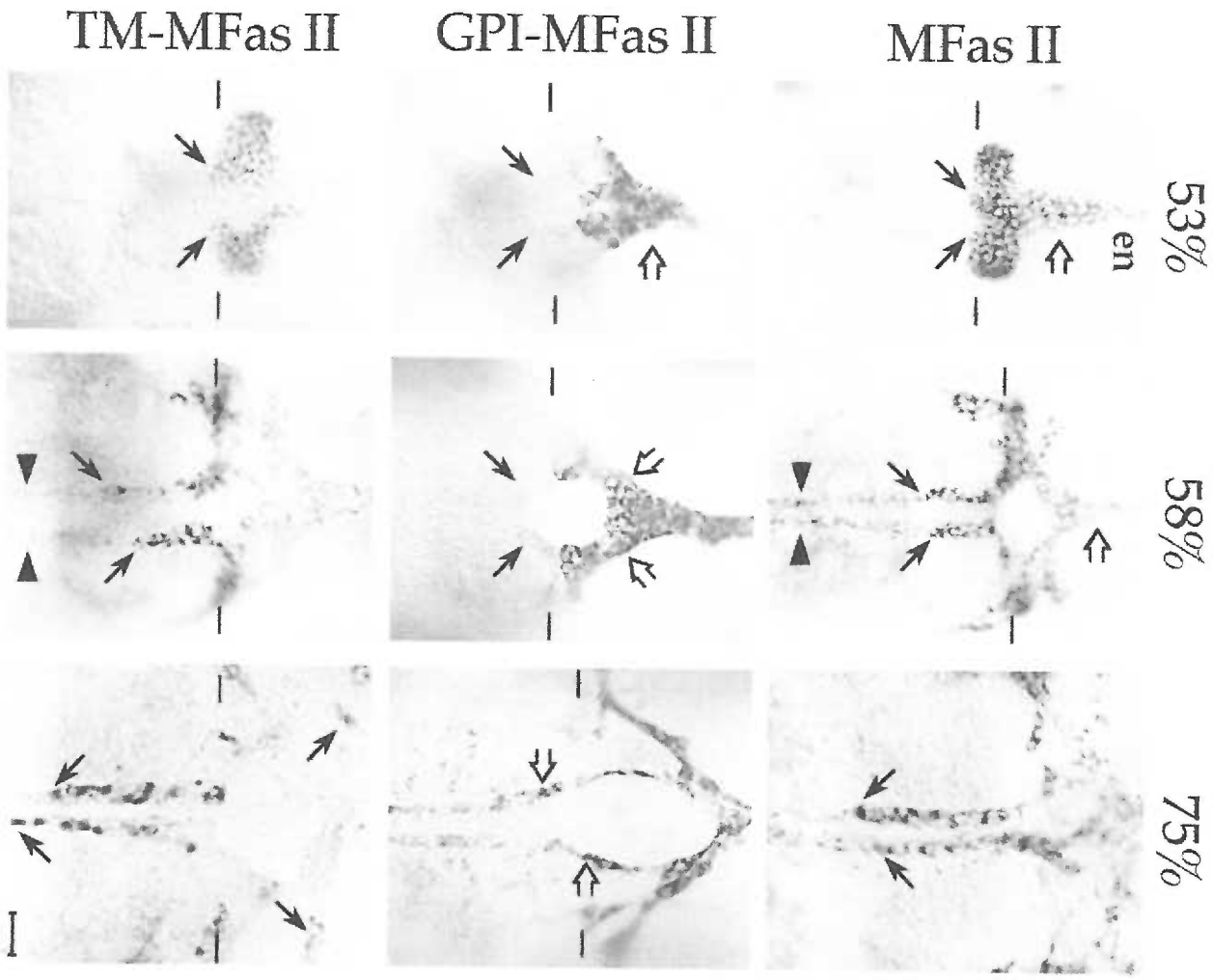
TM-MFas II
GPI-MFas II
MFas II
53%
en
58%
75%

Figure 3. Whole-mount *in situ* hybridization histochemistry reveals isoform-specific patterns of MFas II mRNA expression in the developing ENS. Identically staged embryos (age indicated at top) reacted either with a digoxigenin-labeled probe recognizing both isoforms or with probes against isoform-specific 3' UTR domains (see Fig. 2 legend). At all three ages shown, both the EP cells (solid arrows) and enteric glial cells (open arrows) react strongly with probes recognizing the shared mRNA domain (MFas II). However, by 53% of development, the EP cells express only TM-MFas II-specific mRNA, while the more anterior glial cells express only GPI-MFas II-specific mRNA. During fast migration (58%), strong levels of TM-MFas II expression continue to be present in the migrating EP cells, and fainter levels can be detected in the muscle band pathways (arrowheads; see also Fig. 6). In contrast, the trailing glial cells continue to express strong levels of GPI-MFas II-specific mRNA; the apparent weak staining of the glial cells by the transmembrane-specific probe at this time is the result of high background staining associated with this probe. At 75%, the post-migratory EP cells on both the foregut and midgut continue to express TM-MFas II mRNA (solid arrows; EP cells that have migrated anteriorly onto foregut musculature are out of focus). Similarly, GPI-MFas II expression continues to be co-localized with the glial populations (open arrows) spreading along the pathways that have formed during neuronal migration. **en** = esophageal nerve of the foregut; scale bar = 25 μM.

40%
43%
47%
50%
GPI-Fas II
TM-Fas II
en
ELAV

Figure 4. Whole-mount immunohistochemical staining reveals isoform-specific patterns of MFas II expression during slow migration. By 40% of development, the EP cells have delaminated from the foregut and are juxtaposed with the more anterior population of glial precursors (see Fig. 1). At this stage, both the EP cells and glial precursors stain positively for GPI-MFas II but not TM-MFas II. Solid arrows indicate the posterior edge of the EP cell packet adjacent to the foregut-midgut boundary (shown by paired black lines). From 40-50%, the EP cells spread bilaterally around the foregut (slow migration), during which both the EP cells and the glial cells continue to express GPI-MFas II. Immunostaining is uniform over the entire plasma membrane, including the short filopodia that extend from the EP cells onto the adjacent gut surfaces (arrowheads). During this period, the EP cells but not the glial cells commence the expression of TM-MFas II, with the strongest level of staining localized to the fasciculated axons (arrowhead) that these neurons extend into the esophageal nerve (**en**). Immunostaining an identically staged embryo with the an antiserum against the neuronal-specific protein ELAV (lower panel) matches the pattern of TM-MFas II in the EP cells but not in the more anterior glial cells (open arrows). Scale bar = 25 μM.

MFas II expression within the developing ENS changed dramatically. While the more anterior glial cells continued to express GPI-MFas II (Fig. 5, open arrow), the EP cells expressed TM-MFas II exclusively, with particularly strong staining in the neurons that had begun to align with the midgut muscle bands (55%, solid arrows). Throughout the subsequent period of fast migration (55-65%), all of the EP cells continued to exhibit strong levels of TM-MFas II immunoreactivity. Notably, the most robust staining was associated with the leading processes of the migrating EP cells and their growing axons (Fig. 5, 70%). ELAV staining in identically staged embryos confirmed that the distribution of migratory neurons corresponded to the pattern of TM-MFas II expression seen during this period of development (Fig. 5, bottom row). In contrast, ELAV immunoreactivity was excluded from the more anterior population of glial cells, which continued to express only GPI-MFas II (Fig. 5, top row).

Once neuronal migration is complete in the ENS, mitotically active glial cells gradually enwrap the pathways pioneered by the EP cells (see Fig. 1). By 63% of development, cells expressing GPI-MFas II had begun to spread posteriorly onto the midgut pathways (Fig. 5, open arrows). Although the rate of this third phase of migration was variable, GPI-MFas II-positive cells gradually extended glial-like processes around the post-migratory neurons over the next 20% of development. The morphology and distribution of these cells was identical to the pattern of glial progeny that we previously identified by lineage-tracing techniques (Copenhaver, 1993). In summary, the two isoforms of MFas II exhibited distinct patterns of expression within the developing ENS. GPI-MFas II was expressed by immature neurons and glia during periods when the cells remained tightly adherent or underwent gradual changes in shape and position. By comparison, TM-MFas II was

55%
58%
63%
70%
GPI-Fas II
TM-Fas II
ELAV

Figure 5. Cell type-specific expression of MFas II isoforms during fast migration. By 55% of development, the EP cells no longer express detectable levels of GPI-MFas II (compare with Fig. 4), and strong levels of TM-MFas II are increasingly localized to their leading processes extending onto the midgut (solid arrows). TM-MFas II immunoreactivity is also present in the axons of the EP cells that extend around the glial cells. In contrast, the more anterior glial cells stain strongly for GPI-MFas II but express little or no TM-MFas II (open arrows; compare with neuronal-specific ELAV staining in bottom panels). During fast neuronal migration (55-63%), the EP cells continue to express TM-MFas II exclusively, with the strongest immunostaining associated with their leading processes and growing axons. The glial cells, which subsequently spread along the pathways formed by the migrating EP cells, continue to express GPI-MFas II over the entire surface of their somata and processes (open arrows). Double black bars in each panel indicates the foregut-midgut boundary; scale bar = 25 μM.

expressed exclusively by neurons just prior to and during periods of active motility and was concentrated in the leading processes of migratory neurons or their growing axons.

We previously showed that the muscle bands of the midgut also express MFas II transiently during the fast phase of neuronal migration (Wright *et al.*, 1999). When we applied isoform-specific antisense probes to identically staged embryos, we found that the muscle bands labeled positively with the TM-MFas II probe but not for GPI-MFas II (Fig. 3, 58%, arrowheads). This distinction was also apparent in preparations immunostained with our isoform-specific antibodies, which clearly showed TM-MFas II expression in the muscle bands but not GPI-MFas II (Fig. 6). Thus, only the transmembrane isoform is associated with the pathways followed by the EP cells during their fast, dispersive phase of migration.

By 90% of development, the differentiation of the ENS is essentially complete. The post-migratory EP cells have extended axons along most of the length of the midgut, and their terminal branches have innervated the superficial musculature of both the foregut and midgut (Copenhaver and Taghert, 1989a; see Fig. 1). Persistent expression of TM-MFas II could still be detected in the EP cells at this stage, although the distribution of immunoreactive staining was restricted to their axons and lateral branches (Fig. 7B, D). Strong levels of GPI-MFas II immunoreactivity were also seen in the glial cell population that had spread along the pathways taken by the migratory neurons, providing a clear delineation of the different branches of the enteric plexus on the foregut and midgut (Fig. 7A, C). Unlike the transient expression of fasciclin II that occurs in the insect CNS (Grenningloh *et al.*, 1990; Harrelson and Goodman, 1988; Wright and Copenhaver, submitted for publication), this pattern of isoform-specific expression

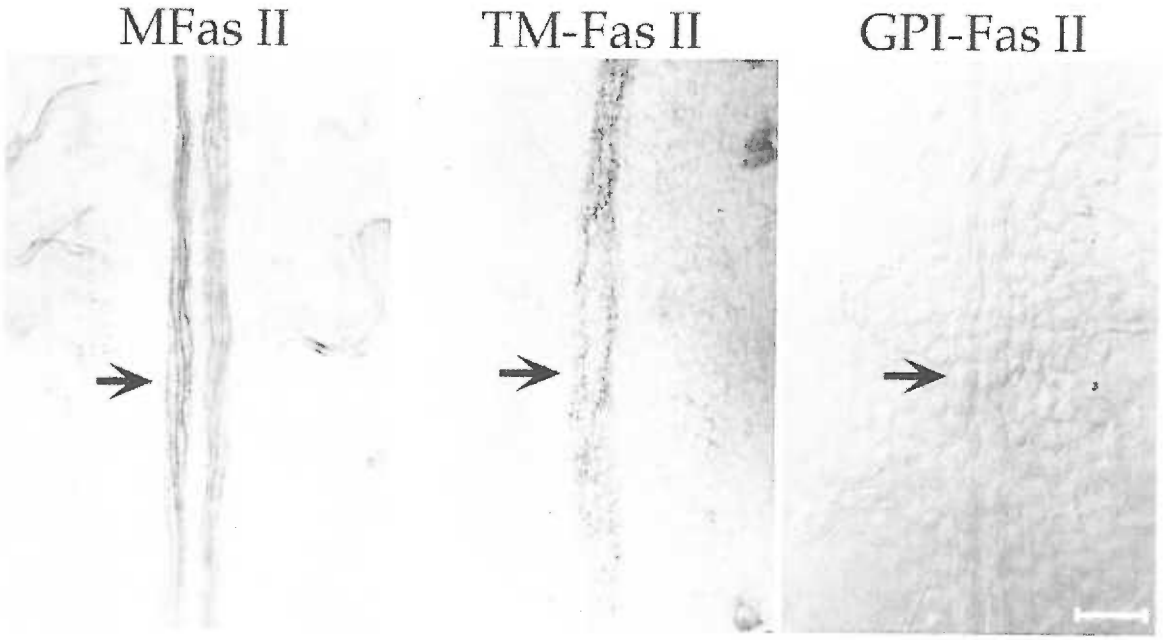
MFas II
TM-Fas II
GPI-Fas II

Figure 6. Transient expression of MFas II in the midgut muscle band pathways. Immunohistochemical staining of the mid-dorsal muscle bands of embryos at 55% of development shows that they react positively with antibodies against the extracellular domain shared by both isoforms (MFas II) and against TM-MFas II but not GPI-MFas II (see also Fig. 3). Preparations were photographed at midgut positions posterior to the foregut-midgut boundary prior to the arrival of the EP cells or their processes. Scale bar = 25 μM.

