Gliolectin is a novel carbohydrate-binding protein expressed by a subset of glia in the embryonic Drosophila nervous system

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SUMMARY

Interactions between embryonic neural cells generate the specific patterns of connectivity observed in nervous systems. Cell surface carbohydrates have been proposed to function in cellular recognition events guiding such interactions. Carbohydrate-binding proteins (lectins) that recognize specific oligosaccharide ligands in embryonic neural tissue provide a molecular mechanism for carbohydrate-mediated cell-cell interactions in neural development. Therefore, we have screened an embryonic Drosophila melanogaster cDNA library, expressed in COS1 cells, for carbohydrate-binding activity. COS1 cells expressing putative Drosophila lectins were identified and recovered based on their adhesion to immobilized preparations of neutral and zwitterionic glycolipids extracted from Drosophila embryos. We have identified an endogenous lectin expressed during Drosophila embryogenesis. The cloned lectin, designated ‘gliolectin’, possesses a novel protein sequence with a calculated molecular mass of 24,993. When expressed in Drosophila S2 cells, the lectin mediates heterophilic cellular aggregation. In embryos, gliolectin is expressed by a subset of glial cells found at the midline of the developing nervous system. Expression is highest during the formation of the Drosophila embryonic axonal commissures, a process requiring midline glial cell function. Immunoprecipitation with a monoclonal antibody against gliolectin yields a protein of Mr=46,600 from Drosophila embryonic membranes, suggesting that post-translational modification of gliolectin is extensive. Epitope-tagged chimeric proteins composed of the amino terminal one-half of gliolectin and the Fc region of human IgG bind a small subset of the total glycolipids extracted from Drosophila embryos, demonstrating that the lectin activity of gliolectin can discriminate between oligosaccharide structures. The presence of gliolectin in the developing Drosophila embryonic nervous system further supports a role for cell surface carbohydrates in cell-cell recognition and indicates that the molecular diversity of animal lectins is not yet completely defined.

Key words: carbohydrate, glycosphingolipid, lectin, glia, gliolectin, Drosophila, nervous system

INTRODUCTION

Cell surface carbohydrates are positioned to mediate cell-cell interactions during development. Composed of the pendant oligosaccharides of glycoproteins, glycolipids and proteoglycans, the glyccalyx forms the interface at which eukaryotic cells recognize and associate with each other. In addition to being localized to the cell surface, carbohydrate expression is regulated, allowing cells to control the surface that they present to their environment and to other cells (Varki, 1993). Within neural tissue, specific distributions of carbohydrate structures have been demonstrated both chemically and immunologically. In vertebrates, for example, the HNK-1 antigen (sulfoglucuronylneolactose) is expressed on neural crest cells and is subsequently found greatly enriched in cells of the peripheral nervous system (Chou et al., 1991). Furthermore, a topographic gradient of expression has been described for a specifically derivatized glycolipid (9-O-acetyl GD3, the Jones antigen) in the developing rat retina (Constantine-Paton et al., 1986; Blum and Barnstable, 1987). In Drosophila melanogaster and other insects, an N-linked carbohydrate of incompletely defined structure, the HRP-epitope, is expressed only within neural tissue (Jan and Jan, 1982; Snow et al., 1987).

For cell surface oligosaccharides to mediate cellular interactions, complementary protein receptors should exist that specifically recognize carbohydrate ligands on apposed cells. Examples of such lectins are found in vertebrates and invertebrates. For instance, the carbohydrate-binding activities of the vertebrate selectins, a family of C-type lectins expressed on endothelial cells, leukocytes and platelets, mediate cellular recognition events between an inflamed vasculature and blood-borne cells (Lasky, 1992). Among the cellular ligands recognized by the selectins are glycolipid oligosaccharides related to the sialyl-Lea structure (Tiemeyer et al., 1991; Brandley et al., 1990; Walsh et al., 1990; Phillips et al., 1990). Inducibly expressed C-type lectins have also been described in invertebrate species, suggesting that the controlled expression of car-
bohhydrates and endogenous lectins is an evolutionarily conserved, or perhaps reinvented, mechanism of cellular recognition (Jomori and Natori, 1991; Takahashi et al., 1985; Giga et al., 1987).

In developing neural tissue several carbohydrate-binding activities have been described; some have been attributed to specific proteins. The rat galectins, RL14 and RL29, are transiently expressed by subsets of dorsal root ganglion cells and may mediate interactions with neural and non-neural cells that bear appropriate lactoseries oligosaccharides (Hynes et al., 1990; Dodd and Jessell, 1985; Regan et al., 1986). Carbohydrate-binding proteins have also been characterized in rat oligodendrocytes and Schwann cells where they are positioned to mediate interactions between these myelinating cells and the axons that they ensheathe (Tiemyer et al., 1990; Needham and Schnaar, 1993).

