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Drosophila Neuroglian: A Member of the Immunoglobulin Superfamily with Extensive Homology to the Vertebrate Neural Adhesion Molecule L1

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Summary

Drosophila neuroglian is an integral membrane glycoprotein that is expressed on a variety of cell types in the Drosophila embryo, including expression on a large subset of glial and neuronal cell bodies in the central and peripheral nervous systems and on the fasciculating axons that extend along them. Neuroglian cDNA clones were isolated by expression cloning. cDNA sequence analysis reveals that neuroglian is a member of the immunoglobulin superfamily. The extracellular portion of the protein consists of six immunoglobulin C2-type domains followed by five fibronectin type III domains. Neuroglian is closely related to the immunoglobulin-like vertebrate neural adhesion molecules and, among them, shows most extensive homology to mouse L1. Its homology to L1 and its embryonic localization suggest that neuroglian may play a role in neural and glial cell adhesion in the developing Drosophila embryo. We report here on the identification of a lethal mutation in the *neuroglian* gene.

Introduction

Studies on the cellular mechanisms of growth cone guidance in the insect central nervous system (CNS) led to the prediction that specific pathways are differentially labeled by surface recognition molecules that guide growth cones along the appropriate glial and axonal surfaces (Raper et al., 1983, 1984; Bastiani et al., 1984; Goodman et al., 1984; Bastiani and Goodman, 1986; Jacobs and Goodman, 1989b). Monoclonal antibodies (MAbs) have been used to identify four different membrane-associated glycoproteins, fasciclin I and fasciclin II in grasshopper (Bastiani et al., 1987) and fasciclin III (Patel et al., 1987) and neuroglian (this report) in Drosophila, that are regionally expressed on subsets of fasciculating axons and interacting glia in the developing nervous system. The neurons and glia that express these proteins display a high affinity for one another; thus these molecules are good candidates for participation in the events of neuronal recognition and adhesion. The genes encoding all four glycoproteins have been cloned and sequenced. Two of the four genes, fasciclin I (in grasshopper and Drosophila; Snow et al., 1988; Zinn et al., 1988) and fasciclin III (Snow et al., 1989), encode novel proteins with no significant homology to sequences in the data bank. The other two, fasciclin II (Snow et al., 1988; Harrelson and Goodman, 1988) and neuroglian (this report), have extensive structural homology to one another and to one of the families of vertebrate neural adhesion molecules, as described below.

Two recent reports described the cloning of the first immunoglobulin (Ig)-like proteins outside of the chordates: grasshopper fasciclin II (Harrelson and Goodman, 1988) and Drosophila amalgam (Seeger et al., 1988). Fasciclin II and amalgam show structural homology to a particular subclass of the Ig superfamily, the neural adhesion molecules, which includes N-CAM (Cunningham et al., 1987; Barthels et al., 1987), MAG (Arquint et al., 1987; Salzer et al., 1987), L1 (Moos et al., 1988), and contactin/F11 (Ranscht, 1988; Brümmendorf et al., 1989). Fasciclin II is expressed on a subset of fasciculating axons in the grasshopper embryo and plays a role in selective fasciculation (Bastiani et al., 1987; Harrelson and Goodman, 1988). Fasciclin II has five Ig C2-type domains (Williams, 1987) and two fibronectin (Fn) type III domains and is most homologous in structure to N-CAM. Drosophila amalgam is expressed on all axons in the developing Drosophila CNS and has one V-type domain and two Ig C2-type domains (Seeger et al., 1988). The structural similarity between these molecules and the vertebrate neural cell adhesion molecules suggests that fasciclin II and amalgam are likely to play roles in neural cell adhesion.

In this paper we report on the characterization, cloning, and sequencing of a third member of the Ig superfamily from insects: Drosophila neuroglian. We also report on the identification of a lethal mutation in the neuroglian gene. Neuroglian is an integral membrane glycoprotein that is widely expressed in the developing Drosophila embryo, particularly on the surfaces of a large subset of neurons and glia that interact with and adhere to one another along the dorsal surface of the developing CNS and within the peripheral nerve roots. Sequence analysis of neuroglian cDNA clones reveals that the extracellular portion of the protein consists of six Ig C2-type domains and five Fn type III domains followed by a transmembrane domain and a short cytoplasmic domain. Neuroglian exhibits extensive structural homology to the mouse neural adhesion molecule L1 and thus, like L1, may play a role in neural and glial cell adhesion.

The close relationship between fasciclin II and N-CAM suggests a common ancestor, before the arthropod–chordate split, with multiple Ig C2-type and Fn type III domains (Harrelson and Goodman, 1988). Similarly, the extensive homology reported here among neuroglian, L1, and contactin/F11 suggests a second common ancestor, before the phyletic split, with a similar multidomain structure.

Results

Neuroglian Is Expressed on a Large Subset of Neurons and Glia in the Developing Drosophila Nervous System

Membrane proteins were isolated from embryonic Dro-

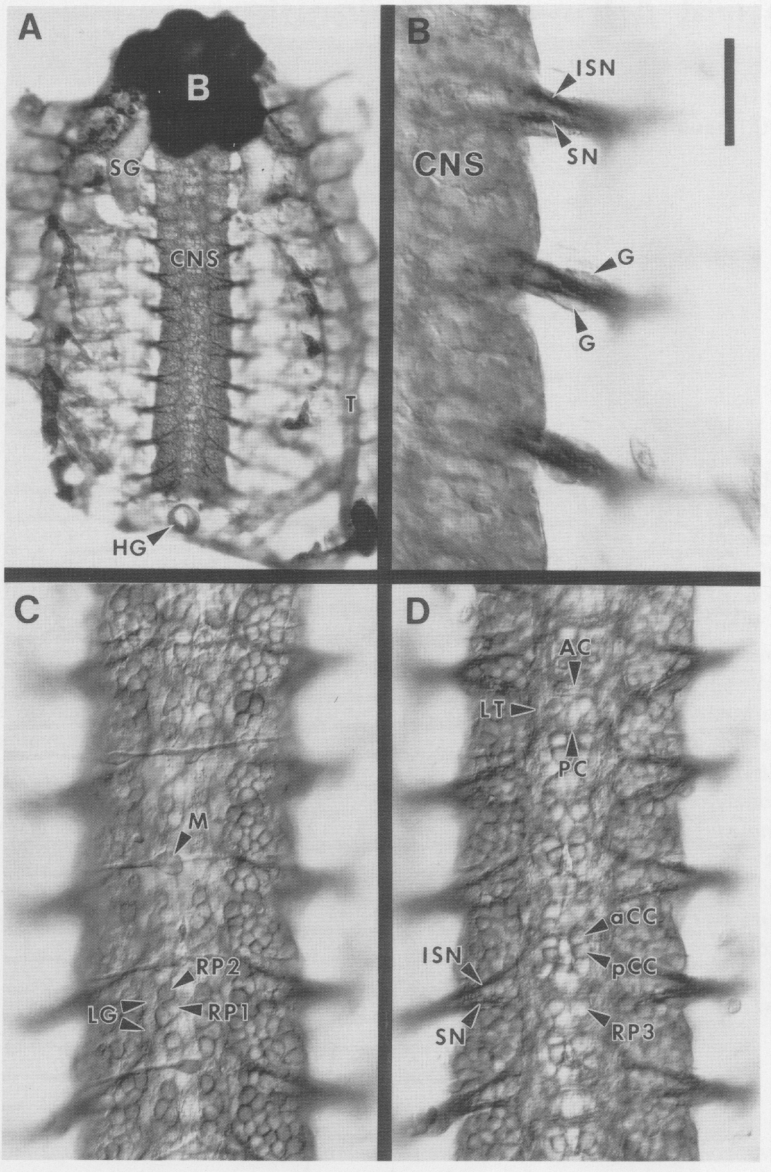


Figure 1. Neuroglian Is Expressed in the Developing Drosophila Nervous System

A 12 hr Drosophila embryo was dissected onto a glass slide and stained with the 1B7 MAb (which recognizes neuroglian protein), followed by visualization with an HRP-conjugated second antibody and HRP immunochemistry.

(A) Neuroglian is expressed on a variety of embryonic tissues including the brain (B), central nervous system (CNS), salivary gland (SG), trachea (T), and hindgut (HG).

(B) The 1B7 MAb intensely stains the axons of the segmental (SN) and intersegmental nerves (ISN). The glia (G) associated with the peripheral nerve roots are also stained, as are most of the cell bodies in the CNS.

