Midline Fasciclin: A *Drosophila* Fasciclin-I-Related Membrane Protein Localized to the CNS Midline Cells and Trachea

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ABSTRACT: ***Drosophila* Fasciclin I is the prototype of a family of vertebrate and invertebrate proteins that mediate cell adhesion and signaling. The *midline fasciclin* gene encodes a second *Drosophila* member of the Fasciclin I family. Midline Fasciclin largely consists of four 150 amino acid repeats characteristic of the Fasciclin I family of proteins. Immunostaining and biochemical analysis using Midline Fasciclin antibodies indicates that it is a membrane-associated protein, although the sequence does not reveal a transmembrane domain. The gene is expressed in a dynamic fashion during embryogenesis in the blastoderm, central nervous system midline cells, and trachea, suggesting it plays multiple developmental roles. Protein localization studies indicate that Midline Fasciclin is found within cell bodies of midline neurons and glia, and on midline axons. Initial cellular analysis of a *midline fasciclin* loss-of-function mutation reveals only weak defects in axonogenesis. However, embryos mutant for both *midline fasciclin* and the *abelson* nonreceptor tyrosine kinase, show more severe defects in axonogenesis that resemble *fasciclin I abelson* double mutant phenotypes.** J Neurobiol 35: 77–93, 1998

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INTRODUCTION

Cell and substrate adhesion molecules play important roles in the development of the nervous system and other tissues. The neural processes in which they participate include neurite outgrowth, axon guidance, axon fasciculation, neuromuscular connectivity, and synaptic plasticity (Goodman, 1996). Adhesion molecules are placed into families based on their structure. The families include Cadherin, Fasciclin I (Fas I), Immunoglobulin (Ig), Integrin, Leucine-rich repeat, as well as others. The function of these molecules in developmental processes has been revealed by cellular, experimental, genetic, and molecular experimentation. Analysis of cell adhesion molecules in *Drosophila* has been primarily undertaken to take advantage of its sophisticated genetics. Nevertheless, although some adhesion molecules have interesting genetic phenotypes, other loss-of-function single-gene mutations often have subtle or no detectable phenotypes. The function of these molecules can still be productively studied by analyzing double mutant combinations, which presumably remove redundant gene function (Elkins et al., 1990b), or by ectopic expression experiments (Chiba et al., 1995; Nose et al., 1992).

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Fas I was originally discovered in *Drosophila*, and related members of the Fas I family of secreted and membrane-associated proteins have subsequently been identified in a number of other organisms. These proteins are involved in a variety of developmental processes including cell adhesion, axon guidance, morphogenesis, and cell proliferation. Characteristic of these proteins is the presence of a repeated domain structure first identified in *Drosophila* Fas I (Zinn et al., 1988). The Fasciclin I Domain (FD) is approximately 150 amino acids (aa) long, and its function is unknown although likely to mediate ligand-receptor or homophilic interactions. The *fasI* gene was originally cloned based on the expression of its protein on subsets of central nervous system (CNS) axons in Grasshopper (Bastiani et al., 1987). It is a glycosyl-phosphotidylinositol (GPI)-linked cell surface protein that can mediate homophilic cell adhesion (Elkins et al., 1990a). It also exists in a secreted form during embryonic development (McAllister et al., 1992).

The *Drosophila* CNS consists of segmentally repeated ganglia in which the two hemiganglia are separated by a discrete set of CNS midline cells. Neurons extend axons that organize into axon bundles. The longitudinal axon bundles connect ganglia along the anterior/posterior axis, and the commissural axon bundles cross the midline to connect each side of the CNS. Formation of commissures involves attraction of axons to the midline, crossing of the midline cells to the contralateral side of the CNS, and proper separation due to the migration of the midline glia (Klämbt et al., 1991). Mechanistic understanding of this process has been approached using molecular, cellular, and genetic analysis. Several studies have shown that the Netrins, Frazzled (the Netrin receptor), Roundabout, and Commissureless all play roles in midline-influenced axon guidance (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Seeger et al., 1993). The possibility that *fasI* is also involved in commissure formation emerged from observations that Fas I protein is initially localized on all commissural axons and midline VUM neurons and their axons (McAllister et al., 1992). Correspondingly, genetic experiments have implicated *fasI* in commissure formation. Null mutant strains of *fasI* are viable and have a normal-appearing CNS. However, a requirement of *fasI* in the formation of commissural axon bundles was demonstrated by constructing a mutant strain lacking both *fasI* and the *abelson* (*abl*) nonreceptor tyrosine kinase, which is also expressed on CNS axons (Gertler et al., 1989). Embryos with single-gene mutations in *abl* also have either a wild-type appearing CNS or show mild defects in axonogenesis (Elkins et al., 1990b). In contrast, the *abl fasI* double mutant embryos show a range of mild and severe CNS axonal defects, the most striking of which is an absence of commissural axons (Elkins et al., 1990b). These results suggest that *fasI* participates in commissure formation and axonogenesis.

Pertinent to its homophilic adhesion function, Fas I is also involved in axon fasciculation and synaptic plasticity. Perturbation of grasshopper Fas I using chromophore-assisted laser inactivation leads to axonal defasciculation phenotypes (Jay and Keshishian, 1990). Recent work has shown that synaptic plasticity at the neuromuscular junction is mediated, in part, by *fasI* (Zhong and Shanley, 1995). Arborization of motor neuron axons on larval muscles is increased by mutations of *fasI* and decreased by *fasI* overexpression. Related results in *Drosophila* have been documented for Fasciclin II, an Ig superfamily member, indicating that multiple adhesion molecules control synaptic arborization (Schuster et al., 1996).

Genes that are expressed in the *Drosophila* CNS midline cells have been extensively studied since the CNS midline cells present a useful system for studying the molecular genetics of CNS neurogenesis and axon guidance. Here we present the cloning of a novel gene, *midline fasciclin* (*mfas*) that encodes a membrane-associated protein with extensive sequence homology to Fas I. Antibody staining and *in situ* hybridization experiments reveal that *mfas* is expressed dynamically in the cellular blastoderm, CNS midline cells, and trachea, suggesting that *mfas* has multiple developmental roles. Presence of *mfas* protein on CNS midline cell bodies and axons suggest roles in axon guidance and synaptic plasticity. Genetic analysis of *mfas* null mutant embryos show mild defects in CNS axonogenesis. However, embryos double mutant for *mfas* and *abl* show more severe defects that resemble *abl fasI* axonal defects.

MATERIALS AND METHODS

Drosophila Strains

Drosophila strains bearing the deficiencies *Df(3R)T-47* (86F1-2; 87A9) and *Df(3R)kar-D1* (87A7-8; 87D1-2) were obtained from the *Drosophila* stock center at the University of Umea, Sweden. *Df(3R)T-47* is maintained

over a MRS balancer chromosome. *Df(3R)kar-D1* contains an inversion, In(3R)AFA (86C; 93D6-7), on the same chromosome, and is kept over a chromosome containing *cu kar Sb*. Canton-S was used as the wild-type strain. The *abl* mutant analyzed was abl^1, which is a severe allele (Henkemeyer et al., 1987). Double mutants were constructed between *Df(3R)T-47* and abl^1, and *Df(3R)kar-D1* and abl^1 by recombination. Mutant chromosomes were balanced over a marked TM3 balancer chromosome containing either a P[*actin-lacZ*] or P[*Ubx-lacZ*] transgene. Staining of embryos with anti-β-galactosidase antibody allowed unambiguous identification of mutant embryos.

Isolation of *mfas* Clones

The initial *mfas* cDNA clone λc6-1 was isolated as a cross-hybridizing clone in a screen of a λgt11 9–12-h embryonic cDNA library (constructed by Kai Zinn) using a genomic DNA fragment within the *Drosophila abrupt* (*ab*) gene (Hu et al., 1995). The sequence, ACACACACAGACACACACACACACACACACA, in λc6-1 has 30 identical nucleotides out of a stretch of 31 in a $(CA)_n$ repeat in the flanking genomic region of *ab*, (ACACTCACAGACACACACACACACACACACA). The λc6-1 clone was used as a probe to obtain 19 additional clones (pNB1-19) from a *Drosophila* embryonic 4–8-h cDNA library (Brown and Kafatos, 1988). Nested deletion series using Exonuclease III were generated from λc6-1 and segments of the two longest clones, pNB2 and pNB4. Both strands of each clone were sequenced with ^{35}S-dATP using a procedure for sequencing double-stranded DNA (USB Sequenase kit). Oligonucleotide primers were synthesized and used to complete sequencing.