The molecular identification of endogenous lectins in developing tissues implicates cell surface carbohydrates in the mediation of cell-cell interactions. The characterization of lectins in Drosophila, an organism amenable to genetic manipulation, presents opportunities for in vivo analyses of carbohydrate function. To realize this potential, we have undertaken the identification of lectins expressed in Drosophila embryos. Here, we report on a novel carbohydrate-binding protein, ‘gliolectin’, identified through an expression cloning scheme based on carbohydrate-mediated cell adhesion. Gliolectin, expressed by a small number of glial cells at the midline of the Drosophila embryonic nervous system, is spatially and temporally positioned to participate in the formation of axonal commissures. Furthermore, the protein’s binding activity discriminates among carbohydrate structures isolated from Drosophila embryos. The limited expression pattern and restricted binding specificity of this novel protein are consistent with gliolectin’s potential function as a cellular recognition molecule in neural development.

**MATERIALS AND METHODS**

**Chemicals**

All solvents were glass redistilled from HPLC grade stocks. The CDMS expression plasmid was obtained from Brian Seed (Harvard) and pBluescript from Stratagene. The plasmid pCOS(LB) was kindly supplied by ATCC. Conjugated anti-species antibodies and Protein A were purchased from Zymed, Inc. and Jackson Immunoresearch. All chemicals and biologicals were of the finest grade available.

**Preparation of glycolipid surfaces for selection by cell adhesion**

The wells of flat-bottomed polystyrene microtiter plates (96-well PVC, Dynatech) were adsorbed with glycolipids as previously described (Blackburn et al., 1986) except that phosphatidylcholine (PC) and cholesterol (C) were added to glycolipid mixtures as carrier. Briefly, enough glycolipid solution in 4:8:3 (chloroform:methanol:water) to give 1 nmol Man equivalents per well of PC:C (1:4, 5 μM total lipid) in 100% ethanol by vigorous sonication and vortex. Following a 1:1 dilution with water, 50 μl of the glycolipid/PC:C mix was added to each microtiter well and the plates were incubated for 90 minutes at ambient temperature to allow adsorption of the lipid. Some wells received PC:C alone for determination of background cell adhesion.

Library enrichment and selection for COS cells expressing lectins by cell adhesion

The day before transfection, COS1 cells were plated into 10 culture
dishes (10 cm diameter) at 2.5×10^5 cells per dish in DMEM supplemented with 10% Nu Serum (Collaborative Research). Transfections were performed by DEAE-Dextran as described by Seed and Aruffo (1987). For metabolic radiolabelling, 25 μCi per dish of carrier-free 32P-O4 (8000-9000 Ci/mmol, Du Pont-New England Nuclear) were added to two randomly selected dishes of library-transfected COS1 cells and also to the two dishes of control-transfected COS1 cells 48 hours post-transfection.

Cell monolayers were prepared for harvest 60-72 hours post-transfection by removing the culture media and briefly washing each dish with 5 ml of Ca2+- and Mg2+-free PBS supplemented with 1 mM EDTA and 0.02% sodium azide. Following removal of this wash, 5 ml of PBS/EDTA/azide was added to each dish and the plates were incubated for 20 minutes at 37°C. Cells were harvested by trituration and the resulting cell suspension was transferred to a centrifuge tube on ice. An additional 5 ml of PBS/EDTA/azide was added to each dish to recover residual cells.

Cells were pelleted, washed by resuspension in ice-cold Ca2+- and Mg2+-free PBS, repelleted and finally resuspended at 1.5×10^5 viable cells/ml (non-radiolabeled library-transfected cells) or 4×10^5 viable cells/ml (radiolabeled cells) in cold Ca2+- and Mg2+-free PBS. Cells were maintained on ice until used, generally less than 30 minutes. Aliquots (50 μl) of cell suspensions were added to the wells of microtiter plates adsorbed with NZ fraction glycolipids and the plates were incubated for 60 minutes at 37°C. Radiolabeled cells, both library transfected and control-transfected populations, were assayed in parallel for their ability to adhere to glycolipid-adsorbed surfaces by the method of Blackburn (1986).

To separate adherent from non-adherent cells, conditions of controlled detachment were employed (McClay et al., 1981). In particular, procedures were adapted to ensure that cells in the microtiter well would not experience passage through an air/water interface, potentially dislodging weakly adherent cells. Centrifugation chambers, originally described by Swank-Hill et al. (1987) for the analysis of cell adhesion to compounds resolved and immobilized on glass-backed thin layer chromatography plates, were similarly constructed for the centrifugation of microtiter plates in a sealed and fluid-filled chamber. The chambers accommodate PVC microtiter plates cut to 4-well by 10-well size.

After incubation with cells, microtiter plates were immersed slowly in a large container of PBS (greater than 4 liters, complete with Ca2+ and Mg2+) previously chilled to 4°C. When completely submerged, a 5x10 cm glass plate was placed over the microtiter well openings. The microtiter plate and its glass plate cover were then slid into a centrifugation chamber which was already immersed in the cold buffer bath. The chamber was then sealed while still submerged, avoiding the introduction of any air bubbles or air pockets. The chamber was removed from the buffer bath and inverted so that a 1 g force opposed putative adhesive interactions between COS1 cells and glycolipid. To remove non-adherent cells, the chamber was then centrifuged at 150 g for 10 minutes at 4°C. After centrifugation, the chamber was resubmerged in the buffer bath and opened. The microtiter and glass plates were slid out together and separated from one another. The microtiter plate was then rotated to upright and slowly lifted out of the buffer bath.