(C) In the plane of focus of the most dorsal aspect of the CNS, staining of the RP1 and RP2 neurons is evident. The longitudinal glia (LG), which lie dorsal to the longitudinal axon tracts, express the antigen. The muscle pioneers (M) are stained.

(D) In a deeper plane of focus, the axons of the longitudinal tracts (LT), anterior (AC) and posterior commissures (PC), and segmental (SN) and intersegmental nerves (ISN) all stain with the antibody. The anterior (aCC) and posterior corner cells (pCC) and the RP3 neuron also stain. Scale bar: (A) 100 μm; (B) 20 μm; (C and D) 30 μm.

sophila nerve cords and used as immunogens for the production of MAbs against neural cell surface proteins. Antibodies were screened histologically on embryonic nerve cords and those antibodies that recognized antigens expressed on subsets of cells in the nervous system were selected and recloned for further characterization. The antigen recognized by the 1B7 MAb is expressed on a variety of different cell types and tissues in the developing Drosophila embryo. Of most interest to us was the observation that the 1B7 antigen is found at high levels on the glia and axons of the peripheral nerve roots. It is also expressed on the glia and neuronal cell bodies, and on a large subset of fasciculating axons that extend along them, at the dorsal surface of the developing CNS. In accordance with its high level of expression on neurons and glia, we have named the protein recognized by the 1B7 MAb, neuroglian.

In Drosophila embryos between 11 hr and 12 hr of development the 1B7 MAb stains most intensely in the brain and on the peripheral nerve roots where axons enter and exit the CNS (Figure 1A). Neuroglian is expressed at high levels on the surfaces of both the glia and axons in the segmental and intersegmental nerve roots (Figure 1B). Within the developing CNS, the surfaces of glia, neuronal cell bodies, and axons express neuroglian (Figures 1C and 1D). A variety of nonneuronal tissues also express neuroglian, including trachea, hindgut, salivary gland, and muscle (Figures 1A and 1C).

The longitudinal glia that prefigure the longitudinal axon tracts in the developing CNS (Jacobs and Goodman, 1989b) express neuroglian, as do a large subset of the neuronal cell bodies that lie along the dorsal surface of the developing CNS, including the identified neurons RP1, RP2, RP3, aCC, and pCC (Jacobs and Goodman,

1989a; Figures 1C and 1D). In a deeper plane of focus (Figure 1D), the protein can be visualized on the commissural (anterior and posterior) and longitudinal axon tracts that form just underneath these glia and neuronal cell bodies. Interestingly, the majority of neuronal cell bodies lie along the ventral surface of the developing CNS, some distance away from the developing axon tracts, and they do not express neuroglian on their surface. The axons of the segmental and intersegmental nerve roots are heavily stained, as are the glia that are associated with these axons as they exit the CNS (Figure 1D).

Immunoelectron microscopic examination of nerve cords stained with the 1B7 MAb allows a more detailed analysis of the pattern of neuroglian expression (Figure 2). A cross section through the nerve cord just posterior to the anterior commissure (Figure 2A) shows the longitudinal axon tracts (dark arrow in Figure 2A), the pair of medial midline glia, several of the longitudinal glia just above the axon tracts, and the cell bodies of the RP1, RP2, and RP3 neurons along the dorsal surface between the midline glia and the longitudinal glia (1, 2, and 3 in Figure 2; see Jacobs and Goodman, 1989a, 1989b). The surfaces of the cell bodies of the RP1, RP2, and RP3 neurons are clearly stained by the antibody, as are the more medial of the longitudinal glial cells that are in contact with the RP neurons (Figure 2A).

The RP1 axon extends contralaterally across the midline and contacts the cell body of its contralateral homolog before turning posteriorly (large open arrow in Figure 2A) and then laterally to extend out the intersegmental nerve (Patel et al., 1987; Jacobs and Goodman, 1989a). The RP1 axon fascicle is heavily stained by the 1B7 MAb; this fascicle extends along the surface of the RP1 cell body and longitudinal glial cells, which also express neuroglian at high levels. Although at this stage of development most of the ventral neuronal cell bodies do not express neuroglian, many of the axons extending dorsally from these cell bodies stain where they enter the dorsal neuropil (dark arrow in Figure 2A). As development proceeds, many of the ventral neuronal cell bodies also begin to express neuroglian.

A sagittal immunoelectron microscopic section through the anterior and posterior commissures reveals that only a subset of the axons in these commissures express neuroglian on their surface (Figure 2B). The three pairs of midline glia (MGA, MGM, and MGP; see Jacobs and Goodman, 1989b) are stained only at regions of contact with labeled axons or cell bodies.

Biochemical Characterization and Purification of Neuroglian Protein

Under reducing conditions, the 1B7 MAb immunoprecipitates three protein bands from iodinated extracts of nerve cord membrane protein (Figure 3A, lane 2). The proteins have apparent molecular masses of 180, 167, and 155 kd. The 167 kd band is predominant. The 1B7 MAb was used to purify neuroglian protein from whole embryo extracts by immunoaffinity chromatography, and the purified protein was used for protein microsequencing experiments and for the production of a neuroglian serum antibody in rat.

On Western blots of 10 hr to 12 hr embryonic membrane proteins, the neuroglian serum antibody detects a pattern of proteins similar to that seen in the immunoprecipitations with the 1B7 MAb (Figure 3B, lane 1). The major band of 167 kd is present, as well as a minor band of 180 kd. A major band of about 50 kd probably represents a degradation product that is detected with the serum antibody but not with the 1B7 MAb. This band is somewhat variable between preparations (compare Figure 3B, lane 1, and Figure 3C, lane 1), and the absence of this band under nonreducing conditions (Figure 3B, lane 2) is consistent with our interpretation that it is a proteolytic fragment that, under nonreducing conditions, remains bound to the high molecular weight component by disulfide bonding. The neuroglian protein migrates somewhat faster under nonreducing conditions (Figure 3B, lane 2), demonstrating that the molecule is not part of a covalently linked multimolecular complex and suggesting the presence of internal disulfide bonding.

Deglycosylation experiments reveal that neuroglian is a glycoprotein. Embryonic membrane proteins were deglycosylated with trifluoromethanesulfonic acid (TFMS) or endoglycosidase H (endo H) and the proteins were analyzed by Western blotting (Figure 3C). The removal of both O-linked and N-linked sugars with TFMS results in a shift in the migration of the protein to a single band of about 155 kd (Figure 3C, lane 2). Removal of N-linked high-mannose oligosaccharides with endo H causes a shift to about 162 kd (Figure 3C, lane 4). These data suggest that native neuroglian probably consists of a core protein of about 155 kd with components of N-linked high-mannose oligosaccharide and O-linked or complex N-linked oligosaccharide.

Identification of cDNA Clones Encoding Neuroglian

The neuroglian serum antibody was used to screen a 9 hr to 12 hr embryonic λgt11 cDNA library (Zinn et al., 1988). Approximately 3×10^5 recombinants were screened and 12 antibody-positive plaques were isolated. Cross-hybridization between the cloned inserts revealed that the 12 clones represent five nonoverlapping classes. The three clones that reacted most intensely with the antiserum fell into the same class by cross-hybridization and detect an embryonic transcript of about 5.1 kb (data not shown). A transcript of this size is expected for a protein the size of neuroglian, and these clones were selected for further characterization.

Three methods were used to verify the identity of putative neuroglian cDNA clones: in situ hybridization to RNA in dissected Drosophila embryos; cytogenetic localization of clones on Drosophila polytene chromosomes, followed by analysis of corresponding genetic deficiencies; and comparison of the deduced amino acid sequence, derived from the neuroglian cDNA sequence, with an amino acid sequence obtained by the microsequencing of purified neuroglian protein.