Overlapping genomic DNA clones containing the *mfas* gene were isolated from a wild-type Oregon-R λfixII genomic library (Stratagene) using two DNA probes from the pNB2 cDNA clone: a 5′ DraI–NotI restriction fragment and a 3′ EcoRI fragment. Restriction enzyme cleavage sites were mapped onto the clones, and *mfas* cDNA sequences were localized by Southern blot hybridization experiments using ^{32}P-labeled cDNA fragments.

Northern Blot Analysis

Northern blot hybridization was performed as described by Sambrook et al. (1989). 3 μg each of embryonic 0–3-, 6–9-, 9–12-, 12–15-, and 15–18-h poly(A)+ RNA was electrophoresed, blotted onto Nytran membrane, and hybridized to a ^{32}P-labeled 2.1 kb EcoRI internal fragment from λc6-1. This fragment contains most of the *mfas* coding sequence.

In Situ Hybridization

Whole-mount embryonic *in situ* hybridization was performed essentially as described by Tautz and Pfeiffle (1989). Digoxigenin (DIG) DNA probes were prepared using a labeling kit (Boehringer–Mannheim), and hybridization was performed at 48°C for 18 h. Embryos were incubated at 22°C for 2 h with anti-DIG-alkaline phosphatase (1:2000). Alkaline phosphatase reactivity was visualized with X-phosphate and NBT as substrates. Stained embryos were mounted in 80% glycerol/PBS, and examined with a Zeiss Axiophot photomicroscope.

Polytene chromosomes for *in situ* hybridization were prepared according to Langer-Safer et al. (1982). Chromosomes were dissected from third instar wild-type larvae in saline, fixed in 45% acetic acid, and hybridized with Biotin-dUTP (Enzo)-labeled cDNA probes at 42 °C for 20 h. Hybridization was detected using HRP-conjugated streptavidin and diaminobenzidene (DAB).

Generation of Mfas Antibodies

Antibodies were generated against *mfas* protein synthesized in a prokaryotic expression system. Protein encoding FD 2–4 was created by cloning a 1-kb Bam HI fragment of *mfas* cDNA into the BamHI site of pQE12 (Qiagen) to yield pQE-mfas. This gene contains a fusion protein of Mfas with a tag of six histidines (his) at the C terminus. pQE-mfas was transformed into the bacterial strain XL1-blue. Production of fusion protein was initiated by administration of IPTG that induces transcription from the pQE-mfas *lacZ* promoter. Synthesized protein was denatured in 8M Guanidine-HCl and purified via the 6Xhis tag using a Ni-NTA agarose column (Hochuli, 1990; Qiagen). Purified soluble fusion protein was injected into both chickens and rats for antisera production. The polyclonal antisera was further affinity purified on a CNBr-activated Sepharose 4B (Pharmacia) column containing purified bacterially synthesized *mfas* protein. Anti-Mfas was eluted in the basic fraction (Harlow and Lane, 1988).

Creation of Heat Shock *mfas* Strains and Protein Analysis

Fly strains able to overexpress *mfas* were generated by transgenic introduction of a *mfas* cDNA clone under heat shock control. The *mfas* DNA was a DraI–NotI fragment of pNB2 containing the entire *mfas* coding sequence, and it was cloned into the pCaSpeRhs P-element vector (Thummel and Pirotta, 1992) creating P[w^+, *hsp70-mfas*]. The P-element construct was then microinjected into w^{1118} embryos together with a Δ2–3 helper P-element, which provides transposase activity (Robertson et al., 1988). Stable transformants were isolated according to eye color, and lines containing two copies of the P-element established. Mfas protein synthesis was induced by incubating appropriately staged embryos at 37°C for 1 h followed by 1 h of recovery at 25°C. The induction of *mfas* expression was confirmed by *in situ* hybridization and antibody staining.

Embryonic protein was prepared and separated into cytosolic and membrane fractions as described by Patel et al., (1987). Embryos were collected, dechorionated, and homogenized in 10-mM Triethanolamine containing 1-mM Phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin, 1 μg/mL leupeptin. The homogenate was centrifuged at 1200 $\times$ G for 10 min at 4°C, and the pellet was resuspended, homogenized, and recentrifuged. The supernatants from the two spins were combined and subjected to centrifugation at 100,000 $\times$ G for 2 h at 4°C. Supernatants were collected, and the pellet was resuspended in PBS with PMSF. Western blot analysis of protein samples was performed by fractionating proteins on SDS–PAGE followed by blotting onto a nitrocellulose membrane (BioRad). The blot was incubated with chicken anti-Mfas antisera at 1:400 dilution in Tris-buffered saline, followed by biotinylated anti-chicken Ig antibody at 1:200 dilution (Vector Labs). Detection was accomplished using a Vector Elite kit (Vector Lab) and DAB as substrate.

Immunocytochemistry

Embryos were collected, fixed, and stained with antibodies as either whole-mount or tungsten needle-dissected preparations (Patel et al., 1987). Primary antibodies were used at the following dilutions: Mab22C10: 1:10, Mab BP102: 1:20, Mab anti-Engrailed: 1:8, and Mfas: 1:200. Antibody reactivity was detected using DAB and the Vector Elite Kit. Stained embryos were cleared in either 100% methylsalicylate or 80% glycerol/PBS.

Chromosomal PCR Deletion Mapping

The absence or presence of *mfas* in *Drosophila* strains with chromosomal deficiencies was determined using a single-embryo polymerase chain reaction (PCR) approach (Hu et al., 1995). Two primer pairs derived from the *mfas* coding sequence, pMa(GTTCTCGAGTCACTCTTTGGCCAGTTCCAG)-pMb(CCGGCTGCAGAAGACATCGAAATCGCCCT) and pMc(TCGTTGAGGATGTTGTCTGC)-pMd(CCGCTCAGAAGAAGACATCG), were utilized to amplify nonoverlapping *mfas* regions. Twenty-four embryos were randomly picked from a cross between heterozygous parents of each strain: (*Df(3R)T-47/MRS* and *Df(3R)kar-D1/cu kar Sb*). Approximately a quarter of the embryos are homozygous mutant. Embryos were individually crushed in 15-μL lysis buffer (10-mM Tris pH 8.0, 25-mM NaCl, 1-mM EDTA, 200-μg/mL Proteinase K). After incubation for 30 min at 37°C, the Proteinase K was inactivated at 95°C for 3 min. One μL of the sample was added to a 15-μL PCR mix (Perkin–Elmer) and subjected to PCR amplification in a Perkin–Elmer 480 thermal cycler. A control PCR reaction was performed in the same tube with a pair of primers derived from the *ab* gene, which resides at 32E1-2. Presence of the *ab* band controls for the quality of DNA and efficiency of the PCR reaction; corresponding absence of the *mfas* bands indicate that the gene is absent from the deficiency chromosome.

RESULTS

Identification and Cloning of the *mfas* Gene

In another study analyzing genes involved in nervous system development, we cloned the *Drosophila abrupt* (*ab*) gene (Hu et al., 1995). This gene is prominently expressed in the embryonic CNS midline, muscle, and epidermal cells. We used a probe from this gene to isolate *ab* cDNA clones, and adventitiously isolated a non-*ab* cDNA clone, λc6-1. Polytene chromosome *in situ* hybridization indicates that this cDNA clone is encoded by a gene residing on the third chromosome (referred to as *mfas*) at cytological location 87A4-9, and is distinct from the *ab* gene, which maps to 32E on the second chromosome. DNA sequence comparisons between *ab* and *mfas* indicate that isolation of the *mfas* clone resulted from cross-hybridization of $(CA)_n$ repeats that lie in the genomic flanking sequences 5′ to the *ab* transcription unit and an intronic region of *mfas* incompletely processed in the clone λc6-1 (this region was absent from all other *mfas* cDNA clones analyzed). Although the expression patterns of the two genes overlap (see below and Hu *et al.*, 1995), it is unknown whether the $(CA)_n$ sequence similarity is functionally relevant.

