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Characterization and Cloning of Fasciclin III: A Glycoprotein Expressed on a Subset of Neurons and Axon Pathways in Drosophila

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Summary

To identify candidates for neuronal recognition molecules in Drosophila, we used monoclonal antibodies to search for surface glycoproteins expressed on subsets of axon bundles (or fascicles) during development. Here we report on the characterization and cloning of fasciclin III, which is expressed on a subset of neurons and axon pathways in the Drosophila embryo. Fasciclin III is also expressed at other times and places including transient segmentally repeated patches in the neuroepithelium and segmentally repeated stripes in the body epidermis. Antisera generated against each of four highly related forms of the protein were used for cDNA expression cloning to identify a single gene, which was confirmed to encode fasciclin III by tissue in situ hybridization and genetic deficiency analysis.

Introduction

One important form of cell recognition during neuronal development is the selective affinity that growth cones display for specific axonal pathways (e.g., Goodman et al., 1984; Bastiani et al., 1985; Kuwada, 1986). For example, in insect embryos, growth cones are confronted with an orthogonal scaffold of longitudinal and commissural axon fascicles (or bundles) in each segment. Although their filopodial extensions contact many fascicles, these growth cones invariably choose to extend along (i.e., fasciculate with) particular bundles of axons, giving rise to stereotyped patterns of selective fasciculation (Raper et al., 1983b; Bastiani et al., 1984).

Previous studies led to the labeled pathways hypothesis, which predicts that axon fascicles in the embryonic neuropil are differentially labeled by surface recognition molecules which are used as guidance cues by neuronal growth cones (Ghysen and Janson, 1980; Goodman et al., 1982; Raper et al., 1983a, 1983b; Bastiani et al., 1984). This model was supported by specific cell ablation experiments in both grasshopper (Raper et al., 1983c, 1984; Bastiani et al., 1986a; du Lac et al., 1986; Doe et al., 1986) and fish (Kuwada, 1986) embryos.

To identify candidates for the recognition molecules implicated by these studies, we generated monoclonal antibodies (MAbs) that recognize surface antigens expressed on subsets of axon fascicles. In a previous study (Harrelson et al., 1986; Bastiani et al., 1986b, 1987), MAbs were used to identify two surface glycoproteins, called fasciclin I and II (70 and 95 kd, respectively), which are expressed on subsets of axon fascicles in the grasshopper embryo. Here we use the 7G10 and 2D5 MAbs to identify a third glycoprotein, called fasciclin III, which is expressed on yet a different subset of axon pathways in the Drosophila embryo. We chose Drosophila for these studies because of the potential to test function using genetic analysis.

The 7G10 and 2D5 MAbs stain the same pattern of cells and immunoprecipitate the same four highly related membrane glycoproteins (80, 66, 59, and 46 kd). Antiserum against each purified protein stains the same subset of cells as do the MAbs. Using these antisera for cDNA expression cloning, we have identified a single gene that maps by in situ hybridization to position 36E1 on the second chromosome. Confirmation that this gene encodes fasciclin III was obtained by both tissue in situ hybridization and analysis of embryos homozygous for deficiencies in the 36E1 region.

Results

Regional Expression of the 7G10/2D5 Antigen on a Subset of Neurons and Axon Pathways in the Drosophila Embryo

To generate MAbs against surface epitopes on subsets of embryonic axons, we used as immunogens membranes from mass-isolated 10–13 hr embryonic central nervous systems (CNS) and membranes from primary embryonic neurons grown in cell culture. MAbs were screened histochemically on the 10–13 hr Drosophila embryonic CNS. At this stage neurons are extending growth cones and axons but the events of synapse formation and terminal differentiation have not yet occurred (Thomas et al., 1984; Goodman et al., 1984).

The 7G10 and 2D5 MAbs recognize an antigen that is regionally expressed (i.e., expressed on restricted regions of a neuron and its processes) on the surface of a small subset of neuronal cell bodies and axons in the developing segmental CNS of the Drosophila embryo (Figure 1). The antigen is also expressed on a subset of non-neuronal (probably glial) cells during this same stage (Figure 1B). Additionally, the antigen is expressed on a subset of axon pathways in the brain and on a subset of sensory neurons in the head (see Figure 5E).

Axon outgrowth begins shortly before hour 10, just as germ-band retraction is ending (Thomas et al., 1984). By hour 12, the segmentally repeated ladder-like arrangement of longitudinal and commissural axon fascicles within the CNS, as well as the peripheral nerves exiting the CNS, has formed (Figure 1D). Each segment contains about 15–20 fascicles (or axon bundles) in the anterior (A) commissure, about 10–15 fascicles in the posterior (P) commissure, and about 15–20 longitudinal fascicles on each side. In addition, several fascicles turn laterally to form both the intersegmental (ISN) and segmental (SN) nerves.

At hour 12, the 7G10/2D5 antigen is expressed on the surface of axons in 5 commissural fascicles (3 in the anterior and 2 in the posterior commissure) and on the con-

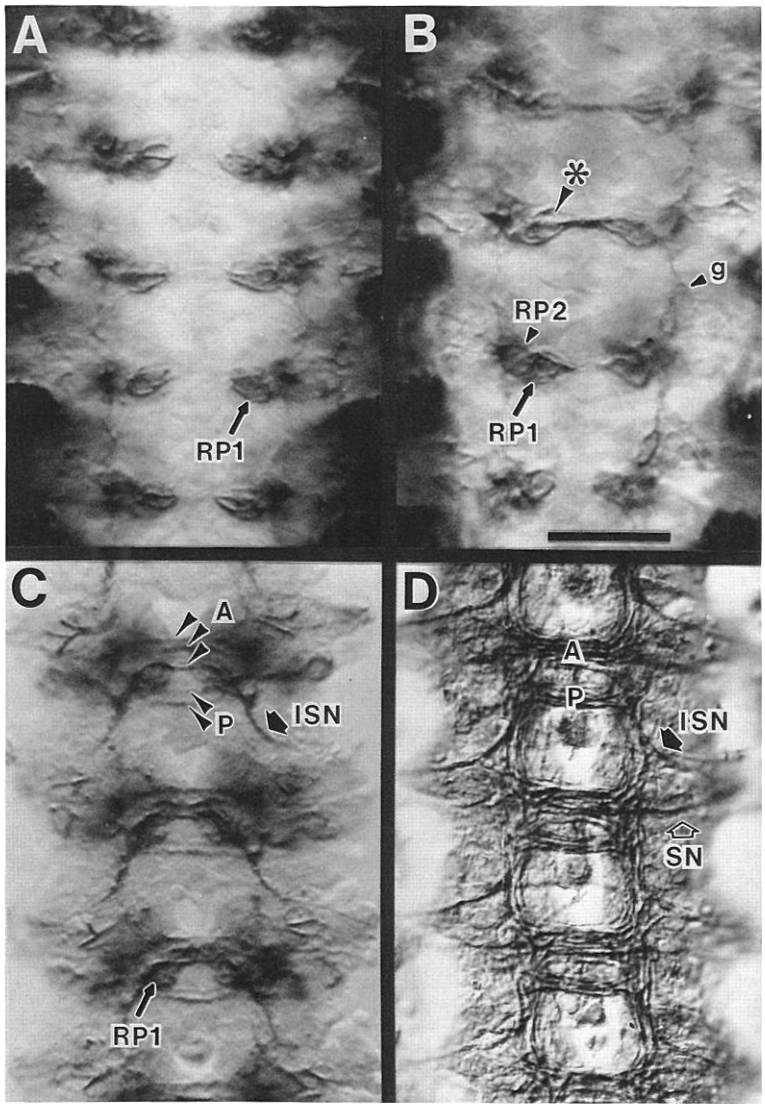

Figure 1. 7G10/2D5 Antigen Is Expressed on a Subset of Embryonic Neurons and Axon Pathways

Photographs of four contiguous segmental neuromeres of the Drosophila CNS (anterior is up) at hour 9:30 (A), hour 10 (B), and hour 12 (C and D) of embryonic development, as shown in whole-mount (A and B) or dissected (C and D) embryos stained with the 7G10 MAb (A, B, and C) or anti-tubulin Ab (D) and HRP immunocytochemistry. (A) Immediately before axonogenesis, the 7G10/2D5 antigen is expressed on the surface of a small group of neurons and glia (g), including the RP1 and RP2 neurons (the RP2 cell body is immediately next to RP1 and partly out of the plane of focus). (B) After the onset of axonogenesis, the 7G10/2D5 antigen is strongly expressed on the RP1 growth cone (asterisk) and only weakly expressed on the RP2 growth cone. (C) By hour 12, the segmentally repeated ladder-like arrangement of longitudinal and commissural axon fascicles within the CNS, as well as the two peripheral nerves exiting each segment, has formed (see D). At this stage, the 7G10/2D5 antigen is expressed on the surface of axons in 5 commissural fascicles (3 in the anterior or A and 2 in the posterior or P commissure) and on the continuation of one of the A commissure fascicles as it turns posteriorly and laterally (as the RP1 fascicle) toward one of the two peripheral nerves: the intersegmental nerve (ISN) (segmental nerve, SN). See text for additional details. (Panel [D], courtesy of J. Thomas.) Scale bar: (A and B) 30 μm, (C and D) 25 μm.

tinuation of one of the anterior commissure fascicles as it turns posteriorly and laterally toward the ISN (Figure 1C). The 7G10/2D5 antigen is also expressed on the surface of a small subset of neuronal cell bodies, including some of the neurons whose axons express the antigen (e.g., RP1; Figure 2) and at least one neuron whose axon does not (RP2; Figure 2). Of the 15 or so 7G10/2D5-positive neuronal cell bodies, only the RP1 and RP2 neurons have been previously well characterized (Thomas et al., 1984; Goodman et al., 1984; Bastiani et al., 1985).