Strand-specific, ^{35}S-labeled RNA probes were prepared from the largest (2.7 kb) of the putative neuroglian cDNA clones. Strand specificity of the probes was determined by detection of the 5.1 kb embryonic transcript on

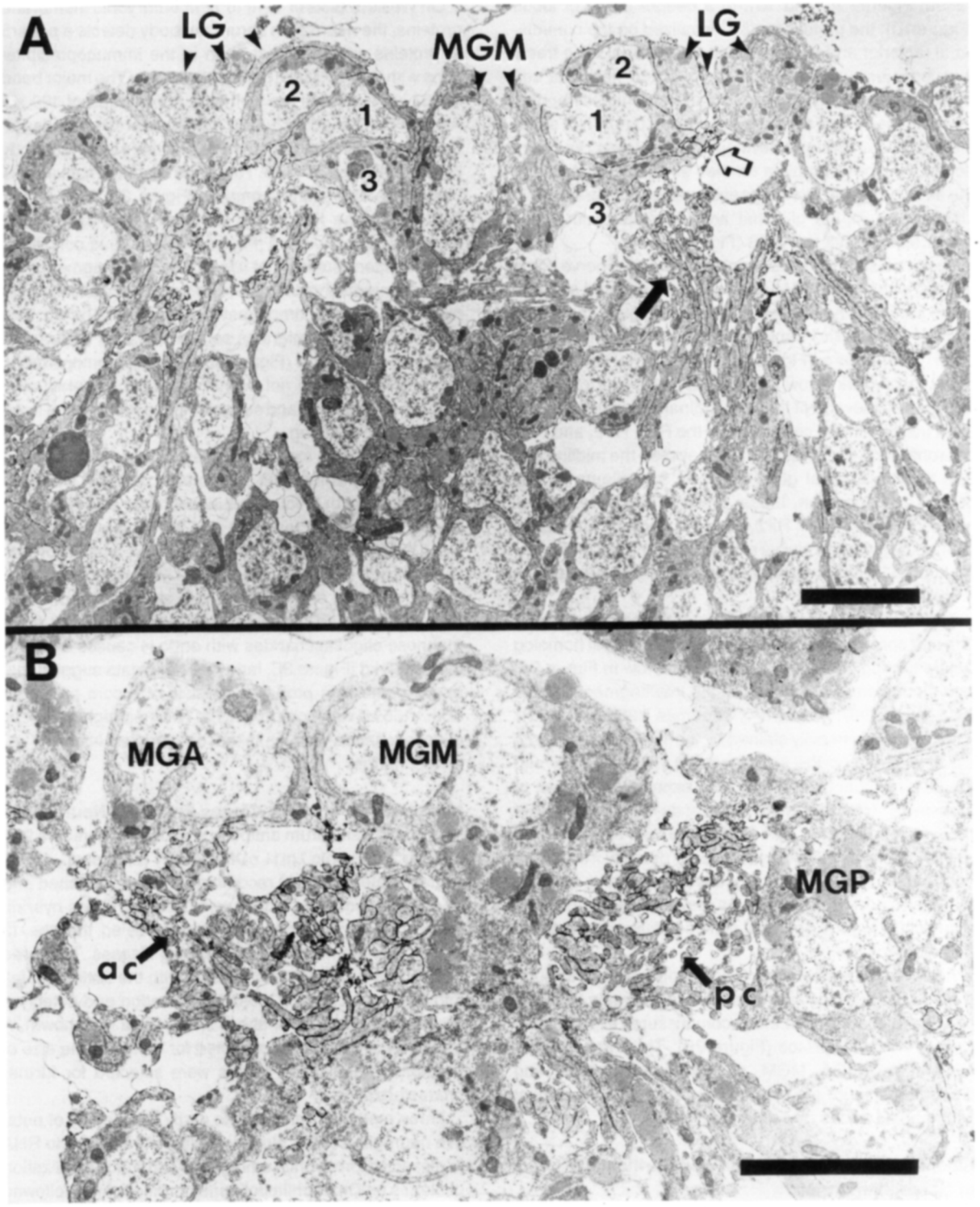


Figure 2. Neuroglian Distribution in the Embryonic CNS Revealed by Immunoelectron Microscopy

In the cross section immediately posterior to the anterior commissure (A) and in sagittal section (B) at 10 hr to 15 hr of development, staining with the 1B7 MAb is visualized with an HRP-conjugated secondary antibody.

(A) The cell bodies of all three RP neurons, RP1, RP2, and RP3 (1, 2, and 3), as well as the RP fascicle containing the RP1 and RP3 axons (open arrow), express neuroglian. In addition, the longitudinal glial cells (LG) and the midline glia (MGM) express antigen only on points of contact with axons or neuronal cell bodies. Although ventral neuronal cell bodies do not express 1B7 antigen at 10 hr to 15 hr of development, their growth cones express the protein where they enter the developing dorsal neuropil (solid arrow).

(B) In sagittal view, many axons of the anterior (ac) and posterior commissures (pc) express the protein, as do the midline glia (MGA, MGM, and MGP) where they contact commissural axons. Note that MGM envelops neuroglian-positive axons in the anterior commissure. Scale bars = 5 μm.

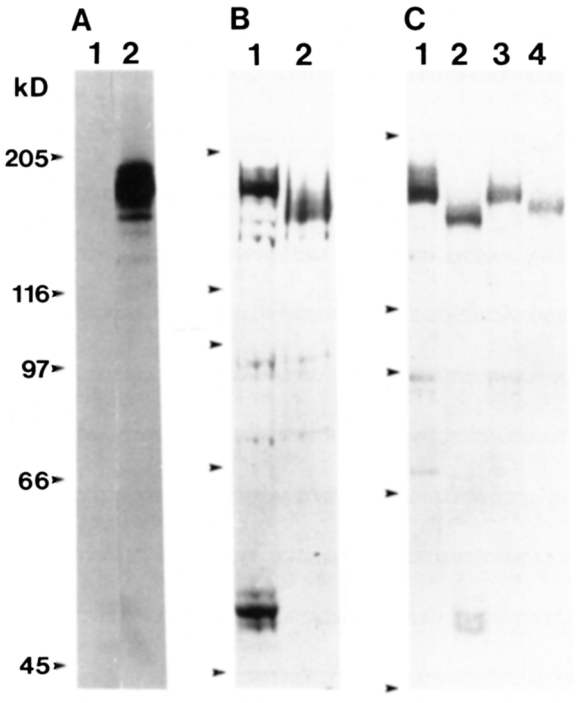


Figure 3. Immunochemical Characterization of Neuroglian

(A) Membranes prepared from 10 hr to 14 hr embryonic nerve cords were labeled with ^{125}I and lactoperoxidase. Membrane proteins were solubilized in NP-40, and immunoprecipitations with normal mouse serum (lane 1) or 1B7 MAb (lane 2) were performed as described in Experimental Procedures. Immunoprecipitates were analyzed on a 7.5% SDS–polyacrylamide gel under reducing conditions. Molecular mass markers are indicated in kilodaltons.

(B) Total membrane proteins from 10 hr to 14 hr embryos were solubilized in SDS sample buffer under reducing (lane 1) and nonreducing (lane 2) conditions. Proteins were separated on a 7.5% SDS–polyacrylamide gel and electroblotted to nitrocellulose. The blot was probed with a 1:1000 dilution of neuroglian rat serum antibody followed by HRP-conjugated second antibody, and was developed with DAB.

(C) Membrane proteins from 10 hr to 14 hr embryos were deglycosylated with TFMS (lane 2) or endo H (lane 4), as described in Experimental Procedures, prior to electrophoretic separation and blotting. The blot was probed as in (B). Lanes 1 and 3 were incubated in deglycosylation buffer without TFMS or endo H.

Northern blots. In situ hybridization to dissected embryos reveals a transcript distribution that is very similar to the pattern of neuroglian antibody staining (data not shown). The hybridization signal is most intense over the brain, hindgut, and peripheral nerve roots, with a weaker signal over other embryonic tissues. Hybridization with an opposite-strand probe of similar specific activity shows no hybridization over any part of the embryo.

In situ hybridization to Drosophila polytene chromosomes localized the cDNA sequences to the X chromosome at cytological location 7F. The genetic deficiencies *Df(1)KA14* and *Df(1)RA2* delete the 7F region and have breakpoints at 7F1–2;8C5 and 7D10;8A4–5, respectively. Stocks bearing these deficiencies were assayed for neuroglian expression by staining embryos with the 1B7 MAb. Approximately one-fourth of the embryos from these stocks lack neuroglian antibody staining (data not shown), as would be predicted for the simple Mendelian segregation of a mutant chromosome carrying a deletion of the neuroglian gene. These results further indicate that the location of the neuroglian gene may be at the overlap of these two deletions, 7F1–2;8A4–5, and demonstrate that the neuroglian gene maps genetically to the same region that was identified by in situ hybridization with the cDNA clones.

