Development 125, 2747-2758 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5198

faint sausage encodes a novel extracellular protein of the immunoglobulin superfamily required for cell migration and the establishment of normal axonal pathways in the *Drosophila* nervous system

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Accepted 28 April; published on WWW 23 June 1998

SUMMARY

We examined the structure of the nervous system in *Drosophila* embryos homozygous for a null mutation in the *faint sausage* (*fas*) gene. In the peripheral nervous system (PNS) of *fas* mutants, neurons fail to delaminate from the ectodermal epithelium; in the central nervous system (CNS), the positions of neuronal cell bodies and glial cells are abnormal and normal axonal pathways do not form. Sequence analysis of *fas* cDNAs revealed that the *fas* protein product has characteristics of an extracellular protein and that it is a novel member of the immunoglobulin (Ig) superfamily. In situ hybridization demonstrated that *fas* transcripts are expressed throughout the embryo but they are in relatively high concentrations in the lateral ectoderm, from which the peripheral nervous system delaminates and in the CNS. Antiserum directed against Fas protein was found to stain neurons but not glia in the CNS. We conclude that *fas* encodes a protein that, in the developing nervous system, is present on the surface of neurons and is essential for nerve cell migration and the establishment of axonal pathways.

Key words: Nervous system, Cell migration, Axonal pathfinding, *Drosophila*, Epithelium

INTRODUCTION

The development of the nervous system involves highly ordered cell movements. One of the earliest of these events is the segregation of neural progenitors in a reproducible spatiotemporal pattern from the ectoderm. Subsequently, these cells migrate along routes to arrive at specific destinations and they divide in stereotypical patterns. The postmitotic neurons differentiate and send out axons that follow specific pathways to form synaptic contacts with their target cells.

Necessary steps toward understanding the mechanisms involved in cell migration and axonal pathfinding are the identification and characterization of the cell surface proteins required for the occurrence of these events. Many such proteins have already been discovered; for example, the radial migrations of neural precursors in vertebrate cortical structures, such as the chick optic tectum or cerebellum, depend on a number of different cell adhesion molecules, including cytotactin and integrin (Galileo et al., 1992; Yamagata et al., 1995; Zheng et al., 1996). Further, a group of adhesion molecules, called Fasciclins, has been shown to be involved in the ordered outgrowth and fasciculation of axons in the developing *Drosophila* nervous system (reviewed in Goodman and Doe, 1993). These molecules, notably Fasciclin II (Harrelson et al., 1988; Grenningloh et al., 1991) and Fasciclin III (Patel et al., 1987; Snow et al., 1989; Chiba et al., 1995) are members of the immunoglobulin (Ig)-like family of cell adhesion molecules, a very large and growing list of proteins that contain at least one but often several repeats of the Ig domain (Edelman, 1970) and often contain other functional domains.

Drosophila is particularly well suited for the analysis of the molecular mechanisms behind cell migration and axonal pathfinding because of the ability to analyze the in vivo functions of specific gene products through genetic analyses. For instance, the analysis of loss-of-function mutations in the *Drosophila* netrins shows that they are required for commissural axon guidance at the ventral midline and also for the proper guidance of motor axons (Mitchell et al., 1996). Alternatively, van Vactor et al. (1993) identified a number of genes that when mutated lead to defects in axonal pathways and target recognition by outgrowing motorneurons. Subsequent analysis of the genes defined by these mutations showed that one of them, *beaten path*, encodes a secreted member of the Ig superfamily that may function in decreasing axon-axon fasciculation, possibly by interfering with CAMs

(Fambrough and Goodman, 1996; Muchegian, 1997; Bazan and Goodman, 1997).

Although significant progress has been made in understanding the mechanisms of axon outgrowth and guidance during *Drosophila* neurogenesis, little is known about the control of neural progenitor cell segregation and migration. To address this problem, we have screened existing collections of mutations for defects in neural progenitor segregation and neuronal migration. As an assay, we stained embryos from each mutant line with the antibody marker mAb22C10 (Zipursky et al., 1984), which stains sensory neurons, thereby allowing us to detect abnormalities in number, position and shape of sensory neurons; these abnormalities mirror changes that had taken place during the specification, division and migration of sensory neural progenitors. We describe here one of the genes identified in this screen, called *faint sausage* (*fas*). Among the most striking aspects of the phenotype resulting from loss of *fas* function are that sensory neurons do not move normally out of the epidermal layer, resulting in defective sensillum differentiation. Further, neurons of the CNS do not migrate to their proper locations during the phase of germ band retraction and CNS condensation, leading to gross abnormalities in cell shape and position; subsequently, patterning defects in axonal pathfinding occur. Combined with our finding that *fas* encodes a novel member of the Ig superfamily that is associated with the cell bodies of most neurons but not glia in the CNS, we propose a model for *fas* function during CNS retraction.

MATERIALS AND METHODS

Fly stocks and genetics

Two *fas* alleles were provided by C. Nüsslein-Volhard in whose screen the *fas* mutant was originally identified (fas^{IIA} and fas^{IC}; Nüsslein-Volhard et al., 1984; Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany), and one allele, fas^{TK}, was isolated in a screen performed in Dr Campos-Ortega's laboratory (Cologne) and was provided to us by Thomas Klein. The fas^{P1411} allele was generated by Karpen and Spradling (1992). We generated the translocation allele, $fas^{G5\text{-}29}$ in a γ-ray mutagenesis experiment. For this screen, *cn bw* males were irradiated with 4000 rad γ-rays and *fas* mutations were recovered with standard F2 screen protocols. Furthermore, three deficiencies for the 50A-50D interval were generated in an X-ray mutagenesis screen. For this screen, males homozygous for a P-element insertion within 0.06 cM of *fas* (line P636; B. Christen and E. Hafen, unpublished) were irradiated with 4000 rad X-rays. 461 progeny that had lost the w^+ marker of the P element were tested for complementation to fas^{IIA}. Three non-complementing mutations were recovered; subsequent analysis of larval polytene chromosomes showed that these were deficiencies for the region (Table 1). To excise the P-element insertion from the *fas* locus, fas^{P1411} was combined with the Δ2-3 99B source of P transposase (Robertson et al., 1988). Of 108 independent excision events isolated, 5 lines were homozygous viable. Lethal revertants were tested for the presence of residual P-element sequences and, of these, fas^{PR95} was found to completely lack any of the original fas^{P1411} P-element insertion. Subsequent Southern analysis showed that fas^{PR95} carries a deletion (Fig. 3C).

Molecular biology

Standard molecular biology procedures were performed as described (Sambrook et al., 1989). Except for northerns, all hybridizations were performed with digoxigenin-labeled probes and were used according to manufacturer's specifications (Boehringer Mannheim). IPCR (Ochman et al., 1988) was performed on P1411 DNA containing the 3′ P-element end using these primers: A: 5′ CGCTCTAGAATTCACTCGCACTTATTGCA 3′ and B: 5′ CCAGAATTCTAACCCTTAGCATGTCCGTG 3′. This product was used to screen a phage genomic library (gift of Dr J. Tamkun, University of California, Santa Cruz), and an initial chromosome walk covering ~30 kb was performed. A 3.5 kb *Sal*I fragment which included the P1411 insertion site was used to screen a 9-12 hour embryonic cDNA library (gift of Dr K. Zinn), which yielded one positive clone, which was used to rescreen the same library and an additional 0-24 hour embryonic cDNA library (Palazzolo et al., 1990). Several overlapping cDNAs were isolated from this screen. Several P1 clones from the region were tested for hybridization to the P1411 insertion site and to the 3′ end of the cDNAs. The locus was found to require three P1 plasmids (DS00096, DS02972 and DS06212; catalogued in the Encyclopedia of Drosophila 2.0) to cover the entire region from the P1411 insertion site to the 3′ end of the cDNAs. Subcloned portions of DS00096 were used as probes on genomic Southern blots to determine the extent of the PR95 deletion and the G5-29 translocation breakpoint. For northern analysis, total RNA was prepared from appropriately aged embryos using TriZOL reagent (Gibco BRL). Poly(A) selection was performed on approximately 2 mg of total RNA samples with an oligo(dT)-cellulose column according to manufacturer's recommendations (Gibco BRL). For northern gels, approximately 5 μg poly(A) RNA was loaded on the gel. Probes were labeled with ^{32}P-dATP and ^{32}P-dCTP.

