

Drosophila Fasciclin I Is a Novel Homophilic Adhesion Molecule That along with Fasciclin III Can Mediate Cell Sorting

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Abstract. Fasciclin I is a membrane-associated glycoprotein that is regionally expressed on a subset of fasciculating axons during neuronal development in insects; it is expressed on apposing cell surfaces, suggesting a role in specific cell adhesion. In this paper we show that *Drosophila* fasciclin I is a novel homophilic cell adhesion molecule. When the nonadhesive *Drosophila* S2 cells are transfected with the fasciclin I cDNA, they form aggregates that are

blocked by antisera against fasciclin I. When cells expressing fasciclin I are mixed with cells expressing fasciclin III, another *Drosophila* homophilic adhesion molecule, the mixture sorts into aggregates homogeneous for either fasciclin I- or fasciclin III-expressing cells. The ability of these two novel adhesion molecules to mediate cell sorting in vitro suggests that they might play a similar role during neuronal development.

DIFFERENTIAL cell adhesion plays a major role in the guidance of neuronal growth cones during development (reviewed by Jessell, 1988). In vertebrates, several neural cell adhesion molecules have been suggested to participate in axonal adhesion and guidance, including N-CAM, L1, contactin/F11, neurofascin, and N-cadherin (e.g., reviews by Jessell, 1988; Takeichi, 1988). Much of what is known about the function of these vertebrate neural cell adhesion molecules comes from antibody blocking experiments in cell culture.

Only recently have neural adhesion molecules begun to be elucidated in *Drosophila*, where there is the opportunity to use genetic analysis to study the role of these molecules during development. Several years ago, mAbs were used to identify four different cell surface glycoproteins, called fasciclin I, fasciclin II, fasciclin III, and neuroglian, that are expressed on different overlapping subsets of fasciculating axons and growth cones during neuronal development in insects (Bastiani et al., 1987; Patel et al., 1987; Bieber et al., 1989). The expression of each of these proteins on the surfaces of neighboring cells at points of close apposition led to the suggestion that these proteins might function in specific neural cell adhesion and/or recognition. The genes encoding all four proteins were cloned and sequenced. Two of them (fasciclin II and neuroglian) are members of the immunoglobulin gene superfamily and are highly related to several well-characterized vertebrate neural cell adhesion

molecules, fasciclin II being most highly related to N-CAM (Harrelson and Goodman, 1988) and neuroglian most highly related to L1 (Bieber et al., 1989). The other two proteins (fasciclin I and fasciclin III) have novel amino acid sequences that are unrelated to any previously described proteins (Zinn et al., 1988; Snow et al., 1989).

Do these four insect proteins function as neural cell adhesion molecules? This question is particularly relevant for fasciclin I and fasciclin III, which, in contrast to fasciclin II and neuroglian, do not share sequence similarity with other known neural cell adhesion molecules. To answer this question, we have used DNA transfection methods to test if these proteins can confer cell aggregation in vitro in the nonadhesive *Drosophila* S2 cells, an experimental paradigm modeled after the studies on vertebrate cadherins begun by Takeichi and his colleagues (Nagafuchi et al., 1987). The first aggregation experiments using transfected *Drosophila* cDNAs showed that fasciclin III can function as a homophilic cell adhesion molecule (Snow et al., 1989). In this study we used this experimental system to show that fasciclin I can also function as a cell adhesion molecule, whose adhesivity is inhibited by addition of antisera raised against fasciclin I.

In addition we show that when the fasciclin I-transfected S2 cells are heterogeneously mixed with the fasciclin III-transfected S2 cells, they undergo cell-type specific sorting into homogeneous aggregates. However, when the fasciclin I-transfected S2 cells are mixed with S1 cells, which endogenously express fasciclin I (and which normally aggregate in vitro), the two cell types do not sort out. These in vitro results lead to the suggestion that these two novel neural cell adhesion molecules (fasciclin I and fasciclin III) might play a similar role in cell sorting during development in vivo, particularly during axonal guidance in which they are ex-

[†] On 5 October 1989, Tom Elkins was killed in an automobile accident. This paper is dedicated to his memory.

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pressed on both specific growth cones and the axon pathways they follow.

Materials and Methods

Culture, Transfection, and Cloning of *Drosophila* S2 Cells

cDNA subcloning was performed by standard methods (Maniatis et al., 1982). Cell transfections were performed using DNA-calcium phosphate coprecipitates as described by Wigler et al. (1979), and the method of Moss (1985). S2 cells were cotransformed with the PC4 plasmid (Jockerst et al., 1988), which confers α -amanitin resistance to the cells. Other details of transformation and cell culture are as in Snow et al. (1989). For cell cloning, cells were titered using a hemocytometer and aliquotted into 96-well tissue culture plates at concentrations of one, three, and five cells/250 μ l/well, in three plates. The plates were wrapped in Saran wrap to prevent desiccation. The media were prepared as follows: S2 cells were grown in Schneider's medium (Gibco Laboratories, Grand Island, NY) plus 15% FCS to 10^7 cells/ml or greater. Cells were then filtered from the conditioned medium. The working medium was 20% conditioned medium in fresh medium containing 15% FCS that had not been heat inactivated.