Conclusive confirmation of the identity of the neuroglian clones comes from comparison of the neuroglian protein sequence, as deduced from the sequence of the cDNA clones, with the amino acid sequence obtained by the microsequencing of purified neuroglian protein. Neuroglian was purified from 10 hr to 20 hr embryo extract by immunoaffinity chromatograghy. The purified protein was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by electroblotting to polyvinylidene difluoride membranes. Membrane-bound protein was stained, the neuroglian band was excised, and the N-terminal sequence was determined directly on the filter-bound protein. The N-terminal sequence was determined for 18 amino acids (Figure 4). The DNA sequence from a full-length neuroglian cDNA clone (discussed below) encodes an amino acid sequence that is identical to the sequence determined for the N-terminus of the protein. This DNA sequence is preceded by a sequence that encodes an initiating methionine and a 22 amino acid hydrophobic signal peptide, as might be expected for the N-terminal coding sequence of an integral membrane protein (Figure 4).

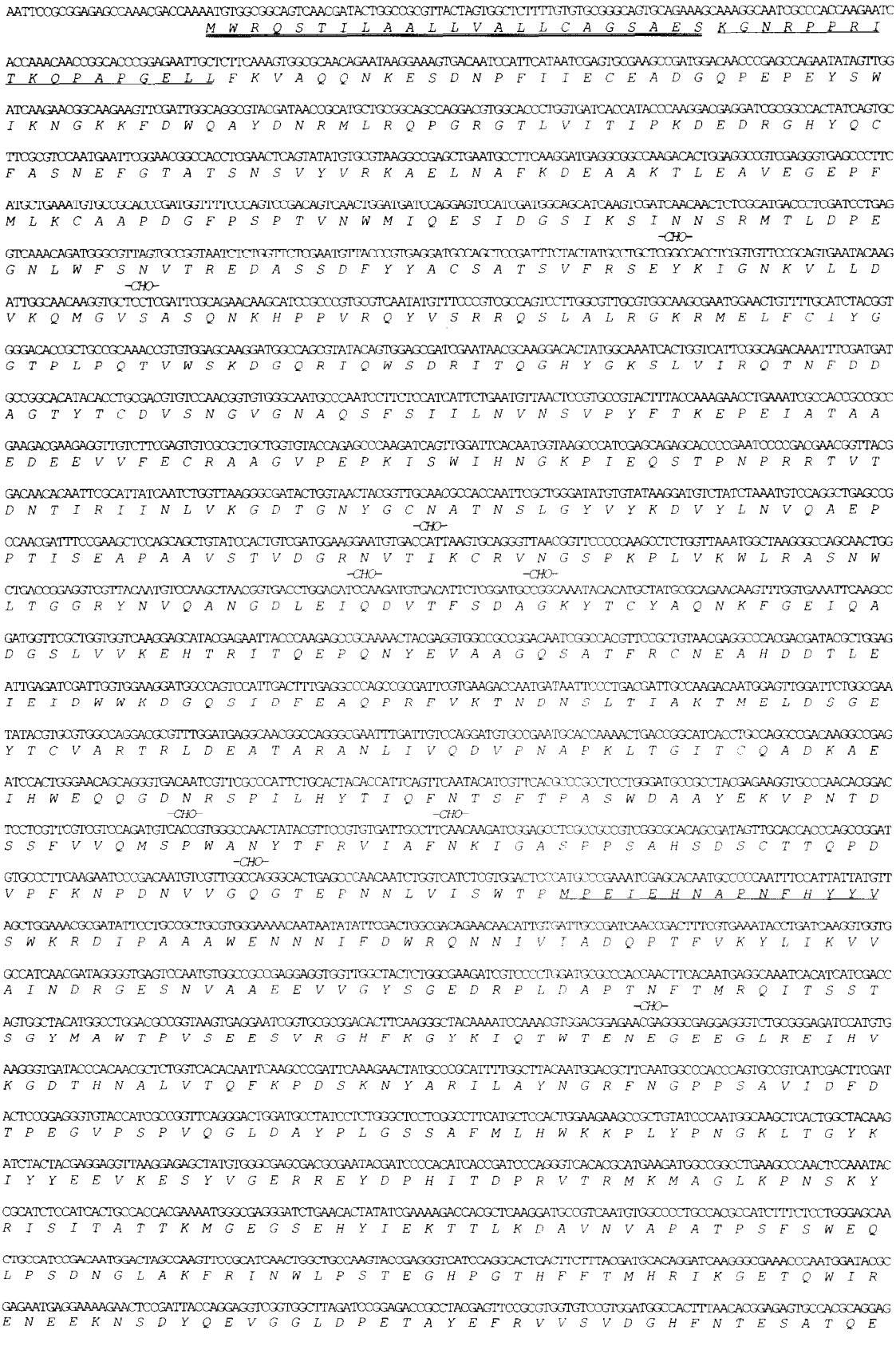
A second peptide sequence was obtained from a cyanogen bromide (CNBr) cleavage fragment of purified neuroglian. Purified protein was cleaved with CNBr and the resulting peptides were separated by SDS–PAGE and electroeluted. The sequence of one of the fragments was determined for the first 15 amino acids (Figure 4). The neuroglian cDNA sequence predicts an amino acid sequence that matches the microsequencing data at 12 of 15 residues. We believe that the differences do not represent variation in the primary amino acid sequence, but rather simply reflect the difficulties in accurately sequencing the small amounts of cleavage fragment that were available (2 of the 3 mismatches are histidines, which are difficult to resolve given the method used here).

In summary, in situ hybridization to embryonic transcripts, genetic deficiency analysis, and comparison of the deduced amino acid sequence with protein microsequencing data clearly demonstrate that the cDNA clones, recovered by expression cloning using the neuroglian serum antibody, do indeed encode the neuroglian protein.

Sequence Analysis of a Neuroglian cDNA Clone

Neuroglian cDNA clones recovered by expression cloning were used as probes to isolate nearly full-length cDNA clones from the 9 hr to 12 hr Drosophila embryonic cDNA library. A 3.9 kb clone was sequenced and the nucleotide and deduced amino acid sequence from this clone is presented in Figure 4.

The cDNA contains an open reading frame that encodes a mature protein of 1216 amino acids. The deduced



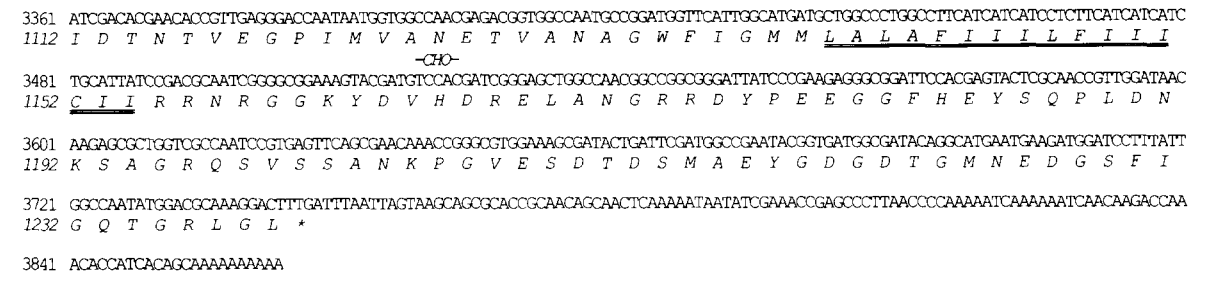


Figure 4. Nucleotide and Deduced Amino Acid Sequence of a Neuroglian cDNA Clone

The nucleotide sequence of the 3.9 kb cDNA clone and the translated sequence of Drosophila neuroglian are presented. The signal peptide and the putative transmembrane domain are double underlined in bold. The two peptide sequences that were determined by protein microsequencing are underlined. Microsequencing data reveal that the N-terminal amino acid of the mature protein is the lysine residue at amino acid position 24. Possible sites for N-linked glycosylation (*-N-X-$\frac{S}{T}$-*) in the extracellular domain are indicated by *-CHO-*.

amino acid sequence predicts a core protein of 145 kd, consistent with the data from deglycosylation experiments. Both of the peptide sequences determined by direct protein sequencing are present in the open reading frame.

The initiating methionine is 23 amino acids upstream of the N-terminal lysine residue of mature neuroglian, as determined by protein microsequencing. This 23 amino acid sequence contains 12 contiguous hydrophobic residues, a characteristic feature of the signal sequence of many integral membrane proteins (von Heijne, 1985). The nucleotide sequence at the initiating methionine residue (CAAAATG) is consistent with the consensus sequence for Drosophila translational start sites ($\frac{C}{A}AA\frac{A}{C}ATG$; Cavener, 1987). The deduced amino acid sequence predicts a mature extracellular domain of 1115 amino acids, followed by a hydrophobic transmembrane domain of 16 amino acids and a cytoplasmic domain of 85 amino acids. The extracellular portion of the protein contains two different types of repeated structural domains. At the N-terminus there are six domains that have a structure typical of Ig domains (Williams and Barclay, 1988). In addition, between the Ig domains and the transmembrane domain there are five repeats that are similar to the type III repeats of Fn (Hynes, 1986).