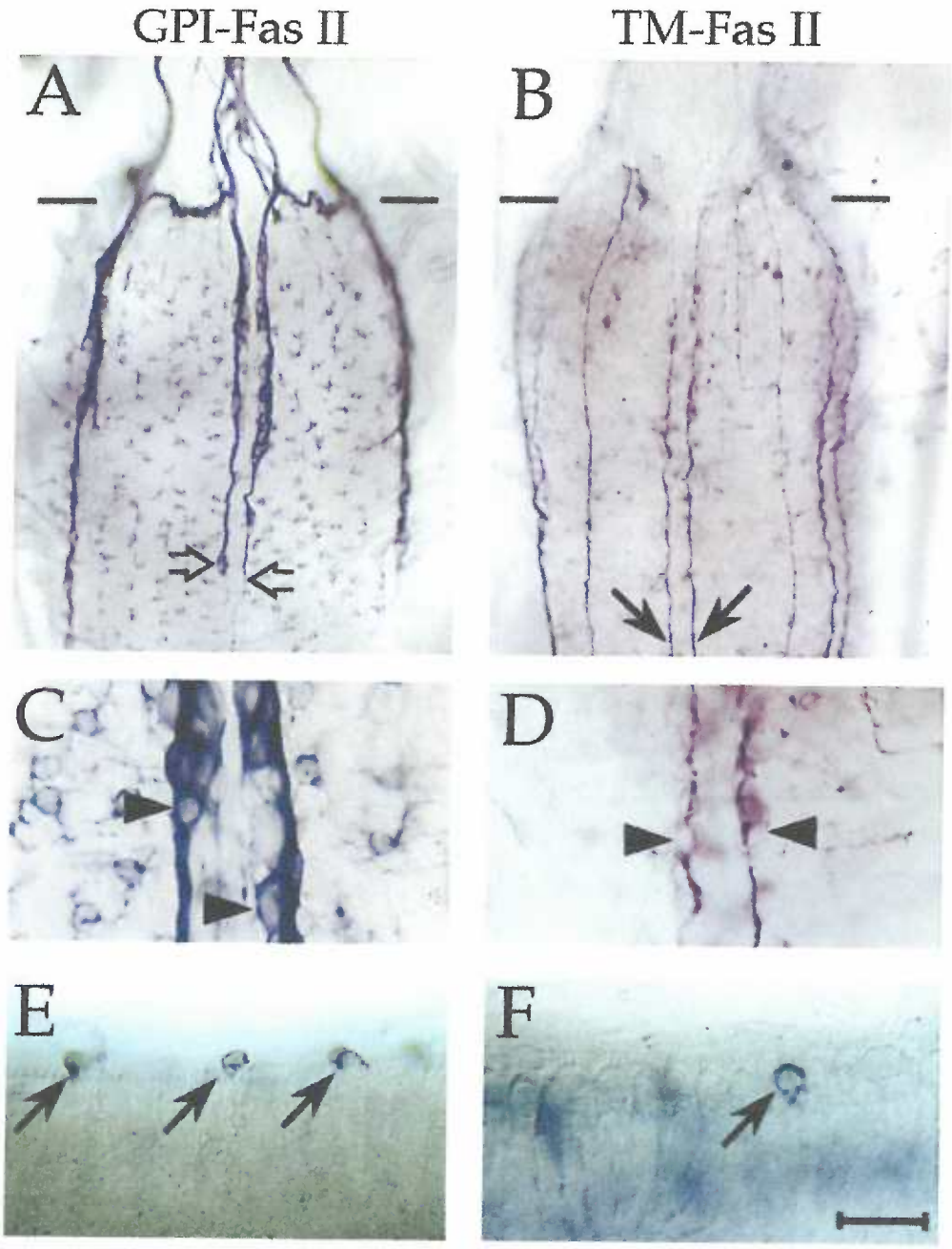
GPI-Fas II
TM-Fas II
A
B
C
D
E
F

Figure 7. Cell type-specific expression of MFas II isoforms persists throughout development. Embryos at 95% of development stained with antibodies against GPI-MFas II (A, C, E) or TM-MFas II (B, D, F) reveal distinct patterns of immunoreactivity corresponding to the extent of glial and neuronal processes, respectively. **A**, low magnification view shows that the glial ensheathment (GPI-MFas II-positive) has enwrapped all of the branches of the ENS on both sides of the foregut-midgut boundary (double black lines) and has extended posteriorly on the midgut (open arrows) to cover the most posterior neuronal somata. **B**, In contrast, TM-MFas II is expressed only by the EP cells and is primarily localized to their posteriorly projecting axons (solid arrows). **C**, higher magnification of panel A shows a detail of the glial ensheathment (GPI-MFas II-positive) enwrapping EP cells on the midgut (arrowheads). **D**, higher magnification of panel B shows strong immunostaining in the axons and lateral branches of the EP cells but only faint staining within their somata (arrowheads). **E**, GPI-MFas II antibodies also label a population of mesodermal cells (arrows) located within the most superficial layer of midgut musculature (also visible in A and C adjacent to the muscle band pathways). **F**. TM-MFas II antibodies label a distinct set of mesodermal cells located within the midgut epithelium (out of focus in B). Scale = 10 μM in A and B; 25 μM in C-F.

persisted into post-natal life (not shown). Intriguingly, the GPI-MFas II-positive glial wrapping never extended beyond the post-migratory somata of the EP cells (Fig. 7A, open arrows), leaving their more posterior axons and terminal branches apparently unsheathed. Some additional mechanism must therefore protect these terminal branches from the high potassium concentrations that are present in the hemolymph (Levine and Truman, 1985).

As has been found in other insects (Grenningloh *et al.*, 1991; Harrelson and Goodman, 1988; Whitlock, 1993), a number of mesodermal cell types also express MFas II at specific developmental stages (Wright and Copenhaver, unpublished data). In particular, two distinct populations of cells intrinsic to the midgut were found to express specific MFas II isoforms towards the end of embryogenesis. A diffuse set of stellate cells interspersed within the superficial musculature of the midgut stained positively for GPI-MFas II (Fig. 7E; also visible in 7A, C), while a smaller number of cells located within the midgut epithelium expressed TM-MFas II (Fig. 7F; out of focus in 7B, D). Whether either of these cell types differentiate into the peptidergic gut endocrine cells of *Manduca* (Copenhaver and Taghert, 1989a; Kingan *et al.*, 1997) remains to be ascertained.

GPI-MFas II helps maintain EP cell adhesion but is not required for slow migration

The marked changes in MFas II expression during ENS development suggested that the two isoforms of this receptor serve distinct functions during neuronal and glial migration. As noted above (Fig. 4), the EP cells remained closely apposed to one another during their initial slow migration around the foregut and expressed GPI-MFas II exclusively. Since PI-PLC

(which cleaves GPI linkages) was previously shown to induce the release of GPI-MFas II but not TM-MFas II from cell membranes (Wright *et al.*, 1999), we exposed the EP cells to PI-PLC to test the role of GPI-MFas II during slow migration. Embryos were isolated at the onset of slow migration (40% of development; see Fig. 1) and treated in culture with a low concentration of PI-PLC. After 12 hr in culture, they were fixed and immunostained with antibodies against GPI-MFas II. As shown in figure 8, the level of GPI-MFas II immunoreactivity in these embryos was substantially reduced, particularly among the neurons at the leading edges of the EP cell packet (compare Fig. 8A, B). In addition, many of the neurons were noticeably more rounded and diffuse in the experimental preparations than in cultured controls, whose cells remained closely adherent (compare Fig. 8C, D). The overall extent of slow migration proceeded normally in both groups of animals, however, resulting in an appropriate bilateral spreading of the EP cells around the foregut. Because PI-PLC should remove all GPI-anchored molecules, it is possible that the loss of adhesivity seen in these preparations reflects the activity of other GPI-linked adhesion receptors in addition to GPI-MFas II. Nevertheless, these results clearly indicate that GPI-MFas II is not required for the slow spreading phase of EP cell migration. Moreover, exposing the EP cells to PI-PLC during fast migration (after 55%; when the neurons express only TM-MFas II) had no effect on their adhesivity or motility (not shown), providing further evidence that this treatment specifically affected interactions mediated by the GPI-linked isoform.

TM-MFas II promotes both EP cell adhesion and fast migration

The results of figures 5 and 7 showed that the expression of TM-MFas II was strongly correlated with neuronal motility, either in the form of rapid

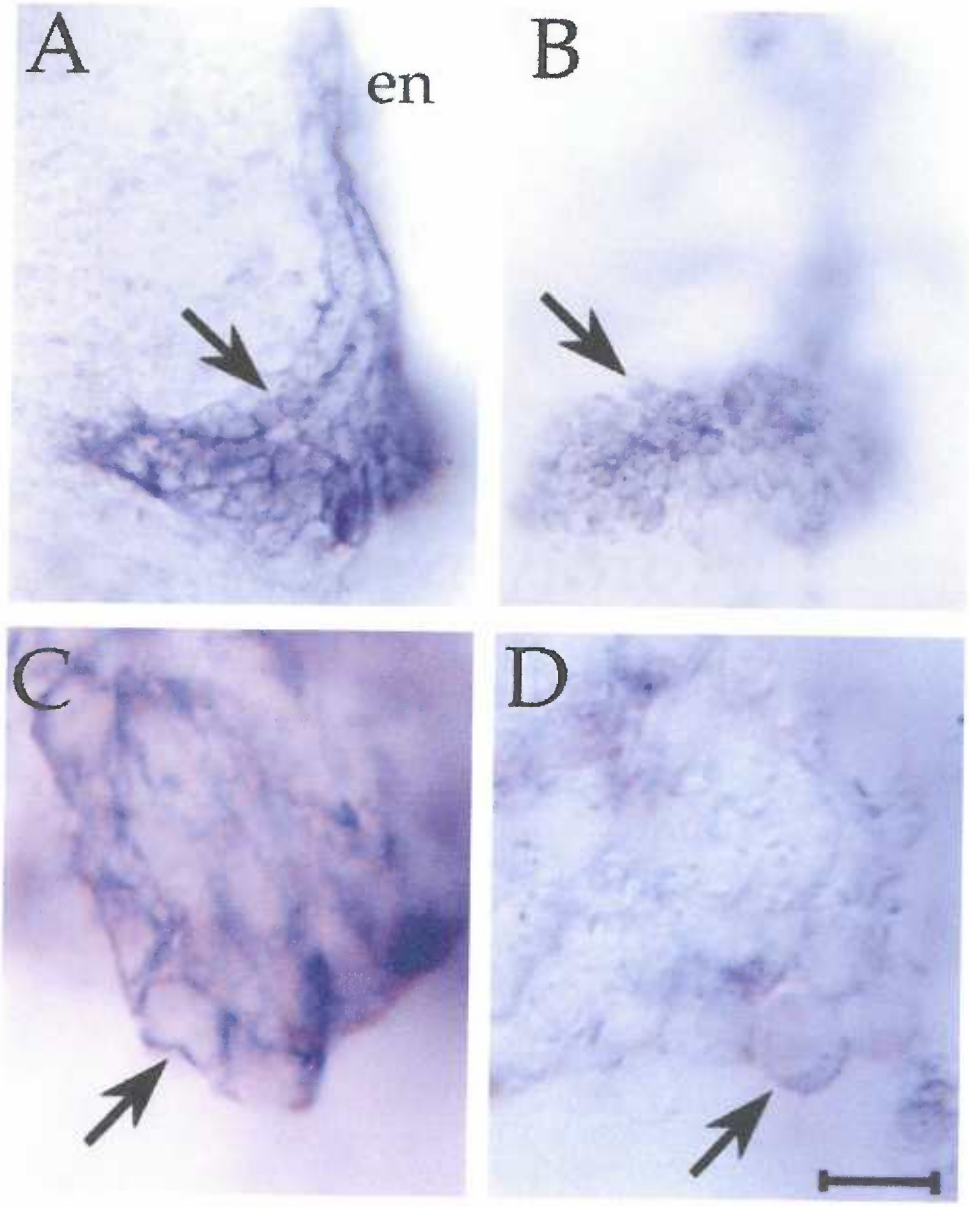
A
en
B
C
D

Figure 8. Removal of GPI-MFas II from the EP cells with PI-PLC disrupts intercellular adhesivity but not slow migration. Embryos were cultured from 40-50% of development in control medium (A, C) or in medium containing PI-PLC (B, D). Panels show lateral views of the EP cells immunostained with anti-GPI-MFas II antibodies (dorsal is to the right, anterior is towards the top of the page). **A**, slow migration of the EP cells round the circumference of the foregut proceeded normally in a cultured control. **B**, slow migration also proceeded normally in an embryo treated with PI-PLC (arrows in A and B indicate similar positions on the lateral foregut). **C**, higher magnification of A, showing that the spreading EP cells remained tightly adherent, with strongest GPI-MFas II expression at the interface between adjacent neurons. **D**, higher magnification of B, showing that PI-PLC caused a loss of GPI-MFas II immunostaining, particularly at the leading edges of the EP cell packet, as well as a substantial reduction in intercellular adhesion and increased cell rounding. **en**, esophageal nerve of the foregut; scale = 25 μM in A-B, 15 μM in C- D.

migration by the EP cells or the outgrowth of their processes. Because the antibody specific for TM-MFas II was generated against the cytoplasmic domain of this isoform, it was not suitable for testing the specific role of TM-MFas II with respect to EP cell motility. As an alternative strategy, several published studies have previously demonstrated that antisense ODNs directed against sequences within the 3' UTR of specific mRNAs can have potent inhibitory effects on protein expression (Chiang *et al.*, 1991; Lipp *et al.*, 1995; Ronnov-Jessen and Petersen, 1996). Accordingly, we designed a panel of four antisense phosphorothioate ODNs against unique sequences within the 3' UTR of TM-MFas II mRNA (ODN-1, -2, -3, & -4; see Fig. 9A), as well as an additional ODN against the 5' end shared by both isoforms (ODN-Start; spanning the translation initiation codon). Each ODN was then applied to the EP cells of cultured embryos at 52-54% of development, shortly after the onset of TM-MFas II expression in the EP cells but before the onset of fast migration (Fig. 9B). The preparations were then allowed to develop for an additional 24 hr prior to fixation and immunostaining.

