Expression and Structural Studies of Fasciclin I, an Insect Cell Adhesion Molecule*  
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Fasciclin I is a lipid-linked cell-surface glycoprotein that can act as a homophilic adhesion molecule in tissue culture cells. It is thought to be involved in growth cone guidance in the embryonic insect nervous system. To facilitate structure-function studies, we have generated Chinese hamster ovary (CHO) cell lines expressing high levels of cell surface grasshopper and Drosophila fasciclin I. Grasshopper fasciclin I released by phospholipase C cleavage was purified on an immunooaffinity column and single crystals were obtained that diffracted to ~5-A resolution. We also generated CHO and Drosophila S2 cell lines that produce a secreted form of fasciclin I. Fasciclin I expressed in S2 cells contains significantly less carbohydrate than the protein expressed in CHO cells, and may therefore be more suitable for crystallization. Biochemical characterization of purified fasciclin I indicates that the extracellular portion exists as a monomer in solution. Circular dichroism studies suggest that fasciclin I is primarily a-helical. Its structure is therefore different from other known cell adhesion molecules, which are predicted to be elongated b-sheet structures. This suggests that fasciclin I may define a new structural motif used to mediate adhesive interactions between cell surfaces.

Neural cell adhesion molecules determine many of the specific cell-cell interactions involved in the patterning of the nervous system. These include the pathway choices made by neuronal growth cones, bundling of neuronal processes, and synapse formation and rearrangement. Relatively little biochemical information concerning the molecular details of the recognition properties of adhesion molecules is available, primarily because they are membrane glycoproteins that are difficult to purify in large quantities. Although some of these molecules have been demonstrated to mediate homophilic adhesion when transfected into cells (1, 2), the possibility of heterophilic interactions with an unknown partner molecule is more difficult to test in a transfection assay. We report the development of an expression system to generate milligram amounts of a soluble form of an insect cell adhesion molecule, fasciclin I, as a first step in a biochemical and structural characterization of this neural adhesion molecule. This expression system could potentially be used to produce any adhesion molecule in a form that can be linked to a solid support and used to affinity-purify possible heterophilic ligand proteins.

Several novel cell adhesion molecules have been identified using monoclonal antibodies against neuronal membrane proteins from grasshoppers and Drosophila embryos. The three fasciclins are expressed on subsets of central nervous system axons and are candidates for molecules involved in growth cone guidance during embryonic development (3). Another adhesion molecule, neuroglian, is more widely expressed on neurons and glia (3). Fasciclin II and neuroglian are members of the immunoglobulin (Ig) superfamily and have domain organizations identical to those of the vertebrate neural cell adhesion molecules N-CAM and L1, respectively (4). Fasciclin III is distantly related to the Ig superfamily (3, 5). Fasciclin I contains four related domains, each of approximately 150 amino acids, that are not similar to other sequences in the current data bases (6). All four of the Drosophila molecules can function as homophilic adhesion molecules in transfected Drosophila Schneider 2 (S2) cells, and cell aggregates expressing different fasciclins sort away from each other (3, 5, 7).

In the embryo, Drosophila fasciclin I (72 kDa) and grasshopper fasciclin I (75 kDa) are expressed on the surfaces of all peripheral nervous system axons, a subset of central nervous system axons, and on some non-neuronal cells (6, 8, 9). Genetic evidence suggests that fasciclin I is involved in guidance. This allows it to be released from the cell surface as a soluble molecule by treatment of intact cells with phosphatidylinositol-specific phospholipase C (PI-PLC). We show that

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The abbreviations used are: Ig, immunoglobulin; CHO, Chinese hamster ovary; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBS-T, TBS-Tween; FPLC, fast protein liquid chromatography; sMK, a minimum essential medium; DSP, 3,3'-dithio-bis-propanoic acid; N-hydroxysuccinimide ester; GPI, glycosyl-phosphatidylinositol; MSX, methionine sulfoximine; PI-PLC, phosphatidylinositol-specific phospholipase C.