Additional *mfas* cDNA clones were isolated from an embryonic 4–8-h plasmid library using the λc6-1 clone as a probe. The two longest cDNA clones, pNB2 and pNB4, are 2.9 and 3.0 kb in length, respectively (Fig. 1). Northern blot analysis of embryonic RNA reveals a broad band of hybridization around 3.0 kb in size (Fig. 2), indicating that clones close to full-length have been isolated. Sequence analysis of four cDNA clones indicates that there are two classes of *mfas* mRNA that differ within the 5′-untranslated region (data not shown). The presence of two forms of *mfas* mRNA is consistent with the broad hybridization band observed by Northern analysis.

Southern blot analysis under moderately stringent hybridization conditions only revealed a single *mfas*-hybridizing gene in the *Drosophila* genome (data not shown). Genomic DNA containing the *mfas* gene was isolated from a wild-type Oregon-R genomic library using an *mfas* cDNA clone re-

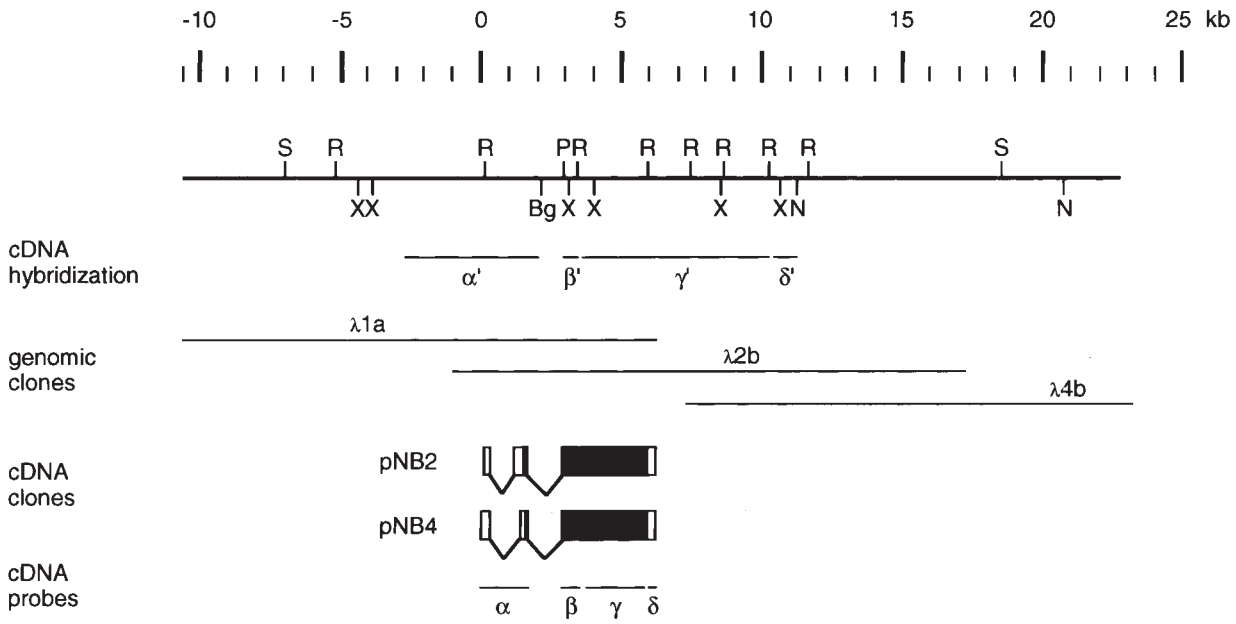


Figure 1 Genomic structure of the *mfas* transcription unit. 33.3 kb of Oregon-R genomic DNA containing the *mfas* gene was isolated as overlapping λfixII genomic clones, λ1a, λ2b, and λ4b, represented as solid lines. The restriction map was constructed by digesting cloned genomic DNA with BglII (Bg), EcoRI (R), NotI (N), SstI (S), and XhoI (X). cDNA clones pNB2 and pNB4 were partially mapped to cloned genomic DNA by a combination of DNA sequencing and Southern blot hybridization; the figure does not indicate the complete exon–intron structure of the *mfas* gene. Exonic regions 1 and 2 of the cDNA clones (probe α) hybridize to genomic DNA fragment α', and exonic region 3 hybridizes to fragments β', γ', and δ' as revealed by hybridization to three nonoverlapping cDNA clone probes (β, γ, and δ'). Sequence analysis of the cDNA and genomic clones indicated the presence of the two introns shown. Open boxes of the cDNA clones indicate untranslated regions, and filled boxes represent coding regions.

striction fragment as probe. The overlapping clones constitute 33.3 kb and were restriction mapped (Fig. 1). Hybridization analysis positions the *mfas* cDNA clone within a 14-kb interval in the contig. The exact boundaries of the *mfas* gene are not known, and no attempt was made to identify and map adjacent transcripts within the cloned genomic region (Glover et al., 1995).

Dynamic Expression of *mfas* during Blastoderm and Gastrula Stage Embryos

Hybridization of a *mfas* cDNA clone probe to a developmental Northern blot of embryonic RNA revealed *mfas* expression throughout much of embryogenesis (6–18 h) with a relative absence of RNA at 0–3 h (Fig. 2). These results suggest that there is little or no maternal *mfas* transcript, but that zygotic transcription is robust throughout embryonic development including all stages of CNS formation.

Embryonic expression of the *mfas* gene was examined in greater spatial and temporal detail by whole-mount *in situ* hybridization. Consistent with the developmental Northern hybridization, there is no obvious maternal contribution of *mfas* since transcripts are not detected in the syncytial blastoderm stage. *mfas* transcription was first detected at the cellular blastoderm stage (stage 5) in all cells except a broad ventral domain about 16–18 cells in width [Fig. 3(A), (B)]. This region corresponds to the primordial mesoderm. Soon after, as gastrulation commences (stage 6), expression along the anterior and posterior axis is periodically reduced in a repeated pattern resulting in expression within seven stripes suggesting a pair-rule gene influence [Fig. 3(C)]. Later, dorsal and lateral staining begins to fade, and the previously uniform *mfas* staining evolves into a graded pattern with strongest expres-

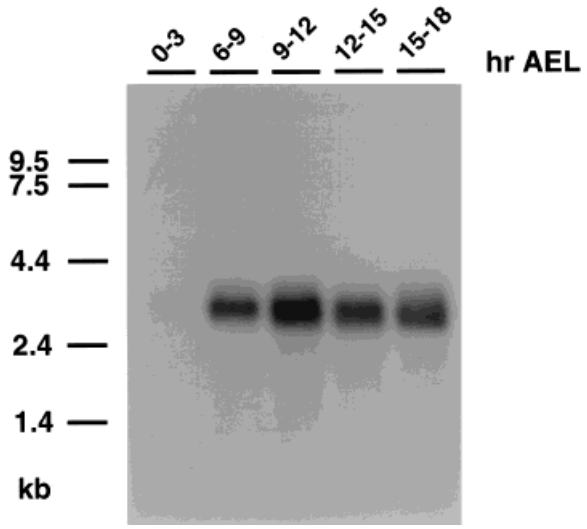


Figure 2 Developmental Northern blot of *mfas* transcripts. Northern blot containing 3 μg of poly(A)+ RNA from indicated embryonic time points [0–3, 6–9, 9–12, 12–15, and 15–18 h after egg laying (AEL)] was hybridized to a *mfas* cDNA probe. One major transcript of 3.0 kb was observed at all time intervals except 0–3 h. The broad appearance of the band is likely due, in part, to the presence of multiple spliced forms that differ by 96 bases. Position of the RNA molecular size markers is shown at left.

sion in the ventral–lateral region [Fig. 3(D)], until all ectodermal staining is barely detectable at the completion of gastrulation (stage 7).

mfas Is Expressed in the Developing CNS Midline and Tracheal Cells

A second phase of *mfas* expression occurs in a variety of cell types from stage 9 to the completion of embryogenesis (stage 17). CNS midline precursor cells form after gastrulation as they meet at the ventral midline of the embryo. Beginning at stage 9, *mfas* RNA appears in all of the CNS midline cells [Fig. 3(E)]. During germband retraction (stages 12–13), the CNS midline cells differentiate, and *mfas* expression continues in these cells (data not shown), which include the midline glia and neurons (Bossing and Technau, 1994; Thomas et al., 1988). After germ band retraction is complete, *mfas* CNS expression is restricted to a subset of midline cells, the midline glia, and only weak expression is observed after stage 14.

mfas is strongly expressed in the developing tracheal system. The embryonic trachea consist of an extensively branched network whose primary function is gas exchange. However, the developing trachea may also play a role in motor and sensory axon growth and pathfinding (Giniger et al., 1993; Van Vactor et al., 1993; Younossi-Hartenstein and Hartenstein, 1993). Tracheal expression of *mfas* begins at stage 11 in the segmentally repeated placodes which are pits of invaginating cells [Fig. 3(E), (F)]. The *mfas* transcript is present in all cells surrounding the tracheal pits, with highest levels in the posterior cells [Fig. 3(F)]. As tracheal cells migrate within the embryo and fuse to form tracheal tubes, *mfas* continues to be expressed.