In animals treated with ODN-Start, overall levels of MFas II immunoreactivity was noticeably reduced in the developing ENS, and there was a substantial amount of defasciculation by the EP cells and their processes away from the muscle band pathways (not shown). In addition, the extent of both fast migration and neuronal outgrowth was significantly reduced in these animals when compared with cultured controls (Fig. 10, histogram A). These results are similar to the effects produced when the EP cells were treated with blocking antibodies against MFas II or MFas II-specific fusion

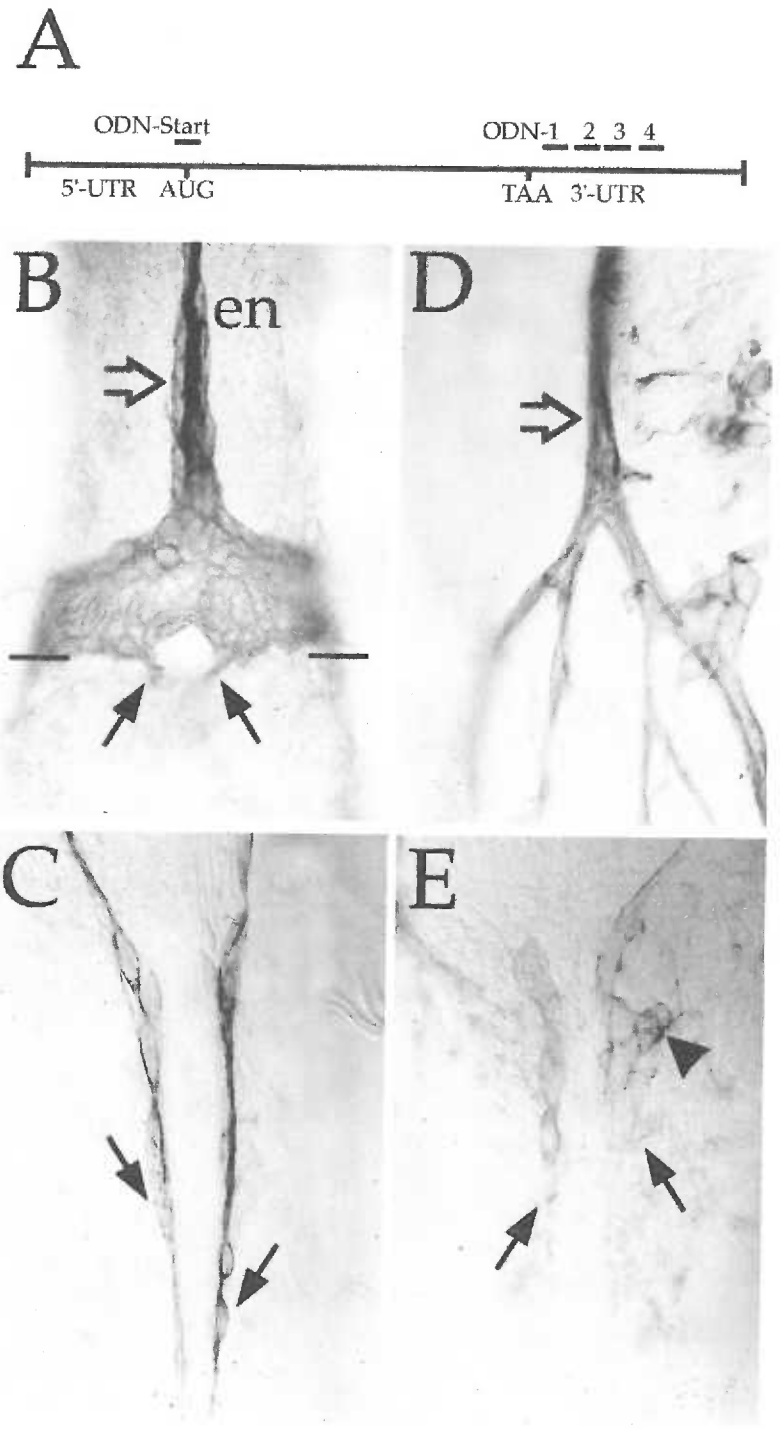
A
ODN-Start
ODN-1 2 3 4
5'-UTR AUG
TAA 3'-UTR
B
en
D
C
E

Figure 9. Antisense ODN treatments that reduce TM-MFas II expression also inhibit EP cell migration. **A**, Diagram showing the positions of the antisense ODNs used in this study with respect to the mRNA sequence encoding TM-Mas II; ODN-Start spans the AUG translation initiation site; ODN-1, 2, 3, and 4 complement unique sequences within the 3' UTR. **B**, photomicrograph of an embryo fixed and immunostained for MFas II at the onset of a culture experiment (52-53% of development); at this stage, the EP cells had commenced the expression of TM-MFas II (see Fig. 4) but were still clustered at the foregut-midgut boundary (black bars), although the neurons had begun to extend short leading processes onto the midgut muscle bands (solid arrows). **C**, cultured embryo treated with control medium exhibited normal EP cell migration and TM-MFas II expression after 24 hr (compare with Fig. 5). **D**, embryo treated with ODN-2 showed no loss of GPI-MFas II expression or errors in cell spreading by the glial cells ensheathing the branches of the enteric plexus on the foregut (open arrows). **E**, embryo treated with ODN-2 showed a substantial loss of TM-MFas II expression in the EP cells and reduced adhesivity between the neurons (solid arrows) and their muscle band pathways. The extent of EP cell migration was also noticeably reduced (see also Fig. 10). **en**, esophageal nerve of the foregut; scale = 25 μM.

proteins directed against the shared extracellular domain of MFas II (Wright *et al.*, 1999). Incubation of the preparations with avidin-HRP followed by reaction with DAB-NiCl prior to anti-MFas II immunostaining (see methods) confirmed that biotinylated ODN-Start had been incorporated into the EP cells (not shown).

Notably, a similar inhibitory effect was obtained when we applied one of the antisense ODNs (ODN-2) directed against the 3' UTR of TM-MFas II (see Fig. 9A). In contrast to cultured control embryos (Fig. 9C) in which EP cell migration and axon outgrowth proceeded normally, many of the treated neurons exhibited a marked loss of adhesivity with their muscle band pathways, accompanied by a substantial reduction in TM-MFas II expression (compare Fig. 9C & E). As in preparations exposed to ODN-Start, EP cell motility was significantly inhibited in the presence of ODN-2 (Fig. 9E, arrows; Fig. 10, histogram B). None of the other ODNs directed against regions within the 3' UTR (ODNs 1, 3, and 4) produced detectable effects on either MFas II expression or migration and outgrowth (histograms C-E). Sense control and scrambled sequence control ODNs (complementary to ODN-2; see methods) also caused no reduction in either EP cell motility or MFas II expression (histograms F & G). In addition, none of the ODNs directed against the 3'-UTR of TM-MFas II (including ODN-2) caused a reduction in the expression of GPI-MFas II within the glial cells ensheathing the foregut branches of the enteric plexus (Fig. 9D), demonstrating that the effects of ODN-2 were isoform-specific. These results indicate that TM-MFas II helps regulate the motile behavior of actively migrating neurons as well as contributing to their adhesive interactions.

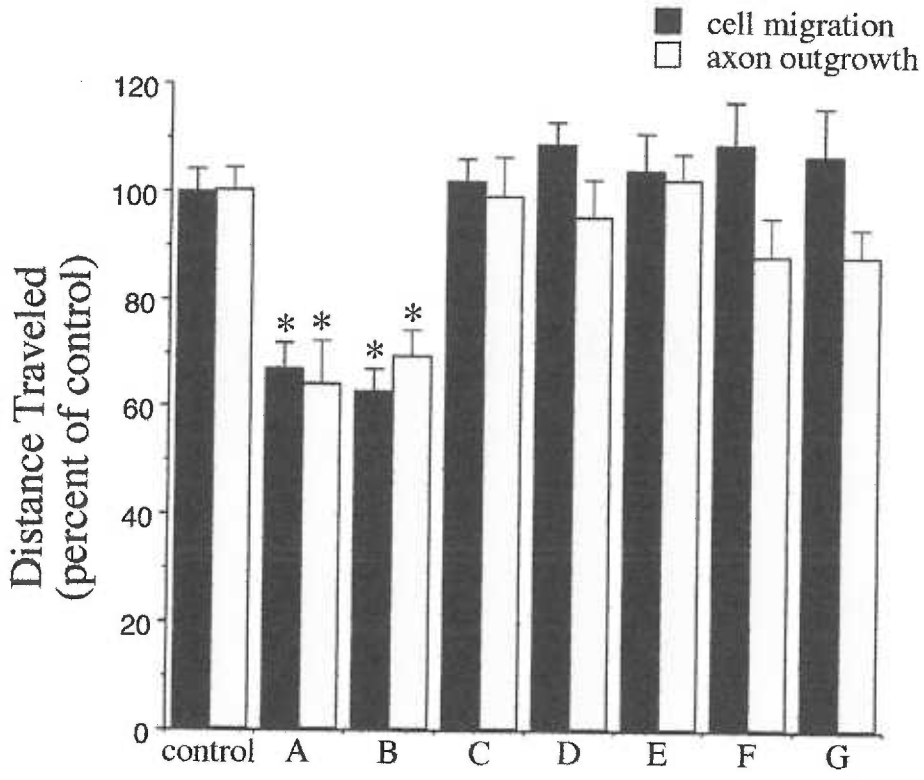
cell migration
axon outgrowth
Distance Traveled
(percent of control)
120
100
80
60
40
20
0
*
*
*
*
control
A
B
C
D
E
F
G

Figure 10. Antisense ODNs that interfere with TM-MFas II expression inhibit fast migration and axonal outgrowth by the EP cells. Embryos were cultured from 52-75% of development in control medium or in medium containing ODNs complementary to MFas II mRNA sequence. Whereas ENS development proceeded normally in cultured controls, both fast migration (black histograms) and axonal outgrowth (gray histograms) were significantly inhibited by ODN-Start (histogram A; recognizing the translation initiation site shared by both isoforms). A similar degree of inhibition was induced by ODN-2 (histogram B; recognizing a sequence in the proximal 3' UTR of TM-MFas II) but not by ODN-1, 3, or 4 (histograms C, D, E; recognizing other sequences in the 3' UTR of TM-MFas II). No effect was seen in embryos treated with ODN-Sense (histogram F; complementary to ODN-2) or with ODN-Scramb. (histogram G; scrambled sequence control for ODN-2). Histograms show the pooled data from four separate experiments; * ≤ 0.01 (Student's 2-tailed T test).

DISCUSSION

A model of isoform-specific roles for MFas II in migration

Our results show that the two isoforms of MFas II are expressed in distinct patterns within the developing ENS that correlate with cell type and motile behavior. Moreover, perturbations of the two isoforms have indicated that they play distinct functional roles during development, suggesting the following model (Fig. 11): upon delamination from the foregut epithelium, both the EP cells and the adjacent glial precursors express GPI-MFas II, which serves a strictly adhesive function. Lacking a cytoplasmic anchoring domain, GPI-MFas II becomes uniformly distributed over the entire plasma membrane. While it helps maintain tight apposition between neighboring cells, it does not regulate the slow spreading migration of the EP cells either positively or negatively. However, the presence of GPI-MFas II may contribute to the subsequent orientation of neuronal subsets onto each of the midgut muscle bands, possibly by allowing neighboring cells to cluster with those neurons that first contact the bands as they form (discussed below).