Sequence analysis

DNA sequencing was performed via oligo walking by the UCLA Biology Core Sequening Facility, or was performed using the transposon facilitated sequencing technique (Strathmann et al., 1991). Sequence analysis was performed with the GCG sequence analysis package (GeneticsComputer Group).

Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed with digoxigenin-labeled RNA probes as described in O'Neill and Bier (1994) with minor modifications. Hybridization was carried out in 100 μl with a probe concentration of 0.5 ng/μl. Post-hybridization washes were: 2× 20 minutes at 55°C in 100% Hyb solution, 2× 20 minutes 55°C Hyb/PBT, 2× 20 minutes 55°C PBT, 2× 10 minutes room temperature PBT. Embryos were mounted in Epon for microscopy or sectioning.

Antibody production

Faint-sausage-GST fusion proteins were constructed in pGEX-2T. PCR primers with added restriction sites were used to amplify a fragment encoding amino acids 40-186 from cDNA FZ1. Induction of protein expression and purification of GST-Fas fusion on glutathione agarose beads (Sigma) was performed as described (Smith and Johnson, 1988). Purified fusion protein was used to immunize five adult female rats according to the standard protocol for the contracted company (Pocono Rabbit Farms and Labs, Inc.).

Immunohistochemistry

Rat anti-Fas antibodies were found to be most effective on heat-fixed embryos (Miller et al., 1989). This antiserum was diluted 1:1000 in PBT+N (Phosphate buffered saline, 0.1% Triton X-100, 10% normal goat serum) for optimal signal/noise ratio. Antibody labelings were performed as described (Rugendorff et al., 1994) using the Vectastain ABC kit (Vector labs) with the exception that secondary antibodies were diluted 1:400 in PBT+N. Other antibodies used in this study were: anti-β-galactosidase (Cappel, dilution 1:800), mAb22C10 (Zipursky et al., 1984; dilution 1:50), anti-Fasciclin II mAb1D5 (Grenningloh et al., 1992; gift of Dr C. Goodman, dilution 1:40), anti-muscle myosin (kindly provided by Dr D. Kiehart, 1:1000 dilution),

anti-RK2 (Campbell et al., 1994; kindly provided by Dr A. Tomlinson, 1:750 dilution), anti-Even skipped (Frasch et al., 1988; kind gift of Dr M. Frasch), anti-Engrailed (DiNardo et al., 1985) and anti-Elav mAb9F8A9 (O'Neill et al., 1994; 1:10 dilution; the latter two antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD).

Western analysis

Groups of 10 embryos, individually scored for their phenotype, were homogenized in 20 μl 2× sample buffer. After boiling samples for 5 minutes, they were centrifuged and stored at −20°C until used. Proteins were separated on a 10% SDS-polyacrylamide gel, then blotted to nitrocellulose overnight. Blots were blocked in 10% nonfat milk in 1× TBS (20 mM Tris pH 7.5, 500 mM NaCl). Blots were then incubated in 20-30 ml anti-Fas antibody diluted 1:1000 in 1× TBS, 1% nonfat milk. After a 2 hour incubation at room temperature, blots were washed 4× 10 minutes at room temperature in 1× TBS, 0.05% Tween-20. Blots were then incubated 1 hour at room temperature in HRP-conjugated anti-rat secondary antibody (Jackson Labs), diluted 1:5000 in 1× TBS, 1% nonfat milk. Blots were washed twice for 10 minutes each wash in 1× TBS, 0.05% Tween-20 before being treated with a chemiluminescent substrate (ECL, Amersham) and exposed to X-ray film.

RESULTS

Loss of *fas* function leads to defects in the delamination of sensory neurons

The sensory nervous system of wild-type embryos is composed of sensilla, small clusters of specialized cells distributed in an invariant pattern over the entire epidermis. The majority of sensilla, specialized for mechanoreception and chemoreception, are visible at the outer surface of the epidermis and are therefore called external sensilla. Each external sensillum consists of one or more subepidermal neurons and a group of accessory cells, all of which are formed by the mitotic division of a sensory organ precursor cell (SOP) located within the epidermis. Following SOP division, the presumptive sensory neuron moves from the epidermis into the interior of the embryo (Fig. 1A), whereas the accessory cells remain within the epidermis and form concentric sheaths around the sensory dendrite (Hartenstein, 1988). Apical processes of the outer two accessory cells (trichogen cell and tormogen cell, respectively) form the stimulus-receiving apparatus of the sensillum.

Table 1. *fas* alleles used in this study. The two EMS-induced alleles fas^{IIA} and fas^{IC} genetically behave as nulls

Name	Mutagen	Strength	Cytology
IIA	EMS	++++	
IC	EMS	++++	
TK	EMS	++	
P1411	P-element	++++	Is(2R)PZ{P[ry+, lacZ]}50C1-4
PR95	P-reversion	++++	
G5-29	γ-rays	+	T(2R;3R)50B7-9;100E/F
Df(2R)AA1	X-rays		Df(2R)50A11-15; 50D4-7
Df(2R)MK1	X-rays		Df(2R)50B3-5; 50D4-7
Df(2R)MK2	X-rays		Df(2R)50A1-5; 50C1-3, T(2R;3L)50A1-5; 62C

In *fas* mutant embryos (for this and the following phenotypic analyses, we refer to the null alleles fas^{IIA} and fas^{PR95}, see below) the movement and shape of sensillum cells are defective (Fig. 1B). After a period of normal SOP division (data not shown), many sensory neurons as visualized by the antibody mAb22C10 are located within the epidermis, instead of subepidermally. Epidermal cells surrounding the sensilla often do not assemble into regular monolayered sheets as in wild type (Fig. 1C) with an apical and basal surface, but pile up into 2-3 layers of irregularly shaped cells (Fig. 1D). Accessory cells of the sensilla fail to form lateral processes that wrap around the sensory dendrite, nor do they form apical processes that become the shaft and socket of the sensillum. Thus, *fas* is necessary for the delamination of the sensory neuron precursor and for the proper differentiation of the sensilla accessory cells.

Loss of *fas* function in the central nervous system leads to defects in cell migration and axonal tract formation

The *Drosophila* CNS develops from an invariant population of progenitor cells (neuroblasts) that segregate from the ectoderm and divide in a reproducible pattern to form a multilayer of neurons. Around the stage when neurons start differentiating (stages 12-14), the CNS primordium undergoes a dramatic compaction. First, the germband (including the CNS primordium) retracts, leading to a more than 50% shortening of the CNS. After germband retraction (stages 13-16) the CNS further shortens until it measures only about 30% of its original length at maximum extension. The reduction in length of the CNS is mainly compensated for by an increase in its dorsoventral axis and a higher packing density. Thus, cells that in the early embryo are arranged one behind the other come to lie closer or even above and/or beside each other in the CNS of mature embryos.

The CNS primordium of *fas* null mutant embryos develops normally until the late extended germ band stage; then, defects in CNS morphogenesis and the expression of differentiation markers become apparent towards the end of germ band retraction (stage 12). Thus, markers for subsets of neuroblasts and their progeny (*svp*, *eve*, *en*; all explained in Doe, 1992) did not show detectable abnormalities in the neuroblast map of early *fas* mutant embryos (data not shown). However, the shortening and thickening of the CNS that normally take place during stages 13-16 fail to occur (compare Fig. 1F and H; schematically summarized in Fig. 7). At the same time, the *fas* mutant CNS remains flattened (in the dorsoventral axis) and widened (in the mediolateral axis; compare Fig. 1G and H). There does not appear in *fas* mutant embryos an obviously decreased number of neurons or glial cells, as visualized with antibodies against the Elav (Fig. 1E,F) and Repo proteins (Fig. 2B,C), respectively. Also, markers for specific subsets of neurons [e.g., Even skipped, expressed in the aCC/pCC pair of neurons (Fig. 2C-F), or Engrailed, expressed in the progeny of the MNB neuroblast (Fig. 1I,J) are expressed in *fas* mutants. However, the repositioning of neurons and glia that normally takes place during CNS condensation fails to occur. For example, longitudinal glial cells (LGCs) remain in segmental clusters, separated by gaps; they also stay at a more lateral level than

their wild-type counterparts (Fig. 2B). Midline cells, such as the MNB progeny, do not form the regular, dense clusters typical for the wild-type embryo (Fig. 1I,J). Thus, the correct specification of NBs does not require *fas*, but the later morphogenetic movements of nerve cord condensation and consequent cell positioning do require *fas*.