One important observation is that the antigen is regionally expressed on only certain portions of the 7G10/2D5-positive neurons. The RP1 neuron expresses the 7G10/2D5 antigen on its cell body and primary axon within the CNS (Figure 1C); however, as the axon exits the CNS in the ISN, the level of the antigen on that portion of the axon dramatically decreases. The RP2 neuron expresses the 7G10/2D5 antigen only on its cell body; its axon, which extends along a 7G10/2D5-negative pathway, does not express the antigen (Figure 2). The neurons with axons extending across in the commissures are typically interneurons whose axons then turn into one of the longitudinal fascicles. Thus, we infer from the staining of 5 different commissural fascicles and none of the longitudinal fascicles that these neurons are likely to express the 7G10/2D5 antigen regionally on only the portions of their axons within these commissural fascicles. Thus, the 7G10/2D5 antigen is not necessarily expressed over the entirety of a neuron, but rather its regional expression correlates with the patterns of axon fasciculation.

Positional Expression of the 7G10/2D5 Antigen at Other Times and Places throughout Development

In addition to its expression on a subset of neurons and axon pathways in the Drosophila embryo, the 7G10/2D5 antigen is also expressed at a variety of other times and places. It is positionally expressed on segmentally repeated patches of cells during neurogenesis (Figures 3A and 4) and outside the developing CNS on segmentally repeated stripes in the epidermis at the segmental

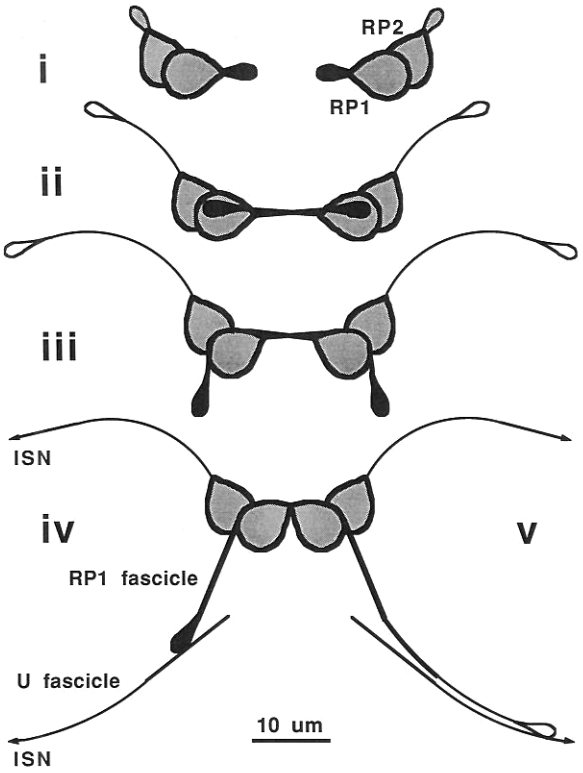

Figure 2. 7G10/2D5 Antigen Is Regionally Expressed during Differentiation of RP1 and RP2 Neurons

Five stages (i–v; from 10:00–10:45 hr) in the differentiation of the RP1 and RP2 neurons in relationship to their regional expression of the 7G10/2D5 antigen (denoted by darkened axons and growth cones and shaded cell bodies). Drawings are based on intracellular injections of Lucifer yellow and immunocytochemistry with the SOX2 and 7G10/2D5 MAbs. The RP1 and RP2 cell bodies begin to express the 7G10/ 2D5 antigen at about the time they first extend growth cones (i). The two bilaterally symmetric RP1 axons extend across the midline, fasciculate with one another, and flatten out on and adhere to each other's cell body. In contrast, the growth cone of the sibling RP2 neuron makes a divergent choice and extends anteriorly and laterally along a 7G10/2D5-negative pathway. The RP2 growth cone is initially weakly positive (i), but shortly thereafter both the RP2 growth cone and the axon are 7G10/2D5-negative (ii). The RP1 growth cone continues to express the antigen as it then turns posteriorly and extends toward the U fascicle (iii and iv). Other 7G10/2D5-positive axons fasciculate with the RP1 axon in the RP1 fascicle. As soon as the two contralateral RP1 growth cones contact one another's cell body, the two cell bodies migrate toward the midline (ii–iv). After the RP1 growth cone contacts the U fascicle (whose axons are 7G10/2D5-negative) and begins to extend laterally out the ISN, the level of antigen expression on that portion of its axon dramatically decreases (v). In the RP1 fascicle, the RP1 axon continues to express the antigen, whereas in the peripheral ISN, it does not.

grooves (Figures 3B, 4A, 4B, 4C, and 4F) (see following sections).

The 7G10/2D5 antigen is also expressed on patches of epithelial cells near the stomodeal and proctodeal invaginations (Figures 3, 5D, and 5F), on the visceral mesoderm that migrates as a sheet around the gut (but not on the somatic mesoderm) (Figures 3A, 4C, and 4E), and on the luminal surface of the salivary gland epithelium. Expression near the stomodeum begins as a narrow patch of epithelium at the midline of the ventral-most tip of the clypeolabrum (Figures 3A, 5D, and 5E). By the end of germ-band retraction, the antigen is expressed on the entire midline of the clypeolabrum and on the lining of the pharynx (Figure 3B). Expression on the visceral mesoderm begins shortly after gastrulation on a single row of cuboidal cells extending along each side of the embryo (Figures 3A, 4C, and 4E). During germ-band shortening, the 7G10/2D5-positive mesodermal cells elongate dorso-ventrally, divide, and begin to migrate dorsally around the gut.

The antigen is not evenly expressed on the entire surface of the 7G10/2D5-positive cells. For example, in the clypeolabrum it is expressed at higher levels where 7G10/ 2D5-positive cells contact one another (e.g., Figure 5D).

Transient Expression of 7G10/2D5 Antigen on Segmentally Repeated Patches of Neuroepithelial Cells and underlying Neuronal Lineages

The 7G10/2D5 antigen is not expressed before or during gastrulation. However, after germ-band extension, the antigen is transiently expressed on segmentally repeated patches of neuroepithelial cells and specific underlying neuronal lineages during the period of neurogenesis (Figures 3A and 4).

During neurogenesis, each hemisegment in the ventral neurogenic region is about 12 cells long and about 6–7 cells wide from midline to lateral edge. A stereotyped array of 26 neuroblasts (NB) delaminate from about 80 neuroepithelial cells in each hemisegment (Doe et al., unpublished results). Each NB divides asymmetrically to generate a characteristic column of progeny, called ganglion mother cells (GMCs), each of which divides once more symmetrically to generate pairs of sibling neurons. These columns of NB progeny add to the layers, which, starting just under the outer epithelial sheet, are the thick NB layer, two thin GMC layers, and several thin layers of neurons (Figure 4D). Although the neuroepithelial sheet in insects appears morphologically uniform, previous experiments have shown that the NBs, GMCs, and neurons that derive from it are all uniquely identified cells (Doe et al., 1985; Doe and Goodman, 1985). The restricted expression of the 7G10/2D5 antigen in the neuroepithelium is consistent with its playing a role in the patterning and specification of cell fates during neurogenesis.

The bilaterally symmetric patches of 7G10/2D5-positive epithelial cells lie just lateral of the midline and nearly half way between the segmental grooves (Figure 3A). The patches of neuroepithelial cells are about 3–4 cells long and 4 cells wide, thus constituting about 20% of the neuroepithelial surface. The antigen is not evenly expressed on the entire surface of the 7G10/2D5-positive neuroepithelial cells. Rather, it is expressed at higher levels where 7G10/2D5-positive cells contact one another (e.g., Figure 4A). The expression of the antigen on neuroepithelial patches is out of phase with the striped epidermal expression at the segmental grooves.

There is little expression of the antigen on the 2–3 NBs that directly underlie the 7G10/2D5-positive patches (Figure 4D). In contrast, expression of the 7G10/2D5 antigen greatly increases on the GMCs under these NBs, and in-