Shortly before fast migration, the EP cells undergo a rapid down-regulation of GPI-MFas II and up-regulation of TM-MFas II, which becomes localized to the leading processes of the neurons. With the onset of MFas II expression by the muscle bands, the dispersive migration of the EP cells along these pathways is facilitated by TM-MFas II. Interference with TM-MFas II expression during this period disrupts the attachment of the EP cells to the muscle bands and neighboring neurons (Fig. 9; and Wright *et al.*, 1999) and inhibits their migration and process outgrowth (Fig. 10). The localized expression of TM-MFas II in the leading processes and axons suggests that it helps regulate the polarized assembly of actin filaments associated with active

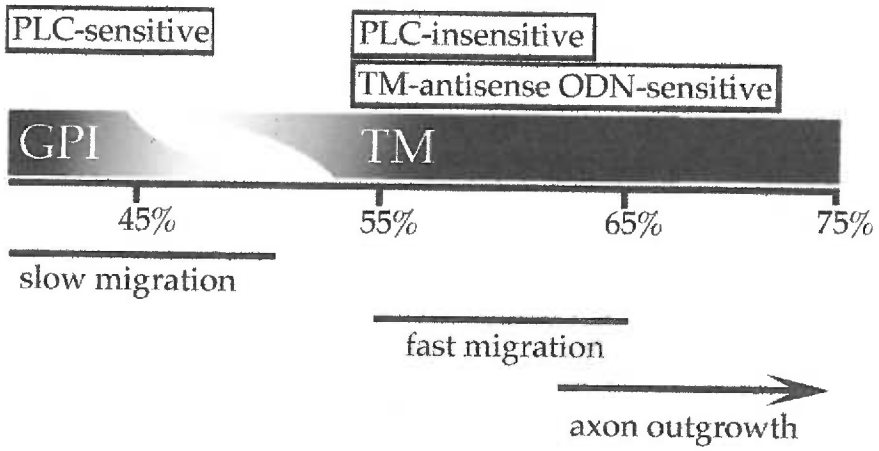
PLC-sensitive
PLC-insensitive
TM-antisense ODN-sensitive
GPI
TM
45%
55%
65%
75%
slow migration
fast migration
axon outgrowth

Figure 11. Summary of the developmental regulation of MFas II isoforms with respect to the phases of EP cell motility. Initially, the EP cells express only GPI-MFas II. They continue to express GPI-MFas II through most of the slow spreading phase of migration (40-55% of development), but this isoform is then rapidly down-regulated shortly before fast migration. At the same time, TM-MFas II is up-regulated during the latter portion of slow migration, so that it is exclusively expressed by the EP cells by the onset of fast migration (55%). The levels of TM-MFas II remain high in the EP cells throughout fast migration and axon outgrowth, with most of the protein becoming concentrated within their most motile regions (first in their leading processes and subsequently in their growing axons). EP cell sensitivity to PI-PLC is confined to the period of slow migration (when GPI-MFas II levels are high), whereas PI-PLC treatments have no effect on the EP cells during fast migration (when they express TM-MFas II exclusively). In contrast, antisense ODNs specific for TM-MFas II disrupt both the fast migration and axon outgrowth of the EP cells (TM-MFas II-positive) but not the migration of the glial cells on the foregut (GPI-MFas II-positive; see figure 9).

neuronal motility, as proposed for NCAM (Beggs *et al.*, 1997; Takei *et al.*, 1999). In contrast to the neurons, the trailing glial precursors continue to express only GPI-MFas II. As with the slow phase of EP cell migration, the subsequent migration of the glial cells along the branches of the enteric plexus involves a slow, spreading movement, during which the glial cells do not disperse but remain tightly adherent to themselves and to the underlying neurons that they ensheath. Thus, the expression of a particular MFas II isoform is both predictive of cell type (neuronal versus glial) and corresponds with the motile behavior displayed by those cells. We have now shown a similar relationship also occurs in other regions of the developing nervous system: TM-MFas II is expressed exclusively by identified sets of neurons (primarily motor neurons) and is localized to their growing axons, while GPI-MFas II is primarily confined to glial cell populations that ensheath the peripheral nerve branches (Wright and Copenhaver, submitted for publication).

Precedent for this model can be found in studies of the vertebrate receptor NCAM. Like MFas II, NCAM is expressed both as GPI-linked and transmembrane isoforms which have been proposed to play distinct roles during axon outgrowth. The predominant transmembrane forms of NCAM (NCAM 140 and 180) are expressed primarily by developing neurons and immature muscle cells (Gegelashvili *et al.*, 1993; Lyons *et al.*, 1992), while GPI-linked NCAM (NCAM 120) is expressed by glial cells but not neurons (Noble *et al.*, 1985; Walsh and Doherty, 1997; but see Rosen *et al.*, 1992). When tested *in vitro*, both NCAM 140 and 120 were found to act as substrates for axon outgrowth (Doherty *et al.*, 1990), and NCAM 140 could also act as a neuronal receptor (enhancing the outgrowth of cells expressing this isoform; Saffell *et al.*, 1994). In contrast, the presence of NCAM 120 (the GPI-linked isoform)

actually inhibited the elongation of processes by cells grown on substrates expressing NCAM (Saffell *et al.*, 1995). Similarly, we found that GPI-MFas II is expressed on tightly adherent cells (primarily on glial cells, but also on the EP cells during their slow spreading phase), while the onset of active neuronal motility was preceded by a rapid switch in isoform expression to TM-MFas II (Fig. 4, 5). Whether persistent expression of the GPI-linked isoform would inhibit neuronal motility (as has been suggested for NCAM 120) can now be tested *in vivo* by expressing ectopic GPI-MFas II in the pre-migratory EP cells; these experiments are currently in progress.

Developmental regulation of MFas II isoform expression

An important conclusion from our current studies is that both isoforms of MFas II can be rapidly regulated at the transcriptional and translational levels of expression. For example, the midgut muscle bands exhibit a rapid onset of TM-MFas II shortly before EP cell migration (between 53-55% of development), and then subsequently lose the expression of MFas II-specific mRNA and protein once migration is complete (between 63-65%; see Fig. 3 and Wright *et al.*, 1999). In like manner, GPI-MFas II expression is rapidly eliminated from the EP cells before the onset of fast migration, while TM-MFas II is steadily up-regulated (see Fig. 3, 5). Studies on a variety of other systems have shown that GPI-linked receptors can be released from cell membranes by endogenous phospholipases (Faivre-Sarrailh and Rougon, 1997; Hortsch and Goodman, 1990; Vaughan, 1996). Phospholipase activity might similarly contribute to the regulation of GPI-MFas II expression by the EP cells, although it would have to be sufficiently localized to avoid perturbing GPI-MFas II on the adjacent glial cells (Fig. 5). Protein turnover

may also be affected by the presence of a PEST sequence (Rechsteiner, 1988), a motif that has been identified in one isoform of fasciclin II in *Drosophila*. TM-MFas II in *Manduca* lacks this motif, but both MFas II isoforms contain multiple ATTTA sites in their 3' UTR regions that may affect mRNA stability (Chen and Shyu, 1995). A similar cluster of ATTTA sites has been proposed to contribute to the rapid regulation of OCAM (Yoshihara *et al.*, 1997), an adhesion receptor related to NCAM that (like fasciclin II) is transiently expressed in the developing nervous system and undergoes activity-dependent down-regulation at synapses (Yoshihara *et al.*, 1993; see Schuster *et al.*, 1996a).

In a number of instances, developmental switching of adhesion receptor isoforms has been proposed to underlie changes in neuronal adhesivity and motility (e.g. Beggs *et al.*, 1997; Walsh and Doherty, 1997). For example, alternate spicing of the VASE exon in NCAM 140 has been shown to enhance the adhesive effects of this isoform while reducing its growth-promoting activity (Doherty *et al.*, 1992). Post-translational modifications of several NCAM isoforms have also been linked to changes in neuronal migration and outgrowth (Hu *et al.*, 1996; Kramer *et al.*, 1997). The rapid switch by the EP cells from the GPI-linked to the transmembrane form of MFas II before fast migration indicates that alterations in membrane attachment may also regulate the function of a particular adhesion receptor. The selective expression of TM-MFas II by the muscle band pathways (Fig. 6) is curious, as both GPI-linked and transmembrane isoforms of MFas II would be expected to act as suitable substrates for migration (c.f.Doherty *et al.*, 1990). However, our manipulations of MFas II in culture have indicated that its expression on the muscle bands is important for the complete coalescence of these bands (Wright *et al.*, 1999; and unpublished observations), an event that

may also require interactions uniquely mediated by the transmembrane isoform.

An emerging feature of many adhesion receptors is their ability to influence neuronal motility by modulating intracellular signaling pathways. For example, transmembrane isoforms of both NCAM and L1 (also an Ig-related adhesion receptor) have been shown to interact either directly or indirectly with non-receptor tyrosine kinases that may in turn regulate cytoskeletal assembly (Beggs *et al.*, 1997; Ignelzi *et al.*, 1994; Walsh and Doherty, 1997). The localization of L1 to growth cones has also been linked with a tyrosine-based sorting signal (Kamiguchi and Lemmon, 1998). Less well understood are the roles that GPI-linked adhesion receptors may play during neuronal differentiation. Whereas some GPI-linked receptors have been implicated in the activation of intracellular signaling events (e.g. Faivre-Sarrailh and Rougon, 1997; Powell *et al.*, 1997; Zisch *et al.*, 1995), it has also been suggested that they might simply act as adhesive molecules capable of stabilizing points of contact (Martin and Kandel, 1996). Potential interactions between either isoform of MFas II and specific signal transduction pathways in the EP cells remain to be explored.

Manipulations of MFas II expression in culture

The experimental manipulations described in this paper support our hypothesis that the two isoforms of MFas II serve distinct functions in the developing ENS. As shown in figure 8, treatment with relatively low concentrations of PI-PLC substantially reduced the expression of GPI-MFas II by the EP cells and caused a substantial loss of intercellular adhesion but did not impede the slow spreading phase of migration. PI-PLC treatments have frequently been used to eliminate GPI-linked receptors in a variety of *in vitro*

and *in vivo* studies (Chang *et al.*, 1992; Karlstrom *et al.*, 1993; Yoshihara *et al.*, 1994), and in previous work, we showed that PI-PLC selectively removed the GPI-linked isoform of MFas II but not the transmembrane isoform from cell membranes (Wright *et al.*, 1999). Because the EP cells may express other GPI-linked receptors besides GPI-MFas II, these results must still be interpreted with some caution. Nevertheless, PI-PLC did not induce any obvious signs of cell death or more widespread disruption of development, supporting the conclusion that the observed effects on EP cell adhesion were due to the loss of GPI-MFas II. We also attempted to determine whether PI-PLC treatments would cause a similar loss of motility by the enteric glia, which also express only the GPI-linked form of MFas II (see Figs. 5, 7). However, antibodies generated against glial markers in other species failed to provide sufficient immunostaining to monitor the morphology of glial cells in the ENS, preventing us from investigating the role of GPI-MFas II on glial migration.

Although removal of GPI-MFas II did not prevent slow migration, it did affect the overall shape of the EP cell packet. In control preparations, the packet became distinctly "scalloped" as sets of neurons gradually oriented onto each of the midgut muscle bands prior to fast migration (Figs. 1, 5). In contrast, the more diffuse cell clusters seen in PI-PLC-treated animals remained relatively unorganized and failed to orient towards the muscle bands; in addition, the short, exploratory filopodia extended by the EP cells during slow migration (Fig. 4) did not form. To test whether this lack of alignment would affect subsequent aspects of EP cell differentiation, we treated embryos at 40% of development with PI-PLC and allowed them to develop for 24 hr in culture (through the periods of both slow and fast migration). Because our culture preparation partially constrains the morphogenetic movements that normally occur during this period

(katatrepsis; Dorn *et al.*, 1987), some distortion in the overall configuration of the ENS occurred in these experiments. Nevertheless, in control animals, we could readily determine that both slow migration (around the gut circumference) and the onset of fast migration (onto the midgut muscle bands) had proceeded normally. However, while slow migration occurred in PI-PLC-treated preparations (as already noted), the EP cells failed to align with or migrate onto the midgut muscle bands. The loss of GPI-MFas II-mediated adhesion may therefore indirectly perturb the subsequent migratory behavior of the EP cells, preventing the neurons from establishing contact with their future pathways. We previously reported that much higher concentrations of PI-PLC caused a partial inhibition of fast migration, while phospholipase B did not (Wright *et al.*, 1999). Our current results indicate that this effect might have been indirect, possibly involving the removal of other proteins besides MFas II, since the EP cells have ceased expressing GPI-MFas II by the onset of this behavior (Fig. 5). As noted above, treatment with PI-PLC after the onset of fast migration had no effect on the motile behavior of the EP cells (unpublished data), consistent with our observations that the neurons only express TM-MFas II during this period of development.

In contrast to manipulations targeting GPI-MFas II, perturbation of TM-Mas II expression affected both the fasciculation and motile behavior of the EP cells. Previously, we showed that blocking antibodies and GST-fusion proteins designed to interfere with MFas II-dependent homophilic interactions caused a loss of EP cell adhesivity and inhibited fast migration (Wright *et al.*, 1999). In our current work, we found that an antisense ODN directed against the 3' UTR of TM-MFas II mRNA (ODN-2) caused a similar inhibition of migration by the EP cells (Fig. 10), accompanied by a reduction in MFas II immunoreactivity and fasciculation (Fig. 9). As the EP cells have now

been shown to express only TM-MFas II during this stage of development (Fig. 3, 5), all of these effects can be attributed to the perturbation of functions mediated by TM-MFas II alone. Of note is that ODN-2 was as effective at inhibiting EP cell migration as ODN-Start (Fig. 10). These results are consistent with previous reports in which ODNs directed against the 3' UTR domains of particular mRNAs inhibited protein expression as effectively as ODNs against their translation initiation sites (Chiang *et al.*, 1991; Lipp *et al.*, 1995; Ronnov-Jessen and Petersen, 1996; Takayama and Inouye, 1990). The fact that only one of the four ODNs designed against the 3' UTR region of TM-MFas II was effective is also consistent with the hypothesis that secondary structure within a particular mRNA region may determine its sensitivity to ODN binding and RNAase H-mediated degradation (Chiang *et al.*, 1991; Ronnov-Jessen and Petersen, 1996). As in our previous studies, interference with MFas II-mediated interactions resulted in only a partial inhibition of EP cell migration. Based on similar results in other systems (Fishman and Hatten, 1993; Goodman *et al.*, 1997; Treubert and Brümmendorf, 1998), it is likely that multiple guidance cues (both positive and negative) may affect the timing and directionality of neuronal motility in this system (Wright *et al.*, 1999). Nevertheless, these results indicate that the expression of TM-MFas II coincident with the fast phase of EP cell migration and outgrowth plays an important role in promoting these developmental events.