Membrane Preparation and Protein Western Blotting

Cells were heat shocked in balanced salt solution (BSS) with 10% BSA to protect against proteolysis, spun at 300 g and chilled on ice, resuspended in PBS with 40 μ g/ml PMSF, and spun again and resuspended in hypotonic buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 μ g/ml PMSF and a protease inhibitor mix as described in Patel et al. (1987). Cells were then homogenized with 8–10 strokes in a tight Dounce homogenizer and spun 10 min at 4,000 g in an Eppendorf microfuge at 4°C. The pellet was resuspended in 1 ml of buffer and spun as before. The supernatants were combined and layered onto 400 μ l of 0.5 M sucrose in hypotonic buffer and spun at 100,000 g for 30 min. The pellet was resuspended in 400 μ l PBS plus PMSF.

Protein electrophoresis was as described by Laemmli (1970). Protein transfer to nitrocellulose was as described by Burnette (1981), and staining of Western blots with horseradish peroxidase-labeled antibodies was as described by Hortsch et al. (1985).

Production of Fusion Protein and Antibodies

The fasciclin I 3.0-kb cDNA (Zinn et al., 1988) was subcloned into the expression vector pRITZT (Nilsson et al., 1985). Fasciclin I-protein A fusion protein was isolated and used as an immunogen in mice and rats. Generation of polyclonal and monoclonal antibodies against fasciclin I will be described in detail elsewhere (M. Hortsch and C. S. Goodman, manuscript submitted for publication).

Immunocytochemistry

For staining living cells, cells were spun for 5 min at 2,000 g, resuspended in 200–500 μ l of BSSG (BSS with 5% normal goat serum; BSS consists of 55 mM NaCl, 40 mM KCl, 15 mM MgSO₄, 10 mM CaCl₂, 20 mM glucose, 50 mM sucrose, 2 mg/ml BSA, 10 mM tricine, pH 6.95), then incubated 30 min. Cells were incubated in monoclonal supernatant (1:1), monoclonal ascites (1:500), or antiserum (1:500) for 2 h at room temperature, spun and resuspended in BSS. Cells were gently agitated for 1 h with two changes of BSS before incubating 30 min in block. Cells were incubated in secondary antibody (goat anti-mouse or goat anti-rat, fluorescein-conjugated, Jackson Laboratories, Bar Harbor, ME) for 1 h, then washed with agitation as before and viewed under a coverslip with epifluorescence. Slides could be stored by sealing the coverslip with nail polish.

Cell Aggregation Assays

Cells were titered using a hemocytometer and cell densities were equilibrated for each experiment to 5×10^6 or 10^7 cells/ml in 0.5 ml BSS. Cells were heat shocked in 24-well tissue culture dishes for 20 min at 37°C and allowed to recover at room temperature for 1 h. The cells were then agitated at 100 rpm on a shaker platform. For determination of cell cluster size, 25 μ l of

1. *Abbreviation used in this paper:* BSS, balanced salt solution.

cells in BSS were put on a microscope slide with an 18-mm square coverslip, which flattened each cell cluster into an adhering monolayer whose cells could be counted. For calculation of the percentage of single cells, cells in BSS were removed from the well and the number of free cells and cells in aggregates were counted as above. In some experiments, the number of free cells and the number of cell clusters were counted in the hemocytometer and the average number of cells per aggregate was determined from a separate aliquot.

Results

Transfection of S2 Cells with Fasciclin I cDNA

We transfected *Drosophila* tissue culture cells with the *Drosophila* fasciclin I cDNA (Zinn et al., 1988). To study the role of fasciclin I in cell aggregation, it was necessary to choose a relatively nonadhesive *Drosophila* cell line that does not aggregate either normally or after heat shock, and which does not naturally express fasciclin I. The Schneider 1 (S1) cells (Schneider, 1972) are unsuitable since they normally aggregate after agitation, and because they express high levels of fasciclin I constitutively (Elkins et al., 1990; Hortsch, M., unpublished results). However, the Schneider 2 (S2) cells normally express very low to undetectable levels of fasciclin I, and do not form aggregates after agitation (Fig. 1; also see Snow et al., 1989). Thus, S2 cells were a good choice for testing the effects of fasciclin I expression from a transfected cDNA, much as has been done for fasciclin III (Snow et al., 1989).