The most dramatic effect caused by loss of *fas* function can be seen in axonal patterning. In wild-type embryos, early differentiating pioneer neurons form a scaffold of longitudinal tracts (connectives) and transverse tracts (commissures) along which later axons fasciculate. *fas* null mutants are characterized by the virtual absence of connectives. The ontogeny of this phenotype has been followed using the FasII antibody, which recognizes most of the pioneer neurons of the connectives (reviewed in Goodman and Doe, 1993; Fig. 2). In the wild type, the first pioneer neurons are aCC (projecting posteriorly and then into the periphery), pCC and vMP2 (projecting anteriorly and forming a medial longitudinal tract), and MP1 and dMP2 (projecting posteriorly and forming a lateral longitudinal tract). All of these cells develop with their cell bodies in close contact with LGCs along which they project their axons. In *fas* mutants, these pioneer neurons develop at abnormal positions and project their axons abnormally. The MPs project their axons peripherally, instead of longitudinally (Fig. 2B,F). Also both pCC and aCC, which can be recognized by their early expression of FasII and by their expression of Even skipped (Doe et al., 1988), project their axons straight laterally instead of longitudinally (Fig. 2F). Later forming axons follow this abnormal trajectory, leaving the CNS devoid of any orderly longitudinal tracts. In addition, the overall amount of axons (i.e. number and length integrated) in a *fas* mutant embryo appears largely reduced. Thus, *fas* function is required for the correct temporal differentiation of neurons in the CNS and for correct pathfinding by pioneer and follower axons.

In summary, loss of *faint sausage* is associated with severe defects in tissue organization and cell movement in the epidermis and CNS. These defects, which become first manifest in the stage during and after germ band retraction with its accompanying cell rearrangements, result in generally abnormal shape and position of neurons. Subsequently, patterning defects in axonal pathfinding occur, possibly as a secondary consequence of the abnormal cell body positions, since Fas is not expressed on the axons themselves (see below).

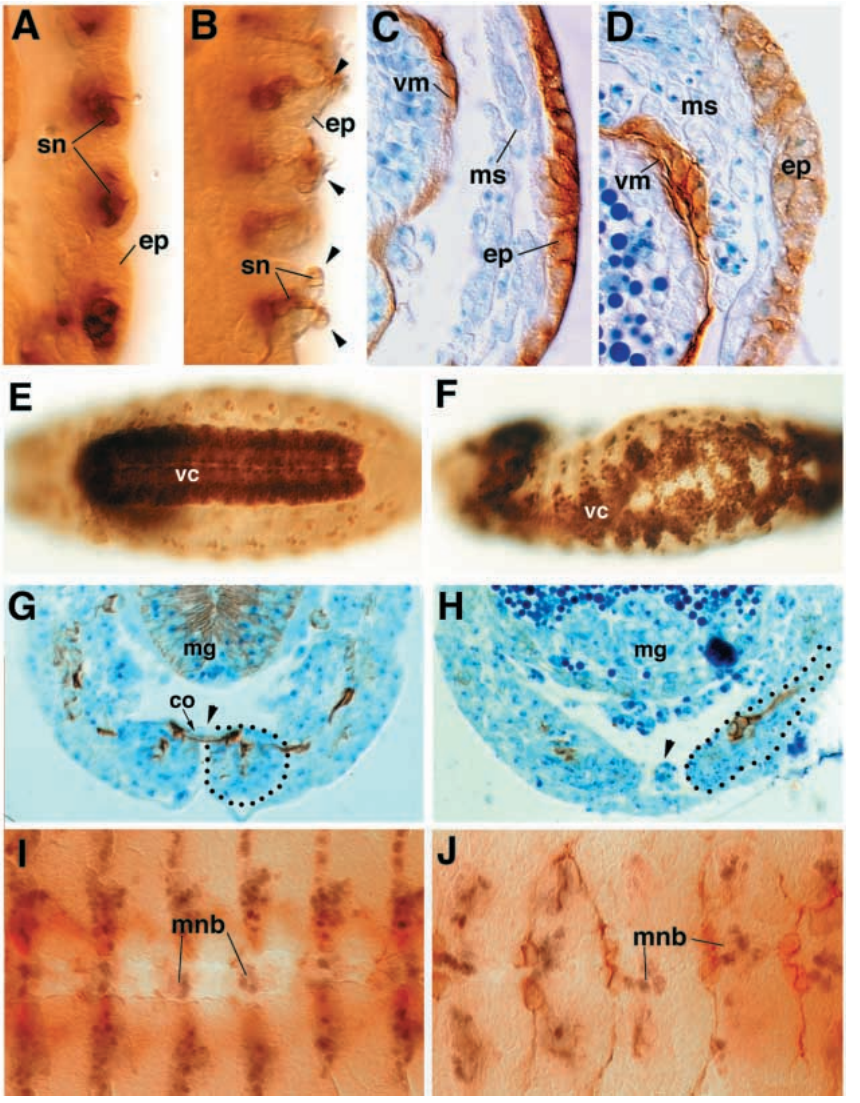

Fig. 1. Morphogenetic defects caused by loss of function of *fas*. (A,B) Whole mounts of stage 16 wild-type (A) and fas^{IIA} (B) embryo, dorsal view, labeled with mAb22C10 to visualize sensory neurons. In wild type, sensory neurons (sn) are beneath the epidermis (ep); in *fas* mutant, there are sensory neurons located at the surface (arrowheads). (C,D) Cross section of stage 15 wild-type (C) and fas^{IIA} (D) embryo, stained with anti-Fas III antibody that labels surface of epidermal cells (ep; preferentially basolateral membrane) and visceral musculature (vm). In the *fas* mutant, epidermal cells are irregularly shaped, form two to three layers instead of one, and express a lower level of Fas III. (E,F) Ventral view of whole mounts of mature wild-type (E) and fas^{IIA} mutant embryo (F) in which CNS was labeled with anti-Elav antibody. In the wild type, neurons of the ventral nerve cord (*vc*) have condensed into a short, compact structure. In *fas* mutant embryos, condensation of the ventral nerve cord fails to occur. (G,H) Cross sections of stage 15 wild-type (G) and fas^{IIA} mutant embryo (H) labeled with anti-FasII antibody which at this stage stains early differentiating CNS neurons and midgut (mg). Sections are counterstained with methylene blue/toluidine blue. In wild type, the ventral nerve cord (vc; surrounded by dotted line on right side of embryo) has condensed and measures approximately 6-8 cell diameters in width and height. In *fas* mutant embryos, the ventral nerve cord remains extremely wide and flat (see dotted outline). Midline structures (arrowhead), including the commissures (co) that tie both sides of the wild-type nerve cord together at this stage, do not differentiate in *fas* mutants. (I,J) Ventral view of whole mounts of stage 13 wild-type (I) and fas^{IIA} mutant embryo (J) labeled with an antibody against the Engrailed-protein, which is expressed in stripe like arrays of neurons, including the progeny of the median neuroblast (mnb). In *fas* mutants, En-positive neurons are present in roughly normal numbers, but fail to form regular segmentally patterned cluster and fail to differentiate.