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grasshopper and Drosophila fasciclin I can be expressed in a GPI-linked form at high levels on the surface of mammalian cells, and in a secreted form when the sequence is truncated before the start of the GPI addition signal. Levels of protein expression were increased using a glutamine synthetase-based amplifiable expression system developed at Celltech, Berkshire, United Kingdom (11). When secreted from or expressed on the surface of Chinese hamster ovary (CHO) cells, the molecule contains an extra 15 kDa of carbohydrate not found on fasciclin I from insect embryos. Milligram quantities of soluble expressed fasciclin I have been purified on an immunoadfinity column, and large single crystals have been obtained. Perhaps because of the excess carbohydrate on the CHO-expressed protein, the crystals do not diffract to sufficient resolution to allow a structure determination by x-ray crystallographic methods. Preliminary studies in which the truncated form of grasshopper fasciclin I was transfected into Drosophila S2 cells show that the resulting secreted protein contains significantly less carbohydrate, which may make it more amenable to crystallization. Soluble fasciclin I expressed in CHO or S2 cells exists as a monomer in solution. This observation suggests that any homophilic interactions it mediates are of low affinity, or involve clusters of proteins on the cell surface. Circular dichroism analysis suggests that the structure of fasciclin I is primarily α-helical. This is in contrast to the predominantly β-sheet structures predicted for adhesion molecules that are members of the immunoglobulin superfamily (1, 2, 12) and/or contain fibronectin type III repeats (2).

MATERIALS AND METHODS

Reagents—Endoglycosidase F/N-glycosidase F was obtained from Boehringer Mannheim. The hybridoma cell line that produces 3B11, a mouse monoclonal antibody against grasshopper fasciclin I (8), and rat anti-grasshopper fasciclin I antiserum were gifts of Michael J. Bastiani (University of Utah). The monoclonal antibody 6D8 against Drosophila fasciclin I (13) was the gift of Corey Goodman (University of California, Berkeley). Goat anti-mouse fluorescein-conjugated IgG and goat anti-mouse IgG-peroxidase conjugate were obtained from Cappel Products. Mouse anti-rat IgG-alkaline phosphatase conjugate for use on Western blots was from Boehringer Mannheim. Methionine sulfoxime (MSX) and phosphacel C were from Sigma. The S2 cell line and the phnseo vector were gifts of Michael Jackson (Scripps Clinic). The PfRmHa3 vector was the gift of Allan Bieber (Purdue University). All other chemicals were reagent-grade.

Construction of Ligand-linked and Soluble Forms of Grasshopper Fasciclin I—Molecular cloning experiments were performed by standard methods (14). Lipid-linked forms of fasciclin I were constructed by introducing full-length cDNA clones encoding grasshopper and Drosophila fasciclin I (6) into the EcoRI site of the polylinker of the expression vector pBJS5 (15), generating plasmids pBJS5.FGI and pBJS5.DFI, respectively. The cDNA clones used for the constructions encoded type IV grasshopper fasciclin I and type II Drosophila fasciclin I (9), respectively. This nomenclature refers to the presence or absence of micro-exon sequences between domains 2 and 3. Type II Drosophila fasciclin I contains the micro-exon sequence SFK, and type IV fasciclin I contains the micro-exon sequence SFK (9). The glutamine synthetase minigene from the Celltech vector pSVLGS was excised using AarI and BamH1, the ends were filled in using T4 polymerase, and the resulting fragment was blunt-ended cloned into the filled-in Xhol site of pBlueScript KS(+) (Stratagene). A 5.5-kilobase Xhol-Saff fragment containing the glutamine synthetase minigene was then cloned into the unique Sall site of pBJS5.DFI to generate pBJS5.GS.DFI or into one of the two Sall sites of pBJS5.GI in a partial digest to obtain pBJS5.GS,GI.