Prominent embryonic expression of *mfas* is also observed in the precursors and differentiated cells of the stomadeal epithelium located on the ventral side of the stomadeal invagination, and at low levels in all of the differentiated epidermal cells found between stages 14 to 17.

Sequence Similarity between *mfas* and *Drosophila Fasciclin I*

Additional insight into the function of the *mfas* gene was revealed by sequence analysis of the two longest cDNA clones (Fig. 1). pNB2 and pNB4 are 2951 and 2991 nucleotides in length, respectively, each representing one of the two classes of *mfas* mRNA. The differences involve the differential use of small, alternative exons of 22 and 118 bp within the 5′ untranslated region. Both cDNA clones share a common open reading frame that is 881 amino acids long (Fig. 4).

Hydrophobicity plot analysis of the protein sequence suggests that Mfas is secreted and/or associated with the cell surface. The N-terminus of Mfas has a positively charged arginine followed by a stretch of 16 predominantly hydrophobic residues, a feature that resembles a membrane signal sequence. There are 9 potential glycosylation sites scattered throughout the second half of the protein. The C-terminal 600 residues of Mfas are composed of four repeated domains. Each domain is approximately 150 residues in length, and characterized by the presence of two well-conserved repeats of 10 and 18 amino acids (see below). There is no obvious Mfas transmembrane domain, suggesting the protein is either secreted or anchored to the membrane by phospholipid. Presence of a glycosyl-phosphotidylinositol (GPI) linkage is generally correlated with 15–20 hydrophobic C-terminal sequences (Englund, 1993), a feature not obviously apparent in Mfas.

The Mfas protein sequence was used to search

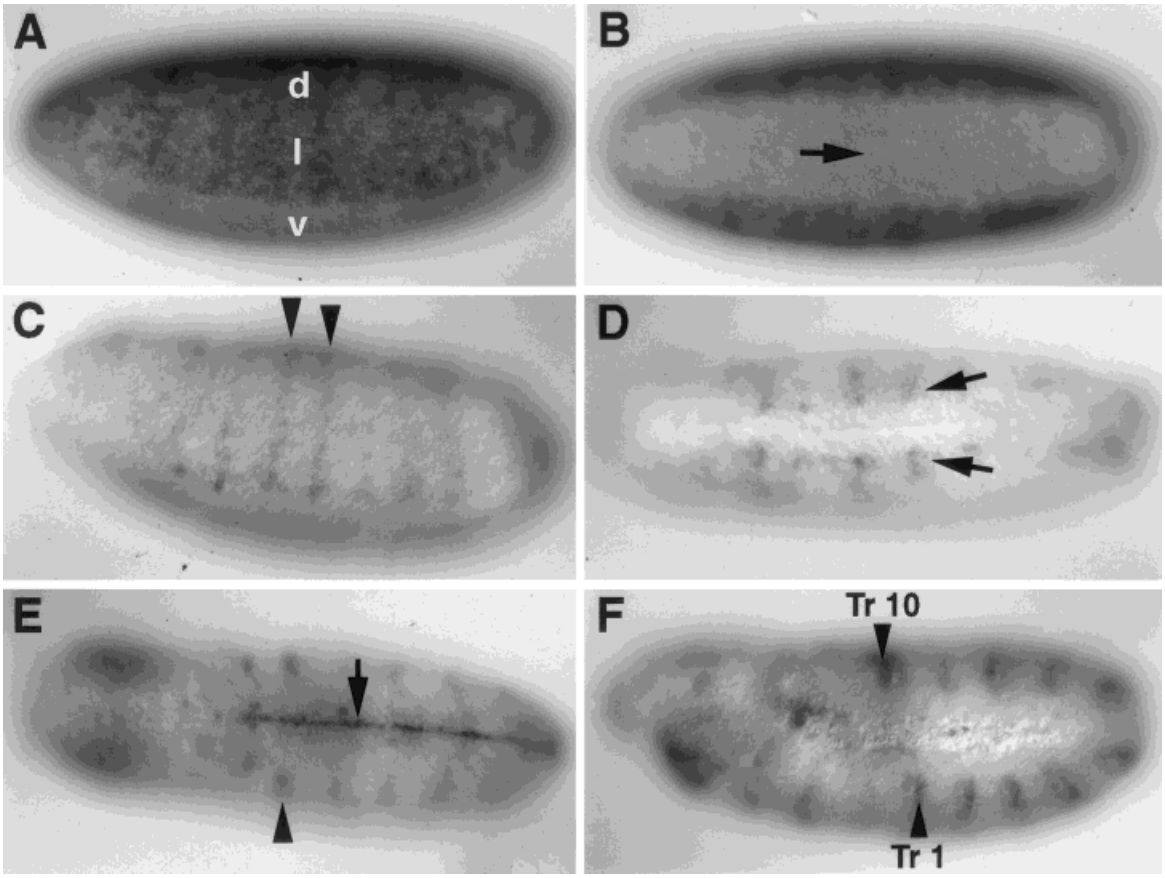
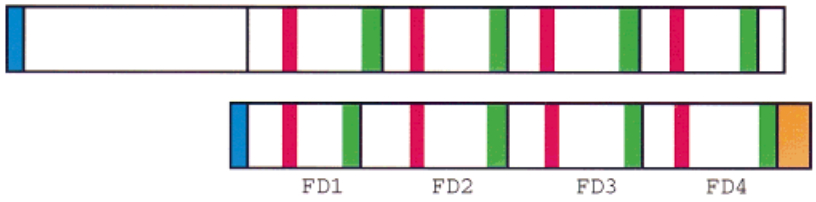


Figure 3 *mfas* transcripts are expressed in a dynamic pattern during gastrulation and in the developing CNS midline and tracheal cells. All embryos are shown as whole-mounts hybridized to a *mfas* cDNA probe. Anterior is to the left in all panels. (A) Sagittal view of a stage 5 blastoderm embryo shows that initial zygotic expression occurs in the lateral (l) and dorsal (d) ectoderm, but is absent from ventral (v) cells. (B) Ventral view of same stage 5 embryo as (A) showing absence of *mfas* transcripts in the ventral, presumptive mesoderm. Arrow points to the ventral midline. (C) Sagittal view of an embryo during gastrulation showing that dorsal *mfas* transcription is quickly fading, and that previously uniform lateral expression is evolving into pair rule stripes (arrowheads). (D) Ventral view of a stage 6 embryo, completing gastrulation, showing reduced lateral ectodermal expression. Strongest expression is adjacent to the presumptive mesoderm (arrows). (E) Strong CNS midline precursor cell staining (arrow) is observed during germband extension. CNS midline cell expression begins at stage 9 and persists to stage 14. Shown is a dorsal view of an early stage 11 embryo. *mfas* transcripts are also present in the tracheal placode (arrowhead). (F) Lateral view of a germband extended embryo at late stage 11, showing *mfas* transcripts present in the 10 tracheal placodes (arrowheads). Expression of *mfas* is more intense in the cells that are posterior to the tracheal opening. Shown are tracheal placode 1 (Tr 1) in segment T2, and tracheal placode 10 (Tr 10) in segment A8. Scale bar: 70 μm.

protein databases using the BLAST algorithm, and comparisons indicate that Mfas belongs to the Fas I protein family (Fig. 4). The family includes Fas I (*Drosophila* and grasshopper) (Zinn et al., 1988), mammalian transforming growth factor-β responsive protein βIG-H3 (Skonier et al., 1992), mammalian Osteoblast Specific Factor 2 (murine and human) (Takeshita et al., 1993), algal-CAM (plant) (Huber and Sumper, 1994), MPB-70 (mycobacteria) (Ulstrup et al., 1995), two related sea urchin proteins, bep-1 and bep-4 (DiCarlo et al., 1990), and an uncharacterized C. elegans protein. Common among this group of proteins is a domain 130–150 amino acids long that coincides with the previously