The pHT4 vector that contains the HSP 70 promoter was chosen to express fasciclin I in the S2 cells (Schneuwly et al., 1987). The entire fasciclin I cDNA (Zinn et al., 1988) was inserted adjacent to the 3' end of the HSP70 promoter. The resulting plasmid was cotransfected into the S2 cells with a selectable plasmid encoding a *Drosophila* α -amanitin resistant RNA polymerase II gene (Jockerst et al., 1989). When cells are grown in the presence of α -amanitin, only transformed cells will survive. In practice, most competent cells will take up both plasmid types (Moss, 1985). Selection was maintained for several cell cycles, until the cell density was $\sim 10^7$ cells/ml. The cell population was then tested for heat shock-inducible fasciclin I expression in two ways.

First, an insoluble membrane fraction was prepared from various cell populations that had been transfected with either the normal pHT4 plasmid or the fasciclin I-containing pHT4 plasmid, and that either had or had not been heat shocked. When protein blots of these preparations were probed with mouse mAbs raised against a fasciclin I fusion protein, fasciclin I protein was detected at very low levels in the control cells that had received only the pHT4 plasmid (Fig. 1, lanes 1 and 2). However, cells that were transfected with the fasciclin I-containing plasmid, and grown with no heat shock, expressed fasciclin I at higher levels than the controls, indicating that the HSP70 promoter allows transcription at low levels even at 23°C. Protein from these fasciclin I-transfected cells prepared 90 min after a 20-min heat shock showed much higher levels of fasciclin I than the same cells without heat shock.

Second, the different populations of cells were examined for surface protein expression by immunofluorescence using the fasciclin I mAb and a fluorescein-conjugated secondary antibody. Only the heat-shocked fasciclin I cells were significantly stained by the antibody. However, the immunofluorescence was observed on the surface of only about one quar-

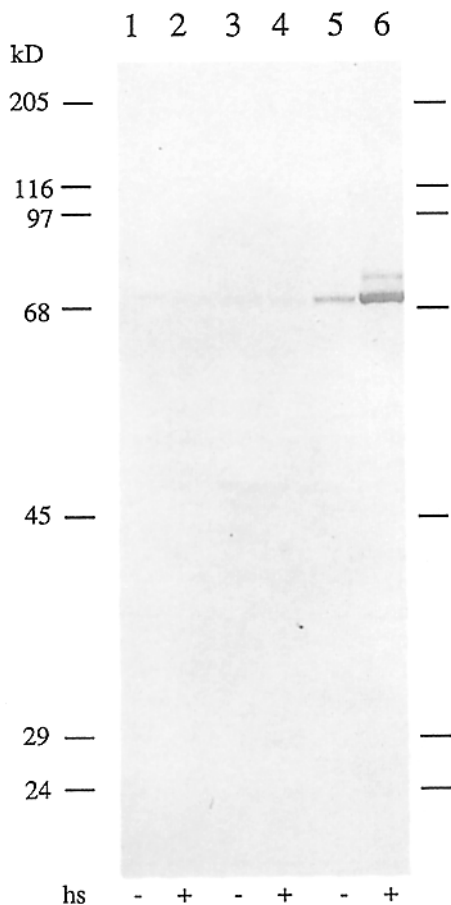


Figure 1. Expression of fasciclin I protein in transfected S2 cells. Western blot of the insoluble membrane pellet from S2 cells using mAb 5H7 raised against fasciclin I. Lanes 1 and 2, protein from cells carrying the plasmid pHT4 without the fasciclin I cDNA, before and after heat shock. Lanes 3 and 4, protein from cells carrying the *sevenless* gene under the control of the actin promoter. Lanes 5 and 6, protein from cells carrying the plasmid pHT4 with the fasciclin I cDNA under the control of the heat shock promoter, before and after heat shock.

ter of these cells, indicating that the cell population was heterogeneous. When these cells were tested for inducible aggregation (described below) only small, sparse aggregates were seen.

Fasciclin I Expression and Aggregation by Clonal Cell Lines

Because the majority of cells in the transfected population did not express fasciclin I, it was possible that the weak aggregation was due to the low numbers of expressing cells. To test this, the cells were recloned to identify a clonal cell line that expressed fasciclin I at high levels on all cells. Although transfected cells can initially contain 1,000 or more copies of a plasmid, this high copy number is lost from the cells over time at a slow rate (Moss, 1985). For this reason, all experiments on the cloned cell lines were performed within 1 mo of recloning.