Sequence similarities within the Ig- and Fn-like domains of neuroglian are presented in Figure 5. The six Ig-like repeats each contain two conserved cysteine residues separated by 46–56 amino acids (Figure 5A). Many amino acids are conserved within the repeats, particularly around the cysteine residues. Several amino acids are conserved in all six neuroglian repeats as well as in the Ig-like repeats of many other Ig-like molecules (Williams and Barclay, 1988). Conserved sequence positions (marked with an asterisk; Figure 5) were initially used to identify the repeated units and have been used to set the register for further alignment of the domains.

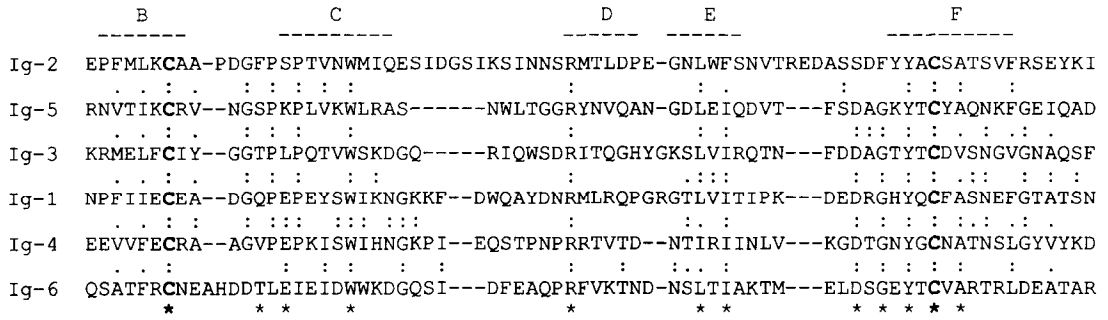
Alignment of the neuroglian Fn-like repeats is presented in Figure 5B. The domains are characterized by conserved tryptophan and tyrosine residues that are separated by 45–53 amino acids. Other amino acids within the domains are also conserved, but the homology between the Fn-like domains is generally less extensive than between the Ig-like domains. The amino acid sequence Arg-Gly-Asp (RGD), which is found in the region of the type III repeats of Fn and is involved in the adhesion of cells to the extracellular matrix, is not found in the neuroglian primary amino acid sequence.

Neuroglian is highly related to the vertebrate neural adhesion molecules with Ig-like structure, particularly mouse L1 (Moos et al., 1988; L1 is equivalent to rat NILE, and chick G4 and Ng-CAM; for review see Jessell, 1988) and chick contactin/F11 (Ranscht, 1988; Brümmendorf et al., 1989). L1 and contactin/F11 are glycoproteins that are expressed on neurons and axons; it has been postulated that they play roles in neuronal cell adhesion and axon fasciculation (Moos et al., 1988; Ranscht, 1988; Brümmendorf et al., 1989). Both molecules contain Ig-like domains and Fn type III repeats similar to those found in neuroglian. Comparison of neuroglian with L1 and contactin, using the FAST.P alignment program (Lipman and Pearson, 1985), yields optimized scores of 1442 and 925, respectively. The homology between neuroglian and these proteins extends throughout most of the extracellular portion of both molecules. The greatest similarity exists between neuroglian and L1, which have greater than 28% amino acid identity over their entire lengths (Figure 6). Besides its homology to L1 and contactin, neuroglian has homology to the other Ig-related neural adhesion molecules from both vertebrates and insects. The optimized FAST.P alignment score with mouse N-CAM is 383 (Barthels et al., 1987), with rat MAG is 198 (Arquint et al., 1987; Salzer et al., 1987), with grasshopper fasciclin II is 182 (Harrelson and Goodman, 1988), and with Drosophila amalgam is 205 (Seeger et al., 1988).

Identification of a Lethal Mutation in the Neuroglian Gene

As described above, the neuroglian gene is located on the X chromosome at cytological location 7F. The genetic deficiencies *Df(1)KA14* and *Df(1)RA2* delete the 7F region and have breakpoints at 7F1–2;8C5 and 7D10;8A4–5, respectively. A screen for lethal point mutations in this region was previously undertaken by Lefevre and Watkins (1986), and ten lethally mutable loci were described that lie within the overlap of these two deficiencies. Antibody staining of

A. Neuroglian Immunoglobulin-Like Domains:



B. Neuroglian Fibronectin-Like Domains:

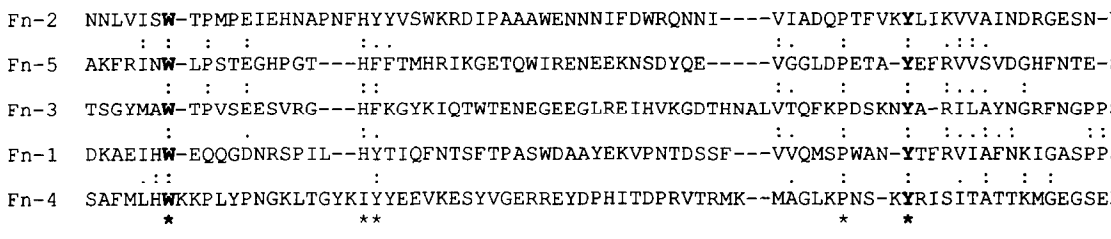


Figure 5. Similarities among the Neuroglian Ig-like and Fn Type III Domains

Sequence similarities among the six Ig-like domains (A) and the five Fn-like domains (B) are presented. The sequences are labeled 1–6, with 1 being the most N-terminal domain and 6 the most C-terminal. The domains were ordered to give the best possible matches using the Needleman and Wunsch (1970) alignment program. The sequences were aligned using the alignment program with adjustments, as described in the text. The conserved cysteine (C) residues of the Ig-like domains and the conserved tryptophan (W) and tyrosine (Y) residues of the Fn-like domains were used to identify the domains and are printed in bold. Certain amino acids (marked with an asterisk) were often conserved between domains and in the domains of other related molecules and were used to set the register for the alignments and to confirm the identity of the domains. The positions of potential β strands within the Ig-like domains are indicated above the domain sequences and are labeled B through F after Williams and Barclay (1988). The β strands A and G have been omitted for simplicity. Double dots between amino acids indicate identities and single dots indicate conservative amino acid changes.

representatives for each of these lethally mutable loci reveals that one of these loci corresponds to the neuroglian gene. Antibody staining experiments demonstrate that the ethyl methanesulfonate–induced mutation designated *l(1)VA142* represents a hypomorphic, lethal mutation in the neuroglian gene (data not shown). Mutant embryos reveal no or little expression of neuroglian in nonneuronal tissues (e.g. trachea, salivary gland, and hindgut) and greatly reduced expression in the nervous system. However, there is still a small amount of neuroglian expression in the CNS, indicating that this is not a complete null allele of neuroglian. The mutation *l(1)VA142* is lethal during the late embryonic and early larval phase of development and exhibits no maternal effect component (Perrimon et al., 1989).

A second lethal mutation, *l(1)RA35* (Lefevre and Watkins, 1986), consists of an inversion with breakpoints at 6E;7F1. Embryonic lethality is associated with the 7F1 breakpoint and mutant embryos completely lack neuroglian expression. We have named lethal mutations in the neuroglian gene, *nrg*.

We have used a variety of nervous system markers for our initial analysis of the phenotype of *nrg* mutant embryos. The most striking observation is that at a gross level, the overall structure of the CNS and PNS, and in particular the peripheral nerve roots and CNS axon pathways, develop in a relatively normal way (Figure 7). Clearly, normally neuroglian-positive axon pathways do not become "unglued" when this Drosophila L1 homolog is genetically deleted, suggesting some redundancy in overall axonal and glial adhesion systems. It will be of interest in the future to examine carefully the behavior of individual neurons and glia at the ultrastructural level in *nrg* mutants alone and in combination with mutations in other neural cell adhesion molecules.

We do not yet know what defect leads to the lethality of *nrg* mutant embryos and larvae. One alternative is that neuroglian might have an essential function in the development of some nonneuronal tissue such as the trachea, hindgut, or salivary gland.