To obtain a secreted form of grasshopper fasciclin I, a stop codon followed by a BamHl site was inserted after residue 684 by site-directed mutagenesis (16). pBJS5.GS,GI was generated by introducing an EcoRV-Ncol fragment containing the modified cDNA into the Xhol (filled-in) and NotI sites of the polylinker of pBJS5.GS (17).

For expression in insect cells, the modified cDNA encoding the truncated form of grasshopper fasciclin I was subcloned into the EcoRI and BamHl sites of the polynucleotide of the expression vector PfRmHa3 (18) to generate pRmHa3.GFI. Cell Culture and Transfection—COS7 cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin (GIBCO or Irvine Scientific). COS7 cells were grown in a minimum essential medium (MEM; Irvine Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. S2 cells were grown in Schneider’s medium (Sigma) supplemented with 12.5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin.

For transient transfections, COS7 cells were transfected by the DEAE-dextran method (12) and selected with 70% confluent plates. A 10% MeSO shock for 2 min following transfection with DEAE-dextran at a concentration of 100-200 units/ml leads to increased expression levels. Three days after transfection, the cells were harvested, washed with phosphate-buffered saline, and suspended in homogenization buffer (20 mM Tris, pH 7.5, 1 mM imidazole, 1 mM diithiothreitol, 1 mM MgCl2, 20 mM sodium diphosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin). The cell extracts were then subjected to a chromatochemical acrylamide transfer assay (21) to determine the effectiveness of the transient transfection, or were prepared for Western blot analysis.

The pBJS5.GS.FGI and pBJS5.GS,GI expression vectors were transfected into CHO cells by a liposome (Lipofectin)-mediated method (Bethesda Research Laboratories). Selection and amplification of the transfectants was done by the protocol established by Celltech. In brief, 200 μl of DNA-Lipofectin complexes (30 ng of DNA plus 80-100 ng of Lipofectin) were introduced into a 10-cm plate with 50% confluent cells cultured in 1% dialyzed fetal bovine serum, aMEM without glutamine. After 18-24 h of incubation at 27°C, the medium was replaced with 10% dialyzed fetal bovine serum, aMEM without glutamine and the incubation continued for an additional 24 h. The cells were then split into six 96-well plates and selected with 25 μg MSX in 10% dialyzed fetal bovine serum, aMEM without glutamine. MSX-resistant clones were isolated 2 weeks later. Positive transfectants were recloned and amplified under 100-500 μg MSX in 10% dialyzed fetal bovine serum, aMEM without glutamine. For cells transfected with pBJS5.GS,GI, the highest expressing cell populations were selected by immunofluorescence with 3B11 or flow cytometry of cells stained with 3B11. For the selection of clones expressing high levels of the secreted form of protein, cell supernatants were filtered through a nitrocellulose paper using a Minifold II slot blot system (Schleicher & Schuell model SRC072/0), and the presence of secreted fasciclin I was verified by immunostaining. The highest expressing cell lines were grown in a Cell Pharm II hollow-fiber bioreactor device (Unisyn Fibertec, Danville, CA).

For stable transfection using the insect expression system, 25 μg of pRmHa3.GS,GI plus 2.5 μg of pPhneo, a selection vector, were cotransfected into S2 cells (106 cells/ml) cultured in 10-cm plate (10 ml of medium) using a calcium phosphate procedure (Stratagene). Two days after transfection, plates were split with 1.0-1.1 ml of 8.0 M G418 (GIBCO). For 2 weeks, the cells were passaged for a few generations and cloned in soft agar. In brief, 8 ml of wild type S2 cells (106 cells/ml) and transformed cells (100-800 cells) in Schneider’s medium, 12.5% fetal bovine serum, penicillin/streptomycin, with 1.0-1.6 mg/ml G418 were pipetted onto one side of a 10-cm bacterial-grade Petri dish, leaving most of the surface of the plate uncovered. Two ml of a 1.5% molten agar solution (48 °C; Difco 142-02) were pipetted onto the opposite side of the Petri plate. The contents of the plate were mixed thoroughly by swirling, and the plates were allowed to solidify and sealed with parafilm. Within 2 weeks of culture at 26°C, clones became visible and were isolated and grown in individual wells of a 96-well plate. Expression of protein was induced for 2-3 days in serum-free medium containing 0.7 mM CuSO4, and supernatants from individual clones were examined by Western blotting or using the Minifold II slot blot system.