Lastly, the expression of MFas II in the ENS is unusual in that high levels of both isoforms persist throughout development and into post-natal life (Fig. 7; and unpublished observations). In contrast, fasciclin II expression occurs only transiently in other regions of the developing insect nervous system, coincident with periods of axonal growth and synaptogenesis (Carr and Taghert, 1988; Grenningloh *et al.*, 1990; Harrelson and Goodman,

1988; Wright & Copenhaver, submitted for publication). The other important exception to this pattern has been described at the insect neuromuscular junction, where regulated changes in fasciclin II expression play a role in activity-dependent plasticity (Goodman *et al.*, 1997; Schuster *et al.*, 1996a). The persistent expression of MFas II in the post-embryonic ENS may therefore reflect an ongoing function that this receptor serves during the continued expansion of the gut and its innervation throughout subsequent life stages.

In summary, our results indicate that the two isoforms of MFas II play distinct roles during the formation of the ENS: GPI-linked MFas II helps maintain intercellular adhesion between expressing cells but does not directly contribute to cellular movements, whereas TM-MFas II promotes the fasciculation of the EP cells with their muscle band pathways and is needed for neuronal motility. We have also shown that the expression of the two isoforms is precisely regulated with respect to cell type and motile behavior, suggesting that isoform switching is an important mechanism in governing the developmental effects of this cell adhesion receptor.

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CELL TYPE-SPECIFIC EXPRESSION OF FASCICLIN II ISOFORMS REVEAL NEURONAL-GLIAL INTERACTIONS DURING PERIPHERAL NERVE GROWTH

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SUMMARY

During the formation of the peripheral nervous system (PNS) in insects, motor neurons extend processes along stereotyped pathways to reach their peripheral muscle targets. Among the cell ahesion receptors known to contribute to this process, fasciclin II has been shown to play a prominent role in axonal fasciculation and synapse formation. In the moth *Manduca*, fasciclin II (MFas II) is expressed both as a transmembrane isoform (TM-MFas II) and glycosyl phosphatidylinositol-linked isoform (GPI-MFas II). Using RNA and antibody probes, we have shown that these two isoforms are expressed in non-overlapping patterns: TM-MFas II is expressed exclusively by neurons and is localized to their most motile regions (axons and growth cones), 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, but in a few key locations, GPI-MFas II-positive glial cells differentiated before to the arrival of the first axons and prefigured their subsequent trajectories. Prior removal of GPI-MFas II disrupted the subsequent outgrowth of axons in these particular locations but not elsewhere in the PNS. Our results suggest that the two isoforms of MFas II play distinct roles with respect to cellular motilty 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 for the differentiation and survival of both cell types. In the developing central nervous systems (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 been shown in turn 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 follow a variety of substrates, including 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, glial scaffolds in

some instances prefigure neuronal tracts before the 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 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 mediates 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 glyocosyl 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). Besides its role in axon guidance, 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). 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, submitted for publication). In addition to these distinct expression patterns, each isoform of MFas II appeared 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 motility but disrupted intercellular adhesivity. We have now investigated whether isoform-specific expression patterns of MFas II occur in the embryonic CNS and PNS. Using isoform-specific riboprobes and antibodies, we have shown that the pattern of TM-MFas II expression alone accounts for the entire pattern of fasciclin II found in the CNS of other insects. We also report the novel observation that the expression of GPI-MFas II is confined to glial populations associated with the developing peripheral nerves. By selective manipulation of GPI-MFas II 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.

METHODS

Embryos were collected from a stable *Manduca sexta* colony as previously described (Copenhaver and Taghert, 1989). When maintained at 25°C, embryos complete development in approximately 100 hours (1 hr is equivalent to 1% of development). Subsets of staged embryos were also maintained at 18 and 20°C to slow the rate of development prior to the onset of an experiment. Sets of internal and external markers characterized in previous work were used to identify particular stages of development (Copenhaver and Taghert, 1989; 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 intact CNS and the complete innervation of the lateral body wall musculature on one side (one intact hemisegment per segment).

For tissue fixation, we tested a number of parameters associated with our standard protocol (Horgan et al., 1994), including the crosslinking reagent (paraformaldehyde or glutaraldehyde), concentration, time of fixation, presence or absence of detergent, and pH of the fixative. For *in situ* hybridization histochemistry, we also tested the relative advantages of dehydration and proteinase K treatment on tissue permeabilization. We selected the fixation protocol which yielded the highest signal sensitivity and lowest 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. Tissue was fixed for 15 minutes, followed by 3 x 5 minute 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, submitted for publication) 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 mRNA 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, submitted).

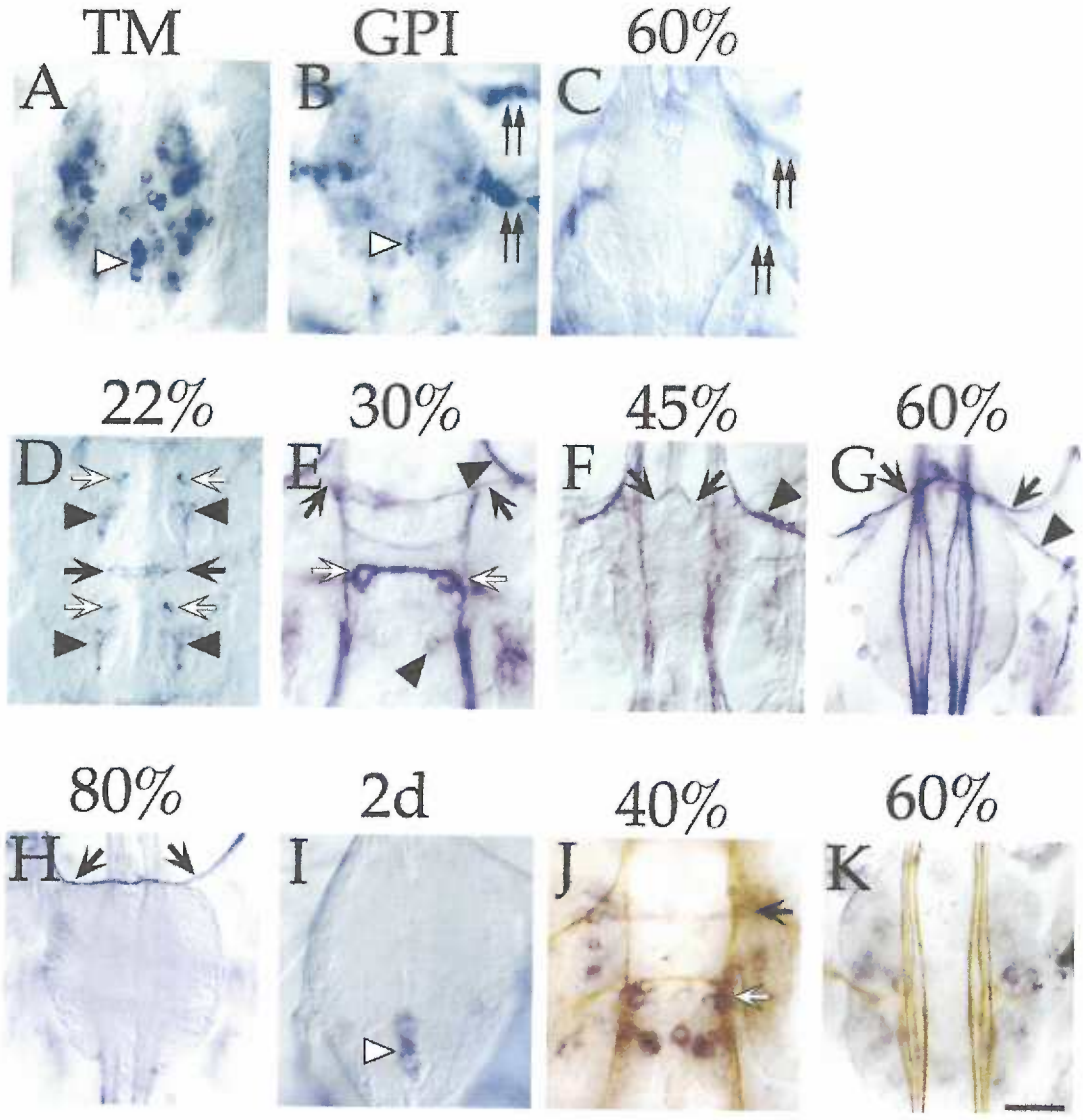
For culture experiments, embryos were prepared essentially as described (Wright et al., 1998), but because the stages investigated in these studies were much younger than in our previous work, the following modifications were introduced: embryos were dechorionated in sterile medium using fine forceps 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. Approximately 10 μl of control or experimental solutions was then introduced into the vicinity of the developing nervous system. Embryos

were cultured overnight at 37°C prior to fixation and immunohistochemical staining (as described above). Phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Boehringer and diluted in sterile medium. Each experimental manipulation was repeated a minimum of three times using replicate sets of identically staged embryos. Analysis of axon outgrowth in cultured preparations was performed using photomicrographic and *camera lucida* techniques.

RESULTS

Isoform-specific expression of MFas II in the central nervous system

Previous work by Goodman and colleagues 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 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 pair of ventral unpaired median (VUM) 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 (including the same VUM cells indicated in figure 1A) consistently stained more strongly (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


TM
GPI
60%
A
B
C
22%
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45%
60%
D
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F
G
80%
2d
40%
60%
H
I
J
K

Figure 1. Developmental expression of MFas II isoforms in the embryonic CNS. Each panel show a representative abdominal ganglion (anterior is towards the top of the page). **A**, *in situ* hybridization histochemistry using a TM-MFas II-specific RNA probe stained a large number of neuronal somata in an abdominal ganglion at 60% of development (white arrowhead indicates a pair of VUM 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 the same VUM cells as in A). **C**, immunohistochemical labeling with a GPI-MFas II-specific antibody produced no detectable staining within the CNS at 45% of development (or any other time during embryogenesis). **D-E**, 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 non-neural cells that prefigure the transverse nerve (black arrows). **E**, TM-MFas II expression 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 exiting 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 two days post-hatch, TM-MFas II expression had reappeared in a pair of VUM 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 potein (brown); arrows in J label the same structures as in E. Scale = 30 μM.

nerve from its origin at the central ganglia (Fig. 1B, arrows) to its peripheral targets (see below).

To determine the distribution of MFas II 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 overall 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 sequence of expression was largely conserved, including characteristic sets of identified neurons and their processes. 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 appeared on 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, G). 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 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 non-neural 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. The most robust staining at 30% of development 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 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. 1D, black arrows). By 45%, TM-MFas II expression was no longer detectable in either these non-neural cells or in 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 (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 levels (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 has declined (Fig. 1H, arrows); however, the elimination of TM-MFas II from axons exiting the ganglia occurred in a proximal-distal gradient, so that expression persisted within their peripheral branches (described below).

Except for the earliest stages of neurogenesis (Figs. 1D, E), 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 (Fig. 1J, K), 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 development. Within 48 hours, however, a new phase of TM-MFas II expression had commenced in the VUM cell somata (Fig. 1I, white arrowhead), neuromodulatory neurons that continue to express TM-MFas II in their somata and terminal processes throughout post-embryonic life (L. Knittel and K. Kent, submitted for publication).

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) and much lower levels of GPI-MFas II mRNA (Fig. 2B). As seen in the ventral ganglia, TM-MFas II protein was excluded from the somata of these neurons but was localized to specific axonal tracts (Fig. 2C) and to a set of bilateral fascicles ascending from the 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 4-8 clustered cells that appeared to be glial precursors, based on their size and position (P.F. Copenhaver, unpublished observations). 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 brain is largely complete.

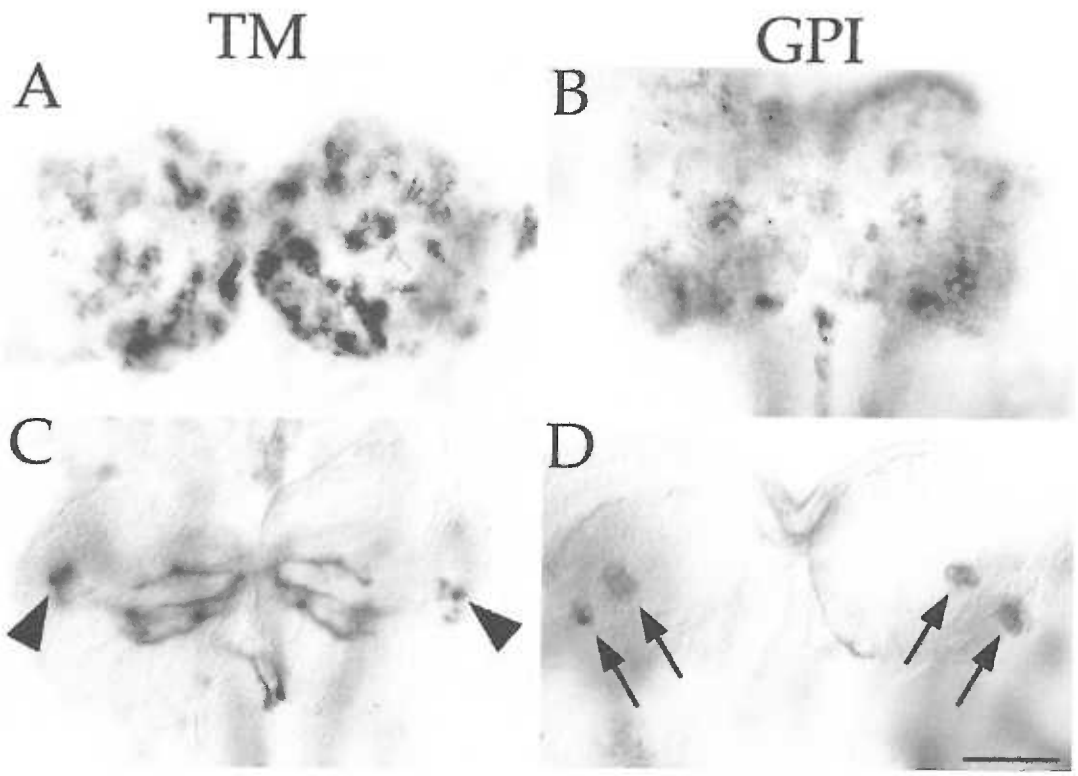
TM
GPI
A
B
C
D

Figure 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 subesophageal ganglion (out of focus below the brain). Arrows in D indicate two bilaterally paired clusters of putative glial precursor cells. Scale = 80 μM.