Immediately after removal from the buffer bath, 200 μl of buffer was removed from each well, leaving approximately 100 μl, and the microtiter plates were allowed to come to ambient temperature. Subsequently 10 μl of 10x Hirt solution (6% SDS, 100 mM EDTA in water) was added to each well and the plate was incubated for 20 minutes at room temperature to liberate plasmid from adherent COS1 cells. The resulting Hirt supernatant was pooled from a total of 80 microtiter wells and enough NaCl was added from a 5 M stock to give 1 M final concentration. Following brief agitation, the solution was placed on ice overnight. The suspension was then centrifuged at 10000 g for 15 minutes at 4°C. DNA in the cleared supernatant was extracted with phenol:chloroform and chloroform, supplemented with glycogen (as carrier) and ethanol precipitated. Precipitated material was resuspended in 200 μl of 10 mM Tris containing 1 mM EDTA pH 7.5 (TE), reextracted with phenol:chloroform and chloroform and then precipitated. The final pellet was resuspended in 25 μl of TE and served as starting material for the next round of selection.

Subsequent rounds of selection were performed in an identical manner except that COS1 cell populations were transfected by spheroplast fusion rather than DEAE-Dextran (Seed and Aruffo, 1987; Sandri-Goldin et al., 1983). To generate spheroplasts for transfection, electroporation-competent E. coli (MC1061/p3) were transformed with 200 ng of plasmid DNA (from the previous round) or with 200 ng of control plasmid (CDM8). The entire suspension of electroporated bacteria was spread on agar plates and the transformed colonies were scraped and converted to spheroplasts for transformation of 6 dishes (10 cm) of COS1 cells that had been plated at 4×10^5 cells per dish 48 hours previously. Approximately 18 hours following fusion, the surviving COS1 cells in 6 dishes were harvested by trypsinization and replated into 8 dishes for an additional 48 hours of growth before being selected by adhesion. COS1 cells in two randomly selected dishes were metabolically radiolabeled by the addition of 32PO4, as described above, 24 hours prior to harvest.

After three rounds of selection, bacteria were transformed with plasmid DNA recovered from adherent COS1 cells and miniprep plasmid DNA was generated from 96 randomly selected colonies. The cloned plasmids were screened for insert size and plasmid integrity by restriction endonuclease digestion with XhoI, HindIII and BamHI. Plasmid clones not demonstrating rearrangement were individually transfected into COS1 cells by DEAE-Dextran (2.5 μg DNA per 10 cm dish) and screened for cell adhesion on Drosophila embryonic glycolipids.

DNA sequencing and other molecular biological techniques

Inserted cDNA was harvested from cloned plasmid by digestion with XhoI. Insert was concatenated, fragmented by sonication, blunted and inserted into M13 phage arms for single-stranded sequencing. DNA from M13 phage clones was sequenced by the dideoxy chain termination method (Sequenase, USB), compiled and analyzed with IntelliJogic sequence analysis software (Sanger et al., 1977). Sequence was gathered from 65 M13 clones, yielding continuous sequence spanning the glioclectin plasmid insert in both directions. At least 4 M13 clones covered all stretches of sequence. Hybridization to polytene chromosomes was performed as described by Mlodzik et al. (1990). Northern analysis, subcloning and other molecular techniques were performed by standard methods (Sambrook et al., 1989).

S2 cell aggregation

Glioclectin cDNA was subcloned into pRmHa3 vector (Bunch et al., 1988) for transfection into S2 cells, a cell line derived from embryonic Drosophila tissue (Schneider and Blumenthal, 1978). S2 cells were cotransfected with a selection vector encoding ß-amanitin resistance and pRmHa3 containing glioclectin in either the forward or reverse orientation. Induction of expression by the addition of cupric ion to culture media and subsequent assay for aggregation by rotation culture were performed as described by Elkins et al. (1990). S2 cells transfected with glioclectin inserted in the reverse orientation in pRmHa3 served as control and were indistinguishable from non-transfected S2 cells in their aggregation behavior.

In situ hybridization

In situ hybridization to whole-mount embryos was performed according to the method of Tautz and Pfeifle (1989) using digoxigenin-dUTP-labeled probes prepared by PCR as described by Patel and Goodman (1992). Hybridization was detected by the deposition of bromochloroindolyl phosphate reaction product with nitroblue tetrazolium at sites of alkaline phosphate-conjugated anti-digoxigenin antibody binding (Boehringer).
Monoclonal antibody production and embryo staining

Fragments of gliolectin were subcloned in-frame into pATH vectors for the production of TrpE fusion proteins in bacteria (Koerner et al., 1991). One fusion protein, containing amino acids P10 to S103 of gliolectin elicited an immune response when injected into Swiss-Webster mice. Hybridoma clones secreting monoclonal antibodies recognizing gliolectin were prepared by standard hybridoma fusion methods (Ogi and Herzenberg, 1980). Two hybridoma lines secreted antibody of interest. The clone 2E4 recognized SDS-denatured and reduced protein by western analysis, while clone IB7 recognized an epitope in fixed embryonic Drosophila tissue. Drosophila embryos were stained with the monoclonal antibodies 1B7 and BP102 as previously described (Klämbt et al., 1991). Staining of IB7 was enhanced by peroxidase-induced precipitation of diaminobenzidine in the presence of NiCl. When embryos were stained with both antibodies, nickel-enhanced IB7 staining was performed first followed by BP102 detected by diaminobenzidine precipitation.