Get Electrophoresis and Western Blot Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (22). Protein transfer after SDS-PAGE to nitrocellulose paper was done at 100 V for 3 h in transfer buffer (20 mM Tris, 190 mM glycine, 100 mM sodium dithiothreitol, 10% methanol) and paper was immersed in 5% nonfat dry milk in TBST solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min and then incubated A. Bieber, unpublished data.
with the primary mAb (6D8) at dilution of 1:1000 with TBST. After 1 h, the blots were washed for 10–15 min three times with TBST, twice with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and incubated with affinity-purified horseradish-peroxidase-conjugated goat anti-mouse IgG or mouse anti-rat (1:200, diluted with TBS, Cappel) for 30 min. After three washes with TBS, two with phosphate-buffered saline, and one with 50 mM Tris, pH 7.6, the blots were visualized using a diaminobenzidine-hydrogen peroxide staining solution (0.06% diaminobenzidine (w/v), 0.03% H2O2 (v/v) in 50 mM Tris, pH 7.6) until the bands were suitably dark (about 5 min). All procedures were carried out at room temperature.

Flow Cytometric Analysis—Tryptophan cells (2 × 10^6 cells/sample) were incubated with mAbs in ascites fluid at a dilution of 1:100 for at least 15 min on ice, washed three times with phosphate-buffered saline, and incubated with fluorescein-conjugated goat anti-mouse IgG. After 15 min, cells were washed three times with phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline containing 0.02% sodium azide, and passed through a 40 μm filter. Flow cytometric analyses were carried out on Ortho Cytofluorograf 50H with a Data General 2150 computer system.

Enzymatic Characterization of Fasciclin I—Protein from transfected cells and from 10–14 h-old Drosophila embryos were deglycosylated with endoglycosidase F carried out according to the manufacturer’s specifications (Boehringer Mannheim). Deglycosylated protein was analyzed on Western blots.

Purified grasshopper fasciclin I from CHO cells was treated with a battery of proteases, including trypsin, chymotrypsin, elastase, and papain. The protein was mixed with trypsin 100 μg/ml in 0.2 M sodium borate, 3 M NaCl, pH 9.0, for immediate neutralization. Generally, about 1 mg of grasshopper fasciclin I. About 0.5 mg of protein was obtained from 1 liter of S2 cells (5 × 10^6 cells/ml) after induction by 0.7 mM copper sulfate for 4 days.

Size-exclusion Chromatography—Following immunofluorimetry chromatography, fasciclin I was concentrated to approximately 0.2–1 mg/ml using a vacuum dialysis apparatus (Schleicher & Schuell) and an Amicon Centricon-30 (M, cutoff of 30,000). 50 μl of the concentrated protein were run on an FPLC Superose 12 column equilibrated with phosphate-buffered saline.

Cross-linking—Purified grasshopper fasciclin I (20 μl at 0.01–0.02 mg/ml) was treated for 30 min at 4 °C with 5,5’-dithio-bis-propanoic acid (DSP; 40 mg/ml as a stock in dimethyldimethylene oxide), a thiol-cleavable homobifunctional cross-linking reagent, at concentrations ranging from 100 to 800 μg/ml. Cross-linking reactions were stopped by addition of glycine to 50 mM, and samples were analyzed by Western blotting an SDS-PAGE gel run under reducing and nonreducing conditions.