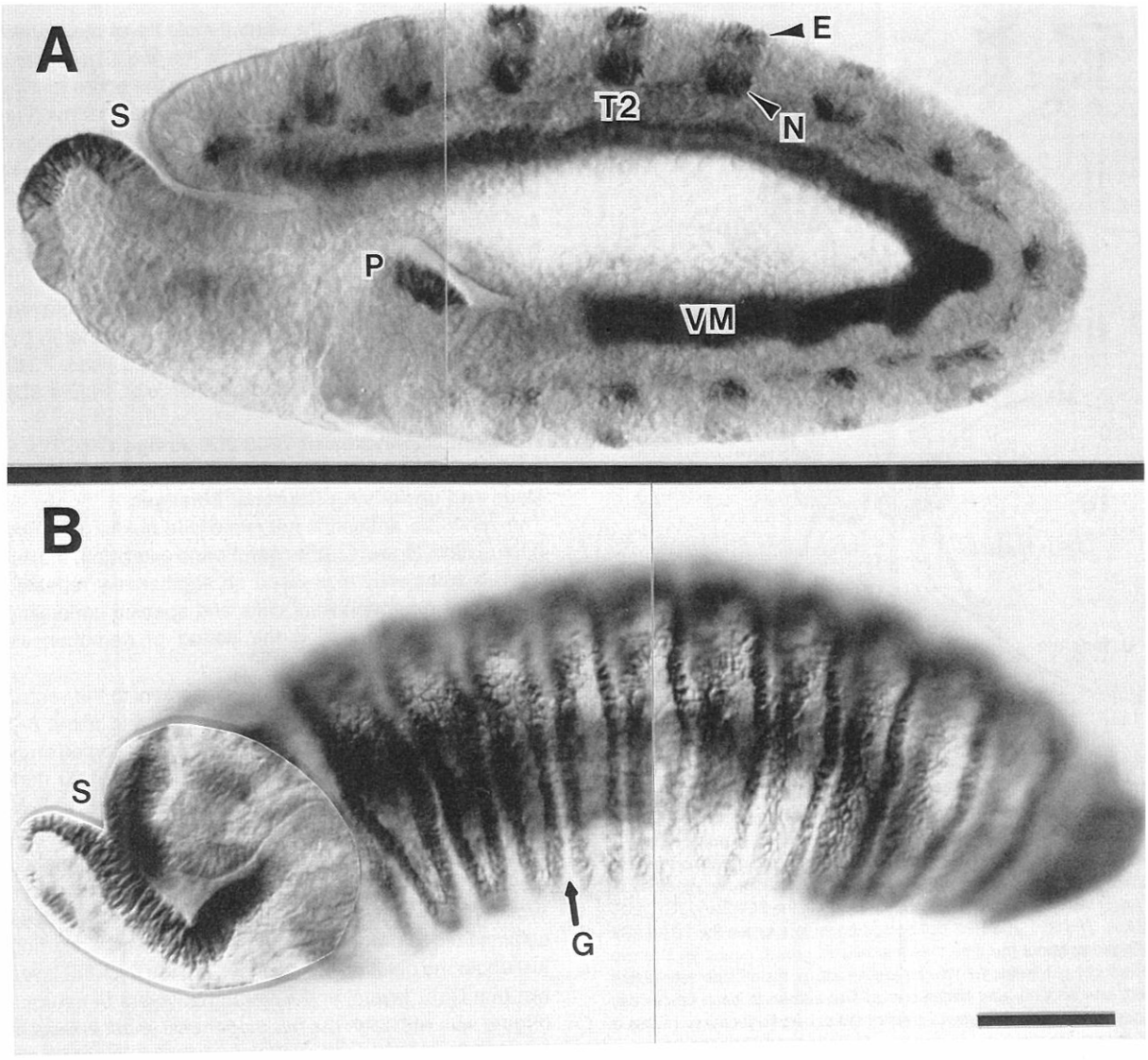

Figure 3. Patterns of Expression of 7G10/2D5 Antigen during Embryogenesis

Photo-montages of whole-mount embryos at hour 7 (A) and hour 10:30 (B) of development stained with the 7G10 MAb and HRP immunocytochemistry (anterior is left, ventral is up). (A) The 7G10/2D5 antigen is expressed in several different tissues in the germ-band-extended embryo, including segmentally repeated patches of neuroepithelial cells (E), underlying neuronal lineages (N; see Figure 4), epithelial patches near the stomodeal (S; this patch is part of the clypeolabrum) and proctodeal (P) invaginations, and the visceral mesoderm (VM, a line of cells extending from the beginning of the cephalic segments to abdominal segment A7) (see Figure 4C). (B) During dorsal closure (and after germ-band shortening), the 7G10/2D5 antigen is expressed in segmentally repeated stripes along and within the segmental grooves (G) of the body epidermis (see Figure 5). In addition, the epithelial expression near the stomodeum (S) has expanded to include the entire lining of the presumptive pharynx. Scale bar: 50 μm.

creases even more on the neurons under these GMCs (Figures 4D and 4E). The patch of 7G10/2D5-positive GMCs and neurons is larger in the thoracic segment, than in the abdominal hemisegments (Figure 3A) and increases during neurogenesis as more neurons are generated (compare Figures 3D and 3E). The antigen is thus a marker of specific NB lineages during neurogenesis.

The 7G10/2D5 antigen is also expressed on a small subset of non-neuronal (probably glial) cells that lie on the inside surface of the neurogenic region just under the basement membrane (see g in Figure 4B, 4D, and 4E). Although the entire inside surface of the neurogenic region is lined by glia (Jacobs and Patel, unpublished results), only those cells in a certain region near the segment border express the antigen, and just as in the neuroepithelium, it is expressed at a much higher level where these 7G10/2D5-positive glial cells contact one another.

The expression of the antigen on neuroepithelial patches is transient. It disappears first in the abdominal segments at around hour 8, but remains at this time in the thoracic segments. As the ventral epithelial cells elongate laterally, the thoracic patches enlarge laterally and then disappear at around hour 10. We do not know the ultimate fate of all of the 7G10/2D5-positive neurons. Although

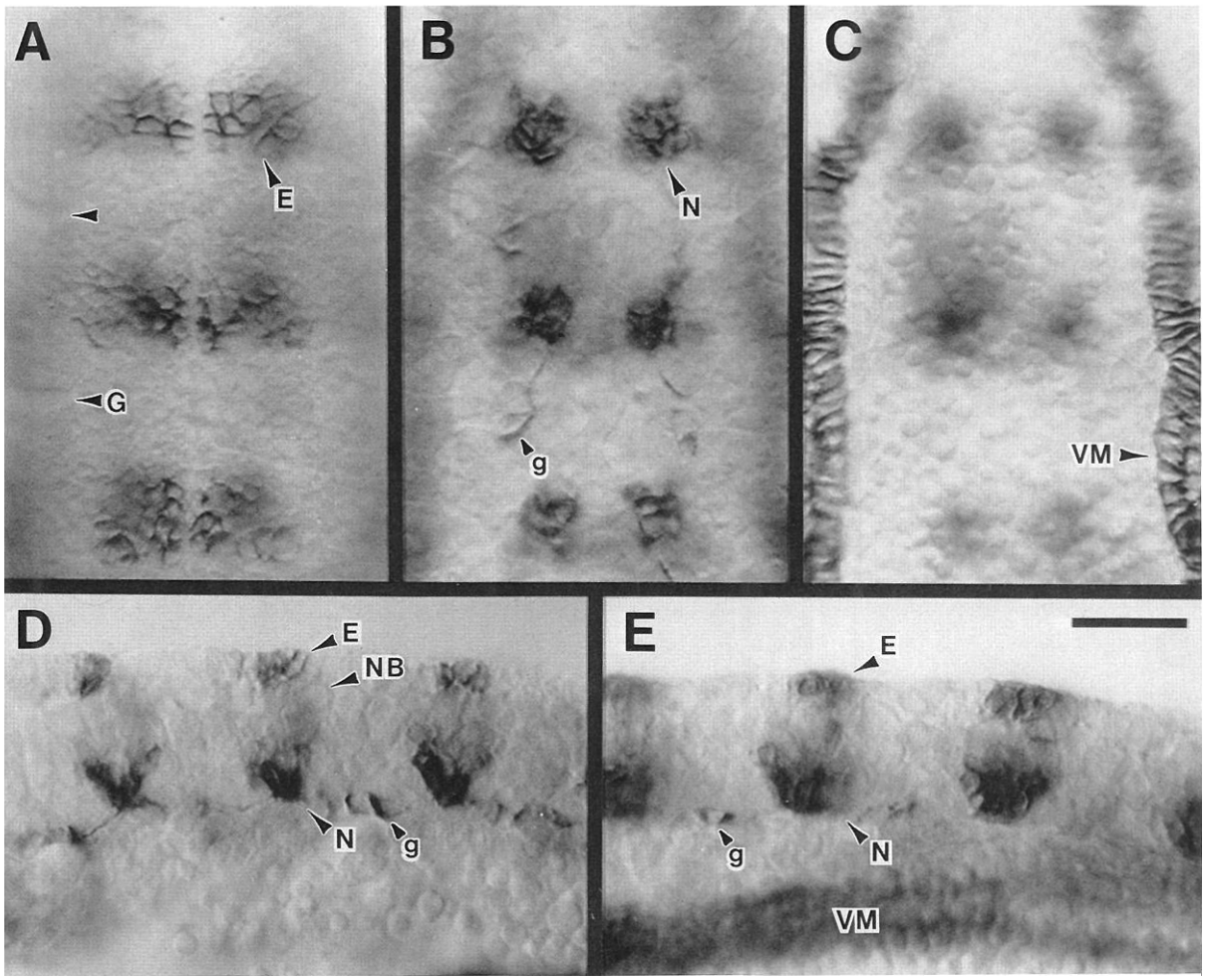

Figure 4. 7G10/2D5 Antigen Is Positionally Expressed during Neurogenesis

Photographs of three contiguous segmental neuromeres of the Drosophila CNS in whole-mount embryos (A–C, anterior is up; D and E, anterior is left, ventral is up) at hour 6 (D) and hour 7 (A–C and E) of embryonic development stained with the 7G10 MAb and HRP immunocytochemistry. (A, B, and C) Three successively deeper focal planes of segments T1–T3 in the same embryo, showing the outer neuroepithelial layer (A), the middle neuronal layer (B), and the inner visceral mesoderm and yolk layer (C). (A) The 7G10/2D5 antigen is expressed on segmentally repeated patches of neuroepithelial cells (E) that lie out of register with the segmental grooves (G). (B) The 7G10/2D5 antigen is expressed on segmentally repeated patches of neurons (N) and glia (g). (C) The 7G10/2D5 antigen is expressed on the bilaterally symmetric rows of visceral mesoderm cells (VM). (D and E) Lateral views of segments T1–T3 at two different stages of neurogenesis show expression of the 7G10/2D5 antigen on the patches of neuroepithelial cells (E), neurons (N), and glia (g), but expression is greatly reduced on the neuroblasts (NB). Note the increase in the size of the patches of 7G10/2D5-positive neurons over time as more neurons of the 7G10/2D5-positive lineage(s) are born. Scale bar: 25 μm.

some of them are likely to extend 7G10/2D5-positive axons during the later stage of axon outgrowth, it is probable that some of the initial 7G10/2D5-positive neurons stop expressing the antigen during axon outgrowth, and some neurons from other lineages begin expressing the antigen during these stages. Thus, there is not an absolute one-for-one correlation between the neurons that express the antigen during these two stages of neuronal development.