Immunoprecipitation

Subcellular fractions were generated from Drosophila embryos aged 0-16 hours by differential centrifugation. Following dechorionation in 50% bleach, approximately 1 g (wet weight) of fresh embryos were extensively washed with cold 50 mM Tris pH 7.4 and transferred to a Dounce glass homogenizer. After the addition of 10 volumes of cold homogenization buffer (1 mM EDTA, 20 μg/ml PMSF, and 1 μg/ml each of peptatin A, leupeptin, chymostatin, antipain, aprotnin, TPCK and TLCK in 10 mM Tris pH 8.0), the embryos were homogenized 7 times with a loose-fitting pestle followed by an additional 10 strokes with a tight-fitting pestle. The resulting homogenate was centrifuged at 250 g for 5 minutes to pellet debris. The supernatant was then spun at 4000 g for 10 minutes at 4°C. The resulting supernatant was transferred to a fresh tube and the pellet was resuspended in an additional 10 volumes of homogenization buffer. Following recen trifugation, the pellet was saved as the nuclear fraction. The combined postnuclear supernatants were centrifuged at 100,000 g for 60 minutes to pellet membranes. The cytosolic fraction (supernatant) was removed. All fractions were stored frozen at ~20°C until used. Protein content in each fraction was determined by the BCA assay (Smith et al., 1985).

Aliquots of subcellular fractions were thawed, boiled in 1% SDS, supplemented to 1% with Triton X-100 and to 0.5% with NP40 and finally adjusted to 1 ml total volume with PBS. Monoclonal supernatant 2E4 (2 ml) and protein A-Sepharose (12.5 μl), added with the mixture was incubated overnight at 4°C. Unbound and non-specifically bound proteins were removed from the protein A-Sepharose beads by repeated washes with 1% Triton X-100, 0.5% NP40 in PBS pH 7.4. Proteins bound by antibody were released from the beads by two incubations in 25 μl of 1% Triton X-100 in 50 mM glycine pH 2.5. The glycine/Triton eluate was neutralized with 2 M Tris pH 8.0 and 10 μl aliquots were reduced, denatured and electrophoresed on 4-20% polyacrylamide gradient gels (Laemmli, 1970). Proteins in the gel were transferred to nitrocellulose and probed with monoclonal 2E4 and peroxidase-conjugated goat anti-mouse (Towbin et al., 1979). Antibody binding was visualized by the deposition of diaminobenzidine in the presence of H2O2.

Epitope-tagged gliolectin constructs

Plasmids encoding L-selectin or the Fc region of human IgG served as starting material for the generation of a cassette vector for epitope tagging gliolectin coding sequence with human IgG Fc (Watson et al., 1990). PCR primers were designed to amplify the signal sequence of L-selectin (amino acid residues Mi-H2, Lasky et al., 1989) within the targeted plasmid and incorporate EcoRI and BamHI sites at the 5’ and 3’ ends, respectively. Similarly, PCR primers were designed to amplify the Fc domain of human IgG2/1 in target plasmid (H/C1r2/Cp3 domains, amino acid residues D103-STOP) and incorporate SalI and HindIII sites at 5’ and 3’ ends, respectively. Primer design resulted in a cysteine-to-valine transformation at residue 103 of human IgG. All PCR amplifications were done with VENT polymerase using the manufacturer’s recommendations. The amplified fragments were digested with appropriate enzymes and subcloned into pCOS(LB) with a stuffer fragment to bridge the BamHI-SalI gap.

Three gliolectin fragments, N (amino acid residues S22-A118), C (E142-Q228) and NC (S22-Q228) were amplified by PCR with primers designed to introduce 5’ BclI (or BamHI for C) and 3’ SalI sites and to maintain reading frame integrity when subcloned into the IgG cassette vector. Primer design introduced no changes in gliolectin coding sequence but introduced a glycine residue following H42 of L-selectin and before the first residue of gliolectin. The resulting vectors were transfected into COS1 cells by DEAE-Dextran as described above. Cell populations were cultured for 72 hours in media containing calf serum from which immunoglobulins had been removed by protein A-Sepharose chromatography. Secreted Ig-chimeras were harvested from culture media by protein A-Sepharose, concentrated by lyophilization and characterized by western analysis with biotinylated goat anti-human antiserum and streptavidin-peroxidase (Zymed).

Binding of epitope-tagged gliolectin to Drosophila embryonic glycolipids

Epitope-tagged gliolectin chimeras harvested from COS1 cell culture media were used as probes in an ELISA-type assay (Foxall et al., 1992). Polystyrene (Probind, Falcon) microtiter plates were adsorbed with 200 pmol Man equivalents per well of glycolipid extract or fraction as described above except that PBS was used as buffer rather than DMEM-Hepes. Following incubation with chimeric protein for 60 minutes at room temperature, microtiter plates were quickly washed three times with PBS (complete with Ca2+ and Mg2+) and probed with preformed complexes of biotinylated goat anti-human-
antiserum and streptavidin-peroxidase (Zymed). Binding was detected by measuring the increase in optical density at 405 nm with H2O2 as substrate in the presence of 2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid). Specific binding was defined as binding of chimera to wells adsorbed with glycolipid in PC:C minus binding to wells adsorbed with PC:C alone.