A



B

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MLRLWACLLLLGSIQIQAVPFYGDFMPHLTHPLPAHRNLFAPLPFAPLAPLEPQDPLHAQASQRMASVWN      70

GPAPLAPQLHSSHLLPLFSSGYDTEFVPLEHQQQPQGRQETAATVPAQTPSGVEQKPFNVDTITTDVNSP     140

NPAIFFQQSFPFFGNEFFNSFGGFGFGAAQEPWWKGPNVCTEKEEDETVASETEAEETVDTFGQERDGVT     210

NVVRSPLFGQFQFSVNSCAEKPNKHVCTKIVNQNGKKKTLTLTRQCCYGYGRPRNADFTTPCEK IDIKD     279

                FD1
VEATASDMGAKQFLESARTAGELADMLGAGSGKKVTLFVPNDAAFMEYRGHHQQENNVEDAKAEASKPSI     349

YKLHAVLGEVQLEDVPNEKLLQTELPDQKIRINSYQLPAALGLEPYRFAVNCVPIEKHDKLSEQALVHTL     419

                          FD2
GGVLKP LTKNLMDIIRERADMSIMRTVLEKTNLSAMLEDDKPVTIFVPTDAAFDKLEPHLRRALKEGRG     488

CASNILKNHLLDLTFCSLATVPGAKTTAYNLLGEPLLLNRTHRAANQTGPTPIYINNLAKIIDADIMGTN     558

                              FD3
GVLHVIDTILP TESALPMTSLMSQKNLTIFQRLLEASGYDDQFDDLDNVTIFAPTDKALQNTEWARMLK     627

EQPELLNHNLDLLEFLNYHVVKPMIKTCDLSEKSLPTVAGSSVRLNLYSTHALFSDVMNRATVNCARLVH     697

                                      FD4
FDDESCGSVLHQVDRALAPP KNNMLKLLEANPNYSKFLELVRKANLTQLLSNDSRSLTLLVPKNDIFEE     766

LNESGEGSKPADPMALVKTHIVEDVVCCAGIIPTNWPFVRSIESISGQHLRITRDRRPKIENAGVTKCDV     836

VATNGILHEINDIIVPR PQRQQQQRPQLIPPGAGYQPQGDFDVFF                             881
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C

		FRa	H-box	FRb
Mfas	FD1	TLFVPNDAAF	IYKLHAV	DKLSEQALVHTLGGVLKP
FasI	FD1	TIFVPTNEAF	HVLYHIT	NSDGKRQIMHIIDEVLEP
Al-CAM	FD1	TFFLPTETAF	ALSYHVL	DVAAGASCINVVDTVLQY
bep-4	FD2	TVLVPTNKAF		AITTNHGVIHVIDRVLVQ
βIG-H3	FD1	TIFAPSNEAW	ALRYHMV	DHHATNGVVHLIDKVIST
C. ele	FD3	TFFAPWNEAF	ILKLHIV	NVAATNGIVHYIERVLGV
MPB-70		TVFAPTNAAF	ILTYHVV	GVSTANATVYMIDSVLMP
OSF-2	FD1	TYFAPSNEAW	ALHSHMV	NQIATNGVVHVIDRVLTQ
Consensus		TIFAPTDEAF	ILXNHIL	DIXATNGVIHXIDXVLXP
		LLV N W	L Y VV	V T L
				V

mentioned repeat unit (referred to as Fas I Domain, FD) [Fig. 4(A), (B)] and is characterized by two conserved stretches of 10 and 18 amino acids, referred to as Fas I Repeat a and b, FRa and FRb, respectively [Fig. 4(A)–(C)] (Skonier et al., 1992; Takeshita et al., 1993). FRa has the consensus of T(I/L)(F/L)(A/V)PT(D/N)EA(F/W). FRb has the consensus sequence: D(I/V)X(A/T)TNGV(I/L/V)HXIDXVLXP, where X indicates lack of consensus. FRa is well-conserved in all members of this family, whereas FRb is less noticeable in algal-CAM, MPB-70, and particular FDs of other family members. There is a third region between the two repeats, referred to as the H-box, that exhibits moderate conservation with the sequence of (I/L)LX(N/Y)H(I/V)(L/V) (Skonier et al., 1992). Overall, the sequence identity is low (often $<25\%$ with significant length variations) between FDs within the same protein and between different proteins outside of the three conserved regions. There is no obvious orthologue of Mfas in other species, nor is conservation particularly high between Mfas and any other protein, including *Drosophila* Fas I.

Mfas Antibodies Detect a Protein Associated with Cell Membranes

Polyclonal antibodies were raised against bacterially synthesized *mfas* protein, and used to examine the *in vivo* distribution and subcellular localization of *mfas* protein in *Drosophila* embryos. Western blot analysis showed that the antibodies detect bacterially expressed *mfas*, but were unable to clearly detect Mfas in embryonic protein extracts (stages 4–15). The most likely reason for its absence was that the embryos contained a relatively low amount of protein. To circumvent this problem, we generated a transgenic fly strain, P[*hsp70-mfas*], in which Mfas synthesis is placed under the control of heat shock. When P[*hsp70-mfas*] embryos were heat shocked and protein extracts analyzed by Western analysis, Mfas antisera detected a protein of 110 kDa molecular weight [Fig. 5(A)]. This protein was not detected using pre-immune serum. The apparent molecular weight of Mfas (110 kDa) is larger than that predicted from its sequence (97 kDa). The difference may be the result of glycosylation given the occurrence of a number of potential glycosylation sites (Fig. 4).

Subcellular localization of Mfas in embryos was examined by cell fractionation techniques. Protein extracts of P[*hsp70-mfas*] embryos were treated with nonionic detergents and centrifuged. This separates cellular proteins into a supernatant fraction that contains cytoplasmic proteins and a pellet fraction that includes membrane-associated proteins. Western analysis of these fractions showed that most of the *mfas* protein was partitioned to the membrane fraction [Fig. 5(B)].

Embryonic Localization of *mfas* Protein

Further analysis of *mfas* protein localization involved immunocytological staining of embryos. It was difficult to visualize *mfas* protein in the blastoderm and gastrulating embryo, a result not surprising since *mfas* transcripts are transient and present at low levels. However, prominent staining was observed in older embryos in the CNS midline cells

Figure 4 Conceptual protein sequence of *mfas* cDNA clone indicates it is related to Fasciclin I. (A) The organization of both Mfas and Fas I proteins are similar. Both have N-terminal signal sequences (blue) and four Fasciclin I Domains (FD1-4). Each domain contains two highly conserved Fas Repeats, FRa (red) and FRb (green). The C-terminal residues of Fas I contains sequences required for glycosyl-phosphotidylinositol association (orange). (B) The predicted 881 aa Mfas protein sequence derived from the putative full-length pNB4 *mfas* cDNA clone is shown using the single letter amino acid code. The boundaries of each FD are indicated by a space. The signal sequence, FRa, and FRb are shown in blue, red, and green, respectively. The H-box residues found in each FD are indicated in cyan. Nine potential N-linked glycosylation sites are shown in magenta. One site overlaps with the FRa in FD3 and is underlined. (C) Comparison of FRa, H-box, and FRb sequences of FD1 or another indicated FD from members of the Fas I protein family are shown with the consensus sequence at the bottom. The consensus is derived from all existing FDs, not just those shown. Presence of a consensus residue in a sequence is colored and ''X'' indicates no consensus residue(s). Proteins included are: Drosophila Mfas, Drosophila FasI, Volvox Al-CAM, sea urchin bep-4, human βIG-H3, C. elegans unknown protein, Mycobacteria MPB-70, and murine OSF-2. This sequence has been deposited with GenBank under accession number AF038842.