Expression of 7G10/2D5 Antigen on Segmentally Repeated Stripes at the Segmental Grooves in the Body Epidermis

When the segmental grooves first form, epidermal cells do not express the antigen, but shortly before the beginning of germ-band retraction, the 7G10/2D5 antigen is expressed on the surface of epidermal cells in the grooves, first in the abdominal segments and then in the thoracic segments. In all segments, antigen expression begins laterally near the boundary of the neurogenic region, rapidly spreads dorsally, and later spreads ventrally. By the end of germ-band shortening, the 7G10/2D5 antigen is expressed in repeated stripes across all of the body segments (Figures 3B and 5A). 7G10/2D5-positive cells can be seen at the bottom and along the sides of the grooves (Figure 5F). In some segments, the 7G10/2D5 antigen is expressed at higher levels on the anterior margins than on the posterior margins of the groove (Figures 3B and 5A). By the time head involution occurs and the grooves

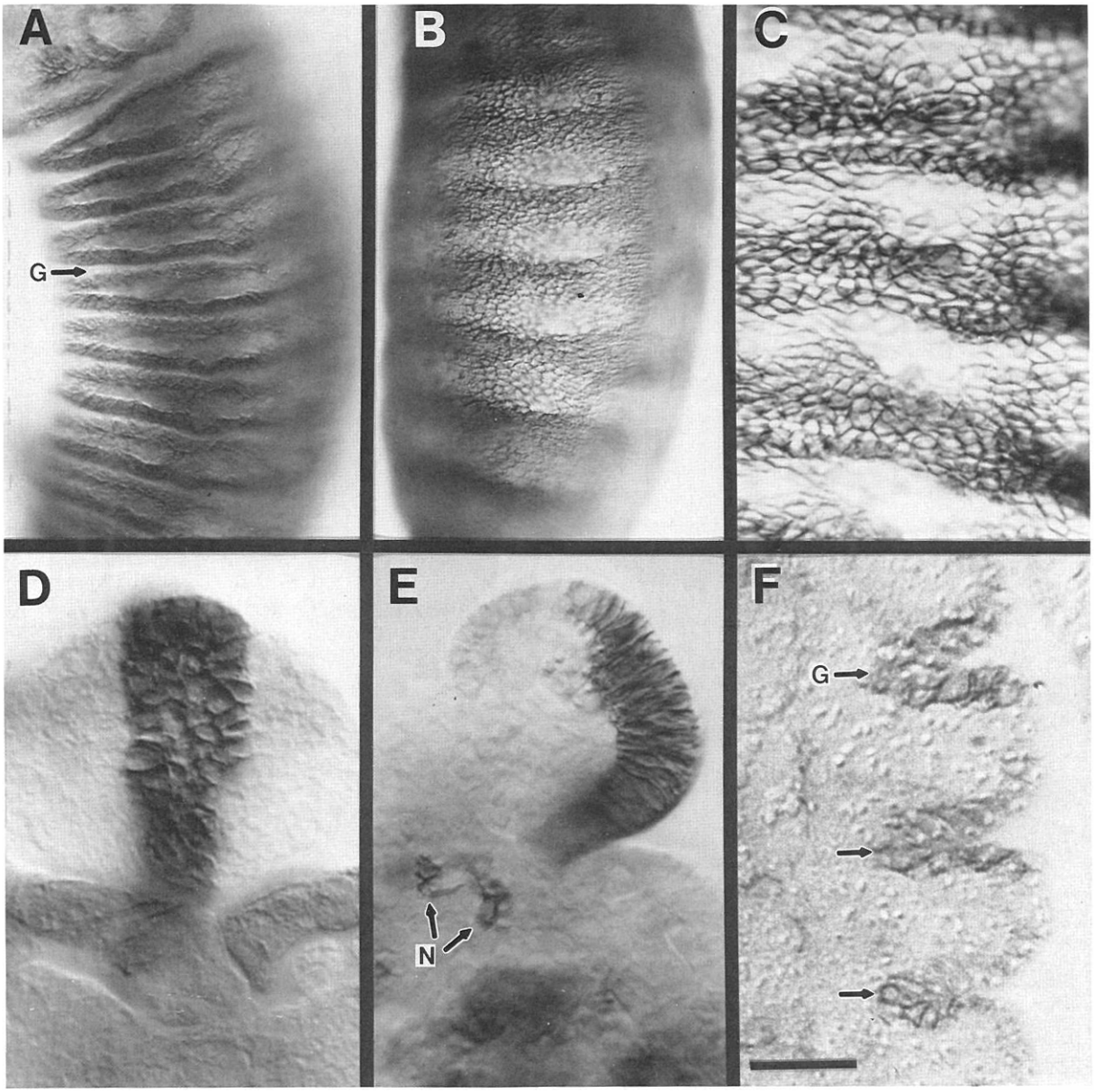

Figure 5. 7G10/2D5 Antigen Is Positionally Expressed in the Body Epidermis

Photographs of whole-mount embryos (A, B, D, and E), dissected epidermis (C), or sectioned embryo (F) (anterior is up, ventral is right in all except [D] and [F]) at hours 10:30 (A and F), 13 (B), 12 (C), and 7 (D and E) of embryonic development stained with the 7G10 MAb and HRP immunocytochemistry. (A) the 7G10/2D5 antigen is expressed along and within the segmental grooves (G), as further shown in the 4 μm plastic section in (F). The antigen is expressed at a higher level along the anterior margin of the grooves in some segments. (B and C) By the time head involution occurs and the grooves smooth out, all epidermal cells express the antigen, although at varying levels that maintain the segmentally repeated striped pattern. (D and E) Ventral and lateral views, respectively, of the staining of a narrow band of epithelial cells in the clypeolabrum near the stomodeal invagination (N marks staining of sensory neurons in the head). Scale bar: (A and B) 50 μm, (C) 25 μm, (D and E) 20 μm, (F) 17 μm.

smooth out, all epidermal cells express the antigen, although at varying levels that maintain the segmentally repeated striped pattern (Figures 5B and 5C).

The segmentally repeated expression of the antigen in the epidermis at the segmental grooves is out of phase with the earlier segmentally repeated patches in the neuroepithelium. By the time neurogenesis is over, the epidermal expression of the antigen at the segmental grooves begins to spread ventrally across the epidermis that was previously the neurogenic region.

The 7G10/2D5 MAbs Immunoprecipitate Four Highly Related Membrane Glycoproteins Called Fasciclin III

The 7G10 and 2D5 MAbs reproducibly immunoprecipitate four different membrane proteins of apparent molecular

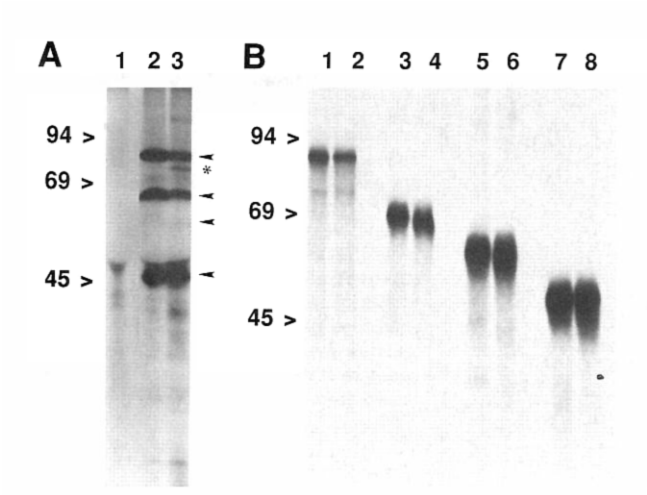

Figure 6. Analysis by SDS–PAGE of the Four Fasciclin III Proteins Recognized by the 7G10 and 2D5 MAbs

(A) Membranes prepared from enriched 10–13 hr embronic nerve cords were labeled with ^{125}I and lactoperoxidase, and the membrane proteins were solubilized with NP40. Immunoprecipitations using normal mouse serum (lane 1), 2D5 MAb (lane 2), or 7G10 MAb (lane 3) were performed as described in Experimental Procedures. The immunoprecipitates were analyzed on a 10% polyacrylamide gel according to Laemmli (1970) under reducing conditions. Solid arrows indicate the positions of the four proteins recognized by the MAbs. The protein marked by the asterisk is probably a degradation product, as it is not observed reproducibly. Molecular weight markers are indicated in kd.
(B) Analysis of the carbohydrate content of the fasciclin III proteins. The fasciclin III proteins were purified from 10–20 hr embryos solubilized in NP40. The individual proteins were purified further by preparative SDS–PAGE and electroelution and labeled with ^{125}I and Chloramine T. Each protein was then treated with PNGase F or treated identically, but without the addition of enzyme as control. Lanes 1, 3, 5, and 7 are the control digestions of the 80, 66, 59, and 46 kd species, respectively. Lanes 2, 4, 6, and 8 represent the results upon addition of PNGase F for the 80, 66, 59, and 46 kd proteins, respectively. The digestion products were analyzed on an 8.5% polyacrylamide gel under reducing conditions.

weights 80, 66, 59, and 46 kd from membranes prepared from the mass-isolated Drosophila embryo CNS (Figure 6). Although one of the four proteins (59 kd) is consistently present in lower relative abundance when isolated from embryo CNS membranes (Figure 6A), this species is present in greater relative abundance in whole embryo lysates (Figure 6B). The four proteins are not covalently linked as indicated by comparison of reduced and nonreduced gels. All four proteins appear to be glycosylated as indicated by their binding to the lectin concanavalin A (data not shown).