Crystallization—Crystallization trials were conducted in hanging drops using the method of vapor diffusion (25). Hanging drops of grasshopper fasciclin I (4.2–4.6 mg/ml in 10 mM Hepes, 0.02% NaN3, pH 7.5, were prepared by mixing equal volumes of the protein and the well precipitant on a coverslip, which was then inverted and equilibrated over a well (24-well plate, Linbro) containing 1 ml of the undiluted precipitant. Crystals were grown in 1.84 M ammonium sulfate, pH 7.5–8.5, or 1.6 M Na,K phosphate, pH 7.5–8.5.

Circular Dichroism—The spectrum of CHO-derived secreted grasshopper fasciclin I (0.21 mg/ml in 5 mM sodium phosphate, pH 7.5) was recorded using a Jasco 781 spectropolarimeter. A cuvette with an 0.1 cm path length was used, and the mean residue ellipticity (degrees·cm²/dmole) was determined assuming a mean residue weight of 141. All spectra were recorded at room temperature from 260 to 185 nm and were determined as the average of five scans.

RESULTS

Expression of GPI-linked and Soluble Forms of Fasciclin I in Mammalian and Insect Cell Lines—To express fasciclin I in mammalian cells, full-length cDNAs encoding the grasshopper and Drosophila fasciclin I proteins (6) were subcloned into the expression vector pBJS (15, 26), which contains a strong SV40/HTLV-1 hybrid promoter, as well as the SV40 origin of replication. The fasciclin I expression plasmids were transiently transfected into COS7 cells, in which plasmids bearing SV40 origins are efficiently replicated. After 3 days, cells transfected with the grasshopper fasciclin I plasmid were analyzed by immunofluorescent staining with the mAb 3B11, which recognizes grasshopper fasciclin I. 1–5% of the cells displayed bright surface staining (data not shown).

COS7 cells transfected with the Drosophila fasciclin I plasmid were treated with PI-PLC, and samples of supernatant and membranes were analyzed for the presence of fasciclin I by Western blotting with the mAb 6D8. Almost all of the fasciclin I appeared in the supernatant fraction, indicating that it could be released from the transfected cells by PI-PLC (data not shown). Because only a limited amount of antiserum capable of recognizing grasshopper fasciclin I on a Western blot was available, a comparable experiment was not performed on COS7 cells transfected with the grasshopper fasciclin I plasmid. However, similar data were obtained for grasshopper fasciclin I expressed in CHO cells (see below).

Hatton was determined that fasciclin I could be expressed on the surface of mammalian cells, stable transformant lines were created in CHO cells. The glutamine synthetase minigene was first subcloned into pBJS.GF1 and pBJS.DF1 to create the plasmids pBJS.GS.GF1 and pBJS.GS.DF1. The glutamine synthetase gene can be used as a selectable marker and genes of gene amplification in the presence of the drug MSX, a system developed at Celltech, Berkshire, United Kingdom (11). The glutamine synthetase-fasciclin I plasmids were introduced into CHO cells, and stable transformant lines were selected and amplified with increasing concentrations of MSX. Analysis of the highest expressing grasshopper fasciclin I line by fluorescence-activated cell sorting using the mAb 3B11...
showed that most of the fasciclin I could be released from these cells by PI-PLC (Fig. 1).

We were unable to analyze the Drosophila fasciclin I-expressing lines by fluorescence-activated cell sorting because none of the existing anti-fasciclin I mAbs, which had been raised against denatured protein expressed in E. coli (13), were capable of staining the surfaces of live cells. Since these mAbs could not be used for purification by affinity chromatography, we did not attempt purification of Drosophila fasciclin I expressed in CHO cells. We have since used native protein released from the CHO-Drosophila fasciclin I line to generate new mAbs that stain live cells and can be used for affinity chromatography,\(^3\) reagents that should be useful for future studies.

