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Major professor

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ANALYSIS OF A FAMILY OF SENSORY, GLIAL, AND CONNECTIVE TISSUE SPECIFIC 130 KD GLYCOPROTEINS

By

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ABSTRACT

ANALYSIS OF A FAMILY OF SENSORY, GLIAL, AND CONNECTIVE TISSUE SPECIFIC 130 KD GLYCOPROTEINS

By

Mary Lynn Bajt

From early development through adulthood, the leech expresses sets and subsets of 130 kD glycoproteins on sensory afferents and glial cells. MAb Laz 6-189 recognizes proteins expressed by all cell types. The glial protein is recognized by mAb Laz 6-297. A 130 kD protein shared by all sensory afferents, we call the "sensory protein", is recognized by mAb Lan 3-2. Three other 130 kD proteins, called the "modality proteins", are recognized by mAbs Laz 2-369, Laz 7-79, and Laz 6-212. In addition, a 130 kD connective tissue specific antigen was identified, recognized by mAb Laz 9-84. We were interested in characterizing the relationship among these various 130 kD glycoproteins and determining whether leech glycoproteins express the L2/HNK-1 and L3, L4, and L5 carbohydrate epitopes.

We postulate that the 130 kD glycoproteins represent a family of related protein cores which are differentially glycosylated. Peptide mapping experiments using limited proteolysis with V8 protease, demonstrated that the sensory, modality, and glial proteins are similar to each other. The 130 kD antigens appear to be differentiated by their carbohydrate moieties. Deglycosylation of antigens immobilized on nitrocellulose, demonstrated that the mAbs specific for the sensory and modality proteins bind to carbohydrate epitopes. In addition, the mAb specific for the sensory protein recognizes a mannose-

containing epitope as shown by sugar blocking studies. The mAbs specific for the modality proteins are not blocked by alpha methyl-mannoside suggesting that the sensory and modality epitopes are different. Recent antibody perturbation studies indicate that the sensory protein carbohydrate epitope is involved in neuronal development. We hypothesize that the modality carbohydrate epitopes also participate in neuronal development.

There is increasing evidence suggesting that carbohydrate structures are involved in cell recognition during neuronal development. The two carbohydrate epitope families, L2/HNK-1 and L3/L4/L5 have been implicated in cell adhesion mechanisms in neuronal development. The epitopes recognized by mAbs L3, L4, and L5 have been identified as mannose-containing glycans. On immunoblots prepared from leech proteins, mAbs L3, L4, and L5 recognize glycoproteins similar to those recognized by leech mAbs Lan 3-2 and Laz 6-189.

In addition, the sulfated glucuronic epitope L2/HNK-1 is expressed in the leech nervous system. However, unlike the L3, L4, and L5 epitopes which are expressed by both neuronal cell bodies and processes, the L2/HNK-1 is largely confined to the cortical cell body layer of the central ganglia. Investigating the roles of the these two carbohydrate epitope families during leech neuronal development, as well as the carbohydrate epitopes expressed by the sensory and modality proteins will allow us to understand how glycosylation modifies cell adhesion.

To my parents, Jack and Sophia Bajt

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Greg, last but certainly not least, you are my joy. Our times together are memories I will always cherish. We don't know what the future has in store for us, but just remember, "whole way far bunch, and more".

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LIST OF ABBREVIATIONS

AMOG	adhesion molecule on glia
CNS	central nervous system
DAB	3,3'-diaminobenzidine
ECA	*Erythrina Cristagalli Agglutinin*
ELISA	enzyme-linked immunosorbent assay
Fab	binding fragment of antibody generated by papain digestion
Fasciclins	adhesion molecules expressed in invertebrates
GC	glycolipid on oligodendrocytes
HRP	horse radish peroxidase
integrins	cell membrane receptors for extracellular matrix molecules
J1	glycoprotein associated with astrocytes
kD	kilodaltons
L1	glycoprotein expressed on neurons and glia
L2/HNK-1	carbohydrate epitope consisting of sulfated glucuronic acid
L3/L4/L5	carbohydrate epitopes consisting of mannose
mAb	monoclonal antibody

mAb Lan 3-2	recognizes surface glycoprotein on sensory afferents in the leech
mAb Lan 3-13	recognizes intracellular protein in glial cells in the leech
mAb Laz 2-369	recognizes surface glycoprotein on subset of sensory afferents in the leech
mAb Laz 6-189	recognizes surface glycoprotein on different neuronal cell types in the leech
mAb Laz 6-212	recognizes surface glycoprotein on subset of sensory afferents in the leech
mAb Laz 6-297	recognizes surface glycoprotein on glial cells in the leech
mAb Laz 7-79	recognizes surface glycoprotein on subset of sensory afferents in the leech
mAb Laz 9-84	recognizes surface glycoprotein on connective tissue in the leech
MAG	myelin associated glycoprotein
N-CAM	neuronal cell adhesion molecule
NILE	nerve growth factor inducible large external glycoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
P_o	glycoprotein on myelin
PSA	polysialic acid
Ran-2	cell surface antigen on type-1 astrocytes

SDS	sodium dodecyl sulfate
TAG-1	transiently expressed axonal surface glycoprotein
Thy-1	glycoprotein on sensory neurons and thymus

INTRODUCTION

During development, neuronal processes must navigate over long distances in order to establish synaptic connections with their appropriate target cells. Cell-cell interactions are essential for the regulative features of development. It is becoming increasingly clear that cell-cell interactions are mediated through cell surface adhesion molecules (Edelman, 1985a; Edelman, 1985b; Rutishauser and Jessel, 1988). Repeated dynamic regulation in the amount, distribution, and expression of the surface molecules influences the development of the nervous system (Edelman, 1984). None of these regulatory mechanisms appears to be fully independent of the others, with their relative contributions varying in time.

As axons travel in search of their targets, they appear to use a combination of these cell surface molecules to interact with the changing local environment. The number and diversity of molecules increases with successive stages of neural development (for review see Jessell, 1988). Consequently, the elimination of the function of any single cell adhesion molecule may result in only minor changes in guidance of individual axons. The relative strength of axon-axon versus axon-substrate adhesion interactions governs the degree of axon fasciculation (for review see Rutishauser and Jessell, 1988). These cell adhesion molecules modulate the strength of axon

fasciculation by allowing growing axons to either enhance fasciculation with other axons or to branch and create new tracts.

Although this area of research has been under investigation for a number of years, the molecular basis for neuronal recognition during growth and synapse formation is still not fully understood. Two problems associated with such studies is the isolation of candidate molecules and the identification of the precise role(s) that they mediate during development (Patterson and Purves, 1982). Recent technical advances in the fields of immunology, cell biology, and genetics have been applied to isolate and characterize cell surface molecules.

The identification of the molecules responsible for cell-cell interactions is of great importance. A large number of different surface molecules that appear to play a role in cell-cell interactions have been identified the last few years (for reviews, see Jessell, 1988; Rutishauser and Jessell, 1988; Linnemann and Bock, 1989). These cell adhesion molecules can be catogorized into classes on the basis of their distributions and functional roles. General cell adhesion molecules are involved in the formation of early neuronal and nonneuronal tissue structures, e.g., neural cell adhesion molecule (N-CAM) (Edelman, 1983). Molecules that are expressed on neurons during particular stages of development are implicated in mediating axon extension and fasciculation, e.g., TAG-1 and L1 (Dodd et al.,1988). Integral surface membrane receptors for extracellular matrix molecules contribute to the general adhesive properties of the neural cell, to cell migration, and to initial axon outgrowth, e.g., integrins (for review see Buck et al., 1986). Cell surface molecules

expressed on distinct subsets of neurons are implicated in target recognition, e.g., retinal tectal marker in the chick (Trisler et al., 1987). In addition, molecules associated with glial cells appear to be involved in certain neuron-glia interactions, e.g., AMOG (Antonicek and Schachner), MAG (Quarles, 1983/84), and J1 (Kruse et al., 1985).

One of the most extensively studied surface molecules is the neural cell adhesion molecule, commonly known as N-CAM (Edelman, 1983; Rutishauser, 1983; Watanabe et al., 1986; Cunningham et al., 1987; Rutishauser et al., 1988). N-CAM is broadly distributed on cells that develop into both neuronal and nonneuronal tissues. N-CAM has been implicated in a variety of developmental events, such as axon guidance, segregation of cells into discrete regions and layers, and the formation and innervation of muscles.

N-CAM is a group of similar proteins comprised of a single polypeptide chain with extensive heterogeneity in both its protein and carbohydrate structures. The different forms of N-CAM are categorized based on their carbohydrate contents and molecular weight of the polypeptide backbone (Hoffman et al., 1982). The carbohydrate composition and structure of N-CAM is unusual because of the high percentage of sialic acid, most of which exists as alpha-2,8-linked linear homopolymers attached to typical N-linked high mannose oligosaccharide cores (Finne et al., 1983).

N-CAM mediates cell-cell adhesion via homophilic binding, N-CAM binding to N-CAM (Rutishauser, 1983). However, the association of proteoglycans with N-CAM suggest that the N-CAM mediated adhesion is more complex than a simple homophilic interaction. N-CAM has binding sites for glycosaminoglycan residues present in

heparin and heparan sulfate which are distinct from the homophilic binding domains (Cole et al., 1986; Cole and Glaser, 1986). Therefore, N-CAM may possess several distinct ligand-binding domains.

In contrast to the ubiquitous distribution of N-CAM, other adhesion molecules such as fasciclins (Patel et al., 1987), TAG-1 (Dodd et al., 1988), and L1 (Rathjen and Schachner, 1984; Moos et al., 1988) demonstrate regional distribution. Fasciclins I, II, III are the best characterized invertebrate axonal glycoproteins (Bastiani et al., 1987; Patel et al., 1987). The expression of individual fasciclins on embryonic grasshopper and *Drosophila* neurons is restricted to specific regions on the neuron and its processes. Fasciclin III is expressed on a subset of neurons and axon pathways in the *Drosophila* embryo. Deletion of the fasciclin III gene appears to results in abnormalities in axon fasciculation (Patel et al., 1987).

The ability of growth cones to extend along axon surfaces underlies selective fasciculation and pathway selection. In rat, two structurally distinct glycoproteins are involved in this process: transiently expressed axonal surface glycoprotein (TAG-1) and L1 (Dodd et al., 1988). TAG-1 is transiently expressed on subsets of embryonic spinal cord axons. The expression of TAG-1 in the spinal cord precedes the appearance of most surface axonal glycoproteins implicated in cell-cell interactions. As the expression of TAG-1 decreases in motor and commissural axons, the appearance of the L1 antigen is initiated. TAG-1 is selectively expressed on the axons, but not the cell bodies of early motor and commissural axons. In contrast, L1 is expressed on the distal axonal segment and not on

neuronal cell bodies, their dendrites, and the proximal axonal segment of motor and commissural neurons. The regional specificity of TAG-1 and L1 provides evidence for the selective expression of distinct proteins to restricted axonal surface areas. The regulation of TAG-1 and L1 on spinal neurons suggests that changes in the expression of axonal membrane glycoproteins on different segments of the same axon contribute to axonal guidance and pathway selection in vertebrates (Dodd et al., 1988).

In addition, L1 has been implicated in mediating granule cell migration in the mouse cerebellar cortex (Rathjen and Schachner, 1984; Fushiki and Schachner, 1986). L1 has been demonstrated to be immunochemically identical to the nerve growth factor-inducible large external glycoprotein, NILE (Bock et al., 1985). While NILE, in turn, has been demonstrated to be the mouse equivalent of chicken Ng-CAM, neuron-glia cell adhesion molecule (Friedlander et al., 1986). The distribution of L1 is more restricted both spatially and temporally compared to N-CAM. L1 expression is related to the developmental stage of neurons and is expressed by only post-mitotic neurons. L1 mediates neuron-neuron binding via homophilic binding (Grumet and Edelman, 1988).

Schwann cells and other glial cells of the peripheral nervous system also express L1 (Faissner et al., 1984; Mirsky et al., 1986). Schwann cells appear to lose L1 shortly after initiation of myelin formation (Martini and Schachner, 1986). In contrast, glial cells of the central nervous system do not express L1 (Salton et al., 1983). L1 mediated neuron-glial binding is heterophilic, i.e. L1 on neurons binds to an unidentified molecule on glial cells (Grumet and Edelman,

1988). The binding sites on L1 for neuron-neuron and neuron-glia interactions appear to be distinct.

Several adhesion molecules associated with glial cells have also been isolated, such as the myelin associated glycoprotein (MAG) and J1 (Figlewicz et al., 1981; Quarles, 1983/84; Kruse et al., 1985). MAG is a constituent of the central and peripheral nervous system myelin sheaths. Because of the localization of MAG in periaxonal membranes and antibody perturbation studies, it has been implicated in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte cell interactions during the myelination process (Quarles, 1983/84; Poltorak et al., 1987). MAG mediates cell-cell adhesion via heterophilic binding (Fahrig et al., 1987). In addition, MAG, like N-CAM, interacts with extracellular matrix components, since it binds heparin and several collagens *in vitro* (Fahrig et al., 1987). Whether these interactions mediate cell-cell adhesion has yet to be determined.

J1, which is associated with astrocytes, mediates neuron-astrocyte adhesion in developing rat brain (Kruse et al., 1985). Therefore, J1 mediates cell-cell adhesion via heterophilic binding. Recently, Antonicek et al. (1987) described a novel glial cell adhesion molecule (AMOG) that also mediates neuron-astrocyte interactions in the developing rat brain. AMOG is associated with the glial cells in the cerebellum during the critical developmental stages of granule neuron migration (Antonicek and Schachner, 1988).

Cell adhesion molecules not only play important roles in the cell-cell contacts between neighboring cells, but also between the cell surface and the extracellular matrix. The cell membrane receptors

that recognize many of the extracellular matrix molecules are collectively known as the integrins. Integrins are a family of integral membrane proteins which contribute significantly to axonal outgrowth, guidance, and selective synapse formation (for review, see Buck et al., 1986; Tamkum et al., 1986; Hynes, 1987). The integrins are cell type specific receptors that interact with specific extracellular matrix glycoproteins such as fibronectin, vitronectin, and laminin (Horwitz et al., 1985; Bozyczko and Horwitz, 1986). The specific integrins have been shown to bind to these ligands at sites encompassing the RGD sequence (arginine-glycine-aspartic acid) (Ruoslahti and Pierschbacher 1987). The integrins are composed of a heterodimer with two membrane-spanning subunits. These adhesion receptors may provide a link between the extracellular matrix and the cytoskeleton (Horwitz et al., 1986).

Arguably, the formation of synaptic connections during neuronal development requires the highest degree of precision of neural cell recognition. Neurons must discriminate between possible target neurons in order to establish functional synaptic connections. Most of the well characterized neuronal surface molecules, such as N-CAM (Edelman, 1984) and L1 (Rathjen and Schachner, 1984), are expressed too globally to account for the precision of recognition needed during synapse formation. The lack of identification of molecules expressed by small subsets of developing neurons has hindered the understanding of the molecular mechanisms involved in this feature of neural cell recognition. To date, only a few such molecules have been identified that are expressed by discrete subpopulations of functionally related neurons, e.g., retinal tectal

marker in the chick (Trisler et al., 1987) and limbic system marker in the rat (Horton and Levitt, 1988).