The fasciclin I cells were recloned from single cells without α -amanitin selection, tested for aggregation, and examined for fasciclin I expression by immunostaining of live

cells. Aggregation assays were performed on the fasciclin I-transfected S2 cells and on two other transfected S2 cell lines: the pHT4-containing cells and cells expressing the *sevenless* protein under the control of the actin promoter, which provides high levels of constitutive expression (provided by M. Simon, University of California, Berkeley). *Sevenless* is a transmembrane protein that, like fasciclin I, contains a large extracellular domain. It contains a cytoplasmic tyrosine kinase domain and is involved in cell signaling (Bowtell et al., 1988; Basler and Hafen, 1988); its well-characterized function during eye development suggests that *sevenless* probably does not function in cell adhesion.

18 clonal cell lines were titered and equilibrated to 10^7 cells/ml before heat shock and agitation. For the aggregation and expression assays, all cells were exposed to 37°C for 20 min, kept at room temperature for 90 min, and then agitated on a shaker for 1 h. Of the lines tested, 10 did not express detectable fasciclin I by immunofluorescence. All 10 of these lines had grown to approximately threefold higher cell density than the other lines, although all lines were grown under identical conditions. Of these 10, 8 were judged to show no aggregation (Fig. 2 A), while the other two formed aggregates of up to ~ 20 cells each that made up only a few percent of the total cells (Fig. 2 B). Two other lines showed some aggregates of up to ~ 100 cells, making up less than half of the total cells. It appeared that up to 15% of the cells in these lines expressed fasciclin I at high levels while the rest of the population did not express detectable levels of the protein at all. Those cells contained within the aggregates all consistently expressed fasciclin I.

The last six lines formed large, numerous aggregates of 100 to several thousand cells each that together composed between 50 to 100% of the total cells (Fig. 2 C). The size of these aggregates grew during continued agitation as the aggregates fused (Fig. 2 D). These cell lines appeared to express fasciclin I in $>95\%$ of the cells. The expression of fasciclin I in the cell lines therefore correlates with the ability of the cells to aggregate.

The aggregates in the clonal lines with the strongest fasciclin I expression were highly adherent and not easily dispersed by pipetting. The cells within the aggregates had large, flat areas of apposition, and there were no visible spaces between cells. When an aggregate was flattened on a microscope slide under a coverslip, cells were forced into extensive monolayers that nevertheless remained contiguous (this behavior allowed the number of cells within an aggregate to be counted).

As a control, cells transfected with the *sevenless* gene were tested that had been recloned within a 2-wk period of the fasciclin I recloning. Western blot analysis of the *sevenless* cells showed that the *sevenless* protein was being expressed (Simon et al., 1989). Cells were tested for aggregation and for *sevenless* expression by immunostaining live cells. Approximately 50% of the cells appeared to express the *sevenless* protein, although $<1\%$ of cells were in aggregates and these were rarely larger than five cells.

Fasciclin I Directly Mediates Cell Adhesion

Although the data thus far presented show that fasciclin I expression leads to cell aggregation, they do not answer the question of whether fasciclin I is itself an adhesion molecule,