RESULTS

A subset of COS cells transfected with an embryonic *Drosophila* cDNA library adheres to zwitterionic glycolipids isolated from *Drosophila* embryos

Glycolipids were extracted from 0-16 hour *Drosophila* embryos to provide a source of endogenous carbohydrate ligands for recognition by putative *Drosophila* lectins. Glycolipid extracts were partitioned, saponified, fractionated and subjected to analyses (to be published elsewhere). Briefly, two populations of glycosphingolipid were generated by DEAE-Sepharose fractionation; a neutral/zwitterionic fraction (NZ fraction) was obtained as the flow-through and acidic species were isolated by salt step-gradient elution. The NZ fraction contained between 16 and 20 major species (visible by chemical detection methods, see Fig. 8B) ranging from di- to octaosylceramides often derivatized with phosphorylethanolamine groups. Using standard methodology, embryonic *Drosophila* glycolipids were readily adsorbed to PVC microtiter wells in the presence of phosphatidylycholine and cholesterol as evidenced by ELISA detection utilizing a monoclonal antibody directed toward a *Drosophila* glycolipid antigenic determinant (results not shown).

COS1 cells transfected with a plasmid cDNA library generated from 7-13 hour embryonic *Drosophila* poly(A)+ mRNA were placed in microtiter wells carrying immobilized NZ fraction glycolipids. This initial population of library-transfected COS1 cells did not demonstrate measurable specific adhesion to glycolipid surfaces (NZ-fraction) relative to carrier lipid (PC:C) surfaces. Specific adhesion is defined as the adhesion of library-transfected COS1 cells minus the adhesion of control plasmid (CDM8)-transfected COS1 cells assayed in parallel. Plasmid was harvested from adherent cells, amplified and retransfected into fresh COS1 cells for another round of enrichment (Seed and Aruffo, 1987). The population of second round transfected cells exhibited measurable specific adhesion which was even more pronounced in the third round (Fig. 1).

Plasmid recovered from adherent COS1 cells at the end of the third round of enrichment generated a library of 5.8×10⁵ bacterial colonies. Miniprep DNA from 96 randomly selected colonies was prepared and digested with various restriction endonucleases to test for plasmid integrity since COS cells may rearrange episomal DNA (Schaeffer et al., 1991). Of the chosen clones, 29 contained nominally unrearranged plasmid. Of these appropriately digestible clones, 16 contained insert less than 300 bp and were not considered further. The remaining 13 clones were transfected individually into COS1 cells whose adhesion to immobilized NZ fraction glycolipids was subsequently measured.

Three clones imparted to COS1 cells the ability to adhere to NZ fraction glycolipids. One of these three yielded significantly greater cell adhesion than the other two and was further characterized. Following analysis of the encoded protein’s expression pattern and carbohydrate-binding activity (see...
Gliolectin also generates adhesion in *Drosophila* S2 cells

To characterize its function in an invertebrate cell type, gliolectin was inductively expressed in S2 cells, a non-adherent cell line derived from embryonic *Drosophila* tissue. When S2 cells expressing gliolectin were placed in rotary culture, aggregates of modest size were seen to form within 2 hours (Fig. 3A,B). Aggregation was not dependent on homophilic interactions between gliolectin molecules on apposing cells since mixed aggregates of transfected and non-transfected cells could be formed (Fig. 3C,D). Rather, this aggregation suggests that gliolectin functions heterophilically in this context by recognizing a glycolipid constituent of the S2 cell surface with sufficient similarity to its embryonic ligand.

Gliolectin cDNA encodes a novel protein

The cloned gliolectin cDNA was 1769 bp in length (Fig. 4). A screen of 7.5×10⁵ plaques of a λgt11 embryonic *Drosophila* cDNA library with the gliolectin cDNA insert as probe yielded 24 hybridizing clones, none of which contained more sequence than did the gliolectin plasmid cDNA insert (Zinn et al., 1988). M13 single-strand sequencing of the gliolectin cDNA insert revealed an open reading frame (ORF) of 684 bp with appropriate codon usage for *Drosophila melanogaster*. No other ORF’s extended for more than 150 bp or exhibited appropriate codon bias. Nucleotides in the putative −1 and −3 positions and the prevalence of adenosine residues from −4 to −10 suggest that translation is unlikely to initiate at M₆ (Cavener, 1987). Database searches at the nucleotide and amino acid level were unsuccessful in identifying significant similarities between gliolectin and any previously catalogued proteins. The GenBank accession number for gliolectin is U42989.