To characterize the CHO-derived grasshopper and Drosophila fasciclin I, cells were metabolically labeled with \(^{35}\)S methionine, and supernatants from cells after PI-PLC treatment were analyzed on a 10% SDS-PAGE gel (Fig. 2A). The PI-PLC-released grasshopper and Drosophila fasciclin I migrated with apparent molecular masses of 90 and 85 kDa respectively, as compared to PI-PLC-released fasciclin I from insect embryos, which migrates with an apparent molecular mass of 75 kDa (grasshopper; Ref. 27) or 72 kDa (Drosophila; Ref. 13). A preparative amount of the 90-kDa band was isolated and subjected to N-terminal amino acid sequencing. The sequence obtained, KGEEKSLKIRDDPDL, exactly corresponds to the N-terminal 16 residues of mature grasshopper fasciclin I (6, 27). The difference in molecular weight between CHO-derived grasshopper and Drosophila fasciclin I is probably due to the presence of six potential N-linked glycosylation sites in the grasshopper fasciclin I sequence, as compared to four in Drosophila fasciclin I (6).

Complex oligosaccharides are added to proteins synthesized in cells from vertebrate hosts, as compared to small oligosaccharide cores that are added to proteins synthesized in insect cells (28, 29). Thus, it seemed likely that the difference in molecular weight between the insect and CHO-derived fasciclin I proteins was due to additional glycosylation in CHO cells. To examine this possibility, PI-PLC-released protein from CHO lines was treated with endoglycosidase F and compared on a Western blot to similarly treated fasciclin I from Drosophila embryos. After deglycosylation, Drosophila fasciclin I from CHO cells comigrates with fasciclin I derived from embryos. Deglycosylated CHO-derived grasshopper fasciclin I also migrates at a similar position, corresponding to an apparent molecular mass of 68 kDa (Fig. 2B).

For generation of ordered crystals, a chemically homogeneous protein is the preferred starting material. In order to eliminate a potential source of heterogeneity in purified fasciclin I caused by the portion of the GPI tail remaining after PI-PLC digestion, a directly secreted form of the protein was also expressed in CHO cells. A stop codon was introduced by site-directed mutagenesis (16) after amino acid 634 of grasshopper fasciclin I, which corresponds to the position presumably encoding the beginning of the GPI linkage site in the fasciclin I cDNA (13, 30), and the CHO expression vector was reconstructed. This plasmid, pBJ.GS.sGf1, was introduced into CHO cells and amplified lines selected as described above. The highest expressing lines were identified by slot blot analysis using the mAb 3B11 or by Western blotting of cell supernatants using the rat anti-fasciclin I antiserum.

\(^3\) W.-C. Wang, unpublished results.

\(^4\) T.-Y. Kung and W.-C. Wang, unpublished results.
The highest expressing CHO clones making the GPI-linked and the secreted versions of grasshopper fasciclin I were grown in a Cell Pharm II hollow fiber bioreactor device (Unisyn Fibertec, San Diego, CA) in the presence of 100 or 300 μM MSX. The expressed protein was isolated from the medium by passing it over a 3B11 mAb immunoaffinity column after a 3-h phospholipase C incubation in the case of the GPI-linked fasciclin I, or with no incubation in the case of the truncated fasciclin I. Approximately 0.7–1.0 mg of pure protein per daily harvest from lines expressing either the GPI-truncated fasciclin I. Approximately 0.7-1.0 mg of pure protein were treated with trypsin, chymotrypsin, papain, and elastase. Fasciclin I was resistant to digestion by all of these proteases (data not shown), suggesting that the protein is folded into a stable structure without obvious flexible linker sequences between its domains.

To examine the stability and possible domain structure of purified fasciclin I, samples of purified protein were treated with trypsin, chymotrypsin, papain, and elastase. Fasciclin I was resistant to digestion by all of these proteases (data not shown), suggesting that the protein is folded into a stable structure without obvious flexible linker sequences between its domains.