Discussion

In this paper we report on the characterization and cloning of Drosophila neuroglian and the identification of a lethal mutation in the neuroglian gene. Neuroglian is a large, cell surface glycoprotein that is expressed on neurons and glia in the developing Drosophila nervous system and on

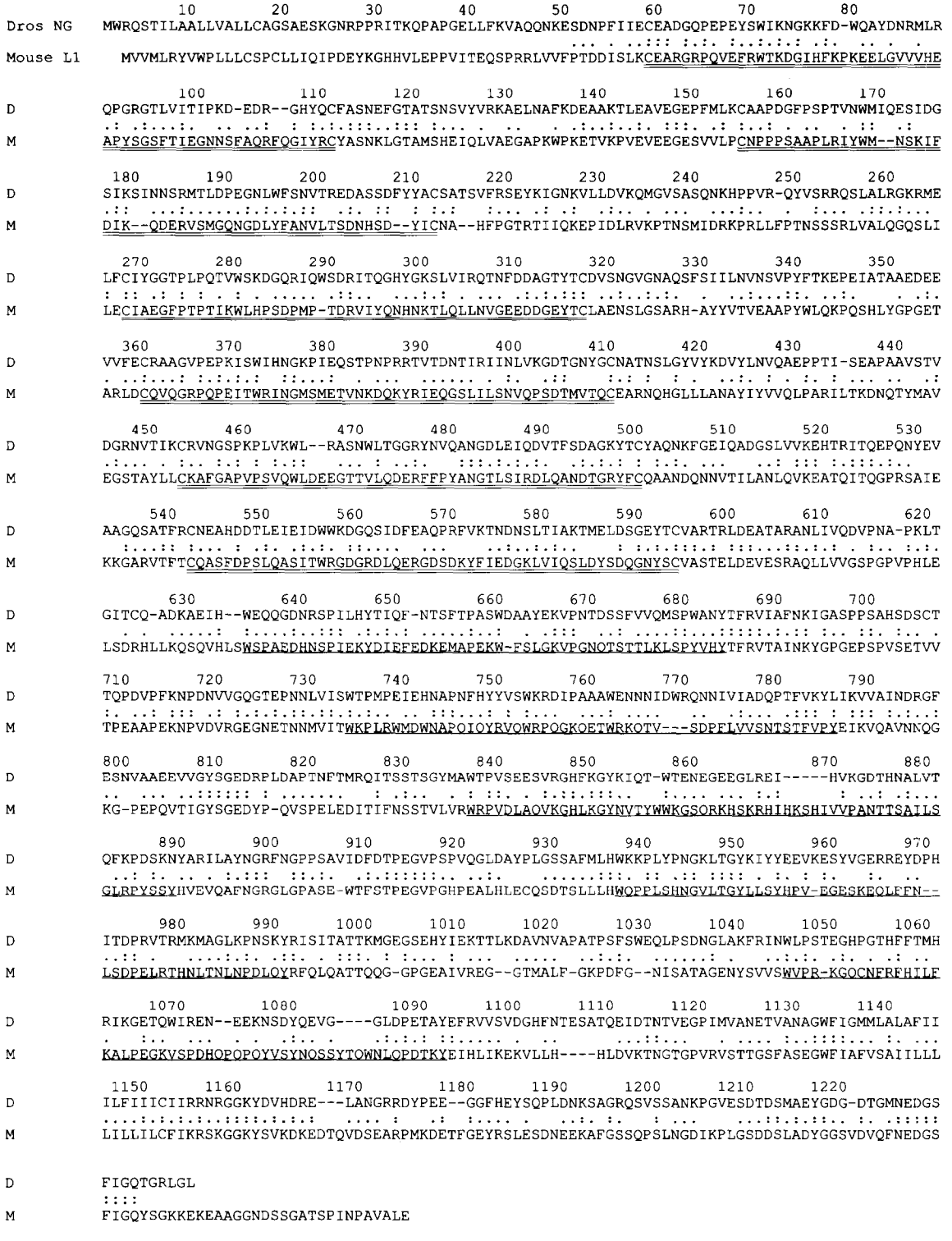


Figure 6. Comparison of the Deduced Amino Acid Sequences of Drosophila Neuroglian and Mouse L1

Deduced amino acid sequences for neuroglian and mouse L1 (Moos et al., 1988) were compared using the FAST.P alignment program (Lipman and Pearson, 1985). Double dots indicate amino acid identities; single dots indicate conservative amino acid changes. The Ig-like domains are double underlined between the conserved cysteine residues. The Fn type III repeats are underlined between the conserved tryptophan and tyrosine residues.

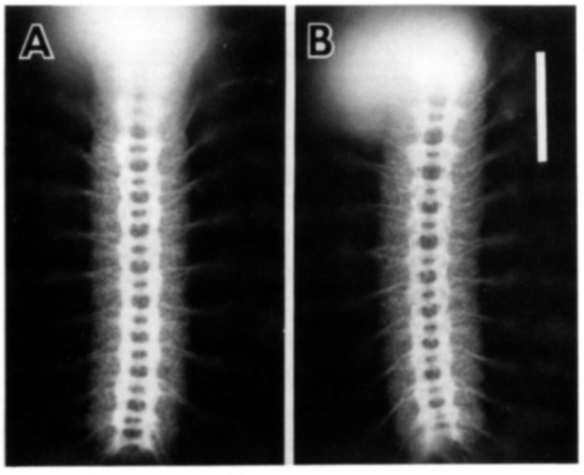


Figure 7. Overall Development of CNS Is Relatively Normal in a Neuroglian Null Mutant

Dorsal views of the developing CNS in dissected wild-type (A) and neuroglian mutant ([B]; *l(1)RA35*) embryos stained as shown with RITC-conjugated anti-horseradish peroxidase (Jan and Jan, 1982). The embryos were also stained with the anti-neuroglian (1B7) MAbs and an FITC-conjugated second antibody to identify the mutant embryos that lack all neuroglian expression (data not shown). At a gross level, the overall structure of the CNS and PNS and, in particular, the peripheral nerve roots and CNS axon pathways develop in a relatively normal way in the *nrg* null mutant embryos. Normally neuroglian-positive axon pathways (see Figure 1) do not become unglued when this Drosophila L1 homolog is genetically deleted, suggesting some redundancy in overall axonal and glial adhesion systems. See text for further discussion. Scale bar = 100 μm.

some other nonneuronal tissues. Neuroglian is a member of the Ig superfamily and is closely related to the Ig-like vertebrate neural adhesion molecules. In particular, neuroglian shows extensive homology to mouse L1 (Moos et al., 1988). The many similarities between neuroglian and L1 suggest that, like L1, neuroglian may be involved in neural and glial cell adhesion.

Biochemical analysis of the neuroglian protein (Figure 3) and sequence analysis of neuroglian cDNA clones (Figure 4) demonstrate that neuroglian is an integral membrane glycoprotein. Neuroglian consists of a core protein of 145–155 kd that is modified by glycosylation to produce the mature forms of the protein (167–180 kd). Mouse L1 consists of a glycoprotein band of about 200 kd that upon deglycosylation yields a single core protein of 150 kd (Faissner et al., 1984, 1985). L1 is also phosphorylated (Faissner et al., 1985), and we have not ruled out the possibility of other forms of posttranslational modification of the neuroglian protein. Both neuroglian and L1 consist of six Ig C2-type domains, five Fn type III domains, a single transmembrane domain, and a short cytoplasmic domain.

Neuroglian and L1 also exhibit similarities in their patterns of expression. Both are expressed on fasciculating axons in the CNS and in the brain. Neuroglian has a wider pattern of expression on neuronal cell bodies, glia, and nonneuronal tissues, although both molecules are also expressed in the gut. L1 has been shown to play roles in neuronal cell adhesion (Keilhauer et al., 1985), neurite outgrowth (Chang et al., 1987), and axon fasciculation (Fischer et al., 1986). The chick homolog of L1, Ng-CAM, is also expressed on neurons and axons. Ng-CAM has been shown to play a role not only in neuron–neuron adhesion but also in neuron–glial cell adhesion (Grumet et al., 1984; Grumet and Edelman, 1988). Given the many similarities between neuroglian and these molecules, it seems likely that neuroglian also plays important roles in neuronal and glial cell adhesion in Drosophila.