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 particularly striking. GPI-MFas II immunoreactivity was absent from the central ganglia (as noted above) but prominent within the glial cells surrounding the peripheral nerves as they exited 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). This distinction was particularly evident within more distal regions of the dorsal nerve, in which the glial population lining the nerve showed strong GPI-MFas II expression over their entire surface (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 clearly visible with Nomarski optics (Fig. 3D, white arrow). *In situ* hybridization labeling confirmed 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 the pattern of MFas II mRNA isoform expression and protein synthesis in the PNS precisely coincide, unlike the CNS in which neurons were found expressing mRNA for both isoforms but only TM-MFas II protein (Fig. 1).

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 contribute to the differentiation of

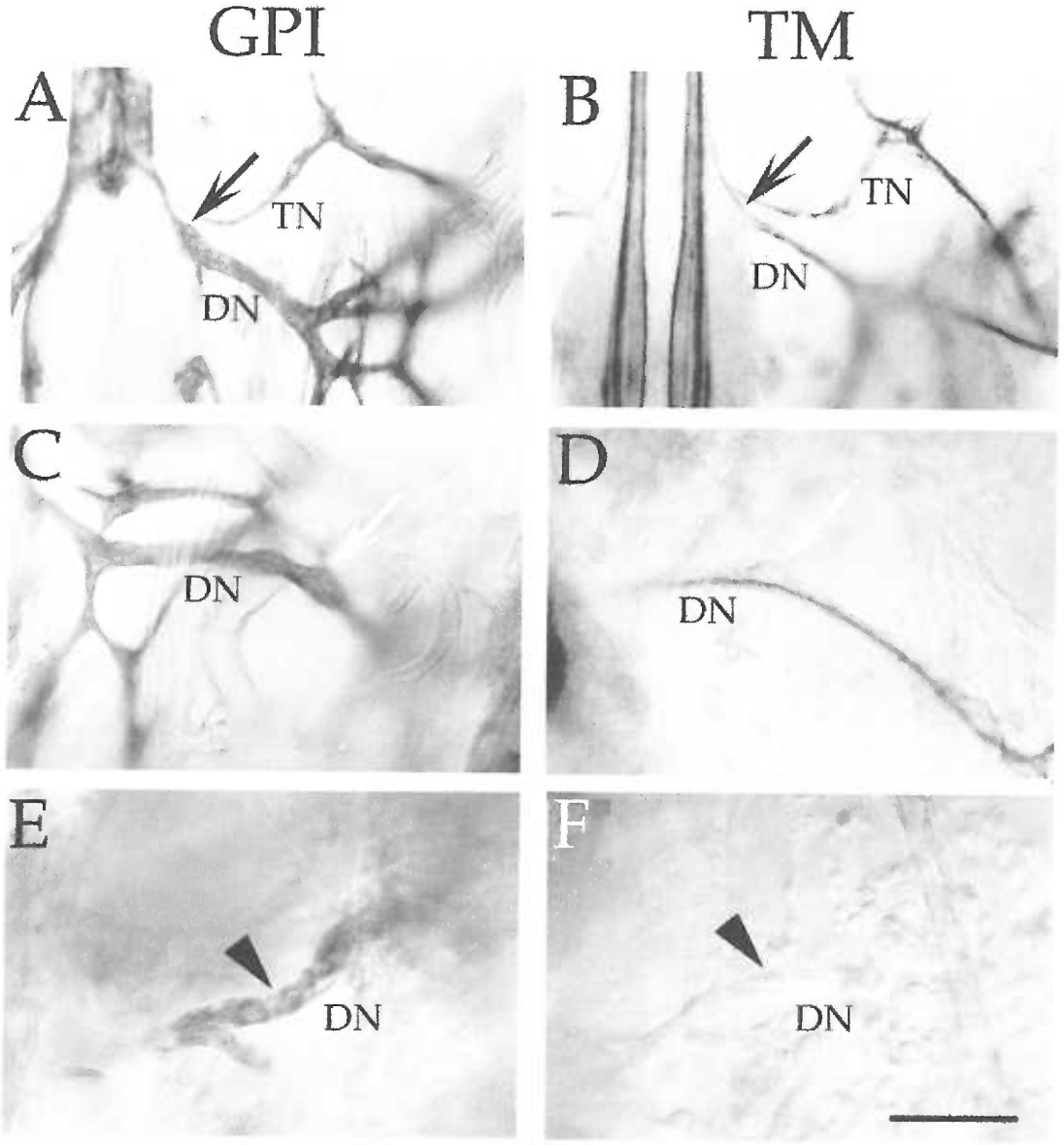
GPI
TM
A
TN
DN
B
TN
DN
C
DN
D
DN
E
DN
F
DN

Figure 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 immmunostaining 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 = 50 μM in A-D, 30 μM in E-F.

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 cohesive population of cells within the mesoderm that had already begun to express GPI-MFas II (Fig. 4A, black arrowhead). These cells subsequently differentiate into a glial "bridge" (Fig. 4C) that prefigures the routes taken by several peripheral nerve branches, as previously shown by Carr and Taghert, 1988). Axons forming the DNa 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 (Fig. 4E, F). Thus, during the establishment of the DNa nerve branch, glial cells expressing GPI-MFas II form a presumptive scaffold structure 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 extends posterior to the spiracle to innervate dorsal musculature (Fig. 5). TM-MFas II-positive growth cones pioneering the DNl at 40% of development had extended 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-

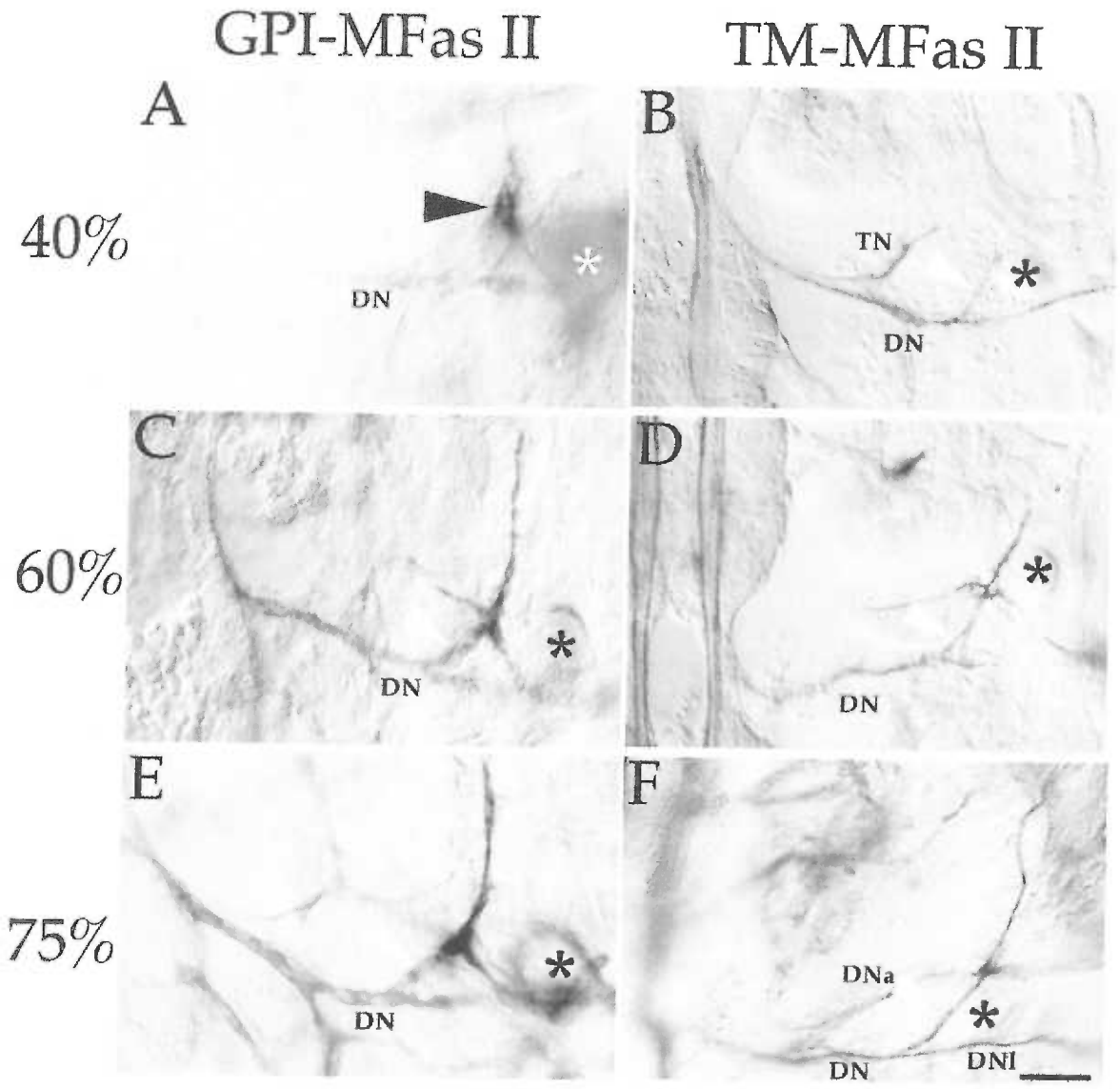
GPI-MFas II
TM-MFas II
A
B
40%
DN
TN
DN
C
D
60%
DN
DN
E
F
75%
DN
DNa
DN
DNl

Figure 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-positve 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). Scale = 30 μM.

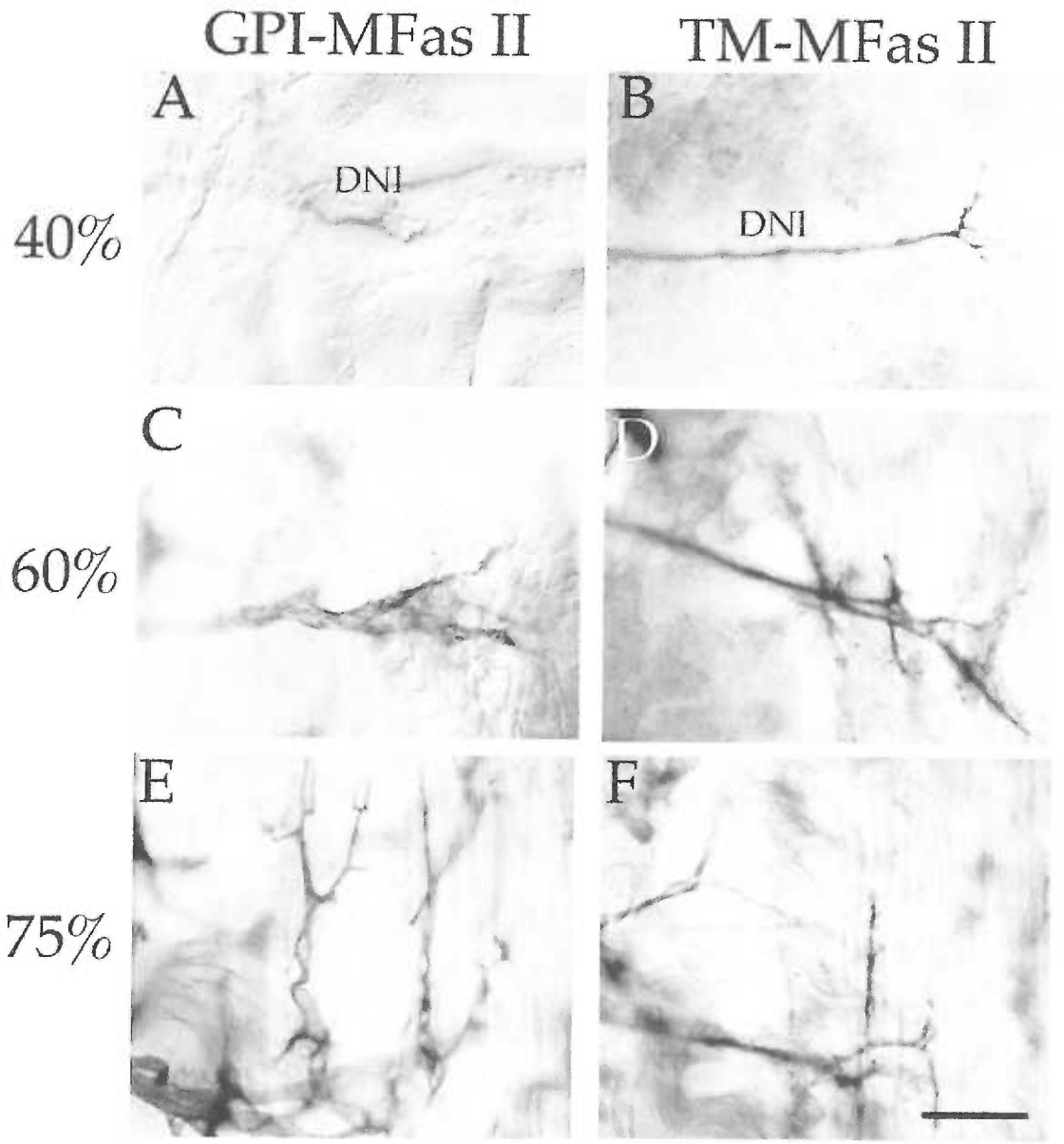
GPI-MFas II
TM-MFas II
A
B
DNI
DNI
40%
C
D
60%
E
F
75%

Figure 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 = 30 μM.