To characterize and compare the four proteins more completely, microgram quantities of each glycoprotein were purified from solubilized Drosophila embryos using affinity chromatography, followed by preparative gel electrophoresis. The gel-purified proteins were used in two ways: to generate antisera in rats against each of the four proteins and for additional biochemical experiments.

We first showed that the four proteins are highly related using two methods. In initial experiments, we used the antisera generated against each of the purified proteins for analysis by Western blot as well as immunoprecipitations. Each antiserum specifically recognized all four proteins (data not shown). While this might indicate similarities in the peptide core, the results could also be explained by a common carbohydrate moiety. To distinguish between these two alternatives, our second approach involved analysis of the four proteins by two-dimensional tryptic peptide maps. Comparison of these four two-dimensional peptide maps indicates that 12 or so characteristic fragments are identical and, thus, that the protein cores are highly related (Figure 7).

While the four proteins are highly related, there are nevertheless significant differences in their protein cores. This was demonstrated using two approaches. First, we treated all four proteins with either trifluoromethanesulfonic acid (TFMS), which has been shown to cleave both N- and O-linked sugars (Edge et al., 1981), or peptide:N-glycosidase F (PNGase F), which cleaves only N-linked sugars (Plummer et al., 1984). In both cases, similar results were obtained. Only the 66 kd and 59 kd species showed significant decreases in mobility when analyzed by SDS–PAGE (Figure 6). This result is inconsistent with the observation that all four proteins bind to concanavalin A. It is possible that the two clearly glycosylated species form complexes with the two apparently nonglycosylated forms. Alternatively, the 80 kd and 46 kd proteins may possess carbohydrate chains that are small in mass compared with the mass of the protein. In this case the change in migration upon cleavage of the sugars might not be detected.

The structural homology among the four proteins could also be explained by proteolytic events occurring during preparation. To address this possibility, we reexamined the two-dimensional peptide maps after much longer exposure times, when many more peptides were visible. While the 12 or so signatory fragments noted above are identical (solid arrows in Figure 7), there are numerous fragments that show significant differences (open arrows in Figure 7). As these samples are digested to completion with trypsin, numerous differences between the proteins would not be expected if they represent proteolysis products of each other. This result suggests that the observed proteins have related but different protein cores that are not derived from each other by proteolysis.

The names fasciclin I and II have been used to refer to two different membrane glycoproteins (70 and 95 kd, respectively) in the grasshopper embryo that are expressed on different subsets of axon pathways (Harrelson et al., 1986; Bastiani et al., 1986b, 1987). As shown below, all four forms of the protein described here are highly related and appear to be encoded by either a single gene or tandemly duplicated genes. Because one or more of these highly related glycoproteins is expressed on a subset of axon pathways in the Drosophila embryo, we call them the fasciclin III glycoproteins.

The antisera against all four forms of the protein specifically recognize the same subsets of cells both within and outside the developing CNS as do the two MAbs (see Figure 9B). Because the antisera are likely to recognize many epitopes common to all four forms of the protein, they do not allow us to distinguish any potential differences in the

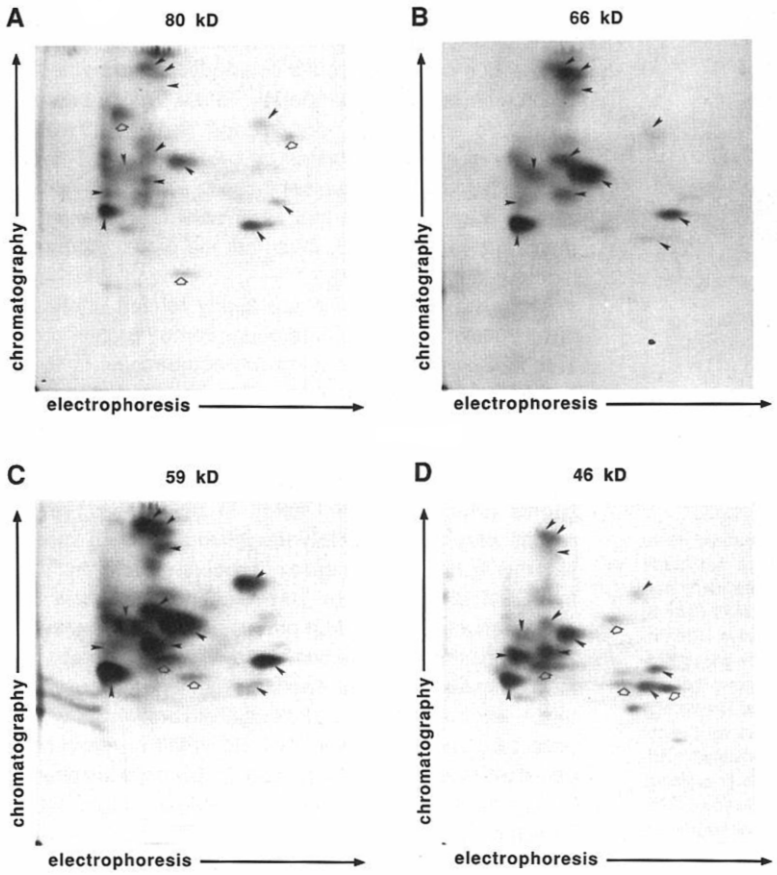

Figure 7. The Four Fasciclin III Proteins Are Structurally Related

The four individual fasciclin proteins were isolated and labeled with ^{125}I (see Figure 7). Each species was digested to completion with trypsin and applied to two-dimensional peptide maps on cellulose-coated TLC plates as described in Experimental Procedures. (A) 80 kd protein, (B) 66 kd protein, (C) 59 kd protein, and (D) 46 kd fasciclin III protein. Peptides common to all of the fasciclin III proteins are indicated by solid arrows. Peptides unique to individual proteins are marked by open arrows. Upon longer exposure of the maps, additional unique peptides present in each digest could be identified.

temporal and spatial expression of each form. Such analysis will have to await immunological probes that distinguish the different proteins.

Isolation of cDNA Clones

Each of the four proteins is N-terminal blocked (P. Snow and J. Schilling, unpublished results); moreover, sufficient protein for the generation of fragments for sequence analysis was not available. However, because we had antisera directed against each of the four highly related proteins, we chose to isolate cDNA clones by screening a 10–13 hr Drosophila embryo CNS λgt11 cDNA library with these antisera. Independent recombinants (4.5×10^5) were screened with the anti–80 kd antiserum, and 7 antibody-positive plaques were isolated.

The 7 cDNA clones (1–7) had inserts of 0.4, 0.5, 0.5, 1.4, 1.4, 1.4, and 1.5 kb, respectively. We rescreened all 7 clones with two of the other antisera and found that clones 2–7 were positive with the anti–59 kd antiserum and clones 4–7 were positive with the anti–46 kd antiserum. We showed that all 7 cDNA clones are highly related by cross-hybridization. Furthermore, all 7 fragments are derived from the same gene (or tandemly duplicated genes) as shown by genomic Southern analysis: the clones hybridize to a single 14 kb BamHI fragment (complete molecular analysis will be presented in a future publication). We used in situ hybridization to the polytene chromosomes with the 1.5 kb insert from cDNA clone 7 to map this gene to position 36E1 on the left arm of the second chromosome (data not shown).

Confirmation of the Identity of the Fasciclin III Gene

Since protein sequence data were unavailable, we used two methods to confirm the identity of the fasciclin III gene: tissue in situ hybridization and genetic deficiency analysis.