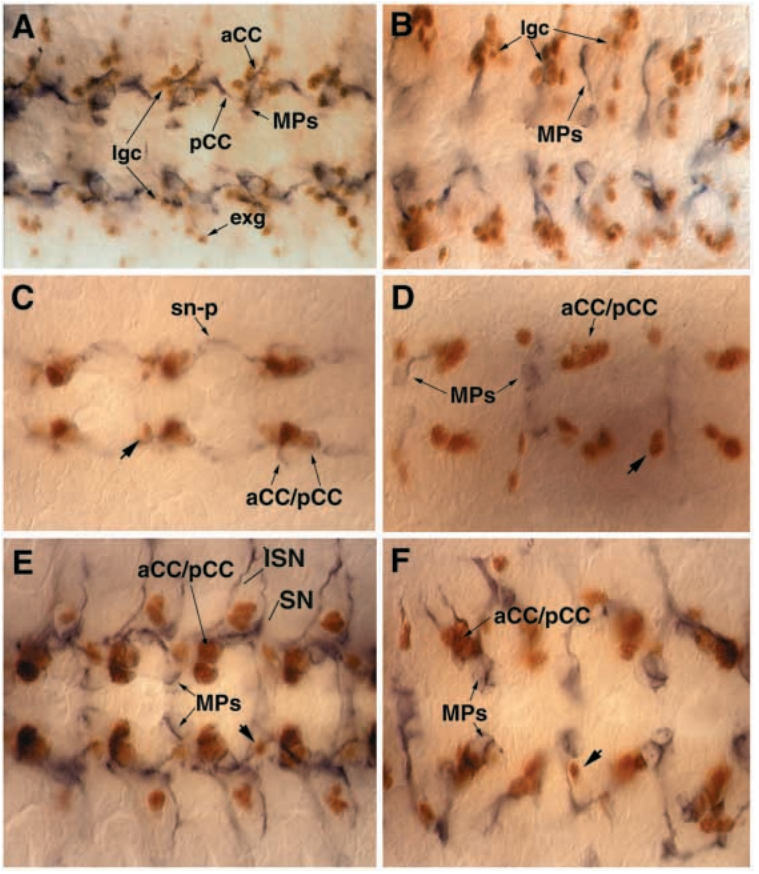

Fig. 2. Axonal pathfinding defects caused by loss of *fas* function. All panels show ventral view of whole mounts of wild-type embryos (A,C,E) and *fas*^{PR95} mutant embryos (B,D,F) labeled with anti-FasII antibody (purple color), which marks early differentiating neurons in the CNS. (A,B) Stage 13. Beside anti-FasII staining of neurons, glia cells are labeled with anti-Repo antibody (brown). In wild type, pioneer neurons [e.g., aCC/pCC; midline precursors (MPs)] appear in direct contact with longitudinal glia cells (lgc), which have migrated in from more lateral positions and which form the substratum for outgrowing longitudinal axon fascicles (pCC growth cone is marked). In *fas* mutant (B), longitudinal glia cells remain at a more lateral level. Pioneer neurons (e.g., MPs) develop far from glia cells and project their growth cones towards them. (C,D) Early stage 12 embryo. Beside anti-FasII, antibody against the Eve gene product that is expressed in aCC/pCC and several other neurons was used (brown color). In wild type (C), Fas II expression comes up in aCC/pCC and a group of cells that we termed 'segmental nerve pioneers' (sn-p). In *fas* mutant (D) of the same stage, the aCC/pCC neurons, recognized by their expression of Eve and position, do not yet express Fas II. Instead, there are other Eve-negative cells, presumably the midline precursors (MPs), that express FasII at this early stage. (E,F) Stage 13. In the wild type (E), segmental clusters of neurons, among them the anterior and posterior corner cells (aCC/pCC) and midline precursor cells (MPs) have differentiated and form pioneer tracts of peripheral nerve roots (ISN, intersegmental nerve; SN, segmental nerve), longitudinal and transverse central axon tracts. In *fas* mutant embryos, all FasII-positive neurons project their axons peripherally.

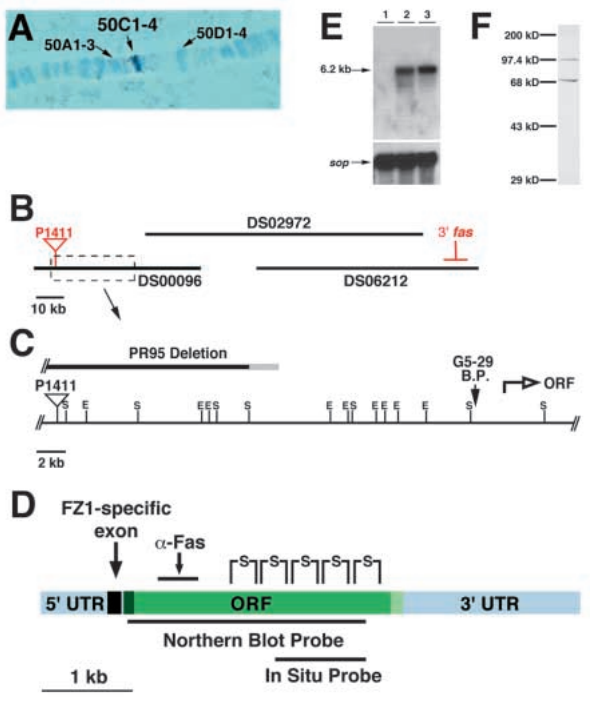

Fig. 3. Molecular analysis of *fas*. (A) In situ hybridization using a *ry*$^{+}$ probe to P1411/+ chromosomes. The P1411 insertion is located on 2R in band 50C1-4. (A-C) The centromeric direction is to the left and telomeric is toward the right. (B) Diagram of three P1 plasmids from 50C1-4 covering the extent of the *fas* locus. This alignment is derived from hybridization data and STS data from the Encyclopedia of Drosophila (release 2.0). The 5′ end of the gene is demarcated by the P1411 insertion site and the location of the 3′ end of the locus was determined by Southern analysis using a probe from the 3′ end of the full-length cDNA. Based on our data, we estimate the *fas* locus to extend over at least 110 kb. (C) An expanded view of the 5′ 35 kb of the *fas* locus. E and S refer to *Eco*RI and *Sal*I restriction sites. The PR95 deletion is indicated; the deletion removes approximately 12 kb downstream from the P1411 insertion and at least 8 kb upstream. The *fas*$^{G5\text{-}29}$ translocation breakpoint (G5-29 B.P.) was mapped by RFLP analysis very near a *Sal*I restriction site 30 kb downstream from the P1411 insertion site. The beginning of the open reading frame lies downstream of the G5-29 translocation breakpoint within the same ~5 kb *Sal*I restriction fragment. The first 0.5-1 kb of the cDNA are likely to comprise 2-3 exons that lie within the interval denoted by the P1411 insertion and the G5-29 breakpoint (not shown). (D) Schematic representation of *fas* cDNAs. All cDNAs conform to this generalized structure, with the exception of cDNA FZ1, which contains a 100 bp sequence (black) not found in other cDNAs in the 5′ UTR and an alternate 5′ splice site for the intron immediately upstream of the additional exon (intron 1). The ORF (green) is approximately 2.5 kb and encodes a protein of 822 amino acids. At the N terminus, there is a putative signal sequence (dark green) and, at the C terminus, there is a putative GPI addition sequence (light green). Five putative Ig domains (S) are found beginning from approximately amino acid 250. The portion labeled α-Fas was the portion used to immunize rats. Also indicated are the portions of cDNAs used as probes for northern analysis and whole-mount in situ hybridizations. (E) Northern analysis of *faint sausage*. Lanes 1-3 contain ~4 μg poly(A) RNA from 0-6 hour embryos, 6-12 hour embryos and 12-24 hour embryos, respectively. The probe used is indicated in D. A single major transcript of 6.2 kb is seen after 6 hours. An overexposure of the blot shows that the 6.2 kb transcript is present at a low level in lane 1. *sop* (Cramton and Laski, 1994) encodes *Drosophila* ribosomal protein S2 and serves as a loading control. (F) Western blot using the anti-Fas antiserum. Two bands are seen in extracts of wild-type embryos: ~80 kDa and ~100 kDa. The 100 kDa band is missing in protein extracts from *fas*^{PR95}/*fas*^{PR95} embryos (not shown) and corresponds to the Fas protein. The 80 kDa band is only seen in western blots; we have not determined the identity of this protein.