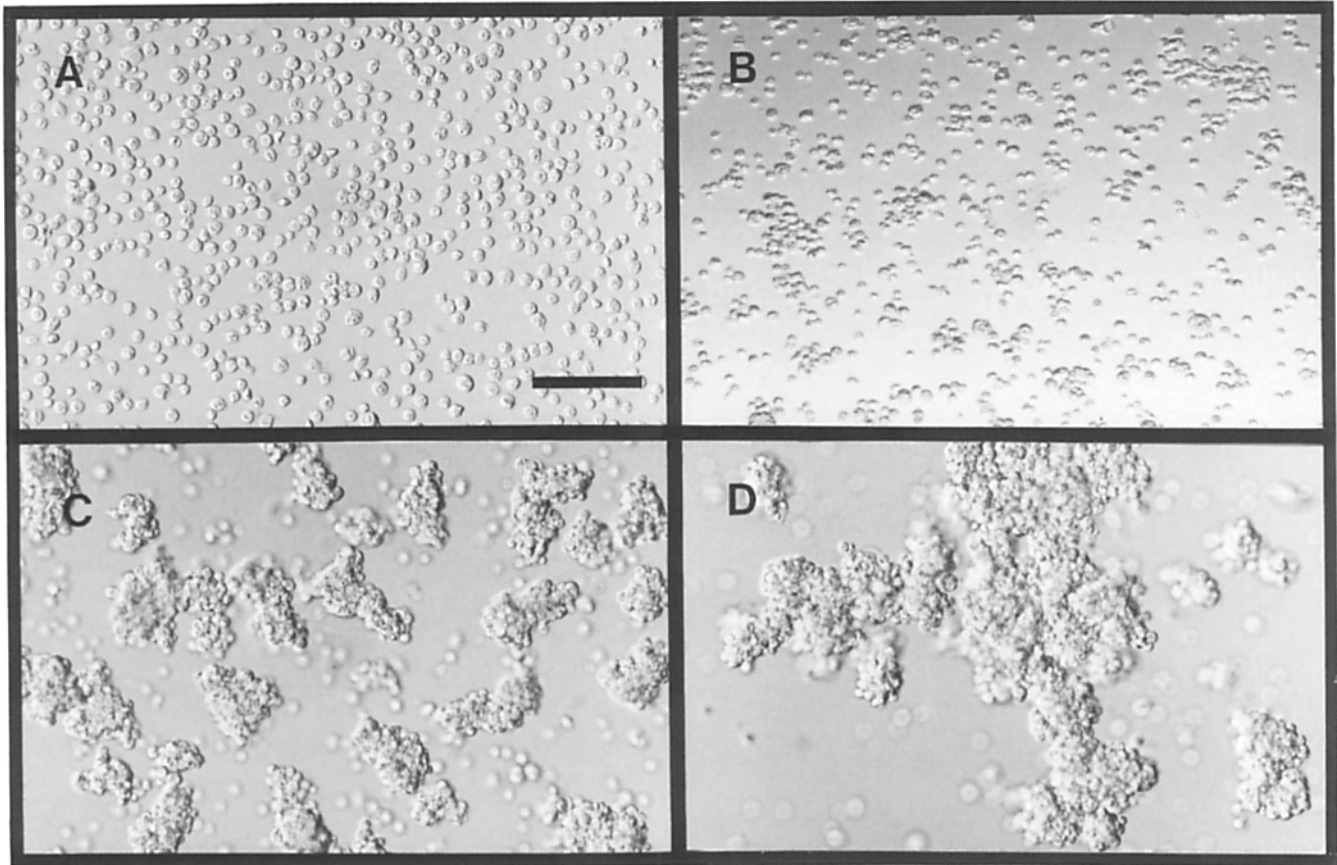


Figure 2. Aggregation of S2 cells transfected with the fasciclin I cDNA. Cells were heat shocked for 20 min, allowed to recover for 90 min, and agitated for 20 min, as described in the text. (A) Cell line 5H6, which does not express fasciclin I. (B) Cell line 1C7, which expresses fasciclin I on only a small fraction of cells. (C and D) Cell line 1B12, which expresses fasciclin I on almost all cells, after 20 (C) and 45 min (D) of agitation. Bar, 100 μm .

or alternatively whether the expression of this protein on the surface of the S2 cells leads to adhesion indirectly by inducing the expression of some other adhesion molecule. Two experiments argue against this second alternative, and support the notion that fasciclin I is itself a cell adhesion molecule.

First, when the cells were allowed to recover from heat shock at room temperature for 30 min to allow enough time for transcription and translation, and then moved at 4°C for the agitation to prevent further transcription and translation, large aggregates still formed (data not shown).

In the second series of experiments, monoclonal and polyclonal antibodies raised against a fasciclin I-protein A hybrid were tested for their ability to inhibit aggregation (see Materials and Methods). Three different mAbs that recognize fasciclin I failed to impede the formation of aggregates; similar negative results were obtained with two other control mAbs that recognize different cell-surface antigens, fasciclin III (Patel et al., 1987) and neuroglian (Bieber et al., 1989). However, cells treated with a 1:100 dilution of a fasciclin I rat antiserum formed smaller and less numerous aggregates than cells treated with the preimmune serum or with any of the mAbs. In order to quantify this effect, the percentage of single cells was measured over time during agitation (see Materials and Methods) (Fig. 3). Although the inhibition of aggregation was not complete, the average of two experiments showed that the percentage of free cells was more than

three times higher after 1 h in antiserum-treated saline than in saline treated with preimmune serum from the same animal. Together, these two experiments (the 4°C experiment and the antiserum inhibition experiment) strongly suggest that fasciclin I is a cell adhesion molecule.

Fasciclin I Mediates Adhesion in a Ca^{2+} -independent, Homophilic Manner

The aggregation observed in the fasciclin I-transfected S2 cells could be due to a homophilic affinity of fasciclin I on one cell surface for fasciclin I on the apposing surface, or alternatively could be due to a heterophilic affinity of fasciclin I for some receptor that is constitutively (and fortuitously) expressed by the S2 cells.

To distinguish between these two alternatives, fasciclin I-transfected cells were mixed with other S2 cells. Since all of the cells tested were S2 cells, the constitutively expressed proteins should have been identical in all the populations. In these experiments, cells were heat shocked separately and either the fasciclin I cells or the other cells were stained with the lipophilic red-fluorescing dye, DiI. The fasciclin I cells were then mixed pairwise with the other cells, allowed to recover from heat shock, and then agitated to induce aggregation. All cells were stained with the yellow-fluorescing nuclear Hoechst dye.