Two stretches of hydrophobicity exist in the ORF, one at the amino terminus (M₁ to P₁₇) and another, flanked by basic residues, approximately halfway through the polypeptide (G₁₂₁ to Q₁₃₈). The amino acid residues to the carboxy-terminal side of the amino-terminal hydrophobic stretch do not exhibit an obvious consensus signal cleavage site (von Heijne, 1986). Five consensus N-linked glycosylation sites can be found, all on the carboxy-terminal side of the second hydrophobic stretch. A very short stretch of OPA repeat, a *Drosophila* repetitive element, is also present at the carboxy terminus (Wharton et al., 1985).

By northern analysis, gliolectin mRNA was not detected below 0.1 μg of RNA (Fig. 5). A screen of the cDNA insert encoding gliolectin generated a 1769 bp base pair novel sequence. M13 phase single-stranded sequencing of the cDNA insert encoding gliolectin generated a 1769 base pair novel sequence. Amino-terminal and internal hydrophobic stretches are doubly underlined; consensus N-linked glycosylation sites are singly underlined. Nucleotide numbers are given relative to the proposed start of translation. Amino acid numbers are shown in bold.
single transcript of 1.7 kb present primarily during embryonic development. Hybridization was slight to larval and pupal stage mRNA preparations and barely detectable to adult mRNA (Fig. 5A). Probes of polytene chromosomes from third instar larval salivary glands revealed a single hybridizing locus on the right arm of the third chromosome at 93F6-8.

Mouse monoclonal antibodies were generated against a bacterially expressed protein composed of the amino-terminal half (P₁₀ to S₁₀₃) of gliolectin fused to TrpE. One particular monoclonal antibody (2E4) proved useful for detection of denatured protein. Western analysis of proteins immunoprecipitated (with monoclonal antibody 2E4) from Drosophila embryonic subcellular fractions revealed the presence of gliolectin primarily in the membrane fraction (Fig. 5B) where it migrated with a relative molecular mass (Mᵣ) of 46.6×10³.

Gliolectin was detected at the surface of transfected cells by staining live, unpermeabilized COS cells with monoclonal antibody 1B7, raised against the amino-terminal half of the molecule. Furthermore, the expressed epitope of a carboxy-terminal epitope-tagged gliolectin (Fc of human IgG) was also accessible on the exterior of the cell (data not shown). Taken together, the immunohistochemical probes, in conjunction with the carboxy-terminal distribution of the 5 N-linked glycosylation consensus sequences and the lack of a clear N-terminal signal-cleavage site, suggest that gliolectin is a type II transmembrane protein with an uncleaved signal-anchor sequence.

Gliolectin is expressed in a subset of midline glial cells during a restricted period of Drosophila embryonic development

In situ hybridization of digoxigenin-labeled DNA probes to whole-mount Drosophila embryos revealed a specific cellular distribution of gliolectin transcripts. Transcript was first detectable in germ-band extended stage 11 embryos (Klämbt et al., 1991) as segmentally repeated clusters of either mes- or neuro-ectodermal cells at the midline (Fig. 6A). As germband retraction occurs, message persists in these midline cells and appears to peak during stages 13 to 14 (Fig. 6B,C). At stage 13, transcript was seen within the ventral nerve cord in clusters of midline cells located dorsally and in a more ventrolaterally positioned pair of bilateral cells (Fig. 6D). Beyond stage 15, transcript was harder to detect although still found faintly at the midline in the condensed nerve cord.

Monoclonal antibody 1B7, generated against TrpE fusion protein, proved useful for localization of gliolectin in fixed embryos. The cellular distribution of 1B7 staining paralleled that seen by in situ hybridization with one exception (Fig. 7A); faint 1B7 staining was seen in small clusters of peripheral ectodermal cells at stage 13 (Fig. 7B). Gliolectin was not detected in imaginal discs by antibody or by in situ hybridization.

To further define the identity and position of the midline cells expressing gliolectin, embryos stained with 1B7 were secondarily probed with monoclonal antibody BP102 which labels all the axon bundles in the embryonic CNS (Fig. 7B). 1B7-stained cells were visible at the midline before BP102 staining was apparent. By late stage 12, 1B7-stained cells were found in close association with the BP102-stained axonal processes of the forming axonal commissures (Fig. 7C). From late in stage 12 through stage 13, gliolectin expression was intimately associated with the anterior commissure in a position consistent with that of the identified midline glial cell pairs, MGA and MGM (Fig. 7C,D) (Klämbt et al., 1991; Jacobs and Goodman, 1989).

Until stage 14, these glial cells are tightly clustered and distinguishing MGA from MGM is difficult. However, a distinct separation between the two pair of cells develops at stage 14/15 as the MGM cells migrate posteriorly, consequently separating anterior from posterior commissure. Although gliolectin expression decreases by stage 15, midline glial cells posterior to the anterior commissure (the position of the MGM cells at this stage) were not stained with 1B7, suggesting that MGA alone expresses gliolectin.