Genetic analysis and cloning of the *fas* locus

At the outset of our study, two alleles, *fas*^{IIA} and *fas*^{IC}, both induced with ethylmethane sulfonate (EMS), existed (Nüsslein-Volhard et al., 1984). Both alleles, in *trans* to a deficiency (see below), genetically behave as null alleles. One further hypomorphic allele, *fas*^{TK}, was isolated in a different EMS screen (T. Klein and J. A. Campos-Ortega, personal communication).

In order to create new alleles of *fas* to aid in our analysis, we performed gamma-ray and X-ray mutageneses and screened for non-complementing lethal P-element insertions (see Materials and Methods). A translocation allele of *fas*, *fas*$^{G5\text{-}29}$, was generated; the translocation breakpoint localized the *fas* locus to the 50B/C region on the right arm of the second chromosome. In addition to the translocation allele, three deficiencies for the region were obtained (Table 1). Complementation testing of lethal P-element lines with insertions in the second chromosome identified a line, P1411 (Karpen and Spradling, 1992), that did not complement *fas*^{IIA} and showed a strong *fas* cuticular phenotype. In situ hybridization to larval polytene chromosomes localized this insertion to 50C1-4 (Fig. 3A). Precise excision of the P-element insertion reverted the mutant phenotype to wild type (data not shown), thereby demonstrating that the P-element insertion was responsible for the phenotype. These data led us to designate P1411 as an allele of *fas* (*fas*^{P1411}). Imprecise excision of the P1411 insertion created the deletion allele *fas*^{PR95} (Table 1). Thus, in total, we identified or generated three new alleles of *fas* and three new deficiencies for the interval containing *fas*.

To clone the *fas* locus, we used inverse PCR (IPCR; Ochman et al., 1988) to amplify approximately 100 base pairs (bp) of genomic DNA flanking the *fas*^{P1411} P-element insertion. This genomic DNA fragment was used to screen two phage genomic DNA libraries (gifts of Drs F. Laski and J. Tamkun), and overlapping clones spanning approximately 30 kbp were isolated. Isolation of full-length cDNAs (see below) showed that our 30 kbp genomic map did not contain the entire locus. Furthermore, the breakpoint of the *fas*$^{G5\text{-}29}$ translocation did not map within this genomic region. Therefore, P1 clones mapping to the 50C1-4 region (Berkeley Drosophila Genome Project) were tested for hybridization to the P1411 insertion site, the 3′ portion of *fas* cDNAs, and to chromosomes carrying the *fas*$^{G5\text{-}29}$ translocation. The results of this analysis suggest that the *fas* locus spans at least 110 kbp, and possibly more than 150 kbp (Fig. 3B,C). A 3.5 kbp genomic fragment flanking the P1411 insertion site was used to screen several cDNA libraries; in addition, the initially isolated cDNA FZ1 was used to rescreen the libraries. A total of 8 overlapping cDNAs with a combined length of 5.9 kbp were isolated. The only difference found between the cDNAs is that cDNA FZ1 contains a 100 bp sequence not found in any other, and the 5′ splice site for the intron immediately upstream of the additional exon (intron 1) in FZ1 is located ~100 bp upstream from that of the first intron of the other cDNAs. These differences are contained within the 5′ untranslated region (UTR); there are no differences between the cDNAs in their open reading frames (ORFs; Fig. 3D).

Three lines of evidence show that the transcription unit that we have identified represents *fas*. First, in situ hybridizations to embryos using cDNA probes and also genomic DNA probes flanking and distal to the P1411 insertion revealed an expression pattern corresponding to that reported by the P1411 insertion. By contrast, in situ hybridizations with 8 kbp of genomic DNA proximal to the P1411 insertion showed no evidence of a transcription unit. Second, by RFLP analysis we mapped the *fas*$^{G5\text{-}29}$ translocation breakpoint 30 kbp downstream (distal) of the P1411 insertion site, within the identified transcription unit (Fig. 3C). Third, an antibody raised against a portion of the identified ORF fails to stain embryos homozygous for the deletion allele, *fas*^{PR95} (Fig. 5). This result is confirmed by western analysis (not shown): a ~100 kDa protein detected in extracts of wild-type embryos is absent from extracts of *fas*^{PR95} homozygotes. Moreover, embryos homozygous for the P1411 insertion show a substantial decrease in the amount of the 100 kDa band, and a similar specific reduction is seen in extracts from embryos homozygous for the independent EMS allele, *fas*^{IIA} (not shown).

fas encodes an extracellular protein containing Ig domains

The ORF contained within the FZ1 cDNA encodes a protein of 822 amino acids with a predicted molecular mass of approximately 90 kDa in the absence of post-translational processing. The N-terminal 30 amino acids have the characteristics of a signal peptide, according to the −3,−1 rule of von Heijne (1986), so Fas is predicted to be extracellular (Fig. 4A). Because the predicted Fas protein does not contain any sequence that would qualify as a transmembrane domain, Fas is either secreted or anchored to the external plasma membrane via the addition of a glycophosphatidyl inositol (GPI) anchor. In seeming support of a GPI-anchored form of Fas, the C-terminal amino acids of the predicted *fas* protein show a short stretch of hydrophobic residues preceded by amino acids conforming to a consensus GPI-addition sequence (Gerber et al., 1992; Kodukula et al., 1993; Fig. 4A). We have not yet determined whether Fas is found in vivo in only one or in both forms, although immunohistochemical stainings suggest that at least a membrane anchored form exists (see below).

The predicted Fas protein sequence was compared to proteins in the NCBI protein databases using the BLAST program; this yielded significant scores from many members of the Ig superfamily. The highest scoring matches include RAGE (Receptor for Advanced Glycosylation Endproducts; Neeper et al., 1992), Cell-CAM 105 (Aurivillius et al., 1990), Contactin (Ranscht, 1988) and IrreC (Ramos et al., 1993). Inspection of the Fas amino acid sequence shows that it contains five putative Ig domains, each of which is characterized by two highly conserved cysteine residues and a tryptophan residue (Edelman, 1970). Ig domains are grouped into three categories: V, C1 and C2 (Williams and Barclay, 1988), the main differences being that V type domains have two more β-strands than the others, and C1 type domains are only found in proteins of the immune system. Comparison of the five Fas Ig domains to the consensus sequence of Vaughn and Bjorkman (1996) suggests that domains 1, 3, 4 and 5 are likely to adopt the V type and domain 2 the C2 type domain topology (Fig. 4B). Comparisons between Fas and the proteins listed above using the FastA program (Pearson and Lipman, 1988) show that Fas shares high degrees of similarity with all of these over limited ranges. However, none of the proteins