Drosophila fasciclin I has been shown to act as a homophilic adhesion molecule in tissue culture cells (7), so it was of interest to determine whether this adhesion activity would correlate with oligomerization in solution. The oligomeric structure of purified grasshopper fasciclin I was examined by gel filtration and cross-linking studies. Concentrated fasciclin I from CHO or S2 cells migrates on a Superose 12 FPLC column in a position corresponding to a monomeric rather than a dimeric or higher oligomeric structure (Fig. 4A). In addition, cross-linking of the S2-derived protein with the homobifunctional reagent DSP did not result in the appearance of dimeric or other oligomeric species on SDS-PAGE gels (Fig. 4B). Thus, our data suggest that the extracellular portion of fasciclin I exists as a monomer in solution. Similar results have been observed for the extracellular portions of the homophilic adhesion molecules N-CAM (32) and cadherins (32, 33).

Crystallization and Structural Characterization of Fasciclin I—Grasshopper fasciclin I purified after PI-PLC cleavage crystallizes in the orthorhombic space group C2221, with unit cell dimensions 156 Å × 352 Å × 168 Å. Based on average volume to mass ratios (34), the asymmetric unit of the crystal is estimated to contain between 2 and 8 molecules (corresponding to solvent contents between 81% and 23%). Typical crystals grow to a size of 0.5 mm × 0.3 mm × 0.3 mm (Fig. 5). Using nickel-filtered CuKα radiation from a rotating anode
was analyzed. As shown in Fig. 6, the CD spectrum of fasciclin within the molecule, thus allowing the estimation of the expression system for crystallization trials. A significant portion of α-helical structure. The far-UV CD spectrum of fasciclin I, the far-UV CD spectrum of fasciclin I was analyzed. As shown in Fig. 6, the CD spectrum of fasciclin I is characterized by a positive peak at 195 nm followed by deep negative doublets at 208 nm and 220 nm, indicating a strong α-helical component. The CD spectrum in this region (35). Based on an equation developed by Greenfield and Fasman (1969), the α-helical content of fasciclin I is calculated as 43% (36). A similar estimate of the percentage helical structure is obtained using the algorithm of Chang (37), in which analysis of the fasciclin I CD spectrum predicts 37% α-helix, 0% β-sheet, 19% β-turn, and 34% irregular structure. Thus, the CD spectrum converts to random coil in the presence of guanidine hydrochloride (data not shown). (iv) We have obtained crystals of fasciclin I. Crystallographic studies suggest that the extracellular portion of grasshopper fasciclin I mediate homophilic adhesion in S2 cells, but +6 does not, or does so much more weakly. The +2 form of grasshopper fasciclin I, which was the form originally cloned (6) and the one that has been used for all expression and aggregation experiments, may be equivalent to Drosophila +6. Thus, this form of fasciclin I may have a function other than, or in addition to, homophilic adhesion. To look for other activities of the purified fasciclin I, we added it to grasshopper embryo cultures in an attempt to perturb the formation of axon tracts. However, no obvious effects were observed.

Despite the lack of a functional assay for grasshopper fasciclin I protein, we are confident that the molecule we have purified is correctly folded. Five lines of evidence support this conclusion. (i) Purified fasciclin I is resistant to digestion by several different proteases (see “Results”), the behavior of a compact globular protein rather than an aberrantly folded or denatured protein. (ii) The purified protein migrates as a sharp peak corresponding to a monomer on a gel filtration chromatography and cross-linking studies suggest that the extracellular portion of grasshopper fasciclin I, which was the form originally cloned (6) and the one that has been used for all expression and aggregation experiments, may be equivalent to Drosophila +6. Thus, this form of fasciclin I may have a function other than, or in addition to, homophilic adhesion. To look for other activities of the purified fasciclin I, we added it to grasshopper embryo cultures in an attempt to perturb the formation of axon tracts. However, no obvious effects were observed.© M. Seeger and C. S. Goodman, personal communication.© B. Condron, M. Bastiani, and W.-C. Wang, unpublished results.
per fasciclin I is a monomer in solution. Thus, we do not observe an oligomerization activity in solution that correlates with the homophilic adhesion activity of Drosophila fasciclin I expressed on the surface of S2 cells (7). Similarly, formation of dimers or polymers of isolated extracellular domains of other homophilic adhesion molecules such as the cadherins (32, 33), or N-CAM (32) have never been observed. These results suggest that the homophilic interactions mediated by these adhesion molecules are of low affinity, and/or involve clusters of proteins on the cell surface. In the case of grasshopper fasciclin I, the lack of oligomerization is consistent with the lack of homophilic adhesion activity displayed by grasshopper fasciclin I in S2 cells.\(^\text{3,5}\)