These cell adhesion molecules are all glycoproteins composed of a broad range of oligosaccharide structures. There is increasing evidence suggesting that these oligosaccharide structures are involved in cell recognition and adhesion processes during development of the nervous system. For example, many of the surface glycoproteins mediate cell-cell interactions through highly charged sialic acid or sulfated epitopes (Rademacher et al., 1988). The percent of polysialic acid (PSA) present on N-CAM affects its interaction with other cells or substrates (Rutishauser et al., 1988). There are two main forms of N-CAM; embryonic and adult which differ in their PSA content (Edelman, 1983; Rutishauser, 1983). Embryonic N-CAM contains a higher percentage of PSA in comparison to the adult N-CAM. Although the PSA molecules are not directly involved in the binding of N-CAM, it does regulate binding by its high negative charge (Rutishauser et al., 1988). The PSA creates an oligosaccharide coating around the cell. Low PSA content would enhance the extent or duration of membrane contact between cells resulting in the ability of other adhesion molecules to interact. High PSA content, because of the large volume occupied by the carbohydrate, would attenuate membrane contact resulting in the inability of other adhesion molecules to interact.

Many of the different cell adhesion molecules share carbohydrate domains as identified by anti-carbohydrate antibodies. On the basis of these shared epitopes, cell adhesion molecules are classified into families. For example, monoclonal L2 antibody binds to a sulfated

glucuronic acid in glycans N-glycosidically linked to N-CAM, L1 (Keilhauser et al., 1985), J1 (Kruse et al., 1985), MAG (Kruse et al., 1984; Chou et al., 1986; Noronha et al., 1986; Poltorak et al., 1987), and integrins (Pesheva et al., 1987). Molecules containing the L2 carbohydrate epitope comprise the L2/HNK-1 family of cell adhesion molecules. HNK-1, a mouse monoclonal antibody raised to a lymphoblastoma and used as a marker for a subset of lymphocytes with natural killer function, has been demonstrated to identity a carbohydrate structure identical to L2 (Kruse et al., 1984). The importance of a cell adhesion family lies in the finding that molecules expressing the epitope are involved in cell surface interactions and that the carbohydrate epitope itself may be functionally involved in these interactions. Since only some of the polypeptides of a particular adhesion molecule express the epitope, the expression of the carbohydrate structure seems to occur independently of the protein backbone (Kucherer et al., 1987).

A second cell adhesion family is characterized by the L3 and L4 mannosidic carbohydrate epitopes, both of which have been implicated in cell-cell and cell-substratum interactions (Kucherer et al., 1987; Fahrig et al., 1989; Streit et al., 1989). The L3 and L4 carrying glycoproteins comprise a new family of adhesion molecules that include some members of the L2/HNK-1 family (L1 and MAG), as well as distinct molecules such as AMOG. Like the L2/HNK-1 epitope, only some of the polypeptides of a particular adhesion molecule express the L3 and L4 epitopes (Kruse et al., 1984; Kucherer et al., 1987).

Our laboratory has been characterizing a group of 130 kD surface glycoproteins which are expressed on restricted sets of neurons and glia in the leech (*Haemopis marmorata, Hirudo medicinalis*) through the use of monoclonal antibodies (summarized in Table 1) (Zipser and McKay, 1981; Flaster et al., 1983; Flanagan et al., 1986). In the leech, many of the early developmental interactions appear to be regulated by these surface glycoproteins (Morell et al., 1989; Zipser et al., 1989). In order to fully comprehend the expression of these surface glycoproteins, a brief description of the leech nervous system is required. The leech is an excellent animal model to use because of the wealth of anatomical information already available for the neurobiologist (for review see Muller et al., 1981; Fernandez, 1987).

The leech nervous system consists of 32 segmental ganglia (Figure 1). The first four ganglia are fused to form the head ganglia, the last seven ganglia are fused to form the tail ganglia, and there are 21 midbody ganglia. Each midbody ganglion consists of approximately 400 cells, with the exception of ganglia 5 and 6 each consisting of approximately 700 cells. Ganglia 5 and 6 innervate the sexual organs in addition to the normal segmental structures. The ganglia are joined by the connective nerves which consist of two large lateral bundles of nerve fibers and a small medial bundle of nerve fibers known as Faivre's nerve. Each segment is innervated via 4 bilaterally paired nerves arising from the central ganglion (Figure 2).

Perhaps the main appeal of the leech is the beauty of the ganglion as it appears under the microscope, with its approximately 400 neurons (Figure 3). These central neurons are very recognizable from segment to segment, specimen to specimen, and species to

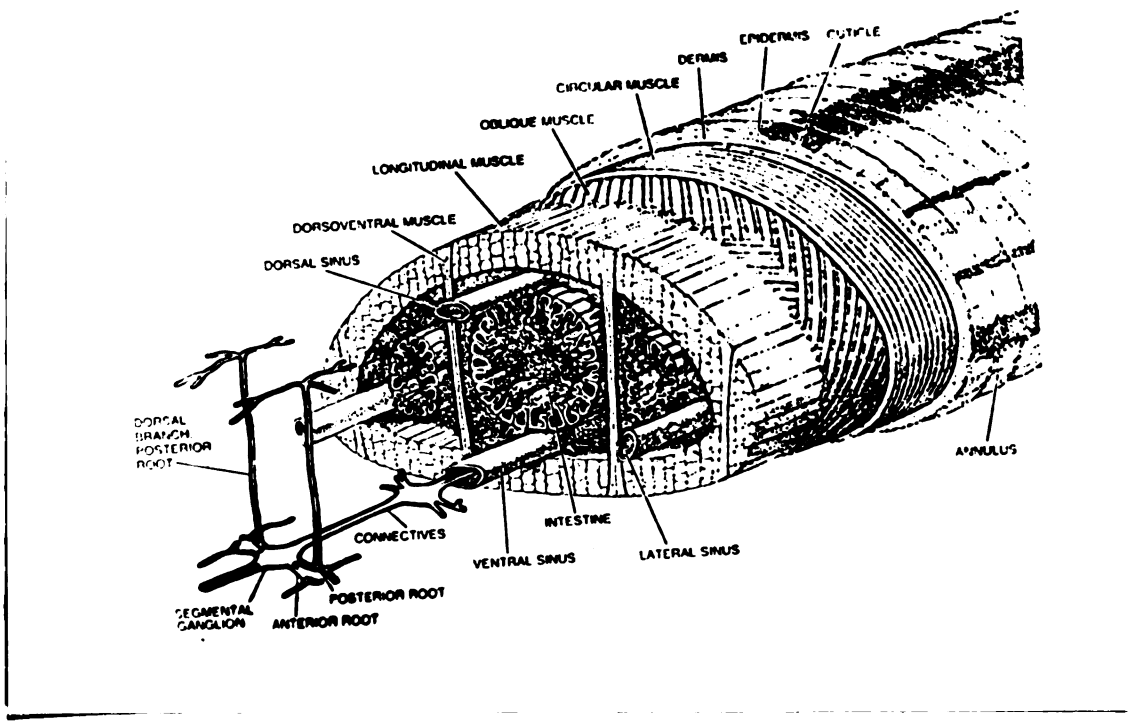
Table 1. Leech Glycoproteins Identified by Monoclonal Antibodies (mAbs)

mAb	Antigen	Molecular Weight (kD)
Lan 3-2	sensory protein	130/103/95
Laz 2-369	modality protein (mechanodectors)	130
Laz 6-212	modality protein (chemodectors)	130
Laz 7-79	modality protein (unknown modality)	130
Laz 6-297	glial protein	130
Laz 9-84	connective tissue protein	130

Figure 1. Diagrammatic representation of whole leech nerve cord. (from J.G. Nicols and D. Van Essen: *The Nervous System of the Leech.* Copyright (1974) by Scientific American, Inc.).


Oral sucker
Brain
Segment
Annulus
Sensillae
Roots
Caudal ganglia
Caudal sucker
Segmental ganglia

Figure 2. Diagrammatic representation of position of the nerve cord within the leech. (from J.G. Nicols and D. Van Essen: *The Nervous System of the Leech.* Copyright (1974) by Scientific American, Inc.).


EPIDERMIS
CUTICLE
DERMIS
CIRCULAR MUSCLE
OBLIQUE MUSCLE
LONGITUDINAL MUSCLE
DORSOVENTRAL MUSCLE
DORSAL SINUS
DORSAL BRANCH POSTERIOR ROOT
ANNULUS
INTESTINE
CONNECTIVES
VENTRAL SINUS
LATERAL SINUS
SEGMENTAL GANGLION
ANTERIOR ROOT
POSTERIOR ROOT

species. The axonal processes and neuritic trees of the central neurons are contained within or pass through the central neuropil. Position, morphology, and electrophysiological properties of three mechanosensory neurons, responsive to touch (T cells), pressure (P cells), and nociception (N cells), are shown in Figure 3. Because of the remarkable stereotypic appearance of these neurons, one can tentatively identify them by simple visual inspection based on their size, shape, and location. The identification can be confirmed by intracellular recordings since each neuron demonstrates characteristic electrophysiological properties (panel at right in Figure 3).

The cell bodies of the sensory neurons are contained both within the CNS and in the periphery. In the periphery, the neurons are located in the skin, gut, and other organs (Figure 4). In the skin, these neurons can occur singly, in small clusters, or in sensory organs known as the "sensillae". Each midbody sensillum contains mechanoreceptors and photoreceptors in addition to cells whose physiological function is not yet known (Mann, 1962). These peripheral sensory neurons project their axons into the CNS 3 tracts. We refer to these axons as the "sensory afferents".

Four of the 130 kD surface glycoproteins identified by our laboratory are expressed by the sensory afferents (Flaster et al., 1983; Hogg et al., 1983; Peinado et al., 1987). The antigen expressed by all if not most of the sensory afferents is recognized by mAb Lan 3-2. We refer to the antigen as the "sensory protein". Three other antigens are expressed by subsets of these same sensory afferents. We refer to the 130 kD glycoproteins of the sensory

Figure 3. Segmental ganglion and action potentials of identified cells in the leech. Mechanosensory neurons labeled T, P, and N which are responsive to touch, pressure, and noxious mechanical stimulation. (Right) Intracellular recordings of T, P, and N cell action potentials induced by depolarizing current through a microelectrode (from K.J. Muller, J.G. Nicholls, and G.S. Stent (eds.): *Neurobioloby of the Leech.* Copyright (1981) by Cold Spring Harbor Laboratory).

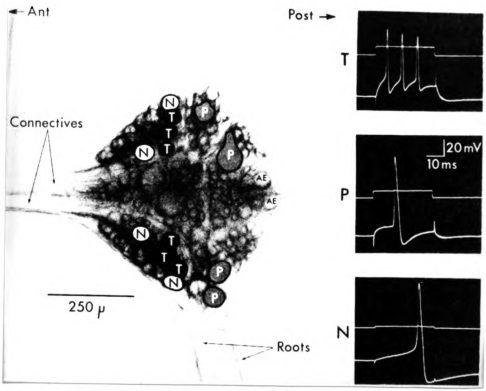
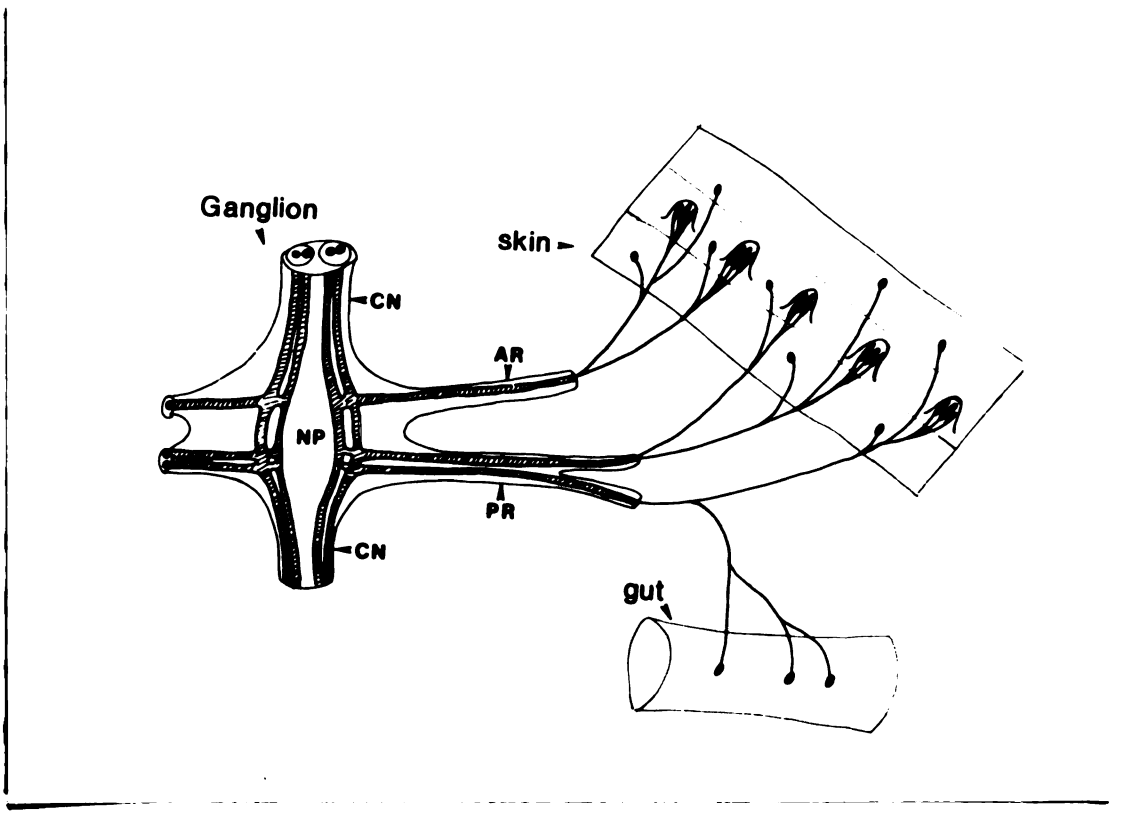

Ant
Post
Connectives
T
P
N
AE
AE
20 mV
10 ms
250 μ
Roots

Figure 4. Diagrammatic representation of midbody segment in the leech. AR, anterior root; PR, posterior root; CN, connective nerve; NP, neuropil (from A. Peinado, E.R. Macagno, and B. Zipser: *A Group of Related Surface Glycoproteins Distinguish Sets and Subsets of Sensory Afferents in the Leech Nervous System.* Copyright (1987) by Brain Research).


Ganglion
skin
CN
AR
NP
PR
CN
gut

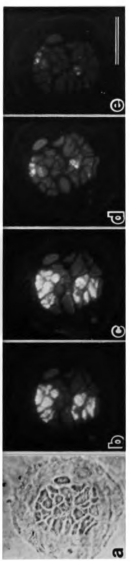
subsets as the "modality proteins". The antigen recognized by mAb Laz 2-369 is expressed by the largest subset of sensory afferents. MAbs Laz 6-212 and Laz 7-79 recognize the antigens expressed by smaller subsets of the sensory afferents. The distribution of these antigens is demonstrated in serial cross sections through a posterior root of a midbody segmental ganglia (Figure 5).