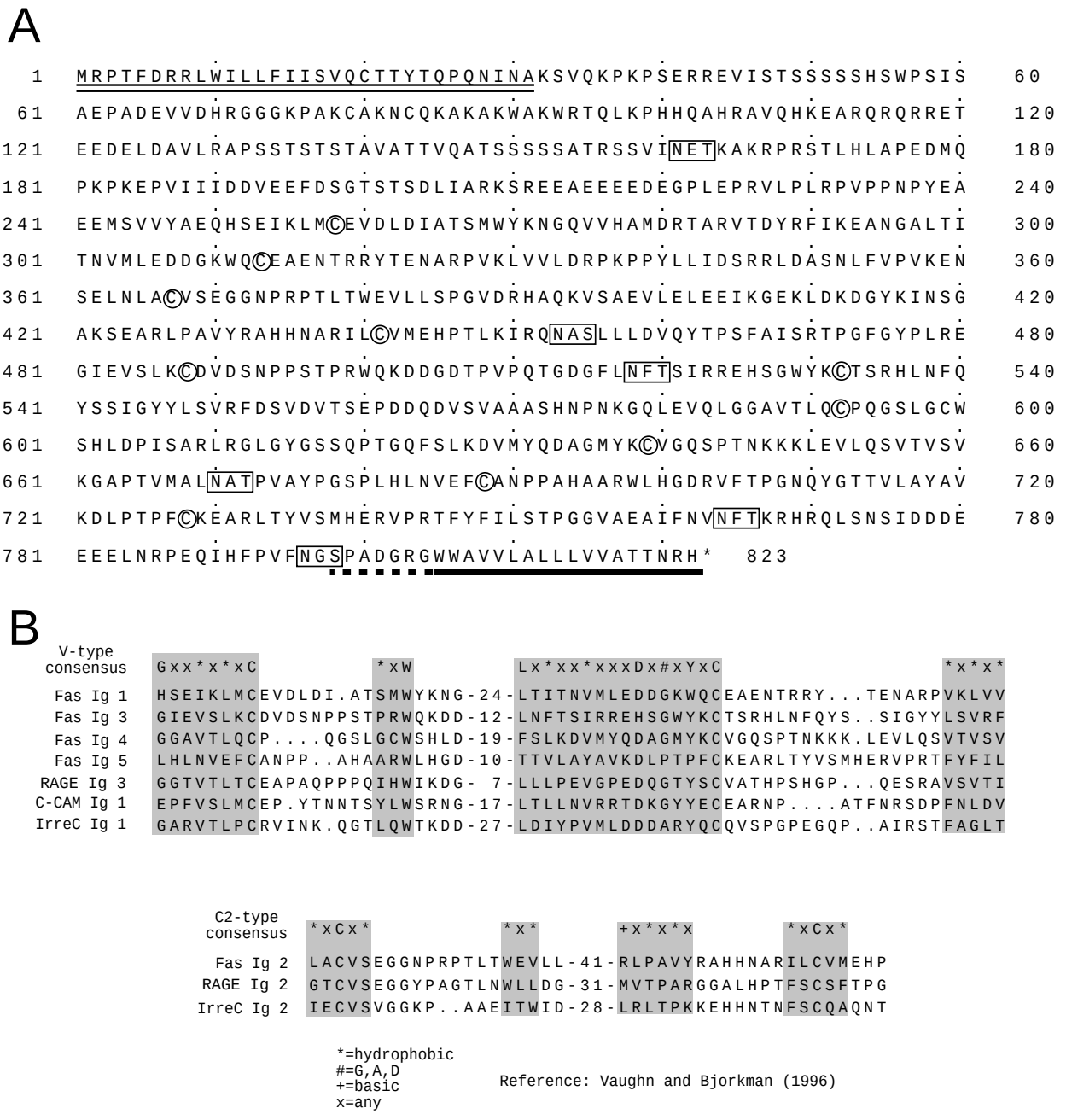

Fig. 4. Characteristics of the Fas protein sequence. (A) The complete amino acid sequence. Indicated are the putative signal sequence (double underline), the putative GPI cleavage/addition sequence (bold underline; the dashed portion reflects uncertainty regarding potential w and w+2 sites), the conserved cysteine residues of the five Ig domains (circled) and potential N-linked glycosylation sites (boxed). (B) Comparison of the five Fas Ig domains with selected domains from similar proteins. Domains were aligned to the consensus sequence from Vaughn and Bjorkman (1996). Fas domains 1, 3, 4 and 5 align best with the V-type consensus sequence. Fas domain 2 best fits the C2-type consensus. The *faint sausage* sequences reported here have been deposited in GenBank under accesion number AF059571.

show more than 30% identity to Fas. Presumably, the significant similarities between the proteins reflect similarities in structures of their respective Ig domains; these other proteins are unlikely to be orthologs of Fas. Thus, Fas represents a novel *Drosophila* member of the Ig superfamily.

fas expression during embryogenesis

Northern analysis using a cDNA probe reveals one major transcript of approximately 6.2 kbp (Fig. 3E), which indicates that the cDNA length of 5.9 kbp is very close to full length. This transcript is expressed from 6-24 hours post-fertilization (hpf), and a lower level of expression from 0-6 hpf was revealed by an overexposure of the blot (data not shown).

The expression of *fas* mRNAs was examined by whole-mount in situ hybridizations using a digoxigenin-labeled

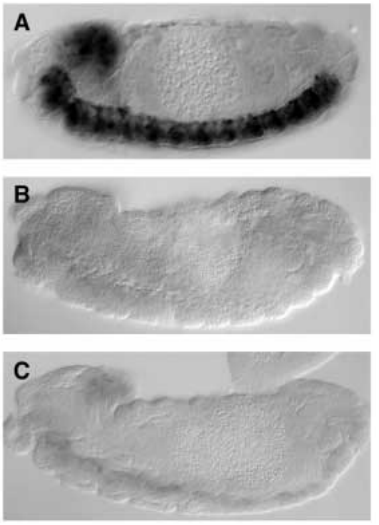

Fig. 5. Anti-Fas antibody is specific for *faint-sausage*. (A) Lateral view of stage 15 wild-type embryo showing Fas expression in CNS. (B,C) Lateral view of stage 15 fas^{P1411} (B; P-element insertion in *fas*-locus) and fas^{IIA} (C; EMS-induced allele) homozygotes. Staining with anti-Fas antibody is barely perceptible.

cDNA probe (Fig. 6A-E). During stage 10 we observed a widespread but weak expression of *fas* in all germ layers. In addition to the widespread expression, localized regions with higher expression levels were observed. In particular, in the ectoderm dorsally, laterally and ventrally, in the middle of each segment, there is a circular spot of *fas* expression corresponding to the region from which many sensillum precursor cells (SOPs) segregate (Younossi-Hartenstein and Hartenstein, 1997; de,ve in Fig. 6A). During later stages (stage 12 onward) *fas* expression becomes concentrated in the ganglion mother cells and neurons forming the CNS (Fig. 6B); at the same time, *fas* expression disappears in all other tissues except for the heart which, like the CNS, expresses *fas* at a high level until late embryonic stages (Fig. 6E). In the developing CNS, most ganglion mother cells and neurons express *fas* at some level throughout embryonic development into the larval period. In each neuromere, at the level of the two commissures, there is a coherent population of cells expressing *fas* more strongly than the remainder of the cells of the neuromere. Apart from this distinction of a 'high level' and 'low level' *fas* domain, the expression appears 'mottled', with individual or small groups of cells expressing at a higher level than their neighbors (Fig. 6C,D).

A polyclonal antiserum raised against a portion of the *fas* protein was used on western blots of embryonic extracts and for whole-mount immunohistochemical stainings of wild-type and *fas* mutant embryos. Specificity of the antiserum for Fas in whole mounts was confirmed by staining embryos homozygous for the deletion allele, fas^{PR95}. These embryos showed no detectable expression of the Fas antigen in comparison to their wild-type siblings (data not shown). The