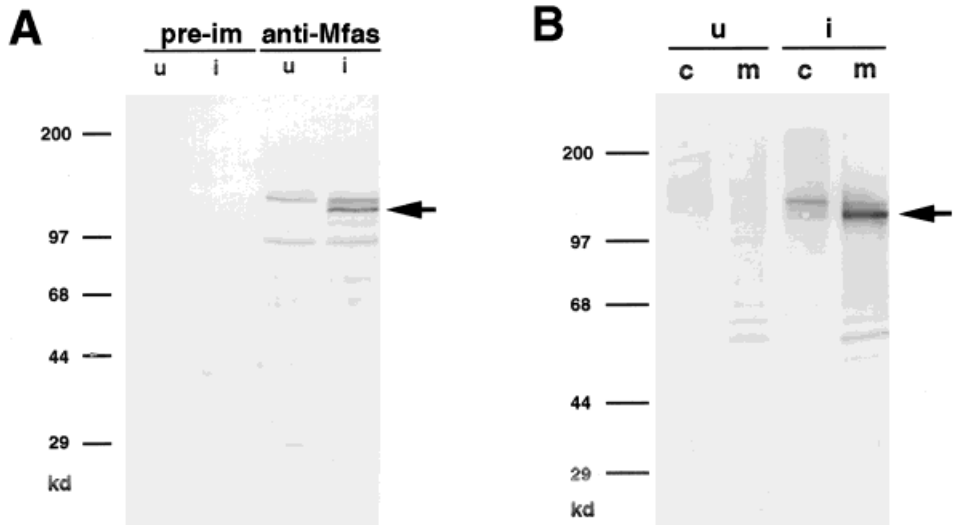

Figure 5 Subcellular fractionation experiments reveal that Mfas is membrane-associated. Embryonic proteins were prepared from a Mfas overproducing *Drosophila* strain. The strain carries two copies of P[*hsp70-mfas*], and heat induction of *mfas* expression involved incubation at 37°C for one h followed by one h recovery at 25°C. (A) Proteins were extracted from heat shock-induced (i) and uninduced (u) embryos, electrophoresed on SDS polyacrylamide gels (PAGE), and analyzed on a Western blot stained with pre-immune sera (pre-im) or anti-Mfas sera. Results indicate the presence of a 110 kDa band in the induced lane incubated with anti-Mfas (arrow). No staining was observed at this position in the pre-immune lanes. (B) Protein extracts from induced and uninduced embryos were partitioned into cytoplasmic (c) and membrane (m) fractions, fractionated by SDS/PAGE, transferred to Western blots, and incubated with anti-Mfas. Results indicate that most of the 110 kDa *mfas* protein was partitioned into the membrane fraction (arrow). Protein molecular weight marker sizes are indicated to the left of each blot.

and trachea, which have relatively high levels of *mfas* transcripts. CNS midline cell staining was first detected at stage 10 and tracheal placode staining at stage 11 [Fig. 6(A), (C)]. In both tissues, the staining has a punctate pericellular pattern suggesting membrane-association [Fig. 6(B), (C)]. Generally, protein expression is coincident with RNA expression, indicating that it does not diffuse in high concentrations over long distances.

During stages 9–11, the CNS midline cell nuclei reside in the neural precursor cell layer, and maintain a cytoplasmic projection to the external ectodermal layer. Denser spots of immunoreactivity in the midline cells were observed in the peripheral ectodermal cell layer occupied by midline cytoplasmic process [Fig. 6(B)] than in the cell nuclei. As neurogenesis proceeds, *mfas* protein distribution is found in a subset of midline cells in the anterior region of each segment, including a pair of glia that are closely associated with the commissural axons. Mfas is also present transiently in a thin axon bundle that exits the CNS during stage 13 (data not shown). These may be the motor axons of the midline VUM or UMI motor neurons; (Bossing and Technau, 1994). However, the low protein levels found on midline cell bodies precludes unequivocal cellular assignment of these axons.

Mfas stains tracheal placodes where those cells invaginate [Fig. 6(C)]. Around the placodes, the cells display an elongated shape streaming towards the tracheal opening prior to their invagination. Mfas in tracheal cells is more enriched on the side facing the tracheal pits [Fig. 6(D)]. Tracheal staining persists after the tracheal cells migrate and form the tracheal tree, and Mfas is present in the main tracheal stem and segmental branches [Fig. 6(D)].

Null Mutations of *mfas* Reveal Subtle Defects in Axonogenesis

Phenotypic analysis of chromosomal deficiencies that lack the *mfas* gene were analyzed for cellular defects in CNS midline cells, tracheal cells, and axon morphology. Polytene *in situ* hybridization

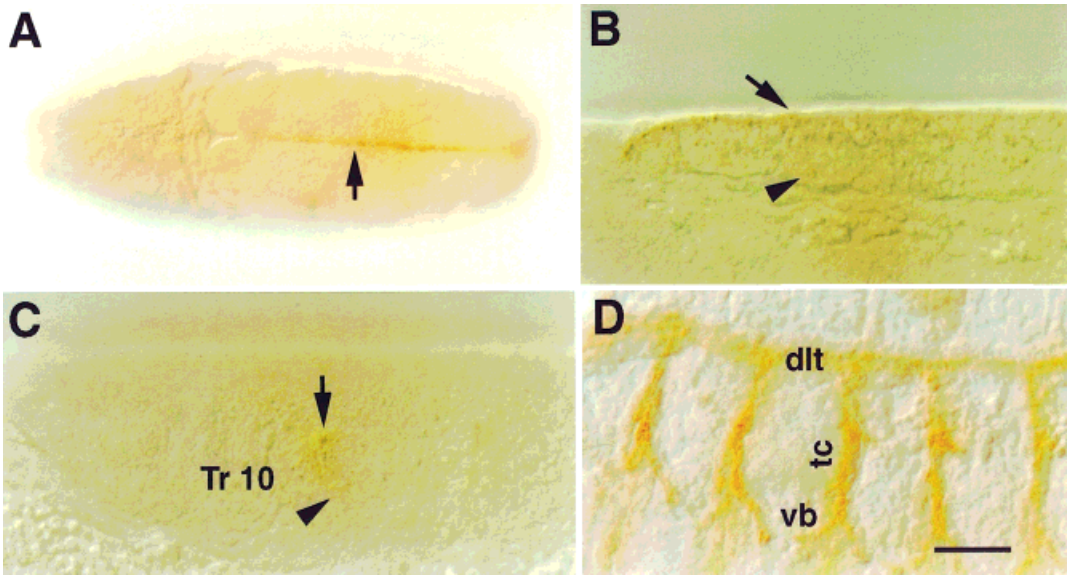

Figure 6 Cellular and subcellular distribution of *mfas* protein during embryogenesis. Staining of whole-mount wild-type embryos with Mfas antisera. All embryos have anterior to the left. (A) Dorsal view of a stage 11 germband-extended embryo showing that *mfas* protein is present in all CNS midline precursor cells (arrow). (B) Sagittal view of a stage 11 embryo showing the dorsal side (top). Immunoreactivity is stronger near the surface of the embryo where CNS midline cell cytoplasmic projections exist (arrow), and weaker in the region where the CNS midline cell nuclei reside (arrowhead). (C) Sagittal view of the dorsal region (top) in a stage 11 embryo showing staining in the tracheal placodes. The tracheal pit of parasegment 10 is indicated with an arrowhead. Tracheal cells have an elongated shape, and the staining is stronger on the side facing the pit (arrow). (D) Staining of a stage 15 embryo showing persistent Mfas expression within the tracheal tree. (dlt) dorsal longitudinal tract, (tc) transverse tract, (vb) ventral branches. Scale bar: 70 μm (A), 15 μm (B and C), and 30 μm (D).

with a *mfas* probe mapped the gene to 87A4-9 on the right arm of chromosome 3. Two chromosomal deficiency strains have deletions of this region: *Df(3R)T-47* (86F1-2; 87A9) and *Df(3R)kar-D1* (87A7-8; 87D1-2). Two lines of evidence indicate that these two deficiencies lack the *mfas* locus. Whole mount *in situ* hybridization using a *mfas* cDNA probe showed that approximately a quarter of the embryos from both the *Df(3R)T-47*/MRS and *Df(3R)kar-D1/cu kar Sb* strains lacked *mfas* transcripts. This is the fraction expected for transcript-null mutations, since one quarter of the embryos are deficiency homozygotes. Additional confirmation was obtained using PCR deletional mapping. Two pair of primers that amplify nonoverlapping fragments within the coding region from wild-type *mfas* genomic DNA failed to yield PCR products from DNA of approximately one quarter of the embryos of each strain. The genetic mapping, which is consistent with the polytene chromosome localization, places the *mfas* gene within chromosomal interval 87A7-9.