Given the highly patterned expression of the fasciclin III glycoproteins at a variety of different times and places during development, we reasoned that if tissue in situ hybridization revealed the same pattern of expression, it would be a strong indication that we had cloned the right gene. We incubated fixed and permeabilized embryos at a variety of different ages with a ^{35}S-labeled probe (Mahoney and Lengyel, 1987) made using the 1.5 kb insert from clone 7 and prepared sections of these embryos for autoradiography. The in situ analysis revealed expression of this gene in stripes in the body epidermis along the segmental grooves (Figure 8A), in the visceral but not somatic mesoderm (Figure 8A), in narrow patches of epithelial cells near the stomodeal (Figure 8C) and proctodeal invaginations, and in segmentally repeated patches in the neurogenic region (data not shown). However, because of limitations in the technique (grain scatter and inability to

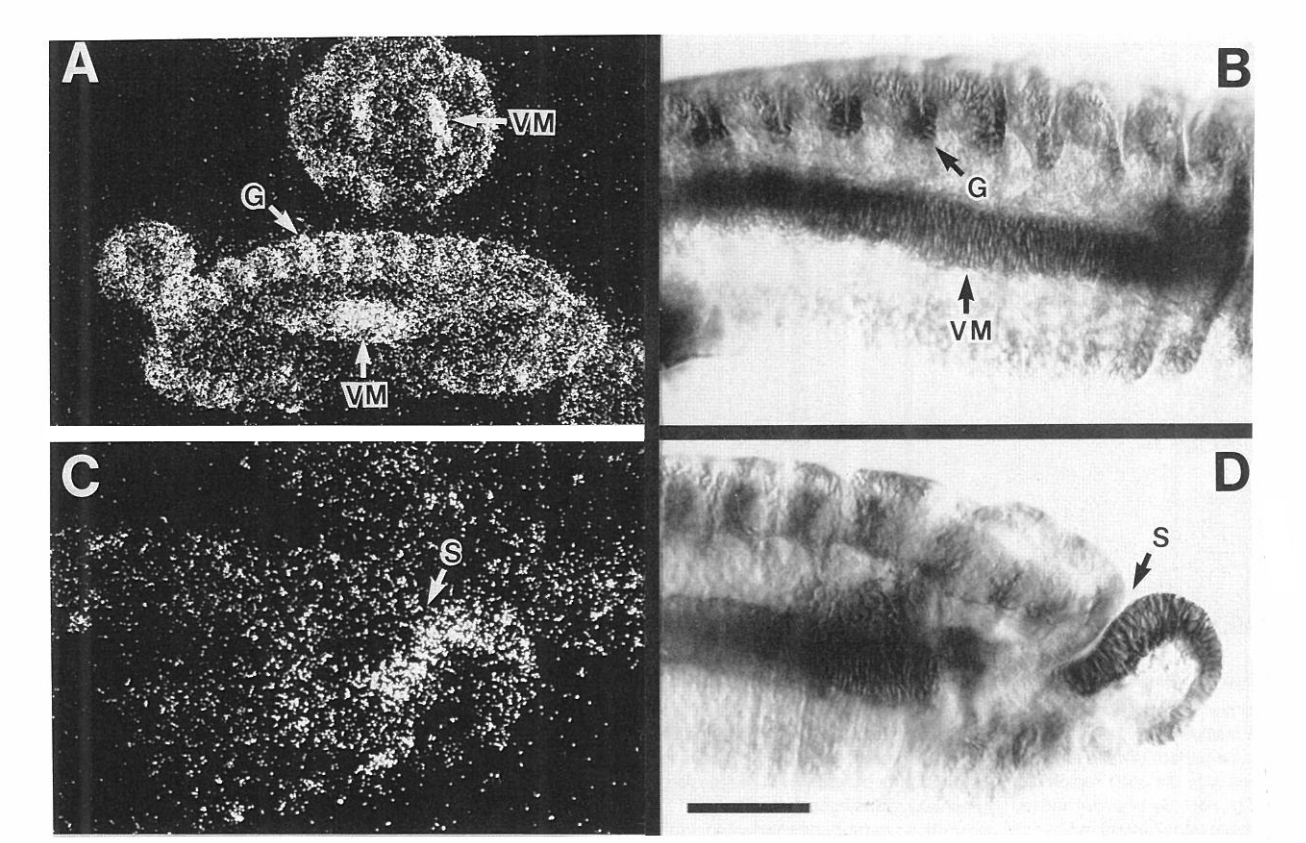

Figure 8. Tissue In Situ Hybridization

Localization of 36E1 cDNA clone transcripts in sections of hour 10:30 embryos (A and C), as compared with fasciclin III expression in whole-mount embryos of the same age at appropriate focal planes (B and D) (anterior is right, ventral is up; in [A] there is a cross section of another embryo). (A and C) Dark field photographs (silver grains are revealed as bright spots) showing transcripts at segmentally repeated stripes at the segmental grooves (G) in the body epidermis, in the visceral mesoderm (VM) migrating dorsally around the gut (e.g., cross-sectioned embryo at top of A), and in the clypeolabral patch of epithelial cells near the stomodeal invagination (S). (B and D) Antibody staining reveals a similar pattern of expression of fasciclin III glycoproteins. Scale bar: (A) 100 μm, (B–D) 50 μm.

identify individual neurons in sectioned material), we could not positively identify grains over individual identified neurons (e.g., RP1 and RP2).

More conclusive confirmation of the identity of the fasciclin III gene came from genetic deficiency analysis. The position 36E1 is flanked distally by *dorsal* and proximally by *dopa decarboxylase*. Previous investigators studying these flanking genes isolated many deficiencies (*Df*) in this region (Wright et al., 1976; Steward and Nusslein-Volhard, 1986). Some of these deficiencies include 36E1 (e.g., *Df(2L)H20*, *Df(2L)H68*, *Df(2L)VA18*, see Table 1) and others delete regions up to but not including 36E1 (e.g., *Df(2L)hk18*). Moreover, Steward and Nusslein-Volhard (1986) performed a near-saturation zygotic and maternal lethal mutagenesis over one of the deficiencies covering this region (*Df(2L)H20*) and generated a detailed complementation matrix of the genes and deficiencies in the 36A–36F region.

We assayed for the presence or absence of fasciclin III in some of these deficiencies by staining embryos with

Table 1. Fasciclin III Expression in Deficiencies in the 36E1 Region

Deficiencies	Breakpoints (Distal; Proximal)	Expression of Fasciclin III
Df(2L)H20	36A6, 7-10, 11; 36E3, 4-F1, 2 (Steward and Nusslein-Volhard, 1986)	–
Df(2L)H68	36B1, 2-C1, 2; 37A1-B1 (Nusslein-Volhard et al., 1984)	–
Df(2L)VA18	36C4-D1; 37C2-5 (T. R. F. Wright, personal communication)	–
Df(2L)hk18	36E4-E6; 37B9-C1 (Brittnacher and Ganetzky, 1983)	+
Df(2L)H2O/Df(2L)VA18	*trans*-heterozygote 36C4-D1; 36E3, 4-F1, 2	–

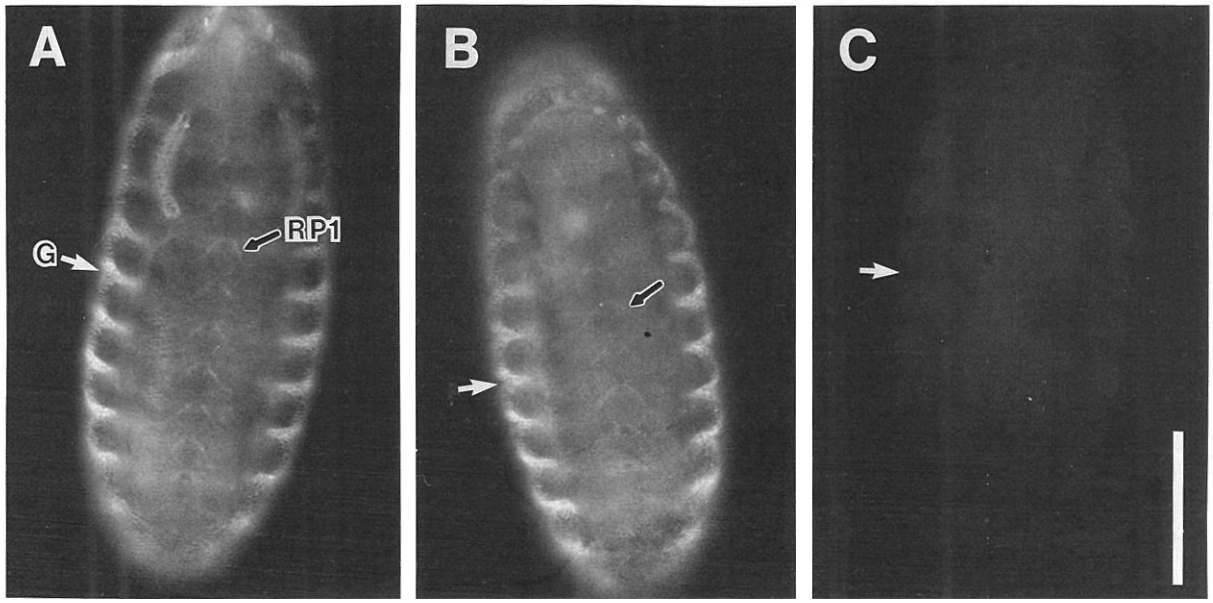

Figure 9. Small Deficiency in 36E1 Region Eliminates Fasciclin III Expression

Immunofluorescence photographs of whole-mount embryos at hour 12 (ventral views focused at level of CNS, anterior is up) showing expression of fasciclin III as revealed by 2D5 MAb (A) and anti–80 kd antiserum (B and C). (A and B) Wild-type embryos. The anti–80 kd antiserum stains the same pattern of cells as the 2D5 MAb. (C) Embryo *trans*-heterozygous for two overlapping deficiencies, *Df(2L)H20* and *Df(2L)VA18*. This small deficiency in the 36E1 region eliminates all forms of fasciclin III expression, including the segmentally repeated subset of fasciclin III–positive axons (e.g., RP1, black arrow) and the segmentally repeated fasciclin III–positive stripes along the segmental grooves (G; white arrow) in the body epidermis. These *trans*-heterozygous embryos undergo germ-band shortening and dorsal closure; moreover, bright field observations (not shown) reveal a superficially normal looking CNS. Scale bar: 100 μm.

both the MAbs and the anti–80 kd antiserum (Figure 9). Those deficiencies coming from either side that included 36E1 eliminate all expression of fasciclin III, whereas those deficiencies that do not include 36E1 have no effect (Table 1). Fasciclin III is also missing in embryos *trans*-heterozygous for two of these overlapping deficiencies, *Df(2L)H20* and *Df(2L)VA18* (generated by mating VA18 females with H20 males) (Figure 9C). According to the Steward and Nusslein-Volhard map, this *trans*-heterozygous deficiency is quite small (Table 1) and eliminates four known genes: *l(2)Bld*, *reduced ocelli*, *ninaD*, and *kelch*.