In addition to these sensory afferent antigens, our fifth 130 kD glycoprotein is expressed by the glial cells surrounding the fiber tracts (Flaster and Zipser, 1983). Only the glial cells that are associated with axon tracts; those in the ganglionic neuropil, root, and connective nerves express the glial cell antigen, while the glial cells enveloping the cell bodies do not.

Recent antibody perturbation studies in leech embryos demonstrated the importance of the 130 kD antigens during synapse formation in the synaptic neuropil (Zipser et al., 1989). Incubating cultured embryos with Lan 3-2 Fab fragments inhibits sensory afferent interactions in the synaptic neuropil while axonal elongation within the peripheral nerves and the central axon tracts was not affected. Incubation of leech embryos with the glial mAb provided evidence for glial antigen involvement during axonal growth (Moore et al., 1988; Morell et al., 1989). The glial antigen appears to have a dual role; guidance of axons and growth promotion.

This dissertation is composed of two chapters, the first one was aimed at characterizing the 130 kD sensory, modality, and glial proteins with respect to their protein core homologies and the nature of their epitopes. The structural characterization discussed in the first chapter concerns only the relationships among the 130 kD leech

Figure 5. Transverse sections through posterior root nerve of midbody ganglion as seen with transmitted light (a) and after immunofluorescent with mAbs Lan 3-2 (b), Laz 2-369 (c), Laz 6-212 (d), Laz 7-79 (e). Scale bar equals 50 um (from A. Peinado, E.R. Macagno, and B.Zipser: *A Group of Related Surface Glycoproteins Distinguish Sets and Subsets of Sensory Afferents in the Leech Nervous System.* Copyright (1987) by Brain Research).


a
b
c
d
e

glycoproteins. In the second chapter, we were interested in determining if the two mammalian carbohydrate epitopes, L2/HNK-1 and L3/L4/L5, are conserved in the leech nervous system.

A clearer understanding of the role of adhesion molecules during development and maintenance of the nervous system may come from information derived from invertebrate systems. Many of the cell adhesion molecules described in invertebrates are structurally and possibly functionally equivalent to those previously characterized in vertebrate systems. For example, the *Drosophila* "position specific antigens", which are involved in development of the imaginal disc, are homologous to integrins (Leptin et al., 1987). The fusion of information obtained from our leech antigens and the vertebrate carbohydrate epitopes may provide a better understanding of the role of adhesion molecules during development.

CHAPTER 1. A Family of 130 kD Leech Surface Antigens: Cell Type Specificity of Neuronal Antigens Encoded via Carbohydrate Domains

INTRODUCTION

The precision of neuronal synapse formation has been postulated to require the interaction of cell type specific surface molecules (Sperry, 1963). Most of the neuronal surface antigens, such as N-CAM (Edelman, 1984) and L1 (Rathjen and Schachner, 1984) are expressed too globally to account for the precision of recognition needed during synapse formation. Only a few molecules have been identified that are expressed by discrete subpopulations on related groups of neurons, e.g. retinal tectal marker in the chick (Trisler et al., 1981; Trisler and Collins, 1987) and limbic system marker in the rat (Horton and Levitt, 1988). Cell type specific antigens also have been identified for different types of glial cells, type-1 astrocyte (Ran-2) and oligodendrocyte (GC) markers in the rat (reviewed in Raff and Miller, 1984) and mesencephalic and cerebellar astrocyte marker in the mouse (Barbin et al., 1988).

We are presently characterizing five different 130 kD surface glycoproteins which are expressed by specific neuronal and glial cells in the leech nervous system (Zipser and McKay, 1981; Flaster et al., 1983; Flaster and Zipser, 1983; Hogg et al., 1983). The neuronal 130 kD glycoproteins are expressed by all or subsets of sensory afferents. Two of the three sensory afferent subsets which express the modality proteins are correlated with different sensory modalities.

The sensory afferents from leech lips which function as chemodetectors (Elliot, 1987) express the 130 kD protein recognized by mAb Laz 6-212 (manuscript in preparation). The uniciliated neurons in the leech crop which are putative gut stretch receptors (Dickinson and Lent, 1987) express the 130 kD protein recognized by mAb Laz 2-369 (Hogg et al., 1983). The modality of the sensory afferents recognized by mAb Laz 7-79 has not yet been determined, but it is not expressed by chemo- or mechanodetectors. Thus, each sensory afferent may be identified by at least two different 130 kD surface glycoproteins, the sensory protein and one of the three modality proteins.

The sensory protein may mediate synaptic recognition within the central nervous system. Incubation of cultured leech embryos with low concentrations of Lan 3-2 Fab fragments inhibits sensory afferent interactions in the synaptic neuropil (Zipser et al., 1989). Axonal elongation within the peripheral nerves and the central axon tracts is not affected. Since the sensory afferents which express the modality proteins have distinct projection patterns within the synaptic neuropil, the modality proteins may also mediate synaptic recognition (Peinado et al., 1987).

As previously stated, the fifth 130 kD protein is glial specific (Flaster and Zipser, 1983). Glial processes expressing the 130 kD protein are juxtaposed to key sites of morphogenic movement and neuronal differentiation. Early in development, the 130 kD glial protein is detected on primordial glial cells prior to sensory afferent differentiation (Cole et al., 1989a). In later development and adulthood, the 130 kD glial protein is detected on macroglial cells. In

the adult nervous system, the expression of the glial protein is regionally regulated. It is detected on the glial cell processes that project along axonal tracts and is absent from the glial cell bodies.

Previously, we have demonstrated that the sensory, modality, and glial proteins contain core fucosylated complex biantennary and high mannose glycans (Flanagan et al., 1986; Cole et al., 1989b). In the present study, we have characterized the 130 kD sensory, modality, and glial proteins with respect to their protein core homologies, and the nature of their epitopes. In addition, we identified a sixth 130 kD protein expressed by the connective tissue surrounding the nervous system.

MATERIALS AND METHODS

Leeches. Leeches (*Haemopis marmorata* and *Hirudo medicinalis*) were purchased from commercial distributors and maintained at 9°C for several months. Nerve cords consisting of supra- and sub-esophageal ganglia and ganglia 1-21 were removed from alcohol anesthetized leeches and stored at -70°C.

Monoclonal antibodies. Monoclonal antibody (mAb) Laz 9-84 was prepared by immunization of BALB/C mice (5 weeks old) with the 130 kD region of a 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAG) derived from leech CNS extracts as previously described (Flaster et al., 1983). Briefly, preparative gels were stained with Coomassie blue, revealing two major bands at 125 and 140 kD. The region between these bands was excised, washed in PBS, homogenized in PBS/adjuvant (0.5 cm wide gel slice/100 ul PBS/100 ul Freund's complete adjuvant), and used as a intra-peritoneal immunogen. Two booster immunization were given at 7 day intervals with proteins obtained by electroelution of SDS-PAG. Proteins were prepared in PBS for tail injections. Spleen cell fusions were performed using methods previously decribed (Hogg et al., 1983). Hybridomas were screened histologically on cross sections of fixed leech ganglia and on immunoblots prepared from leech CNS extracts. MAbs were used as either ascites fluids (5 mg/ml) of Pristane-primed mice injected with cells of the hybridoma clones or as culture supernatants (20 ug/ml). MAbs Lan 3-2, Laz 2-369, Laz

6-212, Laz 7-79, and Laz 6-297 were produced previously (Zipser and McKay, 1981; Flaster et al., 1983; Hogg et al., 1983).

Biochemical methods

Electrophoresis and immunoblotting. Leech nerve cords (CNS) were boiled in sample buffer (Laemmli, 1970) for 3 minutes, centrifuged, and supernatants were separated by electrophoresis on a 7.5% SDS polyacrylamide gel. Immunoblots were prepared as described previously (Garrels, 1979; Towbin, et al., 1979) with modifications. Proteins were transferred onto nitrocellulose membrane (Millipore, 0.45 pore size) at 90 V for 2 hr. The blots were fixed with isopropanol/acetic acid for 30 min, washed three times over 20 min with PBS, and then blocked for a minimum of 2 hr with 10% powdered milk and 0.05% Tween 20 in PBS (blocking solution). Incubation with mAbs (culture supernatants) was performed overnight at 4°C. Excess antibody was rinsed off with PBS and the blots were blocked three times over 45 min at 4°C with the blocking solution. Blots were then incubated for 6 hr at 4°C with ^{125}I-goat antimouse IgG (150,000 cpm/ml blocking solution; ICN Radiochemicals, Costa Mesa, CA), washed for 30 min with the blocking solution, and rinsed for 15 min in PBS. Autoradiography was performed by placing Kodak XAR X-ray film over the dried blot at -70°C for 1-5 days with enhancers or at 22°C for 7-10 days without enhancers. The apparent molecular weights of the leech proteins were estimated using prestained high molecular weight standards (BRL, Gaithersburg, MD) applied to the gel.

Immunoprecipitation. Beads were prepared by incubating 20 ul of swine antimouse IgG (Nordic Immunology, Capistrano Beach, CA) with 100 ul pre-swollen protein A-Sepharose beads (Pharmacia, Piscataway, New Jersey) suspended in 500 ul PBS (pH 8.0) for 6 hr with shaking. This and all subsequent steps were carried out at 4°C. Beads were then carefully washed with PBS (pH 8.0) followed by centrifugation (2 min at 500 rpm). Ten nerve cords were boiled in 400 ul sample buffer (Laemmli, 1970) for 3 min, centrifuged, and the supernatant was used for immunoprecipitation. The supernatant was diluted with PBS pH 8.0 to 3 ml and incubated for 2 hr with swine antimouse IgG conjugated protein A-Sepharose beads to decrease nonspecific binding of proteins to beads. After centrifugation, the supernatant was incubated for 6 hr with 2 ul ascites fluid (mAb: 5 mg/ml), followed by incubation with fresh swine antimouse IgG conjugated protein A-Sepharose beads (100 ul) overnight with shaking. Beads were carefully washed with PBS, centrifuged, and then centrifuged through a sucrose cushion (1M). Beads were resuspended in sample buffer, boiled for 3 min, centrifuged, and supernatants were separated by electrophoresis on a 7.5% SDS polyacrylamide gel.

Peptide Mapping. Leech proteins were prepared as described above and subjected to 7.5% SDS-PAGE. The 130 kD protein band (120-150 kD) was excised from the gel and subjected to limited proteolysis according to the method described by Cleveland et al. (1977). Specifically, the gel slices which contain the 120-150 kD proteins were placed into the sample wells of a second SDS-PAG. The slices were overlayed with dilutions of *Staphylococcus aureus* V8

protease (ICN Immunobiologicals, Lisle, IL). The gel was electrophoresed at constant voltage (90 V) at room temperature until the dye front neared the bottom of the 3% polyacrylamide stacking gel. The power was terminated for 30 min. The power was then restored and the partially digested proteins were separated on a 10% or 15% polyacrylamide resolving gel at constant voltage (320 V) at 5°C. Immunoblotting was conducted as described earlier.

Deglycosylation of immunoblots. CNS extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were fixed with isopropanol/acetic acid for 30 min, washed with PBS, blocked for 2 hr with 3% BSA in PBS, and washed extensively with PBS. Immunoblot strips were preincubated for 30 min at room temperature in 0.17% SDS, 0.2 M sodium phosphate buffer, pH 8.6, 10 mM 1,10-phenanthroline, and 1.25% NP-40. N-Glycanase (60 U/ml; Genzyme Corp., Boston, MA) was then added and the reaction mixture was incubated overnight at room temperature. Control strips were incubated in the same buffer solution without N-Glycanase. Immunoblot strips were washed with PBS and incubated for 2 hr with mAb. Bound mAb was then detected as described above.

Immunocytochemistry. Midbody ganglia were washed in PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1/2 hr, dehydrated, and embedded in polyethylene glycol (PEG; 20% PEG 1450/80% PEG 3350) (Peinado et al., 1987). Nerve cords were sectioned (2-5 um) with glass knives and mounted on 0.1% poly-lysine (Sigma, St. Louis, MO; 150,000-300,000 molecular weight) coated slides. PEG sections were washed three times over 30 min

with 70% and then 95% ethanol. Sections were incubated overnight with mAbs (mAb: 20 ug/ml), briefly washed with PBS, and incubated for 2 hr with HRP conjugated second antibody (goat anti-mouse IgG; Cappel, West Chester, PA, 1:50 PBS/10% fetal calf serum). The sections were reacted with 0.02% 3,3'-diaminobenzidine (DAB) in 0.05 M Tris buffer, pH 7.4, containing 0.015% hydrogen peroxide, rinsed with PBS, dehydrated, cleared in xylene, and embedded in Permount.

Some nerve cords were prestained prior to embedding. Briefly, nerve cords were incubated for 1 hour in protease (5 mg/ml of distilled water) from *Streptomyces griseus* Type XIV (Sigma, St. Louis, MO). Nerve cords were briefly rinsed with PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 hr and incubated overnight with mAbs (mAb: 20 ug/ml). Nerve cords were briefly washed with PBS, incubated for 2 hr with biotinylated Fab fragments (rabbit antimouse IgG; Dako, Santa Barbara, CA 1:100 PBS/10% fetal calf serum), briefly rinsed with PBS, and incubated for 2 hr with horseradish peroxidase avidin D (Vector, Burlingame, CA 1:150 PBS/10% fetal calf serum). Nerve cords were reacted with 0.02% 3,3'-diaminobenzidine (DAB) in 0.05 M Tris buffer, pH 7.4, containing 0.015% hydrogen peroxide, rinsed with PBS, dehydrated, and embedded in JB 4 Immunobed plastic (Polysciences, Inc., Warrington, PA). Embedding with JB 4 was performed according to the supplier's instructions. Nerve cords were sectioned (2-5 um) with glass knives, mounted on 0.1% polylysine (Sigma, St Louis, MO; 150,000-300,000 molecular weight) coated slides, dehydrated, cleared in xylene, and embedded in Permount.