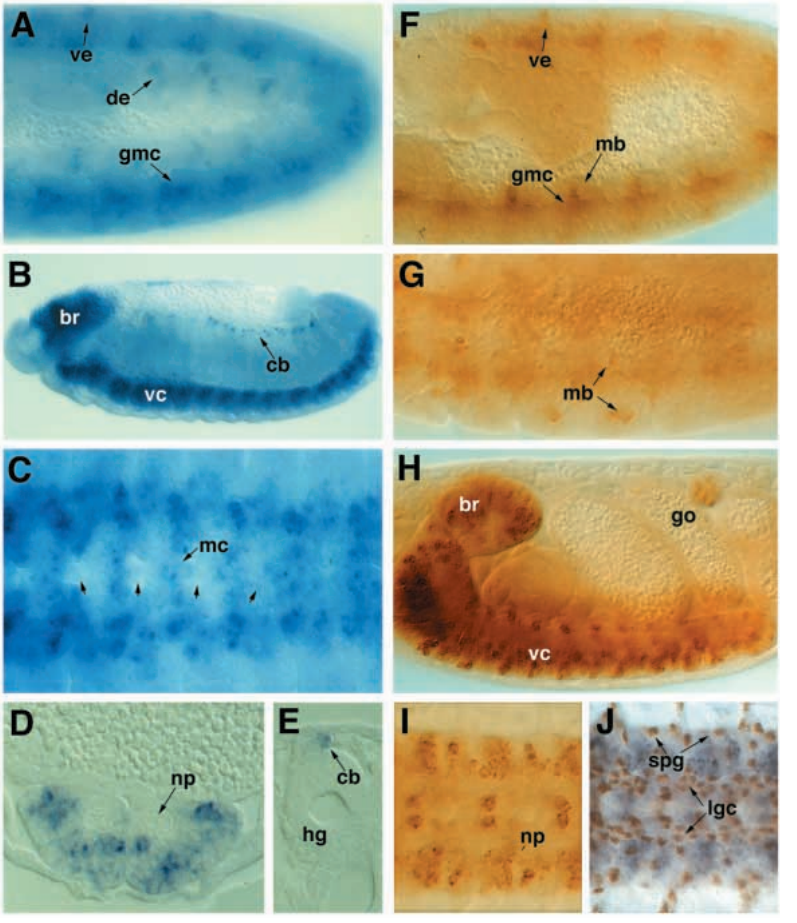

Fig. 6. Pattern of *fas* expression in the embryo. (A-E) Whole mounts (A-C) or sections (D,E) of wild-type embryos labeled by in situ hybridization with a *fas* cDNA (see Fig. 1). (F-J) Whole mounts of wild-type embryos labeled with a polyclonal antiserum against Fas-protein (see Fig. 1). (A) Stage 11, lateral view, showing patchy *fas* expression in ventral (ve) and dorsal ectoderm (de) and ganglion mother cells (gmc). (B) End of germband retraction (stage 12), lateral view, with *fas* transcript strongly concentrated in brain (br), ventral nerve cord (vc) and precursors of the heart (cb, cardioblasts). (C) Stage 13, ventral view. *fas* is expressed in entire CNS primordium, but appears to be concentrated in transverse stripes crossing the midline (mc, midline cells). Small arrows point at gaps between neuromeres. (D,E) Stage 16, cross sections, showing *fas* transcript in neurons of the ventral nerve cord (vc) and cardioblasts (cb) of the heart. hg hindgut; np neuropile. (F) Stage 11, lateral view, with Fas protein expressed on membranes of ectoderm cells (ve), ganglion mother cells and groups of myoblasts (mb). (G) Stage 12, ventrolateral view. Fas expression in clusters of myoblasts shortly before their fusion into multinucleate myocytes. (H,I) Stage 16. (H) Lateral view; Fas is concentrated on somata of the CNS and mesodermal cells forming the gonads (go). (I) Ventral view; Fas is not expressed at detectable levels on axons that form the neuropile (np). (J) Stage 15, ventral view, showing Fas expression in purple and Repo expression in brown. Repo marks glia cells (e.g., lgc, longitudinal glia cells; spg, subperineurial glia cells) which express Fas only at low levels, if at all.

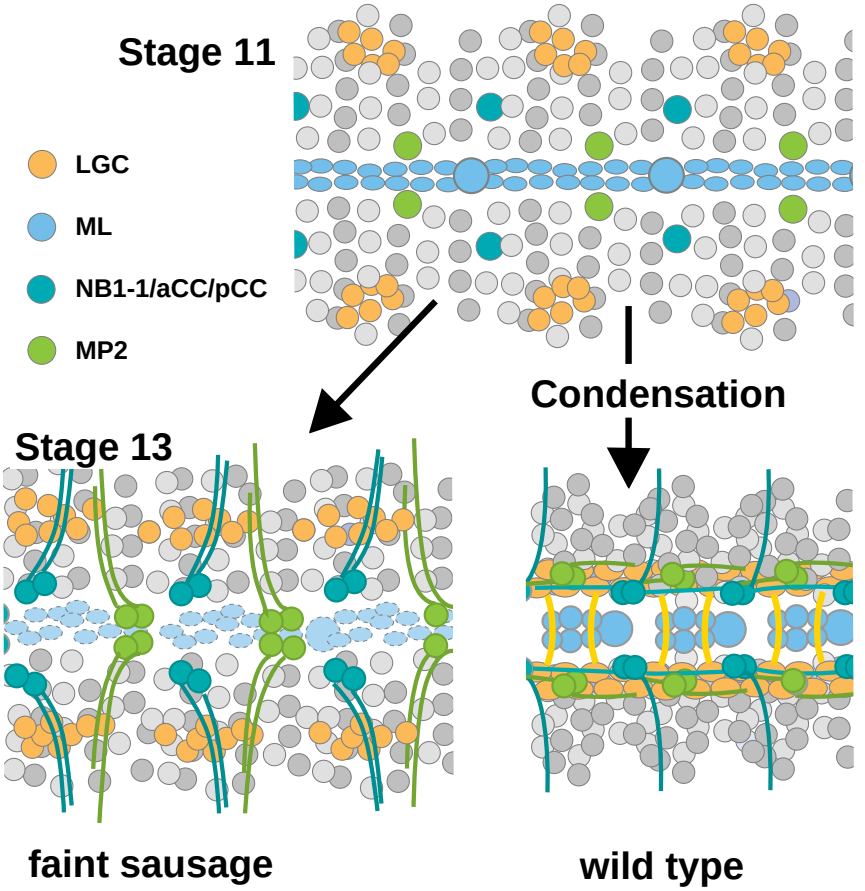

Fig. 7. Defects in neural morphogenesis and axonal pathfinding in *fas* mutant embryo. Top panel schematically depicts neural primordium of stage 11 embryo (identical in wild type and fas mutant), consisting of a layer of neuroblasts/glioblasts (circles) and the midline cells. The position of some identified cells (longitudinal glia cell precursors, MP2 neuroblast, NB-1.1 giving rise to aCC and pCC neurons) has been indicated by differential shading. Beginning with germband retraction, the wild-type CNS condenses, accompanied by movement of individual neurons and glia cells. During this phase, LGCs, MP2, aCC and pCC are brought in close contact with each other. Midline cells condense into compact, segmental clusters. MP2 neurons and pCC form longitudinal pioneer tracts on the track provided by the LGCs. Commissural tracts (yellow) cross the midline clusters. In *fas* mutant embryos, condensation is defective. Neurons and glia cells differentiate at ectopic positions, which may account for the dramatic defects in axonal pathfinding.

antiserum shows two signals on western blots of total embryo protein at ~100 kDa and 80 kDa (Fig. 3F). The 100 kDa band corresponds to Fas; it is absent in extracts of embryos homozygous for the fas^{PR95} deficiency allele (not shown). The 80 kDa species represents a cross-reacting band, since it is not affected by the absence of the *fas* locus in westerns. This band is specific to Westerns, since whole-mount stains of fas^{PR95} homozygotes do not detect any other expression pattern. Additionally, the fas^{P1411} allele and the fas^{IIA} allele show specific reductions in the level of expression of the 100 kDa band; the 80 kDa band is unaffected in these alleles (data not shown, see Fig. 5 for whole mounts). Thus, the *fas* gene encodes a protein of ~100 kDa.

The embryonic Fas expression pattern was studied with this antiserum in whole-mount stainings (Fig. 6F-J); Fas protein expression closely corresponds to the pattern described above for the mRNA, with the exception that protein expression is seen in the somatic cells of the gonads in late embryos, but in situ hybridization fails to detect *fas* mRNA in those cells. Following a weak, widespread expression during stages 10 and 11, Fas is expressed at fairly high levels in the CNS and heart. During early stage 12, there is a distinct expression in multiple clusters of early myoblasts (Fig. 6F,G), as well in the mesodermal precursors of hemocytes, which are located in the head mesoderm (data not shown). In all tissues, Fas is localized in a somewhat 'punctate' pattern at the cell surface (Fig. 6I). Since Fas contains a signal sequence and no transmembrane domain, we hypothesize that this staining represents protein localized to the outer cell surface. Interestingly, in the CNS, there was intense staining of the cortex, which contains neuron cell bodies and glia, while there was no detectable staining in the neuropile, which contains axons (Fig. 6H,I). Double labeling experiments, combining anti-Faint sausage antibody with antibodies against Repo (alias RK2, a homeodomain-containing protein expressed exclusively in glial cells; Campbell et al., 1994; Xiong et al., 1994) indicate that glial cells do not express significant levels of Fas (Fig. 6J). Thus, Faint sausage shows a very dynamic expression pattern, and in the CNS faint sausage appears to be expressed only on neuronal cell bodies.