These results suggest that fasciclin III is encoded by a gene(s) that lies in the 36E region of the second chromosome, exactly where we mapped the gene identified by our cDNA clones. Taken together, the tissue in situ hybridization data and the genetic deficiency analysis confirm the identity of the fasciclin III gene. Moreover, the Southern blot data and genetic deficiency analysis argue that all four forms of the protein are likely to be encoded either by a single gene or by tandemly duplicated genes.

Discussion

The goal of this study was to identify molecular candidates for neuronal recognition molecules in Drosophila for future molecular genetic analysis. In a previous study, MAbs were used to identify two different surface glycoproteins, called fasciclin I and II (70 and 95 kd, respectively), which are expressed on subsets of axon fascicles in the grasshopper embryo (Harrelson et al., 1986; Bastiani et al., 1986b, 1987). Here we used two MAbs, 7G10 and 2D5, to identify another surface glycoprotein, called fasciclin III, which is expressed on yet a different subset of neurons and axon pathways in the Drosophila embryo (Figures 1 and 2). Because of their patterns of expression on different subsets of axon pathways during development, all three glycoproteins (two in grasshopper and one in Drosophila) are good candidates for neuronal recognition molecules.

One step in the generation of neuronal specificity is the selective affinity that many neuronal growth cones display for the surface of specific bundles (or fascicles) of axons as they navigate toward their targets (e.g., Goodman et al., 1984; Bastiani et al., 1985; Kuwada, 1986). These events are called selective fasciculation. The best evidence supporting the notion that fasciclin III may play a role as an axonal recognition molecule involved in these events is its regional expression. The glycoprotein is expressed on particular regions of a neuron and its processes, and not necessarily over the entirety of its surface. Its expression correlates with axon pathways and the patterns of axon fasciculation. Fasciclin III is expressed on the axons in 5 commissural fascicles. It is expressed on the continuation of one of these fascicles as it turns toward the ISN, but is not expressed on these axons as they turn into the longitudinal fascicles.

Fasciclin III comes in four highly related forms of appar-

ent molecular weights 80, 66, 59, and 46 kd; biochemical analysis using two-dimensional peptide maps, Western blots, and deglycosylation experiments suggests that these four proteins have similar but nevertheless distinct protein cores. Each of the four forms of the protein was purified for the generation of specific antisera, and these polyclonal antibodies were used to screen a cDNA expression library. The cDNA clones obtained by this method identified a single gene (or tandemly duplicated genes) at position 36E1 on the left arm of the second chromosome. We confirmed the identity of the fasciclin III gene by tissue in situ hybridization data (Figure 8) and genetic deficiency analysis (Figure 9C; Table 1).

Although we initially identified the 7G10/2D5 antigen (and subsequently the fasciclin III glycoproteins) because of its expression on a subset of neurons and axon pathways in the Drosophila embryo, we soon discovered that fasciclin III is also expressed at a variety of other times and places. It is positionally expressed on segmentally repeated patches of cells during neurogenesis (Figures 3A and 4) and outside the developing CNS on segmentally repeated stripes in the epidermis at the segmental grooves (Figures 3B, 4A, 4B, 4C, and 4F), on patches of epithelial cells near the stomodeal and proctodeal invagination (Figures 3, 5D and 5E), on the visceral mesoderm that migrates as a sheet around the gut (but not on the somatic mesoderm) (Figures 3A, 4C and 4E), and on the luminal surface of the salivary gland epithelium. In most of these cases, the protein is not evenly expressed on the entire surface of the fasciclin III–positive cells, but rather is expressed at higher levels where fasciclin III–positive cells contact one another (e.g., Figures 4A and 5D), suggesting that it may be stabilized at these points of contact.

It is interesting to note that MAbs that recognize the fasciclin III glycoproteins have been identified by two very different antibody screens in two other laboratories. Zipursky et al. (1984) described the 6D6 MAb, which recognizes an interesting pattern of cells in the developing eye disc of Drosophila. Brower et al. (1980) generated MAbs that bind to the surfaces of diploid epithelial cells, particularly in larval imaginal tissues. Our unpublished results show that both of the MAbs described above recognize the same antigen and stain the same pattern of cells in the embryo as the 7G10/2D5 MAbs. Moreover, Brower and coworkers have also described the segmentally repeated expression of the antigen (which they call DENS) in the embryo (A. Gauger, M. Glicksman, R. Salatino, J. Condie, G. Schubiger, and D. Brower, unpublished results).

After germ-band extension, fasciclin III is transiently expressed on segmentally repeated patches of neuroepithelial cells and specific underlying neuronal lineages during neurogenesis (Figures 3A and 4). The fasciclin III protein is a transient marker of specific NB lineages, and its expression is consistent with it playing a role in the patterning and specification of cell fates during neurogenesis (e.g., Doe et al., 1985; Doe and Goodman, 1985).

By the end of germ-band retraction, fasciclin III is expressed in repeated stripes across all of the body segments (Figures 3B and 5A). By the time head involution occurs and the grooves smooth out, all epidermal cells express the surface glycoprotein, although at varying levels that maintain the segmentally repeated striped pattern (Figures 5B and 5C).

The segmentally repeated expression of the protein in the epidermis at the segmental grooves is out of phase with the earlier segmentally repeated patches in the neuroepithelium. After neurogenesis is completed, the epidermal expression of the protein at the segmental grooves begins to spread ventrally across the epidermis that was previously the neurogenic region, concurrent with the extension of the grooves across this region. Thus, at different stages of development, the ventral epidermis has two distinctly different patterns of protein expression, the first presumably involved with neurogenesis and the second with body patterning and morphogenesis.

Thus fasciclin III is the first surface glycoprotein identified in Drosophila that is expressed in a segmentally repeated pattern in the ventral neurogenic region and in the body epidermis. Now that the fasciclin III gene has been cloned, it will be of interest to see whether expression of the fasciclin III gene in these two different segmentally repeated patterns is controlled either directly or indirectly by the nuclear regulatory proteins encoded by many of the previously identified segmentation genes (e.g., Nusslein-Volhard and Wieschaus, 1980; Scott and O'Farrell, 1986).

Fasciclin III is a good candidate for a surface molecule involved in specific cell interactions and recognition, possibly either by mediating or modulating cell adhesion, at a variety of different times and places during development. Within the developing nervous system, its expression on a subset of axon pathways makes fasciclin III a good candidate for a Drosophila neuronal recognition molecule. We hope to test this potential function by genetic analysis.

The fasciclin III gene maps to position 36E1 on the second chromosome. All four forms of the protein are missing in embryos *trans*-heterozygous for two overlapping deficiences, *Df(2L)H20* and *Df(2L)VA18*. According to the detailed complementation matrix map of the 36A–36F region generated by Steward and Nusslein-Volhard (1986), this *trans*-heterozygous deficiency is quite small and eliminates four known genes. Based on this detailed genetic map, we are presently searching for point mutants in the fasciclin III gene. It should soon be possible to specifically delete fasciclin III, and thus examine the consequences of its absence on the development of specific identified neurons and other cells that normally express it.

Experimental Procedures

Membrane Preparation

Embryonic nerve cords were prepared as previously described (Goodman et al., 1984). The enriched nerve cords were resuspended in 10 mM triethanolamine (TEA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml of the following protease inhibitors: antipain, pepstatin, leupeptin, chymostatin, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and Na-p-tosyl-L-lysine chloromethyl ketone (TLCK). The nerve cords were homogenized in a glass Dounce homogenizer and centrifuged at 1200 × g for 10 min at 4°C. The supernatant was reserved, and the pellet was resuspended in the same buffer, rehomogenized, and centrifuged as above. The combined su-

pernatants were centrifuged at 100,000 × g for 2 hr at 4°C. The membrane pellet was resuspended by homogenization in PBS containing 1 mM PMSF at a concentration of 5–10 mg of membrane protein per ml and stored at −70°C.

Generation and Screening of Monoclonal Antibodies

Membranes from mass-isolated Drosophila embryonic CNS (nerve cords) and from mass cultures of primary neurons were used as immunogens for the production of MAbs. Nerve cords were isolated from 10–13 hr embryos (Goodman et al., 1984). Cultures enriched for neurons were prepared using a modified and scaled-up version of procedures described by Shields et al. (1975) and Seecof et al. (1973). Briefly, 4–8 hr embryos (10 g) were dechorionated in 50% bleach, surface sterilized in 75% ethanol, and washed in sterile PBS (pH 7.4). Embryos were then placed into Schneider's media (Gibco) containing 2% fetal calf serum (S + 2% FCS) and homogenized in a Dounce homogenizer at 4°C. Passage through 80 μm Nitex removed vitelline membranes, and the cells were then washed 4 times with S + 2% FCS at 4°C. The cells were diluted to a concentration of 2 × 10^6 cells per ml in culture media (Schneider's media containing 17% fetal calf serum), placed into polyornithine-coated petri dishes, and incubated at 25°C for 12 hr. At this time, the dishes were vigorously agitated to remove nonadherent cells and the culture media was replaced. After an additional 12 hr, cells were harvested by scraping them off into ice cold PBS. Membranes were prepared from both nerve cords and primary cells as described above.