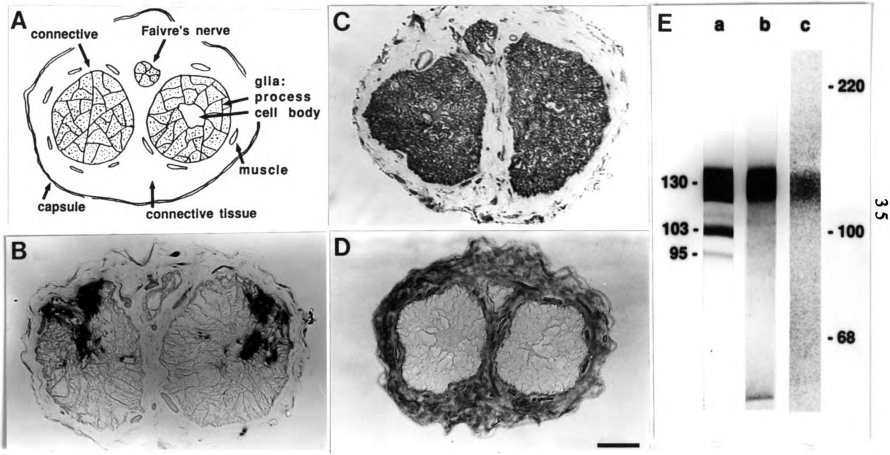
RESULTS

Cell type-specificity of 130 kD proteins

Different cell types in the leech nervous system express unique 130 kD surface proteins, which are identified by monoclonal antibodies (mAbs). The micrographs in Figure 6 are cross sections of a central fiber tract, known as the "connective". Figure 6A is a drawing of the anatomy. The connective was stained with mAbs specific for axons, glial processes, or connective tissue. Figure 6B shows the sensory afferent axons recognized by mAb Lan 3-2 (Zipser and McKay, 1981). The sensory afferent axons comprise approximately 1/3 of all axons in the connective and are located in the dorsal quadrant of each of the lateral tracts. Glial processes permeating all three axon tracts of the connective, the two lateral tracts and the medial tract, are recognized by mAb Laz 6-297 (Figure 6C) (Flaster and Zipser, 1983). Here we report the isolation of an additional 130 kD antigen expressed by the connective tissue, which surrounds the three axon tracts and is recognized by mAb Laz 9-84 (Figure 6D).

The mAbs specific for the sensory axons, glial, and connective tissue react with broad bands centered at 130 kD on immunoblots prepared from leech CNS proteins (Figure 6E). MAb Lan 3-2, which recognizes the sensory axons, also reacts with two lower bands at 103 and 95 kD (lane a).

Figure 6. Cross sections of interganglionic connectives and immunoblots of leech CNS extracts. A: Diagram illustrating the three axonal tracts of the connective. 2 um thick cross sections through the interganglionic connective stained with mAbs B: Lan 3-2, C: Laz 6-297, D: Laz 9-84. Scale bar equals 50 um. E: Immunoblots of leech CNS extracts separated on a 7.5% SDS-PAGE, incubated with mAbs Lan 3-2 (a), Laz 6-297 (b), and Laz 9-84 (c) and visualized by autoradiography. Molecular weights are shown on the right in kilodaltons.

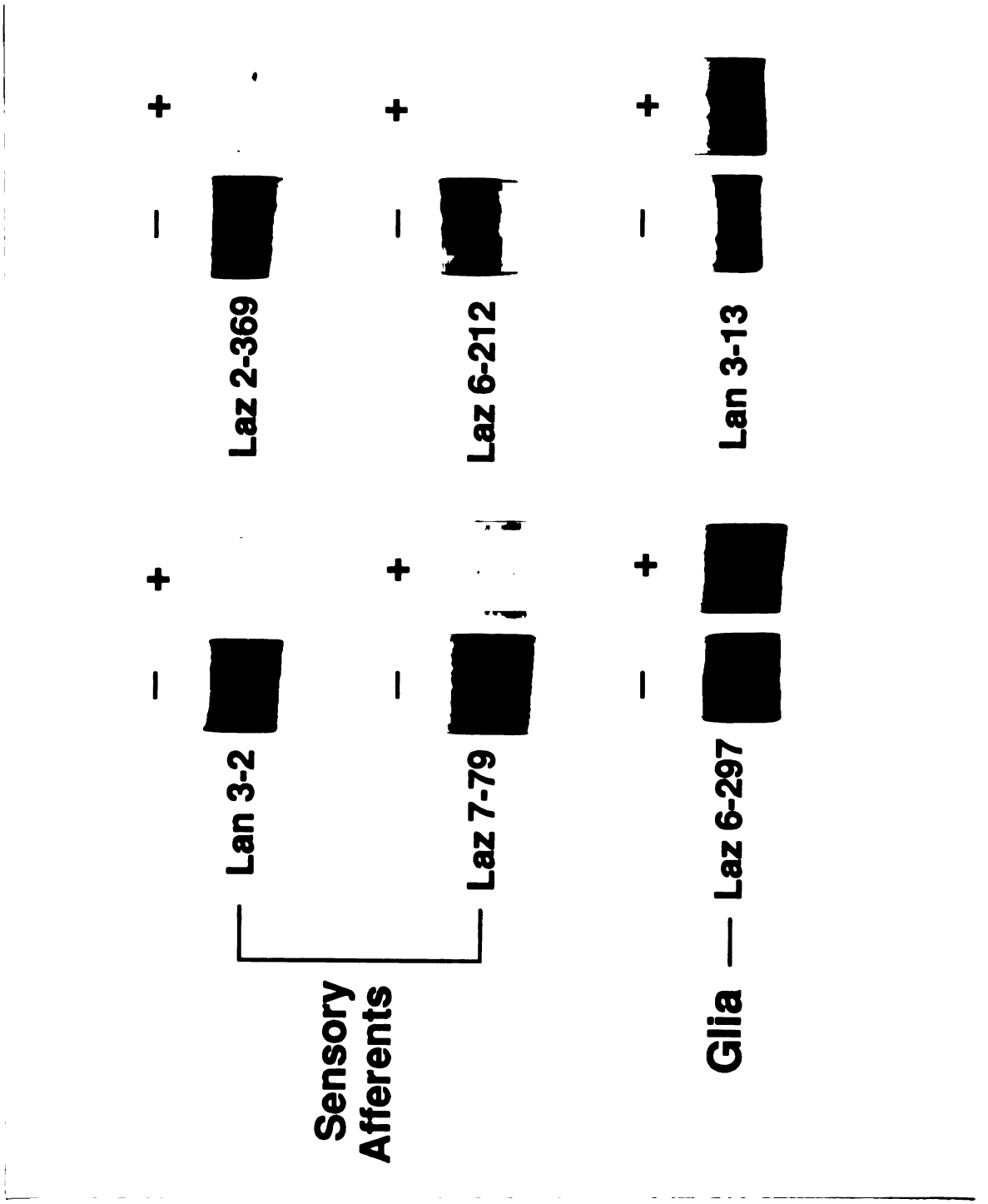

A
connective
Falvre's nerve
glia:
process
cell body
muscle
capsule
connective tissue
B
C
D
E
a
b
c
130 -
103 -
95 -
- 220
- 100
- 68

Neuronal proteins are differentiated by their carbohydrate epitopes

Of interest to us are the epitopes to which the mAbs bind. The fact that the 130 kD proteins are glycosylated raises the possibility that the epitopes recognized by the mAbs are themselves carbohydrate structures. The ability of the mAbs to bind to epitopes on N-linked carbohydrate domains was determined following deglycosylation of the proteins with N-Glycanase. Briefly, the 130 kD protein immobilized on immunoblots was incubated in the presence or absence of N-Glycanase. The immunoblots were then probed with the mAbs and antibody binding was determined by autoradiography (Figure 7). Treatment with N-Glycanase attenuated antibody binding to all of the 130 kD sensory and modality proteins. In contrast, binding of the mAb specific for the 130 kD glial protein was unaffected.

To test for the presence of contaminating proteases, immunoblots were probed with mAb Lan 3-13 which recognizes an epitope sensitive to proteolysis (Flaster and Zipser, 1987). MAb Lan 3-13 binds to an amino acid epitope on a 77 kD protein present on glial cells. N-Glycanase treatment did not inhibit mAb Lan 3-13 binding. This suggests that the attenuation of the antibody binding to the sensory and modality proteins was the result of deglycosylation and not proteolysis. These results demonstrate that all four sensory and modality epitopes are on N-linked carbohydrate domains, while the glial epitope may be either on an O-linked carbohydrate or amino acid domain.

Figure 7. Deglycosylation of proteins immobilized on immunoblots. Immunoblots were prepared from leech CNS extracts and separated on 7.5% SDS-PAGE. Immunoblot strips, 120-150 kD, were incubated overnight in the absence (-) or presence (+) of N-Glycanase. The strips were then incubated with the different mAbs, followed by ^{125}I goat anti-mouse IgG, counted, and visualized by autoradiography. The binding of mAb Lan 3-2 to the 130 kD sensory protein, and the binding of mAbs Laz 2-369, Laz 7-79, Laz 6-212 to 130 kD modality proteins were attenuated 64, 47, 33, and 15%, respectively. The binding of mAb Laz 6-297 to the 130 kD glial protein and the binding of mAb Lan 3-13 to an amino acid epitope on a 77 kD glial was not attenuated.

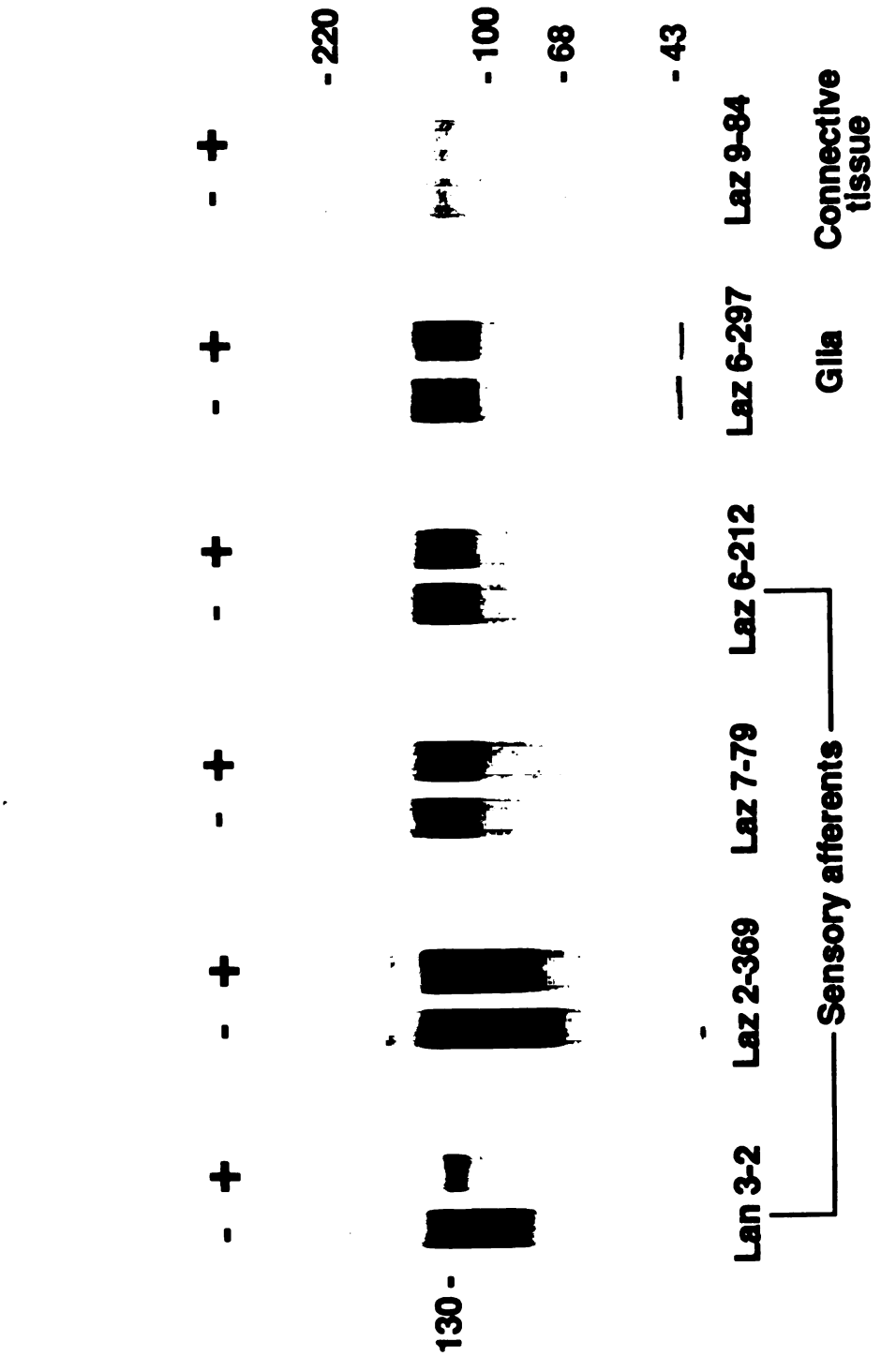

Lan 3-2
Laz 7-79
Sensory Afferents
Laz 2-369
Laz 6-212
Glia — Laz 6-297
Lan 3-13
+
−

To further investigate the nature of the different carbohydrate epitopes, mAbs were incubated in the presence of various sugars and ovalbumin. As previously reported (McKay et al., 1983), preincubating mAb Lan 3-2 with alpha methyl-mannoside inhibited antibody binding to nitrocellulose bound antigen (Figure 8, Lanes 1 and 2). In addition, mAb Lan 3-2 binding was not blocked by ovalbumin or N-acetylgalactosamine (data not shown). The binding of the mAbs specific for the modality, glial, and connective tissue proteins were not inhibited by alpha methyl-mannoside (Figure 8, Lanes 3-12), ovalbumin, or N-acetylgalactosamine. These results suggest that the carbohydrate epitope of the sensory protein is different from those of the modality proteins.

Cross reactivity among neuronal proteins

Before we could immunopurify the proteins for further characterization and possible use in pertubation studies, we needed to determine the amount of cross reactivity exhibited by the various mAbs. Antibody cross reactivities were measured with immunoprecipitated sensory proteins. The immunoprecipitated proteins were subjected to SDS-PAGE and the resulting immunoblots were probed with the mAbs followed by ^{125}I labeled goat anti-mouse IgG. Binding of mAb Lan 3-2 to the 130 kD sensory protein was measured and set to 100%. The cross reactivity of the other mAbs to the Lan 3-2 immunoprecipitated protein was normalized against this value.

Figure 8. Sugar blocking of antibody binding to immunoblots. Immunoblots were prepared from leech CNS extracts and separated on a 7.5% SDS-PAGE. MAbs were preincubated for 30 minutes with 0.5 M alpha methyl-mannoside. Nitrocellulose strips were then incubated with mAb (-) or mAb + 0.5 M alpha methyl-mannoside (+). Nitrocellulose strips were visualized by autoradiography. Molecular weights are shown on the right in kilodaltons.


- +
- +
- +
- +
- +
- +
- 220
- 100
- 68
- 43
130 -
Lan 3-2
Laz 2-369
Laz 7-79
Laz 6-212
Laz 6-297
Laz 9-84
Glia
Connective tissue
Sensory afferents

MAb Laz 2-369 which recognizes the modality protein expressed by the mechanodetectors, cross reacts 114% with the sensory protein identified by mAb Lan 3-2 (Figure 9). MAb Laz 7-79, which binds to sensory afferents of unknown modality, cross reacts 40%. MAb Laz 6-212, which recognizes the modality protein expressed by the chemodetectors, cross reacts 17%. In contrast, the mAbs which recognize the glial and connective proteins do not cross react with the sensory protein.