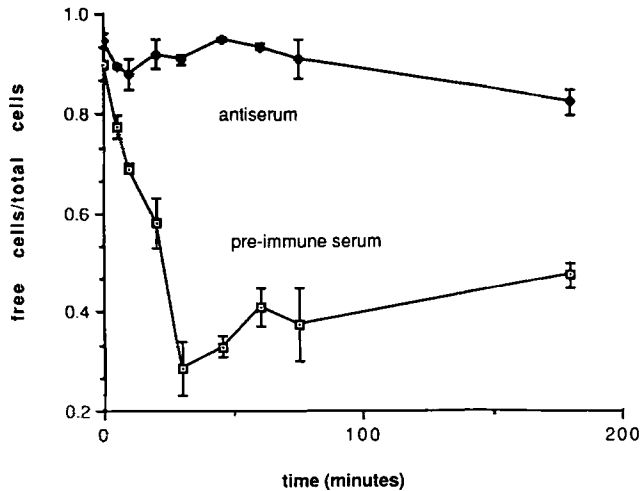


Figure 3. Antiserum against fasciclin I blocks the aggregation of fasciclin I-transfected cells. Rat antiserum against a fasciclin I-protein A fusion protein or preimmune serum from the same animal was added to fasciclin I-transfected S2 cells during the recovery after heat shock, to a final concentration of 1:100. The percentage of total cells that were not part of a cell aggregate was counted at intervals after the start of agitation. When preimmune serum was added to the cells, aggregation occurred rapidly and was complete by ~50 min. When antiserum against fasciclin I was added, most cells did not form aggregates, and the aggregates that were observed did not measurably grow over time.

In the first experiments, fasciclin I cells were mixed with either of two S2 cell types: the original S2 line that had not been transfected, or one that had been transfected with the parental pHT4 plasmid containing the heat shock promoter. In both cases the fasciclin I cells formed large homogeneous aggregates that virtually excluded the other cells. The other cells did not form aggregates and did not adhere to the fasciclin I-transfected cell aggregates (data not shown), supporting the notion that fasciclin I is a homophilic adhesion molecule.

Since other homophilic cell adhesion molecules have been classified by their Ca^{2+} -dependent or Ca^{2+} -independent function, we examined the dependence of fasciclin I-mediated adhesion on Mg^{2+} and Ca^{2+} . Cells were washed and aggregation assays performed in balanced standard saline in the absence of Mg^{2+} or Ca^{2+} and containing EDTA or EGTA, respectively. In each case aggregation was similar to control cells in the presence of divalent cations.

Fasciclin I and Fasciclin III Can Mediate Cell Sorting

Takeichi and colleagues (Nose et al., 1988) have previously shown that L cells expressing E- and P-cadherin segregate from each other when mixed, providing the first direct evidence that the differential expression of cadherins can mediate the selective adhesion and sorting of cells. We wanted to test whether fasciclin I and fasciclin III, two novel neural cell adhesion molecules in *Drosophila* (this report and Snow et al., 1989), could also mediate selective adhesion and cell sorting in vitro. For this experiment, fasciclin I-transfected S2 cells were mixed with fasciclin III-transfected S2 cells (Snow et al., 1989). After a 20-min heat shock and a 90-min recovery to allow for transcription and translation, both cell

lines on their own formed aggregates with similar kinetics: large aggregates are formed within 30 min of agitation. As shown in Fig. 4, A and B, when the two cell lines are mixed together, the mixed cells sorted out in a fashion such that homogeneous aggregates of either fasciclin I- or fasciclin III-expressing cells were observed. Fig. 4 (top) shows all cells, while the bottom shows the same aggregates with only the fasciclin I cells stained with diI. After 30 min of agitation, very large aggregates could be seen that had mixtures of both cell types, but in all of these cases the large aggregates were composed of smaller sectors of hundreds of cells of only one cell type (Fig. 4 B). It may be that fasciclin I- and fasciclin III-expressing S2 cells do have some low affinity for each other. However, even after 48 h of agitation, large homogeneous aggregates as well as the sector boundaries were preserved.

We previously observed that S1 cells naturally express high levels of fasciclin I. Since these cells naturally form aggregates, it seemed possible that this normal aggregation of S1 cells is due in part to the expression of fasciclin I on the surfaces of these cells. To test whether fasciclin I is an adhesive force on the surface of S1 cells, fasciclin I-transfected S2 cells were stained with diI and mixed with S1 cells. After heat shock and agitation, these two cell types formed mixed aggregates in which S1 cells appeared to indiscriminately adhere to themselves and to fasciclin I-transfected S2 cells (Fig. 4 C). That S1 cells appear to adhere to fasciclin I-transfected S2 cells with the same affinity as they do to other S1 cells suggests that fasciclin I is an adhesive force for the normal aggregation of S1 cells; when two different cell lines (S1 and S2 cells) both expressing high levels of fasciclin I are mixed together, they do not sort out. In contrast, when S1 cells are mixed with fasciclin III-transfected S2 cells, they form separate aggregates (data not shown).