MFas II-positive terminal branches of the DNl had begun to extend along them (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 but had extended well beyond them and appeared to delineate the neuromuscular junctions in their entirety (compare Fig. 5E, F). The resulting network of GPI-MFas II-positive glial extensions was considerably more elaborate than the TM-MFas II-positive neuronal axonal terminals (Fig. 5E).

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 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). The results of these studies were unexpected, revealing that the neuronal and glial processes only partially overlapped 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, there were many areas in which the growing glial processes and underlying axons occupied distinct focal planes. In Fig. 6A (which is a montage of four focal planes), the black

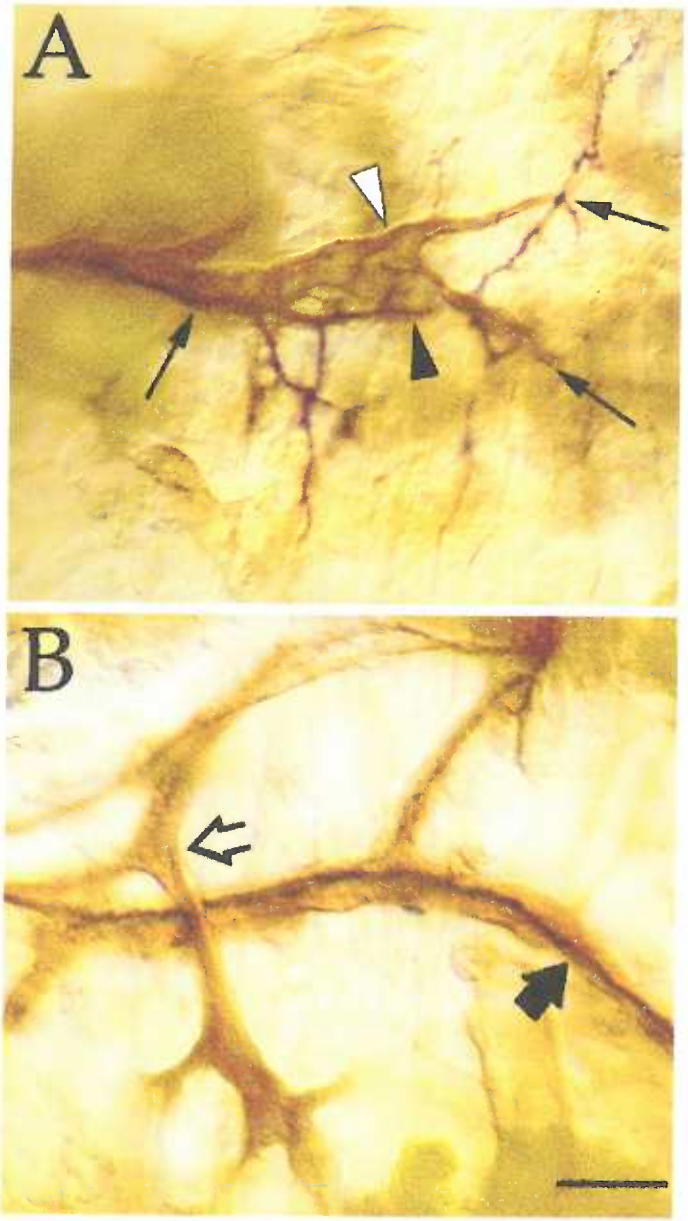
A
B

Figure 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 axons (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. Scale = 20 μM.

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, 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 nerve terminals.

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 neuronal processes into the periphery prior to the differentiation of any glial support. The subsequent outgrowth of glial cells that eventually would ensheath 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 (Fig. 5E, 6B) reflects the selective down-regulation of TM-MFas II at certain terminal branch points or is due to glial ensheathment of MFas II-negative neurons is discussed below.

Guidance of TM-MFas II-positive axons by GPI-MFas II-positive glia

Our observations that neuronal processes expressing TM-MFas II overlap with GPI-MFas II-positive glial cells in a variety of circumstances suggested that neuronal-glial interactions might play a role in axonal and glial pathfinding. In particular, the formation of the glial bridge (expressing GPI-MFas II) prior to the outgrowth of the DNa axons (Fig. 4) raised the possibility that these glia serve as a requisite scaffold that guides these axons towards their correct targets, and that this neuronal-glial interaction might be GPI-MFas II-dependent. We therefore treated cultured embryos with low doses of

PI-PLC at 35% of development (prior to DNa outgrowth) and allowed them to develop overnight at 37°C. We previously 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, submitted). 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 (Fig. 7 B-D, 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 (Fig. 7 B-D, white arrowheads; and Table 1). 37% of treated embryos exhibited a complete failure in the defasciculation of the DNa from the DNl (Fig. 7C). The initial divergence of the DNa proceeded normally in the remaining animals (compare Fig. 7A with 7B, D), 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. 16% of the DNa branches stalled approximately at the position of the bridge and extended abnormal branches in several inappropriate directions (Fig. 7B). More frequently (39% of the time), the DNa branches emerged at variable locations along the DNl and exhibited a "switchback" phenotype, (Fig. 7D), suggesting the presence of an

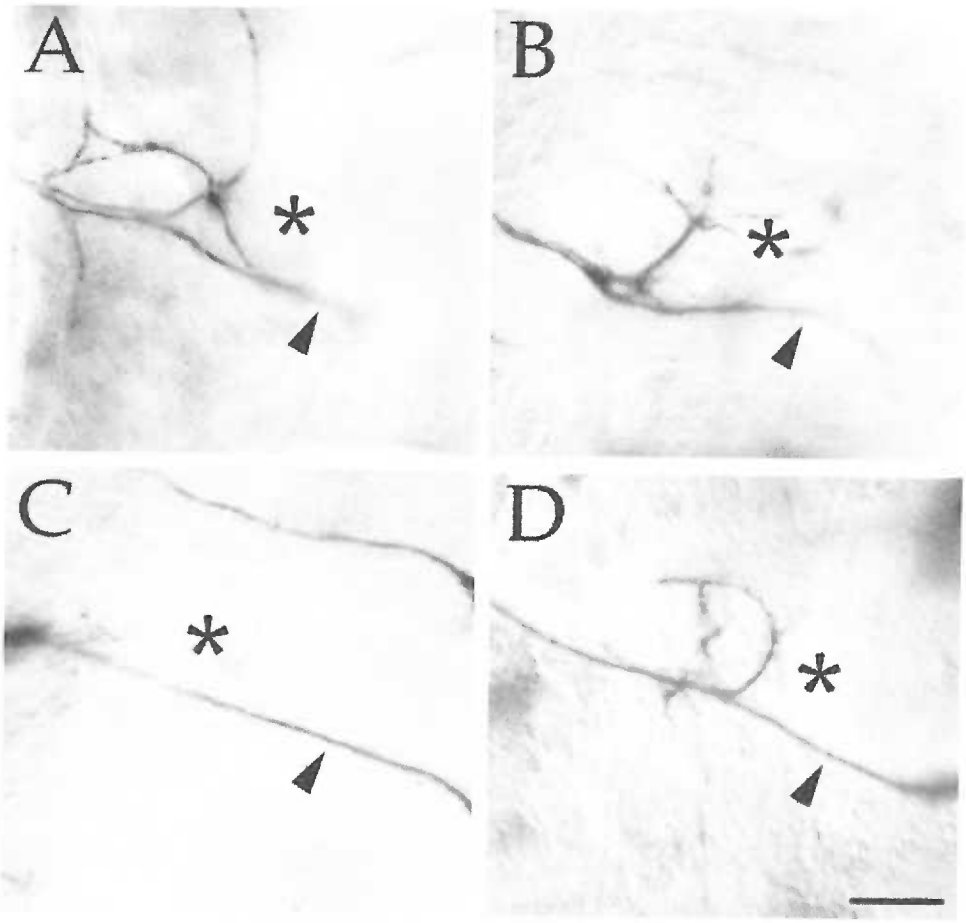
A
B
C
D

Figure 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 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 = 30 μM.

Table 1. Effects of GPI-Mas II removal from the glial bridge by PI-PLC on the trajectory of the DNa nerve branch.

	Normal	Stall	Switchback	No branch
Control	43	0	0	0
PI-PLC	4	8	19	18
Percent of total	8	16	39	37

additional guidance cue in this direction capable of misleading the DNa growth cones when GPI-MFas II expression on the bridge cells is 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 species; unfortunately, none of these reagents provided a convincing independent marker for *Manduca* glia. We were also unable to visualize unstained bridge glia reliably with Nomarski optics, due to the complexity of other mesodermal and ectodermal structures that form in its vicinity. The removal of GPI-MFas II from the glia may therefore disrupt the formation of the DNa directly by eliminating a *bone fide* guidance cue; alternatively, removal of GPI-MFas II may simply disrupt the coalescence of the bridge, thereby precluding the presentation of other guidance cues normally associated with this structure. Nevertheless, our results indicate that the formation of some nerve pathways in the PNS requires axonal guidance by pre-formed glial assemblies, while in other instances, the outgrowth of neuronal and glial processes appear 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 was consistent with 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 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 (Fig. 1D, E). The progressive redistribution of TM-MFas II protein from the somata of expressing neurons into their distal processes was particularly evident in preparations that were double-labeled to show TM-MFas II mRNA and protein expression (Fig. 1J, K). These observations are reminiscent of 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 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. Previous work on the enteric nervous system (ENS) also showed no evidence of mRNA expression for a particular isoform in the absence of detectable protein (Wright and Copenhaver, submitted). 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, submitted).

One possible exception to this cell type-specific pattern of isoform protein expression was seen in the brain (Figure 2D), in which two clusters of large cells within each lobe were labeled strongly with the GPI-MFas II-specific antibody. While these cells are still unidentified, there position, size, and morphology suggest these are glial precursors that later ensheath several neuronal tracts within the brain (based on dye injections and lineage tracing; P. Copenhaver, unpublished observations). Alternatively, these cells might represent adherent clusters of immature neurons that have not yet elaborated processes, perhaps being maintained in a tightly adherent and undifferentiated state until a later stage in development. Expression of GPI-MFas II by an identified set of immature neurons has similarly been detected in the ENS, although these cells then switch MFas II isoforms prior to their migration and differentiation (Wright and Copenhaver, submitted). It is also possible that the labeled brain cells might be expressing both MFas II isoforms but retaining GPI-MFas II on their somata. We and others have found that GPI-coupled proteins are typically distributed over the entire plasma membrane of expressing cells, however, suggesting that this option is unlikely (Wright and Copenhaver, submitted; Varma and Mayor, 1998).

GPI-MFas II expression by peripheral glia provided an unambiguous means of visualizing the distribution of these cells throughout most of embryogenesis (Fig. 3, 4, 6). Although the lack of additional antibodies that recognize *Manduca* glial cells limited some aspects of our analysis, we were able to monitor the interactions between growing axons and their glial partners in some detail. One question that remains unresolved concerns the source of the peripheral glial populations. In *Drosophila*, an identified

population of exit glia (including the segment boundary glia) originate from stem cells in the CNS and delineate the positions at which the peripheral nerves leave the CNS (Jacobs and Goodman, 1989b). Subsequent work has indicated that 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., 1994), and several identified glial structures (including the glial "bridge" that guides the DNa nerve) also appear to arise within surrounding mesodermal tissues prior to the arrival of any growth cones (Fig. 4; and Carr and Taghert, 1988). Glial phenotypes have also been shown to be induced in mesodermal cells by glial-specific transcription factors (Bernardoni et al., 1998), suggesting a combination of ectodermal and mesodermal precursors may contribute to peripheral glial populations in insects. A complete analysis of the glial cells described in this study will require additional markers that can be used to trace their origins in better detail.

The behavior of the glial cells as they spread peripherally was also notable in that they clearly followed additional guidance cues besides the TM-MFas II-positive axons (Fig. 6). Unlike *Drosophila*, in which motor axons 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 post-embryonic life (L. Knittel and K. Kent, submitted). The neuromuscular junction of *Manduca* in this regard is more similar to that of vertebrates than has been reported in fly (Atwood et al., 1993; Rheuben, 1992).