Gliolectin recognizes a subset of Drosophila embryonic glycolipids

Epitope-tagged chimeras of gliolectin were constructed by the in-frame insertion of gliolectin-coding sequence into a cassette vector containing an appropriate signal sequence (amino acid residues M₁₄-H₁₄₂ from L-selectin, signal cleavage occurs at C₁₈₂) and the Fc region of human IgG₁ (H/C H₂/C H₃ domains, amino acid residues D₁₈₄-M₁₉₆). Three constructs, N (amino acid residues S₂₂-A₁₁₈ of gliolectin), C (E₁₄₂-Q₂₂₈) and NC
(S23-Q228), were generated and expressed in COS1 cells. Culture media from transfected COS1 cells contained fusion proteins of the appropriate size that were retained by protein A-sepharose and recognized by anti-human IgG antisera on western blots. The N and NC constructs were also recognized by monoclonal antibody 2E4 (against the amino-terminal half of gliolectin) on western blots. These chimeras were used to probe glycolipids adsorbed in microtiter wells.

Only the N fusion protein demonstrated binding to glycolipids. Binding was detectable in microtiter wells containing the total lipid extract, NZ fraction (extracted lipids that run through an anion exchange column, ie: neutral and zwitterionic species), and A fraction (extracted lipids that are retained by an anion exchange column, ie: acidic components) (Fig. 8A). Binding to the NZ fraction, the same fraction used to initially obtain the gliolectin clone, is restricted to a subset of its glycolipids. Only 2 of 9 fractions, resulting from silicic acid column fractionation of NZ, supported binding of the N fusion protein. Following analytic thin-layer chromatography, chemical detection by a combination of fluorescamine (Fig. 8B), orcinol or napthylethylenediamine demonstrates that these silicic acid fractions all contain unique glycolipid components; although none are homogeneous, certain species predominate in each. The observed binding implies that gliolectin can discriminate between oligosaccharide structures.

**DISCUSSION**

**Cell surface carbohydrates are ligands for endogenous lectins**

More than 25 years ago cell surface carbohydrates were postulated to mediate cell–cell interactions (Roseman, 1970). Subsequent investigations have continued to support a role for glycans in cellular recognition (Varki, 1993). For instance, carbohydrate expression is spatially and temporally regulated; the range of carbohydrate structures expressed at many cell surfaces is modulated during oncogenic transformation, normal development and tissue differentiation (Bolscher et al., 1988; Fenderson et al., 1987; Dodd and Jessell, 1985).

A role for cell surface carbohydrates during invertebrate neural development has also been indicated. Perturbations of neural–specific carbohydrate moieties, by enzymatic removal or antibody blockade in *Hirudo medicinalis* or by genetic mutation in *Drosophila melanogaster*, disrupt sensory axon fasciculation in the CNS (Whitlock, 1993; Song and Zipser, 1995). For cell surface carbohydrates to mediate such cell-cell interactions, it is suggested that cell surface carbohydrates are ligands for endogenous lectins.
interactions, binding proteins (lectins) that recognize glycan structures should exist.

**Gliolectin mediates cell adhesion to glycolipid oligosaccharides in vitro**

The cloning strategy described in this paper was designed to identify endogenous lectins in *Drosophila* based on function, ie: carbohydrate binding. To increase the likelihood of capturing lectin-expressing COS1 cells on glycolipid-adsorbed surfaces, minimal limitations were incorporated into the screen. For instance, rather than adsorbing an individual *Drosophila* glycolipid to microtiter wells, mixtures containing multiple structures were immobilized. Also, during rounds of selection by adhesion, detachment forces of low strength were employed to avoid the loss of transfected COS1 cells whose adhesion was mediated by lectins of low affinity. Under these conditions, a novel clone, designated 'gliolectin,' was identified.

Gliolectin mediates cell adhesion in two contexts. Expressed in COS1 cells, gliolectin generates adhesion to immobilized NZ-fraction glycolipids purified from *Drosophila* embryos. When inductively expressed in the *Drosophila* cell line, S2, gliolectin mediates cellular aggregation. The heterophilic nature of gliolectin binding was demonstrated by the formation of aggregates composed of expressing and non-expressing S2 cells. This result demonstrates that gliolectin expressed on one cell competently binds ligands expressed on another. Aggregation of S2 cells by gliolectin also indicates that this cell line, derived from embryonic *Drosophila* tissue, synthesizes at least one carbohydrate structure with significant similarity to the embryonic ligand. S2 cells may, therefore, provide a useful biochemical source for further ligand characterization.

**Embryonic expression of gliolectin suggests in vivo function**

The temporally and spatially restricted expression of gliolectin indicates potential cellular interactions in which it may be involved during *Drosophila* neural development. The anterior-most midline glial cells begin to express gliolectin just before the neural processes of the forming anterior commissure encounter the MGA (anterior midline glia) cell surface (Klämbt et al., 1991). A glial-glial interaction also occurs within the spatial domain and during the time of gliolectin expression. While maintaining intimate contact with the MGA cells, the MGM cells (medial midline glia) migrate posteriorly resulting in the separation of anterior and posterior commissures (Klämbt et al., 1991). Gliolectin is, therefore, positioned to mediate glia-glia and/or glia-neuron interactions. The significant involvement of the MGA and MGM cells in commissure formation predicts that loss of gliolectin function would disrupt the formation of major axonal pathways, a phenotype to be investigated once gliolectin null or hypomorphic mutants become available.