The neural adhesion molecules that are members of the Ig superfamily have in common a variable number of Ig-like domains at their N-termini. The structure, function, and evolution of Ig-like domains has been the subject of recent reviews (Williams, 1987; Williams and Barclay, 1988). Ig domains typically fold to form a globular structure containing two β sheets, each consisting of three to four antiparallel β strands of 5–10 amino acids each. Intrachain disulfide bonding between the conserved cysteine residues stabilizes the structure. A common characteristic of Ig-related molecules is that they all participate in some form of adhesion or recognition at the surface of the cell. Both homophilic and heterophilic interactions commonly occur between Ig domains. These interactions are probably mediated by determinants exposed at the faces of the β sheets.

Three types of Ig domains have been proposed: V-, C1-, and C2-type domains (Williams and Barclay, 1988). The classifications are based upon the length of the domains between the conserved cysteine residues, the predicted secondary structure of the domains, and the statistical analysis of the conserved amino acids within the domains. V-type domains are usually longer than the C-type domains, with 65–75 amino acids between the cysteine residues. The extra length of the domain allows the formation of β sheets composed of four β strands each. The shorter C-type domains have 55–60 amino acids between the cysteine residues giving rise to β sheets of three and four β strands. C1 and C2 domains are distinguished on the basis of conserved sequence patterns within the domains.

The Ig-like repeats in neuroglian clearly show conserved patterns of protein sequence (Figure 5). Comparison of the neuroglian Ig domains with the domains of other Ig-related molecules, using the Needleman-Wunsch ALIGN program to assess sequence similarities among the domains (Needleman and Wunsch, 1970), indicates that there are significant similarities among all three types of Ig domains but suggests that the Ig domains of neuroglian represent the C2 type. Furthermore, analysis of the neuroglian Ig domains using the Chou and Fasman algorithm to predict secondary protein structure (Chou and Fasman, 1974) suggests the formation of multiple β strands within the neuroglian domains that could allow folding to the characteristic structure of the C-type domain. In Figure 5, the positions of the β strands are indicated above the domain sequences and labeled B through F after Williams and Barclay (1988). The β strands A and G lie outside of the sequences presented and have been omitted. In accordance with their significance to the secondary structure of the domains, the alignments have been ad-

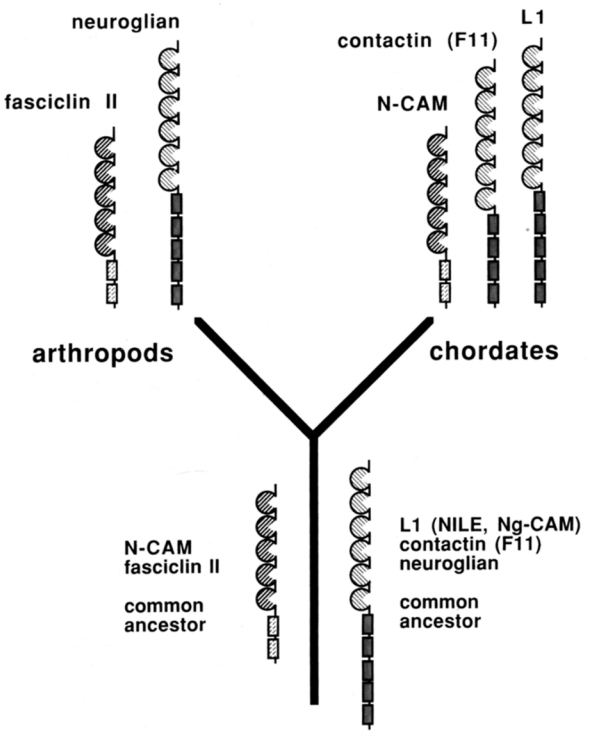


Figure 8. Schematic Model Depicting the Evolution of Some of the Neural Cell Adhesion Molecules in the Immunoglobulin Superfamily before the Split of the Chordates and Arthropods

The proposed evolution of some of the vertebrate and invertebrate Ig-like neural adhesion molecules is presented schematically. The looped structures represent the Ig domains of the molecules and the rectangular structures represent the Fn domains. The shading within the structures denotes their evolutionary origin. The most parsimonious explanation based on sequence analysis is that fasciclin II and N-CAM evolved from a common ancestor before the phyletic split of arthropods and chordates; and that neuroglian, L1, and contactin/F11 arose from a second common ancestor. Thus, there were at least two if not more neural adhesion molecules in primitive organisms, each having multiple Ig type C domains and Fn type III domains. These neural adhesion molecules with both multiple Ig and Fn domains most likely represent the evolutionary ancestors of the Ig superfamily (Williams, 1987; Williams and Barclay, 1988).

	L1	F11	NCAM	Fas II
Neuroglian	**692**	**487**	277	174
L1		**531**	250	157
F11/Contactin			250	114
NCAM				**507**

Figure 9. FAST.P Comparison of Immunoglobulin Domains

The Ig portions of Drosophila neuroglian, mouse L1 (L1), chick contactin/F11 (F11), grasshopper fasciclin II (Fas II), and mouse NCAM are compared using the FAST.P alignment program of Lipman and Pearson (1985). In each case, only the Ig portions of the molecules, beginning 20 amino acids before the N-terminal cysteine residue of the most N-terminal Ig-like domain and ending 20 amino acids after the carboxy-terminal cysteine residue of the most carboxy-terminal Ig-like domain, were included in the comparisons. The simple difference in the number of Ig-like domains within neuroglian, L1, and contactin/F11 (six Ig-like domains), and within fasciclin II and NCAM (five Ig-like domains), is not sufficient to explain the observed differences in FAST.P scores.

justed to exclude gaps from the β strand regions and from other highly conserved regions.

At the amino acid level, Drosophila neuroglian and mouse L1 are 28% identical. At the level of higher order protein structure, both molecules are composed of six Ig-like domains and five Fn type III domains, followed by a transmembrane domain and a short cytoplasmic domain. A similarly close relationship exists between grasshopper fasciclin II and mouse N-CAM (Harrelson and Goodman, 1988). Both are composed of five Ig-like domains and two Fn type III repeats, and they share 25% amino acid identity.

Ig-like molecules appear to diverge quite rapidly. The V-type domain of CD8 retains only 42% amino acid identity between rodent and human (Littman, 1987). Although there is a broad evolutionary span between Drosophila and mouse, the structural similarities and the 28% amino acid identity between neuroglian and L1 suggest a common evolutionary origin. This notion is supported by ALIGN comparison between the Ig domains of neuroglian and L1. For most of the possible alignments, the ALIGN score between any Ig-like domain in neuroglian and the corresponding domain in L1 is higher than the score for the alignment of that domain with the other Ig domains within neuroglian (data not shown). It is widely believed that Ig-related sequences evolved by duplication and divergence from a primordial domain that probably played a role as a cell adhesion molecule (Williams and Barclay, 1988). It seems unlikely that separate evolutionary paths would produce the correspondence between respective domains that is seen with neuroglian and L1.

Figure 8 depicts the proposed evolutionary relationships between neuroglian, L1, contactin/F11, fasciclin II, and N-CAM. Comparison of Ig domains using the FAST.P alignment program reveals that neuroglian is closely related to L1 and contactin/F11, whereas fasciclin II is closely related to N-CAM (Figure 9). Comparison of the entire extracellular region of the proteins including the Fn domains reveals the same set of relationships (data not shown). Thus, whereas fasciclin II appears to have arisen from a common ancestor of fasciclin II and N-CAM (Harrelson and Goodman, 1988), neuroglian appears to have arisen from a second common ancestor of neuroglian, L1, and contactin/F11. The third insect Ig-like molecule, amalgam (Seeger et al., 1988), although related to these neural adhesion molecules, does not fit into either of these two groups.

The discovery of these three members of the Ig superfamily in the developing insect nervous system (fasciclin II [Harrelson and Goodman, 1988], amalgam [Seeger et al., 1988], and neuroglian [this report]) confirms earlier predictions (Williams, 1987; Williams and Barclay, 1988). The data further suggest that the ancestral function of Ig superfamily molecules was as adhesion molecules in the developing nervous system, that these molecules duplicated and diverged with multiple Ig C2-type and Fn type III domains before the split of the arthropod and chordate lines,

and that the molecular mechanisms of neural adhesion and recognition are likely to have been conserved in arthropods and chordates.