Gausz et al., (1981) carried out a saturation ethyl methanesulfonate (EMS) genetic screen for lethal mutations in chromosomal region 86F1-2 to 87B15. Within the chromosomal interval 87A7-9 in which *mfas* is located, there were 5 pupal lethal mutations and one female sterile mutation identified; all were allelic to a single locus, ck^{10}. Recent cloning of the ck^{10} locus indicates that it is the *aurora* gene, which encodes a serine–threonine protein kinase required for centrosome separation (Glover et al., 1995). By elimination, it is unlikely that *mfas* mutations are lethal. The absence of a lethal or severe CNS or tracheal *mfas* mutant phenotype indicates that *mfas* is not required for embryonic or postembryonic survival under laboratory culture conditions.

Both *Df(3R)T-47* and *Df(3R)kar-D1* are deleted for multiple genes in addition to *mfas.* Use of *Df(3R)T-47*/*Df(3R)kar-D1* embryos narrowed the deficiency region to 87A7-9, thus reducing the number of genes mutant in addition to *mfas.* The development of the CNS midline lineage in these *mfas*-deleted mutant embryos was assessed using a

battery of antibodies that stain either a subset or all midline cells. *Drosophila* Single-minded (Sim) antibodies stain all midline precursors from stages 6 to 12 and the midline glia at later stages. Mab22C10 stains CNS and PNS nerve cell bodies and axons, including the midline VUM neurons and axons. Engrailed (En) monoclonal antibodies stain a group of median midline nerve cells. In *DF(3R)T-47/Df(3R)kar-D1* mutant embryos, the CNS midline cells formed normally, and differentiated into midline glia and neurons as shown by staining with anti-Sim and anti-En (data not shown). The VUM neurons are also present, as is the medial axon tract composed of VUM neurons axons. The development of the tracheal system also appears normal, since wild-type-appearing tracheal branches are seen in dissected mutant embryos (data not shown).

The formation of commissural axons is dependent on proper formation and influence of the CNS midline cells (Klämbt et al., 1991; Thomas et al., 1988). Monoclonal antibody BP102 stains CNS axons [Fig. 7(A)], and was used to examine the morphology of CNS axon bundles in *mfas*-deleted embryos [Fig. 7(B)]. The CNS axon scaffold was wild-type in appearance in 26% of the *mfas*-deleted embryos and was weakly defective in the remaining 74% of the embryos ($N = 23$). Most mutant embryos had a mixture of wild-type and mutant ganglia. Mutant embryos showed a disorganization of axons with commissures sometimes fuzzy and poorly separated. This result raises the possibility that *mfas* may play a role in axonogenesis, but does not prove it, since the deletion removing *mfas* may be removing another gene that contributes to the phenotype observed in an *abl* background.

Embryos Containing a Deletion of *mfas* and a Mutation in the *abl* Tyrosine Kinase Show Severe Defects in Axonogenesis

Null mutations of the *fasI* gene are viable and have a wild-type appearing CNS (Elkins et al., 1990b). Mutations in the *abl* nonreceptor tyrosine kinase gene are pupal lethal, and usually show a wild-type CNS, although some embryos have axon scaffold defects (Elkins et al., 1990b). However, double mutants of *fasI* and *abl* have a severely defective CNS axon scaffold (Elkins et al., 1990b), indicating that these genes are able to functionally compensate mutations in the other. Given the molecular and genetic correspondence between *mfas* and *fasI,* a double mutant strain of *abl mfas* was created to test if this strain would reveal a role for *mfas* in axonogenesis.

The CNS phenotype of abl^1 homozygous mutant embryos was reanalyzed. Similar to the previous report (Elkins et al., 1990b), we observed a relatively normal axon scaffold with mild defects. The CNS was wild-type in appearance in 29% of the abl^1 embryos, and mutant in 71% ($N = 14$). The defective abl^1 CNS had thin connectives and commissures [Fig. 7(C)]. The *abl mfas* mutant was created using the *Df(3R)T-47* and *Df(3R)kar-D1* deficiency chromosomes in *trans,* both carrying the abl^1 mutation. When analyzed by MabBP102 staining, 43% of the double mutant embryos showed severe axonal defects in which the longitudinals and commissures were thin or absent [Fig. 7(D)]. The axons were disorganized and bulging. This phenotype may be indicative of a failure of commissural axons to properly navigate to the midline, and closely resembles one of the phenotypes commonly observed in *abl fasI* double mutant embryos (Elkins et al., 1990b). The most severe commissureless phenotype observed in *abl fasI* embryos was not observed in abl^1 *mfas* mutants. CNS scaffolds with thin connectives resembling abl^1 homozygotes were observed in 40% of the embryos and 17% were wild-type in appearance ($N = 30$). The *abl mfas* double mutant embryos show CNS defects that are more severe than either *abl* or *mfas* alone. However, given that both *abl* and *mfas* mutations show mild CNS defects, it is difficult to determine whether the double mutant is synergistic (suggesting gene interaction) or additive.

DISCUSSION

This article describes the identification of a novel *Drosophila* gene, *mfas,* that belongs to the *fasI* gene family. Members of this family generally encode membrane-bound or secreted cell adhesion and cell signaling proteins. Biochemical studies of Mfas reveal that it is also a membrane protein. Expression studies indicate that *mfas* protein is prominently expressed in CNS midline cells, trachea, and epidermis. During axonogenesis, Mfas is localized on midline neuron axons and on the midline cells when they contact and attract lateral CNS growth cones. It is particularly interesting that *mfas* is expressed in both CNS midline precursor and tracheal cells, a correlation observed for a number of other genes, e.g., *breathless* (Klambt et al., 1992) and *drifter* (Anderson et al., 1995). The molecular basis for