Discussion

One of the classical results in developmental biology is the observation that dissociated vertebrate cells from different tissues can reassemble and sort out into organized layers and tissues in culture (e.g., Townes and Holtfreter, 1955; Moscona and Moscona, 1952; Steinberg, 1963). It has been suggested that differential adhesion plays the major role in this ability of cells to reassemble and sort out in culture, and may play an equally important role in organizing cells and tissues during embryonic development. Previous transfection experiments showed that L cells expressing E- and P-cadherin will aggregate and sort out from one another in culture (Nose et al., 1988), thus showing that cadherins can mediate cell sorting. It is still unclear just how many different adhesion molecules are expressed during embryonic development, and how many of these are capable of mediating selective cell adhesion and cell sorting. In the experiments described in this paper, we have shown that two novel cell adhesion molecules in *Drosophila*, fasciclin I and fasciclin III, which do not belong to any of the previously identified families of well-characterized adhesion molecules, can also mediate cell sorting.

Fasciclin I and fasciclin III were initially identified as membrane-associated glycoproteins that are expressed on subsets of fasciculating axons during neuronal development

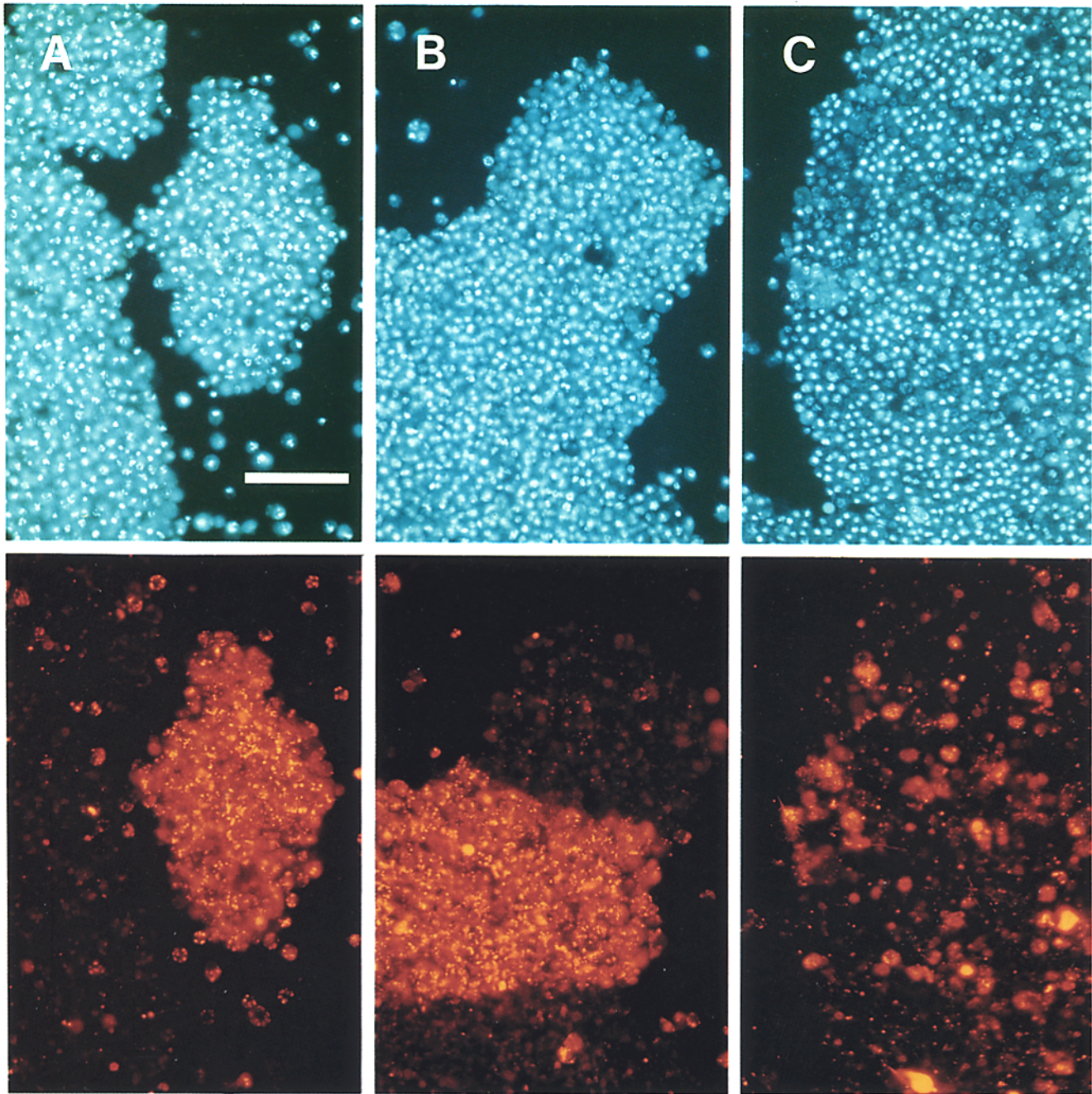


Figure 4. Fasciclin I and fasciclin III can mediate selective cell sorting. (*Top*) all cell nuclei are visualized using Hoechst dye. In the lower panels, only the cells stained with diI are seen. In *A* and *B*, the fasciclin I-transfected S2 cells were stained with diI during heat shock and then mixed with fasciclin III-transfected S2 cells after heat shock. After a 90-min recovery and 30-min agitation, the cell types sorted into cell type-specific homogeneous aggregates. The aggregates remained largely separate early in agitation (*A*) and often formed even larger heterogeneous aggregates later, that were composed of discrete sectors of either fasciclin I- or fasciclin III-expressing cells (*B*). In *C*, S1 cells, which naturally express fasciclin I at high levels, were stained with diI and then mixed with fasciclin I-expressing S2 cells. In this case the two cell types mixed and formed large heterogeneous aggregates. Bar, 100 μ m.

(Bastiani et al., 1987; Patel et al., 1987); the expression of both of these proteins on regions of neurons where they display a high affinity for one another led to the suggestion that these proteins might mediate specific cell adhesion. When the genes encoding both proteins were cloned, their sequences showed no similarity to known proteins; both encode novel protein structures (Zinn et al., 1988; Snow et al., 1989). Fasciclin I is an extrinsic membrane protein that ap-