The neuroglian gene maps to the 7F region of the polytene chromosomes, and deletion of the chromosomal region 7F1–2;8A4–5 abolishes neuroglian expression in Drosophila embryos. Antibody staining experiments demonstrate that the mutations designated *l(1)VA142* and *l(1)RA35* represent lethal alleles of neuroglian (we call this gene *neuroglian* or *nrg*). The mutant *l(1)RA35* appears to be a protein null and lacks all embryonic neuroglian expression. Interestingly, at a gross level of analysis, the embryonic CNS and PNS in *nrg* mutant embryos appear to develop relatively normally (Figure 7) prior to the onset of lethality in the late embryonic and early larval phase of development. We do not yet know what essential function neuroglian plays or in what tissue its absence leads to lethality.

The neural cell adhesion molecule L1 is likely to play a major role in promoting neurite outgrowth and axonal adhesion in the development of the vertebrate nervous system (for reviews see Rathjen, 1988; Jessell, 1988). Here we report on the cloning of neuroglian, a Drosophila protein that has remained highly homologous to vertebrate L1 despite the great evolutionary separation of arthropods and chordates. We also report on the identification of a first protein null mutation in an Ig superfamily adhesion molecule. The surprising result of our initial genetic analysis has been the observation that in the absence of neuroglian the overall structure of the CNS and peripheral nerve roots (locations that normally show a high level of neuroglian expression) appears relatively normal (Figure 7). Given the presumed importance of this adhesion molecule in the developing vertebrate nervous system, one might have predicted a much stronger mutant phenotype.

However, recent studies in vertebrates, in which antibodies were used to perturb neurite outgrowth and axon fasciculation in vitro, suggest that the systems that mediate neural cell adhesion (including L1) are redundant and that perturbation of more than one system is required before major functional disruptions occur (Tomaselli et al., 1986; Chang et al., 1987; Bixby et al., 1987; Tomaselli et al., 1988; Neugebauer et al., 1988). If such redundancy of adhesion systems is indeed at play in Drosophila as it appears to be in vertebrates, then multiple mutations that simultaneously remove more than one cell adhesion system may be necessary to produce gross disruptions of nervous system development. In this light, it is perhaps not so surprising that the *nrg* mutant does not lead to a grossly abnormal CNS in which axon pathways and peripheral nerve roots become unglued and highly disorganized. Rather, it will be of interest in the future to look for more subtle phenotypes in the behavior of individual neurons and glia in *nrg* mutant embryos and gross phenotypes in embryos mutant for *nrg* in combination with mutations in other neural cell adhesion systems. The classical genetic and molecular genetic approaches, which are the strengths of the Drosophila experimental system, should prove ideally suited for the analysis of problems of this type and may help clarify the emerging notion of functional redundancy in neural cell adhesion systems.

Experimental Procedures

Generation and Screening of Monoclonal Antibodies

Membrane proteins from embryonic Drosophila nerve cords were used for the production of MAbs. Embryonic nerve cords were isolated as previously described (Goodman et al., 1984), and the membrane protein fraction was prepared as described by Patel et al. (1987).

BALB/c mice were immunized with intraperitoneal injections containing approximately 1 mg of protein. The primary injection consisted of membrane protein suspended in a 1:1 mixture of phosphate buffered saline (PBS; 2 mM $NaH_2PO_4 \cdot H_2O$, 8 mM Na_2HPO_4, 170 mM NaCl [pH 7.4]) and Freund's complete adjuvant. Mice were given three boosting injections at 3 week intervals. Boosting injections were identical to the primary injection except that incomplete Freund's adjuvant was used. Three days prior to fusion, a final injection in PBS alone was administered. Mouse spleen cells were fused to NS-1 myeloma cells (Oi and Herzenberg, 1980), and hybridoma supernatants were screened by immunofluorescence on isolated nerve cords as previously described (Patel et al., 1987).

Immunocytochemistry

Staged embryos were dissected onto glass slides in PBS, fixed in 3.7% formaldehyde in PEM (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$ [pH 6.95]) for 15 min, and then washed twice for 10 min in PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBT). Embryos were incubated for 20 min in PBT with 5% normal goat serum followed by addition of an equal volume of hybridoma supernatant and incubation overnight at 4°C. Embryos were then washed three times for 10 min in PBT and incubated 20 min in PBT with 5% normal goat serum, followed by incubation in a 1:500 dilution of HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). After washing in PBT, embryos were placed into a 0.3 mg/ml solution of 3,3'-diaminobenzidine (DAB) in PBT containing 0.003% hydrogen peroxide, and the peroxidase reaction was allowed to continue for 5–10 min before it was terminated by washing with PBS. Stained embryos were cleared in a 50%, 75%, and 90% glycerol series prior to mounting.

Electron Microscopy

Embryos between 10 hr and 12 hr of development were dissected onto plastic coverslips in PBS and then fixed in PLP fixative (McLean and Nakane, 1974). Dissections were labeled with primary and HRP-conjugated secondary antibodies, and subsequently reacted in the presence of DAB and hydrogen peroxide. After postfixation with 2% glutaraldehyde, DAB reaction product was intensified using the thiocarbohydrazide method (Tomlinson and Ready, 1987). Dissections were then osmicated, stained with uranyl acetate, dehydrated, and embedded in Epon-Araldite. Ultrathin (70 nm) sections were examined with a Philips EM410 microscope. Tissue shrinkage was less than with glutaraldehyde primary fixation (Jacobs and Goodman, 1989a).

Protein Labeling and Immunoprecipitation

Membrane proteins were labeled with ^{125}I, and immunoprecipitations were performed as previously described (Patel et al., 1987).

Western Blotting

Total membrane proteins from 10 hr to 14 hr Drosophila embryos were prepared as previously described (Patel et al., 1987). Proteins were separated by SDS–PAGE on 7.5% acrylamide gels, and immunoblots were performed using a modification of the procedure developed by Burnette (1981) according to Hortsch et al. (1985).

Deglycosylation

Total membrane proteins from 10 hr to 14 hr embryos were deglycosylated with TFMS as described by Edge et al. (1981). Deglycosylation with endo H was carried out according to the manufacturer's specifications (Boehringer Mannheim Biochemica). Deglycosylated protein was analyzed by immunoblotting as described above.

Protein Purification and the Generation of Antisera

Neuroglian protein was purified by immunoaffinity chromatography

using the 1B7 MAb for construction of the affinity matrix (Schneider et al., 1982; Patel et al., 1987). The protein was further purified by preparative SDS–PAGE and electroelution (Hunkapillar et al., 1983), and used in the production of rat serum antibodies, as previously described (Patel et al., 1987), and for protein microsequencing as described below.

Protein Microsequencing

Protein for microsequencing was initially purified by immunoaffinity chromatography as described above. For sequence determination on CNBr cleavage fragments, purified protein was cleaved with CNBr as described by Snow et al. (1988). The peptide fragments were separated by SDS–PAGE, followed by electroelution and subsequent amino acid analysis on a gas-phase sequenator (Applied Biosystems, model 470A) with an on-line HPLC for analysis of phenylthiohydantoins of the amino acids, using the manufacturer's standard reagents and programs.

To determine the neuroglian N-terminal sequence, affinity-purified protein was separated by SDS–PAGE, followed by electroblotting to a polyvinylidene difluoride membrane (Millipore). The membrane-bound protein was stained with Coomassie blue R-250, and the neuroglian band was excised and sequenced directly. All procedures were as described by Matsudaira (1987).

cDNA Expression Cloning

A 1:1000 dilution of the neuroglian rat serum antibody was used to screen a 9 hr to 12 hr embryonic λgt11 cDNA library (Zinn et al., 1988), as previously described (Patel et al., 1987).

DNA Sequencing

DNA sequencing was carried out according to the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical Corp.), ^{35}S-dATP, and 60 cm buffer gradient gels (Biggin et al., 1983).

A 3.9 kb full-length cDNA in λgt11 was subcloned into the Bluescript KS+ vector (Stratagene) as two EcoRI fragments of approximately 3.5 kb and 0.4 kb. The plasmids were randomly sheared by sonication, and the ends were repaired with T4 DNA polymerase and Klenow fragment (Maniatis et al., 1982). Fragments in the 400 bp–1000 bp size range were electroeluted from a 1% agarose gel and inserted into the SmaI site of M13 mp10 by ligation for 48 hr at 15°C. Ligations were transformed into E. coli strain X-L1 Blue (Stratagene), and recombinant plaques containing cDNA sequences were identified by plaque hybridization to gel-purified cDNA fragments labeled with [α-^{32}P]dATP by oligonucleotide priming (Feinberg and Vogelstein, 1983).

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