Experiments were then undertaken to determine whether the mAbs which recognize neuronal proteins detect epitopes on the glial protein (Figure 10). SDS solubilized glial protein was immuno-precipitated and the percent of cross reactivity of the mAb, specific for the neuronal proteins, was determined as above. Three of the four mAbs specific for the sensory and modality proteins did not appreciably cross react with the glial protein identified by mAb Laz 6-297. The exception was mAb 2-369. These results demonstrate that the mAbs specific for two of the modality proteins only cross react with the sensory protein and not with the glial protein. MAb Laz 2-369 cross reacts with the immunoprecipitated sensory and glial proteins.

Comparison of proteolytic fragments of neuronal and glial 130 kD proteins

To investigate protein core homology among the 130 kD sensory, modality, and glial antigens, peptide mapping studies were

Figure 9. Percent of antibody cross reactivity. Percentage of different mAbs that cross react with the sensory protein. Each column represents the mean and the vertical line indicates 1 standard error of 4 determinations. Immunoblots were prepared from immunoprecipitated SDS solubilized sensory protein and leech CNS extracts. Immunoblots were incubated with the mAbs and visualized by autoradiography. The 130 kD bands were excised and counted. Strips of equal length were excised from the top of each lane, counted, and used to estimate non-specific binding. Control strips not incubated with mAb were also used to determine non-specific binding to the 130 kD antigen. The amount of ^{125}I bound to strips was interpreted as a measure of antigen concentration. MAb Lan 3-2 reactive proteins were set to 100% and the cross reactivity of all other mAbs was normalized against this value.

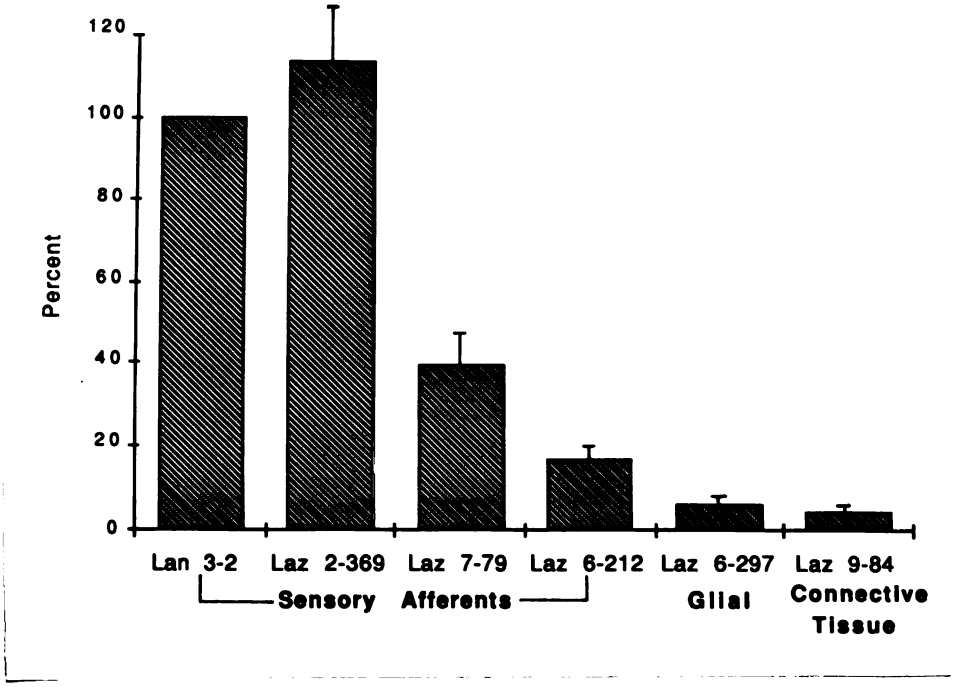
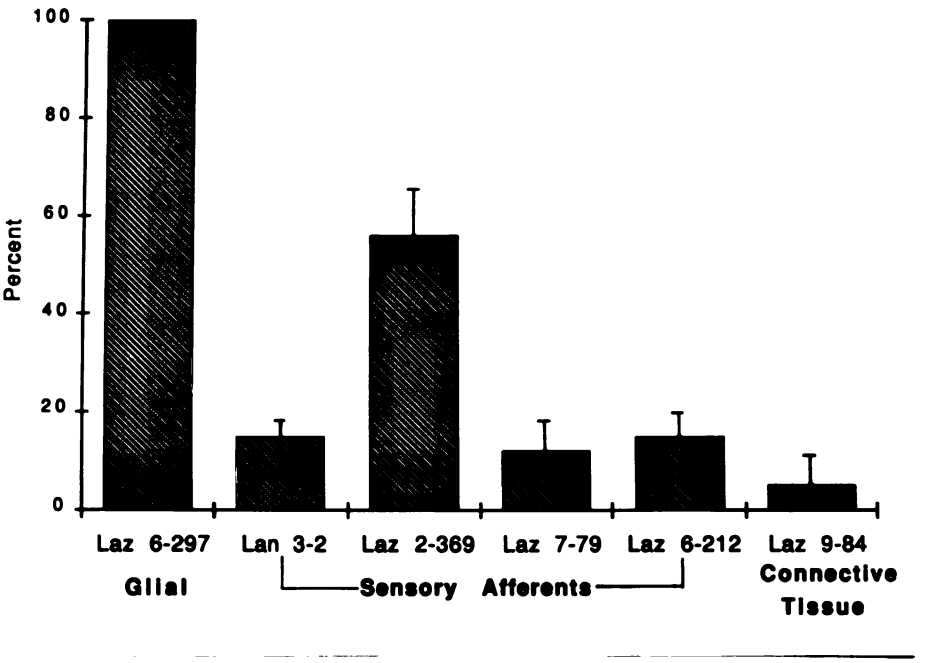

120
100
80
60
40
20
0
Percent
Lan 3-2
Laz 2-369
Laz 7-79
Laz 6-212
Laz 6-297
Laz 9-84
Sensory Afferents
Glial
Connective Tissue

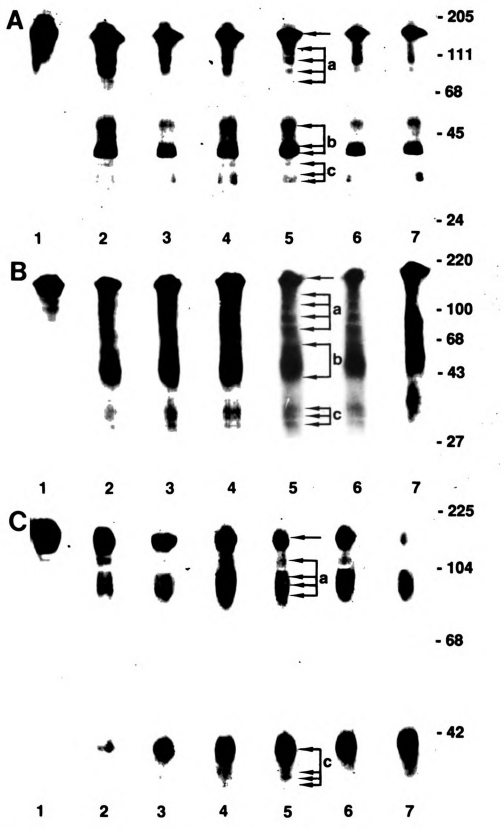
Figure 10. Percent of antibody cross reactivity. Percentage of different mAbs that cross react with the glial protein. Each column represents the mean and the vertical line indicates 1 standard error of 3 determinations. Immunoblots were prepared from immunoprecipitated SDS solubilized sensory or glial proteins and leech CNS extracts. Immunoblots were incubated with the mAbs and visualized by autoradiography. The 130 kD bands were excised and counted. Strips of equal length were excised from the top of each lane, counted, and used to estimate non-specific binding. Control strips not incubated with mAb were also used to determine non-specific binding to the 130 kD antigen. The amount of ^{125}I bound to strips was interpreted as a measure of antigen concentration. MAb Laz 6-297 reactive proteins were set to 100% and the cross reactivity of all other mAbs was normalized against this value.


100
80
60
40
20
0
Percent
Laz 6-297
Glial
Lan 3-2
Laz 2-369
Laz 7-79
Laz 6-212
Sensory Afferents
Laz 9-84
Connective Tissue

undertaken. The 130 kD region (120-140) was excised from a 7.5% acrylamide gel prepared from leech CNS extracts, subjected to digestion with V8 protease, and the fragments were separated on a 15% acrylamide gel. The resulting immunoblots were probed with the different mAbs. The 120-140 kD proteins appear at a higher molecular weight (135-165 kD) on 15% acrylamide gels (Hogg, N. and Zipser, B., personal communications). Glycoproteins are known to behave anomalously during SDS-PAGE when compared to standard proteins (Segrest and Jackson, 1972).

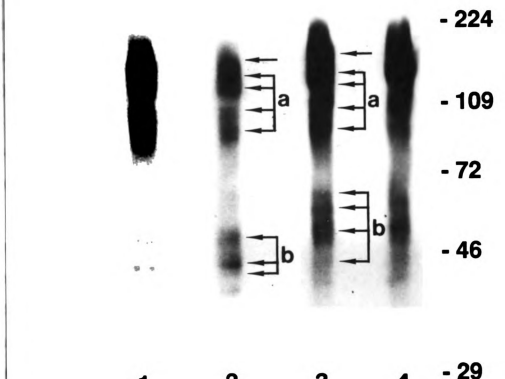
The peptide fragments of both the sensory and modality proteins, recognized by mAbs Lan 3-2 and Laz 2-369 respectively, were clustered in three molecular weight regions, Group a, Group b, and Group c (Figure 11A, B). Within Group a, mAbs Lan 3-2 and Laz 2-369 both recognized four peptide fragments at 115, 100, 85 and 72 kD. Within Group c, they both recognized three peptide fragments between 31 and 35 kD. The peptide fragments within Group b recognized by mAb Laz 2-369 were not resolved, but appeared as a broad band between 42 and 58 kD. In comparison, mAb Lan 3-2 recognized three peptide fragments within Group b at 53, 40, and 38 kD. Similarly, the peptide fragments of the modality protein recognized by mAb Laz 6-212 were clustered in the same three regions (data not shown). In contrast, glial proteins yield proteolytic fragments clustered in two molecular weight regions, Group a and Group c (Figure 11C). Within Groups a and c, mAb Laz 6-297 recognized four peptide fragments at 120, 98, 92, and 86 kD; and four fragments at 37, 35, 33, and 32 kD, respectively.

Figure 11. Peptide mapping of 130 kD sensory, modality, and glial proteins. A: Peptide mapping of the 130 kD sensory protein, recognized by mAb Lan 3-2. The 130 kD band (120-150) was excised from SDS-PAGE and placed into the sample wells of a second 15% SDS-PAGE. The slices were overlayed with 0, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 ug/ml of *Staphylococcus aureus* V8 protease (lanes 1-7, respectively). B: Peptide mapping of the 130 kD modality protein recognized by Laz 2-369. The 130 kD gel slices were overlayed with 0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 ug/ml of V8 protease (lanes 1-7, respectively). C: Peptide mapping of the 130 kD glial protein, recognized by Laz 6-297. The 130 kD gel slices were overlayed with 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ug/ml of V8 protease (lanes 1-7, respectively). Proteins were transferred to nitrocellulose, incubated with mAbs, and visualized by autoradiography. The molecular weights of the peptide fragments were determined from at least 7 peptide mapping experiments for each 130 kD protein. Peptide fragments are clustered in three molecular weight regions; 72-120 (group a), 38-58 (group b), and 31-37 (group c) kD. Molecular weigths are shown to the right in kilodaltons.


A
- 205
- 111
- 68
- 45
- 24
a
b
c
1 2 3 4 5 6 7
B
- 220
- 100
- 68
- 43
- 27
a
b
c
1 2 3 4 5 6 7
C
- 225
- 104
- 68
- 42
a
c
1 2 3 4 5 6 7

The fragments within Group b were resolved further on 10% gels. Differences in peptide fragments can be seen between sensory and modality proteins (Figure 12). Fewer fragments in the Group b region were detected after digestion of the sensory protein than were seen following digestion of the modality protein (recognized by mAb Laz 2-369). MAb Laz 2-369 recognizes two additional bands at 54 and 58 kD. These results suggest that the sensory and modality 130 kD proteins are similar. In addition, the sensory and modality proteins demonstrate a greater degree of similarity compared to the 130 kD glial protein.

Figure 12. Peptide mapping of 130 kD sensory and modality protein. The 130 kD band (120-150) was excised from 7.5% SDS-PAGE and placed into the sample wells of a second 10% SDS-PAGE. The slices were overlayed with 0.2, 1.0, 0.2, and 1.0 ug/ml of *Staphylococcus aureus* V8 protease (Lanes 1-4, respectively). Proteins were transferred to nitrocellulose, incubated with mAbs Lan 3-2 (lanes 1-2) and Laz 2-369 (lanes 3-4), and visualized by autoradiography. The molecular weights of the peptide fragments were determined from 3 peptide mapping experiments. Peptide fragments are clustered in two molecular weight regions, 80-115 (group a) and 41-58 (group b) kD. Molecular weights are shown to the right in kilodaltons.


- 224
- 109
- 72
- 46
- 29
a
a
b
b
1
2
3
4

DISCUSSION

Different cell types in the leech nervous system express unique 130 kD surface proteins, which have been identified by mAbs (Flaster and Zipser, 1983; Peinado et al., 1987). Immunizing mice with SDS PAGE gel bands of the 125 to 140 kD region has proven an effective method for generating mAbs against 130 kD leech proteins (Flaster et al., 1983). Two previous immunizations against such partially purified 130 kD CNS proteins, generated mAbs specific for the five 130 kD sensory, modality, and glial proteins. Performing two additional immunizations, we isolated a mAb reactive with a 130 kD connective tissue protein.

Since the 130 kD sensory, modality, and glial proteins share similar molecular weight and behave as loosely associated peripheral membrane proteins (Cole et al., 1989b), we hypothesize that they form a family of related surface glycoproteins. We examined the nature of the sensory, modality, and glial epitopes. Deglycosylation of the 130 kD sensory and modality proteins demonstrated that the antigenic determinants recognized by the mAbs are composed of N-glycosylated domains. In addition, the binding of the mAb Lan 3-2 to the sensory protein was inhibited by alpha methyl-mannoside. In contrast, alpha methyl-mannoside did not inhibit the binding of the other mAbs to their modality proteins. The epitopes of the modality proteins either contain mannose with more structural information or consist of sugars other than mannose. None of the glycoproteins were blocked with N-acetylgalactosamine, which is consistent with

previous observations that the sensory and modality glycoproteins do not bind to ricin columns (Flanagan et al., 1986). Thus the sensory and modality carbohydrate epitopes are different. In contrast, the glial and connective tissue epitopes are not on N-linked carbohydrate moieties. The mAb specific for the glial and connective tissue protein may bind to an amino acid epitope or alternatively to O-linked carbohydrate moieties.

The results of the cross reactivity experiments suggest that the sensory and modality proteins share related carbohydrate epitopes. The cross reactivity demonstrated by the modality mAbs may be the result of epitopes that are masked or hidden in organized tissue which become exposed during detergent solubilization. Whether these hidden epitopes are relevant in mediating events during neuronal development needs to be determined. This idea of hidden epitopes which become exposed during protein solubilization has been demonstrated previously with respect to AMOG (Antonicek et al., 1987). The L4 epitope has been demonstrated on AMOG solubilized from glial cells, but is not detected on AMOG expressed on the surface of glial cells in tissue culture (Fahrig et al., 1989).