Crystals of purified fasciclin I do not diffract to a resolution sufficient for the determination of an atomic resolution structure. This may be due to the large amount of excess carbohydrate on the CHO-derived protein as compared to the amount of carbohydrate on fasciclin I expressed in insect cells. In an attempt to obtain fasciclin I with the physiological amount of carbohydrate, we expressed a secreted version of grasshopper fasciclin I in Drosophila S2 cells. However, because of low expression of fasciclin I in S2 lines, we have been unable to obtain sufficient protein bearing the normal amount of carbohydrate for extensive crystallization trials.

In the absence of high resolution structure information, an analysis of its circular dichroism spectrum was performed to allow us to compare the secondary structure composition of fasciclin I to that of other adhesion molecules. A number of adhesion molecules mediating interactions in the nervous and the immune systems consist of a series of repeated \(\beta\)-sheet domain structures. For example, the extracellular domains of N-CAM and fasciclin II are composed of five immunoglobulin C2-like domains followed by two fibronectin type III repeats (3, 40). ICAM-1, which interacts with the integrin LFA-1 on T cells, contains five immunoglobulin variable-like domains in its extracellular portion (41). The immunoglobulin variable domain fold has been well-characterized as a two \(\beta\)-sheet structure linked by a disulfide bond, and the crystal structure of a fragment of CD4 (42, 43) illustrates its use by an adhesion molecule that is a member of the immunoglobulin superfamily. Fibronectin type III repeats are also \(\beta\)-sheet structures, as predicted (44) and verified by the recent crystal structure of the human growth hormone receptor (38). A combination of electron microscopic studies (32, 41) with crystallographic studies of CD4 (42, 43, 45) suggest that adhesion molecules that are immunoglobulin superfamily members are likely to have extended structures with flexible linker regions between domains.

The primary sequence of fasciclin I suggests that it consists of four related domains arranged in tandem on a single polypeptide chain, similar to the domain organization of CD4 (46), but the fasciclin I repeats do not have homology to immunoglobulin domains or to any other protein in the current data bases (6). An analysis of the circular dichroism spectrum of purified grasshopper fasciclin I suggests that it is a predominantly \(\alpha\)-helical protein, with little or no contribution from \(\beta\)-structure. Based upon the 48% sequence identity between Drosophila and grasshopper fasciclin I (6), the \(\alpha\)-helical structure demonstrated for the grasshopper protein is expected to be shared by the Drosophila homolog as well (47). These results suggest that the structure of fasciclin I is substantially different than the structures of other vertebrate and vertebrate adhesion molecules, and that they may mediate adhesion through use of a novel structural motif. A human protein related to fasciclin I has been found,\(^\text{2}\) demonstrating the existence of a vertebrate molecule using this structural motif. So far, a function in mediating homophilic adhesion has been demonstrated for two of the three forms of Drosophila fasciclin I, but the functions of the other forms of insect fasciclin I and of the human fasciclin I-related protein are unknown. It is likely that additional molecules will be found in both vertebrate and invertebrate systems that use a helical motif to mediate adhesive interactions, and it will be of interest to define their mode of interaction and function. The experiments described here are the initial steps toward a molecular characterization of this new family of proteins.

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