As shown in figures 5 and 6, we also found that GPI-MFas II-positive glial cells extended substantially beyond the TM-MFas II-positive motor terminals on mature muscles. This pattern did not appear to be due to incomplete TM-MFas II immunostaining, as positively labeled axons exhibited strong levels of immunoreactivity all the way to their terminals on the target muscle. It is possible that TM-MFas II-positive motor neurons may give rise to branches that are TM-MFas II-negative or that down-regulate this receptor upon reaching a particular muscle target. As noted above, we observed the selective localization of TM-MFas II to particular domains of growing axons in both the CNS and PNS (Figs. 1, 3), as has been previously documented in other insects (Bastiani et al., 1987; Grenningloh et al., 1991; Harrelson and Goodman, 1988). Whether the localization of TM-MFas II may be similarly restricted within the peripheral arbor of individual neurons remains to be explored. Alternatively, these additional glial branches may ensheath motor axon terminals that are devoid of TM-MFas II. There may be an additional set of motor neurons that never express MFas II during their outgrowth but nevertheless are ensheathed by GPI-MFas II-positive glia, although all *Drosophila* motor neurons have been reported to express fasciclin II during embryogenesis (Schuster et al., 1996a; Van Vactor et al., 1993). Some of this additional glial elaboration may also reflect the ensheathment of unstained sensory afferent fibers *en route* to the CNS (c.f. Sepp et al., 2000), although the terminal arborizations of sensory neurons in *Manduca* tend to be morphologically and spatially distinct from the motor neuron terminals (Kent et al., 1996). Simultaneous labeling of sensory neurons and the glial sheath in the developing embryo 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 occur between TM-MFas II-positive axons and GPI-MFas II-positive glial cells. The first type, illustrated by the extension of the DNl branch (Figure 5), occurs when axons expressing TM-MFas II navigate 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, which is also reminiscent of the guidance of Schwann cells by axons during vertebrate development. Based on these results, it is not surprising that cleavage of GPI-MFas II from the glial cells prior to the extension of the DNl had no effect on the outgrowth or terminal morphology of this nerve (Fig. 7, black arrowheads; and unpublished observations). Therefore in the case of the DNl, the axons conduct their initial pathfinding independent of glial interactions and provide support for subsequent glial differentiation.

However, 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 figure 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 prior to axonal contact with these glia dramatically altered the development of the DNa branch (Figure 7 and Table 1). The axons

normally forming the DNa were perturbed to some degree in over 90% of the treated preparations, including a complete failure to defasciculate from the DNl 37% of the time. Even when DNa axons did extend into the area normally occupied by the target glia, they typically failed to complete a normal trajectory but instead extended processes in a number of inappropriate directions, including back towards the CNS. 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 pre-specified by glial or other non-neural structures (Singer et al., 1979; see Silver, 1993).

The use of PI-PLC to remove GPI-MFas II has substantial precedent but needs to be interpreted with some caution. A large number of studies have previously employed this strategy to remove GPI-linked receptors both *in vivo* and *in vitro* (e.g. Chang et al., 1988; Karlstrom et al., 1993; Yoshihara et al., 1994), and we have shown that PI-PLC selectively cleaves the GPI-linked form but not the transmembrane form of MFas II from cell membranes (Wright et al., 1999; and submitted). The relatively low doses of PI-PLC used in these studies did not cause any obvious defects in nerves such as the DNl branch that grew independent of substrates expressing GPI-MFas II (Fig. 5). PI-PLC would also be expected to cleave other GPI-linked molecules that might be expressed by the glial cells, so that the effects seen during DNa branch formation might reflect the function of additional receptors besides GPI-MFas II. Nevertheless, these experiments provide support for our hypothesis that expression of this isoform by the glial bridge cells is critical to the formation of this nerve.

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. However, because the removal of GPI-MFas II by PI-PLC also precluded our ability to determine whether the bridge itself was affected by this treatment, we could not reliably visualize these cells in the absence of specific labeling. 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 independent of TM-MFas II-expressing axons (Fig. 3, 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 tyrosine kinases in the *src* family (Beggs et al., 1994; Ignelzi et al., 1994). NCAM 140 has also been shown to interact with focal adhesion kinase (Beggs et al., 1997) and with mitogen-activated protein kinases (Schmid et al., 1999), both of which may modulate cytoskeletal assembly and neuronal growth. A separate line of investigation has lent support for cross-talk between NCAM and fibroblast growth factor-mediated activation of phospholipase C_γ (Saffell et al., 1997), although the potential contribution of different NCAM isoforms to this interaction have not been explored. The cytoplasmic domain of transmembrane apCAM is necessary for the regulation of this isoform during the induction of synaptic plasticity (Bailey et al., 1997) and presumably mediates its association with site-directed actin filament assembly (Thompson et al., 1996). Similarly, a PDZ-binding domain within the cytoplasmic tail of transmembrane fasciclin II has been shown to promote local clustering of this receptor with both the Shaker potassium channel and the membrane-associated guanlyate kinase Discs-large (Thomas et al., 1997; Zito et al., 1997). A variety of intracellular signaling pathways may therefore link transmembrane isoforms of these IgSFs with changes in neuronal motility.

In contrast, the role of the GPI-linked isoforms is less well understood. A number of other GPI-linked cell adhesion receptors in both the nervous and immune systems have been shown to interact with non-receptor tyrosine kinases (Brown, 1993; Casey, 1995; Olive et al., 1995; Zisch et al., 1995), but no such association has been demonstrated for GPI-linked isoforms of the NCAM/apCAM/fasciclin II family. Unlike transmembrane apCAM, the

localization of GPI-linked apCAM is not affected by synaptic activity (Bailey et al., 1997), implying that it might be simply adhesive in nature (Martin and Kandel, 1996). The lack of cytoplasmic interacting domains would similarly preclude GPI-linked fasciclin II from participating in the control of presynaptic plasticity (Schuster et al., 1996b). A more direct approach was taken to test whether different isoforms of NCAM might have different functional capabilities *in vitro*. Ectopic expression of NCAM 140 (a transmembrane isoform) in PC-12 cells enhanced axonal outgrowth, indicating that this isoform can act as an authentic neuronal receptor in cells that normally express it. When NCAM-120 (a GPI-linked isoform) was presented as a substrate molecule, it also could promote neuronal motility (Doherty et al., 1990). However, when NCAM-120 was expressed in PC-12 cells, it actually reduced the extent of outgrowth, leading to the suggestion that the GPI-linked version of an IgSF might normally serve a "dominant negative" role by out-competing other NCAM isoforms for substrate binding sites (Saffell et al., 1995).

In the case of MFas II, a number of our studies using cultured embryos support the notion that 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, submitted). 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 (Fig. 5),

gradually spread along the pathways formed by the neurons and their processes. We also found that the membrane distributions of the two isoforms was similar in the ENS as reported in this paper: GPI-MFas II was 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. This consistent localization of TM-MFas II suggests that it may help regulate the cytoskeletal dynamics associated with motility, 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. Because the two isoforms are largely confined to separate cell types (neuronal vs. glial), it seems unlikely that the normal role for GPI-MFas II is to negatively regulate the growth-promoting effects of TM-MFas II. 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 the two isoforms of fasciclin II in *Manduca* are expressed in dynamic and distinct patterns within the developing nervous system. The pattern of TM-MFas II recapitulates the well-characterized sequence of fasciclin II staining that has been reported in the CNS of other insects. We have also presented novel observations concerning GPI-MFas II, demonstrating that this protein is expressed primarily by peripheral glial cells, although mRNA for both isoforms can be detected in the CNS. Our data support a model whereby the localized expression of TM-Mas II may promote neuronal motility, while GPI-Mas 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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Summary

This thesis reports some findings that are consistent with previous studies of fasciclin II expression in *Drosophila* and grasshopper, as well as NCAM and apCAM. Most notably the pattern of expression of TM-MFas II is essentially conserved between *Manduca, Drosophila,* and grasshopper. In addition, we report novel observations regarding the expression pattern of GPI-MFas II, showing that it is expressed primarily by glia and some non-motile neurons. We suggest a role for GPI-MFas II that is in some ways contradictory to earlier findings regarding NCAM and apCAM, and do not consider GPI-MFas II to be a dominant negative regulator of TM-MFas II activity. Our observations of TM-MFas II expression and function agree with observations in other laboratories: TM-MFas II is a neuronal CAM that participates in the guidance of motile neurons and axonal fasciculation while disruption of TM-MFas II adhesion or expression blocks motility and causes defasciculation.

A particularly interesting finding is that GPI-MFas II is expressed by neurons in the ENS. The significance of this observation is that these neurons express GPI-MFas II only prior to a major period of migration. GPI-MFas II is down-regulated as TM-MFas II expression is up-regulated just before this phase of migration begins. This observation supports a distinct role for GPI-MFas II, as discussed below. Another novel observation is the pattern of isoform expression in the central and peripheral nervous systems. Previous studies did not distinguish between these isoforms, or else focused only on the transmembrane isoform. We show a complete cell type-specific pattern of expression. Motor neurons express TM-MFas II. Glia express GPI-MFas II.

Our use of low concentrations of PI-PLC to enzymatically ablate GPI-MFas II from cell membranes had two profound effects: first, EP cell migration was inhibited, but only if these cells were treated before the onset of TM-MFas II expression. Once these cells began to express TM-MFas II, removal of GPI-MFas II from the cell membrane did not affect migration. Second, the formation of the DNa was extremely affected by removal of GPI-MFas II from the glial "bridge", and this major nerve branch formed improperly in 92% of the cases examined.

The observation that EP cells switch MFas II expression from the GPI-linked form to the transmembrane form just prior to migration led us to speculate that the extracellular domain of MFas II promotes tight intercellular adhesion and that the cytoplasmic domain somehow is involved in both the promotion of motile activity and the localisation of TM-MFas II to motile regions of the neuron. Although GPI-MFas II does not seem to actively promote neuronal motility, it also does not appear to be a dominant negative of the transmembrane isoform (as Saffell et al. suggest for NCAM 120). Furthermore, we found no data supporting any association between GPI-MFas II and intracellular signalling events. We suggest that GPI-MFas II is strictly a CAM, whereas TM-MFas II is a CAM and an active promoter of outgrowth. As stated in the introduction, with many CAMs it is increasingly difficult to separate adhesion from other cellular activities, yet it is possible that this is precisely what is achieved by the domain structure of GPI-MFas II.

In the examples of PI-PLC inhibiting EP cell migration and perturbing DNa formation, we feel that loss of intercellular cohesion adequately accounts for these events, and we suggest possible mechanisms governing this dependence on GPI-MFas II. In the case of the EP cells, it is possible that decreased adhesion causes dissociation of the nascent EP packet, resulting in

displacement of EP cells beyond the range of permissive contact with their normal migratory pathways. Decreased adhesion could also perturb the young "bridge" glia that contact the DNa, such that they fail to retain sufficient association prior to DNa branching and thus can not act as a guidance substrate. It is also possible that cleavage of GPI-MFas II affects the ability of the EP cells to generate exploratory processes. Cleavage of GPI-MFas II from the EP cells induced cell rounding. This change in cellular geometry would be expected to maximize the ratio of volume-to-membrane surface area, thus increasing membrane tension (analogous to "inflation"). Such increases in membrane tension have been shown to inhibit cell spreading and lamellipodial extension (Raucher and Sheetz, 2000). The same effect could be occurring in the "bridge" glia, although we were unable to visualize them following PI-PLC treatment. In either case, the adhesive function of GPI-MFas II could entirely account for its biological activity, and this isoform might not associate with signalling pathways.

In other systems, both transmembrane and GPI-anchored IgSF receptors have been shown to associate with intracellular tyrosine kinase activity. We performed exhaustive analyses but were unable to demonstrate any such association with either MFas II isoform (unpublished data). In addition, when whole embryos were labelled with ^{35}S-labelled amino acids and used for MFas II immunoprecipitation, no coprecipitating proteins could be detected. While this does not prove MFas II isoforms do not associate with intracellular components, it indicates that this technique (immunoprecipitation) is ill-suited to detect any such molecules.

The results of this thesis suggest a number of areas for future research and introduces other applications for this model system. First, technical innovations have extended the use of this model beyond the development of

the enteric nervous system. Using a semi-intact preparation we can investigate features of peripheral nervous system development. Notably, we have seen one significant difference between the mechanisms of axonal extension by enteric vs peripheral motor neurons. The antimetastatic agent CAI effectively blocks EP cell motility in a calcium-dependent manner, yet fails to inhibit the extension of peripheral axons (unpublished observation). Second, the GPI-MFas II-specific antibody could be used as a long term aid to investigate changes in glial cell behavior, owing to its glial specificity. While most studies on the nervous system tend to focus on neurons rather than glia, the role of glia should not be understated. Our data indicate that the formation of the DNa is dependent on the bridge glia, and the relationship between these glia and neurons remains to be unravelled. Finally, the investigation of MFas II is by no means complete. Despite the fact that immunoprecipitation experiments do not detect protein interactions between MFas II and other molecules, the development of other techniques, such as primary cell culture or successful transfection of stable cell lines, could provide invaluable tools to investigate the intracellular function of MFas II and its interactions with signalling molecules.

The study of cell biology occurs at many levels, from protein interactions in isolation, to cells in culture, to *in vivo* research of whole organisms. This model system allows the analysis of cellular interactions with the environment while maintaining a native biological context. It has proven useful in characterizing cellular activities associated with migration and guidance in several publications and should continue to contribute to our deeper understanding of cell biology in the future.

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