The cross reactivity between the 4 sensory and modality epitopes detected in biochemical assays, raised the question of whether the 130 kD proteins were indeed separate protein forms. Results from the peptide mapping experiments suggest that the sensory and modality proteins cores are different but share considerable protein core homology. Both sensory and modality proteins share peptide fragments clustered in three regions, 72-115, 38-58, and 31-35 kD. Separating the peptide fragments of the different sensory and

modality 130 kD proteins on 10% gels demonstrated differences among the neuronal peptide fragments. Thus, this difference in peptide fragments among the sensory and modality proteins speaks in favor of distinct 130 kD sensory and modality proteins. In addition, the sensory and modality proteins demonstrated a greater degree of protein core homology compared to the glial protein. Digestion of the 130 kD glial protein resulted in fragments visualized in the 86-120 and 32-37 kD regions similar to those seen with digestion of the sensory and modality proteins. Digestion of the 130 kD glial protein did not result in any fragments visualized in the 38-58 kD region.

Many biological processes are regulated by protein families, such as the immunoglobulins (Baenziger et al., 1985), glycoprotein hormones (FSH, LH, TSH, and CG) (Baenziger et al., 1985; Rademacher et al., 1988), protein kinases (Hanks et al., 1988), and integrins (Buck et al., 1986; Tamkum et al. 1986; Hynes 1987). In addition, many of the well characterized surface glycoproteins which are involved in neuronal development are members of the immunoglobulin superfamily, such as N-CAM (Cunningham et al., 1987), L1 (Moos et al., 1988), integrins and MAG (Arquint et al., 1987).

The importance of the protein families lies in the elimination of the necessity for large gene pools to regulate biological processes. Molecular diversity can be achieved through gene rearrangement (Tonegawa, 1985) or through post-translational modification of a common core protein (Cunningham et al., 1983). For example, all three major forms of N-CAM (180, 140, and 120 kD) are the result of tissue-specific splicing of RNA species derived from a single copy

gene (Cunningham et al., 1987). Modification in the structure of N-CAM may contribute to the multiple developmental events mediated by N-CAM.

In the leech, the family of cell type-specific 130 kD sensory, modality, and glial glycoproteins may be generated by post-translational modification of similar core proteins. The similar protein cores appear to be differentially glycosylated, thereby generating the functionally different members of the group of 130 kD glycoproteins. We hypothesize that carbohydrate recognition involving the 130 kD glycoproteins may play a role in the formation of the leech nervous system. Carbohydrate coding of sensory neurons has been previously demonstrated in mammalian neuronal systems (reviewed by Rutishauser and Jessel, 1988). In the rat, certain oligosaccharides differentiate between different subsets of dorsal root ganglion cells which project to the spinal cord (Dodd and Jessel, 1985; Dodd et al., 1988).

Our previous antibody perturbation studies suggest that mannose-mediated recognition events are involved in neuronal interactions within the leech CNS (Zipser et al., 1989). In the synaptic neuropil, the processes of functionally distinct neurons are stereotypically positioned and express two different 130 kD epitopes, the sensory epitope and one of the three modality epitopes. These neuroanatomical observations together with our present characterization of these surface proteins, will permit us to further examine the molecular aspects of neuronal interactions within the leech CNS.

CHAPTER 2. Mannose-Containing Epitopes of Neuronal Glycoproteins in Leech and Rat are Related

INTRODUCTION

There is increasing evidence suggesting that carbohydrates are involved in cell recognition and adhesion processes during development. Such diverse events as the compaction of cells in the morula stage (Bayna et al., 1988), lymphocyte homing (Rademacher et al., 1988), and sperm attachment to eggs (Runyan et al., 1988) are dependent on carbohydrates bound either to proteins or lipids. The involvement of carbohydrates during neurogenesis has so far been largely demonstrated through antibody perturbation studies. As previously mentioned, three carbohydrate epitopes called L2/HNK-1, L3 and L4 are expressed on several cell surface molecules in vertebrates (Kruse et al., 1984; Kruse et al., 1985; Kucherer et al., 1987; Pesheva et al., 1987). Blocking these carbohydrate epitopes with antibodies perturbed neuronal migration and axonal projections in developing vertebrate nervous systems (Bronner-Fraser, 1987; Dow et al., 1988; Kunenumd et al., 1988; Fahrig et al., 1989). In addition, the L5 carbohydrate epitope has recently been localized on several vertebrate cell surface molecules (Streit, 1989).

The epitopes implicated in vertebrate neurogenesis are of two general types: L3, L4, and L5 epitopes which contain mannose (Fahrig et al., 1989; Streit, 1989) and the L2/HNK-1 epitope which is highly acidic and consists of sulfated glucuronic acid (Chou et al., 1986; Ariga et al., 1987). Despite the similar characteristic features

and striking co-expression on several glycoproteins, the L3 and L4 epitopes differ from one another as shown by antibody competition experiments (Fahrig et al., 1989). Using antibodies, the related L3 and L4 carbohydrate epitopes have also been demonstrated in *in vitro* assays to mediate cell adhesion in a qualitatively different way than the L2/HNK-1 carbohydrate epitope. We investigated the conservation of these carbohydrate epitopes across phylogeny by reacting their respective monoclonal antibodies (mAbs) with glycoproteins of the leech nervous system.

We have previously identified two different mannose-containing carbohydrate epitopes in the leech nervous system, Lan 3-2 and Laz 6-189. The mannose-containing carbohydrate epitope recognized by mAb Laz 6-189 and is found on proteins which are expressed by different neuronal cell types (McRorie and Zipser, 1988). Here we report that mAbs L3, L4, and L5 also identify mannose epitopes in the leech. In addition, the mammalian sulfated L2/HNK-1 epitope is also expressed in the leech nervous system.

MATERIALS AND METHODS

Leeches. Leeches (*Haemopis marmorata* and *Hirudo medicinalis*) were purchased from commercial distributors and stored at 9°C for several months. Nerve cords (supra- and subesophageal ganglia and ganglia 1-21) were removed from alcohol anesthetized leeches which had been secured in position with pins. The nerve cords were stored frozen at -70°C.

Monoclonal antibodies. Monoclonal Laz 6-189, Lan 3-2, and Laz 9-84 antibodies (mAbs) were obtained from immunization of mice with leech CNS as previously described (Zipser and McKay, 1981; Flaster et al., 1983; Bajt et al., 1989;). MAbs L2, L3, L4 and L5 were obtained as described (Kucherer et al., 1987; Kunemund et al., 1988; Fahrig et al., 1989; Streit et al., submitted). MAbs were used as either ascites fluids of Pristane-primed mice injected with cells of the hybridoma clone or as culture supernatants.

Glycoproteins. The adhesion molecules AMOG, L1, and MAG were isolated as described (Fahrig et al., 1989). Bromelain from pineapple stem, horseradish peroxidase (HRP), ovalbumin from chicken, *Erythrina cristagalli* agglutinin (ECA), and fucosidase from bovine and epididymus were purchased from Sigma (St. Louis, MO). Thy-1 from rat brain and thymus was kindly provided by A.F. Williams (Oxford).

Electrophoresis and immunoblotting. Leech nerve cords (CNS) were boiled in sample buffer (Laemmli, 1970) for 3 minutes, centrifuged,

and supernatants were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. Immunoblots were prepared as described previously (Garrels, 1979; Towbin, et al., 1979). Enzyme-linked immunosorbent assay (ELISA) were performed following the protocol of Bollensen et al., (1988) except that Tween 20 was omitted in all buffers. The monoclonal antibodies were applied as hybridoma culture supernatants (mAb: 20 ug/ml). ELISA in a competition mode was performed by pre-incubating the antibodies with 0.1 M methyl alpha-D-mannopyranoside or methyl alpha-D-galactopyranoside for 30 min at 37°C before adding these mixtures to the antigens coated onto microtiter plastic wells. Second antibodies directed against rat or mouse IgG and IgM coupled to horseradish peroxidase were purchased from Dianova and used at a dilution of 1:1000 for Western blots and 1:7500 for ELISAs.

Immunoprecipitation. Immunoprecipitation was carried out as described in the first chapter. In brief, 20 ul of swine antimouse IgG (Nordic Immunology, Capistrano Beach, CA) were coupled to 100 ul pre-swollen protein A-Sepharose beads (Pharmacia, Piscataway, New Jersey) prepared as suspension in 500 ul PBS (pH 8.0) for 6 hr at 4°C with gentle agitation. Sepharose beads were then carefully washed with PBS (pH 8.0) followed by centrifugation (2 min at 500 rpm). Ten nerve cords were boiled in 400 ul sample buffer (Laemmli, 1970) for 3 min and centrifuged. Following dilution with PBS pH 8.0 to 3 ml, the supernatant was incubated with swine antimouse IgG conjugated protein A-Sepharose beads for 2 hours at 4°C. After centrifugation, the supernatant was incubated with 2 ul ascites fluid

(mAb: 5 mg/ml) for 6 hr at 4°C. The supernatant was then incubated with fresh swine antimouse IgG conjugated protein A-Sepharose beads (100 ul) overnight at 4°C with gentle agitation. The beads were washed extensively with PBS and then centrifuged through a sucrose cushion (1M). The proteins were eluted by boiling for 3 min in sample buffer. Immunoprecipitates were subjected to electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose.

Immunocytochemistry. Immunocytochemistry was carried out as described in the first chapter. In brief, midbody ganglia were washed in PBS and fixed for 1/2 hr with 4% paraformaldehyde in 0.1 M phosphate buffer. Following fixation, the nerve cords were dehydrated with graded alcohols and embedded in polyethylene glycol (PEG; 20% PEG 1450/80% PEG 3350) (Peinado et al., 1987) or JB 4 Immunobed plastic (Polysciences, Inc., Warrington, PA) according to the manufacturer's instructions. The nerve cords were then sectioned at 2-5 um with glass knives and mounted on slides coated with 0.1% polylysine (Sigma; 150,000-300,000 molecular weight). Plastic sections were incubated for 30 min with 0.1% trypsin (Sigma, St. Louis, MO) and rinsed briefly with PBS. PEG sections were washed extensively first with 70% and then 95% alcohol over 30 minutes. Plastic and PEG sections were incubated with mAb supernatants (mAb: 20 ug/ml) overnight. Before incubation with the second antibody, the ganglia were rinsed briefly with PBS. Sections were then incubated for 2 hr with HRP conjugated second antibody (goat antimouse IgG; Cappel, West Chester, PA, 1:50

PBS/10% fetal calf serum). The staining pattern was visualized with 0.02% 3,3'-diaminobenzidine (DAB) in 0.05 M Tris buffer, pH 7.4 and 0.015% hydrogen peroxide. Sections were rinsed with PBS, dehydrated in graded alcohols, cleared in xylene, and mounted in Permount.

In addition, wholemounts of leech midbody segmental ganglia were incubated for 1 hour in 0.5 mg/ml protease (0.5 mg/ml of distilled water) from *Streptomyces griseus* Type XIV (Sigma, St. Louis, MO). Wholemounts were then washed with PBS and fixed for 1/2 hr with 4% paraformaldehyde in 0.1 M phosphate buffer. Following fixation, the wholemounts were incubated overnight with mAb supernatants (mAb: 20 ug/ml). Wholemounts were briefly washed with PBS before and after incubation for 2 hr with biotinylated Fab fragments (rabbit antimouse IgG; Dako 1:100 PBS/10% fetal calf serum). Whole mounts were then incubated with horseradish peroxidase avidin D (Vector 1:150 PBS/10% fetal calf serum) for 2 hr. The staining pattern was visualized with 0.02% 3,3'-diaminobenzidine (DAB) in 0.05 M Tris buffer, pH 7.4 and 0.015% hydrogen peroxide. Wholemounts were rinsed with PBS, dehydrated, and embedded in JB 4 Immunobed plastic.

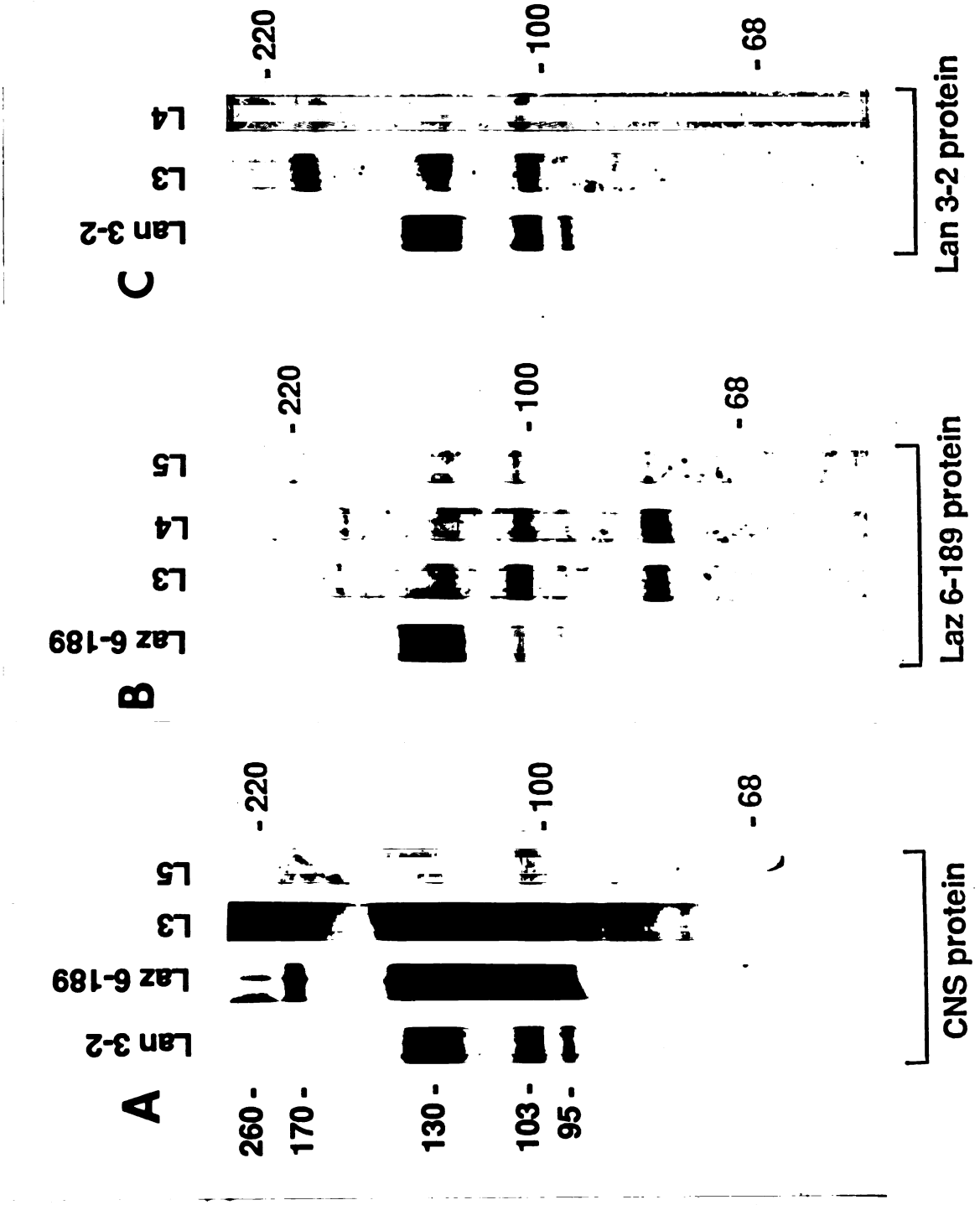
RESULTS

Identification of L3, L4, L5 and L2/HNK-1 epitope-carrying glycoproteins

On immunoblots, the mAbs raised against glycoproteins from the mammalian brain cross react with proteins from the leech nervous system. MAbs L3 and L5 recognize leech glycoproteins ranging from 30 to 260 kD (Figure 13A). MAb L4 reacts with the same bands as mAbs L3 and L5 (data not shown). These antibodies appear to react with the same glycoproteins, but with different intensities of staining. For example, the staining with mAbs L3 and L4 was of comparable intensity, while that with mAb L5 was considerably weaker. Some of the glycoproteins recognized by the mAbs L3, L4, and L5 appear to be of the same molecular weight as a previously identified group of mannose-containing proteins in the leech nervous system. These glycoproteins are identified by mAbs Lan 3-2 and Laz 6-189 (Hogg et al., 1983; Flanagan et al., 1986; McRorie and Zipser, 1988). MAb Laz 6-189 reacts with all members of this group, which include proteins of 260, 170, 130, 103, and 95 kD molecular weight (Figure 13A). MAb Lan 3-2 recognizes only the 130, 103, and 95 kD proteins.