An assessment of gliolectin-mediated cell-cell recognition in vivo should include the identification of its endogenous ligand and placement of that structure on an apposed membrane, perhaps that of extending commissural neurites or the migrating MGM cells. The restricted expression of gliolectin itself suggests that the optimally recognized oligosaccharide structure may be a very minor component in the total glycolipid mixture extracted from *Drosophila* embryos. In fact, if limited to the neurons pioneering the anterior commissure, recovery of this rare glycan would present a formidable technical challenge. The endogenous carbohydrate ligand need not, however, be a unique structure; its secondary expression in ectoderm or mesoderm physically inaccessible to the midline glial cells would not affect the spatial and temporal specificity of gliolectin-mediated recognition in the nervous system.

**Gliolectin binds a subset of *Drosophila* embryonic glycolipids**

The defining characteristic of a lectin is its ability to discriminate between oligosaccharide structures. The binding of a gliolectin/Ig chimera to immobilized *Drosophila* glycoconjugates demonstrates such specificity. Although all of the silicic acid column fractions tested for binding contained more than one component, the two fractions that supported binding (fraction 0 and fraction 11) were composed of a predominate species (greater than 80% by orcinol color yield on TLC plate). Preliminary structural analysis indicates that the major component of fraction 0 is a triafoylceramide (GlcNAc β-4Man β-4Glc-Ceramide) while that of fraction 11 is an hepaosylceramide (GlcNAc β-4Gal β-3GalaNAc α-4GalNAc β-4GlcNAc β-4Man β-4Glc-Ceramide). Other silicic acid column fractions tested for binding activity contained lipids of equal or greater prevalence yet did not support gliolectin binding. Among these were the hexaosyl-, pentaosyl- and tetraosylceramides related to the pre-
dominant species of fraction 11. Gliolectin binding, therefore, requires specific oligosaccharide structural information and is not a result of non-specific or hydrophobic lipid-protein interactions.

The element common to the major lipids of fractions 0 and 11, a non-reducing terminal GlcNac (N-acetylgalactosamine) linked β-1-4 to a hexose, may constitute the minimum structure recognized by gliolectin. However, GlcNac residues in arthropods frequently carry phosphoryl ethanolamine moieties in ester linkage through the 6-position hydroxyl (Wiegandt, 1992). The extent to which phosphoryl ethanolamine derivatization, which we have detected on purified Drosophila glycolipids, affects gliolectin binding remains to be determined.

Alternatively, minor constituents of fractions 0 and 11 could be responsible for the observed binding. A combined genetic and biochemical approach will ultimately be required to assess the in vivo specificity of gliolectin. For instance, identification of genetic interactions between gliolectin hypomorphic alleles and mutant strains blocked at various stages in the synthesis of biochemically defined oligosaccharide structures could implicate specific structural determinants in gliolectin binding and would complement in vitro binding studies.

Investigating gliolectin function in the Drosophila embryo may also suggest a role for the carboxy-terminal portion of the molecule. Gliolectin/Ig chimeras containing the carboxy-terminal half of the molecule were unable to bind glycolipids in vitro. While all fusion proteins (amino-terminal half, carboxy-terminal half or full length) were efficiently produced by COS cells, it is unlikely that Drosophila embryonic post-translational modifications are reproduced by this vertebrate cell line. Since its carboxy-terminal half contains 5 putative N-linked glycosylation sites, carbohydrate alone could account for more than 50% of the mass of this half of gliolectin. In fact, Western-blot analysis indicates that the embryonically expressed form of gliolectin is highly modified (Fig. 5B). Therefore, post-translational processing in a heterologous system could dramatically alter or even mask gliolectin-binding activities.

Cell surface carbohydrates and cell recognition

In the vertebrate inflammatory response, the appropriate capture and trans-endothelial migration of leukocytes relies on an initial cellular recognition event that is subsequently strengthened by recruitment of multiple adhesive and signaling mechanisms. That the initial contact between leukocyte and endothelial cell is mediated by the interaction of cell surface carbohydrates with C-type lectins provides precedent for such a small subset of cells is, therefore, remarkable. Perhaps mechanisms controlling the early establishment of commissural pathways, a putative function for gliolectin, are sufficiently specialized that they are not generally reutilized in other developmental settings. The restricted distribution of gliolectin does not, however, imply that other lectins in Drosophila are similarly disposed. Further characterization of Drosophila lectin and carbohydrate diversity is necessary before conclusions can be drawn regarding the uniqueness of gliolectin.

Current classification schemes for animal lectins (as C-type, I-type or galectin) have been usefully predictive but have also become too confining for the growing cadre of described carbohydrate-binding activities. Lectin activity has been associated with some unexpected molecules. For instance, the endoplasmic reticulum proteins calnexin and calreticulin are neither C-type, I-type nor galectin but bind high-mannose N-linked oligosaccharides of newly synthesized proteins (Hammond et al., 1994). Furthermore, VIP36 and ERGIC-53, proteins involved in targeting and vesicular traffic in animal cells, demonstrate structural similarities to leguminous plant lectins (Fiedler et al., 1994; Fiedler and Simons, 1995). The identification of a novel carbohydrate-binding protein, gliolectin, in Drosophila opens new possibilities for defining the function of cell surface carbohydrates and endogenous lectins during development of the nervous system and indicates that additional undefined lectin families await discovery in many organisms.

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