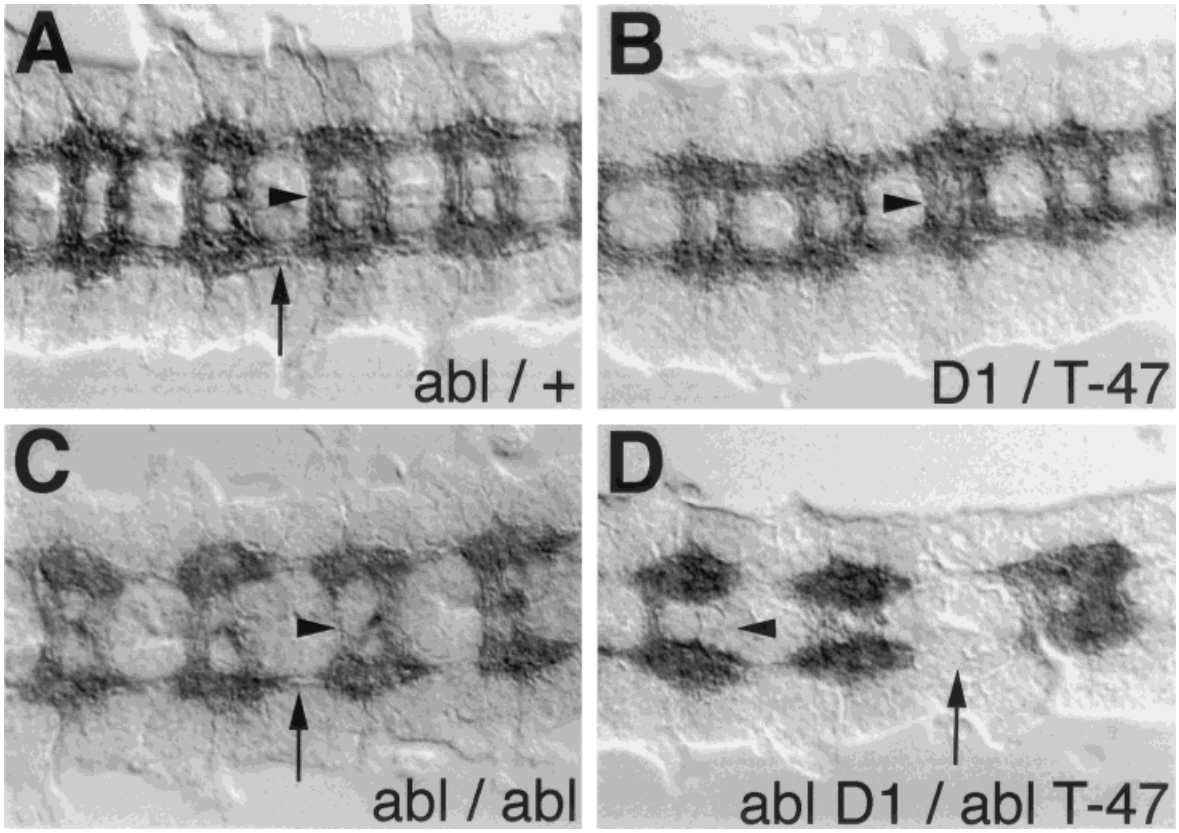

Figure 7 Phenotypic analysis of *mfas* and *abl mfas* mutant embryos. All panels show a dissected CNS from a stage 15 embryo stained with Mab BP102. Anterior is to the left. Mab BP102 stains CNS axons illustrating the longitudinal connectives (arrows) and anterior and posterior commissures (arrowheads). (A) CNS of an $abl^1/+$ embryo showing the characteristic wild-type connectives (arrow) and commissures (arrowhead). (B) CNS of a $Df(3R)D1/Df(3R)T\text{-}47$ embryo, which is deleted for the *mfas* gene, shows a weakly defective CNS. Many ganglia have a normal-looking appearance, but some show disorganization of the axons with fuzzy commissures (arrowhead). (C) CNS of an abl^1/abl^1 mutant embryo. It is common to see embryos with disorganized axons with thin connectives (arrow) and thin commissures (arrowhead). (D) CNS of an $abl^1\ Df(3R)D1/abl^1\ Df(3R)T\text{-}47$ embryo, mutant for *abl* and *mfas*, commonly shows a severely defective and disorganized CNS with thin or absent longitudinal connectives (arrow) and commissures (arrowhead).

this may be the presence within the *mfas* gene of *cis*-control elements that mediate transcriptional activation in the CNS midline cells by Single-minded::Tango heterodimers and Trachealess::Tango heterodimers in the trachea (Sonnenfeld et al., 1997).

Proteins of the Fas I family possess a characteristic FD that consists of two highly conserved stretches of amino acids, FRa and FRb. One function of the FD is to mediate protein–protein interactions. Fas I mediates homophilic cell adhesion when it is expressed in the nonadhesive S2 cell line (Elkins et al., 1990a). Since the Fas I protein is comprised almost entirely of four FDs, the FD is likely involved in the protein–protein interactions that mediate this cell adhesion. Other Fas I family proteins, βIG-H3 and OSF-2, also have four tandem FD repeats, but the relationship between number of FDs and protein function is unclear, since other members have fewer than four FDs. In the most extreme case, the mycobacterial MPB70 protein has only a single FD. The presence of multiple FDs could allow interactions of an individual protein with multiple partners. This would be analogous to proteins of the Immunoglobulin and Epidermal Growth Factor (EGF) superfamilies that often have

variable numbers of Ig domains and EGF repeats that can engage in different functions (Rebay et al., 1991).

Phylogenetically, members of the Fas I gene family are diverse and have been observed in mycobacteria, plants, nematodes, insects, sea urchins, and humans. All encode either secreted or membrane-anchored proteins, and have been implicated in a variety of functions including axon guidance, cell adhesion, proliferation, and morphogenesis. *Drosophila* Fas I exists in both secreted and GPI-anchored forms, is developmentally regulated (McAllister et al., 1992), and a homophilic cell adhesion protein (Elkins et al., 1990a). Genetic experiments have shown that *fasI* function is required for the formation of embryonic axon commissures (Elkins et al., 1990b) and synaptic plasticity (Zhong and Shanley, 1995). βIG-H3 is a mammalian gene whose expression disrupts cell attachment *in vitro,* and attenuates tumor growth in nude mice (Skonier et al., 1992; Skonier et al., 1994). Algal-CAM is expressed in pregastrulation stages of embryonic development (as is *mfas*), is secreted, and monoclonal antibodies raised against it interfere with cell movement and differentiation when introduced into Volvox embryos (Huber and Sumper, 1994). beps are a small family of related, secreted proteins found in early sea urchin embryos. When dissociated sea urchin embryonic cells are incubated in the presence of antibody raised against beps, they fail to reaggregate (Romancino et al., 1992) suggesting a role in cell adhesion. In summary, Fas I-related proteins constitute an evolutionarily conserved group of extracellular membrane-associated and secreted proteins that perform a variety of cell adhesion, growth and differentiation functions.

Based on the sequence similarity of Mfas to Fas I, it is likely that Mfas is a cell signaling or adhesion molecule. Many neural cell adhesion and signaling molecules are expressed in a large number of CNS neurons, but *mfas* CNS expression is restricted to the CNS midline cells. There are a number of possible roles that Mfas protein could perform. Since Mfas is transiently expressed on VUM cell motor neuron axons, the protein may play a role in VUM cell axon guidance or neuromuscular plasticity. The midline expression in both precursor cells and differentiated cell types could also be involved in other functions, such as cell fate, division, and migration. However, one of the most intriguing roles concerns the interaction between CNS midline cells and commissural axons. Genetic analysis suggests that the CNS midline cells contribute both attractive and repulsive signals to axonal growth cones of lateral CNS neurons (Goodman, 1996).

The *fasI* gene is expressed abundantly in the lateral neurons and the CNS midline VUM cell neurons during commissure formation, and the *abl fasI* genetic experiments indicate that *fasI* is required for commissure formation. The appearance of Mfas on midline cells during commissure formation raises the possibility that Mfas and FasI may: (1) interact directly, (2) provide overlapping functions, or (3) contribute distinct functions regarding commissure formation. Consistent with an *mfas* function in commissure formation is the observation that an *mfas* deletion has defects in commissure formation, and *abl mfas* double mutant embryos have severe defects in commissure formation that resemble *abl fasI* mutations. The disorganization and bunching of the axons of commissural neurons, which constitute 90% of the CNS neurons, in *abl mfas* mutant strains could reflect an inability of commissural axons to properly move to the midline. The *mfas* and *abl mfas* double mutant embryos also have thin longitudinal connectives. It is unclear how *mfas* mutants would contribute to this defect, although it is worth noting that *abl* mutant embryos often have thin connectives, suggesting that this defect may be due more to loss of *abl* function rather than *mfas.* Further insight into possible interactions between *fasI* and *mfas* will require creation of *mfas* single gene mutations and phenotypic analysis of *fasI mfas* double mutant strains.

Loss-of-function mutations of neural cell adhesion molecules, e.g., *fasI,* often do not show strong phenotypes. Thus, it is no surprise that deletions of the *mfas* gene reveal normal-appearing CNS midline and tracheal cells, and only weak axon defects. Although a negative result, a saturation EMS mutagenesis screen carried out be Gausz and colleagues (Gausz et al., 1981) failed to identify lethal mutations corresponding to the *mfas* gene, suggesting *mfas* is not required for viability under lab culture conditions. There are several explanations for the absence of strong *mfas* phenotypes: (1) *mfas* expression in certain cell types may be adventitious and nonfunctional, (2) *mfas* mutations can be functionally compensated by redundant genes or by upregulation of a compensatory molecule(s), or (3) some phenotypic functions are too subtle to detect with the reagents and analyses used in this study. There are examples in which cell adhesion proteins whose genes show weak phenotypes have their *in vivo* relevance revealed by double mutant analysis that presumably removes genetically redundant

genes (e.g., *fasI;* Elkins et al., 1990b), or by ectopic expression experiments that create a gain-of-function phenotype (*fasciclinIII;* Chiba et al., 1995; *connectin;* Nose et al., 1992; *semaphorinII;* Matthes et al., 1995). This issue was addressed for *mfas* by constructing a strain mutant for both *mfas* and *abl.* This strain showed severe CNS defects that were more severe than *abl* or *mfas* mutants alone. However, this result does not definitively provide evidence for interactions between *abl* and *mfas.* The result could be reasonably interpreted as either additive or synergistic, and there is still uncertainty if the *mfas* mutant defect is due to absence of *mfas* or some other gene. Additional progress will be made when *mfas* single mutants are available and they can be carefully analyzed for axon guidance, connectivity, and plasticity defects individually and in combination with other mutants. In addition, it will be important to understand how Mfas and FasI function biochemically, and if they interact with receptors.

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