pears to be linked to the membrane via a phosphatidylinositol (P-I)-lipid anchor (Hortsch, M., and C. Goodman, manuscript submitted for publication); fasciclin III, on the other hand, is an integral membrane protein (Snow et al., 1989).

To test whether fasciclin III can mediate cell adhesion, the relatively nonadhesive S2 cells were transfected with the fasciclin III cDNA under control of the heat shock promoter. In this way, it was shown that fasciclin III can mediate cell

adhesion in a Ca²⁺-independent, homophilic manner (Snow et al., 1989). In this paper, the same system was used to show that fasciclin I can similarly mediate cell adhesion in a Ca²⁺-independent, homophilic manner. The mixing of fasciclin I- and fasciclin III-expressing S2 cells shows that these two *Drosophila* molecules can mediate selective cell adhesion and cell sorting. These observations strengthen the suggestion that fasciclin I and fasciclin III might play a similar role during development both in the developing nervous system and in the various other tissues in which they are expressed throughout embryogenesis.

Previous studies in vertebrates have identified several neural cell adhesion molecules that belong to two gene families: N-CAM (Cunningham et al., 1987; Barthels et al., 1987), L1 (Moos et al., 1988), and contactin/F11 (Ranscht, 1988; Brummendorf et al., 1989) are members of a specific subclass of the immunoglobulin superfamily, and N-cadherin (Hatta et al., 1987; Takeichi, 1988) is part of the cadherin family. Studies thus far in insects have identified the genes encoding four neural cell adhesion molecules. Two of the four genes, fasciclin II (Harrelson and Goodman, 1988) and neuroglian (Bieber et al., 1989), are likely to encode neural cell adhesion molecules because they have extensive structural homology to vertebrate neural adhesion molecules in the immunoglobulin superfamily: fasciclin II is most highly related to N-CAM and neuroglian to L1. Fasciclin I (Zinn et al., 1988) and fasciclin III (Snow et al., 1989), on the other hand, possess novel sequences with no significant homology to previously identified adhesion molecules, yet both proteins can mediate selective cell adhesion and robust cell sorting (this study and Snow et al., 1989).

An interesting correlation emerges: of the four insect neural cell adhesion molecules thus far identified, the two that are widely expressed in the developing CNS, fasciclin II and neuroglian (Bastiani et al., 1987; Bieber et al., 1989), are homologous to previously characterized vertebrate neural cell adhesion molecules, whereas the two with the more restricted axonal expression, fasciclin I and fasciclin III (Bastiani et al., 1987; Patel et al., 1987), have novel structures. It is likely that in both insects and vertebrates, there are additional neural cell adhesion molecules on restricted subsets of axon pathways used for growth cone guidance, which, like fasciclin I and fasciclin III, may define new families of cell adhesion molecules.

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