To determine if mAbs L3, L4, and L5 bind to the group of mannose-containing leech glycoproteins, we immunoprecipitated leech CNS proteins with mAb Laz 6-189. Immunoblots prepared from immunoprecipitated proteins were then probed with mAbs Laz 6-189, L3, L4, and L5 (Figure 13B). MAb Laz 6-189 reacts with a

Figure 13. Binding of mAbs Lan 3-2, Laz 6-189, L3, L4, and L5 to leech CNS glycoproteins on immunoblots. Immunoblots were prepared from leech CNS extracts (A) or proteins immunoprecipitated with mAbs Laz 6-189 (B) or Lan 3-2 (C) and separated on 7.5% SDS-PAGE. Molecular weights are shown on the right in kilodaltons.


A
lan 3-2
laz 6-189
L3
L5
260 -
170 -
130 -
103 -
95 -
- 220
- 100
- 68
CNS protein
B
laz 6-189
L3
L4
L5
- 220
- 100
- 68
Laz 6-189 protein
C
lan 3-2
L3
L4
- 220
- 100
- 68
Lan 3-2 protein

broad band centered at 130 kD and the two narrow bands at 103 and 95 kD. MAbs L3, L4 and L5 react with these bands as well as two additional bands at 170 and 75 kD. MAb Laz 6-189 does not appear to recognize the 170 and 75 kD bands on immunoblots, although it immunoprecipitates them. Possibly, these 170 and 75 kD proteins coprecipitate with the mAb Laz 6-189 reactive glycoproteins during immunoprecipitation. However, the samples are boiled in SDS sample buffer prior to immunoprecipitation which should dissociate all proteins from each other. Additionally, upon longer exposures of the autoradiograms, mAb Laz 6-189 does weakly recognizes the 170 kD band. Therefore, it remains possible but unlikely that the 75 kD band may be the result of coprecipitation. In addition, only a subpopulation of the 130 kD protein immunoprecipitated with Laz 6-189 seems to be recognized by mAbs L3, L4, and L5. None of the mAbs react with the 260 kD band suggesting that the 260 kD protein does not immunoprecipitate.

Immunoblots were also prepared from leech CNS proteins immunoprecipitated with mAb Lan 3-2 (Figure 13C). MAb Lan 3-2 stains a broad band at 130 kD and the two narrower bands at 103 and 95 kD. MAbs L3 and L4 react with these bands as well as additional bands at 170 and 85 kD. MAb L5 was not tested. MAb Lan 3-2 does not appear to recognize the 170 and 85 kD bands on immunoblots, although it immunoprecipitates them. Again, mAb Lan 3-2 may coprecipitate these glycoproteins during immunoprecipitation. With longer exposures of the autoradiograms, mAb Lan 3-2 does recognize the 170 kD band, but not the 85 kD band. Therefore, it is possible that the 85 kD band may be the result of

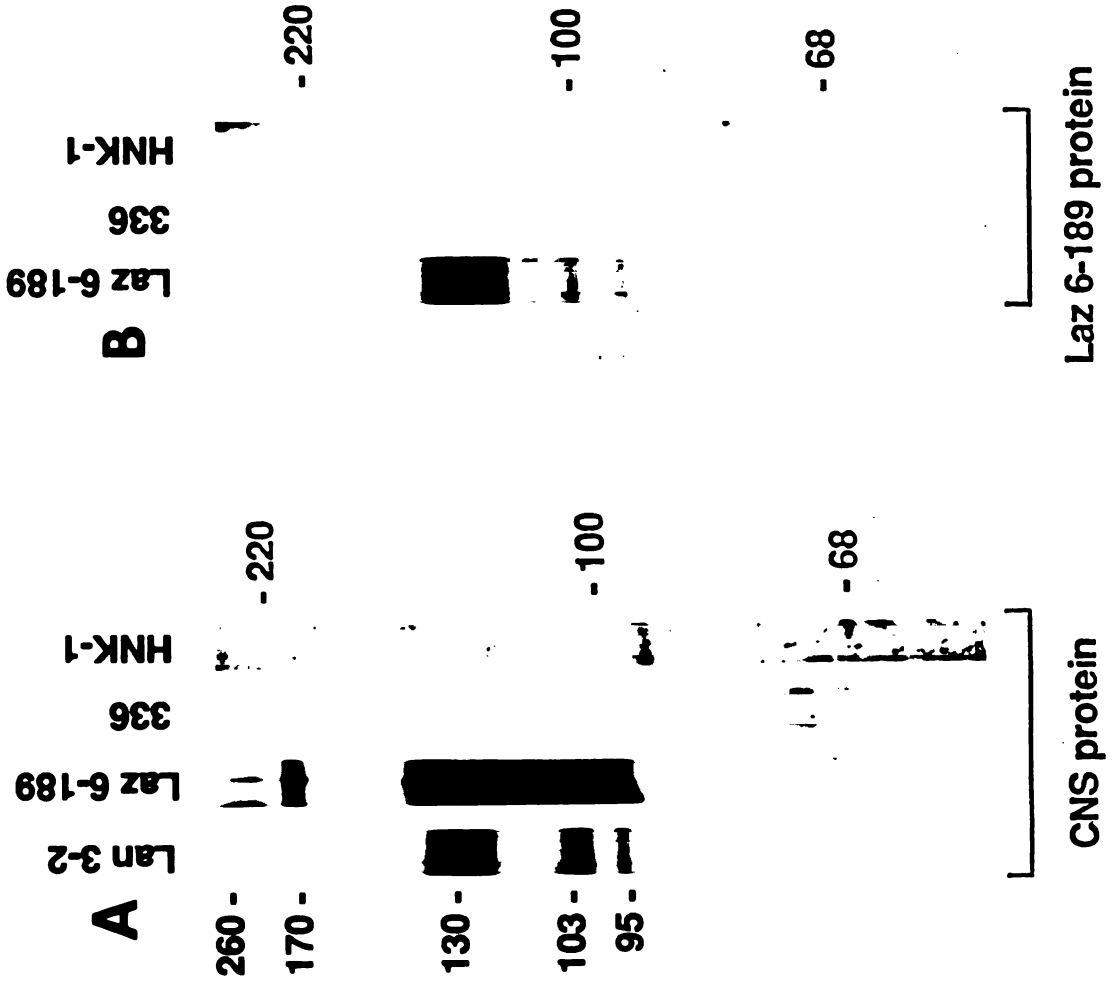
coprecipitation. In the 130 kD region, mAbs L3 and L4 cross react only with a subpopulation of the 130 kD protein immunoprecipitated by Lan 3-2. Thus, the 5 different rat and leech derived mAbs recognize different members of the group of mannose-containing leech glycoproteins.

Immunoblot analysis of glycoproteins from leech CNS were also performed using antibodies that recognize the L2 (mAb 336) and HNK-1 epitopes (mAb HNK-1). In contrast to mAbs L3, L4, and L5, mAbs 336 and HNK-1 bind few, but different glycoproteins of the leech CNS (Figure 14A). MAbs 336 and HNK-1 do not react with any of the proteins immunoprecipitated with mAb Laz 6-189 (Figure 14B). Thus, the 130, 103, and 95 kD proteins appear to lack the highly acidic epitopes to which these rat derived mAbs bind.

Identification of the L3, L4, L5, and L2/HNK-1 epitope-expressing leech cell types

By immunocytochemical analysis, we compared the distributions of the epitopes recognized by the different leech and rat derived mAbs in the leech CNS. The mAbs specific for mannose-containing glycoproteins demonstrate three different types of staining patterns. In cross sections of the central fiber tract, mAb Lan 3-2 stains approximately 1/3 of all axons in the connectives (Figure 15C). These axons are sensory afferents whose cell bodies are located in the skin or in other peripheral organs (Hogg et al., 1983; Peinado et al., 1987). While mAb Laz 6-189 weakly stains all axons, it

Figure 14. Binding of mAbs Lan 3-2, Laz 6-189, 336, and HNK-1 to leech CNS glycoproteins on immunoblots. Immunoblots were prepared from leech CNS extract (A) or proteins immunoprecipitated with mAb Laz 6-189 (B). Molecular weights are shown on the right in kilodaltons.


A
Lan 3-2
Laz 6-189
336
HNK-1
260 -
170 -
130 -
103 -
95 -
220 -
100 -
68 -
CNS protein
B
Laz 6-189
336
HNK-1
220 -
100 -
68 -
Laz 6-189 protein

differentiates the sensory afferents by staining them more intensely (Figure 15D).

In contrast, mAb L3 stains all the axonal tracts within the connective and Faivre's nerve with equal intensity (Figure 15E). MAb L3 also stains CNS capsule and muscle fibers embedded within the connective tissue, but does not stain the connective tissue layer. The extent of the connective tissue layer is visualized with leech derived mAb Laz 9-84 (Figure 15B). MAb L4 stains the same cell types as mAb L3 within the connective (data not shown). In addition, mAbs L3 and L4 stain much stronger in comparison to mAb L5 (Figure 15F). The pattern of variation in histological staining was similar to that demonstrated on Western blots. This variation in staining intensity is also seen in polyethylene glycol (PEG) sections and therefore, is not an artifact associated with postembedding staining of plastic sections.

In cross sections of the central ganglia, mAb L3 stains the cell bodies of central neurons and their projections into the synaptic neuropil (Figure 16A). The peripheral root through which the central and peripheral nerves enter and leave the CNS is also stained with mAb L3. MAbs L4 and L5 stain the same cell types as L3 (data not shown). Again, mAbs L3 and L4 stain much stronger than mAb L5. Thus, leech and rat derived mAbs which react differently with mannose-containing glycoproteins on immunoblots, also demonstrate different staining patterns in the leech nervous system.

The acidic epitopes recognized by mAbs 336 and HNK-1 also have unique distributions in the leech nervous system. MAb 336 intensely stains the cell body layer of the central ganglion (Figure

Figure 15. Cross sections of interganglionic connectives of the leech CNS. Diagrammatic representation of the three axonal tracts of the connective (A). 2-5 um thick cross sections through the interganglionic connective reacted with mAbs Laz 9-84 (B), Lan 3-2 (C), Laz 6-189 (D), L3 (E), and L5 (F). B and F are PEG sections. C, D, and E are JB 4 plastic sections prestained prior to sectioning. Scale bar equals 50 um.

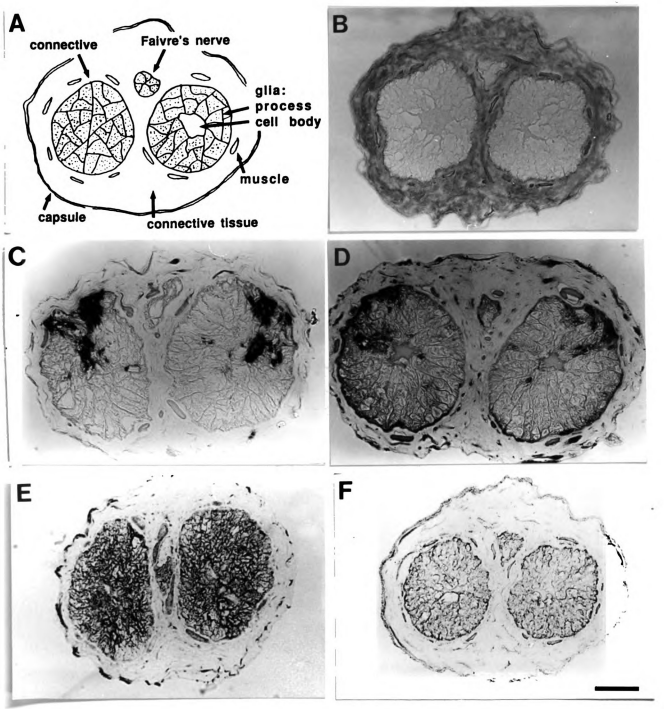
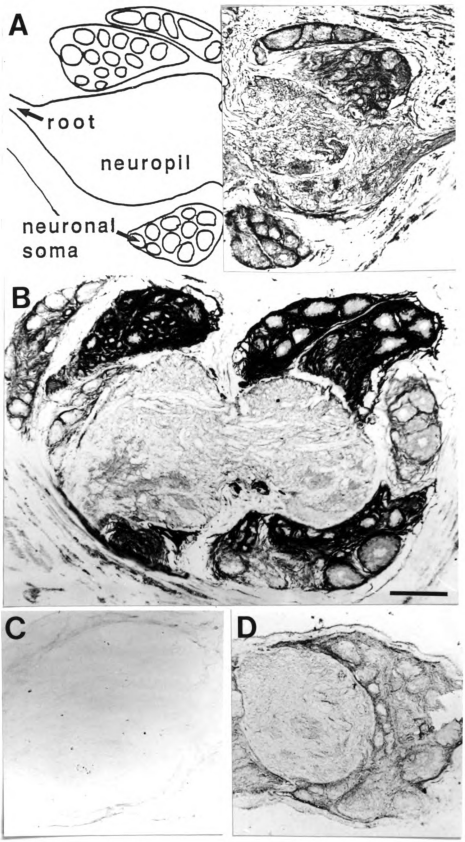

A
connective
Faivre's nerve
glia:
process
cell body
muscle
capsule
connective tissue
B
C
D
E
F

Figure 16. Cross sections of central ganglia of the leech CNS. 2-5 um thick cross sections through the central ganglion reacted with mAbs L3 (A), 336 (B), and HNK-1 (D). As a control, cross section of ganglion was incubated with second antibody only (C). All sections are JB 4 plastic. Scale bar equals 50 um.