In summary, *fas* encodes a novel extracellular protein of approximately 100 kDa that is a member of the immunoglobulin superfamily. The expression pattern of *fas* as revealed by whole-mount in situ hybridization and immunohistochemical staining is as predicted from the analysis of the mutant phenotype, i.e. tissue disorganization and cell movement defects in the epidermis and CNS. Interestingly, subsequent axonal pathfinding defects in mutant embryos appear not to be due to the loss of Fas from growth cones or axons, since Fas does not appear to be localized there. Instead, the fact that Fas is detected on neuronal cell bodies suggests that the observed axonal pathfinding defects are due to improper cell body migration during CNS condensation.

DISCUSSION

faint sausage is required during neuronal migration in *Drosophila*

Numerous studies in vertebrate systems highlight the significant role played by members of the Ig superfamily in several aspects of neural development, in particular neuronal migration, selective fasciculation of axons and promotion of neurite outgrowth. The most common proposal for the mode of functioning of Ig superfamily molecules in these events is through modulation of intercellular adhesion, and the results suggest that the regulation of adhesion molecules during neuronal migration is probably quite complex. For example, adding anti-N-cadherin antibody to explants of bird forebrain hastens radial migration of newly born neurons (Barami et al., 1994). In contrast, the same type of neuron requires a certain level of another adhesion molecule, Ng-CAM, for proper migration; blocking the function of Ng-CAM reduces migration (Barami et al., 1994). The same conclusion can be drawn from experiments knocking out the function of N-CAM-180, an N-CAM isoform widely expressed in the mammalian CNS (Tomasiewicz et al., 1993). Abolishing N-CAM-180 function blocks the migration of olfactory granule neurons, resulting in small and disorganized olfactory bulbs. These findings indicate that Ng-CAM and N-CAM mediate between neuronal precursors and their substratum (e.g., radial glia) a type of adhesion that is necessary for movement to occur.

We have shown that the gene *faint sausage* is required for several aspects of nervous system formation in *Drosophila*. Most notably, *fas* is required for the correct migration of several cell types in the CNS and also for proper ventral nerve cord condensation. Based on the expression pattern of the Fas protein and the unfolding of the *fas* phenotype, we propose the following model for *fas* function. Fas is expressed on the somata of undifferentiated neurons. It promotes adhesion during the phase of nervous system condensation when neurons compact and undergo the complex relocalization movements that are required for later phases of neuronal development, such as axonal pathfinding. Thus, condensation and relocalization create contacts between cells that were formerly separated. Neurons, such as the pioneers aCC/pCC, are brought into direct contact with the substratum upon which their axons grow. In the absence of *fas* function, condensation and neuronal relocalization fail to occur. As a consequence, neurons differentiate at abnormal positions, resulting in profound abnormalities in axonal outgrowth and pathfinding (Fig. 7). This model explains the consistent pathfinding defect observed in *fas* mutant embryos. The pioneers of the longitudinal tracts are not in contact with the LGCs, which remain in lateral positions and also do not form continuous longitudinal 'tracks' as in wild type. This could cause pCC and MP axons to grow laterally, instead of longitudinally. Later forming axons follow this course, so that longitudinal axon tracts and a regular neuropile are never formed. The grossly abnormal pattern and reduced packing density of the CNS may be also responsible for the generally reduced rate of axon formation in older *fas* mutant embryos. Thus, if neurons and/or glia secrete axonal growth-promoting molecules (as has been shown in both vertebrate and invertebrate systems, e.g. Gao et al., 1991; Ebens et al., 1993), the concentration of these molecules at any given position could be dependent on neuronal and glial packing density. Since the density of neurons and glia is reduced in *fas* mutants, concentrations of axonal growth promoting molecules may also be reduced; consequently, the result would be a decrease in axon formation. We cannot entirely rule out a direct involvement of Fas in growth cone guidance by providing a direct interaction between axons and cell bodies; however, this would require that Fas functions through heterophilic interactions since we have not been able to detect Fas on axons or growth cones. Additionally, if Fas were directly involved in growth cone guidance, one might expect that a null phenotype would result in a random directional outgrowth of pioneer axons, which we do not see. Thus, we consider this possibility unlikely.

fas encodes a novel member of the Ig superfamily

We have cloned the *fas* locus and identified a transcript that encodes a novel member of the Ig superfamily. Several lines of evidence show that the transcript that we have identified is *fas*. First, we were able to revert the transposon-induced null allele fas^{P1411} to wild type by genetically excising the P element. This demonstrates that the transposon was responsible for this line's *fas* phenotype. The cDNA that we identified lies within 100 bp distal to the transposon insertion. We found no evidence for the existence of a transcription unit within 8 kb proximal to the transposon insertion. Thus, the only transcription unit that lies close (i.e. within 8 kb) to the fas^{P1411} P-element insertion is the one that we have identified. Second, the expression pattern of the identified transcription unit and that shown by the β-gal reporter gene in the P1411 P element are identical; thus the P1411 reporter construct is reporting the expression of the identified transcription unit. This supports the assertion that there are no other nearby transcription units. Third, the $fas^{G5\text{-}29}$ translocation breakpoint maps to a position 30 kb downstream of the P1411 insertion, within the 5′ UTR of the identified transcription unit. Fourth, polyclonal antibodies raised against a portion of the identified *fas* product fail to stain embryos homozygous for the deletion allele, fas^{PR95}. This antibody also shows an extremely reduced level of staining on embryos homozygous for the independent allele fas^{IIA}. In accordance with these whole-mount results, a 100 kDa band is missing in protein extracts of fas^{PR95} homozygotes and is greatly reduced in fas^{IIA} homozygotes. Therefore, all lines of evidence support the assertion that the identified cDNA is *fas*.

fas encodes an extracellular protein containing five putative Ig domains. All of the proteins that Fas is most similar to have been implicated in cell adhesion in different contexts. For example, RAGE (Neeper et al., 1992) has been shown to be expressed in the rat nervous system where it may bind amphoterin to mediate neurite extension (Hori et al., 1995), and IrreC (Ramos et al., 1993), which is required for correct axonal pathfinding in the adult *Drosophila* visual system, may mediate its function through adhesive interactions (Schneider et al., 1995). It is tempting to speculate that Fas may also provide adhesion between cells as this is consistent with the mutant phenotype. However, in the absence of in vitro adhesion assays, this remains speculation. The deduced amino acid sequence also suggests that Fas may be found in a GPI anchored form. Our immunohistochemical localization experiments and preliminary results of treatments with PIPLC, which cleaves GPI anchors, support this (T. Haag, personal

communication), although we cannot rule out that Fas associates with cell membranes via another protein, nor can we rule out the existence of a secreted form that is found in the extracellular matrix. Future studies in our laboratory will address these issues.

We are grateful to Drs Benzer, Goodman, Laski, Tamkun, Tomlinson and Zipursky for flystocks and reagents. We thank E. Gruszynski-deFeo, G.deFeo, D. Godt, G. Liaw, A. Majumdar and A. Pickup for helpful discussions and technical advice which facilitated this study, and A. Aladadhi and P. Beitkanoun for excellent technical assistance. We thank the Bowling Green and Bloomington Drosophila Stock Centers for sending fly stocks. We appreciate the critical reading of this manuscript by Drs J. Lengyel and U. J. McMahan and members of the Hartenstein laboratory. This work was supported by a USPHS NRSA Traineeship to A. L., a HFSPO fellowship to U. T. and NIH Grant NS29367 to V. H.

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