BALB/c mice were immunized by intraperitoneal injections; each injection contained about 1 mg of protein. Primary injections consisted of nerve cord membranes suspended in a 1:1 mixture of PBS and complete Freund's adjuvant. Mice were given 3 boosts at approximately 3 week intervals. These injections were composed of nerve cord membranes suspended in a 1:1 mixture of PBS and incomplete Freund's adjuvant. Three days before the fusion, a final injection of membrane in just PBS was administered. In the fusion that yielded the 7G10 MAb, the final injection contained nerve cord membrane, whereas for the 2D5 MAb, the final injection used primary neuron culture membrane. Spleen cells were fused with NS-1 myeloma cells (Kohler and Milstein, 1975; Oi and Herzenberg, 1980). Hybridoma supernatants were screened by immunofluorescence on mass-isolated nerve cords (staining protocol described in next section), and hybridoma lines of interest were isolated by single-cell cloning.

FITC and HRP Immunocytochemistry

Immunofluorescence and horseradish peroxidase (HRP) techniques were used to study the distribution of fasciclin III glycoproteins. Dissected nerve cords and mass-isolated nerve cords (in the primary hybridoma screen) were fixed in silicon-welled glass slides for 10 min with 2% paraformaldehyde in Millonig's buffer. Whole mount embryos were prepared essentially by the technique of Mitchison and Sedat (1983). Embryos were dechorionated in 50% bleach, rinsed with water, and placed into a 1:1 mixture of heptane and 4% paraformaldehyde in Millonig's buffer for 10 min. The aqueous layer was removed and replaced with absolute methanol to devitellinize the embryos. From this point on, the same staining procedure was used for dissections, mass-isolated nerve cords, and whole mounts. Embryos were placed into PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBT) for 30 min, then into PBT with 5% normal goat serum (PBT + NGS) for 30 min. This was followed by incubation at 4°C for 12–14 hr in a 1:1 mixture of monoclonal supernatant and PBT + NGS or rat antisera diluted 1:1000 in PBT + NGS. Embryos were then washed (all washings consist of 4 changes of PBT over a 1 hr period) and placed again into PBT + NGS for 30 min.

Embryos for immunofluorescence were incubated for 2 hr at 25°C in a 1:300 dilution (in PBT + NGS) of FITC-conjugated goat anti-mouse IgMAG (Cappel) or FITC-conjugated goat anti-rat IgG (Cappel), as appropriate. After washing, embryos were observed with a Zeiss epifluorescence compound microscope.

Embryos for HRP immunocytochemistry were incubated for 2 hr at 25°C in a 1:200 dilution (in PBT + NGS) of biotin-conjugated, affinity-purified goat anti-mouse IgG (Cappel) or biotin-conjugated, affinity-purified goat anti-rat IgG (Cappel), as appropriate. After washing, embryos were incubated for 1 hr at 25°C in avidin–HRP (Cappel) diluted 1:500 in PBT. Embryos were then placed into a 0.5 mg/ml solution of diaminobenzidine containing 0.003% hydrogen peroxide, and the peroxidase reaction was carried out for 3–5 min. Embryos were cleared by rinsing them 3 times with ethanol and then placing them into methyl salicylate. A Nikon compound microscope with Nomarski optics and Zeiss water immersion lenses was used to view and photograph these embryos.

Membrane Protein Labeling and Immunoprecipitation

Membranes in PBS–PMSF were labeled with ^{125}I using lactoperoxidase (Haustein et al., 1975). After labeling, the proteins were solubilized by incubation for 1 hr on ice in 10 mM TEA, 1% NP 40, 0.5% deoxycholate, and 0.15 M NaCl (pH 8.2) (IPB) containing protease inhibitors as described above. After centrifugation at 100,000 × g for 30 min to remove insoluble material, immunoprecipitations were performed using preformed antibody complexes as described (van Agthoven et al., 1981). The isolated antigens were analyzed using a modification of the system described by Laemmli (1970). Autoradiography was performed using Cronex Lightning Plus screens (Cronex Lightning Plus, Dupont Chemical Co.).

Protein Purification

All solutions contained protease inhibitors as described above. Dechorionated embryos (10–20 hr) were solubilized in IPB by homogenization (5 strokes) in a glass Dounce homogenizer with a Teflon pestle (50 g embryos per 100 ml IPB). The lysate was stirred at 4°C for 1 hr followed by centrifugation at 13,000 × g for 30 min to remove nuclei. Clarification of the lysate was achieved by centrifugation at 100,000 × g for 2 hr at 4°C.

Immunoaffinity absorption of the antigens was performed using columns of protein A–Sepharose (Pharmacia) to which the MAb had been covalently coupled (Schneider et al., 1982). The lysate was passed at 5–10 ml/hr over a 5 ml Sepharose CL-4B column followed by a 1 ml column with the coupled MAb. The affinity column containing the bound antigen was subsequently washed with 10–20 ml of each of the following solutions: IPB; 10 mM TEA, 0.15 M NaCl (TEA–NaCl); TEA–NaCl containing 0.5% deoxycholate; IPB containing 1.2 M NaCl. The antigens were eluted with 10 ml of 50 mM triethylamine, 1% NP 40, and 0.15 M NaCl (pH 11.5) into 1 ml of 1 M TEA (pH 6.5) to neutralize the eluate.

The eluted antigens were further purified by preparative SDS–PAGE and electroelution. The column eluate was treated with 20% trichloroacetic acid for 30 min at 4°C, and the precipitate was collected by centrifugation. After washing 3 times with ice cold acetone, the precipitate was lyophilized and loaded onto a 10% polyacrylamide gel. The procedures for preparative SDS–PAGE and electroelution have been described in detail (Hunkapiller et al., 1983).

Generation of Antisera

Coomassie blue–stained gel slices containing 5–10 μg of protein were soaked in water for 1 hr and homogenized in a glass Dounce homogenizer with a Teflon pestle. The gel slices were mixed with an equal volume of complete Freund's adjuvant and injected intraperitoneally into 2–4 month old male rats. Injections were repeated at 2–4 week intervals, using incomplete Fruend's adjuvant for the boosts. Serum was collected 7–10 days following the boosts.

Biochemical Techniques

Purified proteins were labeled with ^{125}I using Chloramine T (Greenwood et al., 1963). The labeled proteins were isolated on mini-gels and eluted in 10 mM NH_4HCO_3 and 0.1% SDS at 37°C for 12 hr. After lyophilization, the SDS was removed by ion-pair extraction (Konigsberg and Henderson, 1983). After addition of 10 μg of sperm whale myoglobin (Beckman Instruments), the protein was digested with 2 μg of trypsin for 16 hr at 37°C in 0.1 M NH_4HCO_3, followed by the addition of 1 μg of trypsin for an additional 4 hr incubation. Insoluble material was removed by centrifugation for 5 min at 13,000 × g. The supernatant was lyophilized and applied to the first dimension of the peptide map. The solvent system for the first dimension of the peptide maps was pyridine:acetic acid:water (1:10:89 v/v). Electrophoresis on cellulose TLC plates (Merck) was performed for 45 min at 900 V. Chromatography in the second dimension was in pyridine:acetic acid:n-butanol:water (10:3:15:12). Treatment of purified proteins with TFMS

was performed as described (Edge et al., 1981). Digestion of carbohydrates with PNGase F was performed as described by Plummer et al. (1984). Immunoblotting was performed as described by Towbin et al. (1979).

cDNA Expression Cloning

The λgt11 library used was constructed from poly(A)$^+$ RNA from mass-isolated hour 10–13 nerve cords (S. Crews and P. Patton, unpublished data). The λgt11 library was screened using the procedure of Huynh et al. (1985) as modified by Steve Crews (personal communication). The library was plated on RY1090 bacteria with about 25,000 plaques per 150-mm plate and incubated for 3.5 hr at 42°C. Nitrocellulose filters were soaked in 15 mM IPTG, dried, and placed on the bacterial lawns. After an additional 4 hr at 42°C, the filters were removed and rinsed in PBS. Antigen-containing plaques were identified using rat antisera to the 80 kd fasciclin III glycoprotein. The nitrocellulose filters were stained by HRP immunocytochemistry using the same reagents, dilutions, and incubation times described for embryos with the exception that in all steps PBT was replaced by a solution of PBS containing 0.1% bovine serum albumin and 0.05% Tween 20. Positive plaques were purified and also tested for reactivity to the anti-59 kd and anti-46 kd antisera by repeating the above steps.

^{35}S, ^{32}P, and biotinylated probes were made according to the random hexamer priming technique of Feinberg and Vogelstein (1983). Nucleic acid blots (for cross-hybridization and genomic Southerns) were done according to the procedures of Southern (1975). Hybridizations were done in 6× SSC at 68°C and washed in 0.2× SSC at 65°C.

In Situ Hybridization

In situ hybridization techniques were used to localize the isolated cDNA to 36E1 and to visualize the distribution of transcripts in the embryo. Polytene in situ hybridization was done using HRP staining according to the procedures of Levine et al. (1985). The whole-mount incubation procedure of Mahoney and Lengyel (1987) was followed for tissue in situ hybridization with the following changes: ^{35}S-labeled DNA was used and the pronase treatment of embryos was omitted. Autoradiograms were developed after 3 weeks and observed under dark field illumination with a Zeiss microscope.

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