A
root
neuropil
neuronal
soma
B
C
D

16B). In contrast, it weakly stains the axons in the central neuropil and in the peripheral and central fiber tracts. MAb HNK-1 also preferentially stains the cortical cell body layer (Figure 16D). However, the overall reactivity of HNK-1 is weaker than that with mAb 336. In control sections incubated with second antibody only, the cell body and neuropil regions are unstained while the ganglionic capsule is stained lightly (Figure 16C). Therefore, some of the capsule staining with mAbs 336 and HNK-1 may be artifactual.

Identification of leech and rat epitope-carrying glycoproteins

The presence of the Laz 6-189, L3, L4, and L5 epitopes on immunoaffinity purified mouse antigens and several commercially available glycoproteins was quantified by an enzyme-linked immunosorbent assay (ELISA). Microtiter wells were coated with the various glycoproteins and the binding of each mAb was determined by optical density measurements. These experiments allow us to investigate the similarities in the mannose epitopes. The results are summarized in Table 2. The Laz 6-189 epitope is present on L1 and Thy-1, two of the CNS glycoproteins which express the L3, L4, and L5 epitopes. The Laz 6-189 epitope is not present on MAG. In contrast, the L3, L4, and L5 epitopes are expressed on all of the CNS glycoproteins tested; L1, Thy-1, AMOG, and MAG. The presence of the Laz 6-189 epitope on AMOG has not yet been determined.

The Laz 6-189, L3, L4, and L5 epitopes were not restricted to the nervous system. For example, Laz 6-189 epitope is present on

Table 2. **O.D. Values of Binding of Antibodies to Different Glycoproteins Measured in ELISA[a]**

	Laz 6-189	L3	L4	L5
L1[b]	0.33 ± 0.03	0.89 ± 0.18	0.84 ± 0.05	0.74 ± 0.02
+ Man[c]	0.16 ± 0.02	0.05 ± 0.04	0.04 ± 0.00	0.46 ± 0.02
+ Gal[d]	0.26 ± 0.01	0.65 ± 0.03	0.96 ± 0.14	0.51 ± 0.03
Thy-1	0.62 ± 0.12	0.59 ± 0.01	0.73 ± 0.04	0.55 ± 0.02
+ Man	0	0.04 ± 0.18	0.07 ± 0.02	0.23 ± 0.03
+ Gal	0.49 ± 0.01	0.65 ± 0.03	0.71 ± 0.05	0.55 ± 0.04
AMOG	N.D.[e]	1.22 ± 0.10	1.17 ± 0.02	0.64 ± 0.03
+ Man	N.D.	0.16 ± 0.01	0.19 ± 0.01	0.08 ± 0.02
+ Gal	N.D.	0.83 ± 0.03	1.19 ± 0.04	0.65 ± 0.03
MAG	0	0.74 ± 0.04	0.62 ± 0.03	0.13 ± 0.01
+ Man	0	0.09 ± 0.02	0.02 ± 0.01	0.06 ± 0.02
+ Gal	0	0.80 ± 0.06	0.81 ± 0.28	0.13 ± 0.03
ECA	2.27 ± 0.10	0.65 ± 0.04	0.84 ± 0.07	0.68 ± 0.05
+ Man	0.03 ± 0.02	0.52 ± 0.02	0.61 ± 0.04	0.38 ± 0.01
+ Gal	2.25 ± 0.05	0.65 ± 0.04	0.87 ± 0.07	0.53 ± 0.02
HRP	0.95 ± 0.07	0.53 ± 0.03	0.57 ± 0.01	0.48 ± 0.02
+ Man	0	0.39 ± 0.02	0.36 ± 0.01	0.26 ± 0.02
+ Gal	0.77 ± 0.07	0.47 ± 0.02	0.50 ± 0.02	0.47 ± 0.04
Bromelain	0	0.65 ± 0.07	0.75 ± 0.034	0.44 ± 0.02
+ Man	0	0	0.02 ± 0.00	0.05 ± 0.02
+ Gal	0	0.67 ± 0.13	0.77 ± 0.04	0.64 ± 0.11
Ovalbumin	0	0.87 ± 0.09	1.08 ± 0.03	0.69 ± 0.01
+ Man	0	0.12 ± 0.04	0.14 ± 0.03	0.05 ± 0.00
+ Gal	0	0.78 ± 0.03	0.10 ± 0.03	0.76 ± 0.06
Fucosidase I	0	1.20 ± 0.13	1.26 ± 0.08	0.62 ± 0.03
+ Man	0	0.05 ± 0.01	0.01 ± 0.00	0.04 ± 0.02
+ Gal	0	1.23 ± 0.13	1.25 ± 0.09	0.74 ± 0.11
Fucosidase II	0	0.96 ± 0.08	1.07 ± 0.01	0.82 ± 0.02
+ Man	0	0.13 ± 0.04	0.14 ± 0.03	0.07 ± 0.01
+ Gal	0	1.08 ± 0.17	1.19 ± 0.09	0.94 ± 0.10

[a]ELISA's were performed by Dr. Brigitte Schmitz at University of Heidelberg, Fed. Rep. Germany.

[b]Micotiter wells were coated with glycoproteins (2.0-5.0 ug/ml) and absorbance at 405 nm was measured. Numbers represent mean values ± standard deviations from six experiments.

[c]Reactivities of antibodies with glycoproteins in the presence of 0.1 M methyl alpha-D-mannopyranoside.

[d]Reactivities of antibodies with glycoproteins in the presence of 0.1 M methyl alpha-D-galactopyranoside.

[e]Not determined.

Erythrina cristagalli agglutinin (ECA) and horseradish peroxidase (HRP). The L3, L4, and L5 epitopes are present on these glycoproteins as well as fucosidases from bovine kidney and epididymis, ovalbumin from chicken, and bromelain (a protease from pineapple stem). Again, the L3, L4, and L5 epitopes were always found to be co-expressed on all of the glycoproteins tested. The results for these studies demonstrate that the Laz 6-189 epitope is present on some but not all of the glycoproteins which express the L3, L4, and L5 epitopes. This suggests that the epitope recognized by mAb Laz 6-189 is similar but not identical to the epitopes recognized by mAbs L3, L4, and L5.

To confirm the mannosidic nature of the Laz 6-189, L3, L4, and L5 epitopes, the mAbs were preincubated for 30 minutes in the presence of 0.1 M alpha methyl-mannoside or 0.1 M alpha methyl-galactoside and then the binding of the mAbs to the various glycoproteins was determined. Binding of mAbs Laz 6-189, L3, L4, and L5 to epitope-expressing glycoproteins was attenuated by alpha methyl-mannoside. Alpha methyl-galactoside did not attentuate binding of any of the mAbs. These results demonstrate that mAbs Laz 6-189, L3, L4, and L5 bind to mannose-containing epitopes on the glycoproteins tested.

DISCUSSION

In the present study, we examined the expression of two carbohydrate epitope families in the leech. The sulfated glucuronic acid epitope, referred to as the L2/HNK-1 epitope, defines one of these families which includes N-CAM (Kruse et al., 1984), MAG (Poltorak et al, 1987), L1 (Kunemund et al., 1988), J1 (Kruse et al., 1985), and integrin (Pesheva et al., 1987). In this study, we extended this vertebrate epitope family to include glycoproteins of the leech nervous system. MAbs HNK-1 and 336, which recognize the sulfated glucuronic acid epitope, prominently stain the cell body layer of the leech ganglion.

On immunoblots prepared from leech CNS extracts, mAbs HNK-1 and 336 weakly react with different proteins. However, mAbs HNK-1 and 336 are suppose to recognize the same epitope. It is surprising and at present difficult to explain why these antibodies react differently in the leech. Further studies are being performed to explain this discrepancy. For both 336 and HNK-1 mAbs, the weak immunoblot staining does not correlate with the rather strong stain observed histologically in the leech nervous system. One possible explanation for this discrepancy is that the L2/HNK-1 epitopes are also expressed by glycolipids in the cell body layer, and glycolipids would not be detected on immunoblots. This is the case in vertebrates where the L2/HNK-1 epitope is expressed by glycolipids (Chou et al., 1986; Ariga et al., 1987). Presently, little is known about glycolipids in the leech nervous system and further studies are being carried out to clarify this point.

The identification of highly acidic glycans in the leech nervous system is significant, since glycoproteins with acidic glycans have been extensively demonstrated to participate in vertebrate neurogenesis (Rademacher et al., 1988). Three types of acidic glycans have been identified in vertebrates; sulfated, phosphorylated, and those containing sialic acid. The neural cell adhesion molecule N-CAM, for example, expresses not only the sulfated L2/HNK-1 epitope, but also polysialic acid glycans which are thought to regulate the interactions of N-CAM through its steric properties (Rutishauser et al., 1988).

In invertebrates, sialic acid containing glycans have been detected in starfish, but are reported to be absent in lower invertebrates such as the annelids. (Schauer, 1982). Since sialic acid modulates cell adhesion during vertebrate neurogenesis, possibly other acidic glycans could substitute for its role in invertebrate neurogenesis where sialic acid is not detected. Perhaps the acidic L2/HNK-1 epitopes identified in the leech may modulate adhesive interactions during leech development.

The mannosidic epitopes of L3, L4, and L5 define the second family of neural adhesion molecules, which include L1, MAG, AMOG, and P_0, all of which are high mannose or hybrid type glycoproteins (Fahrig et al., 1989; Streit, 1989). In this study, we extended this vertebrate epitope family to include glycoproteins of the leech nervous system. The recognition of leech nervous system glycoproteins by mAbs L3, L4, and L5 raised against rat nervous system is not surprising since many glycoproteins from outside the vertebrate nervous system express the corresponding epitopes

(Fahrig et al., 1989). Some of the glycoproteins are of plant origin, such as horseradish peroxidase and a protease from pinapple stem, bromelain.

The leech glycoproteins which express the L3, L4, and L5 epitopes belong to a characteristic group of nervous system glycoproteins of 260, 170, 130, 103, and 95 kD (Hogg et al., 1983; McRorie and Zipser, 1988). These glycoproteins have been identified previously by two mAbs (Lan 3-2 and Laz 6-189) that were generated against the leech CNS. Both mAbs bind to mannose-containing epitopes (McRorie and Zipser, 1988) MAb Laz 6-189 recognizes the entire group of glycoproteins, while mAb Lan 3-2 binds to a subset of these glycoproteins. MAb Lan 3-2 reacts with the 130, 103, and 95 kD glycoproteins. The difference between reactivities of mAbs Lan 3-2 and Laz 6-189 to the group of mannose-containing glycoproteins, suggests the existence of at least two different types of mannosidic epitopes.

In addition, mAbs L3, L4, and L5 also react differently with the group of mannose containing leech glycoproteins. For example, mAbs L3, L4, and L5 recognize only a subpopulation of the 130 kD proteins immunoprecipitated with mAb Lan 3-2 or Laz 6-189. MAb L3 also binds only to a subpopulation of the 100 kD MAG mammalian glycoprotein (Kucherer et al., 1987). We have previously demonstrated that the 130 kD proteins recognized by mAb Lan 3-2 can be divided into two populations based on phase separation and extraction by hypoosmotic buffer, loosely associated peripheral proteins and integral membrane proteins (Cole et al., 1989b). It is

possible that mAbs L3, L4, and L5 recognize only one of these populations.

The different reactivities of mAbs L3/L4/L5, Lan 3-2 and Laz 6-189 suggest the presence of different types of mannose-containing epitopes in the leech nervous system. This is further supported by the fact that mAbs L3/L4/L5, Lan 3-2, and Laz 6-189 have different staining patterns. MAbs L3, L4, and L5 stain all axons evenly. Although mAb Laz 6-189 stains all axons, is stains the sensory afferents stronger. In contrast, mAb Lan 3-2 is a selective stain for the sensory afferents.

Several glycoproteins present in the vertebrate nervous system provide evidence for the mannose-containing L3, L4, L5, Lan 3-2 and Laz 6-189 epitopes. MAb Laz 6-189 recognizes epitopes on many of the same glycoproteins as L3, L4, and L5, e.g. L1 and Thy-1. Thy-1 from brain and thymus appear to have only mannose-type oligosaccharides in common (Parekh et al., 1987). Thy-1 is primarly expressed in the nervous system in rats and may be involved in transmembrane signaling in sensory neurons (Saleh et al., 1988).

The mAbs L3 and L4 have shown to affect cellular interactions under *in vitro* conditions. In cell adhesion assays, monovalent fragments of L4 strongly inhibit neuron to neuron and neuron to astrocyte adhesion. The mAb L3 has only a minor affect on cell adhesion, but interferes much more strongly with neurite outgrowth and cell migration than mAb L4 when added to the medium of cerebellar microexplants from early postnatal mice (Fahrig et al., 1990). The mannosidic epitope which is recognized by the mAb L5 is predominantly expressed by an astrocyte specific proteoglycan, but

is not yet functionally characterized in detail. It was shown that this proteoglycan binds specifically to collagen type IV (Streit, 1989).

The presence of the L3, L4, and L5 mannosidic epitopes is not surprising because these epitopes are so widely distributed. However, since these epitopes are expressed in the leech, we can now investigate their role(s) during leech neuronal development. Whether these epitopes mediate similar adhesion events as in vertebrate systems remains to be seen. As previously mentioned, the Lan 3-2 mannosidic epitope has been implicated in mediating specific neuronal interactions within the CNS (Zipser et al., 1989). Possibly, the other mannose-containing epitopes, Laz 6-189 and L3, L4, and L5 which are expressed by all neurons, mediate more general adhesive interactions in leech neuronal development.

SUMMARY AND CONCLUSIONS

Sperry orginally postulated that the precision of neuronal pattern formation was regulated by specific surface molecules. However, Sperry's hypothesis (Sperry, 1963) requires too many gene products in relation to the size of the gene pool in order to label the billions of synapses in the mammalian brain. Several solutions to this problem have been proposed. The modulation hypothesis advanced by Edelman (1984) suggests that a small number of adhesion molecules can mediate pattern formation, provided that their binding activities are locally modulated by epigenetic means in a dynamic fashion. Specifically, he postulates that a single molecule, N-CAM which is ubiquitously distributed across neurons and muscles, plays a decisive role in pattern formation.

In the leech, we have identified a group of 130 kD surface glycoproteins. We postulate that some of these constitute a protein family, differentiated by their unique glycosylation. Recent studies indicate that the carbohydrate epitope of the 130 kD sensory protein may be instrumental in neuronal development. To further characterize these glycoproteins we determined whether these glycoproteins were members of one of the known mammalian carbohydrate families, recognized by mAbs L2/HNK-1 and L3, L4, and L5. Both of these carbohydrate families were identified in the leech.

Since these epitope families are expressed in the leech, we can now investigate their function during development. The task of investigating these epitopes is simplified by the fact that so much is known about these epitopes within the vertebrate system. The leech system is simpler than vertebrate systems.and easily manipulated, which will allow us to understand how glycosylation modifies cell adhesion function in an intact system.

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